Tesis Doctoral

"Evaluación de estrategias de control del paludismo"

Pedro Luís ALONSO FERNANDEZ

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RESUMEN

El paludismo o malaria, especialmente la causada por el *Plasmodium falciparum*, continúa siendo una de las principales causas de morbilidad y mortalidad en amplias zonas del mundo. Algo más de dos mil millones de personas viven en los 103 países donde se da la transmisión de la malaria por las hembras de los mosquitos anófeles. Se estima que anualmente se producen alrededor de 300 millones de casos clínicos y entre 1 y 3 millones de muertes. A pesar de la muy amplia distribución geográfica de la transmisión de la malaria, mas del 90% de la mortalidad atribuida a está infección parasitaria se concentra entre los niños menores de cinco años y las mujeres embarazadas en el Africa subsahariana.

El grueso del problema actual de la malaria se localiza en Africa, pero no hace tantos años que la malaria también fue un problema en Europa. La transmisión de la malaria se daba en lugares como Finlandia, y a finales del siglo pasado seguía siendo un grave problema de salud pública en amplias zonas de centro Europa y de las Islas Británicas. La Europa mediterránea ha sido endémica de paludismo hasta la segunda mitad de este siglo. En España, tras la guerra civil y en los primeros años de la década de los 40, se registraron algo mas de 500,000 casos y mas de 5000 muertes. En Cataluña, el delta del Ebro y los humedales del río Llobregat han sido históricamente y hasta bien entrado este siglo unas de las zonas mas maláricas de la península Ibérica.

Durante la década de los cincuenta y sesenta de este siglo, la Organización Mundial de la Salud impulso la campaña de erradicación de la malaria. Mucho se ha debatido sobre

los logros y las deficiencias de este gigantesco esfuerzo. Una discusión detallada queda fuera del ámbito de este trabajo, pero de lo que poca duda cabe es que el continente Africano, y especialmente al sur del Sahara quedo pronto fuera de la campaña mundial. Es probablemente cierto que la situación actual de la malaria en Africa no es substancialmente mejor de lo que fue hace 30 años.

Hoy en día, las estrategias de control de la malaria continúan dependiendo del tratamiento del caso presuntivo con un antipalúdico eficaz. Poco o nulo énfasis se pone en la prevención. Sin embargo, varios son los ángulos u objetivos de posible intervención. Por un lado se puede tratar de eliminar el mosquito vector, bien en su fase larvaria, bien en su fase adulta con insecticidas. También se puede tratar de reducir el contacto entre el humano susceptible y el vector transmisor a través de barreras físicas como los mosquiteros. Finalmente se puede atacar el parásito durante su estancia en el huésped utilizando antipalúdicos en forma de tratamiento o como profilaxis. Una vacuna eficaz, que seria también una estrategia dirigida contra el parásito, es considerada como uno de los griales de la ciencia medica.

Con este punto de partida, nos hemos propuesto evaluar diversas estrategias de control de la malaria en el Africa subsahariana. En el primero de los estudios hemos evaluado el impacto de las mosquiteros impregnadas de insecticida y su impacto sobre la morbilidad y la mortalidad en menores de 5 años. También hemos evaluado el impacto de añadir quimioprofilaxis dirigida a ese grupo de niños. Los resultados son claros. La impregnación de mosquiteros con permetrina a través de un sistema básico de Atención Primaria de Salud en una zona rural de Gambia (Africa occidental) se asocio con una

reducción del 63% en la mortalidad en niños de 1 a 4 años y del 45% en el numero de casos clínicos de malaria. La adición de quimioprofilaxis con Maloprim ® (pirimetamina y dapsona) aumento la protección frente a los episodios clínicos hasta el 97% pero no parece haberse traducido en una mayor protección frente a la mortalidad. Ambas intervenciones fueron fáciles de implementar y muestran un buen perfil de coste eficacia por lo cual se posterior desarrollo y evaluación hacia su aplicación en los programas de control está justificada.

En un segundo estudio, hemos evaluado la seguridad, inmunogenicidad y eficacia en Africa de una vacuna peptídica contra la malaria denominada SPf66. Esta vacuna de subunidades y multiestadío incorpora epítopes de proteínas de estadío sanguíneo asexual así como de estadío pre-eritrocítico. Su urgente evaluación en Africa en niños menores de 5 años, aquellos que sufren las peores consecuencias de la infección malárica, fue solicitada por la Organización Mundial de la Salud a principios de los años 90. En una serie escalonada de ensayos clínicos y de campo realizados en una zona rural del sur de Tanzania, hemos determinado que la vacuna es segura en diversos grupos étnicos y etarios con diversos grados de inmunidad malárica adquirida. La vacuna ha sido inmunogénica en cuanto a que ha producido buenas respuestas humorales de producción de IgG frente al monómero así como frente a los distintos componentes del mismo. También se ha determinado que este péptido ha inducido anticuerpos que por inmunofluorescencia reconocen proteínas nativas del parásito. Finalmente, la vacuna ha demostrado ser eficaz en cuanto a que ha reducido la incidencia de episodios clínicos de malaria en un 31% y la densidad media de parasitación en un 20%.

Estos resultados constituyen la primera demostración de la viabilidad de inducir respuestas inmunes protectoras frente a la malaria en sujetos expuestos a transmisión de la malaria. La protección estimada para está vacuna, aunque modesta, justifica su posterior desarrollo y evaluación como posible herramienta para el control de la malaria.

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1. INTRODUCCION

El paludismo o malaria es una enfermedad causada por parásitos del genero *Plasmodium* que infectan a través de la picadura de las hembras de diversas especies de mosquitos anofelinos. Dentro del genero *Plasmodium*, hay cuatro especies que infectan a los humanos: *P. falciparum, P. vivax, P. ovale* y *P. malariae.* La Organización Mundial de la Salud estima que cuatro mil millones de personas en 90 países viven a riesgo de ser infectados y desarrollar está enfermedad que causa 500 millones de casos clínicos al año y entre uno y tres millones de muertes. A pesar de la muy amplia distribución geográfica de la transmisión de la malaria, el 90% de las muertes por malaria se producen entre niños menores de 5 años y primigrávidas en el Africa subsahariana. El *P. ovale* y el *P. malariae* tienen una muy escasa incidencia clínica en el mundo. El *P. vivax* es una importante causa de enfermedad en amplias zonas de América Latina y de Asía, pero es una relativamente rara causa de muerte. Por el contrario, el *P. falciparum* es el agente causal de una proporción importante de los casos clínicos, pero sobre todo es el responsable de la practica totalidad de los episodios y formas mas severas de malaria y de las muertes por esta infección.

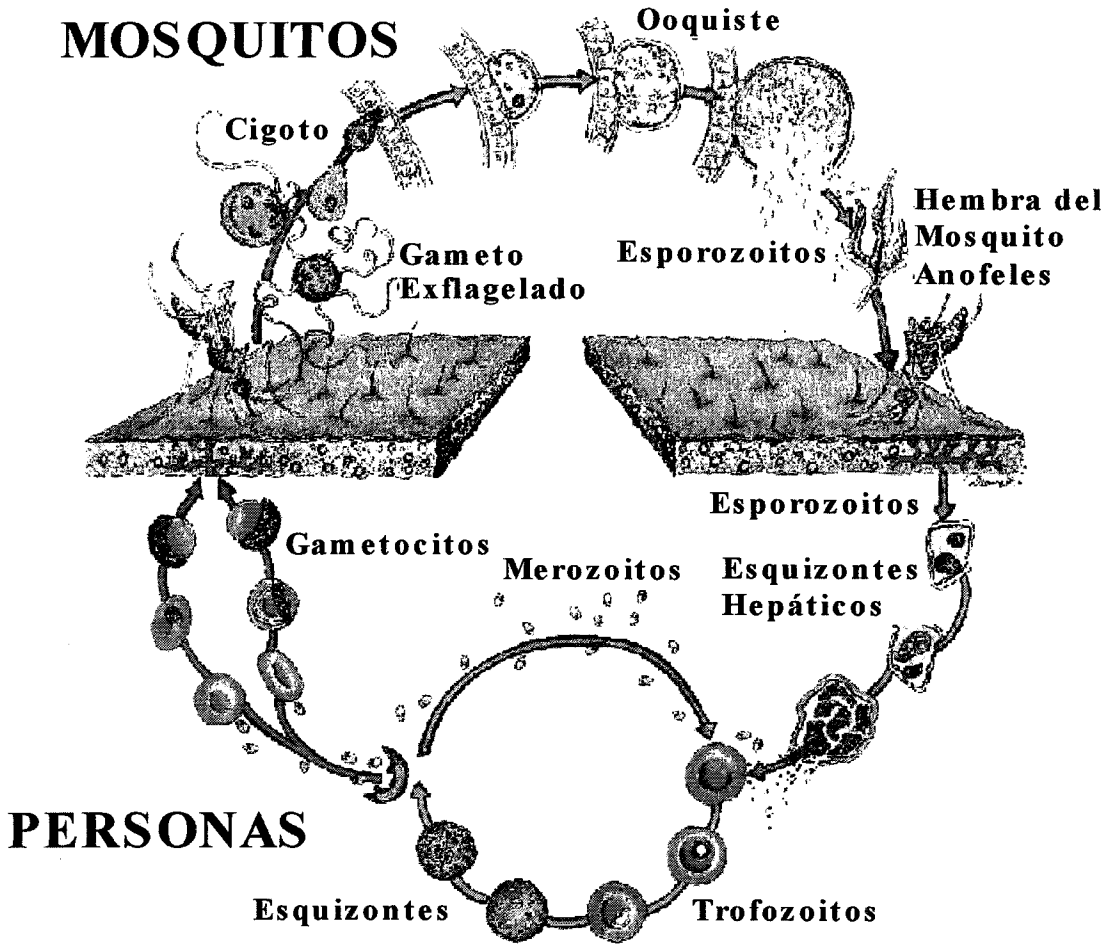
1.1- EL CICLO BIOLOGICO

El ciclo biológico del *P. falciparum* se puede ver en la Figura 1. En resumen, la ingestión de gametocitos por parte de mosquitos hematófogos, permite que emerjan gametos en el intestino del mosquito. Estos se fertilizan y producen ooquinetos mótiles que penetran las paredes del intestino para formar ooquistes. En el interior de estos ooquistes, se producen esporozoitos que migran hacia las glándulas salivares donde terminan de madurar. Allí aguardan a ser inoculadas en el huésped mientras el mosquito se alimenta.

Una vez inoculados los esporozoitos, estos permanecen alrededor de 30 minutos en la sangre hasta lograr infectar los hepatocitos. En el interior de estos, cada esporozoito se desarrolla durante los siguientes 5 a 8 días hasta formar de 30.000 a 50.000 merozoitos. Una vez liberados estos en la sangre, invaden los eritrocitos donde cada uno de ellos se desarrolla durante dos días dando lugar de 6 a 24 nuevos merozoitos. La ruptura del eritrocito infectado libera nuevos merozoitos que continúan el ciclo sanguíneo infectando nuevos eritrocitos. Para permitir la continuación del ciclo biológico permitiendo la transición del parásito desde el huésped humano hasta el mosquito vector, algunos parásitos se diferencian sexualmente en gametos.

Con cierta frecuencia el ciclo del parásito en el humano se clasifica en tres estadíos o fases. El llamado estadío pre-eritrocítico hace referencia a la parte del ciclo desde la inoculación del esporozoito hasta que emergen los merozoitos en sangre tras la ruptura del hepatocito. Está fase no está asociada con ninguna sintomatología clínica, pero si

hay descritas reacciones inmunológicas que controlan la infección. El segundo estádio se le denomina sanguíneo asexual y es el responsable de todas las manifestáciones clínicas y de las cascadas fisiopatológicas responsables de la morbilidad y mortalidad asociadas a esta infección. Finalmente el estádio sexual permite la infección del vector cuando este se alimenta de sangre, y como el estádio pre-eritrocítico, no está asociado con ninguna manifestación clínica.



1.2. EL CONTROL DE LA MALARIA: PASADO Y PRESENTE

En 1997 conmemoramos el primer centenario de la descripción por parte de Ronald Ross de como los mosquitos transmiten la malaria. También a finales del siglo pasado las observaciones de Robert Koch en la isla de Java confirmaban que los humanos, expuestos a la malaria, desarrollan una inmunidad natural. Está inmunidad adquirida implica que el riesgo de malaria clínica, y en particular de la malaria severa y de la muerte por malaria, disminuye con la edad. Estás dos descripciones siguen siendo relevantes hoy en día en cuanto a la definición de las estrategias de control del paludismo. Por un lado confirman el papel de los mosquitos en la transmisión de la malaria y por otro ayudan a definir los grupos de especial riesgo que deben constituir los objetivos principales del control de la malaria.

El esfuerzo de los humanos por controlar o evitar las consecuencias de la malaria son tan antiguas como la propia existencia de la malaria. Ya en la Biblia se describe como en el antiguo Egipto los pescadores del Nilo dormían bajo sus redes de pescar para evitar las fiebres y en la Valencia del siglo XIII estaba prohibido el cultivo del arroz cerca de los núcleos urbanos como forma de prevención de epidemias. Fue sin embargo en los años 50 y 60 de nuestro siglo cuando se realizaron los grandes esfuerzos coordinados por la Organización Mundial de la Salud que se embarco en el programa mundial de erradicación de la malaria. La oportunidad del mismo así como sus logros y fracasos siguen siendo materia de discusión. Lo cierto es que si bien se lograron importantes avances especialmente en América Latina y Asía, la malaria no fue

erradicada del mundo. Esto conllevó que a finales de los años 60 y principios de los 70 se sustituyera el objetivo de la erradicación por el del control.

En esencia, la estrategia mundial de control de la malaria tiene como objetivo el disminuir las consecuencias mas severas de la infección por *P. falciparum*: la malaria severa y la muerte. Estos objetivos deben ser conseguidos con una estrategia flexible basada tanto en la epidemiología de la malaria en la zona y que determina los grupos de riesgo así como en la utilización y eficacia de las herramientas disponibles.

En aquellas zonas del mundo donde se da la transmisión del paludismo con un grado mínimo de intensidad, como lo es la mayor parte del África subsahariana, la morbilidad severa y la mortalidad por *P. falciparum* se concentra en los niños menores de cinco años y las mujeres embarazadas, en especial las primigrávidas. En aquellas otras zonas del mundo donde la transmisión es menos estable, como lo es en términos generales las zonas endémicas de América Latina y Asía, las poblaciones no llegan a desarrollar niveles significativos de inmunidad natural y por lo tanto la morbi-mortalidad se distribuye entre todos los grupos de edad y el determinante fundamental es la exposición a la picadura del mosquito infectivo. Esta exposición es frecuentemente producto de las actividades laborales y así es frecuente que tanto en América Latina como en Asía sean las personas adultas en edad laboral las que mas sufren las consecuencias de la malaria severa.

Hay tres grandes grupos de medidas o herramientas en la lucha contra el paludismo. Podemos actuar contra el mosquito, contra el parásito o bien tratando de interrumpir o disminuir el contacto entre el hombre y el vector.

(i) La lucha anti-vectorial trata de disminuir el numero absoluto de mosquitos infectivos y por lo tanto el riesgo para los humanos y constituyo la piedra angular de las campañas de erradicación de la malaria. En gran medida la lucha contra el vector adulto se basa en la utilización de insecticidas, siendo el mas conocido y utilizado el DDT. Sin embargo su utilización prolongada provoco a menudo el rechazo de las poblaciones y mas importante indujo el desarrollo de mosquitos resistentes a este insecticida. El otro gran objetivo de la lucha anti-vectorial es la larva del mosquito. Por un lado la desecación de zonas pantanosas y la canalización de aguas ha supuesto en algunas zonas la desaparición de los criaderos de las larvas. Quizás uno de los ejemplos mas clásicos sean las grandes obras realizadas en Italia, especialmente en los alrededores de Roma durante los años 30. Finalmente se puede luchar contra las larvas tratando de eliminarlas a través de unos pequeños peces denominados *Gambusia* que tienen una especial avidez por estás. En España, introducidas por la Fundación Rockefeller, jugaron un papel importante en la erradicación de la malaria de la península Ibérica.

La lucha antivectorial ha constituido una pieza central de las estrategias de control, especialmente en las zonas donde la intensidad de transmisión y por consiguiente la tasa básica de reproducción (Ro) es baja. Sin embargo su utilidad ha demostrado ser menor o muy baja en las zonas de mayor endemicidad y especialmente en África.

(ii) Disminución del contacto hombre - vector. Si por lo tanto parece difícil disminuir el numero de mosquitos infectados, especialmente en el África, se puede intentar dificultar y disminuir la posibilidad de contacto entre el mosquito infectivo y el humano susceptible. Está es la base del segundo gran grupo de herramientas de control. Como decíamos al principio de está sección, las mosquiteros son una herramienta de protección individual muy antigua. También lo son las mallas en la puertas y ventanas de las casas y la utilización de repelentes a los mosquitos. La efectividad de todas estás medidas es relativamente baja y depende de su correcta y continua utilización.

(iii) Medidas anti-parasitarias. El tercer gran grupo de herramientas para el control de la malaria lo constituyen las medidas anti-parasítarias. Para luchar contra el parásito contamos con los fármacos administrados al humano infectado. La quinina fue el primer gran fármaco antipalúdico utilizado de forma amplia. La mitad historia mitad leyenda que rodea la identificación y posterior utilización y diseminación por el mundo de este fármaco es especialmente cercana y de interés al mundo hispano parlante. Sin embrago ha sido este siglo XX el que ha presenciado el gran aumento en la identificación y utilización de fármacos antipalúdicos y también en la rápida aparición y diseminación de cepas de parásitos resistentes.

En esencia, podemos utilizar los fármacos de una de dos maneras: como tratamiento de una caso de malaria (tanto confirmado como sospechado), o como profilaxis en la prevención de la infección. Las estrategias actuales de control de la malaria tienen todavía como piedra angular, el tratamiento en los niveles periféricos del sistema de salud. La profilaxis, esto es la toma rutinaria de una antipalúdico eficaz durante los

periodos de tiempo en que se está a riesgo de ser infectado, sigue teniendo una enorme eficacia. Sin embargo solo es utilizada sistemáticamente por los viajeros occidentales a las zonas endémicas. Problemas logísticos y presupuestarios junto a la creciente alarma por el desarrollo y extensión de las resistencias implican que los grupos en mayor riesgo, los niños menores de cinco años y las mujeres embarazadas que viven en las zonas endémicas, raramente se benefician de la profilaxis antipalúdica.

Difícilmente se puede hablar de los fármacos antipalúdicos sin comentar el grave problema que representa la aparición y diseminación de cepas resistentes. En un primer momento fue la resistencia a la cloroquina, la que ha implicado el que este fármaco sea de muy baja utilidad en amplias zonas del mundo endémico. Posteriormente las resistencias han ido apareciendo y diseminándose contra todos los principales antipalúdicos incluyendo las combinaciones sulfadoxina - pirimetamina, la mefloquina, el proguanil y ya también descritas resistencias en el sudeste asiático frente a la quinina y a derivados de la artemisina. Todo este sombrío panorama se agrava por el poco esfuerzo financiero en el desarrollo de nuevos fármacos antipalúdicos por parte de la industria farmacéutica. No es caer en el alarmismo el imaginar que en un periodo de tiempo corto nos podamos encontrar en una situación en que haya una rápida diseminación de cepas resistentes a todos los antipalúdicos conocidos y en la que por lo tanto, nuestra batería terapéutica este enormemente restringida. Las implicaciones que para la salud pública mundial puede tener este escenario son difíciles de imaginar.

Es sobre este sombrío panorama sobre el que debemos plantear el desarrollo de vacunas como el segundo elemento de la lucha antiparasitaria. El esfuerzo por desarrollar

vacunas contra el parásito se remonta a principios de este siglo. Ya a mediados de los años 30 hubo algún intento de ensayar prototipos de vacuna. Sin embargo, la complejidad del ciclo del parásito así como la falta de pruebas *in vitro* o medidas indirectas de protección han implicado que el desarrollo de una vacuna eficaz haya sido mas lento de lo esperado. En concreto, el ciclo del *Plasmodium falciparum*, ofrece múltiples objetivos en su complejo ciclo biológico para el desarrollo de una vacuna. La utilidad de una vacuna, su aplicación y beneficiarios posiblemente dependerá del estadío del parásito contra el que se desarrolle. Tradicionalmente se han clasificado las vacunas de acuerdo al estadío como (i) pre-eritrocíticas, (ii) estadío sanguíneo asexual y (iii) bloqueantes de la transmisión.

Un gran paso adelante fue dado a finales de los años 60 y principios de los 70 con la demostración de que la inmunización de humanos con esporozoitos irradiados inducía una inmunidad esterilizante, especifica del estadío pre-eritrocítico y de corta duración. La posterior descripción de una secuencia repetida de la proteína de circumsporozoito abrió una esperanzas que no fueron confirmadas por los distintos ensayos clínicos.

Nuevas moléculas están siendo desarrolladas frente a los distintos estadíos del parásito y no parece absurdo el pensar que los próximos diez años pueden ver el desarrollo y aplicación a través del Programa Ampliado de Inmunización de una vacuna segura, inmunogénica y eficaz que reduzca la morbilidad y mortalidad por *P. falciparum* entre los grupos mas a riesgo de las zonas endémicas, y que sea asequible a los Sistemas Nacionales de Salud.

4. HIPOTESIS

Objetivo General:

Diseñar y evaluar nuevas medidas de control de la malaria en Africa.

Objetivos específicos:

1. Describir la epidemiología de la malaria en una zona rural de Gambia (Africa Occidental).
2. Describir la morbilidad y mortalidad causada por el *Plasmodium falciparum* en una zona rural de Gambia.
3. Describir los conocimientos sobre la malaria y las actitudes con respecto a la prevención y el tratamiento por parte de los habitantes de una zona rural de Gambia.
4. Diseñar e implementar un esquema de distribución y aplicación de insecticidas para la impregnación de mosquiteros a través de un sistema de atención primaria de salud.
5. Diseñar e implementar un esquema de administración profiláctica de antipalúdicos a través de un sistema de atención primaria de salud.
6. Evaluar el impacto sobre la capacidad vectorial de la utilización de insecticidas en la impregnación de mosquiteros.
7. Evaluar el impacto sobre la morbilidad y la mortalidad de los esquemas de aplicación de insecticidas en los mosquiteros a través de un sistema de atención primaria de salud.
8. Evaluar el efecto de añadir la administración profiláctica de antipalúdicos sobre la morbilidad y mortalidad.
9. Diseñar los estudios de campo tendentes a la evaluación de una vacuna de la malaria en una zona hiperendémica de Tanzania (Africa Oriental).

10. Caracterizar el producto y evaluar su seguridad e inmunogenicidad en sus fases preclínicas.
11. Evaluar la seguridad e inmunogenicidad de la molécula denominada SPf66.
12. Evaluar la eficacia de la SPf66 en la prevención de episodios clínicos de malaria entre niños de 1 a 5 años residentes en una zona rural de Tanzania.

5. MATERIAL Y METODOS

Los estudios epidemiológicos cada vez requieren de equipos mas amplios y a menudo multidisciplinares, además de numeroso personal de apoyo.

Los estudios contenidos en está tesis se han desarrollado en dos países distintos de Africa (Gambia y Tanzania) además de en instalaciones y laboratorios de España, Inglaterra, Colombia y Suiza. Necesariamente han contado con el apoyo de muchas personas a muy distintos niveles. Todos aquellos que han tenido una participación directa desde un punto de vista científico están presentes en la lista de autores de los artículos correspondientes.

Mas allá de las contribuciones científicas recogidas en las autorías, hay que mencionar el apoyo de personal contratado para la realización del proyecto.

En Gambia el trabajo requirió de 25 inqueridores responsables de la vigilancia demográfica y de la monitorización de la morbilidad en las cohortes de niños en estudio. Otros 5 ayudantes de laboratorio permitieron la caracterización entomológica y colaboraron en los estudios sociales. Dos técnicos de laboratorio procesaron las muestras recogidas. Cuatro digitadores aseguraban el procesamiento de todos los formularios y cuestionarios producidos. Dos choferes y un barquero facilitaban el transporte.

Todo este personal trabajaba sobre la estructura ya existente de la estación de campo que el Medical Research Council (MRC) de Gran Bretaña tienen en Farafenni, en la región central de Gambia (Africa occidental). Esta estación de campo opera con una estructura básica de 40 personas, además de viviendas, laboratorio, oficinas centro de digitación y estructura administrativa. A su vez, esta estación de campo (una de las cuatro con las que cuenta el MRC en Gambia), se apoya sobra la base central en Fajara, donde se concentran todos los servicios sanitarios, administrativos, logísticos y de apoyo a todos los grupos de investigación del MRC en el país y que suman mas de 600 personas.

Estos estudios fueron financiados por el programa TDR de la Organización Mundial de la Salud.

Los estudios de Tanzania se realizaron sobre la estructura física y humana ya existente del Ifakara Centre, antigua estación de campo del Instituto Tropical Suizo. Además requirió de la contratación de 15 ayudantes de campo así como de 3 microscopistas y 5 digitadores.

Estos estudios fueron financiado por el programa TDR de la Organización Mundial de la Salud, el Fondo de Investigaciones Sanitarias y la Agencia de Cooperación Internacional de Suiza.

<u>Estudio I:</u> Evaluación de la eficacia de dos estrategias de control de la malaria, basadas en la utilización de mosquiteros impregnados de insecticida y quimioprofilaxis selectiva en una zona rural de Gambia en el Africa Occidental.

Estudio descriptivo de la mortalidad y morbilidad por malaria en la zona de estudio. A malaria control trial using insecticide-treated bednets and targeted chemoprophylaxis in a rural area of The Gambia, West Africa. 2. Mortality and morbidity from malaria in the study area. Alonso PL, Lindsay SW, Armstrong Schellenberg JRM, Gomez P, Hill AG, David PH, Fegan G, Cham and Greenwood BM *Trans R Soc Trop Med Hyg* 1993;**87**, Suppl.2:13-17

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A malaria control trial using insecticide-treated bed nets and targeted chemoprophylaxis in a rural area of The Gambia, West Africa

2. Mortality and morbidity from malaria in the study area

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Abstract

Background data on child mortality and morbidity from malaria were obtained in a new study area in the centre of The Gambia, south of the river, chosen as the site for a malaria intervention trial. Infant and child mortality rates were 120 and 41 per 1000 respectively. Results obtained using post-mortem questionnaires suggested that malaria was an uncommon cause of death in children under the age of one year but responsible for about 40% of deaths in children aged 1–4 years. Ninety-two percent of deaths attributed to malaria occurred during or immediately after the rainy season. Parasite and spleen rates in children aged 1–5 years at the end of the malaria transmission season were 66% and 64% respectively. Malariometric indices were similar in primary health care (PHC) villages, selected as sites for an intervention with insecticide-treated bed nets and targeted chemoprophylaxis, and in smaller, non-PHC, control villages.

Introduction

The epidemiology of malaria has been investigated in various parts of The Gambia over the past 40 years (GREENWOOD & PICKERING, 1993). During this period, malaria has become less prevalent, perhaps due to an increase in the use of antimalarial drugs together with a decline in rainfall, which may have led to a reduction in vector mosquito populations and hence in malaria transmission. Although malaria transmission has not yet become unstable, there are large variations in the intensity of transmission from year to year, and variations between regions and even between villages in the same area (BRADLEY *et al.*, 1986; GREENWOOD, 1989). These variations have implications for the design of malaria control trials, in particular for community-based interventions, such as those employing insecticide-treated bed nets. Variations in intensity of transmission are less important in the evaluation of interventions aimed at individuals, for these can be randomized within villages. Thus, before introducing a controlled community trial of insecticide-impregnated bed nets and malaria chemoprophylaxis in an area of The Gambia which had not been studied previously, we have undertaken a number of investigations to define the pattern of mortality and morbidity from malaria in this area, and to develop and test the tools needed to evaluate the impact of the control measures under investigation.

Materials and Methods

Study area and population

The study area is on the south bank of the River Gambia, east of the town of Soma and approximately 200 km from the coast (Fig. 1). Villages within 10 miles [16 km] of the regional health centre at Mansa Konko were excluded to reduce the influence on the study of treatment provided there.

The study area is one of flat Sudan savanna, with mangrove swamps bordering the river which is still partly saline at this point. The climate is characteristic of the sub-Sahel with a long dry season and a short rainy season from July to October. Rainfall was 1051 mm in 1988 and 887 mm in 1989. Villages in the study area are characteristic of the region, with fenced groups of houses which make up compounds which, in turn, are packed closely together to form villages. Most male villagers are subsistence farmers who grow ground-nuts and coarse grain for cash, while women grow rice. However, males from the Fula ethnic group are also herdsmen, keeping large herds of trypanotolerant Ndama cattle.

Seventy-three villages were included in the study, of which 17 were chosen in 1982 to join the Gambia government's primary health care (PHC) programme because they had a population of 400 or more. The 56 remaining, smaller villages are referred to as non-PHC villages. The total population of the 17 PHC villages in December 1988 was 12 924, and that of the 56 non-PHC villages was 8233. The 2 main ethnic groups present in the study area are Mandinka (51%) and Fula (37%). There were considerable differences in the distribution of ethnic groups between villages. Mandinkas formed 77% of the population of PHC villages but only 10% of the population of non-PHC villages. On the other hand, Fulas comprised 73% of the population of non-PHC villages, but only 14% of the population of PHC villages. Family size, type of housing, economic activity and social behaviour were found to vary with ethnic group, as discussed elsewhere in the supplement (AIKINS *et al.*, 1993).

Demographic surveillance

A full *de jure* census of the total population was carried out during the second half of 1988 and repeated in 1989 and 1990. Trained field assistants mapped all the villages, each of which was given a unique identifying letter or number code. Within each village, compounds were given consecutive numbers. In each compound every resident's name and surname, date of birth, sex and ethnic group were recorded and a unique identification number was issued.

In addition to annual re-enumeration, a retrospective survey of the lifetime fertility and childhood mortality experience of all women of reproductive age was carried out early in 1990 to obtain information on longer-term trends in childhood mortality. The questionnaire included a full birth history together with questions on child survival. This was done in order to determine levels and trends in childhood mortality before the intervention trial and to ascertain whether non-PHC villages were adequate as a control group.

Continuous demographic surveillance was started on 1 July 1988 and was sustained until June 1992. All births, deaths and permanent migrations that occurred in study villages were recorded by village reporters and the infor-

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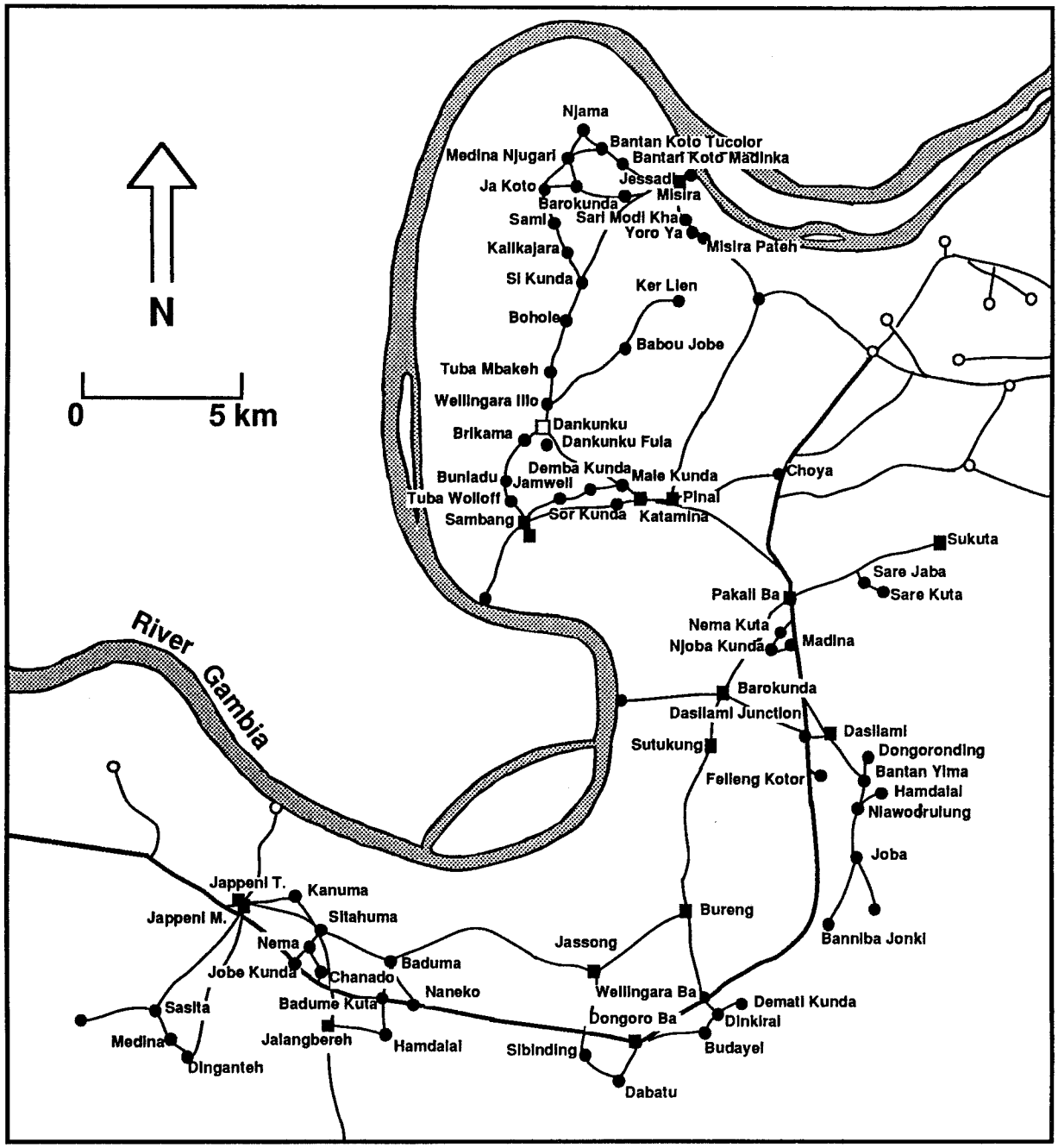

Fig. 1. Map of the study area in The Gambia. Villages included in the study are indicated by closed symbols (PHC villages, ■; non-PHC villages, ●); open symbols indicate villages not forming part of the study.

mation was collected and checked weekly by Medical Research Council (MRC) field assistants. These data were entered regularly into a computer at the MRC field station, Farafenni, so that updated census files could be maintained and checked on a continuous basis.

Ascertainment of cause of death

From 1 July 1988 to June 1991, parents or guardians of all children who died in the study area were interviewed by a senior field assistant using a verbal autopsy technique. This was based on a questionnaire developed by a group of physicians working with patients in local hospitals and villages. The questionnaire started by confirming the identity of the dead child and then determined use of health services before death. Subsequently, an attempt was made to establish the sequence of events leading to death and the symptoms and signs of the last illness noted by the mother or regular guardian were recorded. The senior field assistant then asked directly about the occurrence of 7 major signs and symptoms during the child's final illness and about their duration. A positive response to one of these direct questions led to further in-depth questioning in the appropriate area.

Verbal autopsy forms were reviewed independently by 3 physicians with local experience. Based on the information given by the questionnaire and on that provided by the child's health card, each physician decided on a likely principal cause of death from a list of 10 broad categories, one of which was 'unknown'. A final diagnosis was accepted only when at least 2 of the 3 physicians independently agreed on a probable cause of death. Deaths were generally attributed to malaria if they followed a short febrile episode of acute onset, with or without neurological involvement, without marked gastrointestinal or respiratory symptoms.

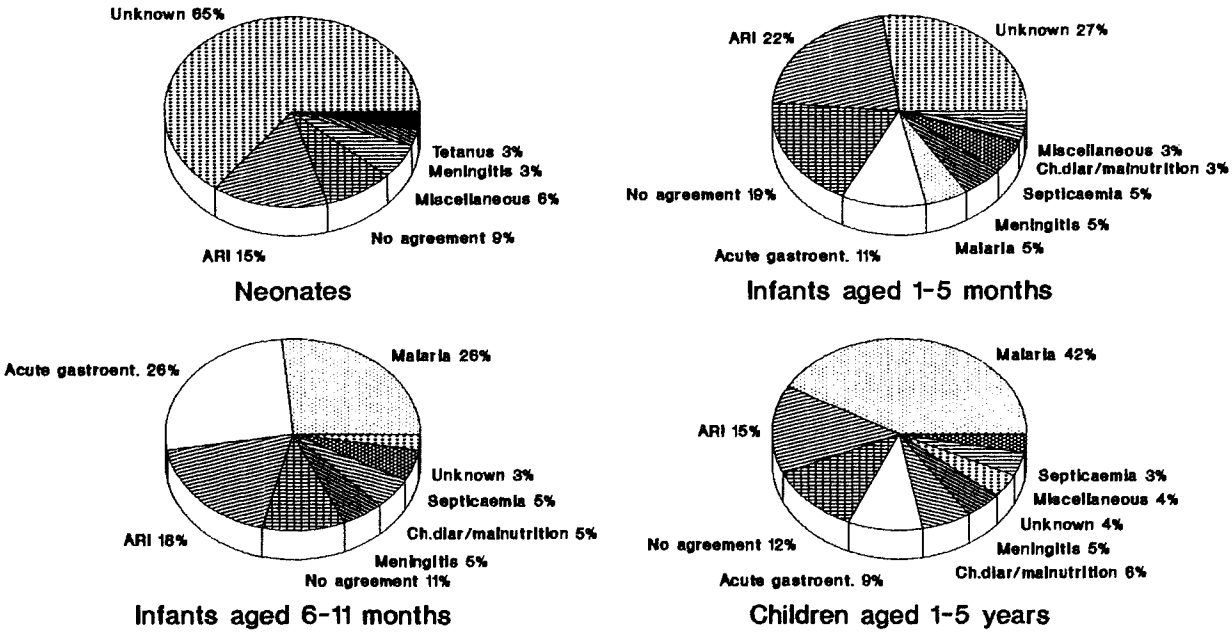

Fig. 2. The influence of age on cause of death in children aged 0–5 years as ascertained by post-mortem questionnaires.

Malaria morbidity

In November 1988 a cross-sectional malaria survey was carried out among children resident in PHC or non-PHC villages. Using the newly created census, a simple random sample of children aged 1–5 years from PHC villages was generated. As non-PHC villages had not been fully enumerated at the time of the cross-sectional survey, cluster samples were chosen in these villages. Analysis of the data allowed for these different forms of sample selection.

Axillary temperatures were measured using an electronic thermometer. All children were examined for splenomegaly in the standing position by the same physician (P. L. A.). Finger-prick blood specimens were collected into Microtainers® by a field worker and thick and thin blood films were prepared directly from the finger.

Laboratory methods

Thick and thin blood films were stained with Giemsa's stain. All films were examined by the same observer. One hundred high power fields (HPF) of each thick film were examined and the number of fields containing one or more parasites recorded. When each HPF contained one or more parasites, the number of parasites per HPF was determined. Ten parasites per HPF correspond to approximately 5000 parasite per μL (GREENWOOD & ARMSTRONG, 1991).

Results

Overall mortality and mortality attributable to malaria

Life tables showed that child survival improved throughout the 1980s and that it was similar in PHC and non-PHC villages (Table 1). During the pre-intervention year, 1 July 1988 to 30 June 1989, 229 deaths were recorded among children aged less than 5 years. The infant mortality rate in PHC and non-PHC villages combined was 120 per 1000 live births; there was no significant difference in mortality between sexes.

Table 1. Infant ($_1q_0$) and under 5 years ($_5q_0$) mortality estimated by life table methods from birth histories collected in 1990 in primary health care (PHC) and non-PHC villages

Approximate date	PHC villages		Non-PHC villages	
	$_1q_0$	$_5q_0$	$_1q_0$	$_5q_0$
1977	0·212	0·378	0·163	0·308
1982	0·157	0·272	0·149	0·256
1987	0·131	0·212	0·127	0·207

Table 2. Place of death of children aged 0–5 years who were resident in primary health care (PHC) or in non-PHC villages

	PHC villages Cause of death[a]		Non-PHC villages Cause of death[a]	
	Malaria	Other	Malaria	Other
Home	35 (71%)	72 (71%)	13 (81%)	51 (72%)
Dispensary/ health centre	9 (18%)	9 (9%)	1 (6%)	4 (6%)
Hospital	2 (4%)	6 (6%)	– –	2 (3%)
Other	3 (6%)	9 (9%)	2 (13%)	8 (11%)

[a]Information on place of death was not known for 6 children resident in PHC villages and 6 resident in non-PHC villages.

Table 3. Utilization of health services for the final illness of 238 children aged 0–5 years resident in primary health care (PHC) or in non-PHC villages

	PHC villages Cause of death[a]		Non-PHC villages Cause of death[a]	
	Malaria	Other	Malaria	Other
Village health worker	18 (37%)	25 (25%)	0 (–)	8 (11%)
Dispenser	11 (22%)	28 (28%)	6 (38%)	22 (31%)
Nurse	14 (29%)	40 (39%)	5 (31%)	15 (21%)
Doctor	12 (25%)	26 (26%)	2 (13%)	10 (14%)
Traditional healer	12 (25%)	23 (23%)	7 (44%)	20 (28%)
Died without seeing anyone	9 (18%)	23 (23%)	3 (19%)	19 (27%)
Died without seeing any 'western' practitioner	13 (27%)	35 (34%)	6 (38%)	29 (41%)
Admitted to hospital	8 (16%)	22 (22%)	2 (13%)	8 (11%)

[a]Information was not available for 6 children in PHC villages and 6 in non-PHC villages

Post-mortem questionnaires were not obtained for 11 cases (5%) because the parents or relatives of the deceased child had migrated from the study area soon after the death of the child. No agreement on diagnosis was reached by physicians in 19 cases (9%). Overall, there were 30 children (13%) for whom a probable cause of death could not be determined.

The place where death occurred and the use of different types of health care before death are shown in Tables 2 and 3. Over 70% of deaths occurred at home in both

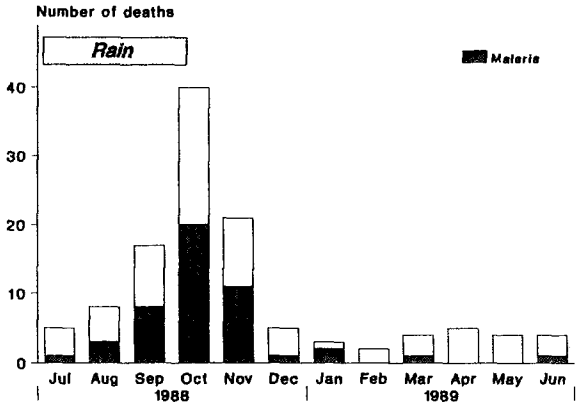

Fig. 3. The influence of season on overall mortality and on mortality attributed to malaria in children aged 1–5 years.

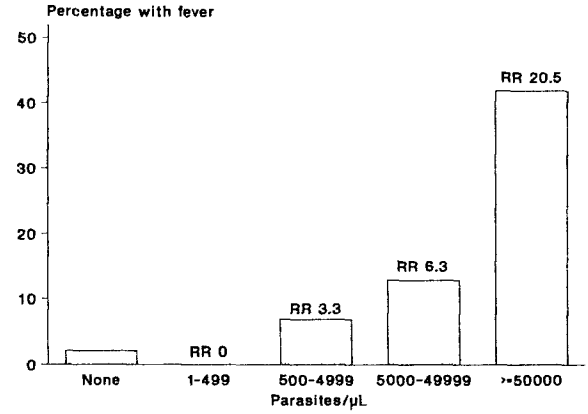

Fig. 4. The influence of parasite density on the prevalence of fever in children aged 1–5 years. The relative risk (RR) of fever in relation to levels of parasitaemia compared to children without parasitaemia is shown.

PHC and non-PHC villages. In PHC villages 29% of children had consulted a village health worker (VHW) during their final illness whilst only 9% of children from non-PHC villages had done so.

During the pre-intervention year, 60 deaths among children less than 5 years old were attributed to malaria, suggesting that malaria was responsible for 26% of all deaths. The mean duration of symptoms for deaths attributed to malaria was 3·3 d (range <1–14 d). The influence of age on deaths from malaria and from other causes is shown in Fig. 2. No death was attributed to malaria among neonates and malaria was an uncommon cause of death among children under the age of 6 months. However, 41% of deaths of children aged 1–4 years were attributed to malaria.

The seasonal distribution of deaths in children aged 1–5 years is shown in Fig. 3. There was no seasonality of deaths among neonates, but a marked excess of deaths during the rainy season occurred among children aged 1–11 months and among those aged 1–4 years. Eighty-one percent of all deaths in children aged 1–4 years (96/118) took place between July and December, 35 of these in October. Ninety-two percent (44/48) of all deaths attributed to malaria among children in this age group occurred during the rainy season or immediately afterwards.

Table 4. Summary of the findings of a cross-sectional malaria survey undertaken in children aged 1–5 years in November 1988 at the end of the malaria transmission season in primary health care (PHC) or in non-PHC villages

	PHC villages[a]	Non-PHC villages[a]
Splenomegaly	64% (60%,68%)[267/416]	67% (61%,73%)[324/481]
Parasitaemia[b]	66% (62%,70%)[281/427]	62% (56%,67%)[306/492]
High parasitaemia[c]	33% (29%,38%)[143/427]	35% (30%,41%)[172/492]
Fever[d]	7% (5%,10%) [32/427]	10% (7%,13%) [51/498]
Fever and parasitaemia	7% (5%,9%) [29/427]	9% (6%,12%) [45/498]
Fever and high parasitaemia	6 (4%,8%) [24/427]	7% (5%,9%) [37/498]
Gametocytaemia	30% (26%,35%)[127/427]	35% (30%,41%)[177/507]

[a]Values are precentages, with 95% confidence intervals (calculated using standard errors corrected for cluster sampling) in parentheses and real numbers in square brackets.
[b]Asexual forms of *P. falciparum* only.
[c]Parasitaemia ≥5000/μL (≥10 parasites per high power field).
[d]Axillary temperature ≥37·5°C.

Malaria morbidity

The results of a cross-sectional survey carried out in November 1988, at the end of the malaria transmission season, are summarized in Table 4. Sixty-four percent of children had malaria parasitaemia. *Plasmodium falciparum* was the predominant species, accounting for 96% of all infections. *P. malariae* alone was found in 9 children (1% of all infections) and *P. ovale* in one case only. Fourteen children (2%) had mixed infections of *P. malariae* and *P. falciparum*. The prevalence of gametocyte carriers in PHC and non-PHC villages was similar and was associated strongly with the prevalence of asexual parasitaemia.

Children with parasitaemia were febrile (axillary temperature ≥37·5°C) significantly more frequently than aparasitaemic children (relative risk=5·0, 95% confidence interval 1·6, 16·2). The relationship between fever and parasite density is shown in Fig. 4.

The relationships of splenomegaly and parasitaemia to age are illustrated in Fig. 5. The prevalence of parasitaemia and splenomegaly increased up to the age of 2 years and then levelled off.

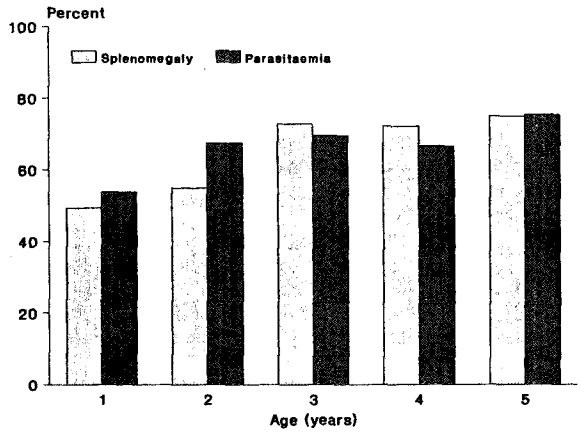

Fig. 5. Spleen and parasite rates in children aged 1–5 years in primary health care villages.

In PHC villages, 93% (396/427) of children were reported to sleep regularly under a net, as were 90% (463/513) of children in non-PHC villages. In both PHC and non-PHC villages, no association was found between bed net use and the presence of parasitaemia (Mantel-Haenszel χ^2 stratified on PHC/non-PHC=0·5; degrees of freedom [d.f.]=1; P=0·50) or high density parasitaemia (Mantel-Haenszel=χ^2 stratified on PHC/non-PHC=0·01; d.f.=1; P=0·91). However, there was a significantly lower prevalence of splenomegaly among those reported to be sleeping under a net than in those who were not (Mantel-Haenszel χ^2 stratified on PHC/non-PHC=4·1; d.f.=1; P=0·04).

Discussion

Infant and childhood mortality remain high in rural areas of The Gambia as shown in this study and in parallel investigations in Upper River Division (A. de Franci-

sco *et al.*, unpublished observations). These high mortality figures are surprising in a country which has operated an active PHC programme since 1982 and which has an impressive vaccination programme, with about 80% of children being fully immunized. It has often been suggested that the high mortality rates found in many rural areas of West Africa are related directly or indirectly to measles (BLACKER *et al.*, 1985). However, in rural Gambia, where vaccination coverage is very high and measles mortality correspondingly low, malaria and acute respiratory infections are now the leading causes of death in children under the age of 5 years.

The contribution made by malaria to under 5 years mortality was found to be higher than that described previously in a neighbouring area on the north bank of the river (GREENWOOD *et al.*, 1987). Clinical and parasitological data also suggested that the new study area had a higher level of malaria transmission during the year in which the survey was carried out than is usually found on the north bank. This finding illustrated again the considerable local variations in the pattern of malaria transmission that may occur between adjacent areas and the need for detailed descriptive pre-intervention studies before intervention trials are undertaken, especially when these are not strictly randomized.

The post-mortem questionnaire technique has helped to establish likely causes of death, particularly in children older than one year. However, the method is less useful in ascertaining causes of death among children less than 6 months old. Nevertheless, information on causes of death generated through the use of post-mortem questionnaires, even in older children, should be viewed with caution and used only as a crude estimate of cause-specific mortality. Differentiation of deaths from malaria and from acute lower respiratory tract infections may be especially difficult (J. Todd *et al.*, unpublished observations).

We found no difference in the prevalence of any malariometric indices between PHC and non-PHC villages, suggesting that the presence of a simple PHC scheme was not a major determinant of malaria morbidity and of overall and malaria-specific mortality. Both the World Health Organization and UNICEF currently recommend treatment of clinical malaria by VHWs and others as their main strategy for the control of malaria in children. However, there have been few studies which have measured the impact of community-based treatment programmes on mortality from malaria. A recent evaluation of this strategy in The Gambia (GREENWOOD *et al.*, 1988) suggested that treatment alone did not have any significant effect on malaria mortality. Our observations support these previous findings; levels of malaria infection in PHC and non-PHC villages were similar despite the fact that children in PHC villages made greater use of the PHC facilities available for them, which included prompt treatment of fever with effective antimalarial drugs. The failure of village-based treatment to reduce mortality and severe morbidity from malaria may be due partly to the fact that in The Gambia, and probably in other parts of Africa, episodes of severe life-threatening malaria are generally brief. Thus, there may be only a short interval between the time at which a mother or guardian first recognizes that the child is sick and the time by which treatment must be given if death is to be prevented. In areas where severe malaria develops as fast as in The Gambia, prompt treatment of clinical cases may be inadequate as a malaria control strategy and additional preventive methods may be required.

Our preliminary study established that the study area on the south bank of the river Gambia had high and seasonal malaria transmission, with high overall and malaria-specific mortality rates. No difference was found in malaria morbidity or mortality between PHC and non-PHC villages, which have shown similar declines in mortality over recent years. Thus, we concluded that the smaller non-PHC villages formed adequate contemporary controls to monitor the impact of interventions aimed at preventing morbidity and mortality from malaria.

Acknowledgements

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Características entomológicas de la zona de estudio. A malaria control trial using insecticide-treated bednets and targeted chemoprophylaxis in a rural area of The Gambia, West Africa. 3. Entomological characteristics of the study area. Lindsay SW, Alonso PL, Armstrong JRM, Hemingway J, Thomas PJ, Shenton FC, Greenwood BM *Trans R Soc Trop Med Hyg* 1993;**87**, Suppl.2:19-23

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A malaria control trial using insecticide-treated bed nets and targeted chemoprophylaxis in a rural area of The Gambia, West Africa

3. Entomological characteristics of the study area

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Abstract

Baseline entomological surveillance was carried out in a rural area of The Gambia during the rainy season in 1988, one year before the implementation of a malaria control programme using insecticide-impregnated nets and targeted chemoprophylaxis in villages with a primary health care (PHC) system. Mosquito collections took place in 6 pairs of settlements each with untreated bed nets; within each pair there was a large PHC village with a resident village health worker (VHW) and traditional birth attendant (TBA) and a smaller non-PHC village without either a VHW or a TBA. The most common vectors in the study area were *Anopheles gambiae sensu stricto* and, to a lesser extent, *An. arabiensis*. These mosquitoes were found in appreciable numbers for at least 4 months of the year (geometric mean/bedroom/night=32·5, 95% confidence interval 18·2–57·3). Numbers of mosquitoes collected in PHC villages or non-PHC villages were not significantly different. Greater numbers of mosquitoes were found in villages closer to the River Gambia than in those further away. Evidence for DDT resistance due to elevated glutathione S-transferase activity was found in one of the 12 villages, but there was no evidence of resistance to organophosphate or carbamate insecticides as suggested by the low esterase levels and carbamate sensitive acetylcholinesterase.

Introduction

In The Gambia, previous small-scale studies using permethrin-impregnated bed nets have shown a reduction in the biting rate of malaria mosquitoes (LINDSAY *et al.*, 1989b). This protection was due largely to mosquitoes being prevented from feeding on people sleeping under treated nets. No living mosquito was found under permethrin-treated nets, although one or more were often found inside untreated nets (LINDSAY *et al.*, 1989b). Permethrin-impregnated bed nets protect people by reducing the number of mosquitoes entering a room in which treated nets are used and by killing or repelling those which land on the treated fabric (LINDSAY *et al.*, 1991). In a study in which whole villages were given permethrin-treated or untreated bed nets, mosquito biting rates were reduced by about 90% in houses whose inhabitants slept under impregnated nets and this resulted in 72% fewer attacks of malaria in children (SNOW *et al.*, 1988). However, part of this success may have been due to the brief and moderate level of malaria transmission which occurred in this area, where elevated numbers of vector mosquitoes were found for only a few weeks of the year. Studies from other parts of the tropics suggest that, in areas of more prolonged and intense transmission, insecticide-treated nets may not affect the prevalence of malaria infection or the incidence of clinical disease (WHO, 1989). Therefore, it was decided to undertake a larger trial of impregnated bed nets in an area of The Gambia with a longer period of malaria transmission.

The present study was carried out on the south bank of the River Gambia, east of the town of Soma. Preliminary mosquito collections from under bed nets in this area in 1987 indicated that this was a region of higher transmission than the area on the north bank of the river where the first 2 Gambian trials of treated nets were undertaken.

The aim of the present investigation was to provide baseline entomological data describing the seasonality of malaria mosquitoes and their infectivity within the study area. In addition, insecticide susceptibility was investigated. Pyrethroids, including permethrin, have so far not been used extensively for mosquito control. However, pyrethroid resistance has been selected for by exposure to agricultural chemicals in *Culex quinquefasciatus* in Saudi Arabia (AMIN & HEMINGWAY, 1989). Cross-resistance to pyrethroids can also occur from earlier DDT selection (RONGSRIYAM & BUSVINE, 1975; MALCOLM, 1988; HEMINGWAY *et al.*, 1989).

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Materials and Methods

Study area

The investigation was carried out in a group of villages on the south bank of the River Gambia, east of Soma town, approximately 100 km from the coast. Details of the study site and population are given by ALONSO *et al.* (1993). Briefly, the study area lies in an area of flat, open Sudan savanna. Typically, large stands of maize, millet and sorghum, which can reach over 3 m in height, are grown around each settlement during the wet season. Beyond the fields and small vegetable gardens, herds of cattle may be tethered at night. Most rice is grown in the marshland bordering the river although some is also cultivated inland in paddies close to villages.

The climate of the area is characteristic of the sub-Sahel with a rainy season, during which most malaria transmission occurs, which lasts from July to the end of October. Total precipitation recorded at Farafenni during the 1988 rainy season was 1051 mm.

Mosquito collections.

Six pairs of villages were selected for the base-line entomological investigations: Jessadi and Barokunda, Male Kunda and Katamina, Pakali Ba and Madina, Dasilami and Niawodrulung, Dongoro Ba and Wellingara Ba, Sitahuma and Jalangbereh (Fig. 1). The first named village in each pair has a resident village health worker and traditional birth attendant and is therefore described as a primary health care (PHC) village. The second named is a neighbouring community smaller in size without these health care facilities (i.e., a non-PHC village).

Mosquitoes were sampled from 4 rooms in each village, with one man sleeping under an untreated net in each room. One room, housing a single man, was selected from houses in each quarter of the village. Two 'knock-down' and 2 light trap catches were made in each village weekly from August to November 1988. 'Knock-down' catches were made using an aerosol of 0·3% D-allethrin and 0·1% D-phenothrin (Target®, Reckitt and Colman). After spraying, mosquitoes were collected off

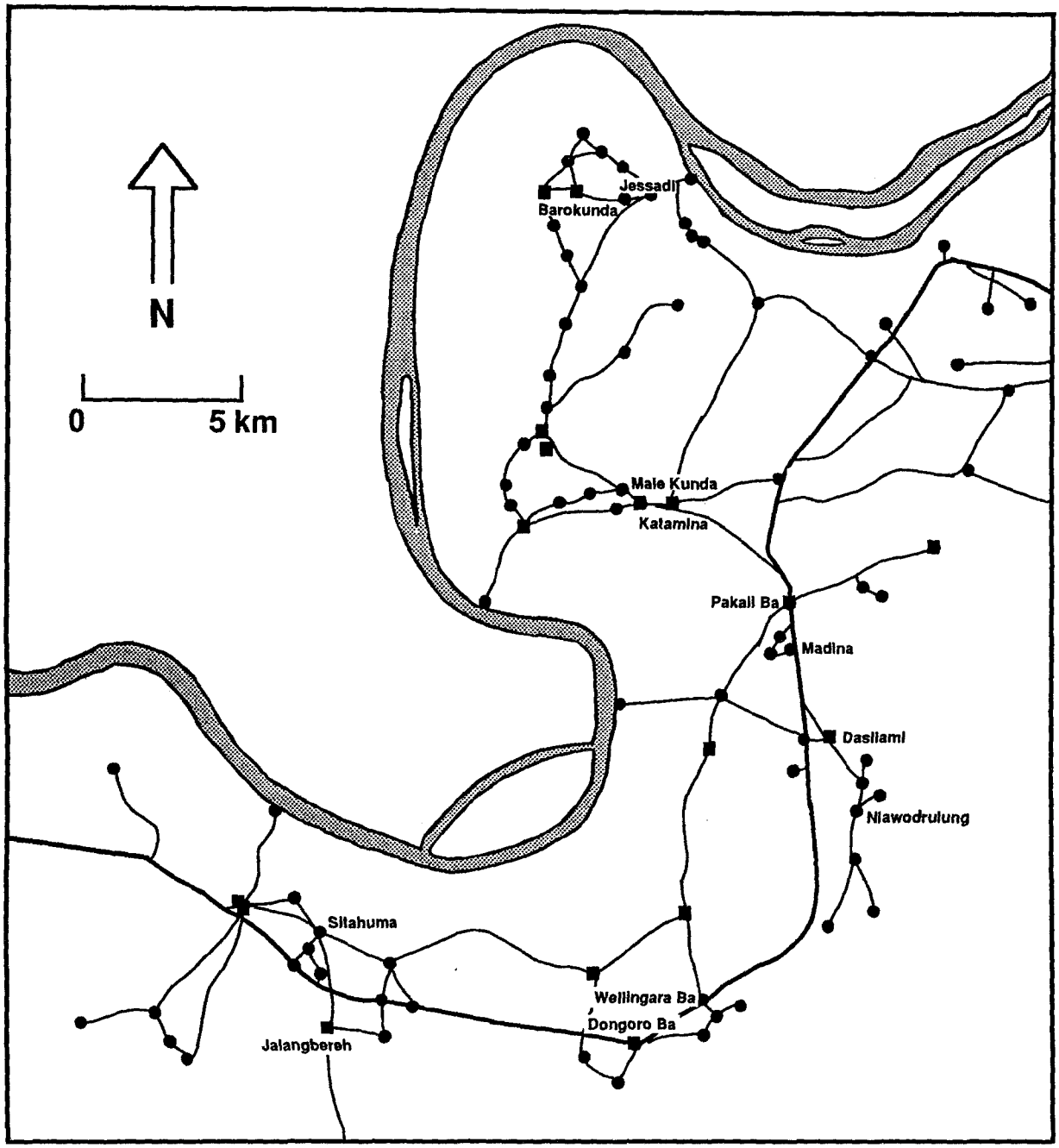

Fig. 1. Location of the 12 villages used for entomological investigations in the study area on the south bank of the River Gambia.

white sheets placed on the floor of the room and from underneath the bed net between 07:00 and 10:00. Light traps were positioned indoors, between the bed net and open eaves, and operated from 19:00 until 07:00. Collections from traps which were not working in the morning were excluded from subsequent data analysis.

Sporozoite assays

The presence of *Plasmodium falciparum* circumsporozoite (CS) antigen, assumed to represent the presence of sporozoites, was detected in pools of the heads and thoraces of 10 dried *Anopheles gambiae sensu lato* using an enzyme-linked immunosorbent assay (ELISA) (WIRTZ *et al.*, 1987). Positive and negative controls were included on each microtitre plate. Positive controls consisted of 10 and 100 pg of a recombinant CS protein. Negative controls were heads and thoraces of pools of 10 male *An. gambiae s.l.*

Mosquito identification and resistance assays

Additional light trap collections were made in houses in the study village in October 1988. Specimens were stunned, snap-frozen in liquid nitrogen, and transported to London for further analysis. Identification of *An. gambiae s.l.* mosquitoes was carried out using deoxyribonucleic acid (DNA) probes as described by GALE & CRAMPTON (1987, 1988). The mosquitoes collected were sorted on dry ice to separate female *An. gambiae s.l.* mosquitoes, from which the tip of the abdomen, containing the spermatheca, was removed and squashed on nitrocellulose filters. These samples were probed to confirm that the mosquito belonged to the *An. gambiae* group, and then probed specifically for *An. arabiensis*. The remainder of the insect was used for resistance detection by biochemical assays.

Resistance assays

Each mosquito was homogenized in 0·5 mL of distilled water and centrifuged at 10 000 *g* for 3 min. The supernatant was used to measure the activity of 3 families of enzymes: acetylcholinesterases, esterases, and glutathione S-transferases. Acetylcholinesterase activity was measured by the Ellman method (ELLMAN *et al.*, 1961), modified for use with microplates (FFRENCH-CONSTANT & BONNING, 1989). Control solutions were prepared by adding 25 µL of the mosquito homogenate to 140 µL of Triton/phosphate buffer (pH 7·2, 0·1 M, 1% Triton X-100®), 10 µL of dithio-bis(2-nitrobenzoic acid) and 25 µL of acetylthiocholine iodide. Test samples were similar to the controls, except for the addition of propoxur, an acetylcholinesterase inhibitor. Reaction rates were recorded simultaneously for both control and test solutions using a UVMax® microtitre plate reader at a wavelength of 420 nm.

Esterase activity was measured as described by PEIRIS & HEMINGWAY (1990). Briefly, 25 µL of homogenate were added to 185 µL of substrate (30 mM 2-naphthyl acetate in acetone diluted 1/100 in 0·02M sodium phosphate buffer, pH 7·4) and left for 15 min at 28°C before the reaction was stopped with 50 µL of fast blue in 3·5% sodium dodecyl sulphate. Each test was carried out in duplicate and absorbances read at 570 nm.

Glutathione S-transferase (GSH) activity was measured by adding 20 µL of homogenate, 100 µL of buffered reduced glutathione (0·06 g reduced glutathione in 100 mL 0·1 m phosphate buffer, pH 6·5) and 5 µL of chlorodinitrobenzene solution (CDNB; 0·06 g CDNB in 10 mL absolute alcohol). The tests were duplicated for each mosquito and the change in absorbance read at 340 nm after 5 min.

Measurements of protein concentration were used to convert the esterase and GSH data into absolute units for comparative purposes. Two hundred µL of mixed Pierce bichinchonic acid reagents A and B were added to 5 µL of homogenate plus 5 µL of sodium phosphate buffer and left at 24°C for 30 min. Protein estimates for each individual mosquito were read off a standard curve constructed from the absorbance values of serially diluted solutions containing bovine serum albumin.

Metabolic studies on DDT labelled with ^{14}C were undertaken in pools of 10 mosquitoes. The homogenate remaining from earlier GSH assays was diluted 1:10 with 0·02 M phosphate buffer, adjusted to pH 7·4 and equilibrated at 28°C for 5 min. Two nmol of [^{14}C]DDT were then injected into the pooled supernatant. Solutions containing all the assay ingredients without mosquito homogenate served as controls. The reaction mixtures were left at 28°C for 6 h before samples were extracted with 3×2 mL of chloroform. Solvent fractions were air-dried and resuspended in 0·4 mL of chloroform before high performance liquid chromatography analysis on an Ultrasphere ODS® reverse phase column with a mobile phase of 90:10 methanol:water at a flow rate of 1 mL/min. All fractions were 'spiked' with a representative sample of non-radioactive substances which were possible metabolites. Collected fractions were counted and metabolites identified from the spiked standards trace detected at 290 nm. Metabolic values of the test solutions were corrected for non-enzymatic breakdown by subtracting the value of the control solution from the test solutions during each experimental run.

Table 1. Species identification of *Anopheles gambiae s.l.* in the Gambia with DNA probes

Village[a]	No. of mosquitoes examined	Number positive *An. gambiae s.s.* and *An. melas*[b]	*An. arabiensis*
Jessadi	41	39 (97·6%)	1 (2·4%)
Barokunda	30	25 (83·3%)	5 (16·7%)
Katamina	39	36 (94·7%)	2 (5·3%)
Male Kunda	32	32 (100%)	0 –
Pakali Ba	40	34 (85·0%)	6 (15·0%)
Madina	42	42 (100%)	6 –
Dasilami	12	12 (100%)	0 –
Niawodurulung	41	40 (100%)	0 –
Dongoro Ba	39	39 (100%)	0 –
Wellingara Ba	40	40 (100%)	0 –
Jalangbereh	47	46 (97·9%)	1 (2·1%)
Sitahuma	149	119 (81·0%)	28 (19·0%)
PHC villages	218	206 (95·4%)	10 (4·6%)
Non-PHC villages	334	298 (90·0%)	33 (10·0%)
All villages	552	504 (92·1%)	43 (7·9%)

[a]The first village in each pair was a primary health care (PHC) village, the second was a non-PHC settlement.

[b]The majority of *An. gambiae s.l.* positive mosquitoes not identified as *An. arabiensis* were assumed to be *An. gambiae s.s.*, with a few *An. melas*.

Data analysis

Counts of mosquitoes were log transformed [ln(n+1)] to normalize the data. Using SAS® software, the mean log number of mosquitoes in each catch was determined for each village during the 1988 rainy season. Confidence intervals (C.I.) for the difference in mosquito numbers between PHC and non-PHC villages were calculated from the difference between means for each pair of villages using the following formula: 95% C.I.=mean difference$\pm t_{5,0\cdot05}$×standard error of mean. These differences were back-transformed to give a confidence interval for the ratio of vectors in PHC and non-PHC villages. Wilcoxon matched signed rank tests were used to test for differences between pairs of villages. The effect of distance from each village to the river on mosquito numbers was assessed using correlation coefficients.

Results

Mosquitoes

Approximately 98% (n=26 635) of the mosquitoes collected using 'knock-down' catches were members of the *An. gambiae* complex, the remainder being culicines and a few other anophelines. Confirmation of *An. gambiae* complex identification was obtained using DNA probes (Table 1). Approximately 8% of the catch in October were *An. arabiensis*, with no significant difference between the proportions of this species found between PHC and non-PHC settlements (T=4; n=4). The remainder of the *An. gambiae s.l.* were probably *An. gambiae s.s.* with a few *An. melas*, although the latter were not differentiated.

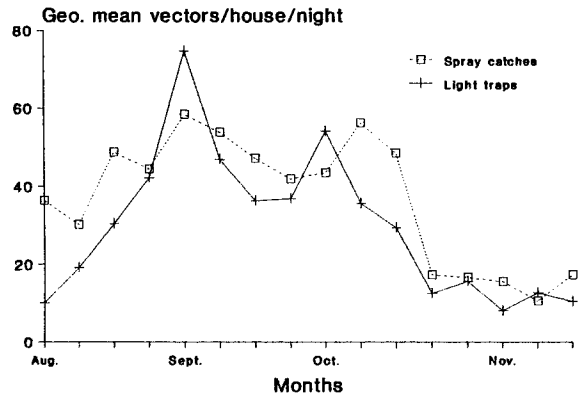

Fig. 2. Seasonal abundance of members of the *An. gambiae* complex sampled using 'knock-down' (spray) and light trap catches.

Variation in vector abundance

High densities of *An. gambiae s.l.* were found in the study villages at the start of the investigation in the middle of August. These remained elevated until the end of the rainy season in mid-October, before declining to a lower level at the end of the study in November (Fig. 2).

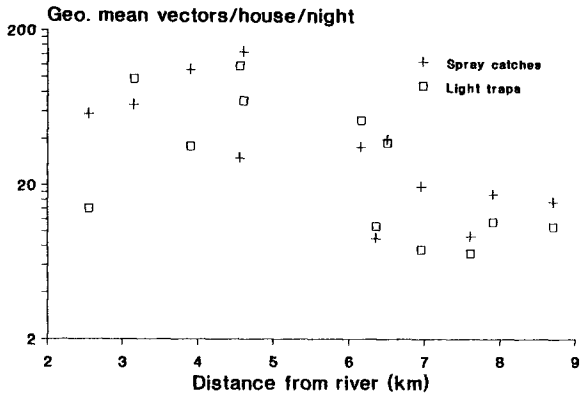

Fig. 3. Relationship between the mean number of *An. gambiae s.l.* collected from a village and the distance of that settlement from the River Gambia.

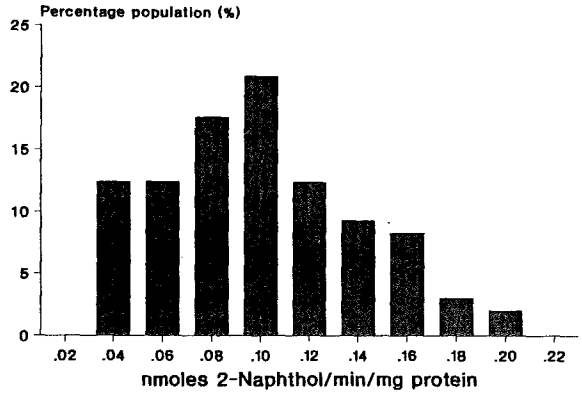

Fig. 4. Esterase activity with 2-napthyl acetate in *An. gambiae s.l.* from each study village.

Table 2. Sporozoite rates of mosquitoes in primary health care (PHC) and in non-PHC villages

Village[a]	No. of mosquitoes examined	No. with sporozoites All	No. with sporozoites High only	Sporozoite rate (%) All	Sporozoite rate (%) High only
Jessadi	3361	180	90	5·36	2·68
Barokunda	5058	250	150	4·94	2·97
Katamina	5920	110	20	1·86	0·34
Male Kunda	6903	390	180	5·65	2·61
Pakali Ba	3274	410	190	12·52	5·80
Madina	2950	220	90	7·46	3·05
Dasalami	1595	20	20	1·25	1·25
Niawodrulung	1322	0	0	0·00	0·00
Dongoro Ba	1058	190	50	17·96	4·73
Wellingara Ba	1375	120	30	8·73	2·18
Jalangbereh	1095	100	10	9·13	0·91
Sitahuma	5397	210	120	3·89	2·22
PHC villages	16620	940	360	5·66	2·17
Non-PHC villages	22688	1260	590	5·55	2·60
All villages	39308	2200	950	5·60	2·42

[a]The first village in each pair was a PHC village, the second a non-PHC settlement.

The geometric mean number of vectors found in each bedroom in the morning as assessed by 'knock-down' catches was 32·5 (95% C.I. 22·9–46·0).

The mean vector densities estimated using 'knock-down' catches was 53% greater in non-PHC settlements than in PHC villages but this difference was not statistically significant (95% CI = +12% to −80%, $T=3$; $n=6$). Similarly, 37% more specimens were caught in light traps in non-PHC villages than in PHC villages but this difference also was not statistically significant (95% CI = +66% to −74%, $T=6$; $n=6$).

There was an inverse relationship between the numbers of mosquitoes in a village and the distance of the settlement from the river (light trap catches, $r=-0·59$; $P=0·04$; spray collections, $r=-0·74$; $P=0·006$) (Fig. 3). Numbers of mosquitoes obtained by 'knock-down' catches or by spray catches in each village were positively correlated ($r=0·68$; 10 degrees of freedom $P<0·02$).

Sporozoite rates

Results from the sporozoite ELISAs for the 12 villages are shown in Table 2. Some weak positive reactions were also obtained which were confirmed on a second test. These had values which suggested that there were fewer than 100 sporozoites in the well. There was no significant difference in the sporozoite rates between PHC and non-PHC settlements (all confirmed positive mosquitoes, $T=9$; $n=6$; mosquitoes with high sporozoite densities only, $T=8$; $n=6$).

Insecticide resistance

GSH activity was low in all mosquitoes tested, except for a few specimens collected at Sitahuma which had an increased level of GSH activity. This suggests that there was DDT resistance in mosquitoes from this village. Pools of the remaining mosquito homogenates from Sitahuma and from the 11 remaining settlements were compared (Table 3). The homogenate of mosquitoes from Sitahuma metabolized significantly more [^{14}C]DDT than did the pooled homogenate made from specimens collected from other villages in the study area.

Acetylcholinesterase (AChE) activity was reduced by around 95% with propoxur, indicating that there was no altered AChE in these insects. The pattern of esterase activity observed with the substrate 2-napthyl acetate (Fig. 4) indicated that the mosquitoes tested ($n=95$) did not contain esterase-based organophosphate or pyrethroid resistance mechanisms.

Table 3. [^{14}C] DDT metabolism in freshwater members of the *An. gambiae* complex

Compound	Percentage radiolabel recovered Low GSH activity[a]	High GSH activity[a]
DDT	90·7+1·5	76·6+1·2
Metabolites		
Dicofol	2·7+0·9	1·8+0·4
DDD	0·4+0·1	0·9+0·3
DDA	1·0+0·6	1·3+0·5
DDE	5·2+1·0	19·4+1·2
Recovery of ^{14}C	89·3+1·0	91·7+10·5

[a]GSH = glutathione S-transferase.

Discussion

The principal vectors in the south bank study area belonged to the *An. gambiae s.l.* complex. Few *An. arabiensis* were identified, suggesting that the major member of the *An. gambiae* complex in the area was *An. gambiae s.s.*, as found during collections made 8 years earlier in Choya, situated in the centre of the present study area (BRYAN *et al.*, 1987). These vectors occurred in large numbers for 3 to 4 months of the wet season, the typical period of malaria transmission. This pattern of seasonal abundance is in marked contrast to that seen in a collection of hamlets to the north of the river, the site of an earlier trial of insecticide-treated bed nets, where vectors were found in appreciable numbers for only 3–8 weeks during the rainy season (LINDSAY *et al.*, 1989a,

1989b). This difference was probably due mainly to the paucity of mosquito breeding sites north of the river because of the small number and size, and the proximity, of rice fields in that area. In contrast, to the south of the river there is more marshland and rice is cultivated more extensively.

On average, we found fewer mosquitoes in PHC villages than in non-PHC villages but there were large variations between villages and differences between means were not statistically significant. Similar variations in mosquito abundance between apparently similar settlements have been observed in studies on the north bank of the river (LINDSAY *et al.*, 1989a, 1989b), although the cause of this variation could not be identified. In the present study, we found an inverse association between the number of mosquitoes in a village and its distance from the river. Presumably this relationship occurred because the main mosquito breeding sites in the south bank area were the marshes adjacent to the river.

In one village, Sitahuma, there was evidence of increased GSH activity which has been shown elsewhere to be associated with resistance to DDT. There was no evidence of organophosphate or carbamate resistance due to altered AChE or elevated esterase. However, the possibility of insecticide resistance associated with a change in oxidase activity cannot be excluded since the number of mosquitoes sampled from each settlement was insufficient to perform direct cytochrome oxidase P450 analysis. On the basis of the current results there was no indication of any pyrethroid resistance, although a kdr nerve insensitivity mechanism or other unknown mechanism of pyrethroid resistance cannot be excluded.

An. gambiae s.l. mosquitoes are highly anthropophilic and endophilic, acting as efficient vectors of malaria, and occur in the south bank study area in appreciable numbers for several months of the rainy season. Thus, this site represents an area of moderate and prolonged transmission and provides a more severe test for the control of malaria using pyrethroid-impregnated nets than did the previous study area on the north bank of the river, where transmission was of shorter duration. Moreover, the presence of a population of vectors apparently susceptible to pyrethroid insecticides made the south bank area a suitable site for assessing the long-term impact of mass community protection against malaria using pyrethroid-treated nets.

Acknowledgements

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Percepciones sobre las causas de la malaria, su tratamiento y prevención en la zona de estudio. A malaria control trial using insecticide-treated bednets and targeted chemoprophylaxis in a rural area of The Gambia, West Africa. 4. Perceptions of the causes of malaria and of its treatment and prevention in the study area. Aikins MK, Pickering H, Alonso PL, D'Alessandro U, Lindsay SW, Todd J, Greenwood BM. *Trans R Soc Trop Med Hyg* 1993;**87**, Suppl.2:25-30

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A malaria control trial using insecticide-treated bed nets and targeted chemoprophylaxis in a rural area of The Gambia, West Africa

4. Perceptions of the causes of malaria and of its treatment and prevention in the study area

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Abstract

Perceptions of the causes of malaria, its treatment and prevention were studied among 996 adults, selected randomly from 73 villages and hamlets in a rural area of The Gambia. Structured questionnaires and other interview techniques were used for data collection. Malaria has no specific name in the study area; it is referred to commonly as *Fula kajewo* (Fula fever). Only 28% of the respondents knew that mosquitoes transmitted malaria. However, most people believed correctly that August to October was the main malaria season. Eighty-six per cent of the subjects were bed net users. The majority of nets were produced locally, usually white in colour and made of sheeting fabrics. Usage of nets was correlated with ethnic group, age and polygamy but not with education, income, occupation or ownership of certain items which indicate high social status. Analysis of expenditure on mosquito coils indicated that non-users of nets spent 43% more on coils than did users. Bed nets have been used for a long time in the study area; 98% of users saw their parents using them during their childhood.

Introduction

In the absence of a vaccine, the only methods of malaria control suitable for implementation in a primary health care (PHC) programme are treatment of clinical attacks as soon as possible, chemoprophylaxis and vector control. Recent studies in The Gambia and other parts of the world have shown that insecticide-impregnated bed nets are a partially effective method of preventing man-vector contact. SNOW *et al.* (1988) showed that the incidence of clinical attacks of malaria was significantly less in Gambian children aged 1–9 years who slept in villages where all bed nets were treated with permethrin than in children who slept in control villages with placebo-treated nets. More recently, ALONSO *et al.* (1991) have shown that overall mortality and mortality attributed to malaria in children aged 1–4 years were reduced by 63% and 70% respectively in villages where impregnated nets were used, suggesting that treated nets could be an effective malaria control method suitable for integration into a PHC system.

The social aspects of bed net usage in The Gambia were first examined by MACCORMACK & SNOW (1986), who found that nearly all Mandinka families in rural areas used bed nets whilst fewer Wollof or Fula families did so. Subsequently, MACCORMACK *et al.* (1989) reported that people had nets not only for protection against mosquitoes but also for privacy in rooms with more than one bed, to ward off other insects, to prevent dirt dropping from the roof on to sleepers and as a protection against cold in the early morning.

The success of insecticide-impregnated net trials has prompted the Gambian government, in conjunction with donor agencies, to extend the programme to the rest of the country and the World Health Organization (WHO) is encouraging further bed net trials in other malarious regions of Africa. However, before large scale trials are started, it is important to learn more about local attitudes to malaria, its treatment and prevention and, in particular, feelings about nets. Such knowledge will help in the planning and implementation of large scale trials and will ensure that the implementation is socially acceptable.

The present study has addressed these questions. It is hoped that the knowledge obtained will be helpful in the formulation of educational and public health programmes and policies on malaria vector control in The Gambia and other parts of Africa.

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Methods

The study area, on the south bank of the River Gambia, is approximately 200 km inland from the coast. The terrain is flat savanna with mangrove and rice swamps near the river (ALONSO *et al.*, 1993). Malaria is transmitted by mosquitoes of the *Anopheles gambiae* complex (LINDSAY *et al.*, 1992). The climate is characteristic of the sub-Sahel with a long dry season, from November to June, and a shorter rainy season. The study area included 73 villages and hamlets which in December 1988 had a total population of 21 157, 51% of whom were Mandinka, 36% Fula, 4% Wollof and 9% from other ethnic groups.

From the 73 study villages and hamlets, 1100 adults aged 20 years and above were sampled randomly from a population census using an SPSS® computer program. By the end of the data collection period 996 (91%) of these subjects had been interviewed, of whom 46% (462) were men. Sixty-one per cent (607) lived in larger PHC villages, with a population of 400 or more, and the remainder lived in smaller non-PHC villages and hamlets. Eighty-five per cent (846) were married, 9% (91) single, 4% (40) widowed and 2% (19) divorced or separated.

A structured questionnaire was used for data collection. This covered socio-economic variables, knowledge about malaria, bed net usage and non-usage, and other methods of malaria control.

Tailors and retailers of bed nets in the study area were interviewed as a supplementary source of information. Additional, qualitative socio-political and economic information was collected through regular field visits to villages and hamlets, group discussions, direct observation of certain behaviour patterns and traditional practices and in-depth interviews with relevant key informants such as village leaders, elders, *marabouts* (local religious healers), women, and youth groups.

Results

Knowledge and attitudes to malaria

Local names for malaria in The Gambia. There is no specific local term for malaria in The Gambia. The most commonly used names for the disease in rural areas are *kadjeh* or *kajewo, kirikiroo, bala maakuey* (Mandinkas), *sibirru* (Wollofs), and *jentinojeh, joforo,* and *jofe* (Fulas). These names generally refer to the symptoms of malaria such as fever, headache, periodic shivering and tiredness. The name *Fula kajewo* is used by nearly all ethnic groups with the exception of the Fulas. This means Fula fever

('Fula hot body'). The belief underlying this name for malaria is that the infection is contracted from cattle; the periodic manifestation of malaria is likened to the movement of cattle to and from the grazing fields. Since Fulas are generally herdsmen, it is believed that they spread the disease to other ethnic groups through pastoral movements, trading and settlement. Only in Mandinka is there a name for malaria which is related to mosquitoes—*susula kurango* (mosquito disease), but this is seldom used in the study area and probably was introduced quite recently.

Knowledge about the cause of malaria. Questions on the cause of malaria showed that, overall, only 28% (274) of the respondents knew that malaria is transmitted by mosquitoes. Other causes given were eating too much *kutcha* (*Hibiscus suranttensis* or *H. asper*) (JAVIS, 1980) in the rainy season, *Allah* (God is believed to be the general cause of all happiness and misery in life), rains (water, time of year and breathing moist vapour), drinking too much fresh cows' milk in the rainy season, or eating mangoes. Men were more knowledgeable about the correct cause of malaria than women ($\chi^2=33{\cdot}1$; degrees of freedom [d.f.]=1; $P=<0{\cdot}001$). Ideas about the cause of malaria did not differ significantly between ethnic groups. All respondents disliked the nuisance of mosquitoes but 72% (722) did not think they caused any ill health. A list of responses according to educational level is shown in Fig. 1. Knowledge of mosquitoes as the cause of malaria increased with education (χ^2 for linear trends=6·6; $P=0{\cdot}01$).

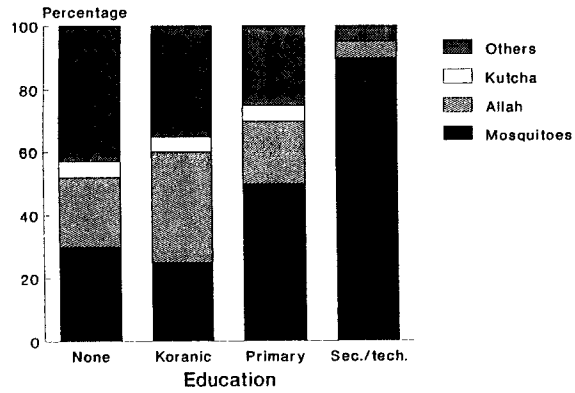

Fig. 1. Knowledge of the cause of malaria according to educational type.

There was little difference in response to the question on the seasonality of malaria transmission. People believed (correctly) that August (33%), September (38%) and October (19%) are the months when most malaria occurs. However, with the exception of January, all other months were mentioned.

Local treatment of malaria

Herbs. Forty-five per cent (449) of the study population used some form of local herbs for the treatment of malaria. Most of these grow wild on the outskirts of the villages and are bitter to taste. Table 1 shows the 5 main herbs used and their general preparation. Other herbs used are baobab fruits (*Adansonia digitata*), *kinkiliba* leaves (*Combretum mircanthum*), mango leaves (*Cordyla pinnata*), *jallo (khaya senegalensis*), and locust beans (*Parkia biglobosa*) (HALLAM 1979; JAVIS 1978, 1980).

Traditional practices. Some other traditional practices are believed to cure malaria. Although these practices were known to most elderly people, few young people knew them well. Traditional treatment of malaria is undertaken mainly by a few village elders.

(i) Treatment of malaria spirit possession. One of the old beliefs in the rural areas is that malaria, particularly in children, is due to possession of the patient by an evil spirit or devil. This belief comes from the periodic shivering experienced by malaria patients. Thus, a cure for malaria is to beat the patient with the wet end of a woman's under-cloth (*fanoo*) that has been dipped in water with the intention of exorcising the malaria evil spirit or devil. The patient is then bathed and wrapped in the same under-cloth (medicine cloth). Respondents emphasized that this treatment has been practised for a long time. However, nowadays, children are usually washed with cold water instead and then given further treatment.

(ii) Cow-pen water treatment. The seemingly 'clean' top layer of stagnant water that collects in cow-pens during the rainy season is used for bathing malaria patients once a day. This 'clean' water has a pungent nitrogenous smell due to the constant deposit of cow's urine and dung, but it is believed to be medicinal. Only 0·3% of villagers (3) used this treatment.

(iii) *Dapewo* treatment. Malaria patients are sometimes bathed with cold water in the early hours of the morning on the path (*dapewo*) used by cows to go for grazing. Some patients are also placed in the midst of cattle at sunset in order to transfer the malaria from the patient to the cattle. Another 0·4% of villagers (4) used this treatment.

Mosquito deterrents

A variety of measures is used to deter mosquitoes.

Mosquito coils and churai. Various brands of mosquito coils (Yotox®, Cock® and Moon Tiger®) are used widely, mainly to smoke out mosquitoes from rooms before people retire for the night. These coils are imported from Asia (particularly China) and are sold very cheaply in The Gambia (5–10 Gambian dalasis per packet of 10 coils) (US $1=9 Gambian dalasis [D]).

Perfumed resins and wood kernels, obtained mainly from *Daniellia oliveli* (SNOW *et al.*, 1987) and known locally as *churai*, are burnt extensively in both rural and urban homes (LINDSAY *et al.*, 1990). *Churai* is used for its fragrance in rooms, but it is used also to kill or drive away mosquitoes and other insects, which it does effectively (LINDSAY & JANNEH, 1989). Fig. 2 shows the estimated expenditure on coils and *churai*. Non-users of bed nets spent 43% more on mosquito coils per month than did users (D12·30 and D8·59 respectively) (Kruskal-Wallis $H=15{\cdot}3$; d.f.=1; $P=0{\cdot}001$).

Table 1. Herbs used for malaria treatment by 996 adult rural Gambians

Herbs	Preparation and usage	No. reporting use
Jumba katango (*Combretum geitonophyllum*)	Leaves boiled in water. Solution taken orally.	120 (12%)
Jamba kasala bah (*Cassia occidentalis*)	Leaves boiled in water. Solution taken orally and for bathing.	75 (8%)
Sanyo jambo (*Pennistum mphoids*)	Leaves boiled or soaked in water. Solution used for bathing.	45 (5%)
Yirindingo kunango (*Azadirachta indica*)	Leaves and bark boiled in water. Solution taken orally and for bathing.	34 (3%)
Sinjango (*Cassia sieberiana*)	Leaves soaked in water or bark boiled in water. Solution taken orally.	23 (2%)

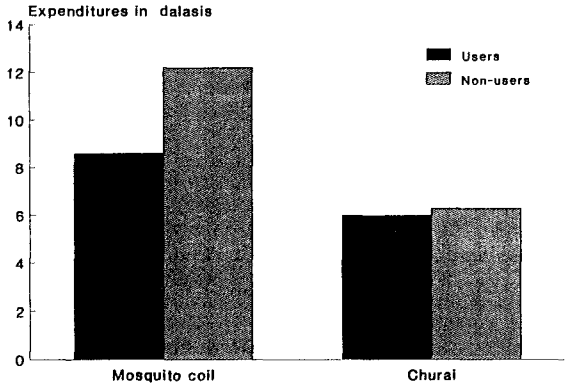

Fig. 2. Estimated monthly expenditure in Gambian dalasis on mosquito coils and *churai* (perfumed resin or wood kernel) in relation to bed net usage.

Some other herbs are also used as repellents; for instance, branches and leaves from *siisilingo jamboolu* (*Lippia cheraliera*), lime (*Citrus aurantifolium*) and *yirindingo-kunango* (*Azadirachta indica*) are spread around in rooms and sitting places. Their scent is believed to repel mosquitoes.

Bed nets. Eighty-six per cent (859) of the adult subjects sampled were bed net users. The sleeping habits of children were difficult to establish because sometimes parents or guardians did not report the true sleeping places of their children. Questionnaire responses showed that 81% of children under 10 years old slept under bed nets. However, direct observations showed that young and adolescent boys often slept without bed nets on verandas, *bantabas* (shady resting places) or other places rather than in rooms. Table 2 shows bed net usage by ethnic group in children under 10 years old; Fula children were less likely to sleep under nets than children of other ethnic groups. Girls used bed nets significantly more frequently than boys ($\chi^2=7{\cdot}9$; d.f.$=1$; $P=0{\cdot}005$).

Table 2. Bednet usage by 1319 Gambian children under 10 years old and 966 Gambian adults, according to ethnic group

Ethnic group	Boys	Girls	Adults
Fula	170/239 (71%)	181/237 (76%)	267/363 (74%)
Mandinka	311/357 (87%)	297/317 (94%)	468/500(94%)
Others	74/87 (85%)	75/82 (91%)	124/133 (93%)
Total	555/683 (81%)	553/636 (87%)	859/996 (86%)

For the past 2 years the PHC villages in the study area have had their nets treated with insecticide (ALONSO *et al.*, 1991). Ninety-three per cent of the 556 respondents (517) in these villages said that they would like their nets to be treated in future. The main reasons given for treating their nets were protection against mosquitoes (72%; 402) and other insects, bed bugs (*Cimex hemipterus*) and household flies (21%, 117). Furthermore, 93% of the villagers (517) said that they were prepared to purchase the insecticide permethrin for treating their nets through village, compound, or individual contributions, if the Government would supply it to the village.

Historical background to the use of bednets in The Gambia

The origin of bed nets (mosquito nets) in West Africa is not clear. Oral tradition does not associate nets with the ancient Ghana and Mali empires from which the Mandinkas of The Gambia are believed to be descended. Mary Kingsley, an explorer, mentioned using bed nets during her expeditions to West Africa between 1893 and 1895 (LINDSAY & GIBSON, 1988), and MACCORMACK (1984) reported that the Fulani and Hausa in the sub-region slept under fine mesh grass mats in pre-colonial

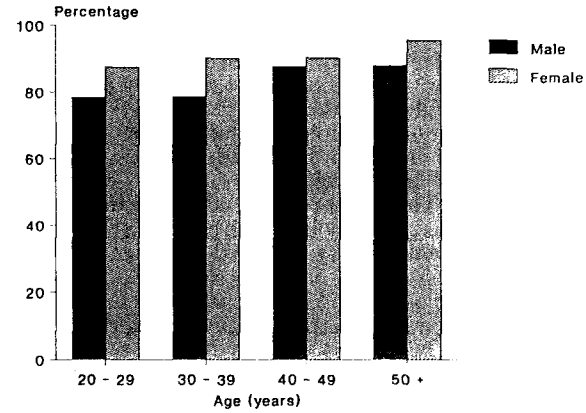

Fig. 3. Percentage of the population using bed nets according to age and sex.

times. Ninety-eight per cent (839) of the bed net users interviewed in the present survey saw their parents using them during their childhood. Further questioning of 65 bed net users aged 70 years and over who saw their parents using nets indicated that the first bed nets, called *dawali* or *wusungo* (conical woven mesh), were used predominantly by Mandinkas. *Wusungo* were hand-woven from fine Mandinka *fataroo fanoo* (locally produced thread) or from *daari fanoo* (imported thread). This practice dates back as far as 1894 when The Gambia became a British protectorate. Thus, the concept of sleeping under a barrier for protection and other reasons became a cultural phenomenon long ago. The primary reason for using nets at that time was the need for uninterrupted sleep, since continuous days of sleeplessness were perceived as one of the causes of fever ('hot body'). With the development of trade links overseas, *sambaakuka* or *perekaanoo* (imported white satin materials) became fashionable for making nets. Currently, bed nets are called *sanke* or *sankewo* (a Mandinka word) throughout The Gambia and are now used widely. The change from *wusungo* to *sankewo* may have occurred some 50 years ago.

Socio-economic determinants affecting the use of bed nets in adults

Age and sex. The use of bed nets by age and sex in the adult study population is shown in Fig. 3. Older people were more likely to use bed nets than younger subjects. In all age groups women used nets more frequently than men (Mantel-Haenszel summary $\chi^2=13{\cdot}98$; d.f.$=3$; $P=<0{\cdot}001$).

Ethnic group. Bed net usage in the adult study population according to ethnic group is shown in Table 2; Fulas

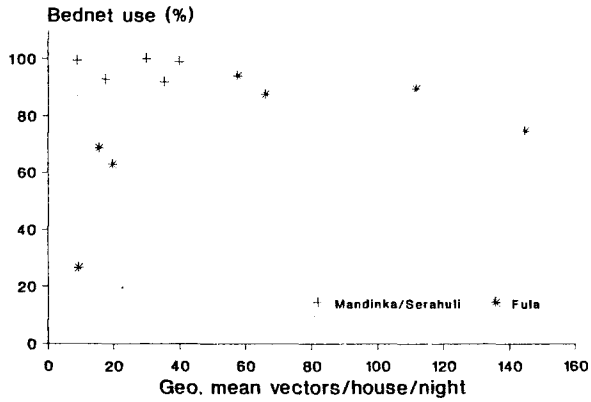

Fig. 4. Bed net usage by village in relation to mosquito density (geometric mean number/house/night) and ethnic group (S. W. Lindsay, unpublished data).

had the lowest percentage of use ($\chi^2=75{\cdot}9$; d.f.=2; $P=<0{\cdot}001$). Fulas are mainly herdsmen and tend to live in smaller villages than members of other ethnic groups. They place their concept of wealth in cattle, which are seldom sold for cash but are used for other social purposes such as bride-price and for maintaining social status, and Fulas pay relatively little attention to their general household requirements and clothing. However, Fulas did use nets when they lived in villages with very high mosquito densities (Fig. 4.)

Education. The level of formal education in the study population was generally low, with only 3% of adults (31) having had some primary school education and a further 2% (22) with secondary or technical education. The majority of people, 76% (755), had had some form of Koranic education, as expected in a predominantly Muslim society like The Gambia. Seventy-five per cent (399) of women and 89% (409) of men had had some education. Villagers with no education used bed nets more frequently than those with Koranic or some form of formal education (χ^2 for linear trend=6·0; d.f.=2; $P=0{\cdot}015$).

Average income. The majority (90%) of the study population were engaged in subsistence farming, with groundnuts, rice and cereals, mainly millet, being grown during the wet season. Ground-nuts were grown predominantly by men, rice by women. Most farmers also kept cattle, sheep, goats and chickens. Large herds of cattle were looked after by professional herdsmen, mostly Fulas. Most of the farmers attended at least one local market (*lumo*) day per week to sell their produce.

In the dry season, the pattern and schedule of work changed with little agricultural activity taking place. Dry season occupations included trading (21%, 210), vegetable gardening (18%, 184), manufacturing (carpentry, soap-making, welding, weaving, pottery and sewing) (4%; 44), building and repairs (3%, 25), religious consultant (*marabout*) (2%; 21) and other jobs such as animal husbandry, fishing, entertaining, labour for wages, teaching, and jewellery making (9%; 93). Of the 42% (419) who had no dry season occupation, 35% (147) depended on the sale of their agricultural produce for a living. The remainder depended on remittances from children or relatives (34%; 144) and the goodwill of other compound members (27%; 115).

Table 3. Average income in Gambian dalasis during the wet season by ethnic group and bed net usage

	Net users		Non-users of nets	
Ethnic group	No.	Income	No.	Income
Fula	241	848·7	91	851·4
Mandinka	428	995·2	27	1472·8
Wollof	30	1060·8	1	1000·0
Others	81	686·9	8	815·3
Total	780	920·4	127	982·4

Table 3 gives average income by ethnic group and bed net usage. Mean income of users in the wet season (D920) was less than that of non-users (D982), but the difference was not statistically significant (Kruskal-Wallis $H=1{\cdot}79$; d.f.=1; $P=0{\cdot}18$). However, income in the dry season showed a significant difference between users (D617) and non-users (D904) (Kruskal-Wallis $H=3{\cdot}98$; d.f.=1; $P=0{\cdot}05$). Overall annual income of bed net users did not differ significantly from that of non-users. Further analysis showed no significant relationship between bet net usage and education, income or occupation.

Ownership of social status items. Indicators of social status and wealth in rural Gambia are livestock (especially cattle), carved or metal beds, bicycles and radio or cassette players. All these items are 'modern' wealth symbols except cattle which are a traditional symbol of wealth. Although people acknowledge ownership, they do not easily disclose the quantity of cattle to 'strangers', since it is believed that this always leads to bad luck and the death of the last cow counted. Analysis showed no difference between ownership of 'modern' items between bed net users and non-users with the exception of radios or cassette players which were owned significantly more frequently by bed net owners than by non-bed net owners (Mantel-Haenszel $\chi^2=13{\cdot}7$; d.f.=2; $P=<0{\cdot}001$).

Marriage. The majority of adults in rural Gambia are married. Women marry comparatively early compared with men. Men of all ethnic groups do not normally share rooms, but women and co-wives often share rooms (called the women's house), especially among Mandinkas. Among couples, 90% (431) of women and 84% (307) of men were bed net users. Moreover, 86% (214) of men with co-wives had a bed net as compared to 80% (93) of men with only one wife. The average numbers of co-wives for female users and non-users of bed nets were 1·3 and 1·0 respectively (Kruskal-Wallis $H=1{\cdot}67$; d.f.=1; $P=0{\cdot}2$). For men, the average numbers of co-wives for users and non-users were 2·0 and 1·9 respectively (Kruskal-Wallis $H=2{\cdot}24$; d.f.=1; $P=0{\cdot}13$). Further analysis showed a statistically significant relationship between the use of nets for privacy by men and the number of people sharing a room ($\chi^2=9{\cdot}4$; d.f.=1; $P=0{\cdot}002$). Thus, polygamous families were more likely than monogamous households to use bed nets for privacy as well as protection against mosquitoes.

Reasons for using or not using bed nets

Reasons for using bed nets. In the wet season, 99% of users of bed nets (852) used them for protection against mosquito bites. Sixty-two per cent (530) of users claimed that bed nets prevented them from having malaria, but only 28% (274) of the total sample acknowledged that mosquitoes caused malaria. When questioned further about alternative or secondary uses of bed nets, the most common responses were that they were used to give privacy, to prevent roof dirt dropping on the bed, and to protect against insects (Fig. 5).

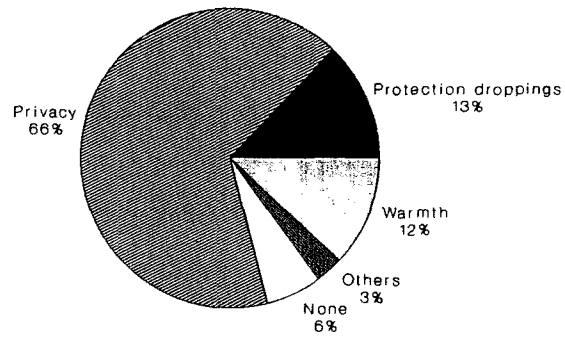

Fig. 5. Uses reported for bed nets apart from protection against mosquitoes.

In the dry season, when most mosquito breeding grounds dry up leading to a large reduction in numbers, 40% (347) of respondents continued to use nets against mosquito bites, 15% (130) used them for warmth in the cool hours before dawn and also for privacy in rooms with more than one bed, 17% (148) out of habit and 15% (152) to prevent dirt, insects, lizard or rat droppings from the thatched roof falling on to their beds. Ten per cent (82) did not use their bed nets in the dry season and 3% (20) used them for other reasons such as 'beautifying' the room and as shelving for keeping clothes and other items out of the reach of children. These findings are similar to those reported by MACCORMACK & SNOW (1986).

Reasons for not using bed nets. The majority of non-users (80; 58%) claimed that they could not afford to buy nets (Table 4). This assertion was unlikely to have been

Table 4. Reasons for not using bed nets given by 137 adult, rural Gambians

Reason	Number
Lack of money	80 (58%)
Spoilt bed nets	35 (26%)
Do not like/need bed nets	13 (9%)
Given bed net to spouse, child or visitor	8 (6%)
Never used bed nets	1 (1%)
Total	137 (100%)

true since most farmers in the harvest season (November to December) have money to spend on jewellery, clothes and adornments. Household and health-related requirements were accorded low priority compared to ceremonial goods and services by this group of people.

Of the 13 subjects who claimed that they did not like or need bed nets, 8 were pastoral Fulas who believed that they would be returning soon to their motherland (northern Nigeria or Futa Jallon, Guinea) and did not intend to travel with cumbersome items like bed nets, beds and other houshold items.

Type of nets in use in the study area

Most bed nets used in rural Gambia are locally sewn by tailors with either second-hand or new fabrics. However, a few imported nets from Asia and Europe, which are relatively expensive, are used. In the main, 4 fabrics are used for bed nets—cotton sheeting, cotton netting, opaque synthetic sheeting, and synthetic netting. The choice of bed net fabric depends on alternative uses of the net and the individual's past bed net experience. Forty-four per cent (377) of the bed nets surveyed were synthetic sheeting, 33% (279) were synthetic netting, 22% (190) cotton sheeting, and only 1% (12) were cotton netting fabric.

A high proportion of subjects (66%) preferred sheeting fabrics because they lasted longer, were strong enough to withstand stress from children playing on the bed, and provided warmth and privacy. Moreover, it is believed that mosquitoes and other smaller insects can pass through netting. On average, bed nets last for 6 years and there was no significant difference between netting and sheeting fabrics in this respect. Most bed nets were white; the rest were blue, pink, brown or cream. A few tailors had multi-coloured nets made from remnants.

Discussion

In a malaria-endemic area like The Gambia one would expect the community to have evolved or adopted some indigenous methods to protect themselves from the disease. Though most people, as this study has shown, did not associate mosquitoes with the transmission of malaria, bed nets have been used for at least 100 years, mainly in the wet season, to prevent mosquitoes disturbing sleep. Perhaps it was also appreciated that bed nets prevented fever.

This study has shown that socio-economic factors that might influence the acquisition of bed nets are not easy to identify. Indicators of economic status, such as ownership of social status items or income, are difficult to define. For instance, when ownership of some identified social status items was used as an index, it showed a positive relationship with bed net usage at first sight. However, further analysis showed that this apparent association was confounded by ethnic group and only the possession of radios or cassette players was positively related to the use of nets. This finding may be related to the promotion of bed nets as a malaria control method by the government by means of radio messages. Again, contrary to expectation, income had an inverse relationship with bed net usage although this association was not statistically significant. Non-users earned higher incomes and spent more (D 12.3 per month) on mosquito coils than users (D8.6). The 4 main socio-economic variables related to bed net usage were ethnic group, marital status, age, and ownership of radios/cassette players.

Despite the fact that bed net usage varied among the ethnic groups, this generalization must be made with caution, because in the study area there were some Fula villages and hamlets with very high rates of usage, especially those with an abundance of mosquitoes (S. W. Lindsay, unpublished observations), and some Mandinka villages with a high proportion of non-users. What is certain is that Fula herdsmen were less likely to use bed nets than settled villagers, due to their pastoral occupation. The life styles and living conditions of Fulas and other ethnic groups (predominantly farmers) differed substantially, affecting each group's priorities. Another plausible reason for Fulas not liking nets might stem from the belief of the other ethnic groups that the Fula spread malaria (*Fula kajewo*). Fulas feel belittled and this may contribute to their refusal to use nets.

People in rural Gambia do not have a common name for malaria, which is normally identified by its symptoms. However, the general season for transmission is well known. It has been observed that local herbs are used sometimes for treatment of malaria when there is an inability to pay for treatment at the nearest PHC post or from a community health nurse. Local herbs and religious consultation (*marabouts*) are the first treatments for most illnesses in some homes. Some of these herbs are well documented as local remedies for malaria in the manual for the training of village health workers and traditional birth attendants produced by the health department of the government of The Gambia.

The majority (93%) of people in the PHC villages found insecticide-treated nets very useful and would like to treat their nets again in future, even if charged a fee. Several non-governmental agencies are involved in the malaria control programme (particularly promotion of insecticide-treated nets) in The Gambia. Although money is hard to obtain at certain times of the year, most people stated that they would be prepared to raise funds for net treatment through their village or compound head or through individual contributions used for funding village projects. The 'Revolving Fund' system operated by the Department of Health, into which medical fees are paid and then used for the purchase of drugs, could be explored as a possible way of funding a national bed net programme.

In a study of this nature, where the community already has an idea about bed nets and their uses, the presence of the research team may, by itself, promote positive changes in behaviour of non-users. Such changes are being observed now. With the intention of the Gambian government to embark on a national impregnated bed net programme to control malaria in the near future, it is recommended that rural communities should be sensitized to the importance of bed nets against mosquitoes and malaria, and advised to purchase or sew bed nets around the harvest season in November and December when they are cheaper and when cash is available.

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A malaria control trial using insecticide-treated bed nets and targeted chemoprophylaxis in a rural area of The Gambia, West Africa

5. Design and implementation of the trial

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Abstract

A large-scale malaria intervention programme using insecticide-treated bed nets and chemoprophylaxis administered to children was introduced into a rural area of The Gambia. The operation was carried out using the existing primary health care (PHC) service in the region. Training of the village health workers, sensitization of the communities, and implementation of net impregnation and the drug delivery programme are described. This delivery system resulted in over 90% of nets being treated with insecticide and 80% of children receiving over 90% of their tablets during the rainy season. There was considerable variation in the distribution of permethrin on a bed net and between individual nets, which is likely to facilitate the spread of insecticide resistance in the local mosquito populations. Bed nets made from heavier fabrics tended to absorb more insecticide than those made from lighter materials. Four months after dipping, 89% of the insecticide had been lost from treated nets. This was probably due mainly to women washing their nets, an activity carried out on average once every 2 months during the rainy season. The high number of insecticide-treated bed nets in the study area demonstrated that a malaria control programme operated through a PHC system can be implemented successfully.

Introduction

The malaria eradication campaigns of the 1950s were complex, centrally managed, vertical programmes that were difficult to sustain. Costs were high as large numbers of employees were involved. In many developing countries, where there is a limited health care infrastructure, vaccination programmes and malaria control campaigns remain as vertical programmes. Elsewhere in the developing world, the implementation and development of primary health care (PHC) programmes has provided an opportunity to alter the management of major health care activities and control programmes from a vertical structure to a horizontal one. In The Gambia, it has been possible to utilize the PHC programme to provide a number of specific health interventions including those directed against malaria. In this paper, we describe the design and conduct of a malaria control trial employing insecticide-treated bed nets and chemoprophylaxis in which the interventions were delivered through the national PHC programme.

Study design

The aims of the study were to determine the effects of 2 interventions, insecticide-treated bed nets and chemoprophylaxis, administered through the Gambian government's existing PHC programme, on mortality and morbidity from malaria in children and also their effects on the vectors of malaria.

Random allocation of individuals to different study groups is the optimum design for most intervention trials but this approach is not desirable for trials of insecticide impregnated bed nets as the use of an impregnated net by one individual may have an effect on the risk of malaria in those sleeping nearby. Thus, for a randomized trial of impregnated nets it is necessary to randomize by village rather than by individual, necessitating a substantially larger sample size than that required for a study employing individual randomization. Because chemoprophylaxis given to only a small proportion of a population is unlikely to have an effect on transmission, individual randomization may be considered appropriate for a study of this intervention.

One way to have studied the effects of the 2 interventions would have been through a factorial design, involving the random allocation of PHC villages to receive or not to receive insecticide and random allocation of children within each village to receive chemoprophylaxis or placebo. A trial conducted along these lines would have resulted in some children from PHC villages sleeping under untreated nets and receiving placebo. As both treated nets and chemoprophylaxis have been shown previously to reduce morbidity from malaria in Gambian children it was considered ethically unacceptable to include this control group. For this reason an alternative design was chosen which relied upon the recruitment of smaller villages not involved in the PHC programme (non-PHC villages) to allow a comparison to be made of the effects of treated nets and untreated nets.

This part of the study was not randomized and, because PHC and non-PHC villages differed in ethnic group distribution and in other ways (Table 1), non-PHC villages did not provide an ideal control for PHC villages. However, preliminary studies indicated that child mortality and morbidity from malaria were similar in the 2 groups so that non-PHC villages provided acceptable controls (ALONSO *et al.*, 1993). Within PHC villages, randomization to chemoprophylaxis or placebo groups was undertaken on an individual basis in a 'double blind' manner with neither the study families nor the project field staff being aware of the active preparation. Thus, after intervention, the trial design gave 3 groups of study children as indicated in Table 1.

Mortality was assessed through demographic surveillance in all 3 groups for one year before and one year after the interventions were implemented. In order to assess whether mortality levels and trends had been similar in groups IIa and IIb (PHC villages) to that in group I (non-PHC villages) in the years preceding the study, indirect demographic techniques were used to estimate recent child mortality levels.

Morbidity was assessed through cross-sectional surveys undertaken at the end of the malaria transmission season in all 3 groups and by active case detection achieved through weekly morbidity surveys of all

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Table 1. Study groups before and after the interventions

Group	Village Population	PHC[a]	Treatment of malaria Dispenser	VHW[b]	Chemoprophylaxis by VHW[b]	Insecticide-treated nets by PHC[a]
Pre-intervention						
I	<400	No	Yes	No	No	No
II	>400	Yes	Yes	Yes	No	No
Post-intervention						
I	<400	No	Yes	No	No	No
IIa	>400	Yes	Yes	Yes	Yes	Yes
IIb	>400	Yes	Yes	Yes	No	Yes

[a]Primary health care programme.
[b]Village health worker.

children in the age group 1–6 years resident in PHC villages and in a subset of children resident in non-PHC villages.

Entomological monitoring of the effect of the interventions was done during the malaria transmission seasons before and after the interventions were implemented in 12 villages, 6 PHC villages and 6 non-PHC villages.

Materials and Methods

The Gambia's primary health care programme

In 1982, the government of The Gambia, through the Ministry of Health, initiated a national PHC programme. The scheme is organized at 3 levels: central, regional and village.

Villages with a population of 400 people or more were invited to join the scheme. Each village then selected a village health worker (VHW) and a traditional birth attendant (TBA) from among its residents. Both individuals attended an 8 weeks training course at the Regional Health Team's headquarters and in key villages. VHW training concentrated on basic preventive and curative medicine, including the treatment of all febrile cases with chloroquine during the rainy season. The Ministry of Health issued an initial 3 months stock of basic drugs to each VHW; replacements were purchased from the Government's regional medical stores using funds obtained from the sale of drugs to patients. The TBA training course covered basic obstetric procedures, including the practice of good hygiene during delivery, proper care of the umbilical cord, and the administration of drugs to prevent post-partum haemorrhage. Whilst the position of the VHW is a new one in the community, that of the TBA is, as the name suggests, an old one and commands great respect in Gambian society. Each village community is meant to support the VHW and TBA by cash payments helping them with their farming, or by other means decided by the community.

Supervision of the VHW and the TBA is provided by a community health nurse (CHN). These nurses are usually recruited from rural areas, and receive 18 months health-care training at the CHN school in The Gambia. Then they are based at key village subdispensaries, where their main function is to support and supervise 4–8 VHWs. The CHN forms the main link between the community, their VHWs and the formal sector. In turn, the CHN is supervised and supported by a regional public health nurse, who is part of the Regional Health Team. This team also includes a regional medical officer and other support staff.

The director of health services is responsible for the implementation and management of all health programmes. Further management and supervision of the programme are carried out by a Health Planning Unit and a Health Planning Committee. However, at the community level, management and supervision rely on active community participation which is central to a village-based PHC programme. The community is no longer seen as passive recipients of health care, but rather as active participants in health development. Village Development Committees were formed in all PHC villages to co-ordinate, mobilize and manage the resources for PHC.

Training and sensitization

A malaria control officer (MCO) was seconded from the Medical and Health Department to co-ordinate training and implementation of the different control strategies. He was based at the regional health team's headquarters in Mansa Konko, and provided with a motor cycle. Following discussions with the assistant director of preventive services, the PHC unit's training staff, key village CHNs and the MCO, it was agreed that the best strategy for implementing the malaria control programme was to focus on village-based health care workers and on the women of each village.

In The Gambia, women of a similar age form groups or *kafos* to carry out activities such as the organization of community gardens, ceremonies and trade. These groups are usually led by a highly respected woman. It was considered important to involve women in the control programme for a number of reasons. Firstly, the interventions are aimed mainly at children, who are always looked after by a woman, usually the mother but sometimes another female relative. Secondly, dipping bed nets in insecticide is hard manual work and many of the VHWs are old men who would find this activity too strenuous. Lastly, dipping, which in many ways resembles washing, may be considered by many to be women's work. Because the role of women seemed central to the success of the dipping programme a Gambian woman from the staff of the Medical Research Council (MRC) worked with the MCO to explain the scheme to women in the villages.

The MCO visited all PHC villages and explained the aims of the programme to VHWs and TBAs and sought their advice on how best to implement the scheme. He and the female MRC staff member then held meetings with village leaders, including the head of the women's group in each village. This was followed by village meetings which the entire community was invited to attend. These talks were held to explain the control programme, to encourage the participation of the community in the programme, and to demonstrate how to dip bed nets in insecticide. Senior members of the community often had their nets impregnated during preliminary meetings in order to obtain their support and thus to motivate the rest of the community.

The MCO trained first the CHNs and then VHWs and TBAs on the procedures of impregnating nets with insecticide at the headquarters of the Regional Health Team. VHWs were also trained how to distribute weekly chemoprophylaxis tablets to children, and how to maintain a compliance ledger (GREENWOOD *et al.*, 1987). Most VHWs could read and write in Arabic, and those who were illiterate were helped by a literate village schoolboy.

Distribution of tablets

VHWs distributed Maloprim® (pyrimethamine+dap-

sone) and placebo tablets at the village health post every Wednesday morning for a 20-week period, which covered the main malaria transmission season. Wednesday was chosen as women usually stayed home on that day rather than work in the fields. Mothers or carers were asked to bring their children to the health post during the morning, when the VHW would be available to distribute the drugs. Children who were older than 6 months on 1 July 1989, but who would not reach 6 years by 31 December 1989, were individually allocated at random to receive either 2 white (Maloprim®) or 2 pink tablets (placebo). Each quarter-strength Maloprim® tablet contained 6·25 mg of pyrimethamine and 25 mg of dapsone. Children were issued with a colour-coded identification card which also served as a compliance ledger. VHWs issued tablets only to children who presented at the health post with their identification card. The VHW ensured that the child swallowed the tablets in his presence and then marked the child's card to show that the child had received them. VHWs also maintained a separate note of each child's compliance in a book kept at the health post.

At the end of the transmission season, the total number of tablets each child had taken was recorded. Percentage compliance for each child was calculated by dividing the number of tablets the child had taken by the number the child should have consumed, taking into account migration or death. Thus, for a child who migrated after 15 weeks of the study, having taken 20 tablets, compliance was considered to be 20/30=67%. Compliance was also checked through a random sample of urines collected from study children. The presence of dapsone was checked using an enzyme-linked immunosorbent assay (GREENWOOD *et al.*, 1986).

Impregnation of bed nets

In each PHC village, the VHW asked women to wash their nets the day before impregnation. Dipping began on a Wednesday morning (5 July, 1989) and was organized by the VHW, usually supported by the TBA and the head of the women's group. The VHW was also responsible for measuring the dose of insecticide. The 2 senior women in each village helped motivate and direct the impregnation of nets by other women. The dipping team was supervised by a CHN under the overall direction of the MCO.

On entry into each compound (a collection of related households usually delineated by a fence) the TBA and head of the women's group arranged for all women to collect their bed nets and fetch water from a well. The target dose of permethrin was 500 mg/m^2. The protocol for dipping each net was as follows. The VHW measured 40 mL of permethrin (25% emulsifiable concentrate; ICI) into an empty evaporated milk can calibrated previously for this purpose. This quantity was poured into a large plastic washing bowl, one litre of water was added from a plastic drinking cup of that capacity, and the mixture was stirred. When large numbers of nets were to be dipped, sufficient solution was prepared to dip up to 5 nets at one time. All items in the dipping procedure, except the insecticide, were purchased locally. Women from each compound dipped their family's nets, wrung out any excess fluid, and left the nets to dry on a mattress. The women then washed their hands with soap and water provided by the VHW. The head woman checked that all nets in the compound were treated and marked each with an indelible pen. All marked nets were counted subsequently to assess how many nets had been treated in each village.

Residents of PHC villages were advised not to wash their nets during the rainy season, whilst no such recommendation was made to the people in non-PHC villages.

Collection of bed net samples

The quantity of permethrin on bed nets was determined from samples of 58 bed nets in the PHC villages within one month of impregnation and 4 months later. In each village, the number of bed nets sampled was proportional to the total number of nets in the settlement. Within each village nets were selected at random and approximately 20 cm^2 sections of material were cut from the flap, top and side of each net. The owners of damaged nets were given new insecticide-treated bed nets. Each fabric sample was wrapped in aluminium foil and stored at 4°C until the chemical analysis was performed.

Chemical analyses

The quantity of permethrin on and in fibres of netting samples was determined using gas liquid chromatography (GLC). Insecticide was extracted by agitating each fabric sample in 25 mL hexane for one h. The amount of permethrin in each solution was then determined using an HP 5890A® one-column capillary chromatograph (Hewlett Packard) and flame ionization detection. One microlitre samples together with 1 μL of pentadecane as an internal standard were injected into a 12 M DB1® column (0·32 mm internal diameter, 0·12 μm film thickness; Jones Chromatography). Helium was used as the carrier gas at a flow rate of 6 mL/min. The oven temperature was increased from 100°C in 3 steps; first to 225°C at a rate of 30°C/min, second to 255°C at a rate of 10°C/min, and finally to 300°C at a rate of 30°C/min. The total running time was 9 min. The amount of permethrin was determined by calibration with standard solutions of 50, 100, 250 and 500 p.p.m. containing the same concentration of internal standard as the control.

Fabric identification

Bed net samples taken immediately after dipping were examined under a microscope by a fibres expert to classify the fabric according to its chemical composition, yarn type and fabric construction.

Frequency of washing bed nets

The numbers of treated and untreated bed nets were counted in all PHC villages. All insecticide-treated bed nets in PHC villages were marked twice: once with a permanent ink mark for identification and once with a water-soluble mark for recording how frequently nets were washed. Bed nets were inspected in 3 surveys carried out at the beginning of September, during the first week of October, and in the middle of November. Washed nets, recognized by the disappearance of the water-soluble mark, were counted and re-marked with a water-soluble pen.

A similar, but more intensive, system was used to monitor washing of bed nets in 6 PHC villages and in 6 neighbouring non-PHC villages more closely. Twenty randomly selected bed nets were inspected weekly, and washed nets identified by the disappearance of the water-soluble mark.

VHW interviews

A questionnaire survey was administered to 16 VHWs in the study villages in November, 4 months after impregnation. The men were questioned about the dipping procedure and the problems they experienced during its implementation.

Results

Chemoprophylaxis

Compliance with drug administration was generally high. The distribution of compliance was skewed; 1511 children (79·6%) took their tablets on more than 90% of the occasions on which they should have done so (90% compliance), while 44 children (2·3%) did so on less than 10% of the required occasions. Compliance was not significantly related to age (χ^2=3·1; degrees of freedom [d.f]=2; P>0·2) or to sex (χ^2=2·8; d.f.=1; P=0·1). However there were significant differences in the levels of compliance between different ethnic groups. Wollofs

and Serahulays had the highest rate, 118/198 (62%) of their children having a compliance level of 95% or more whilst Fula children had the lowest level of compliance, 199/310 (36%).

Compliance with drug administration was similar among children whose nets were treated and among those whose nets were not (mean compliances 43·0% and 45·3% respectively; $\chi^2=0{\cdot}17$; d.f.=1; $P=0{\cdot}68$). However, children without a bed net had a significantly lower level of compliance than children who slept under an insecticide-treated bed net (20·3% vs. 45·3; $\chi^2=16{\cdot}8$; d.f.=1; $P<0{\cdot}001$).

Net impregnation

Eighty-eight percent (5380/6093) of all bed nets in PHC villages were treated during the 3 weeks when insecticide was available; 80% of the nets were treated within the first 2 d of the programme. Ninety-six per cent (1745/1814) of all children in PHC villages slept regularly under a bed net, 95% (1659/1745) of which were treated with insecticide. Thus, 92% (1659/1814) of children in these villages slept regularly under a treated net. Although there were differences in coverage between villages, most communities had a coverage greater than 90%, and 6 had 100% coverage.

Frequency of washing of bed nets

Impregnated bed nets of 1584 study children were seen during both the follow-up surveys and the water-soluble ink marks examined; 52·9% (838/1584) of nets had been washed at least once a month.

Results from a detailed monitoring of bed nets in 6 pairs of villages are shown in Table 2. Bed nets were washed less frequently in PHC villages than in those outside the scheme. On average, bed nets were washed at monthly intervals in non-PHC villages and about every 2 months in PHC villages. However, there was variation in washing behaviour between villages.

Table 2. Frequency of bed net washing in 12 villages in the study area

Village[a]	No. of nets studied	No. of washes/month Median	Range
JSD	20	0·8	0–2·3
BKD	20	2·0	1–3·8
KTM	21	0	0–0·7
MKD	21	0·8	0–1·7
PKB	20	0	0–1·0
MNA	21	1·5	0–4·0
DSM	22	0	0–1·2
NWL	20	0·6	0–1·5
DGB	25	0·3	0–1·5
WGB	24	0·2	0–2·0
JBH	20	0·7	0–2·1
SHM	22	0·9	0–4·0
PHC villages	128	0	0–2·3
Non-PHC villages	128	0·9	0–4·0

[a]The first village in each pair was a primary health care (PHC) settlement, the second was a control non-PHC village; village names are given in full by LINDSAY *et al.* (1993).

Fabric identification

Analysis of fabric samples obtained shortly after impregnation showed that 59% (101) were made of polyester, 15% (26) of nylon, and 10% (17) of cotton. The remainder were made of viscose (6), acetate (3), acrylic (2), and mixtures of all these fibres (17). One sample was missing.

Polyester nets were further classified according to the type of yarn and the method of construction. In order of increasing density of material these were 53% (53) flat warp-knitted netting, 20% (20) woven sheeting nets made from continuous yarns and staple spun yarn, 19% (19) thick woven sheets made only of flat-continuous filaments, and 8·9% (9) weft-knitted heavily bulked fibres.

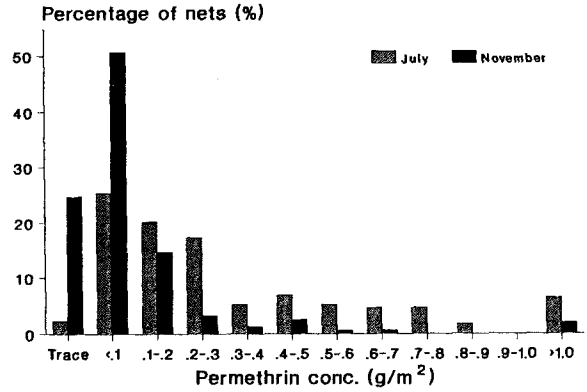

Fig. 1. Frequency distribution of the permethrin content of bed nets treated by villagers measured shortly after impregnation and four months later.

Amount of permethrin on bed nets

During the treatment of 5380 bed nets a total of 383 L of 25% permethrin was used, averaging approximately 1183 mg/m^2. The geometric mean amount of permethrin on bed net samples taken shortly after dipping was 179 mg/m^2 ($n=173$, 95% confidence interval [C.I.]=147, 219 mg/m^2), as assessed by GLC analysis. This amount declined significantly (89% reduction) on net samples taken 4 months after impregnation (mean concentration=20 mg/m^2, $n=164$, 95% C.I.=14,27 mg/m^2; $t=12{\cdot}2$; d.f.=288, $P<0{\cdot}001$) (Fig. 1).

There were significant variations in the permethrin content between samples taken from different parts of the same net shortly after dipping (mean range within an individual net=285 mg/m^2; $t=39{\cdot}4$; d.f.=57; $P<0{\cdot}001$). The variation in permethrin concentration was greater between individual nets than within nets ($F=2{\cdot}2$; d.f.=57, 115; $P<0{\cdot}01$). However, the variation between nets could not be attributed to differences between villages, nor between the different types of fabrics used ($F=1{\cdot}7$, d.f.=13, 44; $P<0{\cdot}10$ and $F=2{\cdot}7$; d.f.=3, 44; $P<0{\cdot}10$.).

Cotton absorbed more permethrin than nylon or polyester (Table 3), but the differences in permethrin absorption by the different kinds of material were not statistically significant ($F=2{\cdot}7$; d.f.=3,155). Different kinds of polyester absorbed similar amounts of permethrin.

Questionnaire survey

Four months after dipping the bed nets, 14 of 16

Table 3. Permethrin concentration on different types of bed net

Material	No.	Permethrin content (mg/m^2)[a]
Cotton	17	413·6 (194–884)
Nylon	26	116·6 (63–215)
Other	28	179·6 (99–325)
Polyester[b]		
All knits	101	169·2 (124–231)
Heavy	9	219·4 (95–508)
Medium	20	172·9 (98–304)
Light	19	176·8 (99–315)
Netting	53	158·1 (112–223)

[a]Geometric mean (95% confidence interval in parentheses).
[b]See text for detailed description of polyester nets.

VHWs remembered the correct dosage for treating bed nets (i.e., one cup of insecticide and one litre of water). However, only 5 kept this dosage constant; most made up more solution when this was needed to saturate a net completely. In 15 of 16 villages VHWs had measured out the insecticide; in one village this was done by a TBA. In 10 villages TBAs dipped the nets and in 8 they arranged them for drying on the beds. In every village the nets were dried on mattresses. Marking of the nets was done most frequently by the head of the women's group. In half the villages dipping was performed by the VHW, TBA and head woman alone. In 5 villages nets were impregnated by the dipping team together with the village women and in 2 villages nets were dipped only by the women. All VHWs ensured that people washed their hands after handling the insecticide. In one village the VHW dipped the bed nets in his village with the assistance of the village reporter only. Four VHWs said that some villagers had complained about the dipping. Some villagers complained that (i) mosquitoes still entered the house, (ii) the insecticide smelt badly for the first few days after dipping, and (iii) they believed the insecticide to be harmful to their health.

Discussion

The high coverage levels obtained in the impregnation of bed nets and in drug administration suggested that village-based PHC workers, supervised by an MCO, were capable of implementing simple malaria control interventions effectively. The active participation of women, and particularly the motivation and leadership of the head of the women's group and the TBA, were critical in attaining high coverage. Although VHWs made a contribution, they did not generally have a leading role in the implementation. Based on these results, the newly created role of MCO is considered to have been a success. The high coverage attained in the interventions was also related to the receptivity of the community to this novel approach to malaria control.

Previous methods for impregnating bed nets with insecticide were complicated and time consuming, requiring nets to be sized and the quantity of insecticide calculated individually for each net in order to produce a standard treatment dosage (SCHRECK & SELF, 1985). Since then several simpler methods have been used, often employing a large container so that more than one net could be dipped at the same time. In an earlier trial of insecticide-treated nets in The Gambia, a dustbin was used for dipping nets (SNOW *et al.*, 1988). However, bins are expensive and not widely available locally. Moreover, dippers bending over the large vats of insecticide solution are exposed to volatile solvents which could be a health hazard for some individuals. In The Gambia, only small amounts of insecticide were used at any one time and the wide-mouthed bowls used for dipping the nets probably contributed to the dispersal of noxious vapours.

During the impregnation of nets more insecticide was used than expected. The insecticide dilution, allowing for some loss of insecticide due to dripping, was calculated to give a target dose of 500 mg/m^2 for a lightweight net made of synthetic netting. However, most nets were made from heavier material which absorbed more insecticide solution.

Fig. 2. Net impregnation in a Gambian village.

Despite the higher volumes of insecticide used, the average dose of permethrin on bed nets was only 180 mg/m^2 when samples were collected shortly after dipping. Similar findings have also been reported when nets were dipped by scientists under more rigorous conditions (LINDSAY *et al.*, 1991a; MILLER *et al.*, 1991). The loss of permethrin from nets in the present study is likely to have been due to a number of causes including (i) absorption of insecticide by the mattress, (ii) poor absorption and adsorption of insecticide by netting, (iii) the patchy distribution of insecticide on netting, (iv) spillage and leakage during impregnation, and (v) diversion of insecticide to other uses. Drying nets on mattresses probably contributed most to the loss of insecticide. However, this method of drying nets was largely responsible for the killing of bedbugs and other nuisance arthropods (LINDSAY *et al.*, 1989), a feature of net impregnation which was appreciated greatly by the community.

The method of insecticide application employed had one serious disadvantage: treated bed nets were not uniformly covered with the same quantity of insecticide. There was a large variation in the amount of insecticide absorbed by different bed nets, with nets made of thicker material generally taking up more chemical than lighter weight fabrics. Moreover, insecticide distribution on nets was patchy due partly to the oily consistency of the permethrin solution. Low levels of permethrin on both washed and unwashed nets may make the emergence of insecticide resistance in vectors more likely by selecting for resistant heterozygotes.

Despite the facts that village women were asked not to wash their treated bed nets during the study, and 85% of women knew that washing the net would reduce the strength of the insecticide, more than 50% washed their nets. However, on average, women in PHC villages tended to wash their nets less often than women in the control villages with untreated nets. Washing probably contributed greatly to the loss of insecticide from the fabric noted during the rainy season, as previous work in The Gambia has indicated (SNOW *et al.*, 1987; LINDSAY *et al.*, 1991b; MILLER *et al.*, 1991; PLEASS *et al.*, in press). This behaviour is a cause for concern, particularly as more nets were washed at the end of the transmission season when most deaths from malaria occur. Future control programmes need to reinforce the deleterious effect of washing treated nets. More than 70% of the population felt that the treated nets were either good or very good at keeping mosquitoes away, a view supported by entomological findings during this study (LINDSAY *et al.*, 1993).

The few children who did not sleep under a net were more likely to be poor compliers in drug administration than net users. This finding suggests that ownership of a bed net may be related to a more positive attitude to health and that children who do not sleep under a net in PHC villages are generally at an increased risk from malaria.

The enthusiasm of the health care workers and of the MCO may decline over time, especially as there is a growing tendency to overburden VHWs with new interventions and programmes that adopt a more horizontal structure. This tendency is likely to be increased with the success of this malaria control programme. VHWs and TBAs are voluntary workers and, in practice, villages rarely compensate them adequately for the time and effort they devote to their health activities. Whether effective malaria control can be sustained through The Gambia's PHC programme remains to be established.

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A malaria control trial using insecticide-treated bed nets and targeted chemoprophylaxis in a rural area of The Gambia, West Africa

6. The impact of the interventions on mortality and morbidity from malaria

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Abstract

The effects of insecticide-impregnated bed nets on mortality and morbidity from malaria have been investigated during one malaria transmission season in a group of rural Gambian children aged 6 months to 5 years. Sleeping under impregnated nets was associated with an overall reduction in mortality of about 60% in children aged 1–4 years. Mortality was not reduced further by chemoprophylaxis with Maloprim® given weekly by village health workers throughout the rainy season. Episodes of fever associated with malaria parasitaemia were reduced by 45% among children who slept under impregnated nets. The addition of chemoprophylaxis provided substantial additional benefit against clinical attacks of malaria; 158 episodes were recorded among 946 children who slept under impregnated nets but who also received chemoprophylaxis. Chemoprophylaxis reduced the prevalence of splenomegaly and parasitaemia at the end of the malaria transmission season by 63% and 83% respectively. Thus, insecticide-impregnated bed nets provided significant protection in children against overall mortality, mortality attributed to malaria, clinical attacks of malaria, and malaria infection. The addition of chemoprophylaxis provided substantial additional protection against clinical attacks of malaria and malaria infection but not against death.

Introduction

Until a safe, effective and affordable vaccine becomes widely available malaria control in Africa will continue to rely on antimalarial drugs and on reduction of human–vector contact.

Provision of facilities for the prompt treatment of presumptive cases of malaria with an effective drug remains the cornerstone of malaria control strategies in sub-Saharan Africa. However, in studies undertaken in The Gambia and in Kenya, this strategy had no significant effect on mortality in children (SPENCER *et al.*, 1987; GREENWOOD *et al.*, 1988). An alternative to presumptive treatment is the regular distribution of antimalarial drugs as prophylactics to the groups at highest risk through appropriate primary health care (PHC) schemes. The efficacy of such a strategy depends upon its operational feasibility and sustainability. In The Gambia, targeted chemoprophylaxis, integrated into a PHC programme, was successful in producing large reductions in morbidity and mortality from malaria in young children (GREENWOOD *et al.*, 1988; MENON *et al.*, 1990).

Reduction of human–vector contact has traditionally relied on house spraying with residual insecticides. In many malarious areas this strategy has had limited success because of the exophilic behaviour of vector mosquitoes, resistance to insecticides, poor co-operation by the population and because of financial and other organizational constraints. Thus, new vector control technologies are needed which are locally appropriate. In many parts of Africa, the success of strategies to reduce human–vector contact will depend largely upon the local intensity of malaria transmission, the behaviour of the vector and on whether schemes can be implemented through established PHC programmes. Bed nets have been used for protection from mosquitoes for many years (LINDSAY & GIBSON, 1988) but there have been few evaluations of their effect on malaria. In a trial carried out in The Gambia, conventional bed nets failed to reduce significantly the incidence of clinical episodes of malaria (SNOW *et al.*, 1988a). However, there has recently been growing interest in the use of insecticide impregnation of mosquito nets as a means of increasing their potential to reduce human–vector contact. This old idea is now perceived as a potentially effective malaria control strategy which could be integrated into PHC programmes.

Results from previous trials that evaluated the efficacy of insecticide-treated bed nets in preventing malaria infection and disease are often difficult to interpret and complicated by variations in the epidemiology of malaria between study areas (WHO, 1989; ROZENDAAL, 1989). In The Gambia, impregnated bed nets reduced the incidence of clinical episodes of malaria when used to protect an individual or a whole village (SNOW *et al.*, 1987, 1988b). They were easy to distribute and were well accepted by the local population.

The Gambia has an effective village-based PHC programme which can be used to deliver malaria control strategies once these have been shown to be effective. The aim of our trial has been to evaluate the feasibility and impact of treating existing bed nets with insecticide through an established PHC structure. A further aim has been to determine whether chemoprophylaxis is of additional benefit in preventing morbidity and mortality among children who sleep under a treated net.

Subjects and methods

Study area and population

The study was carried out in a rural area of The Gambia on the south bank of the River Gambia, east of the town of Soma. The study population of approximately 20 000 comprised mostly subsistence farmers belonging to the Mandinka and Fula ethnic groups. The climate of the area is characteristic of the sub-Sahel with a rainy season from July to October during which most malaria transmission occurs. Further details of the study area and population are given elsewhere (ALONSO *et al.*, 1993a).

Study design and implementation

The design of the study and the way it was implemented have been described previously (ALONSO *et al.*, 1993b). In brief, bed nets in 17 PHC villages were impregnated with permethrin (target dose 0·5 g/m^2, dose achieved 0·2 g/m^2) at the beginning of the 1989 malaria

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transmission season; 92% coverage was achieved. In addition, children aged 6 months to 5 years were individually allocated at random to receive weekly chemoprophylaxis with Maloprim® (pyrimethamine 12·5 mg+ dapsone 50 mg) or placebo given by village health workers (VHWs) throughout the rainy season. Mortality and morbidity from malaria were compared in these 2 groups of children and in a third group of children of the same age who lived in neighbouring non-PHC villages and who slept without nets, or under untreated nets, and who did not receive chemoprophylaxis.

Mortality and morbidity surveillance

Mortality surveillance using village reporters and the verbal autopsy technique was undertaken as described previously (ALONSO *et al.*, 1993a). Morbidity surveillance comprised cross-sectional surveys undertaken at the beginning and at the end of the malaria transmission season and active case detection by weekly morbidity surveys.

A sample of children aged 6 months to 5 years was selected in both PHC and non-PHC villages using information obtained from a census. These children were asked to take part in 2 cross-sectional surveys undertaken in June and November 1989, i.e. before, and at the end of, the main period of malaria transmission. Children were examined by a physician (P.L.A.) who recorded the presence or absence of splenomegaly with the child in the standing position. Axillary temperature was measured with an electronic thermometer and a finger-prick blood sample was obtained for measurement of packed cell volume (PCV) and for the preparation of thick and thin blood films. Blood films were stained with Giemsa's stain and examined as described previously (ALONSO *et al.*, 1993a). Children who had malaria parasitaemia received treatment with chloroquine (25 mg/kg) within 24 h.

All children aged 6 months to 5 years in the PHC villages, and all children in the same age group from 6 of the non-PHC control villages, were visited weekly throughout the main malaria transmission season. Each child was visited on the same day each week, either in the early morning or in the late afternoon. The mother was interviewed and a short questionnaire completed on the health of the child and the use of health services during the previous week. The child's axillary temperature was then recorded with an electronic thermometer. A blood film was taken if the child's temperature was 37·5°C or higher.

In PHC villages, mothers of febrile children were advised to take their child to the VHW for treatment. Febrile children from the 6 non-PHC villages who were found to have peripheral parasitaemia were given prompt treatment with chloroquine by Medical Research Council (MRC) field assistants.

During September 1989, a stratified cross-sectional survey of chloroquine consumption was carried out. Urine samples were tested for chloroquine using an enzyme-linked immunosorbent assay (ELISA) (SHENTON *et al.*, 1988).

Statistical methods

For the June and November cross-sectional surveys, a stratified, simple random sample was drawn from the census for both PHC and non-PHC villages. The sampling fractions were approximately 1:3 in PHC villages and 1:4 in non-PHC villages. The 8 strata comprised the 4 districts into which the study area was divided and 2 age groups (dates of birth: 1 July 1986–31 December 1988 and 1 January 1984–30 June 1986 respectively). Since both samples were self-weighting, and because the within-stratum variances were the same as between-stratum variances, an unweighted analysis was carried out. The standard errors and confidence intervals presented were corrected for the finite population correction.

The results of weekly morbidity surveys were used to calculate the incidence rate of clinical episodes of malaria. Rates were calculated using 2 alternative definitions of a clinical episode: (i) fever (axillary temperature ≥37·5°C) with any parasitaemia and (ii) fever with parasitaemia of 5000 per μL or greater. Rates were calculated per 1000 child-weeks at risk. Children found to have fever and parasitaemia were removed subsequently from the denominator (number at risk) and the numerator (number of episodes). A relative rate (RR) was used to compare children in PHC villages who had been individually allocated at random to receive Maloprim® or placebo and from this figure the protective efficacy of chemoprophylaxis was calculated as 1−RR.

To compare the incidence of malaria in children in PHC villages receiving weekly placebo and in children from 6 paired neighbouring, non-PHC villages, rates were calculated for each village and the 2 groups compared using Wilcoxon's rank sum test. The confidence intervals for the protective efficacy are calculated from the mean and standard error of log rate ratios of each pair.

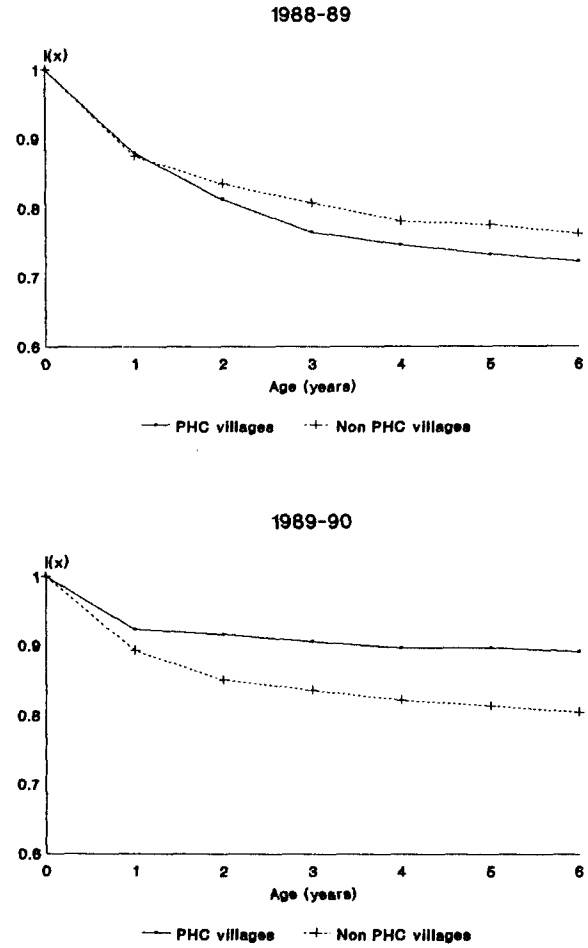

Fig. 1. The cumulative probability of survival to the age x, l (x), among children in primary health care (PHC) and in non-PHC villages before and after the introduction of malaria control measures in PHC villages in The Gambia, 1988–1989 and 1989–1990.

Results

Mortality

The impact of the 2 interventions on overall mortality and on cause-specific mortality has been presented previously (ALONSO *et al.*, 1991); it is summarized in life table format in Fig. 1, which compares the cumulative probabilities of surviving to age *x* in PHC and non-PHC villages during the year in which insecticide-treated bed nets and chemoprophylaxis were introduced into PHC villages, with the probabilities of survival during the previous year. During the pre-intervention year mortality

Table 1. Mortality rates for infants (deaths/1000 live births) and children aged 1–4 years (deaths/1000 per year), and probability of dying by age 5 (5q0), for one year before and one year after the introduction of interventions into primary health care (PHC) villages

Age (years)	Villages PHC	Non-PHC	Rate ratio PHC/non-PHC[a]	P^b
Pre-intervention				
<1	115·5 (65/563)[c]	127·1 (46/362)[c]	0·91 (0·6–1·3)	NS[e]
1–4	47·6 (81/1700)[d]	31·5 (37/1176)[d]	1·51 (1·0–2·2)	0·03
5q0	267·5	224·6	–	–
Post-intervention				
<1	73·5 (41/558)[c]	105·1 (37/352)[c]	0·7 (0·5–1·1)	NS[e]
1–4	9·0 (16/1787)[d]	24·2 (30/1240)[d]	0·37 (0·2–0·7)	0·001
5q0	104·4	186·7	–	–

[a]95% confidence interval in parentheses.
[b]χ^2 test.
[c]Deaths/live births.
[d]Deaths/approximate mid-year population.
[e]Not significant.

was higher in PHC than in non-PHC villages. Following the introduction of the malaria control measures into PHC villages, this situation was reversed. Mortality rates during the pre-intervention and post-intervention years are shown for infants and for children aged 1–4 years in Table 1, together with probabilities of dying by age 5 years. There were 19 deaths among children under 5 years old resident in PHC villages and allocated to receive either Maloprim® or placebo during the post-intervention year. Ten of these children had received Maloprim® (mortality rate 10·5 per 1000) and 9 had received placebo (mortality rate 9·5 per 1000) (χ^2=0·05; degrees of freedom [d.f.]=1; P=0·83) (RR=0·91, 95% confidence interval=0·37,2·22). The effects of season on mortality in intervention and in control villages are shown in Fig. 2. The reduction in mortality recorded in PHC villages during the post-intervention year was most marked during the rainy season.

The post-mortem questionnaire technique was used to try to establish causes of death among study children. The overall reduction in mortality observed after inter-

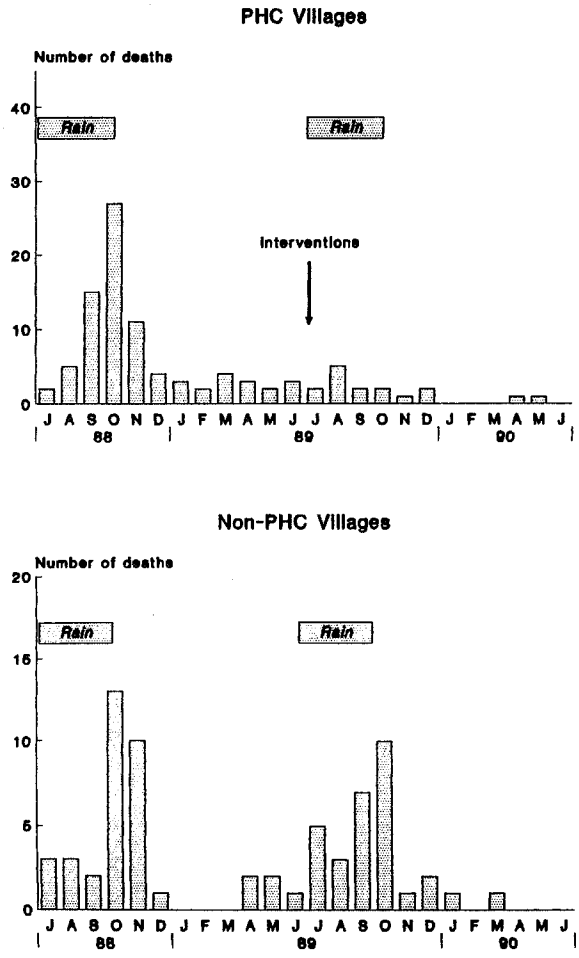

Fig. 2. The seasonal pattern of deaths in children aged 1–4 years in primary health care (PHC) villages and in non-PHC villages before and after the introduction of malaria control measures into PHC villages.

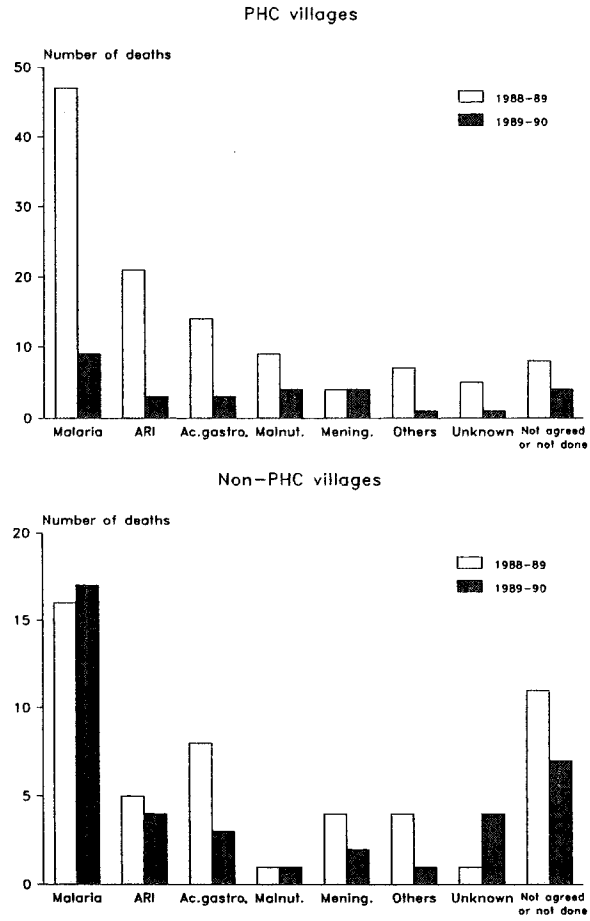

Fig. 3. Cause-specific mortality in children aged 6 months to 5 years in primary health care (PHC) villages and non-PHC villages, before (1988–1989) and after (1989–1990) the introduction of malaria control measures into PHC villages. ARI=acute respiratory infections, Ac. gastro.=acute gastroenteritis, Mening.=meningitis.

Table 2. Mortality rates attributable to malaria for infants (deaths/1000 live births) and children aged 1–4 years (deaths/1000 per year) for one year before and one year after the introduction of interventions into primary health care (PHC) villages

Age (years)	PHC	Non-PHC	Rate ratio PHC/non-PHC[a]	*P*
Pre-intervention				
<1	19·5 (11/563)[b]	2·8 (1/362)[b]	7·07 (0·9–54·6)	0·03[d]
1–4	20·6 (35/1700)[c]	11·1 (13/1176)[c]	1·86 (1·0– 3·5)	0·05[e]
Post-intervention				
<1	3·6 (2/558)[b]	2·8 (1/352)[b]	1·26 (0·1–13·9)	NS[d]
1–4	3·4 (6/1787)[c]	11·3 (14/1240)[c]	0·3 (0·1– 0·8)	0·01[e]

[a]95% confidence interval in parentheses.
[b]Deaths/live births.
[c]Deaths/approximate mid-year population.
[d]Fishers exact test; NS=not significant.
[e]χ^2 test.

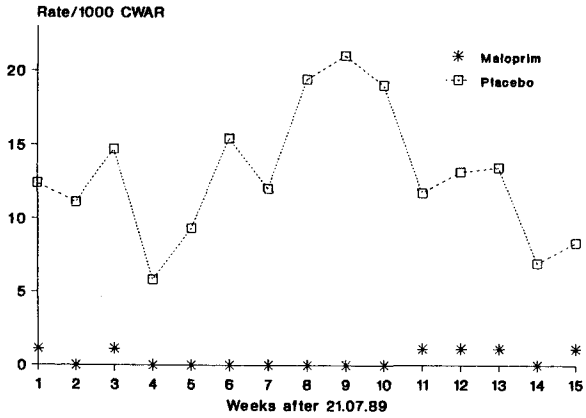

Fig. 4. The incidence of clinical attacks of malaria (fever+parasitaemia) in children aged 3–59 months who slept under impregnated nets and who received either Maloprim® or placebo; CWAR=child-weeks at risk.

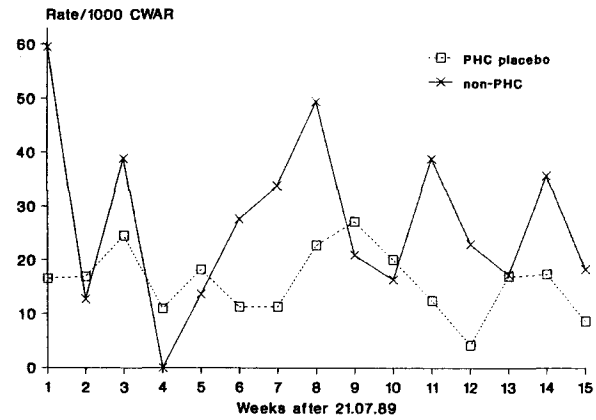

Fig. 5. The incidence of clinical attacks of malaria (fever+parasitaemia) in children who slept under impregnated nets but did not receive chemoprophylaxis and in children who slept under non-impregnated nets or who did not use nets; CWAR=child-weeks at risk.

Table 3. The results of weekly morbidity surveys of children aged 6 months to 5 years who slept under insecticide-treated bed nets and received chemoprophylaxis with Maloprim® or a placebo

	Maloprim®	Placebo
Total no. of children	952	946
Child-weeks at risk	13445	12227
Temperature <37·5°C	13115	11814
Temperature ≥37·5°C	330	413
Children with fever		
Blood film collected	317	405
No. of parasites	311	247
Parasitaemia <5000 μL	2	50
Parasitaemia ≥5000 μL	4	108
Rates per 1000 child-weeks at risk		
Fever	24·5	33·8
Fever+low parasitaemia	0·4	12·9
Fever+high parasitaemia	0·3	8·8
Protective efficacies[a]		
Fever	27% (16%,37%)	
Fever+low parasitaemia	97% (92%,99%)	
Fever+high parasitaemia	97% (91%,99%)	

[a]Protective efficacy=100 (1−relative rate); 95% confidence intervals in parentheses.

vention among children in PHC villages was accompanied by a marked reduction in deaths attributed to malaria (Table 2). However, in PHC villages there were reductions also in deaths attributed to other causes, particularly acute lower respiratory tract infections, which were not seen in control non-PHC villages (Fig. 3).

Morbidity surveillance

The incidence of clinical episodes of malaria among children in PHC villages who received either weekly Maloprim® or placebo is illustrated in Fig. 4. and summarized in Table 3. Among children who slept under insecticide-treated bed nets, the addition of chemoprophylaxis resulted in a 97% protective efficacy against clinical episodes associated with any parasitaemia or with high density parasitaemia.

The incidence of clinical episodes was also compared

Table 4. The results of weekly morbidity surveillance of children in 6 paired primary health care (PHC) villages and non-PHC villages. Only children in PHC villages who received placebo are included in this analysis

Village pairs	Fever[a]+parasitaemia[b] Rate ratio[c]	Protective efficacy	Fever[a]+high parasitaemia[b] Rate ratio[c]	Protective efficacy
1	0·784	22%	0·915	8%
2	0·579	42%	0·155	84%
3	0·193	81%	0·257	74%
4	0·489	51%	0·579	42%
5	0·734	27%	0·650	35%
6	0·872	13%	0·659	34%
Overall protective efficacy[d]	45% (14%,65%)		54% (21%,74%)	
Wilcoxon rank sum test	*P*<0·01		*P*<0·01	

[a]Temperature ≥37·5°C.
[b]*P. falciparum* parasitaemia only; high parasitaemia: ≥10 parasites/high power field (≥5000/μL approximately).
[c]Rate ratio=PHC/non-PHC values.
[d]Protective efficacy was calculated from the mean log rate ratio of village pairs.

Table 5. Findings in a cross sectional survey undertaken in primary health care (PHC) villages and non-PHC villages in June 1989 before the malaria transmission season[a]

	Villages		
	PHC		Non-PHC
	Maloprim	Placebo	
Splenomegaly	28% (24%,31%)[103/374]	28% (24%,32%)[100/359]	28% (24%,32%) [96/344]
Parasitaemia[b]	32% (28%,36%)[124/390]	28% (24%,32%)[107/381]	33% (29%,38%)[117/353]
High parasitaemia[b]	5% (3%,6%) [18/390]	5% (3%,7%) [18/381]	3% (1%,5%) [11/353]
Fever[c]	10% (8%,13%) [40/390]	9% (6%,11%) [33/383]	12% (9%,15%) [43/354]
Fever[c]+parasitaemia[b]	3% (2%,5%) [12/390]	2% (1%,4%) [9/383]	5% (3%,7%) [18/354]
Fever[c]+high parasitaemia[b]	1% (0%,2%) [2/390]	1% (0%,2%) [3/383]	0% (0%,1%) [0/354]
Packed cell volume	32·6% (32·2%,32·9%)	32·7% (32·3%,33·2%)	32·9% (32·5%,33·4%)

[a]Means with 95% confidence intervals in parentheses and actual numbers in square brackets.
[b]*P. falciparum* parasitaemia only; high parasitaemia: ≥10 parasites/high power field (≥5000/μL approximately).
[c]Temperature ≥37·5°C.

Table 6. Findings in a cross-sectional survey undertaken in primary health care (PHC) villages and non-PHC villages in November 1989 at the end of the malaria transmission season following the introduction of malaria control interventions into PHC villages[a]

	Villages		
	PHC		Non-PHC
	Maloprim	Placebo	
Splenomegaly	14% (11%,17%)[50/359]	37% (33%,42%)[129/346]	57% (52%,62%)[166/290]
Parasitaemia[b]	3% (2%,5%) [12/367]	26% (22%,30%) [94/357]	43% (38%,48%)[129/300]
High parasitaemia	11% (0%,1%) [2/367]	9% (7%,12%) [33/357]	18% (14%,22%) [53/300]
Fever[c]	0% (0%,1%) [1/367]	2% (1%,3%) [6/357]	9% (6%,12%) [28/302]
Fever[c]+parasitaemia[b]	0% (0%,1%) [0/367]	1% (0%,3%) [5/357]	7% (4%,9%) [20/302]
Fever[c]+high parasitaemia[b]	0% (0%,1%) [0/367]	1% (0%,3%) [4/357]	6% (4%,8%) [18/302]
Gametocytes[d]	2% (1%,4%) [8/367]	8% (5%,11%) [29/357]	15% (11%,19%) [45/300]
Packed cell volume	33·4% (33·0%, 33·8%)	32·2% (31·9%, 32·6%)	30·6% (30·1%, 31·1%)
Change (June–November)	+0·80% (+1·23%,+0·38%)	−0·52% (−0·04%,−1·00%)	−2·41% (−1·88%,−2·94%)

[a]Means with 95% confidence intervals in parentheses and actual numbers in square brackets.
[b]*P. falciparum* parasitaemia only; high parasitaemia: ≥10 parasites/high power field (≥5000/μL approximately).
[c]Temperature ≥37·5°C.
[d]*P. falciparum* gametocytes only.

between children from PHC villages who received placebo and children from neighbouring non-PHC villages The results are illustrated in Fig. 5 and summarized in Table 4. The protective efficacy of insecticide-treated bed nets compared with untreated nets was 45% for clinical episodes associated with any parasitaemia and 54% for clinical episodes associated with high density parasitaemia.

Pre- and post-intervention clinical surveys

A summary of the results of the 2 cross-sectional surveys are presented in Tables 5 and 6. In the pre-intervention (dry season) survey, there was no difference in the prevalence of any malariometric index between the 2 groups of children from PHC villages who had been allocated to receive either weekly Maloprim® or placebo during the forthcoming malaria transmission season, or between these children and those resident in non-PHC villages. In the post-intervention survey, there were significant differences in all the measured indices between the 2 groups of children who received weekly chemoprophylaxis, the prevalence of splenomegaly was reduced by 63%, and the prevalence of parasitaemia and high parasitaemia by 88% and 94%, respectively, compared with children who received placebo.

Following the interventions there were also significant differences in the prevalence of malariometric indices between children in non-PHC villages and children in PHC

Table 7. Malaria among users of untreated bed nets or treated bed nets and among non-users of bed nets in primary health care villages

	No nets[a]	Nets not dipped	Nets dipped[b]
Frequency[c]	3·2% (31/960)	4·4% (42/960)	86·4% (829/960)
Parasitaemia[d]	54·5% (6/11) *	29·4% (5/17)	24·4% (75/307)
Fever[e]+parasitaemia[f]	24·3 (9/371)**	17·3 (9/520)	12·3 (135/11017)
Fever[e]+high parasitaemia[f]	18·9 (7/371)***	7·7 (4/520)	8·4 (93/11017)

[a]Significance of difference between no nets and dipped nets is indicated thus: *Fisher's exact test, $P=0·03$; **Relative rate=2·0 (95% CI: 1·0,3·9) ($\chi^2=4·1$; d.f.=1; $P=0·04$); ***Relative rate=2·2 (95% CI: 1·0,4·8) ($\chi^2=4·5$; d.f.=1; $P=0·03$).
[b]Only children who received placebo are included.
[c]Status not known for 58 children (6·0%).
[d]Prevalence of *P. falciparum* asexual parasitaemia at the November 1989 survey.
[e]Temperature ≥37·5°C.
[f]Episodes/1000 child-weeks at risk; high parasitaemia ≥10 parasites/high power field (≥5000/μL approximately).

villages. The most relevant comparison was between children from non-PHC villages and children from PHC villages who received placebo. The prevalence of splenomegaly among children in PHC villages who slept under impregnated nets and who received weekly placebo was reduced by 35% compared to children in non-PHC villages, while the prevalence of parasitaemia and of high density parasitaemia were reduced by 39% and 48% respectively.

Bed net use and malaria

In PHC villages there were some children who did not use a net and others whose nets were not dipped. The results of the weekly morbidity surveillance and the cross-sectional surveys in these groups of children are presented in Table 7. The incidence of clinical malaria was similar among children who had a treated bed net and among those with an untreated bed net ($\chi^2=1{\cdot}0$; d.f.$=1$; $P=0{\cdot}31$). However, children who did not use a net had significantly more malaria than children using dipped nets ($\chi^2=4{\cdot}1$; d.f.$=1$; $P=0{\cdot}04$).

In the June cross-sectional survey, there was no difference in the prevalence of splenomegaly or parasitaemia between children resident in PHC villages who had a net and in those who did not. However, in the November survey, children without nets had a significantly higher prevalence of parasitaemia than children with dipped nets (Fisher's exact test, $P=0{\cdot}03$) (Table 7). There was no difference in the prevalence of parasitaemia or splenomegaly between children with treated and untreated nets living in PHC villages.

The relation between the condition of the net and morbidity from malaria was also investigated. At the time when bed nets were counted, the condition of the net was assessed and classified into one of 3 broad categories: intact, holed with fewer than 5 holes, and holed with 5 or more holes. In the June survey, there was no relationship between the condition of the net and the prevalence of parasitaemia. However, in November, the prevalence of splenomegaly and parasitaemia increased as the condition of the net worsened (χ^2 for trend$=3{\cdot}8$; $P=0{\cdot}05$; $\chi^2=5{\cdot}56$; $P=0{\cdot}02$ respectively). No significant trend was found between the incidence of clinical malaria and the condition of the net.

Washing of bed nets and malaria

The incidence of clinical malaria and the prevalence of malariometric indices were similar in children whose insecticide-treated bed nets were washed during the malaria transmission season and in children whose treated nets had not been washed (Table 8).

Compliance with chemoprophylaxis and malaria

Compliance with drug administration was monitored as described previously (ALONSO *et al.*, 1993b). Compliance with drug administration was not associated significantly with age or sex, nor was it significantly different in the children who died compared with those who were alive and resident in the study area at the end of the season ($\chi^2=0{\cdot}02$; d.f.$=1$; $P>0{\cdot}20$).

Table 8. The effect of washing of bed nets on malaria morbidity at the end of the rainy season

	Unwashed	Washed once	Washed twice
Fever+parasitaemia[a]	14·0	9·6	12·5
Fever+high parasitaemia[b]	9·2	7·4	8·9
Spleen rate[c]	35%	35%	36%
Parasite rate[d]	25%	24%	21%
High parasite rate[e]	7%	9%	7%

[a]Episodes/1000 child-weeks at risk. χ^2 for trend$=1{\cdot}1$; d.f.$=1$; $P=0{\cdot}29$. χ^2 for heterogeneity$=3{\cdot}7$; d.f.$=2$; $P=0{\cdot}17$. Fever: temperature $\geq 37{\cdot}5$°C; *P. falciparum* parasitaemia only.
[b]Episodes/1000 child-weeks at risk. χ^2 test for trend$=0{\cdot}2$; d.f.$=1$; $P=0{\cdot}69$. χ^2 for heterogeneity$=0{\cdot}9$; d.f.$=2$; $P=0{\cdot}65$. Fever: temperature $\geq 37{\cdot}5$°C; high parasitaemia: *P. falciparum* only, ≥ 10 parasites/high power field ($\geq 5000/\mu$L approximately).
[c]χ^2 for trend$=0{\cdot}0$, $P=0{\cdot}99$.
[d]χ^2 for trend$=0{\cdot}2$, $P=0{\cdot}64$.
[e]χ^2 for trend$=0{\cdot}03$, $P=0{\cdot}86$.

Chloroquine consumption

During September 1989, a stratified cross-sectional survey was carried out to obtain urine samples from children in each of the 3 study groups. Chloroquine was demonstrated by ELISA in 19% (33/171) of samples obtained from children in the PHC-Maloprim® group, in 30% (51/169) from those in the PHC-placebo group, and in 35 % (54/156) from those in the non-PHC group (Maloprim® vs placebo $\chi 2=5{\cdot}4$; d.f.$=1$; $P=0{\cdot}02$; placebo PHC vs non-PHC $\chi^2=0{\cdot}7$; d.f.$=1$; not significant).

Discussion

We found a sharp reduction in child mortality following the introduction of the combined malaria intervention of insecticide-treated bed nets and targeted chemoprophylaxis. This was probably due to the introduction of treated bed nets, since children who received chemoprophylaxis had no apparent additional protection from death, regardless of the level of compliance. However, caution is needed in accepting this interpretation. In 1991, interventions with chemoprophylaxis and treated nets were repeated in PHC villages. During the 1991 rainy season, when malaria transmission was less intense, there were fewer deaths in both PHC and non-PHC villages than during the previous year but a similar selective advantage for PHC villages compared to non-PHC villages was observed. However, in contrast to the finding in 1990, children who received chemoprophylaxis and slept under a treated net had less chance of dying than those who slept under a treated net and received placebo (P. L. Alonso *et al.*, paper in preparation).

The reduction in overall mortality produced by the interventions was greater than expected. During the pre-intervention year, malaria was thought to be responsible for approximately 40% of deaths among children aged 1–4 years but the interventions produced a reduction in mortality close to 60%. There are a number of possible explanations for this finding. Firstly, it is possible that the verbal autopsy technique underestimates the contribution of malaria to mortality. Recent studies undertaken in another part of The Gambia (J. Todd *et al.*, unpublished observations) have shown that malaria frequently gives rise to a raised respiratory rate so that it is likely that some malaria deaths were, incorrectly, considered as deaths from acute respiratory infection. Secondly, it could be argued that the fall in mortality seen in PHC villages was due to concomitant improvements in health care or living standards in the intervention villages associated with the trial. This seems unlikely as the fall in mortality closely followed the introduction of the interventions and was restricted to the malaria transmission season. Thirdly, it is possible that insecticide-treated bed nets are a non-specific intervention effective against other vector-born diseases. This also seems unlikely as sleeping sickness has disappeared from The Gambia, kala-azar is extremely uncommon, and arbovirus infections are rarely fatal except during epidemics. A reduction in flies, and hence in enteric infections, is a possibility, but seems unlikely. Lastly, impregnated nets may have been more effective than expected because malaria is an important indirect cause of death in The Gambia. The fact that, following the introduction of the interventions, a large reduction was seen, not only in malaria deaths but also in deaths attributed to acute respiratory infections, supports this view. Similarly, in Guyana, successful malaria eradication campaigns led to a reduction in deaths from pneumonia (GIGLIOLI, 1972).

In field trials, there is legitimate concern about the impact that the observers may have on the results. We do

not believe that active weekly morbidity surveillance had any significant impact on mortality. Children in PHC villages received antimalarial treatment only from a VHW or health centre. Moreover, urine tests indicated that the level of chloroquine consumption was lowest among PHC children who received weekly Maloprim®, and highest among children from non-PHC villages. The use of health services in both PHC and non-PHC villages remained similar to that described in the pre-intervention year; after intervention, 65% and 67% of deaths in non-PHC and PHC villages respectively occurred at home.

The impact of the interventions on morbidity and on the prevalence of malaria infection has been assessed by means of cross-sectional malaria surveys and through a system of active case detection. Insecticide-treated bed nets reduced the incidence of fever episodes associated with parasitaemia by 45% and were even more effective at reducing those associated with high parasitaemia. These findings are similar to those reported by SNOW *et al.* (1988b) in a nearby area. The slightly lower protection we found may have been due to a higher intensity of transmission in the new study area or to the fact that, in the present trial, net impregnation was done through the PHC scheme and not by the investigators. Among children in PHC villages who slept under treated bed nets, the addition of chemoprophylaxis had a dramatic effect on the incidence of clinical episodes of malaria.

Results from the cross-sectional survey carried out in June, before the malaria transmission season began, showed no difference in the prevalence of malaria between PHC and non-PHC villages. Following introduction of the interventions, significant differences emerged. Insecticide-treated bed nets reduced the prevalence of splenomegaly and malaria parasitaemia by about 40%, and the prevalence of high density parasitaemia by 50%. Among the group of children protected by an insecticide-treated bed net, the addition of malaria chemoprophylaxis led to even greater reductions in these indices. Measurement of the PCV is a good indicator of malaria morbidity in The Gambia. In our trial, the mean PCV of children resident in non-PHC villages fell by an average of 2·4% over the malaria season. Children who slept under a treated net experienced only a minor drop, while those who received additional prophylaxis increased their mean PCV during the malaria transmission season.

The results of the cross-sectional surveys and the weekly surveillance suggested that the combination of the 2 interventions was so effective that, unlike chemoprophylaxis alone (GREENWOOD, 1991), the combined interventions might impair the development of natural immunity.

Permethrin, the pyrethroid insecticide used in this trial, is removed by washing and it was estimated that half of the amount on the net was lost after each wash (LINDSAY *et al.* 1991). At the time when the trial was discussed with the study population, and also when the interventions were implemented, villagers were asked to refrain from washing their nets after treatment with insecticide until the end of the malaria transmission season. This recommendation was followed partially, and the frequency with which people usually washed their nets was reduced. However, more than 50% of nets were washed at least once during the transmission season. None the less, the incidence of malaria was not increased among those who had washed their nets. Similarly, there was no evidence that the protective efficacy of the treated bed nets was reduced over time. In a previous Gambian trial, SNOW *et al.* (1988b) found that insecticide-treated nets were more effective at the beginning of the transmission season than at the end, but this might have been due to a change in the pressure of infection rather than to a decline in the insecticidal property of the nets. It is possible that, even after washing, treated nets have sufficient insecticide remaining to have a protective effect against mosquitoes seeking a bloodmeal. Alternatively, the impregnation of the mattress, on which bed nets were dried after treatment, may have been sufficient to deter mosquitoes from entering the house to feed.

We found that, in PHC villages, the small group of children whose bed nets were not treated with insecticide had a similar incidence of malaria to those whose nets were treated, whereas those with no nets at all had a higher prevalence of malaria parasitaemia. This effect may have been due to confounding factors; for example, children without nets had a lower compliance with chemoprophylaxis than those with nets. However, it is possible that mass treatment of bed nets with insecticide provides a degree of community protection which extends to those with treated nets that have been washed and to those with untreated nets, but not to those without nets. It is possible that children in PHC villages without a net were at an increased risk of malaria due to deflection of mosquitoes.

Approximately one-third of the children who were protected directly or indirectly by insecticide treated bed nets suffered a malaria episode during the transmission season, while children who were additionally receiving Maloprim® had practically no malaria. However, both groups of children experienced very similar levels of mortality, suggesting that insecticide-treated bed nets have a greater protective effect against life-threatening malaria infections than against uncomplicated infections. Insecticide-treated bed nets are only moderately effective at reducing the prevalence of infection; they are better at reducing clinical episodes and most effective at preventing deaths. How this graded effect is achieved is uncertain, nor has the exact way in which insecticide-treated nets achieve their protective effect been determined.

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El impacto de los mosquiteros impregnados de permetrina sobre los vectores de la malaria. A malaria control trial using insecticide-treated bednets and targeted chemoprophylaxis in a rural area of The Gambia, West Africa. 7. Impact of permethrin-impregnated bednets on malaria vectors. Lindsay SW, Alonso PL, Armstrong JRM, Hemingway J, Adiamah JH, Shenton FC, Jawara M, Greenwood BM. *Trans R Soc Trop Med Hyg* 1993;**87**, Suppl.2:45-51

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A malaria control trial using insecticide-treated bed nets and targeted chemoprophylaxis in a rural area of The Gambia, West Africa

7. Impact of permethrin-impregnated bed nets on malaria vectors

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Abstract

The impact of permethrin-impregnated bed nets on malaria vectors was studied in 6 pairs of villages during the rainy season in 1989. In each pair, the residents of one village had their nets treated whilst those of the other remained untreated. Routine collections of mosquitoes were made outdoors in the early evening using human-biting collections, and indoors with insecticide sprays, light traps and by searches under bed nets. Mosquitoes of the *Anopheles gambiae* complex, *An. gambiae sensu stricto*, *An. arabiensis* and *An. melas*, were present in large numbers for 5 months of the study period. These mosquitoes were susceptible to permethrin as judged by bioassay results. Outdoor human-biting rates in the early evening in communities with treated bed nets were similar to those in communities with untreated nets. In villages with treated bed nets most biting occurred outdoors in the early evening with little taking place under impregnated nets. The insecticidal activity of permethrin-impregnated bed nets, dipped by the local population, provided good individual protection against mosquitoes throughout the rainy season and bed nets remained effective even when washed up to 3 times. There was little to suggest that the use of insecticide-treated nets reduced the survival of mosquito populations in villages with impregnated nets. The absence of the expected village-wide effects of net impregnation may have resulted from the circulation of mosquitoes between villages with treated and untreated nets. The proportion of mosquitoes which fed on humans did not differ significantly between villages with treated and untreated nets. Permethrin-impregnated bed nets proved an effective barrier against vectors when people were under their nets, but had no apparent effect on biting outdoors before individuals retired to bed.

Introduction

Sleeping under a permethrin-impregnated bed net is a highly effective means of protection from bites of night-biting mosquitoes. The insecticide formulation used for treating nets can deter mosquitoes from entering houses and cause mosquitoes landing on treated netting to be killed or irritated and repelled (DARRIET *et al.*, 1984; LINES *et al.*, 1987; ROZENDAAL, 1989; MILLER *et al.*, 1991; LINDSAY *et al.*, 1992a). Trials of insecticide-treated bed nets in many parts of the tropics have shown that this form of personal protection operates well against several different malaria vectors. Moreover, the degree of protection afforded to a community can be enhanced by using insecticide-impregnated bed nets on a large scale (CURTIS *et al.*, 1990; MAGESA *et al.*, 1991). In such circumstances, the high mortality among mosquitoes attracted to these nets can cause a reduction in the population of mosquitoes in a village and in the mean age and proportion of sporozoite-bearing mosquitoes. This malaria control strategy has been effective in reducing morbidity in areas of relatively low transmission (SNOW *et al.*, 1988; CURTIS *et al.*, 1990) but was less successful in places where transmission was more intense (ROZENDAAL, 1989; CURTIS *et al.*, 1990; WHO, 1990).

The present study describes a series of entomological investigations designed to assess the effects of permethrin-treated bed nets in an area of seasonal malaria transmission in The Gambia. A description of the malaria vectors in this area during the rainy season immediately before this intervention has been given by LINDSAY *et al.* (1993). The study population consisted of 21 157 people in 73 villages in a rural area on the south bank of the River Gambia. Bed nets in 17 villages were impregnated with a pyrethroid insecticide, permethrin, whilst the remaining communities had untreated nets and served as controls. In addition, half of the children aged 6 months to 5 years in villages with treated nets were given chemoprophylaxis with Maloprim® weekly. The other half received a placebo. This intervention resulted in a 63% reduction in mortality in children aged 1–4 years (ALONSO *et al.*, 1993c). This protection against mortality was probably a result of using insecticide-treated nets as there was no significant additional effect when children were given weekly chemoprophylaxis.

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Materials and Methods

Study design

Descriptions of the study area, the adult vector populations in the villages and the implementation of the control programme have been described elsewhere (ALONSO *et al.*, 1993a, 1993b; LINDSAY *et al.*, 1993). Entomological investigations were carried out in 6 pairs of villages: Jessadi and Barokanda, Katamina and Male Kunda, Pakali Ba and Madina, Dasilami and Niawoorulung, Dongoro Ba and Wellingara Ba, and Jalangbereh and Sitahuma. The first named village in each pair of settlements was a primary health care (PHC) village, where basic health care was provided by a village health worker and a traditional birth attendant. During the intervention year, most people in these villages slept under bed nets treated with permethrin (25% emulsifiable concentrate, ICI Public Health) at a target dose of 0·5 g/m^2 (the actual mean permethrin dose was 0·2 g/m^2; ALONSO *et al.*, 1993b). An adjacent village without health care facilities (non-PHC village), where residents slept under untreated nets, served as a control for each intervention village.

Environmental studies

Meterological recordings were made using standard methods (METEROLOGICAL OFFICE, 1982) at Pakali Ba throughout the study.

The salinity of water collected from the River Gambia was measured using a refractometer (Aguafauna®, Chemlab Scientific Products). One litre samples of water were collected along the river and from a major tributary, the Sofaniama Bolon, at Bai Tenda, Baro Kunda-Tenda, Pakali Ba, Sambang Tenda, Kani Kunda Wharf Town, Balanghar Wharf Town and Jessadi Wharf Town. Collections were made close to the bank at the time of the

highest tide at the end of the rainy season during the period of maximum mosquito production.

Entomological surveillance

Routine mosquito catches were made using 4 different sampling techniques: human-biting catches, light trap collections, 'knock-down' sampling, and bed net searches, from June until the end of December 1989. Four men were employed as collectors in each of the 12 villages. These men received weekly chloroquine prophylaxis and slept under an untreated bed net in a room on their own, in a separate quarter of each village. Each man collected mosquitoes outdoors between 19:30 and 23:00 on one evening each week. Each person sat alone outside his house and collected insects landing on his exposed limbs using an aspirator and torchlight. Further collections were made indoors in the bedroom of each collector using a CDC light trap on one night each week and with an aerosol of insecticide on another night, as described elsewhere (LINDSAY *et al.*, 1993).

Twenty bed nets were selected at random from each PHC village (n=120) in June 1989 and sampled at weekly intervals until the end of the study. Nets were searched between 07:00 and 10:00 with data being used in the analysis only if the net was found with both entry flaps closed. Bed nets were marked with a water-soluble pen and inspected each week for evidence of washing. Washed nets were identified by the disappearance of the ink label and were marked again after each inspection.

Mosquito identification

Female mosquitoes were identified morphologically and scored as unfed, blood-fed or gravid. Identification of members of the *Anopheles gambiae* complex was carried out using deoxyribonuclec acid (DNA) probes on specimens collected from light traps in October 1989. DNA extracted from the tip of the abdomen of each species was amplified using a polymerase chain reaction (PASKEWITZ & COLLINS, 1989). The products of this reaction were probed with species-specific probes (supplied by Dr Frank Collins, Centers for Disease Control, Atlanta, Georgia, USA) on 0·8% agarose plates and visually inspected under ultraviolet light (PASKEWITZ & COLLINS, 1989).

Blood meal and sporozoite analysis

Mosquito bloodmeals and sporozoite positive mosquitoes were identified as described by LINDSAY *et al.* (1989b).

Bioassays

Assays were carried out to determine the insecticidal activity of permethrin on bed nets. Six randomly chosen nets were selected from each PHC village with insecticide-treated nets, 4–5 months after dipping. Mosquitoes were collected using window traps fitted to bedrooms of men sleeping under untreated nets. Using a World Health Organization (WHO) cone, 9–59 unfed *An. gambiae sensu lato* were exposed to each treated bed net, and 28–43 mosquitoes exposed to an untreated sample of polyester netting which served as a concurrent control. For all tests mosquitoes were exposed for 3 min in batches not exceeding 15 and were then transferred to paper cups and maintained on 10% sucrose. Mortality was recorded after 24 h.

Susceptibility of mosquitoes to permethrin was determined using WHO test kits with mosquitoes collected from exit traps in Sitahuma, Pakali Ba and Male Kunda. Unfed *An. gambiae s.l.* were exposed to 0·25% permethrin test paper for 10, 30, 60 and 120 min. Additional tests were carried out on mosquitoes collected from Sitahuma using 4% DDT test paper and exposures of 15, 30, 60 and 120 min.

Statistical analysis

Logarithmic transformations were applied to data when appropriate. SAS® software was used to calculate the mean logarithm of the number of vectors in each catch for each village during the 1989 rainy season. This was carried out for collections using light traps, sprays and outdoor human bait catches. Confidence intervals for the differences between the numbers of mosquitoes found in different pairs of villages were calculated as described by LINDSAY *et al.* (1993). Comparisons between years were made using data gathered during the same period in 1988 and 1989.

Human-biting rates per night for people with untreated nets were estimated by multiplying the goemetric mean number of blood-fed *An. gambiae s.l.* collected in each house in a village using spray catches by the human blood index (HBI) for each village. Seasonal inoculation rates for people with unimpregnated nets were calculated by multiplying the human-biting rates by the number of days in the season (150) and the mean seasonal sporozoite rate. Inoculation rates in the pre-intervention year were calculated as described above, assuming that the HBI in each village was the same in 1988 as in 1989.

Bioassay data collected when the control mortality exceeded 20% were excluded from subsequent data analysis.

Results

Seasonal changes in climate

The climate in the study area is typical of the sub-Sahel, with rain falling only from June to October. Temperature, relative humidity and rainfall in 1989 are shown in Fig. 1. Although saltwater enters the River Gambia in the western extremity of the study area during the dry season the salinity of water collected from the river during the middle of the wet season never rose above 3 g/L NaCl. Thus, the river water could be tol-

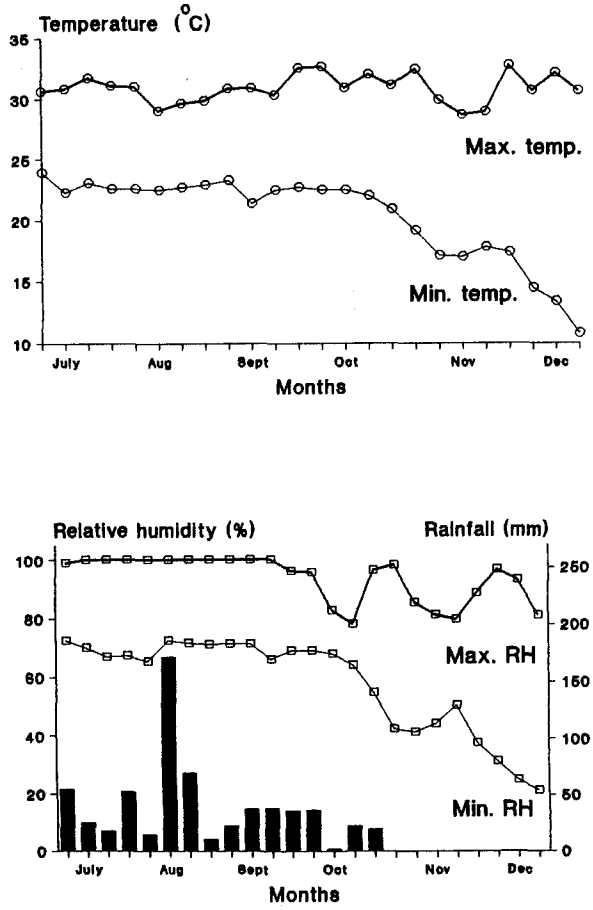

Fig. 1. Weather recorded at Pakali Ba in the centre of the study area during the period of entomological surveillance; RH = relative humidity.

Table 1. Identification of members of the *An. gambiae* complex using DNA probes

Village[a]	No. of mosquitoes sampled	Species *An. gambiae*	*An. arabiensis*	*An. melas*	Unknown
JSD	37	14 (87·5%)	0	2 (12·5%)	21
BKD	291	6 (76·2%)	4 (19·0%)	1 (4·8%)	8
KTM	100	54 (72·0%)	18 (24·0%)	3 (4·0%)	25
MKD	–	–	–	–	–
PKB	40	12 (50·0%)	12 (50·0%)	0	16
MNA	–	–	–	–	–
DSM	–	–	–	–	–
NWL	–	–	–	–	–
DGB	100	67 (77·9%)	19 (22·1%)	0	14
WGB	29	5 (38·5%)	8 (61·5%)	0	16
JBH	100	57 (100%)	0	0	43
SHM	100	28 (36·4%)	13 (16·7%)	37 (47·4%)	22
Total with treated nets	377	204 (79·1%)	49 (19·0%)	5 (1·9%)	119
Total with untreated nets	158	49 (43·8%)	25 (22·3%)	38 (33·9%)	46

[a]The first village in each pair was an intervention village; the second was a control village. Village names are given in full in the text.

erated by larvae of freshwater members of the *An. gambiae* complex.

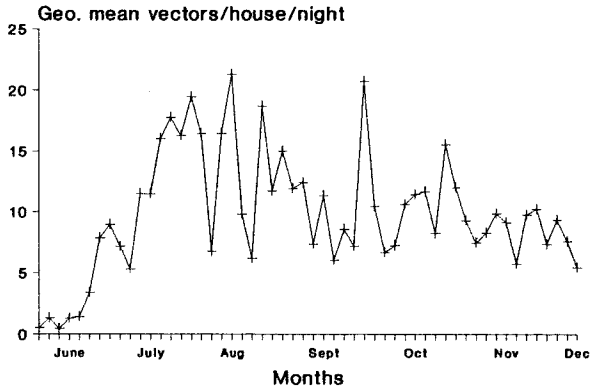

Fig. 2. Seasonal abundance of *An. gambiae s.l.* assessed from 'knock-down' collections.

Mosquito abundance

Among 68 023 mosquitoes collected using light traps during the study, 48 375 (71%) were anophelines, of which 44 887 (93%) were *An. gambiae s.l. An. gambiae sensu stricto* (68%) was the most common member of the *An. gambiae* complex found in the study area, followed by *An. arabiensis* (20%). *An. melas* occurred in a village close to the river in the western part of the study area (Table 1). Following the start of the rainy season in May the numbers of *An. gambiae s.l.* rose to a plateau and remained high until December (Fig. 2).

Generally, there was a significant inverse relationship between the numbers of *An. gambiae s.l.* collected from each village and the distance of the settlement from the river (light traps, $r=-0{\cdot}72$; $n=12$; $P=0{\cdot}01$; spray catches, $r=-0{\cdot}84$; $n=12$; $P=0{\cdot}001$; human-biting collections, $r=-0{\cdot}57$; $n=12$; $P=0{\cdot}06$). The proportion of mosquitoes which had fed on people (HBI) was inversely associated with the number of blood-fed *An. gambiae s.l.* collected using spray catches ($r=-0{\cdot}83$; $n=12$; $P<0{\cdot}001$) and directly related to the proportion of humans relative to cattle in each village ($r=0{\cdot}64$; $n=12$; $P<0{\cdot}05$) (Fig. 3).

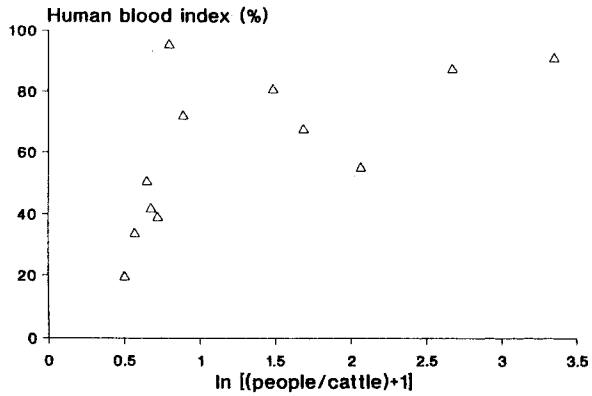

Fig. 3. Relationship between the human blood index of malaria vectors collected indoors and the relative abundance of men and cattle in each village ($\log_n$[people/cattle]+1).

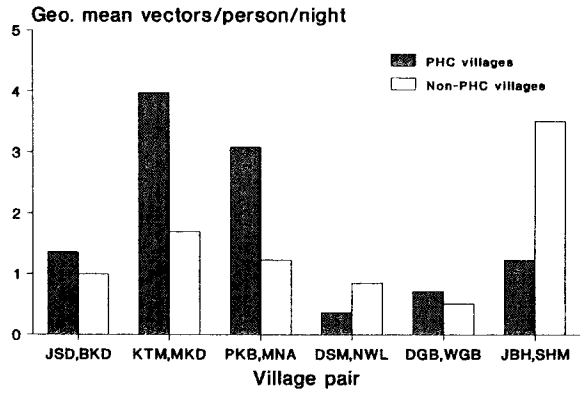

Fig. 4. Geometric mean numbers of *An. gambiae s.l.* collected landing on human baits in the early evening during the year of the interventions (1989).

Effects of permethrin-impregnated bed nets

Comparisons between mosquito numbers in villages with impregnated bed nets and those with untreated nets are shown in Figs 4–6 and summarized in Table 2. Although fewer mosquitoes were collected in PHC villages than in non-PHC villages in both years, the difference reached significance at the 5% level only for spray catches in 1989. Human-biting catches made outdoors in the early evening were similar in PHC and non-PHC villages in 1989 (6% more mosquitoes in PHC villages than in non-PHC villages).

Significantly fewer live mosquitoes were collected

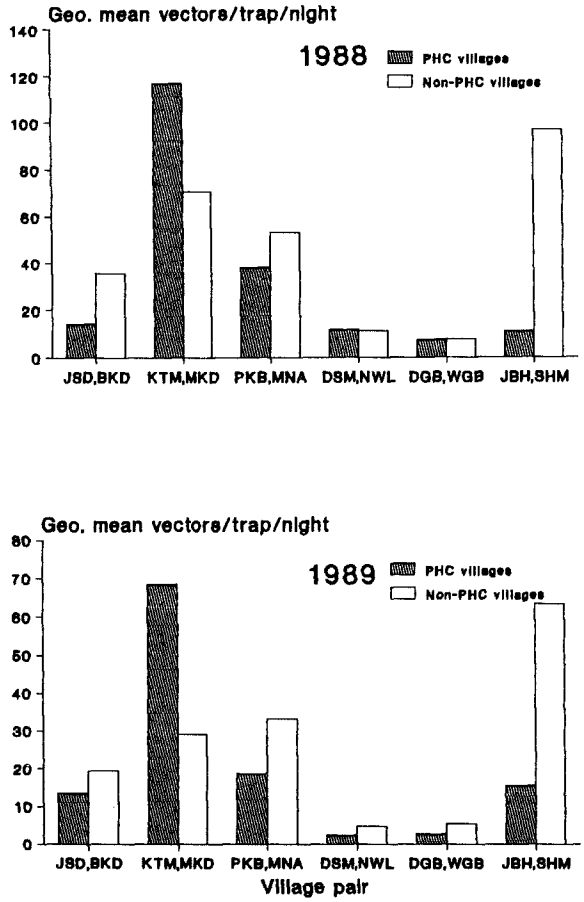

Fig. 5. Geometric mean numbers of malaria mosquitoes sampled with light traps in the study villages during the pre-intervention (upper) and intervention (lower) years (1988 and 1989).

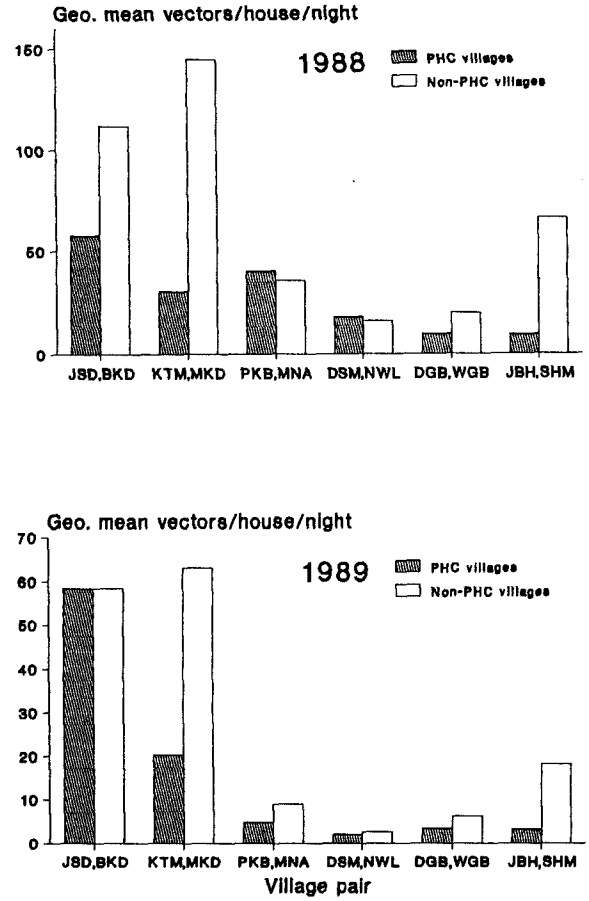

Fig. 6. Geometric mean numbers of *An. gambiae* complex mosquitoes collected with insecticide spray catches from villages during the pre-intervention and intervention years (1988 and 1989).

Table 2. Relationship between mosquito numbers in six pairs of intervention (PHC) and control (non-PHC) villages in the year before the start of a mosquito control programme in PHC villages using insecticide-treated bed nets and during the intervention year

Collection method	Pre-intervention year[a] PHC:non-PHC villages[b]	Significance[c]	Intervention year PHC:non-PHC villages[b]	Significance[c]
Light traps	0·63:1 (0·24–1·66:1)	n.s.	0·66:1 (0·32–1·40:1)	n.s.
Spray catches	0·47:1 (0·20–1·12:1)	n.s.	0·43:1 (0·25–0·73:1)	$P<0·05$
Human biting collections	– –	–	1·06:1 (0·61–1·83:1)	n.s.

[a]Data from LINDSAY *et al.* (1993).
[b]95% confidence interval in parentheses.
[c]Wilcoxon matched-paired signed-ranks test; n.s.=not significant.

Table 3. Number of occasions on which live *An. gambiae s.l.* mosquitoes were found under 120 bed nets searched each week during the rainy season in primary health care (PHC) villages, in which most nets were treated, and in non-PHC villages, in which the only treated nets were those taken there by people who moved from PHC villages

	Villages			
	PHC Nets		Non-PHC Nets	
	Treated	Untreated	Treated	Untreated
Mosquitoes				
Present	2 (0·1%)	15 (14·6%)	1 (3·8%)	765 (48·3%)
Absent	1497 (99·9%)	88 (85·4%)	25 (96·2%)	819 (51·7%)
Total	1499	103	26	1584

from under insecticide-treated bed nets compared with untreated nets, both in villages with treated nets and in those with untreated ones (Mantel-Haenszel $\chi^2=83·0$; degrees of freedom (d.f.)=1; $P<0·001$) (Table 3). Significantly fewer human-fed mosquitoes were collected from homes with untreated bed nets in villages with treated nets compared to those with untreated nets (57% reduction, $T=0$; $n=6$; $P<0·005$).

There was no significant difference between the HBI in villages with treated and untreated nets (Table 4) after adjusting for differences in levels of vector abundance ($T=6$; $n=6$; not significant [n.s.]).

Sporozoite rates were similar in villages with treated nets and those with untreated nets ($T=8$; $n=6$; n.s.) (Table 4); they were lower overall in 1989 than in 1988 before any bed nets had been treated (paired t test, $t=4·1$; d.f.=11; $P=0·002$). The drop in sporozoite rates between the pre-intervention and intervention years was similar in the intervention villages and their paired comparison villages ($T=7$; $n=6$; n.s.). Estimated inoculation rates were lower in villages with treated nets than in those with untreated ones, although this difference was

Table 4. The proportions of mosquitoes which had fed on human blood and of infective mosquitoes determined by a visual inspection of ELISA plates in 1989

Village[a]	No. of mosquitoes fed on human blood	Human blood index	No. of infective mosquitoes	Sporozoite rate
JSD	249/509	41·8%	40/4875	0·82%
BKD	339/849	33·8%	82/10255	0·80%
KTM	189/421	39·0%	40/11500	0·35%
MKD	257/1041	19·6%	47/13141	0·36%
PKB	99/105	87·6%	25/4030	0·62%
MNA	190/240	72·1%	43/5450	0·79%
DSM	41/59	67·8%	4/403	0·99%
NWL	81/85	95·3%	4/994	0·40%
DGB	39/74	50·6%	11/844	1·30%
WGB	89/110	80·9%	11/1447	0·76%
JBH	94/103	91·3%	13/2927	0·44%
SHM	292/496	55·3%	74/12324	0·60%
Total with treated nets	711/1271	55·9%	133/24579	0·54%
Total with untreated nets	1248/2821	44·2%	261/43611	0·60%

[a]The first village in each pair was an intervention village with treated nets; the second was a control village. Village names are given in full in the text.

Table 5. Estimates of sporozoite inoculation rates in primary health care (PHC villages) and in non-PHC villages before and after intervention

	Pre-intervention year (1988)		Post-intervention year (1989)	
Village[a]	Bites/ adult/ season	Infective bites/ adult/ season[b]	Bites/ adult/ season	Infective bites/ adult/ season
JSD	2380	64	1125	9
BKD	3701	110	2493	20
KTM	1076	4	672	2
MKD	2692	70	1257	5
PKB	3253	99	406	3
MNA	2376	177	693	6
DSM	1160	15	151	2
NWL	1481	0	291	1
DGB	448	80	197	3
WGB	1553	34	550	4
JBH	769	70	296	1
SHM	3366	75	960	6
Geometric mean in PHC villages	1222	20	373	3
Geometric mean in non-PHC villages	2383	33	833	5

[a]The first village in each pair was an intervention village; the second was a control village. Village names are given in full in the text.
[b]Human blood index was not determined in 1988; inoculation rates have been calculated on the assumption that it was the same as in 1989.

of borderline significance ($T=1$; $n=6$; $P=0·06$) (Table 5).

Bioassays

Treated bed nets killed significantly more mosquitoes than untreated nets ($t=3·7$; d.f.=34; $P<0·001$). However, there was considerable variation between the killing ability of individual nets impregnated with permethrin. For example, the percentage mortality of mosquitoes exposed to treated nets which had not been washed was 42% (95% confidence interval [CI]=28%,57%). There was no significant linear decrease in the mortality of mosquitoes exposed to treated netting as the number of washes increased ($F=2·1$; d.f.=1 and 54; n.s.).

Susceptibility of mosquitoes to permethrin impregnated paper is shown in Table 6. The 95% confidence intervals of probit regression lines fitted to the data suggested that the mosquito populations from the 3 villages tested were similar, although the 50% lethal exposure time (LT_{50}) calculated for Male Kunda ($LT_{50}=10·6$ min, 95% C.I.=5·7,14·6 min) was significantly less than that for Pakali Ba ($LT_{50}=19·3$ min, 95% C.I.=15·6,22·7 min). The LT_{50} for mosquitoes from Sitahuma was 17·7 min (95% C.I.=13·2,21·6 min) compared with 15·9 min (95% C.I.-13·6,18·1 min.) for all the villages combined. Mosquitoes collected from Sitahuma and exposed to DDT impregnated paper had an $LT_{50}=14·5$ min (95% C.I.=11·3,16·9 min), with no mosquito surviving 60 min exposure.

Discussion

Malaria transmission in The Gambia is confined to the rainy season. On the north bank of the river appreciable numbers of vectors are found for only a few weeks each year (SNOW *et al.*, 1987; LINDSAY *et al.*, 1989a, 1989b). However, in the present study area on the south bank of the river the transmission period lasted about 5 months.

The predominant vectors in the study area were night-biting mosquitoes belonging to the *An. gambiae* complex. Of these, *An. gambiae s.s.* were more common than both *An. arabiensis* an *An. melas*, the last species being confined largely to the western fringe of the study area where the breeding sites are brackish during the early part of the rainy season. Although no major larval survey was carried out, the principal mosquito breeding sites in the area were probably the large, mainly freshwater, marshes bordering the River Gambia and its tributary, the Sofaniama Bolon, as the highest numbers of mosquitoes were found in villages nearest to the river in both 1988 (LINDSAY *et al.*, 1993) and 1989.

In contrast to the results of other workers (CURTIS *et al.*, 1990; MAGESA *et al.*, 1991) there was little to suggest that treating the majority of bed nets in a village with permethrin reduced the survival of mosquitoes. Firstly, there was no indication of a substantial decrease in mosquito numbers caught indoors in villages with treated bed nets compared to those with untreated nets. On average, PHC villages had a similar proportion of mosquitoes relative to non-PHC villages in the intervention year compared with the pre-intervention year. Using light traps, 37% fewer mosquitoes were collected in PHC villages than in non-PHC villages during the pre-intervention year, and 34% fewer during the intervention year.

Table 6. Mortality of *An. gambiae s.l.* exposed to paper impregnated with 0·25% permethrin and to untreated paper

	Village			
Exposure[a] (min)	Sitahuma	Pakali Ba	Male Kunda	Total
15	24/52 (46·1%)	22/60 (36·7%)	38/60 (63·3%)	84/172 (48·8%)
30	41/60 (68·3%)	50/68 (73·5%)	51/61 (83·6%)	142/189 (75·1%)
60	38/41 (92·7%)	58/64 (90·6%)	55/60 (91·7%)	151/165 (91·5%)
120	46/46 (100%)	64/64 (100%)	62/62 (100%)	172/172 (100%)
Untreated	2/77 (2·6%)	1/73 (1·4%)	0/65 –	3/215 (1·4%)

[a]Exposure temperature 23–32°C.

Corresponding values for insecticide spray catches were 53% and 57%. It is unlikely that in PHC villages mosquitoes deterred from entering homes with treated nets accumulated in sentinel homes with untreated nets. Studies carried out using experimental huts in The Gambia suggested that *An. gambiae s.l.* deterred from entering a hut with a permethrin-treated bed net would not be displaced to feed in adjacent huts where occupants slept under untreated nets (LINDSAY *et al.*, 1992). Secondly, the HBI of mosquitoes collected indoors in sentinel houses was similar in villages with treated bed nets and in those with untreated ones. However, this index was shown to be related to the numbers of mosquitoes in each settlement and the availability of alternative host species. Proportionately fewer mosquitoes fed on people in villages where there were large numbers of mosquitoes than in those with few vectors. This relationship may have arisen because people in villages with large numbers of mosquitoes protected themselves from biting mosquitoes better than those in villages where there were few vectors. For instance, people may go to bed earlier in villages where there are many mosquitoes. The HBI was also related to the relative availability of people and cattle in each village, with proportionately more human-fed mosquitoes occurring in settlements in which people outnumbered cattle. A similar relationship was described in Nigeria with *An. arabiensis*, although not with *An. gambiae s.s.* (WHITE & ROSEN, 1973). The readiness of Gambian *An. gambiae s.s.* to feed on species other than humans may have been due to the widespread use of bed nets which made it more difficult for a mosquito to obtain a human blood meal (LINDSAY *et al.*, 1989b). Lastly, there was no detectable reduction in sporozoite rates in mosquitoes collected from villages with permethrin-treated bed nets compared to those with untreated nets.

In the present study, the inability to demonstrate a 'mass killing' effect may have been due to the mixing of mosquito populations between villages with treated and untreated nets. A study carried out in a village nearby suggested that large numbers of mosquitoes could travel distances of 1–2 km (LINDSAY *et al.*, 1991b) and subsequent studies using mark–release–recapture methods have demonstrated substantial circulation of mosquitoes between villages in the study area (M. Thomson & M. Quinones, unpublished data). Thus, the possibility remains that the use of permethrin-treated bed nets reduced mosquito survival throughout the study area. This hypothesis is supported by the overall reduction in sporozoite rates and mosquito numbers during the intervention year compared with the previous year. However, this suggestion needs to be treated with caution since these results may have been due to the lower rainfall in 1989 than in 1988, with a consequent reduction in mosquito production.

Although at least one live *An. gambiae s.l.* was found in nearly half of all searches of untreated bed nets, they were found on only 3 occasions during 1525 searches under insecticide-treated nets. Thus, it is likely that those who slept under treated nets received far fewer mosquito bites than those who did not, despite the fact that the treatment of bed nets during this study was not optimal. Nets were treated by the villagers themselves, which resulted in a large variation of insecticide dosages between and within bed nets, and many of the nets were washed, which reduced the amount of permethrin remaining on the netting (ALONSO *et al.*, 1993b).

Results from outdoor biting collections on humans are difficult to interpret since similar collections were not made in the pre-intervention year. Whilst indoor biting rates were lower overall in PHC villages than non-PHC villages in both years, outdoor biting rates were similar in both groups of villages. As a result of the small sample size of 6 pairs of villages and the large variation in mosquito numbers between pairs, only the largest differences were significant. However, future research should consider the possibility that the use of insecticide-treated bed nets may increase the biting rates of people outdoors in the early evening. This phenomenon might occur if mosquitoes deterred from entering homes with permethrin-impregnated bed nets (LINDSAY *et al.*, 1991a; MILLER *et al.*, 1991) shifted their biting cycle from the early hours of the morning to early evening, as has been shown following DDT indoor spraying (SLOOF, 1964; TAYLOR, 1975) and following the use of permethrin-impregnated bed nets in Papua New Guinea (CHARLWOOD & GRAVES, 1987). However, no consistent effect of this kind was found in a recent study in Tanzania (MAGESA *et al.*, 1991).

Permethrin-treated nets proved extremely effective at protecting Gambian children from clinical attacks of malaria and reducing mortality (ALONSO *et al.*, 1993c). This was probably due to the action of treated nets in preventing mosquitoes entering or feeding through the nets. The outdoor biting rates obtained using men as baits may have overestimated the number of bites children received outdoors since, compared with adults, children (i) are smaller, and thus less attractive to mosquitoes, (ii) were unrestrained and could move to avoid biting mosquitoes, (iii) often slept outdoors in the early evening on raised platforms surrounded by adults, (iv) were covered frequently with a sheet or tied to their mothers' back with a cloth, or (v) went to bed earlier, particularly on nights when there were large numbers of mosquitoes.

The possible effects on malaria transmission of the administration of Maloprim® to half the children in PHC villages in the study area are uncertain. Children taking Maloprim® comprised only 10% of the total population of the villages with treated nets. However, in endemic areas, the prevalence rate of gametocytes and gametocyte density are usually highest in subjects in this age group (BRUCE-CHWATT 1951; MUIRHEAD-THOMSON, 1954; MOLINEAUX & GRAMMICIA, 1980).

Tests carried out in which mosquitoes were exposed to treated netting demonstrated a wide range of insecticidal activity between nets, which probably reflected the uneven distribution of permethrin on bed nets (ALONSO *et al.*, 1993b). Areas of netting with high dosages of insecticide may have been responsible for the finding that washing nets did not reduce killing of mosquitoes. This was a surprising result since there is overwhelming evidence to show that permethrin is washed out readily from treated netting (SNOW *et al.*, 1987; LINDSAY *et al.*, 1991c; MILLER *et al.*, 1991; NJUNWA *et al.*, 1991). However, patches of high dosages of permethrin on netting will still be potent after several washes. The patchy distribution of permethrin deposits on netting may have import ant consequences for future control programmes since it may make the emergence of resistance among vectors more likely by allowing selective survival of resistant heterozygotes. However, to date, bioassays have shown that *An. gambiae s.l.* was susceptible to permethrin in the study area. Moreover, mosquitoes were also susceptible to DDT, as assessed by bioassays, despite evidence for enhanced DDT metabolism in mosquitoes obtained from one of the study villages in 1988 (LINDSAY *et al.*, 1993).

Permethrin-treated nets provided an effective barrier against malaria mosquitoes. However, evidence for enhanced protection in villages where most people used treated nets was not found, possibly due to the circulation of mosquitoes between settlements with treated and untreated bed nets. Thus, the use of insecticide-treated bed nets throughout all villages in an area may be required before greater control can be achieved.

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Coste efectividad de la impregnación de mosquiteros solo o en combinación con la quimioprofilaxis en la prevención de la mortalidad y la morbilidad por malaria en niños gambianos. A malaria control trial using insecticide-treated bednets and targeted chemoprophylaxis in a rural area of The Gambia, West Africa. 8. Cost effectiveness of bednet impregnation alone or combined with chemoprophylaxis in preventing mortality and morbidity from malaria in Gambian children. Picard J, Aikins M, Alonso PL, Armstrong JRM, Greenwood BM, Mills A. *Trans R Soc Trop Med Hyg* 1993;**87**, Suppl.2:53-57

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A malaria control trial using insecticide-treated bed nets and targeted chemoprophylaxis in a rural area of The Gambia, West Africa

8. Cost-effectiveness of bed net impregnation alone or combined with chemoprophylaxis in preventing mortality and morbidity from malaria in Gambian children

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Abstract

In The Gambia, insecticide impregnation of bed nets, used alone or combined with Maloprim®, reduced morbidity and mortality from malaria amongst children between one and 4 years of age. Taking expenditure of both time and money by public authorities and village volunteers into account, the costs and cost-effectiveness of each intervention were estimated. Bed net impregnation alone and the combined strategy cost US $5·65 and US $7·49 per child-year protected respectively (1990 figures). Insecticide (and drugs) accounted for more than 80% of the costs of each intervention strategy. They were both highly cost-effective. Estimated costs per death and per clinical episode of malaria averted were US $188 and US $28 for bed net impregnation and $257 and $19 for impregnation combined with chemoprophylaxis. Estimated costs per healthy year of life saved, discounted at 3%, were US $7·90 and US $10·84.

Introduction

Effective control of malaria has proved difficult in tropical, rural Africa, where the disease is responsible for a high proportion of deaths of young children. Technically promising means to curb its incidence are therefore welcome. Malaria is predominantly a poor country's disease. Thus, it is very important that the economic as well as the epidemiological effects of novel control strategies should be evaluated so that scarce resources can be distributed most efficiently. In recent trials in The Gambia, villagers supervized by primary health care (PHC) workers have impregnated their bed nets with an insecticide, permethrin. Deaths amongst children under 5 years old were reduced markedly (ALONSO *et al.*, 1993b). So too were the number of clinical malaria episodes amongst children in the same age group. Clinical episodes were reduced even further by weekly doses of Maloprim® (pyrimethamine+dapsone) administered by PHC workers during the rainy season. However, chemoprophylaxis had no additional effect on mortality (ALONSO *et al.*, 1993b).

The results of this trial suggested that net impregnation alone, or combined with chemoprophylaxis, offers effective means of controlling childhood malaria in The Gambia. In this paper, the economics of these strategies are considered. Estimates are made of the cost-effectiveness of each option in averting deaths and malaria episodes amongst children between one and 4 years old, in saving discounted healthy years of life, and in protecting a child for one year.

Methods

Interventions

Interventions were made in a rural area of The Gambia over 2 successive rainy seasons (1989 and 1990). Each year, nets in 17 villages within the PHC system were impregnated. Children aged 6–59 months in these PHC villages also received either weekly Maloprim® or a placebo. Morbidity and mortality surveys were conducted amongst these children and amongst children living in villages outside the PHC system where no intervention was made. The children in these non-PHC villages acted as controls. Further details of how the interventions were carried out are provided by ALONSO *et al.* (1993a). Permethrin, Maloprim® and placebo were supplied to study villages by UK Medical Research Council (MRC) staff. Staff of the Gambian Medical and Health Department were responsible for the training and supervision of lower level officials and of village health workers (VHWs), traditional birth attendants (TBAs), and the leaders of the Women's Development Council (WDC). VHWs, TBAs and WDC leaders arranged the net impregnation in each village (in the last weeks of the dry season), while VHWs, often assisted by another villager, were responsible for the Maloprim® or placebo administration during the rainy season. Morbidity and mortality surveillance was the responsibility of MRC field workers and reporters, so their research costs could be distinguished from the project's implementation cost.

Costs

The implementation costs were borne both by public authorities and by village volunteers and reflected investment of time as well as money. The estimated values of this time, including the volunteers' contributions, are included in the estimates given here. The average market exchange rates during this period are used throughout. All amounts are expressed as their 1990 equivalents ($=US dollars).

Representative, full-time employees of the national health care system were interviewed during the second year. The work times spent on the project each year and by each level in the health care hierarchy were estimated. The value of their time was taken as the sum of their gross wage and employers' national insurance contributions. The transport and subsistence allowances paid to VHWs, TBAs and WDC leaders attending training sessions outside their villages were taken to reflect the cost of their participation at the session.

In each instance, estimates were made also of the time that would have been necessary to impregnate nets without giving chemoprophylaxis. The differences between the actual and hypothetical estimates were attributed to the distribution of chemoprophylaxis. The second year's estimates were taken as annually recurrent costs. Any additional time spent in the first year was defined as investment in the project's introduction. Such investment was made only in the formal public sector, where its lifetime was assumed to be 2 years, reflecting recent staff turnover rates.

The annual equivalent value of this investment was

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calculated using a discount factor of 6%—the difference between the prevailing Treasury Bill and (consumer price) inflation rates. Investments in plastic utensils and vehicles were treated in the same way. The costs of vehicles and equipment used only in part for the scheme were estimated by apportioning their annual costs (including tax and insurance) by the fraction of the annual mileage accounted for by the scheme.

VHWs, TBAs and WDC leaders in each village were interviewed soon after the net dipping was completed. The total numbers of hours spent by each person in net impregnation were calculated. VHWs and their helpers were interviewed again at the end of each of the first 3 Maloprim®/placebo distribution rounds, yielding estimates of the total time spent by them in drug distribution.

Throughout the 1990 season, interviews were also conducted with the carers of all children who died (n=35) and of confirmed non-fatal malaria cases identified in the course of the morbidity survey (n=45). These furnished estimates of the average expenditure incurred by carers in the pursuit of treatment and the average time spent by them seeking treatment for their charges. Amongst the fatal cases, estimates were made also of the average expense of ceremonies following death and of the average number of adult work hours lost during mourning.

Marginal values of different categories of villagers' work time were estimated. (The marginal value of time worked reflects the value of time occupied by treating nets better than the average value of all work time.) Past agronomic work (PUETZ *et al.*, 1990) provided estimates of the marginal value of working on ground-nuts, coarse grains or traditional rice fields during the wet season at Gambian dalasi (D)1·58/h ($0·20/h), D1·35/h ($0·17/h), and D0·68/h ($0·09/h) respectively. (Ignoring changes in the crops' relative producer prices, each figure was inflated from its 1986 equivalent by the rise in the only published agricultural producer price, that of ground-nuts.)

Since ground-nut and coarse grain cultivation are traditionally male occupations, the time of the exclusively male VHWs and their helpers was valued at D1·58/h if they would otherwise have been working on ground-nuts and D1·35/h if they would have been doing other work. On the other hand, rice fields are traditionally a woman's preserve. Since the mother went with the child on 70% of the trips in pursuit of treatment for which the identity of the child's companion was known, the carer's time in seeking treatment was valued at D0·68/h. Moreover, because during the dry season VHWs are believed to have more lucrative occupations than (female) TBAs and WDC leaders, though all are less productive than in the wet season, the value of the net dippers' time was assumed to be D1/h ($0·13/h) for men and D0·5/h ($0·06/h) for women. The value of mourners' time was set at D1/h, a value between the 2 sexes' wet season marginal values of labour.

These various estimates were then combined to produce the annual implementation costs of 2 possible interventions: impregnating nets, and impregnating nets and supplying Maloprim® to all eligible children in PHC villages. The first implementation's costs were assumed to be equal to the costs incurred by simply impregnating nets; the second's to the full implementation costs plus the cost of the additional Maloprim® necessary to substitute the active preparation for the placebo. Estimates were also made of the resources saved by preventing childhood malaria (saved treatment and caring expenses and averted funeral and mourning costs).

Effects

Field staff visited study children weekly and, if appropriate, made blood films (ALONSO *et al.*, 1993b). Since malaria episodes may last less than 7 d, some may not have coincided with the field worker's visit. However, only the confirmed episodes, together with all reported deaths, were considered in the economic calculations.

The numbers of deaths and episodes averted by net impregnation alone and by net impregnation combined with Maloprim® were calculated by comparing the rates of each event amongst children in the relevant PHC group with those in the non-PHC villages. For example, the number of deaths averted by net impregnation and chemoprophylaxis was estimated as the difference in the non-PHC and Maloprim® PHC groups' fatality rates multiplied by the number of children enrolled in the project in PHC villages (n=1898). The estimates of implementation cost and of effectiveness were then combined to yield implementation cost-effectiveness estimates.

The estimated values of resources released by reducing the incidence of malaria (treatment and caring costs) are reported separately. They could, alternatively, be subtracted from the implementation cost-effectiveness figures to yield numbers which take the value of spared resources into account. The values are, however, so small that the additional complexity seems unnecessary. Only the implementation costs are included in all the cost-effectiveness estimates reported below.

Results

The total annual costs of impregnating nets in the PHC villages and of combining net impregnation with chemoprophylaxis were $10 714 and $14 207 respectively. Thus, the additional cost of chemoprophylaxis

Table 1. Breakdown of costs of net impregnation alone[a]

Capital investment (annualized costs, 6% discount rate)			
Training government employees, initial sensitization, and training dippers	42·90%		$73·87
Plastic ware	39·31%		$67·70
Ledgers	17·79%		$30·63
Total annualized capital cost	100·00%	1·61%	$172·20
Recurrent costs			
Retraining government employees, resensitizing in the villages and supervision	3·38%		$356·44
Insecticide delivery	0·33%		$35·16
Malaria control officer's transport	14·25%		$1501·85
Dipper's time	0·86%		$90·23
Insecticide	81·18%		$8558·38
Total annual recurrent costs	100·00%	98·39%	$10542·05
Total costs (recurrent and capital)		100·00%	$10714·25

[a]All costs are in US dollars (1990).

Table 2. Additional costs of adding chemoprophylaxis to net impregnation[a]

Capital investment (annualized costs, 6% discount rate)				
Training government employees, initial sensitization and training VHWs[b]	100·00%			$129·22
Total annualized capital cost	100·00%	3·70%		$129·22
Recurrent costs				
Retraining government employees, resensitizing in the villages, supervising drug distribution	4·66%			$156·87
VHWs[b] and their helpers' time	5·93%			$199·37
Maloprim®	89·41%			$3007·30
Total additional annual recurrent costs	100·00%	96·30%		$3363·54
Total additional costs		100·00%	24·58%	$3492·76
Total costs of net impregnation[c] and chemoprophylaxis			100·00%	$14207·01

[a]All costs are in US dollars (1990).
[b]Village health workers.
[c]From Table 1.

was relatively small at $3493. In either case, permethrin, or permethrin and Maloprim® together, accounted for approximately 80% of the total annual cost (Tables 1 and 2). The estimated costs per child-year protected were $5·65 for bed net impregnation alone and $7·49 for the combined strategy.

The estimated numbers of deaths averted by the 2 interventions amongst protected children in PHC villages are shown in Table 3. A summary of the estimated implementation costs together with the implied implementation cost/effectiveness ratios are shown also. Averting a death by impregnating bed nets cost an estimated $188, while the corresponding implementation cost per malaria episode averted was $28. Because it had little effect on mortality but a marked effect on morbidity, adding chemoprophylaxis to the intervention had opposite effects on these 2 figures. Thus, the implementation cost per death averted rose to $257 while the implementation cost per case averted fell to $19. The additional cost per additional case prevented by adding chemoprophylaxis was $10.

Table 3. Cost, effectiveness and cost-effectiveness of bed net impregnation and impregnation combined with chemoprophylaxis[a]

Costs per death and case averted	
Bed net impregnation alone	
Implementation cost	$10714·25
Deaths averted	57·13
Cases averted	378·14
Cost/death averted	$187·53
Cost/case averted	$28·33
Bed net impregnation and chemoprophylaxis	
Implementation cost	$14207·01
Deaths averted	55·25
Cases averted	732·02
Cost/death averted	$257·13
Cost/case averted	$19·41
Marginal cost-effectiveness of chemoprophylaxis	
Additional cost	$3492·76
Additional deaths averted	−1·88
Additional cases averted	353·87
Cost/additional death averted	−$1857·96
Cost/additional case averted	$9·87
Cost per discounted healthy life year gained	
Life expectancy at death (years)	43
Median age at death (years)	0·92
Difference (years)	42·08
Discount rate	0·03
DHLYs[b] gained/death averted	23·73
Cost/DHLY[b] saved	
Net impregnation	$7·90
Net impregnation and chemoprophylaxis	$10·84
Cost per child year protected	
Bed net impregnation alone	$5·65
Bed net impregnation and chemoprophylaxis	$7·49

[a]All costs in US dollars (1990).
[b]Discounted healthy life year(s).

The implementation cost/effectiveness ratios of both interventions are necessarily sensitive to changes in estimated effectiveness. An increase by a given factor in the number of deaths (or cases) averted reduces the cost per death (or case) averted by the reciprocal of the same factor. For example, if the number of deaths and episodes averted by each intervention were reduced by 10%, each implementation cost/effectiveness ratio would be increased by 11%.

The implementation cost/effectiveness ratios are also particularly sensitive to changes in the price of permethrin. The effects of such price changes are shown in the Figure. If, for example, the price of permethrin (25% emulsifiable concentrate) were reduced to $15 per litre, the costs per death and per episode averted by net impregnation alone would be $143 and $22. The corresponding figures for the combined strategy would be $211 and $16.

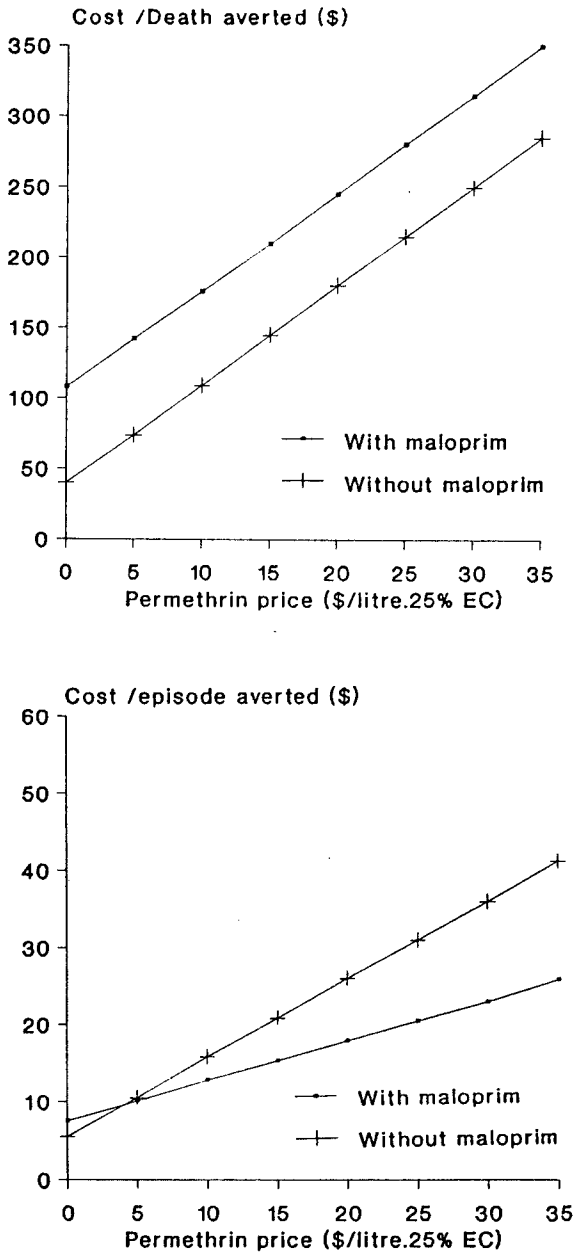

Figure. The sensitivity of cost-effectiveness to variations in the prices of permethrin and of Maloprim®; costs are in US dollars. 25% EC=25% emulsified concentrate of permethrin (ICI Public Health).

The median age of children who died was 11 months. If malaria is assumed to kill randomly in the absence of either intervention, then the life expectancy of those children whose death was averted would be equal to their cohort's average—43 years. If morbidity in the intervening years is ignored, then these children each gain 42·1 years of healthy life or, discounting at the 3% rate used in other calculations of this kind (JAMISON & MOSLEY, 1992), 23·7 years of discounted healthy life. Thus, the costs of net impregnation and of the combined strategy are $7·90 and $10·84 per discounted healthy life year saved respectively . (If 6% is used instead of 3%, the figures become $12·31 and $16·88.)

The average costs (including time) spent by carers seeking, preparing and administering treatment to sick

children who survived were D5·68 ($0·72; $n=33$) per episode. The equivalent figure for children who died was D24·47 ($3·10; $n=25$). Bereaved families also faced further expenses. They spent an average of D159 ($20·17; $n=35$) on the ceremonies that followed death. The estimated value of the average loss of work time through mourning was larger still—D576 ($73·09; $n=35$); very wide kinships reportedly ceased work over extended periods. The deaths and the corresponding expenditures are not, of course, prevented but delayed. Their net present values are reduced accordingly. If, for example, they are postponed 40 years, then, at a discount rate of 6%, their equivalent is just 9·7% of their current cost. For both dying and surviving patients, mean costs of treatment were lower in PHC villages, perhaps reflecting its easier accessibility. However, in our sample, the differences were not significant. In comparison, the value of the additional work time spent caring for the ill was negligible. This is a consequence of the brevity of typical fatal and non-fatal episodes (some 3 or 4 d) and of the youth of the patients (even when well they required considerable caring time). Thus, the value of additional work-time spent caring for sick children remains minuscule relative to the implementation cost per death or per episode averted, even if implausibly protracted additional caring times are assumed.

Discussion

The study's policy implications depend on its reliability, on the particular ratios that are used for comparison with other findings, on the results of such comparisons, and on their applicability to other situations. Each of these factors will be considered in turn.

The accuracy of the effectiveness estimates was limited by practical necessity in 4 ways. Firstly, some 36 children moved out of the area before the study's completion or were otherwise not included in the mortality rate calculation. Secondly, differences in mortality and morbidity rates between PHC and non-PHC villages may vary with the severity of transmission during the season in question. Thirdly, these differences may be due to factors other than the interventions. Finally, the proportion of unconfirmed cases is likely to grow as the average duration of each episode falls (and fewer coincide with the field assistants' visits). In PHC villages appropriate treatment was probably administered more promptly. Thus, the ratio of unconfirmed to confirmed cases may have been higher in PHC villages, and estimates of cases averted based on confirmed cases alone may be too high. The mortality estimates do not suffer from the latter weakness.

Any imprecision in the effectiveness estimates is probably outweighed by inaccuracies in the cost estimates. The very high proportion of the costs attributable to chemicals renders estimated cost and cost-effectiveness ratios exquisitely sensitive to permethrin and Maloprim® prices. By the same token, estimated costs are relatively insensitive to other price changes. For example, the predominance of annually recurrent costs ensures that cost-effectiveness numbers are insensitive to real discount rate changes. Furthermore, since the chemicals are used in approximately the same amounts per child protected, there are few technical returns to scale. That is, physical resources used per child are insensitive to the size of the programme. However, the unit price of permethrin delivered to tropical ports is likely to be highly sensitive to the quantity ordered. Here the price quoted for the small amount required for the present study has been used. If the programme were to be repeated on a larger scale, and if the permethrin price fell accordingly, the costs and cost/effectiveness ratios would decline markedly.

Implementation cost/effectiveness ratios may not be the most appropriate basis for health planners' decisions. If the control of 2 diseases has similar implementation cost/effectiveness ratios and one condition's treatment is more expensive, then the health budget is more efficiently spent on the control of the more expensively treated disease, so saving treatment costs. However, such refined cost-effectiveness estimates are likely to become relevant only when an alternative option for health planners is known with confidence to have an implementation cost-effectiveness so close that differences in treatment cost alter their ranking. Similarly, the value of avoiding the average costs of caring for patients should in principle be included in the health planner's decision making. However, in practice they are likely to be too small to affect conclusions. Nevertheless, the costs of ceremonies and mourning brought forward by a premature death are large, regardless of its cause. They should not affect health planners' choices between diseases with similar case-fatality rates, but are comparatively so large that they should perhaps influence allocation of resources between control of diseases with high case fatality rates and control of more benign conditions.

Direct comparison with the results of other intervention trials is hindered by the scarcity of comparable figures. What data there are suggest that net impregnation is a highly cost-effective means of averting childhood death in The Gambia. For instance, WALSH & WARREN (1979) found an average cost of averting child and infant deaths by mosquito control to be over 3·5 times greater. Indeed, even on the comparatively small scale at which the Gambian project was implemented, net impregnation may match immunization's cost per death averted. BARNUM (1980) and BARNUM *et al.* (1980) found that the implementation cost per death averted by immunization in Indonesia was similar at around $180 and in Kenya only moderately lower at $120. Similarly, the cost of saving discounted years of healthy life by net impregnation compares favourably with other interventions; on this measure net impregnation ranks with bacillus Calmette-Guérin immunization in high risk environments amongst the most cost-effective preventative interventions, surpassed only by measles immunization and vitamin A supplementation (JAMISON & MOSLEY, 1992). Meanwhile, at around $28, the cost of averting an episode by net impregnation in The Gambia is about one-tenth of the sum spent in the Garki project using case detection and treatment and vector control (MOLINEAUX & GRAMICCIA, 1980).

Adding chemoprophylaxis to a net impregnation project may increase the estimated cost per death averted but, in The Gambia, it sharply reduced the estimated cost per episode averted: once the expense of the impregnation has been incurred, the additional cost per additional episode averted is around $10. At this level it matches the lowest estimates of the cost of averting diarrhoea episodes by promoting breast feeding (PHILLIPS *et al.*, 1987).

Thus, comparison of the cost-effectiveness numbers derived in this paper with the other available figures suggests that bed net impregnation is an efficient means of improving the health of rural Gambian children. Moreover, it has the additional advantage of protecting individuals from the nuisance of bedbugs, head lice and ticks (LINDSAY *et al.*, 1989).

The high prevalence of bed net use in rural areas of The Gambia is probably unusual; in other countries nets may have to be bought before they can be dipped. Moreover, more nets may be necessary than there are children to protect; in the PHC villages there were approximately 2·3 nets per child under 5 years old. If new nets were to be imported from south-east Asia, cost $3·70 and last 6 years, then their purchase would increase the estimated annual implementation costs of net impregnation by 31%, taking the cost per death and per episode averted to $245 and to $37 respectively. The costs of the combined strategy would rise by 23%, and its corresponding cost-effectiveness figures to $317 per death and $24 per episode averted. If, instead, a representative price of locally made nets (D77, $9·77) is used, the annual costs of net

impregnation rise 81% and the cost per death and per episode averted rise to $339 and $51 respectively. The corresponding figures for the combined strategy would then be 61%, $414 and $31 respectively. At these levels both strategies remain attractive. But the assumption of equal effectiveness is clearly a weak one, especially if bed net use is strange to the population.

In The Gambia and the neighbouring sub-Sahel, transmission of malaria is restricted largely to the rainy season and shortly afterwards and adults enjoy clinical immunity to the disease. Elsewhere transmission patterns and the extent of natural immunity are different. This may affect the relative cost-effectiveness not only of net impregnation and other interventions but also of alternative insecticides. Thus, further studies in other representative areas will be necessary to confirm the economic efficiency of bed net impregnation and to define the most appropriate procedure in each instance.

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Estudio II: Estudio de la seguridad, inmunogenicidad y eficacia de la vacuna de la malaria denominada SPf66 en niños tanzanos de 1 a 5 años de edad.

Diseño del ensayo clínico. A trial of the synthetic malaria vaccine SPf66 in Tanzania: rationale and design. Alonso PL, Tanner M, Smith T, Hayes RJ, Armstrong Schellenberg JRM, Lopez MC, Bastos de Azevedo I, Menendez C, Lyimo E, Weiss N, Kilama WL, Teuscher T *Vaccine* 1994;**12**:181-186

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A trial of the synthetic malaria vaccine SPf66 in Tanzania: rationale and design

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The development of a safe, affordable and effective malaria vaccine to form part of control schemes in malaria endemic countries is a priority for researchers and public health officials. SPf66 is the first malaria vaccine to have shown partial protection against natural challenge in a phase III trial carried out in a hypoendemic area of Colombia. This paper describes the rationale and design of the first field trial of SPf66 outside South America, and the first to be conducted in an area of high perennial transmission.

Keywords: Malaria vaccine; phase III; Africa

Malaria remains one of the most important infectious diseases of humankind, responsible each year for around 100 million clinical cases and about a million deaths, the latter mostly among young children in sub-Saharan Africa. The development of an effective vaccine against the most important malaria parasite, *Plasmodium falciparum*, would be a major public health advance, but has been hindered by the complexity of the life cycle of the parasite. Nevertheless, several molecules from the pre-erythrocytic, asexual and sexual stages have been identified as potential vaccine candidates, aimed at eliciting protection against infection, clinical illness and/or transmission[1].

The first attempt to immunize humans against sporozoites was carried out as long ago as 1936[2], but it was in the 1970s that the findings in the sporozoite-immunized rodent and simian models were successfully applied to human malarias. In studies at the University of Maryland and the Naval Medical Research Institute in the USA, human volunteers immunized with irradiated sporozoites were shown to be protected against *P. falciparum* and *P. vivax* infection[3–5]. Since then, the pre-erythrocytic stage has been the target of intensive research, development and testing. However, human trials using recombinant or chemically synthesized vaccines have shown no evidence of protection[6–8].

Although considerable research has been carried out on blood stage antigens, a number of which are considered to be promising vaccine candidates, comparatively few human trials have been performed. Subunit vaccines combining pre-erythrocytic and asexual stage antigens have also been developed. These have been the only vaccines to undergo large-scale field trials.

The first such subunit multi-stage malaria vaccine to undergo phase III field trials was SPf66. This is a synthetic peptide consisting of amino acid sequences derived from three asexual stage proteins (85, 55 and 35 kDa) linked by pNANP sequences derived from the circumsporozoite protein of the *P. falciparum* parasite. The molecule was designed and synthesized by Dr M.E. Patarroyo[9,10] at the Instituto de Inmunologia, Hospital San Juan de Dios, Bogota, Colombia. Field trials carried out in Venezuela[11] and in Colombia[12] established the safety and immunogenicity of SPf66. However, limitations in the design of these trials precluded a clear demonstration of protective efficacy.

In June 1990, a WHO/PAHO *ad hoc* committee visited Bogota and concluded that the chemical production of the SPf66 peptide was of high quality, and that the safety and immunogenicity of the product were well established. However, the evidence regarding the protective efficacy of SPf66 was inconclusive, and the committee recommended that randomized placebo-controlled trials should be carried out urgently among children living in areas of high transmission, for example in Africa[13].

Following this recommendation, Dr Patarroyo asked the Spanish Science Council (Consejo Superior de Investigaciones Cientificas: CSIC) to promote the development and execution of an independent field trial of SPf66 in an area of high malaria transmission in Africa. In January 1992, the Swiss Tropical Institute, the Ifakara Centre/National Institute of Medical Research of Tanzania, the London School of Hygiene and Tropical Medicine and the Spanish Science Council (CSIC) agreed to jointly develop a protocol and carry out a trial of SPf66 in the Kilombero District of Tanzania, where the Ifakara Centre, the former Swiss Tropical Institute Field

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Laboratory, an affiliate of the National Institute of Medical Research has been conducting research on malaria transmission, control and immunoparasitology since the 1960s[14–17]. This trial is now in progress.

Meanwhile, research continued in South America, and a randomized placebo-controlled trial was completed in La Tola, Colombia[18]. This provided the first published evidence from a phase III trial showing that parenteral immunization with SPf66 can reduce the risk of clinical malaria in populations subject to natural exposure.

These results have provoked considerable interest in the independent trial in Tanzania, which is the first phase III trial of SPf66 outside South America, and the first to be conducted in a highly endemic area with year-round transmission. This paper describes and discusses the vaccine product and study design which are being used for this field trial.

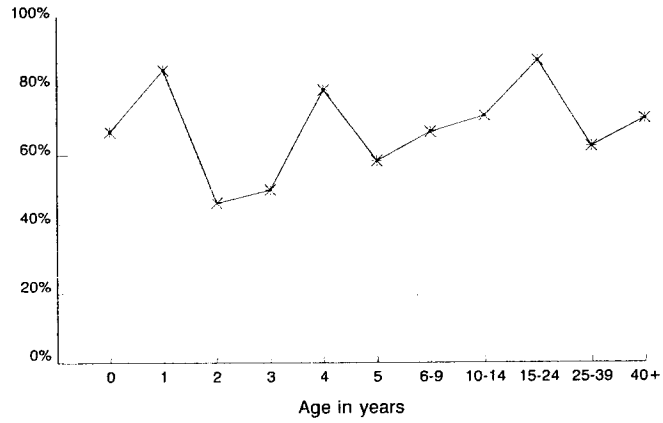


Figure 1 Prevalence of antibodies against SPf66 and constituent peptides

THE VACCINE

SPf66 is a synthetic hybrid polymer solubilized in sterile saline solution and adsorbed onto aluminium hydroxide. The monomer unit is a chemically synthesized peptide of 45 amino acids. The monomer unit is allowed to polymerize under controlled conditions linking monomers end to end through disulfide bonds and side to side through sulfoxyl bonds.

The product in use in Tanzania is SPf66 of GMP grade, peptide batch 09, synthesized in September 1991 at the Instituto de Inmunologia, Hospital San Juan de Dios, Bogota. The polymer underwent quality testing at CSIC Laboratories in Madrid and Granada, Spain, and was found to comply fully with the specifications established by Dr Patarroyo. The polymer was then formulated and bottled at Llorente SA, Madrid. The finished product was shown to be atoxic, sterile and immunogenic in rodents and rabbits, and is under clinical trial licence by the Direccion General de Farmacia, Ministerio de Sanidad y Consumo, Madrid, Spain. Further information on the peptide and its formulation is provided elsewhere (Lopez *et al.*, in preparation).

The vaccine has been bottled in individual clear glass syringes at two different volumes, 0.5 ml (2 mg peptide) for children 5 years and above, and 0.25 ml (1 mg peptide) for children aged 1 to 4 years in order to ensure full binding. The syringes are packed in individual amber blisters and shaken before opening. The vaccine is administered by subcutaneous injection on days 0, 30 and 180. Tetanus toxoid (Lorente SA batch 10/92, 16792/1/10) is used as a placebo on the first dose while aluminium hydroxide is used on the second and third doses. The placebo has been bottled and packed in identical volumes, syringes and blisters to those of the vaccine.

THE STUDY SITE

The phase III trial will be carried out in the village of Idete, 20 mg west of the town of Ifakara, in the Kilombero District of Morogoro Region, Tanzania. The village is in the Kilombero River plain, and most inhabitants are subsistence farmers growing maize and natural irrigation rice. Malaria transmission, especially of *P. falciparum*, is intense. *Anopheles gambiae* (sensu lato) and *A. funestus* are the two main vectors. Although mosquito densities are highly seasonal, transmission occurs all year round, and parasite prevalence shows no marked seasonality[17]. It is estimated that the number of infective bites per person per year exceeds 300.

Facility-based early detection and treatment with chloroquine is the main strategy of malaria control in the district. This has led to a high consumption of anti-malarial drugs. About 15–20% of parasites show R-II chloroquine resistance[19].

Levels of natural antibodies to the SPf66 construct have been determined in sera obtained in the course of other studies from an age-stratified random sample of individuals living in nearby communities in the same district. Antibody levels were measured using a FAST (Falcon Assay Screening Test)–enzyme-linked immunosorbent assay technique[20]. These analyses established that individuals living in the area and subject to natural challenge recognize the SPf66 construct (*Figure 1*).

THE INSTITUTIONS

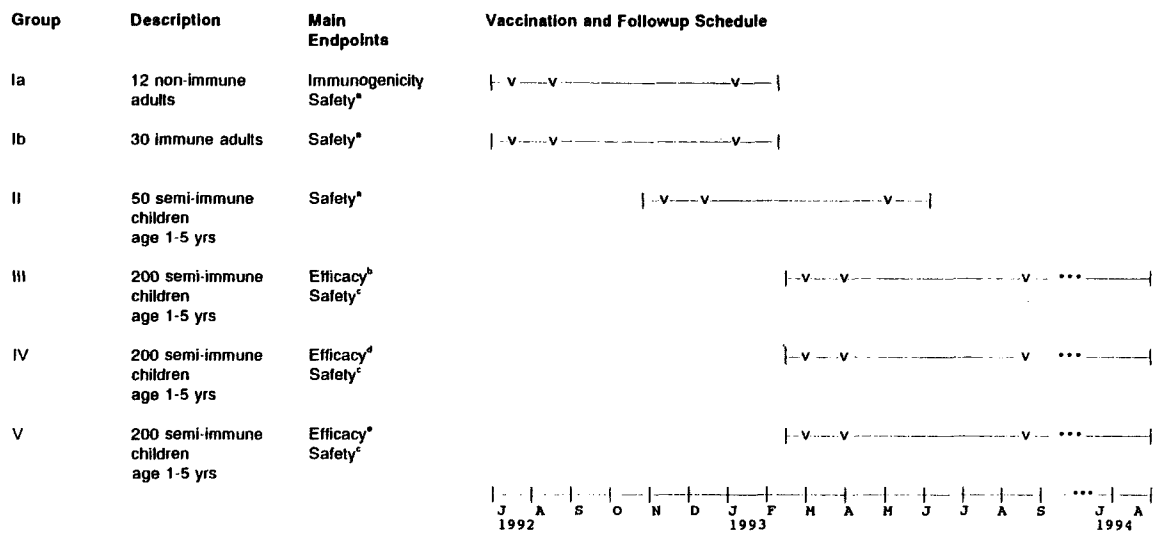
The trial is the result of a collaboration between the Ifakara Centre and the National Institute of Medical Research, Tanzania, the Swiss Tropical Institute, Basel, Switzerland, the London School of Hygiene and Tropical Medicine, UK, the Instituto de Parasitologia (CSIC) Granada, Spain and the Fundacio per a la Recerca Biomedica, Hospital Clinic i Provincial, Barcelona, Spain.

The Ifakara Centre, an affiliate of the Tanzanian National Institute of Medical Research, implements and administers the trial, while the collaborating institutions provide professional and technical support. The protocol has been reviewed, approved and is being funded by all the participating institutions together with the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

Three principal investigators (T.T., M.T. and P.L.A.) jointly bear responsibility for the adequate execution of all aspects of the trial.

STUDY DESIGN AND SCHEDULE OF THE TRIAL

The Tanzanian study design corresponds broadly with published recommendations[21] and with comprehensive



[a]Detailed clinical observation daily for 10 days following each dose; clinical laboratory testing on all blood samples.
[b]Passive case detection (PCD); Incidence of parasitaemia between dose 2 and dose 3.
[c]Local and systemic reactions monitored after each dose.
[d]PCD; Incidence of parasitaemia following dose 3; Immunogenicity one year after dose 3.
[e]PCD; Active Case Detection (ACD); Immunogenicity following each dose.

Figure 2 Study time plan and follow-up schedules

WHO guidelines for the evaluation of vaccines against *P. falciparum* malaria[22,23]. The study protocol was developed jointly by the investigators, and agreed and signed by all investigators and trial monitors. The protocol details both technical and organizational aspects of the trial.

The major purpose of a field trial of a new malaria vaccine is to produce unambiguous evaluation of the protection conferred against malaria and of any adverse effects of vaccination. We aim to achieve this by a randomized double-blind placebo-controlled trial of vaccine safety and efficacy to be undertaken in 600 children aged 1–5 years living in the study village.

SPf66 has undergone extensive testing in trials carried out in South America, and has been shown to be safe, with mild reactions occurring in less than 6% of individuals after three doses[9]. However, it is possible that it might induce a higher incidence of side-effects when administered to children living in a highly endemic area, who may have been exposed to malaria infection many times prior to vaccination. We therefore conducted two preliminary studies to assess safety and immunogenicity in this Tanzanian population before embarking on the main trial. Both preliminary studies were randomized, double-blind and placebo-controlled. Continuation from each stage of the trial to the next is conditional on the prior approval of the trial monitors.

Preliminary testing and assessment of safety and immunogenicity

The first of the preliminary studies commenced in July 1992. Thirty healthy male adult volunteers from Ifakara town (Group Ib, *Figure 2*) were recruited, screened and, if eligible, immunized with vaccine or placebo. Detailed and intensive clinical observation for 10 days following each dose was supplemented with clinical laboratory tests performed on blood samples collected at baseline and at defined intervals. These included haematocrit, full blood count, glycaemia, liver function, creatinine, proteinuria and auto-antibodies. In addition, 12 non-immune adult male Caucasian volunteers (Group Ia, *Figure 2*) were immunized and followed in the same way. The main objectives were to evaluate the safety of the vaccine in adults in this area (Group Ib) and the immunogenicity of the product in Ifakara after transport and storage (Group Ia). The final results of these studies will be available shortly (Teuscher *et al.*, in preparation).

After scrutiny of the initial results from Groups Ia and Ib, the trial monitors approved commencement of the second preliminary study. This began in November 1992, and involved 50 children aged 1–5 years from Ifakara town (Group II). The objective was to evaluate the safety of the vaccine in children in this area. The study procedures were as for Groups Ia and Ib (Teuscher *et al.*, *Vaccine*, 1994, in press).

Phase III trial: Objectives and endpoints

On the basis of findings from Groups Ia, Ib and II, the trial monitors approved commencement of the main phase III trial in the village of Idete in February 1993. The primary objective of this trial is to determine whether immunization with three doses of SPf66 reduces the incidence of clinical episodes attributable to malaria or the prevalence and intensity of parasitaemia in Tanzanian children aged 1–5 years, and to estimate the level of protection achieved. Secondary objectives are (i) to measure any immediate or delayed side-effects associated with the administration of SPf66 in a semi-immune population and (ii) to assess the immunogenicity of each dose of SPf66.

A critical aspect in the design of malaria vaccine trials is the choice of appropriate endpoints against which to assess the efficacy of the vaccine. Since there are no *in vitro* assays that are predictive of protective immunity in humans, evaluation of efficacy must be based on the diagnosis of malaria infection or disease in the individuals under study. Vaccines acting against different stages of the parasite life cycle may require different endpoints. Thus pre-erythrocytic vaccines might aim to prevent parasitaemia, and the incidence of new infections may be

the simplest endpoint to evaluate. Vaccines against the asexual blood stages of the parasite might have less impact in preventing parasitaemia, but may inhibit parasite levels such that signs and symptoms of malaria are less likely to develop, or such that the duration and severity of 'clinical malaria' are modified.

To assess the impact of the vaccine on clinical malaria, a suitable definition of this endpoint has to be developed. In areas where malaria is not highly endemic, and in non-immune migrants to endemic areas, the diagnosis of clinical malaria is relatively straightforward, and is made in those developing fever and other signs and symptoms suggestive of malaria, and who have malaria parasites in their blood. However, in areas of high malarial endemicity, where there is a high prevalence of parasitaemia among asymptomatic individuals, the definition is problematic. To assume that a child who presents with fever and who has parasitaemia is ill from malaria is not valid, and will result in over-diagnosis. Such a definition lacks specificity, and in a vaccine trial would be expected to bias estimates of efficacy towards zero.

A more specific definition can be obtained by defining clinical malaria as fever together with a high parasite density, since such parasitaemias are much less common in asymptomatic individuals. In the Idete trial, appropriate cut-offs to define high parasite density will be determined during the course of the trial by comparing densities in fever cases with those in asymptomatic children of the same age[24].

In order to evaluate all the required endpoints, the 600 children in the Idete trial have been randomized by household into three follow-up groups (Groups III, IV and V) which will undergo differing follow-up schedules (*Figure 2*).

Clinical malaria episodes will be recorded through both active case detection (ACD) and passive case detection (PCD). PCD will be carried out by screening all children in the three study groups (III, IV and V) when their parents or guardians take them to the local dispensary reporting that they are sick. All children with an axillary temperature of 37.5°C or higher, or with a history of fever during the past 24 hours, will have thick and thin blood films made for malaria parasites, and the packed cell volume determined as a measure of disease severity.

ACD will be carried out in a subsample of 200 children (Group V). All children in this group will be visited weekly from the time they receive the second dose until the end of the study. A brief morbidity questionnaire will be administered and the axillary temperature recorded. If the temperature is 37.5°C or higher, or if fever during the past 24 hours is reported, thick and thin blood films will be prepared. All fever cases detected by ACD or PCD are treated at Idete dispensary following national guidelines.

SPf66 includes the NANP repeat sequence of the circumsporozoite coat protein of *P. falciparum*. Although unlikely[6,7,8], it is possible that SPf66 might have an anti-sporozoite effect. All study children will receive a curative dose of sulfaxodine–pyrimethamine before each vaccination in order to optimize immune response to the vaccine. This will allow us to measure the incidence of subsequent *P. falciparum* infection in defined subgroups of children (*Figure 2*).

To address the secondary objectives of the study, the occurrence of any side-effects that may be associated with the vaccine will be recorded by continuous monitoring of children presenting to the dispensary or to the hospital in Ifakara town. Antibody response to the vaccine will be measured in all children at baseline and after the third dose and at other times in specified subgroups of children, allowing evaluation of the immunogenicity of each dose and of the correlation between antibody response and protective efficacy.

Chloroquine consumption as a measure of the drug pressure in the study village will be monitored by testing urine samples from a subsample of the study children, using a high performance thin layer chromatography assay[25]. In order to quantify the pressure of infectious challenge, the sporozoite inoculation rate will also be estimated.

Randomization and study size

The vaccine syringes, which are individually numbered, were randomized to either vaccine or placebo, and allocated sequentially to the children in each follow-up group. Random allocation of study children to the three follow-up groups (III, IV and V) was done by household. The randomization was performed by the trial monitors, who also hold the randomization code.

Sample size estimates have been calculated for each of the endpoints (efficacy, immunogenicity, safety). Allowing for withdrawals and refusals, 600 children will give the study more than 90% power to detect a 50% reduction in the incidence and prevalence of overall and high density parasitaemia, or a 50% reduction in the incidence of clinical malaria during the year following administration of the third dose, at a 5% significance level.

Ethical considerations

Three essential issues must be addressed when considering a trial in humans of a new vaccine. Firstly, the need for such a vaccine must be established, and the need to test it in the proposed population. Malaria is a major cause of morbidity and mortality in children in sub-Saharan Africa, and a safe effective vaccine would therefore have major public health significance. Moreover, given the ecological, sociological, political and economic conditions prevalent in this region, a blood stage vaccine that decreased the severe consequences of malaria infection without impairing the development and boosting of natural immunity may have advantages over alternative types of vaccine. The safety and efficacy of SPf66 have not yet been evaluated in Africa, where levels of endemicity are much higher than those found in South America.

Secondly, the investigators and their collaborators should be satisfied with all data provided on the product, including pre-clinical and clinical data, especially those referring to safety and toxicity.

Thirdly, all trials should be carried out under the guidance and approval of recognized ethical committees. The ethical committees of all collaborating institutions and of the World Health Organization have reviewed and approved the study protocol.

Furthermore, the trial is being monitored by an independent group of trial monitors appointed by the participating institutions and funding agencies, who have approved the study protocol and performed the randomization and who hold the code, continuously

assess the progress of the trial and its results, and are able to terminate the trial at any point, or to prevent the progression of the trial from one stage to the next. This group incorporates clinical, statistical, bioethical and malariological expertise.

Participation in all parts of the study is subject to the informed consent of the individuals, and in the case of children, of their parents or guardians. Clear and unambiguous information on procedures and any possible risks is presented in the local language and in a way that is understood by all those from whom consent has been sought. Full understanding of the most important features and implications of participation is checked individually through key questions.

CONCLUSIONS

Over the past few years, several candidate malaria vaccines have been developed and tested in humans. Most have targeted the pre-erythrocytic stages of *P. falciparum* in the hope of eliciting an immune response that prevents infection. If such a vaccine were completely effective, it would destroy all sporozoites and liver stages of the parasite before they could mature to cause a blood-stage infection, and therefore prevent all clinical manifestations of malaria and its transmission. However, such a vaccine would be useful for residents of malaria endemic areas only if it provided long-lasting protection, or if immunity could be boosted by natural exposure to malaria, neither of which seem likely. It is envisaged that if an effective pre-erythrocytic vaccine becomes available, its main value will be for tourists and other short-term visitors.

In contrast, a blood-stage vaccine is intended to control parasite numbers after their release from the liver, thus preventing or reducing malaria-related morbidity and mortality. Immunity provided by a blood-stage vaccine might be long-lasting, might not signficantly reduce transmission and might be boosted by natural exposure to malaria. If such a vaccine were safe, effective and affordable, it would constitute a valuable malaria control tool for populations living in malaria endemic areas, and particularly in sub-Saharan Africa where more than 90% of the world's malaria cases and deaths occur.

The investigators conducting the vaccine trial in Tanzania are concerned with the development and scientific evaluation of malaria control tools, including vaccines, that are of potential use to the populations of malaria endemic countries. SPf66 has been shown to induce partial protection in an area of Colombia that is of low endemicity by African standards. The vaccine now needs to be evaluated in a range of epidemiological and geographical conditions, covering different intensities of transmission and allowing for antigenic variability of the parasite. The Tanzanian trial will therefore complement, rather than replicate, the results of the Colombian studies, providing data on the potential of SPf66 as a malaria vaccine in conditions of intense year-round malaria transmission.

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The trial monitors are C. Alonso Bedate, CSIC, Centro de Biologia Molecular, Universidad Autonoma de Madrid, Spain; M.E. Molyneux (WHO/TDR monitor), Liverpool School of Tropical Medicine, UK; W.F.K. Mpanju, Commonwealth Regional Health Community Secretariat, Arusha, Tanzania; P.G. Smith, London School of Hygiene and Tropical Medicine, UK.

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Caracterización de la molécula quimérica SPf66 usada como vacuna de malaria.

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Characterization of SPf(66)n: a chimeric molecule used as a malaria vaccine

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SPf66 is a chemically synthesized 45 amino acid peptide derived from fractions of four different proteins of Plasmodium falciparum *(83, 55 and 35 kDa and CS, the circumsporozoite protein) that elicits a protective immune response against malaria. In this paper we show the characterization of the SPf(66)n in batch 9 to be used in a field trial in young children at Ifakara in Tanzania. The analysis of SPf(66)n indicates that it is highly soluble in water and that the amino acid composition and sequence corresponds to that designed for the synthesis of the polypeptide. The packed product has a molecular weight ranging from 10 to 25 kDa. It is pure, free of metallic contaminants, atoxic and stable at 4°C. The antibodies raised against this product in rabbits recognize the individual antigenic determinants of the molecule and the native epitopes of merozoites.*

Keywords: SPf(66)n; synthetic malaria vaccine; immunogenicity; formulation

Plasmodium falciparum is one of the aetiological agents of malaria. This parasitic disease, endemic in many areas of the world, at present affects more than 300 million people. There is an estimated 3 million deaths due to this disease annually. In spite of the technological developments in the fields of immunology, protein chemistry and molecular biology and the enormous efforts made by a great number of researchers, the development of a vaccine effective against malaria has been a slower process than one could have hoped. The Colombian research group under the direction of Dr Manuel E. Patarroyo has developed a synthetic molecule based on epitopes from various antigenic proteins of the merozoite form of *P. falciparum*[1,2]. Various experimental laboratory studies and field trials have shown that this molecule is safe, immunogenic and that it induces protection[3–9]. This particular vaccine integrates only certain antigenic determinants of the parasite proteins. Peptide-based vaccines, like SPf(66)n, may allow the design of molecules that include relevant B and/or T epitopes which can stimulate appropriate immune responses while excluding the fraction of a particular protein which might trigger unwanted effects.

Following the recommendations of a WHO *ad hoc* committee and the OPS (Pan American Health Organization, June 1990) a double-blind randomized placebo-controlled protocol for a phase III efficacy trial was developed in collaboration with various European and African scientific institutions. The trial is being carried out at the Ifakara centre in Tanzania (Africa) with children aged between 1 and 5 years[10]. The present study reports the results of the chemical and immunological characterization of the SPf(66)n molecule being used in Tanzania.

MATERIALS AND METHODS

Synthesis of the SPf(66)n molecule

The molecule was produced under GMP conditions in the Laboratory of Chemistry of the Instituto de Immunologia, Hospital de San Juan, Bogota, Colombia. The molecule has been constructed by chemical linking of specific fragments of the 83, 55 and 35 kDa proteins of the parasite and the Asn-Ala-Asn-Pro sequence of the circumsporozoite (CS) protein. The molecule was synthesized using the solid-phase synthesis technique described by Merrifield[11] and modified by Houghten[12] as a single batch of 7 g (batch 9). In the hybrid molecule Cys residues were present in the carboxy and amino terminals to allow for polymerization under oxidation conditions. The polymerized product is called SPf(66)n. For comparative studies the SPf(66)n molecules from

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batches 7 and 8 were also used in order to determine the similarities or differences between batches.

Analysis of the amino acid composition and sequence of SPf(66)n

Reverse-phase high-performance liquid chromatography (h.p.l.c.) was used to determine the composition of the amino acids of the SPf(66)n polymer (batch 9). Before h.p.l.c. analysis, hydrolysis of the polymer was carried out at 110°C in 6 M HCl in 24 h. After neutralization with 0.1 M borax until alkaline, the OPT (*o*-phthaldialdehyde) derivatives were obtained using OPT as reagent. A C-18 column (Pharmacia) was used. The amino acids were detected by fluorescence. *Nor*-leucine was used as an internal standard. The retention time of the individual amino acid was determined using synthetic standards (Sigma). The amino acid composition of SPf66 (batches 7, 8 and 9) was also determined in an Aminoacid Analyzer (Kontron Analytical) following standard conditions. The Edman Begg technique[13] was used to determine the sequence of amino acids (Applied Biosystems Protein Analyzer) in a peptide synthesized without Cys in the amino and carboxy ends which was produced simultaneously with SPf(66)n containing Cys at both ends. The absence of Cys at both ends facilitates the sequence process because the formation of polymers and amides is avoided.

Antigenicity and immunogenicity of SPf(66)n

Two rabbits were inoculated with 750 μg of SPf(66)n (packed product). The control rabbit was inoculated with the corresponding dose of aluminium hydroxide in saline solution. The inoculation pattern was 0, 15, 30, 60, 80 and 100 days. The first dose was injected subcutaneously in the axillary region in ten different points. The rest of the injections were intramuscular with two equal aliquots in each leg.

The reactivity against SPf(66)n batch 9 and the individual epitopes forming the molecule in sera induced by SPf(66)n (packed product) was determined on days 50, 70, 90, 100, 110, 120 and 150 by the FAST-ELISA (Falcon Assay Screening Test-ELISA: Becton Dickinson Labware, Linco Park, NJ) technique according to the method described by Salcedo *et al.*[4]. Blood was obtained from the auricular artery. The reactivity of the sera obtained on days 110 and 120 was also analysed against SPf(66)n from batches 7 and 8. The antigen concentration was 1 μg/well. Absorbances ≥ 0.1 were considered positive. The 0.1 cut-off point was determined by addition of 5 standard deviations (s.d.) to the mean value obtained with preimmune sera.

Indirect immunofluorescence assays (IFA)

Late-stage schizonts from a continuous *P. falciparum* culture (FCB-2 strain), presenting a 10% parasitaemia[14], synchronized according to the method of Lambros and Vandenberg[15], collected and washed in sterile PBS (phosphate buffer 0.15 M, containing 0.15 M NaCl, pH 7.2) were used. The pellet of infected cells was suspended in fetal bovine serum (FBS): PBS (1:1 v/v) and left out to settle dry on the slide. The parasites were blocked for 10 min with 1% non-fat milk and incubated for 30 min with the sera induced by SPf(66)n (packed product). The reactivity was visualized by fluorescence microscopy using the $F(ab')_2$ fragment of a goat anti-rabbit IgG:FITC conjugate at a dilution of 1/100. Preimmune sera from rabbits were used as negative controls.

Chemical characterization

The solubility of the molecule in water was determined by the method of Lowry *et al.*[16] and spectrophotometry at 280 nm. Both methods were also used to determine the adsorption percentage of the polymer to $Al(OH)_3$. The degree of polymerization of SPf(66)n (batch 9) and the molecular weight were determined in 15% polyacrylamide SDS–PAGE in non-reducing conditions using standard proteins as markers. The molecule was labelled *in vitro* with [^{35}S]-methionine using the technique described by Browder *et al.*[17].

The purity of the SPf(66)n polymer was assayed by means of total reflection X-ray fluorescence analysis[18]. The purity of the packed product was also analysed by reverse-phase h.p.l.c. The SPf(66)n molecule (batches 7, 8 and 9) was also analysed by h.p.l.c. molecular exclusion. The detection was performed at 220 nm with an absorbance unit full scale (AUFS) sensitivity of 0.4.

Formulation and packing

First, 5.5 g of the SPf(66)n molecule (batch 9) were dissolved in 962 ml of 0.5% apyrogen saline solution (pH 6.7). After magnetic shaking for 10 min the solution was filtered through a 0.2 μm Millipore cartridge. Aliquots were taken to determine the protein concentration of the filtrate. Next, 192 μl of 10% phosphoric acid were added to readjust the solution to the initial pH value. Then, 413 ml of aluminium hydroxide, containing 6.67 mg ml^{-1} of Al, were added to the polymer solution and shaken for 1 h. The pH was readjusted to 6.8 with 4.5 ml of 0.5% NaOH. After centrifugation at 2000 rev min^{-1} (700*g*) at 4°C, aliquots from the supernatant were taken to determine the percentage of the polymer adsorbed to aluminium. The formulation and packing of the product in syringes were done at the Instituto Llorente (Madrid, Spain).

Toxicity and sterility

Twenty mice (Swiss strain) divided into five equal groups were used in the toxicity studies. Three groups were inoculated with 0.08, 0.16 and 0.24 mg of the SPf(66)n (packed product), respectively. The control groups were inoculated with aluminium hydroxide or with saline solution alone. The weight and the temperature of all mice were monitored daily for a period of 21 days. A histopathological analysis was done on day 21. Basic toxicity tests following the WHO and the Ph. European USP XXI norms were also conducted in guinea-pigs and mice on day 7 after inoculation of the product. The presence of organisms such as bacteria, yeast and fungi in the packed product was determined by addition of the product to specific culture media: thioglycolated broth (for micro-organisms in general); Saboureaud agar (for fungi and yeast); hydrolysed soya-casein and blood agar (for Gram-positive and Gram-negative bacteria). An enzymatic immunoassay diagnostic kit (Boehringer Mannheim® Cat. No. 1296744) was used to detect the presence of mycoplasms. Contaminating particles in ten randomly chosen samples of the packed product were also determined by two different observers.

RESULTS

Amino acid composition and sequence of SPf(66)

The data from the quantitative analysis of the amino acid content of the packed SPf(66)n (batch 9) product indicated that the composition corresponded to the sequence designed for the synthesis of the polypeptide (*Table 1*). These data are identical to those obtained 9 months earlier by analysis of the same polypeptide performed 10 days postsynthesis in a Kontron Analytical Aminoacid Analyzer (results not shown) showing the presence of Cys and Pro. The amino acid composition from batches 7 and 8, synthesized 24 and 12 months earlier respectively than batch 9, was also determined and gave identical results. The data indicate, therefore, that the synthesis process is highly reproducible, providing fundamental evidence of the absence of intermediary molecules in the final product. The absence of intermediary products during the synthesis was also shown by h.p.l.c. (95% purity). The similarity between the SPf(66)n polypeptides from batches 7, 8 and 9 and the purity of the products were further suggested by reverse-phase h.p.l.c. analysis. *Figure 1* B_1, B_2 and B_3 show that only one peak was detected and that the retention time of the product from each batch was the same.

Analysis of the amino acid sequence of the SPf66 molecule (batch 9), showed it to be identical to the designed one[3]. The sequence of the polypeptide is: Gly-Asp-Glu-Leu-Glu-Ala-Glu-Thr-Gln-Asn-Val-Tyr-Ala-Ala-Pro-Asn-Ala-Asn-Pro-Tyr-Ser-Leu-Phe-Gln-Lys-Lys-Glu-Lys-Met-Val-Leu-Pro-Asn-Ala-Asn-Pro-Pro-Ala-Asn-Lys-Lys-Asn-Ala-Gly. The presence of Asp, Asn, Glu and Gln in the proper site within the molecule resolves the limitations imposed by the amino acid composition analysis in which Asp-Asn and Glu-Gln can not be resolved.

Molecular weight determination of SPf(66)n

Figure 2 shows the migration of ^{35}S SPf(66)n (batch 9) in SDS–PAGE. There is a clear predominance of dimeric and trimeric molecues with a molecular weight of around 15 kDa. Protein bands of different intensities are also detected in positions corresponding approximately to molecular weights of 20 and 25 kDa. The faint protein band of approximately 5 kDa may represent the monomer. The range of the molecular weight from batches 7, 8 and 9 was further confirmed by h.p.l.c. molecular exclusion (*Figure 3*). The peak at a retention time of around 41 could represent dimeric and trimeric units while that at around 38 could be due to the larger size units. It is likely, therefore, that the degree of polymerization of SPf(66)n from batches 7, 8 and 9 is similar (*Figure 3*). Similar results were obtained when the SPf(66)n molecule from the packed product was analysed by the same method.

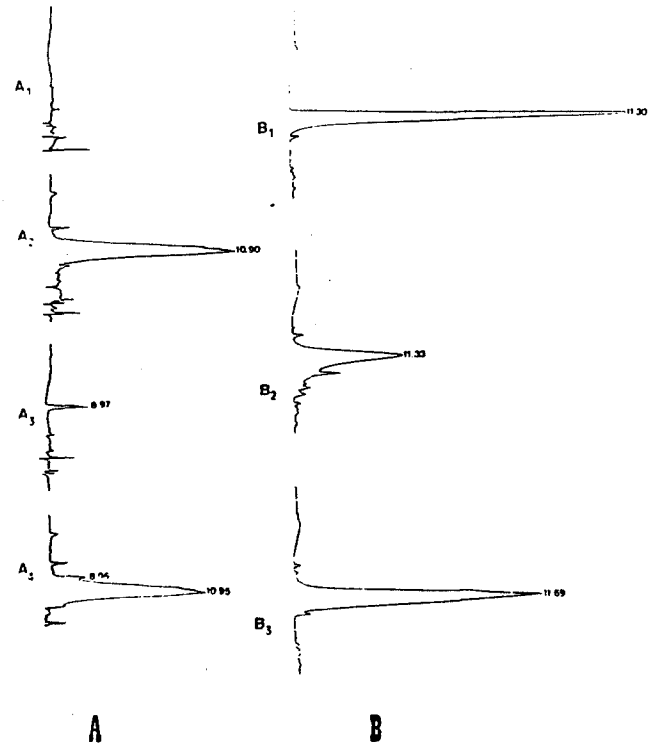

Figure 1 Reverse-phase h.p.l.c. of SPf(66)n. A C1–C8 Pro RPC HR 5/10 column (Pharmacia) was used with the following mobile phases: phase A: 0.1% trifluoroacetic acid in double-distilled water; phase B: 0.1% trifluoroacetic acid in acetonitrile. Elution: 0–100% B gradient in 30 min and a flow of 0.3 ml min^{-1}. The detection was done at 220 nm and a sensitivity of 0.1 (A) and 0.2 (B) AUFS. A_1, negative control; A_2, 100 μg of the packed SPf(66)n product; A_3, 1 μl of anisole; A_4, 100 μg of SPf(66)n + 1 μl anisole. B_1, SPf(66)n batch 7; B_2, batch 8; B_3, batch 9

Table 1 Amino acid content of the SPf(66)n molecule (packed product) obtained by reverse-phase h.p.l.c.

Amino acid	Retention time	Concentration (pmol)	Concentration (%)	Theoretical formula
Asp + Asn	2.45–3.07	161.31	7.80	8
Glu + Gln	5.85 + 5.05	84.19	4.10	6
Ser	7.79	23.71	1.15	1
Gly	11.51	51.70	2.35	2
Thr	12.05	20.68	1.00	1
Ala	15.73	153.03	7.40	7
Tyr	15.75	31.50	1.53	2
Met	20.50	28.61	1.38	1
Val	20.98	34.52	1.68	2
Phe	22.61	29.03	1.40	1
Leu	24.29–25.28	55.83	2.70	3
Lys	27.22	88.49	4.20	4

The name of the amino acid is indicated in the abbredivated form (glutamic acid and glutamine, and aspartic acid and asparagine elute together). A reverse-phase C18 column (Pharmacia) was used with the following mobile phases: (A) NaH_2PO_4 (0.05 M, 0H 5.5) containing 20% methanol and (B) NaH_2PO_4 (0.05 M, pH 5.5) containing 80% methanol. Elution: 0–10% B in 10 min, 10–85% B in 30 min, and 85–0% B in 5 min and 10 min re-equilibration. The amino acids were detected by fluorescence. The excitation wavelength was 365 nm and the detection was at 455 nm

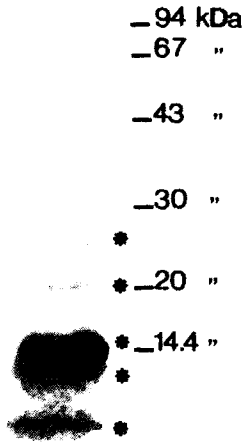

Figure 2 15% polyacrylamide electrophoresis of the SPf(66)n molecule labelled *in vitro* with ^{35}S methionine. An amount of 10 μg of the product was loaded into the gel. The radioactivity was visualized using an autoradiographic film. The exposure time was 60 h. The molecular weight standards are indicated at the left-hand side of the figure

Chemical characteristics of SPf(66)n

The lyophilized SPf(66)n is an amorphous white solid. Its solubility in water, obtained by manual shaking, is 8–9 mg ml^{-1}. The X-ray fluorescence determination showed any significant levels of metallic contaminants present in SPf(66)n (*Table 2*). The high level of S detected by this technique is due to the presence of Cys. Given that anisole is one of the major potential contaminants of synthetic polypeptides, occurring during the process of synthesis, reverse-phase h.p.l.c. analysis of the final packed SPf(66)n product was carried out in the presence and absence of anisole. *Figure 1* A_2 and A_3 show that no traces of anisole were detected in the final packed product. When the product was submitted to h.p.l.c. in the presence of anisole peaks corresponding to the anisole and to the polymer were observed (*Figure 1* A_4).

Formulation and packing of SPf(66)n

The formulated and packed product has a whitish appearance which sediments when it is left to stand. The percentage of adsorption of the polymer to aluminium was 78.9%. The concentration of aluminium in the packed product was 1.90 mg ml^{-1}. The number of syringes for adult doses of the formulated product was 950 and the number of syringes for children's doses was 1300. The mean actual volume of all the syringes was 0.530 ± 0.026 ml and 0.260 ± 0.013 ml as deduced from a sample of 10% of all the samples.

Toxicity

None of the animals (mice and guinea-pigs) inoculated with the product experienced any signs of weight loss or mortality during the 21 and 7 days respectively following injection with SPf(66)n. Twenty-one days after the molecule was injected the control mice experienced an average weight increase of 6.1 ± 0.72 g. The animals injected with the SPf(66)n molecule experienced an average increase of 6.3 ± 0.64 g. No histopathological signs of irregularities were observed in any of the organs from the animals inoculated with the SPf(66)n product. The temperature of the mice was 36.5–37°C in the control group as well as in the inoculated ones. Contamination of the product with bacteria, fungi or yeast was excluded as there was no growth of these micro-organisms in specific media inoculated with randomly taken samples of the final packed product. The absence of mycoplasms in the packed product was also confirmed. Contaminating particles were not detected.

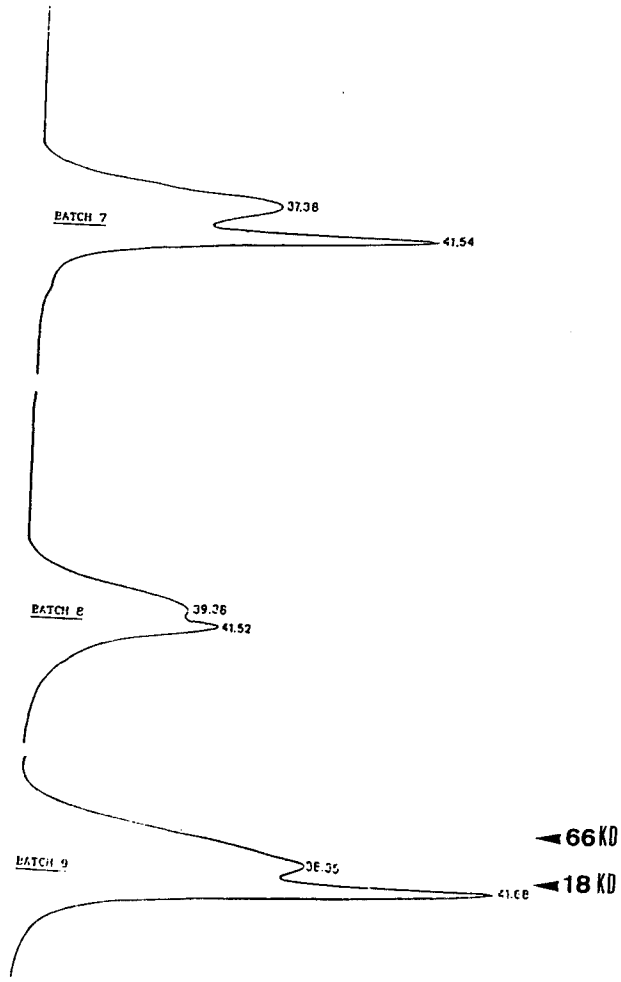

Figure 3 Molecular-exclusion h.p.l.c. of SPf(66)n. The quantity injected was 100 μg. The retention times of SPf(66)n are indicated above the peaks. Arrows indicate the retention times of the markers used (bovine β-lactoglobulin, 18 kDa and BSA, 66 kDa). A Superose 12 HR 10/30 column (Pharmacia) was used with a NaH_2PO_4, 0.05 M; NaCl, 0.15 M (pH 7.2) buffer as mobile phase at a flow rate of 0.4 ml min^{-1}. The detection was done at 220 nm and a sensitivity of 0.4 AUFS

Table 2 Levels of metallic ions detected per mg of SPf(66)n (batch 9)

Ion	Amount detected
Mn	16 ng
Fe	9 ng
Co	1 ng
Ni	25 ng
Cu	6 ng
Ca	0.6 μg
Cl	0.4 μg
Pb	0
Hg	0
S	23.3 μg

Trace elements were determined by means of total reflection X-ray fluorescence analysis. An X-ray generator, a fine focus tube and a multiple reflection module (EXTRA II) were used (Seifert & Co.). The energy-dispersive spectrometer with the Si(Li)-detector and the software were provided by Link Analytical Ltd (System AN-10000). Stroncio was used as the internal standard

Immunogenicity and antigenicity of SPf(66)n

It was observed that the SPf(66)n molecule from batch 9 is capable of inducing antibody titres of 12 800 against the whole molecule 20 days after the third dose. The titre increased to 25 600 10 days after the fourth and fifth doses. The antibody titre fell to 6400 20 days after the fifth dose. The antibody titre increased again to 25 600 10 days after a new dose of SPf(66)n was administered. The titre decreased to 6400 10 days later, remaining stable for at least 1 month. *Table 3* shows, moreover, that the antibodies raised by the SPf(66)n (packed product) recognize not only the SPf(66)n molecules from batch 9 but also those from batches 7 and 8 and that they do it at an identical dilution.

We have also analysed whether the anti-SPf(66)n antibodies raised from batch 9 recognize the individual fractions of the 83, 55, 35 kDa and the CS protein which form SPf(66)n. Titres of $\geqslant 400$ were detected only against the peptide of the 55 kDa protein and Asn-Ala-Asn-Pro (NANP) from the CS. Titres of 200 and 100 against the peptides from the 83 and 35 kDa protein, respectively, were detected only 10 days after the fifth and sixth doses. All these data indicate, therefore, that the SPf(66)n molecule is capable of inducing antibodies against itself and against the individual peptides which form the hybrid molecule. *Figure 4* shows, moreover, that the antibodies present in the sera from animals inoculated with SPf(66)n (packed product) recognize epitopes of the merozoite form of the parasite as detected by IFA.

DISCUSSION

The results presented in this paper show that the amino acid composition of SPf(66)n from batch 9 is that expected from its theoretical amino acid sequence[3] and that the SPf(66)n molecule from batches 7 and 8, independently synthesized within a period of 2 years, is identical to that of batch 9. This conclusion was further confirmed by reverse-phase h.p.l.c., which showed that the molecules from the three batches have identical behaviour and that no truncated intermediary products are generated during the synthesis (other peaks besides the main one would have been detected otherwise). The absence of intermediary products occurring during the synthesis was also revealed by analysis of the amino acid composition. The amino acid sequence analysis of the polypeptide showed, moreover, that the fragments from the 83, 55 and 35 kDa proteins and the NANP sequence from the CS protein, which have been described for the SPf(66)n molecule, are present in that product.

The absence of contaminant metallic ions was guaranteed by X-ray fluorescence spectrometry. The reverse-phase h.p.l.c. excluded, moreover, the presence of anisole in the packed product. The SPf(66)n molecule was seen to be highly soluble in water near to neutral pH. The packed product is atoxic and apyrogenic. We believe that the SPf(66)n molecules are highly stable for at least 2 years because the molecules from batches 7, 8 and 9 have similar reverse-phase and molecular-exclusion h.p.l.c. behaviour, amino acid composition and antigenic reactivity. The result of the analysis performed 9 months after formulation on the packed product (batch 9) maintained at 4°C further indicated that the packing process did not affect the quality of the product.

The molecular-exclusion h.p.l.c. studies of the SPf(66)n molecules from batches 7, 8 and 9 and the SDS–PAGE analysis showed that SPf(66)n has similar polymerization in all the batches, with a molecular weight ranging from 10 to 25 kDa. Thus, the molecules present in SPf(66)n could be of high enough molecular weight to behave as their own carriers. The results of the immunogenic and antigenic properties of SPf(66)n revealed that the polymerization of the molecule did not affect the structure of the individual epitopes present in the molecule because antibodies raised against the entire molecule recognize individual epitopes. The positive reactivity of anti-SPf(66)n induced by the packed product (batch 9) against SPf(66)n from batches 7 and 8 indicates that the same antigenic determinants are present in the molecules from all batches. Titres of 25 600 against SPf(66)n were reached 10 days after the fourth and fifth doses. This titre falls to a level of 6400 10 days later. Other authors have reported higher titres of antibodies against both the polymer and

Table 3 Reactivity of the anti-SPf(66)n raised by the packed product against SPf(66)n from batches 7, 8 and 9 and against the individual epitopes

		Anti-peptide					
Date	SPf(66)n (batch 9)	755	758	760	NANP	SPf(66)n (batch 7)	SPf(66)n (batch 8)
Preimmune	0	0	0	0	0	0	0
20 days post third dose	12 800	400	0	0	400	ND	ND
10 days post fourth dose	25 600	400	0	0	800	ND	ND
10 days post fifth dose	25 600	400	100	100	1 600	ND	ND
20 days post fifth dose	6 400	200	0	0	1 600	ND	ND
10 days post sixth dose	25 600	200	100	200	3 200	25 600	25 600
20 days post sixth dose	6 400	100	0	100	1 600	6 400	6 400
50 days post sixth dose	6 400	100	0	0	1 600	ND	ND

Anti-peptide 760 [CG**YSLFQKEKMVL**GC], 758 [CGYGG**PANKKNA**GC], 755 [CG**DELEAETQNVYAA**GC] and NANP [CGNAN**PNANP**NAN**PNANP**GC]
Numbers indicate the antibody titres
The amino acids present in the packed SPf(66)n product are indicated by heavy type
ND, not determined

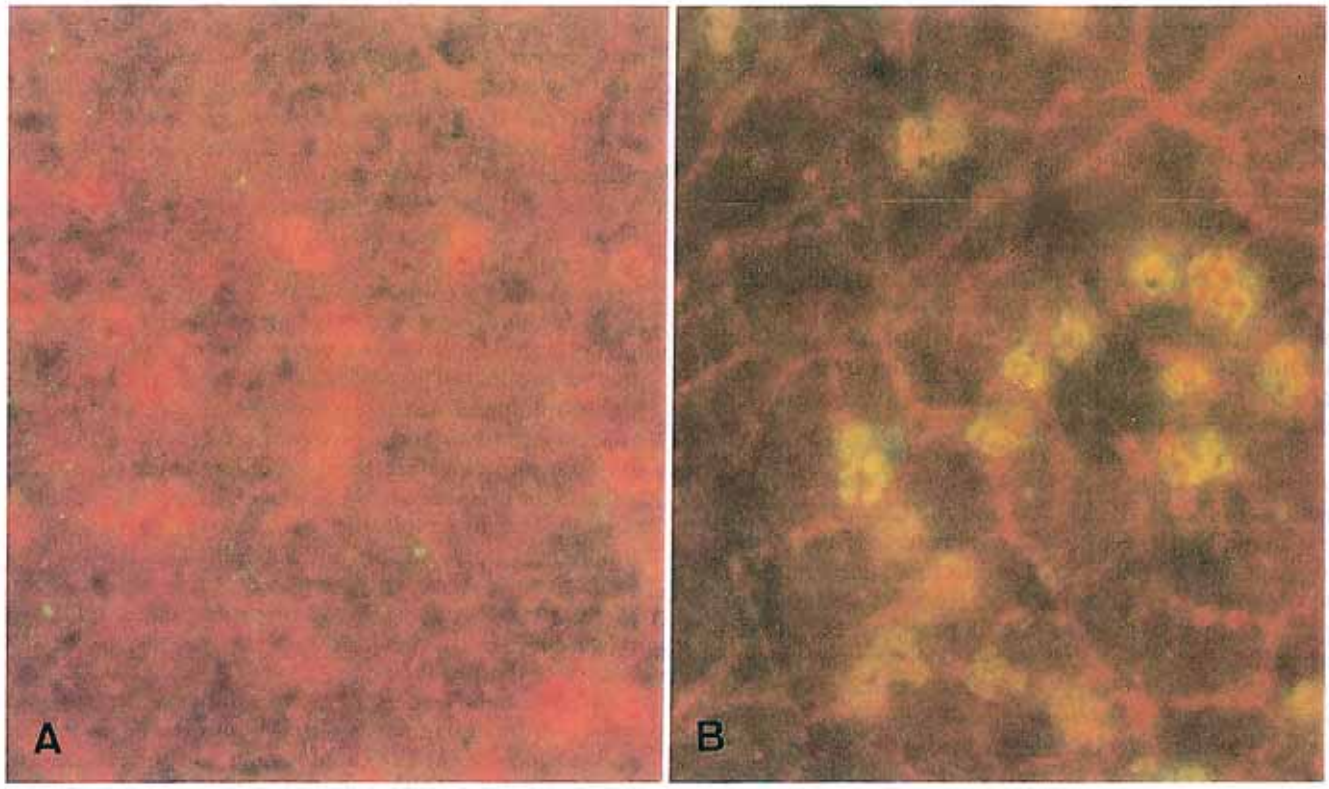

Figure 4 Immunofluorescence staining of parasites. (A) Parasites incubated with preimmune sera. (B) Parasites incubated with sera obtained from rabbits 10 days after the fifth inoculation of the packed SPf(66)n product at a dilution of 1/80

its constituents using the same SPf(66)n molecule using complete and incomplete Freund's adjuvant[19]. The lower titre values obtained in our case are probably due to the inoculation of the packed product in the absence of Freund's adjuvants, in an attempt to mimic the real situation of immunization in humans in which the vaccine was administered in the absence of adjuvants except aluminium hydroxide. This fact may be of crucial importance regarding vaccination because the antibodies raised against the packed SPf(66)n product in the absence of Freund's adjuvant recognize native epitopes in merozoites by IFA, in contrast to the data reported by Millet *et al.*[19].

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Evaluación de la seguridad e inmunogenicidad de la vacuna en Tanzania. SPf66, a chemically synthesized subunit malaria vaccine, is safe and immunogenic in Tanzanians exposed to intense and perennial malaria transmission. Teuscher T, Armstrong Schellenberg JRM, Bastos de Azevedo I, Hurt N, Smith T, Hayes R, Masanja H, Silva Y, Lopez MC, Kitua A, Kilama W, Tanner M and Alonso PL. *Vaccine* 1994;**12**:328-336

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SPf66, a chemically synthesized subunit malaria vaccine, is safe and immunogenic in Tanzanians exposed to intense malaria transmission

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As part of the first trial of the SPf66 malaria vaccine in Africa, three randomized double-blind placebo-controlled studies of SPf66 have been conducted in a highly endemic area of Tanzania. The objectives were to confirm that the product is immunogenic and safe in highly exposed individuals. Results from ten male adult expatriates indicated that the product used in Tanzania is at least as immunogenic as that used in Colombia. No major side-effects were observed in indigenous SPf66 recipients (18 adults, and 25 children aged 1–4 years). Anti-SPf66 antibody titres in all groups showed clear responses to three doses of the vaccine.

Keywords: Malaria; SPf66 malaria vaccine; Tanzania; safety; immunogenicity

The synthetic malaria vaccine, SPf66, developed in Colombia by Dr Manuel Patarroyo[1,2] has been shown to be safe and immunogenic in several studies in Latin America[1,3–5]. Results from a recent field trial carried out in an area of low malaria transmission in Colombia have indicated that it is also partially protective against clinical episodes of *Plasmodium falciparum* malaria in both adults and children[6].

However, most of the world's malaria infections, clinical episodes and deaths occur in subsaharan Africa[7]. The higher intensity of *P. falciparum* transmission and the existence of greater degrees of naturally acquired immunity, among other factors, might influence the efficacy of a malaria vaccine. Consequently, an independent, randomized, double-blind, placebo-controlled phase III trial to evaluate the efficacy of the SPf66 vaccine in Tanzania was launched. The rationale and design of this first trial in Africa have been presented elsewhere[2,8,9].

Not only the efficacy but also the tolerability and safety of SPf66 could be different in African children from that reported among non-immune Colombians. In advance of the large-scale vaccine efficacy evaluation in children, it was therefore considered necessary to determine the incidence and severity of hypersensitivity reactions or any other adverse effects in this population following vaccination with SPf66 and exposure to malaria. The immune response to SPf66 was assessed in a group of non-immune adults to confirm the immunogenicity of the product. Finally, it was also important to determine whether the product elicits a humoral immune response amongst individuals exposed to intense malaria transmission and with varying degrees of pre-existing immunity. This paper reports the results of these preliminary safety and immunogenicity studies.

MATERIALS AND METHODS

Study area

Ifakara town is in the Kilombero river valley in Southern Tanzania (08°S; 32°E). The setting and population characteristics have been described elsewhere[10]. Malaria is transmitted by *Anopheles gambiae s.l.* and *Anopheles funestus*. *P. falciparum* accounts for 80% of all malaria infections, and in a nearby village the average inoculation rate for this species has been estimated to be over 300 infectious bites per person per year[11]. Parasite prevalence shows no seasonality and by the age of 6 months 80% of infants are already infected.

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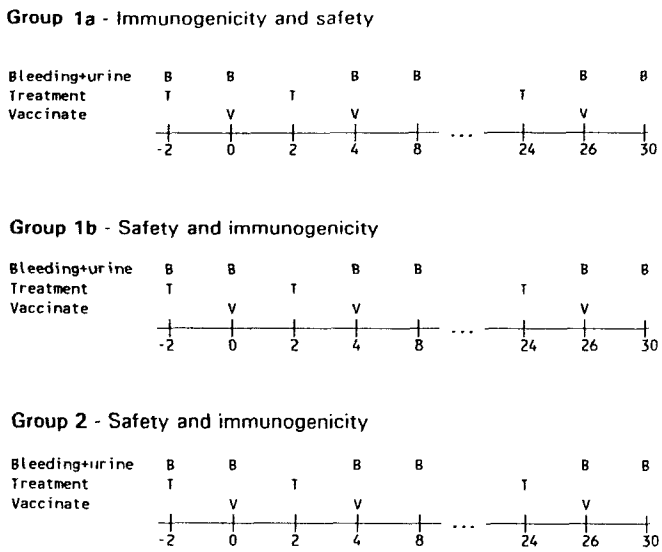

Figure 1 Schedules for vaccination, treatment, and bleeding (all time points presented as weeks pre- or postadministration of first dose: week −2 for groups 1a and 1b, 13 July 1992; for group 2, 26 October 1992). Bleeding+urine: at every occasion, haematology, biochemistry, anti-SPf66 antibody and urine were assayed. Autoantibody was screened for positivity at weeks 0, 8 and 30. Most of group 1a took regular malaria prophylaxis: those who did not, together with all of groups 1b and 2, were treated with sulfadoxine–pyrimethamine

Study design

The preliminary testing of safety and immunogenicity was carried out through three double-blind, randomized, placebo-controlled studies (*Figure 1*).

The study groups and objectives were as follows:

Group 1a – Evaluation of immunogenicity in non-immune adults. To test the immunogenicity of the product, 12 male Caucasian expatriates aged 18–55 were recruited (10 SPf66, two placebo).

Group 1b – Evaluation of safety in semi-immune adults. As a preliminary test of safety before vaccinating children, 30 male adult volunters aged 18–55 were recruited from the staff of St Francis Designated District Hospital and the Medical Assistant Training Centre in Ifakara (20 SPf66, ten placebo).

Group 2 – Evaluation of safety in semi-immune children. Fifty local children (male and female) aged 1–5 were recruited from families in the neighbourhood of Ifakara Centre (25 SPf66, 25 placebo)*.

Screening and informed consent

Two weeks before the first immunization round, volunteers were screened by a physician for the following inclusion criteria:

- Appropriate age range;
- No history of allergic predisposition leading to medical consultation and treatment;
- No acute condition warranting hospital admission;
- No chronic condition, such as epilepsy or sickle cell disease, which might make the subject unsuitable for enrolment;
- Packed cell volume $\geqslant 25\%$;
- No evidence of impaired liver or kidney function.

Concurrently, written informed consent endorsed by the relevant ethical committees was individually requested (in English for group 1a, in Kiswahili for groups 1b and 2) from adult volunteers or children's guardians by a procedure outlined in the study protocol[8]. Volunteers were given an identification card, including a photograph, for use during follow-up.

In order to apply each immunization to individuals free of blood-stage *P. falciparum* infection and to evaluate the safety of the regime planned for the efficacy field trial, all group 1b and 2 volunteers received a single therapeutic dose of 25 mg sulfadoxine and 0.75 mg pyrimethamine (SPM) per kilogram bodyweight 2 weeks before each immunization.

Volunteers could be withdrawn at any time during the studies for the following reasons: any systemic side-effect requiring more than symptomatic treatment; evidence of impaired liver or kidney function; movement away from the study area; or their own request.

Immunization

In all these trials SPf66 and placebo administration were planned for weeks 0, 4 and 26 (*Figure 1*).

The vaccine used was SPf66 peptide adsorbed to aluminium hydroxide. The placebo consisted of aluminium hydroxide, with tetanus toxoid (Llorente S.A. batch 10/92, 16679/1/10) added only to the first dose. The SPf66 peptide was of GMP grade, part of batch 09, synthesized at the Instituto de Inmunologia de Bogota, Colombia. The vaccine was formulated at Llorente S.A., Madrid, Spain and is under a clinical trial licence of the Ministry of Health, Spain, according to EC guidelines.

SPf66 vaccine and placebo were bottled in identical clear glass syringes at two different volumes, 0.5 ml (2 mg peptide) for adults and older children, and half that dose for children less than 5 years. SPf66 and placebo were injected subcutaneously into the left upper deltoid area for the first and third dose, and right deltoid for the second dose. Further details on the product used are given elsewhere[8,9].

Follow-up and laboratory methods

The main purpose of the follow-up was to compare the incidence of side-effects, paticularly immediate or delayed-type, or Arthus (type III), hypersensitivity reactions among SPf66 and placebo recipients.

Clinical follow-up. Volunteers were kept under observation by medically trained staff in a hospital ward for 18 to 24 h following each immunization. Medical staff and resuscitation equipment were continuously available. Examinations were made 0.5, 1 and 2 h after immunization, in the evening of the day of immunization and the next morning before discharge. Information relating to symptoms and signs of local and systemic allergic side-effects was collected and a morbidity questionnaire was applied. After discharge, a similar questionnaire was administered daily to the volunteers at their homes for a further 10 days. All volunteers were encouraged to

*This group was enrolled only after the external trial monitors concluded that the first two doses had been safe in group 1b volunteers and immunogenic in group 1a. Similarly, the monitors' approval (based on evaluation of the safety of the third dose in groups 1a and 1b) was required before the third dose was administered to group 2

attend the Ifakara Centre clinic at any time should any intercurrent illness occur.

Laboratory methods. Blood and urine samples were obtained from all volunteers following the schedule shown in *Figure 1*. Blood samples were obtained by venepuncture for group 1a and 1b and by finger prick (microtainer brand serum separator, Becton Dickinson) in group 2. Serum samples were cryopreserved at −70°C before carrying out biochemical and immunological assays. When the quantity of serum available was limited, priority was given to immunogenicity evaluation for group 1a and to biochemical and autoantibody determinations for groups 1b and 2.

Haematological parameters (white blood cell count, red blood cell count, haemoglobin, packed cell volume, platelets) were measured with a semiautomated cell counter (SYSMEX F88® microcell counter, TOA Medical Electronics Co. Ltd, Kobe, Japan). Internal quality control standards supplied by the producer were run daily (Cellcheck®, Histan®, Eightcheck 3WP®) Liver and kidney function tests (total bilirubin, aspartate transaminase (AST), alanine transaminase (ALT), glucose and creatinine) were measured in an automated dry chemical photometer (Kodak Ektachem DT II® system, Eastman Kodak Co.). Daily quality control was carried out with a standard sample provided by the manufacturer (Kodatrol DT®). Normal values provided by the manufacturers were used for the haematological and biochemical tests (see footnote to *Table 4*). Proteinuria was assayed using dipsticks (Albustick®), Readings of + and over were considered positive. Anti-dsDNA antibody was assayed by a commercial ELISA kit (Progen®). Titres of greater than 50 units were considered positive. Quality control was performed with a control sample provided by the kit manufacturer.

IgG levels against the whole polymeric SPf66 peptide were determined by FAST ELISA (Falcon Assay Screening Test-ELISA, Becton Dickinson Labware, Oxrand, CA, USA). The system was established and run as described by Salcedo *et al.*[12]. The cut-off for positivity was an absorbance of 0.100, which is the mean absorbance plus five standard deviations obtained from 200 sera of malaria-free individuals from Venezuela, Colombia and Spain. Sera were first screened for reactivity in triplicate by two operators at 1:100 dilution. Titres of less than 100 were considered negative. Positive sera were then titrated. Positive and negative controls were run on each plate and a determination of antigen available on the FAST ELISA lids was performed.

For the determination of IFA titres, late stage schizonts from a continuous *P. falciparum* culture (FCB-2) strain[13], presenting a 10% parasitaemia, synchronized according to the method of Lambros and Vandenberg[14], collected and washed in sterile PBS (0.15 M phosphate buffer containing 0.15 M NaCl pH 7.2) were used. The pellet of infected red blood cells was resuspended in PBS–fetal bovine serum 1:1, applied to microscope slides and allowed to dry, blocked for 10 min with 1% non-fat milk and incubated for 30 min with the trial human sera at dilutions of 1/100, 1/200, 1/400, 1/800, 1/1600, 1/3200, 1/6400 and 1/12 800. The reactivity was visualized under a fluorescence microscope using an affinity-isolated FITC-conjugated $F(ab')_2$ fragment of goat-anti-human IgG (gamma chain) at a dilution of 1:50.

Statistical methods

Geometric mean end titre dilutions (GMT) were calculated separately for SPf66 and placebo recipients in each group using a $\log(x+1)$ transformation.

For each individual and each dose, the index of response was defined as the ratio of the end titre 4 weeks after vaccination to the titre at week 0. Negative sera were assigned a titre of 50 for this purpose. Thus an index of response of 1 suggests no response; an index of less than 1 suggests a drop in titre and an index greater than 1 suggests an increase in titre. Geometric mean indices of response and corresponding 95% confidence intervals (95% CI) were calculated for each study group by vaccination status using a log transformation. For groups 1b and 2, the SPf66 and placebo groups were compared using Student's *t* test.

RESULTS

Compliance and withdrawals

All 12 volunteers in group 1a completed the vaccination and follow-up schedules, as shown in *Table 1*. For logistic reasons volunteers in this group received the third dose at week 25 instead of 26 as originally intended.

Of the 30 individuals found eligible in group 1b, one decided not to take part in the study before receiving dose 1 and a second was withdrawn because of abnormal liver function. The remaining 28 received the first two doses. An additional volunteer withdrew before the third dose as he moved away from Ifakara in search of rubies (*Table 1*).

Fifty semi-immune children were recruited to group 2. Their compliance, age and sex distribution are shown in *Tables 1* and *2*. After review of their clinical and laboratory follow-up data collected at dose 1 (week 0), the clinical monitors withdrew 14 children, four in the SPf66 and ten in the placebo group. Six of these children (two SPf66, four placebo) were withdrawn because of elevated AST levels ($>50\ U\ l^{-1}$). A further two children allocated to SPf66 were withdrawn because of illness: one

Table 1 Number of volunteers receiving doses 1, 2 and 3 of either SPf66 or placebo

	Group 1a		Group 1b		Group 2	
Dose	SPf66	Placebo	SPf66	Placebo	SPf66	Placebo
1	10	2	18	10	25	25
2	10	2	18	10	21	15
3	10	2	18	9	21	15

Table 2 Age and sex distribution of group 2 study children at the time of receiving the first dose

Age (years)	SPf66	Placebo	Total
1	7	5	12
2	5	8	13
3	5	4	9
4	7	6	13
5	1	2	3
Total	25	25	50
Sex:			
Male	17	7	24
Female	8	18	26

of them suffered a febrile illness and generalized pruritus and the second was admitted to hospital with a diagnosis of pneumonia. The reasons for the remaining six withdrawals of children randomized to placebo were: temporary absence (2), recent admission for meningitis (1), generalized pruritus after the first dose (1), persistent proteinuria (+ +) (1), parent's request (1). The remaining 36 children completed the full immunization schedule.

Group 1a

Three individuals (two SPf66, one placebo) had pre-existing anti-SPf66 antibodies (1:200, 1:400 and 1:400, respectively). The dynamics of antibody production and the effect of immunization with each dose of SPf66 can be seen in *Figure 2a*. All SPf66 recipients produced anti-SPf66 antibody titres of at least 1:200 after the second dose and of 1:3200 or greater after the third dose. Relative to baseline values, there was no significant titre increase after dose 1, but two doses induced a 15-fold (95% CI 6–35) and three doses a 110-fold (95% CI 43–283) increase in an individual's anti-SPf66 antibody titre. This group's cumulative exposure to total malaria antigens remained minimal as indicated by very low IFAT titres (week 30: 9/9 SPf66 and 2/2 placebo recipients IFAT-negative).

One of the two placebo recipients did not develop any anti-SPf66 antibodies. The second placebo recipient had preimmunization antibody levels which increased during the trial period (week 0: 1:400, week 4: 1:800, week 8: 1:3200; week 24: 1:6400; week 30: 1:25 600). However, this volunteer's IFAT titres remained negative at all time points. This volunteer was not under regular malaria chemoprophylaxis and suffered a severe episode of *P. falciparum* malaria requiring hospital admission between week 24 and week 30.

Approximately half of all SPf66 recipients reported mild local pain after each dose. Moderate or severe erythema, induration, pruritus and pain were reported only after dose 2 and 3, in four individuals who were all SPf66 recipients. The most pronounced reaction was in a volunteer aged 33 who developed local erythema, pruritus and local and contralateral induration immediately following the third dose but did not require treatment. The same volunteer had received a full course of postexposure rabies prophylaxis (containing 0.01% Thiomersal as preservative) following a wild-dog bite at the injection site shortly after receiving the first dose of immunization.

Group 1b

Clinical follow-up information on local and systemic side-effects among SPf66 and placebo volunteers is summarized in *Table 3*. Completeness of observations was similar in the two groups (range: SPf66 84–100%; placebo 88–97%). No generalized side-effects such as shock, bronchospasm, wheezing or vomiting were observed. Mild local pain (after each of the three doses) and mild local induration (at the first dose) were reported more frequently among the SPf66 group. Local pain and induration of moderate or severe intensity were only noted among SPf66 recipients, at the second and third doses. No ulcerations at the injection site occurred in either group. No appreciable differences were seen between the vaccine and placebo groups at each dose for other side-effects.

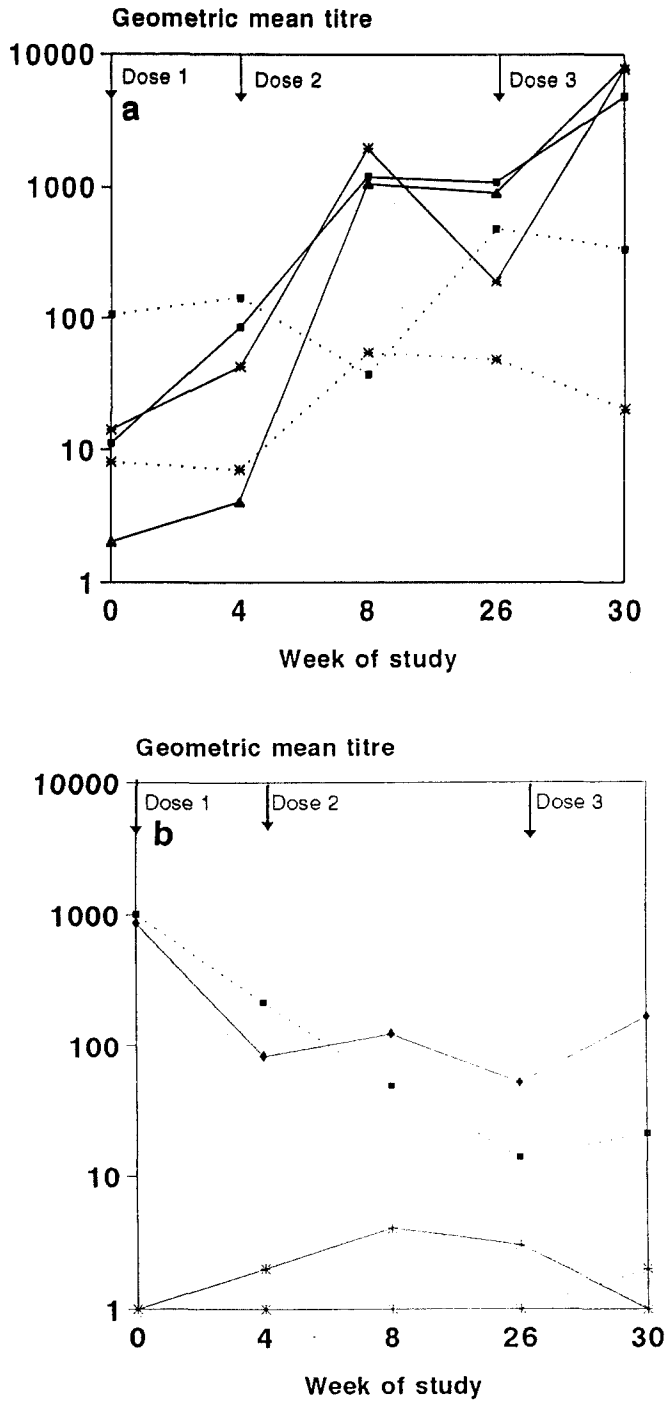

Figure 2 (a) SPf66 antibody titre development (log($x+1$) transformation) in response to three doses of SPf66 vaccine in groups 1a, 1b and 2 in comparison to placebo recipients: ▲, group 1a; ■, group 1b; *, group 2; —, vaccine; ····, placebo. (b) IFA titre development (log($x+1$)) in response to three doses of SPf66 vaccine in groups 1b and 2 in comparison to placebo recipients (group 1a data not shown): ◆, ■, group 1b; *, group 2; —, vaccine; ····, placebo

Following the third dose, a volunteer in the SPf66 group developed local and contralateral induration with contralateral pruritus and rash. No treatment was required for this syndrome.

Biochemical and haematological abnormalities and results of autoantibody testing (*Table 4*) were similar in SPf66 and placebo recipients. AST levels were elevated in 23% of all samples and distributed similarly between the two intervention groups. No volunteer developed autoantibodies.

Table 3 Group 1b: incidence of signs and symptoms among SPf66 and placebo volunteers following each dose (number of individuals with abnormality/total number of individuals observed (*n/N*))

	Dose 1		Dose 2		Dose 3	
Symptoms and signs	SPf66	Placebo	SPf66	Placebo	SPf66	Placebo
Injection site inflammation						
Local erythema	2/18	1/10	1/18	1/10	0/18	0/9
Local pain	14/18	4/10	12/18[c]	4/10	11/18[d]	2/9
Local induration	7/18	2/10	2/18[c]	1/10	1/18[c]	0/9
Local pruritus	1/18	2/10	0/18	0/10	1/18	0/9
Alternative injection site inflammation						
Contralateral induration	0/18	0/10	0/18	0/10	1/18[c]	0/9
Contralateral pruritus	0/18	0/10	0/18	0/10	1/18[c]	0/9
Systemic allergic reactions						
Rash			0/18	0/10	1/18	0/9
Generalized pruritis	0/18	0/10	1/18	0/10	0/18	0/9
Cough	2/18	1/10	0/18	0/10	0/18	0/9
Larynx oedema, bronchospasm, shock, clammy skin	0/18	0/10	0/18	0/10	0/18	0/9
Malaise	1/18	0/10				
Fever ($\geq$37.5°C)	3/18	0/10	1/18	0/10	0/18	0/9
Reported fever	1/18	0/10	1/18	0/10	0/18	0/9
Other[a]	1/18	3/10	0/18	0/10	0/18	2/9
No complaints for period	3/18	3/10	5/18	6/10	6/18	6/9
Completeness of data[b] (%) (*n/N*)	84 (3632/4320)	88 (2109/2400)	100 (4302/4320)	95 (2271/2400)	87 (3753/4320)	97 (2090/2160)

[a]Dose 1: one episode of headache of 1 day duration; one episode of headache of 2 days duration; one episode of headache; one episode of cold of 1 day duration; Dose 3: one episode of flu; one episode of headache
[b]Number of recorded observations/total intended observations (15 per person for each sign or symptom)
[c]One of which was moderate
[d]Two of which were severe

Table 4 Groups 1b and 2: number of relevant biochemical and autoantibody findings obtained at screening and follow-up among adult and child SPf66 and placebo volunteers (number of individuals with abnormal result/total number of individuals tested (*n/N*))

	Baseline (week 0)		4 weeks after dose 1 (week 4)		4 weeks after dose 2 (week 8)		4 weeks after dose 3 (week 30)	
	SPf66	Placebo	SPf66	Placebo	SPf66	Placebo	SPf66	Placebo
Group 1b								
AST Hi	2/18	3/10	2/18	3/10	1/18	2/10	6/18	6/9
ALT Hi	0/18	1/10	0/18	2/10	0/18	1/10	0/18	0/9
Proteinuria[a]	0/18	0/10	0/17	1/9	2/18	0/10	0/17	0/9
Anti-dsDNA antibody[c]	2/18[b]	0/10			1/18	0/10	1/16	0/8
Group 2								
AST Hi	11/25	13/25	13/24	9/25	19/21	11/14	17/21	13/14
ALT Hi	0/25	1/25	0/23	1/25	0/21	1/15	4/20	3/15
Proteinuria	2/25	0/25	0/24	0/22	1/20	2/15	0/20	0/14
Anti-dsDNA antibody[c]	0/21	0/18			0/20	0/13	0/20	0/15

Normal values used were: white blood cell count 3000–10 000 μl^{-1}; red blood cell count 4–6.25 $\times 10^6$ μl^{-1}; haemoglobin 8.0–18.0 g/100 ml; packed cell volume 0.25–0.52; platelets 130 000–400 000 μl^{-1}; creatinine 25–133 μmol l^{-1}; glucose 2.0–6.2 mmol l^{-1}; total bilirubin 3.4–25.7 μmol l^{-1}; AST 5–35 U l^{-1}; ALT 7–56 U l^{-1}; proteinuria 0–(+); anti-dsDNA $\leq$50 U ml^{-1}
[a]Proteinuria was measured at week −2 instead of week 0
[b]One individual remained positive throughout trial; one became negative at week 8 and negative at week 30
[c]Autoantibody was screened for positivity at weeks 0, 8 and 20

None of the differences in safety parameters between vaccine and placebo groups reached statistical significance.

At screening, 14 out of 28 volunteers had anti-SPf66 antibody at a dilution of 1:100. The pattern and dynamics of anti-SPf66 antibody production following vaccination with SPf66 were similar to that seen in group 1a (*Figure 2a*). The geometric mean index of response after each dose is summarized in *Table 5*. At week 30 anti-SPf66 antibody was detected in 17/17 SPf66 and 7/9 placebo recipients but the final titres were much higher in the SPf66 recipients (range of titres: 800–51 200; GMT: 4811) than among those who had received placebo (range of titres: 400–12 800; GMT: 800). IFAT results suggested a continuous exposure to naturally occurring *P. falciparum* infection (*Figure 2b*) and GMTs were similar in SPf66 and placebo recipients at week 30 (*t*-test: $p=0.12$).

Group 2

Clinical follow-up information on local and systemic side-effects among SPf66 and placebo volunteers is summarized in *Table 6*. Completeness of observations was similar in the SPf66 and placebo group (range: SPf66 81–97%; placebo 82–100%). Incidence of side-effects at the local injection site was generally similar in the two intervention groups after each of the three doses. There

Table 5 Geometric mean index of response: within-individual change of IgG anti-SPf66 antibody titres after each dose among SPf66 and placebo recipients who received all three doses (index of response defined as titre 4 weeks after dose/titre at week 0[a])

	Response index (95% CI) 4 weeks after:					
	Dose 1		Dose 2		Dose 3	
	SPf66	Placebo	SPf66	Placebo	SPf66	Placebo
Group 1a	1.4 (0.6, 2.9) $N=9$		14.9 (6.3, 35.4) $N=10$		109.7 (42.6, 282.7) $N=9$	
Group 1b	2.0 (1.1, 3.5) $N=18$	1.4 (0.5, 3.5) $N=9$	11.8 (5.4, 25.8) $N=18$	0.9 (0.3, 2.3) $N=9$	36.2 (17.7, 74.0) $N=17$	3.2 (1.7, 5.9) $N=9$
Student's t	0.70		4.00		4.50	
Degrees of freedom	25		25		24	
p	0.47		<0.001		<0.001	
Group 2	1.9 (1.0, 3.7) $N=18$	0.6 (0.4, 1.0) $N=15$	13.8 (7.2, 26.5) $N=14$	1.3 (0.5, 3.5) $N=11$	61.1 (37.2, 100.4) $N=15$	0.7 (0.2, 2.2) $N=12$
Student's t test	2.7		4.1		7.9	
Degrees of freedom	31		23		25	
p	0.01		<0.001		<0.001	

N = number of children
Student's t tests compare SPf66 with placebo recipients in each group
[a]Due to insufficient serum quantity for group 2 at week 0, week −2 sample was used

Table 6 Group 2: incidence of signs and symptoms among SPf66 and placebo volunteers following each dose (number of individuals with abnormality/total number of individuals observed (*n/N*)

	Dose 1		Dose 2		Dose 3	
Symptoms and signs	SPf66	Placebo	SPf66	Placebo	SPf66	Placebo
Injection site inflammation						
Local pain	3/25	2/25	4/21	1/15	6/21	2/15
Local induration[a]	3/25	2/25	0/21	0/15	7/21[g]	3/15
Swelling[b]	0/25	1/25	2/21	1/15	4/21	0/15
Local pruritus[a]	0/25	0/25	0/21	0/15	1/21	0/15
Alternative injection site inflammation						
Contralateral induration[a]	0/25	1/25	0/21	0/15	1/21	0/15
Systemic allergic reactions						
Rash	0/25	1/25	0/21	0/15	1/21[f]	0/15
Generalized pruritus[a]	1/25	1/25	0/21	0/15	0/21	0/15
Scratching[b]	1/25	0/25	0/21	0/15	0/21	1/15
Wheeze	0/25	0/25	0/21	0/15	4/21	3/15
Cough	7/25	3/25	3/21	2/15	7/21	5/15[f]
Vomiting	3/25	1/25	1/21	1/15	1/21	2/15[f]
Diarrhoea[b]	4/25	4/25	4/21	1/15	2/21	2/15
Fever (≥37.5°C)	8/25	5/25	4/21	1/15	11/21	5/15
Reported fever	5/25[f]	5/25	5/21	2/15	8/21	4/15[f]
No impairment of normal behaviour and activities[c] (%)	17/25 (68)	19/25 (76)	16/21 (76)	11/15 (73)	14/21 (67)	12/15 (80)
Other[d]	2/25	2/25	3/21	2/15	7/21	5/15
Completeness of data[e] (%) (*n/N*)	97 (6331/6500)	100 (6494/6500)	81 (4441/5460)	82 (3193/3900)	96 (5226/5460)	97 (3798/3900)

[a]Observations made at hospital only
[b]Observations made only during community follow-up
[c]Well: walking or crawling, playing, laughing, eating, feeding, not crying more than usual
[d]*Dose 1: SPf66* – Nasal discharge (2 h after vaccination) and constipation (day 1); diarrhoea and recurrence of rash present before vaccination (day 9). Placebo – Swollen eyelid (day 1); perioral and genital vesicular eruption (days 5–7). *Dose 2: SPf66* – Broken finger (day 7); perioral impetigo (day 6); rhinitis (day 5). Placebo – Broken hand (day 5); eye pain (day 10). *Dose 3: SPf66* – Hot at night (days 2 and 3) and flu (day 8); rhinitis (day 9); flu (days 2, 3 and 7) and feels cold (days 7 and 9); flu (day 10); abdominal pain (day 9); feels cold (day 6), and rhinitis and upper respiratory tract infection (day 7); slight cough (days 1 and 5). Placebo – Hot at night (day 3) and flu (day 8); upper respiratory tract infection (day 10); slight cough (days 3 and 6) and flu (day 5); slight cough (days 3 and 9) and flu (day 9); slight cough (day 1)
[e]Number of recorded observations/total intended observations (five per person for each sign or symptom recorded in hospital, and ten per person for each sign or symptom recorded during community follow-up)
[f]One moderate episode
[g]Two moderate episodes

is possibly a trend for a greater incidence of induration and swelling, which resolved without medication, in SPf66 recipients after the third dose. One SPf66 recipient developed local and contralateral induration resolving without medication. No erythema, contralateral pruritus or injection site ulceration were observed. In approximately 30% of children in both SPf66 and placebo groups, some impairment of well-being (*Table 6*) occurred during follow-up after each dose. Two children were withdrawn (one SPf66, one placebo) after they developed

generalized pruritus within 12 h of receiving the first dose. A single dose of diphenhydramine was given and no further medication was required.

Results from relevant biochemical and autoantibody testing are shown in *Table 4*. No child developed autoantibody after immunization with SPf66. Haematological parameters were normal and, in particular, no transient thrombocytopenia was observed. AST levels were elevated in 63% of all samples distributed similarly between the two intervention groups. Significant proteinuria occurred in one child (placebo) with an intercurrent illness, who was withdrawn after the first dose.

As with group 1b, none of the differences in safety parameters between vaccine and placebo groups reached statistical significance.

At screening, 33/50 children had anti-SPf66 antibody titres of at least 1:100. Group 2 showed a similar response to that seen in groups 1a and 1b (*Table 5*, *Figures 2a* and *3*). After three doses, anti-SPf66 antibody titres increased 61-fold (95% CI 37–100) in SPf66 recipients compared to baseline levels. At week 30, 15/15 SPf66 and 6/12 placebo recipients with three doses had detectable anti-SPf66 antibody but the final titres were much higher in the SPf66 group (range: 800–51 200; GMT: 7699) than in the placebo group (range: 0–3200; GMT: 150). The IFAT response observed at week 8 (*Figure 2b*) was not significantly different between SPf66 and placebo recipients ($p = 0.28$). In these analyses, sera from week −2 were used to determine the baseline titre as the serum quantity available from week 0 was insufficient for ELISA. Antibody titres in groups 1a and 1b did not show any difference between week −2 and week 0.

DISCUSSION

This study has provided evidence of the tolerability and safety of the SPf66 vaccine when given to young semi-imune children in an area of intense perennial transmission in Africa. High levels of previous exposure to *P. falciparum* do not appear to enhance susceptibility to allergic reactions to SPf66. However, we provide some evidence that SPf66 does induce a higher incidence of local pain and induration than aluminium hydroxide alone. All three contralateral indurations took place in volunteers who had received two or three doses of SPf66. The mechanism and significance of this unusual side-effect remain unclear.

The inclusion of a placebo group in the trial was crucial in enabling us to differentiate SPf66-related side-effects from other common events. High overall frequencies of localized side-effects were recorded, but this was the case in both SPf66 and placebo recipients. Possible explanations are the intense follow-up schedule (15 observations per person), an adjuvant-related effect, or high background rates of signs or of perceived symptoms. There could also have been some late responses to the SPM which was given 2 weeks before inoculation. Generalized pruritus occurred in 2/50 children during the first 24 h after the first dose. One of these children received SPf66 and one placebo, and therefore this cannot be attributed to the vaccine.

When allowance is made for the background levels

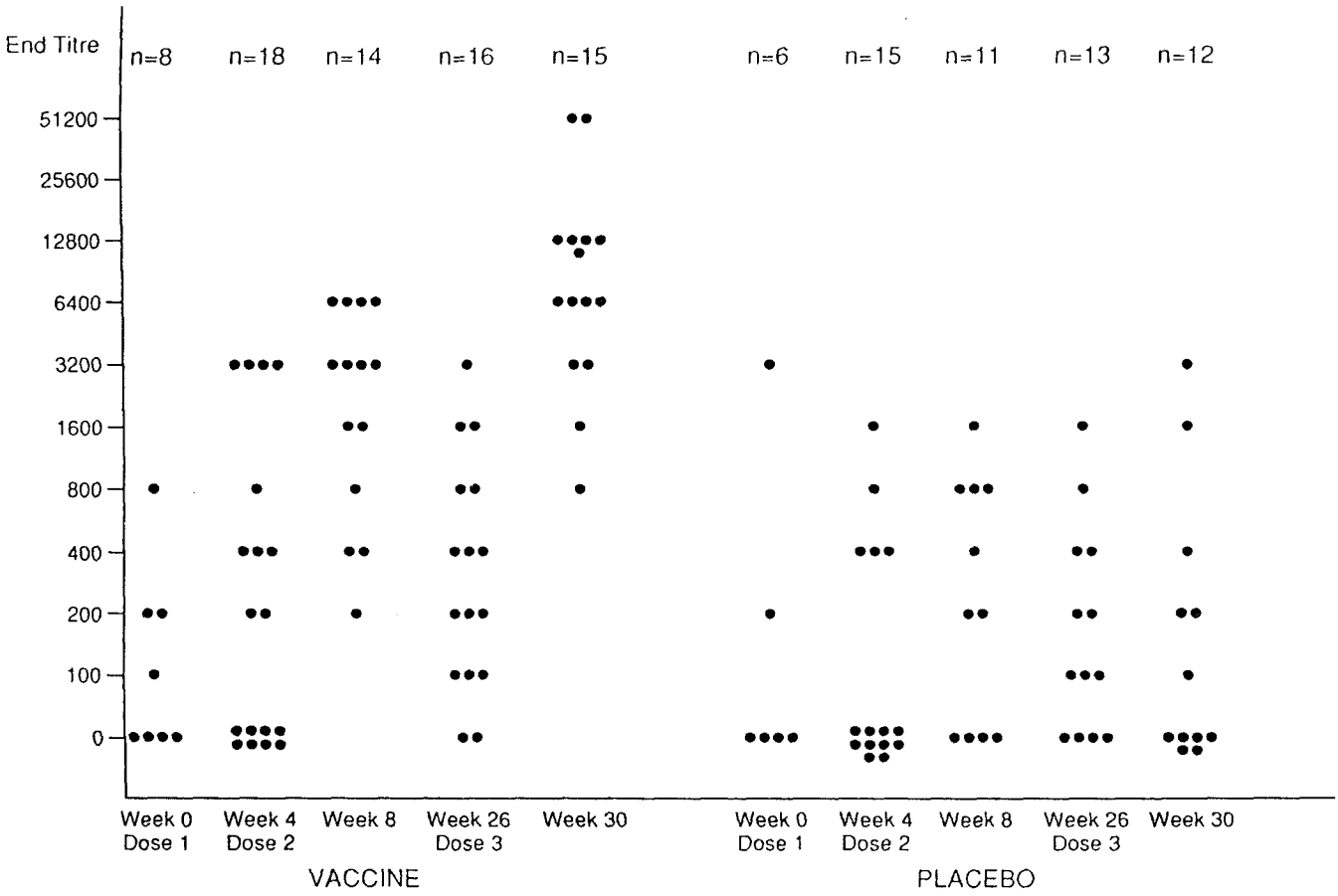

Figure 3 Group 2: SPf66 antibody titre development in response to three doses of SPf66 vaccine in comparison to placebo recipients

observed with the placebo, the frequencies of localized side-effects were similar to those recorded in other trials of synthetic or genetically engineered malaria vaccines. When such vaccines have been applied to non-immune subjects a few, transient, self-limiting episodes of hypersensitivity, but no generalized anaphylaxis, were recorded. The safety results for SPf66[4–6,15] have been similar to those for other vaccines[16–19]. In contrast to some of the results from Colombia[6], we observed an increase in local reactions with successive rounds of immunization with SPf66. In addition it must be borne in mind that rare SPf66-related side-effects could not be excluded unless a very large study were conducted.

As reported from South America[5], neither haematological nor biochemical abnormalities were associated with SPf66 vaccination. We found no evidence for autoimmune antibody stimulation. Both in children and semi-immune adults, placebo recipients experienced similar frequencies of liver function (AST) abnormalities to those in SPf66 recipients. These frequencies were much the same as those recorded in an uncontrolled study of SPf66 in Colombia[4] and in a placebo-controlled study of a recombinant vaccine in Nigeria[20]. Some of the elevated levels in our study may have been related to the treatment with SPM, which is known to cause liver enzyme abnormalities[21].

The prevalence of naturally acquired antimalarial IgG is high among populations in Kilombero district[8,22,23], reflecting the high malaria exposure. More than 40% of children in group 2 had preimmunization anti-SPf66 IgG titres of 1:100 or more, which is much higher than the 3.3% reported from South America[4]. This prevalence is lower than that previously found in Kikwawila, a village outside Ifakara[8]. A rather lower exposure (as indicated also by the relatively low IFAT titres, *Figure 2b*) in the semi-urban setting of Ifakara might be the reason.

All group 1a adults, most of whom were non-immune to malaria and therefore similar to Colombian or US volunteers, seroconverted after two doses of aluminium hydroxide-adsorbed SPf66 polymer. These findings are in marked contrast to the 33–76% antibody response rate reported from Colombia[5,6], or the 67% among US soldiers[24]. Indeed, regardless of pre-existing anti-SPf66 antibody levels, all volunteers in all three groups who received SPf66 showed a measurable increase in anti-SPf66 IgG and a clear dose-response effect. The mean response in SPf66 recipients was several orders of magnitude higher than that in placebo recipients. Therefore the increase in titres cannot be due to non-specific immune activation induced by the adjuvant, as has been suggested recently[25]. A clear boosting effect with the third dose, similar to that in Colombia[26], was also observed. Titre elevations were higher in group 1a than in groups 1b and 2. This may merely reflect the lower initial levels in group 1a, but the differences between the three SPf66 groups were in any case not statistically significant.

We have demonstrated that young children living in an area of intense perennial transmission in Africa have a clear and substantial antibody response to three doses of SPf66, and that this malaria vaccine is well tolerated and safe when given to such children. The trials in Colombia failed to establish a correlation between anti-SPf66 antibody titres and clinical protection[6,12]. Therefore, our immunogenicity results should not be seen as indicative of clinical efficacy, but as evidence that the SPf66 vaccine for use in the phase III trial in Kilombero was adequately synthesized, formulated and delivered. The phase III field trial, which is now in progress, will provide further information on the safety and immunogenicity of the vaccine in larger numbers of children. However, the primary objective of that trial is to estimate the level of protective efficacy of SPf66 against clinical malaria when given to young children in an area of high malaria endemicity.

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Articles

Randomised trial of efficacy of SPf66 vaccine against *Plasmodium falciparum* malaria in children in southern Tanzania

P L Alonso, T Smith, J R M Armstrong Schellenberg, H Masanja, S Mwankusye, H Urassa, I Bastos de Azevedo, J Chongela, S Kobero, C Menendez, N Hurt, M C Thomas, E Lyimo, N A Weiss, R Hayes, A Y Kitua, M C Lopez, W L Kilama, T Teuscher, M Tanner

Summary

Effective, safe antimalarial vaccines have proved elusive. The synthetic polypeptide SPf66 vaccine is based on pre-erythrocytic and asexual blood-stage proteins of *Plasmodium falciparum*. We report here a randomised double-blind placebo-controlled trial of the efficacy of the SPf66 vaccine against clinical *P falciparum* malaria in Idete, southern Tanzania, an area of intense perennial malaria transmission.

586 children aged 1–5 years received three doses of vaccine (n=274) or placebo (n=312). The incidence and density of parasitaemia were assessed through repeated cross-sectional surveys on subgroups of children. Morbidity was monitored over a 1 year period through passive case detection in all children plus active case detection in a subgroup of 191. An episode of clinical malaria was defined as measured fever (≥37·5°C) and parasite density >20 000/μL.

No severe side-effects were seen and the frequency of mild side-effects after the third dose was less than 6%. The vaccine was highly immunogenic and after three doses all vaccine recipients had detectable anti-SPf66 antibodies: the geometric mean index of response was 8·3 in the vaccine group and 0·7 in the placebo group. The incidence of parasitaemia was similar in both groups. 123 children had at least one episode of clinical malaria during the follow-up period after the third dose and annual incidence rates were 0·25 in the vaccine group and 0·35 in the placebo group. Estimated vaccine efficacy was 31% (95% confidence interval 0–52%; p=0·046). After the third dose there were 6 deaths among the study cohort (1 vaccine, 5 placebo). This study confirms that SPf66 is safe, immunogenic and reduces the risk of clinical malaria among children exposed to intense *P falciparum* transmission.

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Introduction

Malaria, especially that caused by *Plasmodium falciparum*, is the most important parasitic disease of man. It causes more than 400 million clinical cases and between 1 million and 3 million deaths per year, mainly among young children and pregnant women in sub-Saharan Africa.[1] Malaria is endemic in 103 countries, in which over half the world's population live.

The search for a vaccine against malaria has been long, partly due to the complex life cycle of the parasite, an incomplete understanding of the mechanisms of effective immunity, and a lack of surrogate measures of protection and of animal models. Various molecules from the pre-erythrocytic, asexual blood, and sexual stages have been characterised, and some may be promising vaccine candidates. However, the processes of antigen selection and vaccine development are complex and the best methods for selecting vaccines for trials remain unknown. Although the first attempt to immunise man was in 1936[2] it was not until the 1970s that findings in rodent and simian models were successfully applied to human malarias, and volunteers immunised with irradiated sporozoites were shown to be protected against *P falciparum* and *P vivax* infection.[3] However, recombinant and synthetic vaccine candidates derived from pre-erythrocytic stage antigens, in particular the repeat sequence of the circumsporozoite protein, have not been protective in human trials.[4-6]

Vaccines derived from asexual-blood-stage antigens are of special interest in Africa, because they may mimic the development of natural immunity in children living in endemic areas. Furthermore, such vaccines may induce long-term immunity because of natural boosting. The chimaeric protein SPf66 was the first such vaccine to be tested in man. SPf66 is a synthetic hybrid polymer solubilised in sterile saline solution and adsorbed onto aluminium hydroxide. The monomer unit is a chemically synthesised peptide of 45 aminoacids which contains aminoacid sequences derived from three asexual-blood-stage proteins (83, 55, and 35 kDa) linked by Pro-Asn-Ala-Asn-Pro (PNANP) sequences derived from the circumsporozoite protein of *P falciparum*.[7] SPf66 has been shown to be safe, immunogenic, and protective against *P falciparum* malaria in populations living in low endemicity areas of South America.[8-12]

The Kilombero SPf66 trial in southern Tanzania is the first trial of the vaccine outside Latin America, and the first in an area of intense perennial transmission. Preliminary studies to evaluate the safety and immunogenicity of the vaccine among small groups of non-immune and semi-immune adults and children began in July, 1992 (groups Ia, Ib, and II). No adverse effects were found and the vaccine was well tolerated and

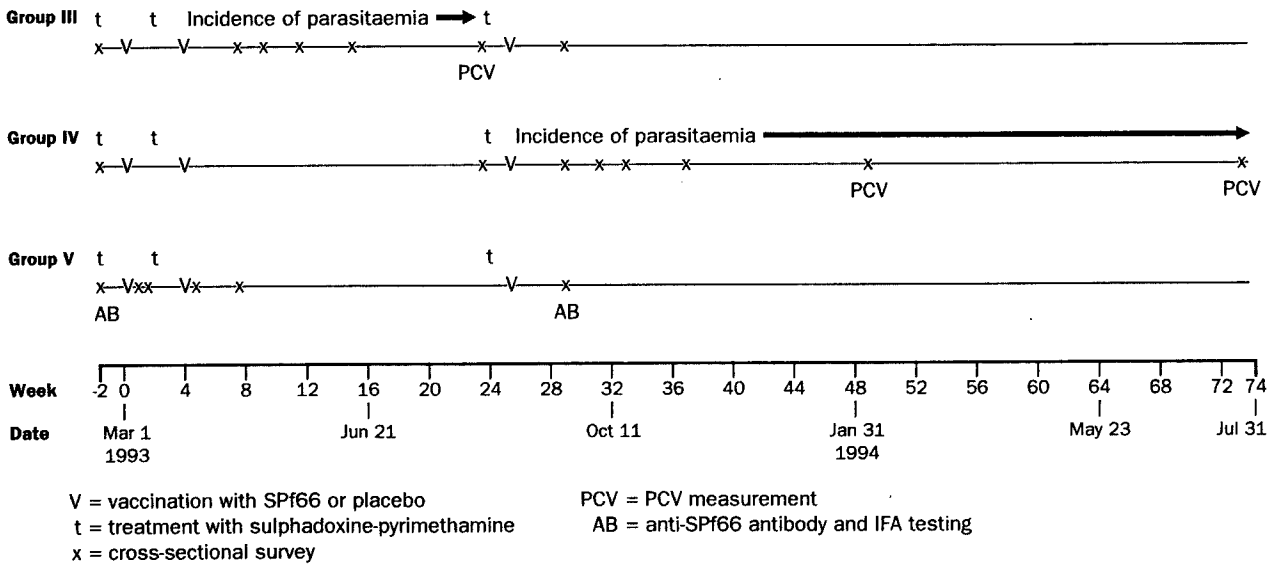

Figure 1: **Schedules for vaccination, treatment, and follow-up**
Groups I and II took part only in preliminary studies of safety and immunogenicity. Group III (n=176), IV (n=219), V (n=191); passive case detection only in groups III and IV, passive plus active in group V.

immunogenic.[13] A phase III trial was then launched to determine the efficacy of the vaccine in preventing clinical malaria in African children living in this area. The rationale and design of these trials are described elsewhere.[14] This paper is the first report of efficacy results from the phase III trial.

Population and methods

Study area and population

The study was done in the village of Idete (08° 5' S; 36° 30' E), about 20 km west of Ifakara, southern Tanzania. The village lies south of the Udzungwa Mountains, at the northern edge of the alluvial plain of the Kilombero River at 270 m. The main rains start in March and extend through to May, and the short rains occur in December and January. A cool dry season follows the long rains, in June and July.[15] Annual rainfall (August, 1993 to July, 1994) was 1417 mm.

The Kilombero Valley is an area of intense and perennial malaria transmission. *Anopheles gambiae* sp and *A funestus* are the two main vectors. Although mosquito densities and exposure are seasonal, the prevalence of asymptomatic *P falciparum* parasitaemia is high and shows no marked seasonality. 80% of infants are infected by age 6 months and data from an adjacent village indicate that, on average, everyone receives more than 300 infective bites per year.[16] Transmission of the three other species of human malarias is low and unstable. Malaria control in the district is based on prompt diagnosis and chloroquine treatment at health facilities. Chloroquine consumption is high, and 15–20% of parasite strains show RII resistance.[17]

Idete is typical of villages in the area, with widely scattered houses which have mud walls, thatched roofs, small windows, and little or no ventilation. Most villagers are subsistence farmers growing maize and naturally irrigated rice. There are few cattle because of trypanosomiasis. People sleep on the floor or on string beds with woven straw mats. Few villagers use bed nets, insecticides, or other anti-mosquito measures. The only health facility in Idete is a government dispensary, which is responsible for curative and preventive cure.

A census done between October, 1992, and February, 1993, established that Idete had 868 households. 4758 residents were enumerated of whom 45% were less than 15 years and 49·6% were male. All pre-school children were issued with photocards which included their full name and identification number, date of birth, and place of residence. Parents or relatives were asked to show their child's photocard at all contacts with the health services or the study team. A duplicate copy of all cards was kept to ensure positive identification even if the child's card was forgotten or lost.

Study design

The primary objective of this randomised double-blind, placebo controlled trial was to determine whether three doses of SPf66 reduce the incidence of clinical episodes attributable to malaria or the prevalence and intensity of parasitaemia in children aged 1–5 years, and to estimate the protection achieved. The target sample size of 600 children was chosen to give the study at least 90% power to detect a 50% reduction in the incidence of clinical malaria during the year after administration of the third dose, at a 5% significance level. An analytical plan written by the investigators was agreed with four independent trial monitors before the code was broken.

Children were randomised individually to receive vaccine or placebo. The code, drawn up and held by the monitors, was released to the investigators on Aug 8, 1994, when a copy of the study data had reached the monitors. Children were further randomised by household of residence to one of three follow-up groups (figure 1), with target sample sizes of 200 per group. Parasitological surveillance was carried out through repeated cross-sectional surveys in groups III and IV to estimate the efficacy of the vaccine in reducing the incidence, prevalence, and intensity of parasitaemia. Active case detection was done among children in group V and children from all three groups were monitored for morbidity through passive case detection.

Vaccine

The vaccine used was SPf66 peptide of Good Manufacturing Practice grade, part of batch 09, synthesised at the Instituto de Inmunologia, Bogota, Colombia. The vaccine was formulated at Llorente SA, Madrid, Spain, and is under a clinical trial licence of the Direcion General de Farmacia, Ministerio de Sanidad y Consumo, Spain. The placebo was aluminium hydroxide, with tetanus toxoid (Llorente SA batch 10/92, 16679/1/10) added to the first dose. Further details of the product are presented elsewhere.[18] SPf66 vaccine and placebo were bottled in identical clear glass syringes at two different volumes, 0·5 mL (2 mg peptide) for children 5 years of age and half that dose for younger children. The syringes were individually packed in amber blisters which were coded with numbers individually randomised to vaccine or placebo. The vaccine was transported and stored at 4°C. The physicochemical characteristics and immunogenicity of unused vaccine which had travelled several times to the field were unaltered (unpublished).

Screening, informed consent, and immunisation

Institutional and national ethical clearances were obtained from the Medical Research Coordinating Committee of the Tanzanian National Institute for Medical Research and from all participating institutions and sponsors. The trial started in February, 1993, following the monitors' confirmation that the vaccine had been safe and immunogenic in preliminary studies.[13]

Village meetings were held to explain the purpose of the trial. Following initial consent, children were called in small groups to key posts throughout the village for screening. Mothers or guardians were given a copy of the consent form in Kiswahili, which included information on procedures and potential risks involved with the trial, including the use of a placebo control. This was read out by a trained senior field assistant who answered any questions the mothers had. The children's identity and date of birth were checked, a brief medical history was taken, and a physical examination was done. Children were considered eligible if they had no history of allergies leading to medical consultation and treatment, and no acute condition warranting hospital admission or any chronic condition which might make them unsuitable. A fingerprick blood sample was taken into a heparinised microtainer (Becton Dickinson), from which a microcapillary tube was filled, and the packed cell volume (PCV) was measured. If the PCV was less than 25% the child was not enrolled. Children were given a single dose of sulfadoxine-pyrimethamine (25 mg sulfadoxine and 0·75 mg pyrimethamine per kg body weight) before every immunisation in order to clear blood stage *P falciparum* infections. Children who satisfied all inclusion criteria were asked to attend for immunisation 2 weeks later. On that day, the informed consent form was again read to mothers or guardians. Their understanding of the implications of trial participation was checked by their answers to three questions before the form was signed by the reader and a village elder, who acted as a witness.

Children were reviewed by a physician to check for exclusion criteria. Syringes were drawn from a cold box and the number of the vial was recorded and checked by a witness. SPf66 and placebo were injected subcutaneously with a 25G needle in the left upper deltoid area for the first and third dose, and right deltoid for the second. Vaccine doses were planned for weeks 0, 4, and 26 (figure 1). After each dose, children remained under medical surveillance for one hour with immediate access to portable resuscitation equipment, and were regularly monitored for side-effects. Children then returned home and parents were asked to report subsequent side-effects to the trial medical team on call at Idete dispensary.

Clinical and parasitological follow-up

Clinical and parasitological follow-up comprised both active and passive case detection and cross-sectional malariological surveys.

Passive case detection, based at Idete dispensary, was started in February, 1993. Idete dispensary was refurbished by the project, and supplies of essential drugs were provided throughout the study. All children attending the dispensary because of a perceived illness were screened by project medical personnel, who provided round-the-clock cover. Children were identified, and axillary temperatures checked with an electronic thermometer (MBO, Munich, Germany). If the temperature was 37·5°C or greater or if fever during the previous 24 h was reported, a fingerprick blood sample was collected into a heparinised serum separator microtainer for PCV measurement, and thick and thin blood films were prepared. The children were then diagnosed and treated by the routine medical services at the dispensary. Quality control by senior project personnel included random checks on children leaving the dispensary, to confirm the accuracy of the system. Hospital admissions of study children at the St Francis Designated District Hospital, Ifakara, were monitored daily to detect severe illness and side-effects.

Parasitological surveillance was carried out through cross-sectional surveys in groups III and IV, to estimate the efficacy of the vaccine in reducing the incidence, prevalence, and intensity of parasitaemia. Children in group III had blood samples taken through fingerprick at defined time-points between doses 2 and 3 and samples from children in group IV were taken at similar times after the third dose (figure 1). At these surveys, any sick children were referred to the dispensary for diagnosis and treatment.

—	Vaccine (n=274)	Placebo (n=312)
Demographic data		
Male	145 *(52·9%)*	154 *(49·4%)*
Mean (SD) age at vaccination (yr)	3·4 (1·5)	3·5 (1·5)
No aged under 18 mo at vaccination	38 (13·9%)	28 (9·0%)
Mean (interquartile range) distance from dispensary (km)	1·9 (0·9–4·8)	1·6 (0·8–3·1)
Parasitaemia		
Prevalence of *P falciparum* parasitaemia	253/272 (93%)	286/312 (92%)
Median density (parasites/μL)*	3852	4603
Prevalence of *P malariae* parasitaemia	22/272 (8%)	35/312 (11%)
Hb genotype		
AA	195/248 (79%)	223/272 (82%)
AF	15/248 (6%)	16/272 (6%)
AS	38/248 (15%)	33/272 (12%)
Chloroquine detectable in urine†	14/92 (15%)	16/98 (16%)
Antibodies		
Geometric mean (95% CI)‡ anti-SPf66 titre	226 (144–357)	239 (153–372)
Geometric mean (95% CI)‡ anti-*P falciparum* titre	33 (17–64)	44 (25–77)

*Median of positive slides; †from random sample of children; ‡measured 2 weeks after enrolment.

Table 1: **Characteristics of study cohort on enrolment**

All children in group V were followed by active case detection (figure 1). Children were visited at home weekly by project field assistants, from 4 weeks after the second dose. A brief morbidity questionnaire was administered and the axillary temperature recorded with an electronic thermometer. If fever in the previous 24 h was reported or if the temperature was 37·5°C or higher, thick and thin blood films were prepared, and the mother was advised to take the child to the dispensary. Quality control included weekly repeat visits by senior project personnel to a 10% random sample of children and weekly reallocation of field assistants to different village areas. Thermometers were checked in a water bath against a standard mercury thermometer every fortnight.

As a measure of drug pressure the level of chloroquine consumption was monitored throughout the study period. Urine samples were collected from group V children at week −2 and week 48, and urine from 50 children was collected every month through a randomised procedure that ensured that at least one sample from each child was collected over a one year period.

Cross-sectional surveys and active case detection ensured close demographic surveillance during the study period. Children who did not have any contact with the study team for more than one month were visited by a project field assistant to check residence.

Immunogenicity and laboratory methods

Blood samples were taken from group V children on the day of the first dose (week 0) and 4 weeks after the third dose to determine the immune response. Blood was collected by fingerprick into heparinised microtainers, centrifuged at 3000 rpm for 3 min, separated, and stored without preservatives at −70°C.

No serological assays were done until the code was broken. IgG levels against the whole polymeric SPf66 peptide were measured by the Falcon Assay Screening Test ELISA (Becton Dickinson Labware, Oxrand, CA, USA).[19] Immunofluorescent antibody titres were also measured.[13]

Thick and thin blood films were air dried, stained with Giemsa, and read on a light microscope (Wild-Heerbrug, Switzerland) with a ×50 oil immersion lens and ×10 eyepieces. Parasite density was assessed by counting the number of asexual stage parasites per 200 leucocytes. Slides were declared negative only after 200 leucocytes had been read. Parasite numbers were converted to a count/μL by assuming a standard leucocyte count of 8000/μL.[20] All slides were read twice independently, and a

Weeks from first dose	Vaccine		Placebo	
	No positive*	Density†	No positive*	Density†
After dose 2				
10	20/42 (48%)	1860 (599-8446)	25/53 (47%)	4210 (366-23 915)
12	29/42 (69%)	3746 (894-11 546)	38/53 (72%)	1882 (419-10 647)
16	39/42 (93%)	1984 (424-10 213)	51/53 (96%)	3871 (1180-12 092)
24	42/42 (100%)	2892 (934-12 696)	52/53 (98%)	1850 (500-9876)
After dose 3				
32	11/65 (17%)	1657 (131-5675)	11/62 (18%)	2245 (1197-13 028)
34	24/65 (37%)	745 (272-5722)	28/62 (45%)	1580 (189-8127)
38	47/65 (72%)	982 (365-5815)	42/62 (68%)	2000 (586-5539)
50	55/65 (85%)	2299 (608-5788)	51/62 (82%)	2946 (902-11 350)
73	63/65 (97%)	1716 (642-6676)	59/62 (95%)	3660 (1466-8783)

*Numbers positive represent cumulative incidence in those who were negative 4 weeks after each dose and who had complete data
†Median of positive slides among those who had been negative 4 weeks after each dose. Inter (first to third) quartile range shown in parentheses.

Table 2: **Cumulative incidence and density (parasites/μL) of parasitemia among incident cases after second and third dose of vaccination**

third time if the ratio of densities from the first two was greater than 1·5 or smaller than 0·67 or if there was a discrepancy in positivity. If less than 30 parasites were counted a third reading was done if the difference in the number of parasites was greater than 10. The definitive result was based on the majority verdict for positivity and the geometric mean of the positive densities for positive slides.

PCV was measured in heparinised microcapillary tubes using a microhaematocrit centrifuge. Urine samples were tested for chloroquine by thin-layer chromatography.[21] Haemoglobin cellulose acetate electrophoresis (Helena, USA) was done by standard procedures.

Case definitions, data processing, statistical methods

Data were double-entered using a menu-driven system written in FoxPro v2·0 (Fox Software). All records were routinely checked for range and consistency at Ifakara centre. Analysis was done with SAS (SAS Institute, Cary, NC, USA) and EGRET (SERC, Seattle, WA, USA) software.

Humoral immune responses to vaccination were summarised using an index of response, defined as the ratio of the titre 4 weeks after the third dose to the titre at week 0.[13] Negative sera were assigned a titre of 50 for this purpose. Geometric mean titres were calculated using a $\log_e(\times+1)$ transformation.

For both passive and active case detection the primary outcome was defined before breaking the code. Because asymptomatic parasitaemia is common in this population, a clinical episode of malaria was defined as an axillary temperature of 37·5°C or more together with a *P falciparum* parasite density over 20 000/μL. This case definition was determined by modelling the relation between fever risk and parasite density among passively detected cases from weeks 16–24 (June to August, 1993), regardless of vaccine status, using logistic regression.[22,23] Estimates of sensitivity and specificity were made for a range of cut-offs in parasite density. Lack of specificity would result in a biased estimate of vaccine efficacy, and lack of sensitivity would result in a loss of power. The estimates of sensitivity and specificity for the chosen case-definition were 83% and 82%, respectively.

Vaccine efficacy based on comparison of incidence rates of clinical episodes between vaccine and placebo was calculated using the standard formula $1-(I_v/I_p)$, where I_v and I_p are the incidence rates in vaccine and placebo groups, respectively. The primary analysis considered passive case detection follow-up starting four weeks after dose 3. Subsidiary analyses included those for the period between 4 weeks after dose 2 up to dose 3, as well as corresponding analysis of active case detections. Incidence rates were calculated by dividing the number of children with episodes by total child days at risk: children did not contribute either to the numerator or denominator following an episode. Confidence intervals (CI) for vaccine efficacy with and without adjustment for confounders were calculated by Poisson regression (passive case detection) and logistic regression (active detection). Kaplan Meier survival curves were used to display the timing of malaria episodes: these show the proportion of children without an episode over each follow-up period.

The effect of the vaccine on incidence of parasitaemia was assessed only in those children who were parasite negative at the first cross-sectional survey following vaccination. Cumulative incidences of parasitaemia following the 2nd and 3rd dose were compared separately. Cox regression models[24] were used to estimate efficacy against infection adjusting for age. Incidence and density of parasitaemia in the vaccine and placebo groups was compared among those children who had incident infections—ie, those who were aparasitaemic at week 8 (after dose 2) or week 30 (after dose 3). Incidence analysis was further restricted to those children who had complete data for all the cross-sectional surveys.

Results

Screening and immunisation

The census identified 789 children who would be aged between 1 and 5 years on March 1, 1993. The 672 children living nearest the centre of the village were asked to come to be screened. 5 children were too sick to attend and 3 parents refused. Of the 664 children screened, 8 were excluded (1 was on tuberculosis treatment, 1 had a history of asthma, and 6 had a PCV less than 25). On the day of first vaccination, a further 25 children were excluded (16 were temporarily away, 1 had migrated, 4 refused to take part, and 1 had died, and 3 were excluded on medical grounds [1 pneumonia, 1 epilepsy, and 1 mixed mitral valve disease]). 631 children received the first dose, and 624 (99%) and 588 (93%), respectively, of those received the second and third doses. Thus 43 children received the first but not the third dose: 4 refused (1 vaccine/3 placebo), 2 were withdrawn due to illness (0/2), 6 died (2/4), and 31 moved away (17/14). 1 child received doses 1 and 3 but not dose 2, and 1 child was found subsequent to vaccination to have been aged wrongly and has been excluded from the analysis.

Study cohort

The main analysis of efficacy is restricted to the 586 children (274 vaccine, 312 placebo) who were aged 1-5 years on March 1, 1993 and who received all three doses. These children form the study cohort (table 1), and the two groups are well balanced except for age and distance between home and dispensary. The imbalance between the groups in age is small but could confound estimates of efficacy because the risk of clinical malaria diminishes rapidly with age. The imbalance in the distance between home and dispensary is also small but this too has a confounding effect on estimates of efficacy from passive

Episodes* per child	Vaccine (n=274)	Placebo (n=312)
Between 2nd and 3rd dose		
0	222 (81·0%)	238 (76·3%)
1	46 (16·8%)	62 (19·9%)
2	6 (2·2%)	10 (3·2)
3	0	2
Total episodes	58	88
After 3rd dose		
0	225 (82·1%)	238 (76·3%)
1	33 (12·0%)	52 (16·7%)
2	9 (3·3%)	18 (5·8%)
3	6 (2·2%)	2
4	1	2
Total episodes	73	102

*An episode is defined by fever ≥37·5°C and parasites >20 000/μL.

Table 3: **Number of clinical episodes of *P falciparum* per child as detected by passive case detection**

case detection because the risk of being diagnosed with malaria diminishes with distance from the dispensary. Both these confounding factors were taken into account when calculating adjusted estimates of vaccine efficacy.

After the third dose there were 20 withdrawals: 19 children moved away (10 vaccine, 9 placebo) and 1 refused to continue to participate (placebo).

Adverse effects

No severe adverse effects of vaccination were recorded and no children required medical care for the mild effects seen. After the first dose there were 25 children with mild or moderate induration (12 vaccine/13 placebo); after the second dose two contralateral indurations were noted (0/2); and after the third dose 5 children had erythema (3/2), 17 had induration (12/5), and 1 (1/0) had contralateral induration.

The number of immunised children attending the dispensary during the 2 days following each dose was similar (eg, for dose 3 3·6% in the vaccine group *vs* 3·2% in the placebo group; p=0·77).

Immunogenicity

The purpose of the limited serological data presented is simply to confirm vaccine immunogenicity. The relation between measured immune response and clinical protection has not yet been assessed. The prevalence of detectable anti-*P falciparum* antibodies (IFAT) at baseline was similar in vaccine and placebo groups (49/81 [60%] *vs* 67/102 [66%]; p=0·47). The baseline prevalence of anti-SPf66 IgG antibodies was 74/81 (91%) among vaccinees and 90/102 (88%) among those receiving placebo (p=0·49). Geometric mean titres were also similar (table 1).

4 weeks after dose 3, all vaccine recipients assessed (74/74) and 88% (80/91) of placebo recipients had detectable SPf66 antibody (geometric mean titres 2782 in the vaccine group and 161 in the placebo). The geometric mean index of response was 8·3 in the vaccine group and 0·7 in the placebo group (p<0·001). IFA titres in the vaccine group also increased substantially after three doses of vaccine (geometric mean index of response 1·8 *vs* 0·7 [p<0·001]; geometric mean titres 207 and 17).

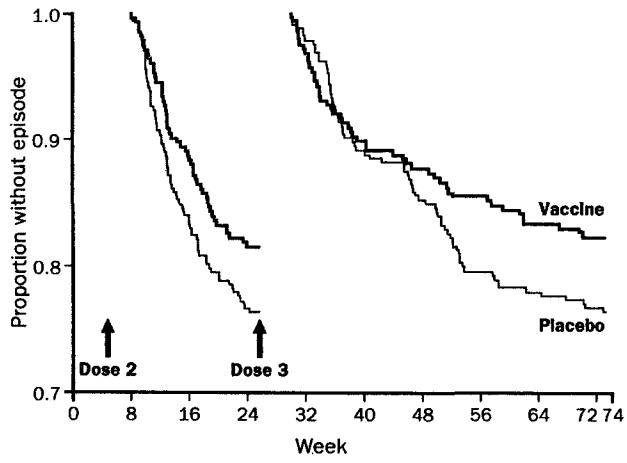

Figure 2: **Kaplan Meier survival curves established from passive case detection before and after the third dose**

Mortality and hospital admissions

6 cohort children (1 vaccine/5 placebo) died during the 48 weeks of follow-up after the third dose. A further 6 children who had received first dose (2/4) died before the third dose was due at week 26. Thus 12 children died during the study: 5 deaths were in hospital. Hospital records and interviews with relatives suggest that 6 of the deaths were due to malaria (1 vaccine *vs* 5 placebo).

32 cohort children were admitted to the paediatric wards of St Francis Hospital during the full 17 months of follow-up (13 vaccine/19 placebo). Malaria was diagnosed in 10 (4/6).

Cross-sectional surveys

Table 2 shows the cumulative incidence of infection and median parasite density for incident infections separately for the periods after the second (group III) and third doses (group IV). Incidence rates were similar among vaccine and placebo recipients after both the second and third doses (age-adjusted hazard ratios 1·0 and 1·0). After the third dose, median parasite densities were lower among vaccinees than among placebo recipients. However, this difference was significant only at the last cross-sectional survey (p=0·03).

From cross-sectional surveys (figure 1) mean PCV was similar in vaccine and placebo recipients after the second and third doses (31·7% vaccine/31·8% placebo at week 24, 33·9%/33·5% at week 50, and 31·8%/31·9% at week 73). The proportion of children who had PCV of less than 25% was also similar in vaccine and placebo recipients after both second and third doses (not shown).

Chloroquine levels in urine were similar in vaccine and placebo recipients throughout the study. Chloroquine was found in 40/211 (19·0%) urine samples from SPf66 recipients and 47/239 (19·7%) of placebo recipients.

Passive and active case detection

The numbers of clinical episodes seen at the dispensary between second and third dose and after the third dose are shown in table 3. Vacinees had fewer episodes than placebo recipients. Analyses of vaccine efficacy for the periods between second and third dose and after the third dose are shown in table 4. For the purpose of assessing efficacy, follow-up started 4 weeks after vaccination. The primary analysis, of first or only episodes of fever ≥37·5°C with parasitaemia over 20 000/μL after dose 3, gave a vaccine efficacy of 31% (95% CI 0–52%). After two doses of SPf66, the vaccine efficacy was 22% (95% CI −12% to 46%). Kaplan Meier survival curves for the

Period of follow-up	Case definition	Vaccine			Placebo			RR	Vaccine efficacy (95% CI)			
		First (or only) episodes	Child-days at risk	Annual incidence rate	First (or only) episodes	Child-days at risk	Annual incidence rate		Unadjusted	p	Adjusted*	p
Between 2nd and 3rd dose:	Fever† and >20 000 parasites/μL	52	30 464	0·62	74	33 164	0·81	0·76	24% (−8, 47)	0·13	22% (−12, 46)	0·17
After dose 3:	Fever† and >20 000 parasites/μL	49	72 052	0·25	74	78 046	0·35	0·72	28% (−3, 50)	0·069	31% (0, 52)	0·045
	Fever† and >0 parasites/μL	90	65 309	0·50	130	68 656	0·69	0·73	27% (5, 45)	0·018	25% (2, 43)	0·034
	Fever† and no parasites‡	32	76 406	0·15	32	85 973	0·14	1·13				

*Adjusted for age at episode and distance to dispensary.
*†≥37·5°C.
‡Shown for comparison.

Table 4: **Annual incidence of first clinical episodes of *P falciparum* and vaccine efficacy as established by passive case detection**

period between dose 2 and dose 3 and for the period following the third dose (figure 2) show the proportion of children who had not yet had a clinical episode by follow-up time. The estimates of efficacy were similar when time at risk excluded children for 28 days after they had been given antimalarials at the dispensary (not shown).

A subsidiary analysis considered first episodes satisfying the primary case definition from 4 weeks after dose 2 up to the end of the follow-up period. 109 placebo recipients experienced at least one episode compared with 79 SPf66 recipients. This gave an unadjusted efficacy of 23% (95% CI −3% to 42%). An intention-to-treat analysis, including all children randomised at first dose and all time at risk from 4 weeks after 2nd dose up to the first episode, also gave a vaccine efficacy estimate of 23% (95% CI −2% to 42%).

There were 1369 attendances at the dispensary among vaccine recipients and 1610 in the placebo group during the full study period from March 1, 1993 to July 31, 1994 (annual incidence 3·6 and 3·7 visits per year, respectively). The incidence of aparasitaemic fever (table 4) was also similar in the vaccine and placebo groups.

The mean PCV among malaria cases diagnosed at the dispensary was similar in the vaccine and placebo groups, both in episodes between the second and third dose (31·8% vaccine/31·6% placebo) and in those after the third dose (32·4%/32·0%). Furthermore, the proportion of children with a PCV of less than 25% was also similar among the vaccine and placebo groups (between second and third doses, 9% [5/55] and 8% [6/73], respectively; after the third dose, 9% [6/66] and 5% [5/91], respectively).

Active case detection in Group V children (n=191) revealed 29 episodes (10 vaccine/19 placebo) of fever ≥37·5°C together with parasitaemia over 20 000/μL during 6947 home visits (3222/3725) in the follow-up period after dose 3. The prevalence of cases was 0·31% among vaccinees and 0·51% in the placebo group giving a crude vaccine efficacy of 39%. When analysis was restricted to first or only episodes the age-adjusted estimate of vaccine efficacy was 18% (95% CI −88% to 64%; 10 *vs* 15 children). Between second and third doses, 12 episodes were recorded in 1404 visits to SPf66 recipients (0·85%) and 14 episodes in 1723 visits among placebo recipients (0·81%) corresponding to a crude vaccine efficacy of −5%.

Discussion

This trial confirms that vaccination with the chimaeric protein SPf66 reduces the risk of malaria among children highly exposed to natural infection. The results complement those from Latin America, by providing evidence that the vaccine can induce partly effective immunity at the top end of the spectrum of malaria transmission intensity. The vaccine was safe in children highly exposed to *P falciparum*, the incidence of mild or moderate local side-effects being low with no adverse effects requiring medical treatment. The vaccine was also highly immunogenic, leading to anti-SPf66 IgG titres similar to those observed in our preliminary studies,[13] even though pre-existing antibodies against *P falciparum* were common.

The vaccine incorporates the PNANP repeat sequence of the circumsporozoite protein. Despite the high immunogenicity of this vaccine the incidence of *P falciparum* infections was similar in SPf66 and control groups, indicating that the vaccine does not induce effective anti-sporozoite immunity. In contrast, the asexual blood stage antigens in the vaccine appear to have induced or boosted immunity which reduced the incidence of clinical episodes of malaria. The increase in immunofluorescence antibodies after three doses suggests that anti-SPf66 antibodies raised after immunisation with SPf66 recognise native epitopes of *P falciparum* merozoites.

The vaccine efficacy estimate was 31%, lower than the 50% level that the trial was designed to detect and resulting in wide confidence intervals. However, the primary case definition is less than 100% specific so vaccine efficacy is likely to be underestimated. Case definitions with lower parasitaemia cut-offs are even less specific and likely to result in greater bias. Too few episodes were recorded to establish clearly the duration or age dependence of protection induced by the vaccine. Indeed active case detection picked up far fewer episodes than we expected probably due to the improved health services in the village. Moreover, the trial was not intended (and did not have the power) to establish the impact of the vaccine on severe life-threatening malaria or death, though it is of interest that only 1 of the 6 deaths in the study cohort was in a vaccinee. We were not able to assess the severity of malaria episodes revealed by passive

case detection. Our primary measure, the PCV, seems not to have been a good marker of malaria severity in this study since PCV was similar in symptomless children and those with clinical episodes.

SPf66 appears to be effective against *P falciparum* in both South America and Africa. The best estimates of efficacy in both areas are much higher than the prevalence of any one strain of parasites,[25] a result which suggests that the mechanisms mediating protection must be effective against many strains. The vaccine may therefore mimic naturally acquired strain-transcending immunity.

In further analyses we will look at the effect of the vaccine on parasite densities and evaluate protection in relation to age and serological response. The partial protection reported in this paper in itself should encourage investigations to understand better the mechanisms involved, with the aim of improving efficacy. Our results suggest that two doses may give some protection, so further work on the vaccination schedule may be required. They also argue in favour of the approach to antigen selection followed by Patarroyo, and the use of synthetic chimaeric proteins as immunogens. In the absence of surrogate parasitological or immunological markers of protection, our results also highlight the importance of clinical and field trials in vaccine development.

The potential of SPf66 vaccine as a public health measure in Africa will be debated. Current malaria control strategies rely on partly effective tools such as chemotherapy for suspected cases and measures to reduce man-vector contact. The estimated efficacy of SPf66 is lower than that of most vaccines in use for other infections. However, since the burden of malaria morbidity and mortality is vast, measures with a moderate efficacy merit development.

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This trial was independently monitored by Prof C Alonso Bedate (CSIC/Centro de Biologia Molecular, Madrid), Prof M Molyneux (Liverpool School of Tropical Medicine and WHO/TDR), Dr W Mpanju (Commonwealth Secretariat, Arusha), and Prof P G Smith (London School of Hygiene and Tropical Medicine), who provided invaluable support and guidance.

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Duration of Protection and Age-Dependence of the Effects of the SPf66 Malaria Vaccine in African Children Exposed to Intense Transmission of *Plasmodium falciparum*

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The SPf66 synthetic vaccine is safe and partly efficacious against *Plasmodium falciparum* malaria among children 1–5 years old. The estimated vaccine efficacy [VE] for all clinical episodes over a period of 18 months after the third dose is 25% (95% confidence interval [CI], 1%–44%; $P = .044$). The observed temporal variations in efficacy could have been due to chance (likelihood ratio $\chi^2 = 13.8$, 8 *df*; $P = .086$). Efficacy against clinical malaria did not vary significantly with age ($\chi^2 = 1.07$, 4 *df*; $P = .90$). Overall parasite density was 21% lower in vaccine recipients than in the placebo group (95% CI, 0%–38%; $P = .044$). Further development of SPf66 may require trials to evaluate safety, immunogenicity, and efficacy when administered in the first year of life, together with other vaccines contained in the Expanded Programme of Immunization schedule.

Malaria, especially that caused by *Plasmodium falciparum*, is the most important parasitic disease of humans. Estimates of the burden of disease and death are imprecise but include >300 million clinical episodes and 1–3 million deaths per year. Even though malaria has a wide geographic distribution and is endemic in >100 countries, >90% of the world's burden of disease and death concentrates among young children and primigravid pregnant women living in sub-Saharan Africa.

Development of vaccines against *P. falciparum* offers multiple potential targets in all stages of the parasite's life cycle, and technological developments allow for the possibility of combining antigens from either one or several stages. Each target or combination of targets may possibly imply different outcomes. In the absence of a pre–erythrocytic stage vaccine that induces sterilizing lifelong immunity (clearly the most desirable option), vaccines derived from asexual–blood stage antigens may be of particular interest in Africa, because they may mimic the development of natural anti-parasite and clinical immunity in children living in endemic areas [1].

SPf66 is a synthetic hybrid polymer, solubilized in saline solution and adsorbed onto aluminium hydroxide. The monomer unit is a chemically synthesized peptide of 45 amino acids that contains sequences derived from three asexual–blood stage proteins (83, 55, and 35 kDa) linked by repeat sequences from the circumsporozoite protein of the *P. falciparum* parasite [2]. SPf66 has been shown to be safe and partially protective against *P. falciparum* malaria in populations >1 year of age living in low-endemicity areas of South America [3, 4, 5]. In the La Tola trial in Colombia [3], SPf66 was efficacious for at least 1 year after the third dose, and there was a suggestion that efficacy was higher among young children and older adults. However, all of the effects of SPf66, including safety, immunogenicity, and short- and long-term efficacy, could vary with different transmission levels.

The first safety, immunogenicity, and efficacy results of SPf66 from Africa came from a trial in the Kilombero Valley, an area of intense perennial transmission of *P. falciparum* in southern Tanzania [6, 7]. In the study, the efficacy analysis of the SPf66 vaccine in 1- to 5-year-old children indicated that three doses reduced the risk of first or only episodes of clinical malaria by 31% (95% confidence interval [CI], 0%–52%; $P = .045$) [7]. Clinical malaria was defined as fever (axillary temperature ≥37.5°C) with parasite density >20,000/μL. The efficacy against all episodes of fever and parasitemia (a less specific case definition of clinical malaria), though lower than that against hyperparasitemic episodes, was 25% (95% CI, 2%–43%; $P = .03$). Thus, the vaccine had partial efficacy against clinical malaria during 1 year following administration of the third dose. Longer-term protection remained to be established,

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The protocol was reviewed and approved by the ethical committees of all participating institutions, the World Health Organization, and the Tanzanian Commission for Science and Technology (UTAFITI NSR/RCA 90). Detailed written informed consent was obtained from the parents and guardians of children.

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including a theoretically possible rebound in morbidity among vaccine recipients if immunity induced by the vaccine decreased over time. More recently, a trial among nonimmune Gambian infants, who were 6- to 11-months old at the time of the first dose, has failed to document significant levels of protection (vaccine efficacy [VE] = 8%; 95% CI, −18%–29%) [8].

Here we report further analyses of the data from the Kilombero SPf66 trial. We examine whether vaccine efficacy extends beyond the first year of follow-up, whether there is evidence to suggest that efficacy varies with age, and the effect of the vaccine on parasite density in more detail.

Methods

Study area and period. The study was done in the village of Idete in the Kilombero river plain in southern Tanzania. Details of the study area and study design have been presented elsewhere [9, 10]. In brief, the Kilombero Valley is an area of intense and perennial malaria transmission. Most inhabitants are subsistence farmers growing maize and natural irrigation rice. The Kilombero SPf66 vaccine trial was a randomized double-blind, placebo-controlled, community-based efficacy trial. After screening procedures were completed, 586 children aged 1–5 years received three doses of either SPf66 or placebo.

The main analyses presented here are based on the period starting 4 weeks after the administration of the third dose of vaccine (September 1993). The previously reported analyses [7] considered follow-up for 48 weeks, until the end of July 1994. We now describe results including an extended follow-up for a further 26 weeks, terminating on 31 January 1995. Although the randomization code was broken in early August 1994 for the analysis, the study population and field team remained blind for the extra period of follow-up, while further supplies of SPf66 were prepared and transported to Tanzania. In the first week of February 1995, SPf66 was offered to children who had previously received placebo, as agreed between the village and the investigators at the beginning of the trial.

Parasitologic and clinical surveillance. Cross-sectional surveys, at which blood films for parasitologic examination were taken, were done on a group of 219 children during weeks 30, 32, 34, 38, 50, 73, and 99 of the follow-up (4, 6, 8, 12, 24, 47, and 73 weeks, respectively, after the third dose). Similar surveys were also done between the second and third doses on a different subgroup of 176 children at weeks 8, 10, 12, 16, and 24. All 586 children making up the complete study cohorts were seen at a baseline survey 2 weeks before receiving the first dose [10]. Before each dose of either vaccine or placebo, all study children received one dose of sulfadoxine-pyrimethamine (25 mg of sulfadoxine and 0.75 mg of pyrimethamine/kg of body weight).

Passive case detection of clinical episodes was done at the only village dispensary as described [7, 10]. In brief, each patient attending the dispensary for a perceived illness was screened by project medical personnel, who provided 24-h coverage. Children were identified using the census and individual identification cards, and axillary temperatures were checked with an electronic thermometer (MBO, Munich). If the temperature was ≥37.5°C, a fingerprick blood sample was collected and thick and thin blood films prepared. Children were then cared for by the routine medical services at the dispensary. Clinically suspected malaria episodes were treated, following national standard guidelines, with 25 mg/kg chloroquine.

Statistical methods. Analysis of risk of clinical malaria considered all episodes satisfying the primary case definition (>20,000 parasites/μL and an axillary temperature ≥37.5°C). Note that this differs from previous analysis [7], which were restricted to first or only episodes. For the present analyses, time at risk was computed in weeks. Any week that included days at risk was included. To avoid the inclusion of treatment failures and recrudescence as repeated events, second events <28 days after an episode were excluded. Children were not considered at risk for those 28 days.

The proportion of the weeks at risk in which an episode occurred was analyzed by logistic regression using the EGRET software package [11]. Because there were small imbalances between vaccine and placebo groups with respect to age and distance from the dispensary [7], all analyses allowed for these factors as defined in the analytical plan [7]. Analysis also considered effects of vaccination status and of time periods of ~8 weeks. The age-dependence of the effect of vaccination was tested by including a term representing age for vaccine recipients and 0 for placebo recipients. Changes in vaccine efficacy over time were tested similarly.

The above analyses assume that multiple episodes in the same child occur independently of each other. To test whether this assumption affected the conclusions, a logistic-normal regression model [12] was fitted, which allowed for variation in susceptibility between children. Adjusting for this potential source of variation did not improve the fit of the model, and we therefore report tests and CIs derived from the original analysis.

Parasite positivity was analyzed by logistic regression in relation to age group, vaccination status, and time period of the survey. Allowance for variation between children in susceptibility again did not improve the fit of the model.

Analyses of parasite densities were related to vaccination status, survey, and age using least squares regression models. Aging of the cohort meant that the age distribution was different at each survey. Age-specific average parasite densities allowing for survey variation were computed by the method of least squares means [13]. The same method was used to calculate survey-specific average adjusted for age.

Parasite densities on successive slides from the same child are correlated. Models that allowed for this were fitted by residual maximum likelihood [14] using the SAS MIXED procedure [15]. Statistical significance test was determined by likelihood ratio (LR) tests.

Results

Clinical episodes. During the additional 6 months of follow-up from 1 August 1994 to 31 January 1995, there were no deaths among cohort children. Twenty-nine episodes of clinical malaria satisfying the primary case definition (10 vaccine and 19 placebo) were detected through passive case detection. Between 4 weeks after the third dose and the end of follow-up 18 months later, a total of 121 clinical episodes were

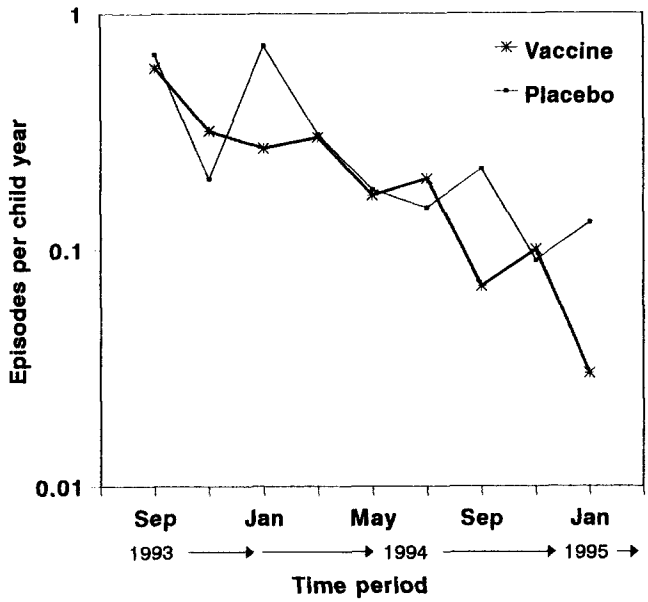

Figure 1. Incidence of clinical episodes among SPf66 vaccine and placebo cohorts after third dose by 8-week time period.

recorded in placebo recipients (0.30 episodes/child-year) and 83 in SPf66 recipients (0.23 episodes/child-year). The age- and distance-adjusted estimate of vaccine efficacy was 25% (95% CI, 1%–44%; LR χ^2 = 4.04, 1 *df*; P = .044). The incidence of clinical episodes, among both vaccine and placebo recipients, showed a decline with time (figure 1). Age-standardized incidence estimates indicated that the decline in incidence over the period of follow-up could not be entirely accounted for by the aging of the cohort (age- and distance-adjusted tests of the difference in risk between 2-month time periods, LR χ^2 = 43.4, 8 *df*; $P < .001$). Although vaccine efficacy appeared to vary over time and to be restricted to several periods (figure 1), there was no clear trend, and these variations could have been due to chance (test of heterogeneity in efficacy between time periods, LR χ^2 = 13.8, 8 *df*; P = .086).

Risk of clinical malaria decreased steeply with age (table 1 and figure 2). Incidence of clinical malaria was lower among vaccine recipients than among placebo recipients in all age groups. The corresponding vaccine efficacy estimates by age are also presented in table 1. There was no evidence of vaccine efficacy changing with age (LR test for heterogeneity, χ^2 = 1.07, 4 *df*; P = .90; LR test for trend, χ^2 = 0.26, 1 *df*; P = .69). In children <2.5 years of age, the vaccine efficacy of 29% corresponds to a time period–standardized reduction in risk from 0.92 to 0.65 episodes per child-year (i.e., a substantial reduction in risk of 0.27 prevented episodes per child-year). The corresponding reduction in children >5 years old, for whom the estimated VE was 43%, was from 0.12 to 0.07 episodes per child-year (0.05 prevented episodes).

Parasite positivity. Treatment with sulfadoxine-pyrimethamine before each dose of vaccine aimed to optimize the response to the vaccine. It consequently also ensured that follow-up began with a low prevalence of parasitemia in both vaccine and placebo groups (figure 3). Prevalence increased rapidly with time, gradually tending toward levels similar to that at screening (92%). The difference between overall parasite positivity in vaccine and placebo groups was small and not statistically significant (LR χ^2 = 2.25, 1 *df*; P = .13). The prevalence of patent parasitemia averaged over all surveys declined with age (LR χ^2 = 16.74, 4 *df*; P = .002), but this decline was not very steep. There was no evidence that the effect of vaccination varied with age (LR χ^2 = 0.26, 1 *df*; P = .6).

Parasite densities. Overall, parasite density after the third dose was 21% lower in vaccine recipients than in the placebo group (95% CI: 0%–38%; F statistic = 4.06, 1740 *df*; P = .044). Parasite densities decreased with age, both after the third dose (figure 4A, table 2) and between the second and third doses (figure 4B). Allowance for differences between surveys did not account for the age trends (LR test for effect of age adjusted for survey, χ^2 = 28.16, 4 *df*; $P < .001$). After the third dose, parasite density was much more strongly age-dependent among vaccine recipients than among placebo recipients. In the youngest age group, the densities in children receiving three doses of vaccine were higher than in placebo recipients, but the reverse was true for older children. The interaction between a linear effect of age and vaccination status was statistically highly significant (LR χ^2 = 12.6, 1 *df*; $P < .001$).

Discussion

Eighteen months after the third dose of SPf66 was given in the Kilombero trial, there is evidence to suggest that vaccine efficacy against clinical malaria was sustained. Previous analyses of this trial indicated that the vaccine is safe and immunogenic and reduced the risk of first or only episodes of clinical malaria by a best estimate of 31% during a 1-year period of follow-up after the third dose. By including a further 6 months of follow-up, as well as all malaria episodes and not just first or only episodes, we estimate vaccine efficacy to be 25% (95% CI, 1%–44%). This figure therefore represents the reduction in the burden of malaria attributable to vaccination in this group of children over a period of 18 months after the third dose.

None of the published trials were designed to look at variations in efficacy over time, and observed data must be interpreted with caution. The La Tola trial [3] suggested some degree of variation in vaccine efficacy over time. However, there was evidence of efficacy in all time periods and a suggestion that efficacy was highest in the first and last 3-month period. The apparent temporal variations in the trial of Las Majadas in Venezuela [4] are very difficult to interpret given the nonrandomized nature of the trial.

In the Tanzania trial (figure 1), there is a suggestion that vaccine efficacy has been limited to certain time periods (January and September 1994 and January 1995). Although a number of hypotheses related to transmission intensity or seasonal cir-

Table 1. Annual incidence of clinical episodes by age group among SPf66 vaccine and placebo recipients and corresponding vaccine efficacy estimates.

	Vaccine			Placebo			
Age, months	No. of episodes	Weeks at risk	Adjustd incidence rate*	No. of episodes	Weeks at risk	Adjusted incidence rate*	Vaccine efficacy, % (95% confidence interval)
<30	24	1508	0.65	27	1220	0.92	29 (−24, 59)
30–35	20	1900	0.56	24	1793	0.62	9 (−66, 50)
36–47	19	3843	0.30	29	4653	0.31	5 (−70, 47)
48–59	11	3557	0.17	20	4021	0.25	32 (−42, 68)
≥60	9	7765	0.07	21	9145	0.12	43 (−25, 74)

* Estimated incidence for fifth 8-week period of follow-up, adjusted for distance from dispensary.

culation of certain parasite strains could be postulated, the apparent variations in vaccine efficacy over time may have been simply due to chance. Nonetheless, one of those periods when differences between vaccine and placebo recipients appear to be more noticeable is precisely at the end of the follow-up. This would support the suggestion that the vaccine remains efficacious for at least 18 months after the third dose. It would also argue against decreasing vaccine efficacy or rebound in morbidity among vaccine recipients.

The data also highlight the difficulty of establishing long-term protection afforded by an asexual-stage vaccine in an area of high transmission where the age pattern of disease is strongly shifted toward the younger age groups. Indeed, while children <30 months old had nearly 1 clinical episode detected through passive case detection every year, children >60 months old had just over 0.1 episodes per year. Other authors have argued that even in areas of very high transmission, there are relatively small differences in the incidence of malaria among children aged 0–6 years and subject to a very intense and active follow-up [16]. These children have, on average, 4 episodes per year. While this may be true, our data clearly show that the risk of clinical malaria cases that attend the dispensary and fulfill a definition of clinical malaria with a high specificity decreases steeply with age in this area of high and perennial transmission. As previously argued [17], it may be that the cases seen at the dispensary and therefore ascertained through passive case detection represent the more severe episodes in the clinical spectrum of malaria, while many other mild, self-limited, non-severe cases go unreported.

The trial was not designed, and therefore had little power, to demonstrate differences in vaccine efficacy between age groups. In any case, the data suggest that clinical efficacy is not restricted to children of any specific age group or that the efficacy depends on age. Younger children seem to be protected

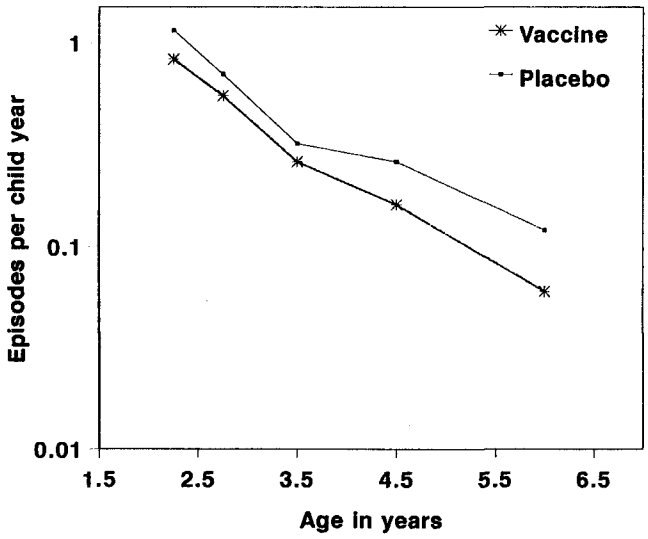

Figure 2. Incidence of clinical episodes among SPf66 vaccine and placebo recipients after third dose by age at time of episode.

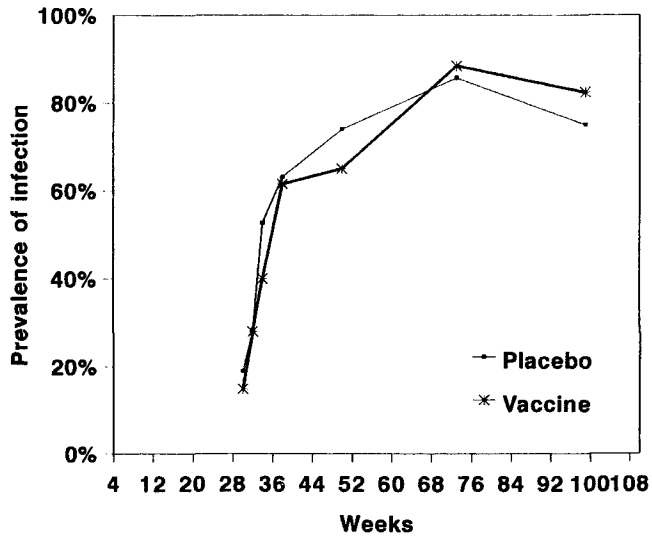

Figure 3. Prevalence of parasitemia by time period among SPf66 vaccine and placebo recipients.

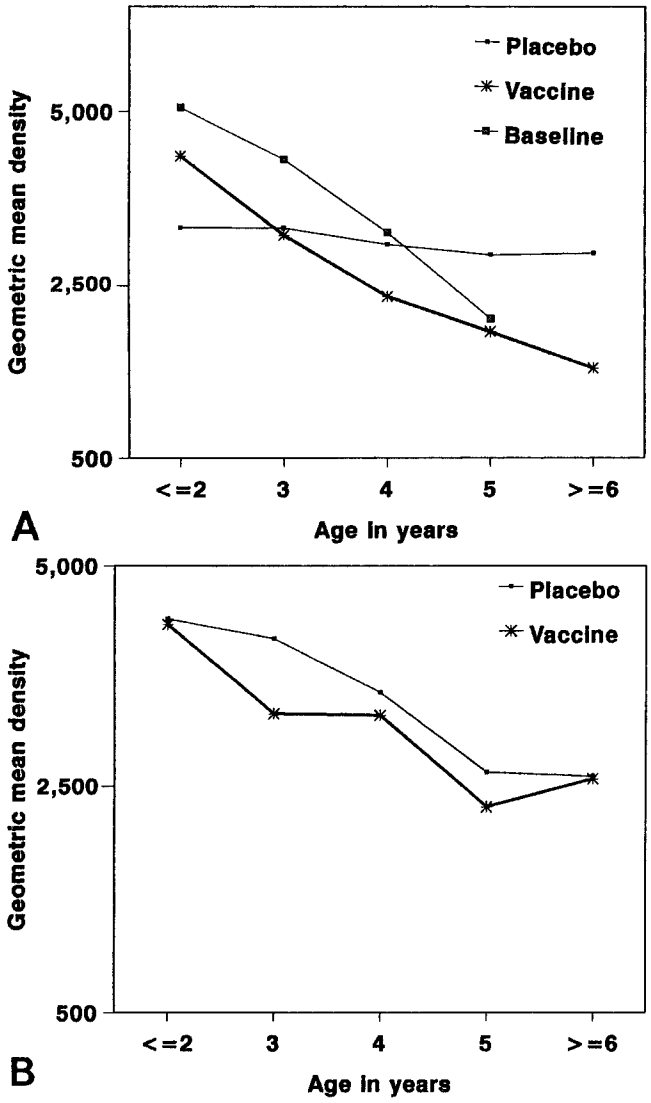

Figure 4. Geometric mean parasite densities by age at time of episode after third dose of SPf66 vaccine (**A**) and between second and third dose (**B**).

to a similar degree as older ones. The impact of even a modest efficacy of 30% in the younger age groups is large, given the high incidence of malaria in this age group, and corresponds to 0.27 prevented episodes per child-year.

A primary effect of SPf66 on asexual-stage parasites was anticipated because the vaccine includes amino acid sequences derived from merozoite membrane proteins. In the original artificial challenge experiments in Colombia in both monkeys and humans [18, 19], self-limiting symptomatic infections were observed in some immunized individuals, implying that a primary effect of SPf66 may be to reduce densities of blood-stage parasites. In our trial, there is evidence of the vaccine reducing parasite densities after the third dose by ~20%.

When comparing parasite densities between SPf66 and placebo recipients in different age groups, it appears that three doses of vaccine were associated with a reduction in parasite densities in the older children and with an apparent negative effect in the youngest children. However, the comparison of the age-dependence of densities after the third dose with those in the baseline survey (figure 4A) and between the second and third dose suggests that rather than the vaccine group showing an unusually strong relationship between age and density, the placebo group shows an unexpectedly weak age-dependence of densities. The reason for this temporary decrease in age-dependence is unclear. It may be a consequence of repeated sulfadoxine-pyrimethamine treatment. This resulted in an extended period of reduced antigenic stimulation in the placebo group at the same time as the vaccine group was being immunized with SPf66. However, this may help explain the lower parasite densities seen in the young children receiving placebo but can hardly explain the higher parasite densities in older children in the placebo group compared to baseline data.

Recently reported results from a trial of SPf66 among nonimmune Gambian infants 6- to 11-months old at the time of first dose have not documented significant levels of protection [8]. The results raise further questions on the potential public health relevance of the vaccine. Efficacy estimates were based on a subgroup analysis of the study children and a short 3.5-month period of follow-up. The authors suggest that the lack of a significant level of protection documented in the SPf66 trial in Gambia may have been due to the short follow-up and that it may take the vaccine this long to show any apparent effect. While this may be true, data on this point are lacking.

Some of the differences in the estimated efficacies between the trial in Gambia and all other trials may include the design and its emphasis on active case detection, the ages and level of preimmunity of the study children, and the specificity of the case definitions of the primary end point. It is also possible that the apparently contradictory results between Gambia and all other sites may have been due to chance, as the upper 95% confidence bound overlaps with the lower bound from the Tanzanian trial. These results also highlight the need for adequate coordination of trials that ensure complementarity and comparability.

The benefit of any malaria vaccine in sub-Saharan Africa will depend mainly on its effectiveness in preventing severe disease and death. Its applicability is likely to depend on the feasibility of delivering it through the Expanded Programme of Immunization. Recent estimates suggest that a vaccine with a 30% efficacy against death may be a highly cost-effective public health intervention in countries of sub-Saharan Africa in which malaria is endemic if it can be delivered through the existing Expanded Programme of Immunization (Evans D, personal communication). A further large-scale study to investigate these questions and complete the process of vaccine development for SPf66 is required [20]. Also, further work to understand the reason for the different estimates of efficacy between different trial sites is needed. The comprehensive approach to all these questions as well as a systematic review of

Table 2. Geometric mean parasite densities by age group and treatment group between second and third dose and after third dose of either vaccine or placebo.

	Vaccine		Placebo				
Time point, age (years)	*n*	Parasite density	*n*	Parasite density	Lower 95% confidence limit*	Ratio of densities*	Upper 95% confidence limit*
Between second and third dose							
≤2	80	3708	112	3817	0.56	0.97	1.69
3	42	2334	62	3449	0.34	0.68	1.36
4	55	2315	62	2608	0.49	0.91	1.68
5	40	1441	52	1725	0.42	0.82	1.62
≥6	14	1666	19	1689	0.34	1.00	2.90
After third dose							
≤2	100	3699	74	2288	0.98	1.67	2.84
3	77	2174	92	2284	0.50	0.82	1.32
4	72	1443	83	2046	0.41	0.69	1.14
5	52	1146	54	1910	0.29	0.54	1.00
≥6	61	904	88	1930	0.28	0.45	0.73

* Allowing for differences between surveys in average densities.

all published and ongoing work on this vaccine will clarify the potential of SPf66 as a potential public health tool and help in the process of development of new vaccines with increased efficacy.

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Immune responses to *Plasmodium falciparum* antigens during a malaria vaccine trial in Tanzanian children

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SUMMARY

Among Tanzanian children living in an area of intense and perennial malaria transmission, prevalence of naturally acquired IgG antibodies that recognize SPf66, NANP, p190 and a 19 kDa fragment of the merozoite surface protein-1 (MSP-1) is high and increases with age. This possibly reflects the high level of natural exposure of the children to P. falciparum. *The prevalences of IgG antibodies that recognize the three putative merozoite derived sequences contained in the malaria vaccine SPf66 (83·1, 55·1 and 35·1) is low but also show some age dependence. Three doses of the SPf66 vaccine induce a strong IgG antibody response against both the SPf66 construct, NANP and the three individual peptides. Vaccination with SPf66 did not result in an increase of anti19 kDa fragment antibodies. This reflects the specificity of the humoral immune response induced by the SPf66 construct. Among vaccinated children, antibody titres against SPf66 decreased over time following the third dose. However, 18 months after the third dose, SPf66 recipients still had significantly higher IgG titres and stimulation indices of peripheral blood mononuclear cells (PBMC) than placebo recipients. Within the vaccine group, there is a trend for increasing anti-SPf66 IgG titre to be associated with decreasing risk of clinical malaria but this was not statistically significant. Results also show the difficulties of establishing whether antibody responses are related to protection in field trials in endemic areas.*

Keywords *malaria, vaccine, immune responses, protection*

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INTRODUCTION

The earliest systematic evidence of man's capacity to develop immunity against malaria comes from epidemiological studies carried out by Koch in Java during the latter part of the 19th century (Ross 1910). These studies showed how in endemic areas, the clinical and parasitological manifestations of disease declined with age. Sinton (1939) considered that there were two stages to the development of naturally acquired immunity. The first was the development of an antitoxic immunity, and the second was the consolidation of antiparasitic mechanisms. The slowness with which antiparasitic mechanisms consolidate has often been taken as evidence that plasmodial populations in hyperendemic areas comprise many antigenically distinct species and strains, most of which have to be experienced before an effective antiparasite immunity is acquired (Hackett 1941). However, during malariotherapy, good evidence was produced that partial clinical protection after challenge was evident not only to the homologous strain, but also towards heterologous strains of the same species (Nicole & Steel 1926). This was further reinforced by the observations that in passive transfer studies, IgG from West African immune serum protected against *P. falciparum* parasites of East African origin (McGregor *et al.* 1963). Finally, volunteers immunized with irradiated sporozoites and shown to be protected against *P. falciparum* or *P. vivax* infection (Clyde *et al.* 1975) confirmed that immunity induced by vaccination was strictly species and stage specific, but also effective against geographically remote isolates within each species.

There is solid evidence that antibodies play an important role in mediating protection. In passive transfer studies (Cohen *et al.* 1961), the 7-S gammaglobulin (IgG) fraction of sera from immune West African adults, administered in

large doses to young children with clinical malaria, reduced parasitaemia & promoted clinical recovery. Also, IgG of cord blood obtained from Nigerian infants was shown to be effective in the treatment of acute malaria in older children (Edozien 1962). Finally, there is also some good evidence that IgG has an antiparasitic effect and not an antitoxic effect, as the clinical improvements during the passive transfer studies were not observed without prior reduction in parasitaemia (McGregor 1964). A number of observational studies have also described associations between presence of antibodies against certain *P. falciparum* antigens, including SPf66 and reduced risk of clinical malaria (Al-Yaman *et al.* 1995).

Vaccines against malaria may in future become important tools as part of comprehensive control programs. The search for a vaccine against malaria has been long, partly due to the complex life cycle of the parasite, an incomplete understanding of the mechanisms of effective immunity, and a lack of surrogate measures of protection and of adequate animal models. Various molecules from the preerythrocytic, asexual blood and sexual stages have been characterized and some may be promising vaccine candidates. The chimeric protein SPf66 was the first asexual blood stage vaccine to show efficacy in phase III human trials (Tanner *et al.* 1995, Facer & Tanner 1997). The monomer unit of SPf66 is a chemically synthesized peptide of 45 amino acids which contains sequences thought to be derived from three asexual blood stage proteins (83·1, 55·1 and 35·1 kDa) linked by PNANP sequences derived from the circumsporozoite protein of the *P. falciparum* parasite (Moreno & Patarroyo 1989). The 83·1 fragment (part of Block 1 of MSP-1) derives from a well characterized blood stage protein. SPf66 has been shown to be safe, immunogenic and partially protective against *P. falciparum* malaria in populations older than 1 year of age living in both low endemicity areas of South America (Patarroyo *et al.* 1988, Amador *et al.* 1992, Valero *et al.* 1993, Noya *et al.* 1994, Sempertegui *et al.* 1994, Valero *et al.* 1996) and high endemicity areas of Tanzania (Alonso *et al.* 1994b, Teuscher *et al.* 1994). Further trials in The Gambia failed to establish protection among 6–11 months old infants (D'Alessandro *et al.* 1995). More recently, in a trial carried out in Thailand with a US produced version of SPf66 previously shown to be less immunogenic than the Colombian vaccine (Leach *et al.* 1995), the vaccine failed to reduce the risk of malaria among children aged 2–15 years living in a refugee camp (Nosten *et al.* 1996).

At present there is little information on the mode of action of SPf66 (Tanner *et al.* 1995). Understanding the mechanisms of action might help in understanding the variation in efficacy estimates obtained in different epidemiological settings, and more importantly it may help to develop surrogate measures of protection. This in turn could simplify the process of malaria vaccine development and reduce the number of clinical phase III trials needed. In this paper we report on further analysis of the humoral and cellular responses to the vaccine, its individual components and other *P. falciparum* epitopes during the phase III trial carried out in Tanzania (Alonso *et al.* 1994b).

METHODS

Study design, clinical surveillance and analysis

The Kilombero SPf66 vaccine trial was carried out in the village of Idete in the Kilombero river plain in southern Tanzania. Details of the study area and study design have been presented elsewhere (Alonso *et al.* 1994a). In brief, the Kilombero Valley is an area of intense and perennial malaria transmission. Most inhabitants are subsistence farmers growing maize and natural irrigation rice. The Kilombero SPf66 vaccine trial was a randomized double blind, placebo controlled community-based efficacy trial. Following informed consent and screening procedures, 586 children aged 1–5 years received three doses of either SPf66 or placebo, each preceded by a single dose of sulphadoxine-pyrimethamine to clear parasites. The follow-up period of 12 months following the third dose of the vaccine corresponds to the one described earlier in detail (Alonso *et al.* 1994b).

Passive Case Detection of clinical episodes was carried out at the only village dispensary as previously described. In brief, every patient attending the dispensary for a perceived illness were screened by project medical personnel, who provided round-the-clock coverage. Children were identified using the census and individual identification cards, and the axillary temperature checked with an electronic thermometer (MBO, Munich, Germany). If the temperature was $>37{\cdot}5°C$, a fingerprick blood sample was collected and thick and thin blood films prepared. Children were then cared for by the routine medical services at the dispensary. Clinically suspected malaria episodes were treated following national standard guidelines with 25 mg/kg of chloroquine. As for previous analyses (Alonso *et al.* 1994b, 1996) an episode of clinical malaria was defined when a child presented at the dispensary with an axillary temperature $\geq 37{\cdot}5°C$ and $>20\,000$ parasites/ul in the blood smear.

The analysis considered time at risk from four weeks after the third dose up to the first episode satisfying this case definition. Poisson regression was used to test the relationship of the incidence of clinical malaria to postvaccination titres, allowing for possible age and distance confounding

(as in Alonso *et al.* 1994b). Separate analyses were conducted for placebo and vaccine recipients.

Laboratory methods

IgG antibody levels against the SPf66 polymeric construct and the three individual epitopes of merozoite origin (83·1, 55·1 and 35·1) were measured on three sets of serum samples collected at screening (preimmunization), four-weeks after third dose and 18 months after the third dose. The IgG levels against SPf66 were determined by FAST ELISA (Falcon Assay Screening Test-ELISA, Becton Dickinson Labware, Linco Park NJ, USA), according to the conditions previously described (Teuscher *et al.* 1994, Lopez *et al.* 1994). The IgG levels against the 760 [CG***YSLFQKEKMVL***GC], 758 [CGYGG***PANKKNA***GC] and 755 [CG***DELEATQNVYAA***GC] peptides (in bold are the actual components present in the SPf66 monomer), were also determined by FAST ELISA.

The 760 peptide contains amino acid sequences derived from the 83·1 fragment of the N-terminal region of MSP-1 (Block 1), while peptide 755 and 758 contain sequences from the 55·1 kDa and 35·1 kDa proteins. For these three peptides, the amount was 5 μg/well. The microtitre plates were incubated overnight with lids and shaking at room temperature. The peptide-coated lids were then washed by an automatic and homogeneous system of intense pulverization (Boch) with PBS-Tween 20 (0·15 M phosphate buffer, 0·15 M NaCl, 0·5% Tween 20, pH 7·2) and with deionized water. The lids were then left to dry at room temperature during two h. The reactivity with the sera was assessed whilst shaking for 15 min at room temperature. The sera dilutions were prepared in PBS, 0·5% Tween 20 and 5% nonfat milk. Finally the lids were incubated with goat antihuman IgG peroxidase conjugated (Tago) at a dilution of 1/2000 in PBS, 0·5% Tween 20, 5% nonfat milk, in shaking for one h. After every step, the lids were washed as described above. The reactivity was visualized by adding 100 ml of TMB peroxidase substrate: H_2O_2 (1:1, v:v) (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA) to the wells and incubating for a further 15 min at room temperature.

The optical densities were determined at a wavelength of 620 nm using a Multiskan Plus plate reader (Labsystem). The cut-off points for positivity were an optical density of 0·025, 0·035 and 0·065 for the 83·1 55·1 and 35·1 peptides, respectively. The above mentioned cut-off points were determined by adding five standard deviations to the mean values obtained for each peptide with 100 sera of malaria-free individuals from Spain, Colombia and Venezuela. Sera were first screened for reactivity in triplicate by two operators at 1/32 and 1/64 dilutions. Titres of less than 32 were considered negative. Positive sera were then titrated. Two positive and negative control sera (against SPf66 and the specific peptides, respectively) were run on each plate and a determination of antigen available on the lids was performed. Negative samples were assigned a nominal titre of half the minimum dilution for the analyses.

In addition IgG was assessed against the synthetic peptide, $(NANP)_{50}$ (Etlinger *et al.* 1988); the recombinant peptide p190 (a 190 kDa fragment from the N-terminal end of the merozoite surface protein-1 (MSP-1) of the MAD-20 strain of *P. falciparum*, expressed in *E. coli*); and the conserved 19 kDa peptide from the C-terminal end of MSP-1 (Kumar *et al.* 1995). The ELISA systems to detect antibodies against these antigens were as follows: Microtitre plates (Dynatech, Immulon, M 129 B/II/IV) were coated at room temperature overnight with 100 μl antigen/well diluted in PBS 7·2 (2–5 μg/ml). Plates were washed (deionized water plus 0·05% tween 20) three times for 15 s and blocked with PBS + 5% milk powder at 37°C for one h. The plates were subsequently washed again and 100 μl/well of serum dilutions (PBS-tween plus 5% milk powder) were added. Incubation at room temperature lasted 30 min on a plate shaker. After washing (three times) 100 μl/well ICN IgG (h + l)HRP conjugate (dilution 1:13′000 in PBS-tween plus 5% milk powder) was added. The plates were incubated on a shaker at room temperature for another 30 min. Subsequently, the plates were washed three times and rinsed once with distilled water. Enzymatic reaction was assayed using 10 mg OPD per 50 ml of substrate buffer (pH 5·0, with 10 μl of 30% H_2O_2 at 100 μl per well. The reaction was stopped with 8 M H_2SO_4 when the positive control reached an optical density (OD) of 1·0. OD was measured at 492 nm on an ELISA reader (Titerek Multiskan MCC/340). Crude OD-values were transformed into standardized OD (mean OD of negative sera in coated wells minus mean OD of no sera in coated wells) minus (mean OD of negative sera in uncoated wells minus mean OD of no sera in uncoated wells). Standardized OD-values were then adjusted relative to the plate-specific OD of the positive control.

Proliferation assays were performed in a random sample of immunized children 18 months after the third dose. Peripheral blood mononuclear cells (PBMC) were isolated from 5 to 7 ml EDTA-blood by density gradient centrifugation on Leucoprep tubes according to the instructions of the manufacturer (Becton-Dickinson, Lincoln Park, NJ, USA). After removing the plasma, PBMC were washed twice in Ca^{++}, Mg^{++} free Hanks' balanced solution (HBSS) by centrifugation at 500 *g* for 7 min at room temperature. After resuspension in Iscove's modified Dulbecco medium (IMDM) supplemented with 2 mM L-glutamine, 100 U/mL penicillin-streptomycin, 5×10^{-5} β-mercaptoethanol and 10% heat inactivated foetal calf serum, 10^5 viable PBMC

were aliquotted into round bottomed 96-well Microtitre plates (200 μl/well). The optimal antigen concentration was determined by titration experiments (100 μmol for SPf66 was the optimal antigen concentration determined in previous titration pilot experiments (data not shown)). Control wells were cultured by replacing the antigen with the same volume (20 μl) of culture medium. Cultures were incubated for seven days. In the last 18 h of cultivation, the cells were pulsed with 1μCi ^{3}H-thymidine/well. After harvesting on filter mats, the cells were liquid scintillation counted. The proliferative responses were expressed as geometric mean counts per min (cpm) of the quadruplicate samples. The stimulation index (SI) was calculated as the ratio of geometric mean cpm of test samples divided by the geometric mean cpm of unstimulated control samples. At day seven of cultivation the means of triplicate control samples ranged between 996 and 1622 cpm.

The stimulation assays were performed on several days according to sample collection. To reduce technical and methodological inconsistencies, a second cell-stimulation with phytohemaglutinin (PHA) was carried out with all samples over three days as internal control. Only cultures with a stimulation index of 15 or higher with PHA were included in the evaluation of PBMC stimulation by SPf66.

RESULTS

Pre-immunization antibody levels

Table 1 shows the prevalence and geometric means of antibody levels for SPf66 and its three individual putative merozoite-derived epitopes. For each epitope the levels were similar in placebo and vaccine recipients (t-statistic for comparison of log-transformed total SPf66 data 1·13, 564.d.f., $P = 0·26$).

There was a small but significant correlation of increasing antibody titres with increasing age for SPf66, the 55·1 and the 35·1 epitopes, but no age trend for the 83·1 (Table 1). For SPf66 every one year increase in age was associated with an average 17% increase in antibody titres (95% CI 9·1%, 26%; $P < 0·001$). Titres against the SPf66 construct and all individual epitopes were highly correlated with each other. Spearman rank correlation coefficients ranged from 0·33 to 0·55 (with P-values $< 0·0001$ throughout).

Antibody levels four weeks after the third dose

Table 2 shows the number of samples assayed four weeks after the third dose and the prevalence and geometric means of antibody levels separately for the vaccine and placebo recipients. Geometric mean titres and prevalence had increased substantially for all merozoite epitopes contained in SPf66 which were assayed in the vaccine group, but had declined slightly in the placebo group. There were consequently large differences in both prevalence and geometric mean titres between placebo and vaccine recipients. These differences were reflected in the geometric mean indices of response, which were all less than unity for placebo recipients and ranged from 1·66 for the 35·1 peptide to 6·93 for the SPf66 construct.

In the placebo group, antibody titres four weeks after the third dose were correlated with those at baseline. Similarly to baseline data, antibodies also increased with age. After adjusting for baseline antibodies, each year increase in age was corresponded to a 12% increase in titres (95% CI 0·6%, 24·6%; $P = 0·04$). In the vaccine group, there was a trend for antibody titres to decrease with age. After adjusting for baseline antibodies, each year age was associated with a 11·2% decrease in the SPf66 titre after dose three (95% CI – 77·0%, 1·1%; $P = 0·07$). A similar pattern is observed when analysing the age specific index of response. For each year of age, there was a 21·4% decrease in the index of response (95% CI – 32·5%, – 8·5%; $P = 0·002$) defined as the ratio of postvaccination to prevaccination titres (Teuscher *et al.* 1994).

Correlations between postimmunization titres of different antibodies in the placebo group were similar to those seen preimmunization. In the vaccine group, the rank correlations between postimmunization titres were even higher than those preimmunization varying between 0·52 and 0·80.

Table 1 Pre-immunization prevalence, mean antibody levels against SPf66 and its three blood stage components and age dependence

Peptide	No. with detectable antibody/ No. tested (%)	Geometric Mean titre (95% CI)	r (*P*-value)	SPf66 *vs* placebo z (*P*-value)
83·1	53/534 (10)	17·5 (17·1, 18·0)	0·05 (0·24)	– 1·16 (0·25)
55·1	124/483 (26)	22·1 (20·8, 23·4)	0·21 (0·0001)	– 1·11 (0·27)
35·1	34/361 (9)	17·6 (17·0, 18·2)	0·12 (0·026)	0·29 (0·77)
SPf66	517/566 (91)	426·3 (382·9, 474·6)	0·19 (0·0001)	– 1·24 (0·21)

r: Spearman correlation between antibody titres and age
z: Wilcoxon Test

Table 2 Prevalence, age dependence and geometric mean postimmunization IgG levels against several *P. falciparum* antigens four weeks after the third dose (Table 2a) and index of response (Table 2b), among vaccine and placebo recipients

Table 2a

	Vaccine			Placebo			
Peptide	*n*/N (%)	IgG level (95% CI)	r (*P*)	*n*/N (%)	IgG level (95% CI)	r (*P*)	z (*P*-value)
83·1 [1]	142/234 (60·7)	49·5 (41·5, 59·0)	−0·08 (0·23)	16/262 (6·1)	17·0 (16·5,17·5)	0·09 (0·13)	13·1 (<0·0001)
55·1 [1]	159/217 (73·3)	81·3 (66·6, 99·4)	−0·02 (0·75)	41/255 (16·1)	20·2 (18·7,21·9)	0·20 (0·011)	12·9 (<0·0001)
35·1 [1]	55/158 (34·8)	30·5 (25·2, 36·9)	0·14 (0·08)	16/202 (7·9)	17·3 (16·6,17·9)	0·13 (0·06)	6·6 (<0·0001)
SPf66 [1]	236/237 (99·6)	2855·1 (2355·6, 3460·4)	−0·07 (0·27)	222/264 (84·1)	309·3 (259·7, 368·3)	0·20 (0·001)	13·7 (<0·0001)
$(NANP)_{50}$ [2]	188/231 (81·4)	0·208 (0·149, 0·291)	0·27 (0·0001)	191/261 (73·2)	0·114 (0·085, 0·152)	0·42 (0·0001)	2·60 (0·009)
p190 [2]	204/231 (88·3)	0·437 (0·299, 0·639)	0·08 (0·2)	203/261 (77·8)	0·196 (0·135, 0·283)	0·31 (0·0001)	3·03 (0·002)
19 kDa [2]	153/231 (66·2)	0·082 (0·058, 0·117)	0·13 (0·05)	176/261 (67·4)	0·082 (0·059, 0·113)	0·14 (0·02)	0·04 (0·97)

Table 2b

Peptide	Vaccine No.	Vaccine Index of response (95% CI)	Placebo No.	Placebo Index of response (95% CI)
83·1	214	2·76 (2·31, 3·29)	236	0·93 (0·88, 0·98)
55·1	176	3·40 (2·70, 4·30)	211	0·87 (0·78, 0·97)
35·1	107	1·66 (1·30, 2·11)	122	0·99 (0·91, 1·08)
SPf66	228	6·93 (5·49, 8·76)	255	0·66 (0·55, 0·78)

1: Antibody levels expressed as titres; 2: Antibody levels expressed as standardized optical densities (OD); r: Spearman correlation between antibody levels and age; z: Wilcoxon Z statistic testing titre difference between placebo and vaccine; *n*/N: number with detectable antibodies/total number tested (%); Index of response: ratio of postvaccination to prevaccination titres (Teuscher *et al.* 1994).

Prevalence of anti-NANP, antip190 and anti19 kDa IgG were high, increased with age, and were similar among both vaccine and placebo recipients. However, there were large and statistically significant differences in geometric mean titration units between the two groups for anti-NANP and antip190 antibodies, while there were no differences for the 19 kDa protein.

Immune responses 18 months after the third dose

Eighteen months after the third dose, anti SPf66 IgG was measured in 78 placebo recipients and 58 vaccine recipients. Titres had decreased in the vaccine group (geometric mean = 330·4; SD = 56), but were still significantly higher than among placebo recipients (geometric mean = 183·0; SD = 22·6) (Figure 1). Anti-SPf66 titres in placebo recipients were age dependent, but not among vaccine recipients ($r = 0·33$; $P = 0·003$ and $r = 0·001$; $P = 0·996$, respectively)

Cellular reactivity to SPf66 in 3·5–5 year-old children was measured by *in vitro* thymidine incorporation into PBMC. Stimulation Indices (SI) among 20 vaccine recipients were higher than those from placebo recipients (geometric means 2·2 and 0·9, respectively). In the vaccine group 11 out of 20 assays had SI higher than 2·0, and 5 of them were higher than 4·0. None of the assays from the placebo group was higher than 2·0 (Figure 2)

Relation between antibody titres and clinical malaria

The relation between the risk of malaria and the post dose 3 antibody titre was analysed using Poisson regression models. The regression coefficient for the effect of the log of the final anti-SPf66 titre on risk was −0·18 (s.e. 0·10) for the placebo group and −0·10 (s.e. 0·10) for the vaccine group. The test of whether the gradient differed between vaccine and placebo groups found no significant difference (LR $\chi^2 = 0·2$, 1 d.f. $P = 0·6$). The corresponding analyses for the individual peptides, both in the vaccine and placebo group, also suggested reduced risk with increased IgG levels (Table 3).

Similar Poisson regression models were applied to the risk of malaria and the antibody titres against p190, NANP and 19 kDa. There was a significant correlation between anti-NANP antibodies and risk of malaria in the placebo

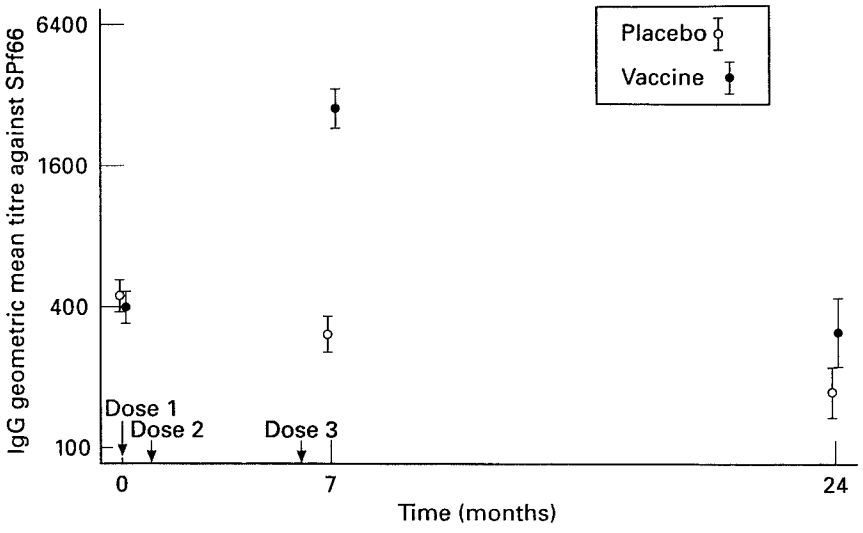

Figure 1 Geometric mean of total IgG anti-SPf66 titres. Each bar represents ± 2 SE around the geometric mean value. For the vaccine, group the bars are displaced horizontally slightly to allow better resolution from placebo.

group. The regression coefficients do not suggest any reduced risk of clinical malaria with increasing titres of antip190 or anti19 kDa.

The analysis of incidence of clinical malaria was repeated using the index of response instead of the post vaccination antibody titres. There was little trend for total anti-SPf66 IgG. The log index of response accounted for negligible variation over and above the vaccination status (LR $\chi^2 = 0{\cdot}008$, 1 d.f. $P = 0{\cdot}9$) in a Poisson regression model. The estimated vaccine efficacy was barely changed by the inclusion of the log index of response as a term in the model. Because prevalence of antibodies to the individual peptides at baseline was low, analysis of incidence of clinical malaria in relation to individual indices of response gave very similar results as those for final titres.

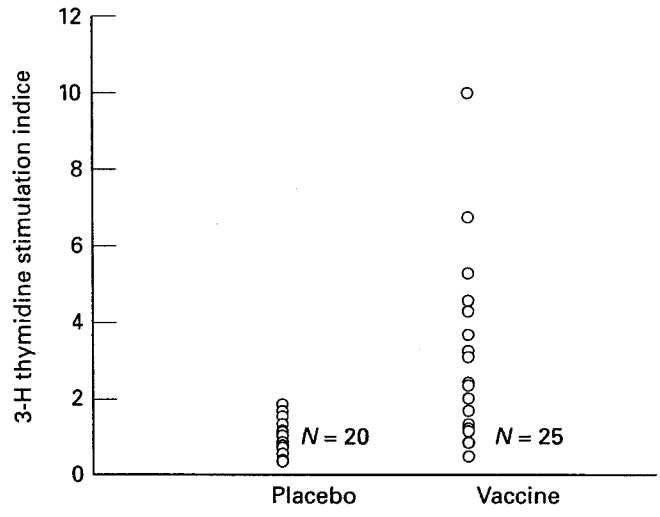

Figure 2 Lymphoproliferation assay 18 months after third dose.

DISCUSSION

Natural exposure to *P. falciparum* leads to the production of specific antibodies against SPf66 among children and adults living in endemic areas (Millet *et al.* 1992, Alonso *et al.* 1994a, Teuscher *et al.* 1994, Al-Yaman *et al.* 1995). This was also observed in the cohorts of this study. Preimmunization anti-SPf66 IgG titres are high and increase with age. They may partly reflect the high prevalence and age dependence of anti-NANP antibodies, also a component of SPf66.

Prevalence and mean titres of IgG antibodies to the individual merozoite derived epitopes contained in SPf66 and induced by natural exposure are generally low. In contrast, the prevalence of antip190 and of anti19 kDa IgG, a portion of the C-terminal part of MSP-1 but not part of SPf66, is high and similar to that reported from another area with similar intensity of transmission (Udhayakumar *et al.* 1995)

Following immunization, there was an increase in IgG antibodies against the SPf66 construct as well as against all individual epitopes contained in SPf66, including NANP. There was also an increase in antibodies against the p190 antigen. This probably represented predominantly antibodies against the 83·1 portion as there was no concomitant increase of antibodies against the 19 kDa antigen. The slight decline in antibodies in the placebo group after three doses is possibly due to the repeated sulphadoxine-pyrimethamine administration (part of the trial design, see Alonso *et al.* 1994a) resulting in reduced antigenic stimulation during the period of vaccination. Previous studies have shown that suppressive drug dosage at monthly intervals can lead to a reduced rate of acquisition of malarial antibodies (Harland *et al.* 1975), and in Nigeria, effective malaria control over a period of 70 weeks was shown to depress antibody levels at

Table 3 Analysis of Poisson regressions testing the relationship between log antibody level and incidence of clinical episodes adjusted for age and distance

	Placebo			Vaccine		
log (titre)	Coeff	(SE)	*P*-value	Coeff	(SE)	*P*-value
83·1	−0·65	(0·72)	0·3	−0·07	(0·11)	0·5
55·1	−0·20	(0·28)	0·4	−0·09	(0·11)	0·4
35·1	−18·7	(407·01)	>0·9	−0·09	(0·17)	0·6
SPf66	−0·18	(0·10)	0·077	−0·10	(0·10)	0·3
NANP	−0·15	(0·07)	0·028	−0·10	(0·07)	0·15
p190	−0·05	(0·05)	0·3	0·01	(0·05)	0·9
19 kDa	−0·04	(0·06)	0·5	−0·09	(0·08)	0·2

P-values by LR test.

all ages (Molineaux & Gramiccia 1980). In these studies, the more profound falls occurred in young children, thus confirming earlier observations that antibody persistence in the absence of repeated stimulation is less in children than in adults.

Among SPf66 recipients, the immune response was not homogeneous among the different age groups. Younger children had a greater antibody response than older ones, and this was the case for both the SPf66 construct and the individual peptides. One of several possible explanations for this observation is that the older children may already have reached a saturation level of antibody.

Antibody levels appear to decline over time and there does not appear to be substantial natural boosting. Nonetheless two years after the first dose, there is still a significant difference between vaccine and placebo recipients. Similarly, lymphoproliferative responses as assessed in a small number of children two years after the first dose confirm the potential of these synthetic peptides to stimulate specific cellular responses, and the persistence of these responses over time. PBMC from a majority of semi-immune adults living in the same area and having high antibody titres against SPf66 react strongly to SPf66 stimulation *in vitro* (unpublished results) indicating that priming by SPf66 still functioned 18 months after sensitization.

In a system where natural immunity is likely to comprise both responses which are partially protective, and others which merely reflect exposure to the parasite, it is difficult to establish criteria that define or characterize from an immunological point of view, a vaccine-induced protective response. This is especially so if a vaccine induces antibodies that are indistinguishable from those acquired through natural exposure, and when there are still no reliable *in vitro* tests or surrogate measurements of protection. Basically, one would expect decreasing risk of clinical malaria with increasing levels of protective antibodies among vaccinated individuals. The present study shows a tendency for all antibodies tested in this direction. However, none of the statistical tests for trend were significant. Using this criterion, we conclude that the study did not have sufficient power to determine whether antibody is or is not protective.

The observation of a trend among vaccinated individuals is neither the only, nor a sufficient condition to establish whether antibody-mediated protection occurred. On the one hand, since the regression coefficients of morbidity risk on antibody titres were similar in both placebo and vaccine groups, it seems likely that natural and vaccine-induced antibody do not have different protective efficacies. On the other hand, since the trend within the vaccine group is not statistically significant we cannot exclude the possibility that the vaccine induced antibody has no protective effect at all. Indeed, trends in both vaccine and placebo groups could easily arise if antibody titres merely reflected previous exposure to *P. falciparum*, and were related to protection only because that exposure also induced other protective responses which were not measured. It seems likely that such confounding accounts for the strong negative association with clinical malaria of anti-NANP antibodies, as these antibodies are thought to have little protective efficacy (Hoffman *et al.* 1987).

The results presented in this paper are therefore consistent with SPf66 induced IgG antibodies being involved in mediating protection against clinical malaria. Serum from SPf66 vaccinated individuals has been shown to inhibit parasite growth *in vitro* (Salcedo *et al.* 1991). However, other trials in which protection by SPf66 has been established were also not designed to analyse the antibody effects and could not show relations between antibody levels and protection (Rodriguez *et al.* 1990, Valero *et al.* 1993, Sempertegui *et al.* 1994). This result could also be explained by (i) the relatively low immunogenicity of the vaccine in these trials together with the small number of cases detected, (ii) serological assays that did not include full titration and (iii) a possible confounding by previous exposure that might have affect vaccine-specific immune responsiveness.

The present study did not have enough power either to test whether antibodies against the 83·1, 55·1 or 35·1 peptides may contribute to protection. The 83·1 fragment from the N-terminal region of MSP-1 (block 1) contains highly conserved sequences and at least one conserved T-cell epitope has been described (Sinigaglia *et al.* 1988). The specificity of the association between antibodies against 83·1 and 55·1 epitopes and reduced risk of clinical malaria, is further strengthened by the lack of protection associated with antibodies against the 19 kDa fragment of MSP-1. This cleavage product of the 42 kDa fragment in Block 17,

contains two EGF domains, is retained in the intracellular ring stage and its sequence is highly conserved. Antibodies to this fragment have been shown to inhibit parasite development *in vitro* (Blackman *et al.* 1990) and have been shown to mediate solid protection in immunization experiments with *P. yoelii* (Daly & Long 1993). Presence of anti19 kDa antibodies have also been described to be associated with a reduced risk of clinical malaria (Riley *et al.* 1993).

SPf66 has been shown to reduce the risk of *P. falciparum* malaria in areas geographically far apart (Valero *et al.* 1993, Alonso *et al.* 1994b, 1996). The best estimates of efficacy in both areas are too high to be explained if the vaccine specifically acts against certain genotypes of parasites. Moreover, SPf66 had no effect on the allelic families of MSP-1 parasites in infected individuals (Beck *et al.* 1996). This suggests that SPf66 mediated protection may be effective against a broad variety of *P. falciparum* genotypes. In this respect, highly conserved epitopes which appear to be poorly immunogenic through natural exposure such as the 83·1 fragment of MSP-1, may be important in mediating strain-transcending immunity. This would also help explain the slow development of naturally acquired immunity, essentially dependent on these epitopes, while strain or allele-specific immunity develops faster but may be short-lived (Müller *et al.* 1989).

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RESUMEN Y CONCLUSIONES

Estudio I

Resumen de resultados:

1. Se ha desarrollado un amplio estudio entre mas de 20.000 personas residentes en 73 poblados de una zona rural del centro de Gambia.

2. 17 de estos poblados contaban con un pequeño puesto de atención primaria de salud (APS).

3. Durante el año de descripción previo a la intervención se estimo que

   3.1. la mortalidad en menores de 1 año era de 120 / 1000 nacidos vivos

   3.2. la mortalidad en niños de 1 a 4 años era de 41 / 1000

   3.3. la mortalidad en niños de 1 a 4 años era significativamente mayor entre los residentes en los poblados APS

   3.4. las autopsias verbales sugerían que aproximadamente el 25 % de las muertes de niños menores de 5 años se debían a la malaria, y que estás se concentraban durante los meses de lluvia e inmediatamente después de las mismas

   3.5. la prevalencia de parasitemia por *P. falciparum* al final de la época de lluvias entre niños de 1 a 5 años era del 64%

3.6. la prevalencia de esplenomegalia al final de la época de lluvias entre niños de 1 a 5 años era del 66%

3.7. el *Anopheles gambiae sensu stricto* era el principal vector transmisor en el área donde también se encontraba el *Anopheles arabiensis*

3.8. la media geométrica de mosquitos por dormitorio y por noche durante los cuatro meses de transmisión era de 33

3.9. El índice esporozoítico en los mosquitos era del 2.4%

3.10. En la población anofelina no había evidencia de resistencia a los piretroides pero si al DDT

4. Se introdujo un esquema de impregnación con permetrina de las mosquiteros ya existentes en los 17 poblados de APS.
5. Los niños de esos mismos poblados y con edades de 6 meses a 6 años fueron aleatorizados a recibir semanalmente quimioprofilaxis con Maloprim ® (pirimetamina 6.3 mg + dapsona 50 mg) o placebo a través de la estructura de APS durante 20 semanas tras el inicio de las lluvias.
6. El 88% de las mosquiteros existentes en los poblados de APS fueron impregnadas con permetrina.
7. El 50% de las mosquiteros fueron lavadas al menos una vez después de la impregnación.
8. Si bien la dosis diana de permetrina era de 0.5 mg/m^2, la concentración obtenida fue de 0.2 mg/m^2.
9. Todas las actividades de implementación fueron coordinadas por un oficial de control de malaria del Ministerio de Salud de Gambia.

10. Durante el año posterior a la implementación de las medidas de control:

10.1. la mortalidad entre niños de 1 a 4 años de edad residentes en los poblados de APS se redujo en un 63%

10.2. entre los niños residentes en los poblados de APS, no hubo diferencias en la mortalidad entre los que recibían quimioprofilaxis con Maloprim y los que recibían placebo

10.3. la incidencia de episodios clínicos de malaria se redujo en un 45% entre los que dormían bajo mosquiteros impregnadas de insecticida

10.4. la adición de quimioprofilaxis redujo la incidencia de episodios clínicos en un 97%

10.5. dormir bajo una mosquitera impregnada de insecticida también estuvo asociado con una reducción en la prevalencia de esplenomegalia, de parasitemia y de anemia y con un aumento significativo en el hematocrito medio

10.6. la utilización de mosquiteros impregnados de insecticida no redujo de forma perceptible ni el numero de mosquitos ni la proporción de ellos infectados, aunque si se documento un claro efecto de protección personal al ser raro encontrar mosquitos bajo las mosquiteros impregnadas

11. A pesar de que el 86% de la población adulta de la zona utiliza una mosquitera para dormir, solo un 28% saben que los mosquitos transmiten la malaria.

12. Los mosquiteros parecen haber sido utilizados desde hace mas de 100 años, al menos por los Mandinkas.

13. Las dos estrategias de control parecen haber sido altamente coste eficaces. La impregnación de mosquiteros costaba 5.65 dólares americanos por niño - año de protección. La combinación de impregnación de la mosquitera y la profilaxis dirigida suponía un coste de 7.34 dólares americanos por niño - año de protección.

14. El coste de la prevención de una muerte fue de 188 dólares para la estrategia basada en la impregnación y de 257 dólares en la combinación de impregnación y profilaxis dirigida.

15. Los costes respectivos para la prevención de un episodio clínico fueron de 28 dólares y 19 dólares respectivamente.

Conclusiones:

Los mosquiteros impregnados de insecticida constituyen una estrategia de control de la malaria coste eficaz y fácilmente aplicable a través de un esquema de atención primaria de salud que reduce la mortalidad en los niños menores de 5 años.

La adición de profilaxis antipalúdica a la impregnación de los mosquiteros aumentó sustancialmente la prevención de episodios clínicos de malaria, pero no parece haber disminuido la mortalidad.

Estos resultados constituyen la base sobre la que evaluar en distintas situaciones ecológicas el papel de los mosquiteros impregnados de insecticida en el control de la malaria en el Africa subsahariana.

Estudio II

Resumen de resultados

1. La vacuna de la malaria denominada SPf66 está constituida por un péptido sintético polimerizado, diluido en solución salina y absorbido en hidróxido de aluminio.
2. La unidad monomérica es un péptido de 45 aminoácidos que corresponden a secuencias de aminoácidos derivadas de 3 proteínas de estadío sanguíneo asexual unidas entre si por una secuencia repetida de la proteína de circumsporozoito. Para posibilitar su polimerización cuenta con glicinas y cisteinas en sus extremos amino y carboxi terminal.
3. De las tres secuencias de aminoácidos derivadas de proteínas putativas de estadío sanguíneo asexual, solo una corresponde a una proteína bien caracterizada: la MSA1
4. La antigenicidad e inmunogenicidad de diversos lotes de la vacuna parece razonablemente conservada.
5. En estudios preclínicos en ratones y conejos la vacuna cumplió con los perfiles requeridos de inmunogenicidad y de ausencia de toxicidad.
6. Se diseño una secuencia de estudios para determinar la seguridad, inmunogenicidad y eficacia protectora de la vacuna en una zona rural de Tanzania. Está serie de estudios constituyen el primer ejemplo de evaluación sistemática y rigurosa de una vacuna de la malaria en Africa.
7. La evaluación preliminar de la seguridad, reactogenicidad e inmunogenicidad de la vacuna se realizo en 12 varones adultos caucásicos no inmunes y entre 30 varones adultos hiperinmunes. En ambos casos la vacuna fue segura e inmunogénica.

8. La confirmación de estos resultados permitió comenzar la evaluación de la seguridad e inmunogenicidad en 50 niños tanzanos semi-inmunes. De nuevo la vacuna fue segura y cumplió los requisitos preestablecidos de inmunogenicidad.
9. Estos resultados permitieron comenzar el ensayo de campo, con asignación aleatoria, controlado con placebo y a doble ciego para evaluar la eficacia de la vacuna entre 600 niños de 1 a 5 años viviendo en el poblado de Idete, una de las zonas de mas alta intensidad de exposición natural al *Plasmodium falciparum* y estimada en algo mas de 300 picaduras infectivas por persona y año.
10. La incidencia de reacciones adversas en el lugar de inyección o de reacciones sistémicas fue similar entre el grupo vacuna y el grupo placebo, confirmando una vez mas el buen perfil de seguridad de este producto.
11. La inmunización se asoció con un aumento significativo en el título de IgG anti SPf66 y en el título de anticuerpos inmunofluorescentes, sugiriendo que la inmunización induce anticuerpos que reconocen proteínas nativas del parásito.
12. Se diseñó un plan analítico que fue discutido y aprobado por los co-investigadores y por el Comité de Seguimiento para la Seguridad y los Datos (Data and Safety Monitoring Board) antes de la finalización del ensayo.
13. Durante los doce meses de seguimiento del estudio, comenzando un mes después de la administración de la tercera dosis hubo 73 episodios en el grupo vacuna y 102 en el grupo placebo.
14. Las estimaciones de eficacia para el primer o único episodio de malaria, ajustadas por regresión de Poisson para desequilibrios en la distribución de factores de confusión fue del 31% (95% CI: 0, 52).

15. La evaluación prolongada de la eficacia de la vacuna sugiere que la eficacia frente al número total de episodios de malaria fue del 25% (95% CI: 1, 44) sin evidencia de que disminuyera con el tiempo.
16. La inmunización con SPf66 también se asocio con una disminución de un 21% (95% CI: 0, 38) en la densidad media de parasitación.
17. En los sueros preinmunes, la prevalencia de anticuerpos IgG era del 91% para la SPf66, de 10% para el fragmento 83.1 de 26% para el fragmento 55.1, de 9% para el 35.1 y del 73% para el fragmento PNANP del circumsporozoito.
18. Tras la inmunización hubo un aumento significativo en la prevalencia de cada uno de estos anticuerpos así como en la de anticuerpos inmunofluorescentes frente a lisado de parásito.
19. Hay una tendencia a que los títulos de anticuerpos frente al péptido SPf66 y el fragmento 83.1 estén asociados con un menor riesgo de malaria, pero está relación no llega a ser estadísticamente significativa.

Conclusiones:

La vacuna denominada SPf66 parece ser segura e inmunogénica en diversos grupos poblacionales y etarios sujetos a distintos niveles de preexposición.

Está vacuna peptídica induce anticuerpos específicos frente a cada uno de sus componentes. Estos anticuerpos reconocen proteínas nativas del parásito.

La inmunización con SPf66 se asoció a una reducción moderada y estadísticamente significativa en el numero de episodios clínicos de malaria y en la densidad media de parasitemia en niños sujetos a un alto grado de exposición natural a *P. falciparum*.

Está serie de ensayos constituyen la primera evidencia de la capacidad de inducir inmunidad protectora frente a la malaria en niños africanos sujetos a transmisión natural de la malaria.

La protección obtenida es moderada, pero considerada suficiente como para avalar el subsiguiente desarrollo y evaluación del producto como posible herramienta para el control de la malaria en Africa.

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