0023-6837/03/8307-1013$03.00/0
LABORATORY INVESTIGATION

Vol. 83, No. 7, p. 1013, 2003
Printed in U.S.A.

Polyreactive Autoantibodies Purified from Human Intravenous Immunoglobulins Prevent the Development of Experimental Autoimmune Diseases

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SUMMARY: Intravenous immunoglobulins (IVIg) are therapeutic preparations of normal human polyclonal Ig G (IgG) that exert immunomodulatory effects in patients with autoimmune or systemic inflammatory diseases. Two different IgG subfractions were evaluated for their respective immunomodulatory effects in the treatment of experimental autoimmune diseases: a fraction enriched in antibodies that recognize the $F(ab')_2$ portion of IVIg and a fraction of natural polyreactive autoantibodies purified on a dinitrophenyl (DNP)-Affiprep immunoadsorbent. A very small fraction of IgG interacting with DNP but not with $F(ab')_2$ fragments expressed an increased ability to bind to self-antigens. The anti-DNP fraction, but not the anti-idiotype fraction, protected against inflammation observed in collagen-induced arthritis and experimental autoimmune encephalomyelitis in rats. Furthermore, it was able to reduce the occurrence of spontaneous diabetes mellitus in nonobese diabetic mice at lower concentrations than unfractionated IVIg. The therapeutic benefit of the anti-DNP fraction was associated with the inhibition of secretion of proinflammatory cytokines and stimulation of secretion of IL-1 receptor antagonist. Our results provide evidence that polyreactive autoantibodies play a role in the protective effect of IVIg in experimental models of autoimmune diseases in which inflammatory reactions are part of the disease process. (*Lab Invest 2003, 83:1013–1023*).

Intravenous immunoglobulins (IVIg) are therapeutic preparations of normal human polyclonal Ig G (IgG) that are obtained from pools of plasma from a large number of healthy blood donors. IVIg contain immune antibodies directed toward external antigens and natural autoantibodies that recognize intracellular, cell-surface, and circulating antigens, as well as V regions of antibodies (idiotypes) (Kazatchkine et al, 1994). Natural autoantibodies are produced without deliberate immunization (Coutinho et al, 1995) and are thought to be generated by positively selected autoreactive B cells (Hayakawa et al, 1999). They are polyreactive and express variable affinity for self-antigens (Berneman et al, 1992; Lacroix-Desmazes et al, 1998). In addition, almost all human and murine natural m-antibodies tested thus far react with a large panel of ligands, including the dinitrophenyl (DNP) hapten (Berneman et al, 1992; Dighiero et al, 1983; Seigneurin et al, 1988).

DOI: 10.1097/01.LAB.0000077982.70800.02

Received January 31, 2003.
Youri Chanseaud is a recipient of a grant from the French Ministry of Research. This work was supported by INSERM (CreS No. 4CR08F).
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Originally used as a substitutive therapy for primary antibody deficiencies, IVIg has more recently been proven to be beneficial in autoimmune and systemic inflammatory diseases (Dalakas, 1997; Dwyer, 1992; Kazatchkine and Kaveri, 2001). The mechanisms behind the immunomodulating effects of IVIg are now better understood and implicate both Fc and V regions of infused IgG (Larroche et al, 2002). IVIg interact with complement components and with Fc receptors on the surface of phagocytes (Larroche et al, 2002). In an experimental model of idiopathic thrombocytopenic purpura, IVIg were recently shown to prevent platelet destruction mediated by pathogenic autoantibodies by inducing expression of the inhibitory receptor FcγRIIB on splenic macrophages (Samuelsson et al, 2001). Moreover, by blocking Fc receptors of the neonate (FcRn) that enable IgG to enter the cells by pinocytosis and thereby protect them against catabolism, IVIg could be responsible for the accelerated rate of IgG catabolism in patients with antibody-mediated autoimmune disorders (Yu and Lennon, 1999). IVIg also modulate cytokine production as well as cell activation and proliferation (van Schaik et al, 1992) through the recognition of a large number of cell-surface molecules, such as V regions of B- and T-cell receptors, CD4, CD5, HLA class-I molecules, Fas, and Arg–Gly–Asp (RGD) sequence that is part of many adhesive extracellular-matrix proteins (Dalakas,

Table 1. Reactivity with Self and Nonself Antigens of Anti-F(ab')$_2$ and Anti-DNP-Lysine Eluates Obtained after Affinity Chromatography Purification of IVIg

Fractions and conditions of elution	Recovery, %	Polymers, %	Mean enrichment of eluates as compared to IVIg[a]					
			Tetanus toxin	F(ab')$_2$	DNP-albumin	Actin	Myosin	MBP	Tubulin
Anti-F(ab')$_2$ fraction glycine-HCl pH = 3.25	0.39 ± 0.05	6.0 ± 2.8	1.7 ± 0.25	71 ± 15	65 ± 27	67 ± 27	46 ± 20	32 ± 9	54 ± 19
Anti-F(ab')$_2$ fraction glycine-HCl pH = 2.8 + 0.15 M NaCl	0.38 ± 0.09	0.86 ± 1.24	1.0 ± 0.2	67.1 ± 15.2	14.7 ± 4.1	16.9 ± 3.9	9.6 ± 5.2	7.7 ± 3.8	8.1 ± 1.7
Anti-DNP-lysine fraction 2 M NaI pH 7	0.13 ± 0.02	2.1 ± 0.8	4.8 ± 2.2	4.5 ± 1.3	235 ± 100	158 ± 64	110 ± 33	94 ± 66	238 ± 108

DNP, dinitrophenyl; IVIg, intravenous immunoglobulin; MBP, myelin basic protein.
[a] Mean of five eluates.

1997; Dwyer, 1992; Kazatchkine and Kaveri, 2001; Larroche et al, 2002; Rhoades et al, 2000). IVIg may also neutralize circulating autoantibodies and modulate T- (Marchalonis et al, 1993) and B-cell repertoires (Hayakawa et al, 1999) through V region–dependent interactions.

IVIg contain anti-idiotype antibodies that are able to interact with natural and disease-associated autoantibodies (Kaveri et al, 1993; Rossi et al, 1991). A fraction of IVIg containing anti-idiotype antibodies was previously obtained by affinity purification of F(ab')$_2$ fragments of IVIg onto Sepharose-bound F(ab')$_2$ fragments of IVIg prepared from the same source (Dietrich et al, 1993). A greater proportion of autoantibodies and complementary (anti-idiotype) antibodies to autoantibodies were observed in this fraction compared with intact IVIg (Dietrich et al, 1993). It was thus thought to be potentially more effective than IVIg for the therapeutic control of autoimmune diseases. Polyreactive autoantibodies can also be obtained by affinity purification of normal human IgG using a DNP-Sepharose column (Berneman et al, 1993). To discriminate between immunomodulating effects due to polyreactive autoantibodies or anti-idiotype antibodies, we isolated and characterized the immunomodulatory properties of an anti-F(ab')$_2$ fraction (anti-idiotype autoantibodies) and an anti-DNP fraction (natural polyreactive autoantibodies). We observed that the anti-DNP IgG fraction, which represent a very small portion of IVIg, expressed increased ability to bind to self-antigens and protected against inflammation associated with several experimental autoimmune diseases, whereas the same effect was not observed with the anti-F(ab')$_2$ fraction. Therapeutic effect of the anti-DNP fraction was associated with inhibition of secretion of proinflammatory cytokines and stimulation of secretion of the anti-inflammatory cytokine IL-1 receptor antagonist (IL-1ra).

Results

Preparation and Reactivity of an Anti-F(ab')$_2$–Enriched Fraction from IVIg

IVIg were affinity purified on an IVIg-coupled N-hydroxysuccinimide (NHS) Affigel column, and elution was performed with glycine-HCl at pH 3.25. The mean reactivity of five eluates is presented in Table 1. The amount of IgG retained was 0.39%, and the level of antibody enrichment for F(ab')$_2$ fragments was 71. This fraction was 32- to 67-fold more reactive than intact IVIg for all autoantigen sources tested. In contrast, reactivity with the tetanus toxoid (TT) was not enriched at all. Molecular weight analysis by high pressure liquid chromatography revealed the presence of a significant percentage of polymers (6%) that was not present in unfractionated IgG (<1%). To decrease the amount of polymers, 0.15 M NaCl was added during the elution with glycine-HCl performed at pH 2.8; the amount of polymers was thus reduced to 0.86%, while the level of F(ab')$_2$ enrichment was not modified (67.1). Surprisingly, however, a 4-fold reduc-

tion in the reactivity with DNP, actin, myosin, myelin basic protein (MBP), and tubulin was observed. The fraction containing a low percentage of polymers is referred to as the "anti-F(ab')$_2$ fraction" and was used for in vitro and in vivo functional studies.

Preparation and Reactivity of Anti–DNP-Lysine IgG Fraction from IVIg

Experiments were performed with a column of DNP-lysine coupled NHS-Affiprep, and elution of bound IVIg was performed with 2 M NaI pH 7.0. This agent was selected instead of glycine-HCl because of its higher chaotropic activity, which allowed elution to take place at neutral pH. In these conditions we obtained a low level of polymers (2.1%). We observed that a small amount of the loaded unfractionated IVIg was retained (0.13%) and that the eluted fraction was enriched 235-fold in anti-DNP activity and expressed strong reactivity with autoantigens ($\times$94 to $\times$238) but not with F(ab')$_2$ fragments of IVIg or the TT (Table 1). The eluate is referred to as the "anti-DNP-fraction."

Unfractionated IVIg, anti-F(ab')$_2$, and anti-DNP fractions obtained from IVIg tested at an IgG concentration of 200 μg/ml recognized more than 20 protein bands in normal human liver and muscle tissue extracts as assessed by quantitative immunoblotting. However, some of the immunoreactivity peaks were higher in the case of the anti-DNP fraction than observed in the case of unfractionated IVIg or anti-F(ab')$_2$ fraction (data not shown).

Taken together, these findings clearly demonstrated that the anti-F(ab')$_2$ and anti-DNP fractions did not express the same antibody repertoires.

Collagen-Induced Arthritis (CIA) in Rats

Injection of 250 to 350 μg/kg of collagen resulted in the development of arthritis in 80% to 90% of Lewis rats. Preliminary experiments (data not shown) demonstrated that ip injection of 500 mg/kg of IVIg protected against CIA with the same efficacy either by ip or iv route. A dose of 50 mg/kg of IVIg did not reduce arthritis severity but significantly delayed disease onset (Fig. 1, A to C). No effect was observed at 5 mg/kg and 0.5 mg/kg IVIg (Fig. 1A). Administration of 500 mg/kg of human serum albumin (HSA) ip had no effect on disease development (data not shown).

The anti-F(ab')$_2$ fraction at 50 mg/kg protected against the disease significantly ($p < 0.05$) as compared with the untreated group but not better than 50 mg/kg IVIg (Fig. 2B). The dose of 5 mg/kg of anti-F(ab')$_2$ fraction was as inefficient as IVIg (Fig. 1, A and B). However, in the group that received the anti-DNP fraction, the disease severity was significantly reduced either at 50 mg/kg ($p < 0.01$) or 5 mg/kg ($p < 0.05$) as compared with the control group (Fig. 1C). The protection obtained with the anti-DNP fraction was significantly better than that observed with 50 mg/kg IVIg. The results indicated that the anti-DNP fraction provided better protection than intact IVIg or the anti-F(ab')$_2$ fraction.

Experimental Autoimmune Encephalomyelitis (EAE) in Rats

No therapeutic effect was observed when unfractionated IVIg or the anti-DNP fraction were injected ip from Day 0 to Day 8 at 500 mg/kg and 50 mg/kg, respec-

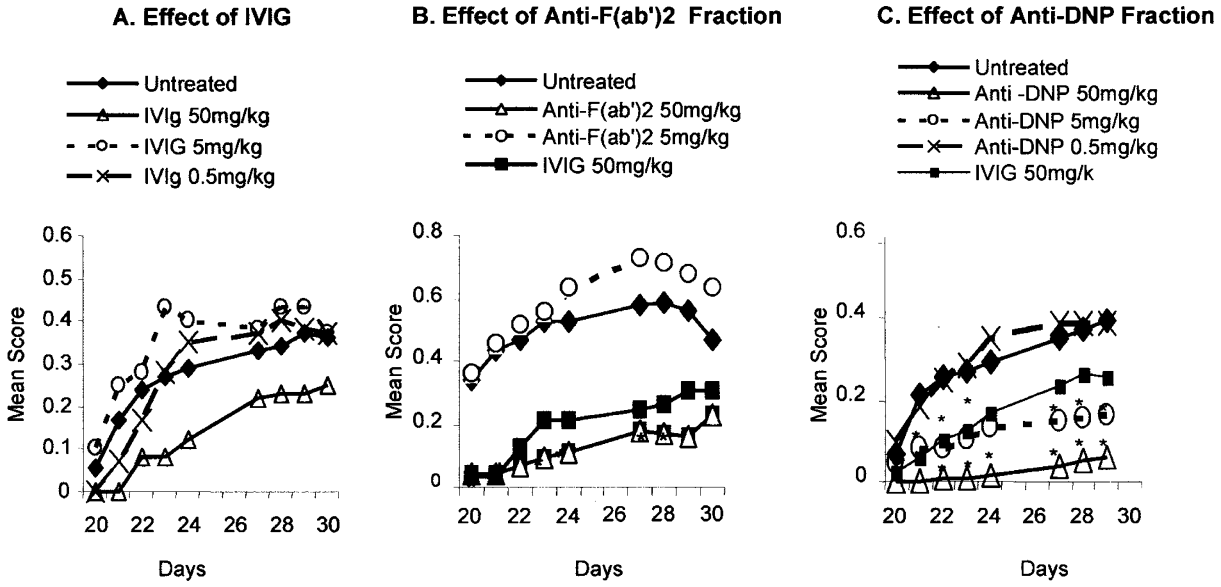

Figure 1.

Antidinitrophenyl fraction protected better than anti-F(ab')$_2$ fraction and intravenous immunoglobulin (IVIg) against collagen-induced arthritis. Dark-Agouti rats were immunized with collagen, and the mean arthritic score was measured. (A) Mean arthritic score of 12 animals either left untreated or treated ip from Day 0 to Day 3 with four injections of IVIg at doses of 50, 5, or 0.5 mg/kg. (B) Mean arthritic score of six to eight animals either left untreated or treated from Day 0 to Day 3 ip with four injections of anti-F(ab')$_2$ fraction at the doses of 50 or 5 mg/kg or of IVIg at the dose of 50 mg/kg. (C) Mean arthritic score of 12 animals either left untreated or treated ip from Day 0 to Day 3 with four injections of anti-DNP fraction at the doses of 50, 5, or 0.5 mg/kg or of IVIg at the dose of 50 mg/kg. Significant ($p < 0.05$) compared with the untreated group using Student's *t* test.

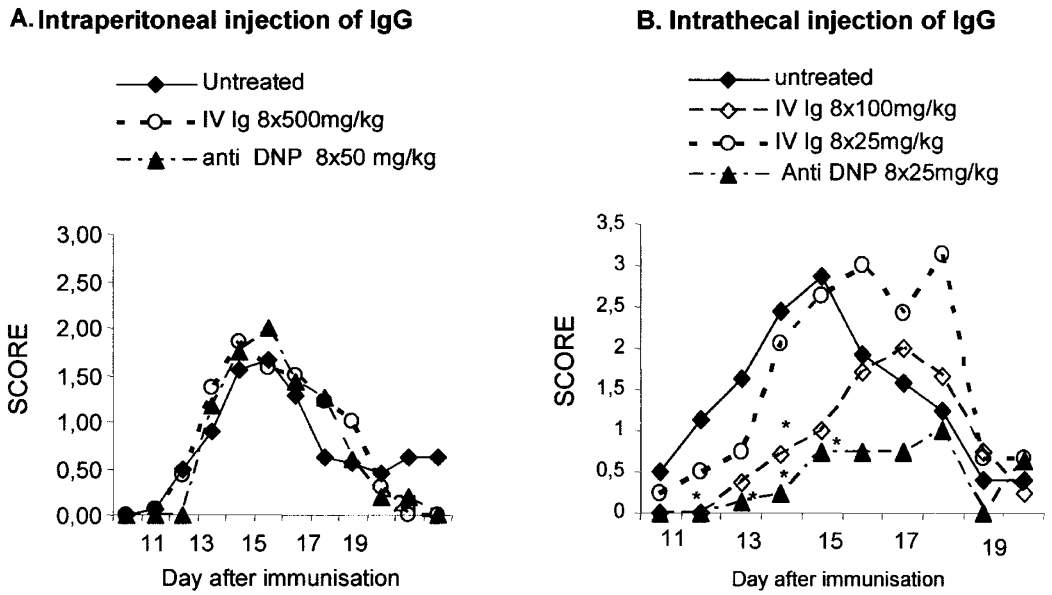

Figure 2.

Anti-dinitrophenyl (DNP) fraction protected better than intravenous immunoglobulin (IVIg) against experimental autoimmune encephalomyelitis (EAE) when injected intrathecally. Female Lewis rats were immunized with 1 μg of myelin basic protein, and the EAE score was measured. (A) Mean score of eight animals either left untreated or treated ip from Day 0 to Day 7 with eight injections of IVIg at the dose of 500 mg/kg or of the anti-DNP fraction at the dose of 50 mg/kg. (B) Mean score of eight animals either left untreated or treated intrathecally from Day 0 to Day 7 with eight injections of IVIg at the doses of 100 or 25 mg/kg or of the anti-DNP fraction at the dose of 25 mg/kg. Significant ($p < 0.05$) compared with the untreated group using Student's t test.

tively (Fig. 2A). In a subsequent experiment, 50 μl of IVIg and the anti-DNP fraction were injected intrathecally. The dose of 25 mg/kg of the anti-DNP fraction significantly inhibited ($p < 0.05$) the disease compared with untreated mice, whereas only a delay in symptoms was observed with intact IVIg at a dose of 100 mg/kg but not of 25 mg/kg (Fig. 2B).

Type I Diabetes Mellitus in Non-obese Diabetic (NOD) Mice

The effect of IgG given at birth was monitored in female NOD mice on the spontaneous development of diabetes; in male NOD mice, because only 20% of mice displayed diabetic symptoms, cyclophosphamide (CYC) was administered to promote the onset of disease by abrogating suppressor mechanism (Andersson et al, 1991).

CYC-Induced Diabetes in Male NOD Mice

Male mice were injected ip with CYC twice at 8 and 10 weeks of age. Fifty percent of untreated or 60% of HSA-treated NOD mice developed diabetes mellitus (Fig. 3A). Early treatment with 1 mg IVIg or 0.1 mg of the anti-DNP fraction significantly reduced the incidence of diabetes mellitus to 17% ($p = 0.0002$) and 21% ($p = 0.009$), respectively. In contrast, 0.1 mg of the anti-F(ab')$_2$ fraction did not significantly reduce the incidence of diabetes mellitus (43%, $p = 0.10$).

Spontaneous Development of Diabetes Mellitus in Female NOD Mice

Seventy-two percent of untreated female NOD mice were diabetic at 40 weeks of age (Fig. 3B), whereas only 43% ($p = 0.014$) and 37% ($p = 0.004$) were diabetic after IVIg (1 mg) or anti-DNP fraction (0.1 mg) treatment, respectively. The anti-F(ab')$_2$ fraction (0.1 mg) did not protect from the occurrence of diabetes mellitus because 60% of mice were diabetic ($p = 0.49$). Surprisingly, HSA significantly reduced the incidence of the disease ($p < 0.013$) as well as IVIg.

Influence of IVIg and Anti-DNP and Anti-F(ab')$_2$ Fractions on the Proliferation and Cytokine Secretion Occurring During Mixed Lymphocyte Culture (MLC)

Human peripheral blood mononuclear cell (PBMC) proliferation in MLC was reduced in a dose-dependent fashion in IVIg containing cultures as assessed by thymidine incorporation (Fig. 4). The inhibition was greater in the presence of the two fractions, the most effective being the anti-DNP fraction. The dose of IgG necessary to achieve 50% inhibition of proliferation was 75 μg for IVIg, 15 μg for anti-F(ab')$_2$, and 8 μg for anti-DNP fractions.

The secretion of IL-2, IL-1β, IL-4, IFN-γ, and TNF-α by PBMC was reduced in a dose-dependent fashion in the 72-hour supernatants of IVIg containing MLC cultures (Fig. 5). The anti-DNP fraction exhibited a stronger capacity to inhibit the secretion of most cytokines with an intermediate inhibitory values for anti-F(ab')$_2$ fraction. Moreover, the anti-DNP fraction exerted a strong capacity to stimulate IL-1ra secretion, which was not the case for unfractionated IVIg and anti-F(ab')$_2$ fraction. To identify IL-1ra–producing cells, we positively selected CD14$^+$ PBMC onto specific beads. However, isolated CD14$^+$ cells were activated by the selection process and secreted important amounts of IL-1ra in the absence of IgG. We

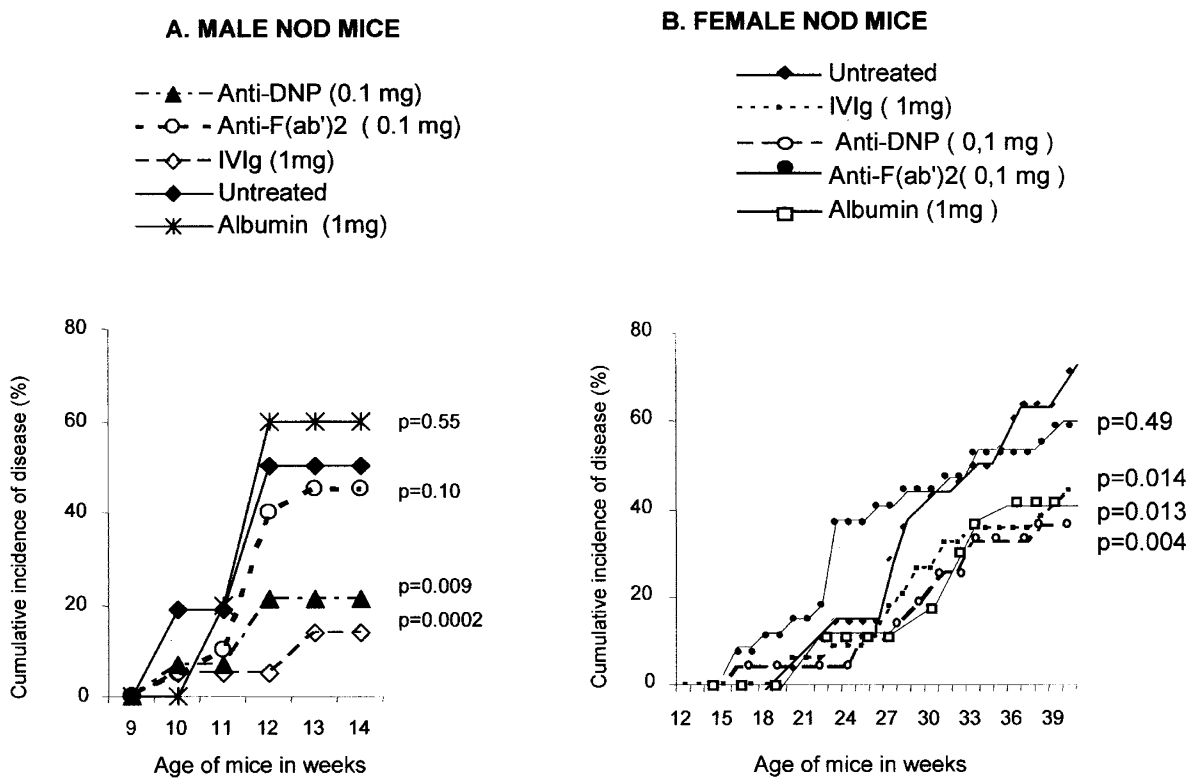

Figure 3.

Protection against diabetes in nonobese diabetic (NOD) mice is achieved by intravenous immunoglobulin (IVIg) and anti-DNP fraction but not by anti-F(ab')$_2$ fraction. Cumulative incidence of diabetes in NOD mice injected ip with IVIg, IVIg fractions, or human serum albumin. Treatment was initiated within 24 hours after birth and continued for 4 weeks. A mouse was considered positive when glycemia exceed 3 g/l. (A) Diabetes was induced in male NOD mice by the ip injection of 200 mg/kg of cyclophosphamide at 8 and 10 weeks of age. Untreated mice, $n = 16$; mice treated with 1 mg albumin, $n = 5$; 1 mg IVIg, $n = 21$; 0.1 mg anti-F(ab')$_2$ fraction, $n = 20$; 0.1 mg anti-DNP fraction, $n = 14$. (B) Diabetes developed spontaneously in female NOD mice. Untreated mice, $n = 20$; mice treated with 1 mg albumin, $n = 17$; 1 mg IVIg, $n = 27$; 0.1 mg anti-F(ab')$_2$ fraction, $n = 27$; 0.1 mg anti-DNP fraction, $n = 28$. Statistical analysis was performed using the logrank chi-square test and compared experimental groups to the untreated group.

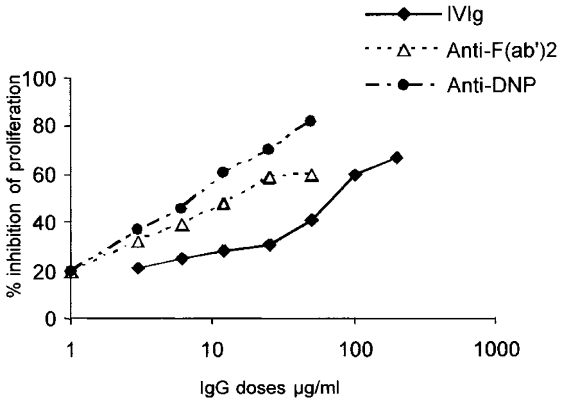

Figure 4.

Anti-dinitrophenyl (DNP) and anti-F(ab')$_2$ IgG fractions inhibited more strongly than intravenous immunoglobulin (IVIg) the lymphocyte proliferation observed during mixed lymphocyte culture. Peripheral blood lymphocyte (PBL) from two unrelated healthy donors were cultured together for 72 hours in the presence of various concentrations of anti-DNP, anti-F(ab')$_2$ fractions, and IVIg; thymidine was added during an additional 6 hours of culture. Radioactivity was measured, and results are presented in percent inhibition of thymidine incorporation as a function of IgG concentration. Results represented the mean of two to three experiments using different preparations of the fractions.

further isolated $CD3^+$ and $CD3^-$ cell populations from PBMC and stimulated them with ConA in the presence of different doses of unfractionated IVIg or anti-DNP fraction. We observed that the $CD3^-$ but not the $CD3^+$ population secreted high amounts of IL-1ra in the presence of the anti-DNP fraction but not in the presence of unfractionated IVIg (Fig. 6). We then cultured the myelomonocytic human cell line THP1 and detected higher amounts of IL-1ra in cell culture supernatants in the presence of 50, 25, and 12 μg/ml IgG of anti-DNP fraction than in the presence of similar IgG concentrations of anti-F(ab')$_2$ fraction (Fig. 7). Moreover, unfractionated IVIg did not stimulate IL-1ra production by THP1 cells at similar dosages.

Anticytokine, Anti-Fas, and Anti-CD4 Autoantibodies in IVIg, Anti-DNP, and Anti-F(ab')$_2$ Fractions

The anti-DNP fraction expressed a higher degree of enrichment in anti-CD4 and anti-Fas autoantibodies and in autoantibodies directed toward IL-1β, IL-2, IFN-γ, TNF-α, and IL-1ra as compared with IVIg (Table 2). The degree of enrichment in autoantibodies of the anti-F(ab')$_2$ fraction with reference to IVIg was much lower than that of the anti-DNP fraction, with the exception of anti-CD4 autoantibody. In addition, no IL-1β, IL-2, IFN-γ, TNF-α, IL-1ra, and transforming growth factor-β were detected in IVIg and fractions by ELISA assays using anticytokine antibodies–coated plates (data not shown).

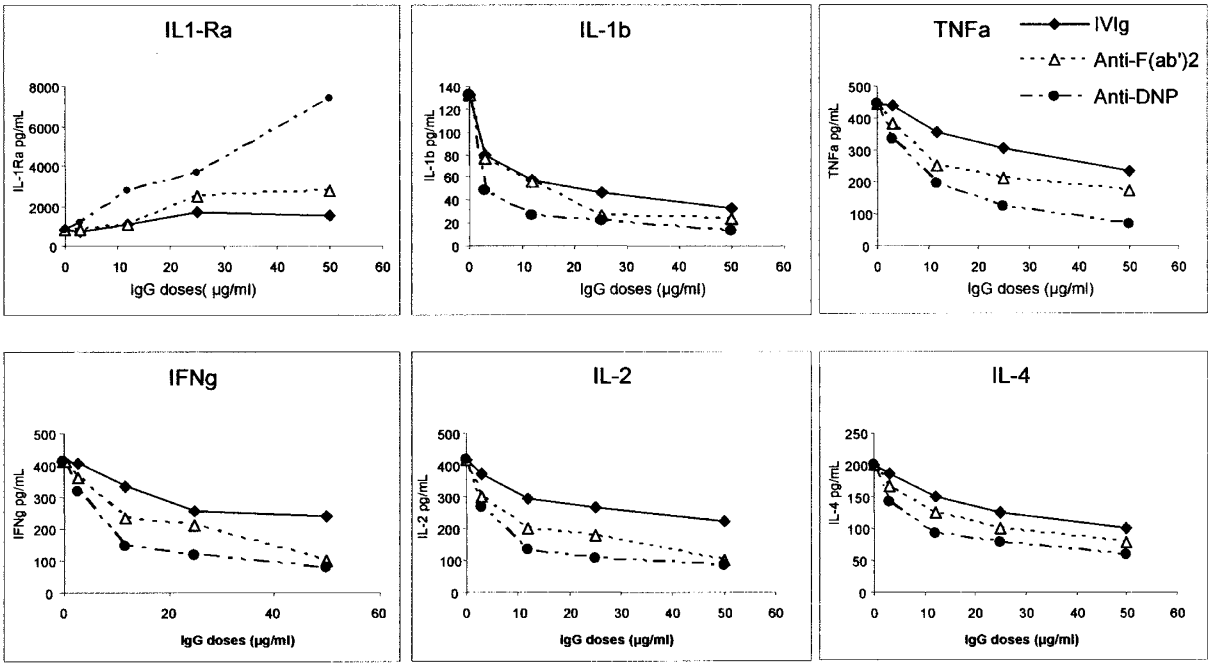

Figure 5.

Anti-dinitrophenyl (DNP) and anti-F(ab')$_2$ fractions inhibited the secretion of IL-1β, TNF-α, IFN-γ, IL-4, and IL-2 more efficiently than intravenous immunoglobulin (IVIg). Anti-DNP fraction but not anti-F(ab')$_2$ fraction stimulated the production of IL-1 receptor antagonist. Cytokine levels were assayed by ELISA in the 72-hour supernatants of mixed lymphocyte culture performed in the presence of various concentrations of IgG from IVIg and fractions. The results are expressed as the mean of two to three experiments using different preparations of the fractions.

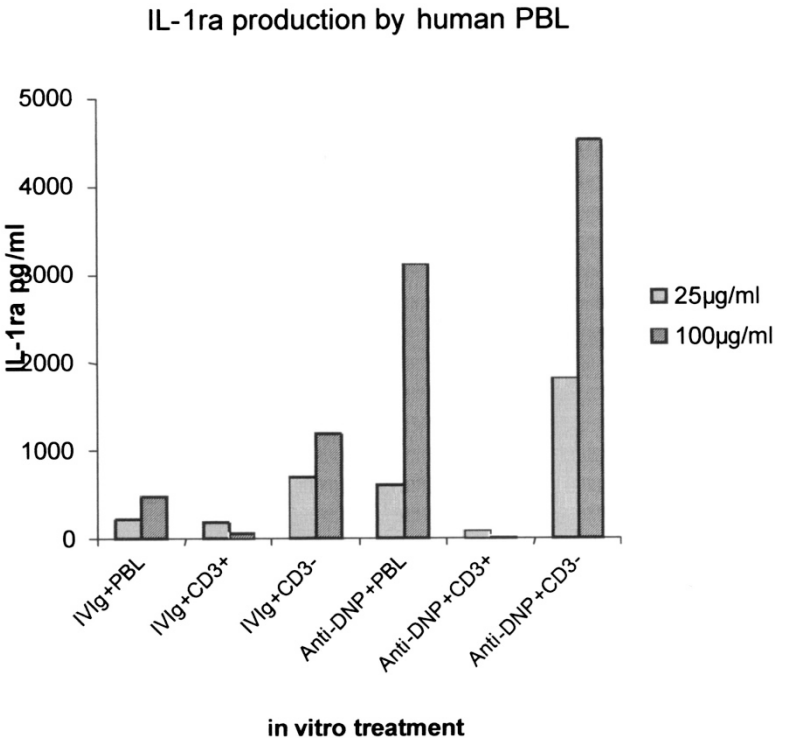

Figure 6.

Anti-dinitrophenyl (DNP) fraction but not IVIg increased in a dose-dependent fashion the secretion of IL-1 receptor antagonist (IL-1ra) by T-cell depleted peripheral blood mononuclear cells (PBMCs). IL-1ra level was assayed by ELISA in the 72-hour supernatants of ConA-stimulated PBMC in the presence of various concentrations of IgG from IVIg and anti-DNP fraction. A typical experiment is presented.

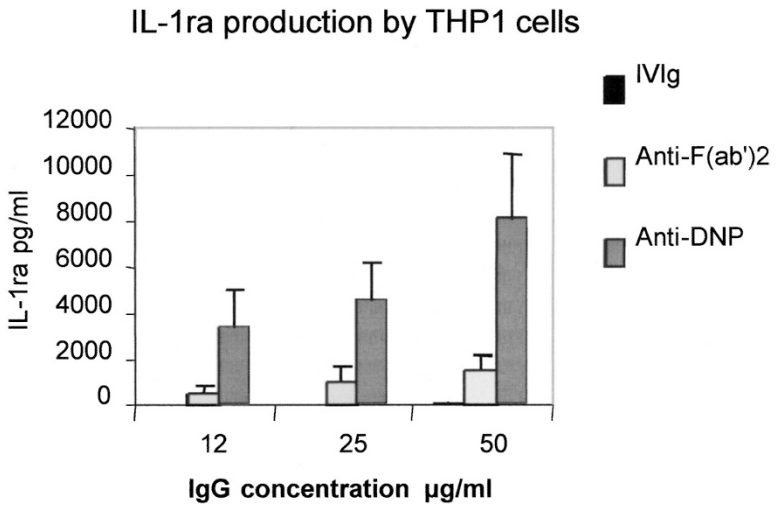

Figure 7.
Anti-DNP fraction but not anti-F(ab')$_2$ fraction or intravenous immunoglobulin (IVIg) increased in a dose-dependent fashion the secretion of IL-1 receptor antagonist (IL-1ra) by THP1 cell line. IL-1ra level was assayed by ELISA in the 72-hour supernatants of THP1 cells in the presence of various concentrations of IgG from IVIg and fractions. The results are expressed as the mean of two to three experiments using different preparations of the fractions.

Table 2. Presence of Autoantibodies Against Immunoregulatory Molecules in Affinity Purified Anti-F(ab')$_2$ and Anti-DNP-Lysine Enriched Fractions of IVIg

Immune molecules	Mean[a] ± SD level of enrichment in autoantibodies in the fractions compared to IVIg	
	Anti-F(ab')$_2$ fraction	Anti-DNP fraction
Fas	6.0 ± 1.0	31.0 ± 9.0
CD4	14.2 ± 5.5	16.5 ± 1.9
IL-2	1.4 ± 1.2	23.3 ± 2.7
TNF-α	1.0 ± 0.9	26.0 ± 5.0
IL-1-β	1.6 ± 1.1	37.2 ± 9.2
INF-γ	2.9 ± 0.8	39.6 ± 6.4
IL-1ra	1.1 ± 1.0	16.5 ± 3.5

DNP, dinitrophenyl; IVIg, intravenous immunoglobulin.
[a] Mean of two to three different fractions.

Discussion

Our results indicate that a very small fraction of IVIg interacting with DNP but not with F(ab')$_2$ fragments of IVIg expressed an increased ability to bind to self-antigens and not to the TT.

The anti-F(ab')$_2$ fraction eluted in the presence of NaCl and containing a low percentage of polymers was strongly enriched in antibodies recognizing F(ab')$_2$ fragments but only weakly reactive with self-antigens and the DNP hapten. Because intact IVIg was used to affinity purify the anti-F(ab')$_2$ fraction, we cannot exclude the possibility that some of the antibodies in this fraction could recognize Fc fragments of IgG; however, very few anti-Fcγ antibodies were detected in IVIg, and we think that the majority of retained IgG were anti-F(ab')$_2$ antibodies.

Conversely, the anti-DNP fraction did not bind to F(ab')$_2$ fragments of IVIg but was highly enriched in IgG reactive with self-antigens, ie, in natural autoantibodies(Berneman et al, 1992; Dighiero et al, 1983). It was previously demonstrated that the IVIg fraction that binds to the DNP hapten is enriched in polyreactive autoantibodies (Berneman et al, 1993) and that DNP competes with autoantigens for the binding of specific autoantibodies (Druet et al, 1994). The binding of antibodies in this fraction to DNP was inhibited in the presence of actin, myosin, and tubulin, indicating that these autoantigens share the same binding site on IgG molecules as the DNP hapten. In addition, because the eluate of a DNP-Affiprep-NHS column loaded with F(ab')$_2$ fragments of IVIg was strongly enriched in anti-DNP activity (data not shown), we believe that the recognition of DNP may be F(ab')$_2$ dependent.

The percentage of anti-DNP IgG retained on the NHS-Affiprep column is very small (0.13%) in comparison to the previously reported 30% of IVIg binding to DNP coupled to Sepharose (Rossi et al, 1991). However, Farah observed that only 0.4% of IgG was retained when an amino-ethyl cellulose gel coupled to DNP was loaded with IVIg (Farah, 1973). The differences in adsorption and elution conditions and in the immunoadsorbent used could help explain the discrepancy between our results and those previously reported in the literature (Berneman et al, 1993; Farah, 1973).

We have observed that the two antibody fractions purified from IVIg exert mutually exclusive antibody activities: the anti-DNP fraction bound to self-antigens but not to F(ab')$_2$ fragments of IVIg and the anti-F(ab')$_2$ fraction interacted weakly to self-antigens. These results differed from those obtained by Dietrich et al (1993), who reported that a fraction of IVIg obtained by affinity purification onto Sepharose-bound F(ab')$_2$ fragments of IVIg contained antibodies connected through V regions and also expressed a higher degree of autoreactivity than unfractionated IVIg. Two expla-

nations can be proposed to account for this discrepancy. Firstly, in our hands Sepharose gel nonspecifically adsorbed self-reactive IgG, whereas Affigel and Affiprep gels exerted negligible nonspecific IgG adsorption (data not shown), and we speculate that the autoantibody activity of this anti-F(ab')$_2$–connected fraction may result at least in part from Sepharose nonspecific binding of IgG. Secondly, because we and others have observed that the amount of polymers increased as the pH lowered and that the degree of polyreactivity was correlated with the amount of polymers (MacMahon and O'Kennedy, 2000), we did not use glycine-HCl pH 2.8 alone as Dietrich et al (1993) did, but we added NaCl during acid elution of IVIg-coupled Affigel. This process prevented the formation of polymers and considerably reduced the polyreactivity of the anti-F(ab')$_2$ fraction.

As previously shown, we confirmed that IVIg prevented the occurrence of CIA (Ulmansky and Napastek, 1995) and EAE (Achiron et al, 2000) in rats and diabetes mellitus in NOD mice (Andersson et al, 1991; Fosgren et al, 1991). Before injecting fractions into animals, we first compared iv and ip routes of administration of IVIg in CIA and observed for both routes the same strong protective effect at 500 mg/kg. Moreover, by measuring serum IgG concentrations after ip and iv IVIg infusion, we obtained similar results at 6 hours, whereas at 3 hours higher IgG concentration was measured when IVIg were administered by iv route. These results validate the ip route used in the CIA model. In the EAE model, ip administration of IVIg had no protective effect, suggesting that IgG did not efficiently cross the blood-brain barrier. Consequently, IgG was administrated intrathecally.

Data indicated that the anti-DNP fraction but not the anti-F(ab')$_2$ fraction protected against inflammation associated with CIA in rats and reduced the occurrence of diabetes in NOD mice at concentrations 10-fold lower than unfractionated IVIg. The anti-DNP fraction also reduced more effectively EAE symptoms than unfractionated IVIg. The mechanisms that have been proposed to explain the beneficial effect of IVIg in autoimmune and systemic inflammatory diseases include IVIg interactions with Fc receptors, complement proteins and cell-surface molecules, neutralization of circulating autoantibodies, and modulation of cytokine production and cell proliferation (Kazatchkine et al, 2001; Yu and Lennon, 1999).

Two Fc-dependent mechanisms have recently been described that contribute to the therapeutic effect of IVIg in autoantibody-mediated diseases, ie, increased IgG catabolism (Yu and Lennon, 1999) and induction of the inhibitory receptor Fc-γ RIIB expression on macrophages surface (Samuelsson et al, 2001). In our models, we observed that low amounts (5 mg/kg in CIA, 25 mg/kg in EAE, and 0.1 mg in newborn NOD mice) of natural autoantibody-enriched fraction protected from the development of autoimmune experimental diseases, whereas similar doses of IVIg did not. Therefore, we do not think that these results can only be explained by an effect through Fc-γ receptors and stress the role of variable regions of anti-DNP IgG as an explanation to biological effects observed in vivo and in vitro. Conversely, we postulated that the anti-idiotype fraction failed to protect better than IVIg because our experimental models of autoimmune diseases were T-cell dependent.

Many authors have demonstrated that IVIg up-regulate IL-1ra (Andersson et al, 1994; Arend and Leung, 1994; Ruiz de Souza et al, 1995) and down-regulate IL-1 synthesis and release (Okitsu-Negishi et al, 1994) in vitro and in vivo, thereby explaining their major anti-inflammatory effect in certain autoimmune and systemic inflammatory diseases. Our data provide evidence that the anti-DNP fraction, and to a lesser degree the anti-F(ab')$_2$ fraction, inhibited lymphocyte proliferation and secretion of IL-2, IL-1β, IL-4, IFN-γ, and TNF-α during MLC in a greater proportion than IVIg. Although the data on Th1 lymphokines in the literature are concordant with the down-regulation of IL-2 and IFN-γ by IVIg in vitro, conflicting results have been reported concerning IL-4 and TNF-α. The main difference between anti-DNP and anti-F(ab')$_2$ fractions was the unique ability of the anti-DNP fraction to stimulate the secretion of high amounts of IL-1ra. These data were confirmed in experiments showing that the THP1 myelomonocytic cell line produces a high amount of IL-1ra in the presence of the anti-DNP fraction but not in the presence of the anti-F(ab')$_2$ fraction or total IVIg. IL-1ra is a strong natural anti-inflammatory molecule that is produced by monocytes and binds to the IL-1β receptor. Furthermore, we found that the amounts of naturally occurring autoantibodies recognizing various cell-surface molecules and cytokines already described to be present in IVIg (Kazatchkine and Kaveri, 2001; Larroche et al, 2002) were increased in the anti-DNP fraction. Thus, the combination of an inhibition in proinflammatory cytokine secretion such as IL-1β, IFN-γ, and TNF-α; a stimulation of IL-1ra secretion; and the presence of natural anticytokine antibodies (Abe et al, 1994) could explain the therapeutic effect of the anti-DNP fraction compared with IVIg in experimental autoimmune diseases in which inflammatory reactions are part of the disease process. However, the detailed mechanism by which IgG presents in the anti-DNP fraction modulate cytokine production remains to be determined.

The active fraction in IVIg corresponds to a very small percentage of IgG and probably is not representative of the total immunomodulatory effect of IVIg. Natural polyreactive autoantibodies may play a role in the protective effect of IVIg and could potentially represent a new generation of IVIg treatments with the theoretical benefit of inducing similar responses with much lower doses of IgG.

Materials and Methods

Animals

All animals used were bred and maintained in our animal facility. Two- to three-month-old male Dark Agouti and female Lewis rats (mean weight, 180 to 200 g) were purchased from Janvier (Le Genest St Isle,

France). NOD (Ltd) mice were purchased from Iffa Credo (L'Arbresle, France).

IgG

IVIg (Tegeline) was prepared by LFB (Les Ulis, France) as previously described (Malgras et al, 1970) from a pool of plasma from more than 5000 healthy blood donors.

Affinity Chromatography

IVIg were coupled to Affigel-NHS (Bio-Rad, Hercules, California) immunoadsorbent at a concentration of 5 mg/ml of gel. Two grams of IVIg in PBS were loaded and circulated on a 2-L column for 4 hours at 22° C. Retained Ig were eluted with glycine-HCl at various pH values (2.8 to 3.5) with or without 0.15 M NaCl and immediately brought to pH 4 with NaOH. This fraction is referred to as the "anti-F(ab')$_2$ fraction."

DNP-lysine (Sigma, St Quentin Falavier, France) was coupled to Affiprep-NHS (Bio-Rad) at a concentration of 5 mg/ml of gel. Six grams of IVIg were loaded and circulated on a 2-L column for 4 hours at 22° C. Retained Ig were eluted with 2 M NaI pH 7.0 and further desalted on a G25 Sephadex column. This fraction is referred to as the "anti-DNP fraction."

The eluates were concentrated on 50-kd membranes (Macrosep, Filtron; Pall Life Sciences, Ann Harbor, Michigan) and frozen at −80° C until use. The percentage of monomers, dimers, polymers, and fragments were determined by high pressure liquid chromatography gel filtration on a G 3000 TSK column (Merk, Darmstad, Germany).

Detection of Antibody Reactivities with Self- and Nonself-Antigens by ELISA and Western Blotting

The eluates were assessed by ELISA for antibody reactivity against DNP-albumin (Sigma), F(ab')$_2$ fragments of IVIg (Tegeline, LFB), calf actin (Sigma), calf myosin (Sigma), guinea pig MBP (Sigma), pig tubulin (Institut Pasteur, Paris, France), and TT (Pasteur Merieux, Lyon, France). Ninety-six–well ELISA plates were coated overnight at 37° C with F(ab')$_2$ or 1 hour at 37° C and subsequently overnight at 4° C with all other antigens. The plates were saturated with 1% milk (for F(ab')$_2$) or 1% BSA (for other antigens) and incubated for 1 hour at 22° C. Increasing amounts of IVIg and of the various eluates diluted in PBS-gelatin were added to the wells and incubated for 2 hours at 37° C or 22° C. After extensive washing, plates were incubated with peroxydase-labeled γ-chain specific goat antihuman IgG antibody and then revealed with o-phenylenediamine. The enzyme reaction was stopped after 5 minutes and optical density measured at 490 nm (Labsystem, Cergy Pontoise, France). The results were expressed as a ratio of enrichment as compared with unfractionated IVIg.

IgG reactivity of unfractionated IVIg and the anti-DNP and anti-F(ab')$_2$ fractions with liver and muscle extracts were analyzed using a quantitative immunoblotting technique as described by Mouthon et al (1995).

MLC, THP1 Culture, and Cytokine Secretion

Peripheral blood was collected from two healthy donors and mononuclear cells (PBMC) were separated on ficoll density gradient centrifugation. Bidirectional MLCs were established between donor A (1.5 10^5 cells/well) and donor B (1.5 10^5 cells/well) in a total volume of 200 μL in 96-well flat-bottom tissue culture plates. Cells were cultured at 37° C in 5% CO_2 in RPMI 1640 supplemented with 1 mmol/L L-glutamine and 10% FCS in the presence of serially diluted IgG from IVIg and fractions, and supernatants were collected after 72 hours for cytokine measurement. For proliferation assay, 200 μL of fresh medium was added, and cultures were pulsed with 1 μCi of ^{3}H-thymidine for an additional 6 hours. Cells were harvested, and ^{3}H-thymidine incorporation was determined in a beta counter (Kontron, Plaisir, France). Purification of $CD3^+$, $CD3^-$, or $CD14^+$ cell populations from PBMC was performed using microbeads and magnetic column selection (MACS, Miltenyi Biotech, Germany).

The myelomonocytic cell line THP1 (ATCC, TIB 202) was cultured in 1640 RPMI medium supplemented with 10% FCS at the concentration of 10^5 cells per ml in 9-cm^2 culture Petri dishes in the presence of serially diluted IgG from IVIg and fractions. After 72 hours of culture, cells were numbered and supernatants collected for IL-1ra measurement.

Cytokine ELISA Assays

IL-1ra, IL-1β, IL-2, IFN-γ, IL-4, and TNF-α were quantitated using ELISA kits (R&D Systems, Abingdon, United Kingdom) according to the manufacturer's instructions. The amount of cytokines was expressed in pg/ml, and the minimum detectable concentrations were 150 pg/mL for IL-1ra, 5 pg/mL for IL-1β, and 20 pg/mL for IL-2, IL-4, IFN-γ, and TNF-α. MLC culture supernatants were harvested and tested undiluted to determine cytokine concentration. THP1 culture supernatants were serially diluted and tested to determine IL-1ra concentration.

Detection of Anticytokine, Anti-Fas and Anti-CD4 Neutralizing Antibodies

Serially diluted IVIg and anti-DNP and anti-F(ab')$_2$ fractions were incubated for 1 hour at 37° C in culture plates coated with recombinant IL-1ra (100 ng/ml), IL-1β (10 ng/ml), IL-2 (20 ng/ml), IFN-γ (20 ng/ml), TNF-α (20 ng/ml) (R&D Systems), recombinant human Fas (1 μg/ml) (R&D Systems), and CD4 (100 ng/ml of purified protein) (a gift from Dr. Klatzman). The presence of antibodies against these molecules was revealed in ELISA by peroxydase-labeled rabbit antihuman IgG as described above. Results were presented as the level of enrichment in autoantibodies in anti-DNP and anti-F(ab')$_2$ fractions compared with unfractionated IVIg.

Collagen-Induced Arthritis

Type II bovine collagen (Morwell, Zurich, Switzerland) was dissolved vol/vol in 0.1 M acetic acid and emulsified in incomplete Freund adjuvant (DIFCO, Detroit, Michigan). One ml emulsion containing 250 to 350 μg collagen was injected intradermally at the base of the tail and on the back above each leg. Each experimental group included six to eight rats, and experiments were performed twice.

The rats were examined daily for inflammatory signs such as erythema and swelling (Ulmansky and Naparstek, 1995). The ankle, tarsus, and metatarsus joints were scored as follows: 0 = unaffected, 1 = joint erythema, 2 = localized or moderate joint swelling, 3 = serious swelling and edematous aspect, and 4 = deformity of the joints. The total score for each animal was calculated as an arthritis index (maximum 48). The IVIg and affinity purified fractions were injected ip at 200, 50, 5, or 0.5 mg/kg for 4 days starting the day of immunization. HSA was used as a control.

Experimental Autoimmune Encephalomyelitis

One μg of MBP (Sigma) was emulsified in complete Freund adjuvant plus mycobacterium at the dose of 400 μg per animal (DIFCO) and injected in each hind footpad of female Lewis rats. The following validated clinical severity scale was used: 0 = normal, 1 = decreased tail tone, 2 = tail paralysis, 3 = paraparesis, 4 = paraplegia, and 5 = moribund (Achiron et al, 2000).

The IVIg and anti-DNP fraction were administered at different dosages from Day 0 to Day 8 either by ip or intrathecal route.

Insulin-Dependent Diabetes Mellitus in NOD Mice

Newborn NOD mice were injected three times a week ip with 1 mg unfractionated IVIg or 0.1 mg of the anti-DNP or anti-F(ab')$_2$ fraction and 1 mg HSA as a control. The treatment was initiated within 24 hours after birth and continued for 4 weeks.

Diabetes mellitus that developed spontaneously in 60% of female NOD mice was monitored from 8 to 40 weeks of age by measuring blood glucose concentration using the glucometer Eprit (Bayer Diagnostics, Munich, Germany) colorimetric assay. Animals were considered diabetic when blood glucose concentration exceeded 3 g/l for 2 weeks consecutively (Andersson et al, 1991).

Because only 20% of male NOD mice spontaneously developed insulin-dependent diabetes mellitus, the disease was accelerated by injection of CYC given at the dose of 200 mg/kg ip twice, at 8 and 10 weeks of age (Yasunami and Bach, 1988). Blood glucose level was measured after retro-orbital bleeding before the second CYC injection and every week thereafter until 15 weeks of age.

Acknowledgements

We thank S. Avrameas, W.H. Fridman, and M.D. Kazatchkine for their advice and active participation in discussions. Animal experiments were done in our animal facilities (LFB, Les Ulis, accreditation No. 91639) by G. Clarisse (No. 03697) and M. Bruley-Rosset (No. 91–9) according to European Council directives No. 86/609 concerning the protection of animals used for experimental and research purposes and to current laboratory procedures.

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