Inhibition of Atherosclerosis by T-Cell Depletion in Normocholesterolemic Rabbits Immunized With Heat Shock Protein 65

Bernhard Metzler, Manuel Mayr, Hermann Dietrich, Mahaur Singh, Evelyn Wiebe, Qingbo Xu, Georg Wick

Abstract—Previous studies in our laboratory have shown that arteriosclerotic changes can be induced in normocholesterolemic rabbits by immunization with mycobacterial heat shock protein (hsp) 65. To further investigate the immunologic mechanisms underlying such vascular lesions, 39 male New Zealand White rabbits were treated by triple immunization with fortified Freund’s complete adjuvant containing 5 mg/mL Mycobacterium tuberculosis as a source of hsp65 and simultaneous immunosuppressive therapy twice per week with either anti-CD3 monoclonal antibody (1 mg/kg) and prednisolone (1 mg/kg) or prednisolone (1 mg/kg) alone. Sixteen weeks after the first immunization the animals were killed, and as expected, severe arteriosclerotic lesions in the intima of the aortic arch were found in 9 of 10 immunized rabbits. However, only 1 of 10 rabbits immunized and immunosuppressed with the combined anti-CD3 monoclonal antibody and prednisolone treatment showed a single moderate lesion in the aorta, whereas 5 of 9 rabbits immunized and immunosuppressed by prednisolone treatment alone showed lesions, albeit mild. In conclusion, the early inflammatory stages of arteriosclerotic lesions induced by immunization with hsp65 can be inhibited by immunosuppressive therapy with anti-CD3 monoclonal antibody. (Arterioscler Thromb Vasc Biol. 1999;19:1905-1911.)

Key Words: arteriosclerosis • immunosuppression • heat shock proteins • stress proteins • lymphocytes

It has been established by various groups that arterosclerotic lesions in humans and rabbits contain large numbers of T lymphocytes,\textsuperscript{1-5} half of which express major histocompatibility class (MHC) class II antigens and some of which also express interleukin-2 receptors,\textsuperscript{6} indicating a state of activation. The presence of T cells in arteriosclerotic lesions could be important, because these cells can secrete factors chemotactic for monocytes/macrophages and smooth muscle cells, determine the differentiation and function of B cells and monocytes/macrophages, and modulate lipoprotein uptake by the latter.\textsuperscript{7,8} However, the antigen(s) responsible for this immunooactivation has not been identified. Two major candidates have been proposed: (1) Oxidized LDL (oxLDL) has been shown to be a chemokine for T lymphocytes\textsuperscript{9} and to activate T lymphocytes derived from arteriosclerotic lesions\textsuperscript{10} by an MHC class II–dependent mechanism.\textsuperscript{11} Interestingly, immunization of hypercholesterolemic rabbits with oxLDL has been shown to protect these animals from atherosclerosis development.\textsuperscript{12,13} (2) Heat shock protein 65 (hsp65), as we have shown, may be an (auto)antigen initiating the pathogenesis of atherosclerosis,\textsuperscript{14-17} and several lines of evidence support this hypothesis. First, T-cell proliferation to hsp65 was found not only in lesion-derived cells from hsp65-immunized rabbits but also in nonimmunized rabbits fed a cholesterol-rich diet exclusively.\textsuperscript{18} Second, hsp65 immunization of normocholesterolemic New Zealand White rabbits leads to formation of arteriosclerotic lesions demonstrating the typical features of their human counterparts, ie, inflammatory cell accumulation and smooth muscle cell proliferation, but without foam cells. In addition, a combination of immunization with hsp65-containing material and a cholesterol-rich diet led to the development of complicated arteriosclerotic lesions, including foam cells, exactly paralleling the situation in classic human lesions.\textsuperscript{18}

Therefore, the question arose as to whether T lymphocytes play a key role in the induction of arteriosclerosis in normocholesterolemic rabbits immunized with hsp65-containing material. In the present experiment, we addressed this issue by immunosuppression with monoclonal antibodies (mAbs) against CD3 lymphocytes, antibodies known to result in rapid lymphopenia and impaired T-cell immune response.\textsuperscript{19,20}

We provide evidence herein that the formation of arteriosclerotic lesions by immunization with hsp65-containing material can be abolished by immunosuppression with an anti-CD3 mAb plus prednisolone.
TABLE 1. Rabbit Experiment Schedule and Arteriosclerotic Lesions in Aortas

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Treatment</th>
<th>Antigen</th>
<th>Immunization, Times</th>
<th>Incidence/No. of Animals</th>
<th>Lesion Area, mm²/Aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>Control</td>
<td></td>
<td>...</td>
<td>1/10</td>
<td>0.4±1.2</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>Immunization</td>
<td></td>
<td>FCA</td>
<td>3</td>
<td>9/10</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>Immunization + anti-CD3 mAb + prednisolone</td>
<td>FCA</td>
<td>3</td>
<td>1/10</td>
<td>0.9±3.0</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>Immunization + prednisolone</td>
<td></td>
<td>FCA</td>
<td>3</td>
<td>5/9</td>
</tr>
</tbody>
</table>

Values are mean±SD per aorta in each group. In groups 2, 3, and 4, FCA supplemented with M tuberculosis (5 mg/rabbit) was used for 3 immunizations at 5-week intervals. Group 3 received 2 intravenous injections per week of the combination anti-CD3 mAb (1 mg/kg) and prednisolone (1 mg/kg), and group 4 received 2 intravenous injections per week of prednisolone (1 mg/kg). All animals were killed 16 weeks after the first immunization. All macroscopically detectable arteriosclerotic lesions of the aortic intima were documented on a plastic template and quantified by computerized planimetry.

*Aortic lesions of the group immunized with FCA are significantly different from the FCA-immunized rabbits treated with the combination of anti-CD3 mAb and prednisolone and from controls (P<0.01).

Methods

Animals, Reagons, and Immunization

Thirty-nine male New Zealand White rabbits, 10 to 11 weeks old and weighing between 1800 and 2200 g, were obtained from Savo/Charles River Co (Kisslegg im Allgäu, Germany). All animals were selected for serum cholesterol levels <2.6 mmol/L (100 mg/dL) with a relative humidity of 60%. They received water ad libitum and were fed a normal, standard diet (TT75, Tagger & Co). The animals were separated into 4 groups. Ten rabbits were immunized with Freund’s complete adjuvant (FCA) fortified with 5 mg/mL Mycobacterium tuberculosis, FCA (lot No. 3114-33-8 containing 0.5 mg/mL Mycobacterium butyricum) and heat-killed M tuberculosis H37Ra (lot No. 3114-33-7) purchased from Difco Laboratories. Recombinant Mycobacterium bovis hsp65 (BCG 65K, batch No. ma11B) was a gift from Dr van Embden, National Institute of Public and Environmental Protection, Bilthoven, The Netherlands. (The production of hsp65 was supported by the United Nations Development Program/World Bank/World Health Organization special program for research and training in tropical diseases.) Immunization schedules were detailed previously.24 In brief, rabbits received 3 intracutaneous injections from the nape downward at 5-week intervals (Table 1). Each milliliter of emulsion (1 mL per rabbit) consisted of 0.5 mL of PBS, pH 7.2, and 0.5 mL of FCA (15 mg of mycobacteria per rabbit). The 1-mL emulsion was always administered at 4 sites. Before the first intracutaneous immunization, 2 groups of rabbits were immunosuppressed with either an anti-CD3 mAb (1 mg/kg) and prednisolone (1 mg/kg) or prednisolone (1 mg/kg) alone for 5 subsequent days. These 2 groups were then treated twice per week with the same dose as before immunization, determined to be appropriate based on preliminary experiments. The group treated with prednisolone alone (1 mg/kg twice per week) served as a control because the anti-CD3 mAb must be combined with another immunosuppressive agent to prevent a possible anaphylactic reaction due to formation of antibodies against the injected anti-CD3 mAb. Without additional application of prednisolone, the rapid depletion of T lymphocytes by intravenous application of anti-CD3 mAb would wane within ~2 weeks.25 Ten animals served as untreated controls. All experimental animal procedures were done in accordance with institutional guidelines.

Production and Purification of mAbs

The mAb-producing anti-CD3 hybridoma (clone L11/135: catalog No. TIB 188) was obtained from the American Type Culture Collection (ATCC, Manassas, Va). This mouse antibody is of the IgG1 subclass and is specific for CD3 on all rabbit T cells.23 Antibody-containing ascites fluid was produced by injecting these cells into the peritoneal cavities of pristane-primed BALB/c mice, and precipitation of the immunoglobulin fraction was performed with saturated ammonium sulfate.

Blood Cholesterol Determination

Blood samples (1 to 2 mL) were collected from the central ear artery of rabbits after a 16-hour fast. Serum cholesterol values were measured at 4-week intervals by using an enzymatic procedure (catalog No. 352-100, Sigma). In brief, 10 µL of serum was added to 1 mL of solution from a cholesterol test kit and incubated for 18 minutes at room temperature, followed by photometric measurement at a wavelength of 500 nm (Dynatech Laboratories Inc). Serum cholesterol levels of all rabbits before treatment were <100 mg/dL (2.6 mmol/L).

Blood Glucose Determination

Blood samples (1 to 2 mL) were collected from the central ear artery of rabbits after a 16-hour fast. Serum glucose values were measured at 4-week intervals by using an enzymatic procedure (catalog No. 315-100, Sigma). In brief, 5 µL of serum was added to 1 mL of solution from a glucose test kit and incubated for 18 minutes at room temperature, followed by photometric measurement at a wavelength of 505 nm (Dynatech Laboratories Inc). Serum glucose levels of all rabbits before treatment were <75 mg/dL (4.1 mmol/L).

Enzyme-Linked Immunosorbent Assay

Determination of anti-hsp65 antibodies was performed essentially as described.24 In brief, recombinant hsp65 (1 µg/mL) was coated onto flat-bottom ELISA plate wells (Petta-Plastic 1.1041E) overnight at 4°C. After being washed with PBS supplemented with Tween 20 (0.05%, vol/vol) and blocked with 1% BSA (Sigma) in PBS, rabbit serum was added in appropriate dilutions and incubated for 1 hour at room temperature. A horseradish peroxidase–labeled swine anti-rabbit immunoglobulin conjugate (catalog No. P217, Dako) was then added, and the plates were incubated for 1 hour at room temperature followed by 4 washes with PBS/Tween. Finally, 100 µL of citrate phosphate buffer (0.1 mol/L, pH 4.2) containing 0.53 mg/mL 2,2'-azino[3-(ethylbenzthiazoline-6-sulfonic acid (Sigma) was added, and absorbance was measured after 30 minutes with a Microelisa Autoreader (Dynatech Laboratories Inc) at 410 nm.

Lymphocyte Culture

Before and 8 and 16 weeks after the first immunization, heparinized blood (15 IU/mL of preservative-free heparin, Immuno AG) was obtained from the central ear artery and diluted 1:2 in RPMI 1640 (Seromed). Blood mononuclear cells were isolated by density gradient centrifugation on Lymphoprep (d = 1.077 g/mL, Nycomed Pharma AS) as described previously.25 Peripheral mononuclear cells (10⁷ per well) were cultivated in duplicate in round-bottom microtiter plates (Falcon 3077) in 0.2 mL of RPMI 1640 supplemented with 5% fresh autologous serum, 5 × 10⁻⁵ mol/L 2-mercaptoethanol, streptomycin (100 µg/mL), penicillin (100 IU/mL), and 2 µg/mL concanavalin A (Pharmacia) or antigens in appropriate dilutions, including a purified protein derivative of mycobacteria (PPD, Statens Seruminstitut). The proliferative response of the cells was determined by measuring the incorporation of [³H]thymidine (1 µCi per well; specific activity 5 Ci/mmol, 185...
GBq/mmol; Amersham) during the last 8 hours of a 48-hour culture at 37°C and 5% CO₂.

Fluorescence-Activated Cell Sorting (FACS)
Analysis of Blood Mononuclear Cells
For flow cytometric analysis, 2x10⁶ peripheral blood mononuclear cells were incubated in PBS containing 1% BSA in a total volume of 100 µL with predetermined, appropriately diluted mAbs against a rabbit pan-T cell surface marker (L11/135, ATCC), Ia antigen (2C4; ATCC catalog No. CRL 1760), and CD4 (catalog No. MCA 799, Serotec, Oxford, UK) for 60 minutes at 37°C. After 3 washes with PBS–1% BSA, the cells were incubated with FITC-conjugated rabbit anti-mouse immunoglobulin for 1 hour and washed again. Fluorescence measurements were performed in an FACSScan (Becton Dickinson and Co). Details of FACS settings and methods of quantification of fluorescence intensity for the labeled cells are described elsewhere.²⁵,²⁶

Arteriosclerotic Lesion Measurement
Animals were killed by heart puncture under ketamine (25 mg/kg) and xylazine (5 to 10 mg/kg) anesthesia. Some serum was used immediately for supplementation of cell culture medium, and the rest was stored frozen at −70°C. The aortas were carefully removed intact from the aortic arch to the iliac bifurcation and cut longitudinally for documentation of macroscopically visible intimal lesions on a plastic template.²¹ The total surface area of the aortas covered by lesions was determined by computerized planimetry (IBM PC-AT 486 image analyzer; Optimas 4.1 program, Bioscan) after scanning the templates (GT8000 Epson) at a resolution of 400 dots per inch in black-and-white mode. All drawings of the lesions and planimetric evaluations were done by the same operator. These data were used to calculate the intimal surface area (mm²) affected by arteriosclerotic lesions.

Several portions of uninvolved and lesioned aortic intimas from each group were subdivided and processed for conventional histology. Tissue fragments were fixed in 4% phosphate-buffered (pH 7.2) formaldehyde, embedded in paraffin, and sectioned for hematoxylin–eosin (HE) staining.

Statistics
Statistical tests were carried out with the Mann-Whitney U test for analysis of arteriosclerotic lesions, antibody titers, and proliferative cell responses and paired Student’s t test for comparison of blood cholesterol and glucose levels (StatView SE+Graphics for the Macintosh computer, Abacus Concepts, Inc).

Results
Macroscopic and Microscopic Assessment of Arteriosclerotic Lesions
Sixteen weeks after the first immunization, 9 of 10 rabbits in the FCA-immunized group showed arteriosclerotic lesions in their aortic intimas similar to our previous observations.¹⁸,²¹ The number of lesions present in the individual rabbits varied from 1 to 7. Only 1 of 10 rabbits from the group receiving 2 intravenous injections per week of combined anti-CD3 mAb and prednisolone developed arteriosclerotic lesions. Moreover, 1 of 10 rabbits from the nonimmunized control group showed minor arteriosclerotic lesions. In the group treated with 2 injections of prednisolone (1 mg/kg) only per week, 5 of 9 rabbits developed arteriosclerotic lesions, mainly located in the aortic arch. Quantification of lesion areas in the aortic intima revealed a significant increase (P<0.01) in FCA-immunized animals (Table 1). In fact, no significant quantitative difference in lesion areas between control and immunosuppressed animals (anti-CD3 and prednisolone, Table 1) was found. As shown previously,²¹ the histological characteristics of immunization-induced lesions included intimal thickening with mononuclear cell infiltration, migration of smooth muscle cells, and extracellular matrix deposition, but an absence of foam cells (Figure 1B). The exact immunohistochemical description of these arteriosclerotic lesions has been detailed previously.²¹ The arch portions of all aortas, even those without macroscopically apparent lesions after 16 weeks of treatment, were examined microscopically on serial cross sections. Infiltrating mononuclear cells were occasionally found in the aortic intimas of group 4 rabbits, with some extracellular matrix deposition (Figure 1C), whereas aortas of group 3 animals (Figure 1D) appeared similar to those of normal rabbits (Figure 1A).

It has been demonstrated that T lymphocytes are present in arteriosclerotic lesions and are induced by either immunization or a cholesterol-rich diet.⁵,²¹ In the present experiment, we also found a number of T lymphocytes in the lesions of immunized rabbits, but not in the normal vessel wall (Figure 2).

Serum Antibodies to hsp65
To determine serum antibody levels, ELISA plates were coated with recombinant mycobacterial hsp65, ovalbumin, PPD, or mouse immunoglobulins. The data summarized in Tables 2 and 3 show significantly increased antibody titers against hsp65 and PPD in FCA-immunized rabbits. The antibody titer against the control antigen ovalbumin remained low (≤1:10) in all groups (data not shown). The anti-hsp65 and anti-PPD titers in the immunized group immunosuppressed with anti-CD3 mAb and prednisolone remained significantly lower than positive controls. The anti–mouse immunoglobulin titer in the group immunosuppressed with anti-CD3 mAb and prednisolone rose to 3830±2340 (not shown in these Tables) at the end of the experiment, but


without symptoms of serum sickness. In all other groups, this titer remained low.

**Blood Cholesterol and Blood Glucose Levels**
Blood cholesterol levels in rabbits immunized with FCA remained below 2.6 mmol/L (100 mg/dL), ie, the same range as untreated controls, confirming previous results indicating that immunization alone does not elevate blood cholesterol levels in rabbits receiving a normal diet.21

Owing to the glucose-liberating effect of glucocorticoids, blood glucose levels in rabbits treated with prednisolone alone or with combined anti-CD3 mAb and prednisolone after immunization with FCA rose progressively from the beginning of the immunosuppressive therapy to the end of the experiment (Figure 3). However, the blood glucose level of the FCA-immunized group treated with prednisolone alone was significantly higher than that in FCA-immunized rabbits without immunosuppressive therapy (7.27±1.23 versus 4.65±0.91 mmol/L, P<0.01).

**Peripheral T-Cell Response**
Figure 4 summarizes the results of proliferation assays of blood mononuclear cells cultivated for 48 hours in the presence or absence of various antigens or with the T-cell mitogen concanavalin A as a positive control, which strongly stimulated the cells of all rabbits. As expected, the cells of immunized animals reacted specifically with hsp65 and PPD, the open arrow denotes positive T lymphocytes. Original magnification ×250.

with FCA-immunized animals. The cells of FCA-immunized animals also showed a higher proliferative response to hsp65 or PPD than those in medium without antigen or with ovalbumin.

The spleen cell response was determined in the same manner as the peripheral mononuclear cell response. Although the effect of immunosuppression with anti-CD3 mAb and prednisolone was also clearly evident in spleen cells, it was less distinct than that seen with peripheral mononuclear cells (data not shown).

**FACS Analysis of Blood Mononuclear Cells**
FACS analysis was used to test the effect of various forms of immunosuppressive treatment on the composition of peripheral blood mononuclear cell populations. The viability of labeled peripheral blood mononuclear cells was determined by FACS scatter analysis, and only living cells were gated and subjected to immunofluorescence measurements. Flow cytometric studies showed that treatment with anti-CD3 mAb significantly reduced CD3+ and CD4+ peripheral blood mononuclear cells (P<0.02) as well as the Ia+ peripheral blood mononuclear cells, albeit at a lower level (Table 4).

**Discussion**
Our clinical and experimental data provide ample evidence that atherosclerosis can start as an inflammatory, immunologic disease induced by an autoimmune reaction against hsp60. Arteriosclerosis is characterized by vascular areas containing mononuclear and proliferating smooth muscle cells as well as extracellular matrix components, resulting in hardening and thickening of the arterial wall. Atherosclerotic lesions are localized in the intima and additionally contain foam cells and deposits of cholesterol crystals, manifested as fatty streaks. Most humans possess hsp65-reactive humoral antibodies and T cells, which afford vital protection from potentially dangerous microorganisms expressing cross-reactive antigens of the hsp60 family of heat shock proteins. These polyclonal effector mechanisms allow for recognition of various hsp65 epitopes, several of which provide the basis for potential pathogenetic cross-reactions with human hsp60.

The role of T lymphocytes in atherogenesis remains controversial, with different data published by laboratories using various animal models.27–30 Hansson et al33

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**TABLE 2. Anti-hsp65 Antibody Titer of Rabbit Sera**

<table>
<thead>
<tr>
<th>Group</th>
<th>Preimmune</th>
<th>4 Weeks</th>
<th>8 Weeks</th>
<th>12 Weeks</th>
<th>16 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>≥20</td>
<td>≥20*</td>
<td>≥20*</td>
<td>≥20*</td>
<td>≥20*</td>
</tr>
<tr>
<td>FCA</td>
<td>≥20</td>
<td>2355±1924</td>
<td>13125±8500</td>
<td>16 000±5477</td>
<td>11 250±6291</td>
</tr>
<tr>
<td>FCA+prednisolone</td>
<td>≥20</td>
<td>2268±1616</td>
<td>4000±1369*</td>
<td>9000±6519*</td>
<td>8000±7373</td>
</tr>
<tr>
<td>FCA+anti-CD3 mAb+prednisolone</td>
<td>≥20</td>
<td>288±208*</td>
<td>1370±1300*</td>
<td>2660±1789*</td>
<td>1832±1819*</td>
</tr>
</tbody>
</table>

Values are mean±SD of antibody titers. Antibody titers of rabbit sera were determined with an ELISA by using a log dilution from 1:20 at the starting point and at 4, 8, 12, and 16 weeks after the first immunization. Cutoff point for a positive titer was OD<sub>410</sub>=0.20.

*Significant difference from FCA-immunized group, P<0.05. Statistical evaluation was performed by ANOVA.
reported that rats in which T lymphocytes were eliminated by an mAb developed even larger proliferative arterial lesions after balloon-catheter injury. On the other hand, some data support the important role of T lymphocytes in atherogenesis, because T lymphocytes have been shown to be prominent components of early lesions, not only in human atherosclerotic plaques1 but also in apoE-deficient mice.24 Moreover, Emeson et al27 showed atherosclerosis inhibition in CD4 T cell–ablated and nude (nu/nu) C57BL/6 hyperlipidemic mice.

In contrast to our approach with normocholesterolemic rabbits, previous published experiments in this field involved feeding the animals a hypercholesterolemic diet,27–30 as is the case for studies showing enhanced development of atherosclerosis after immunosuppression with cyclosporin A28,29 or in immune-deficient mice.30 Because cyclosporin A can also increase arterial blood pressure,35 damage endothelial cells,36 induce elevated plasma total cholesterol levels,37 and increase endothelin production,38 this chemical seems to be unsuitable for investigations on the role of T cells in atherogenesis.

It is also becoming evident that immunity against modified LDL, in contrast to immune reactions against hsp65/60, may be protective. Thus, immunization of hypercholesterolemic rabbits with oxLDL protects them against cholesterol-induced arteriosclerosis,12,13 although the mechanism by which it exerts this effect remains unknown. One theory is the elimination of oxidatively modified circulating LDL by high-titer antibodies against oxLDL.

The presumed mechanism by which hsp65 immunization of rabbits leads to formation of arteriosclerotic lesions differs from the mechanism by which oxLDL immunization reduces arteriosclerosis, ie, an autoimmune cross-reaction between antigenically highly conserved heat shock protein. We speculate that these 2 phenomena are not contradictory, although the mechanisms and animal models are different. In humans, the 2 phenomena could coincide. The hsp60 or portions thereof are present on the surface of stressed endothelial cells and serve as targets for circulating lymphocytes and antibodies against hsp65/60 provoked by immunization with bacterial hsp65.39,40 Schett et al41 showed that these anti-hsp65/60 antibodies can mediate both complement-mediated cytotox-

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunization With/Without Immunosuppression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 Weeks</td>
</tr>
<tr>
<td>Control</td>
<td>≤20</td>
</tr>
<tr>
<td>FCA</td>
<td>1768 ± 668</td>
</tr>
<tr>
<td>FCA + prednisolone</td>
<td>1585 ± 610</td>
</tr>
<tr>
<td>FCA + anti-CD3 mAb + prednisolone</td>
<td>960 ± 369*</td>
</tr>
</tbody>
</table>

Values are mean ± SD of antibody titers. Antibody titers of rabbit sera were determined with an ELISA by using a log dilution from 1:20 at the starting point and at 4, 8, 12, and 16 weeks after the first immunization. Cutoff point for a positive titer was OD_{410} > 0.2.

*Significant difference from FCA-immunized group, P < 0.05. Statistical evaluation was performed by ANOVA.

Figure 3. Effect of immunization and immunosuppression on serum glucose. Line plot showing effect of immunization and the different kinds of immunosuppression on serum glucose. Serum glucose values of 10 or 9 rabbits from each group were pooled. Serum glucose values of rabbits immunized with FCA (△) were found to be similar to controls (○). Serum glucose levels of rabbits immunized with FCA (△) were significantly higher after ~12 weeks of immunosuppressive treatment than those without immunosuppressive therapy (○, △), whereas serum glucose levels of rabbits immunosuppressed by anti-CD3 mAb and prednisolone (□) were not significantly elevated, *P < 0.05.

Figure 4. Proliferative blood cell response. Peripheral blood mononuclear cells were isolated over Lymphoprep 8 (□) and 16 (●) weeks after the first immunization. The cells (10^5 per well) were cultivated in duplicate in round-bottom microtiter plates in 0.2 mL of RPMI 1640 containing 5% autologous serum and antigen supplement (ovalbumin 30 μg/mL, PPD 30 μg/mL, or recombinant hsp65 5 μg/mL). The proliferative response of cells was determined by measuring the incorporation of [3H]thymidine (1 μCi per well) that was added for the last 8 hours of the 48-hour culture period. Columns show [3H]thymidine uptake ± SD and are means of 5 rabbits from each group. *The [3H]thymidine uptake in the groups immunized with FCA and intravenously treated with the combination of anti-CD3 mAb and prednisolone or prednisolone alone are significantly different from the group immunized with FCA only, *P < 0.02.
mononuclear cells preexist in the intima of unaffected arteries of children. Inhibition of the first step, by immunosuppression, is thus of particular importance and probably of potent clinical relevance. Recently, the prominent role of inflammation in the progression of arteriosclerosis in humans was described in a large study on the protective effect of aspirin. The present data suggest that the progression of arteriosclerosis development could be delayed or even prevented by immunosuppression. We are now investigating the effects of immunosuppression in rabbits in which arteriosclerosis is induced by a high-cholesterol diet only.

**Acknowledgments**

This study was supported by grants P12213-MED from the Austrian Science Fund, P5651 from the Inbliumfonds of the Austrian National Bank, the State of Vorarlberg and the State of Tyrol (to G. Wick). We thank A. Jennewein and G. Sturm for excellent technical assistance, E. Rainer for help in animal experimention, and T. Ottl for the preparation of photographs.

**References**


**TABLE 4. CD3⁺, CD4⁺, and La⁺ Populations in Peripheral Blood**

<table>
<thead>
<tr>
<th>Populations and Groups</th>
<th>Preimmune</th>
<th>8 Weeks</th>
<th>16 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD3⁺ PBMCs, %</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>63±4</td>
<td>72±1.4</td>
<td></td>
</tr>
<tr>
<td>Anti-CD3 mAb + prednisolone</td>
<td>69±4</td>
<td>21.5±5.5</td>
<td>20±5.6†</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>48±5.6</td>
<td>63±4.2</td>
<td></td>
</tr>
<tr>
<td><strong>CD4⁺ PBMCs, %</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>32±7.5</td>
<td>32.5±3.5</td>
<td></td>
</tr>
<tr>
<td>Anti-CD3 mAb + prednisolone</td>
<td>32±4</td>
<td>15.5±2.1</td>
<td>7.5±2.1†</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>31±1.4</td>
<td>31.8±2.6</td>
<td></td>
</tr>
<tr>
<td><strong>La⁺ PBMCs, %</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>19.5±3.5</td>
<td>24.4±2.6</td>
<td></td>
</tr>
<tr>
<td>Anti-CD3 mAb + prednisolone</td>
<td>25±3</td>
<td>10.7±1.8</td>
<td>15.2±0.5</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>8.4±1.9</td>
<td>10.7±4.5</td>
<td></td>
</tr>
</tbody>
</table>

**After intravenous injection of anti-CD3 mAb and prednisolone or prednisolone alone, the percentage of CD3⁺, CD4⁺, and La⁺ peripheral blood mononuclear cells (PBMCs) was determined by FACScan analysis at the beginning of the experiment, 8 weeks after the first immunization, and at the end of the experiment.**

†Significant difference from control group, P<0.02.

‡Significant difference from control group and prednisolone-treated group, P<0.02.


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doi: 10.1161/01.ATV.19.8.1905
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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