American Journal of Pathology, Vol. 142, No. 6, June 1993

Accelerated Atherosclerosis in Hyperlipidemic C57BL/6 Mice Treated with Cyclosporin A

Eugene E. Emeson and Mei-Ling Shen

Department of Pathology, University of Illinois College of Medicine, Chicago, Illinois

We and others have demonstrated that T lymphocytes are prominent components of atherosclerotic lesions. We hypothesized that if T cells were necessary for the development of atherosclerosis it would be possible to demonstrate its prevention or retardation in T-cell-suppressed mice. To test this hypothesis, CyA, a potent suppressor of T-cell activation, was used to treat C57BL/6 mice undergoing lipid hyperalimentation. Mice receiving normal mouse chow were completely free of atherosclerotic lesions. In mice receiving the atherogenic diet plus control oil injections, lesions of the aorta and coronary arteries were observed at 135 days and increased progressively in area until 310 days. Somewhat surprisingly, mice given the atherogenic diet plus CyA injections displayed even larger lesions at all three observed time intervals. Although CyA did suppress T-cell reactivity sufficiently to obtain the expected prolongation of skin allografts, it did not suppress the development or progression of atherosclerotic lesions. (Am J Pathol 1993, 142:1906–1915)

The common presence of T cells in atherosclerotic lesions at all stages of development has now been firmly established by several groups of investigators.[1–6] In our own study of over 200 aortas and coronary arteries of children and young adults (ages 15–32) dying of acute trauma, we showed that T cells and macrophage-derived foam cells were present in the subintimal spaces of over 75% of all aortic samples and were far more numerous in lesion-prone areas (dorsal aorta) than lesion-resistant areas (ventral aorta) (Emeson et al, unpublished data).[3] These mononuclear cells were also commonly seen in eccentric lesions of the coronary arteries. In other studies Hansson et al[7] have shown that approximately one-third of the T lymphocytes within atherosclerotic lesions were activated, as indicated by their expression of class II antigens, interleukin-2 receptors, or γ-interferon (IFN-γ). Since T lymphocytes have been shown to be necessary participants in the pathogenesis of a wide variety of autoimmune, infectious, and neoplastic diseases, it is important to clarify the role of these cells in the initiation and progression of atherosclerotic lesions. The best approach to this problem would be to examine the evolution of the lesion in T-cell-deficient or T-cell-suppressed animal models.

Cyclosporin A (CyA) is a potent T-cell suppressor that has been used to suppress allograft rejection, graft-*versus*-host disease, and a wide variety of autoimmune diseases (reviewed in Ref. 8). Two recent studies have examined the effects of this drug on the response of the vessel wall to injury and have yielded conflicting results. In the studies of Jonasson et al,[9] CyA suppressed the intimal proliferative vascular smooth muscle cell response to balloon injury *in vivo* in rats. In contrast, Ferns et al[10] presented evidence in rabbits that CyA increases intimal thickening in selectively de-endothelialized carotid arteries, suggesting that this immunosuppressive agent may enhance the progression of this lesion. Therefore, the role of T cells requires clarification. Furthermore, since the vascular response to injury is an acute model of intimal injury and not necessarily relevant to atherosclerosis, we thought it appropriate to re-examine this question in the more relevant chronic lesions of animals undergoing lipid hyperalimentation.

To conduct these studies we have selected the hyperlipidemic murine model of atherogenesis recently developed by Paigen et al.[11,12] We believe that this model is especially valuable for the above kinds of studies for the following reasons: 1) The atherosclerotic lesions which develop in this model are very similar pathologically to those in the human, are reproducible, and evolve over a 4–8-month period of time.

Supported by funds from NIH Biomedical Research Support grant 2 507 RR05 369-28, the University of Illinois at Chicago Campus Research Board, Office of Vice-Chancellor for Research, and funds from the Department of Pathology.

Accepted for publication December 7, 1992.

Address reprint requests to Dr. Eugene E. Emeson, University of Illinois College of Medicine, Department of Pathology, M/C 847, 1853 West Polk Street, Chicago, IL 60612.

2) The atherogenic diet used in this model is similar in percentage total fat content (although the cholesterol subfraction is much higher) to the diet consumed by humans. 3) The plasma lipoprotein levels induced by the atherogenic diet are similar to those in humans consuming a typical western diet. 4) The immune system in mice is extremely well defined, and there are many tools available such as monoclonal antibodies to manipulate it. 5) There are many genetic models of immune deficiency which can be used to investigate the role of immunity in atherogenesis. The disadvantages of this model include the facts that the lesions are quite focal (ie, localized to the ascending aorta and the proximal segments of the coronary arteries) and that they generally lack some of the features of the more advanced complicated lesions of humans such as calcification, hemorrhage, and thrombosis. In addition, the atherogenic diet we utilized contains 0.5% sodium cholate, a potential angiotoxin.

Since we hypothesized that T lymphocytes are necessary participants in atherogenesis, we anticipated that the CyA treatment would prevent or retard the evolution of atherosclerotic lesions. To our surprise, we discovered that the CyA treatment did not retard atherogenesis but, in fact, probably enhanced it.

Materials and Methods

Experimental Design

Three groups of animals were studied as summarized in Figure 1. The animals of all three groups were started on the atherogenic diet at day 0. In group A the mice were treated with CyA from day 0 until the termination of the experiment on day 135. Similarly, the mice in group B were treated with CyA from day 0 to day 168. These two groups of animals thus constituted the immediate treatment group. In contrast, in group C, the delayed treatment group, the CyA treatment was not initiated until the 168th day and was continued until the termination of the experiment at day 310. Groups A and B, the immediate treatment groups, were used to determine the effects of CyA on the development of relatively early fatty streak lesions, while group C, the delayed treatment group, was employed to ascertain the effects of CyA on the progression of the fatty streak to more advanced lesions such as the fibrous plaque. Control groups used for each of the three groups included mice given the atherogenic diet plus injections of olive oil (vehicle for CyA) and mice given a normal diet plus CyA or olive oil or no injection. At the end of each of the time intervals blood was obtained from each of the mice by retro-orbital puncture under light ether anesthesia, and the mice were killed by cervical dislocation. Their hearts, aortas, livers, kidneys, and spleens were then dissected out, fixed in 4% buffered formaldehyde, and processed for histology as described below.

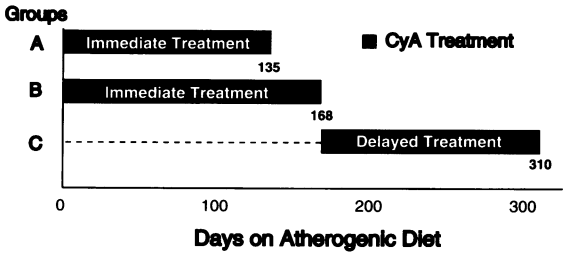

Figure 1. *Experimental design showing time line of the three major groups of mice (Groups A, B and C) killed at 135, 168, and 310 days after the inception of the atherogenic or control diets. Each group consisted of subgroups of mice fed the atherogenic or normal diets and treated with CyA or the control lipid. Mice in groups A and B were treated with CyA or control lipid from days 0 to 135 or 168 days, respectively, and thus could collectively be designated the immediate treatment group. With group C, the initiation of the CyA or control lipid injections were delayed until the 168th day and continued until the mice were killed at 310 days. This latter group is termed the delayed treatment group.*

Animals

The C57BL/6J female mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and were started on the experimental protocol at 8 to 10 weeks of age. One-half of the mice (representing all experimental and control groups) were maintained under pathogen-free conditions to avoid infection by mouse hepatitis virus, Sendai virus, and other opportunistic microorganisms. These mice were housed in sterile micro isolator cages (Lab Products, Inc., Mayfield, NJ) and transferred weekly into clean sterile cages. The transfer and treatment of animals was performed in a vertical laminar flow Class II-type hood. The normal mouse chow and water were sterile. The remaining mice (also representing all experimental and control groups) were maintained under standard laboratory conditions. Since there were no significant differences in the data obtained from the two differently maintained mouse populations, the results were combined for our final analyses.

Atherogenic Diet

The atherogenic diet was purchased from the Teklad Test Diets (Madison, WI). This diet was made by mixing a diet containing 30% cocoa butter, 5% cholesterol, 2% sodium cholate, 30% casein, 5% alphacel, 4% vitamin mixture, 6.5% sucrose, 6.5%

dextrin, and 0.5% cholic acid with Purina breeder chow in a ratio of 1:3 parts, respectively, and then pelleting it. This diet has 15% total fat content and a cholesterol content of 1.25%, thus being comparable in percentage total fat content (although the cholesterol subfraction is much higher) to the diet consumed by western humans. This diet could not be sterilized because of the probable degradation of lipids and other important nutritional components during the procedure. It was stored at 4 C prior to use.

Administration of Cyclosporin A

The CyA, obtained from Sandoz, Ltd. (East Hanover, NJ) was diluted in olive oil, and 0.05 ml was administered subcutaneously to each mouse at a dose of 20 mg/kg/day or 40 mg/kg every other day. This dose has been shown by others[13] to suppress both cellular and humoral immunity in C57BL/6 mice.

Plasma Lipids

Total plasma cholesterol was measured by an enzymatic method using 4-aminoantipyrine as a color substrate[14] and the Express 550 Analyzer (Ciba Corning Diagnostic Corp, Oberlin, OH) to read the absorbance. High-density lipid (HDL) cholesterol was measured the same way after selectively precipitating out the low-density lipid (LDL) and very low-density lipid (VLDL) with tungstic acid and magnesium.[15] LDL plus VLDL was calculated by subtracting the HDL cholesterol from the total cholesterol. A two-sided *t*-test for unpaired samples or two-way analysis of variance was used to compare groups (Statview statistical program software for the Macintosh Computer by Brain Power, Inc., Calabasa, CA).

Quantitative Assessment of Atherosclerotic Lesions

The method we used to evaluate the aortic and coronary atherosclerotic lesions was a modification of the method described by Paigen et al.[11] The mice were killed by exsanguination under ether anesthesia, and their hearts and aorta were immediately removed, rinsed in saline to remove the blood, and fixed in buffered 4% formaldehyde. Although this method of fixation as compared to perfusion at physiological pressure could affect plaque size, we believe that since we have treated the hearts from all groups (including the control groups) identically, it is valid to compare the relative sizes of lesions between groups. Each heart was cut just below the beginning of the aortic sinuses on a plane parallel with a plane formed by drawing a line between the lower tips of the right and left atria. The lower portion of each heart was discarded. The upper portion was mounted on a cryostat with OCT, with the above-identified plane parallel to the plane of cutting so as to obtain true cross-sections of the aorta. Ten-μm sections were then cut and discarded until we were able to locate the most caudal portion of the aortic sinus by examining unstained sections. Once this section was located, serial 10-μm sectioning was continued along the ascending aorta until the valve cusps were no longer visible, a procedure which involved 40 to 50 sections covering 400 to 500 μm. A schematic outline of this procedure and the method used to place the sections on two polylysine-D-coated slides is shown in Figure 2. The first section was placed in the upper left-hand corner of slide A and the second section in the same location of slide B. Thenceforth we alternated the placement of sections with all odd-numbered sections (1, 3, 5, 7) placed on slide A and all even-numbered (2, 4, 6, 8) on slide B. Slide A was stained with Oil Red O and a hematoxylin counterstain, and slide B was stained with hematoxylin and eosin (H&E). This approach permitted us to correlate the lipid distribution of lesions stained with Oil Red O with the morphological details better appreciated with the H&E-stained sections. To quantitate the degree of atherosclerosis in each mouse, the areas of the atherosclerotic lesions of the five circle sections of the Oil Red O-stained slides at 100-μm

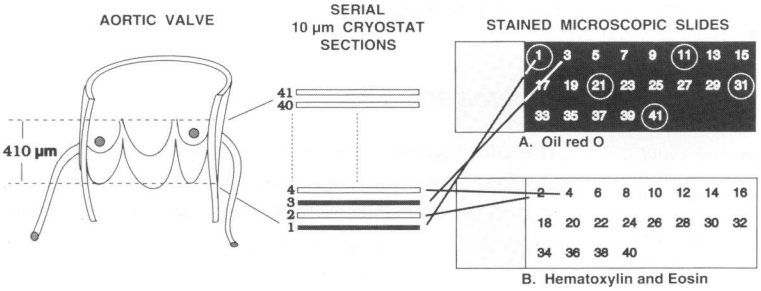

Figure 2. *Sampling procedure showing a schematic outline of the location from which the sections were cut, how they were systematically placed on glass slides, how they were stained, and how the circled sections at 100-μm intervals were selected for morphometric analysis.*

intervals (sections 1, 11, 21, 31, and 41) were examined (Figure 2). If any of the designated sections were folded or torn the section preceding or following it was used. The area of the lesions within each Oil Red O section was determined by point counting using a squared grid ocular graticule (Graticules, Ltd., Townbridge, England) at ×40 magnification.[16,17] The two-dimensional area of the lesions per section was then determined by the formula

$$a = p \cdot \mu^2 \qquad (1)$$

where a is the area in μm^2, P is the number of points falling within the lesions, and μ is the distance between two neighboring points (ie, μ^2 will equal the area associated with each point). In our system μ = 50 μm as determined by a reference grid and therefore $\mu^2 = 2500\ \mu m^2$ at ×40 magnification. The aorta of each mouse provided five independent data points for evaluation. The mean value of the five points were then used as the final value for each mouse. A two-sided *t*-test for unpaired samples or two-way analysis of variance was used to compare groups (Statview statistical program software for the Macintosh Computer by Brain Power, Inc.).

Skin Allografts

The skin allografting was performed and evaluated by slight modifications of the methods previously described in detail by Norin and Emeson.[18] Donor skin was obtained aseptically from the abdomens of shaved female BALB/c mice, and the panniculus adiposus layer was removed by careful dissection. Grafts (1.5 × 1.5 cm) were cut and secured onto the prepared dorsum of 12 of the C57BL/6 mice in the experimental protocol (310-day group) with 5-O silk interrupted sutures. The grafts were applied approximately 6 weeks prior to the termination of the experiment. The allografts were inspected daily for visual and tactile signs of rejection. A graft was considered rejected when 90% of the surface was no longer viable. The Mann-Whitney U-test was used to compare the CyA-treated with the control (oil-treated) groups of mice (Statview statistical program).

Results

Lesion Size

The kinetics of the effects of the atherogenic diet and CyA on lesion area is summarized in Figure 3. The aortas and coronary arteries of mice in all groups of mice receiving a normal diet were completely free of atherosclerotic lesions. In marked contrast, in mice receiving the atherogenic diet plus the control oil injection, typical atherosclerotic lesions of the ascending aorta were first observed at 135 days and increased progressively in size and number up to 310 days, the last observed time interval. Somewhat surprisingly, mice given the atherogenic diet plus CyA injections displayed larger lesion areas than those given the atherogenic diet plus control corn oil at all of the three observed time intervals. Statistical analyses of these data indicate that the differences in mean areas between the CyA-treated and control oil-treated groups were significant when looking at both components of the immediate treatment groups, ie, the 135- and 165-day groups, together ($P < 0.02$) and when looking at the 135-day group alone ($P < 0.04$). The differences in mean areas at 310 days, ie, the delayed treatment group, were large but not significant ($P > 0.10$). If one looked at all of the differences at all three time intervals together, they were significant at the $P < 0.05$ level. For all of the above statistical analyses the data was log transformed to reduce the differences in variance among the different time intervals.

The histological features of lesions from 135, 168, and 310 days are demonstrated in Figures 4, 5, and 6, respectively. Early lesions (135 days) displayed a marked thickening of the subendothelial intimal space associated with an extensive mononuclear infiltration consisting of foam cells and what appear to be lymphocytes. Focal areas of necrotic debris (Figure 4C) and deposition of connective tissue matrix (Figure 4C) were also present. Intermediate lesions (165 days) were similar to the early lesions but less cellular (Figure 5A, 5B, 5C). Late lesions (310 days) covered a much greater surface area of the aortic intima, were far less cellular, and contained a great deal more homogeneous pink (in

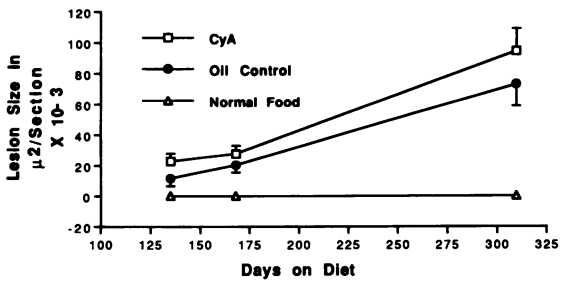

Figure 3. *Kinetics of effects of atherogenic diet and CyA treatment on the area of lesions in μ^2/section × 10^{-3}. The numbers of mice in each group at each time interval starting at 135 days are as follows: CyA 8,6,6, oil 7,7,5, and normal diet 10,6,4.*

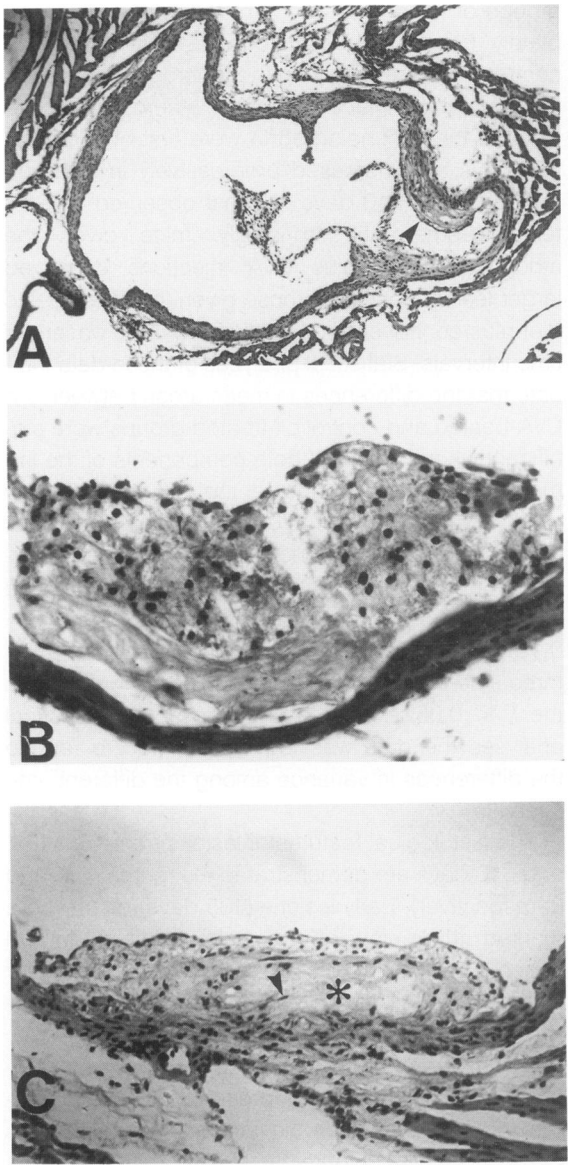

Figure 4. *135-day lesions.* **A**, *low-power view through the upper portion of the aortic valve of a control oil-treated mouse showing a large fibrofatty lesion (arrowhead) encroaching on the takeoff of a coronary artery. H&E, ×60.* **B, C**, *high-power views of fatty streak lesions from the aortas of both control oil-treated* (**B**) *and CyA-treated* (**C**) *mice displaying marked mononuclear cell infiltration and prominent foam cells. A spindle-shaped myointimal cell (arrowhead) appears to be emigrating from the media to the intimal space, and a lipid core (*) with what appears to be a central area of necrosis or degeneration of foam cells is seen in* **C**. **B**, *H&E, ×300;* **C**, *H&E, ×200.*

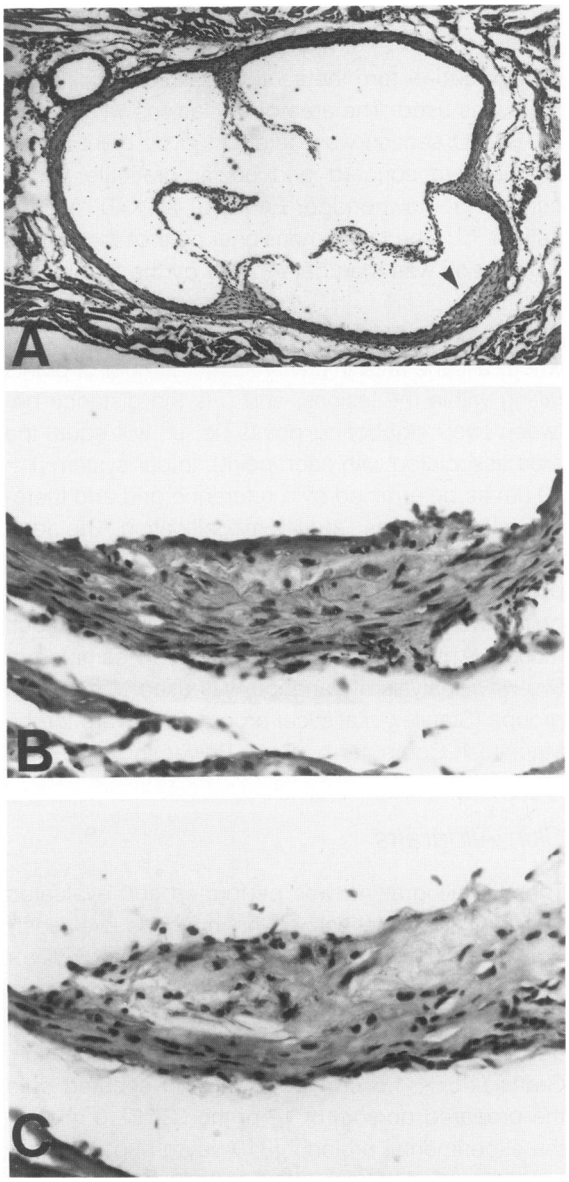

Figure 5. *168-day lesions.* **A**, *low-power view through the midportion of the aortic valve of a control oil-treated mouse displaying a small fibrous lesion (arrowhead). H&E, ×60.* **B**, *high-power view of this lesion showing a focal area of thickened intimal containing a few mononuclear cells. H&E, ×264.* **C**, *high-power view of a similar lesion from a CyA-treated animal showing a thickened intima and a moderate degree of cellularity. H&E, ×288.*

H&E sections) extracellular matrix material (Figure 6A, 6B, 6C). Some of the late lesions displayed prominent cholesterol clefts (Figure 6D) or fibrous caps consisting of multiple layers of spindle cells (Figure 6E). Involvement of the coronary arteries was seen in all three groups of mice (Figures 4A, 6F) but was far more common in the 310-day group. Some of the lesions from all groups also revealed spindle-shaped myointimal cells (probable smooth muscle cells) emigrating from the media to the intimal space (Figures 4A, 6F).

Total, HDL, and LDL Plus VLDL Cholesterol Levels

The total, HDL, and LDL plus VLDL cholesterol levels of mice fed normal mouse chow and receiving

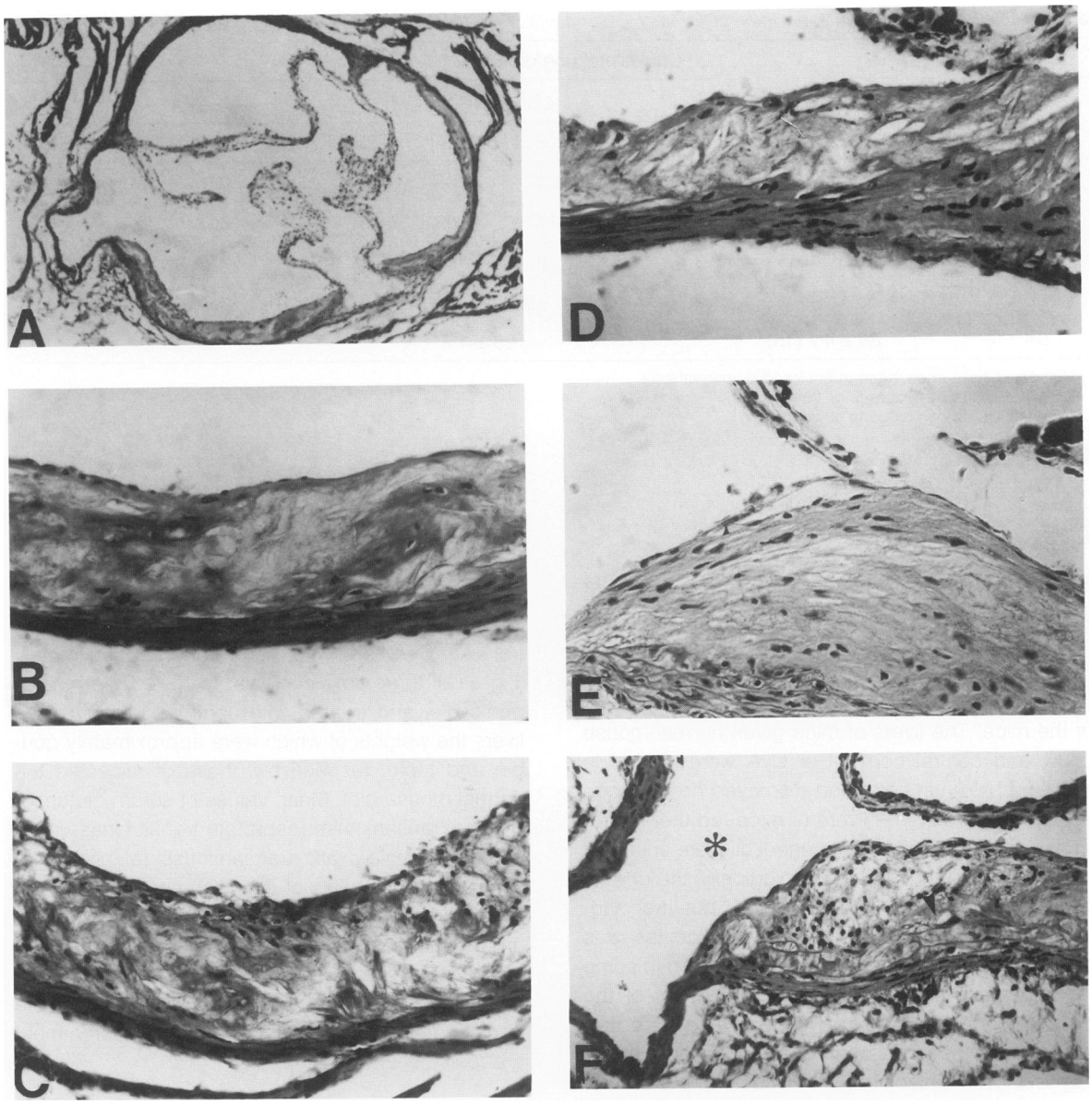

Figure 6. *310-day lesions.* **A**, *low-power view through the midportion of the aortic valve of a CyA-treated mouse showing two fibrofatty acellular lesions covering approximately 50% of the aortic surface. H&E, ×48.* **B**, *high-power view illustrating the acellular nature of these lesions, which consist primarily of an extracellular deposition of lipids and connective tissue matrix. H&E, ×264.* **C**, *high-power view of a similar lesion from an control oil-treated animal. H&E, ×240.* **D**, *high-power view through a similar lesion from another CyA-treated mouse showing multiple prominent cholesterol clefts. H&E, ×325.* **E**, *high-power view of a fibrofatty lesion from a CyA-treated mouse showing a large fibrous cap consisting of multiple layers of spindle cells. The intimal space beneath the fibrous cap contains scattered spindle cells, a few mononuclear cells, and deposits of extracellular lipid and connective tissue matrix. H&E, ×325.* **F**, *large fibrofatty lesion encroaching upon a coronary artery orifice (*). Note the prominent mononuclear cell infiltration and extracellular deposition of lipid and connective tissue matrix. Several elongated nuclei (probably of smooth muscle cell origin) are seen deep in the intimal space (arrowhead). The edge of an aortic valve is seen in the upper right side corner. H&E, ×200.*

no treatment were 68 ± 4.7, 29 ± 4.7, and 39 ± 3 mg/dl, respectively, levels comparable with those reported by other investigators[13] (Table 1). Treating the mice with CyA did not alter these lipid levels. The effects of feeding the mice the atherogenic diet on total and LDL plus VLDL cholesterol levels was dramatic, especially in mice treated with CyA. The exceptionally high total and LDL plus VLDL values in the 1500 mg/dl range observed in the 135- and 168-day (immediate treatment) groups were quite surprising and have not been reported in mice by other investigators.

The HDL levels were not significantly altered by the atherogenic diet or the CyA treatment.

Table 1. *Effect of Cyclosporin A on Total, LDL Plus HDL and HDL Plasma Cholesterol in Mice Fed an Atherogenic Diet*

	Cholesterol (mg/dl) ± SE											
Days on diet	Total				LDL ± VLDL				HDL			
	CyA	±SE	Oil	±SE	CyA	±SE	Oil	±SE	CyA	±SE	Oil	±SE
135	1560* (7)	237	141 (7)	7.9	1530*	242	105	7.9	26	8.0	36	4.0
168	1460* (2)	141	198 (6)	39	1450*	133	159	40.9	11	8.0	40	7.9
310	225* (5)	10.8	159 (5)	6.3	185†	16.3	145	3.7	40†	10.6	14	4.7
0‡	67 (11)	5.1	72 (9)	8	26	3.6	25	4.8	41	3.6	45	4.5
0§	68 ± 4.7 (15)				29 ± 4.7				39 ± 3.0			

Number of mice in each group enclosed by parentheses.
* $P \leq 0.01$ when compared to oil controls.
† $P \leq 0.05$ when compared to oil controls.
‡ Age-matched controls.
§ No treatment.

Liver

To assess the possible role of CyA-induced liver damage as a possible mechanism for the markedly increased total and LDL cholesterol levels in the CyA-treated mice, we obtained histological sections, liver weights, and liver chemistries from most of the mice. The livers of mice given normal mouse chow and control corn oil or CyA were normal in size and brown in color and displayed no histological abnormalities. The livers of mice fed the atherogenic diet and treated with control oil were enlarged and yellowish-tan in color. Histologically, the normal hepatic architecture was preserved, but the cytoplasm of most of the individual hepatocytes was distended by coalesced fat droplets, which in some cells displaced the nuclei to the periphery of the cells (fatty change). There were a few scattered areas of focal necrosis characterized by degenerating liver cells associated with a focal accumulation of polymorphonuclear leukocytes or mononuclear cells or both. The livers of mice given the atherogenic diet plus CyA treatment showed similar changes, but the changes were more extensive.

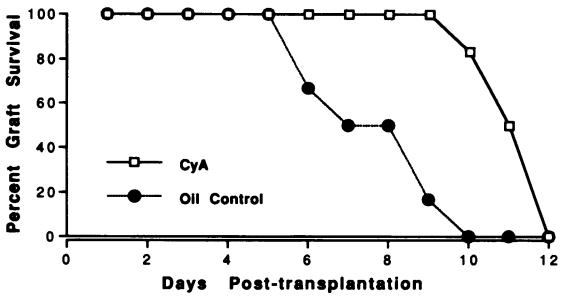

Figure 7. *Survival of BALB/C skin allografts on C57BL/6 mice treated with CyA or control oil. There are six mice in each group.*

There was no evidence of intrahepatic bile stasis in the livers of any of the treatment groups. The extent of the liver changes were reflected in their weights. Mice fed normal mouse chow and treated with control oil or CyA had livers that weighed approximately 1 g at all three time intervals. Mice fed the atherogenic diet and treated with control oil or CyA had livers the weights of which were approximately double and triple, respectively, those of mice fed the normal mouse diet. Mean values of serum glutamic-pyruvic transaminase (aspartate transaminase) and serum glutamic-oxalic transaminase (alanine transaminase), indicators of liver cell necrosis, did not differ significantly among the various treatment groups, although there was a great deal of scatter of individual data points. Mean serum albumin levels, indicators of liver cell synthetic capacity, were also comparable in value among the treatment groups.

Other Significant Side Effects

We did not observe any other significant side effects that could have influenced the observed findings. Food consumption and weight curves, for example, did not differ significantly between treatment groups.

Skin Allografts

To assess the efficacy of the CyA treatment in our experiments, we skin-grafted 12 of the mice in the 310-day group using H-2-incompatible BALB/c abdominal skin as the allografts. The CyA treatment prolonged the skin grafts an average of 3.5 days in both the normally fed and hyperlipidemic mice. The

differences in survival curves (Figure 7) have been shown to be highly significant ($P < 0.01$) by the Mann-Whitney U-test. These results are consistent with those of others using CyA to suppress the rejection of H-2-incompatible trunk skin.[19]

Discussion

On the basis of on our own[3] as well as other studies[1,2,4–6] demonstrating the prominence of T cells in the atherosclerotic lesions of human and experimental animals,[20] we hypothesized that T cells were important participants in the pathogenesis of these lesions and not merely "innocent bystanders." To test this hypothesis we attempted to inhibit atherogenesis in our mouse model by treating them with the potent T-cell suppressor CyA. The results of these studies, however, did not support our hypothesis. Although the dose of CyA used was sufficient to obtain the expected prolongation of skin allografts, this dose of CyA did not suppress the development or progression of atherosclerotic lesions.

Other investigators have also attempted to study the vascular response to injury in immunodeficient animals. The previously cited studies of Jonasson et al[9] and Ferns et al[10] on the effects of CyA on the vascular response to injury yielded conflicting results. In the studies of Jonasson et al the CyA suppressed the lesion by reducing smooth muscle cell proliferation. More recent studies by Thyberg and Hansson[21] suggest that the inhibitory effect of CyA on vascular smooth muscle cell proliferation *in vivo* is due, at least in part, to a direct effect of the CyA on these cells. In the studies of Fern et al[10] the CyA enhanced the progression of the lesion. In their experiments there was a significant elevation of total cholesterol in both the CyA-treated and control vehicle (Cremophor-EL) animal groups. Ferns et al attributed the conflicting results of the two CyA experiments to the differences in animal species used (rabbit *versus* rat), method of vascular injury (selective endothelial damage as opposed to balloon injury, which also may injure the underlying medial smooth muscle cells), and the doses of CyA (15 mg/kg *versus* 30 mg/kg). Our studies in the hyperlipidemic mouse model appear to be in agreement with those of Ferns et al. Nevertheless, we believe that there are several cogent reasons why it is not possible to evaluate the role of T cells in atherogenesis based on the results of these three CyA studies. First of all, CyA does not completely suppress T-cell immunity, as indicated by the numerous rejection crises that still occur in CyA-treated allograft recipients.[8] It is also possible that CyA could selectively effect a subset of T lymphocytes, resulting in a net enhancement of the immune-mediated processes involved in atherogenesis. Metzger and Peterson[22] have demonstrated that the T-cell-dependent[23] pulmonary granuloma response induced by *Schistosoma mansoni* eggs in mice is enhanced by CyA treatment. They suggested and presented indirect evidence that this effect was due, at least in part, to an inhibition of the generation of CD8 suppressor cell by CyA. Similarly, other investigators have shown that CyA can augment the pathologic responses in three experimental autoimmune disease models, the spontaneous autoimmune thyroiditis of obese chickens,[24] collagen arthritis in rats,[25] and organ specific autoimmunity in neonatally treated mice.[26] In the latter studies, the investigators suggested that the neonatal CsA treatment caused a selective deficiency of T suppressor cells that are normally involved in the down-regulation of resistant self-reacting lymphocytes that are relatively CyA resistant. Finally, there are multiple nonimmunological side effects of CyA that could have profound effects on lesion development and progression. These include its ability to directly inhibit vascular smooth muscle cell proliferation,[21] to increase plasma lipid levels,[27] to induce hypertension,[28] to damage endothelial cells,[29] and to alter cytokine[30–32] and endothelin production.[33] Endothelin is a peptide synthesized by endothelial cells that stimulates vascular SMC proliferation *in vitro* and arterial hypertension *in vivo*. CyA induces the synthesis of endothelin *in vitro* by vascular endothelial cells.[33]

In other relevant studies Hansson et al[34] have presented evidence in their balloon catheter and nylon filament models of vascular injury in rats that T cells inhibit the vascular response to injury. They showed that in rats depleted of T cells by monoclonal antibodies or in athymic nude (*rnu/rnu*) rats the lesions were larger than those in appropriate controls. They suggested that the lack of IFN-γ production in the T-deficient rats removed an inhibitory factor in the repair process, since treating normal rats with balloon catheter injuries with IFN-γ resulted in smaller lesions and IFN-γ inhibits the proliferation of vascular smooth muscle cells *in vitro* and *in vivo*.[21,34] Similarly, Fukuo et al[35] demonstrated an increase in atherosclerosis in the aortas of cholesterol-fed, thymectomized guinea pigs. These latter investigators also suggested a mechanism involving IFN-γ. These studies again differ from those of Ferns et al,[36] who showed no significant differences in size of balloon catheter-induced

lesions in *rnu/rnu* rats and their *rnu/+* heterozygote controls. Hansson et al[34] concluded that the studies of Ferns et al were not valid because "These studies were done in rats that were older and, therefore, probably contained circulating T cells." Indeed, the nude rat, like its murine counterpart, does posses both mature and precursor T cells that increase in number with age.[37,38] An additional consideration is the fact that although the vascular injury model is a good one for the study of lesions resulting from angioplasty, it may not be a valid one for the study of naturally occurring atherosclerosis.

The marked increases in total and LDL plus VLDL cholesterol induced by CyA in the 135- and 165-day mice are remarkable and have not been previously noted. The more modest increases in the CyA-treated 88- and 310-day mice have been observed in both experimental animals[39,40] and humans treated with CyA.[27,41] Although the causes of these alterations in plasma lipids are unknown, several mechanisms have been suggested, including: 1) a CyA-induced reduction in the synthesis and secretion of bile, which in turn decreases the excretion of cholesterol;[42,43] 2) a CyA-induced decrease in lipid breakdown due to a decrease in lipoprotein lipase activity;[44] and 3) CyA may interfere with LDL receptor function.[45] Of interest is the fact that the CyA-induced elevation in total and LDL plus VLDL cholesterol was only seen in the groups of mice fed the high-cholesterol atherogenic diet and not in the groups fed normal mouse chow. This suggests that it may be possible to control the lipid-altering effects of CyA in patients by using low-cholesterol diets.

Although most of the above evidence favors the idea that T cells are not essential participants in the pathogenesis of the atherosclerotic lesion, we believe that the final verdict is not in because of the limitations of the models tested. We are currently studying other, possibly more relevant, T-cell-deficient murine models to further evaluate our hypothesis.

Acknowledgments

The authors gratefully acknowledge the following: Robert J. Buschmann, Ph.D., and Minu K. Patel, Ph. D., for their help in data analysis; Jack A. Maggiore, M.S., and Robert Williams, Ph.D., for performing the liver chemistry studies; and Amelia M. Bartholomew, M.D., for her help in performing the skin allograft studies.

References

1. Jonasson L, Holm J, Skalli O, Bonders G, Hansson GK: Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. Arteriosclerosis 1986, 6:131–138
2. Gown AM, Tsukada T, Ross R: Human atherosclerosis II. Immunocytochemical analysis of the cellular composition of human atherosclerotic lesions. Am J Pathol 1986, 125:191–207
3. Emeson EE, Robertson AL: T lymphocytes in aortic and coronary intimas: Their potential role in atherogenesis. Am J Pathol 1988, 130:369–376
4. van der Wal AC, Das PK, van de Berg DB, van der Loos CM, Becker AE: Atherosclerotic lesions in human. *In situ* immunophenotypic analysis suggesting an immune mediated response. Lab Invest 1989, 61: 166–170
5. Munro JM, van der Walt JD, Munro CS, Chalmers JAC, Vox EL: An immunohistochemical analysis of human aortic fatty streaks. Hum Pathol 1987, 18:375–380
6. Xu Q, Oberhuber G, Gruschwitz M, Wick G: Immunology of atherosclerosis: Cellular composition and major histocompatibility complex class II antigen expression in aortic intima, fatty streaks, and atherosclerotic plaques in young and aged human specimens. Clinical Immunol Immunopathol 1990, 56:344–359
7. Hansson GK, Holm J, Jonasson L: Detection of Activated T lymphocytes in the human atherosclerotic plaque. Am J Pathol 1989, 135:169–175
8. Kahan BD: Cyclosporine. N Engl. J. Med 1989, 321: 1725–1738
9. Jonasson L, Holm J, Hansson GK: Cyclosporin A inhibits smooth muscle proliferation in the vascular response to injury. Proc Natl Acad Sci USA 1988, 85: 2303–2306
10. Ferns M, Reidy M, Ross R: Vascular effects of CyA *in vivo* and *in vitro*. Am J Pathol 1990, 137:403–413
11. Paigen B, Morrow A, Holmes PA, Mitchell D, Williams RA: Quantitative assessment of atherosclerotic lesions in mice. Atherosclerosis 1987, 68:231–240
12. Ishida BY, Paigen B: Atherosclerosis in the mouse. Genetic Factors in Atherosclerosis: Approaches and Model Systems. Edited by AJ Lusis, SR Sparks. Basel, Switzerland, Karger, 1989, pp 189–222
13. Ishida O, Ochi M, Miyamoto Y, Ikuta Y, Akiyama M: Suppression by cyclosporine of cellular and humoral reactivity after peripheral nerve allografts in mice. Transplantation 1989, 48:824–828
14. Tietz NW: Fundamentals of Clinical Chemistry. Philadelphia, W. B. Saunders, 1987, p 473
15. Lopes-Virella MF, Stone P, Ellis S, Colwell JA: Cholesterol determination in high-density lipoproteins separated by three different methods. Clin Chem 1977, 23: 882–884
16. Aherne WA, Dunnill MS: Morphometry. London, Edward Arnold Ltd, 1982, p 75
17. Gundersen HJG, Bendtsen TF, Korbo L, Marcussen N, Moller A, Nielson K, Nyengaard JR, Pakkenberg B,

Sorensen FB, Vesterby A, West MJ: Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. Acta Pathol Microbiol Immunol Scand 1988, 96:379–394
18. Norin AJ, Emeson EE: Effects of restoring lethally irradiated mice with anti-thy 1.2-treated bone marrow: Graft-vs-host, host-vs-graft, and mitogen reactivity. J Immunol 1978, 120:754–758
19. Auchincloss H, Winn HJ: Murine CD8+ T cell helper function is particularly sensitive to cyclosporine suppression *in vivo*. J Immunol 1989, 143:3940–3943
20. Hansson GK, Seifert PS, Olsson G, Bondjers G: Immunohistochemical detection of macrophages and T lymphocytes in atherosclerotic lesions of cholesterol-fed rabbits. Arterioscler Thromb 1991, 11:745–750
21. Thyberg J, Hansson GK: Cyclosporine A inhibits induction of DNA synthesis by PDGF and othe peptide mitogens in cultured rat aortic smooth muscle cells and dermal fibroblasts. Growth Factors 1991, 4:209–219
22. Metzger JM, Peterson LB: Cyclosporin A enhances the pulmonary granuloma response induced by *Schistosoma mansoni* eggs. Immunopharmacology 1988, 15:103–116
23. Damian RT: Immunity in schistosomiasis: A holistic view. Contemp Top Immunobiol 1984, 12:359–420
24. Wick G, Muller PU, Schwarz S: Effect of cyclorsporin A on spontaneous autoimmune thyroiditis of obese strain (OS) chickens. Eur J Immunol 1982, 12:877–881
25. Kaibara N, Hotokebuchi T, Takagishi K, Katsuki I: Paradoxical effects of cyclosporin A on collagen arthritis in rats. J Exp Med 1983, 158:2007–2015
26. Sakaguchi S, Sakaguchi N: Organ specific autoimmune disease induced in mice by elimination of T cell subsets. V. Neonatal administration of cyclosporin A causes autoimmune disease. J Immunol 1989, 142: 471–480
27. Ballantyne DM, Podet EJ, Patsch WP, Harati Y, Appel V, Gotto AM Jr, Young JB: Effects of cyclosporine therapy on plasma lipoprotein levels. JAMA 1989, 262: 53–56
28. Lustig S, Stern N, Golub MS, Eggena P, Barrett J, Lee DBN: Experimental cyclosporin hypertension: characterization of the rat model. Transplant Proc 1987, 21: 950–951
29. Zoja C, Furci L, Ghilardi F, Zilio P, Benigini A, Remuzz G: Cyclosporin-induced endothelial cell injury. Lab Invest 1986, 55:455–462
30. Granelli-Piperno A: Lymphokine gene expression *in vivo* is inhibited by cyclosporine A. J Exp Med 1990, 171:533–544
31. Nguyen DT, Eskandari MK, DeForge LE, Raiford CL, Strieter RM, Kunkel SL, Remick DG: Cyclosporin A modulation of tumor necrosis factor gene expression and effects *in vitro* and *in vivo*. J Immunol 1990, 144: 3822–3828
32. Reisman L, Cooper D, Lieberman KV, Martinelli GP: Cyclosporine suppresses IL-1 production by isolated human monocytes and by the human histiocytoma cell line, THP-1. Transplant Proc 1990, 22:1744–1746
33. Bunchman TE, Brookshire CA: Cyclosporine-induced synthesis of endothelin by cultured human endothelial cells. J Clin Invest 1991, 88:310–314
34. Hansson GK, Holm J, Holm S, Fotev Z, Hedrich J-J, Fingerle J: T lymphocytes inhibit the vascular response to injury. Proc Natl Acad Sci USA 1991, 88: 10530–10534
35. Fukuo Y, Nagashima M, Kobayashi Y, Terashi A: Thymus function as initiation of atherosclerosis: The effect of experimental atherosclerosis. Ann NY Acad Sci 1990, 598:572–574
36. Ferns GAA, Reidy MA, Ross R: Balloon catheter deendothelialization of the nude rat carotid. Response to injury in the absence of functional T lymphocytes. Am J Pathol 1991, 138:1045–1057
37. Schwinzer R, Hedrich HJ, Wonigeit K: The alloreactive potential of T-like cells from athymic nude rats (*rnu/rnu*). Transplant Proc 1987, 19:285–287
38. Schwinzer R, Hedrich HJ, Wonigeit K: T cell differentiation in athymic nude rats (*rnu/rnu*): Demonstration of a distorted T cell subset structure by flow cytometry analysis. Eur J Immunol 1989, 19:1841–1847
39. Evans GO: Hypomagnesaemia, hypoalbuminaemia and plasma lipid changes in rats following the oral administration of ciclosporin. Comp Biochem Physiol 1988, 89:375–376
40. Jevnikar AM, Petric R, Holub BJ, Philbrick DJ, Clark WF: Effect of cyclosporine on plasma lipids and modification with dietary fish oil. Transplantation 1988, 46: 722–725
41. Stamler JS, Vaughan DE, Rudd MA, Mudge GH, Kirshenbaum J, Young P, Alexander RW, Loscalzo J: Frequency of hypercholesterolemia after cardiac transplantation. Am J Cardiol 1988, 62:1268–1272
42. Princen HMG, Meijer P, Hofstee B, Havekes LM, Kuipers F, Vonk RJ: Effects of cyclosporine A on LDL receptor activity and bile acid synthesis in hepatocyte monolayer cultures and *in vivo* in the rat (abstract). Hepatology 1987, 7:1109
43. Rotolo FS, Branum GD, Bowers BA, Myers WC: Effects of cyclosporine on bile secretion in rats. Am J Surg 1986, 151:35–40
44. Derfler K, Hayde M Balcke P, Widhalm K: Pathological decreases in lipoprotein lipase activity in renal transplant recipients immunosuppressed with cyclosporin. Dial Transplant 1988, 3:569–570
45. De Groen PC: Cyclosporine, low-density lipoprotein, and cholesterol. Mayo Clin Proc 1988, 63:1012–1021