*Am J Physiol Heart Circ Physiol* 285: H899–H906, 2003;
10.1152/ajpheart.00926.2002.

# Immunoglobulin treatment suppresses atherosclerosis in apolipoprotein E-deficient mice via the Fc portion

**Zuyi Yuan,[1] Chiharu Kishimoto,[1] Hideto Sano,[2] Keisuke Shioji,[1] Yang Xu,[2] and Masayuki Yokode[2]**

[1]*Department of Cardiovascular Medicine and* [2]*Department of Geriatric Medicine, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan*

Submitted 28 October 2002; accepted in final form 24 March 2003

**Yuan, Zuyi, Chiharu Kishimoto, Hideto Sano, Keisuke Shioji, Yang Xu, and Masayuki Yokode.** Immunoglobulin treatment suppresses atherosclerosis in apolipoprotein E-deficient mice via the Fc portion. *Am J Physiol Heart Circ Physiol* 285: H899–H906, 2003; 10.1152/ajpheart.00926.2002.—Atherosclerosis is associated with immune activation. Immunoglobulin is used for the treatment of immune-mediated diseases. The mechanisms and importance of the Fc portion of immunoglobulin upon experimental atherosclerosis in apolipoprotein E-deficient mice were examined. Experimental atherosclerosis was induced in mice fed a high-fat diet containing 0.3% cholesterol. Over 8, 12, and 16 wk, on alternate days, mice were treated with an intraperitoneal injection of either 1 $g \cdot kg^{-1} \cdot day^{-1}$ of human intact immunoglobulin or $F(ab')_2$ fragments of human immunoglobulin. Fatty streak formation and fibrofatty plaques were markedly suppressed in mice that received intact immunoglobulin for 8, 12, and 16 wk. In contrast, atherosclerotic lesions were not ameliorated in mice that received $F(ab')_2$ fragments. Immunohistochemical analysis revealed that macrophage accumulation in the fatty streak lesions was suppressed in mice received intact immunoglobulin but not in those that received $F(ab')_2$ fragments. In addition, the cytotoxic activities of splenocytes from immunoglobulin-treated mice, but not from $F(ab')_2$ fragment-treated mice, were significantly suppressed compared with those from human serum albumin-treated mice. Differences in lesion area did not correlate with any significant alterations in serum lipid levels. Immunoglobulin therapy markedly suppressed atherosclerosis due to Fc receptor-mediated anti-inflammatory and immunomodulating actions. The antiatherosclerotic effects of immunoglobulin may be related to the suppression of cytotoxic activity of atherogenic T cells and the reduction of macrophage accumulation in the lesions.

knockout mice; Fc receptor

ATHEROSCLEROSIS IS ASSOCIATED with immune activation and with systemic immune responses and signs of inflammation (8, 18, 24). The lesions contain a large number of immune cells, particularly macrophages and T cells (11, 30). Histopathological and clinical investigations point to inflammatory/immune activation of plaques as a cause of acute coronary syndromes, and seroepidemiological studies have suggested links between atherosclerosis and microbial infections (5, 21). Congenital deficiency of macrophages, lymphocytes, and the $Th_1$ effector pathway, caused by crossbreeding apolipoprotein (apo) E knockout (KO) mice with *op*/*op* mutant mice (28), recombinase acting gene-1 KO mice (3), and interferon-γ receptor KO mice (7), respectively, have resulted in the reduction of lesions. We (22) have previously reported that blockade of *c-fms*, a receptor for macrophage colony stimulating factor, caused marked suppression of atherogenesis in apo E-deficient mice, where macrophage differentiation was impaired. These results suggest that immunomodulation may be used to treat or prevent atherosclerosis.

Therapy with immunoglobulin has been investigated in a wide range of immune-mediated disorders (6, 13, 27). The mode of action of immunoglobulin is still unclear and may involve both Fc and V region-dependent mechanisms: blockage of Fc receptors on macrophages and effector cells, anti-inflammatory effects by attenuation of complement-mediated damage, regulation of the production of cytokines, or inhibition of lymphocyte proliferation. Several of these mechanisms might be beneficial in atherosclerosis (23). Here, we evaluated whether administration of immunoglobulin could affect the development of atherosclerosis, and the antiatherosclerotic mechanisms conferred by immunoglobulin were also investigated. For this purpose, 6-wk-old apo E-deficient mice were fed a high-fat diet and injected with 1 $g \cdot kg^{-1} \cdot day^{-1}$ of intact immunoglobulin or $F(ab')_2$ fragments intraperitoneally on alternate days. The fatty streaks and fibrofatty plaques were markedly reduced by the immunoglobulin treatment, but $F(ab')_2$ fragments failed to reduce atherosclerosis. The antiatherosclerotic effects of immunoglobulin may be related not only to suppression of the cytotoxic activity of atherogenic T cells from atherosclerotic mice but to a reduction of the macrophage accumulation in the lesions.

## MATERIALS AND METHODS

### *Experimental Atherosclerosis*

The apo E-deficient 129ola × C57BL/6 hybrid mice were generous gifts of Dr. Edward M. Rubin (University of California, Berkeley, CA). These mice were mated with C57BL/6

Address for reprint requests and other correspondence: C. Kishimoto, Dept. of Cardiovascular Medicine, Graduate School of Medicine, Kyoto Univ., 54 Kawaracho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan (E-mail: kkishi@kuhp.kyoto-u.ac.jp).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

  

mice to produce $F_1$ hybrids. The $F_1$ apo $E^{+/-}$ mice were then backcrossed to C57BL/6 mice for 10 generations. Mice homogeneous for the apo E-null allele on a C57BL/6 background were subsequently generated. Male mice were subjected to subsequent experiments. The mice were kept in a temperature-controlled facility on a 14:10-h light-dark cycle with free access to food and water. After being weaned at 4 wk of age, mice were fed a normal chow diet (CMF, Oriental Yeast) until 6 wk of age, when the animals were switched to a high-fat diet containing 20% fat and 0.3% cholesterol as previously described (Fig. 1) (22).

We performed animal experiments in accordance with the Declaration of Helsinki, and these were approved by our institutional ethics committee for animal experiments.

### *Immunoglobulin Treatment*

*Protocol I.* In 6-wk-old male apo E-deficient mice, intact immunoglobulin (Venoglobulin-IH, Mitsubishi Pharma; a polyethylene glycol-treated human immunoglobulin) was administered intraperitoneally on alternate days at a dose of 1 $g \cdot kg^{-1} \cdot day^{-1}$ for 8 wk (fatty streak stage: intact immunoglobulin, $n = 5$; controls, $n = 6$) or for 12 and 16 wk (fibrofatty plaque stage: intact immunoglobulin, $n = 4$ for 12 wk and $n = 5$ for 16 wk; controls, $n = 4$ for 12 wk and $n = 6$ for 16 wk) (Fig. 1). Littermate controls were injected with 1 $g \cdot kg^{-1} \cdot day^{-1}$ of human serum albumin (HSA) intraperitoneally.

*Protocol II.* To determine the mechanisms and importance of the Fc portion of immunoglobulin on the development of atherosclerosis, mice were intraperitoneally injected on alternate days at a dose of 1 $g \cdot kg^{-1} \cdot day^{-1}$ with either intact immunoglobulin or $F(ab')_2$ fragments of human immunoglobulin (Gamma-Venin, Aventis; a polyethylene glycol-treated human immunoglobulin) for 8 wk [intact immunoglobulin, $n = 6$; $F(ab')_2$ fragments, $n = 6$; controls, $n = 6$] or 16 wk [intact immunoglobulin, $n = 5$; $F(ab')_2$ fragments, $n = 5$; controls, $n = 5$]. As shown in previous studies (15, 23, 27, 32), immunoglobulin antigenicity between humans and mice did not appear to be a problem. The molecular weight of $F(ab')_2$ fragments is not completely equal to that of intact immunoglobulin at the same dosage. In addition, both intact immunoglobulin and $F(ab')_2$ fragments have the same molecular structure in the Fab region of immunoglobulin except that the $F(ab')_2$ fragments have no Fc region.

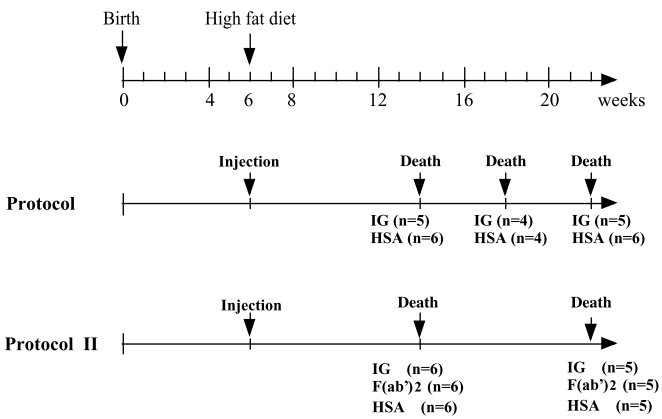


Fig. 1. Treatment protocol. After being weaned at 4 wk of age, apolipoprotein (apo) E-deficient mice were fed a normal chow diet until 6 wk of age, when the mice were switched to a high-fat diet. In *protocol I*, apo E-deficient mice were injected with immunoglobulin (IG) or human serum albumin (HSA) for 8, 12, or 16 wk. In *protocol II*, apo E-deficient mice were injected with IG, $F(ab')_2$ fragments, or HSA for 8 or 16 wk.

### *Tissue Processing*

Mice were killed by bleeding with puncture of the right ventricle. The blood was collected and allowed to clot. After the serum was separated, lipid profiles were analyzed, as described in *Serum Lipid Measurement*. The vasculature was perfused with sterile PBS. The root of the aorta was dissected under a macroscope and frozen in OCT embedding medium for serial cryosectioning covering 1.0 mm of the root. The first section was harvested when the first cusp became visible in the lumen of the aorta. Four sections of 10 $\mu$m thickness were harvested per slide, and thus 20 slides per mouse were prepared. All sections were immersed for 2 min in 60% isopropanol, stained for 15 min in a saturated oil red-O solution at 37°C, counterstained with hematoxylin, and then mounted under coverslips with glycerol gelatin.

### *Quantitation of Atherosclerotic Lesions*

The oil red-O-stained sections were analyzed at a magnification of ×10, as previously described (22, 23, 26). The image was captured directly from the RGB camera attached to a light microscope and displayed on a microcomputer to quantify the cross-sectional surface area of the lesion and the cross-sectional surface area of the vessel. The fraction area of the lesion was calculated by dividing the whole vessel area including the lumen, intima, media, and adventitia, as previously described (23). For each animal, 20 sections, i.e., every fourth section, were examined, and the mean of the fraction area was calculated and expressed as a percentage.

### *Immunohistochemistry*

Aortic root cryosections from mice treated with immunoglobulin, $F(ab')_2$ fragments, or HSA for 8 wk were also processed for immunohistochemistry as described previously with minor modifications (14, 27). In brief, anti-macrophage (anti-Mϕ, M3/84, 1:400, PharMingen), anti-CD4 (GK1.5, 1:50, PharMingen), anti-CD8 (53–6.7, 1:50, PharMingen), anti-I-$A^b$ (25–9-17, 1:25, PharMingen), and anti-ICAM-1 (M-19, 1:100, Santa Cruz Biotechnology) antibodies were applied to acetone-fixed cryosections. After being washed, the sections were then exposed to a second antibody (horseradish peroxidase-conjugated antibodies), and the antibody binding was visualized with diaminobenzidine. Sections were counterstained with 1% methyl green. The percentage of positively stained cells per infiltrating cells in the lesions was calculated for each antibody as previously described (14, 23). That is, lesions of the aortic root were analyzed. Data were obtained by dividing the number of positively stained cells by all methyl green-stained cells inside the internal elastic lamina. Three to five random microscopic fields were analyzed at ×200.

### *Serum Lipid Measurement*

Serum was separated by centrifugation and stored at −80°C. Serum total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), and triglyceride (TG) levels were measured with assay kits (Wako) according to the manufacturer's instructions.

### *Cell Culture*

Spleens from mice treated with immunoglobulin, $F(ab')_2$ fragments, or HSA for 16 wk in *protocol II* were harvested, and single-cell suspensions were obtained by passing

through a stainless steel mesh screen. Cells were suspended in RPMI-1640 supplemented with 10% fetal calf serum, 1% sodium pyruvate, 1% nonessential amino acids, $5 \times 10^{-5}$ M 2-mercaptoethanol, and a penicillin-streptomycin mixture.

F-2 cells, a murine endothelial cell line established from an ultraviolet-induced tumor, and rat aorta smooth muscle cells (SMCs) were maintained in 10% fetal calf serum-supplemented DMEM at 37°C in 5% $CO_2$.

*Cytotoxicity Assays*

Splenocytes from mice treated with immunoglobulin, $F(ab')_2$ fragments, or HSA for 16 wk in *protocol II* were used as effector cells. SMCs and F-2 cells ($2 \times 10^4$ cells/well) plated in 96-microwell plates were labeled with 37 kBq/well sodium chromate ($^{51}Cr$, Amersham International) for 1 h. After being labeled, target cells were washed with PBS three times, and splenocytes were incubated at the effector-target ratios of 50:1, 100:1, and 200:1 for 4 h. The supernatant was collected, and the radioactivity of $^{51}Cr$ release into the supernatant was measured by a gamma counter. The percentage of cytotoxicity was calculated using the following formula

$$\%\text{Cytotoxicity} = (E - S)/(M - S) \times 100$$

where E is counts per minute (cpm) released in the presence of effector cells, S is the spontaneous cpm released from target cells incubated in medium, and M is the maximal cpm released from target cells incubated with 2% Triton X-100.

*Statistical Analysis*

Values are expressed as means ± SD. Statistical analysis of the data was determined by *t*-test or by one-way ANOVA, followed by the Fisher protected least-significant-difference test. A value of $P < 0.05$ was considered statistically significant.

## RESULTS

*Effects of Immunoglobulin on Physiological Parameters*

As shown in Table 1, treatment with immunoglobulin did not significantly modify the serum lipid profiles in *protocols I* or *II*. The HDL-C-to-TC ratio was also similar in all groups of mice. In addition, immunoglobulin did not reduce or increase body weights.

*Effects of Immunoglobulin on Fatty Streak and Fibrofatty Plaque Formation*

Apo E-deficient mice were injected with immunoglobulin or HSA and kept on a cholesterol-rich diet for 8 wk to induce fatty streak formation and for 12 and 16 wk to form fibrofatty plaques, respectively. The surface area covered by fatty streak and fibrofatty plaque lesions was quantified in oil red-O-stained samples, and specimens from immunoglobulin-treated mice were compared with HSA-treated controls. Controls developed extensive lesions in the root of the aorta in both stages (Fig. 2). In mice treated with immunoglobulin, as shown in Table 2, the fraction area of lesion was markedly reduced (4.2 ± 2.0% vs. 13.6 ± 4.8% in controls, $P < 0.01$ for 8-wk treatment; 7.1 ± 2.9% vs 18.3 ± 4.0% in controls, $P < 0.01$ for 12-wk treatment; 8.1 ± 2.7% vs. 21.5 ± 3.9% in controls, $P < 0.01$ for 16-wk treatment).

*Effects of the Fc Portion of Immunoglobulin on Atherosclerosis*

In *protocol II*, mice were kept on the high-fat diet for 8 or 16 wk to permit the formation of atherosclerotic lesions, and immunoglobulin, $F(ab')_2$ fragments, or HSA injection were given for 8 or 16 wk. Control treatment resulted in extensive fatty streak formation and advanced fibrofatty plaques in HSA-treated mice (Fig. 3). In mice treated with immunoglobulin, the fractions of the cross-sectional area covered by lesions were markedly reduced (4.6 ± 3.1% vs. 12.9 ± 4.4% in controls, $P < 0.01$ for 8-wk treatment; 9.6 ± 3.7% vs. 24.4 ± 6.8% in controls, $P < 0.01$ for 16-wk treatment). In contrast, $F(ab')_2$ fragment treatment did not reduce the lesion areas significantly (11.2 ± 3.7% for 8-wk treatment, 22.8 ± 5.7% for 16-wk treatment, $P$ = not significant vs. HSA each; Table 2).

Table 1. *Physiological parameters*

| | *n* | TC, mg/dl | HDL-C, mg/dl | HDL-C-to-TC Ratio | TG, mg/dl | Body Weight, g |
|---|---|---|---|---|---|---|
| | | | *Protocol I* | | | |
| 8 wk | | | | | | |
| HSA | 6 | 787.7 ± 142.5 | 46.6 ± 4.6 | 0.059 ± 0.029 | 6.2 ± 4.8 | 23.7 ± 0.7 |
| Immunoglobulin | 5 | 802.2 ± 122.9 | 50.1 ± 2.4 | 0.063 ± 0.022 | 9.0 ± 6.8 | 23.2 ± 0.6 |
| 16 wk | | | | | | |
| HSA | 6 | 776.2 ± 140.7 | 51.2 ± 4.7 | 0.065 ± 0.040 | 5.6 ± 4.6 | 25.9 ± 0.8 |
| Immunoglobulin | 5 | 698.7 ± 99.9 | 52.1 ± 3.4 | 0.071 ± 0.041 | 5.0 ± 6.5 | 25.7 ± 0.9 |
| | | | *Protocol II* | | | |
| 8 wk | | | | | | |
| HSA | 3 | 733.6 ± 126.9 | 50.9 ± 4.9 | 0.069 ± 0.031 | 5.0 ± 5.0 | 23.1 ± 0.4 |
| Immunoglobulin | 3 | 701.1 ± 111.3 | 49.4 ± 2.4 | 0.070 ± 0.038 | 8.3 ± 6.8 | 23.0 ± 0.8 |
| $F(ab')_2$ | 6 | 687.8 ± 131.6 | 49.9 ± 6.5 | 0.068 ± 0.042 | 7.6 ± 5.9 | 22.8 ± 0.5 |

Values are means ± SD; *n* = no. of mice. Treatment of intact immunoglobulin or $F(ab')_2$ fragments did not significantly modify the serum lipid profiles in *protocols I* and *II*. TC, total cholesterol; HDL-C, high-density lipoprotein-cholesterol; TG, triglycerides; HSA, human serum albumin.

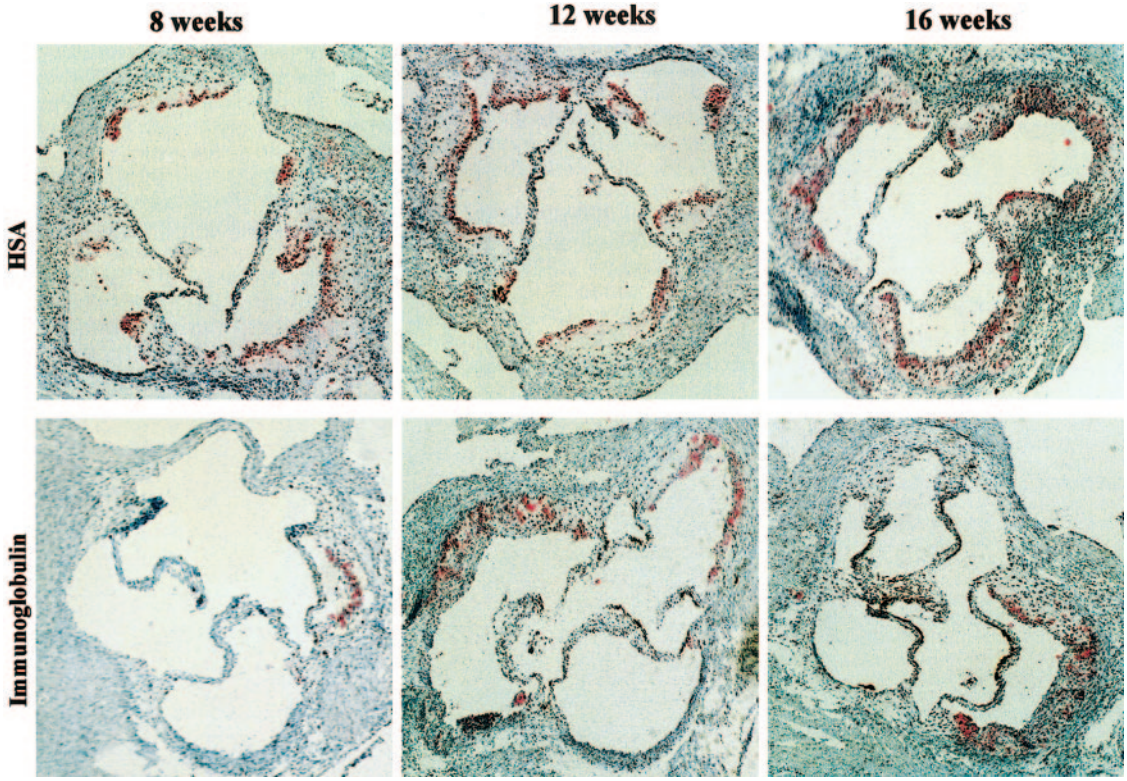


Fig. 2. Effects of IG on fatty streak and fibrofatty plaque formation. Apo E-deficient mice were fed a high-fat diet for 8, 12, or 16 wk and injected with IG or HSA (*protocol I*). The aortic root was cryosectioned and stained for lipids with oil red-O. Atherosclerotic lesions of IG-treated mice are smaller and cover a smaller fraction of the inner circumference of the aortic root than those of HSA-treated control mice. Sections are of 600 μm distance from the cusps. Magnification, ×40.

Table 2. *Quantitation of atherosclerotic lesions in protocols I (fatty streak stage) and II (fibrofatty plaque stage)*

| | *n* | Fraction Area, % |
|---|---|---|
| | *Protocol I* | |
| 8 wk | | |
| HSA | 6 | 13.6 ± 4.8 |
| Immunoglobulin | 5 | 4.2 ± 2.0* |
| 12 wk | | |
| HSA | 4 | 18.3 ± 4.0 |
| Immunoglobulin | 4 | 7.1 ± 2.9* |
| 16 wk | | |
| HSA | 6 | 21.5 ± 3.9 |
| Immunoglobulin | 5 | 8.1 ± 2.7* |
| | *Protocol II* | |
| 8 wk | | |
| HSA | 6 | 12.9 ± 4.4 |
| Immunoglobulin | 6 | 4.6 ± 3.1* |
| $F(ab')_2$ | 6 | 11.2 ± 3.7 |
| 16 wk | | |
| HSA | 5 | 24.4 ± 6.8 |
| Immunoglobulin | 5 | 9.6 ± 3.7* |
| $F(ab')_2$ | 5 | 22.8 ± 5.7 |

Values are means ± SD; *n* = no. of mice. Fatty streak formation and fibrofatty plaque were markedly suppressed in mice that received intact immunoglobulin for 8, 12, and 16 wk. In contrast, atherosclerotic lesions were not ameliorated in mice that received $F(ab')_2$ fragments. *$P < 0.01$ vs. HSA.

### *Effects of the Fc Portion of Immunoglobulin on Inflammatory Cell Infiltration in Fatty Streak Lesions*

The inflammatory cell infiltrations were assessed immunohistologically by the number of Mϕ, $CD4^+$, $CD8^+$, and $I\text{-}A^{b+}$ cells divided by the number of methyl green-stained cells. The percentage of Mϕ-positive cells was significantly reduced in the immunoglobulin-treated group compared with the control group (Fig. 4 and Table 3). Treatment with $F(ab')_2$ fragments did not suppress the frequency of Mϕ compared with the control group. ICAM-1 was expressed on the intimal cells just below plaques in the control groups. Treatment with intact immunoglobulin, but not with $F(ab')_2$ fragments, reduced the expression of ICAM-1 in the lesions.

### *Fc Portion of Immunoglobulin Suppresses the Cytotoxic Activity of Splenocytes from Atherosclerotic Mice*

The cytotoxic activities of splenocytes against SMCs and F-2 cells from atherosclerotic mice were examined. At an effector-to-target ratio of 200:1, the cytotoxic activities in immunoglobulin-treated mice were significantly suppressed compared with those in HSA-treated mice (Fig. 5). $F(ab')_2$ fragment treatment did not suppress the cytotoxic activities of splenocytes compared with control mice. Accordingly, the antiathero-

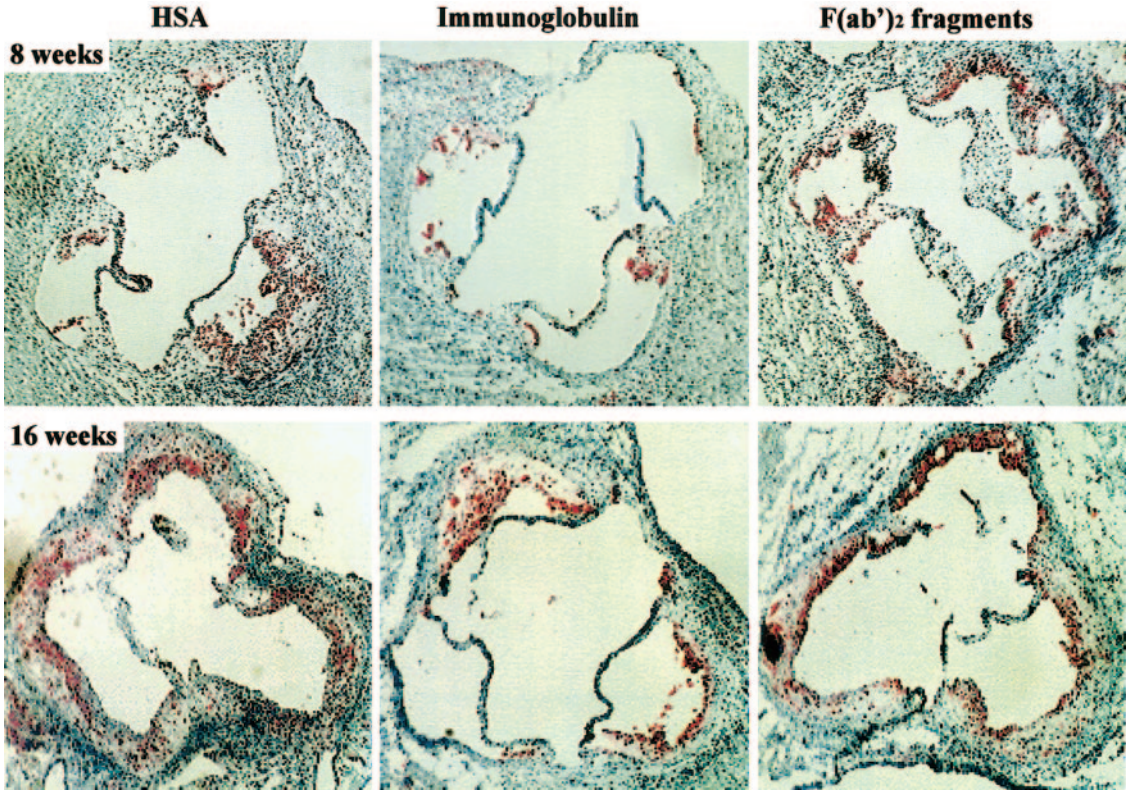


Fig. 3. Effects of IG and F(ab′)$_2$ fragments on fatty streak formation. Apo E-deficient mice were fed a high-fat diet for 8 or 16 wk and injected with IG, F(ab′)$_2$ fragments, or HSA (*protocol II*). The aortic root was cryosectioned and stained for lipids with oil red-O. Atherosclerotic lesions of IG-treated mice are smaller and cover a smaller fraction of the inner circumference of the aortic root than those of HSA-treated control mice. However, lesions of F(ab′)$_2$ fragment-treated mice are almost the same size as those of HSA-treated control mice. Sections are of 600 μm distance from the cusps. Magnification, ×40.

sclerotic effect of immunoglobulin may be due, at least in part, to the inhibition of cytotoxic activity of T cell-enriched splenocytes from atherosclerotic mice.

## DISCUSSION

The present study demonstrates marked suppression of the severity of atherosclerosis in experimental atherosclerotic mice treated with immunoglobulin. Immunoglobulin therapy did not modify the serum lipid profiles. A previous study (23) has reported that immunoglobulin therapy suppressed atherosclerosis in apo E KO mice and indicated that immunoglobulin may act by modulating T cell activity and antibody production. In our study, intact immunoglobulin, but not F(ab′)$_2$ fragments, inhibited atherosclerosis during both the fatty streak and plaque phases. In addition, suppression of Mϕ accumulation in lesions was present in mice treated with intact immunoglobulin but not in mice treated with F(ab′)$_2$ fragments. Because the significant role of Mϕ in the development of early atherosclerosis has already been demonstrated (3, 29, 34), and we (22) have already reported that administration of an antagonistic rat monoclonal antibody against murine *c-fms* could prevent accumulation of macrophages in the aortic intima and thereby protect atherogenesis in apo E-deficient mice, suppression of one antigen-present-

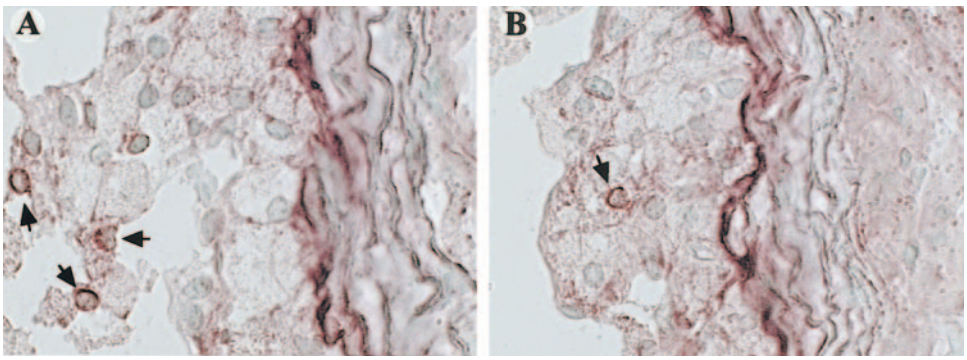


Fig. 4. Effects of IG and F(ab′)$_2$ fragments on macrophage (Mϕ) accumulation in the lesions. Frequency of Mϕ in IG-treated mice (*B*) is markedly decreased compared with HSA-treated control mice (*A*). Arrows indicate positive cells. Magnification, ×400.

Table 3. *Effect of immunoglobulin and F(ab′)$_2$ fragments on inflammatory cell infiltrates of lesions*

| | HSA | Immunoglobulin | F(ab′)$_2$ |
|---|---|---|---|
| Mϕ, % | 11.3 ± 3.0 | 3.5 ± 1.3* | 9.8 ± 1.7 |
| CD4$^+$, % | 16.0 ± 3.4 | 12.0 ± 3.1 | 11.5 ± 3.9 |
| CD8$^+$, % | 5.0 ± 2.2 | 5.3 ± 1.5 | 5.0 ± 2.3 |
| I-A$^{b+}$, % | 4.0 ± 1.8 | 4.3 ± 1.5 | 4.5 ± 2.1 |

Values are means ± SD. Lesions of the aortic root were analyzed. Data were obtained by dividing the number of positively stained cells by all methyl green-stained cells inside the internal elastic lamina. Three to five random microscopic fields were analyzed at ×200. ICAM-1 was expressed on the intimal cells just below plaques in the control groups. Treatment with intact immunoglobulin, but not with F(ab′)$_2$ fragments, reduced the expression of ICAM-1 in the lesions. Mϕ, macrophages. *$P < 0.01$ vs. HSA.

ing cell, Mϕ, may lead to a reduction in the severity of atherosclerosis. Also, reduced ICAM-1 expression in the lesions was demonstrated by the intact immunoglobulin treatment. Because ICAM-1 expression is regulated through nuclear factor (NF)-κB, the results suggest that the treatment may attenuate proinflammatory NF-κB signaling in the artery wall. Our study clearly indicates that the Fc portion of immunoglobulin plays an important role in the antiatherosclerotic action and hence in the reduction of the disease.

CD4$^+$ T cells play an important role in atherosclerosis (7, 11, 30), and transfer of T cell-enriched splenocytes from atherosclerotic mice aggravates atherosclerosis in immunodeficient apo E KO mice (34). In the present study, the cytotoxicity assay indicated that the cytotoxic activities of splenocytes against endothelial cells and SMCs in immunoglobulin-treated mice were significantly suppressed compared with those in HSA-treated mice. F(ab′)$_2$ fragment treatment did not suppress the cytotoxic activities of splenocytes compared with the control mice. Therefore, the antiatherosclerotic effect of immunoglobulin may be due, at least in part, to the inhibition of cytotoxic activity of T cell-enriched splenocytes from atherosclerotic mice, and this action may be mediated via the Fc portion of immunoglobulin.

Fc receptors act as trigger molecules for inflammatory, allergic, endocytotic, and inhibitory activities of immune effector cells (4). It has been demonstrated that human monocyte-derived macrophages uptake pathogens [e.g., oxidized low-density lipoprotein-containing immune complexes (oxLDL-ICs)] and lead not only to transformation of macrophages into foam cells but also to macrophage activation and release of cytokines. Both processes depend on the engagement of Fcγ receptor I by oxLDL-ICs (9, 19). Most recently, it has been shown that Fc receptors play a pivotal role in experimental neointimal vascular hyperplasia via the immunoreceptor tyrosine-based activation motif/immunoreceptor tyrosine-based inhibition motif (16, 33). We and others have reported that F(ab′)$_2$ fragments did not ameliorate experimental myocarditis in rats (27) and that the anti-inflammatory activity of immunoglobulin was mediated through the inhibitory Fc receptor (13, 25). In the present study, treatment with intact immunoglobulin, but not with F(ab′)$_2$ fragments, markedly suppressed the progression of atherosclerosis. In a previous in vitro study, we (27) have demonstrated that intact immunoglobulin, but not F(ab′)$_2$ fragments, downregulated CD32 (Fcγ receptor II) expression in U937 cells. Our study adds additional information on the precise mode of antiatherosclerotic action of immunoglobulin, that is, via the Fc portion. It may be reasonably hypothesized that injected immunoglobulin may block the Fc receptor of immune effector cells from binding to oxLDL-ICs, resulting in inhibiting T cell expansion and macrophage activation. Taken together, it appears that the Fc portion of immunoglobulin may play a role in the pathogenesis of antiatherosclerosis.

In addition to the immunomodulatory effects, several functions of immunoglobulin have been proposed. Microorganisms have recently been implicated in the pathogenesis of atherosclerosis (5), and there is strong evidence for an association between infections, such as

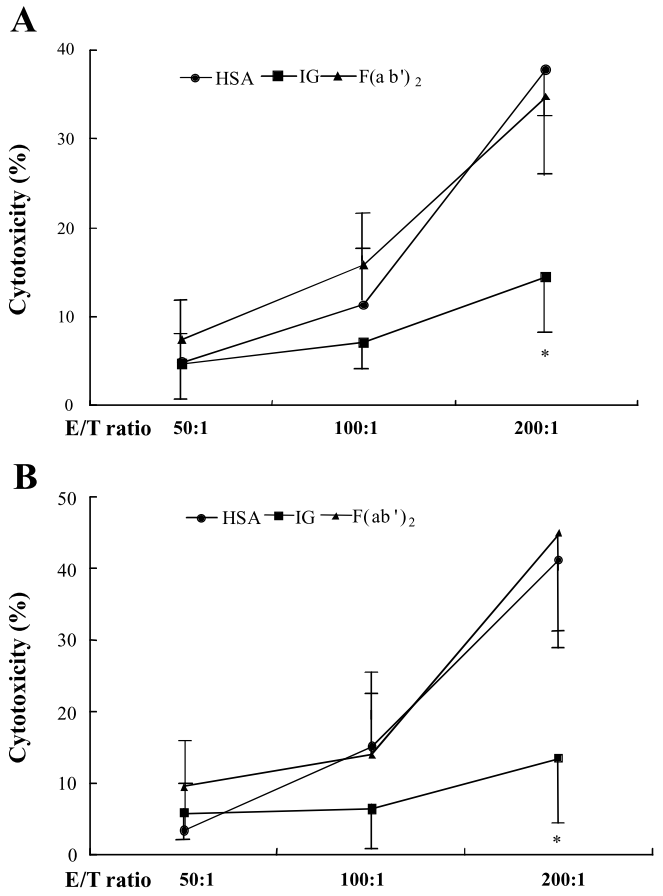


Fig. 5. Effects of IG and F(ab′)$_2$ fragments on cytotoxic activities of splenocytes from atherosclerotic mice. Effector (E) splenocytes were from mice treated with IG, F(ab′)$_2$ fragments, or HSA for 16 wk. Target (T) smooth muscle cells (*A*) and F-2 cells (*B*) were incubated at the effector-to-target (E/T) ratios of 50:1, 100:1, and 200:1. At the E/T ratio of 200:1, the cytotoxic activities of splenocytes from IG-treated mice were significantly suppressed compared with those from HSA-treated mice. F(ab′)$_2$ fragments treatment did not suppress the cytotoxic activities of splenocytes compared with the control mice. Means ± SD cytotoxicity (in %) of splenocytes in 4 individual wells are shown. *$P < 0.01$ vs. HSA.

*Chlamydia pneumoniae*, *Helicobacter pylori*, and cytomegalovirus, and atherosclerosis in humans (20). Immunoglobulin preparations contain many different antibody and autoantibody specificities that can neutralize a large number of pathogens and autopathogens (10, 12). Antibodies directed against several soluble and membrane molecules have also been identified in immunoglobulin preparations (2, 17). It is suggested that expression of cytokines may be blocked not only by the anticytokine antibodies included in immunoglobulin but also by the anti-inflammatory action mediated through the inhibitory Fc receptor (1, 31). The possibility of antipathogen and anticytokine antibody effects in the current study is probably very low because $F(ab')_2$ fragments, the antigen-binding portion of immunoglobulin, did not suppress the disease progression, which, theoretically, may possess anticytokine and antipathogen antibodies. Although experiments directly examining the effects of the Fc portion of immunoglobulin itself upon suppression of atherosclerotic lesions were not performed in the present study, it was clearly demonstrated that the antiatherosclerotic effects of immunoglobulin may due to Fc receptor-mediated anti-inflammatory and immunomodulating actions, because intact immunoglobulin, but not $F(ab')_2$ fragments, suppressed the atherosclerosis.

In conclusion, the present study provides evidence that intact immunoglobulin therapy markedly suppresses atherosclerosis due to Fc receptor-mediated anti-inflammatory action. The effects were not associated with a reduction of high serum lipid levels. The antiatherosclerotic effects of immunoglobulin may be related to the suppression of cytotoxic activity of atherogenic T cells and a reduction of macrophage accumulation in the lesions. The findings of the present study may yield important insights into an immunological approach in the treatment and future clinical use of this therapy in human atherosclerosis.

The authors thank M. Nimata for technical help, Mitsubishi Pharma, Japan, for supplying the human immunoglobulin preparation, and Aventis, Japan, for supplying the human $F(ab')_2$ fragments of immunoglobulin.

## DISCLOSURES

This study was supported in part by research grants from the Japanese Education of Science and Welfare and Shimizu Immunology Foundation.

## REFERENCES

1. **Bendtzen K, Hansen MB, Ross C, and Svenson M.** High-avidity autoantibodies to cytokines. *Immunol Today* 19: 209–211, 1998.
2. **Blasczyk R, Westhoff U, and Grosse-Wilde H.** Soluble CD4, CD8, and HLA molecules in commercial immunoglobulin preparations. *Lancet* 341: 789–790, 1993.
3. **Dansky HM, Charlton SA, Harper MM, and Smith JD.** T and B lymphocytes play a minor role in atherosclerosis plaque formation in the apolipoprotein E-deficient mouse. *Proc Natl Acad Sci USA* 94: 4642–4646, 1997.
4. **Deo YM, Graziano RF, Repp R, and van de Winkel JG.** Clinical significance of IgG Fc receptors and FcγR-directed immunotherapies. *Immunol Today* 18: 127–135, 1997.
5. **Espinola-Klein C, Rupprecht HJ, Blankenberg S, Bickel C, Kopp H, Rippin G, Victor A, Hafner G, Schlumberger W, and Meyer J.** Impact of infectious burden on extent and long-term prognosis of atherosclerosis. *Circulation* 105: 15–21, 2002.
6. **Godeau B, Chevret S, Varet B, Lefrere F, Zini JM, Bassompierre F, Cheze S, Legouffe E, Hulin C, Grange MJ, Fain O, and Bierling P.** Intravenous immunoglobulin or high-dose methylprednisolone, with or without oral prednisone, for adults with untreated severe autoimmune thrombocytopenic purpura: a randomized, multicentre trial. *Lancet* 359: 23–29, 2002.
7. **Gupta S, Pablo AM, Jiang X, Wang N, Tall AR, and Schindler C.** IFN-γ potentiates atherosclerosis in Apo E knock-out mice. *J Clin Invest* 99: 2752–2761, 1997.
8. **Hansson GK.** Immune mechanisms in atherosclerosis. *Arterioscler Thromb Vasc Biol* 21: 1876–1890, 2001.
9. **Huang Y, Jaffa A, Koskinen S, Takei A, and Lopes-Virella MF.** Oxidized LDL-containing immune complexes induce Fc gamma receptor I-mediated mitogen-activated protein kinase activation in THP-1 macrophages. *Arterioscler Thromb Vasc Biol* 19: 1600–1607, 1999.
10. **Hurez V, Kaveri SV, Mouhoub A, Dietrich G, Mani JC, Klatzmann D, and Kazatchkine MD.** Anti-CD 4 activity of normal human immunoglobulin G for therapeutic use? *Ther Immunol* 1: 269–277, 1994.
11. **Jonasson L, Holm J, Skalli O, Bondjers G, and Hansson GK.** Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. *Atherosclerosis* 6: 131–138, 1986.
12. **Kaveri S, Vassilev T, Hurez V, Lengagne R, Lefranc C, Cot S, Pouletty P, Glotz D, and Kazatchkine MD.** Antibodies to a conserved region of HLA class I molecules, capable of modulating CD8 T cell-mediated function, are present in pooled normal immunoglobulin for therapeutic use. *J Clin Invest* 97: 865–869, 1996.
13. **Kazatchkine MD and Kaveri SV.** Immunomodulation of autoimmune and inflammatory diseases with intravenous immune globulin. *N Engl J Med* 345: 747–755, 2001.
14. **Kishimoto C and Abelmann WH.** In vivo significance of T cell in the development of coxsackievirus B3 myocarditis in mice: antigen-specific T cells aggravate cardiac injury. *Circ Res* 67: 589–598, 1990.
15. **Kishimoto C, Takamatsu N, Kawamata H, Shinohara H, and Ochiai H.** Immunoglobulin treatment ameliorates murine myocarditis associated with reduction of neurohumoral activity and improvement of extracellular matrix change. *J Am Coll Cardiol* 36: 1979–1984, 2000.
16. **Konishi H, Katoh Y, Takaya N, Kashiwakura Y, Itoh S, Ra C, and Daida H.** Platelets activated by collagen through immunoreceptor tyrosine-based activation motif play pivotal role in initiation and generation of neointimal hyperplasia after vascular injury. *Circulation* 105: 912–916, 2002.
17. **Lam L, Whitsett CF, McNicholl JM, Hodge TW, and Hooper J.** Immunologically active proteins in intravenous immunoglobulin. *Lancet* 346: 678, 1993.
18. **Libby P and Hansson GK.** Involvement of the immune system in human atherogenesis: current knowledge and unanswered questions. *Lab Invest* 64: 5–15, 1991.
19. **Lopes-Virella MF, Binzafar N, Rackley S, Takei A, La Via M, and Virella G.** The uptake of LDL-IC by human macrophages: predominant involvement of the Fc gamma RI receptor. *Atherosclerosis* 135: 161–170, 1997.
20. **Mayr M, Kiechl S, Willeit J, Wick G, and Xu Q.** Infections, immunity, and atherosclerosis: associations of antibodies to *Chlamydia pneumoniae*, *Helicobacter pylori*, and cytomegalovirus with immune reactions to heat-shock protein 60 and carotid or femoral atherosclerosis. *Circulation* 102: 833–839, 2000.
21. **Memon RA, Staprans I, Noor M, Holleran WM, Uchida Y, Moser AH, Feingold KR, and Grunfeld C.** Infection and inflammation induce LDL oxidation in vivo. *Arterioscler Thromb Vasc Biol* 20: 1536–1542, 2000.
22. **Murayama T, Yokode M, Kataoka H, Imabayashi T, Yoshida H, Sano H, Nishikawa S, Nishikawa S, and Kita T.** Intraperitoneal administration of anti-c-*fms* monoclonal antibody prevents initial events of atherogenesis but does not reduce

the size of advanced lesions in apolipoprotein E-deficient mice. *Circulation* 99: 1740–1746, 1999.
23. **Nicoletti A, Kaveri S, Caligiuri G, Bariety J, and Hansson GK.** Immunoglobulin treatment reduces atherosclerosis in apo E knockout mice. *J Clin Invest* 102: 910–918, 1998.
24. **Ross R.** Atherosclerosis: an inflammatory disease. *N Engl J Med* 340: 115–126, 1999.
25. **Samuelsson A, Towers TL, and Ravetch JV.** Anti-inflammatory activity of IVIG mediated through the inhibitory Fc receptor. *Science* 291: 484–486, 2001.
26. **Sano H, Sudo T, Yokode M, Murayama T, Kataoka H, Takakura N, Nishikawa S, Nishikawa SI, and Kita T.** Functional blockade of platelet-derived growth factor receptor-β but not of receptor-α prevents vascular smooth muscle cell accumulation in fibrous cap lesions in apolipoprotein E-deficient mice. *Circulation* 103: 2955–2960, 2001.
27. **Shioji K, Kishimoto C, and Sasayama S.** Fc receptor-mediated inhibitory effect of immunoglobulin therapy on autoimmune giant cell myocarditis: concomitant suppression of the expression of dendritic cells. *Circ Res* 89: 540–546, 2001.
28. **Smith JD, Trogan E, Ginsberg M, Grigaux C, Tian J, and Miyata M.** Decreased atherosclerosis in mice deficient in both macrophage colony-stimulating factor (*op*) and apolipoprotein E. *Proc Natl Acad Sci USA* 92: 8264–8268, 1995.
29. **Song L, Leung C, and Schindler C.** Lymphocytes are important in early atherosclerosis. *J Clin Invest* 108: 251–259, 2001.
30. **Stemme S, Faber B, Holm J, Wiklund O, Witztum JL, and Hansson GK.** T lymphocytes from human atherosclerotic plaques recognize oxidized low density lipoprotein. *Proc Natl Acad Sci USA* 92: 3893–3897, 1995.
31. **Svenson M, Hansen MB, Ross C, Diamant M, Rieneck K, Nielsen H, and Bendtzen K.** Antibody to granulocyte-macrophage colony-stimulating factor is a dominant anti-cytokine activity in human IgG preparations. *Blood* 91: 2054–2061, 1998.
32. **Takada H, Kishimoto C, and Hiraoka Y.** Therapy with immunoglobulin suppresses myocarditis in a murine coxsackievirus B3 model: antiviral and anti-inflammatory effects. *Circulation* 92: 1604–1611, 1995.
33. **Watson SP and Gibbins J.** Collagen receptor signaling in platelets: extending the role of the ITAM. *Immunol Today* 19: 260–264, 1998.
34. **Zhou X, Nicoletti A, Elhage R, and Hansson GK.** Transfer of $CD4^+$ T cells aggravates atherosclerosis in immunodeficient apolipoprotein E knockout mice. *Circulation* 102: 2919–2922, 2000.