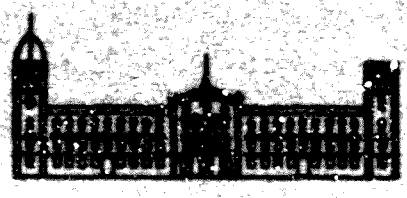

UNIVERSITAT DE BARCELONA
DEPARTAMENT DE CIÈNCIES FISIOLÒGIQUES HUMANES I DE LA NUTRICIÓ
FACULTAT DE FARMÀCIA

IMMUNOTERÀPIA DE L'ARTRITIS ADJUVANT AMB ANTICOSSOS MONOCLONALS ANTILIMFOCÍTICS

TESI PRESENTADA PER OPTAR AL GRAU DE DOCTOR EN FARMÀCIA

PROGRAMA DE DOCTORAT:
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BARCELONA 1995

Article 4

ADMINISTRATION OF A NON-DEPLETING ANTI-CD4 MONOCLONAL ANTIBODY (W3/25) PREVENTS ADJUVANT ARTHRITIS, EVEN UPON RECHALLENGE. PARALLEL ADMINISTRATION OF A DEPLETING ANTI-CD8 MONOCLONAL ANTIBODY (OX8) DOES NOT MODIFY THE EFFECT OF W3/25

Cellular Immunology 165:177-182 (1995)

RESUM

Objectiu: Estudiar la implicació dels limfòcits $CD4^+$ i $CD8^+$ en el desenvolupament de l'AA mitjançant el tractament preventiu amb AcMo dirigits contra aquestes subpoblacions limfocítiques.

Material i mètodes: L'AA es va induir en rates Wistar mitjançant una única injecció de *Mycobacterium butyricum*. Durant el període de latència de la patologia, les rates van ser tractades amb l'AcMo W3/25 (anti-CD4), amb l'AcMo OX8 (anti-CD8) o amb el dos AcMo conjuntament. Posteriorment, es va realitzar una reinducció de l'AA als grups que no havien desenvolupat la patologia. La gravetat de l'artritis es va valorar mitjançant l'índex d'artritis. Els efectes dels AcMo a nivell cel·lular es van avaluar per citometria de flux. Els nivells plasmàtics d'AcMo administrat i d'anticossos dirigits contra el micobacteri es van mesurar per tècniques d'ELISA.

Resultats: La immunoteràpia preventiva amb l'AcMo W3/25 administrat sol o conjuntament amb l'AcMo OX8 va inhibir el desenvolupament del procés inflamatori de l'AA, fins i tot després d'una reinducció de la patologia. En canvi, l'administració de l'AcMo OX8, tot i que va aconseguir una deplecció completa de la subpoblació $CD8^+$, no va modificar el curs de l'AA. L'administració de l'AcMo W3/25 va produir majoritàriament la modulació de la molècula CD4 i no la deplecció dels limfòcits T $CD4^+$. Els nivells d'anticossos antimicobacteri en els grups tractats amb AcMo van ser similars als desenvolupats pel grup artrític control.

Conclusions: Un AcMo anti-CD4 no deplecionant, el W3/25, és capaç de prevenir el desenvolupament de l'AA, fins i tot enfront d'una segona inducció tardana. Aquest efecte és independent de la producció d'anticossos antimicobacteri. Els limfòcits T $CD8^+$ no són rellevants en el desenvolupament de l'AA i no semblen exercir un paper regulador dels limfòcits T $CD4^+$.

CELLULAR IMMUNOLOGY 165, 177–182 (1995)

Administration of a Nondepleting Anti-CD4 Monoclonal Antibody (W3/25) Prevents Adjuvant Arthritis, Even upon Rechallenge: Paralliel Administration of a Depleting Anti-CD8 Monoclonal Antibody (OX8) Does Not Modify the Effect of W3/25

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The aim of this study was to determine the effects of the anti-CD4 monoclonal antibody (mAb) W3/25, found to be nondepleting, on the onset of rat adjuvant arthritis (AA), and, in addition, to ascertain whether depletion of CD8$^+$ cells during the same period could interfere with those effects. Female Wistar rats in which AA had been induced were treated with W3/25 and/or OX8 (anti-rat CD8) mAb during the latency period of arthritis. W3/25 alone or in combination with OX8 prevented the inflammatory process of AA. When the protected groups were rechallenged with a second dose of *Mycobacterium butyricum* no arthritis was observed. Protected and nonprotected arthritic animals developed the same anti-mycobacteria antibody levels as the arthritic control group. This study indicates that a nondepleting anti-CD4 mAb can prevent AA, while CD8$^+$ lymphocytes do not appear relevant for the development of AA and do not seem to have a regulatory role for CD4$^+$ cells. © 1995 Academic Press, Inc.

INTRODUCTION

The discovery of an effective treatment for an inflammatory arthritic disease requires an intimate knowledge of the cells and molecules which initiate and maintain the arthritic process. In human studies, it is difficult to investigate what happens before the clinical phase of the disease, because the first events of human arthritis are usually asymptomatic. The use of experimental models of arthritis solves such problems. Although several studies have attempted to clarify the arthritic process (1, 2), further studies *in vivo* are needed to elucidate the complexities of the cellular and molecular mechanisms responsible for the initiation of arthritic diseases.

Adjuvant arthritis (AA)[1] is an experimental model of arthritis commonly used to study new anti-inflammatory and anti-arthritic drugs. Although the pathogenesis of AA is unknown, a strong T-lymphocyte dependence has been described (3–5) and the beneficial effects in AA of W3/13, an anti-pan T monoclonal antibody (mAb) (6), R73, an anti-$\alpha\beta$-TCR mAb (7), and OX35, a depleting anti-CD4 mAb (8), have been reported.

In the present study, another anti-CD4 mAb, W3/25, was found to be nondepleting and its potential immunomodulatory effects on the onset of rat AA were ascertained. Moreover, in order to determine whether depletion of CD8$^+$ cells could interfer in these effects, both mAb, W3/25 + OX8 were administered together during the latency phase of AA. When the protective capability of W3/25 administered alone or in combination with OX8 had been demonstrated, a rechallenge of AA was performed on those groups treated with W3/25 or with W3/25 + OX8, long after the antibody has been cleared. In addition, we studied the effect upon humoral response to mycobacteria in protected and nonprotected arthritic animals.

MATERIALS AND METHODS

Arthritis induction. Female Wistar rats (180–200 g, Charles River, Spain) were injected intradermally in the tail base with 0.1 ml of an oil suspension of heat-killed *Mycobacterium butyricum* (Mb) (5 mg/ml, Difco Laboratories, Detroit, MI). For determination of the arthritic score, lesions of the four paws were each graded from 0 to 4 according to the extent of erythema and edema of the periarticular tissue as described by Wood *et al.* (9). All scorings were performed blind.

mAb. W3/25 (mouse IgG1, anti-rat CD4) (10) and OX8 (mouse IgG1, anti-rat CD8) (11) hybridoma (ECACC) were used. 124-1D1 (mouse IgG1, anti-human CD7), used as a control isotype mAb, was a kind gift from Dr. Vilella (Hospital Clinic Provincial, Barce-

[1] Abbreviations used: AA, adjuvant arthritis; mAb, monoclonal antibody; Mb, *Mycobacterium butyricum*; MFI, mean fluorescence intensity; PBL, peripheral blood lymphocytes.

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lona, Spain). Antibodies were obtained from ascitic liquid, purified by protein A column, and quantified spectrophotometrically. Antibodies were administered using PBS as vehicle and the delivery volume was 2 ml per injection.

mAb administration protocols. Rats were distributed in six groups of seven animals each. Three groups were treated i.p. with 3 mg of W3/25 mAb, 1 mg of OX8 mAb, or both together (3 mg W3/25 + 1 mg OX8) on Day 0, before arthritis induction, and on Days 2, 4, 7, 9, 11, and 14 thereafter. A group of healthy animals, a group of arthritic rats (treated only with PBS), and a group of arthritic rats treated with 3 mg of the isotype control mAb were used as controls. Doses of W3/25 and OX8 mAb were chosen in pilot experiments, on the basis of their ability to apparently eliminate blood $CD4^+$ and $CD8^+$ lymphocytes for at least 72 hr.

Reinduction. On Day 59, i.e., 45 days after the last administration of mAb, both W3/25- and W3/25 + OX8-treated groups were rechallenged with the same dose of Mb as in the arthritis induction (see above). Arthritic score was measured until Day 86.

Determination of T lymphocyte subpopulations. On Days 8, 17, 24, 30, 38, and 49 post-induction, blood samples were taken by retroorbital puncture and collected in EDTA-coated Multivette tubes (Sardstedt, Spain). Before staining, erythrocytes were lysed with NH_4Cl and lymphocyte subsets were determined by indirect immunofluorescence and flow cytometry as described previously (12). Briefly, cells were incubated with anti-rat CD4 (W3/25), anti-rat CD8 (OX8), or with an irrelevant mAb as a negative control. FITC-conjugated goat $F(ab')_2$ anti-mouse IgG (Sigma Immunochemicals, Spain) was added as a second antibody. Cells were analyzed on a Epics flow cytometer (Coulter Corp., U.S.A.). Forward/side scatter gates were set to include all viable lymphocytes by staining T- and B-lymphocytes together. Three thousand events were analyzed using the Epics Elite flow cytometry workstation program version 3.25 (Coulter) and the results were displayed as histograms.

Mouse IgG1 levels in plasma. An enzyme-linked immunosorbent assay (ELISA) was performed. Polystyrene microELISA plates (Labsystems, Spain) were incubated overnight with 5 μg/ml rabbit anti-mouse IgG1 (Dako, Glostrup, Denmark) at room temperature. Following incubation of test samples, peroxidase-conjugated goat anti-mouse (Fab specific) IgG (Sigma Immunochemicals, Spain) was added. Both antibodies were adsorbed with rat serum proteins. Mouse IgG1 from ICN ImmunoBiologicals (U.S.A.) was used as standard.

Anti-Mb antibodies. Plasma levels of anti-Mb antibodies were measured as described previously (13). Since a standard was not available, a pool dilution obtained from arthritic control animals was added to each plate. This pool was arbitrarily assigned 1000 U/ml of anti-Mb antibodies.

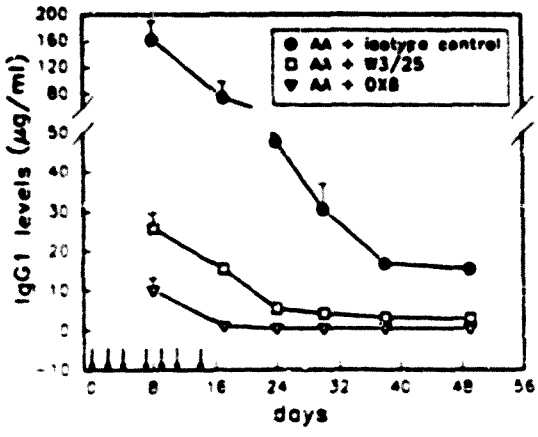

FIG. 1. Mouse IgG1 levels detected in rat plasma following administration of mAb against T-lymphocyte subpopulations during the latency period of AA. Values are expressed as means + SEM. Arrows indicate days of mAb administration.

Ig assay in vitro. In order to determine antibody production *in vitro*, PBL from healthy rats treated or not with W3/25 mAb were stimulated with pokeweed mitogen (PWM, 10 μg/ml, Gibco). After 7 days of incubation at 37°C, supernatants from the wells were harvested and stored at −20°C. IgG determinations were performed using standard ELISA techniques.

Statistical analysis. Differences between groups in all the parameters studied were analyzed by means of the Mann–Whitney *U* test, using the CSS Statistica (Stat Soft) program. Significant differences were accepted for $P < 0.05$.

RESULTS

Plasma levels of administered mAb. Plasma levels of mouse IgG1 from mAb-treated groups are summarized in Fig. 1. After four doses of mAb, plasma levels of W3/25 were 1/5 of those observed for the isotype control group treated with the same amount of mAb, indicating that most of W3/25 mAb was linked to $CD4^+$ cells. Three days after finishing treatment, plasma W3/25 or OX8 antibody levels fell markedly, and 10 days after stopping mAb administration, no free mAb was detected in plasma.

Percentage of $CD4^+$ and $CD8^+$ lymphocytes. $CD4^+$ and $CD8^+$ populations expressed as relative percentages of total gated lymphocytes are represented in Fig. 2. $CD4^+$ cells in healthy and arthritic control animals represented about 50% of total lymphocytes (Fig. 2A). The percentage of $CD8^+$ cells in the arthritic control

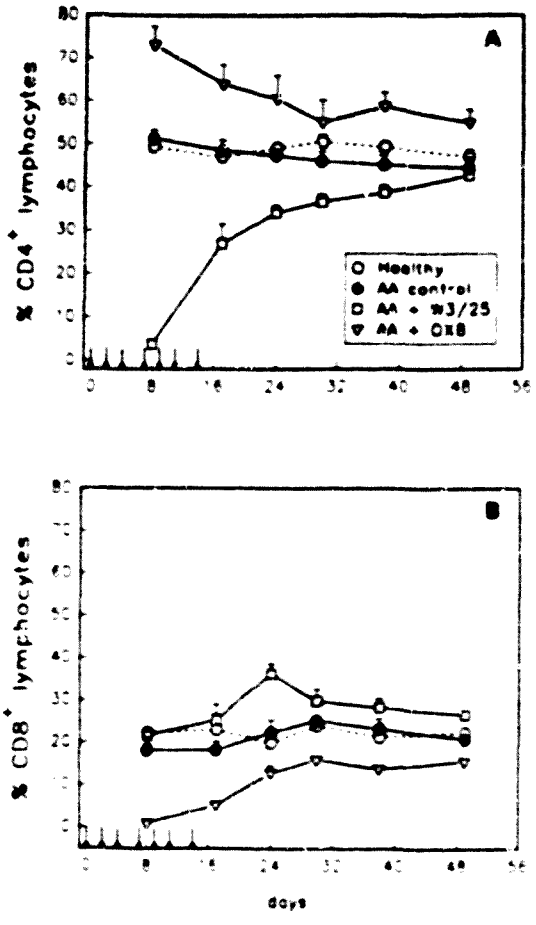

FIG. 2. Time course of percentages of CD4+ (A) and CD8+ (B) T lymphocytes following administration of mAb against T lymphocyte subpopulations during the latency period of AA. Values are expressed as means + SEM. Arrows indicate days of mAb administration.

group was significantly lower on Days 8 and 17 than in healthy animals ($P < 0.05$) (Fig. 2B).

On Day 8, following four doses of mAb, no CD4+ cells with normal expression of CD4 molecule were detected in PBL from W3/25-treated animals (Figs. 2A and 3E). On Day 17, i.e., 3 days after the last dose of W3/25, CD4+ cells were first detected but normal CD4+ cell percentage was not achieved until Day 49 (Fig. 2A). The percentage of CD8+ cells in W3/25-treated animals was similar to that of the arthritic control group on Days 8 and 17, and it was significantly higher on Days 24, 38, and 49 ($P < 0.05$) (Fig. 2B).

OX8-treated animals presented no CD8+ cells in PBL, after four doses (Fig. 2B and 3O). After the last dose of OX8, the percentage of CD8+ cells began to rise, although it was not completely restored at the end of the study. A significant increase in the percentage of CD4+ cells was observed in the OX8-treated group in comparison with arthritic control animals throughout the period studied ($P < 0.05$) (Fig. 2A). These findings indicate that OX8 mAb administration produced a depletion of CD8+ cells in PBL.

Expression of the CD4 molecule on PBL from W3/25-treated animals. On Day 8, CD8+ cell percentage in W3/25-treated animals did not increase although CD4+ cells were not detected (Fig. 2B). This result suggests the down-regulation of CD4 antigen expression in W3/25-treated animals.

Further analysis of histograms obtained after staining with anti-CD4 mAb revealed that W3/25-treated animals presented a wider negative peak than that from nontreated animals (Fig. 3E). This peak could be due to lymphocytes with a low expression of CD4 molecule appearing unseparated from nonstained cells. Histograms from Days 17 and 24 (Fig. 3H and 3K) showed a progressive separation between positive and negative peaks, demonstrating the reappearance of CD4 molecule on T lymphocytes.

In order to quantify the CD4 antigen expression on lymphocytes from W3/25-treated animals, data from flow cytometry were expressed as mean fluorescence intensity (MFI) of the whole lymphocyte population. The MFI obtained through negative control histograms

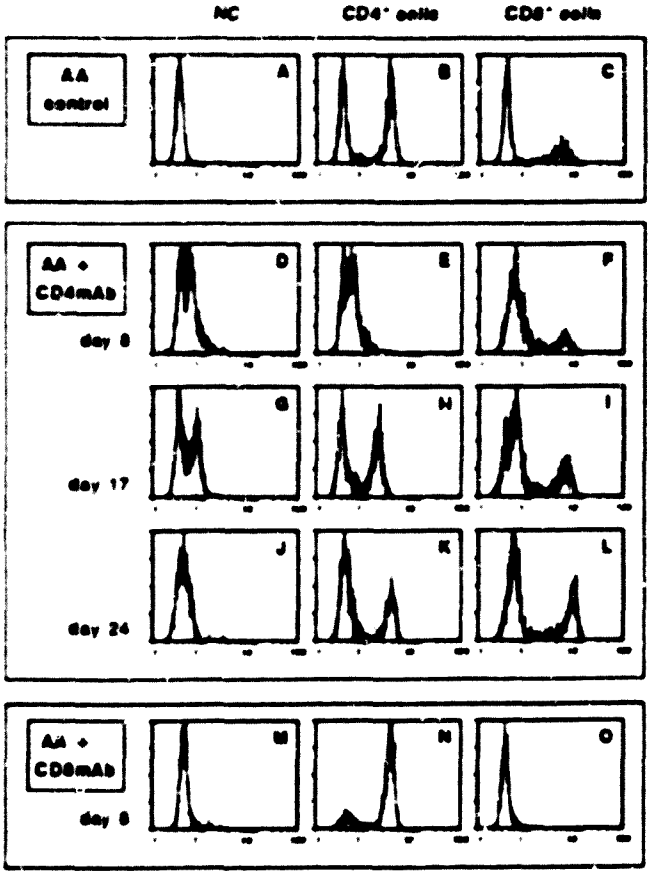

FIG. 3. Frequency distribution of the fluorescence intensities obtained after staining with an irrelevant IgG1 negative control (left column) or the anti-CD4 mAb (middle column) or the anti-CD8 mAb (right column) and the second antibody. A representative rat from the arthritic control group (A–C), W3/25-treated group (D–L), and OX8-treated group (M–O) is shown.

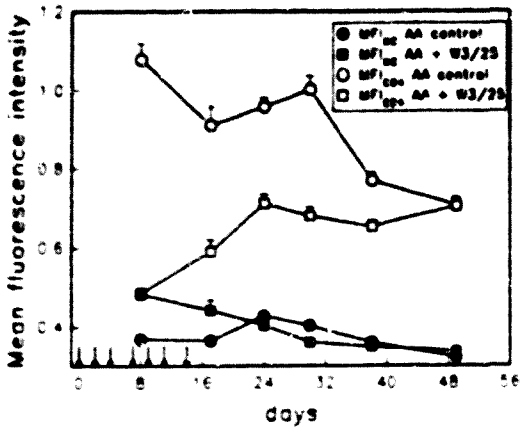

FIG. 4. Time course of mean fluorescence intensity from negative control staining (MFI_{NC}) and staining with anti-CD4 mAb (MFI_{CD4}) for the arthritic control group and W3/25-treated group. MFI were obtained from the whole lymphocyte population in every staining. Values are expressed as means ± SEM. Arrows indicate days of mAb administration.

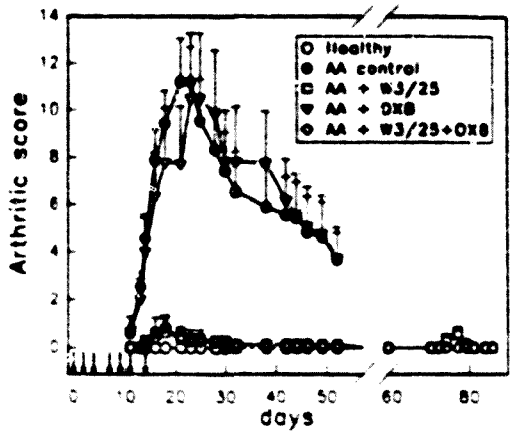

FIG. 5. Time course of arthritic score following administration of mAb against T-lymphocyte subpopulations during the latency period of AA. On Day 59, W3/25-treated animals were rechallenged and inflammation was studied until Day 86. Values are expressed as means ± SEM. Arrows indicate days of mAb administration.

(MFI_{NC}) or CD4$^+$ histograms (MFI_{CD4}) from W3/25-treated rats and from arthritic control rats are represented in Fig. 4. On Day 8, MFI_{CD4} values from W3/25-treated animals were lower than those from arthritic control animals, but higher than MFI_{NC} from arthritic control animals. These results confirm the decreased CD4 expression and show incomplete disappearance of CD4 molecules on the lymphocyte surface.

MFI_{NC} from W3/25-treated animals was significantly higher than MFI_{NC} from the arthritic control group on Days 8–17 ($P < 0.01$), indicating that W3/25 mAb administered *in vivo* was linked to CD4$^+$ cells, as can also be observed in individual histograms (Fig. 3D and 3G). On Day 8, MFI_{NC} and MFI_{CD4} from W3/25-treated rats were identical, which demonstrates the complete saturation of CD4 molecules after mAb administration *in vivo* (Fig. 4). This finding is also observed when comparing Figs. 3D and 3E.

Effect of mAb administration on arthritis development. Arthritic scores from all groups are represented in Fig. 5. In comparison to healthy animals, arthritic control rats showed a full-blown arthritic syndrome throughout the course of the experiment. Animals treated with the isotype control antibody followed a similar time course to the arthritic control group throughout the study (data not shown).

Administration of W3/25 suppressed the development of arthritis, showing significant differences with the arthritic control group throughout the period studied ($P < 0.01$ on Days 14–42, $P < 0.05$ on Days 44–52). Animals remained protected even when no W3/25 mAb levels were detected in plasma and when T cells recovered the normal expression of CD4 molecule.

When rats were treated with W3/25 + OX8 mAb together, the same protective effect was observed as when W3/25 mAb was administered alone. Administration of the OX8 mAb alone during the latency phase of AA failed to modify the development or the severity of the disease in comparison to arthritic control animals.

Arthritis reinduction. Groups protected against arthritis development were rechallenged with the same initial dose of Mb on Day 59. Only 1/14 animals developed a mild transient inflammation, and the rest were free of any sign of arthritis (Fig. 5).

Antibody response in groups in which AA was induced. Plasma anti-Mb antibody levels from all animals which received mycobacteria suspension are represented in Fig. 6. No significant differences were detected in any of the groups studied and the time course

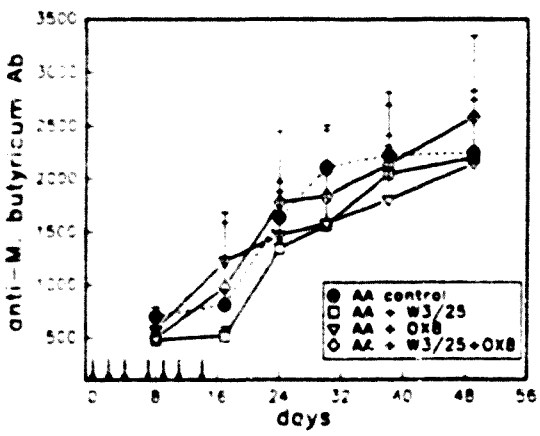

FIG. 6. Anti-Mb antibody levels (U/ml) detected in plasma by ELISA following administration of mAb against T-lymphocyte subpopulations during the latency period of AA. Values are expressed as means ± SEM. Arrows indicate days of mAb administration.

of anti-Mb antibody levels was similar in protected and arthritic groups.

Because the W3/25-treated animals developed a similar anti-Mb antibody response to that of arthritic control animals, PBL from W3/25-treated animals were stimulated with PWM in order to determine antibody production *in vitro*. Ig levels were similar when culture media were obtained from CD4 down-regulated PBL and when culture media were obtained from control cells (data not shown).

DISCUSSION

Five major findings emerge from the present study: (i) Administration of W3/25, an anti-CD4 mAb, during the latency period of AA prevented the development of articular inflammation, whereas administration of OX8 did not ameliorate or aggravate the time course of the disease. (ii) Administration of W3/25 + OX8 mAb together during the latency period prevented the development of AA to the same extent as W3/25 alone. (iii) The protective effect of W3/25, administered alone or together with OX8, remained even upon rechallenge, long after the antibody has been cleared. (iv) Administration of W3/25, an anti-CD4 mAb, led to a down-regulation of lymphocyte CD4 antigen expression. (v) The down-regulation of CD4 antigen expression and the depletion of $CD8^+$ cells did not affect the production of antibodies against mycobacteria in mAb-treated animals in comparison to the arthritic control group.

Following administration of W3/25 and OX8, the effects of these mAb on PBL were studied by flow cytometry. OX8 mAb produced a complete depletion of peripheral blood $CD8^+$ lymphocytes, which agrees with the first description of Holmdahl *et al.* (14). However, administration of W3/25, an anti-CD4 mAb, produced a very different effect to that observed for the OX8 mAb. W3/25 mAb led to a decrease in CD4 antigen expression or down-regulation in $CD4^+$ lymphocytes, as demonstrated by the total absence of gated lymphocytes with normal expression of CD4 and no relative increase in $CD8^+$ cell percentage. After stopping the administration of W3/25, a progressive recovery of CD4 expression on T cell surface occurred and around 10 days after the last dose, normal expression of CD4 molecule was observed in PBL. Although W3/25 mAb has been used by several authors (15, 16) this down-regulatory effect has not yet been described.

On the other hand, the effects of OX8 and W3/25 mAb on AA development were established. In previous studies, a decrease in CD4/CD8 ratio was observed in PBL of arthritic animals respect to healthy animals (17) and an increased number of $CD8^+$ cells was found in arthritic synovial membranes in comparison with healthy tissues (18). However, administration of the OX8 mAb, which depleted $CD8^+$ cells, did not aggravate rat AA. The ineffectiveness of OX8 in AA agrees with results from Larsson *et al.* (6), who administered OX8 mAb to arthritic animals in a less extensive protocol. These findings show that $OX8^+$ cells do not appear to have an essential role in the pathogenesis of AA.

In contrast, administration of W3/25 mAb suppressed the development of arthritis and this effect remained even when T cells recovered normal expression of CD4 molecule on their surface and when the rats were rechallenged with Mb. These results indicate a major role of $CD4^+$ T-lymphocytes in the pathogenesis of AA and suggest that the down-regulation of CD4 molecules hinders $CD4^+$ T-lymphocyte response, resulting in a reduced release of lymphokines and other inflammatory mediators. This powerful immunomodulatory effect of W3/25 mAb has not been described previously in AA, although Van der Broek *et al.* (15) reported the inhibition of streptococcal cell wall-induced arthritis by this anti-CD4 mAb. On the other hand, Billingham *et al.* (8) successfully inhibited AA development by administering OX35, a mAb that produces depletion of $CD4^+$ cells. The immunomodulatory effect of anti-CD4 mAb seems therefore to be independent of whether depletion of $CD4^+$ cells or down-regulation of CD4 antigen expression is induced. In mice, the preventive effect of anti-CD4 mAb has also been demonstrated in collagen-induced arthritis (19–21), but the mAb used in those studies were reported as depleting ones.

Administration of W3/25 + OX8 mAb together produced a similar result to that obtained after the administration of W3/25 mAb alone. OX8 mAb did not diminish the preventive effect of W3/25, suggesting that $CD8^+$ cells do not regulate $CD4^+$ cells.

Because W3/25 mAb produced a tolerance against to AA, the antibody response to Mb was measured. Surprisingly, anti-mycobacteria antibodies achieved the same plasma levels for nontreated animals as for W3/25- or and OX8-treated rats. This striking result led us to test immunoglobulin production *in vitro*, and, as in the experiment *in vivo*, no differences were found between normal $CD4^+$ cells and down-regulated $CD4^+$ cells. Therefore, the modulation of the CD4 molecule interferes only in some responses of CD4 lymphocytes. These results agree with those from Mannie *et al.* (22), who reported that W3/25 mAb inhibits IL-2 production of T cells *in vitro*, but not IL-4 release. Using other anti-CD4 mAb against mice or rat lymphocytes, a different susceptibility of TH1 and TH2 cell subsets to mAb has been indicated *in vivo* (23, 24). This different response of $CD4^+$ T lymphocyte subsets could explain the preventive effect of W3/25 mAb in AA and also the fact that the treatment does not interfere with the production of antibodies against Mb. The lack of the effect of anti-CD4 mAb in antibody response was reported in collagen-induced arthritis by Williams *et al.* (21). In contrast, some authors, using other autoimmune models, describe a decrease in antibody formation after anti-CD4 administration in some autoimmune experimen-

tal diseases (20, 25) and also after W3/25 mAb injection in a model of autoimmune glomerulonephritis (16).

In conclusion we demonstrate a potent immunomodulatory effect of a nondepleting anti-CD4 mAb in AA. Moreover, the prevention of AA with W3/25 remained even when normal CD4$^+$ cells were restored and it was independent of the antibody production against the "arthritogen" Mb. On the other hand, CD8$^+$ T-lymphocytes do not appear relevant for the development of AA and do not seem to have a regulatory role for CD4$^+$ cells.

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Article 5

TREATMENT WITH AN ANTI-CD4 MONOCLONAL ANTIBODY STRONGLY AMELIORATES ESTABLISHED RAT ADJUVANT ARTHRITIS

Clinical and Experimental Immunology (en premsa)

RESUM

Objectiu: Estudiar la implicació dels limfòcits T CD4$^+$ i CD8$^+$ en la perpetuació de l'AA. Determinar la possible efectivitat terapèutica dels AcMo dirigits contra aquestes subpoblacions limfocítiques en l'AA establerta.

Material i mètodes: L'AA es va induir en rates Wistar mitjançant una única injecció de *Mycobacterium butyricum*. El dia 14 postinducció, amb l'artritis ja establerta, es va iniciar un tractament amb l'AcMo W3/25 (anti-CD4), l'AcMo OX8 (anti-CD8) o els dos AcMo conjuntament. El seguiment de la inflamació es va realitzar mitjançant el volum articular. Les poblacions limfocítiques CD4$^+$ i CD8$^+$ es van analitzar per citometria de flux. Els nivells plasmàtics d'AcMo administrat, d'anticossos dirigits contra el micobacteri i d'anticossos produïts en resposta a l'AcMo administrat es van mesurar per tècniques d'ELISA.

Resultats: El tractament amb l'AcMo W3/25 va revertir el procés inflamatori ja establert. Aquest efecte, però, va ser transitori, ja que va desaparèixer després de 6 dosis. El tractament combinat W3/25+OX8 va produir els mateixos efectes antiinflamatoris que en administrar només W3/25. L'administració de l'AcMo OX8 no va aconseguir modificar el procés inflamatori ja existent, però sí que va incrementar els nivells d'anticossos antimicobacteri respecte als del grup artrític control.

Conclusions: El procés inflamatori de l'AA es pot revertir mitjançant el tractament amb un AcMo anti-CD4 no depleccionant, el W3/25. Mentre que els limfòcits T CD4$^+$ intervenen en la cronificació de l'AA, els limfòcits T CD8$^+$ no són rellevants en la perpetuació del procés inflamatori ni exerceixen un paper regulador sobre els limfòcits T CD4$^+$ responsables de la resposta inflamatòria. Ara bé, la potenciació en la producció d'anticossos antimicobacteri produïda per l'AcMo OX8, suggereix que els limfòcits T CD8$^+$ tenen un efecte regulador sobre les cèl·lules responsables de la resposta humoral dirigida contra el micobacteri.

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Treatment with an anti-CD4 monoclonal antibody strongly ameliorates established rat adjuvant arthritis

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SUMMARY

Some experimental arthritic diseases can be prevented by treatment with anti-CD4 MoAbs. Trials with ongoing disease have not been successful so far. The aim of this study was to ascertain whether W3/25 could reverse adjuvant arthritis (AA), when beginning treatment on day 14, i.e. when the disease was established. Moreover, one group of animals treated with the anti-CD4 MoAb received OX8 MoAb at the same time, thus depleting $CD8^+$ cells from circulation. During treatment with W3/25, a strong amelioration of inflammatory signals was observed, as assessed by means of paw volume increase and arthritic score. However, when treatment stopped, a rebound to arthritis signals occurred. The parallel depletion of $CD8^+$ cells did not modify these effects, thus the combined treatment W3/25 + OX8 gave the same amelioration as treatment with W3/25 alone. These findings indicate that $CD4^+$ cells play an important role in perpetuating rat AA. Moreover, $CD8^+$ cells do not seem to have a regulatory role in the $CD4^+$ cells responsible for the inflammatory response.

Keywords immunotherapy monoclonal antibody CD4 CD8 arthritis

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation within the joint, associated with synovitis and erosion of cartilage and bone. A strategy for the treatment of autoimmune diseases such as RA is to administer MoAbs to block the immunological responses or inflammatory cascades. One advantage of MoAbs over conventional therapy is their potential for great specificity, which allows them to act selectively on a specific lymphocyte subset *in vivo*.

Many observations suggest that T lymphocytes contribute significantly to the disease process in RA [1–4]. Several anti-CD4 MoAbs have been shown to be successful in treating RA [5–11], although some randomized studies have given negative results [12–14]. Moreover, the immunological mechanisms upon which anti-CD4 therapy is based are still not fully understood. For that reason, animal experiments are required to optimize immunomodulatory protocols and to identify the mechanism of operation of an anti-CD4 MoAb. On the other hand, several studies have been performed on collagen induced arthritis (CIA) in mice using anti-CD4 MoAbs [15–17], and although these treatments were found to prevent the induction of CIA, they were ineffective in ameliorating established arthritis.

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Adjuvant arthritis (AA) is an experimental autoimmune disease in rats which shares certain clinical and immunological features with RA in humans. This experimental model has been commonly used to study new anti-inflammatory and anti-arthritic drugs. Some studies have demonstrated the ability of a depleting IgG2a anti-CD4 MoAb, OX35, to prevent rat AA [18] and, in previous studies, we have also been successful in this, using a non-depleting IgG1 anti-CD4 MoAb, even when $CD8^+$ cells were depleted from circulation [19]. However, these treatments were begun on the day of arthritis induction, before clinical illness.

To determine whether W3/25 could reverse as well as prevent AA, rats with established arthritis were treated intraperitoneally with this MoAb, beginning treatment on day 14 post-induction. Moreover, one group of animals treated with W3/25 received OX8 MoAb at the same time, thus depleting $CD8^+$ cells from circulation. This form of therapy, applied when the disease is established, would act not on the induction but on the effector mechanisms, thereby interrupting both the immune and inflammatory disease processes, resembling what really happens when treating RA.

MATERIALS AND METHODS

Animals

Female Wistar rats (Charles River, Spain) weighing 180–200 g were housed in plastic cages with food and water *ad libitum*.

Temperature (20 ± 2°C), relative humidity (55%) and a 12-h light-dark cycle were constantly monitored. Animals were allowed 1 week to adjust to the housing conditions before the experiments began.

Induction and assessment of AA

For arthritis induction, rats were injected intradermally in the tail base with 0·1 ml of a liquid vaseline suspension of heat-killed *Mycobacterium butyricum* (Mb) (5 mg/ml; Difco Labs, Detroit, MI), on day 0. Hind-paw oedema was measured using a water plethysmometer (LI 7500; Letica, Spain). For determination of the arthritic score, lesions of the four paws were each graded from 0 to 4 according to the extent of erythema and oedema of the joint [20]. All scorings were performed blind.

Monoclonal antibodies

W3/25 (mouse IgG1, anti-rat CD4) [21] and OX8 (mouse IgG1, anti-rat CD8) [22] hybridoma (ECACC) were used. 124 1D1 (mouse IgG1, anti-human CD7) was used as a control isotype MoAb, and was a kind gift from Dr Vilella (Hospital Clinic Provincial, Barcelona, Spain). After culturing the hybridoma, ascites was induced in BALB/c mice (Charles River) by standard methods. IgG from ascitic liquid was purified by protein-A column and its concentration was quantified spectrophotometrically. Antibodies were administered using PBS as a vehicle, and the delivery volume was 2 ml per injection.

Treatment protocols

On day 14 post-induction, before beginning treatment, only those rats in which the disease was clearly established were selected and distributed in six groups of seven animals each, leading to groups with equal mean of arthritic score. Three groups were treated intraperitoneally with either 3 mg of W3/25 MoAb, 1 mg of OX8 MoAb, or both together (3 mg W3/25 + 1 mg OX8). A group of healthy animals, a group of arthritic rats (treated only with PBS) and a group of arthritic rats treated with 3 mg of the isotype control MoAb were used as controls. Doses of W3/25 and OX8 MoAbs were chosen in pilot experiments on the basis of their apparent ability to eliminate blood $CD4^+$ and $CD8^+$ lymphocytes for at least 72 h. The MoAbs were administered 3 days/week from day 14 until day 28, giving a total of seven doses.

Flow cytometry from peripheral blood

On days 18, 25, 31, 38 and 50 post-induction, blood samples were taken by retroorbital puncture and collected in EDTA-coated Multivette tubes (Sardstedt, Spain). Samples were then treated with an erythrocyte lysing solution of NH_4Cl and blood lymphocyte subsets were determined by indirect immunofluorescence and flow cytometry, as described previously [23]. Briefly, cells were incubated with anti-rat CD4 (W3/25), anti-rat CD8 (OX8) or with an irrelevant MoAb as a negative control. FITC-conjugated goat $F(ab')_2$ anti-mouse IgG (Sigma Immunochemicals, Spain) was added as a second antibody. Cells were analysed on a Epics Elite flow cytometer (Coulter Corp., Hialeah, FL). Additionally, two-colour CD5/CD4 and CD5/CD8 staining was performed using an unlabelled MoAb, followed by PE-conjugated goat anti-mouse IgG (Sigma). After adding 10 μg/ml of normal mouse IgG, the staining was completed with FITC-conjugated MoAb directed to the second marker.

Mouse IgG1 levels in plasma

An ELISA was performed. Polystyrene microELISA plates (Labsystems, Spain) were incubated overnight with 5 μg/ml rabbit anti-mouse IgG1 (Dako, Glostrup, Denmark) at room temperature. Following incubation of test samples, peroxidase-conjugated goat anti-mouse (Fab-specific) IgG (Sigma) was added. Both antibodies were adsorbed with rat serum proteins. Mouse IgG1 from ICN ImmunoBiologicals (USA) was used as a standard.

Anti-mouse antibody response

To determine the immune response against W3/25 MoAbs, plasma were analysed by means of an ELISA technique. W3/25-coated plates (1 μg/ml) were incubated with plasma dilutions and later with peroxidase-conjugated rabbit anti-rat IgG (Dako), previously adsorbed with mouse sera. A pool of sera from five- and seven-fold treated rats was used as a standard. This pool was arbitrarily assigned 800 U/ml of anti-mouse antibody.

Anti-Mb antibody

Plasma levels of anti-Mb antibody were measured as described previously [24]. Since a standard was not available, a pool dilution from arthritic control animals was added to each plate. This pool was arbitrarily assigned 1000 U/ml of anti-Mb antibody.

Expression of results and statistical analysis

Hind-paw inflammation is expressed as volume increase compared with articular volume measured on day 0. The effects of treatment on paw volume were measured as the area under curve (AUC) and as the percentage of swelling inhibition. Differences between groups in all the parameters studied were analysed by means of the Mann–Whitney *U*-test. Significant differences were accepted for $P < 0.05$.

RESULTS

Effects of treatment on inflammation

Hind-paw volume from all studied groups is represented in Fig. 1. Arthritic scores gave similar results to those obtained measuring hind paw volume (data not shown). Arthritic control rats showed a full-blown arthritic syndrome throughout the course of the experiment, showing a significantly higher paw volume than healthy animals from day 14 until the end of the study ($P < 0.01$). Animals treated with the isotype control antibody followed a similar time course to the arthritic control group throughout the period studied (data not shown).

Treatment with anti-CD4 MoAbs had efficiently diminished the articular swelling by day 18, when only two doses of MoAbs had been administered, showing a 70% of swelling inhibition with respect to the arthritic control group (Fig. 1). The hind paw volume of anti-CD4-treated animals was significantly lower than that of the arthritic control group until day 25, i.e. 3 days before the end of the treatment ($P < 0.05$; Fig. 1). Percentages of swelling inhibition were between 70% and 80% during this period. The AUC of the W3/25-treated group from day 14 until day 28 was significantly lower than that from the arthritic control group ($P < 0.006$). On day 28, although animals were still receiving the last dose of treatment, a rebound to the arthritis signals was observed. On day 32, hind paw volume

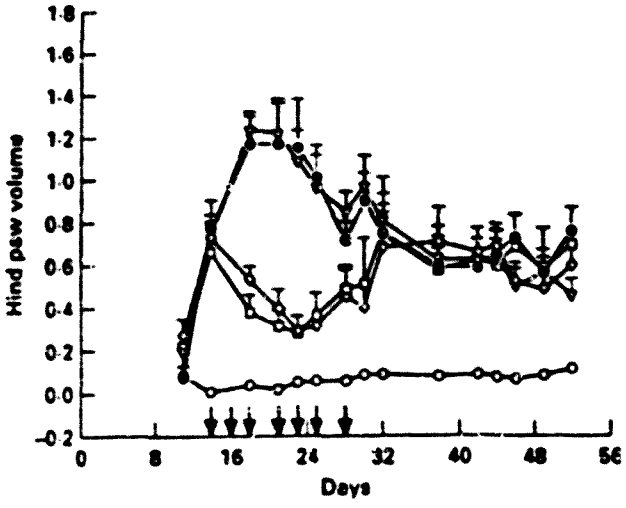

Fig. 1. Time course of hind-paw volume increase following administration of MoAbs against T lymphocyte subpopulations in established disease. Values are expressed as means + s.e.m. Arrows indicate days of MoAb administration. ○, Healthy; ●, adjuvent arthritis (AA) control; □, AA + W3/25; ▽, AA + OX8; ◇, AA + W3/25 + OX8.

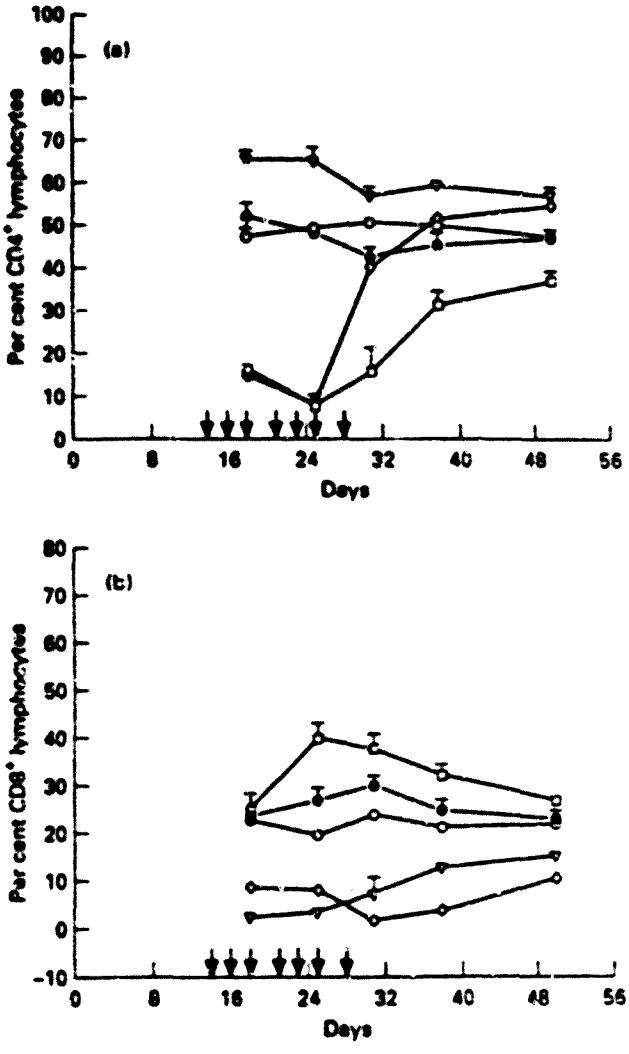

Fig. 2. Time course of percentages of $CD4^+$ (a) and $CD8^+$ (b) T lymphocytes following administration of MoAbs against T lymphocyte subpopulations in established disease. Values are expressed as means + s.e.m. Arrows indicate days of MoAb administration. ○, Healthy; ●, adjuvent arthritis (AA); control, □, AA + W3/25; ▽, AA + OX8; ◇, AA + W3/25 + OX8.

from W3/25-treated animals achieved arthritic control group values and followed the same time course during the rest of the study.

Treatment with W3/25 + OX8 together showed a similar effect to that observed with W3/25 alone. The effectiveness of this combined treatment was noticeable on day 18 (swelling inhibition of 57%), and remained significant until day 30 ($P < 0.05$). The AUC obtained following this combined treatment from day 14 until day 28 was significantly lower than that from the arthritic control group ($P < 0.006$). In this treatment a rebound to arthritis signals was also observed, achieving arthritic control values on day 32.

Treatment with OX8 alone was unable to modify the disease throughout the period studied, following the same time course as the arthritic control group.

$CD4^+$ and $CD8^+$ lymphocytes

Percentages of $CD4^+$ and $CD8^+$ cells with respect to total gated lymphocytes from peripheral blood of treated and control animals were studied at intervals of 6–10 days throughout the study (Fig. 2). $CD4^+$ cells represented about 50% of total lymphocytes in healthy and arthritic control groups (Fig. 2a). The percentage of $CD8^+$ cells in healthy animals was about 20% (Fig. 2b), and arthritic control animals presented significant increased $CD8^+$ cell values on days 25 and 38 in comparison with healthy animals.

Treatment with W3/25 produced the down-regulation of CD4 molecule as observed, after two doses of MoAbs, by a marked decrease in $CD4^+$ cells with normal expression of CD4 (Fig. 2a). The CD5/CD4 double staining (Fig. 3) showed that treatment with W3/25 did not modify the percentage of $CD5^+$ cells, while $CD5^+CD4^+$ cells were not detected. In addition, this finding was not accompanied with a proportional increase in the $CD5^+CD8^+$ population. When analysing the $CD5^+CD8^-$ population before and after treatment, no significant changes were observed.

The percentage of $CD4^+$ lymphocytes increased after stopping W3/25 treatment, although control values were not reached at the end of the period studied, showing significant differences even on day 50 ($P < 0.05$). Although on day 18 $CD8^+$ cell percentage from W3/25-treated animals was similar to that of the arthritic control group, on days 25 and 38 this percentage was higher than in the arthritic control group ($P < 0.05$).

Treatment with OX8 caused a significant and sustained increase of about 15% in the number of $CD4^+$ cells in comparison with arthritic control animals throughout the period studied (Fig. 2a; $P < 0.05$). Moreover, OX8-treated animals presented no $CD8^+$ cells, and after stopping treatment, $CD8^+$ cells progressively and slowly reappeared (Fig. 2b; $P < 0.01$ with respect to the arthritic control group throughout the study).

When rats were treated with W3/25 + OX8 together, the same percentage of $CD4^+$ cells as in animals treated with W3/25 alone was observed during treatment. However, once

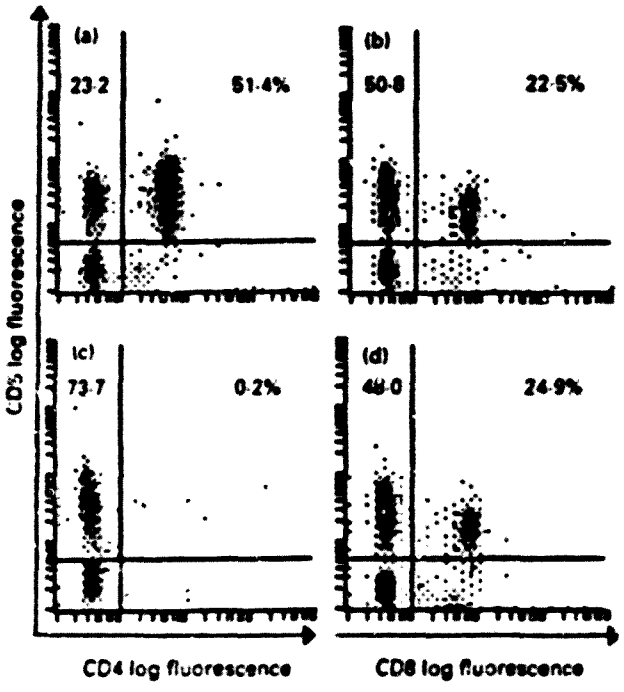

Fig. 3. Two-colour fluorocytometric analyses of rat peripheral blood lymphocytes obtained before (a,b) and after (c,d) W3/25 administration. Correlation of CD5 with CD4 (a,c) and CD5 with CD8 expression

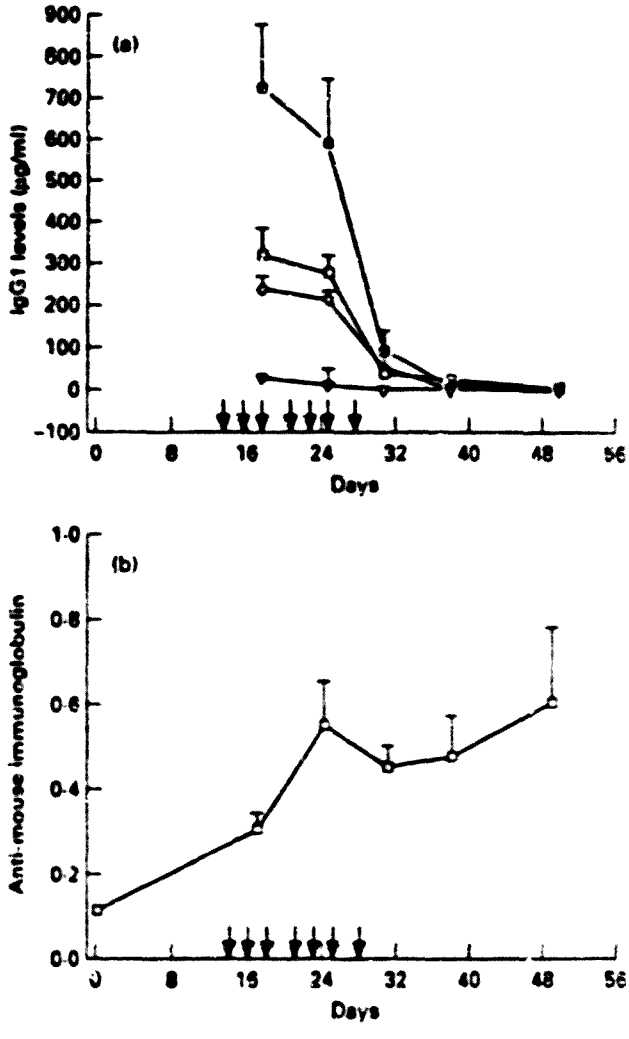

Fig 4. Mouse IgG1 levels (a) and anti-mouse antibody levels (b) detected in rat plasma following administration of MoAbs against T lymphocyte subpopulations in established disease. Values are expressed as means + s.e.m. Arrows indicate days of MoAb administration ■, Adjuvant arthritis (AA) + control isotype; □, AA + W3/25, ▽, AA + OX8, ◇, AA + W3/25 + OX8.

treatment stopped, $CD4^+$ lymphocytes with normal expression of CD4 quickly increased, reaching arthritic control values on day 31, i.e. 3 days after stopping treatment, and presenting a tendency to achieve OX8-treated group values thereafter. The combined treatment led to a curve of percentage of $CD8^+$ cells similar to that observed in OX8-treated animals, although $CD8^+$ cell recovery was slower.

Plasma levels of administered MoAbs and anti-mouse antibody response

Plasma levels of mouse IgG1 from treatment are represented in Fig. 4a. On day 18, after two doses of MoAbs, plasma levels of W3/25 were less than half of isotype control MoAb levels, indicating that more than half of the W3/25 administered was not free in plasma, but linked to $CD4^+$ cells.

Animals treated with W3/25 + OX8 presented slightly lower plasma levels of IgG1 than W3/25-treated animals throughout the study, thus indicating that the anti-CD8 MoAbs was not free in plasma. OX8-treated animals showed very low levels of IgG1 during treatment. On day 31, i.e. 3 days after stopping treatment, IgG1 levels fell markedly in all groups, achieving non-detectable values on day 50.

W3/25-treated animals developed an anti-mouse antibody response, showing high levels from day 24, i.e. after receiving five doses of the MoAb, until the end of the studied period (Fig. 4b).

Anti-Mb antibody

Arthritic control animals presented high levels of antibody against Mb throughout the study, although treatments applied even increased these antibody levels (Fig. 5). In the W3/25-treated group, levels of anti-Mb antibody were significantly higher than those of the arthritic control group from day 38 until the end of the period studied ($P < 0.05$). When treatment consisted of W3/25 + OX8, levels of anti-Mb antibody were slightly higher than those from W3/25 treatment during almost all the study, and significantly higher than those from the arthritic control group ($P < 0.01$ on days 25 and 38, $P < 0.02$ on days 17 and 49).

When $CD8^+$ cells were depleted from circulation in OX8-treated animals, anti-Mb antibody increased markedly to values which were almost four-fold those of the other groups ($P < 0.01$ with respect to arthritic control group on days 25–38 and $P < 0.02$ on day 49).

DISCUSSION

The first conclusion of this study is that treatment with anti-CD4 MoAbs can ameliorate established AA. It is now clear that T lymphocytes play a crucial role in AA, as demonstrated by induction of arthritis either by transfer of lymphocytes [25,26] or by the clones [27]. Moreover, Billingham *et al.* [18]

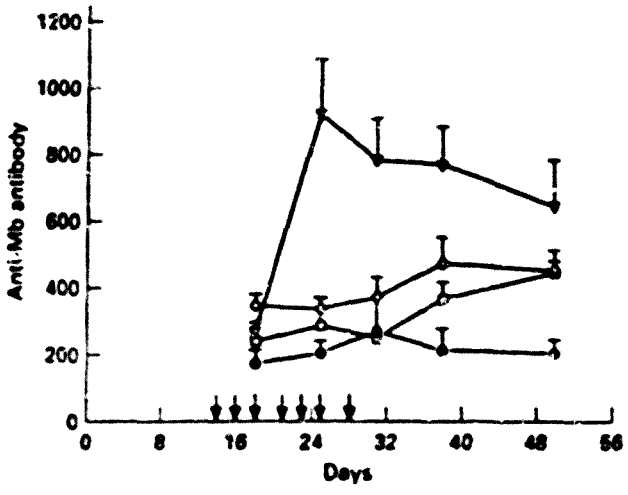

Fig. 5. Anti-*Mycobacterium butyricum* (Mb) antibody levels (U/ml) detected in plasma by ELISA following administration of MoAbs against T lymphocyte subpopulations in established disease. Values are expressed as means + s.e.m. Arrows indicate days of MoAb administration. ●, Adjuvant arthritis (AA) control; □, AA + W3/25; ▽, AA + OX8; ◇, AA + W3/25 + OX8.

reported the prevention of AA using OX35, a depleting anti-CD4 MoAb, and in previous studies we observed that W3/25, an anti-CD4 MoAb that led to a down-regulation of CD4 molecules, could also prevent the development of AA [19]. In both cases, the prevention was maintained throughout the study, and a rechallenge performed when $CD4^+$ cells were recovered was unsuccessful.

Here we demonstrate that treatment with W3/25 MoAb to rats with a full-blown arthritic syndrome reduced most inflammatory signs after only two doses. However, this effect was only transient, lasting during the maximum of inflammation and disappearing after six doses. Afterwards, W3/25-treated rats followed the same course as the arthritic control group. The inefficacy of last doses of the anti-CD4 MoAbs could be due to an anti-mouse response developed by the treated rats, which blocked the subsequent doses. These results suggest that interference with CD4 molecule can be an effective method to reverse chronic inflammation, but this interference should be continuous, and therefore an antibody that does not act as an immunogen is needed.

To our knowledge, the high capability of W3/25 in ameliorating AA has not been described. Van den Broek *et al.* [28] reported a decrease in paw swelling when administering W3/25 to rats with established streptococcal cell wall-induced arthritis. In contrast, anti-CD4 MoAbs were unable to ameliorate established CIA in mice [15-17] or in rats [29].

Immunotherapy with W3/25 mostly produced a decrease in CD4 antigen or down-regulation in $CD4^+$ lymphocytes, as demonstrated by the total absence of $CD5^+CD4^+$ lymphocytes, accompanied by a non-proportional increase in $CD5^+CD8^+$ cell percentage. However, lymphocyte percentages from days 25 and 30 showed a higher proportion of $CD8^+$ cells than those from the arthritic control group, which could indicate a partial depletion of $CD4^+$ lymphocytes. The $CD5^+CD8^+$ double staining from cells obtained before and after treatment has shown that depletion of $CD4^+$ cells was a minor effect of W3/25 MoAb treatment.

Anti-CD4 therapy did not affect the production of anti-Mb antibody, thus suggesting a different effect of W3/25 on Th1 and Th2 activities. These results agree with experiments performed *in vitro* [30]. However, when treatment stopped, a higher anti-Mb response was observed in W3/25-treated animals with respect to the arthritic control group. This suggests that the reappearance of $CD4^+$ cells with normal expression of CD4 enhances the humoral response.

A second conclusion of this study is the absence of effect of OX8 MoAb upon established AA. The dose of OX8 given to arthritic rats practically eliminated $CD8^+$ cells from peripheral blood during treatment. Depletion of these cells was evidenced by quantifying this subpopulation, and also by the concomitant increase in $CD4^+$ cells. Nevertheless, the disappearance of $CD8^+$ lymphocytes did not influence the chronic inflammatory response. Antibodies against CD8 molecule are also ineffective in the prevention of rat AA [31] and mouse CIA [16]. $CD8^+$ cells do not seem therefore to regulate the inflammatory response, either in the initiation or in the perpetuation of an arthritic disease.

On the other hand, the depletion of $CD8^+$ cells in peripheral blood produced a significant increase in specific antibody response. Anti-Mb antibody levels rose quickly after four doses of OX8 to levels that were almost four-fold those of the arthritic control group. Therefore, $CD8^+$ lymphocytes have an effect upon those T lymphocytes which direct immunoglobulin synthesis, but not upon those T lymphocytes involved in the inflammatory response. The question therefore arises as to whether T suppressor lymphocytes act on Th2 cells but not on Th1 cells. Further analysis should be performed to answer this question.

A third form of therapy was assayed in this study: combining W3/25 and OX8. The same effect as treatment with W3/25 alone resulted. The $CD8^+$ cell depletion together with CD4 modulation did not modify the ameliorating effect from W3/25 alone. On the other hand, the reappearance of $CD8^+$ cells in animals treated with W3/25 + OX8 was slower than that observed for OX8-treated animals, thus suggesting an influence of CD4 down-regulation on this process. The high anti-Mb antibody levels achieved by OX8-treated animals were not observed when $CD4^+$ cells were modulated at the same time as $CD8^+$ cells were depleted. This suggest that, when $CD8^+$ cells are depleted, the ability of modulated $CD4^+$ cells to increase the antibody response was lower than that of normal $CD4^+$ cells.

In conclusion, we have demonstrated that established rat AA can be strongly ameliorated by treatment with an anti-CD4 MoAb, which indicates an essential role of $CD4^+$ cells in the perpetuation of this disease. Moreover, $CD8^+$ cells do not seem to have the regulatory role of those $CD4^+$ cells which are responsible for the inflammatory response, although $CD8^+$ lymphocytes regulate those $CD4^+$ cells responsible for the antibody response against the inducer agent.

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DISCUSSIÓ

L'artritis adjuvant és una patologia induïda en rata mitjançant l'administració, via intradèrmica, d'adjuvant complet de Freund. Aquest model va ser induït per primera vegada per Stoerk i col. (1954) i perfeccionat més tard per Pearson (1956). L'AA es considera un model experimental d'AR humana i s'utilitza tant per a l'estudi i investigació d'agents antiinflamatoris com per intentar esbrinar els mecanismes implicats en la patogènesi del procés artrític. En aquest sentit, té importància establir els màxims paral·lelismes entre la malaltia humana i la patologia experimental.

L'AA ha estat objecte d'estudi en el nostre grup de recerca des d'un punt de vista immunològic, fet que ha permès la detecció i quantificació d'autoanticossos contra Ig (Castell i col., 1985 i 1986; Franch i col., 1994d), contra components del cartílag articular (Pantaleoni i col., 1991; Franch i col., 1994b), així com autoanticossos dirigits contra filaments intermedis del citosquelet (Franch i col., 1994c), alteracions també presents en l'AR. D'altra banda, també s'han constatat desequilibris entre les diferents poblacions limfocítiques presents en sang perifèrica (Franch i col., 1994a) i en teixits limfoides (Carol i col, 1995; Rodríguez-Palmero i col., 1995).

Un dels objectius d'aquest treball va ser de tipus metodològic i va consistir en un estudi comparatiu dels efectes, sobre les poblacions limfocítiques de rata, de tres mètodes de lisi d'eritròcits, utilitzats normalment per a sang humana (lisi amb clorur amònic, amb reactiu de Becton & Dickinson i mitjançant el sistema Coulter Q-prep) i dels efectes d'un gradient de sedimentació Ficoll-Isopaque adaptat a rata (Franch i col., 1993).

El gradient de sedimentació Ficoll-Isopaque adaptat a rata va proporcionar els percentatges més baixos de limfòcits T, fet que va suggerir la pèrdua selectiva d'alguns d'aquests limfòcits. D'altra banda, però, el gradient és l'únic mètode dels quatre estudiats que permet separar els limfòcits dels granulòcits.

En relació als dos mètodes comercials, la lisi amb el reactiu de Becton & Dickinson i la lisi mitjançant el sistema Coulter Q-prep, en ambdós casos es van obtenir uns valors percentuals de limfòcits T i B intermitjos i comparables. El principal avantatge d'aquests dos mètodes és la seva rapidesa, però en contrapartida, els dos mètodes requereixen reactius específics que impliquen un cost elevat i, a més a més, el sistema Coulter Q-prep també requereix un aparell específic per processar les mostres.

Quan es va aplicar el clorur amònic com a mètode de lisi d'eritròcits el percentatge de limfòcits B va ser inferior a l'obtingut pels altres mètodes, suggerint la pèrdua selectiva d'alguns limfòcits B com a resultat de la susceptibilitat d'aquests a la solució de clorur amònic. Malgrat aquest efecte sobre els limfòcits B, la lisi amb clorur amònic no afecta selectivament cap subpoblació de limfòcits T i representa un mètode de baix cost i fàcil

aplicabilitat. A causa del nostre interès específic centrat en l'anàlisi de limfòcits T, i en base als avantatges ja esmentats que presentava el mètode de lisi amb clorur amònic, aquest va ser l'escollit per avaluar l'efecte en els limfòcits T dels AcMo administrats en la *immunoteràpia*.

El primer objectiu no metodològic del present treball va consistir en esbrinar quins tipus cel·lulars estan implicats en el procés artrític a nivell local. L'interès va sorgir de resultats previs que van mostrar un desequilibri entre les poblacions limfocítiques de sang perifèrica de rates amb AA. Es va realitzar un estudi immunohistoquímic de la membrana sinovial de genoll de rates amb AA i d'entre tots els marcadors utilitzats, el teixit sinovial sa només va presentar cèl·lules $CD4^+$ i Ia^+ en la capa íntima, corresponent possiblement a macròfags (Jefferies i col., 1985) i a cèl·lules dendrítiques (Steinmann i Nussenzweig, 1980), respectivament. Set dies després de la inducció de l'AA, durant la fase de latència de la patologia, els canvis observats van ser mínims, mentre que a partir del dia 14, les alteracions tissulars van comportar la proliferació de macròfags i cèl·lules dendrítiques i la infiltració d'altres tipus cel·lulars.

Es van trobar alguns limfòcits T (cèl·lules $CD5^+$) a nivell de la subíntima durant els dies 14-21 postinducció, però aquest fet no es va reproduir en tots els teixits estudiats i, a més a més, els limfòcits no estaven distribuïts en cúmuls al voltant dels vasos, tal i com s'ha descrit per a l'AR (Iguchi i col., 1986). En relació a l'AA, tot i que els primers estudis histològics realitzats per Pearson i col. (1961) també descriuen la presència d'infiltracions de limfòcits en aquest model, estudis posteriors realitzats també per tècniques d'histologia convencional no fan referència a la presència d'aquests infiltrats, coincidint amb els nostres resultats (Mohr i Wild, 1976; Taurog i col., 1988).

La població de cèl·lules $CD4^+$ es va trobar augmentada en els teixits sinovials artrítics. La majoria d'aquestes cèl·lules, en base a la seva mida, a la tinció citoplasmàtica observada amb l'AcMo anti-CD4 i després de comparar els resultats amb l'AcMo ED2 (marcador de macròfags; Dijkstra i col., 1987), es van identificar com a macròfags.

A l'anàlisi immunohistoquímica també es va detectar la presència de cèl·lules $CD8^+$ en la subíntima sinovial, fet que no coincidia amb l'escassesa o absència de limfòcits T revelats amb un AcMo anti-CD5 i que reflectia, per tant, la presència de cèl·lules $CD8^+CD5^-$. Aquest resultat va suggerir la possibilitat que fossin limfòcits T-γ/δ, alguns dels quals són $CD5^-$ $CD4^-CD8^+$ (Goodman i Lefrancois, 1988). Els limfòcits T-γ/δ representen una població minoritària de limfòcits T que expressen un receptor per a l'antigen estructuralment diferent al convencional, és a dir, expressen un TCR format per una cadena γ i una δ (Raulet, 1989). Les funcions dels limfòcits T-γ/δ *in vivo* encara no es coneixen, però s'ha descrit un augment d'aquestes cèl·lules en sang perifèrica i en líquid sinovial de malalts amb AR (Brennan i col., 1988 i 1989). A més a més, s'ha demostrat que els limfòcits T-γ/δ proliferen davant

d'antígens micobacterians (Haregewoin i col., 1989; Janis i col., 1989) i també s'ha descrit la seva possible implicació en la patogènesi de l'AIC en ratolí (Peterman i col., 1993).

L'any 1994, Kühnlein i col. van produir i caracteritzar un AcMo dirigit contra el TCR-γ/δ de rata. Aquests autors van caracteritzar els limfòcits T-γ/δ presents en ganglis limfàtics, en melsa i en intestí prim de rates i van mostrar que el fenotip d'aquests limfòcits era variable i depenia de la seva localització. Per tant, resultava factible que la població $CD5^-CD8^+$ observada en el teixit sinovial artrític correspongués a limfòcits T-γ/δ, els quals, com ja s'ha esmentat, semblen ser importants en alguns processos artrítics.

En resum, l'estudi de les poblacions cel·lulars presents en la membrana sinovial inflamada de rates amb AA va revelar un augment en el nombre de cèl·lules mononuclears a partir del dia 14 postinducció, sense la presència de cúmuls de limfòcits T $CD5^+$. Aquests fets van suggerir que la sinovitis a l'AA estava causada majoritàriament per macròfags, més que per limfòcits, si bé quedava per aclarir si l'increment en cèl·lules $CD8^+$ reflectia la presència de limfòcits T-γ/δ. Cal esmentar que aquest increment en les cèl·lules $CD8^+$ no va disminuir en membranes sinovials procedents d'animals tractats amb dexametasona, les quals eren similars a les sinòvies control pel que fa a les altres poblacions cel·lulars.

Per tal d'analitzar la implicació dels limfòcits T-γ/δ en el desenvolupament de l'AA es va portar a terme una immunoteràpia amb l'AcMo V65, dirigit contra el TCR-γ/δ de rata.

Es van desenvolupar diferents protocols d'administració de l'AcMo V65: un tractament preventiu, realitzat a animals nounats durant tot el període previ a la inducció de l'AA, i dos tractaments curatius, iniciats abans o durant el màxim d'inflamació, respectivament. Tots els tractaments amb l'AcMo V65 van conduir a la presència, en sang i ganglis limfàtics, d'una població de limfòcits amb una expressió antigènica disminuïda de les molècules de TCR-γ/δ i de CD3 en la seva superfície, fet que indica que l'AcMo V65 va produir la *down-regulation* del receptor i la comodulació de la molècula CD3. Ara bé, el percentatge d'aquesta subpoblació amb el TCR modulat era inferior al percentatge de limfòcits T-γ/δ detectats abans del tractament, suggerint que algunes cèl·lules T-γ/δ s'havien depleccionat o havien perdut totalment l'expressió del seu TCR.

Malgrat l'efectivitat i especificitat de l'AcMo V65 demostrada sobre els limfòcits T-γ/δ i que els nivells detectats d'AcMo en plasma van ser saturants, cap dels protocols assajats en la immunoteràpia amb l'AcMo V65 va aconseguir prevenir el desenvolupament de la patologia, ni modificar la simptomatologia clínica ni les alteracions hematològiques de la mateixa. L'avaluació histològica de l'articulació del turmell va mostrar, però, que el tractament amb l'AcMo V65 iniciat abans del màxim d'inflamació (dies 12, 15 i 18 postinducció) va produir un grau de destrucció articular significativament superior al dels animals artrítics control. Aquest fet va suggerir que els limfòcits T-γ/δ exerceixen un paper protector a nivell local en una determinada fase de la patologia.

Aquest efecte fase-depenent dels limfòcits T-γ/δ a l'AA, és semblant al descrit en algunes patologies experimentals inflamatòries en ratolí, com l'AIC (Peterman i col., 1993) i la listeriosi (Mombaerts i col., 1993; Fu i col., 1994), on s'ha demostrat que els limfòcits T-γ/δ es localitzen en els infiltrats inflamatoris tissulars només en fases secundàries o tardanes de la patologia.

Es va pensar que la propietat moduladora dels limfòcits T-γ/δ a l'AA podia estar mitjançada per la regulació de la resposta enfront d'antígens micobacterians. De fet, s'ha demostrat que una proteïna d'estrès tèrmic (hsp) d'origen micobacterià, la hsp-60, i pèptids derivats d'aquesta proteïna tenen un efecte protector en l'AA (Anderton i col., 1995). Així, els limfòcits T-γ/δ podien exercir la seva acció protectora mitjançant la reacció amb la hsp-60. Fins ara, però, l'únic clon de limfòcits T caracteritzat, que respon a la hsp-60 i que és capaç de transferir propietats antiartrítiques en l'AA, expressa el fenotip TCR-α/β^+CD4$^+$ (Anderton i col., 1995). Aquests resultats i el fet que el tractament amb V65 no modifiqués els nivells d'anticossos antimicobacteri en els animals tractats, descarten la possibilitat que el mecanisme d'acció del tractament amb l'AcMo anti-TCR-γ/δ sigui mediat principalment per la resposta enfront del micobacteri.

Un altre possible mecanisme de l'acció protectora dels limfòcits T-γ/δ podia ser indirecte, mitjançant la neutralització de la capacitat proartritogènica dels limfòcits T-α/β (Yoshino i col., 1990a i b). Aquesta possibilitat està basada en estudis desenvolupats en ratolí que demostren que la modulació dels limfòcits T-γ/δ afecta la reactivitat dels limfòcits T-α/β, i suggereixen que la població T-γ/δ està implicada en la regulació de l'activació dels limfòcits T-α/β *in vivo*. En el nostre cas, les dades obtingudes per citometria de flux durant el tractament amb l'AcMo anti-TCR-γ/δ mostren que ni el nombre ni l'expressió fenotípica dels limfòcits T-α/β estan modificats.

Després de descartar la intervenció directa dels limfòcits T-γ/δ en la patogènesi del procés artrític experimental, vàrem centrar l'atenció cap als limfòcits T-α/β, concretament cap a la població CD4$^+$ i la CD8$^+$. Estudis de transferència realitzats per Holoshitz i col. (1983) i Taurog i col. (1983) havien demostrat un paper essencial dels limfòcits T CD4$^+$ en el desenvolupament de l'AA. Quedava per veure, però, si els limfòcits T CD8$^+$ exercien algun efecte complementari o capaç de modular el dels limfòcits T CD4$^+$.

Per estudiar la implicació de les subpoblacions limfocítiques CD4$^+$ i CD8$^+$ en el desenvolupament de l'artritis, es va realitzar una immunoteràpia preventiva amb AcMo dirigits contra ambdós tipus cel·lulars durant la fase de latència de la patologia.

Durant els períodes de tractament amb els AcMo W3/25 i OX8 es van estudiar els seus efectes a nivell cel·lular, mitjançant l'anàlisi dels limfòcits T CD4$^+$ i CD8$^+$ per citometria de flux. L'AcMo OX8 va produir la deplecció completa dels limfòcits T CD8$^+$ de sang perifèrica, tal com s'havia descrit prèviament (Holmdahl i col., 1985; Larsson i col., 1985).

En canvi, l'administració de l'AcMo W3/25 va produir majoritàriament la modulació o *down-regulation* de la molècula CD4 de la superfície dels limfòcits T $CD4^+$. Aquest efecte, no s'havia descrit anteriorment per a l'AcMo W3/25.

La immunoteràpia preventiva amb l'AcMo W3/25 va inhibir completament l'artritis, mentre que l'administració de l'AcMo OX8 no va produir cap efecte. A més a més, l'administració conjunta de l'AcMo OX8 amb el W3/25 no va modificar l'efecte inhibidor d'aquest últim. Cal esmentar que l'efecte preventiu de l'AcMo W3/25 va romandre fins i tot quan els limfòcits T ja havien recuperat l'expressió normal de CD4 i va ser efectiu en protegir els animals d'una segona inducció amb *Mycobacterium butyricum*.

Així, malgrat l'augment de limfòcits T $CD8^+$ en sang perifèrica (Franch i col., 1994a) i l'increment del nombre de cèl·lules $CD8^+$ en el teixit sinovial artrític, aquesta població cel·lular no sembla exercir un paper rellevant en la patogènesi de l'AA.

Els resultats obtinguts indiquen que els limfòcits T $CD4^+$ estan implicats en la fase de desenvolupament de l'AA i suggereixen que la *down-regulation* de la molècula CD4 comporta un reducció en l'alliberament de limfocines i altres mediadors inflamatoris dels limfòcits T $CD4^+$. L'efecte preventiu de l'AcMo W3/25 no havia estat descrit prèviament en l'AA, tot i que Van den Broek i col. (1992) havien publicat la inhibició de l'artritis produïda per SCW emprant aquest mateix AcMo. D'altra banda, Billingham i col. (1990) havien descrit que un AcMo anti-CD4 amb capacitat depleccionant, l'OX35, era capaç de prevenir el desenvolupament de l'AA. Així, l'efecte beneficiós dels AcMo anti-CD4 sobre el desenvolupament de l'AA sembla que és independent del seu efecte a nivell cel·lular, és a dir, deplecció o modulació de la molècula CD4.

L'AcMo W3/25 no va modificar els nivells d'anticossos antimicobacteri respecte el grup artrític control, indicant que la *down-regulation* de la molècula CD4 només interfereix algunes funcions dels limfòcits T $CD4^+$. Aquests resultats suggereixen que l'AcMo W3/25 té un efecte diferent sobre les activitats Th1 i Th2, és a dir, les responsables del procés inflamatori i les responsables de la producció d'anticossos, respectivament. Aquesta hipòtesi concorda amb els estudis de Papp i col. (1992) que descriuen la inhibició de la producció de IL-2 però no de IL-4 per un AcMo anti-CD4.

A partir de l'any 1987, es va començar a aplicar el tractament amb AcMo anti-CD4 a malalts amb AR. Aquest fet resulta sorprenent ja que prèviament no s'havia descrit l'eficàcia d'aquesta teràpia en cap model experimental d'artritis. No va ser fins l'any 1992, que Van den Broek i col. van descriure l'eficàcia terapèutica d'un AcMo anti-CD4 sobre l'artritis induïda per SCW. D'altra banda, el tractament resultava inefectiu en l'AIC en ratolí (Ranges i col., 1985; Hom i col., 1988; Williams i col., 1994) i en rata (Goldschmidt i Holmdahl, 1994).

El tractament de rates amb AA ja establerta emprant l'AcMo W3/25, a la mateixa dosi que la utilitzada en el tractament preventiu, va ser capaç de revertir la inflamació. Juntament amb el tractament amb W3/25, es va realitzar una teràpia amb OX8 sol o addicionat al W3/25. La deplecció dels limfòcits T CD8$^+$, aconseguida en ambdós casos, no va interferir ni en el desenvolupament de l'artritis establerta ni en l'efecte positiu del W3/25, mostrant novament la poca rellevància dels limfòcits T CD8$^+$ en la cronificació del procés artrític. De totes maneres, cal remarcar que la deplecció dels limfòcits T CD8$^+$ va conduir a un gran increment en els nivells d'anticossos antimicobacteri. Per tant, durant la fase crònica de l'AA, els limfòcits T CD8$^+$ exerceixen un paper regulador sobre els limfòcits involucrats en la síntesi d'anticossos.

Els efectes beneficiosos observats amb l'AcMo W3/25 en els nostres experiments sobre AA establerta van ser transitoris i van desaparèixer després de 6 dosis. La ineficàcia de les últimes dosis pot ser atribuïda a la resposta antiratolí que es va detectar en les rates tractades amb W3/25. En aquest sentit, la interferència amb la molècula CD4 pot esdevenir un mètode efectiu per revertir la inflamació crònica, però és necessari que aquesta interferència sigui contínua i, per tant, és molt important que l'AcMo a emprar no actuï com a immunogen. Per tant, és de gran importància disposar de grans quantitats d'anticossos humanitzats per al tractament de l'AR humana.

En resum, en aquest treball s'ha demostrat la implicació dels limfòcits T CD4$^+$ o col·laboradors no només en la fase de desenvolupament del procés artrític, sinó també en el procés de cronificació, fet que permet considerar els limfòcits T CD4$^+$ com a possibles dianes d'un tractament en l'AR humana. Cal tenir en compte, però, que aquest tractament s'ha de realitzar de forma contínua i amb molècules que no estimulin una resposta immunitària. A més a més, s'ha demostrat que els limfòcits T-γ/δ juguen un paper secundari en el desenvolupament de l'AA i que aquest paper és més aviat protector, modulant l'acció exercida per altres tipus cel·lulars en una determinada fase de la patologia. D'altra banda, els limfòcits T CD8$^+$ no estan implicats en el desenvolupament de l'artritis, ni tan sols com a moduladors de l'acció dels limfòcits T CD4$^+$ artritogènics. Per últim, malgrat la rellevància dels limfòcits T col·laboradors en el procés artrític, la presència d'aquestes cèl·lules a nivell inflamatori local és mínima, suggerint un efecte regulat majoritàriament per citocines des dels òrgans limfoides.

CONCLUSIONS

1. L'estudi immunohistoquímic del teixit sinovial de genoll de rates amb artritis adjuvant va mostrar absència de cúmuls de limfòcits T, tot i que es van detectar algunes cèl·lules $CD5^+$ els dies de màxima inflamació articular. D'altra banda, es va observar un augment de cèl·lules mononuclears $CD4^+$ en la membrana sinovial inflamada. Aquests dos fets suggereixen que els macròfags poden ser el tipus cel·lular majoritàriament responsable de la sinovitis en l'AA.

2. La membrana sinovial de genoll dels animals artrítics va mostrar un augment en la població $CD8^+$. Aquest increment i la restringida presència de limfòcits T ($CD5^+$) són indicatius de la presència de cèl·lules $CD8^+CD5^-$ en la sinòvia inflamada.

3. La membrana sinovial dels animals artrítics va presentar un increment en les cèl·lules $OX39^+$, que ja va ser manifest durant la fase de latència, i un augment en el nombre de cèl·lules $OX6^+$ i $OX33^+$ a partir de l'establiment de la patologia.

4. El tractament amb dexametasona (0,3 mg/kg/dia, p.o) va revertir la sinovitis observada en els animals artrítics i va restaurar, a excepció de les cèl·lules $CD8^+$, els increments de les poblacions cel·lulars detectats en sinòvies inflamades.

5. La immunoteràpia de l'artritis adjuvant amb l'AcMo anti-TCR-γ/δ de rata, V65, administrat de forma preventiva des del naixement o bé de forma terapèutica, abans o durant el màxim d'inflamació, no va modificar la simptomatologia clínica ni les alteracions hematològiques de l'AA, fet que indica que els limfòcits T-γ/δ no intervenen en la seva patogènia.

6. El tractament de l'artritis adjuvant amb l'AcMo anti-TCR-γ/δ iniciat abans del màxim d'inflamació va potenciar, en fases tardanes de la patologia, el grau de destrucció articular present en els animals artrítics, suggerint un paper protector fase-depenent dels limfòcits T-γ/δ.

7. L'administració de l'AcMo anti-TCR-γ/δ va provocar la desaparició de l'expressió normal del TCR en els limfòcits T-γ/δ de sang i ganglis limfàtics. En una part de la població cel·lular T-γ/δ es va detectar la *down-regulation* del TCR mitjançant la presència de limfòcits amb expressió reduïda del receptor.

8. L'administració de l'AcMo anti-TCR-γ/δ no va modificar el percentatge de limfòcits T-α/β ni l'expressió antigènica del TCR-α/β.

9. El gradient de sedimentació Ficoll-Isopaque adaptat a rata i els tres mètodes de lisi d'eritròcits estudiats (lisi amb clorur amònic, amb reactiu de Becton & Dickinson i el sistema Coulter Q-prep) són, els quatre, mètodes potencialment vàlids i aplicables en l'aïllament i caracterització fenotípica de limfòcits de sang perifèrica de rata.

10. El gradient de densitat Ficoll-Isopaque adaptat a rata és el mètode que proporciona uns percentatges de limfòcits $CD5^+$, $CD4^+$ i $CD25^+$ més baixos, suggerint una pèrdua selectiva d'alguns limfòcits T. Amb el mètode de lisi amb clorur amònic s'obtenen els percentatges més baixos de limfòcits B, la qual cosa suggereix que els limfòcits B són sensibles a les condicions d'aquest mètode.

11. L'administració de set dosis de l'AcMo anti-CD4 W3/25 durant la fase de latència de l'AA va prevenir el desenvolupament de la inflamació articular, fet que confirma el paper essencial dels limfòcits T $CD4^+$ en la patogènesi de l'AA.

12. L'administració de l'AcMo anti-CD8 OX8 durant la fase de latència de l'AA no va afectar el desenvolupament de la inflamació articular. A més a més, l'administració conjunta de l'AcMo OX8 amb l'AcMo W3/25 no va modificar l'efectivitat de l'AcMo W3/25, la qual cosa indica que els limfòcits T $CD8^+$ no intervenen directament ni exerceixen un paper regulador sobre els limfòcits T $CD4^+$ en la patogènesi de l'AA.

13. El tractament preventiu amb l'AcMo W3/25 sol o conjuntament amb l'AcMo OX8 va induir un estat de resistència enfront d'una segona inducció de la patologia, realitzada 45 dies després de l'última administració d'AcMo.

14. L'administració de l'AcMo W3/25 produeix majoritàriament una *down-regulation* de la molècula CD4 en la superfície dels limfòcits T de sang perifèrica.

15. L'administració de l'AcMo W3/25 sol o conjuntament amb l'AcMo OX8 durant la fase de latència de l'AA no va modificar la producció d'anticossos dirigits contra l'agent inductor. Aquest fet demostra que la *down-regulation* de la molècula CD4 no interfereix el desenvolupament de la resposta immunitària humoral.

16. El tractament amb l'AcMo anti-CD4 W3/25 iniciat un cop instaurada la patologia és capaç de revertir la inflamació articular ja establerta, fet que indica una implicació directa dels limfòcits $CD4^+$ en la perpetuació de l'AA.

17. L'efectivitat terapèutica de l'AcMo W3/25, que ja és aparent després de l'administració de dues dosis, desapareix després de la sisena dosi. Aquesta pèrdua d'efectivitat es pot atribuir a la presència d'anticossos dirigits contra l'AcMo administrat, detectats en el plasma dels animals tractats.

18. El tractament amb l'AcMo anti-CD8 OX8 iniciat quan la patologia ja és establerta no modifica el curs de l'AA. A més a més, la teràpia combinada W3/25+OX8 produeix els mateixos efectes beneficiosos que l'administració de l'AcMo W3/25.

19. La deplecció dels limfòcits $CD8^+$, conseqüent al tractament curatiu amb l'AcMo OX8, s'acompanya d'un fort increment dels nivells d'anticossos anti-*Mycobacterium butyricum*. Aquests resultats mostren la influència dels limfòcits T $CD8^+$ en la regulació de la resposta immunitària humoral. Aquest fet, però, no ha interferit en el desenvolupament de la resposta inflamatòria.

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