Fc Receptor–Mediated Inhibitory Effect of Immunoglobulin Therapy on Autoimmune Giant Cell Myocarditis
Concomitant Suppression of the Expression of Dendritic Cells

Keisuke Shioji, Chiharu Kishimoto, Shigetake Sasayama

Abstract—In the present study, the mechanisms and importance of the Fc portion of immunoglobulin in experimental giant cell myocarditis were examined. Giant cell myocarditis was induced in rats by immunization of porcine cardiac myosin. Human intact immunoglobulin (1 g·kg⁻¹·d⁻¹) or F(ab')₂ fragments of human immunoglobulin (1 g·kg⁻¹·d⁻¹) were administered intraperitoneally daily on days 1 to 21. Intact immunoglobulin administration significantly ameliorated myocarditis, but F(ab')₂ fragments did not. The ribonuclease protection assay revealed that therapy with intact immunoglobulin, but not F(ab')₂ fragments, suppressed the mRNA expressions of inflammatory and proinflammatory cytokines. Immunohistochemical analysis showed that therapy with intact immunoglobulin, but not F(ab')₂ fragments, suppressed dendritic cell (DC) expression during both the early and the subsequent fulminant phases. Moreover, the early treatment of intact immunoglobulin until the 11th day or 14th day, when the expression of DCs had already been completed, failed to ameliorate the condition. An in vitro study showed that intact immunoglobulin, but not F(ab')₂ fragments, suppressed the lipopolysaccharide-induced interleukin-1β production associated with the downregulation of CD32 antigen (Fcγ receptor II) expression. Thus, intact immunoglobulin therapy markedly suppressed myocarditis as a result of Fc receptor–mediated anti-inflammatory action, and the suppression of the disease was associated with the suppression of DCs, ie, the suppression of the initial antigen-priming process in experimental giant cell myocarditis. (Circ Res. 2001;89:540-546.)

Key Words: myocarditis ■ immunoglobulin ■ dendritic cells ■ cytokines ■ Fc receptors

Giant cell myocarditis is frequently fatal. Because the disease is occasionally associated with various autoimmune diseases, autoimmune mechanisms were suggested to be involved in its pathogenesis.¹ The therapeutic efficacy of high-dose immunoglobulin has been reported in inflammatory and autoimmune diseases, eg, Kawasaki disease,² idopathic thrombocytopenic purpura,³ and peripartum cardiomyopathy.⁴ We have previously reported that immunoglobulin therapy suppresses acute viral myocarditis as a result of an anti-viral effect, an anti-inflammatory effect, and the improvement of extracellular matrix changes.⁵,⁶ Most recently, a novel mechanism of action of immunoglobulin was proposed to be due to anti-inflammatory activities through the inhibitory Fc receptors (FcRs).⁷

It has been suggested that T-cell–mediated autoimmune diseases are the result of inappropriate antigen presentation of either a self-antigen or an antigen with the capacity to mimic a self-antigen in the peripheral lymphoid tissues.⁸ In fact, immunosuppressive agents, such as 15-deoxyspergualin, FK-506, and leflunomide, were confirmed to be effective in the suppression of the initial antigen-priming process in experimental autoimmune myocarditis.⁹⁻¹¹ Dendritic cells (DCs) are specialized antigen-presenting cells (APCs) with FcRs that possess the capacity to activate naïve T cells.¹² Accordingly, DCs appear to play an important role during the initial antigen-priming process of myocarditis.¹¹,¹³ Therefore, to clarify the FcR-mediated effects, we investigated the effects of immunoglobulin on autoimmune giant cell myocarditis with the analyses of immunologic behaviors of DCs and myocardial cytokines.

Materials and Methods

In Vivo Study

Immunization

Autoimmune myocarditis was induced as previously described.¹⁴ Six- to 7-week-old Lewis rats (Shimizu Laboratory Supplies Co, Ltd) were injected subcutaneously in their foot pads with porcine cardiac myosin (1 mg/mL M0531, Sigma Chemical Co) mixed with Freund’s complete adjuvant (FCA) supplemented with Mycobacterium tuberculosis H37Ra (No. 3113-60, DIFCO) on days 1 and 7.
Figure 1. Representative histopathology and immunohistochemistry for surface markers of myocardial infiltrating cells. A and B, Hematoxylin and eosin (H-E) staining on tissues from rats immunized with myosin and injected with PBS. On day 15, several infiltrating inflammatory cells (arrow) are observed (A). On day 21, extensive injury of myocytes with various kinds of inflammatory changes, including multinucleated giant cells (arrow and inset), is observed (B). Original magnification ×100 (inset ×320). C through F, Immunohistochemistry for OX6 (C), ED1 (D), ED2 (E), and W3/25 (F) on tissues from rats immunized with myosin, injected with PBS, and killed on day 15. Almost of all the inflammatory cells in panel A show immunoreactivity for anti-OX6 antibody (C). Arrows indicate positive staining cells. Original magnification ×200. All sections are serial.

TABLE 1. Histological Analysis for Experiment I

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n indicates number of rats; HW/BW, ratio of heart weight to body weight. Values are mean±SD.

*P<0.01 vs rats immunized with myosin and injected with PBS; †P<0.01 and ‡P<0.05 vs rats immunized with myosin and injected with F(ab’)2 fragments (1-way ANOVA, Fisher protected least significant difference test).

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Immunoglobulin Treatment

Experiment I

In the rats immunized with porcine myosin or FCA, intact immunoglobulin (Venoglobulin-IH, a polyethylene glycol–treated human immunoglobulin, Welfide Corp) or F(ab’)2 fragments (GammaVenin, Aventis Corp) of human immunoglobulin were administered intraperitoneally daily at a dose of 1 g · kg⁻¹ · d⁻¹ from day 1 to day 15 [intact immunoglobulin and myosin, n=6; F(ab’)2 fragments and myosin, n=4] and to day 21 [intact immunoglobulin and myosin, n=13; intact immunoglobulin and FCA alone, n=4; F(ab’)2 fragments and myosin, n=10; F(ab’)2 fragments and FCA alone, n=4]. As determined from previous studies, the dose used was 1 g · kg⁻¹ · d⁻¹. Immunoglobulin antigenicity between different species did not appear to be a problem. In addition, both agents have the same chemical structure as the Fab portion of immunoglobulin. Littermate controls were injected with PBS intraperitoneally and killed on day 15 (myosin, n=6; FCA alone, n=3) or day 21 (myosin, n=16; FCA alone, n=4).

Experiment II

To clarify the importance of the suppression of the initial self-antigen process by immunoglobulin treatment on the basis of the findings of experiment I, in which the expression of DCs, the initiators of immune responses, reached maximum at approximately day 15, experiment II was conducted.

Early Treatment

This protocol aimed to suppress the initial self-antigen-priming process during the course of the disease. That is, intact immunoglobulin was administered daily to the rats immunized with myosin at the same dose as given in experiment I from day 1 to day 8 (n=5), to day 11 (n=5), and to day 14 (n=4), and the rats were killed on day 21. Control rats were injected with PBS from day 1 to day 8 (n=7), to day 11 (n=6), and to day 14 (n=6), and the rats were killed on day 21. Two rats in each group were also killed on day 8, day 11, and day 14 for the pathological examination.

Late Treatment

This protocol aimed to investigate the effects of intact immunoglobulin on the disease severity after completion of the initial self-antigen–priming process. That is, intact immunoglobulin was administered daily to the rats immunized with myosin at the same dose as...
in experiment I from day 15 to day 21, and the rats were killed on day 21 (n=8). Control rats were injected with PBS during these periods and killed on day 21 (n=7).

**Histopathology**

At euthanasia, macroscopic findings and pericardial effusion were graded on a scale of 0 to 2, as previously described. Microscopic findings for cellular infiltration and myocardial necrosis were graded on a scale of 0 to 3, as previously described. 

**Immunohistochemistry**

Immunohistochemistry for surface markers was performed, as previously described. The primary antibodies (Serotec) used were as follows: OX62 antibody to recognize an integrin or integrin-like molecule present on DCs and γδ T cells, V65 antibody to specifically detect γδ T cells, OX6 antibody to recognize major histocompatibility complex (MHC) class II–expressing cells, including DCs, monocytes, and B lymphocytes, ED1 antibody to detect inflammatory macrophages, ED2 antibody to detect tissue macrophages, W3/25 antibody to detect helper T lymphocytes and macrophages, and OX8 antibody to detect cytotoxic-suppressor T lymphocytes. W3/25 antibody is regarded as being directed against the rat homologue of CD4.

**Ribonuclease Protection Assay**

mRNA was extracted from the myocardium by using TRIZol (GIBCO-BRL), and cytokine mRNA levels were measured with
RiboQuant Multi-Probe template sets, In Vitro transcription kits, and ribonuclease protection assay (RPA) kits (PharMingen) according to the PharMingen/RiboQuant protocol. The NIH Image system was used to quantify the pixel intensity of macrophage inhibitory factor (MIF) cytokine bands, which were divided by the intensities in their L32 bands in the same lanes for normalization.

In Vitro Study
U937 cells, human monoblast cells bearing FcRs, were stimulated with 10 ng/mL lipopolysaccharide (LPS) of Escherichia coli (Sigma). Forty-eight hours later, interleukin (IL)-1β in the medium was assayed by using antibody-sandwich ELISA.

For the analysis of surface markers, the collected cells were incubated with an appropriate dilution of primary antibodies: CD16 (FcγRIII, Ancell), CD32 (FcγRII, Serotec), and CD64 (FcγRI, Ancell). They were incubated with the fluorescein isothiocyanate–conjugated F(ab′)2 secondary antibody (Serotec), and 1 \times 10^5 cells per trial were analyzed with a FACSscan cytometer (Becton Dickinson) by use of CELLQuest.

Intact human immunoglobulin or the F(ab′)2 fragments were added to the medium 30 minutes before LPS stimulation. The doses of the agents used in the present study were derived from the methods of Andersson and Andersson.

Statistical Analysis
Values were expressed as the mean ± SD. Statistical analyses of the data were performed by 1-way ANOVA and were reanalyzed with the Fisher protected least significant difference test to characterize significant differences between groups in the in vitro study and experiment I; the Student t test was used in experiment II. A value of P<0.05 was considered statistically significant.

Results
FcR-Mediated Inhibitory Effect and Suppression of Expression of DCs by Immunoglobulin Treatment In Vivo (Experiment I)
Histopathology and Heart Weight/Body Weight Ratio
None of the rats died throughout the entire period. On day 15, the hearts showed a normal appearance macroscopically except for one of six rats. However, several infiltrating inflammatory cells surrounding small vessels among cardiomyocytes were observed microscopically in all the rats (Figure 1A). In rats treated with intact immunoglobulin, no evidence of myocarditis was shown on day 15. In rats treated with F(ab′)2 fragments, several infiltrating inflammatory cells were observed microscopically. Table 1 shows histological analysis for experiment I.

On day 21, 11 of 16 hearts showed severe and diffuse discolored myocarditis with massive pericardial effusion. Extensive injury of the myocytes with inflammatory changes and multinucleated giant cells were observed microscopically (Figure 1B). Treatment with intact immunoglobulin, but not F(ab′)2 fragments, reduced the expression of cytokine mRNAs. IFN-γ indicates interferon-γ. L32 is a housekeeping gene. A representative finding of 3 distinct experiments is shown.

Immunohistochemistry of Surface Markers
On day 15, in rats immunized with myosin and injected with PBS, almost of all the infiltrating inflammatory cells showed immunoreactivity for anti-OX6 antibody (Figure 1C). ED1-positive cells (Figure 1D) and ED2-positive cells (Figure 1E) were observed, but only a few cells showed very weak...
immunoreactivity for anti-W3/25 (Figure 1F) or anti-OX8 (data not shown) antibody. Several OX62-positive cells infiltrated into the perivascular cardiac tissue (Figure 2A); all were DCs, because no anti–V65-positive cells were detected (data not shown). Accordingly, MHC class II–positive myeloid cells, including DCs, play a pivotal role during the early phase. Treatment with intact immunoglobulin (Figures 2B and 3A), but not F(\(ab'\))\(_2\) fragments (Figures 2C and 3A), reduced the number of OX62-positive cells.

On day 21, OX62-positive cells were scattered in inflammatory foci (Figure 2D), and only a few V65-positive cells were detected (data not shown). The number of OX6-positive cells was markedly increased (Figure 3C). The ratio of OX62-positive cells to OX6-positive cells in the heart immunized with myosin and injected with PBS on day 15 and day 21 was 0.46 ± 0.23 and 0.042 ± 0.029, respectively. Accordingly, in the natural course of the disease, the proportion of OX62-positive cells in the MHC class II–expressing cells was decreased during the fulminant phase (day 21) compared with the early phase (day 15). Treatment with intact immunoglobulin (Figures 2E and 3B), but not F(\(ab'\))\(_2\) fragments (Figures 2F and 3B), reduced the numbers of OX62-positive cells.

**Ribonuclease Protection Assay**

On day 15 (Figure 4A), the mRNA expression of cytokines in intact hearts immunized with FCA alone was found only for MIF, which is released as a proinflammatory cytokine.\(^2\) In rats immunized with myosin and injected with PBS, mRNA expression of MIF was enhanced by 3.1-fold relative to intact hearts. However, in rats immunized with myosin and treated with intact immunoglobulin, the mRNA expression of MIF was not enhanced (1.1-fold relative to intact hearts).

On day 21 (Figure 4B), in intact hearts immunized with FCA alone and injected with PBS, mRNA expression of cytokines was detected only for MIF. In rats immunized with myosin and injected with PBS, mRNAs of Th1 cytokines (such as IL-18), Th2 cytokines (such as IL-6 and IL-10), and proinflammatory cytokines (such as MIF, IL-1\(\alpha\), IL-1\(\beta\), and IL-1Ra) were markedly upregulated, and mRNA expression of IL-12p35, IL-12p40, and interferon-\(\gamma\) was slightly upregulated. Treatment with immunoglobulin, but not F(\(ab'\))\(_2\) fragments, reduced the expression of cytokine mRNAs.

**FcR-Mediated Inhibitory Effect of Immunoglobulin In Vitro**

IL-1\(\beta\) production was increased by LPS stimulation in U937 cells (0.7 ± 0.8 pg/mL for controls, n = 4; 9.1 ± 1.5 pg/mL for LPS, n = 4 [\(P<0.01\)]). Intact immunoglobulin (7.0 ± 1.4 pg/mL for 0.6 mg, n = 4 [\(P=NS\)]; 5.5 ± 1.7 pg/mL for 6.0 mg, n = 4 [\(P<0.05\)]) but not F(\(ab'\))\(_2\) fragments (8.9 ± 1.5 pg/mL for 0.4 mg, n = 4 [\(P=NS\)]; 8.8 ± 5.0 pg/mL for 4.0 mg/mL, n = 4 [\(P=NS\)]) suppressed LPS-induced IL-1\(\beta\) production in a dose-dependent manner (Figure 5A).

In U937 cells, CD32 was expressed and upregulated by LPS stimulation (Figure 5B). The expression of CD16 and CD64 was not varied from the baseline at any time point (data not shown). Intact immunoglobulin downregulated LPS-induced CD32 expression, and the median fluorescence intensity (MFI) relative to the control was decreased by the treatment (143 ± 26% for LPS, n = 4; 101 ± 18% for LPS plus intact immunoglobulin, n = 4 [\(P<0.05\)]. However, the LPS-induced CD32 expression was not changed by the treatment of F(\(ab'\))\(_2\) fragments [143 ± 26% for LPS, n = 4; 125 ± 11% for LPS plus F(\(ab'\))\(_2\) fragments, n = 4 (\(P=NS\)].

**Importance of Suppression of Initial Self-Antigen Priming Process by Intact Immunoglobulin Treatment (Experiment II)**

**Early Treatment**

The treatment from day 1 to day 8 failed to decrease the severity of myocarditis (Table 2). Treatment with intact
immunoglobulin from day 1 to both day 11 and day 14 decreased the severity of myocarditis, as assessed by measuring the heart weight/body weight ratio and histological scores. On day 8, OX62-positive cells were rarely detected in rats injected with PBS or in rats treated with intact immunoglobulin. On day 11, only a few OX62-positive cells had infiltrated the cardiomyocytes in rats injected with PBS but not the rats treated with intact immunoglobulin. On day 14, several OX62-positive cells were observed among cardiomyocytes in rats injected with PBS but not in rats treated with intact immunoglobulin. Accordingly, the early treatment of intact immunoglobulin from day 1 to day 11 or day 14, but not day 8, suppressed the expression of DCs, i.e., the initial antigen-priming process, leading to the suppression of myocarditis.

Late Treatment
Treatment with intact immunoglobulin did not decrease the severity of myocarditis (Table 2). Thus, the late treatment after the completion of DC expression did not cause a reduction in the severity of myocarditis.

Discussion
In the present study, it was shown that intact immunoglobulin, but not F(ab’)2 fragments, markedly suppressed both the severity of the disease and cytokine mRNA expression in experimental giant cell myocarditis in vivo. Intact immunoglobulin, but not F(ab’)2 fragments, suppressed LPS-induced IL-1β production associated with downregulation of CD32 expression in U937 cells in vitro. Accordingly, FcR-mediated inhibitory effects actually play an important role in the reduction of inflammatory cytokine production in vitro and in vivo. In addition, intact immunoglobulin, but not F(ab’)2 fragments, suppressed the DC expression during both the early and fulminant phases in vivo. An additional experiment confirmed that the suppression of DC expression during the early phase of the disease, when DCs operate actively, caused a reduction in myocarditis. The effect of immunoglobulin may be associated with the suppression of the expression of DCs, i.e., the suppression of the initial antigen-priming process in experimental giant cell myocarditis.

FcγRs act as trigger molecules for inflammatory, allergic, endocytic, and inhibitory activities of immune effector cells. It has been reported that F(ab’)2 fragments do not ameliorate experimental allergic encephalomyelitis in rats and that the anti-inflammatory activity of immunoglobulin is mediated through the inhibitory FcR. In the present study, an in vivo study showed that intact immunoglobulin administration, but not the administration of F(ab’)2 fragments, completely suppressed the severity of the disease and the mRNA expression of cytokines (Figure 4). An in vitro study showed that intact immunoglobulin, but not F(ab’)2 fragments, suppressed LPS-induced the IL-1β production associated with the downregulation of CD32 (FcγRII) expression. It is suggested that mRNA expression of cytokines may be blocked not only by anti-cytokine antibodies included in immunoglobulin used in the present study but also by the anti-inflammatory action mediated through the inhibitory FcR. The former possibility may be slight, because F(ab’)2 fragments did not suppress mRNA expression of cytokines, which theoretically may possess anti-cytokine antibodies. We confirmed that the anti-inflammatory action of intact immunoglobulin in murine myocarditis induced by encephalomyocarditis virus was due to the reduction of the plasma level of interferon-γ and soluble intercellular adhesion molecule-1 but not to antiviral effects. Accordingly, the present study added further information that intact immunoglobulin treatment suppressed not only viral myocarditis but giant cell myocarditis by the anti-inflammatory action.

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<th>Peritoneal Injection</th>
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n indicates number of rats. Values are mean±SD. Early treatment of intact immunoglobulin from day 1 to both day 11 and day 14 suppressed the initial antigen-priming process, leading to the suppression of myocarditis. However, the late phase (the phase after completion of antigen-priming) treatment of intact immunoglobulin did not cause the reduction of severity of myocarditis. *P<0.05 and †P<0.01 vs rats immunized with myosin injected with PBS (Student t test).
The present study provided evidence that almost of all the infiltrating inflammatory cells were MHC class II–positive myeloid cells, including DCs during the early phase. The proportion of DCs in the MHC class II–expressing cells was higher during the early phase compared with during the fulminant phase (Figure 3). DCs are functionally specialized APCs and efficient stimulators of B and T cells. Mature DCs express high MHC class II molecules that are 10- to 100-fold greater on DCs than on other APCs, such as B cells and monocytes.\textsuperscript{8,12} Intact immunoglobulin administration, but not the administration of F(ab\textsuperscript{\prime})\textsubscript{2} fragments, may suppress the activation of DCs because immature DCs are well equipped to capture antigens and have antigen-capturing Fc\textgamma and Fc\epsilon receptors\textsuperscript{12}; ie, exogenous native and intact immunoglobulin may bind to FcRs on DCs and prevent internalization of the antigen, and as a result, intact immunoglobulin with the Fc portion may prevent DCs from processing antigens to form MHC peptide complexes. The results of experiment II confirmed that the early treatment of immunoglobulin by the time DCs infiltrated to cardiomyocytes caused a reduction in myocarditis. Accordingly, the effect of immunoglobulin may be associated with the suppression of the initial antigen-priming process in experimental giant cell myocarditis.

We have previously reported that immunoglobulin suppresses coxsackievirus B3 myocarditis by an antiviral antibody included in the agent.\textsuperscript{5} However, immunoglobulin treatment failed to ameliorate myocarditis in an Intervention in Myocarditis and Acute Cardiomyopathy With Immune Globulin (IMAC) trial of human myocarditis.\textsuperscript{23} One reason may be that patients with dilated cardiomyopathy with noninflammatory causes occupied a large part of that trial. From the present findings, it may be that immunoglobulin therapy, if it is initiated early, is effective against human giant cell myocarditis, a disease with no present effective therapy other than transplantation.\textsuperscript{1} In conclusion, the present study provided evidence that intact immunoglobulin therapy markedly suppressed myocarditis because of the FcR-mediated anti-inflammatory action and the concomitant suppression of the initial antigen-priming process in experimental autoimmune myocarditis. The findings of the present study may yield important insights into both the clinical use of this therapy for human immune or autoimmune myocarditis and the future studies of FcR-mediated therapy for immune or autoimmune diseases.

Acknowledgments

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References

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