Intravenous Immunoglobulin Treatment of Experimental T Cell–mediated Autoimmune Disease

Upregulation of T Cell Proliferation and Downregulation of Tumor Necrosis Factor α Secretion

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Abstract

It has been reported previously that intravenous administration of normal human immunoglobulins (IVIg) to human patients can suppress the clinical signs of certain autoimmune diseases. However, the mechanism(s) by which normal Ig interferes with the various disorders and the scheduling of treatment have been poorly delineated. To study these questions, we examined IVIg treatment of two experimentally induced T cell autoimmune diseases in rats: experimental autoimmune encephalomyelitis (EAE) and adjuvant arthritis (AA). We now report that IVIg treatment (0.4 g/kg) inhibited the active induction of both EAE and AA, and that this treatment did not affect the acquisition of resistance to reinduction of EAE. The importance of the site of administration and schedule of treatment were studied in the AA model. Ig was effective when given intravenously, but not when administrated subcutaneously or intraperitoneally. IVIg treatment was effective when given daily from immunization to outbreak of disease; but it was also effective when given once at the time of immunization or once 2 wk after induction of AA, just at the clinical outbreak of disease. Administration of IVIg between immunization and outbreak of AA was less effective. Prevention of disease by IVIg occurred despite the presence of T cell reactivity to the specific antigens in the disease. In fact, IVIg administrated to naive rats activated T cell reactivity to some self-antigens. Nevertheless, IVIg treatment led to decreased production of the inflammatory cytokine TNFα. Thus, IVIg treatment may exert its therapeutic power not by inhibiting T cell recognition of self-antigens, but by inhibiting the biological consequences of T cell recognition. (*J. Clin. Invest.* 1994. 93:600–605.) Key words: autoimmunity • immunoglobulin • adjuvant arthritis • experimental autoimmune encephalomyelitis • tumor necrosis factor

Introduction

Normal human immunoglobulin administered intravenously (IVIg)[1] has been reported to be effective in treating autoimmune diseases such as idiopathic thrombocytopenic purpura, autoimmune hemolytic anemia, immune neutropenia, aplastic anemia, Guillain-Barré syndrome, and myasthenia gravis (1–6). However, well-designed, placebo-controlled double-blind studies are scarce and most of the reports relate to a small number of patients and were uncontrolled open trials. The clinical usage of IVIg has been recommended for acute autoimmune thrombocytopenic purpura of childhood and Kawasaki's syndrome (7), and for the Guillain-Barré syndrome (8).

How these diseases are suppressed by IVIg is not clear, but the treatment is thought to involve regulation of the autoantibodies that cause the particular disorder (9, 10). However, a recent study suggests that IVIg may also be effective in an autoimmune disease probably mediated by T cells; it was shown that the number of acute exacerbations in patients with relapsing-remitting multiple sclerosis (MS) was reduced by long-term treatment with IVIg. Furthermore, it was demonstrated that this mode of treatment improved significantly the functional neurologic status of the patients and was well tolerated with no apparent side effects (11).

This study was undertaken to investigate the mode of action of IVIg in experimental models of T cell–mediated diseases: experimental autoimmune encephalomyelitis (EAE; 12), an animal model of MS, and adjuvant arthritis (AA), a chronic inflammatory autoimmune disease of the joints resembling human rheumatoid arthritis (13, 14). AA is induced in Lewis rats by immunization with *Mycobacterium tuberculosis* (MT) in oil, and EAE is induced in rats by immunization with myelin basic protein (BP) in CFA. Both diseases have been shown to be caused by clones of antigen-specific CD4^{+} T cells (15, 16).

We report here that IVIg treatment inhibited the active induction of both EAE and AA. The site of inoculation of the Ig and its schedule of administration were important. Inhibition of disease was associated with upregulation of T cell–proliferative responses together with downregulation of secretion of the inflammatory cytokine TNFα.

Methods

Animals. Inbred Lewis rats, 6–8 wk of age, were supplied by the Animal Breeding Center of the Weizmann Institute. Rats were matched for age and sex in each experiment.

Antigens and reagents. MT H37Ra and CFA were purchased from Difco Laboratories Inc. (Detroit, MI). Guinea pig BP was prepared as previously described (15). Synthetic peptides of BP were synthesized according to the published sequence of the protein. BSA, OVA, and

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1. *Abbreviations used in this paper:* AA, adjuvant arthritis; BP, myelin basic protein; EAE, experimental autoimmune encephalomyelitis; hsp, heat shock protein; IVIg intravenous immunoglobulin; MS, multiple sclerosis; MT, *Mycobacterium tuberculosis;* SI, stimulation index.

maltose (present in the diluent of the IVIg) were purchased from Sigma Chemical Co. (St. Louis, MO).

Induction of EAE. Groups of 5–10 rats were inoculated subcutaneously in each hind foot pad with 0.05 ml containing 25 μg of BP and 200 μg of MT emulsified in equal volumes of incomplete Freund's adjuvant and PBS. Clinical signs of EAE appeared 11–12 d after induction. The degree of clinical disease was scored as follows: 0, no signs; 1, decrease in tail tonicity; 2, paralysis of hind limbs; 3, paralysis of all four limbs; 4, quadriplegic animal in a moribund state.

Induction of AA. Groups of 5–12 Lewis rats were inoculated subcutaneously at the base of the tail with 0.1 ml of incomplete Freund's adjuvant supplemented with 1 mg of MT. Clinical signs of AA appeared 12–14 d later and were scored by judging the swelling and redness of each of the four limbs on a scale of 1–4 as described (13, 14). The scores of the four limbs were summed to produce a maximum score of 16.

To prevent inadvertent observer bias, the individual who measured disease incidence or severity of EAE or AA was unaware of the identities of the various groups, as well as to their treatment status.

IVIg treatment. Human IgG in a sterile 4.5–5.5% solution in 9–11% maltose (Gamimune N; Miles Inc., Cutter Biological, Promedico, Israel) was inoculated intravenously into a tail vein, or as described in the text, at a dose of 0.4 g/Kg per dose. Controls included BSA administered at a dose of 0.4 g/Kg per dose, and 10% maltose, 0.5 ml/dose, given intravenously, subcutaneously, or intraperitoneally.

T cell proliferative responses. 10 d after induction of EAE or AA, or after treatment with IVIg, splenocytes and lymph node cells were isolated from treated or control rats and incubated in flat-bottomed microtiter plates in quadruplicate wells. Each well contained 2×10^5 cells in 0.2 ml of proliferation medium (DMEM; GIBCO BRL, Gaithersburg, MD) supplemented with 1% syngeneic fresh rat serum, 5×10^{-5} M 2-mercaptoethanol, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin, with the following antigens: MT, 10 μg/ml; the 65-kD heat shock protein of MT (MT-hsp65), 5 μg/ml; the 70-kD hsp of MT (MT-hsp70), 5 μg/ml (kindly donated by J. Kamerbeek and J. Van Embden, The Netherlands); BP, 10 μg/ml; the encephalitogenic peptide of BP 71–90, 10 μg/ml; Con A, 2.5 μg/ml; OVA, 10 μg/ml; or BSA, 20 μg/ml. After 72 h of incubation at 37°C in a 10% CO_2 humidified incubator, each well was pulsed with 1 μCi of [^{3}H]thymidine for 16 h. The cultures were then harvested on fiberglass filters and thymidine incorporation was measured using a liquid scintillation counter. The proliferative response was expressed as the stimulation index (SI; mean cpm of test wells divided by mean cpm of control wells without antigen). The standard deviations of the mean cpm were always < 10% of the means.

Measurement of TNFα secretion. Spleen cells were obtained either from untreated rats or from rats 10 d after treatment. Suspensions of splenocytes were then placed in microtiter plates in quadruplicate and were either activated with the T cell mitogen Con A or untreated. The culture plates were incubated at 37°C in a humidified incubator for 3 h. Subsequently, the contents of the wells were collected, centrifuged, and the supernatants were assayed for TNFα secretion as previously described (17). Briefly, test supernatants were added to cultures of clone-7 cells sensitive to killing by TNFα. Death of these cells was determined by the release of neutral red dye. The concentration of TNFα in test medium was determined by comparing the degree of killing of clone-7 cells to that produced by a titration curve obtained using known amounts of recombinant TNFα. The levels of TNFα were expressed in picograms per milliliter. The specific activity of the recombinant TNFα used was 6×10^7 U/mg; thus, 100 pg/ml is equivalent to 6 IU.

Statistical analysis. Student's *t* test was used to evaluate differences between the various groups.

Results

IVIg inhibits EAE. Fig. 1 shows the course of EAE induced in groups of five rats that were untreated or were treated intrave-

nously daily from induction to day 12 by IVIg, PBS, BSA, or maltose solution. It can be seen that IVIg treatment reduced the severity of EAE, whereas control IV treatments with BSA or maltose did not.

EAE in Lewis rats is usually marked by spontaneous remission followed by resistance to subsequent attempts to reinduce EAE (18). Therefore, we wished to investigate whether IVIg treatment that inhibited the development of EAE might also inhibit the acquisition of resistance to EAE upon a secondary challenge. Table I shows that IVIg treatment inhibited the primary induction of EAE; but the treated rats still manifested resistance to a secondary induction of EAE even though it was done without a second course of IVIg. Thus, IVIg treatment does not appear to interfere with the acquisition of the regulatory mechanisms induced by primary EAE.

IVIg inhibits AA. Fig. 2 shows the effects on the induction of AA of treating rats daily for 14 d with IVIg, BSA, or maltose. It can be seen that IVIg treatment markedly inhibited the development of AA; the other treatments had no effect on the course of disease, which was similar to that manifested by untreated control rats.

Route of administration. To test whether the site of inoculation of Ig influenced the outcome, we treated rats daily for 14 d with Ig given intravenously, subcutaneously, or intraperitoneally. Fig. 3 shows that the intravenous route of treatment was the most effective. Thus, Ig is best given intravenously.

Time of administration. In the above experiments IVIg administrated daily for 14 d was found to be effective in inhibiting the induction of AA. The 14-d period of administration covered all the phases of induction of AA, from the first contact with the MT antigen through the generation of the effector T cells and the onset of joint inflammation. We therefore wished to see whether IVIg had to be given throughout the whole 14-d period and whether there were particular time points at which the AA process was particularly sensitive to treatment. Fig. 4 shows the results of an experiment in which rats were treated with only a single injection of IVIg administered either on day 0 (the day of immunization) or on day 14 (appearance of clinical disease). It can be seen that both schedules of treatment were effective. Fig. 5 shows that the single dose at day 14 inhib-
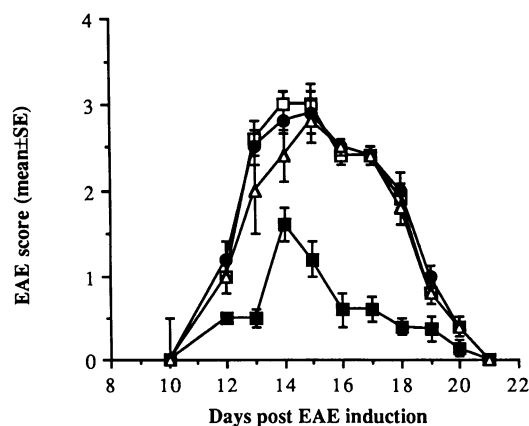

Figure 1. IVIg inhibits EAE. EAE was induced in Lewis rats, six per group, which were then either untreated (PBS; *open squares*) or treated daily (until day 12) with intravenous administration of BSA (*open triangles*), maltose (*filled circles*), or IVIg (*filled squares*). This is one experiment representative of the four done.

Table I. IVIg Treatment Does Not Inhibit the Acquisition of Resistance to EAE

Group	Treatment	Primary induction of EAE	Incidence	Secondary induction of EAE	Incidence
			%		%
1	None	No	—	Yes	7/7 (100)
2	None	Yes	10/10 (100)	Yes	0/10 (0)
3	IVIg	Yes	2/12 (17)	Yes	0/12 (0)

EAE was induced in Lewis rats that were then either untreated or treated daily (until day 12) with IVIg. 2 mo later, when all the rats had recovered from EAE, the test rats (groups 2 and 3), as well as naive group of rats (group 1), were challenged with BP/CFA. None of the groups were treated with IVIg at the time of secondary induction.

ited AA when given intravenously, but not when given subcutaneously.

To learn if the middle of the induction period was also sensitive to a single administration of IVIg, rats were treated on days 0, 5, or 7 after MT immunization. Fig. 6 shows that the IVIg doses given on days 5 or 7 were less effective than was the dose of IVIg given on day 0. Thus, it appears that sensitivity to IVIg is more marked at the extremities of the induction of AA, on day 0 and 14, than it is in the middle of the induction period. In other words, there appear to be certain phases of the immune response that are particularly susceptible to regulation by IVIg.

The experiment illustrated in Fig. 7 shows that unpropitious timing of treatment may actually negate the benefit of IVIg. In this experiment, rats were treated by a single dose of IVIg on day 0, or by five daily doses of IVIg beginning on day 0 and ending on day 4. It can be seen that the single day 0 dose suppressed AA, but the additional four doses only abrogated the benefit of the single treatment on day 0. Hence, the timing of administration is important.

IVIg activates T cells. During the course of these experiments, we observed that IVIg treatment of rats influenced the size of the spleen. This is tabulated in Table II. The average
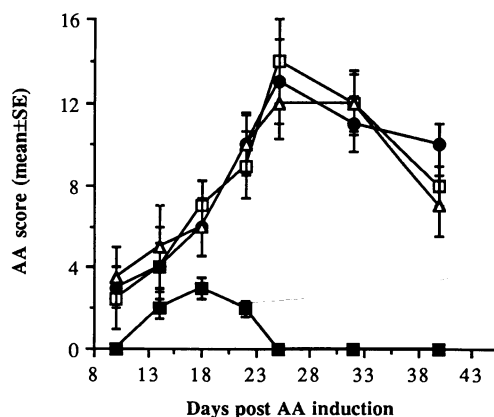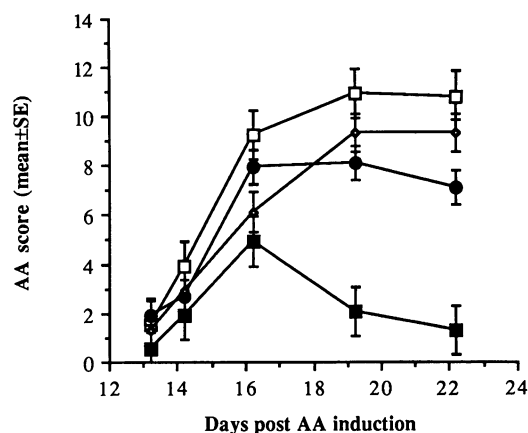

Figure 3. The route of administration of Ig affects its therapeutic effects. Ig was administered daily for 14 d into MT inoculated Lewis rats. The compound was administered intraperitoneally (*open diamonds*), subcutaneously (*filled circles*), or intravenously (*filled squares*). Untreated rats (*open squares*) served as a control group. Each group contained 10 rats. This is one experiment representative of the five done.

spleen weight of naive female Lewis rats was 550 mg. The weights of the spleen were increased 32 and 27% by administration of IVIg alone or by induction of AA using MT. However, the combination of IVIg and MT in the same rats caused an enlargement in spleen weight of 177%. The spleens of the rats with severe AA were less than half the size of the spleens of rats with AA suppressed by IVIg. This alerted us to the possibility that IVIg might activate rather than depress the immune system.

We therefore investigated the T cell–proliferative responses to a variety of antigens in naive rats or in immunized rats that had been treated with IVIg or with BSA. Naive rats immunized with BP in adjuvant developed EAE that was inhibited by treatment with IVIg, but that was not affected by BSA (see Fig. 1). Table III shows the T cell responses of various groups of rats. All rats showed strong responses to the T cell mitogen Con A and none responded to the control antigens BSA or OVA. Note
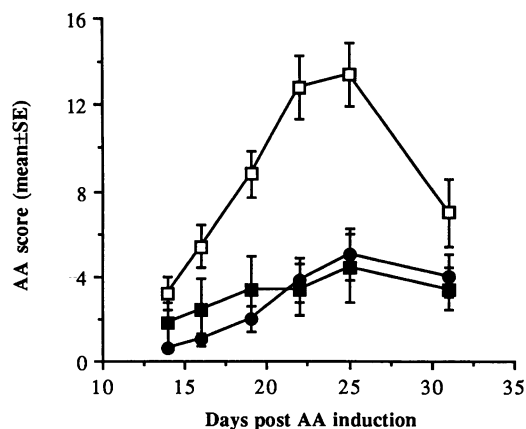

Figure 2. IVIg inhibits AA. Lewis rats, 12 per group, were inoculated with MT to induce AA. The rats were then treated daily with intravenous administration of BSA (*open triangles*), maltose (*filled circles*), PBS (*open squares*), or IVIg (*filled squares*). This is one experiment representative of the three done.

Figure 4. Effect of a single dose of IVIg. AA was induced in Lewis rats, eight rats per group, and the rats were treated with a single intravenous injection of Ig administered either on day 0 (*filled circles*) or on day 14 (*filled squares*). Untreated rats served as a control group (*open squares*). This is one experiment representative of the three done.
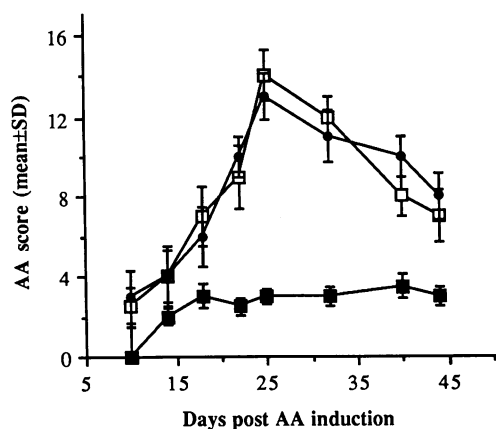
Figure 5. A single intravenous administration of Ig on day 14 abrogates AA. Lewis rats, six per group, were inoculated with MT to induce AA and treated on day 14 with Ig given either intravenously (*filled squares*) or subcutaneously (*filled circles*). Untreated rats served as a control group (*open squares*). This is one experiment representative of the five done.

Figure 7. Unpropitious timing of treatment may negate the benefit of IVIg. Rats, six per group, were treated by a single dose of IVIg on day 0 of AA induction (*filled squares*), or by five daily administrations of the IVIg until day 4 (*filled circles*). Untreated rats served as a control group (*open squares*). This is one experiment representative of the five done.

that treatment of naive rats with IVIg activated a proliferative T cell response to MT and to BP. Induction of EAE was associated with T cell responses to MT, to BP, and to its 71–90 encephalitogenic peptide. Treatment with BSA did not inhibit these responses, just as it failed to depress EAE. Indeed, administration of BSA by the intravenous route did not activate an anti-BSA-proliferative response. However, treatment with IVIg, which did inhibit clinical EAE (Fig. 1), did not depress the responses to MT, to BP, or to its 71–90 encephalitogenic peptide epitope (Table III). In fact, the responses to the disease-associated antigens were actually higher in the IVIg-treated rats than they were in the rats developing severe EAE. Thus, the suppression of EAE by IVIg treatment was accompanied by strong T cell responses to the target antigens of the encephalitogenic T cells and to the MT antigens in the adjuvant.

Comparable findings were obtained in AA experiments. Table IV shows that treatment of naive rats with IVIg caused increased responses to MT, to hsp70, to hsp65, and to the
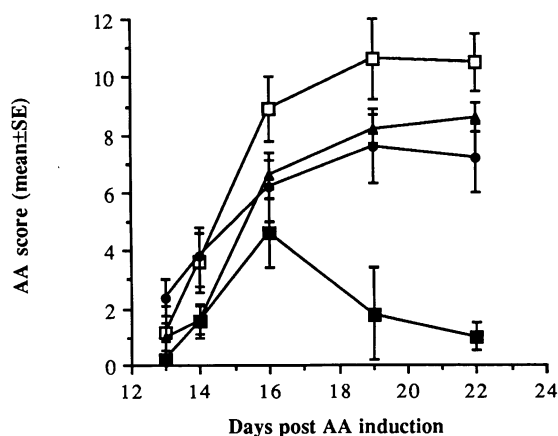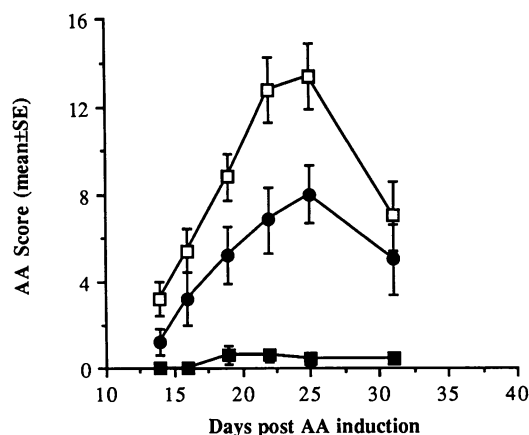
hsp65 peptide 180–188, which is a target for arthritogenic T cells (14). The responses to these antigens also appeared in rats immunized to MT and developing AA. However, treatment of the rats with IVIg, which suppressed the development of AA (Fig. 2), was associated with unchanged or augmented responses to these antigens (Table IV). Thus, the mechanism by which IVIg suppresses AA is not through inhibition of T cell recognition or proliferative responses to the relevant antigens.

IVIg inhibits TNFα secretion. An autoimmune disease is caused by destructive inflammation in the target organ and not merely by recognition of target antigens; antigen recognition is necessary but not sufficient. It is conceivable, therefore, that inhibition of disease by IVIg could result from inhibition of the mediation of inflammation. Fig. 8 shows the effect of IVIg treatment on the secretion of the key inflammatory cytokine TNFα. Groups of Lewis rats were either treated or not treated with IVIg daily for 10 d after induction of AA by MT. In addition, two groups of rats served as controls: one was untreated and the other was treated with IVIg. The spleens of the rats were removed and tested for their production of TNFα in the presence or absence of stimulation by the T cell mitogen Con A.

It can be seen that the basal unstimulated secretion of TNFα was somewhat increased by the administration of IVIg, by the induction of AA, and by the combination of both. However, the Con A–stimulated secretion of TNFα was markedly

Figure 6. Ig administered in the middle of the induction period of AA does not inhibit AA. Lewis rats, seven per group, were inoculated with MT and then treated with IVIg on day 0 (*filled squares*), day 5 (*filled circles*), or day 7 (*filled triangles*). Untreated rats served as a control group (*open squares*). This is one experiment representative of the three done.

Table II. Weights of Spleens Obtained from Treated Rats

Treatment of rats	Weight of spleens	Percent increase in weight
	mean mg±SD	
None	550±58	—
IVIg	730±85*	32
MT	700±115	27
MT + IVIg	1525±96*	177

Lewis rats (10 rats per group) were either untreated or treated by the indicated reagents. The weights of the removed spleens were assessed 24 d after AA induction by MT or after a course of IVIg daily for 14 d or after both treatments. *$P < 0.05$ of experimental vs. control group (None). This is one experiment representative of the 5 done.

Table III. IVIg Treatment of EAE Enhances Proliferative Responses to Antigens

Group	Disease	Treatment	Con A	BSA	OVA	MT	BP	BP peptide 71–90
					SI			
1	Naive	None	28	0.6	0.8	0.6	1	0.8
1	Naive	IVIg	20	1	1	6	3*	1
3	EAE	None	13	1	1	12*	6*	5*
4	EAE	BSA	19	1	1	7*	4*	4*
5	EAE	IVIg	11	1	1	14*	7*	5*

Lymph node cells were obtained on day 9 from naive or EAE rats, five rats per group, treated or untreated with BSA or IVIg. The cells were activated with the various antigens or Con A mitogen to induced proliferative responses measured as the stimulation index (SI). The background cpm (in the absence of added antigen) were in the range of 0.7–1.5×10^3 cpm. The SEMs for each group were in the range of ±0.3–1.6. This is one experiment representative of the three done. * $P < 0.005$ compared with control group 1.

inhibited in rats given IVIg alone and in the MT-immunized rats that had been treated with IVIg. Thus, the inhibition of disease was associated with inhibition of TNFα secretion, rather than with inhibition of antigen recognition (Tables III and IV).

Discussion

It is remarkable that IVIg therapy has been so widely used clinically despite the paucity of information about its mechanism of action on the immune system (7). The fact that IVIg improved the functional neurologic status of MS patients (11) led us to examine IVIg in T cell–mediated autoimmune diseases induced in rats. Indeed, this study indicates that much may be learned by attending to animal models. It was of interest that IVIg could be used to suppress both EAE and AA (Figs. 1 and 2), experimentally induced diseases in rats that are mediated primarily by CD4$^+$ T cells (15, 16). Based on its mode of clinical usage, it was not surprising that Ig treatment was observed to be most effective when given intravenously (Fig. 3). The reason for the ineffectiveness of the intraperitoneal or subcutaneous routes is not known at the present time; however, it might be that a delay in the absorption of the effective immunoglobulins or their anatomic compartmentalization might determine the outcome of treatment.

The timing of the IVIg treatment was critical: the effect of 2 wk of daily treatment with IVIg could be mimicked by a single dose of IVIg (Fig. 4) given either on the day of immunization with MT (day 0) or at the outbreak of AA (day 14), but not when given between the immunization and the appearance of clinical disease (days 5 or 7; Fig. 6). In fact, the benefit of a single dose of IVIg given at immunization was partly lost by continuing treatment for an additional 4 d (Fig. 7). Thus, IVIg has the capacity to neutralize itself at certain dose schedules. The cause for this paradoxical effect of IVIg requires further research; however, its implications for the clinical scheduling of IVIg treatment are great.

Investigation of the effects of IVIg on T cell behavior in vitro produced two observations: intact or enhanced T cell-proliferative responses to relevant antigens and depressed production of TNFα in response to a T cell mitogen (Tables III and IV, and Fig. 8). These findings indicate that IVIg does not work by inhibiting T cell recognition of antigens. IVIg also does not appear to depress the processing and presentation of antigens by antigen-presenting cells since the proliferative responses we measured depended on both APC and T cell populations obtained from the treated rats. Rather, the therapeutic effect of IVIg could be explained by regulation of a biologic mediator, TNFα, elaborated as a consequence of T cell activation (19). It remains to be elucidated whether secretion of cytokines other than TNFα is also inhibited by IVIg.

How normal human Ig can interact with rat T cells and influence both their proliferative behavior and their cytokine production is not clear. Perhaps normal Ig contains antibodies

Table IV. IVIg Treatment of AA Enhances Proliferative Responses to Antigens

Group	Disease	Treatment	Con A	MT	hsp70	hsp65	Peptide 180–188
				SI			
1	Naive	None	25	0.6	0.8	1	0.8
2	Naive	IVIg	22	20*	2	2	3
3	Naive	BSA	24	1	1	1	1
4	AA	None	21	7*	2	2	4*
5	AA	IVIg	26	16*	10*	5*	3

Lewis rats were either untreated (naive) or injected with MT to induce AA. Some groups of rats, four rats per group, were treated daily with IVIg or BSA. The lymph nodes were harvested on day 9 and their proliferative responses, expressed as SI, were assessed. The background cpm (in the absence of added antigen) were in the range of 0.5–1.2×10^3 cpm. The SEMs for each group were in the range of ±0.3–2.3. This is one experiment representative of the four done. * $P < 0.001$ compared with control group 1.
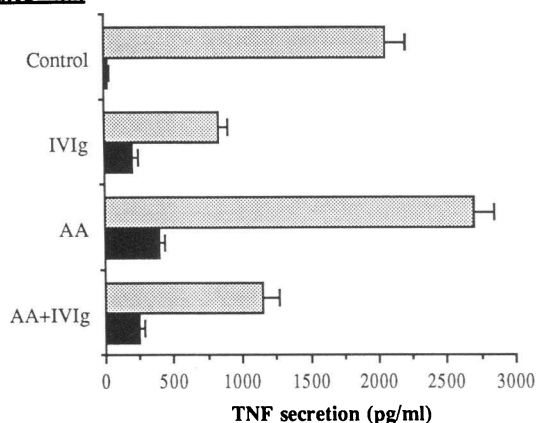
Animals treated with:

Figure 8. IVIg treatment of rats affects the secretion of TNFα. Spleen cells were obtained from either untreated control rats, or from rats treated with IVIg, with MT to induce AA, or with both MT and IVIg. Treatment with IVIg was for a consecutive 9 d. The splenocytes were seeded in microtiter plates and were either activated with Con A (*gray bars*) or not activated (*black bars*). The contents of the wells were assayed for TNFα secretion. Each group contained four rats. This is one experiment representative of the three done.

that can bind to T cells directly. It is also possible that the activation of T cells by Ig is indirect and is mediated by other cells, such as macrophages, which may be activated by antibodies present in the IVIg.

It is interesting that naive rats responded to IVIg treatment by enhanced responses to the MT and BP antigens involved in the diseases AA and EAE, but not to the OVA or BSA antigens. This suggests a degree of specificity in the stimulatory effect of IVIg on T cell activation. Guinea pig basic protein probably acts as a self-antigen in the rat, and the MT antigen involved in AA is thought to induce disease by mimicking a self-antigen present in the joints (20). This raises the possibility that IVIg treatment might activate certain autoreactive T cells. Several observations indicate that naive animals are populated naturally with T cells that can recognize dominant self-antigens, including hsp65 and BP. The collection of naturally autoimmune T lymphocytes has been proposed to form an immunological homunculus that functions to regulate autoimmunity (21, 22). Therefore, activation of naturally autoimmune T cells by IVIg might enhance regulatory mechanisms by a kind of endogenous T cell vaccination (23). However, more work must be done to investigate this possibility; the effect of IVIg on regulatory T cells is only one of the possible avenues of research opened up by the present observations. Although this investigation does not solve the riddle of the basic mechanisms underlying the effects of IVIg treatment, it does present new immunological observations related to these mechanisms. In general, it is clear from this study that normal Ig contains antibodies that are capable of interacting in complex ways with the circuits that regulate the mediators of autoimmune diseases.

Acknowledgments

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