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

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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 atherosclerotic lesions in humans and rabbits contain large numbers of T lymphocytes,[1–5] half of which express major histocompatibility class (MHC) class II antigens and some of which also express interleukin-2 receptors,[6] indicating a state of activation. The presence of T cells in atherosclerotic 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.[7,8] However, the antigen(s) responsible for this immunoactivation has not been identified. Two major candidates have been proposed: (1) Oxidized LDL (oxLDL) has been shown to be a chemokine for T lymphocytes[9] and to activate T lymphocytes derived from atherosclerotic lesions[10] by an MHC class II–dependent mechanism.[11] Interestingly, immunization of hypercholesterolemic rabbits with oxLDL has been shown to protect these animals from atherosclerosis development.[12,13] (2) Heat shock protein 65 (hsp65), as we have shown, may be an (auto)antigen initiating the pathogenesis of atherosclerosis,[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.[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 atherosclerotic lesions, including foam cells, exactly paralleling the situation in classic human lesions.[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.[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

Group	n	Treatment	Antigen	Immunization, Times	Incidence/No. of Animals	Lesion Area, mm²/Aorta
1	10	Control	...	...	1/10	0.4±1.2
2	10	Immunization	FCA	3	9/10	28±20.4*
3	10	Immunization+anti-CD3 mAb+prednisolone	FCA	3	1/10	0.9±3.0
4	9	Immunization+prednisolone	FCA	3	5/9	17.9±19.2

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.

*The 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, Reagents, 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 an average value of 1.34±0.44 mmol/L and were individually housed under conventional conditions in wire-bottom cages at 22°C with a relative humidity of 60%. They received water ad libitum and were fed a normal, standard diet (T775, 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-) were purchased from Difco Laboratories. Recombinant *Mycobacterium bovis* hsp65 (BCG 65K, batch No. mA-11B) 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.[18,21] 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.[22] 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 (Petra-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′-azinobis(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 over Lymphoprep ($d=1.077$ g/mL, Nycomed Pharma AS) as described previously.[25]

Peripheral mononuclear cells (10^5 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% CO_2.

Fluorescence-Activated Cell Sorting (FACS) Analysis of Blood Mononuclear Cells

For flow cytometric analysis, 2×10^5 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 FACScan (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
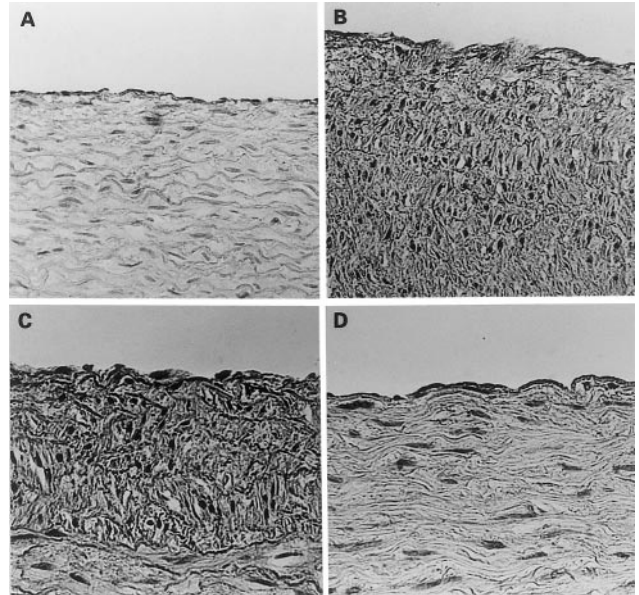

Figure 1. Morphology of lesions of FCA- and immunosuppressive drug–treated rabbits. Photomicrographs of HE-stained paraffin sections of aortas from rabbits immunized with hsp65-containing material with or without simultaneous immunosuppressive therapy. A, Aorta of an nonimmunized rabbit; B, aorta of a rabbit immunized with hsp65-containing material; C, aorta of an immunized rabbit immunosuppressed with prednisolone; and D, an immunized rabbit immunosuppressed with anti-CD3 mAb and prednisolone. Magnification ×250, A and B; ×400, C and D.

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.[6,21] 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 ($\leq$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
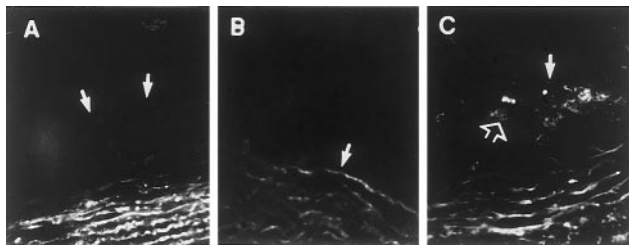
Figure 2. Detection of T lymphocytes in aortic lesions by indirect immunofluorescence. Sections derived from arteriosclerotic lesions (A and C) and from the normal vessel wall (B) were labeled with an mAb (L11/135, B and C) or with normal mouse immunoglobulins (A) as negative control. The sections were incubated with rabbit anti-mouse immunoglobulins conjugated with FITC. Solid arrows indicate the endothelium, and the open arrow denotes positive T lymphocytes. Original magnification ×250.

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, ie, hsp65-containing material, but not with ovalbumin. The peripheral lymphocyte cell response of FCA (ie, hsp65)-immunized rabbits treated with anti-CD3 mAb and prednisolone was significantly decreased ($P < 0.02$) compared

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] The opinion that T lymphocytes do not play a priming role in atherogenesis is supported by the fact that T cell–deficient mice develop atherosclerotic lesions to the same extent as do normal mice after receiving a cholesterol-rich diet.[30–32] Hansson et al[33]

TABLE 2. Anti-hsp65 Antibody Titer of Rabbit Sera

| Group | Preimmune | Immunization With/Without Immunosuppression | | | |
		4 Weeks	8 Weeks	12 Weeks	16 Weeks
Control	≤20	≤20*	≤20*	≤20*	≤20*
FCA	≤20	2355±1924	13 125±8500	16 000±5477	11 250±6291
FCA+prednisolone	≤20	2268±1616	4000±1369*	9000±6519*	8000±7373
FCA+anti-CD3 mAb+prednisolone	≤20	288±208*	1370±1300*	2660±1789*	1832±1819*

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 $OD_{410} > 0.20$.

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

TABLE 3. Anti-PPD Antibody Titers of Rabbit Sera

Group	Preimmune	Immunization With/Without Immunosuppression			
		4 Weeks	8 Weeks	12 Weeks	16 Weeks
Control	≤20	≤20*	≤20*	≤20*	≤20*
FCA	≤20	1768±668	48 000±17 888	96 000±35 777	120 000±46 188
FCA+prednisolone	≤20	1585±610	36 000±7984	40 000±24 494*	34 000±13 416*
FCA+anti-CD3 mAb+ prednisolone	≤20	960±369*	14 500±7984*	27 500±15 000*	22 500±12 583*

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 $OD_{410} > 0.20$.

*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 plaques[1] but also in apoE-deficient mice.[34] Moreover, Emeson et al[27] 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 also the case for studies showing enhanced development of arteriosclerosis after immunosuppression with cyclosporin A[28,29] 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 al[41] showed that these anti-hsp65/60 antibodies can mediate both complement-mediated cytotox-
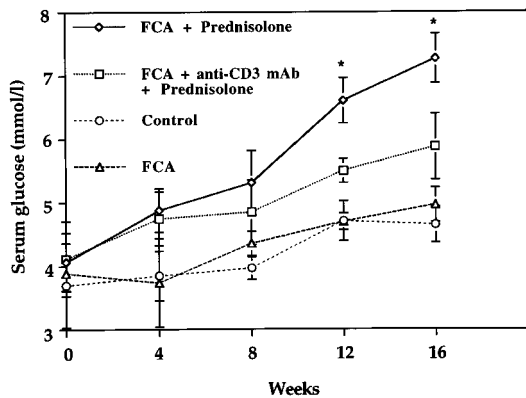
Figure 3. Effect of immunization and immunosuppression on serum glucose. Line plot showing effect of immunization and the different kinds of immunosuppression on serum glucose. Serum glucose values of 10 or 9 rabbits from each group were pooled. Serum glucose values of rabbits immunized with FCA (△) were found to be similar to controls (○). Serum glucose levels of immunized rabbits immunosuppressed by prednisolone (◇) 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, *$P<0.05$.
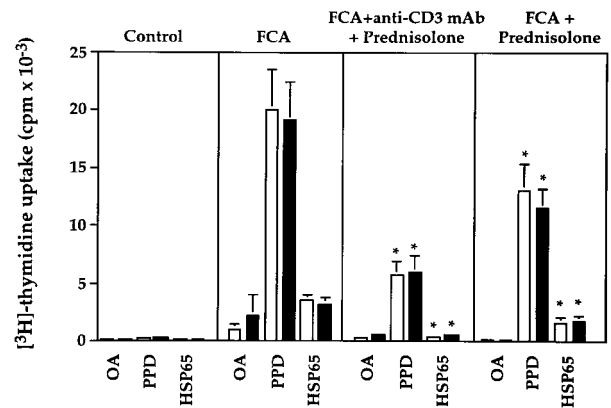
Figure 4. Proliferative blood cell response. Peripheral blood mononuclear cells were isolated over Lymphoprep 8 (□) and 16 (■) weeks after the first immunization. The cells (10^5 per well) were cultivated in duplicate in round-bottom microtiter plates in 0.2 mL of RPMI 1640 containing 5% autologous serum 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, *$P<0.02$.

TABLE 4. CD3$^+$, CD4$^+$, and Ia$^+$ Populations in Peripheral Blood

Populations and Groups	Preimmune	8 Weeks	16 Weeks
CD3$^+$ PBMCs, %			
Control		63±4.9	72±1.4
Anti-CD3 mAb+prednisolone	69±4	21.5±5*	20±5.6†
Prednisolone		48±5.6	63±4.2
CD4$^+$ PBMCs, %			
Control		32.7±5	32.5±3.5
Anti-CD3 mAb+prednisolone	32±4	15.5±2.1†	7.5±2.1†
Prednisolone		31±1.4	31.8±2.6
Ia$^+$ PBMCs, %			
Control		19.5±3.5	24.4±2.6
Anti-CD3 mAb+prednisolone	25±3	10.7±1.8	15.2±0.5
Prednisolone		8.4±1.9	10.7±4.5

After intravenous injection of anti-CD3 mAb and prednisolone or prednisolone alone, the percentage of CD3$^+$, CD4$^+$, and Ia$^+$ peripheral blood mononuclear cells (PBMCs) was determined by FACS analysis at the beginning, 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, $P<0.02$.

icity and antibody-dependent cellular cytotoxicity of stressed (eg, by heat, tumor necrosis factor, etc) but not unstressed endothelial cells. Furthermore, we have shown that a cholesterol-rich diet and simultaneous immunization with hsp65 synergistically leads to more severe atherosclerotic lesions than does a cholesterol-rich diet alone and that arteriosclerotic lesions provoked by hsp65 immunization are reversible, whereas those arising as a consequence of hypercholesterolemia are not.[42] It has been shown that long-term treatment with glucocorticosteroids does not influence heat shock protein expression in the aortic wall of animals.[43]

To further scrutinize our hypothesis that T cells support the development of arteriosclerotic lesions in this model, we analyzed the effect of immunosuppression on the formation of arteriosclerotic lesions by selective depletion of CD3$^+$ lymphocytes with mAbs. Although immunosuppression with prednisolone alone led to decreased T lymphocytes (Table 4) and moderately decreased antibody titers against hsp65 and PPD, this treatment alone did not inhibit development of arteriosclerotic lesions. The most likely explanation, beyond the incomplete depletion of lymphocytes, is the significant rise of blood glucose levels provoked by long-term administration of prednisolone (Figure 3). Because diabetes is a classic risk factor for arteriosclerosis, this result is not surprising. Interestingly, blood glucose levels did not increase significantly in the group treated with anti-CD3 mAb and prednisolone, but so far, we have no explanation for this phenomenon. Finally, we provide data herein that the inflammatory lesions provoked in normocholesterolemic rabbits by immunization with hsp65-containing material can be almost completely abolished by intravenous administration of anti-CD3 mAb, which depletes mature T lymphocytes.

We hypothesize that the first stage of atherosclerosis is inflammatory in nature, and with age, is aggravated by high cholesterol levels to form typical atherosclerotic plaques. The sequence of this hypothesis is supported by the fact that mononuclear cells preexist in the intima of unaffected arteries of children.[17] 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.[44] 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 atherosclerosis is induced by a high-cholesterol diet only.

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Correction

We published "Inhibition of Arteriosclerosis by T-Cell Depletion in Normocholesterolemic Rabbits Immunized With Heat Shock Protein 65" (*Arterioscler Thromb Vasc Biol*. 1998;19:1905-1911) under the consideration that treatment of normocholesterolemic rabbits with a monoclonal antibody against rabbit T-cells prevents the development of arteriosclerosis after immunization with mycobacterial heat shock protein 65. The antibody used for this study was produced from the hybridoma line L11/135 obtained from the American Type Culture Collection (ATCC-No. TIB 188). In the ATCC specification, this hybridoma was described as producing antibodies reactive with rabbit T-cells. In the reference on this clone given in the ATCC specification sheet,[1] a panel of six monoclonal antibodies used against cell surface glycoproteins of a rabbit T-lymphocyte line was studied. This paper explicitly states that in functional studies only the L11/135-bearing cells responded to the T-cell mitogens concanavalin A and phytohemagglutinin and to allogenic splenocytes.

However, we became aware recently that this antibody, defined as pan-T-specific, recognized the equivalent of CD43/leukosialin,[2] an antigen that is strongly expressed by all T-cells, but also weakly stains monocytes and macrophages. Although the experimental data that we presented (ie, the severe quantitative and functional suppression of T-cells in the antibody-treated animals) are solid and reproducible, we are at present not able to assign the atherosclerosis-inhibiting effect to either a depletion of T-cells or the inhibition of the adhesion to and subsequent transmigration of monocytes through the vascular endothelium.[3] CD3 and CD43 are jointly present in a large complex in a mild detergent lysate of T-cells, and it is suggested to add this as a co-stimulatory molecule in CD3/T-cell receptor signalling.[4] Unfortunately, a pure anti-rabbit-CD3 monoclonal antibody that binds to extra cellular domains of the CD3-complex and can therefore be used for in vivo experiments is not available. We apologize for any confusion this may have caused.

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