



Intravenous immunoglobulin prevents experimental autoimmune myositis in SJL mice by reducing anti-myosin antibody and by blocking complement deposition

J Wada, N Shintani, K Kikutani, T Nakae, T Yamauchi, and K Takechi

Drug Discovery Laboratories, Pharmaceutical Research Division, Welfide Corporation, Hirakata, Osaka, Japan

Correspondence: Junko Wada, Drug Discovery Laboratories, Pharmaceutical Research Division, Welfide Corporation, 2-25-1 Shodai-Ohtani, Hirakata, Osaka 573-1153, Japan E-mail: junko@welfide.co.jp

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ABSTRACT

High-dose intravenous immunoglobulin (IVIG) therapy has been effective in many autoimmune and systemic inflammatory diseases including polymyositis (PM) and dermatomyositis (DM). In the present study we evaluated the efficacy of IVIG using experimental models of PM and DM. An experimental autoimmune myositis (EAM) model was produced in SJL/J mice by an immunization with rabbit myosin B (MB) fraction. In this model, the plasma level of anti-MB antibody was elevated, and mouse IgG and complement C3 were deposited in the muscle fibres. Administration of IVIG dose-dependently reduced the incidences of necrotic and inflammatory changes in the skeletal muscle. IVIG treatment also decreased the elevation of anti-MB antibody level, as well as the deposition of IgG and C3. We next evaluated the effect of IVIG in adoptive EAM mice made by an intravenous injection of lymph node cells previously stimulated with MB. Adoptive EAM mice showed similar lesions in skeletal muscle as EAM mice and IVIG inhibited the lesion development. *In vitro* experiments demonstrated that IVIG inhibited complement-mediated lysis of human erythrocytes sensitized with anti-human erythrocyte antibodies. The binding of C1q, C4 and C3 to the same cells was also inhibited by IVIG. Taken together these findings suggest that IVIG prevents the development of myositis in EAM and adoptive EAM models by several mechanisms, such as reducing anti-myosin antibody and by blocking

complement activation. Our present findings might account for the clinical efficacy of IVIG in PM and DM patients.

Keywords: IVIG, experimental autoimmune myositis, complement, autoantibody, T lymphocyte

INTRODUCTION

Polymyositis (PM) and dermatomyositis (DM) are chronic, immune-mediated inflammatory diseases. The major pathological characteristics are muscle weakness with elevated serum levels of creatine kinase, and degenerative changes are observed in their skeletal muscles. Autoantibodies against myosin and nuclear substances are prominent in their serum, and infiltration of lymphocytes and macrophages beneath the basement membrane of muscle fibres has been observed [1]. In addition, deposition of terminal C5b-C9 membrane attack complex (MAC) in intramuscular capillaries has been reported in DM patients, which may lead to the destruction of endothelial cells followed by the development of microinfarcts in the muscle fascicles and perifascicular atrophy [2]. These immunological disorders are thought to work synergistically and eventually cause progression of the muscle degeneration. Various combinations of immunosuppressive agents have been used in uncontrolled clinical studies, but in many cases the patients became resistant to these remedies [1–3].

Similar to the other autoimmune diseases, high-dose intravenous immunoglobulin (IVIG) therapy has beneficial effects against steroid-resistant and refractory PM and DM patients [3]. IVIG administrations resulted in significant improvement of muscle strength, reduction in serum creatine kinase levels, down-regulation of muscular ICAM-1, MHC-I and TGF- β and reduction of immune-complex deposition in the intramuscular capillaries [4–6].

Several mechanisms have been proposed for the immunomodulatory effect of IVIG [7,8]: (1) inhibition of complement activities (2) neutralization of autoantibodies by anti-idiotypic antibodies (3) modulation of lymphocyte functions and cytokine synthesis (4) blockade of Fc-receptors on phagocytic cells. To assess which of the above and/or unknown mechanisms are involved in its ameliorative effect in PM and DM patients, we attempt to define the characteristics using animal models.

Mouse experimental autoimmune myositis (EAM) has been widely used for the study of PM and DM [9–11]. In this model, SJL/J mice immunized with rabbit myosin B (MB) fraction develop several symptoms similar to human PM and DM, such as

inflammatory cell infiltration, anti-MB antibody production, and IgG and complement depositions. In the present study, we report the inhibitory effects of IVIG on the muscle lesion, circulating autoantibody levels and deposition of complement in the EAM model. In addition we describe the effect of IVIG in adoptive EAM model, which was made by the transfer of lymphocytes from EAM mice to homologous mice. In addition we describe the effect of IVIG in adoptive EAM model, which was made by the transfer of lymphocytes from EAM mice to homologous mice.

MATERIALS AND METHODS

Animals

Male SJL/J mice were obtained from Jackson Laboratory (Bar Harbor, ME).

EAM model

Rabbit skeletal muscle MB fraction was prepared as described previously [12]. Rabbit MB fractions (4.2 mg/ml) were emulsified with equal volumes of complete Freund's adjuvant. SJL/J mice were injected s.c. with MB/CFA (0.3 ml/mouse) in the back on days 0, 7, 14 and 21. IVIG (Venoglobulin-IH, Welfide, Co., Osaka, Japan) (100, 200, 400, or 800 mg/kg/day) was administered i.v. into the tail vein for 5 consecutive days from each day of the immunization. On day 49, mice were sacrificed and both thigh quadriceps were extirpated. Tissue specimens were fixed with 10% buffered formalin solution and embedded in paraffin. These sections (4 μ m) were stained with haematoxylin and eosin. Two thousand muscle fibres were examined in each animal to assess the number of fibres with necrotic or inflammatory pathological changes. The necrotic and inflammatory fibres were characterized by pale amorphous cytoplasm and the presence of more than five inflammatory cells infiltrated around the muscle fibre, respectively.

Adoptive EAM model

The EAM mice were sacrificed on day 28. Axillary, inguinal and popliteal lymph nodes, spleens and serum were collected. Spleen cells were haemolysed with Tris-NH₄Cl, suspended in Dulbecco's modified eagle medium (DMEM)(Life Technologies, Inc., Grand Island, NY) supplemented with 5.5×10^{-5} M 2-mercaptoethanol, 25 mM HEPES, 50 units/ml penicillin and 50 μ g/ml streptomycin at 3×10^7 cells/ml and seeded into 6-well plate (1 ml/well). After 2 h of incubation at 37°C under 5% CO₂, cells were washed three times to remove nonadherent cells. Lymph node cells (3.6×10^7 cells/6 ml/well) were added to this plate and cultured with 25 μ g/ml MB and 1%

autologous serum. Cells were collected on day 2, and dead cells were removed by density gradient centrifugation using Lympholyte-M (Cedarlane, Ontario, Canada). Viable cells were collected and cultured at 2×10^6 cells/ml in DMEM supplemented with IL-2 (20 JRU/ml)(KOHJIN BIO, Co., Saitama, Japan) and 1% autologous serum. On day 5, cells were collected and viable cells were separated. Phenotype of unstimulated cells (day 0) and MB-stimulated (days 2 and 5) lymph node cells were analysed by flow cytometry.

After the 5 days of stimulation MB-activated cells (5×10^7 cells/mouse) were injected into SJL/J mice i.v. to make an adoptive EAM model. To activate the transferred cells, IL-2 (500 JRU/mouse) was administered i.p. twice a day for 5 days. IVIG (100, 200, 400, or 800 mg/kg/day) was administered i.v. for 5 days from the day when cells were transferred. On day 7 mice were sacrificed and histological evaluations were carried out as described above.

Measurement of plasma anti-MB antibodies and immunohistochemical analysis in EAM mice

The mouse EAM model was made as described above except that immunization was carried out only on days 0 and 21. IVIG (400 mg/kg/day) was administered i.v. for 5 consecutive days from day 21. Plasma was collected every week for 12 weeks from the day of the first immunization. The plasma level of anti-MB antibody was measured by ELISA using 96-well microtiter plates coated with mouse MB, and peroxidase-labelled anti-mouse IgG + IgM (Chemicon International, Inc., Temecula, CA). Anti-MB antibody level was expressed in arbitrary units against in-house standard plasma. Standard pooled plasma was prepared from 30 mice obtained 5 weeks after the MB immunization and its titre was defined as 1000 units/ml. Tissue specimens from thigh muscles were prepared 6, 8, 10 and 12 weeks later from the day of the first immunization.

The muscle tissue samples were frozen with liquid nitrogen and embedded in O.C.T. compound. They were cut 6 μ m thick on a cryostat, air-dried, fixed with cold acetone for 10 min, and stained immunohistochemically with counter staining by haematoxyline to visualize the nuclei. For detection of immunohistochemical localization of mouse IgG, sections were incubated with horseradish peroxidase (HRP)-labelled anti-mouse IgG antibody (Chemicon International, Inc.). HRP-labelled normal goat IgG was used as a negative control. For detection of mouse C3, sections were incubated with goat IgG anti-mouse C3 antibody (Cappel Labs., Durham, NC), washed and then incubated with HRP-labelled anti-goat IgG (Chemicon International, Inc.). Normal goat IgG was used as a negative control. Binding of HRP-labelled

antibodies was detected using DAB substrate kit (Vector Laboratories, Inc., Burlingame, CA). Results were expressed on a score of 0–3+, depending on the intensity of deposits.

Erythrocytes, sera and IgGs

Erythrocytes were prepared from whole blood of healthy donors and stored in Alsever's solution (Sigma, St Louis, MO) at 4°C. Sera from the same healthy donors were collected and stored at –80°C.

Veronal buffer solution of Venoglobulin-IH, Gamma-Venin P (Aventis Pharma AG, Frankfurt, Germany), Human IgG Fc fragment (Jackson Immunoresearch Laboratories, Inc. West Grove, PA) and human serum albumin (HSA) (Welfide Co.) were used. To remove endogeneous Abs against allogenic antigens on erythrocytes, the IgGs and HSA were incubated with erythrocytes for 30 min on ice before use.

Preparation of antibody (Ab)-sensitized erythrocyte (EA)

One milliliter of erythrocytes was incubated with 9 ml of GVBE (veronal buffer containing 10 mM EDTA and 0.1% gelatin, pH 7.5) for 15 min at 37°C, washed with GVB²⁺ (veronal buffer containing 0.5 mM MgCl₂, 0.15 mM CaCl₂ and 0.1% gelatin, pH 7.5) three times, and the cell number was adjusted to 1 × 10⁹ cells/ml in GVB²⁺ buffer. Washed erythrocytes were sensitized with an equal volume of rabbit anti-erythrocytes antibody (Organon Teknika, Co., Durham, NC) in GVB²⁺ buffer for 20 min at 37°C.

Measurement of haemolysis

Fifty microliters of EA (2.5 × 10⁷ cells) was incubated with 100 µl of GVB²⁺ containing IgG or HSA and 50 µl of autologous serum diluted 2-fold with GGVB²⁺ (veronal buffer containing 0.5 mM MgCl₂, 0.15 mM CaCl₂, 0.1% gelatin and 2.5% glucose, pH 7.5) at 37°C for 1 h in a shaking water bath. Cells were quickly chilled on ice and centrifuged for 5 min at 1100 ×g at 4°C. Absorbance at 405 nm of the supernatant was measured.

Measurement of C1q, C3 and C4 binding on Ab-sensitized erythrocytes

Radioiodination of goat anti-C1q, C3 and C4 antibodies (Organon Teknika) was carried out with Iodobeads (Pierce, Rockford, IL) according to the manufacturer's instructions. Free iodine was removed by small-scale gel filtration in a Bio-Spin 6 column (Bio-Rad Laboratories, Hercules, CA). C3 and C4 binding was measured as

follows. Fifty microliters of EA (2.5×10^7 cells) was incubated with 100 μ l of GVB²⁺ containing IgG or HSA and 50 μ l of autologous serum diluted 10-fold with GGVB²⁺ at 37°C for 5 min in a shaking water bath. Then 2 ml of cold GVBE was added to stop the reaction. Cells were washed twice with ice-cold GVBE, resuspended in 100 μ l of radiolabeled anti-C3 or C4 antibodies in GVBE and incubated on ice for 1 h. After washing the cells twice with ice-cold GVBE, the radioactivities of the cells were counted by gamma spectrometer. C1q binding was measured as described above, except for using GGVB²⁺ instead of GVBE and using anti-C1q antibody for detection.

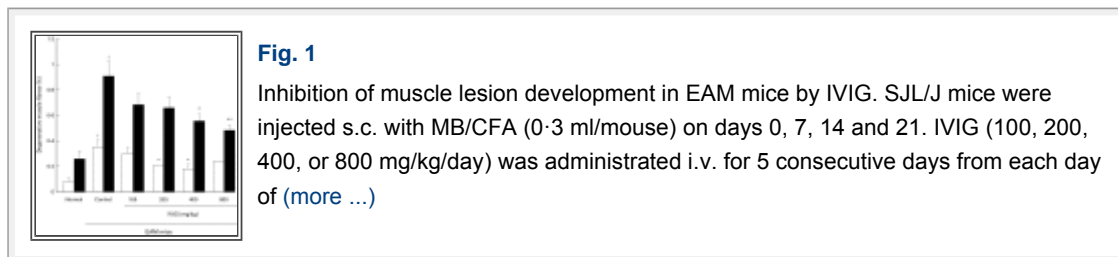
Statistical analysis

Data of histological analysis are expressed as the mean \pm s and statistically significant differences between control group and treated groups were determined by analysis of variances (ANOVA), followed by Dunnett's multiple comparison test. The nonparametric Wilcoxon test was used when comparing anti-MB titres between control and IVIG treated group. In complement assay *in vitro*, Student's *t*-test was used. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of IVIG on muscle fibre degeneration in the mouse EAM model

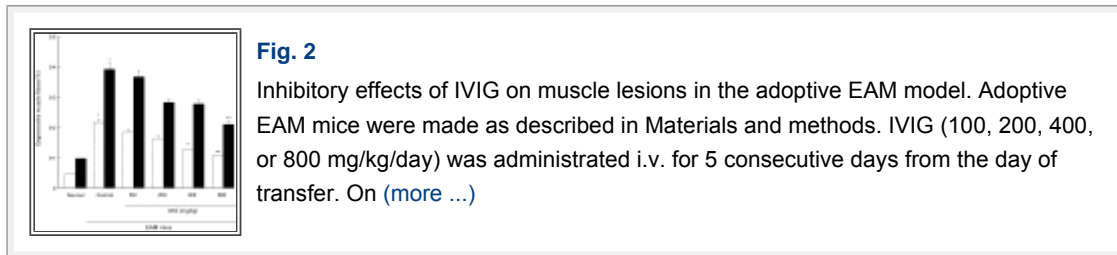
The EAM model was made in SJL/J mice by immunization with rabbit MB fraction. In this model, pathological changes were observed in skeletal muscle fibres, such as necrosis in muscle fibres and infiltration of inflammatory cells, which resembled human PM and DM. Incidences of necrotic and inflammatory changes in the lower limbs of EAM mice were low but significantly higher than those of untreated mice ([Fig. 1](#)). Administration of IVIG dose-dependently suppressed the incidences and significant effects were observed at 200 and 400 mg/kg (necrotic changes), or at 400 and 800 mg/kg (inflammatory changes).



Effect of IVIG on adoptive EAM model

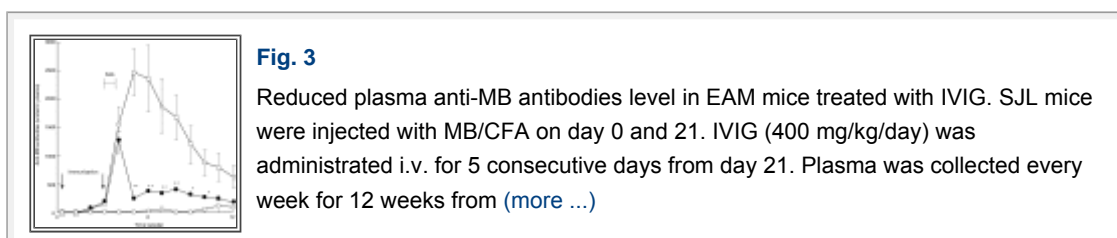
Adoptive EAM model was made by intravenous injection of activated cells into homologous mice. To obtain the *in vitro* activated T cells, lymph node cells from the EAM mice were cocultured with antigen presenting cells derived from the same mice in the presence of antigen (MB) and IL-2. Surface phenotype and activation markers of the cells before and after antigen treatment were examined to assure that desirable activation had occurred. The population of CD3 and CD4 positive cells increased significantly from 70.5 to 91.1% and from 38.6 to 69.4%, respectively. The population of CD25 positive cells in the CD4-positive cells also increased from 12.5 to 50.8%.

Histological examination of the adoptive EAM mice showed that the mice developed degenerative changes in muscle fibres similar to the EAM model, indicating that the transferred cells caused the pathological changes. IVIG treatment suppressed the necrotic and inflammatory changes in a dose-dependent manner ([Fig. 2](#)) at a similar level of dosage to the EAM model ([Fig. 1](#)).

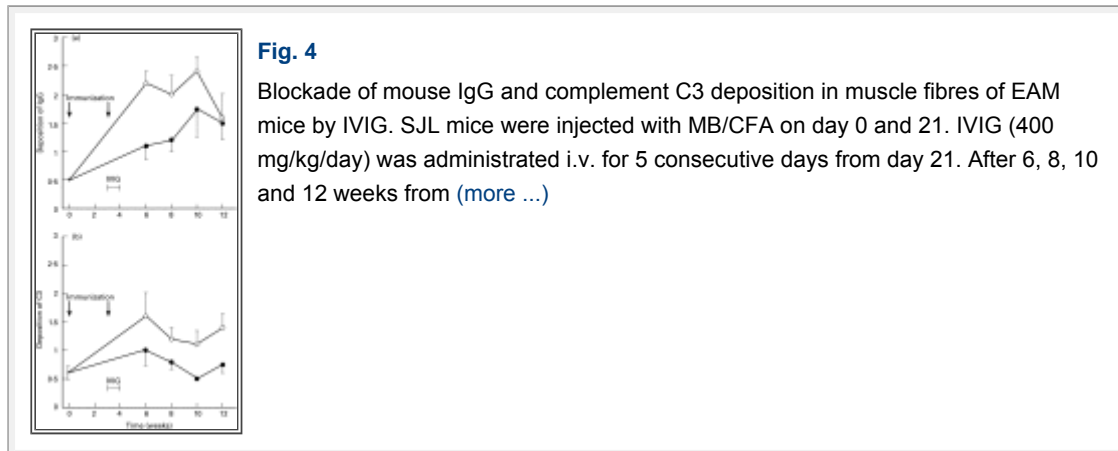


Suppression of the production and deposition of antibodies by IVIG in the mouse EAM model

High level of circulating autoantibodies and deposition of both autoantibodies and complement C3 in skeletal muscles have been observed in human PM and DM, and they are thought to cause destructive changes in muscle fibres. These immunological changes were also found in the current EAM model. In SJL/J mice immunized with MB/CFA on days 0 and 21, plasma levels of anti-MB antibody were markedly elevated after the second immunization, reached a peak and gradually decreased ([Fig. 3](#)). Interestingly, although mice were immunized with rabbit MB, antibody against mouse MB was produced. Administration of IVIG remarkably decreased the highest level and duration of the high titres.

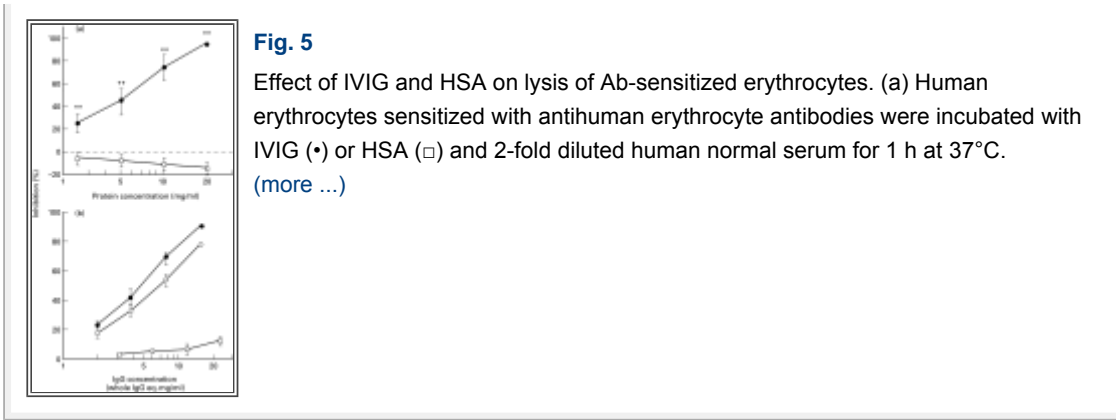


Depositions of mouse IgG and complement C3 on the muscle fibres were also found in the EAM model, and the incidences were reduced by IVIG treatment ([Fig. 4](#)). These findings suggest that IVIG suppresses degenerative changes in muscle fibres in EAM mice, by depressing the production of anti-MB antibody and its deposition as an immune complex in the muscle fibres.

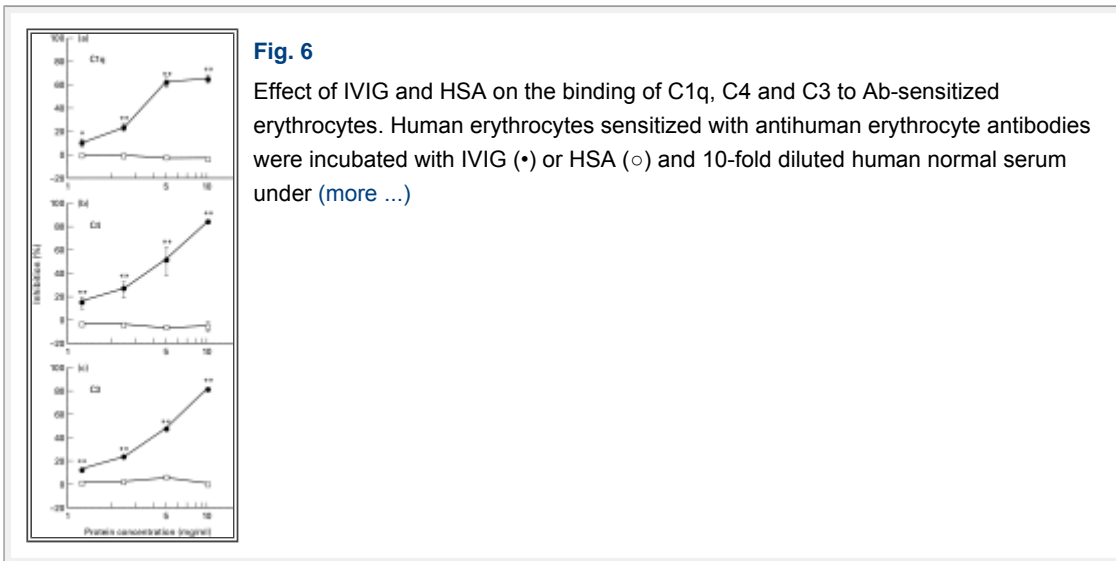


In vitro effect of IVIG on complement function

To elucidate the effect of IVIG on the activation of complement system, it was incubated with human erythrocytes sensitized with antihuman erythrocyte antibodies and human complements. As the complement source, autologous serum of the erythrocyte donor was used to avoid allogenic reaction. IVIG inhibited lysis of the sensitized erythrocytes in a dose-dependent manner while HSA did not exhibit any effect ([Fig. 5a](#)). We next compared the inhibitory effects of whole IgG (Venoglobulin-IH), Fc fragment, and F(ab')₂ fragment (Gamma-Venin) to clarify what part of the IgG molecule was responsible for the inhibition. As shown in [Fig. 5b](#), the Fc fragment but not the F(ab')₂ fragment revealed the same level of inhibition as whole IgG. This suggests that the inhibitory effect of IgG is mainly due to the Fc portion of the molecule.



IVIG also showed dose-dependent inhibition of the binding of C1q, C4 and C3 to Ab-sensitized erythrocytes in similar manners (Fig. 6). The inhibition of C1q binding strongly suggests that IVIG inhibited complement activation through the classical pathway.



DISCUSSION

Many clinical studies have shown that high-dose IVIG is effective in the treatment of autoimmune disorders including PM and DM [13–15]. From *in vitro* and *in vivo* experiments several modes of action of IVIG have been proposed [6,7]. However, the mechanisms of development of the muscular lesions and their amelioration by IVIG have remained unclear, thus, animal experiments are required to obtain a better understanding of the mechanism. To achieve this, we first examined the effect of IVIG in the mouse EAM model, according to a protocol similar to that of clinical study of DM and PM patients. We showed here that IVIG treatment was effective in the EAM model. It inhibited both necrotic and inflammatory changes in the skeletal muscle of

EAM mice at approximately 400 mg/kg of administration, that is a dosage widely used for the treatment of autoimmune diseases [16,17]. In the EAM mice, nonspecific activation of the immune system by injection of a foreign protein (human IVIG) must not be responsible for the effect, because mouse IgG instead of human IgG showed a similar effect in this model (data not shown).

Matsubara and Okuma [18] reported that muscular lesions similar to those of EAM mice could be generated by the transfer of the lymphocytes from mice immunized with rabbit MB into homologous mice. In order to examine the importance of cell-mediated mechanisms in the development of myositis, we generated adoptive EAM model by an *in vitro* immunization of lymphocytes from the EAM mice and transferring the lymphocytes into homologous mice. The adoptive EAM mice showed necrotic and inflammatory changes in muscle fibres similar to the EAM mice. Although the differences between the EAM and adoptive EAM models has not been well studied, it is suggested that antigen specific T cells play important roles in the development of the muscular lesions. Treatment of the adoptive EAM mice with IVIG also inhibited the development of muscular lesion development. Taking into consideration that IVIG administration was effective in both models, it might decrease the incidence of muscular lesions through cellular and humoral mechanisms.

In concert with the development of the muscular lesions, plasma anti-mouse MB antibody level increased in the EAM mice. Depositions of both mouse IgG and complement C3 in the muscle fibres were also observed. Although anti-MB antibody was generated in response to the injection of the rabbit MB fraction, the above observations suggest that anti-MB antibody may act like an autoimmune antibody of human diseases. It may attack muscle fibres of the host animal and cause degenerative changes, with a simultaneous activation of the complement system. IVIG treatment markedly suppressed the increase of anti-MB antibody, as well as the deposition of IgG and C3 in the muscle fibres, suggesting that IVIG interfered with the deposition of antibodies on antigens by decreasing the titre of autoantibodies and the following complement attack to the target tissue. This idea is supported with the findings that IVIG inhibited the production of antibodies from B cells *in vitro* experiments [19–21]. Another possible mechanism by which IVIG decreases the anti-MB antibody level is drawn from the observations that many kinds of anti-ideotypic antibodies have been found in IVIG, such as antibodies against anti-Factor VIII antibodies, anti-thyroglobulin autoantibodies, anti-neuroblastoma antibodies, anti-GPIIb/IIIa autoantibodies, and anti-DNA autoantibodies [22–24]. Therefore, anti-ideotypic antibodies to anti-MB antibodies may be present in the immunoglobulin preparation and have decreased the antibody titre.

We next evaluated the effect of IVIG on complement activation by *in vitro* studies. As reported previously [24–26], IVIG inhibited the lysis of the Ab-sensitized erythrocytes by human complements. IVIG also inhibited the binding of C1q, C4 and C3 to the Ab-sensitized erythrocytes in a dose-dependent manner, which was in consistent with findings that IgG binds to C3 [27,28], and also binds C1q [25,29,30]. The effect of IgG on C1q binding may be somewhat controversial, because Basta *et al.* [31] showed that IVIG did not affect C1q uptake onto Ab-sensitized human erythrocytes. However we showed here that Fc fragment inhibited the lysis of the Ab-sensitized erythrocytes by human complements as strong as whole IgG, demonstrating that IVIG inhibited lysis through the inhibition of C1q binding. The idea that IVIG inhibited the classical pathway was also supported by an alternative pathway haemolytic assay using rabbit erythrocytes and human serum. In this assay IVIG inhibited lysis of rabbit erythrocytes by human complement about 30% at a concentration of 20 mg/ml (data not shown), which was much weaker than that of Ab-sensitized human erythrocytes (Fig. 5). Taking together these results it is suggested that IVIG interfered the binding of C1q to immune complex.

We have shown here that IVIG suppresses the development of muscle lesions of EAM and adoptive EAM mice. While further studies should allow better understandings, our current findings should accelerate the elucidation of the molecular mechanisms involved in the development of muscle lesions in PM and DM. In addition, the observed effect of IVIG on EAM mice may provide the rational reason for its clinical use in PM and DM patients.

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