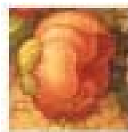
TESIS DOCTORAL

Estudio De La Respuesta Inmune Humoral Post-Trasplante Renal: "Rechazo Agudo Humoral"



Marta Crespo Barrio

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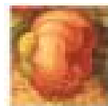

Directores: Manuel A. Pascual y Josep M. Campistol

"...traigamos a la memoria la sensata recomendación de nuestros mayores
cuando nos aconsejaban guardar lo que no era necesario porque,
más pronto o más tarde,
encontraríamos ahí lo que, sin saberlo entonces,
nos acabaría haciendo falta."

José Saramago, *La caverna*

A **Daniel** sobre todo.
siempre presente,
incluso en mis ausencias

A la Lda. Barrio,
a la que de niña tomaba
la lección.
Mi **madre**.

A mi **padre**,
y nuestras charlas transoceánicas.

A mi **hermano**, por existir
Y a **elisa**.

A **J. Pascual** y **M. Pascual** cuya amistad y
casualidades alteraron mi formación, y, en definitiva,
mi vida

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ABREVIATURAS

HLA: Antígenos leucocitarios de histocompatibilidad.

ADS: Anticuerpos donante-específicos.

CPT: Capilares peritubulares.

MGH: Massachusetts General Hospital.

PRA: Panel reacting antibodies o anticuerpos citotóxicos frente al panel.

CCTT: Cooperative Clinical Trials in Transplantation.

RAH: Rechazo agudo humoral.

PF: Plasmaféresis.

CDC: Citotoxicidad dependiente de complemento.

AHG: Linfocitotoxicidad aumentada con antiinmunoglobulina humana.

ADCC: Citotoxicidad celular dependiente de anticuerpos.

INTRODUCCIÓN

REVISIÓN HISTÓRICA

El trasplante renal constituye en la actualidad la opción terapéutica más adecuada para los enfermos con insuficiencia renal crónica terminal. Muchos obstáculos, unos superados y otros aún no, de corte técnico pero sobre todo inmunológico, han surgido desde el primer alotrasplante renal realizado con éxito en 1954 (1). El hecho de que el primer éxito clínico se realizara siendo donante y receptor individuos genéticamente idénticos pone de manifiesto el importante papel que juegan en este campo las "diferencias antigénicas", responsables del rechazo del injerto.

A lo largo de los años 60, varios autores relacionaron el "rechazo hiperagudo" de alotrasplantes renales con la existencia de anticuerpos preformados en el suero del receptor frente a antígenos del donante (2-5), un tipo de rechazo ya insinuado por la experimentación con xenoinjertos en décadas previas. En el campo del alotrasplante, estos antígenos fueron identificados progresivamente como pertenecientes al sistema sanguíneo ABO (conocidos por los trabajos de Landsteiner en 1901) o al Sistema Mayor de Histocompatibilidad (descubrimiento de Dausset en 1952, figura 1). El estudio histológico de los injertos destruidos de forma prácticamente inevitable en minutos u horas tras el trasplante en presencia de anticuerpos donante-específicos reveló la presencia de un intenso infiltrado intersticial constituido por neutrófilos y trombos de fibrina en capilares, asociados en ocasiones con necrosis fibrinoide arterial. La ausencia de infiltrado mononuclear sugería una patogenia diferente de la atribuida al clásico "rechazo agudo celular". La aplicación de la compatibilidad ABO y la indicación de obtener pruebas cruzadas "negativas" pretrasplante recomendada desde 1969 (6) -es decir, la demostración de la ausencia de anticuerpos en el suero del receptor contra los

linfocitos del donante- han hecho prácticamente desaparecer el rechazo hiperagudo. Sin embargo, estos casos iniciales permitieron conocer y describir con detalle los datos clínicos, inmunológicos e histológicos básicos de este tipo de rechazo, puramente humoral, en el riñón trasplantado.

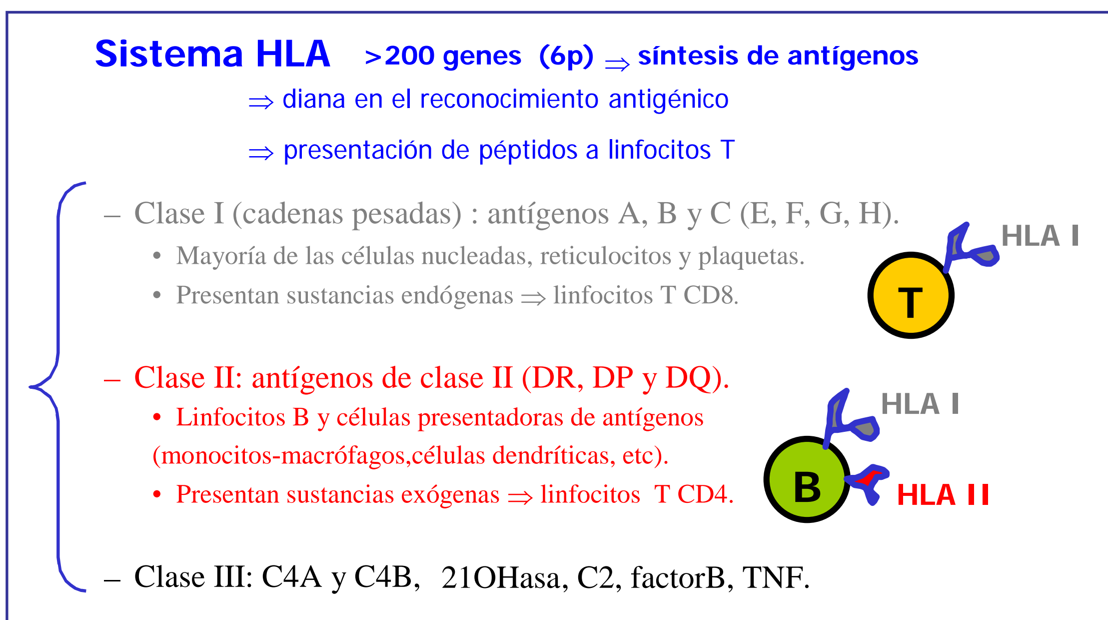


Figura 1.- Sistema mayor de histocompatibilidad en humanos: genes que codifican los antígenos HLA o antígenos humanos leucocitarios.

En 1970, M. Jeannet y col. señalaron que una respuesta humoral de novo frente al donante en el periodo post-trasplante renal (es decir, una prueba cruzada positiva post-trasplante tras una prueba cruzada "negativa" pre-trasplante) puede también producir un rechazo severo (7). Exponen su experiencia, según la cual, de 16 enfermos que desarrollaron anticuerpos específicos frente al donante en los primeros días o semanas post-trasplante renal, 12 perdieron el injerto, con una presencia significativa de lesiones vasculares obliterantes a nivel histológico. En comparación sólo se produjeron dos pérdidas en 12 enfermos sin respuesta humoral de novo. Este estudio sugirió que la aparición de anticuerpos específicos frente a antígenos del

donante en el periodo inmediato post-trasplante se asocia a una disfunción severa y a una lesión tisular, que comprometen la supervivencia del injerto.

Si bien los mecanismos por los cuales la inmunidad humoral producía la destrucción del injerto no estaban claros, algunos estudios proponían que la unión de los anticuerpos donante-específicos a antígenos presentes en la pared vascular activaban la cascada del sistema del complemento con atracción de polimorfonucleares y plaquetas (3,7). Algunas experiencias de la época, que evidenciaron consumo de complemento durante los episodios de rechazo agudo, apoyaban esta hipótesis (8).

Una vez conocida y superada la barrera del rechazo hiperagudo (6), la comunidad científica trasplantadora fue perdiendo interés en el estudio de la participación de los anticuerpos y el complemento en el daño del injerto. Como había ocurrido previamente, también en años posteriores los estudios se han centrado de forma preferente en el importante papel que juega la inmunidad celular en el rechazo agudo del injerto renal (9,10). Diversas experiencias iniciales en el campo del trasplante demostraron la presencia de un infiltrado de células mononucleares en injertos rechazados, así como la capacidad de provocar rechazo agudo con la transferencia de células linfoides (11,12). La incapacidad para producir rechazo en ratas timectomizadas en el periodo neonatal o ratas genéticamente atímicas estableció el papel crítico de los linfocitos T (13,14). Estos y otros experimentos han permitido aclarar progresivamente muchos detalles de la respuesta celular inmunológica del huésped frente al aloinjerto, desencadenada por la identificación del mismo como "extraño", debido fundamentalmente a la existencia de los antígenos de histocompatibilidad (HLA en humanos). Se trata de un proceso fundamentalmente mediado por linfocitos T en el que participan múltiples mecanismos de daño. Se conocen dos vías de reconocimiento de los HLA "extraños", directa e indirecta: los linfocitos T (CD4 y CD8) reconocen a través de sus receptores (TCR) los antígenos HLA que las células presentadoras de

antígenos, del donante o propias respectivamente, portan en las moléculas HLA de clase II y clase I de su membrana (Figura 2). La vía directa de reconocimiento parece jugar un papel fundamental en el rechazo agudo celular, y la vía indirecta más bien en el rechazo crónico. Receptores de membrana y múltiples señales co-estimuladoras permiten la interacción entre estas células. Los linfocitos T CD4 o colaboradores (frente a la subpoblación CD8 o citotóxica) ocupan un lugar central en el arranque del rechazo agudo, puesto que sintetizan la mayor parte de las citoquinas involucradas, sustancias con actividad local necesarias para estimular la respuesta inmune. Podemos diferenciar dos subpoblaciones de linfocitos colaboradores por su actividad secretora, TH1 y TH2. Entre las citoquinas que secreta la subpoblación TH1 destaca la interleukina 2, un potente factor de crecimiento autocrino que induce proliferación de las células T, expansión clonal de estas y otras células, y producción de más citoquinas. De esta manera (Figura 2), los linfocitos T CD4 reclutan y favorecen la participación de más linfocitos CD4, linfocitos CD8,

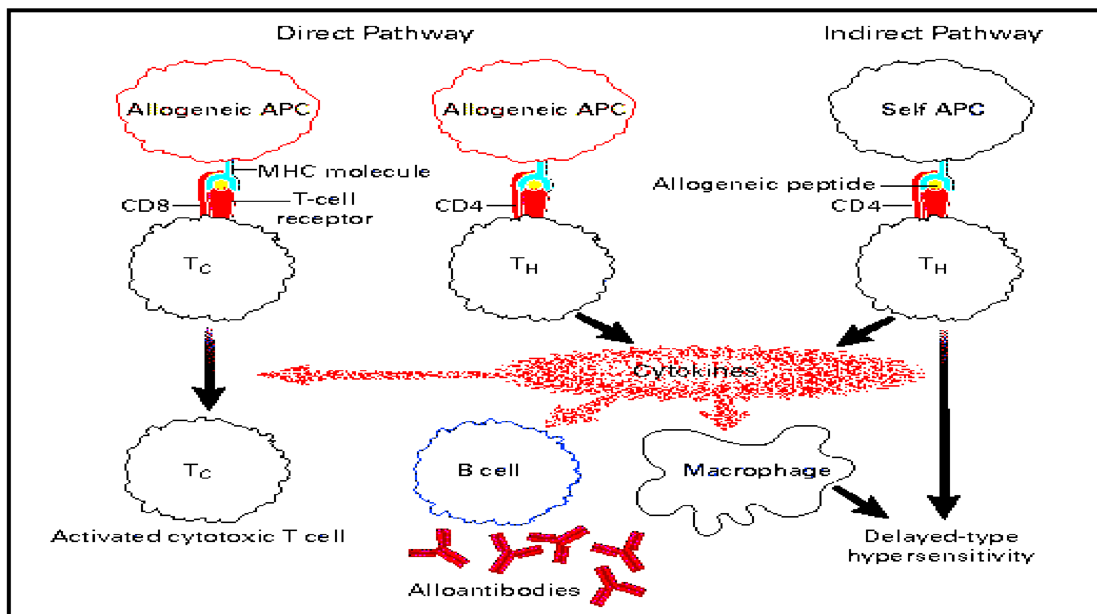


Figura 2.- Vías de reconocimiento de aloantígenos y mecanismos de rechazo. (Tomada de Sayegh MH, Turka LA. The role of T-cell costimulatory activation pathways in transplant rejection. N Engl J Med 1998; 338(25): 1813.)

células B (que producen anticuerpos frente a los antígenos HLA extraños), macrófagos y células NK. Los linfocitos CD8 provocan apoptosis de las células del donante por medio de perforinas, granzima B y la interacción FAS-FAS ligando. Las células NK probablemente actúan de manera similar, y los linfocitos CD4 y los macrófagos participan en una respuesta de hipersensibilidad retardada. Si bien la subpoblación de linfocitos CD4 TH1 participa en la expansión clonal y la citotoxicidad, la subpoblación TH2 parece más relacionada con la producción de inmunoglobulinas y la memoria inmunológica (9,10,15).

La incidencia de rechazo agudo celular ha ido disminuyendo progresivamente gracias al desarrollo de fármacos inmunosupresores, que aunque aún relativamente inespecíficos, tratan especialmente de controlar la activación de las células T, la producción de citoquinas y/o la expansión clonal (Figura 3)(16,17). A principios de los años 80, la introducción de la ciclosporina en la clínica, como primer inhibidor de la calcineurina (paso limitante previo a la activación intranuclear de la transcripción de los genes responsables de la

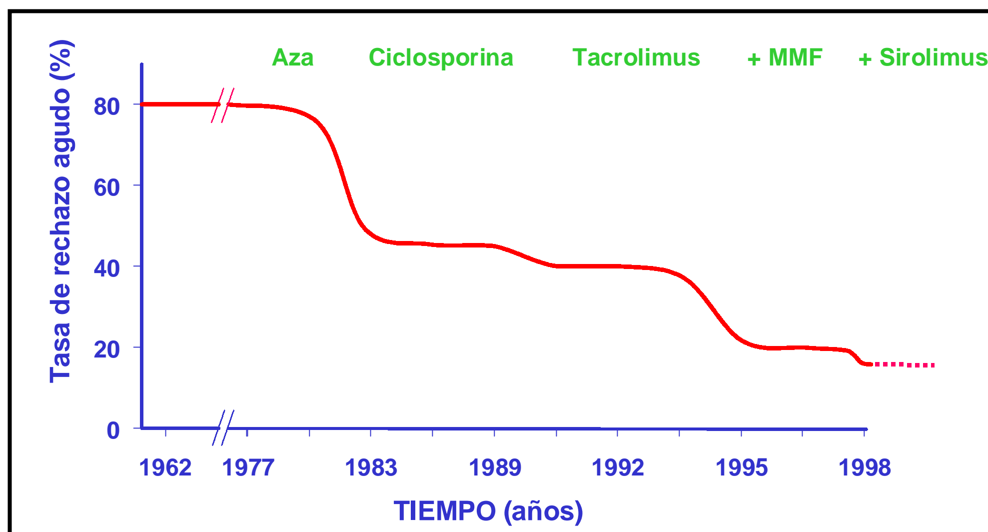


Figura 3.- Evolución de la tasa de rechazo agudo post-trasplante renal.

síntesis de citoquinas), revolucionó el pronóstico a corto plazo del injerto renal. En la década siguiente se han añadido al arsenal inmunosupresor el tacrólimus, también inhibidor de la calcineurina, el micofenolato mofetil, nuevo antimetabolito más específico, y el sirolimus, que inhibe la proliferación de las células T secundaria a la activación mediada por la interleukina 2 (Figura 4). El desarrollo de anticuerpos mono (OKT3, antiCD25) y policlonales (ALG, ATG o timoglobulina) y su uso como terapia de inducción también ha contribuido a disminuir la tasa de rechazo agudo, o a controlarlo cuando se emplean como tratamiento del mismo (15). Sin embargo, hay que resaltar que algunos episodios de rechazo agudo continúan siendo refractarios al tratamiento convencional dirigido a controlar la inmunidad celular.

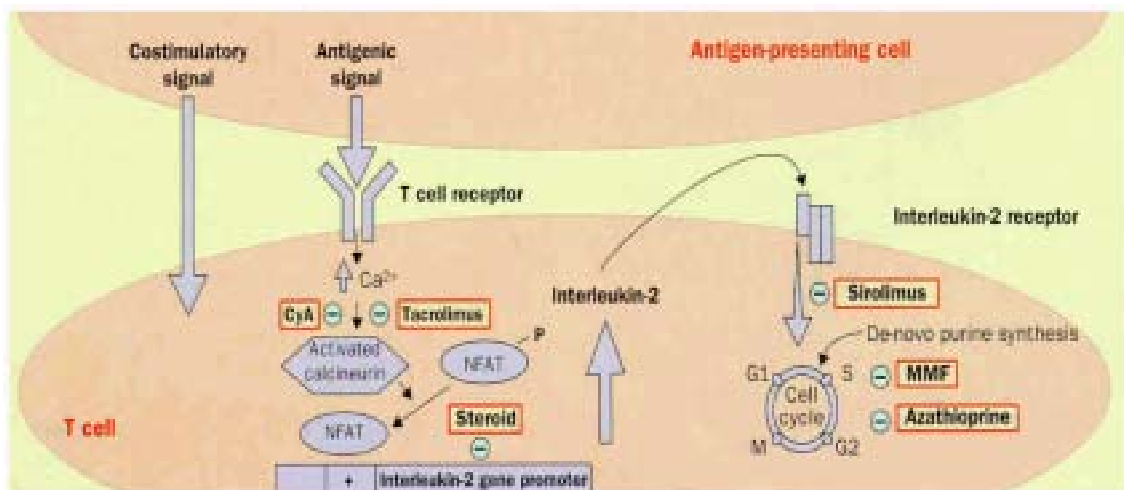


Figura 4.- Mecanismos de acción de los fármacos inmunosupresores de base. (Tomada de Denton MD, Magee CC, Sayegh MH. Immunosuppressive strategies in transplantion. Lancet 1999; 353: 1083-1091)

Este último hecho, a pesar del importante avance en el control de los mecanismos celulares del rechazo agudo, y la escasez relativa de injertos han impulsado la investigación en campos limitados de forma importante por la respuesta humoral, como el uso de injertos ABO incompatibles (18,19), o el xenotrasplante (20). Por otra parte, el mejor conocimiento de la interrelación

entre inmunidad celular y humoral (anticuerpos, citoquinas, etc) ha permitido relanzar el estudio de los fenómenos humorales a nivel clínico y básico.

Durante los años 80, algunos grupos investigaron la relación entre la aparición de anticuerpos anti-HLA post-trasplante, no siempre donante-específicos (es decir estudio del porcentaje de anticuerpos frente a un panel heterogéneo de antígenos HLA (PRA) en lugar de usar pruebas cruzadas donante-específicas), y el pronóstico del injerto renal, utilizando técnicas distintas, sueros recogidos en distintos momentos post-trasplante y sólo en contadas ocasiones incluyendo valoración histológica (21-25). Muchos de estos estudios encontraron un peor pronóstico de los rechazos agudos asociados con la aparición de anticuerpos.

Pero fue el grupo de P. Halloran, el que a principios de los años 90 profundizó en el interés por los mecanismos humorales del rechazo y señaló que el rechazo agudo asociado con el desarrollo de anticuerpos donante-específicos (ADS) de novo es una entidad clínico-patológica que implica mal pronóstico (26, 27). En una primera comunicación, estos autores describieron el curso clínico de siete pacientes que presentaron rechazo agudo mediado por anticuerpos frente a antígenos HLA de clase I del donante. Incluyeron cuatro enfermos que desarrollaron ADS de novo post-trasplante y tres enfermos con anticuerpos pre-existentes no detectados en el estudio inmediato pre-trasplante, cuya disfunción no se comportó como rechazo hiperagudo. Los siete enfermos presentaron un rápido deterioro de la función del injerto durante la primera semana post-trasplante, con lesiones histológicas diferenciadas – sin infiltrado mononuclear o poco prominente- y un pésimo pronóstico (5/7 perdieron el injerto a pesar de tratamiento antilinfocitario) (26).

En un estudio prospectivo posterior compararon 13 receptores de trasplante renal con ADS anti-HLA de clase I de novo post-trasplante y 51 receptores sin anticuerpos. Las siguientes diferencias entre ambos grupos

resultaron significativas: incidencia de rechazo (100% de los enfermos con ADS frente a sólo 41% de los receptores sin ADS), características clínicas del rechazo (aparición precoz, oliguria y necesidad de diálisis en los primeros), lesiones histológicas (daño endotelial en la microcirculación, infiltrado por neutrófilos en capilares peritubulares (CPT) o glomérulos y depósitos de fibrina en glomérulos o vasos) y pronóstico (5/13 injertos perdidos en el primer grupo y 2/51 en el segundo) (27).

El mismo grupo analizó detalladamente las características histológicas del "rechazo mediado por anticuerpos" basándose en la clasificación de Banff de 1993 (28, 29). De 44 pacientes con rechazo agudo confirmado por biopsia según la clasificación de Banff, 24 habían desarrollado ADS post-trasplante y 20 no presentaban ADS. Encontraron una mayor incidencia de vasculitis severa y glomerulitis en los enfermos con ADS post-trasplante, así como una mayor presencia de trombos de fibrina, necrosis fibrinoide, dilatación de capilares peritubulares, infartos y, sobre todo, polimorfonucleares en CPT (Figura 5). Sin

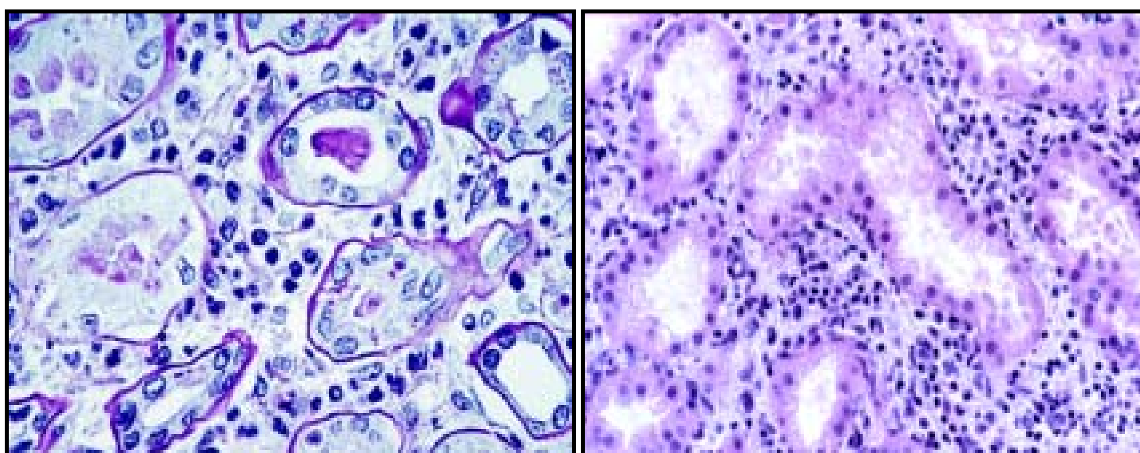


Figura 5.- Microscopio óptico. **Izda)** Rechazo agudo humoral: infitración por neutrófilos de capilares peritubulares. **Dcha)** Rechazo agudo celular: infiltración tubular por células mononucleares. (Imágenes cedidas por S. Mauiyyedi, MGH)

embargo, las biopsias de enfermos sin ADS mostraban tubulitis moderada-severa con mayor frecuencia (95%) que las de los enfermos con ADS (50%). A pesar de la presumible participación de mecanismos humorales de rechazo agudo en los pacientes con ADS, no encontraron diferencias significativas en la detección de IgM, IgG, IgA, λ, κ, C1q, C3, C4, albúmina o fibrinógeno por inmunofluorescencia, ni en los hallazgos obtenidos con el microscopio electrónico.

ESTUDIOS	Tipo Estudio y Objetivo	Nº enfermos estudiados	Pérdida injerto	Datos de interés
Jeannet y col. 1970	Retrospectivo Comparativo	16 XM+ postTX 12 XM- postTX	- 12/16 - 2/12	• Significado de la aparición de ADS post-TX
Halloran y col. 1990	Retrospectivo Descriptivo	7 con ADS anti- HLA I	- 5/7	• Descripción de una serie de enfermos con rechazo mediado por ADS
Halloran y col. 1992	Prospectivo Comparativo	13 con ADS anti-HLA I 51 sin ADS	- 5/13 - 2/51	• No permite establecer incidencia • Sólo 29/64 tienen XM con células B
Trpkov y col. 1996	Retrospectivo Comparativo	24 con ADS anti-HLA I 20 sin ADS	-12/24 - 3/20	• Datos histológicos diferenciados, a veces sin datos de RAC. • Sesgo por selección

Tabla 1. Estudios relevantes sobre el impacto de la aparición de ADS post-trasplante renal. TX= Trasplante. XM= Prueba cruzada. RAC= Rechazo agudo celular.

INTERÉS DEL GRUPO DE TRABAJO POR EL TEMA

A partir de 1995, en la Unidad de Trasplante del Massachusetts General Hospital (MGH) se inició el estudio prospectivo de la presencia de ADS en casos de rechazo agudo severo. A finales del año 1994, una enferma hipersensibilizada (PRA máximo con células T: 100%, PRA pre-trasplante con células T: 68%) con pruebas cruzadas negativas pre-trasplante (prospectivas y retrospectivas con células T y B, por citotoxicidad y citometría de flujo) presentó función retardada y pérdida precoz del injerto, a pesar de inducción con terapia antilinfocitaria. Con los sueros correspondientes al día ocho post-trasplante y las células T y B del donante se obtuvieron pruebas cruzadas positivas. El estudio histológico mostraba importante infiltración por neutrófilos en CPT, además de rechazo agudo célular tipo 2 según la clasificación del Cooperative Clinical Trials in Transplantation (CCTT, tabla 2) (30).

Tipo 1	Infiltrado mononuclear >5% de la corteza renal, al menos 3 túbulos con tubulitis en 10hpf. de las áreas más afectadas, y al menos dos de los tres siguientes datos: edema, linfocitos activados o daño tubular.
Tipo 2	Endotelialitis arterial o arteriolar, con o sin datos de rechazo agudo tipo 1.
Tipo 3	Necrosis fibrinoide arterial o inflamación transmural, con o sin trombosis, necrosis del parénquima o hemorragia.

Tabla 2.- Clasificación histológica del rechazo agudo post-trasplante renal del Cooperative Clinical Trials in Transplantation (CCTT).

Entre diciembre de 1995 y febrero de 1997 y de forma prospectiva, diagnosticaron una serie de cinco enfermos de "rechazo mediado por

anticuerpos" o **"rechazo agudo humoral" (RAH)**. El diagnóstico de **RAH** se realizó ante la existencia de pruebas cruzadas donante-específicas positivas post-trasplante coincidiendo con un rechazo agudo refractario (córtico-resistente y resistente a tratamiento antilinfocitario), que además a nivel histológico presentaba infiltración por polimorfonucleares en CPT y glomérulos, trombos de fibrina en arteriolas y glomérulos, vasculitis y/o necrosis fibrinoide de los vasos (31).

Diversos estudios realizados hasta el momento admitían un pésimo pronóstico a corto plazo de los injertos que presentan rechazo agudo asociado a la presencia de ADS anti-HLA, con una supervivencia anual entre 15 y 50% (21,26,29,30,32). Algunos grupos habían sugerido un posible efecto beneficioso del uso de plasmaféresis en estos casos (24,32,33), así como en enfermedades autoinmunes mediadas por anticuerpos o como posibilidad de acceso al trasplante para receptores hipersensibilizados (34-37). Por otro lado, nuevos fármacos inmunosupresores, como el tacrólimus y el micofenolato mofetil, demostraban disminuir las tasas de rechazo agudo y de rechazo agudo severo (15, 17). Por tanto, M. Pascual y col. del MGH propusieron como tratamiento alternativo del **RAH** la combinación de plasmaféresis (PF) y rescate con los nuevos fármacos inmunosupresores tacrólimus y micofenolato (31). Los cinco enfermos antes referidos fueron sometidos a una serie de entre cuatro y siete sesiones diarias de PF, monitorizada por la respuesta clínica y los títulos de ADS (Figura 6). Una enferma recibió una nueva serie de 5 sesiones debido a un repetido deterioro de la función renal coincidente con un nuevo ascenso en el título de ADS. Además, todos los enfermos recibieron al menos una dosis de 0,4 g/kg de gammaglobulina policlonal tras la última sesión de PF con objeto de prevenir infecciones. Se había propuesto el uso de este derivado sanguíneo en casos semejantes, aunque a dosis mayores (38-41). La respuesta a este tratamiento fue excelente y estos cinco enfermos presentaban una buena función renal con creatininas que oscilaban entre 0,9 y 1,8 mg/dl entre 370 y 790 días post-trasplante.

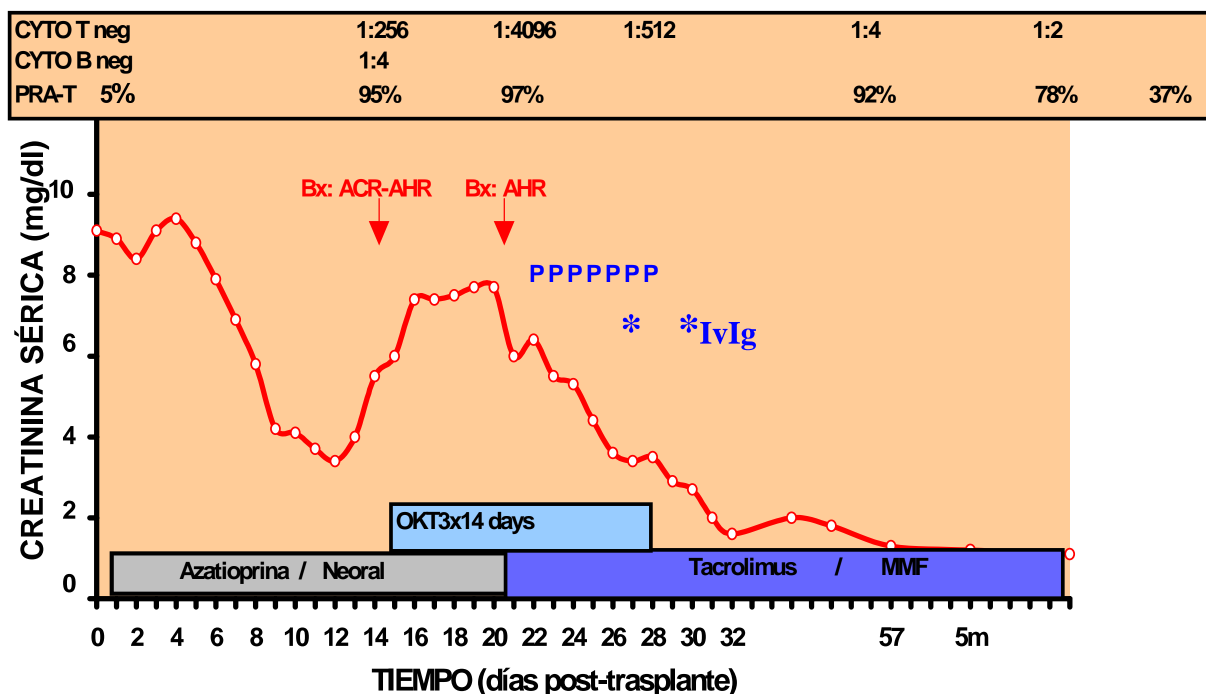


Figura 6.- Evolución y tratamiento de un enfermo que sufrió rechazo agudo humoral. CYTO= prueba cruzada por linfocitotoxicidad. P= plasmaféresis. *= Inmunoglobulina policlonal.

En definitiva esta experiencia sugería que aunque el pronóstico del injerto con **RAH** ha sido tradicionalmente significativamente peor que el del injerto que sufre rechazo agudo celular, la supervivencia del injerto renal a medio plazo, cuando se controla la respuesta humoral, puede resultar comparable (29, 31).

Las implicaciones terapéuticas estimularon al equipo de Patología del MGH en la búsqueda de criterios patológicos más precisos para el diagnóstico del **RAH**. Retomaron los interesantes trabajos de Feucht y col. que relacionaban la presencia de la fracción C4d del complemento en CPT en las biopsias renales con la existencia de rechazo agudo severo, especialmente en enfermos hipersensibilizados (42), o con disfunción precoz del injerto renal (43), sugiriendo mecanismos humorales de daño tisular. Se procedió a complementar el estudio histológico estándar de 16 biopsias de diez enfermos

diagnosticados de **RAH** con estudios de inmunofluorescencia para analizar la existencia de depósitos de C4d, C3 e IgM en CPT, como marcadores de daño humoral. Todas las biopsias de **RAH** presentaban depósitos prominentes de C4d en capilares peritubulares, en comparación con sólo una biopsia de 24 enfermos con rechazo agudo celular, toxicidad por ciclosporina o sin daño histológico (Figura 7). Esta última biopsia con datos de nefrotoxicidad por ciclosporina correspondía a un enfermo en cuyo suero peri-biopsia de forma retrospectiva se demostró la existencia de ADS, que además presentó datos histológicos sugestivos de **RAH** en una biopsia posterior (44). Este caso incrementa el valor de la detección de C4d, ya que al margen de resultar más sensible y específico que los criterios histológicos tradicionales de diagnóstico de **RAH**, parece que el C4d en CPT podría ser un marcador precoz de daño humoral.

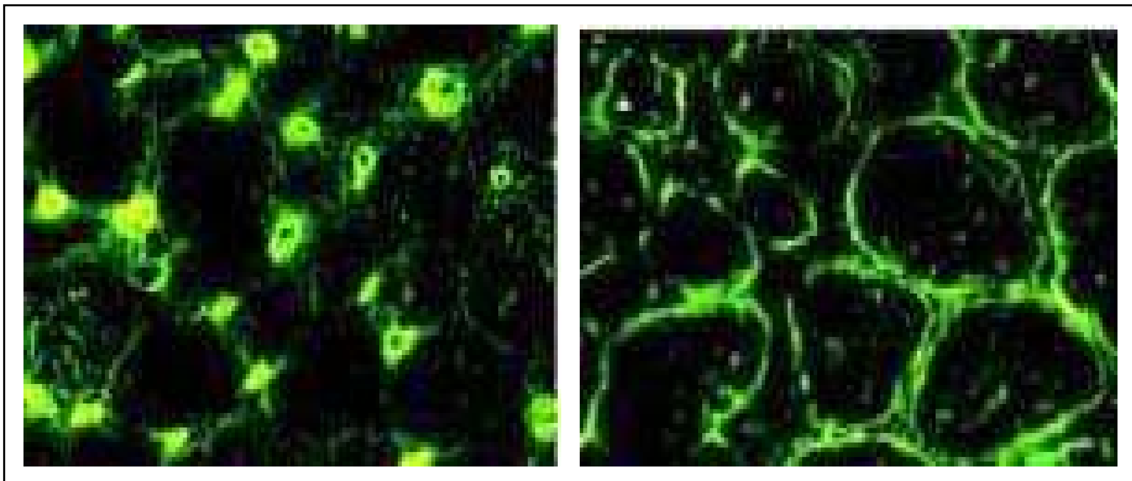


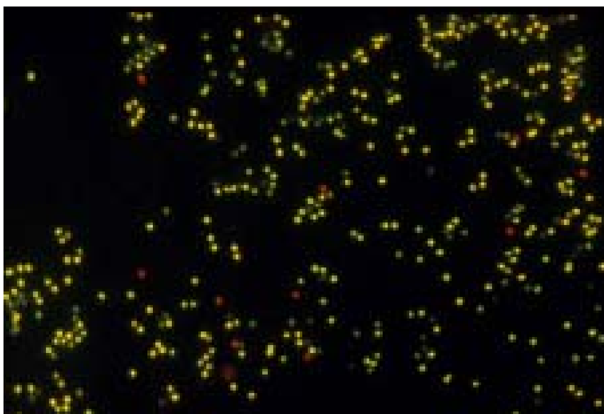
FIGURA 7.- (Fotografías cedidas por B. Collins, MGH)

Izda) Rechazo agudo humoral: tinción difusa y brillante de C4d en capilares peritubulares.

Dcha) Rechazo celular agudo: C4d únicamente en membranas basales tubulares.

ANTICUERPOS ANTI-HLA DONANTE-ESPECÍFICOS

Hoy en día múltiples técnicas permiten estudiar la presencia en el suero del receptor de anticuerpos frente a los antígenos HLA del donante. Fundamentalmente se distingue entre técnicas directas e indirectas. Las primeras detectan anticuerpos capaces de fijar complemento y son variaciones de la técnica básica de linfocitotoxicidad (CDC o citotoxicidad dependiente de complemento) (45). Se basan en la capacidad del suero del presunto receptor de lisar las células T y/o B del donante con la ayuda de complemento exógeno (de conejo), si contiene anticuerpos específicos frente a los antígenos HLA del donante (Figura 8). Las variaciones implican lavados de las células T y/o B del donante antes de añadir el complemento y/o prolongación de los tiempos de



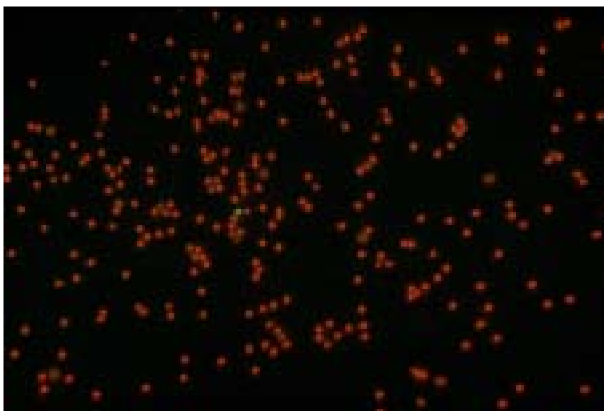


Figura 8.- En las pruebas cruzadas (XM) realizadas por CDC se enfrentan linfocitos del donante y sueros del receptor en distintos pocillos. En cada pocillo se evalúa el porcentaje de muerte celular tras la adición de suero del receptor, complemento de conejo, tinte, etc y se asigna un número:

1=0-10% de muerte celular
2=11-20%
4=21-50%
6=51-80%
8=81-100%
0=lectura no valida.

1 y 2= negativo
4, 6 y 8= positivo

En nuestro laboratorio utilizamos diacetato de fluoresceina que tiñe células vivas (verde) y bromuro de etidio que tiñe los núcleos de las células dañadas (rojo).

Estas imágenes corresponden a

- 1 = XM negativo (superior)
- 8 = XM positivo (inferior)

(Imágenes cedidas por S. Saidman)

incubación, para mejorar la sensibilidad. Los lavados o el uso de ditiotreitol permiten distinguir si la lisis se debe a anticuerpos de tipo IgG o IgM.

Métodos indirectos, como la linfocitotoxicidad aumentada con antiinmunoglobulina humana (CDC-AHG, figura 8) y la citometría de flujo, permiten detectar anticuerpos no fijadores de complemento, niveles bajos de anticuerpos anti-HLA o anticuerpos CDC negativos – absorción positivos (fenómeno CYNAP) (46-49). La técnica clásica de linfocitotoxicidad se desencadena por la activación de C1q, paso inicial de la vía clásica del complemento, que necesita la unión de una IgM o dos IgG. La técnica CDC-AHG utiliza la adición al suero humano de un anticuerpo anti-cadena ligera humana κ para amplificar la reacción de citotoxicidad (Figura 8) (47,48). La citometría de flujo detecta anticuerpos unidos a la membrana de células; usa

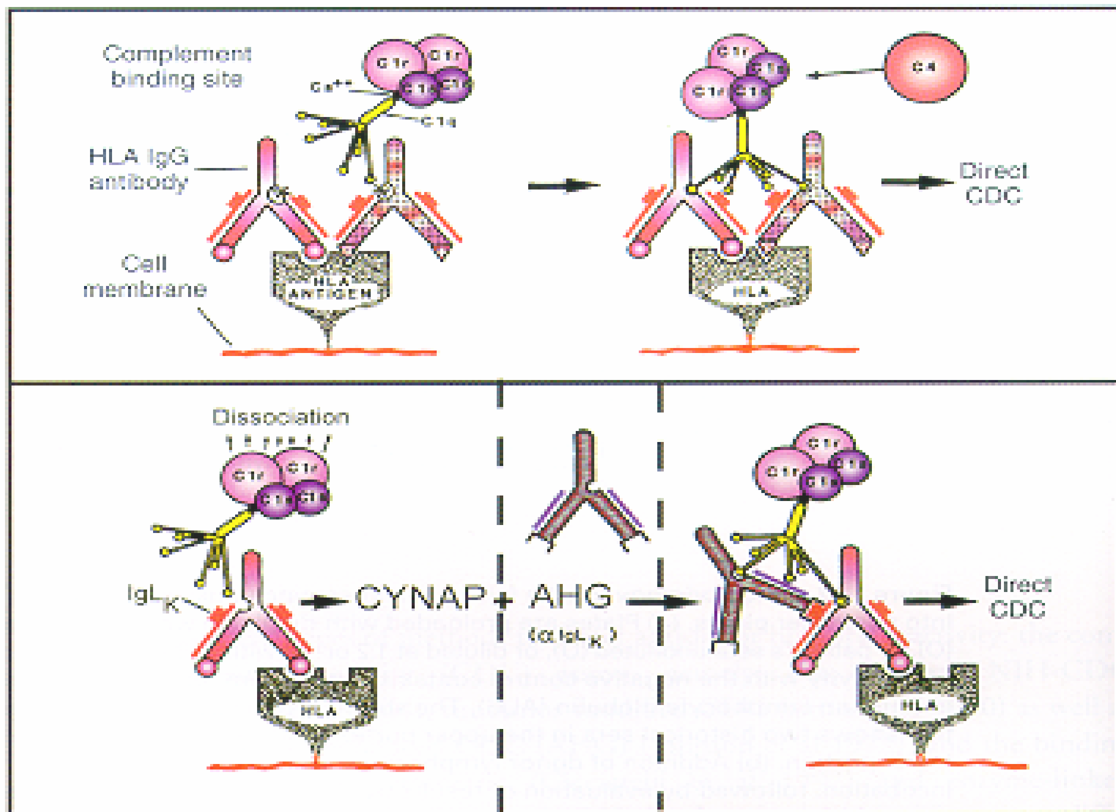


FIGURA 9. Ilustración comparada de los mecanismos de actuación de la CDC clásica y la CDC-AHG. (Tomada de The complement system. Mayer MM, Sci Am 1973, 229: 54-66)

IgG antiinmunoglobulina humana para detectar anticuerpos (IgG o IgM), y anticuerpos anti-CD3 y anti-CD19 para identificar células T y B respectivamente. Existen otras variaciones de esta técnica también aplicadas a la realización de la prueba cruzada donante-receptor (49). Aunque algunos grupos sugieren que la citometría de flujo es una técnica más sensible que la AHG, estas dos técnicas no están bien estandarizadas y los resultados varían entre distintos laboratorios (50). En el laboratorio de histocompatibilidad del MGH estos métodos han resultado comparables (51).

Si bien clásicamente se utilizaban técnicas de linfocitotoxicidad como método de screening de la presencia de anticuerpos anti-HLA pre-trasplante renal (estudio de PRA), hoy en día se están introduciendo también en este campo la citometría de flujo y técnicas de enzimoinmunoanálisis (ELISA) (52). El estudio de los PRA (tasa y especificidad) es una herramienta importante en el momento de seleccionar un receptor adecuado para un injerto.

Detección de ADS Pre-Trasplante

La presencia de anticuerpos frente a los antígenos HLA de clase I del donante en el suero del receptor, es decir, una prueba cruzada donante-específica positiva pre-trasplante con linfocitos T, es una contraindicación para la realización de ese trasplante renal (6). Algunos autores defienden que una prueba cruzada positiva pretrasplante con células B a títulos altos también es nociva cuando se debe a especificidades de tipo HLA (HLA de clase II) y no a autoanticuerpos (53), pero en muchos centros ni las pruebas cruzadas ni los PRA con linfocitos B se realizan de forma rutinaria (54). También existe cierta evidencia del efecto negativo de la presencia de anticuerpos de tipo IgM frente a antígenos HLA del donante en la supervivencia del injerto renal (55), aunque hay que resaltar que en la mayoría de las ocasiones estos anticuerpos, sobre todo cuando son de baja afinidad o reaccionan sólo con células B, son autoanticuerpos y por tanto no suponen un obstáculo para el alotrasplante (45).

Detección de ADS Post-Trasplante

El estudio de ADS post-trasplante no está incluido en la práctica clínica habitual, aunque existen cada vez más evidencias que sugieren su utilidad en casos seleccionados. La determinación de PRA post-trasplante por citotoxicidad, citometría de flujo o ELISA puede ser útil para identificar pacientes con riesgo de presentar rechazo mediado por anticuerpos o **RAH**, dado que habitualmente la aparición o incremento de PRA post-trasplante es debida a inmunoreactividad frente al injerto (21,56,57). Sin embargo, dado que el **RAH** parece ocurrir sobre todo en receptores hipersensibilizados (PRA>50-75%), es difícil determinar la especificidad de los nuevos anticuerpos añadidos a PRA previamente elevados (26, 27, 31). Por supuesto, la existencia de PRA= 0% con células T y B hace improbable la presencia de ADS. Nosotros propugnamos la realización de una prueba cruzada donante-específica para la realización del diagnóstico de **RAH** en el contexto adecuado: rechazo refractario con datos histológicos ya comentados sugestivos de daño humoral (29,31,44). En caso de no disponer de células del donante, se pueden seleccionar cuidadosamente células subrogadas que compartan las disidentidades HLA del donante, especialmente para monitorizar cambios en el título de anticuerpos o como guía terapéutica (31). Hay que tener en cuenta que es más complicado realizar pruebas cruzadas post-trasplante cuando se está administrando terapia anti-linfocitaria, dado el riesgo de falsos positivos. Este, sin embargo, es probablemente el momento preciso para realizar el diagnóstico en la mayor parte de las ocasiones. En el laboratorio de Histocompatibilidad del MGH se retira el OKT3 del suero del receptor que lo recibe utilizando un anticuerpo monoclonal de oveja anti-ratón (Figura 9) (58). Los sueros de enfermos que reciben otro tipo de tratamiento anti-linfocitario se estudian con citometría de flujo, asegurando que el anticuerpo secundario anti-inmunoglobulina no presente reacción cruzada con la especie en la que se produjo el anticuerpo policlonal.

	1	2	3	4	5	6	7	8	9	10	11	12	
Positive Controls	Class II [illegible]	Class II [illegible]	Class I [illegible]	Class I [illegible]	Class I [illegible]	[illegible]	[illegible]		[illegible]				F
Neg. C (NHS)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]							E
1:8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]							D
1:4	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]							C
1:2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]							B
1:1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]							A
Serum Date	PRETR 9-11-95		POSTTR 10-11-95 OKT3 treat.		10-11-95 no treated								

PRETATION OF RESULTS/ COMMENTS:

T cell XM compatible

	1	2	3	4	5	6	7	8	9	10	11	12	
Positive Controls	Class II [illegible]	Class II [illegible]	Class I [illegible]	Class I [illegible]	Class I [illegible]	[illegible] 66							F
Neg. C (NHS)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]							E
1:8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]							D
1:4	[illegible]	2 [illegible]	0 [illegible]	4 2	4 1	2 1							C
1:2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]							B
1:1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]							A
Serum Date	PRETR		POSTTR - OKT3 10-11-95 treated		10-11-95 not treated								

INTERPRETATION OF RESULTS/ COMMENTS:

B cell XM compatible

FIGURA 9. Hojas de trabajo: pruebas cruzadas con linfocitos T (arriba) y B (abajo) y células del donante. Suero post-trasplante utilizado: con y sin tratamiento para retirar el OKT3.

La prueba cruzada con el suero del receptor en tratamiento con OKT3 y células T es positiva. Sin embargo, es negativa cuando se retira el OKT3 de este mismo suero. El OKT3 no influye en la prueba cruzada con células B.

Existe cierta controversia en cuanto al papel deletéreo directo que juegan los ADS que aparecen post-trasplante, porque si bien varios autores han comunicado su efecto negativo (21,24,26,27,29,31,32,59,60), otros (utilizando citometría de flujo) han sugerido que la aparición de ADS de novo post-trasplante puede ser un epifenómeno presente en el momento del rechazo agudo (25, 61).

El grupo de P. Halloran ha apoyado la relevancia clínica únicamente de la aparición de ADS anti-HLA de clase I, basándose en series de casos relativamente seleccionadas (26,27,59). El grupo de HE. Feucht, que ha estudiado los depósitos de C4d en tejido renal, inicialmente utilizó sueros pre-trasplante con la intención de detectar ADS que pudieran predecir el daño mediado por anticuerpos. No han encontrado ADS pre-trasplante utilizando técnicas convencionales, pero sí han demostrado la presencia de anticuerpos frente a líneas célulares linfoblastoides DR homocigotas para los antígenos DR del donante con citometría de flujo utilizando sueros pre-trasplante (62). Es decir, han destacado la relevancia de la existencia de anticuerpos frente a los antígenos HLA de clase II del donante.

Nuestra experiencia preliminar sugiere que tanto ADS frente a antígenos HLA de clase I como ADS frente a antígenos de clase II se asocian a **RAH** (31, 44), cuestión que nos proponemos abordar con detalle en la presente tesis doctoral.

DEPÓSITOS DE LA FRACCIÓN C4d DEL COMPLEMENTO

La fracción C4d del complemento es un producto de la degradación de la partícula C4 de la vía clásica de activación del complemento. El primer componente de esta vía es C1, un complejo de tres proteínas: C1q, C1r y C1s. La activación del complemento generalmente se inicia cuando C1q se une a anticuerpos, generalmente del tipo IgM o la mayor parte de las subclases de IgG, adheridos a antígenos presentes en la superficie celular. C1q activa C1r y este a su vez C1s. C1s escinde C4 en C4a y C4b; esta partícula es capaz de unirse a una molécula de C2 y sensibilizarla a la activación inducida por C1s, para dividirse también en C2a y C2b. El complejo C4b-C2b constituye la C3 convertasa, paso clave en la activación de la vía final común del complemento (63) (Figura 10).

La inactivación de este complejo puede ser mediada por la C4 binding-protein (C4BP) o por el decay-accelerating factor (DAF). Cuando C4BP se une a C4b, esta partícula se hace especialmente sensible a la acción del llamado factor I. El factor I inactiva C4b, escindiéndolo en C4c y C4d (63). C4d contiene un grupo tiol ester, que condiciona su unión covalente al lugar de depósito (64).

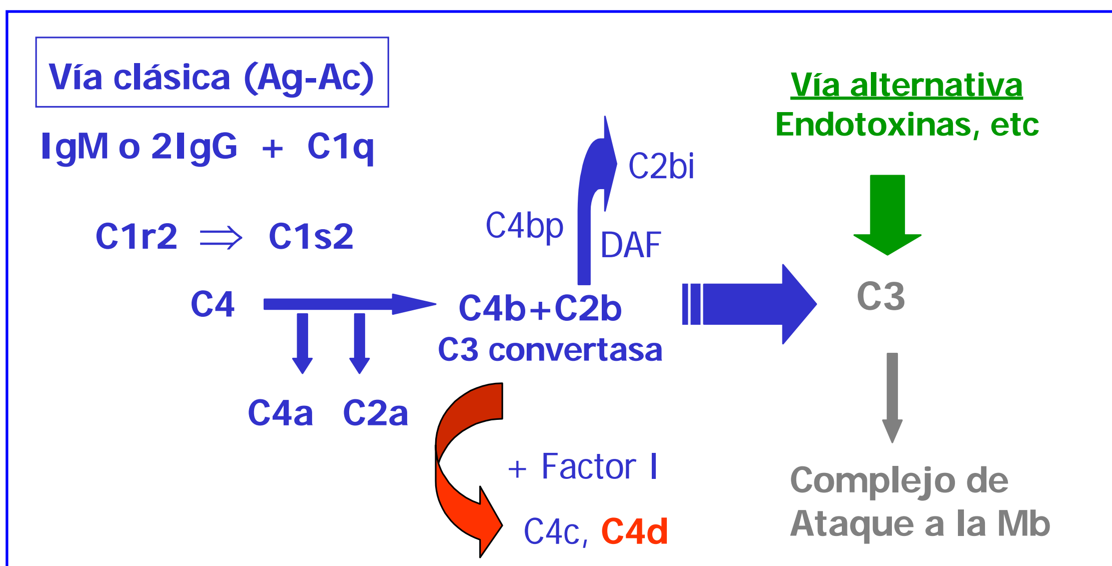


Figura 10. Vía clásica de activación del complemento. Producción de C4d. Mb= membrana

Es posible detectar C4d de forma constante en los glomérulos – a nivel mesangial sobre todo, pero también en la membrana basal- y algunos vasos arteriolares de riñones normales, probablemente como vestigio de mecanismos fisiológicos de aclaramiento de inmunocomplejos. Estudios de elución han demostrado su unión covalente a los tejidos (65). Utilizando **técnicas de inmunohistoquímica**, el grupo de Feucht fue el primero en evidenciar un depósito importante de C4d en vasos intersticiales, especialmente en CPT de injertos con rechazo agudo, además del clásico depósito glomerular, fundamentalmente en enfermos hipersensibilizados pre-trasplante. La coexistencia de inmunoglobulinas y C4 binding-protein en varias de esas biopsias apoya una activación local del complemento mediada por anticuerpos (42). Este grupo encontró una menor supervivencia a corto plazo de los injertos que mostraban depósitos de C4d en CPT durante los primeros meses post-trasplante, y especularon que este marcador distinguiría "rechazos celulares puros" de "rechazos mixtos" con mecanismos humorales de lesión tisular (43). En estudios consecutivos se centraron en la búsqueda de anticuerpos pre-trasplante que pudieran predecir el daño humoral, utilizando líneas celulares linfoblásticas (62,66,67), sin emplear sueros contemporáneos al momento de la biopsia. En definitiva, concluyeron que el hallazgo de C4d y/o la detección de anticuerpos anti-HLA pretrasplante suponen un factor de riesgo y permiten resaltar mecanismos humorales de rechazo no detectables de otra manera. Sin embargo no detectaron ADS post-trasplante, es decir, no caracterizaron una entidad diagnóstica.

El estudio inicial del MGH sobre la presencia de depósitos difusos de C4d en CPT corticales de injertos renales, detectada por **técnicas de inmunofluorescencia**, encontró una buena correlación con la aparición de ADS de novo post-trasplante renal (44). La detección de C4d en CPT resultó aparentemente más específica que los datos histológicos convencionales, ya que se observaron neutrófilos en CPT o necrosis fibrinoide arterial, parámetros previamente correlacionados con la existencia de ADS, en casos de rechazo

agudo celular (es decir, rechazo no mediado por anticuerpos). La presencia de C4d en glomérulos, arterias, arteriolas o tubulos resultó sin embargo inespecífica. De forma paralela, en este primer estudio se utilizaron técnicas de inmunofluorescencia para detectar células endoteliales (con Ulex europeaus aglutinina I) y membrana basal vascular (con anticuerpo monoclonal anti-colágeno tipo IV) (Figura 11). Estas técnicas mostraron que el C4d se distribuía con el colágeno tipo IV, es decir en la membrana basal capilar de todos los CPT, incluso en zonas que no mostraban reactividad con Ulex europeaus aglutinina I, sugiriendo que no todo el C4d se une al endotelio. Es decir, la destrucción del endotelio por daño humoral no elimina los depósitos de C4d y éste persiste si aún existe pared capilar. El fracaso en la detección de C3 o inmunoglobulinas en estos casos podría ser debido a modulación del sistema y/o a la desaparición de las células endoteliales objeto del daño (44).

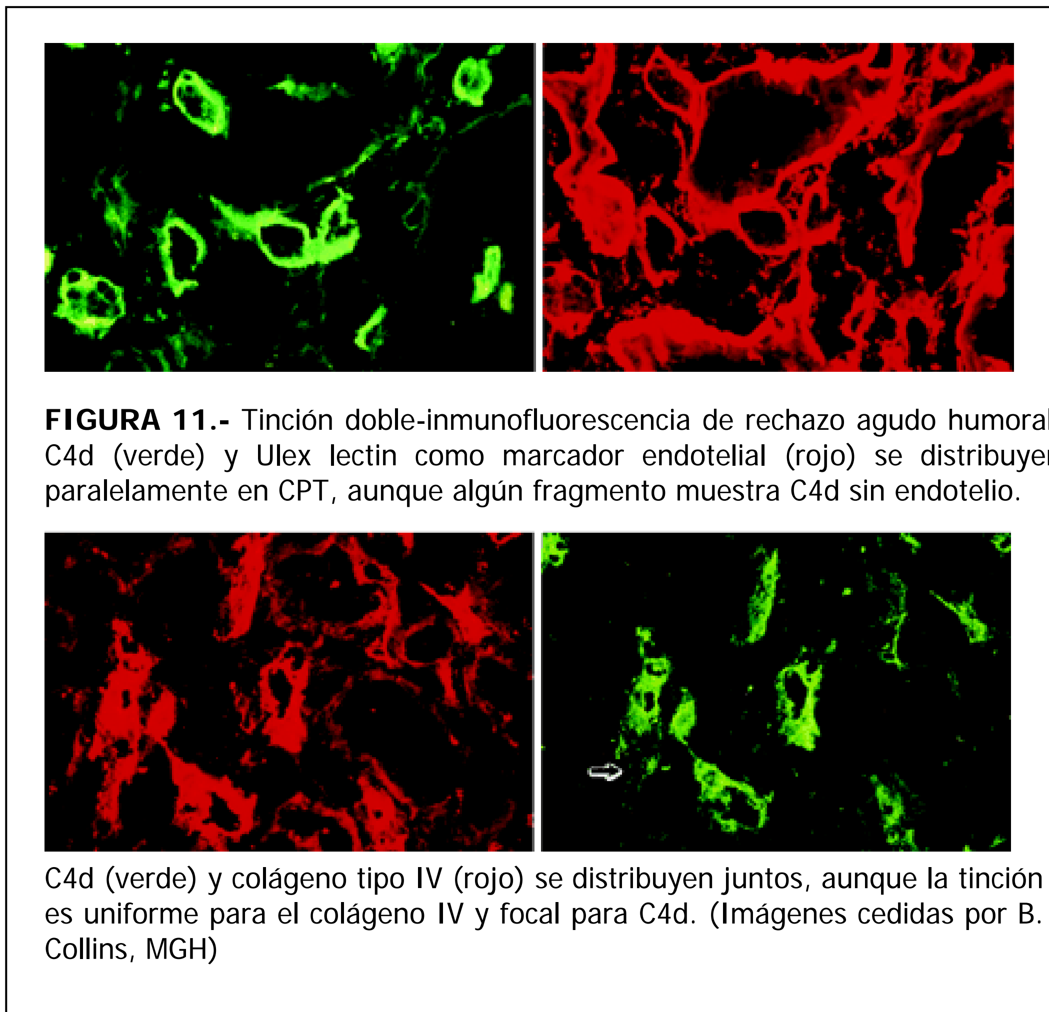


FIGURA 11.- Tinción doble-inmunofluorescencia de rechazo agudo humoral. C4d (verde) y Ulex lectin como marcador endotelial (rojo) se distribuyen paralelamente en CPT, aunque algún fragmento muestra C4d sin endotelio.

C4d (verde) y colágeno tipo IV (rojo) se distribuyen juntos, aunque la tinción es uniforme para el colágeno IV y focal para C4d. (Imágenes cedidas por B. Collins, MGH)

La presencia de antígenos HLA de clase I y II en las células endoteliales del donante en permanente contacto con la sangre del receptor convierte los vasos en el sitio propicio donde pueden desencadenarse los fenómenos humorales (68). Los CPT son la estructura vascular más representada en las biopsias renales, aunque no la única (69). Se desconoce por qué razón los CPT son la diana fundamental de los ADS. La correlación entre la existencia de C4d en CPT y de ADS en suero frente a la presencia constante de C4d en glomérulos sin ADS podría ser consecuencia de una diferente actividad de los mecanismos de regulación (inhibidores de la activación) o de los mecanismos de aclaramiento del complemento.

Se ha detectado también C4d en CPT en casos de rechazo hiperagudo por incompatibilidad ABO (70). Por tanto, el depósito difuso de C4d en CPT es secundario a la activación de mecanismos humorales de rechazo, independientemente de los anticuerpos involucrados. Por consiguiente, será interesante en este estudio sistemático de población que planteamos precisar la correlación C4d-ADS antiHLA y/o la posible presencia de otro tipo de anticuerpos.

FISIOPATOLOGÍA DEL DAÑO MEDIADO POR ADS

Los linfocitos B, al transformarse en células plasmáticas, son capaces de sintetizar anticuerpos, es decir inmunoglobulinas capaces de unirse de forma específica a otras moléculas concretas. En la reacción inmunológica post-trasplante la activación de las células B depende de tres tipos de señales. Por un lado, la unión del antígeno a la inmunoglobulina de membrana o receptor de la célula B (BCR), por otro una segunda señal que las células T envían a las células B a través de la interacción CD40 ligando-CD40 y finalmente la liberación de citoquinas por parte de los linfocitos T. Un proceso de apoptosis mantiene una regulación estrecha del desarrollo de las células B activadas y de memoria (63). Existe la posibilidad de que las células B puedan activarse sin la intervención de las células T en algunos casos de antígenos no proteicos.

Existen anticuerpos naturales producidos por linfocitos B primarios, como las isohemaglutininas frente al sistema ABO, sintetizadas sin contacto previo con el antígeno, y anticuerpos secundarios derivados de los linfocitos B de memoria, que requieren un contacto previo con el antígeno, como los anti-HLA. La aparición de anticuerpos anti-HLA se desencadena tras transfusiones de hematíes (no desleucotizadas), embarazos y trasplante de órganos, y también parece que algunas infecciones virales son capaces de producir sensibilización. Un segundo contacto con antígenos HLA presentes en un órgano puede por tanto desencadenar daño tisular. Los anticuerpos pueden producir este daño dependiendo de variables como especificidad, subclase, concentración, etc. (33). La lesión puede ser consecutiva a la activación del sistema del complemento (que produce contracción de músculo liso, quimiotaxis y fagocitosis) o a citotoxicidad celular dependiente de anticuerpos (ADCC: las células NK son capaces de destruir células recubiertas por anticuerpos sin intervención de las células T) (71).

El papel patogénico de los ADS y el complemento ha sido demostrado en animales. Un ejemplo precoz lo constituyen los experimentos de Winn y col. que mostraron que injertos cutáneos de ratón en ratas inmunosuprimidas podían ser destruidos por la transfusión de antisueros específicos (72, 73). Este fenómeno se abolía con el bloqueo del sistema del complemento en las ratas receptoras (73). Experiencias con ratas deficientes en complemento que no sufrían rechazo hiperagudo tras la transfusión de ADS, a diferencia de las ratas control, apoyan también estos datos (74,75). En humanos existen múltiples ejemplos ya comentados del papel patogénico de la presencia de ADS pre-trasplante renal (2-6), y de la lesión histológica asociada con infitración por neutrófilos, trombosis, etc, aunque sin estudios que evidenciaran la presencia de complemento. La activación del complemento (lisis y liberación de anafilotoxinas) es responsable del reclutamiento celular y la lesión histológica.

Sin embargo la existencia de daño mediado por la aparición de ADS de novo continúa resultando polémica, sobre todo debido al uso de diferentes técnicas en la detección de los ADS. TC Fuller y col. sugieren que una prueba cruzada positiva por técnica directa pre-trasplante anticipa el rechazo hiperagudo por la presencia de suficiente cantidad y calidad de anticuerpos necesarios para fijar complemento. Sin embargo, según estos autores, la detección de ADS por técnica indirecta se correlacionaría con la aparición de "rechazo agudo acelerado", ya que estos anticuerpos ocasionarían ADCC (48). Plantean que la detección de ADS por distintas técnicas puede reflejar diferentes mecanismos de daño mediado por anticuerpos. Por otro lado, JC Scornik y col. opinan que el significado de la presencia de ADS depende de la expansión clonal de células B existente en el receptor (53). Así, cuando la expansión clonal es mínima y la detección de ADS se obtiene con citometría de flujo, el significado de la presencia de estos anticuerpos no implicaría mal pronóstico. Además, niveles bajos de anticuerpos pueden no activar el complemento lo suficiente como para contrarrestar las proteínas reguladoras presentes en la superficie de las células endoteliales (75). Otros autores

sugieren que el daño mediado por ADS puede depender de la variabilidad inter e intra-individual en la expresión de los antígenos HLA del injerto (53,76).

NOMENCLATURA Y DEFINICIÓN

Tras los datos expuestos hasta el momento, tenemos que señalar que es importante definir claramente la entidad **"rechazo agudo humoral"** o "rechazo agudo mediado por anticuerpos". Se han utilizado varios términos, más o menos afortunados, que han contribuido a la confusión en este campo. Se ha empleado el término "rechazo agudo vascular" para etiquetar al rechazo agudo severo, atribuyéndo una patogenia humoral (61,62,77). Sin embargo, el "rechazo agudo vascular" no es necesariamente secundario a mecanismos humorales, puede también corresponder a una lesión mediada únicamente por linfocitos T (30,31,78,79). En concreto, la lesión de endarteritis o endotelialitis, un tipo de "rechazo vascular" (rechazo tipo II para las clasificaciones de Banff (79) y CCTT (30)), puede ser debido a mecanismos celulares en ausencia de ADS (80). Aún existe cierta controversia con respecto a los mecanismos inmunológicos en el caso de rechazo vascular con presencia de necrosis fibrinoide arterial (44).

Se han usado asimismo los términos "rechazo agudo acelerado" o "rechazo hiperagudo tardío" para hacer referencia al **"rechazo agudo humoral"**, aludiendo a la forma clínica de presentación y no al mecanismo patogénico. Algunos rechazos agudos celulares pueden presentarse como "rechazos acelerados" probablemente debido a una respuesta celular anamnéstica frente a los antígenos HLA del donante, y sin producción de ADS detectable pre o post-trasplante. Por otra parte, aunque en nuestra experiencia el **"rechazo agudo humoral"** tiende a presentarse de forma precoz post-trasplante, en ocasiones se produce a partir de la segunda semana (31).

Creemos que el término **"rechazo agudo humoral"** resulta más preciso para etiquetar este cuadro clínico-patológico al que nos referimos.

Además, distinguimos entre rechazo hiperagudo y **"rechazo agudo humoral"**, ya que el primero se asocia a niveles detectables de ADS en el suero del receptor utilizando técnicas convencionales en el momento del trasplante renal (prueba cruzada positiva pre-trasplante), que se unen al endotelio y activan el complemento, conduciendo a una rápida pérdida del injerto, habitualmente en minutos u horas. Sin embargo, aunque el momento y la presentación clínica del rechazo son diferentes, probablemente ambos rechazos comparten mecanismos fisiopatológicos. En el rechazo hiperagudo, la extensa activación del complemento por los ADS excede la capacidad de las partículas reguladoras de membrana y circulantes de evitar la lesión endotelial, y acaba transformando la superficie vascular en procoagulante. Experimentos con ratas deficientes en C6 y con inhibidores del complemento en las que el daño consecutivo a la transferencia pasiva de ADS es abolido o retrasado han demostrado la importancia que el complemento y los ADS juegan en el rechazo hiperagudo (74,81).

Proponemos y utilizamos como punto de partida para nuestros estudios los siguientes criterios clínicos, serológicos e histológicos para la definición de **"rechazo agudo humoral"**:

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1. **Criterios clínicos:** **Evidencia de disfunción renal severa, típicamente córtico-resistente y con frecuencia resistente al tratamiento antilinfocitario.**
2. **Criterios serológicos:** **Demostración de ADS en el momento del rechazo, indetectables pre-trasplante.**
3. **Criterios histológicos:** **Presencia de C4d de forma difusa en capilares peri-tubulares. Pueden existir otros elementos sugestivos en la biopsia renal, como neutrófilos en CPT, glomerulitis, microtrombos en arteriolas o glomérulos, necrosis fibrinoide, trombosis o infartos.**

HIPÓTESIS

El **rechazo agudo humoral** es per se una entidad clínica y patológica, que es posible y conviene identificar adecuadamente, dado que supone un porcentaje significativo de las pérdidas precoces del injerto post-trasplante renal que podemos evitar con pautas terapéuticas no convencionales.

Nos proponemos profundizar en el conocimiento de los mecanismos humorales del rechazo agudo revisando la conveniencia de los criterios expuestos en la introducción para el diagnóstico de **rechazo agudo humoral**, evaluando la incidencia de esta patología en nuestra población receptora de un injerto renal y analizando con detalle las características clínicas, serológicas e histológicas de estos episodios.

OBJETIVOS

OBJETIVO PRINCIPAL

Ampliar y perfeccionar el conocimiento del rechazo agudo humoral post-trasplante renal.

OBJETIVOS ESPECÍFICOS

1) Determinar la incidencia de **rechazo agudo humoral** en la población receptora de trasplante renal de un centro hospitalario universitario.
2) Estudiar las características clínicas de estos episodios, así como los factores de riesgo asociados al desarrollo de **rechazo agudo**

humoral: edad, sexo, raza, enfermedad de base, tipo y duración del tratamiento sustitutivo anterior de la función renal, número de trasplantes previos, grado de incompatibilidad HLA, grado de sensibilización.

3) Analizar los anticuerpos involucrados (tipo y títulos). Estudiar la evolución en el tiempo de los títulos de anticuerpos - relacionada o no con la modificación del tratamiento inmunosupresor-; así como su correlación clínico-patológica, la evolución a medio plazo y la aparición o no de un posible mecanismo de "acomodación" (permanencia de los anticuerpos sin aparente influencia sobre la función del injerto).
4) Evaluar la validez de los datos histológicos relacionados con el **rechazo agudo humoral,** tomando la detección de la fracción C4d del complemento en capilares peritubulares como "gold standard". Identificar características anatomo-patológicas que puedan implicar un pronóstico diferenciado dentro de este cuadro.
5) Evaluar el pronóstico de los injertos renales que sufren **rechazo agudo humoral.** Valorar la eficacia del tratamiento con plasmaféresis, tacrólimus y MMF, en términos de pronóstico de la función del injerto, a corto y medio plazo.

TRABAJOS ORIGINALES PUBLICADOS

- De novo production of anti-HLA donor specific antibodies during acute renal allograft rejection. M Crespo, M Pascual, S Mauiyyedi, AB Collins, N Tolkoff-Rubin, FL Delmonico, AB Cosimi, RB Colvin and SL Saidman. **Transplantation 1999; 67 (7): S87 (abstract)**

- Acute humoral rejection in renal allograft recipients: I. Incidence, serology and clinical characteristics. M Crespo, M Pascual, N Tolkoff-Rubin, S Mauiyyedi, AB Collins, D Fitzpatrick, ML Farrell, WW Williams, FL Delmonico, AB Cosimi, RB Colvin, SL Saidman. **Transplantation 2001, 71: 652 –658.**

- Acute Humoral Rejection in Kidney Transplantation: II. Morphology, Immunopathology and Pathologic Classification**.** S Mauiyyedi, M Crespo, AB Collins, EE Schneeberger, MA. Pascual, SL Saidman, NE Tolkoff-Rubin, WW. Williams, FL Delmonico A B Cosimi, RB Colvin. **J Am Soc Nephrol 2002, 13: 779-787.**

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DE NOVO PRODUCTION OF ANTI-HLA DONOR SPECIFIC ANTIBODIES DURING ACUTE RENAL ALLOGRAFT REJECTION

Acute humoral responses after kidney transplantation play an important role in allograft survival. Detection of circulating donor specific antibodies (DSA) is a major tool in the diagnosis of acute humoral rejection (AHR). We determined the incidence of post-transplant DSA to analyze its specificity in the diagnosis of AHR. Between November 1994 and September 1998, 216 kidney transplants were performed at our institution. We initially identified 10 cases of AHR (4.6%) according to strict criteria: 1) steroid and anti-lymphocyte antibody resistance, 2) typical pathological findings including neutrophils and C4d complement deposition in peritubular capillaries and 3) *de novo* anti-HLA DSA at the time of rejection. We searched for DSA in 47 cases from 4 other groups: severe (steroid-resistant) acute cellular rejection (sACR, n=21), mild (steroid-responsive) ACR (mACR, n=10), acute renal dysfunction not due to rejection (ARD, n=9) and no renal dysfunction (NO, n=7). In 22 cases the DSA work-up was requested to rule out AHR. In the remaining cases, post-transplant crossmatches (XM) were performed by protocol. Immunosuppression included cyclosporine (CsA) – prednisone (P) - azathioprine (n=44), CsA-P-mycophenolate mofetil (MMF) (n=9), tacrolimus-P-MMF (n=3) and cyclophosphamide-P (n=1). AHG enhanced cytotoxic T cell XM, standard cytotoxic B cell XM at 37°C and flow cytometric XM (T cell, IgG) were performed. The groups were comparable in terms of age, race, underlying renal disease and type of donor (cadaver vs. living). The number of highly sensitized patients (pre-transplant PRA >50%) was higher in the AHR (90%) vs sACR (5%), mACR (0%), ARD (22%) and NO groups (14%). In the AHR group all patients had positive post-transplant XM with either anti-HLA class I (n= 8) or class II (n= 2) IgG antibodies. DSA were also found in 3/21 sACR and 1/9 ARD patients. Retrospective staining for C4d showed deposits in peritubular capillaries in these four patients, indicating a humoral component to their allograft dysfunction. Graft survival rate at six months was lower in AHR (60%) as compared to sACR (85.7%), mACR (100%), ARD (88.9% due to one death) and NO (100%). In conclusion, we found that *de novo* production of anti-HLA DSA is specific for AHR and is associated with complement mediated allograft injury. AHR tends to occur in pre-sensitized patients, and was associated with lower graft survival. A donor specific crossmatch should be performed in cases of acute refractory rejection, so that aggressive treatment for AHR including plasma exchange and tacrolimus-MMF rescue can be initiated if necessary.

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ESTUDIO PILOTO: "De novo production of anti-HLA donor specific antibodies during acute renal allograft rejection" (Transplantation 1999; 67 (7): S87 (abstract))

(Información más completa para la presentación definitiva del trabajo en el Congress of the American Society of Transplant Physicians, Chicago 1999)

Partiendo de las experiencias previas de la Unidad de Trasplante del MGH, revisamos los estudios de ADS post-trasplante realizados en 62/244 enfermos trasplantados entre noviembre de 1994 y febrero de 1999 (161 sueros estudiados) con objeto de averiguar el valor de la detección de ADS post-trasplante (datos parciales recogidos en el abstract citado) (Crespo 99). Diez de esos enfermos habían sido diagnósticados de **RAH** prospectivamente, los otros 52 habían sufrido presumiblemente rechazo agudo celular córtico-resistente (sAR, n=22), rechazo agudo celular córtico-sensible (mAR, n=11), disfunción aguda no secundaria a rechazo (ARD, n=12) o ningún problema (NO, n=7), desde el punto de vista clínico, e histológico cuando existía biopsia renal. Sólo tres enfermos con rechazo agudo córtico-resistente y uno con una biopsia compatible con nefrotoxicidad por ciclosporina (caso comentado en la introducción) habían desarrollado ADS en el momento de la disfunción renal (Tabla 3). Esos cuatro enfermos también presentaban depósitos difusos de la fracción C4d del complemento en CPT en las biopsias correspondientes. Se trataba por supuesto de una población seleccionada (35 enfermos habían sido estudiados prospectivamente para descartar respuestas humorales post-trasplante y 27 retrospectivamente por motivo de estudio). Este análisis nos permitió concluir que la detección de ADS de novo en suero y de C4d en el tejido renal son marcadores de mecanismos humorales de rechazo severo, con cierta frecuencia asociados a mecanismos celulares clásicos de rechazo.

	RAH n=10	sAR N=22	mAR n=11	ARD n=12	NO n=7
Post-tx ADS	10/10	3/22*	0/11	1/12*	0/7
Re- tx	4/10	2/22	1/11	4/12	2/7
Diferencias HLA	3,8±1,2	4,3±1,0	3,3±1,6	3,0±2,4	2,0±2,0
PRA> 50% - pico	8/10	1/22	0/11	3/12	1/7
- pretx	7/10	1/22	0/11	2/12	1/7
Día de RA (media)	8±3	13±8	17±2	-	-
Injertos funcionantes a los 6 meses (%)	70	86,4	100	91,9	100
Creatinina (mg/dl) 6 meses (media)	1,9±0,6 (n=6)	1,5±0,3 (n=18)	1,3±0,2 (n=11)	1,6±0,2 (n=8)	1,3±0,3 (n=7)

* En el estudio retrospectivo, estos 4 enfermos presentaban también depósitos difusos de C4d en CPT.

Tabla 3.- Datos más relevantes del estudio preliminar sobre la presencia de ADS en el periodo inicial post-trasplante renal en distintos grupos de enfermos según la evolución clínica.
sAR = Rechazo agudo córtico-resistente. mAR= Rechazo agudo córtico-sensible. ARD = Disfunción renal aguda (función retrasada del injerto, toxicidad por ciclosporina). NO= No disfunción.

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ACUTE HUMORAL REJECTION IN RENAL ALLOGRAFT RECIPIENTS: I. INCIDENCE, SEROLOGY AND CLINICAL CHARACTERISTICS[1]

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Background. **Acute rejection (AR) associated with de novo production of donor-specific antibodies (DSA) is a clinicopathological entity that carries a poor prognosis (acute humoral rejection, AHR). The aim of this study was to determine the incidence and clinical characteristics of AHR in renal allograft recipients, and to further analyze the antibodies involved.**

Methods. **During a 4-year period, 232 renal transplants (Tx) were performed at our institution. Assays for DSA included T and B cell cytotoxic and/or flow cytometric cross-matches and cytotoxic antibody screens (PRA). C4d complement staining was performed on frozen biopsy tissue.**

Results. **A total of 81 patients (35%) suffered at least one episode of AR within the first 3 months: 51 had steroid-insensitive AR whereas the remaining 30 had steroid-sensitive AR. No DSA were found in patients with steroid-sensitive AR. In contrast, circulating DSA were found in 19/51 patients (37%) with steroid-insensitive AR, and widespread C4d deposits in peritubular capillaries were present in 18 of these 19 (95%). In at least three cases, antibodies were against donor HLA class II antigens. DSA were not found in the remaining 32 patients but C4d staining was positive in 2 of 32. The DSA/C4d positive (n=18) and DSA/C4d negative (n=30) groups differed in pre-Tx PRA levels, percentage of re-Tx patients, refractoriness to antilymphocyte therapy, and outcome. Plasmapheresis and tacrolimus-mycophenolate mofetil rescue reversed rejection in 9 of 10 recipients with refractory AHR.**

Conclusion. **More than one-third of the patients with steroid-insensitive AR had evidence of AHR, often resistant to antilymphocyte therapy. Most cases (95%) with DSA at the time of rejection had widespread C4d deposits in peritubular capillaries, suggesting a pathogenic role of the circulating alloantibody. Combined DSA testing and C4d staining provides a useful approach for the early diagnosis of AHR, a condition that often necessitates a more intensive therapeutic rescue regimen.**

INTRODUCTION

In recent years, acute renal allograft rejection associated with the development of donor specific antibodies (DSA) has emerged as a clinicopathological entity that carries a poor prognosis (acute humoral rejection, AHR) (*1–7*). Tubulitis and endothelialitis characterize T cell-mediated rejection (acute cellular rejection, ACR). In contrast, typical pathological features of AHR by light microscopy include neutrophils in peritubular capillaries, vasculitis, and fibrinoid necrosis in vessel walls (*6–10*) although no single histological feature is considered to be diagnostic.

The risk of graft loss has typically been high in recipients with AHR, with 1-year graft survival rates varying between 15 and 50% despite intensive conventional immunosuppressive therapy (*1–4, 11, 12*). We have recently reported that a new approach combining plasmapheresis and "tacrolimus-mycophenolate mofetil (MMF) rescue" can more consistently suppress DSA production and prevent allograft loss in these patients, and, therefore, has the potential to improve the outcome of AHR (*4*).

The significance of DSA in the diagnosis of renal allograft rejection has been unclear, with 38–100% of the reported patients with DSA suffering from rejection at the time (*1, 13, 14*). This variability is likely related to differences in the sensitivity and specificity of the assays used to detect DSA, and demonstrates the need for more specific assays for the diagnosis of AHR. Supporting the initial work of Feucht et al.(*15*), we recently found that peritubular capillary (PTC) staining for C4d, a durable split product of C4 that indicates activation of the classical pathway of complement, is the marker that best identifies AHR in renal allograft biopsies (*10*). C4d remains covalently bound to nearby endothelium or basement membrane collagen, thereby providing in situ pathological evidence of an anti-donor humoral response.

The purpose of our study was to determine the incidence of AHR in our transplant population, and to identify its clinical characteristics. We reviewed our experience over a 4-year period (1995–1999) and performed both sensitive and specific

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techniques for antibody detection, and correlated them with the presence of widespread C4d staining in PTC to identify patients with AHR.

PATIENTS AND METHODS

Patient population and immunosuppression. Between July 1995 and July 1999, 232 kidney transplants (127 cadaver, 75 living related, and 30 living unrelated) were performed in 143 males and 88 females (1 patient received two transplants). Demographics of the population are shown in Table 1. We excluded 10 recipients who received combined kidney-pancreas (n=5) or kidney-liver transplants (n=5), and 2 patients who underwent tolerance-induction study protocols (*16*) from the analysis. Baseline immunosuppression included cyclosporine and prednisone, with or without azathioprine, between July 1995 and May 1997 (*17*); and cyclosporine, prednisone, and mycophenolate mofetil between June 1997 and July 1999. Clinically diagnosed episodes of acute rejection considered in this study were biopsy-confirmed in 89% of cases, using criteria published previously (*18*). AR was initially treated with daily intravenous methylprednisolone (500 mg), and if no significant improvement in allograft function was seen within 2 or 3 days ("steroid insensitive" rejection), monoclonal (OKT3 mAb or anti-CD2 mAb), or polyclonal antilymphocyte therapy (ATGAM) was administered for 7 to 15 days. Absence of improvement in allograft function was defined by no decrease in serum creatinine from its peak value during the rejection episode. Clinical information was collected by chart review and using the Transplantation Unit database.

Detection of DSA. Pre- and post-TX DSA tests performed included complement-dependent-cytotoxic (CDC) and flow cytometric assays. For CDC, T and B cells were isolated using immunomagnetic beads (Dynal, Lake Success, NY). Four wash antihuman-globulin-enhanced (AHG) CDC assays were done with T cells and standard and four wash CDC at 37°C with B cells as previously described (*19, 20*). IgG was differentiated from IgM using dithiothreitol (DTT). For patients receiving OKT3, the murine monoclonal antibody was removed using immunomagnetic beads bound with sheep anti-mouse Ig (*21*). For patients receiving ATG, cross-match tests were performed by two-color flow cytometry using phycoerythrin-labeled anti-CD3 (T cells) or anti-CD19 (B cells) mAb and affinity purified fluorescein-isothiocyanate-conjugated goat-antihuman IgG (Fab) that did not cross-react with horse or mouse immunoglobulin. Selected patients were tested by both cytotoxicity and flow cytometry assays. We have found that our AHG and flow cytometry assays have similar sensitivity (*22*).

For pretransplant cross-match, sera from the previous 6 months (for sensitized, retransplant, or living donor recipients) or the most recent serum only (for unsensitized recipients of cadaveric transplants) were tested against peripheral blood lymphocytes from living donors or lymphocytes from cadaver lymph nodes or spleen. Panel-reactive antibodies (PRA) against T and B cells were determined by cytotoxicity using local frozen cell panels (40 and 20 cells, respectively).

Pretransplant PRA were determined monthly with T cells and quarterly with B cells. Posttransplant PRA were determined when donor or appropriate surrogate cells were not available for cross-match and to identify HLA specificity of DSA in select patients. In some cases, HLA specificities were determined or confirmed with purified HLA antigens using an ELISA technique (LAT, One Lambda Inc., Canoga Park, CA). Posttransplant assays for DSA testing were performed either prospectively (n=38 patients) using sera collected at the time of rejection or retrospectively (n=40 patients) with sera stored at −30°C at the time of rejection. Three patients did not have sera available for DSA testing. Donor cells were stored at −85°C at the time of the transplant or were collected fresh from some living donors.

Pathology. Renal biopsies were obtained at the time of rejection from 72 patients and 70 had sufficient frozen tissue available for immunopathological studies. Paraffin-embedded tissue sections (2–3 μm) were stained with hematoxylin and eosin and periodic acid-Schiff stains. Direct immunofluorescence using mono-specific rabbit antisera against human IgG, IgM, IgA, C3, albumin, fibrin/fibrinogen, and a sensitive three-step immunofluorescence technique for C4d staining was performed on frozen kidney tissue as described (*10*). The details of the pathologic studies are described in a second report (Mauiyyedi et al., manuscript in preparation).

Statistical analysis. Parametric variables are expressed as mean±SD, and nonparametric variables are expressed as medians and ranges. Statistical tests were performed with the assistance of the SAS package program, and included χ^2 for qualitative variables; ANOVA with test post hoc (Scheffe test) for the relationship between qualitative and quantitative parameters; and Mann-Whitney and Kruskal-Wallis H for nonparametric variables.

RESULTS

During the study period, 81 of the 232 renal allograft recipients (35%) suffered at least one episode of acute rejection within the first 3 months posttransplantation. The AR episode proved to be steroid insensitive in 51 of these 81 cases (sAR group: 63% of total AR cases and 22% of the total study population). Nine of these 51 patients suffered two episodes of acute rejection within the first 3 months posttransplant

TABLE 1. **Demographics for renal transplant recipients with and without acute rejection (AR)**

	No rejection n=151	Steroid-insensitive rejection (sAR) n=51	Steroid-sensitive rejection (mild AR) n=30
Age (mean±SD)	47.5±12.6[a]	42.8±11.9	37.7±13.2[a]
Sex			
% Males	65	53	60
Race			
% Caucasians	78	84	77
Etiology of ESRD (%)			
Glomerular	41	29	23
Diabetic	18	16	13
Others	40	55	63
Mo dialysis (median, range)	18 (0–264)	23 (0–276)[b]	9.5 (0–68)[b]
% Retransplants	14	18	7
Type of donor			
% Cadaveric donors	55	59	40

[a] $P<0.05$ for no rejection versus mild rejection.
[b] $P<0.05$ for steroid insensitive versus mild rejection.

and at least one of the episodes was steroid insensitive. The remaining 30 patients (mild acute rejection group: 37% of AR) had mild, steroid-sensitive rejection episodes. As detailed in Table 1, there were no significant differences in sex, race, etiology of end-stage renal disease, and time on dialysis before transplantation between patients with no AR and those with sAR or mild steroid-sensitive AR. Age was lower in patients with mild AR versus those with no AR. Patients with sAR were on dialysis longer pretransplant than were patients with mild AR.

Steroid-Insensitive Acute Rejection Group

Pretransplant assays for DSA. All patients (n=51) received their transplants with negative immediate pretransplant T cell cross-matches by AHG CDC. At the time of rejection in these recipients, we retested the pretransplant samples, 28 by AHG and 23 by both flow cytometry and AHG. We found that 2/51 patients had very weakly positive retrospective T cell cross-matches with pretransplant sera using frozen instead of fresh donor cells (*23*). Both flow cytometry and AHG CDC confirmed these. Forty-four of 51 patients had negative B cell CDC cross-match as well and the remaining patients had pretransplant PRA-B equal to 0%.

Two primary transplant recipients had very weakly positive flow cross-matches (one T, one B) but CDC was consistently negative and neither developed posttransplant DSA. One highly sensitized recipient had a borderline positive AHG cross-match, but was negative by flow with both T and B cells.

Posttransplant assays for DSA. A total of 165 serum samples from these 51 patients were tested. Sera were collected at the time of acute rejection, except for one patient tested within 2 weeks and one patient tested within 1 month of the rejection episode. All 51 patients had a DSA study performed: 26 by cytotoxicity, 14 by flow, and 11 by both techniques. We found DSA in serum samples at the time of rejection in 19 of these 51 recipients (37% of patients in the steroid-insensitive AR group). Seventeen of 19 developed IgG DSA. At the time of the first positive cross-match, eight were positive with both T and B cells, five were positive with T cells only (B cells were negative or untested), and four were positive with B cells only (T cells were negative). Two of 19 had IgM only against donor B cells. We could determine DSA specificity for 10/19 patients using PRA tests: 6 recipients had anti-HLA class I DSA, 3 had anti-HLA class II, and 1 had a positive T cell cross-match but only antibody specificity against class II antigens could be defined. These and other immunological data are shown in Table 2. The remaining 32 patients were not found to have DSA at the time of rejection. Thirty-one had negative T cell cross-matches and one had PRA-T equal to 0%, 26 had negative B cell cross-matches, and 5 had PRA-B equal to 0%.

C4d staining. The test was performed in biopsies taken to diagnose the cause of the renal allograft dysfunction, except for one patient whose only available frozen tissue was obtained approximately 1 month after the rejection episode. All 17 patients who developed IgG DSA at the time of rejection had widespread and diffuse C4d deposits in PTC in their biopsies. One of the two recipients who formed IgM DSA against donor B cells showed widespread C4d deposits in PTC. The other patient had typical pathological features of AHR by light microscopy (neutrophils in PTC and interstitium, fibrinoid necrosis of arterioles, and acute tubular injury), but only demonstrated focal C4d deposits in PTC.

In the 32 recipients who did not develop DSA after the

TABLE 2. Cross-match results and antibody specificity in patients with posttransplant DSA

Patient	Pretransplant				Donor mismatches	Posttransplant				
	XM T	XM B	% PRA T	% PRA B[a]		XM T[b]	XM B[b]	% PRA T	% PRA B	Antibody specificity
1	+w	–	57	5	A2, 29, B61; DR14	+	–	100	ND	Multi
2	–	–	79	0	A1, 3; B8, 38; DR13, 17	+	+	ND	ND	ND
3	–	–	89	ND	A1; B7, 35; DR3, 8	+	+	98	ND	Multi
4	–	–	0	ND	A3; B7; DR2	+	+	63	ND	B7 CREG
5	–	–	5	0	A30, 68; B55 (Bw6); DR2	+	+	95	ND	Bw6
6	–	–	60	65	A3, 31; B51, 35; DR15	+	ND	85	65	New B5 CREG
7	–[c]	–	100	ND	B16	+	+	100	ND	Multi
8	–	–	0	0	A25; B60; DR1	–	+	5	83	DR1, 2, 10+
9	–	–	78	ND	A24 (9), 29; DR1	+	+	97	ND	New A9
10	–	–	95	87	DR103, 13	–[d]	+	82	100	New DR1, 13
11	–	–	5	11	A29, 32; B7, 70; DR3	–	+[e]	0	40[e]	?
12	–	–	14	0	A2; B18, 27; DR11	+w	–	15	ND	?
13	–	–	0	0	A2, 23; B51, 57; DR4, 7	–	+	10	50[e]	DQ2, DR7
14	–	ND	0	0	A2; B39, 51; DR1, 11	+	ND	0	30	DR1
15	–	–	0	0	A23, 24; DR1	–	+[e]	3	14[e]	?
16	–	–	90	ND	A31; B35, 51; DR4, 7	+	+	100	ND	Multi
17	+w	–	25	5	A2; B57, 60; DR4, 7	+	–	90	90	A2, B7 CREGS
18	–	–	0	0	A2, 30; B53, 72; DR1, 11	–	+w	0	5	?
19	–	–	13	10	A33; B35; DR4, 13	+	+	95	ND	Multi

XM, Cross-match; +w, weakly positive; ND, not determined; CREG, cross-reactive group.
[a] Sera were adsorbed with pooled platelets if needed.
[b] At time of initial positive XM.
[c] XM was wk with AHG, neg by flow.
[d] T cell XM was negative with surrogate cell; donor had no class I mismatches.
[e] Became negative or reduced with DTT treatment (IgM antibody).

transplant, two patients had widespread C4d deposits in PTC (6%). These two patients had no detectable serum antibodies against donor T or B cells as tested by flow cytometry and cytotoxicity, respectively. The serum from one of these patients was further tested against a panel of keratinocytes in an attempt to identify non-HLA antibodies, but no such antibodies could be detected (*24*). The renal biopsy pathology of both these patients was similar to the other C4d+ AHR cases, with prominent neutrophils in PTC, glomerular capillaries, and tubules. One of these patients lost the graft six weeks posttransplant, and the nephrectomy specimen had severe fibrinoid necrosis of arterioles and endarteritis, in addition to capillary neutrophils.

Clinical features of patients with acute humoral rejection versus acute cellular rejection. The detection of both serum DSA and C4d deposits in PTC at the time of rejection allowed us to define 18 recipients as having primarily AHR (DSA+C4d+) and 30 recipients, with severe acute cellular rejection (severe ACR) who had no apparent humoral component (DSA−C4d−). Table 3 compares the clinical and immunological data in patients with AHR versus those with severe ACR, and also shows the characteristics of mild acute rejection patients. The number of HLA mismatches was similar. However, recipients in the AHR group were significantly more frequently sensitized, as measured by historical and current PRAs, ($P<0.05$), and more patients in this group were recipients of re-transplants (7/18 vs. 2/30, $P<0.05$). Cadaveric donors were more common in the AHR group than in the severe ACR group ($P<0.05$). The onset of rejection in the AHR group was somewhat earlier; however, this did not reach statistical significance. The percentage of recipients with delayed graft function and the percentage of patients receiving antilymphocyte therapy as induction immunosuppression did not differ in the two groups. Moreover, there was no significant difference in the incidence of AHR between patients receiving different baseline immunosuppression regimens (cyclosporine and prednisone, with or without azathioprine, versus cyclosporine, prednisone, MMF). Both of the AHR patients with a retrospective weak positive pretransplant cross-match (Table 2, patients 1 and 17) had primary nonfunction (the grafts never functioned).

Rejection was more often resistant to antilymphocyte therapy (no decrease in serum creatinine after 3 to 4 days of treatment) in recipients suffering AHR than in the severe ACR group (67 vs. 3%, $P<0.001$) and their graft outcome was significantly worse (78 vs. 97%, $P<0.05$). However, serum creatinine values at 6 months for functioning grafts were not significantly different.

We found that three patients with steroid-insensitive acute rejection did not fulfill the strict definition of either AHR (DSA+C4d+) or severe ACR (DSA−C4d−). One patient developed an IgM DSA but did not have widespread C4d deposits in PTC (Table 2, patient 11), and two patients had widespread C4d deposits but no detectable serum DSA. The patient who developed IgM DSA of unclear specificity presented with refractory acute rejection and pathological features of AHR, and he was treated according to the protocol below. Of the two patients with C4d and no DSA, one lost the graft due to ongoing rejection at 6 weeks despite OKT3 and ATG treatment, although the other patient has good allograft function 16 months posttransplant (serum creatinine 1.6 mg/dl).

Treatment of rejection associated with DSA. Thirteen of the 19 patients (67%) found to have circulating DSA at the time of acute graft dysfunction had a clinical picture of refractory acute rejection, that is a rejection episode resistant to both steroid boluses and antilymphocyte therapy. Primary nonfunction occurred in 2 of the 13 patients (as mentioned above). One patient, the initial case to be identified, did not receive the protocol treatment described below and lost the graft at 6 months due to ongoing rejection despite sequential courses of therapy with OKT3 and ATG.

In 10 consecutive recipients with refractory AHR, a regimen of plasmapheresis (PPh) with "tacrolimus-mycophenolate mofetil (MMF) rescue" was initiated (*4*). Daily PPh (five sessions, 1.3 volume per exchange), followed by alternate day

TABLE 3. Clinical and immunological data for patients with acute rejection

	Steroid insensitive rejection[a]		Steroid sensitive rejection
	AHR (DSA+, C4d+) n=18	Severe ACR (DSA−, C4d−) n=30	Mild acute rejection (mAR) n=30
No. mismatched HLA antigens (median, range)	4 (1–6)	4 (0–6)	4 (0–6)
% 0 mm donors	0	13.3	6.7
Pretransplant PRA (median, range)			
Peak %	56.5 (0–100)[b]	3 (0–100)	3 (0–100)
Current %	19.5 (0–100)[b]	0 (0–100)	0 (0–51)
% Retransplants	38.9[b]	6.7	6.7
% Cadaveric donors	77.8[b]	43.3	40.0
Day of onset of AR (median, range)	7 (1–22)	9 (2–37)	9.5 (2–68)
% Patients with DGF	22.2[c]	20.0	0
% Patients with induction AL therapy	16.7	3.3	3.3
% Refractory to AL therapy	67[b]	3	na
% Grafts functioning at 6 months	77.8[b]	96.7	96.7
Serum creatinine at 6 months (mean±SD)	2.1±1.4[c]	1.6±0.6	1.3±0.3

DGF, Delayed graft function; AL, antilymphocyte therapy.

[a] Does not include two patients who were DSA−C4d+ or one patient who was DSA+C4d−.

[b] $P<0.05$ when comparing AHR to severe ACR or mild acute rejection.

[c] $P<0.05$ when comparing AHR with mild acute rejection.

PPh (five sessions) if necessary, were combined with tacrolimus (mean 0.11 mg/kg/day, with target plasma trough levels of 10–15 ng/ml) and MMF (2 g/day initial dose). Sequential DSA titers were measured throughout the treatment regimen. This therapeutic strategy significantly decreased circulating DSA over 2 to 4 weeks, with reversal of the rejection process in 9/10 patients. One patient (Table 2, #4) suffered from recurrent AHR, i.e., another episode of renal dysfunction with increase in DSA titers, and was successfully treated with additional PPh (*4*). At the end of PPh, polyclonal immunoglobulin (0.4 g/kg) was administered i.v. to limit the risk of infectious complications. The total number of PPh treatments was guided by response to therapy (improvement in allograft function) and serum levels of DSA. Of note, in one patient low levels of DSA were detected for 6 months after PPh, yet renal function had returned to normal.

Currently, with a mean follow-up of 29 months (range 9 months to 4.2 years), patient and graft survival are 100 and 80% (mean serum creatinine 1.5±0.4 mg/dl), respectively. The only graft losses were due to refractory AHR and anti-glomerular basement membrane disease at day 10 (third transplant in a recipient with underlying Alport's disease) and to allograft glomerulopathy associated with CMV viremia at day 290. Pulmonary cryptococcal infection occurred in one patient, and was successfully treated by lobectomy and prolonged fluconazole administration at 3 years posttransplant. There were no neoplastic complications in patients who received PPh and tacrolimus-MMF rescue.

Six of the 19 patients with AHR responded to i.v. methylprednisolone boluses and antilymphocyte therapy and therefore did not receive the therapeutic rescue regimen. Five of the six currently have good allograft function and one died (with a functioning graft) of metastatic bladder cancer 40 months posttransplant. Of note, low titers of DSA were present at the time of rejection in five of these six patients. This was in contrast to patients with refractory AHR, where only 2 of 13 had low titer antibodies.

Steroid-sensitive acute rejection group

Thirty of 81 patients with AR presented with steroid-sensitive acute rejection episodes (Table 1). Sera taken at the time of rejection were available in 27 patients; 23 had negative T cell cross-matches and 4 had PRA-T equal to 0% at the time of rejection; 18 had negative B cell cross-matches, and 9 had PRA-B equal to 0%. A total of 21 recipients had allograft biopsies at the time of rejection, and 19 had frozen tissue available for C4d staining. One biopsy showed C4d staining in PTC, but unfortunately there was no available serum sample to test for DSA in this patient.

Clinical and immunological data for this group are shown in Table 3. Patients with mild acute rejection were similar to the steroid insensitive ACR group. However, none had delayed graft function, an unusual finding because the incidence of DGF in our renal transplant population has always approximated 20% in cyclosporin A-treated patients.

DISCUSSION

In this study, we determined the incidence and clinical characteristics of AHR in kidney transplant recipients over a 4-year period, by using sensitive and specific cross-match techniques and analyzing C4d deposits in renal allograft biopsies from patients with AR. We have also extended our previously reported observations with the use of PPh-tacrolimus-MMF rescue therapy in the subset of patients who present with refractory AHR, a condition that typically carries a poor prognosis (*1–4, 11, 12*).

We found an overall incidence of AHR of 7.7% in our renal transplant recipients, as defined by acute rejection associated with DSA. Of the 19 patients who were found to have DSA in posttransplant sera, 18 (95%) also had prominent and diffuse C4d deposits in allograft biopsy PTC, a marker of antibody-mediated rejection (*10*). This contrasts with patients who developed AR but without serum DSA, who were rarely (6% of biopsies) found to have C4d in PTC. These results confirm that C4d staining of allograft biopsies provides a useful molecular tool to identify the presence of humoral mechanisms of rejection at the time of graft dysfunction. Previous studies have noted the central role of complement activation in allograft damage mediated by DSA (*25–27*) and therefore the presence of C4d deposition in the biopsies can be taken as evidence supporting a pathogenic role of the circulating DSA.

The typical clinical presentation of AHR was that of early (within the first week) severe rejection (n=11), poorly responsive to both methylprednisolone boluses and antilymphocyte therapy. This is consistent with previous observations, in which AHR carried a poor prognosis with a risk of early graft loss varying from 50 to 85% (*1–3, 6, 11, 28*). The second most frequent clinical presentation in our series was that of "classic" acute rejection (n=7), i.e., allograft dysfunction occurring after the first week posttransplant.

Four AHR cases had delayed graft function or primary nonfunction. We and others have suggested that delayed graft function or primary nonfunction due to AHR is probably associated with low levels of DSA undetected by the pretransplant cross-match (*5, 7, 29*), but that become apparent in recipient sera a few days posttransplantation. This was confirmed in the two patients with primary nonfunction, in whom retrospective cross-matches using frozen cells demonstrated very low levels of DSA (*23*). These two graft failures did not meet the definition of "hyperacute rejection" (graft loss occurring in minutes or hours), as renal scans revealed persistence of renal blood flow in the early days posttransplant. However, the pathogenic mechanisms of hyperacute rejection or AHR presenting as primary nonfunction are likely to be similar, that is local complement activation after DSA binding to the graft vasculature.

There were also rare cases of primary transplant recipients with no apparent evidence of sensitization but who developed humoral rejection early posttransplant. One example is patient 8, an apparently unsensitized male who received a kidney transplant from his mother, and who was diagnosed with severe rejection and high titer DSA within the first week. Another example is patient 4, who received a kidney from her 32-year-old daughter and developed DSA 7 days posttransplant despite having no detectable antibodies pretransplant. This case is similar to reports of accelerated rejection in previously pregnant women who received cadaver donor kidneys that shared mismatched paternal antigens (*30*).

One striking finding of our study is that more than one-third of patients with steroid-insensitive acute rejection had evidence of AHR. This emphasizes that screening for de novo

production of DSA should be performed in all renal recipients with steroid-insensitive AR. Six of the 19 patients with DSA were identified retrospectively, demonstrating that this type of AR is often associated with acute cellular rejection and therefore is not always detected by classic pathology (see companion paper, Mauiyyedi et al., manuscript in preparation).

Higher historical and pretransplant sensitization, and a previous failed allograft, were found to be risk factors for AHR, suggesting that an anamnestic humoral response against donor antigens may play a role in the pathogenesis of this type of rejection. In addition, it also suggests that an overall state of enhanced humoral alloreactivity may facilitate a donor-specific response, or alternatively such patients may be "high responders" who are more likely to develop antibody in response to an allograft. More cadaver donors were in the AHR group compared to the other two rejection groups, consistent with the hypothesis that ischemic damage increases the immunogenicity of the transplanted organ (*31*).

In our series, 6 of the 19 patients with DSA initially presented with B cell positive/T cell negative cross-matches. Some authors have noted that anti-HLA class I DSA are predominantly involved in causing AHR (*1, 2, 7*). However, we could determine the specificity in three of the six, and all were directed against mismatched donor class II antigens, suggesting that in at least three cases, anti-HLA class II DSA played a major role in the AHR process. One of the donors was mismatched only for class II antigens, confirming that class II DSA were the only alloantibodies involved in this case. However, half (three of six) of these patients developed weak positive T cell cross-matches within a few days, thus making it impossible to discern if anti-HLA class I DSA were or were not also playing a role in the initial rejection process.

Two patients (nos.11 and 15) developed IgM antibody of unclear specificity against donor B cells. These antibodies may not have been directed against any HLA or other antigens present on the kidney, and therefore it cannot be concluded that the IgM was directly involved in the rejection process. These patients, as well as the two patients with widespread C4d deposition yet no detectable DSA may instead have had non-HLA antibodies against endothelial specific antigens (*24, 32–34*) that could have caused the humoral rejection and complement deposition. However, another explanation may be that these patients had HLA antibodies below the level of detection of our assays, perhaps because most were absorbed by the kidney and therefore not present in the circulation.

In 10 patients with refractory AHR identified prospectively, we used PPh and tacrolimus-MMF rescue in an attempt to remove and suppress production of DSA. With this combined therapeutic approach, successful initial reversal of AHR was achieved in 9 of 10 patients. It should be noted that at the end of the PPh treatment, polyclonal i.v. immunoglobulin (IVIg; 0.4 g/kg) was administered to prevent infectious complications. As other groups have reported that high-dose (2 g/kg) polyclonal immunoglobulin can control AHR (*35*), a contribution of the IVIg cannot be ruled out.

We have found that the combination of tacrolimus and MMF is highly effective in preventing a rebound in antibody synthesis after the initial removal of DSA by PPh. This observation in patients with refractory AHR has been confirmed in the treatment of chronic rejection associated with DSA, in which rescue with tacrolimus and MMF alone (without PPh) can effectively suppress anti-donor antibody production (Pascual M, unpublished results). Interestingly, MMF has been shown to inhibit Ab production by B cells in vitro and in vivo, and this property appears to be useful in the control of humoral responses in humans (*36*).

From these observations, a strict definition of AHR in renal transplantation can be proposed which would incorporate the following features. 1) Evidence of acute allograft dysfunction, typically steroid-insensitive rejection, requiring the addition of more intensive immunosuppression, such as antilymphocyte therapy, and often resistant to it. 2) The presence of widespread C4d deposits in peritubular capillaries (*10*). In addition, other pathological features such as neutrophils in peritubular capillaries and interstitium, neutrophilic tubulitis or glomerulitis, microthrombi in arterioles and glomeruli, arteritis with fibrinoid necrosis, thrombosis, and infarction are more prevalent in this kind of rejection (*5,6,8,9*) (Maiuyyedi et al., manuscript in preparation). 3) Demonstration of previously undetected DSA in the recipient serum at the time of rejection. Most frequently DSA are IgG anti-HLA class I, but IgG anti-class II or IgM DSA may also be associated with AHR. In some instances low titers of DSA, which were undetected at the time of transplant with routine cross-match techniques, become apparent in serum in the early days posttransplant. Rarely, DSA not reactive with HLA antigens on donor lymphocytes may cause a clinicopathological picture consistent with AHR (*24, 32–34*).

However, we suggest that from a clinical and therapeutic perspective, the presence of DSA in serum only or C4d deposits in PTC only in a patient with severe allograft dysfunction may justify an aggressive approach, because in these patients ongoing humoral mechanisms of rejection are likely. Combining DSA testing in serum and C4d staining of biopsy tissue provides a useful approach to diagnose AHR, a condition that often necessitates an intensive therapeutic rescue regimen. Currently at our institution, as soon as the diagnosis of AHR has been established (steroid-insensitive acute rejection, C4d+, DSA+), we initiate a therapeutic regimen that includes antilymphocyte therapy, tacrolimus-mycophenolate rescue and daily PPh for 5 consecutive days, followed by alternate day PPh if necessary for up to five additional procedures.

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Acute Humoral Rejection in Kidney Transplantation: II. Morphology, Immunopathology, and Pathologic Classification

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Abstract. The incidence of acute humoral rejection (AHR) in renal allograft biopsies has been difficult to determine because widely accepted diagnostic criteria have not been established. C4d deposition in peritubular capillaries (PTC) of renal allografts has been proposed as a useful marker for AHR. This study was designed to test the relative value of C4d staining, histology, and serology in the diagnosis of AHR. Of 232 consecutive kidney transplants performed at a single institution from July 1995 to July 1999, all patients (n = 67) who developed acute rejection within the first 3 mo and had a renal biopsy with available frozen tissue at acute rejection onset, as well as posttransplant sera within 30 d of the biopsy, were included in this study. Hematoxylin and eosin and periodic acid-Schiff stained sections were scored for glomerular, vascular, and tubulointerstitial pathology. C4d staining of cryostat sections was done by a sensitive three-layer immunofluorescence method. Donor-specific antibodies (DSA) were detected in posttransplant recipient sera using antihuman-globulin–enhanced T cell and B cell cytotoxicity assays and/or flow cytometry. Widespread C4d staining in PTC was present in 30% (20 of 67) of all acute rejection biopsies. The initial histologic diagnoses of the $C4d^+$ acute rejection cases were as follows: AHR only, 30%; acute cellular rejection (ACR) and AHR, 45%; ACR (CCTT types 1 or 2) alone, 15%; and acute tubular injury (ATI), 10%. The distinguishing morphologic features in $C4d^+$ *versus* $C4d^-$ acute rejection cases included the following: neutrophils in PTC, 65% *versus* 9%; neutrophilic glomerulitis, 55% *versus* 4%; neutrophilic tubulitis, 55% *versus* 9%; severe ATI, 75% *versus* 9%; and fibrinoid necrosis in glomeruli, 20% *versus* 0%, or arteries, 25% *versus* 0%; all $P < 0.01$. Mononuclear cell tubulitis was more common in the $C4d^-$ group (70% *versus* 100%; $P < 0.01$). No significant difference between $C4d^+$ and $C4d^-$ acute rejection was noted for endarteritis, 25% *versus* 32%; interstitial inflammation (mean % cortex), 27.2 ± 27% *versus* 38 ± 21%; interstitial hemorrhage, 25% *versus* 15%; or infarcts, 5% *versus* 2%. DSA were present in 90% (18 of 20) of the $C4d^+$ cases compared with 2% (1 of 47) in the $C4d^-$ acute rejection cases ($P < 0.001$). The pathology of the $C4d^+$ but DSA^- cases was not distinguishable from the $C4d^+$, DSA^+ cases. The $C4d^+$ DSA^- cases may be due to non-HLA antibodies or subthreshold levels of DSA. The sensitivity of C4d staining is 95% in the diagnosis of AHR compared with the donor-specific antibody test (90%). Overall, eight grafts were lost to acute rejection in the first year, of which 75% (6 of 8) had AHR. The 1-yr graft failure rate was 27% (4 of 15) for those AHR cases with only capillary neutrophils *versus* 40% (2 of 5) for those who also had fibrinoid necrosis of arteries. In comparison, the 1-yr graft failure rates were 3% and 7%, respectively, in ACR 1 (Banff/CCTT type 1) and ACR 2 (Banff/CCTT type 2) $C4d^-$ groups. A substantial fraction (30%) of biopsy-confirmed acute rejection episodes have a component of AHR as judged by C4d staining; most (90%), but not all, have detectable DSA. AHR may be overlooked in the presence of ACR or ATI by histology or negative serology, arguing for routine C4d staining of renal allograft biopsies. Because AHR has a distinct therapy and prognosis, we propose that it should be classified separately from ACR, with further sub-classification into AHR 1 (neutrophilic capillary involvement) and AHR 2 (arterial fibrinoid necrosis).

Acute rejection of renal allografts is considered primarily a T cell–mediated process (acute cellular rejection, ACR). The role of humoral mechanisms, although well recognized in the setting of hyperacute rejection, ABO-incompatible transplants, and xenograft rejection, has received less attention in the evaluation of acute allograft rejection. In fact, diagnostic criteria for acute humoral rejection (AHR) are not well established in the current classification systems for renal allograft rejection (2,3).

Nearly 20 yr after the observation by Jeannet *et al.* (4) that posttransplant, *de novo*, donor-specific antibodies are associated with a poor outcome, this area is attracting renewed attention. Circulating cytotoxic antidonor HLA class I antibod-

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ies can be detected in about 23 to 38% of patients with ACR (5,6). These rejection episodes typically have an aggressive clinical course. Feucht *et al.* (7,8) first proposed the use of C4d staining of peritubular capillaries (PTC) in the renal allograft biopsies to identify patients with severe cellular rejection and associated C4d with pretransplant panel reactive alloantibodies. Whether C4d was correlated with donor-specific antibodies or pathology in their studies was not established. C4d is a fragment of complement component C4 released during activation of the classical complement pathway by antigen-antibody complexes (9). Because C4d contains an internal thiolester bond, it binds covalently to tissue elements at the local site of activation and is potentially, therefore, a durable marker of antibody-mediated injury.

We recently reported a series of patients selected for circulating *de novo* donor-specific antibodies and biopsy features regarded as typical of AHR, including peritubular capillary/glomerular neutrophils with or without fibrinoid necrosis (10). In all these cases we demonstrated the conspicuous deposition of C4d in PTC of renal allograft biopsies and proposed that C4d is a specific *in situ* marker of antibody-mediated rejection. In that study, the possibility of AHR in the absence of typical morphologic features or positive HLA serology was raised but was not addressed.

Although morphologic features (6,10,11,12,13) may sometimes distinguish AHR from ACR, we suspected that the diagnosis might be missed if the typical features of AHR are focal or if cellular rejection is also present. We designed the current study to test the relative value of C4d staining (immunofluorescence microscopy) as well as histology and serology (circulating *de novo* donor-specific antibodies) in the diagnosis of AHR in all patients who underwent renal allograft biopsies for suspected acute rejection. The serology and the clinical characteristics of these patients have been reported previously (14). In this report, the morphologic features of $C4d^+$ acute rejection are compared with $C4d^-$ acute rejection, and diagnostic criteria for classification of acute rejection in renal allografts are proposed.

Materials and Methods

Patients

Of 232 consecutive renal transplants performed at the Massachusetts General Hospital over a 4-yr period (July 1995 to July 1999), 81 patients suffered at least one episode of clinical acute rejection in the first 3 mo after transplantation. Clinical acute rejection was suspected in cases with acute allograft dysfunction with normal or subtherapeutic levels of cyclosporine and normal findings by renal ultrasound. Of these, all patients who had undergone a renal biopsy at onset of acute allograft dysfunction with: (*1*) available frozen tissue for immunofluorescence microscopy and (*2*) available posttransplant serum samples within 30 d of the biopsy for donor specific antibody testing were included in this study ($n = 67$). The remaining cases (14 of 81) with clinical acute rejection were excluded because nine patients had not undergone a renal allograft biopsy, four patients lacked frozen tissue for C4d staining, and one patient lacked serum to test for donor specific antibodies.

Clinical data were gathered from our patient and pathology databases and review of medical records. For outcome analysis and clinicopathologic correlation, all patient data up to July 31, 2000, were included. Serum creatinines were observed and compared between the groups at the time of biopsy, at 6 mo, and 1 yr after biopsy. The graft failure rate at 1 yr was calculated. Graft survival was compared between the $C4d^+$ and $C4d^-$ groups.

Histology

Renal allograft biopsies were processed for routine light microscopy. Hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) stained sections of the renal allograft biopsies were examined for (*1*) ACR, using standard diagnostic criteria as outlined by the Cooperative Clinical Trials in Transplantation (CCTT) classification system and incorporated into the revised Banff criteria (2,3) and (*2*) AHR. Morphologic criteria for AHR are not established; therefore, we used provisional criteria of neutrophils in PTC and fibrinoid necrosis, as have been previously observed in AHR (10,11,12). However, we then surveyed a broad range of histologic features that might correlate with independent evidence of AHR (C4d and donor-specific antibodies) in all cases. Coded samples from the initial renal allograft biopsy with available frozen tissue that correlated with acute allograft dysfunction were scored by one of the authors (SM). The criteria used to score the cases included neutrophil counts in PTC per high power field (hpf) in ten ×40 fields (field diameter, 0.55 mm) using an Olympus BX41 microscope (Tokyo, Japan). A case was considered positive for the presence of PTC neutrophils when, on average, ≥2 neutrophils per hpf in PTC were identified in 10 consecutive ×40 hpf. Similarly, a case was considered positive for neutrophilic glomerulitis when, on average, ≥1 neutrophil per glomerulus was identified. The presence of one tubule with intraepithelial neutrophils in 10 high power fields was sufficient for neutrophilic tubulitis. The presence or absence of fibrinoid necrosis and thrombi in glomeruli and arteries, endarteritis, mononuclear cell tubulitis, tubular injury, interstitial infiltrates, interstitial hemorrhage, and cortical infarction were also recorded.

Immunofluorescence

Unfixed frozen sections of the renal allograft biopsies were stained for C4d with a sensitive, three-step immunofluorescence technique developed in our laboratory and described previously (10). Briefly, 4-μm-thick frozen sections were prewashed in phosphate-buffered saline (pH 7.2) and then incubated in 100 mg/ml Avidin D (Vector Labs, Burlingame, CA) to block endogenous biotin. Sections were washed, and excess avidin was bound by adding 10 mg/ml d-biotin (Sigma Chemical, St. Louis, MO). Monoclonal antibody to C4d (clone 10–11; Biogenesis, Sandown, NH) was applied for 30 min. Sections were washed and incubated sequentially with biotinylated horse anti-mouse IgG (1:100) (Vector Labs), and then after washing, with FITC-streptavidin (1:50) (Biomeda, Foster City, CA), each for 30 min. Sections were examined by an Olympus BX60 (Tokyo, Japan) epiillumination fluorescence microscope at ×40 and scored for C4d staining in PTC without knowledge of the clinical or pathologic diagnoses. Staining for C4d was considered positive ($C4d^+$) when the PTC were diffusely (all high-power fields) and brightly stained (excluding areas of necrosis). Focal areas and no staining of PTC for C4d were considered C4d-negative ($C4d^-$). All subsequent biopsies ($n = 11$) on these patients performed up to July 31, 2000, were evaluated for changes in the pattern of C4d staining. Routine immunofluorescence studies were also done by using polyclonal antibodies to IgG, IgM, IgA, C3, fibrin, and albumin by standard methods (15), and staining patterns in PTC were observed; these were negative in PTC of nearly all the acute rejection cases; therefore, they are not further discussed.

Screening for Circulating Donor-Specific Antibodies

Circulating donor-specific antibodies posttransplant were identified by using T and B cell cytotoxicity assays and/or flow cytometry as described previously in detail (14,16). Pretransplant donor-specific antibodies were tested in all patients by antihuman globulin cytotoxicity assay.

Statistical Analyses

Data are expressed as mean ± SD. Statistical significance was assessed by ANOVA for continuous data variables and Fischer's exact test or χ^2 test for nominal data variables. The results were considered significant with $P < 0.05$. Graft survival was estimated by the Kaplan Meier method. All statistical analyses were performed with StatView 4.5 for Windows (Abacus Concepts Inc. Berkley, CA).

Results

By immunofluorescence microscopy, 30% (20 of 67) of all acute rejection renal allograft biopsy samples revealed diffuse and bright C4d deposition in PTC (Figure 1). The initial morphologic diagnoses of the acute rejection cases in the C4d$^+$ and C4d$^-$ groups based on the histology, without knowledge of C4d staining or serology results are shown in Table 1.

The histologic features (Table 2) that distinguished C4d$^+$ acute rejection cases from C4d$^-$ acute rejection included by objective counts neutrophils in PTC and glomerular capillaries, neutrophilic tubulitis, and fibrinoid necrosis of arteries and glomeruli (Figures 1 and 2). The overall mean of the mean number of neutrophils per hpf in PTC was higher in the C4d$^+$ cases compared with the C4d$^-$ cases (4.1 ± 5.2 *versus* 1.0 ± 2.6; $P < 0.001$). More than one neutrophil per peritubular capillary was commonly seen in the C4d$^+$ cases. The overall mean of the mean number of neutrophils per glomerulus was higher in the C4d$^+$ cases compared with the C4d$^-$ cases (1.2 ± 1.0 *versus* 0.2 ± 0.4; $P < 0.001$). Usual mononuclear cell tubulitis was more common in the C4d$^-$ acute rejection cases. No statistically significant difference existed between the C4d$^+$ and C4d$^-$ acute rejection groups for endarteritis, interstitial hemorrhage, cortical infarcts, or the percent cortical involvement by an interstitial mononuclear cell infiltrate (Table 2).

C4d staining in PTC strongly correlated with the presence of posttransplant donor-specific antibodies: 90% (18 of 20) of the C4d$^+$ acute rejection cases had circulating donor-specific antibodies compared with 2% in C4d$^-$ acute rejection cases ($P < 0.001$) (Table 3). In 10% of the C4d$^+$ acute rejection cases, donor-specific antibodies were not detected, but the pathology was similar to other C4d$^+$, donor-specific antibody–positive, acute rejection cases, *e.g.*, abundant neutrophils in PTC and glomeruli. In the C4d$^-$ acute rejection group, 46 of 47 cases were negative for donor-specific antibodies; the remaining patient from this group had weak IgM donor-specific antibodies with focal, weak C4d staining of PTC. All patients had been tested pretransplantation for donor specific antibodies and were negative. Pretransplantation crossmatches were repeated retrospectively in the C4d$^+$ acute rejection cases; only two patients were shown to have weakly positive pretransplantation crossmatches, but all other patients remained negative as discussed in part I (14).

Sensitivity and specificity were calculated with either donor-specific antibodies or C4d as the criterion in diagnosing AHR. When serum donor-specific antibodies were used to define the diagnosis of AHR in these patients with acute rejection, C4d deposition in PTC by immunofluorescence microscopy achieved a sensitivity and specificity of 95% and 96%, respectively (Table 4). In comparison, the presence of neutrophils in PTC or glomeruli was less sensitive and specific for diagnosing AHR. Although the presence of arterial fibrinoid necrosis showed high sensitivity (100%) for diagnosing AHR, its absence did not exclude AHR (specificity, 75%). When C4d was used as the criterion for diagnosis of AHR, the sensitivity of donor-specific antibodies was 90% and the specificity was 98%.

Certain clinical features distinguished the C4d$^+$ and C4d$^-$ groups. The mean serum creatinines at biopsy were higher in the C4d$^+$ *versus* C4d$^-$ acute rejection cases ($P = 0.003$; Table 5). The 1-yr graft loss was 30% (6 of 20) in the C4d$^+$ acute rejection group, compared with 4% (2 of 45) in the C4d$^-$ acute rejection group ($P = 0.007$). The 1-yr graft failure rate (Table 5) was 40% (2 of 5) in those AHR cases that had arterial fibrinoid necrosis in the initial biopsy, compared with 27% (4 of 15) in the remaining AHR cases without arterial fibrinoid necrosis. In contrast, the 1-yr graft failure rate was 7% (1 of 15) and 3% (1 of 30) in ACR cases with and without endarteritis, respectively. The one patient who lost the graft in the ACR group without endarteritis had developed thrombotic microangiopathy secondary to calcineurin inhibitor toxicity subsequent to the rejection episode. In this series, no patient lost a graft due to rejection after the initial biopsy showed only ACR without endarteritis or a humoral component.

The outcome and pathology suggest classifying AHR into two categories (Table 6): those with involvement of PTC and glomerular capillaries by neutrophils (AHR type 1) and those with additional arterial fibrinoid necrosis (AHR type 2). C4d$^+$ cases with either combined AHR and ACR morphology or ACR-only morphology are best grouped with the AHR cases because their outcome correlates with the presence of C4d rather than an additional component of ACR.

The cumulative renal allograft survival was worse in the C4d$^+$ acute rejection group compared with the C4d$^-$ acute rejection group, with a trend for the worse prognosis in those C4d$^+$ AHR cases that had arterial fibrinoid necrosis (AHR 2) (Figure 3). However, the majority of the grafts in all the acute rejection groups that survived the first year after transplantation were stable and functioning at the end of a mean follow-up of 36.1 ± 15.3 mo after transplantation.

Follow-up biopsies in AHR cases ($n = 11$) showed that C4d presence and intensity correlated with persistence of circulating donor-specific antibodies. The time of disappearance of C4d staining after treatment was not systematically determined, because rebiopsies were done for graft dysfunction. Biopsies within 30 d of the initial C4d$^+$ biopsy showed persistent C4d deposition in PTC in 9 of 10 cases; one case was C4d$^-$ 17 d after the initial C4d$^+$ biopsy. Biopsies taken after

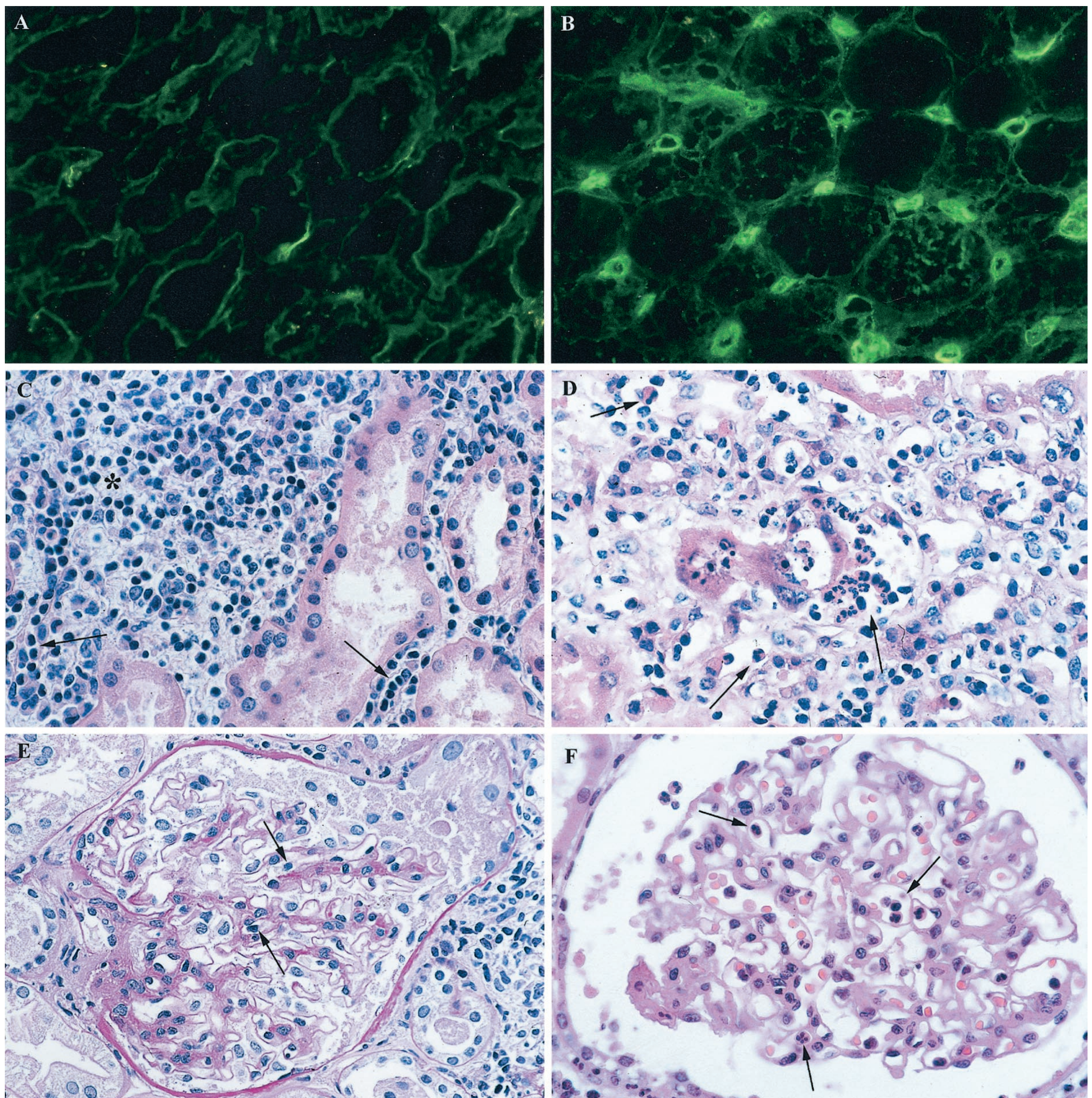

Figure 1. (A) Acute cellular rejection (ACR): no staining for C4d is seen in peritubular capillaries. (B) Acute humoral rejection (AHR): widespread and bright staining for C4d is present in the peritubular capillaries that are interspersed in between the silhouettes of tubules. (C) ACR: mononuclear cells are present in the interstitium (*) and in peritubular capillaries (arrows). (D) AHR: abundant neutrophils are present in dilated peritubular capillaries (arrows). (E) ACR: scattered mononuclear cells are present in glomerular capillaries (arrows). (F) AHR: neutrophils are present in glomerular capillaries (arrows). Staining: C4d-FITC in A and B; Hematoxylin and eosin (H&E) in C, D, and F; and periodic acid-Schiff (PAS) in E. Magnifications: ×400 in A through D; ×450 in E and F.

30 d were negative in 3 of 4 cases (at days 50, 80, 390); one biopsy at 180 d was $C4d^+$ and had chronic rejection. We conclude that C4d is not permanent and disappears within 2 to 3 wk of the loss of donor-specific antibodies. Persistence (or recurrence) of C4d staining in PTC is associated with active, chronic rejection.

Discussion

In this study, we demonstrate that a substantial fraction (30%) of all biopsy-confirmed acute renal allograft rejection episodes have a component of AHR as judged by C4d deposition in PTC. Circulating donor-specific antibodies and characteristic histopathologic morphology are also usually present

Table 1. Histologic diagnoses of C4d$^+$ and C4d$^-$ acute rejection[a]

	C4d$^+$ (n = 20)	C4d$^-$ (n = 47)
AHR	30%	0
AHR and ACR	45%	9%[b]
ACR 1	10%[c]	68%[d]
ACR 2	5%	23%
ATI	10%	0

[a] AHR, acute humoral rejection; ACR, acute cellular rejection; ACR 1, tubulointerstitial, type 1; ACR 2, with endarteritis; ATI, acute tubular injury.
[b] Patients had ACR 2 and abundant neutrophils in peritubular capillaries (≥2 neutrophils per hpf).
[c] 50% (1 of 2) were "suspicious for ACR" by Banff criteria.
[d] 34% (11 of 32) were "suspicious for ACR" by Banff criteria.

Table 2. Morphology: C4d$^+$ vs C4d$^-$ acute rejection[a]

	C4d$^+$ (n = 20)	C4d$^-$ (n = 47)	P
Neutrophils in			
PTC[b]	65	9	<0.0001
glomeruli[c]	55	4	<0.001
tubules[d]	55	9	0.0001
Fibrinoid necrosis			
arteries[d]	25	0[f]	0.001
glomeruli[d]	20	0	<0.006
Fibrin thrombi			
arteries[d]	0	0	
glomeruli[d]	20	0	<0.006
Endarteritis[d]	25	32	0.7 (ns)
MNC tubulitis[d]	70	100	<0.001
Acute tubular injury[d]	75	9	<0.0001
focal necrotic tubules[d]	40	2	0.0002
Interstitial inflammation[e]	27.2 ± 27	38 ± 21	0.09 (ns)
hemorrhage[d]	25	15	0.4 (ns)
Cortical infarction[d]	5	2	0.5 (ns)

[a] PTC, peritubular capillaries; MNC, mononuclear cells.
[b] Percent (%) cases with an average of ≥2 neutrophils per high-power field in peritubular capillaries in ten ×40 (field diameter, 0.55 mm) fields.
[c] Percent (%) cases with an average of ≥1 neutrophil per glomerulus.
[d] Percent (%) cases.
[e] Mean percent cortex involved.
[f] Fibrinoid necrosis in an arteriole only was seen in one case.

in these patients. However, we also demonstrated that 25% of the AHR cases would not have been recognized without the C4d stain; 15% showed only ACR morphology and 10% acute tubular injury. C4d staining of renal allografts is therefore valuable in the recognition of AHR, especially when features of ACR or only acute tubular injury are present.

The histologic features associated with C4d deposition in PTC in our cases are similar to those reported in patients with acute rejection and circulating class I cytotoxic anti-donor HLA antibodies (11). The differences include more frequent PTC neutrophils in our series (65% *versus* 46%) and fewer cases of cortical infarction and fibrin thrombi than were described in the report by Trpkov *et al* (11). Although a similar number of humoral rejection cases have arterial fibrinoid necrosis in the two series (25% and 24%), more frequent endarteritis was described in their class I antibody–negative acute rejection cases (75%) than in our series of C4d$^-$ acute rejection cases (32%). The increased frequency of AHR in our series (30% *versus* 20%) is due in part to our inclusion of cases with donor-specific antibodies reactive to class II antigens as well as the donor-specific antibody–negative cases.

In our patients, when the antigen could be identified, the alloantibodies reacted with donor HLA class I in 60%, HLA class II in 30%, and combined HLA class I and II in 10%, as previously reported (14). This is consistent with published reports indicating that circulating antibodies in AHR are most commonly to donor HLA class I antigens (5) but that some have only reactivity against HLA class II antigens (17,18). Two C4d$^+$ AHR cases (10%) in our series did not have detectable donor-specific antibodies to lymphocytes; however, their biopsies were pathologically similar to the donor-specific antibody positive, C4d$^+$, AHR cases with abundant neutrophils in PTC and glomeruli. Non-HLA antibodies, antiendothelial antibodies that have been described for example (19), might explain the C4d$^+$, donor-specific antibody–negative cases. No statistically significant correlation between any specific pathologic feature (*e.g.*, fibrinoid necrosis) and HLA reactivity of the donor-specific antibodies was detected; however, such a possibility cannot be excluded. C4d deposition in PTC of renal allograft biopsies may, therefore, have diagnostic value in the absence of demonstrable donor-specific antibodies.

In our series, two patients with widespread C4d staining of PTC showed predominantly acute tubular injury on initial biopsy. Both had circulating donor-specific antibodies. Later biopsies performed in one of these cases showed typical morphologic features of AHR with abundant neutrophils in PTC and glomeruli as well as fibrinoid necrosis. In prospective studies, we have found no (10) or focal (2) staining for C4 d in PTC in 12 cases of acute tubular necrosis (0 of 12; Mauiyyedi S, *et al.*, unpublished data); delayed graft function was present in 58% of these cases. Thus, our data differ substantially from that of Feucht *et al.* (20), who found C4d deposition in PTC of 60% of their ATN cases and 60% of recurrent glomerulonephritis. Their series also had a higher frequency of C4d$^+$ acute rejection. We believe that the differences are due in part to the inclusion of cases with "focal" C4d staining of PTC in their "positive for humoral rejection" group. Our criteria for "positive for C4d" interpretation requires widespread and bright staining of PTC, which avoids this potential pitfall. Other technical factors in the performance of the test may also be contributory (such as fixation of tissue, variable intensity of staining in immunohistochemistry, and antibody titer).

The present data permit a better definition of AHR as a pathologic entity that can be incorporated into the diagnostic

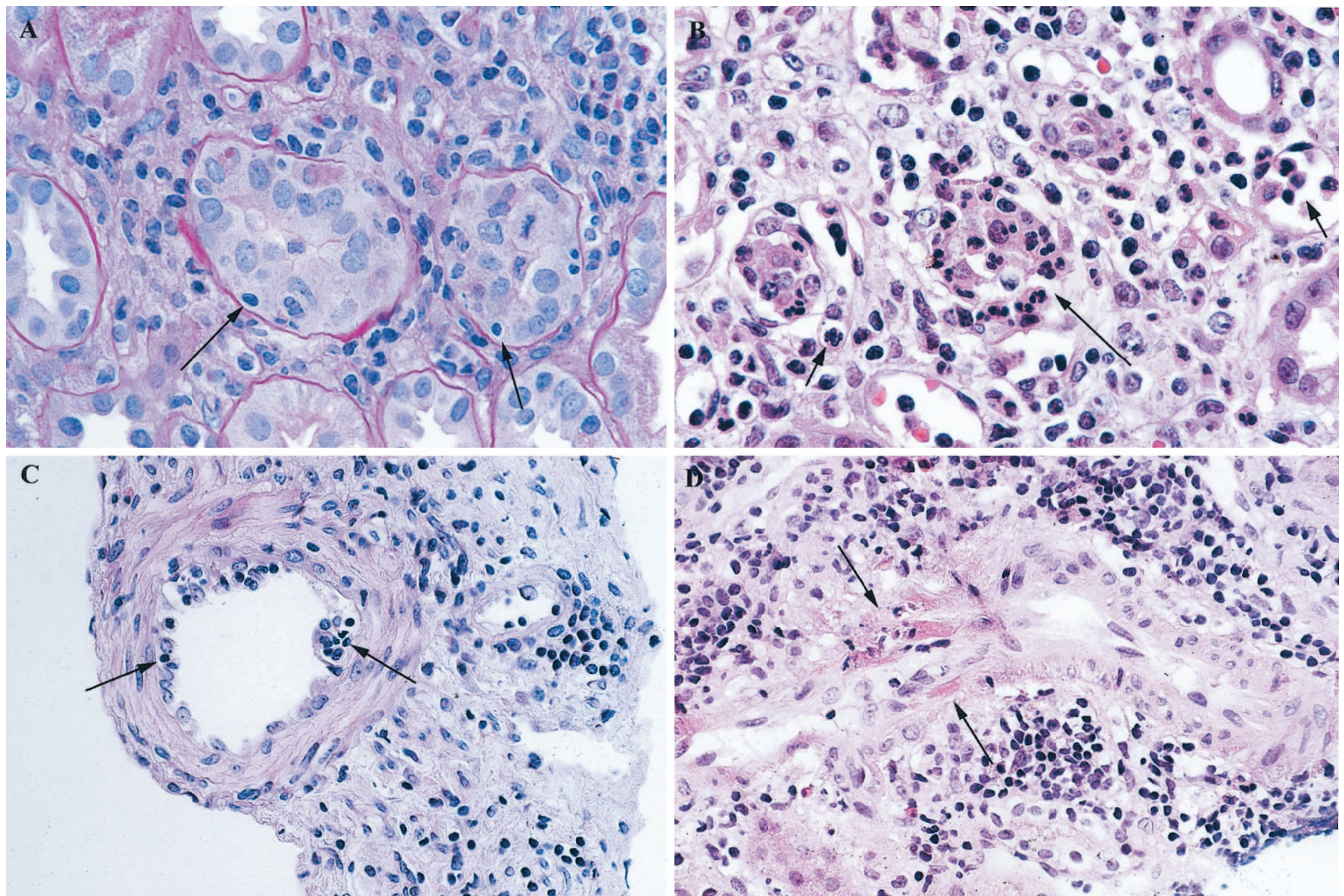

Figure 2. (A) ACR: mononuclear cell tubulitis with intraepithelial lymphocytes in tubules (arrows). (B) AHR: neutrophilic tubulitis with neutrophils invading tubular epithelium (long arrow) and neutrophils in peritubular capillaries (short arrows). (C) ACR: endarteritis with mononuclear cells underneath the endothelium of an artery (arrows). (D) AHR, fibrinoid necrosis of arterial wall (arrows). Staining: PAS in A; H&E in B through D. Magnifications: ×450 in A and B; ×400 in C and D.

Table 3. C4d deposition correlates with donor-specific antibodies in acute rejection

Acute Rejection	*n*	Donor-Specific Antibodies
$C4d^+$	20	18 (90%)
$C4d^-$	47	1 (2%)[a,b]

[a] Weak IgM anti-donor antibodies.
[b] $P < 0.0001$.

Table 4. Sensitivity and specificity of C4d in PTC, histology, and donor-specific antibodies in the diagnosis of acute humoral rejection[a]

	Sensitivity	Specificity
Serum donor-specific antibodies as criterion for AHR		
C4d in PTC	95%	96%
neutrophils in PTC	76%	86%
neutrophils in glomeruli	47%	91%
arterial fibrinoid necrosis	100%	75%
C4d in PTC as criterion for AHR		
Donor-specific antibodies in serum	90%	98%

[a] PTC, peritubular capillaries.

schema of acute rejection. Three types of acute rejection are currently recognized by the CCTT and the revised Banff classifications, based entirely on histologic criteria: type 1 is characterized by a mononuclear cell interstitial infiltrate and tubulitis, type 2 with endarteritis, and type 3 with fibrinoid arterial necrosis (2,3). Types 1 and 2 are believed to be T cell–mediated on the basis of immunophenotype, experimental studies, and response to anti-T cell agents, *e.g.* OKT3 (21,22,23). Animal studies, including T cell transfer experiments, and the ability of B cell knockout mice to develop typical endarteritis in allografts support this hypothesis (22). In contrast, fibrinoid necrosis of arteries, which is highly associated with circulating anti-donor antibodies in this and previous studies (11), typically does not respond to anti-T cell therapy and has a substantially worse prognosis (21). In the past this was the only form of AHR widely recognized (24). In addition to this category, we now delineate another morphologic type of acute rejection that is humorally mediated and is centered on graft peritubular and glomerular capillaries.

Table 5. Clinical follow-up after biopsy diagnosis of acute rejection[a]

Variable	$C4d^-$		$C4d^+$	
	Without Endarteritis	With Endarteritis	No Arterial Fibrinoid Necrosis	With Arterial Fibrinoid Necrosis
n	32[b]	15	15	5
Post Tx day of bx	14 ± 9.3	15 ± 14.2	12.6 ± 7.4	20.2 ± 16.3
Bx Cr (mg/dl)	3.0 ± 1.0	3.9 ± 3.5	7.0 ± 4.5	5.8 ± 3.3
6m Cr[c] (mg/dl)	1.6 ± 0.6	1.2 ± 0.3	1.8 ± 0.6	1.5 ± 0.3
12m Cr[c] (mg/dl)	1.4 ± 0.4	1.2 ± 0.2	1.5 ± 0.4	1.6 ± 0.4
GF at 1 yr (% cases)	3	7	27	40

[a] $C4d^-$, acute cellular rejection; $C4d^+$, acute humoral rejection; Tx, renal transplant; Bx, at renal allograft biopsy; Cr, mean creatinine; 6m, at 6 months after biopsy; 12m, at 12 months after biopsy; GF, graft failure at 1 year after transplantation.
[b] One patient died with functioning graft, and one patient was lost to follow-up <1 mo after renal transplantation.
[c] Excluding failed grafts.

Because AHR has a distinctive pathogenetic mechanism (antibody/complement), comprises about 30% of the acute rejection biopsies, and has a substantially worse prognosis, we believe that AHR deserves to be classified separately. Our observations suggest that the diagnostic categories can be grouped by pathogenesis and prognosis. The proposed classification (Table 6) retains the morphologic criteria used by the revised Banff/CCTT for ACR types 1 and 2 and moves the former type 3 acute rejection to the more appropriate and specific AHR group. Thus, two forms of AHR can be recognized histologically: type 1 AHR with capillary inflammation, *i.e.*, neutrophils in peritubular and glomerular capillaries, and type 2 AHR with arterial fibrinoid necrosis. The proposed classification adds immunophenotypic criteria to the above, with ACR being $C4d^-$ and AHR being $C4d^+$. One might propose a separate category for $C4d^+$, donor-specific antibody–positive cases with acute tubular necrosis, but in our experience to date, this has been a transient precursor to typical AHR, and we include these in type 1 AHR. The frequency and outcome of the biopsies so classified in this study are given in Table 6. The presence of C4d in PTC clearly alters the prognosis of what may otherwise appear to be ordinary ACR. We believe therefore that the subset of the $C4d^+$ acute rejection biopsies that show morphologic features of both ACR as well as AHR should be classified as AHR because their prognosis resembles "pure" AHR rather than "pure" ACR.

The criteria for AHR we proposed previously were acute graft dysfunction, C4d in PTC, and circulating donor-specific antibodies (14). These criteria identified 90% of the AHR cases but failed in 10%, as discussed above. We did not use histologic criteria then because no one feature could be ascribed to AHR consistently. However, for a purely pathologic classification, relevant morphologic features from the renal biopsy are essential even if limited. We now propose that combining histologic and immunopathologic data gives the most sensitive and specific diagnosis of AHR according to the pathologic criteria in Table 7. Because of the confusing data in the literature that C4d deposition (at least focally) can occur in ischemic injury (20), we recommend that, for now at least, the definitive diagnosis of AHR requires the demonstration of circulating donor-specific antibodies, even though this is not as sensitive as C4d in our series. If only two of the three criteria are met, then the diagnosis would be considered "suspicious for AHR." The threshold number of neutrophils needed to diagnose AHR on the basis of morphology has not been analyzed in detail. For the purposes of this article and the diagnostic criteria, we used an average of ≥2 neutrophils per high-power field in PTC in 10 consecutive high-power fields and an average of ≥1 neutrophil per glomerulus to diagnose AHR. It is, however, clear that, even though on average the mean neutrophils per hpf in PTC is increased in AHR, there is some overlap. Fibrinoid necrosis should be in an artery as an indicator of AHR, because arteriolar fibrinoid necrosis can be seen in other processes such as thrombotic microangiopathy and severe hypertension. The histologic criteria of acute in-

Table 6. Classification, frequency, and outcome of acute renal allograft rejection

Classification	*n*	Frequency[a]	Graft Loss at 1 yr
Acute cellular rejection[b]			
type 1 (tubulointerstitial)	32	48%	3% (1 of 30)[c]
type 2 (endarteritis)	15	22%	7% (1 of 15)
Acute humoral rejection[d]			
type 1 (capillary)[e]	15	22%	27% (4 of 15)
type 2 (arterial fibrinoid necrosis)	5	7%	40% (2 of 5)

[a] Frequency in patients with biopsies of first rejection episode. In the present series, overall 31% (72 of 232) of patients had an episode of acute rejection diagnosed on biopsy.
[b] C4d negative in peritubular capillaries.
[c] One patient died with functioning graft, and one patient was lost to follow up <1 mo after renal transplantation.
[d] Includes patients with combined humoral and cellular rejection.
[e] Includes two patients whose initial biopsy showed acute tubular injury.

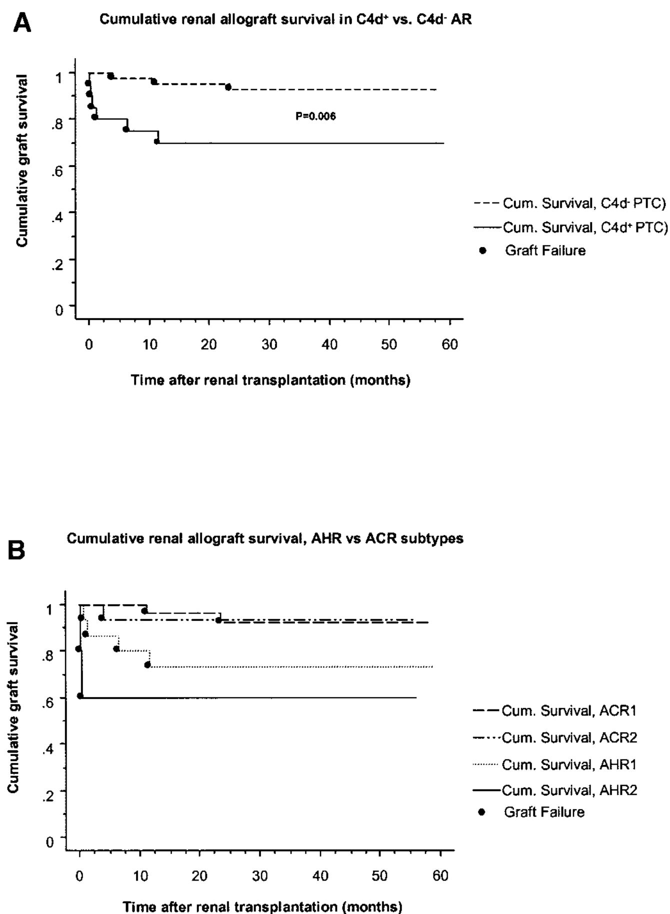

Figure 3. Cumulative renal allograft survival estimated in C4d$^+$ and C4d$^-$ acute rejection groups diagnosed within 3 mo of transplantation (Kaplan Meier method). (A) Worse renal allograft survival in the C4d$^+$ group compared with C4d$^-$ group (●, graft failure at mo after transplantation; $P = 0.006$). (B) Cumulative renal allograft survival of AHR and ACR subtypes reveal a trend for the worse survival in the AHR 2 group (●, graft failure at mo after transplantation; ACR *versus* AHR, $P = 0.006$; ACR 1 *versus* ACR 2, $P = 0.9$; AHR 1 *versus* AHR 2, $P = 0.4$).

flammation or injury separate acute from chronic humoral rejection because the latter lacks these features (25).

Several special caveats of C4d staining should be emphasized. First, only PTC staining should be considered, because C4d in normal kidneys can always be found in glomeruli and often in arterial intima and the tubular basement membranes. In contrast, PTC uniformly lack C4d. Second, infarcted tissue can be negative for C4d despite positive staining for C4d in viable areas. Third, PTC neutrophils or arterial fibrinoid necrosis can rarely occur with absent or only focal staining of PTC for C4d; such cases should probably be classified as suspicious for AHR. For example, weak C4d staining was identified in one of our cases that had PTC neutrophils in the initial biopsy and arterial fibrinoid necrosis in a subsequent biopsy, with weak IgM donor-specific antibodies in serum of undetermined specificity. The nature of such findings remains unknown, but it may represent AHR mediated by antibodies to antigens predominantly expressed in arteries. The presence of PTC neutrophils alone as a diagnostic criteria will lead to overcalling AHR, as seen in 9% of our C4d$^-$ ACR cases.

Table 7. Pathologic criteria for acute humoral rejection[a]

1. C4d deposition in peritubular capillaries[b]
2. At least one of the following:[c]
   a. Neutrophils in peritubular capillaries
   b. Arterial fibrinoid necrosis
   c. Acute tubular injury
3. Circulating donor specific antibodies

[a] Cases that also meet the criteria of type 1 or 2 acute cellular rejection (Banff/CCTT) are considered to have both processes.

[b] Bright and diffusely positive staining for C4d in peritubular capillaries.

[c] Neutrophils in peritubular capillaries: on average ≥2 neutrophils per high power field in peritubular capillaries in 10 consecutive ×40 (field diameter, 0.55 mm) fields; fibrinoid necrosis in an artery: larger than an arteriole; acute tubular injury: loss of brush borders, flattened epithelium, apoptosis.

If only two of the three numbered criteria are present, the term "suspicious for AHR" is recommended (for example, when donor specific antibodies are not tested).

In our series of patients, the overall graft loss at 1 yr is 4% for ACR and 30% for AHR. These data are quite compatible with that of Halloran *et al.* (6), who reported 3.9% graft loss after a rejection episode without anti-class I antibodies and 38.5% after rejection associated with class I antibodies. It is notable that 75% (6 of 8) of the overall 1-yr graft losses in our patients are in the AHR group. Renal allograft function after successful treatment of AHR was similar to that observed in the ACR cases, confirming the observation of Halloran's group that those with rejection associated with anti-class I antibodies who recover have a similar prognosis as those without antibodies (6). The relatively better 1-yr graft survival rate (70%) in our AHR group, compared with others that have reported poor graft survival rates of about 16 to 50% (11,26), may be due in part to our treatment approach with plasmapheresis, tacrolimus, and mycophenolate mofetil (13,14). Chronic rejection is associated with donor-specific antibodies, and we have detected C4d and donor-specific antibodies in patients with morphologic evidence of chronic rejection (25). Whether AHR promotes chronic rejection is unknown. Further studies are warranted to answer this question.

In summary, the present pathologic data support the recognition of a distinct and common type of acute renal allograft rejection mediated by specific anti-donor antibodies that react with graft endothelium leading to the deposition of complement, notably C4d. This form of rejection, termed "acute humoral rejection," has a typical but variable morphology and a distinctly worse prognosis compared with T cell–mediated rejection. Incorporation of AHR as a diagnostic category, including its two variants (capillary and arterial), should be important in clinical management and the analysis of clinical trials.

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Acute Humoral Rejection in Kidney Transplantation

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Historical Background

Initial Experience—1960s-1970s

The detrimental effect of preformed donor specific antibodies (DSA) became apparent in the early clinical trials of renal transplantation. Several observations emphasized that, in the presence of pre-existing DSA ("positive crossmatch" at the time of renal transplantation), destruction of grafted kidneys occurred almost inevitably within minutes or hours following transplantation.[1-3] This condition was termed "hyperacute" allograft rejection to emphasize the rapidity of the rejection process. Histopathologic examination of the failed allografts revealed intense interstitial neutrophilic infiltrate and fibrin thrombi in capillaries associated in some cases with fibrinoid necrosis of small arteries.[1,2] The absence of a mononuclear cell infiltrate suggested that hyperacute rejection resulted from a different pathogenesis than the "classic" acute cellular rejection. It became clear then that either preformed alloantibodies to antigens of the major histocompatibility complex (MHC) or isohemagglutinins directed against major blood group antigens could result in "hyperacute rejection".[1-4] This experience encouraged the requirement for demonstration of a pretransplant "negative" crossmatch and, in most instances, ABO compatibility for successful kidney transplantation.[3,4]

In 1970, M. Jeannet et al, of our Unit, reported that kidney transplant recipients who had negative crossmatches at the time of transplantation but developed DSA in the early post-transplant period also suffered severe rejection and allograft injury.[5] Twelve of 16 patients with de novo DSA against their grafts experienced poor clinical outcomes, compared to only 2 of 12 recipients in whom antibody responses to the graft were not demonstrated. Additionally, a correlation was found between the presence of DSA and histological lesions of severe vascular damage, i.e., obliterative vascular lesions. These initial observations indicated that, in addition to cellular immune responses, de novo production of specific antibodies against a donor organ can lead to severe graft injury and/or graft destruction (acute humoral rejection, AHR). Thus, it was predicted that control of humoral responses with immunosuppressive agents would be a necessary goal in renal transplantation.

Although, the mechanisms by which humoral responses were associated with graft destruction remained unclear, initial hypotheses suggested that these antibodies might bind to endothelial cell antigens and activate the complement system, resulting in the attraction of granulocytes and platelets with associated tissue damage.[1,5] Consistent with this interpretation was the evidence that complement C2 was consumed from the serum during acute transplant rejection.[6]

Renewed Interest in Humoral Responses—the 1990s

Despite this earlier period of excitement and interest, the pathogenic effect of DSA and complement did not receive as much attention for several years, as most efforts were directed towards studying the role of T cells in acute allograft rejection.[7-9] A renewed focus upon the importance of DSA post-transplant was presented by P. Halloran et al in the early 1990s, who established that acute rejection associated with the development of de novo DSA is a clinico-pathologic entity carrying a poor prognosis.[10,11] In their initial communication, the authors described the clinical course of seven patients with "pure" anti-HLA class I antibody-mediated injury. Included were four recipients with de novo DSA post-transplantation and three recipients with preformed DSA, which had not been detected prior to transplantation. All seven patients with serological detection of anti-HLA class I antibodies had rapid deterioration of graft function, within the first week post-transplant, distinct pathologic features on allograft biopsies and an ominous outcome (5/7 lost their grafts).[10] In a subsequent report, they compared 13 patients with de novo anti-HLA class I DSA to 51 consecutive renal transplant recipients with no DSA after transplantation. These two groups were differentiated by rejection incidence (100% vs. 41%), clinical picture of the dysfunction (early onset, oliguria, higher creatinine peak and need of dialysis), pathologic features (more vascular damage) and outcome (5/13 vs 2/51 grafts lost).[11] The same group analyzed in detail the pathologic features characterizing "antibody-mediated rejection" based on the Banff classification from 1993.[12,13] Biopsies from 24 patients with de novo DSA at the time of rejection demonstrated more severe vascular injury (endothelial proliferation, thrombi, fibrinoid necrosis or infarction) and glomerulitis, but less frequent tubulitis than observed in biopsies from 20 patients with acute rejection but without de novo DSA. Importantly, the presence of neutrophils in peritubular capillaries was significantly more prevalent ($p<0.003$) in the group with de novo DSA. Despite the presumed role of intragraft humoral mechanisms of rejection in the recipients with DSA, no significant differences in immunofluorescence detection of IgM, IgG, C3 or fibrinogen deposits were observed in the specimens from these two groups.

At our institution, we recently observed a series of renal allograft recipients with AHR. We confirmed the previous pathological features and found that peritubular staining for the complement fraction C4d, a split product of C4 of the classical pathway, is highly specific and reliable in the diagnosis of acute humoral rejection (AHR).[14] In our study, all renal biopsies (n=16) from ten

patients with AHR showed prominent and diffuse C4d deposits in peritubular capillaries, localized in basement membranes and in the endothelium. In contrast, biopsies from 14 patients with histopathologic characteristics of acute cellular rejection revealed only trace or absence of C4d. These observations support the suggestion by H. Feucht et al in 1993 that C4d staining in biopsies from renal allografts with early dysfunction might be prognostic of allograft failure. They found that 18 of 43 grafts (42%) with diffuse C4d capillary staining were lost compared to only 4/42 (10%) with no C4d deposits in capillaries.[15] Feucht proposed that complement C4d deposition was probably evidence for an otherwise undetectable humoral immune reaction against graft endothelial cells. Later, the same group reported that 44 of 86 patients with early graft dysfunction showed anti-HLA class II reactivity pretransplantation and that finding correlated with capillary C4d staining in post-transplant biopsies; however, DSA detection after transplant was not reported.[16]

Definition

It is essential that "acute humoral rejection" or "alloantibody-mediated acute rejection" (interchangeable terms) be precisely defined with the characteristic features described above. Unfortunately many other terms, which may or may not be appropriate to refer to this type of rejection, have introduced some confusion into the field. Some reports have described severe acute rejection, both in kidney and other solid organ transplant recipients, as "acute vascular rejection".[16-19] However the term "acute vascular rejection" is not necessarily restricted to an antibody-mediated process, as it can also reflect a T cell-mediated pathology.[19-22] In particular endarteritis or endothelialitis, a form of "vascular rejection", can be exclusively due to cell-mediated immunity in the absence of DSA.[23] Other terms, such as "accelerated rejection" or "delayed hyperacute rejection" have also been used to refer to humoral rejection, but these denominations allude to the clinical presentation of AHR (see below), not the pathological process. Some early acute cellular rejection episodes can clinically present as "accelerated rejection" likely due to an anamnestic cellular response against donor HLA antigens, without humoral sensitization detectable before or after transplantation.

In order to better characterize and define AHR, we propose utilizing the following criteria for its diagnosis:[21,24]

1. Evidence of allograft dysfunction, typically steroid-resistant rejection requiring addition of more intensive immunosuppression, such as anti-lymphocyte therapy. Unfortunately AHR has often proved to be refractory to treatment with anti-lymphocyte therapy as well.[9-11,21,24,25]
2. Pathologic features of widespread C4d deposits in peritubular capillaries.[14] In addition, other features such as neutrophils in peritubular capillaries, glomerulitis, microthrombi in arterioles and glomeruli, arteritis with fibrinoid necrosis, thrombosis and infarction may be found.[12,14,19,20,22] In the revised Banff 97 classification, "Acute/active rejection, type III" (with transmural arteritis) is considered to be either a reflection of severe acute cellular rejection or of AHR.[22]
3. Demonstration of previously undetected DSA in the recipient serum at the time of rejection. Most frequently DSA are IgG anti-HLA class I or class II, although high titers of IgM DSA may also be associated with AHR. In some instances low titers of DSA, which were undetected at the time of transplant with routine crossmatch techniques, are demonstrated retrospectively only with more sensitive assays.[10] Rarely anti-endothelial antibodies, that is DSA not reactive with HLA antigens on donor lymphocytes, may cause a clinicopathologic picture consistent with AHR.[26-28]

We distinguish AHR from hyperacute rejection, as hyperacute rejection is associated with detectable levels of DSA in recipient's serum at the time of kidney transplantation, resulting in a very rapid graft loss (usually minutes or hours). However, although the timing and clinical presentation of the rejection may be different, the pathogenic mechanisms of hyperacute rejection and AHR may share similarities.

Clinical Presentation

Approximately 5-10% of renal allograft recipients treated with "cyclosporine-prednisone-azathioprine" as induction immunosuppression experience AHR.[24,25,29] We have recently reviewed our experience over a four-year period (1995-1999) and found a similar incidence of AHR after renal transplantation (ref. 24 and M.Crespo et al, manuscript in preparation). We and others have observed three distinct patterns of renal dysfunction in the renal allograft recipient with AHR.[9,21,24]

Delayed Graft Function (DGF)

Most often DGF is due to graft ischemic injury (resulting in ischemic acute tubular necrosis), that may require dialysis during the immediate post-transplant period. When DGF is ischemic in origin, spontaneous improvement in kidney function occurs within days or weeks, similar to the resolution of acute tubular necrosis in native kidneys.[30,31] However, DGF may be due to immunological factors as it has been observed that recipients of second or third transplants and highly sensitized patients are at increased risk for DGF.[32] As discussed earlier, complement C4d staining of allograft biopsies from recipients with DGF may be a very useful tool to identify DSA-mediated tissue injury; it may also be of prognostic value.[15,33] When DGF is due to AHR, intensive immunosuppressive therapy should be initiated (see below), since the risk of allograft loss in these patients is very high.[15,16] We and others hypothesize that DGF due to AHR is probably associated with low levels of DSA undetected by the pre-transplant crossmatch,[16] which become apparent in recipient's sera a few days post-transplant.

Early Severe Acute Rejection

The more common presentation of AHR is that of severe renal dysfunction with progressive oliguria or even anuria that develops during the first post-transplant week, following an initial period of good allograft function. This type of rejection is likely due to an anamnestic humoral response against donor antigens, as the generation of immunoglobulins from newly activated plasma cells should not be expected earlier than about six or seven days after primary antigenic stimulation. Elevated panel reactive antibodies (PRA) before or at the time of transplantation are frequently found in these recipients' sera, suggesting that an overall state of enhanced humoral alloreactivity is present.[11,21,34] Of note, high PRA pretransplant are not necessarily associated with a positive historical crossmatch against the current donor. Clinically, such rejection episodes are commonly resistant to intravenous methylprednisolone boluses and often resistant to anti-lymphocyte therapy. Typical histopathologic features are present in allograft biopsies (see above); in some instances, the presence of neutrophils in peritubular capillaries and interstitial edema can be the only features suggesting the diagnosis of AHR. A donor-specific crossmatch at the time of rejection demonstrating de novo production of DSA in the recipient's serum and a positive C4d staining in perritubular capillaries confirm the diagnosis of AHR.

"Classic" Acute Rejection

Acute rejection occurring after the first week post-transplantation is usually due to cellular mechanisms (acute cellular rejection), but rarely it can be due to humoral or mixed (cellular and humoral) mechanisms. It typically presents as acute renal dysfunction with a decrease in urine output and weight gain, often without other clinical findings, such as graft tenderness or fever. In mixed cases the renal biopsy demonstrates a mononuclear infiltrate with tubulitis and/or endothelialitis (cellular component) together with the presence of neutrophils in peritubular capillaries (humoral component). In our experience, C4d staining revealed by immunofluorescence has been the key marker implicating a humoral component in the rejection process. These episodes are also resistant to steroids and frequently to anti-lymphocyte therapy. Again, demonstration of de novo DSA in the recipient's serum confirms the diagnosis so that appropriate therapy can be initiated.

Donor Specific Antibodies: Detection and Pathogenic Role

Multiple techniques are currently available for the detection of DSA. There are a number of variations of the basic direct lymphocytotoxic techniques that detect complement-fixing antibodies (CDC or complement-dependent cytotoxicity).[35] These include washing the cells prior to addition of complement and/or prolonging the incubation times, both of which increase the sensitivity of the assays. Indirect techniques such as antiglobulin-augmented lymphocytotoxicity (AHG)[36,37] and flow cytometry[38] can also detect non-complement fixing, very low levels of anti-HLA antibodies or CDC negative-adsorption positive antibodies. Although flow cytometry has been reported to be more sensitive than AHG, these techniques are not yet well standardized and results vary among different laboratories.[39-41]

Detection of DSA Pretransplantation

The presence of DSA against HLA class I antigens pre-transplant, that is a positive crossmatch with T cells, precludes transplantation.[5] Several authors propose that a high titer positive crossmatch with B cells pretransplant (DSA against HLA class II) is deleterious when it is HLA specific and not due to auto-antibodies,[42] but B-cell crossmatches are not performed routinely for first transplants at many centers.[43] There is also evidence suggesting that IgM antibodies against donor HLA antigens in a pretransplant crossmatch may have a negative effect on kidney allograft survival.[44] However it is important to emphasize that IgM antibodies, especially those that show low affinity or react only with B cells, are most often auto-antibodies or other non-HLA directed antibodies and, therefore, not clinically relevant.[35]

DSA Post-transplantation

The detection of IgG DSA either anti-class I or anti-class II early after renal transplantation identifies a group of patients who present with AHR.[9-12,21,24] The crossmatch result, however, must be analyzed carefully, as non-HLA antibodies may not be clinically relevant. DSA against class II HLA can be associated with AHR, but they appear to be less frequent than class I antibodies.[21,24] In our experience only 3/19 patients with AHR showed a defined DSA specificity against class II donor antigens. However it is possible that additional patients had class II DSA masked by the class I DSA which were present. Interestingly, one of these 19 patients developed only a high titer of IgM DSA and this patient showed all the features of AHR, including C4d deposition in peritubular capillaries.

Determining panel reactive antibodies (PRA) after transplantation either by cytotoxicity, flow cytometry or ELISA may be useful to identify patients at risk for developing AHR, as usually the de novo appearance or incremental rise in PRA after transplantation is due to immune reactivity to the graft.[45-47] But as AHR frequently occurs in highly sensitized recipients (PRA>50%),[11,21,34] it is often difficult to define the specificity of newly formed antibodies superimposed on a high baseline pre-transplant PRA. Therefore, in the appropriate clinico-pathological setting a donor-specific-crossmatch should be performed. Of note, a negative PRA against both T and B cells (PRA=0%) renders unlikely the presence of DSA. When donor cells are not available, surrogate cells chosen to share mismatched HLA antigens with the donor can be very useful, especially for monitoring changes in titer of DSA and as a guide to therapy.[21] In such instances, pretransplant sera should always be tested with the surrogate cells to ensure that antibodies against other HLA antigens on the surrogate cell were not present. Performance of cytotoxic crossmatches or PRA after transplantation is difficult in the presence of anti-lymphocyte therapy (risk of false positive). In our center, we remove OKT3 when necessary using a sheep anti-mouse monoclonal antibody.[21,48] Samples from patients on other types of antilymphocyte therapy (mainly polyclonal antilymphocyte sera) are processed by flow cytometry, but it is necessary to ensure that the labeled anti-Ig secondary antibody used must not cross react with the species in which the anti-lymphocyte reagent was produced (from mouse, horse or rabbit).

The pathogenic role of DSA and complement has been demonstrated in animals. An early example of such studies can be found in the experiments of Winn et al who showed that test skin grafts surviving upon immunosuppressed recipients could be damaged by transfused specific antisera.[49,50] Eliminating complement from these recipients by various means completely blocked the otherwise impressive effects of antiserum transfer.[50] Similar findings have been reported following passive transfer of DSA in complement sufficient rats, while complement deficient rats were protected from hyperacute rejection.[51,52] These observations reemphasized the central role of the complement system after DSA fixation to the allograft. Some controversy remains in the clinical field as to the direct role of DSA, as many authors have reported a deleterious effect of DSA after transplant,[5,9-12,21,24,25,29,45,53] while others (using flow cytometry) have suggested that de novo DSA production may be an epiphenomenon occurring at the time of acute rejection.[18,34] TC Fuller et al hypothesized that a positive direct crossmatch pretransplant results in hyperacute rejection due to the presence of sufficient alloantibody populations required to fix complement. These authors suggested that DSA identified pretransplantation only by indirect techniques (flow or AHG) correlates with "acute accelerated rejection" post-transplantation, as these antibodies may exhibit an antibody-dependent NK cellular cytotoxic (ADCC) mechanism of damage.[37] This suggests that detection of DSA by different techniques may reflect different mechanisms of antibody damage (complement, ADCC). JC Scornik et al also proposed that the significance of DSA may depend on the degree of clonal B-cell expansion in the patient. When clonal expansion is minimal and DSA is detected only by flow cytometry with current sera or by CDC with historical sera, DSA probably does not herald a bad prognosis.[54] Therefore, in some instances detection of low titers of DSA by flow cytometry post-transplant may indicate minimal sensitization with insufficient clonal expansion to result in AHR. In addition, low levels of antibodies may not activate complement strongly enough to overcome the regulatory proteins expressed on the endothelial cell surface.[52] Finally other authors have suggested that response against DSA might depend on the intra- and interindividual different expression of HLA antigens in the graft.[55,56]

Prognosis

As noted above, AHR as a clinico-pathologic entity has been only recently characterized,[9-11,21,24] and, therefore, precise data regarding its prognosis are still accumulating. The reported one-year graft survival rate after an episode of AHR has been only in the range of 15 to 50%[12,25,29,45] despite intensive treatment with conventional immunosuppressive therapy. S. Martin et al reported a series of 55 transplant recipients in whom donor standard specific cytotoxic crossmatches were performed after transplantation: 35 patients of 47 with positive post-transplant crossmatches lost their grafts compared to only 2/8 with negative crossmatches.[45] In another study by P. Halloran et al, 5 of 13 grafts with antibody-mediated rejection were lost.[11] In the experience of P. Lobo et al, 13 of 17 renal transplant recipients with de novo DSA at the time of acute rejection lost their grafts within a three-month period.[25] Overall, it appears that the risk of allograft failure is particularly increased within 3 months of the rejection episode.[12,25,29] These data emphasize that the mechanisms of tissue injury associated with AHR are unique and that this process is much less responsive to the treatment than classic cell-mediated rejection. Interestingly, early AHR, if reversed successfully, does not necessarily predispose to chronic rejection.[5,9,29] P. Campbell et al reported that recipients with AHR, who did not lose their grafts within the first three months, had favorable long-term survival of the graft without chronic rejection.[29] Whether this observation indicates that adequate reversal of the AHR episode arrests the production of DSA, or whether in some cases an "accommodation phenomenon" takes place, remains to be studied. Whatever the precise explanation, these data underline the importance of a prompt diagnosis of AHR and the possibility of a favorable prognosis if effective therapy is administered.

Diagnosis and New Approaches to Therapy

As already mentioned, AHR should be suspected in the presence of clinically severe rejection, in allograft recipients with oliguria and rapidly deteriorating renal function not responding to standard therapy.[9-11,21,24] In addition, DGF in a highly sensitized patient or a recipient of a retransplant should raise the possibility of AHR. In these clinical settings a renal allograft biopsy is mandatory. If typical pathological features of AHR are present by light microscopy, in particular neutrophils in peritubular capillaries, the diagnosis should be confirmed by both staining for C4d and by performing a post-transplant crossmatch with donor T and B cells. A renal ultrasound helps to rule out other reasons for graft dysfunction, such as obstruction, and a renal nuclear scan or a duplex ultrasound reveals persistence of renal blood flow, although perfusion to the allograft can be diminished in AHR.

As mentioned above, the treatment of AHR represents a continuing challenge for the transplant team, since AHR is often resistant to conventional immunosuppressive protocols. Although the optimal therapy remains undefined, recent studies emphasize the need for a combined therapeutic approach.[21,25]

Removal or Suppression of Alloantibody

The role of alloantibody removal in the treatment of AHR remains controversial as most series are small and lack appropriate controls. The rationale for utilizing plasmapheresis or immunoabsorption to treat AHR is based upon efforts to remove alloantibodies prior to transplantation in order to prevent hyperacute rejection in highly sensitized patients.[57-61]

We have recently evaluated plasmapheresis combined with "tacrolimus-mycophenolate rescue" in the effort to improve the outcome of AHR.[21] In this study, the number and duration of plasmapheresis treatments was guided by response to therapy (improvement in renal function) and serum levels of DSA (see Fig. 1). Our current protocol includes five daily sessions of plasmapheresis, followed by five additional treatments on alternate days if necessary. At the end of plasmapheresis treatments, polyclonal immunoglobulin (0.4 g/Kg) is administered to prevent infectious complications. Others have used immunoadsorption with staphylococcal protein A in patients with AHR to achieve a rapid decrease in the titer of circulating immunoglobulins.[62] Here it is important to emphasize that removal of antibody needs to be combined with intense immunosuppression to prevent a rebound in immunoglobulin synthesis.[9,21]

Another interesting approach that has been evaluated recently to control AHR in renal and cardiac allograft recipients is the administration of high-dose (2 g/Kg) polyclonal immunoglobulin (Ig).[63] Intravenous Ig has well-known immunomodulatory properties, including reticuloendothelial blockade, inhibition of complement-mediated endothelial injury and a decrease in antibody synthesis.[63-66] Intravenous Ig may also block HLA specific antibodies through an anti-idiotypic mechanism.[63,64,67] The use of high-dose Ig is therefore an attractive potential addition to protocols designed to treat AHR. However the high cost and limited supply of Ig, and concerns regarding potential complications (mainly allergic reactions and transmission of infections), may limit its widespread use in organ transplantation.

Immunosuppression

Since the early 1990s, newer immunosuppressive agents, such as tacrolimus and mycophenolate mofetil (MMF), have become available for organ transplantation. Interestingly both drugs have been used successfully as "rescue" agents in severe allograft rejection in kidney transplantation.[68-71] As compared to azathioprine, MMF has the advantage that it can inhibit antibody production by B cells in vitro and in vivo.[72,73] This property suggests that MMF should have superior efficacy in reversing AHR. In the past cyclophosphamide has been used in highly sensitized patients with variable efficacy.[57-61] Studies comparing MMF to cyclophosphamide, however, are lacking and therefore definite conclusions cannot be made as to which drug should be used preferentially. Our current regimen includes a combination of tacrolimus and MMF, in addition to plasmapheresis, for the treatment of AHR. Other groups have combined plasmapheresis with high-dose tacrolimus and antilymphocyte therapy, but without MMF.[74] This approach may be limited by tacrolimus nephrotoxicity and neurotoxicity, which in our experience is commonly observed with tacrolimus levels greater than 20 ng/ml.

Finally, although antilymphocyte therapy alone is generally insufficient to reverse AHR, a role for it to contribute to the successful reversal of AHR cannot be ruled out. Indeed it has been well established that, at least in their initial stages, humoral responses are T-cell-dependent.[9,55]

New Perspectives

Clearly new agents that prevent and/or allow successful reversal of AHR are needed. The effects of new immunosuppressive drugs such as anti-interleukin-2 receptor monoclonal antibodies or rapamycin on DSA production remain to be clarified. These two classes of drugs have been shown to decrease the incidence of acute rejection in renal transplant recipients.[75-77] However, their potential usefulness to control anti-donor humoral responses has not been established in humans.

Another interesting new strategy to treat AHR is the use of complement inhibitors. In animal models of antibody-mediated rejection, specific inhibition of the complement system

is a very effective approach to treat AHR, without the need for antibody removal by extracorporeal techniques.[78] In addition to the early experimental work performed in the 1970s,[49,50,79] the critical role of complement in humoral rejection has been demonstrated more recently by using soluble complement receptor type 1 (CR1), which resulted in delaying hyperacute xenograft rejection (pig to primate model) from one hour to 5-7 days.[80] Soluble CR1 (sCR1 or TP10), a recombinant form of endogenous soluble complement receptor 1 (CR1, CD35, C3b/C4b receptor), is a very efficient inhibitor of both classical and alternative pathways of the complement cascade.[81,82] The presence of C4d in peritubular capillaries of renal recipients with AHR indicates that the classical pathway is activated, presumably due to the binding of DSA to the graft endothelial cells.[14] Complement activation results in neutrophil recruitment with subsequent tissue damage. In the future, administration of complement inhibitors such as sCR1 may allow treating AHR without the need for antibody removal. This new class of drugs, already undergoing clinical trials in allograft recipients,[83] may become an important addition to the armamentarium of anti-rejection agents in organ transplantation.

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Abbreviations

PE: plasmapheresis; DSA: donor specific antibody; PRA: panel reactive antibodies; CyA: Cyclosporine; AZA: azathioprine; Bx: biopsy; AHR: acute humoral rejection.

FIGURE 1

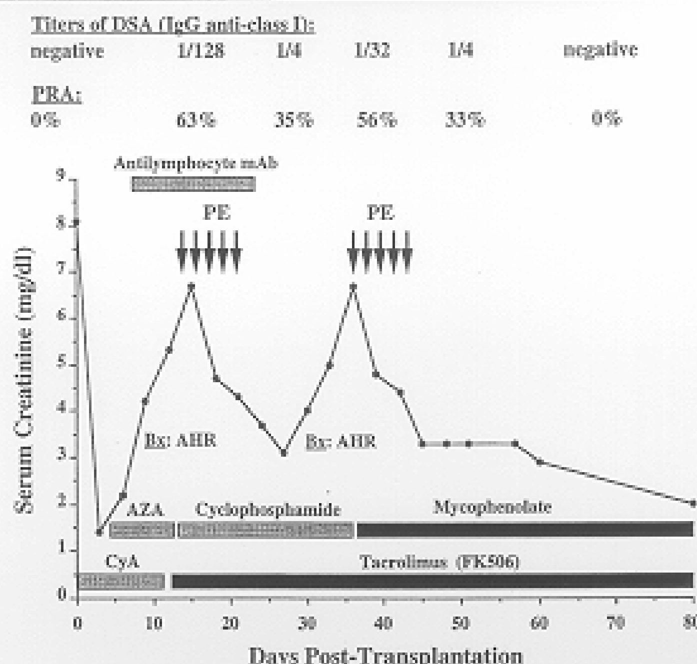

Fig. 1.This figure was adapted from Ref. 21, with permission from Lippincott Williams and Wilkins. The figure depicts the post-transplantation course of a 57 year-old female who received a living-related transplant from her daughter. The kidney allograft had excellent initial function but one week later marked renal dysfunction occurred and a renal biopsy revealed AHR, with diffuse C4d staining of peritubular capillaries. A repeat crossmatch demonstrated the presence of anti-HLA class I DSA (IgG) in patient's serum. DSA was directed against the B7 antigen present on the daughter's paternal haplotype. Renal dysfunction persisted despite administration of methylprednisolone pulses, antilymphocyte therapy and initiation of tacrolimus and cyclophosphamide. Plasmapheresis (PE) significantly decreased circulating DSA and was associated with an initial improvement in renal function. Subsequently, recurrent renal dysfunction occurred in association with a new rise in the titers of circulating DSA, and a repeat biopsy again revealed pathological features of AHR. A second series of five plasmapheresis, together with mycophenolate mofetil rescue, resulted in a sustained decrease in DSA titers and complete reversal of the rejection episode. Currently, three years after transplantation, the patient is doing well and the serum creatinine is 1.9 mg/dl.

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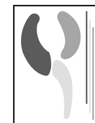

EDITORIAL

Progress in understanding humoral rejection in kidney transplantation: implications for patient management

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Until recently, most studies on the mechanisms of renal allograft rejection have focused on the central role of T cells and of other cellular mechanisms of tissue injury. Over the years, it has been established that CD4 T cells are crucial in initiating most acute rejection episodes, and that alloactivated CD4T cells, cytotoxic CD8T cells, monocytes/macrophages and NK cells play a major role in cell-mediated mechanisms that eventually result in allograft destruction[1]. These research efforts in the cellular immunity of organ transplantation have been illustrated in a recent study by Li y cols., in with measurement of urinary mRNA encoding perforin and granzyme B was used as a noninvasive means of diagnosing acute renal allograft rejection[2]. Perforin and granzyme B are two proteins that are present in the cytoplasmic granules of cytotoxic T cells and NK cells which are an integral part of the effector mechanisms of cell-mediated allograft rejection.

In recent years, however, it has become increasingly appreciated that detection of anti-HLA donor specific antibodies (DSA) *de novo* after transplantation is associated with specific «humoral syndromes» which are due predominantly, or in part, to antibody-mediated effector mechanisms of tissue injury. The identification of the complement fragment C4d as a specific marker for humoral rejection in peritubular capillaries of renal allograft biopsies has helped to define and characterize these syndromes, which we have recently termed *acute humoral rejection* (AHR) and *chronic humoral rejection* (CHR)[3-7]. In this article, the three different clinical settings in which humoral immunity appears to play an important role in clinical kidney transplantation are reviewed. In addition, new therapeutic approaches to control the production or the detrimental consequences of alloantibodies are discussed in the light of our recent studies and those of others.

HYPERACUTE REJECTION

The rejection of an allograft within minutes to hours after transplantation is termed «hyperacute rejection» (HAR). HAR is generally mediated purely by humoral mechanisms, that is by the binding of recipient's DSA to the donor graft vasculature which triggers complement activation. Both preformed DSA to HLA antigens or anti-ABO isohemagglutinins can result in «hyperacute rejection»[8,9]. Preformed anti-HLA DSA have generally been induced by previous exposure to alloantigens through prior transplantations, pregnancies or multiple blood transfusions[10]. Anti-ABO blood group natural antibodies are present in recipients who receive a blood group-incompatible transplant[11].

The detrimental effect of preformed DSA became apparent in the 1960s. In the presence of pre-existing DSA («positive crossmatch» at the time of renal transplantation), hyperacute destruction of grafted kidneys occurred almost inevitably[8,9]. The histopathologic findings of HAR revealed intense neutro-

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philic infiltrate, edema, focal interstitial hemorrhage and thrombosis, and fibrin thrombi in capillaires associated in some cases with fibrinoid necrosis of small arteries. Importantly, the absence of a mononuclear cell infiltrate indicated that HAR was not due to cell-mediated immunity. This initial experience stimulated the requirement for demonstration of a pre-transplant «negative» crossmatch and, in most centers, ABO compatibility to perform kidney transplantation[12,13]. HAR has become a very rare clinical event in kidney transplantation. Renewed interest in HAR (and in humoral rejection in general) has originated from the field of discordant xenotransplantation, in which natural xenoantibodies are responsible for complement-mediated HAR that has proved difficult to overcome. Removal of circulating natural xenoantibodies and/or inhibition of complement activation are two methods that have been succesfully used to prevent HAR in pig to primate models[14].

ACUTE HUMORAL REJECTION

In the early 1990s, P. Halloran y cols., proposed that acute rejection associated with the development of *de novo* anti-HLA DSA in recipient's serum (i. e. AHR) is a clinico-pathologic entity carrying a poor prognosis[15,16]. In a subsequent report, these authors analyzed the histopathologic features of AHR. Neutrophils in peritubular capillaires, glomerulitis, fibrin thrombi, infarction, severe vasculitis and fibrinoid necrosis in vessels walls were found to correlate with circulating DSA against HLA class I antigens[17]. These findings are distinct from those of cell-mediated acute rejection (without circulating DSA), which is characterized predominantly by a mononuclear cellular infiltrate with tubulitis and/or endothelialitis[18]. In the past, the terms «accelerated rejection», «delayed hyperacute rejection» or «acute vascular rejection» have been used to describe what most likely was AHR in a majority of cases[5]. However, it should be noted that these other terms may be confusing. For example, «acute vascular rejection» or «accelerated rejection» are entities not necessarily restricted to an antibody-mediated process, as they can also reflect T cell-mediated injury[3,18]. In particular endarteritis or endothelialitis, a form of «vascular rejection», can be exclusively due to cell-mediated immunity[5]. For these reasons, we prefer to keep the term «AHR» which is based on pathogenic mechanisms.

In 1999, B. Collins y cols., demonstrated that staining of allograft biopsies for the fragment C4d, a split product of complement C4, is a reliable method to identify AHR[4]. It was found that widespread C4d deposits in cortical peritubular capillaries correlated with the detection of *de novo* anti-HLA DSA in recipient's serum *(de novo* positive crossmatch). After activation of the classical pathway of complement by antibodies, the fragment C4d is released and remains covalently bound to the nearby endothelium or basement membrane collagen, thereby providing *in situ* pathologic evidence of antibody-mediated injury. These observations extended prior work by H. Feucht y cols., in 1993. These authors found that allografts with early dysfunction whose biopsies demonstrated capillary deposition of C4d were at a singnificantly increased risk of failure when compared to allografts with no C4d[19]. In their study, however, repeat posttransplant crossmatches were not performed so that the presence of *de novo* circulating DSA was not assessed.

Clinically, AHR typically presents as early and severe allograft dysfunction[3,5,6]. The risk of allograft failure (50-80%) is particularly increased in the first three months posttransplant[17,20,21]. We have recently analyzed the Massachusetts General Hospital experience over a four-year period and found an incidence of AHR within the first three months after renal transplantation of 7.7%, that is 20-25% of all acute rejections had a humoral component[6]. Most often, DSA were IgG against HLA class I antigens, but in some cases specificities against HLA class II or IgM antibodies could be defined. In approximately half of the patients with AHR, the rejection was resistant to both steroid and antilymphocyte therapy. A higher level of pretransplantation sensitization and a history of a previous failed allograft were found to be significant risk factors for AHR, suggesting that a specific anamnestic humoral response against donor antigens plays an important role in its pathogenesis[6]. We have also diagnosed AHR in two patients receiving cyclosporine, prednisone and mycophenolate mofetil, in whom the dosage of cyclosporine was decreased to subtherapeutic levels because of cyclosporine toxicity as well as in two hepatitis C-infected recipients following the introduction of interferon therapy (M. Pascual, unpublished observations). Finally, it should be emphasized that not uncommonly, histopathologic findings of acute cellular rejection may be present in biopsies with AHR («mixed» cellular and humoral pattern). The identification of C4d deposits in peritubular capillaries can be the only pathologic feature indicating the humoral component of the rejection process.

CHRONIC HUMORAL REJECTION

In addition to non-immunological factors, both cellular and humoral immune mechanisms play a key

role in the pathogenesis of chronic rejection (CR), a condition also termed «chronic allograft nephropathy» (CAN)[22]. In particular, the presence in serum of alloantibodies to donor HLA class I or class II antigens has been associated with CR/CAN, possibly manifesting alloresponsiveness via the indirect pathway of allograft recognition[1,7,23-25]. Posttransplant production of alloantibodies can predate the clinical manifestations of CR/CAN, implicating humoral mechanisms as a possible cause of CR/CAN[23]. Recent studies indicate that C4d deposits in peritubular capillaries are not only found in patients with AHR but also in a subset of patients with CR/CAN[7]. Approximately 60% of biopsies with histologic criteria for CR/CAN (transplant arteriopathy and/or chronic transplant glomerulopathy) had C4d deposits in peritubular capillaires. In most cases, this was accompanied by detectable DSA in the patient's serum[7]. To determine the relative contribution of humoral mechanisms of rejection to late allograft failure, we studied the prevalence of CHR in patients with chronic renal allograft dysfunction of all causes. C4d deposits in PTC were found in 13% of renal recipients who received an allograft biopsy for chronic allograft dysfunction[26]. Contrary to AHR, it does not appear that pretransplant sensitization is a risk factor for the development of CHR. In our experience, non compliance or underimmunosuppression is often found to be a cause for the development of CHR, suggesting that clinical trials evaluating withdrawal of calcineurin inhibitors or steroids should monitor DSA production.

NEW THERAPEUTIC APPROACHES TO CONTROL HUMORAL REJECTION

Since 1995, we have evaluated a new therapeutic approach consisting of «Plasmapheresis combined with tacrolimus-mycophenolate rescue» (PPh-TAC-MMF rescue) for renal recipients suffering from AHR refractory to both steroid and antilymphocyte therapy[3,6]. During a 4-year period, 232 renal transplants were performed under cyclosporine-based immunosuppression. In 10/232 (4.4%) consecutively studied patients with «refractory» AHR, the protocol of PPh-TAC-MMF rescue was initiated. Ths therapeutic strategy significantly decreased circulating DSA over a period of 3 to 6 weeks with reversal of rejection in 9/10 patients. At the end of plasmapheresis, polyclonal immunoglobulin was administered intravenously to limit the risk of infectious complications. With a mean follow-up of 42 months, patient and graft survival are 100% and 80%, respectively. Long-term monitoring of DSA titers revealed persistently undetectable levels of DSA in all patients with functioning allografts. In contrast, DSA was demonstrated in both patients with failed allografts[27]. These data suggest that a therapeutic strategy using «PPh-TAC-MMF rescue» can consistently prevent allograft loss and improve the outcome of early refractory AHR.

These observations on the control of humoral responses in patients with refractory AHR have been recently extended to the treatment of CHR, i.e. CR/CAN associated with DSA and C4d deposits in PTC[24]. We found that in recipients with CHR, rescue therapy with TAC and MMF alone (i.e., without plasmapheresis) resulted in a progressive and sustained decrease in DSA titers, with stabilization of renal allograft function. In two patients, DSA became undetectable after six to nine months of therapy, and repeat biopsies performed at 12 months revealed a decrease or absence of C4d deposits in PTC[24]. These preliminary findings confirm that suppression of alloantibody production is possible with the combination of TAC-MMF, and this effect may be of value for the treatment or prevention of CR/CAN.

Similar therapeutic strategies may also be useful to prevent HAR and thus allow kidney transplantation in highly sensitized patients. In Spain and in the US, approximately 10-15% of kidney transplant recipients are highly sensitized[28,29]. A rapid «desensitization» protocol was recently evaluated by Montgomery y cols.[30]. Successful desensitization was achieved in four highly sensitized patients who had positive cross-matches against their potential living donors. The protocol consisted of PPh and intravenous immunoglobulin (IVIg) with concomitant administration of TAC-MMF-prednisone, initiated several days or weeks before the planned transplantation. This was continued until a negative cross-match was achieved. All four patients developed episodes of AHR in the first month posttransplant, but these were successfully reversed with additional PPh and IVIg. In another similar study, 11 out of 15 patients with a pretransplant positive cross-match against their living donor could be successfully desensitized[31]. Pretransplant, the patients received PPh three times weekly over two consecutive weeks, in combination with IVIg and TAC-MMF-prednisone. These patients underwent successful transplantation with OKT3 induction and continuation of TAC-MMF. Relatively low initial titers of DSA were predictive of successful attainment of a negative crossmatch. Suppression of HLA-specific antibodies by the administration of high-dose IVIg has also been proposed as a means to desensitize patients awaiting transplantation[32,33]. IVIg has well-known immunomodulatory properties, including inhibition of complement activation and a

decrease in antibody synthesis, and has also been shown to be beneficial in the treatment of AHR in renal and cardiac allograft recipients[34]. Thus, we believe that incorporating IVIg in protocols of combining PPh-TAC-MMF probably provides additive effects that may be useful to decrease DSA synthesis.

In the upcoming years, it is likely that the effects of other newer immunosuppresive drugs on *in vivo* alloantibody synthesis will be clarified. Blocking the interleukin-2 receptor with daclizumab has already been shown to reduce the formation of anti-HLA antibodies posttransplant in cardiac transplant patients[35]. Rapamycin (sirolimus) suppresses immunoglobulin synthesis *in vitro*[36], thus it can be speculated that combining tacrolimus with sirolimus may offer an interesting alternative to tacrolimus-MMF regimen to control humoral responses in humans[37]. Finally, the role of complement inhibitors remains to be defined in solid organ transplantation. Indeed, specific inhibition of the complement system may allow preventing or treating the deleterious consequences of DSA without the need of antibody removal. Soluble CR1 (sCR1 or TP10), a recombinant form of endogenous soluble complement receptor 1 (CR1, CD35, C3b/C4b receptor), is a very efficient inhibitor of both the classical and alternative pathways of complement[14, 38, 39]. The presence of C4d in peritubular capillaries of renal recipients with humoral rejection indicates that the classical pathway is activated due to the binding of DSA to the graft endothelial cells. In the near future, induction therapy or posttransplant administration of complement inhibitors may open new avenues in transplantation. This new class of drugs, already undergoing phase I clinical trials in allograft recipients may become an important addition to the pharmacologic armamentarium used in organ transplantation and other immune-mediated diseases[40].

SUMMARY AND CONCLUSION

After more than 40 years of clinical renal transplantation, the contribution of humoral immunity to the pathogenesis of allograft rejection is progressively being clarified. With the advent of a new generation of immunosuppressive agents, the production and consequences of anti-donor alloantibodies can now be controlled. In the upcoming years, immunosuppressive regimens that will specifically control both T- and B-cell responses may further improve long-term allograft survival, if the immunosuppresive efficacy of such regimens is not hampered by an increase in infectious, neoplastic or cardio-vascular complications.

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CONCLUSIONES

- El **rechazo agudo humoral** es una entidad clínica y patológica diferenciada que sugerimos diagnosticar con los siguientes criterios:

    1) CLÍNICOS: Disfunción renal severa precoz tipicamente córtico-resistente y con frecuencia resistente al tratamiento anti-linfocitario convencional.

    2) SEROLÓGICOS: Acompañada de la aparición de anticuerpos donante-específicos en sueros contemporáneos al momento del rechazo, no detectables pre-trasplante. Si bien los anticuerpos donante-específicos son habitualmente IgG anti-HLA de clase I o de clase II, no podemos descartar un posible papel patogénico de anticuerpos de tipo IgM.

    3) HISTOLÓGICOS: Presencia de depósitos difusos de C4d en capilares peritubulares.

    La presencia de sólo dos de estos tres criterios permitiría establecer el diagnóstico de **"probable rechazo agudo humoral"**, en cuyo caso probablemente resulta acertado aplicar la misma pauta terapéutica (86,87).

- La incidencia de **rechazo agudo humoral** en la población estudiada (MGH, 1995-99) es de 7.7%. Es decir, hasta en un tercio de los enfermos que sufrieron rechazo agudo córtico-resistente, existen mecanismos humorales de lesión del injerto (86).

- Los factores de riesgo claramente identificados son: sensibilización (pre-trasplante o histórica) y retrasplante renal (86)

- El estudio histológico tradicional resulta con frecuencia insuficiente para diagnosticar el **rechazo agudo humoral**, aunque la presencia de neutrofilos en capilares peritubulares y/o glomerulares, la necrosis fibrinoide arterial o

glomerular y la presencia de trombos de fibrina en glomérulos resultan datos sugestivos. En virtud de estos parámetros sugerimos distinguir entre **rechazo agudo humoral tipo 1** (con afectación capilar) y **rechazo agudo humoral tipo 2** (con afectación arterial) con severidad y pronóstico diferenciados (87).

- Reconocemos tres formas clínicas de presentación de **rechazo agudo humoral:** rechazo agudo precoz, rechazo agudo clásico y función retrasada del injerto. Es particularmente importante realizar un estudio serológico y/o de inmunofluorescencia en estos dos últimos tipos de presentación para poder reconocer mecanismos humorales de rechazo, inadvertidos de otra manera (85).

- Podrían existir casos leves de **rechazo agudo humoral,** aunque su incidencia es muy baja en nuestra experiencia (84,86).

- Nuestro estudio apoya la ausencia de mecanismos de acomodación (persistencia de anticuerpos donante-específicos a medio plazo con función renal adecuada) tras un episodio de **rechazo agudo humoral**.

- El rescate con plasmaféresis, tacrólimus y micofenolato muestra una eficacia elevada en los casos severos de **rechazo agudo humoral**. Parece aconsejable asociar la administración de gamaglobulina policlonal al tratamiento, como medida profiláctica e inmunomoduladora. Nuevos fármacos recientemente incorporados al mercado y otros en fase de estudio clínico podrían constituir alternativas eficaces en el control de las respuestas humorales post-trasplante en el futuro (31,86).

- La posibilidad clínica de revertir el **rechazo agudo humoral** debe estímular el estudio de pautas de desensibilización pre-trasplante en una subpoblación de receptores con gran dificultad para acceder a un re-trasplante.

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