Activation of CD4+ T Cells in the Presence of a Nondepleting Monoclonal Antibody to CD4 Induces a Th2-Type Response In Vitro

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Summary

In vitro experiments using purified rat CD4+ T cells in primary and secondary mixed leukocyte cultures (MLC) have been carried out to explore the mechanism of inhibition of cell-mediated autoimmune disease in the rat by a nondepleting monoclonal antibody (mAb) to CD4. Previous work has shown that W3/25, a mouse anti-rat CD4 mAb of immunoglobulin G1 isotype, completely prevents the development of the paralysis associated with experimental allergic encephalomyelitis (EAE) in Lewis rats, but does so without eliminating the encephalitogenic T cells. The in vitro experiments described in this study have shown that when CD4+ T cells were activated in the presence of the anti-CD4 mAb in a primary MLC, the synthesis of interferon (IFN) γ, but not interleukin (IL) 2, was completely inhibited. After secondary stimulation, now in the absence of the mAb, the synthesis of IL-4 and IL-13 mRNA was greatly enhanced compared with that observed from CD4+ T cells derived from primary cultures in which the mAb was omitted. As IL-4 and IL-13 are known to antagonize cell-mediated immune reactions, and as EAE is cell-mediated disease, the data suggest that the W3/25 mAb controls EAE by modifying the cytokine repertoire of T cells that respond to the encephalitogen. The capacity for the mAb to suppress IFN-γ synthesis provides, in part, an explanation for this change in cytokine production. These findings are discussed in terms of what is known of the factors that determine which cytokine genes are expressed on T cell activation. Possible implications for the evolution of T cell responses in human immunodeficiency virus infection are also discussed.

C D4 is a 55-kD Ig superfamily membrane glycoprotein primarily expressed on the population of thymocytes and mature T lymphocytes that recognize peptide determinants associated with MHC class II molecules on APC (1). The binding of CD4 on T cells to MHC class II is thought to stabilize the T cell receptor–MHC class II interaction and to provide appropriate costimulatory signals (2). However, in rats and humans, but not mice, CD4 is also expressed on macrophages, where, in contrast to the situation with T cells, its function is not known. Mature CD4+ T cells have been shown to be phenotypically and functionally heterogeneous, differing in their ability to respond to alloantigen, mediate lethal graft versus host disease, or provide help for secondary antibody responses (3–5).

Modulating the activity of CD4+ T cells by using mAbs to the CD4 molecule has proved extremely effective in preventing graft rejection and autoimmune disease in animals (for reviews see references 6 and 7), and has recently led to the use of anti-CD4 mAbs in the therapy of human autoimmune diseases, particularly multiple sclerosis and RA (8, 9). Anti-CD4 mAbs can be classified as either depleting or nondepleting depending on their cytotoxic activity, and although

attention has focused on anti-CD4 mAbs that kill their target cells as a form of general immunosuppression, nondepleting antibodies have also proved effective as therapeutic agents.

A nondepleting mAb to rat CD4 (W3/25), known to inhibit CD4+ T cell activation in vitro (10), has been shown to be extremely effective in Lewis rats in the prophylaxis and therapy of experimental allergic encephalomyelitis (EAE)[1], a cell-mediated autoimmune disease of the central nervous system induced by immunization with myelin basic protein (MBP) (11). Depending on the time of administration, W3/25 mAb can either halt the course of active EAE or completely prevent the onset of disease (11, 12). W3/25 mAb–protected rats have normal levels of CD4+ T cells and are a good source of T cells capable of adoptive transfer of EAE to naive recipients after in vitro activation with MBP (12), confirm-

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ing that the MBP-reactive T cells are not killed by the antibody and are not anergic.

The ability to treat disease without killing the target cell makes nondepleting anti-CD4 mAbs particularly attractive for therapy; however, to date it remains unclear how these mAbs provide their therapeutic effects. The aim of this study was to develop a system with which to examine how W3/25 mAb modifies the activity of CD4$^+$ T cells to prevent EAE. For this purpose, the influence of W3/25 mAb on the allogeneic MLC was analyzed. It was found that cytokine gene expression after secondary stimulation was strongly affected by the presence of the anti-CD4 mAb in the primary culture. The results provide a possible explanation for the capacity of W3/25 mAb to control cell-mediated immunity in vivo and suggest criteria for the selection of anti-CD4 mAbs for therapeutic use in humans. They also suggest a mechanism whereby human immunodeficiency virus (HIV) can promote its own expansion in vivo.

Materials and Methods

Animals. Male inbred PVG (RT1^c) and DA (RT1^a) strain rats were obtained specific pathogen free (SPF) from the MRC Cellular Immunology Unit, University of Oxford, and used on the day of removal from the SPF unit.

Antibodies. W3/25 (IgG1, mouse anti–rat CD4 domain 1) (13) IgG was purified from ascites by ion exchange chromatography (Sepharose Q/FPLC; Pharmacia LKB, Uppsala, Sweden) using a 0.15–1.5-M NaCl gradient. The eluted antibody was dialyzed against PBS, filter sterilized, and stored at −20°C. The OX6 (IgG1, mouse anti–rat MHC class II) (14), OX8 (IgG1, mouse anti–rat CD8) (15), OX12 (IgG2a, mouse anti–rat Ig κ chain) (16), OX19 (IgG1, mouse anti–rat CD5) (17), OX21 (IgG1, mouse anti–human C3b inactivator) (18), and W3/13 (IgG1, anti–rat CD43) (13) mAbs were used as tissue culture supernatants, or where indicated as purified IgG prepared from ascites as described above. OX81 (IgG1), a mouse neutralizing mAb to rat IL-4 (Fowell, D., M. Puklavec, S. Simmonds, and D. Mason, to be published), was used as purified IgG prepared from ascites as described above. Rabbit anti–mouse IgG cross-reacting with rat IgG (RAMR) was purified from sera of rabbits immunized with mouse IgG by affinity chromatography on rat IgG Sepharose 4B (Pharmacia LKB).

Preparation of CD4$^+$ Responder Cells. CD4$^+$ T cells were purified from thoracic duct lymphocytes (TDL) of PVG rats by rosette depletion as previously described (19). Briefly, TDL obtained by cannulation (20) were washed in PBS containing 0.2% BSA (PBS/BSA) and incubated for 1 h on ice with a mixture of the OX6, OX8, and OX12 mAbs. Labeled cells were then rosette depleted by incubating with RAMR-coated SRBC followed by brief centrifugation, and the supernatants were recovered and cleared of erythrocytes by hypotonic lysis and washing in PBS/BSA. The remaining cells were consistently >99% pure CD4$^+$ T cells as assessed by flow cytometry (FACScan®; Becton Dickinson & Co., Mountain View, CA).

Preparation of Allogeneic Stimulator Cells. Unfractionated spleen cells (SpC) from DA rats were prepared as single-cell suspensions by gently disrupting the tissue through stainless steel mesh into PBS/BSA. Debris was removed by filtration through lens tissue, and the cells were washed and irradiated with 25 Gy of ^{137}Cs irradiation.

Primary MLC. Primary (1°) MLC was carried out as follows. PVG CD4$^+$ responder cells and DA stimulator SpC were resuspended in RPMI-1640 medium supplemented with 2 mM glutamine, 2.5 × 10^{-5} M 2-ME, 1 mM sodium pyruvate, and antibiotics, with heat-inactivated FCS added at a final concentration of 10% (complete RPMI). Responder and stimulator cells were mixed in 96-well round-bottom tissue culture plates in a final volume of 200 μl, incubated for 72 h at 37°C in 5% CO$_2$, and then pulsed for 18 h with 0.5 μCi [^{3}H]thymidine. Cells were then harvested and assayed for radiolabel incorporation by liquid scintillation counting (1211 Rackbeta; Pharmacia LKB) and the results were expressed as mean cpm of triplicate wells. At various times throughout the culture, supernatants were harvested for cytokine analysis and cells harvested for mRNA preparation (see below). Stimulator cell doses were kept constant at 5 × 10^5 cells/well and responder cell doses were usually 2 × 10^6 cells per well, except where indicated. mAbs W3/25 or OX21 IgG were added at the beginning of the cultures at a final concentration of 5 μg/ml, except where otherwise indicated.

For cytokine supplementation of 1° MLC, cytokines were included from the beginning of the cultures at final concentrations of 50 U/ml for IL-2 and IL-4, or 100 U/ml for IFN-γ (see below for details of recombinant cytokines).

IL-2 Expansion and Secondary MLC. After 1° MLC activation, cells for secondary (2°) MLC stimulation were washed in warm complete RPMI and resuspended to a concentration of 10^6/ml in complete RPMI containing 50 U/ml of recombinant rat IL-2 (see below for details). After an expansion phase of 72 h in IL-2 at 37°C, the cells were washed, adjusted to 10^7 cells/ml, and 100 μl was mixed with 100 μl of irradiated DA SpC diluted to 5 × 10^6 cells/ml. Subsequently, at various times, cell proliferation was assessed as described above, supernatants were harvested for cytokine analysis, and cells were harvested for mRNA preparation (see below).

Recombinant Cytokines and Cytokine Assays. IL-2 production was assessed using proliferation of the CTLL-2 cell line as previously described (21). Briefly, tissue culture supernatants were added to 2 × 10^4 CTLL cells at a final concentration of 10% and incubated for 18 h at 37°C. The cells were then pulsed with 0.5 μCi of [^{3}H]thymidine for 6 h, harvested, and radiolabel incorporation was determined by liquid scintillation counting. Values are expressed as units per milliliter of IL-2 as derived from a standard curve constructed using a commercial preparation of recombinant human IL-2 (Boehringer Mannheim GmbH, Mannheim, Germany). Recombinant rat IL-2 for use in cell culture was obtained as serum-free tissue culture supernatant (10^4 U/ml) grown from a transfected CHO cell line (22). The supernatant was dialyzed against PBS and filter sterilized before use.

IFN-γ levels were determined by an antigen capture ELISA using 96-well microtiter plates coated overnight at 4°C with 10 μg/ml of an anti–rat IFN-γ mAb (DB-1) and blocked for 30 min with 1% BSA in PBS. Undiluted tissue culture supernatants (50 μl/well) followed by rabbit anti–mouse IFN-γ antiserum that cross-reacts with rat IFN-γ (diluted 1:200) and a swine anti–rabbit IgG–alkaline phosphatase–conjugated antiserum diluted 1:1,000 (Dakopatts, Glostrup, Denmark) were sequentially incubated for 2 h at room temperature, separated by washes with PBS containing 0.05% Tween 20. Antisera were diluted in PBS containing 14% normal mouse serum, 5% FCS, 0.05% Tween 20, and 10 mM NaN$_3$. OD at 405 nm (Titretek Multiskan MCC/340; Labsystems, Helsinki, Finland) was then determined after adding the enzyme substrate 4-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) at 5 mg/ml for 45 min at room temperature. Values are expressed as units per milliliter of IFN-γ derived from a standard curve constructed using

serial dilutions of purified rat recombinant IFN-γ of known concentration. The DB-1 mAb and recombinant rat IFN-γ were kindly provided by Dr. P. van der Meide (TNO, Rijswijk, The Netherlands). The rabbit anti–mouse IFN-γ antiserum was provided by Dr. J. Tite (Wellcome Research Laboratories, Beckenham, UK).

Detection of IL-4 was achieved by a bioassay to determine upregulation of expression of MHC class II molecules on B cells. Rat B cells were purified from TDL by direct rosetting using W3/13 mAb-labeled SRBC (19). 50 μl of tissue culture supernatant was added to 5 × 10⁵ B cells in 96-well tissue culture plates and made to a final volume of 200 μl. After incubating for 18 h at 37°C, the cells were washed and labeled with ¹²⁵I-OX6 IgG (1.5 × 10⁵ cpm) for 1 h at 4°C. After washing, bound radiolabel was measured by gamma counting (1261 Multigamma; Pharmacia LKB) using 15-s counts per tube. Assay specificity was determined by means of a neutralizing mAb to rat IL-4 (OX81) added at the beginning of the 18-h culture period at 100 μg/ml IgG final concentration. Values are expressed as units per milliliter of IL-4 derived from a standard curve constructed using serial dilutions of recombinant rat IL-4 obtained as tissue culture supernatant (10⁴ U/ml) from a transfected CHO cell line (22). 1 U was defined as that concentration of IL-4 that gave 50% of maximal induction of MHC class II on B cells, as assessed by the ¹²⁵I-OX6 radioimmunoassay.

Reverse Transcriptase PCR.   Reverse transcriptase (RT)-PCR was performed as follows. Total RNA was prepared from 2 × 10⁶ MLC-stimulated lymphocytes by RNAzol B® extraction according to the manufacturer's instructions (Biogenesis, Poole, UK) and mRNA reverse transcribed to cDNA using oligo-dT priming and murine Moloney leukemia virus reverse transcriptase (Gibco Laboratories, Paisley, UK) in a final volume of 40 μl as described (23). For semiquantitative PCR analysis of cytokine mRNA levels, 10-fold dilutions of the cDNA were amplified in 50-μl reaction volumes as previously described (23) using 2.5 mM MgCl₂ for IL-4 and β-actin or 3.0 mM for IL-13. Primer sequences for rat IL-4 and β-actin have been previously described (23), and for rat IL-13 were 5′ CAGGGAGCTTATCGAGGAGC 3′and 3′ CGAGTTAGTAGG-ACTTTTGAAG 5′, based on the published sequence for rat IL-13 (24). Primers were designed to produce amplified products of 378 bp for IL-4, 279 bp for IL-13, and 607 bp for β-actin. Cycle conditions were 93°C for 1 min, 60°C for 2 min, and 72°C for 3 min using 35 cycles for IL-4 and IL-13 or 20 cycles for β-actin. 10 μl of amplified product was then separated by electrophoresis on 1.5% agarose minigels, visualized by ethidium bromide staining, and saved as a digital image (Appligene Imager; Appligene Inc., Pleasanton, CA).

Results

Effect of W3/25 Anti-CD4 mAb on CD4⁺ T Cell Proliferation in the 1° MLC.   Previous studies have shown that the W3/25 mAb is a potent inhibitor of T cell activation in the MLC (10), but it has also been shown that this inhibition is not complete at high responder cell numbers (Mason, D., and S. Simmonds, unpublished observations). These findings suggested the presence of a population of CD4⁺ T cells that were at least partly refractory to the inhibitory actions of W3/25 mAb in vitro, and this effect was confirmed in this study. As shown in Fig. 1 A, the mAb was a potent inhibitor of the MLC at low responder cell doses (1.25–5 × 10⁵ cells/well), where almost 100% suppression was observed. In contrast, as CD4⁺ responder T cell numbers were increased in excess of 5 × 10⁵ cells per well, a significant de-
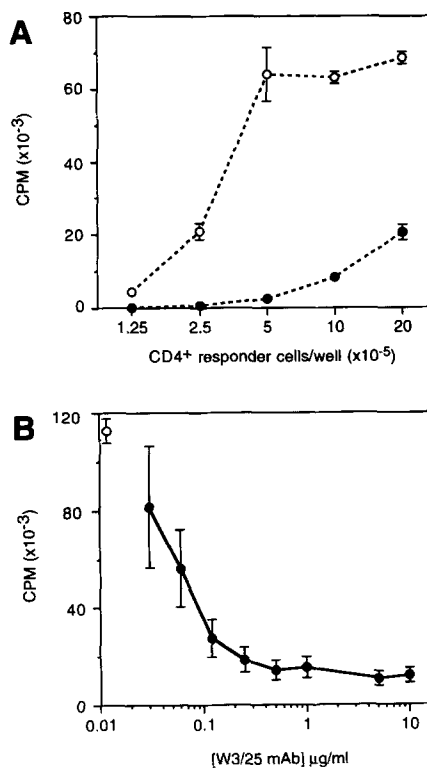

Figure 1.   Inhibition of cell proliferation in the 1° MLC by W3/25 mAb. (*A*) Increasing numbers of purified PVG CD4⁺ responders were stimulated in an allogeneic 1° MLC using a constant number of irradiated DA SpC as stimulators (5 × 10⁵ cells/well). All cultures were incubated for a total of 90 h, and proliferation was determined by [³H]thymidine uptake during the final 18 h of culture. W3/25 IgG was added at the beginning of the cultures at 5 μg/ml (*solid circles*), while control cultures contained the isotype-matched OX21 mAb (*open circles*). Results are expressed as mean cpm ± SE of triplicate wells of a representative experiment, and similar results have been observed in at least three independent experiments. (*B*) Increasing concentrations of W3/25 mAb IgG were titrated into a 1° MLC using 2 × 10⁶ purified PVG CD4⁺ responders and 5 × 10⁵ irradiated DA SpC stimulators per well. The open circle indicates the proliferation observed when W3/25 mAb was replaced by 10 μg/ml of OX21 mAb in the cultures. Results are expressed as described above and repeated on three independent occasions.

gree of cellular proliferation was observed, reaching levels 20–30% of that of control cultures at the highest cell dose.

To exclude the possibility that this proliferation was simply due to limiting amounts of W3/25 mAb, increasing concentrations of the mAb were titrated into an MLC established at high responder cell numbers (Fig. 1 B). Maximal inhibition of proliferation was achieved at an mAb concentration of ∼0.5 μg/ml, after which point increasing the concentration had no additional effect. A concentration of 5 μg/ml mAb was routinely used in further assays, this being a level at least 10 times in excess of that required for maximal inhibition of proliferation at high responder cell doses. Furthermore, flow cytometric analysis of blast cells at the end of the culture period indicated that the W3/25 mAb had uniformly bound all CD4⁺CD5⁺ T cells and had not modulated cell surface expression of the CD4 antigen (data not shown).

Cytokine Production by CD4⁺ T Cells Activated in the Presence of Anti-CD4 mAb in the 1° MLC. As discussed in the introduction, the previous observation that the nondepleting W3/25 mAb was able to very effectively control EAE in rats given an encephalitogenic immunization with MBP suggested that the mAb was inducing a regulatory mechanism capable of suppressing cell-mediated immune responses. The present finding that some CD4⁺ T cells were refractory to the inhibitory effects of W3/25 mAb in vitro has raised the possibility that such refractory cells might provide the regulatory mechanism capable of controlling EAE in vivo. The primary aim of this study was to determine whether the CD4⁺ T cells that proliferated in the presence of W3/25 mAb in the MLC possessed a cytokine repertoire compatible with this hypothesis.

To examine this question, 1° MLCs were established at high responder cell doses (2 × 10⁶ cells per well) in the presence or absence of W3/25 mAb, and supernatants were examined for the presence of cytokines indicative of a Th1- (IL-2, IFN-γ) or a Th2- (IL-4, IL-10, IL-13) type CD4⁺ T cell response (25) (Fig. 2). Levels of IL-2 produced by cells grown in the presence of W3/25 mAb, although reduced when compared with control cultures, did tend to reflect the degree of cell proliferation. Thus, IL-2 was detectable by 48 h of culture in the presence of W3/25 mAb (before signs of cellular proliferation), and levels continued to increase in parallel with the degree of proliferation in a similar manner to that seen in uninhibited control cultures (Fig. 2 C). This level of production of IL-2 was not affected by increasing the concentration of mAb in the cultures (Fig. 2 D). In addition, CD4⁺ T cells activated in the presence of W3/25

mAb displayed normal levels of IL-2 receptor, as demonstrated by flow cytometric analysis (data not shown).

In contrast, IFN-γ was virtually undetectable at all times of culture (Fig. 2 A) when mAb concentrations were >1 μg/ml (Fig. 2 B), despite a significant degree of cell proliferation after 90 h (~20 × 10³ cpm). This inhibition could not have simply been a consequence of a lower level of cell proliferation in the cultures containing W3/25 mAb because if IFN-γ production were proportional to cell proliferation, then after 90 h the inhibited cultures should have produced ~50 U/ml of IFN-γ. This complete suppression of IFN-γ production has been consistently observed in MLCs inhibited by W3/25 mAb. As subsequent experiments showed (see below), the inhibition of IFN-γ synthesis by W3/25 mAb in the 1° MLC had profound effects on the subsequent synthesis of IL-4 after 2° MLC stimulation.

For IL-4, IL-10, and IL-13, mRNA encoding these cytokines was detectable after 1° MLC activation, as determined by semiquantitative RT-PCR. However, the message levels for these cytokines were similar in both W3/25 mAb–treated and control cultures (data not shown). Furthermore, bioassays for IL-4 showed that these 1° MLCs produced little of this cytokine (data not shown).

Effects of Exogenous IL-4 on CD4⁺ T Cells Activated in the 1° MLC in the Presence of Anti-CD4 mAb. Experiments were performed to determine the effects of adding various cytokines to the 1° MLC on the subsequent proliferation of CD4⁺ T cells. Supplementing cultures containing W3/25 mAb with IL-4 produced a significant increase (~300%) in the level of T cell proliferation compared with control cultures not containing the mAb (Fig. 3). This effect was much larger than

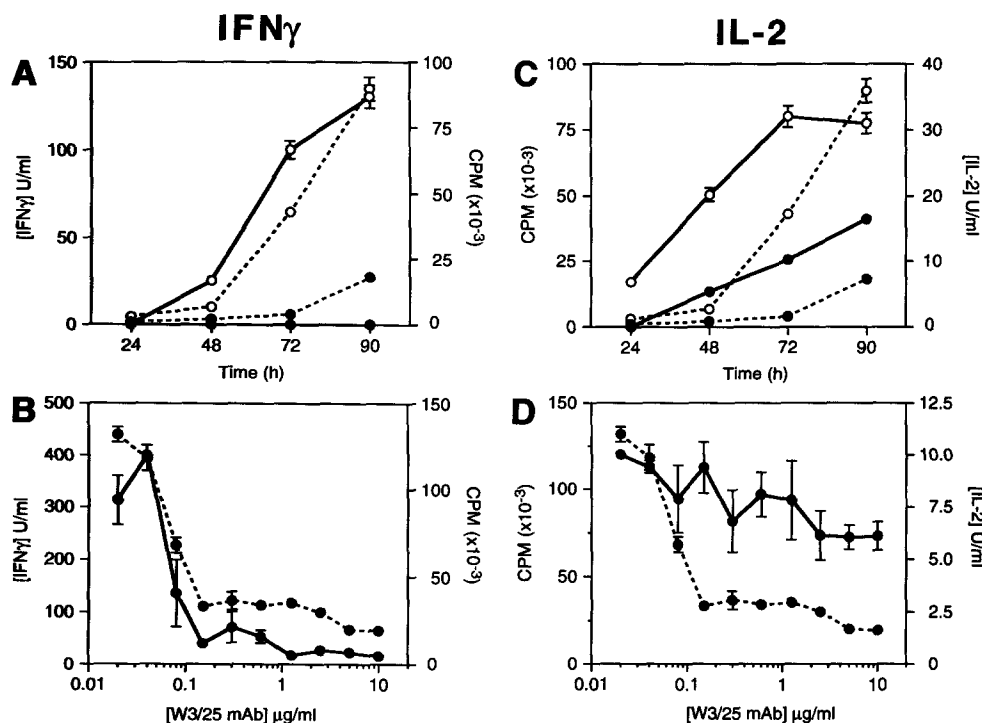**IFNγ**

IL-2

Figure 2. IL-2 and IFN-γ production during the 1° MLC. MLCs were established using 2 × 10⁶ PVG CD4⁺ responders and 5 × 10⁵ irradiated DA SpC stimulators per well in the presence of 5 μg/ml W3/25 mAb and samples of tissue culture supernatant taken at the indicated times for analysis of (A) IFN-γ and (C) IL-2 protein as described in Materials and Methods. Alternatively, W3/25 mAb was titrated into the cultures from 0.2 to 10 μg/ml, and supernatants were analyzed after 90 h for (B) IFN-γ and (D) IL-2. Solid lines indicate cytokine production (IFN-γ or IL-2), and broken lines indicate cell proliferation. Solid circles represent cultures grown in the presence of W3/25 mAb, while open circles represent control cultures containing 5 μg/ml OX21 mAb. The mean proliferation (CPM) or cytokine concentration (U/ml) ± SE of triplicate wells from a representative of at least three independent experiments is shown.

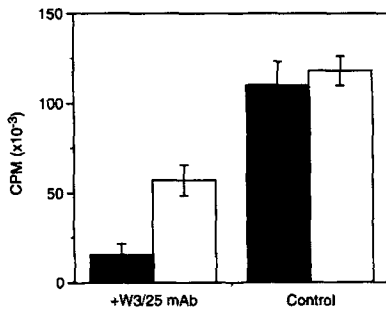**Figure 3.** Influence of supplementing the 1° MLC with IL-4. 1° MLCs were established at 2 × 10^{6} CD4+ responders and 5 × 10^{5} irradiated DA SpC stimulators per well in the presence of 5 μg/ml W3/25 IgG or controls containing 5 μg/ml OX21 IgG. Recombinant rat IL-4 was added at the beginning of some of the cultures at a final concentration of 50 U/ml (*open bars*), and the proliferation of these cells was compared with that of cells grown in the absence of added IL-4 (*solid bars*). Results are expressed as the mean cpm ± SE of triplicate wells of three experiments.

Figure 4. Semiquantitative RT-PCR analysis of IL-4 and IL-13 mRNA production after 2° MLC. CD4+ T cells stimulated for 90 h in a 1° MLC (2 × 10^{6} PVG responders, 5 × 10^{5} DA SpC stimulators per well) in the presence (+) or absence (−) of 5 μg/ml W3/25 mAb were expanded in IL-2 for 3 d and then restimulated in a 2° MLC in the absence of W3/25 mAb using 10[6] responders and 5 × 10^{5} DA SpC stimulators per well. cDNA was reverse transcribed from mRNA prepared at 24, 48, and 72 h of 2° MLC culture and diluted in 10-fold steps from neat to 1:100. PCR was then performed using oligonucleotide primers specific for rat IL-4, IL-13, and β-actin, and the products were separated on agarose gels and analyzed as described in Materials and Methods. Representative gels of three independent experiments are shown.

that observed when cultures were supplemented with IFN-γ or IL-2, which both induced a <50% increase in proliferation when added in the presence of W3/25 mAb (data not shown). Supplementing control cultures not containing W3/25 mAb with these cytokines produced no significant increases in proliferation, presumably because endogenous IL-2 and IFN-γ synthesis was already inducing optimum T cell proliferation. This finding indicated that the addition of the W3/25 mAb in the 1° MLC cultures resulted in responsiveness to IL-4 but, as the bioassays had already indicated, little IL-4 synthesis. This conclusion was further supported by the observation that incorporating the OX81 anti–rat IL-4 mAb in W3/25 mAb–treated 1° MLCs had no appreciable effect on cell proliferation (data not shown).

Cytokine Production after 2° MLC. To determine whether the presence of W3/25 mAb in the 1° MLC had any significant effects on subsequent cytokine synthesis, cells activated in the presence of the mAb in a 1° MLC were restimulated in a 2° MLC and cytokine synthesis analyzed. When assessed by semiquantitative RT-PCR analysis, mRNA encoding IL-4 could be detected at high levels at all times of the 2° MLC when W3/25 mAb was included in the 1° MLC (Fig. 4). In contrast, after 24 h of culture, IL-4 mRNA was no longer detectable in 2° MLCs of cells stimulated in the 1° MLC in the absence of W3/25 mAb, despite equal quantities of cDNA, as indicated by the similar β-actin mRNA levels (Fig. 4). Similarly, production of IL-13 mRNA was dramatically increased in 2° MLCs of cells inhibited by W3/25 mAb in the 1° MLC, to levels ~100-fold greater than those seen in control cultures (Fig. 4). In contrast, however, levels of mRNA encoding IL-10 were similar between W3/25 mAb–treated cultures and controls at each time point (data not shown).

To confirm that the cytokine mRNA for IL-4 was translated into secreted protein, 2° MLC supernatants of cells treated with W3/25 mAb in the 1° MLC were assayed for their ability to upregulate MHC class II expression on B cells. 24-h and 48-h supernatants from these cultures induced significant upregulation of MHC class II, which was blocked with OX81,
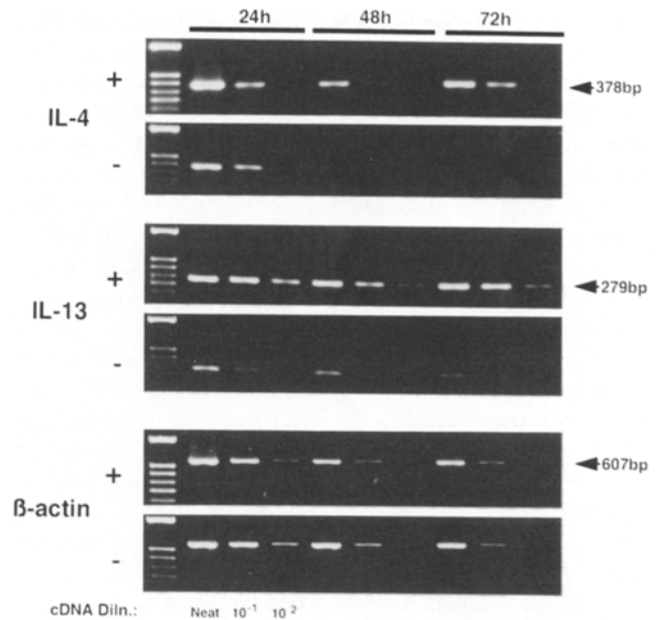
a neutralizing mAb to rat IL-4, suggesting that the up-regulation observed was due to IL-4 and not to IL-13 (Fig. 5 *A*). However, the possibility that OX81 also reacted with rat IL-13 cannot be excluded, this being theoretically possible since IL-4 and IL-13 share a common receptor element (26). Consistent with the mRNA data, cultures not containing W3/25 mAb in the 1° MLC stimulation produced no significant up-regulation of MHC class II on B cells (Fig. 5 *A*). In contrast, similarly high levels of both IL-2 and IFN-γ were produced after 24 or 48 h of secondary stimulation, regardless of whether or not the cells had seen the W3/25 mAb in the 1° MLC (Fig. 5 *B*).

In these experiments, the starting population of CD4+ T cells, although highly purified, was nevertheless heterogeneous in that it contained a mixture of activated cells, as well as naive and memory cells. To generate a more homogeneous starting population of responder cells and to examine the contribution of activated cells to the observed up-regulation of IL-4 production, purified whole CD4+ T cells were depleted of activated (OX39+) cells by rosetting and subjected to 1° and 2° MLC in the presence or absence of W3/25mAb. It was found that removal of activated cells did not reduce, and if anything increased, the levels of IL-4 produced after 2° MLC, when the cells were primarily activated in the presence of W3/25 mAb (data not shown). Further subdivision of the
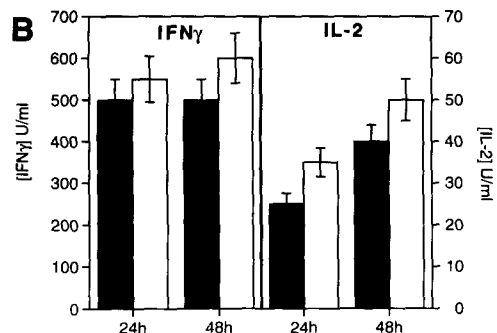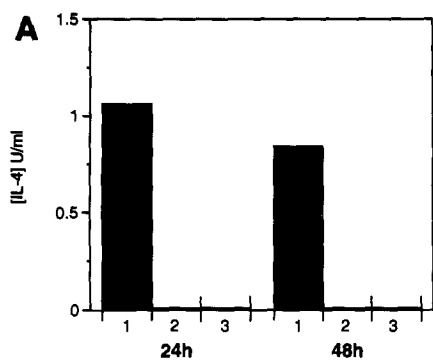
Figure 5. IL-2, IL-4, and IFN-γ protein production after 2° MLC. CD4+ T cells were activated in 2° MLCs after exposure to W3/25 mAb in the 1° MLC as described for Fig. 4, and supernatants were harvested at 24 and 48 h and analyzed for the presence of cytokines. (*A*) Analysis of IL-4 by MHC class II up-regulation on B cells. (column 1) W3/25 mAb added to the 1° MLC; (column 2) OX21 mAb added to the 1° MLC; (column 3) OX81 anti–IL-4 mAb added to the bioassay for the supernatants of column 1. (*B*) Analysis of IL-2 and IFN-γ by ELISA. Solid bars show W3/25 mAb added to the 1° MLC; open bars show OX21 mAb added to the 1° MLC. Results are expressed as mean units per milliliter ± SE of triplicate wells for one representative out of three experiments.

CD4+ T cell starting population is now required to determine the nature of these IL-4–producing cells.

Effect of Adding IFN-γ or Neutralizing IL-4 in the 1° MLC on Cytokine Expression in the 2° MLC.   As previously illustrated, the inclusion of W3/25 mAb in the 1° MLC resulted in reduced T cell proliferation and the complete suppression of IFN-γ synthesis (Fig. 2), while supplementing these cultures with exogenous IFN-γ resulted in a modest increase in cell proliferation. However, as shown by RT-PCR and MHC class II up-regulation on B cells, 1° MLCs supplemented with a mixture of W3/25 mAb and IFN-γ failed to show the sustained expression of mRNA for IL-4 (Fig. 6) and IL-4 protein synthesis (data not shown) that were characteristic of cultures treated with W3/25 mAb alone (Figs. 4 and 5). Furthermore, addition of the neutalizing antibody to IL-4 (OX81) together with the W3/25 mAb in the 1° MLC cultures had no effect on IL-4 production in the 2° MLC (data not shown). Thus, it appears that the enhanced IL-4 synthesis observed on 2° MLC stimulation of cells treated with W3/25 mAb in the 1° MLC is not dependent on the presence of IL-4 but rather depends, at least in part, on the ability of the mAb to suppress IFN-γ production.
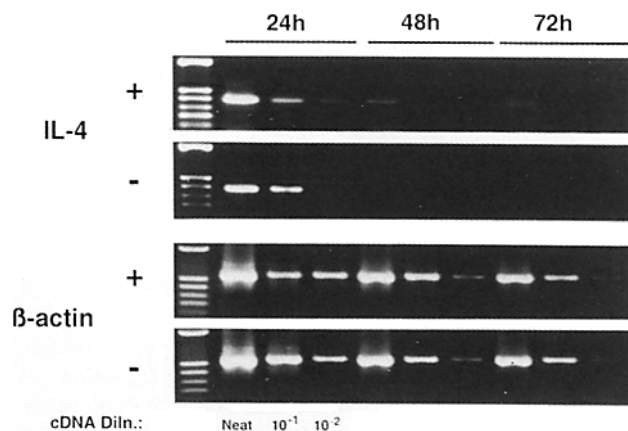

Figure 6. RT-PCR analysis of IL-4 production in the 2° MLC after supplementing the 1° MLC with IFN-γ. CD4+ T cells were stimulated in a 1° MLC in the presence (+) or absence (−) of 5 μg/ml W3/25 mAb and in the presence of 100 U/ml of recombinant rat IFN-γ and then restimulated in a 2° MLC in the absence of both W3/25 mAb and exogenous IFN-γ. RNA was isolated at the times indicated, reverse transcribed to cDNA, diluted, and analyzed by PCR for IL-4 and β-actin mRNA as described for Fig. 4.

Discussion

These studies have analyzed the effect of the anti-CD4 mAb W3/25 on CD4+ T cell activated in vitro in terms of cell proliferation and cytokine production by means of the 1° and 2° MLC in an attempt to explain the ability of the mAb to inhibit cell-mediated immunity in vivo. In terms of cytokine production, the most striking effects of incorporating W3/25 mAb during cell activation were the complete inhibition of IFN-γ (but not IL-2) production in the 1° MLC (Fig. 2) and the greatly enhanced levels of IL-4 and IL-13 synthesis after 2° MLC stimulation (Fig. 4). IL-4 and IL-13 are known to antagonize the effects of cell-mediated immunity in vivo (26, 27), so the above finding suggests an explanation for the capacity of this antibody to control EAE in vivo. This interpretation is supported by recently published observations that the suppression of allograft rejection in the rat by a nondepleting anti-CD4 mAb was associated with a decrease in Th1 cytokines and maintenance of Th2 cytokine production by graft-infiltrating cells (28, 29), and by the observations of Mannie and associates (30), who demonstrated a positive correlation between IL-4 synthesis and resistance to W3/25 mAb inhibition in vitro in MBP-specific T cell lines and hybrids. Interestingly, the four- to fivefold increase in the production of IL-4 in the 2° MLC in these experiments was not matched by a significant reduction in IFN-γ synthesis upon 2° MLC stimulation, indicating that the suppression of IFN-γ synthesis seen in the 1° MLC was reversible. These in vitro observations of the reversibility of the suppression of IFN-γ production provide an explanation for the in vivo effects of the mAb when used to treat EAE, specifically, that splenocytes recovered from protected animals are able to passively transfer EAE when secondarily stimulated in vitro with MBP (12).

The mAb's mode of action in enhancing IL-4 and IL-13 synthesis in vitro is not fully understood. As shown in Fig. 2 B, the inclusion of W3/25 mAb in the 1° MLC completely inhibited IFN-γ synthesis, and this inhibition may have accounted for the enhanced IL-4 synthesis in the 2° MLC. Significantly, the supplementation of 1° MLCs containing W3/25 mAb with IFN-γ strongly inhibited IL-4 synthesis in the subsequent secondary activation. Consistent with this observation, it has been reported that IFN-γ promotes the selection of Th1 clones over Th2 clones in mouse T cell cultures (31).

If the addition of W3/25 mAb to the MLC favors the activation of cells that produce IL-4 because it inhibits IFN-γ synthesis, then the question arises as to why it should have this effect. It has been noted (32) that the activation of Th1 T cells that respond to a given peptide requires that the peptide interacts with relatively high affinity with the relevant MHC molecules on the APC, and that relatively high concentrations of peptide are available. These observations suggest that compared with a Th2 cell, an individual Th1 cell requires a greater number of peptide–MHC interactions for activation to occur. If the blocking of CD4–MHC class II interactions by an anti-CD4 mAb generates a suboptimal activation signal for CD4⁺ T cells (as appears to be the case because most cells responded poorly in the presence of W3/25 mAb), then it may be that only Th2-type cells proliferate in the 1° MLC when the anti-CD4 mAb is present. A similar explanation has been advanced to account for the observations reported for rat allografts (28, 29). However, whether the CD4⁺ T cells proliferating in the presence of the W3/25 mAb represented a distinct subpopulation of cells refractory to the effects of the mAb, or alternatively that all the CD4⁺ T cells were responding, but at a reduced rate of proliferation, is not yet clear, and further experiments are necessary to resolve this point. Furthermore, it remains to be determined whether the cells that proliferate in the presence of the anti-CD4 mAb in the 1° MLC are responsible for the enhanced synthesis of IL-4 and IL-13 in the 2° MLC. In any event, the fact that increasing the mAb concentration to levels >0.5 μg/ml induced no further inhibition of the MLC (Fig. 1 B), in conjunction with the observation that the mAb was uniformly labeling all cells, suggested that the cells proliferating in the presence of the W3/25 mAb at or above this antibody concentration were not using the CD4 antigen as a costimulatory molecule, at least in its normal physiological role. In this respect, it may be noted that although at least the great majority of CD4⁺ T cells that proliferated in cultures not containing W3/25 mAb were responding to MHC class II antigens, at present we have no direct evidence that the CD4⁺ T cells responding in the presence of W3/25 mAb in the 1° MLC are similarly restricted. The fact that the mAb had a potent inhibitory effect in the 1° MLC does not exclude the cytoplasmic portion of the CD4 molecule from playing a role in the activation of those T cells that proliferate in the presence of the mAb, nor does it exclude the possibility that these cells were recognizing non–class II MHC molecules.

It has been shown (33; Ramirez, F., manuscript in preparation) that corticosteroids promote in vitro the development of T cells that secrete IL-4 and IL-13 upon activation. The acute paralytic phase of EAE is associated with a greatly elevated level of circulating corticosterone, and this transient burst of steroid release has been shown to bring about the spontaneous remission that is characteristic of EAE in the rat (34). Given the effects of corticosteroids on IL-4 and IL-13 synthesis referred to above, and the capacity for these cytokines to antagonize cell-mediated immune responses, it has been suggested that the corticosterone release that induces this remission is also responsible for the subsequent refractory phase of EAE (35). The current data showing that W3/25 mAb also enhances IL-4 synthesis raise the possibility that the use of this antibody to treat EAE in the rat may produce effects on cytokine synthesis that augment those induced by endogenous corticosterone release.

Finally, there is evidence that the pathogenesis of HIV-1 infection in humans is associated with a predominance of CD4⁺ T cells secreting Th2 cytokines over those secreting Th1 cytokines (36), and that the virus actively promotes this cytokine switch and preferentially replicates in Th2-type cells (37). To infect CD4⁺ T cells, HIV-1 uses the envelope glycoprotein gp120 to bind CD4 in a region that overlaps the binding site of CD4 for the MHC class II molecule (38). Interestingly, significant amounts of gp120 are shed from the viral surface, and several groups have shown that this soluble protein is able to bind CD4 and block the interaction with MHC class II (39, 40). Consequently, the question arises as to whether or not soluble gp120 produced during the course of HIV infection is capable of blocking the CD4/MHC class II interaction in vivo with the effect of promoting the generation of Th2-type CD4⁺ T cells, which constitute an environment favored by the virus for replication. The effects of gp120 on human T cell differentiation in vitro are under investigation.

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