Reversal of experimental allergic encephalomyelitis with non-mitogenic, non-depleting anti-CD3 mAb therapy with a preferential effect on Th1 cells that is augmented by IL-4

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Abstract

This study examined whether therapy with a non-mitogenic, non-activating anti-CD3 mAb (G4.18) alone, or in combination with the Th2 cytokines, could inhibit induction or facilitate recovery from experimental allergic encephalomyelitis (EAE) in Lewis rats. G4.18, but not rIL-4, rIL-5 or anti-IL-4 mAb, reduced the severity and accelerated recovery from active EAE. A combination of rIL-4 with G4.18 was more effective than G4.18 alone. The infiltrate of CD4+ and CD8+ T cells, B cells, dendritic cells, and macrophages in the brain stem was less with combined G4.18 and IL-4 than G4.18 therapy or no treatment. Residual cells had preferential sparing of Th1 cytokines IL-5 and transforming growth factor-β with loss of Th1 markers IL-2, IFN-γ and IL-12Rβ2, and the Th2 cytokine IL-4 as well as macrophage cytokines IL-10 and tumor necrosis factor-α. Lymph nodes draining the site of immunization had less mRNA for Th1 cytokines, but Th2 and Th1 cytokine expression was spared. Treatment with G4.18, rIL-4 or rIL-5 from the time of immunization had no effect on the course of active EAE. MRC OX-81, a mAb that blocks IL-4, delayed onset by 2 days, but had no effect on severity of active EAE. G4.18 also inhibited the ability of activated T cells from rats with active EAE to transfer passive EAE. This study demonstrated that T cell-mediated inflammation was rapidly reversed by a non-activating anti-CD3 mAb that blocked effector Th1 cells, and spared cells expressing Th2 and Th1 cytokines.

Introduction

Experimental allergic encephalomyelitis (EAE) is an animal model of acute demyelination that has been used to study the mechanisms of central nervous system injury as seen in multiple sclerosis (1,2). EAE can be induced by active immunization with myelin basic protein (MBP) (3), with immunogenic peptides of MBP (4) or by the passive transfer of activated T cells and clones reactive to MBP peptides (5). The mechanisms by which injury to the central nervous system occurs include infiltration of MBP-reactive CD4+ T cells (6) and release of cytokines, especially IFN-γ (7) and tumor necrosis factor (TNF)-α (8). This is followed by infiltration of activated macrophages that are thought to mediate demyelination (9). A variety of therapies that inhibit T cell function have been shown to reduce inflammation, demyelination and clinical symptoms in EAE. This inhibition of T cell functions leads to the prevention of induction of EAE, as well as reversal of established EAE. These therapies include the administration of mAb to CD4 (10–12) and VLA-4, which block T cell migration across the endothelium (13). Therapies directed at specific TCR V, D and J regions
have also been used to block disease (14,15). An alternate therapeutic approach has been used to inhibit effector cytokines, and in particular the Th1 cytokines IFN-γ (16) and TNF-α (17,18).

The inflammation in EAE is thought to be mediated by Th1 cells, that are activated by IL-12 (19) and produce IL-2, IFN-γ and TNF-α, but not Th2 cytokines (20). Induction of Th2 cells is promoted by IL-4, and these cells produce IL-4, IL-5, IL-6, IL-10 and IL-13, but not Th1 cytokines (21). Th1 cells, such as IFN-γ, inhibit the generation of Th2 cells (22), and the Th2 cytokines IL-4 and IL-10 inhibit Th1 cells (23). The role of Th2 cells in EAE is less clear, but these cells may be active in both the mediation and regulation of EAE (24). Recombinant IL-4 therapy has been shown to delay and limit the development of passive EAE induced by the transfer of encephalitogenic Th1 clones (25). They enhance Th2 responses, and inhibit Th1 responses through production of IL-4 and IL-10 (24,26).

Although earlier studies had shown that clones producing Th1 cytokines, especially TNF-α, were best able to mediate EAE (27), other studies suggest that Th1 effector clones can also mediate EAE (28). Th3 cells, which produce transforming growth factor (TGF)-β, are regulatory cells that can transfer tolerance to EAE and are induced in oral tolerance to EAE by feeding with MBP (29). In Th1-mediated autoimmune colitis (30,31) and NOD mice (32), tolerance may be mediated by Th1 regulatory cells that produce IL-5, IL-10 and TGF-β, but not IL-4 or other Th2 cytokines. The role of Th1 cells in the regulation of EAE is unknown.

In multiple sclerosis, anti-CD3 mAb therapy was found to exacerbate disease symptoms, which was postulated to be due to the release of pro-inflammatory cytokines, IFN-γ and TNF-α (33). Anti-CD3 mAb therapy is widely used in organ transplantation models to prevent and reverse rejection, which is also predominantly Th1 mediated (34). Most anti-CD3 mAb activate Th1 cells to release cytokines including IL-2, IFN-γ and TNF-α (35,36). This effect is mediated by the Fc of the mAb binding to antigen-presenting cells (APC), and the facilitation of T cell activation by mAb binding to CD3 and inducing proliferation (37,38). Anti-CD3 mAb which do not bind Fc receptors because of their iso-type, or through the Fc portion being altered by enzymatic digestion (F(ab')2 fragments) or genetic modification, do not induce activation or proliferation of T cells (37–39). These non-mitogenic anti-CD3 mAb selectively block Th1 cells, but not Th2 cells, both in vivo (40) and in vitro (41,42). In vivo treatment of rodents with these mAb can induce transplantation tolerance (43,44) and re-establish tolerance to insulin-dependent diabetes in NOD mice (45).

In this study, we examined if G4.18, a non-mitogenic mouse anti-CD3 mAb of the IgG3 isotype (44), could prevent induction or reverse established EAE in Lewis rats. In vivo, G4.18 induces tolerance to fully allogeneic cardiac allografts in rats, but only causes a minor depletion of circulating T cells (<20%) and transient modulation of the TCR–CD3 complex from the cell surface (44). G4.18 therapy is not associated with T cell activation or release of cytokines, but preferentially inhibits Th1 cytokines and spares Th2 cytokine induction (40). We examined if G4.18 also inhibits Th1 cells in EAE.

### Methods

#### Animals

Lewis (RT-1l) rats were bred and maintained in the Animal House, Liverpool Hospital. BALB/c mice were purchased from the animal breeding facility of the University of NSW (Little Bay, NSW). All animals were fed standard chow and given water ad libitum. Experiments were approved by the Animal Ethics Committee of the University of NSW.

#### Disease induction and monitoring

Active EAE was induced in 12-week-old female Lewis rats immunized with 50 µg of guinea pig MBP emulsified with incomplete Freund's adjuvant (Sigma-Aldrich, St Louis, MO) and 5 mg/ml of heat killed Mycobacterium tuberculosis H37RA (Difco, Detroit, MI). Groups of at least six animals were immunized and treated with G4.18, MRC OX-81 mAb or rIL-4 alone or in combination. Controls included isotype-matched mAb without reactivity to rats, supernatant from non-transfected CHO-K1 cell lines or rIL-5.

All rats were clinically assessed and weighed daily. The animals were graded for disease severity as: 0, normal; 0.5, partial loss of tail tone; 1, complete loss of tail tone; 2, hind limb weakness; 3, hind limb paralysis and front limb weakness; 4, hind and front limb paralysis; 5, moribund or dead. Onset of active EAE was normally at about day 11 post-immunization when there was an abrupt onset of paralysis and marked weight loss. Clinical recovery occurred between days 18 and 30, with gradual weight gain. Due to a variation in the time of onset of disease and the day of peak activity, disease activity was assessed as the mean of the daily results for clinical score and percent weight loss of each animal over days 12–18. Group data was expressed both as the mean ± SD of the daily mean values for days 12–18 inclusive and the mean of the maximal mean clinical score for each animal. At least two animals in each group were sacrificed at day 14 and 18 post-immunization, when brain stems and popliteal lymph nodes (PLN) were collected for immunohistology and analysis of cytokine mRNA by RT-PCR.

#### Production and administration of mAb

mAb used included G4.18 (mouse IgG3) (44) and MRC OX-81 (mouse IgG1), a mAb that blocks rat IL-4 function (46) (a kind gift of Dr Don Mason, MRC Cellular Immunology Unit, Oxford, UK). Isotype control mAb with no reactivity to rats, supernatant from non-transfected CHO-K1 cell lines or rIL-5.

### Results

#### Assays

G4.18 was grown in ascites of BALB/c mice primed by i.p. injection of incomplete Freund's adjuvant (Difco, Detroit, MI) 3 days prior to injection of clones. Ascites were purified on a DEAE–Sepharose column (Pharmacia, Uppsala, Sweden) and antibody concentration was determined by radial immunodiffusion. All mAb preparations had <0.06 U/ml of endotoxin as determined by a Limulus amebocyte lysate assay (Coatest Gel-LAL; Chromogenix, Möndal, Sweden).

#### Animals

Animals were treated with G4.18, BCLA8, MRC OX-81 or A6 mAb at a dose of 7 mg/kg daily by i.p. injection for 14 days or control BCLA8 mAb were given for 14 days, starting...
at day 0, 7 or 12 after immunization. With this dosage, there is sufficient MRC OX-81 mAb in serum 8 days after a single injection to block 10,000 U of rIL-4 function in vivo (48). This dose of MRC OX-81 also inhibits IL-4 function in vivo; it reduces inflammation in experimental uveoretinitis (49,50) and blocks IgG isotype switching in alloimmune responses (48).

**Rat rIL-4 production**

rIL-4 was obtained from the supernatant of cultures of CHO-K1 cell lines stably transfected with rat IL-4 cDNA sequence subcloned into the pEE6. MCMV-GS expression vector (kindly provided by N. Barclay, MRC Cellular Immunology Unit, Oxford, UK). DMEM-F12 medium (Gibco) was supplemented with 10% FCS and 0.05 mM L-methionine sulfoximine (Sigma) as a selection marker. Final cultures were serum free, and supernatant was concentrated 25 times using a Sartocron micro-cross flow filtration unit (Sartorius, Gottingen, Germany) and stored at −70°C. rIL-4 activity was assayed for its ability to induce class II MHC on B cells, as described (51). One unit of rIL-4 was that which induces 50% of maximal class II MHC expression on 5×10^5 B cells. rIL-4 preparations were assayed for endotoxin and only those with <0.06 U/ml were used. rIL-4 was given i.p. daily at 30 μg (10^4 U)/kg for 14 days, either from the day of immunization or the day of onset of disease, usually day 12 post-immunization. Control animals were treated with either supernatant from non-transfected CHO-K1 cell lines or rat rIL-5 produced by transfected CHO-K1 cells in a manner similar to rIL-4 (51). rIL-5 activity was assessed by its ability to support an IL-5 dependent line, as described (52); 5 μg/day of rIL-5, equivalent to 3000 U, was given daily for 14 days.

**Staining of mononuclear cells in blood and tissues with mAb to identify subsets**

Anti-rat mAb used included G4.18 (CD3), MRC OX-35 (CD4), MRC OX-12 (κ chain of Ig), MRC OX-6 (RT-1B, MHC II), MRC OX-8 (CD8), MRC OX-33 (CD45RA, B cell), MRC OX-22 (CD45RC), MRC OX-39 (IL-2 receptor, α chain), MRC OX-34 (CD2) and R7.3 (TCRβ) (PharMingen, San Diego, CA), and ED1 (macrophage) was obtained from Serotec (Oxford, UK). Tissue specimens were stained with an indirect immunoperoxidase technique as described (53). Single-cell suspensions of mononuclear cells were stained with a direct immunofluorescent method and analyzed on a FACScan, as described (53).

**Semi-quantitative PCR**

Methods for RNA extraction, reverse transcription and PCR for cytokines have been described (53). cDNA samples were similarly diluted in diethyl pyrocarbonate-treated water as neat (N), 1/10, 1/20, 1/40 and 1/80. PCR used 1 μl of cDNA, 0.5 U of Taq Polymerase (Biotech International, Perth, Australia), 1.5 mM of MgCl₂ and 125 μM dNTP (Promega, Madison, WI) using specific primers for rat IL-2, IL-10, IL-4, IFN-γ, TNF-α and TGF-β, as described (54). IL-12Rβ2 primers were designed from regions of mouse and human genes with high similarity, and were 5′-ctt ata tct gtt atg aaa tca ggt-3′ and 5′-ctg tca cag ctt tca tca ata-3′ (55,56). Each cDNA sample was analyzed in duplicate, with a positive (cDNA from concanavalin A-activated lymphocytes) and negative (no reverse transcriptase) control included.

All samples were assayed using GAPDH primers, a housekeeping gene, to confirm that the concentration of template cDNA was uniform in all samples. Standard PCR conditions consisted of an initial 3 min denaturation (94°C) followed by 30 s each of denaturation (94°C) and primer annealing (60°C), and 50 s extension (72°C) cycles, then a final extension at 72°C for 4 min, performed on a Corbett thermal cycler (Corbett Research, Sydney, Australia). The number of cycles for each primer set was between 23 and 35 cycles, and was determined to ensure the dilutions were tested on the linear phase of amplification. A quarter of total PCR products were run on 6% polyacrylamide gels with pUC19/HpaI-digested mol. wt marker, stained with ethidium bromide, and photographed under UV light using a Kodak DC40 camera and Digital Science software (Rochester, NY).

**ELISA assay of antibody to MBP**

Serum from all animals collected at day 0, 3, 7, 10, 14, 18 and 21 was used in ELISA assays to detect antibody responses against MBP. Ninety-six-well plates were coated overnight at 4°C with MBP (25 μg/ml) in coating buffer (0.15% Na₂CO₃, 0.29% NaHCO₃, pH 9.5). Plates were washed twice before triplicate samples of serum at 1/200 dilution were added, incubated for 2 h, and then washed and reacted with horseradish peroxidase-conjugated rabbit anti-rat Ig (Dako) diluted at 1/2000. The plates were again washed twice then reacted with phosphatase substrate (Sigma) and read on a microplate reader (BioRad, Hercules, CA) at 450 nm. All data were expressed as a mean of triplicate values and SE < 20% of the mean. All assays included positive controls of hyperimmunized sera from rats repeatedly immunized with complete Freund’s adjuvant (CFA)/MBP after recovery and were collected at day 50 post-immunization. Results were expressed as a percentage of the positive control.

**Passive EAE**

To examine the effects of G4.18 on EAE induced by passive transfer of activated cells, spleens from Lewis rats immunized 10 days prior with MBP/CFA were cultured with concanavalin A (2 μg/ml) for 4 days in DMEM (Difco) and 10% FCS. Then, 3.6×10⁷ cultured cells were injected i.v. into three groups of normal Lewis rats. One group was treated with G4.18 7 mg/kg/day from day of transfer for 14 days. Controls were treated with BLCA8 (Ig isotype control) from day of transfer for 14 days. A third group was treated with G4.18 from day of onset of clinical disease (day 5) until 19 days.

**Statistics**

Significant differences in maximal disease and weight loss between different groups of animals were assessed using the Mann–Whitney U-test and P < 0.05 was considered a significant difference. Data was expressed as mean ± SD.

**Results**

**Effect of early anti-CD3 therapy on induction of active EAE**

Groups of Lewis rats were immunized with MBP in CFA and immediately treated with G4.18 or BLCA8 control mAb (day
cells showed immediate modulation of CD3–TCRαβ receptors, with a reduction in CD3+ cells from 65 to <10% and a reduction in TCRβ+ cells from 70 to <50% within 24 h of administration. No circulating cells had mouse Ig on the surface, indicating that the TCRβ–CD3 receptor had been modulated off the cell surface. There was no reduction in CD2+ cells (72 versus 70%); minor reduction in CD4+ (64 versus 56%) and CD8+ cells (15 versus 13%). TCR–CD3 modulation was observed only in the first 24 h of therapy, after which the number of CD3+ and TCRβ+ cells stained were similar to that in untreated controls. This data was not shown, as it is similar to a previous report (44).

To examine what effect of G4.18 given at the time of immunization on the response against MBP, the PLN draining the site of immunization was examined. At day 14 post-immunization, G4.18-treated nodes were enlarged (56 ± 22 mg, n = 8) compared to normal lymph nodes (4.2 ± 0.84 mg, n = 6), but of similar size to those from rats immunized with CFA without MBP (57 ± 6 mg, n = 3). MBP/CFA-immunized untreated control PLN were significantly larger than all other groups (87.8 ± 14 mg, n = 6, P ≤ 0.009 for normal, 0.001 for CFA and 0.01 for G4.18 treated). G4.18-treated PLN had less CD4+ (20 ± 7 versus 58 ± 10%) and CD8+ (10.5 ± 3.5 versus 33.5 ± 10%) T cell infiltration compared to untreated EAE controls.

In RT-PCR of mRNA extracted from G4.18-treated PLN at day 3 post-immunization there was less T_{h}1 cytokines IL-2

We examined if failure of early G4.18 therapy was due to development of anti-idiotype antibodies. These were demonstrated by co-incubation of sera from G4.18-treated rats with FITC-labeled G4.18. The sera with anti-idiotype antibodies blocked labeled G4.18 binding to normal T cells, as demonstrated by the lack of a positive peak (Fig. 2). Anti-idiotype antibodies developed by day 10 and were undetectable prior to this. This suggests the effect of G4.18 was blocked by anti-idiotype antibodies and may have accounted for the failure of treatment commencing on the day of immunization to inhibit the development of active EAE. When treatment was commenced at day 7, onset of disease was delayed, but occurred whilst animals were still receiving G4.18, but after anti-idiotype antibodies developed. These findings suggest the anti-CD3 mAb only blocked activated T cell function and did not inhibit the generation of activated T cells to MBP.

The effects of G4.18 on T cells and their activation were examined. FACS analysis of peripheral blood mononuclear cells showed immediate modulation of CD3–TCRαβ receptors, with a reduction in CD3+ cells from 65 to <10% and a reduction in TCRβ+ cells from 70 to <50% within 24 h of administration. No circulating cells had mouse Ig on the surface, indicating that the TCRβ–CD3 receptor had been modulated off the cell surface. There was no reduction in CD2+ cells (72 versus 70%); minor reduction in CD4+ (64 versus 56%) and CD8+ cells (15 versus 13%). TCR–CD3 modulation was observed only in the first 24 h of therapy, after which the number of CD3+ and TCRβ+ cells stained were similar to that in untreated controls. This data was not shown, as it is similar to a previous report (44).

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The effects of G4.18 on T cells and their activation were examined. FACS analysis of peripheral blood mononuclear
and IFN-γ, as well as the marker of T\textsubscript{h}1 cells IL-12Rβ2 than in control (Fig. 3A). T\textsubscript{h}2 cytokines IL-4 and IL-5 mRNA were more abundant in G4.18-treated than untreated controls. The levels of macrophage cytokines IL-10 and TNF-α as well as the T\textsubscript{h}3 cytokine TGF-β were comparable in all groups, as were control GAPDH levels. At day 8 there was no difference in T\textsubscript{h}1 marker mRNA in G4.18-treated compared to untreated controls. However, increased IL-4 and a stronger IL-5 band in the neat dilution was observed in G4.18-treated PLN (Fig. 3B). Again, there was no difference in IL-10, TNF-α and TGF-β levels. Thus, G4.18 therapy given from the time of immunization was associated with an early reduction of T\textsubscript{h}1 cytokines and enhanced induction of T\textsubscript{h}2 cytokines.

**Effect of therapy with rIL-4, rIL-5 or blocking mAb to IL-4 on EAE induction**

Given the preferential induction of T\textsubscript{h}2 and inhibition of T\textsubscript{h}1 cytokines by G4.18, we examined if treatment with T\textsubscript{h}2 cytokines could alter the course of EAE. Treatment with rIL-4 for 12 days from the day of immunization had no effect on the clinical course of disease (Fig. 1C). With rIL-4 treatment, the weight loss observed in untreated controls did not occur due to the development of ascites, which were found to contain up to 25% eosinophils. The clinical course of disease seen with rIL-4 was comparable to that of CHO-K1 supernatant or rIL-5-treated controls. These latter groups had weight loss similar to that observed in untreated EAE and did not develop ascites.

Treatment with MRC OX-81 delayed the onset of clinical EAE by 2 days, but there was no difference in the severity of disease, its clinical course or the weight loss observed when compared to untreated control animals (Fig. 1D). The isotype control A6 mAb-treated group had an identical course to CHO-K1 supernatant and untreated controls.

**Effect of G4.18 commenced at time of onset of clinical symptoms in active EAE**

To examine if G4.18 could reverse EAE at the time of onset of clinical symptoms, treatment with G4.18 was started on day 12 post-immunization, when the majority of rats had signs of clinical disease. All groups were stratified to match for clinical score at the time of treatment. The combined results of four experiments are summarized in Table 1. All but two of the 103 rats immunized developed clinical signs of EAE, indicating that the immunization schedule was reliable and effective. Once treated with G4.18, rats that had developed EAE did not progress clinically; their disease stabilized the following day and they started to recover within 2 days (Fig. 4). The number of days of illness when rats had clinical symptoms was significantly reduced with G4.18 therapy compared to untreated controls. In the peak disease activity period from 12 to 18 days, clinical score and weight loss for each days were also significantly less in G4.18-treated rats compared to controls. This was due to the earlier recovery of G4.18-treated rats. The mean of the maximal disease activity in all G4.18-treated groups was also significantly less than all control groups (\( P < 0.01 \)). Nearly all G4.18-treated rats had recovered by day 18, but control rats had a mild disease that persisted throughout the period of observation and up to day 30 post-immunization (Fig. 4). Thus, G4.18 therapy reduced the peak severity of EAE and led to recovery 2–3 days sooner than in untreated controls. Isotype control mAb (BCLA8)-treated rats had similar disease severity, clinical score and weight loss to that of untreated controls, which indicated that G4.18's effects are related to its T cell specificity.

**Effect of rIL-4 and anti-IL-4 mAb therapy alone or with G4.18 on established active EAE**

As G4.18 has been demonstrated to inhibit T\textsubscript{h}1 cells, while sparing T\textsubscript{h}2 cells, we examined if the T\textsubscript{h}2 cytokine IL-4 was critical in the effect of G4.18. Neither treatment with rIL-4 alone nor MRC OX-81 alone from day 12 post-immunization had a significant effect on the clinical course of EAE compared to untreated controls (Table 1). Days of illness, the mean of the mean disease activity and the mean maximal disease were not significantly different to the control group. There was a tendency for rIL-4-treated animals to have lower maximal disease and to recover a day earlier than the control animals (Fig. 4). As found with the early rIL-4 therapy groups, weight loss was less severe due to the development of ascites. Control groups treated with rIL-5 or with supernatant from non-transfected CHO-K1 cells had a similar course of EAE to untreated controls (Table 1).

Combining rIL-4 and G4.18 therapy significantly shortened the days of illness and reduced the mean of daily disease activity compared to G4.18 or rIL-4 treatment alone (Table 1).
Fig. 3. Representative semi-quantitative RT-PCR results in the draining PLN of G4.18 treatment from day 0. (A) Day 3 post-immunization. (B) Day 8 post-immunization. cDNA was diluted from neat (N), 1/10, 1/20 and 1/40 with dilution factor shown along the base. Duplicate PCR reactions were performed at each dilution. Product was confirmed by expected base pairs (bp). GAPDH levels were similar in all samples (data not shown).

and Fig. 4). There was no difference in mean maximal score in the G4.18/rIL-4- and G4.18-treated groups. Controls given G4.18 therapy combined with control CHO-K1 supernatant had a similar clinical course to rats treated with G4.18 alone (Fig. 4). Combining MRC OX-81 with G4.18 therapy had no effect on EAE remission induced by G4.18. There was no significant difference in days of illness, mean of the mean daily disease activity or mean of the mean daily weight loss. Controls treated with G4.18 and A6, an isotype-matched control for MRC OX-81, were also comparable to G4.18 treatment alone. Taken together, these studies showed that a combination of rIL-4 with G4.18 was more effective than G4.18 therapy alone. The effect of G4.18 on recovery from active EAE was not dependent upon IL-4 as blocking this cytokine with MRC OX-81 had no impact on the effect of G4.18.

Examination of the effects of G4.18 alone and with rIL-4 on mononuclear cell infiltrates in brainstem

Mononuclear cell infiltrates in brain stem were examined at day 14, when there was first evidence of recovery, and day 18, when there was complete recovery, this corresponded to 2 and 6 days after commencement of therapy (Table 2). Untreated controls showed a large infiltration of TCRαβ+, CD4+, CD8+, B cells, dendritic cells and macrophages at both day 14 and 18. This infiltration persisted for up to 30 days (data not shown). Normal brain stem showed no positive
Table 1. Comparison of various treatments with mAb and cytokines at onset of disease

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<th>Treatment from onset of EAE</th>
<th>Disease incidence</th>
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<th>Mean of mean daily activity day 12–18</th>
<th>Maximum clinical score (mean±SD)</th>
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Comparison of various treatments with mAb and cytokines commencing day 12 post-immunization. Disease incidence is given as the number of rats with clinical signs of EAE/total number in a group. Days of illness is number of days each rat had signs of clinical paralysis. Mean disease activity (day 12–18) and mean maximal score are given as mean±SD. Compared to controls there was significantly less weight loss in G4.18 (P<0.001), G4.18/IL-4 (P<0.0001), G4.18/IL-4 (P<0.0001), G4.18/CHO-K1 supernatant (P<0.001) but not IL-4 alone (P=NS). Compared to G4.18, only G4.18/IL-4 had less weight loss (P<0.0001). Compared to control, the mean clinical score was also significantly less in G4.18 (P<0.0001), G4.18/IL-4 (P<0.0001) and G4.18/CHO-K1 supernatant (P<0.005). G4.18 combined rIL-4 treated had significantly lower mean clinical score than G4.18 treated (P=0.02), G14.8 and CHO-K1 supernatant (P=0.035), and G4.18 and OX-81 (P=0.011).

G4.18-treated rats had significantly fewer cells of all types by day 14, with even fewer cells at day 18. At day 18, no B cells, dendritic cells or CD5+ T cells were observed (Table 2). With either MRC OX-81 or rIL-4 therapies alone, the cellular infiltrate in brain stem was comparable to controls at day 14 or 18. Rats given combined MRC OX-81 with G4.18 therapy also had a similar cellular infiltrate to those with G4.18 therapy alone. However, rIL-4 combined with G4.18 rats had less infiltrate than those given G4.18 treatment alone. In particular, TCRβ+ cells, CD8+ and B cells were undetectable at day 14 and 18, except for a small residual TCRβ+ infiltrate at day 14. Macrophages were also markedly reduced compared to G4.18 treatment alone. Thus, the benefit of combined G4.18/rIL-4 therapy was confirmed by reduction of inflammatory response in brain stem.

**Fig. 4.** Comparison of clinical course of EAE treated with mAb or cytokines from day 12. (A) Clinical score and (B) weight changes of untreated controls (▲, n = 20), G4.18 and CHO-K1 supernatant (□, n = 18), G4.18 and rIL-4 (●, n = 12), and rIL-4 treatment alone (△, n = 5).

staining for any lymphocyte markers used, thus all cells stained in other groups are an inflammatory infiltrate. The number of CD4+ cells exceeded that of TCRβ+ cells in all samples, as rat macrophages also express CD4.

**Fig. 5.** Reversal of EAE by G4.18 and G4.18/rIL-4 on the cytokines expressed by the mononuclear cell infiltrates in brainstem.

RT-PCR was used to examine cytokine mRNA levels for the brain stem at day 14 and 18 of rats treated from day 12. GAPDH levels were similar in all samples (Fig. 5). Tn1 cytokine mRNA (IL-2, IFN-γ and IL-12Rβ2) was detected in brain stems of controls but not in G4.18- and G4.18/rIL-4-treated rats at day 14 (Fig. 5) and 18 (data not shown). This was consistent with the marked reduction in brain stem cellular infiltration found with G4.18 and combined G4.18/rIL-4 therapy. Tn2 cytokines, IL-4 and IL-10, were detected in untreated controls, but in G4.18 or rIL-4/G4.18-treated rats no IL-4 and only low levels of IL-10 were detected. IL-5 was different in that at day 14 there were higher levels of IL-5 in both G4.18 and G4.18/IL-4-treated rats compared to controls (P = 0.035 and 0.015 respectively). At day 18, the IL-5 mRNA levels were similar in G4.18-treated and control animals. As the T cell infiltrate in rIL-4/G4.18-treated brain stems was trivial, these results suggested that the residual cells predominantly produced IL-5. A marked reduction in the macrophage cytokines TNF-
Table 2. Leukocyte subsets in spinal cord at day 14 and 18 post-immunization of treatment at onset of disease

<table>
<thead>
<tr>
<th>mAb</th>
<th>Receptor</th>
<th>Control</th>
<th>IL-4</th>
<th>OX-81</th>
<th>G4.18</th>
<th>G4.18/OX-81</th>
<th>G4.18/IL-4</th>
<th>Normal brainstem</th>
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<tr>
<td>Day 14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ED1</td>
<td>macrophages</td>
<td>124±10.4</td>
<td>147.8±38</td>
<td>143.6±9</td>
<td>36.8±8</td>
<td>46.6±9</td>
<td>15.5±3.8</td>
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<td>R73</td>
<td>TCRβ</td>
<td>83±20</td>
<td>106±13</td>
<td>68±12</td>
<td>30±2</td>
<td>40±4.5</td>
<td>3±1.5</td>
<td>0</td>
</tr>
<tr>
<td>W3/25</td>
<td>CD4</td>
<td>57.8±9.5</td>
<td>96±8</td>
<td>70±6</td>
<td>20±7</td>
<td>33±6</td>
<td>10±4.8</td>
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<tr>
<td>OX-19</td>
<td>CD5</td>
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<td>30±10</td>
<td>29±6.5</td>
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<td>0</td>
<td>0</td>
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<td>CD45RA</td>
<td>24±5.5</td>
<td>44±5</td>
<td>21±6</td>
<td>10±3</td>
<td>0</td>
<td>0</td>
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<td>OX-62</td>
<td>dendritic</td>
<td>15±2</td>
<td>N/A</td>
<td>19±4</td>
<td>N/A</td>
<td>4.5±1</td>
<td>3.4±0.8</td>
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<tr>
<td>ED1</td>
<td>macrophages</td>
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<td>95.2±10</td>
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<td>36±11</td>
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<td>OX-62</td>
<td>dendritic cells</td>
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<td>13.5±1.5</td>
<td>7.6±1.1</td>
<td>0</td>
<td>2.5±1</td>
<td>1.5±0.5</td>
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</table>

Cellular infiltrate present in the brain stem were compared for different preventative treatments at day 14 or 18 post-immunization. The results expressed as mean±SD of four high-power fields per specimen (×40 magnification). In normal brain CFA-treated rats, there were no cells stained with any of the mAb used.

α and IL-10 was also found with G4.18 and rIL-4/G4.18 treatment compared to controls. TGF-β mRNA was comparable at both days 14 and 18 for all groups. These results show that there was sparing of IL-5 and TGF-β with G4.18 and G4.18/IL-4 treatment, while T<sub>H</sub>1, other T<sub>H</sub>2 and macrophage cytokines were reduced or not detected, consistent with depletion of T cells and macrophages.

To examine the effect of treatment on the site of immunization, PLN mRNA was also assayed at day 14 and 18 (Fig. 6). This site was not confounded by the depletion of mononuclear cells in the brain stem that resulted from therapy. The size of these nodes in all groups was less at day 14 than at day 18. This may relate to depletion of the lymph node at the time of onset of disease, followed by a second phase of enlargement. The GAPDH levels were similar in all samples (data not shown). mRNA levels for all cytokines was higher at day 18 than at day 14. There was no difference in T<sub>H</sub>1 markers between controls and G4.18-treated at day 14, but a slight reduction in mRNA for IL-2, IFN-γ and IL-12Rβ2 in the rIL-4/G4.18-treated compared to G4.18 and control (Fig. 6). IL-4 levels were increased in both G4.18 and G4.18/rIL-4 groups compared to controls. IL-5 had stronger bands in G4.18 at both days 14 and 18 compared to controls and rIL-4/G4.18-treated rats. There were comparable levels of IL-10, TNF-α and TGF-β at both day 14 and 18 in all groups.

Effect of G4.18 and rIL-4 late treatment on anti-MBP response

Anti-MBP antibodies were not detected at days 0, 3 and 7, but was marked at day 10, and persisted until day 14 and 21 in all groups, including the late G4.18- and G4.18/IL-4-treated groups. The IgG1 response was significantly greater with late G4.18 (69.9 ± 14.1%, n = 6), rIL-4 (71.3 ± 8%, n = 6) and rIL-4/G4.18 (74 ± 14.7%, n = 6) therapies, compared to controls (56.4 ± 7.7%, n = 6, P = 0.018, 0.02 and 0.004 respectively), consistent with the IL-4/T<sub>H</sub>2 effect on Ig isotype switching. There was no change in the IgG2a and IgG2b response with any therapy. Thus, rIL-4 or G4.18 therapy did not reduce the complement fixing T<sub>H</sub>1 isotypes, but increased the T<sub>H</sub>2-dependent IgG1 isotype.
Fig. 6. Representative semi-quantitative RT-PCR results of PLN draining site of immunization from rats treated with G4.18 or G4.18 and rIL-4 (G4.18/IL-4) from day 12. Day 18 post-immunization. cDNA was diluted from neat (N), 1/10, 1/20 and 1/40 with dilution factor shown along the base. Duplicate PCR reactions were performed at each dilution. Product was confirmed by expected length (bp). GAPDH levels were similar in all samples (data not shown).

The effect of G4.18 on passive EAE

As the G4.18 effect appeared to be on activated T cells, we tested its effect on passive EAE that was induced by transfer of activated spleen cells from Lewis rats immunized with MBP/CFA. The adoptive hosts developed clinical signs of EAE by day 5 and maximal disease by day 6 with recovery by day 8. G4.18 given from time of transfer of cells markedly reduced severity of EAE with only one out of six rat developing any clinical disease with mean maximal disease of 1.5 compared to control treated with isotype-matched non-functional mAb (BCLA8) which developed severe clinical disease (3 ± 0.35). G4.18-treated rats at onset of clinical disease has reduced severity (1.9 ± 0.6, *P* = 0.02) and accelerated recovery compared to the control group (Fig. 7).

Discussion

These studies demonstrate that G4.18 (a non-mitogenic, non-activating anti-CD3 mAb) can rapidly reverse established active EAE. Our findings are consistent with the final mediator of injury being T<sub>H</sub>1 cells, which once activated were inhibited by G4.18 therapy. The lower number of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, along with B cells, dendritic cells and macrophages in the brains stem was consistent with G4.18 mAb either inhibiting migration of T cells or affecting the in situ survival of these cells. In particular, G4.18 probably inhibited the cytokine production necessary to recruit and activate macrophages, as well as other cells, to the site of inflammation in the brain.

Early G4.18 treatment from the time of immunization had no effect on the clinical development of active EAE. There was an early inhibition of T<sub>H</sub>1 cell cytokine mRNA expression at day 3; however, this appears to have only delayed induction, as T<sub>H</sub>1 cytokine expression was comparable to untreated controls by day 8. At both time points (day 3 and 8), mRNA for the T<sub>H</sub>2 cytokines IL-4 and IL-5, but not IL-10, was higher in G4.18-treated compared to untreated controls. This is
consistent with the reported inability of anti-CD3 mAb to block T\(_{h2}\) cell function, both with T\(_{h2}\) clones in vitro (41,42) and by the sparing of T\(_{h2}\) cells in allograft tolerance induction in vivo (40). The failure of the mAb to maintain this blocking function or to effect onset of disease when given from the time of immunization was found to be due to the development of anti-idiotypic antibodies. These antibodies neutralized the function of G4.18, preventing the mAb binding to CD3 and enhancing clearance of the mAb from the circulation. Delaying treatment by commencing 7 days after immunization only delayed the course of EAE until anti-G4.18 antibodies developed to G4.18. These results suggest G4.18 mAb may have a limited ability to block activation of naive T cells, but works by blocking T\(_{h1}\) effector cell function (40). The findings that G4.18 blocks induction of passive EAE, as well as reversing clinical disease with late treatment, is also consistent with a dominant effect of anti-CD3 mAb being on activated T cell function.

The most dramatic effects with G4.18 were found when treatment was commenced after the onset of active or on passive EAE, when there are activated T cells. This effect was shown to be specific for G4.18, in that isotype-matched mouse IgG3 mAb (BCLA8) which is non-reactive to rat antigen had no effect on the clinical course of either active or passive EAE. G4.18 therapy was associated with less cellular infiltrate into the brain stem, which paralleled earlier clinical recovery of active EAE. There was a persistent infiltrate of CD4\(^+\) T cells and macrophages for >1 week after spontaneous recovery of EAE in untreated control (data not shown). A major infiltrate of T cells and macrophages precedes onset of clinically active EAE (data not shown), thus the G4.18 leads to a reduction of central nervous system infiltrate. The remaining cells after G4.18 treatment did not express mRNA for T\(_{h1}\) cell markers including IL-2, IFN-\(\gamma\) and IL-12R\(\beta2\), but there was continued expression of IL-5 and TGF-\(\beta\). The macrophage cytokines TNF-\(\alpha\) and IL-10 were also markedly reduced compared to untreated controls, consistent with reduction of macrophages from the brain stem. The absolute readings did not compensate for the marked loss in all mononuclear cells in the infiltrate, which makes the persistent expression of IL-5 and TGF-\(\beta\) a much more significant finding.

This data is consistent with the relative sparing of T\(_{h1}\) cells but not T\(_{h1}\) or T\(_{h2}\) cells in the brainstem. T\(_{h1}\) cells are a recently described subset of T cells that have been shown to have regulatory function in autoimmune colitis (30,31) and immune-mediated non-obese diabetes (32). These cells have been cultured in vitro by repeated antigen stimulation with IL-10 and antigen, characterized by high IL-5, IL-10 and TGF-\(\beta\) levels with low IL-2 and no IL-4. These cells proliferate slowly compared to other T cell phenotypes. In our experiments, both IL-5 and TGF-\(\beta\) were preserved, but IL-10 was markedly reduced. This was most likely related to the high expression of IL-10 by macrophages in the rat (37,58). The key T\(_{h2}\) cytokine, IL-4, was not preserved in the brainstem but was in the PLN. This is consistent with G4.18’s previously described selective ability to inhibit activated T\(_{h1}\) cells but spare T\(_{h2}\) cells in vivo and in vitro (40). In this study, the effect of G4.18 was examined in vivo, and thus determined the overall trends in cytokine profiles not the number of T\(_{h1}\) and T\(_{h2}\) cells. Studies of individual cell cytokines would require in vitro stimulation in the absence of G4.18 that may have changed the profile, thus these studies were not performed.

Studies with rIL-4 failed to demonstrate that treatment with this cytokine alone could affect the course of EAE, irrespective of whether treatment was instituted at induction or at the onset of clinical EAE. This is consistent with some reports (23), but at variance with others in the mouse and rat, that showed delayed onset and reduced severity of EAE with IL-4 therapy (25,29). Likewise, treatment with rIL-5, or supernatant from non-transfected CHO-K1 cell lines, had no effect on EAE. The rIL-4-treated rats developed an enhanced IgG1 response to MBP and mRNA for IL-4 was increased in lymph nodes, consistent with induction of T\(_{h2}\) responses. rIL-4 prepared and given in a similar manner in our laboratory has been shown to delay organ allograft rejection in DA rats (51) and inhibit induction of Heymann nephritis in Lewis rats (manuscript in preparation), both associated with enhanced T\(_{h2}\) cytokine expression and increased IgG1 with reduced IgG2a and IgG2b responses. Thus, our protocol is able to significantly modify T\(_{h1}\)/T\(_{h2}\) responses in Lewis rats. There is no ready explanation for the variation in reports on the efficacy of rIL-4 in modifying EAE (23,24,26). A similar protocol of administration of rIL-4 used in our study failed to inhibit T\(_{h1}\)-mediated uveitis in Lewis rats (49). Differences in dose, time of administration and the models used may account for the conflicting reports of rIL-4 treatment on EAE. Whilst, T\(_{h2}\) have been considered to regulate EAE, this is not always the case as T\(_{h2}\) clones can mediate EAE (28). The dosage of rIL-4 was close to the maximum tolerated by rats and caused ascites with an inflammatory infiltrate of eosinophils. Higher doses of rIL-4 caused a high mortality in Lewis rats (data not included). rIL-4 combined with G4.18 enhanced the effect of the mAb in that a further reduction in the severity and duration of EAE was observed. The combined treatment was associated with a lesser mononuclear cell infiltrate in the brain stem than in G4.18-treated rats. There was also enhanced up-regulation of IL-4 mRNA in the draining lymph nodes, but not in the persisting mononuclear cell infiltrate. The modest delay in onset of active EAE with blocking anti-IL-4 mAb therapy suggests T\(_{h2}\) responses may have contributed to part of the early injury in active EAE in this model.

The efficacy of G4.18 was not dependent upon IL-4, as treatment with MRC OX-81 which blocks IL-4 function in vivo (46,41) did not affect the recovery of EAE induced by G4.18. Thus, G4.18’s effects were most likely due to inhibition of activated T\(_{h1}\) cells. Secondary effects were the sparing of cells expressing T\(_{h2}\) and T\(_{h1}\) cytok\(\text{s}\), which may have promoted the re-establishment of tolerance. Such regulatory cell-mediated tolerance may have prevented relapse of EAE once the anti-CD3 mAb was blocked by the development of anti-idiotypic antibodies. Late G4.18 treatment groups were observed for up to 50 days and never developed major relapses, unlike groups treated from day 7, which relapsed when anti-idiotypic antibodies to G4.18 developed.

It is possible that with late treatment, regulatory cells had time to develop and these could prevent relapse. The specific effects of anti-CD3 mAb on activated T\(_{h1}\) cells have been identified in clones grown in vitro (42). Anti-CD3 mAb affect specific signaling pathways required for activation of T\(_{h1}\) but not T\(_{h2}\) cells (38,59). Our results suggested that anti-CD3...
mAb may also spare T\(_1\) cells. Thus, there are two potential regulatory cell subsets that could have promoted recovery from EAE induced by anti-CD3 mAb. The effect of rIL-4 in enhancing recovery suggested that T\(_1\)2 cells may contribute to recovery in this model. The precise role of T\(_1\)1 cells or TGF-\(\beta\)-producing T\(_1\)3 cells in the anti-CD3 mAb effect requires further investigations. These studies suggest that a non-mitogenic, non-activating anti-CD3 mAb may have potential in treating T\(_1\)1-mediated autoimmune diseases in man, including demyelinating diseases. The effects observed are similar to those observed in NOD mice, where onset of diabetes is stopped by non-mitogenic anti-CD3 mAb (60). Furthermore, anti-CD3 therapy may be enhanced by co-administration of rIL-4.

Acknowledgements

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Abbreviations

CFA  complete Freund’s adjuvant  
EAE  experimental autoimmune encephalomyelitis  
MBP  myelin basic protein  
PLN  popliteal lymph node  
TGF  transforming growth factor  
TNF  tumor necrosis factor

References


