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Suppression of Experimental Autoimmune Encephalomyelitis by Intravenously Administered Polyclonal Immunoglobulins

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Experimental autoimmune encephalomyelitis (EAE) was induced in Lewis rats either by active immunization with myelin basic protein (MBP) or by adoptive transfer using anti-MBP specific $CD4^+$ T cells. Treatment with human polyclonal immunoglobulins (IgG) effectively suppressed active EAE. Time-dependent experiments demonstrated that the effect of IgG was manifested only when treatment was given immediately after immunization; administration from day 7 after disease induction did not suppress the disease. In the adoptive transfer model of EAE, IgG had no effect *in vivo*. However, pretreatment *in vitro* of the antigen-specific T-cells with IgG inhibited their ability to mediate adoptive EAE, as it did in active EAE. Similarly, *in vitro* IgG pretreatment of the antigen-specific T-cells suppressed the proliferative response to MBP. Fluorescent Activated Cell Sorter (FACS) analysis demonstrated the binding of IgG to activated T-cell lines that was inhibited by soluble Fc molecules. The differential effects of IgG on active EAE and on the adoptive transfer of EAE suggest that IgG *in vivo* can suppress disease by acting during the early phase of the immune response which involves naive T cells. The inhibition of T-cell proliferation and adoptive transfer of EAE by incubation of T cells *in vitro* appears to require higher concentrations of IgG than those obtained *in vivo*.

Introduction

Since its introduction over a decade ago, iv immunoglobulin (IgG) has demonstrated beneficial therapeutic activity in a variety of autoimmune disorders such as idiopathic thrombocytopenic purpura, autoimmune hemolytic anemia, immune neutropenia, aplastic anemia, Guillain Barré syndrome and myasthenia gravis [1–5]. Several studies recently reported that IgG is a safe and effective treatment to reduce the frequency of exacerbations in relapsing–remitting multiple sclerosis (MS) [6–8].

Understanding the mechanism(s) of action of IgG is important in order to explain its immunomodulatory effects and to provide a rationale for its application in the management of MS. IgG appears to provide large amounts of immunoregulatory substances that have the capacity to regulate the immune system in various ways: blockade and down-regulation of phagocytic functions via Fc receptors [9], antibodies directed to the T-cell receptor, the CD4 molecule, and major histocompatibility complex [10], regulation of idiotype–anti-idiotype networks [11, 12], suppression of idiotype synthesis [13], and suppression of proinflammatory cytokines [14–16].

We have evaluated the effect of human IgG for iv use on the onset and progression of experimental autoimmune encephalomyelitis (EAE), a T-cell-mediated autoimmune disease inducible in Lewis rats either by immunization with myelin basic protein (MBP), or by adoptive transfer of MBP-sensitized T cells [17]. We have shown previously that treatment of Lewis rats with intravenous IgG suppressed EAE induced by MBP, and significantly reduced TNF-α secretion by rat spleen cells [18]. The aim of the present study was to investigate further the immunomodulatory effects of IgG in EAE.

Materials and Methods

Animals

Female Lewis rats were obtained from the animal breeding centre at the Weizmann Institute of Science (Rehovot, Israel). Animals were 6–8 weeks old (body weight of 180–200 g). Each experimental group consisted of 6–8 rats.

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Active induction of EAE

Animals were inoculated sc in the hind foot pads with an emulsion of 25 μg of guinea-pig MBP in complete Freund's adjuvant (CFA) containing 200 μg of Mycobacterium tuberculosis (Difco, Detroit, MI, USA) in 0.1 ml of oil.

Adoptive transfer of EAE

Activated anti-MBP $CD4^+$ encephalitogenic T cells, derived from spinal cord, were administrated ip (2×10^6 per rat).

Preparation of cell suspensions and T cell lines

Rats were killed during acute EAE by ether anesthesia. The spinal cords were extruded from the vertebral column, and lymphocytes were obtained by gentle grinding of the spinal cord tissue. The homogenate was then subjected to three cycles of Ficoll gradient separation, and lymphocytes derived from the spinal cord (SC line) were recovered from the pellet. After the harvest of spinal cord cells the cells were seeded in 96-well round-bottomed microtitre plates, 250–500 cells per well, in propagation medium with irradiated thymocytes as antigen-presenting cells (APC) (3,000 rad, 10^5/well), and MBP (20 μg/ml) [19]. The cultures were maintained for 7 days. Wells showing the highest proliferation rate were transferred to 24-well plates to be expanded by repeated stimulation with guinea-pigs MBP (10 μg/ml) and using irradiated thymocytes as APC (5×10^6/ml in 24-well plates and 10^7/ml in 10-ml plates) every 10–12 days. After five rounds of stimulation, activated T-cell blasts were separated from cell debris by centrifugation on Ficoll gradient and 2×10^6 blasts were injected ip to each rat.

Pretreatment incubation of encephalitogenic T cells with IgG

Activated encephalitogenic T cells, prepared as described above, were incubated (37°C, 60 min) with IgG (1–10 mg/ml). The cells were then washed twice and injected ip (2×10^6 per rat).

Clinical assessment of EAE

Clinical signs of EAE appeared 5 and 12 days after passive and active disease induction, respectively. The degree of clinical disease was scored as follows: 0=no signs; 1=loss of tail tonicity; 2=paralysis of hind limbs; 3=paralysis of all four limbs; 4=quadriplegic animal, in a moribund state; 5=death caused by EAE.

IgG treatment

Each rat received IgG in a sterile 5.5% solution of human protein in 10% maltose (Gamimune N, Bayer, Promedico, Israel), iv to a tail vein at a dose of 0.4 g/kg body weight/day. Treatment was given in several schedules after active induction of EAE as follows: from the day of disease induction (day 0) for 4 days or for 14 consecutive days, and from day 7 after disease induction to day 14. In passive EAE, IgG was injected iv from day 0, for 5 or for 12 consecutive days, and from day 6 after disease induction to day 12. Control animals were either not treated after immunization or received bovine serum albumin (BSA) iv at a dose of 0.4 g/kg body weight/day, at similar schedules after immunization as were the IgG-treated animals.

Analysis of IgG-binding to the MBP-specific T cell line

The binding of IgG to anti-MBP-specific, anti-ovalbumin specific, and non-activated anti-MBP-specific T cell lines was determined by indirect immunofluorescence. The T cell lines were incubated (4°C, 45 min) with IgG at increasing concentrations (10–100 μg/ml). Next, the T cells were washed twice and incubated (30 min, 4°C) with FITC-conjugated IgG goat anti-human antibodies (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA, USA). The labelled cells were then washed extensively, fixed with PBS containing 3% formaldehyde and subjected to flow cytometric analysis in a FACScan Flow Cytometer (Beckton-Dickenson, CA, USA). In the experiment of IgG blockade by soluble Fc, we used rat Fc fragments (Jackson ImmunoResearch) at 50 μg/ml concentration, later followed by IgG (10 and 100 μg/ml). IgG binding was examined by Fab FITC conjugated anti-human Fab (Jackson ImmunoResearch).

Proliferative response of MBP-specific T cell line after pretreatment with IgG

T-cell-line cells (5×10^4) were seeded in 96 round-bottom microtitre wells with 5×10^5 irradiated (3,000 rad) thymocytes as accessory APC. Cells were incubated (37°C, 180 min) with 1–10 mg/ml human IgG, washed twice and tested for reactivity to increasing concentrations of MBP. The proliferation assay was performed in stimulation medium composed of Dulbecco's modified Eagle's medium (DMEM), supplemented with 1% syngeneic fresh rat serum, 5×10^{-5} M 2-mercaptoethanol, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Following 72 h of incubation at 37°C in a humidified atmosphere plus 7% CO_2, each well was pulsed with 1 μCi [3H]thymidine for 16 h. The cultures were then harvested (MicroMate 196 cell harvester; Packard Instrument Co., Meriden, CT, USA) and cpm were determined (Matrix 96 direct beta counter, using avalanche gas [98.7% helium; 1.3% C4H10] ionization

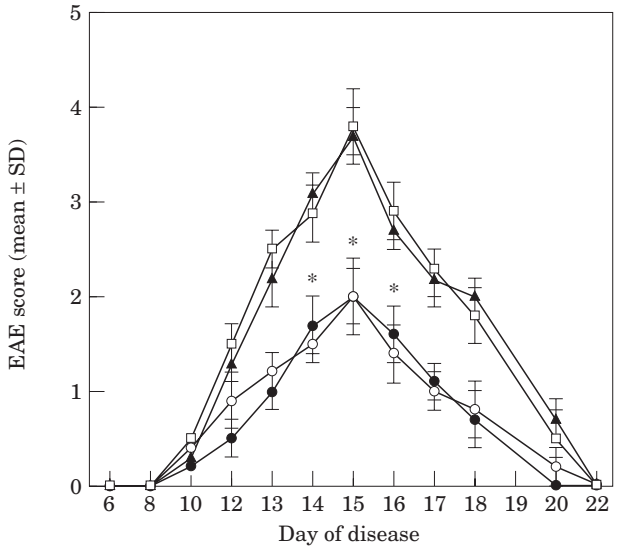

Figure 1. IgG treatment inhibits active EAE. Treatment with IgG from the day of disease induction (day 0) for either 4 days (—○—) or 14 days (—●—) significantly (*P<0.01) suppressed EAE. No beneficial effect appeared when the treatment was administered from day 7 (—▲—) after disease induction, compared to untreated control rats (—□—).

detectors; Packard Instruments Co.). The background proliferation of the T-cell line and the accessory cells following IgG treatment was determined as the thymidine incorporation of the line cells (5×10^4 cells/well) with irradiated thymic APCs (5×10^5 cells/well), on the last 18 h on day 3 of the proliferation assay.

Statistical analysis

Data are represented as mean±SD. Clinical scores were compared using MANOVA for repeated measures. The Mann-Whitney U-test was used for the comparison of proliferative responses. Disease severity (EAE score), duration, and incidence were compared for the significance of differences by Student's t-test. P<0.05 was considered significant.

Results

Effect of IgG treatment on active EAE

Active EAE was induced with MBP (day 0) followed by iv IgG given at three time schedules: days 0–4, days 0–14, or days 7–14. The effect of IgG was evident when treatment was given on days 0–4, or days 0–14 concomitantly with MBP inoculation. No suppression was seen when treatment was given from days 7–14 after EAE induction (Figure 1). IgG treatment starting at day 0 reduced the incidence of active EAE; only 61% of the IgG treated rats (days 0–4) and 58% of the IgG treated rats (days 0–14) developed the disease, while the incidence of EAE in the IgG-treated animals (days 7–14) was 100% (Table 1). The mean maximal EAE score in IgG treated animals was 1.6 compared with a mean score of 2.8–3.0 in the control groups (P<0.05). Duration of EAE was 7 days in IgG treated rats, while it lasted 9 days in control animals. Disease onset was not affected by IgG treatment (Table 1). Thus, MBP-induced active EAE was significantly suppressed by the concomitant iv administration of IgG.

Effect of IgG treatment on adoptive EAE

Intraperitoneal injection of activated encephalitogenic T cells (day 0) was followed by iv IgG treatment as follows: days 0–5, days 0–12, and days 6–12. None of these treatments modified either the duration of EAE, the clinical score (Figure 2), or the disease incidence and mortality rate (data not shown). Thus, iv administration of IgG did not suppress the ability of a T-cell line to induce adoptive transfer of EAE.

IgG pretreatment affects the encephalitogenic potential of a T-cell line in adoptive EAE

Since iv administration of IgG to Lewis rats *in vivo* did not suppress adoptive EAE, we analysed whether *in vitro* incubation of encephalitogenic T cells with IgG could modify the disease course. *In vitro* treatment of the T-cell line with various concentrations of IgG was followed by ip injection of the cells to Lewis rats. Pretreatment of the cells with 1 mg/ml did not suppress the disease. However, pretreatment of the encephalitogenic T cells with IgG at doses of 5 or 10 mg/ml resulted in a significant suppression of adoptive EAE (Figure 3). Treatment of the T cells with BSA, 10 mg/ml, had no effect on their ability to mediate disease (data not shown). These experiments demonstrate that IgG has an inhibitory effect on activated encephalitogenic T cells and suppressed their ability to mediate adoptive transfer EAE.

IgG-binding to T-cell lines

In order to understand the mechanism(s) underlying the inhibition of encephalitogenic T cells by IgG *in vitro*, we analysed the interaction of IgG with different T cell lines. FACS analysis demonstrated that IgG bound to the surface of the anti-MBP activated T-cell line (Figure 4A). At 10 µg/ml IgG about 85% of the T cells were positively stained, and at 50–100 µg/ml IgG, all the T cells were positively stained and the mean fluorescence intensity increased further. Binding of IgG to an activated anti-ovalbumin T cell line is also evident (Figure 4B). At 10 µg/ml IgG 74% of the T cells were positively stained, and at 50 µg/ml 90% of the T cells were stained and the mean fluorescence intensity increased further. IgG binding to a non-activated, MBP-specific T cell line was negligible as only 2–4% of the cells were stained by an IgG concentration of 10–100 µg/ml (Figure 4C). These results suggest that the *in vitro* binding of IgG to T cells is dependent on T-cell activation, and is not specific to anti-MBP T cell lines. To examine the role of Fc

Table 1. *Effect of IgG treatment on active EAE*

Treatment	Incidence (%)	Mortality (day)	Onset (days)	Mean EAE score	*n* rats
None (control)	100	30	12.2±2.1	3.0±1.1	(40)
IVIg (0–4)	61	0	11.4±1.8	1.5±0.6*	(20)
IVIg (0–14)	58	0	12.1±2.3	1.6±0.4*	(40)
IVIg (7–14)	100	0	11.8±2.6	2.9±0.7	(20)
Maltose 10%	100	0	12.4±2.1	2.9±1.5	(10)
BSA	100	0	12.2±1.7	2.8±1.2	(10)

Following EAE induction, rats were either left untreated or treated with IVIg, Maltose 10%, or BSA.
*$P<0.05$ *vs* untreated group.

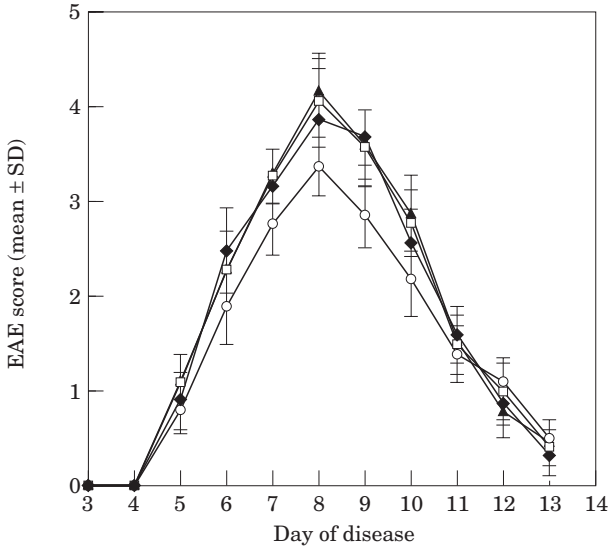

Figure 2. IgG treatment does not inhibit adoptive EAE. Treatment of Lewis rats with IgG on days 0–5 (—◆—), 0–12 (—○—) and 6–12 (—▲—) after disease induction did not suppress the disease, compared to untreated control rats (—□—).

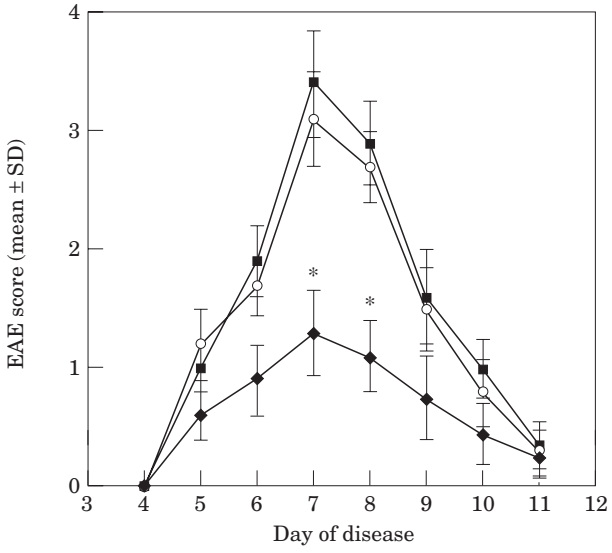

Figure 3. Pretreatment of activated anti-MBP T-cells with IgG suppress adoptive EAE. Pretreatment of activated T cells with 10 mg/ml IgG (—◆—) suppressed the disease significantly (*$P<0.05$), while T-cell pretreatment with 1 mg/ml IgG (—○—) was ineffective compared to untreated control rats (—■—).

receptors in IgG binding to activated T cells, we stained activated T cells with 10 and 100 µg/ml IgG after blocking with soluble rat Fc. As seen in Figure 5, soluble Fc treatment (at 50 µg/ml concentration) decreased the binding of IgG to activated T cells: the decrease was more pronounced at 10 µg/ml IgG (Figure 5A) but could be seen also at 100 µg/ml (Figure 5B).

Effect of IgG on the proliferation of the T cell line

The proliferative response of anti-MBP specific T cell line was analysed with 2, 10, and 20 µg/ml of MBP. The low dose of MBP (2 µg/ml) induced a modest proliferative response that was not inhibited by IgG (1–10 mg/ml), suggesting that IgG was not cytotoxic to the cells. The lack of toxicity of IgG on the cells was inferred from the lack of a decrease in background CPM upon incubation with varying concentrations of IgG, as the incorporated CPM correlates with viability.

Preincubation of the T-cell-line with 5 or 10 mg/ml IgG inhibited the cell proliferation significantly ($P<0.05$, $P<0.01$, respectively). No inhibitory effect was seen with a low dose (1 mg/ml) of IgG. This effect was only observed in T cells exposed to 10 or 20 µg/ml of MBP, and not at the concentration 2 µg/ml (Figure 6A). Background proliferation of the T-cell-line (SC line) and the accessory cells (APC) was not suppressed by IgG also suggesting that IgG pretreatment, *per se*, was not cytotoxic to the cells (Figure 6B). Concanavalin A induced an increase in intracellular calcium that was not inhibited by pretreatment with different concentrations of IgG (data not shown). In addition, IgG pretreatment inhibited the proliferative response of an anti-ovalbumin-specific T cell line (data not shown). Thus, IgG inhibited the proliferative response induced by either antigen. This suggests that the inhibition is irrespective of TCR specificity and probably involves common T-cell surface receptors and signal transduction regulatory mechanisms.

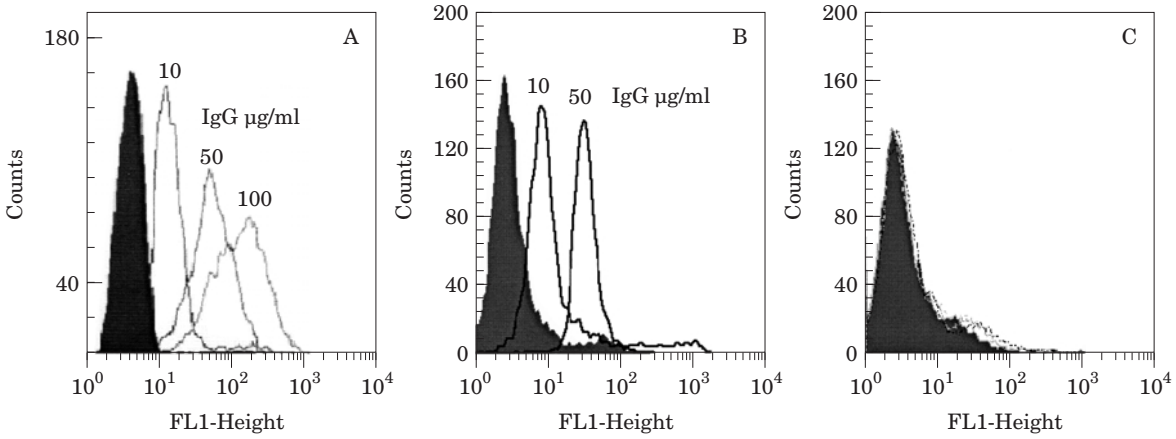

Figure 4. FACS analysis of IgG binding to various T-cell lines. T-cell lines were incubated with the indicated concentration of IgG for 45 min at 4°C. (A) Activated MBP specific T cells. (B) Activated ovalbumin-specific T cells. (C) Non-activated MBP-specific T cells. Dark histograms show control cells.

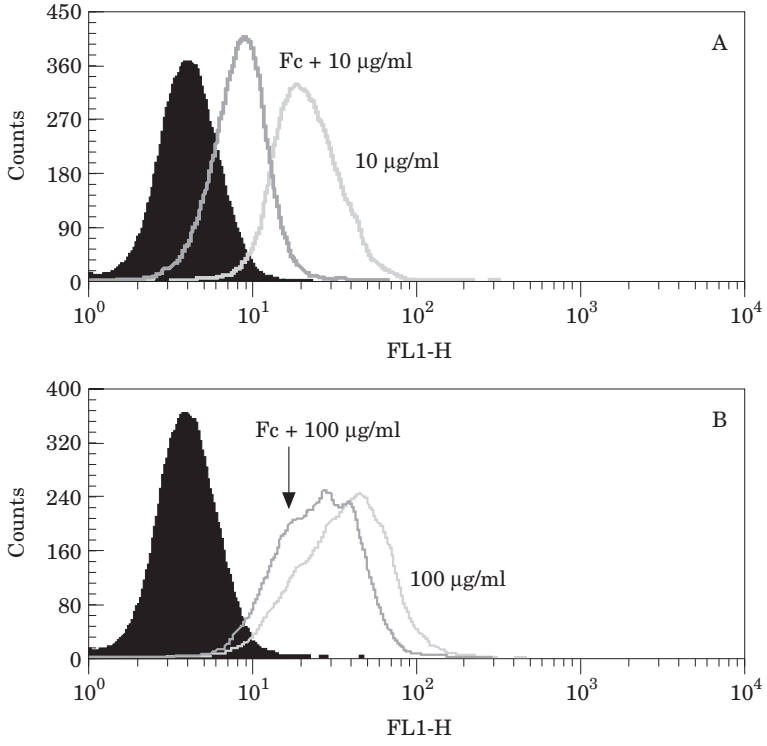

Figure 5. Blocking of IgG binding to activated T cells by soluble Fc. Cell lines reactive to MBP were stimulated for 3 days with antigen. Cells were incubated for 30 min at 4°C with 50 µg/ml Fc. After washing cells were incubated with 10 or 100 µg/ml IgG. The binding was then examined by staining with FITC conjugated Fab anti human Fab. The histograms in A show the effect of Fc on 10 µg/ml IgG; and B in 100 µg/ml IgG.

Discussion

Intravenous immunoglobulin treatment has been reported to be beneficial in a large number of autoimmune diseases, whether mediated by antibodies or by T cells [20]. In the present study, we found that the administration of pooled human polyclonal IgG effectively suppressed and decreased the frequency and clinical signs of active EAE, an animal model for multiple sclerosis. The regulatory effects of a human IgG preparation in the Lewis rat is in agreement with earlier reports on their beneficial role across the species barrier [21, 22]. This regulatory effect was achieved in active EAE either by iv IgG treatment in the first 0–4 days after disease induction, or when IgG treatment was administered for 14 consecutive days. However, no effect on active EAE was observed when IgG was administered during days 7–14 after immunization. Thus, the failure to achieve suppression of active EAE after 7 days, indicates that IgG treatment has an effective window mainly during the initial phase of the immune response.

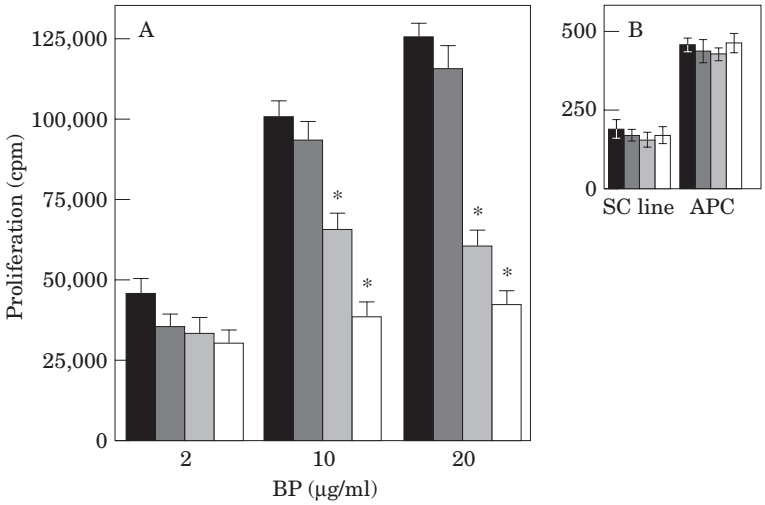

Figure 6. IgG inhibits T-cell proliferation. (A) Preincubation of the T-cell line with 5 and 10 mg/ml IgG significantly inhibited (*) ($P<0.05$, $P<0.01$, respectively) cell proliferation to MBP. No inhibitory effect was seen with a low dose (1 mg/ml) of IgG. The inhibition was only observed in T cells exposed to 10 or 20 μg/ml of MBP, not with a concentration of 2 μg/ml of MBP. Control designate the absence of IgG. (B) Background proliferation of the anti-MBP T-cell line (SC line) and the accessory cells (APC) was not suppressed by IgG. Data is represented as means±SD. Control (■), IgG 1 mg/ml (▩), IgG 5 mg/ml (□), IgG 10 mg/ml (□).

The findings that pretreatment of activated T cells *in vitro* with relatively high concentrations of IgG inhibited the transfer of adoptive EAE, suggests that IgG may also operate at a later stage of the immune response, after T-cell activation. Indeed, FACS analysis experiments demonstrated that IgG binds to the surface of anti-MBP activated T cells, but not to the resting, non-activated anti-MBP T cells. IgG binds also to an activated anti-ovalbumin T-cell line. Thus, IgG binds to surface receptors induced during cell activation with antigen. Receptors for the Fc portion of immunoglobulins (FcR) have been reported to be induced upon T-cell activation, disappeared during rest, and re-appeared upon re-activation of the cells by specific antigens [23, 24]. Thus, we speculate that IgG may control T-lymphocyte activation through a mechanism which requires binding to the FcR expressed on activated T cells [25]. Our results (Figure 5) show that binding of IgG to activated T cells was inhibited by pretreatment of the cells with soluble Fc. However, the involvement of other T-cell surface molecules is possible, and other mechanisms are also plausible.

Our experiments also demonstrated that IgG prevented T-cell-line proliferation to antigen. Thus, IgG may induce (*in vitro*) functional inactivation of T cells. This inactivation may be due to various antibodies within the IgG preparation that bind to different T-cell surface sites [10]. This *in vitro* inhibition, however, requires higher concentrations of IgG.

The IgG dose administered *in vivo* in adoptive EAE was inadequate to inhibit the T-cell mediated disease. Actually, the distribution of IgG *in vivo* among the various tissue compartments is probably very complex, and we cannot compare *in vitro* and *in vivo* results. In any case, the efficacy of the same dose of IgG in active EAE, which was evident only when administered early, suggests that there may be interference with immune system activation at multiple levels during the pathogenesis of EAE.

The mechanism of inhibition of iv IgG on T-cell proliferation seen in our experiments is not clear. Recent work has demonstrated the presence of anti-FAS antibodies capable of inducing apoptosis in T cells [26]. A similar effect of iv IgG on inhibition of T-cell activation to S-antigen was reported in rats with experimental autoimmune uveoretinitis [22]. This work found that T lymphocytes obtained from iv IgG-treated rats failed to secrete IL-2 and proliferate in response to S-antigen, an effect which was not related to induction of suppressor T cells [22]. Recent work testing the effect of iv IgG on human T-cell proliferation documented inhibition of superantigen stimulation and IL-4 production [27]. Intravenous IgG was also reported to inhibit endothelial cell proliferation as well as the effects of proinflammatory cytokines on endothelial cells [28]. Inhibition of human lymphocyte cytokine synthesis by iv IgG was found to be mediated by the effects of the on accessory cells [29]. Thus, the inhibition of T-cell activation may be mediated by several mechanisms including blocking of surface molecules on T cells [30], effect on antigen presenting cells [29], inhibition of cytokine synthesis [18] and direct induction of apoptosis [26].

To conclude, IgG treatment was effective in active EAE and interfered mainly in the early induction phase of the immune response. In adoptive transfer of EAE, *in vivo* administration of IgG did not inhibit the disease. However, *in vitro* incubation of activated T cells with high concentrations of IgG was associated with inhibition of the T-cell proliferation to the antigen, and with a reduction of T-cell ability to mediate adoptive EAE.

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