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Short Communication

Evidence for a Local Immune Response in Atherosclerosis

$CD4^+$ T Cells Infiltrate Lesions of Apolipoprotein-E-Deficient Mice

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It has been suggested that immune responses are involved in the development of atherosclerosis. We have evaluated this possibility by analyzing immunocompetent cells in a murine model of the disease. Apolipoprotein E knockout (apoE −/−) mice are genetically hypercholesterolemic due to targeted disruption of the apolipoprotein E gene and develop severe atherosclerosis. Such mice were fed either standard pellets or a diet containing 1.25% cholesterol. Lesions were analyzed from mice at 9 and 16 weeks of age. Immunohistochemical staining of fatty streaks showed that $CD4^+$ T cells were frequent, both in clusters and as single cells. In advanced atherosclerotic plaques, $CD4^+$ T cells were prominent in the fibrous cap and subendothelially, whereas $CD8^+$ T cells were sparse. The CD25 subunit of the interleukin-2 receptor, which is a marker for activated T cells, was expressed in CD4-rich areas and the major histocompatibility complex class II antigen, I-A^b, which is induced by cytokines released from activated T cells, was also found in the lesions. These data indicate that $CD4^+$ T cells participate in the formation of atherosclerotic lesions in genetically hypercholesterolemic apoE −/− mice. They suggest that immune activation is part of the disease process, and we speculate that a direct link may exist between cholesterol accumulation and T cell activation, possibly by autoimmune responses to modified lipoproteins. (Am J Pathol 1996, 149:359–366)

Atherosclerosis is a complex disease, the etiology of which involves both genetic and environmental factors. Histopathological analysis of human atherosclerosis suggests that lesion formation represents an inflammatory-proliferative response to lipid metabolic disturbances in regions of the vasculature exposed to hemodynamic strain.[1–3] Human atherosclerotic plaques contain significant amounts of T lymphocytes, many of which are in an activated state.[4–9] This suggests that an immune response may be involved in the pathogenesis of atherosclerosis.[3,10–12] This notion is also supported by the expression of immune cytokines[7,13–15] and of cytokine-induced genes such as HLA-DR in human plaques.[16] Our recent observation that plaque T cells of human plaques respond immunospecifically to oxidized low density lipoprotein[17] suggests that autoimmunity may play a role in the development of the atherosclerotic plaque. This is also supported by the finding of autoimmune T cells responding to heat shock protein 60 in plaques of hypercholesterolemic rabbits.[18]

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Table 1. *Monoclonal Antibodies Used in This Study*

Antigen	Cell type	Hybridoma	Antibody type	Supplier
CD4	MHC-II-restricted T cells	H129.19 (L3T4)	Rat IgG	PharMingen (San Diego, CA)
CD8a	MHC-I-restricted T cells	53–6.7 (Ly-2)	Rat IgG	PharMingen
BM8	Macrophages	BM8	Rat IgG	Boehringer Mannheim (Mannheim, Germany)
F4/80	Macrophages	MCA497	Rat IgG	Serotec (Oxford, UK)
I-A^b	MHC-II-expressing cells	KH74	Biotin-mouse IgG2a κ	PharMingen
CD25	Activated T (IL-2R)	3C7	Rat IgG2b κ	PharMingen

IL-2R, interleukin-2 receptor.

Immune responses are under genetic control and might explain some of the genetic influence in atherosclerosis. In addition, immune-derived cytokines are important regulators of metabolic events and repair processes in a variety of physiological and pathological conditions. It will therefore be important to determine whether an autoimmune response in the atherosclerotic plaque activates regulatory loops that either enhance or counteract the disease process. Recent studies of the response to mechanical arterial injury in rats and the development of fatty lesions in rabbits and mice show that suppression or elimination of immunocompetent cells aggravate lesion formation.[19–22] Data in the literature are, however, conflicting as others have observed aggravating effects of $CD4^+$ cells after mechanical injury[23] and ameliorating effects of immunosuppressive drugs on cholesterol-induced atherosclerosis.[24] This underlines the need for genetically homogeneous animal models with relatively standardized lesion development.

We have analyzed a transgenic model for rapid atherosclerosis development, the apolipoprotein E knockout (apoE −/−) mouse.[25–27] Our immunohistochemical analysis of aortic lesions of these mice revealed a prominent involvement of T lymphocytes, particularly of the CD4 subtype, and suggests that immune activation occurs in atherosclerotic lesions caused by genetic hypercholesterolemia.

Materials and Methods

Animals

ApoE −/− mice[25] bred into a C57BL/6J background (strain C57BL/6J-Apoe^{tm1Unc129}) were obtained from Jackson Laboratory (Bar Harbor, ME). Six-week-old male mice were fed either a high-cholesterol diet with 1.25% cholesterol or standard mouse pellets (ND; see below). Groups of four animals were sacrificed under carbon dioxide anesthesia after 3, 5, or 10 weeks on these diets.

Diets

The high-cholesterol diet was prepared as pellets by AB AnalyCen (Lidköping, Sweden). It contained 25.7% protein and 8.75% fats and was based on corn starch, casein, glucose, saccharose, cocoa butter, cellulose, minerals, cholesterol, and a vitamin mix. The cholesterol content was 1.25% and the energy content 14.3 MJ/kg. The normal diet (Brood Stock Feed R3) was purchased from B&K (Sollentuna, Sweden). It contained 5.0% fats, its cholesterol content was <0.05%, and the energy content 13.0 MJ/kg.

Immunohistochemistry

The heart and thoracic aorta were dissected out and snap-frozen in *n*-heptane chilled with liquid nitrogen. Cryostat sections were cut from the level of the aortic valves and distally. They were collected on poly-D-lysine-coated glass slides, fixed for 5 minutes in ice-cold acetone, dried, and preincubated in 2% dry milk in Tris-buffered saline (140 mmol/L NaCl, 15 mmol/L Tris-HCl, pH 7.2). Serial sections were incubated for 60 minutes at room temperature or overnight at 4°C with the battery of monoclonal antibodies shown in Table 1. All antibodies were used at optimal dilutions determined by staining of spleen sections, and all dilutions were made in Tris-buffered saline/milk. After rinsing, sections treated with unconjugated monoclonals were incubated with biotinylated $F(ab')_2$ fragments of goat anti-rat IgG (Vector Laboratories, Burlingame, CA), rinsed, and incubated in 0.3% H_2O_2 in 30% methanol followed by additional rinses and an avidin DH/biotinylated peroxidase complex (Vector). When biotinylated monoclonals were used, the sections were treated with H_2O_2 followed by the avidin-biotin complex after in-

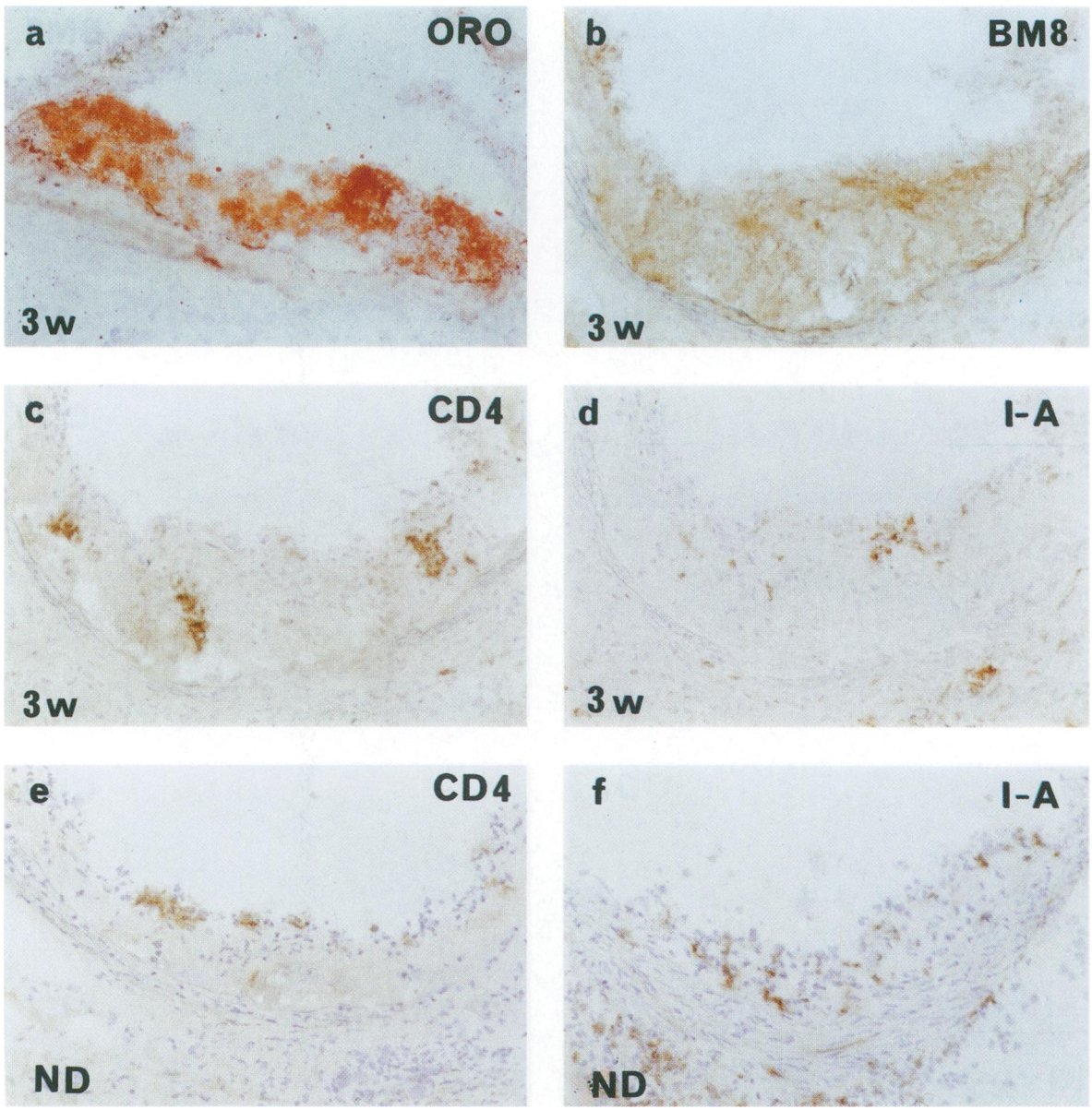

Figure 1. *Immunohistochemical analysis of fatty streaks in apoE −/− mice.* **a** *to* **d:** *Nine-week-old mice fed a high-cholesterol diet for 3 weeks (3w).* **e** *to* **f:** *Sixteen-week-old mice fed a normal diet (ND). Antibody binding was visualized by the avidin-biotin-peroxidase detection system. Magnification, ×100.* **a:** *Oil Red O staining shows lipid accumulation.* **b:** *BM8 staining for macrophages, which are abundant throughout the lesion.* **c:** *CD4 staining for T lymphocytes. Notice clustering of $CD4^+$ cells in the lesions.* **d:** *$I\text{-}A^b$ staining visualizes MHC class II expression, which appears clustered in the fatty streak and also can be detected in some of the cells in the media and adventitia.* **e:** *CD4 staining for T lymphocytes in fatty streak of mouse fed standard pellets. $CD4^+$ cells are frequent in the subendothelial part of the lesion.* **f:** *$I\text{-}A^b$ staining of the same fatty streak shown in* **e**. *$I\text{-}A^+$ cells are found both in the intima, media, and adventitia.*

cubation with biotinylated monoclonal. Staining was visualized with a diaminobenzidine/H_2O_2 substrate solution, and sections were counterstained with hematoxylin. Parallel sections were stained with Oil Red O to visualize lipid deposits. As controls, sections were stained without exposure to any primary antibody. In addition, the different staining patterns using the different antibodies confirmed the specificities of the reagents employed in this study.

Results

Composition of Lesions in ApoE −/− Mice

Lesions developed rapidly in apoE −/− mice fed standard pellets, and their formation was dramatically enhanced by cholesterol feeding. As reported,[27] they started at the root of the aorta and progressed distally. Figures 1a and 2a show representative, Oil-Red-O-

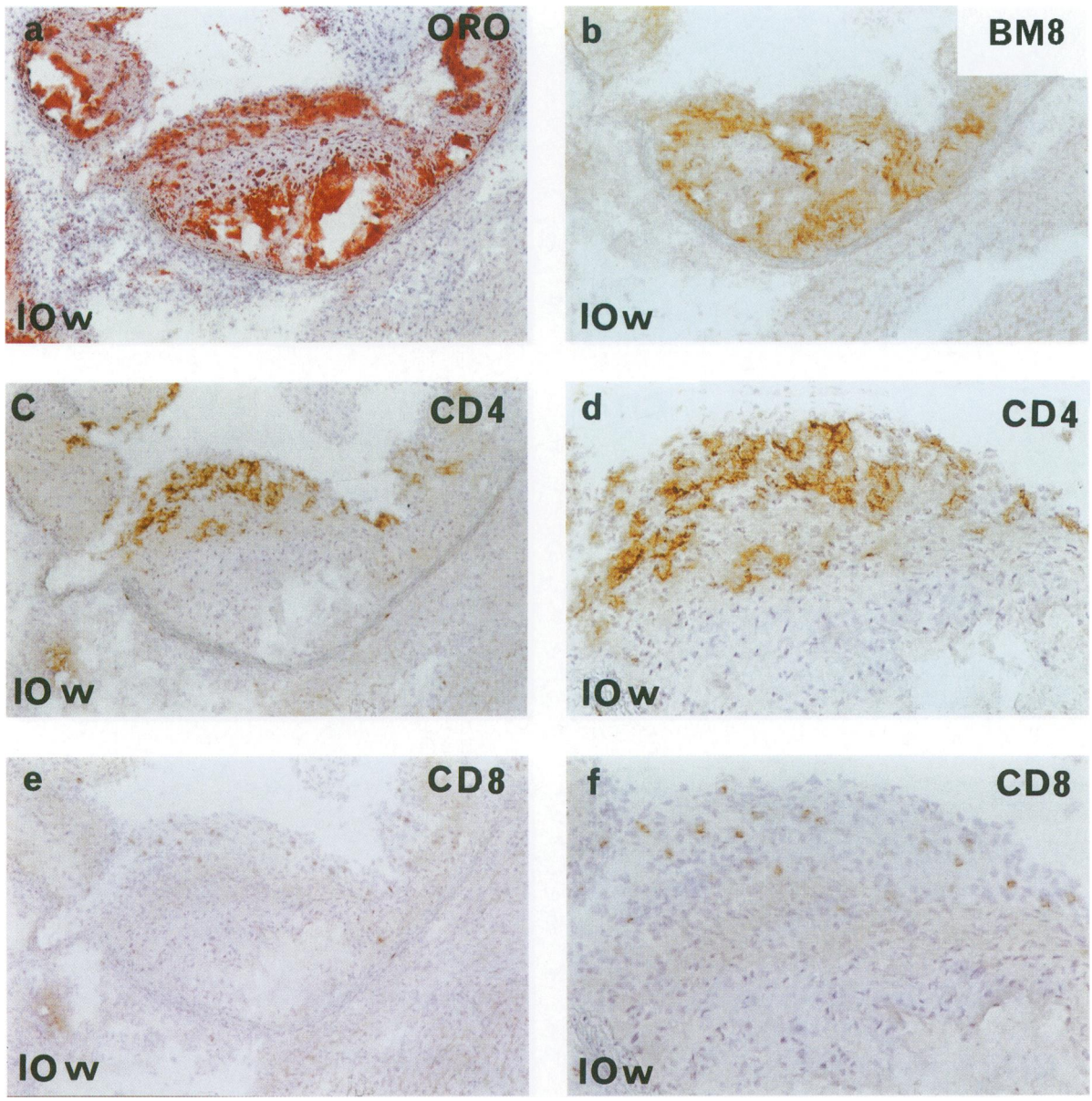

Figure 2. *Immunohistochemical analysis of atherosclerotic plaques in apoE −/− mice. Mice were analyzed at 16 weeks of age after having been fed a high-cholesterol diet for 10 weeks. Staining was performed as described in the legend to Figure 1.* a: *Oil Red O staining for lipids. Notice that this plaque has a lipid-rich, necrotic core, a fibrous cap, and a subendothelial zone infiltrated with lipids. Magnification, ×100.* b: *BM8 staining for macrophages, which are frequent throughout the plaque. ×100.* c: *$CD4^+$ T cells occur predominantly in the cap region. ×100.* d: *At higher magnification, clusters of $CD4^+$ cells can be seen in the subendothelium. ×200.* e: *$CD8^+$ T cells are much less frequent than $CD4^+$ cells but appear with the same distribution. ×100.* f: *At high magnification, $CD8^+$ T cells are found as single cells throughout the cap and subendothelial zone. ×200.*

stained lesions from the aortic root of mice fed 1.25% cholesterol for 3 (Figure 1a) or 10 (Figure 2a) weeks.

Lesions started as fatty streaks filled with foam cells that stained with the macrophage markers BM8 and F4/80 (Figure 1b). Fatty streaks were found in all mice fed a high-cholesterol diet for 3 weeks (age of animals, 9 weeks) as well as in 16-week-old mice fed standard pellets.

With lesion progression, an amorphous, necrotic core region developed and became surrounded by a cap of spindle-shaped cells that were negative for macrophage markers. $BM8^+$ cells were now found at the edge of the necrotic core and also subendothelially (Figure 2b). With the appearance of distinctive core and cap regions, lesions were considered to represent atherosclerotic plaques. Such plaques were found in all mice fed the high-cholesterol diet for 10 weeks (age of animals, 16 weeks). Less advanced lesions were found in several of the mice fed standard pellets at 16 weeks of age. BM8 and F4/80

Table 2. *Immunological Characteristics of Lesions in ApoE −/− Mice*

Diet	Age/time on diet (weeks)	CD4:CD8 ratio	BM8:CD4 ratio	I-A:total*	Lesion type
Normal	9/9	ND	ND	0.06	Minute fatty streaks
Normal	16/16	5.3	2.4	0.08	Large fatty streaks
HC	9/3	4.0	1.3	0.09	Large fatty streaks
HC	16/10	4.9	1.4	0.17	Atherosclerotic plaques

Data were obtained by counting lesions at the aortic root from three mice of each group at ×200 magnification. ND, not done; HC, hypercholesterolemic diet with 1.25% cholesterol and 15% total fat.
* Values represent I-A-positive cells divided by all hematoxylin-stained cells inside the internal elastic lamina.

stained the same cells and regions of lesions, with BM8 providing the strongest staining intensity (data not shown).

Immunological Characterization of Lesions

Lesions were stained with antibodies to CD4 and CD8 to reveal T cell infiltrates. As shown in Figure 1c, Figure 2, c and d, and Table 2, lesions at all stages of development contained large numbers of $CD4^+$ T cells. In fatty streaks, these cells were interspersed between foam cells and often aggregated in clusters suggestive of clonal proliferation (Figure 1c). $CD4^+$ T cells were observed in fatty streaks of mice fed standard pellets as well as in mice fed the cholesterol-rich diet (Figure 1e). In atherosclerotic plaques, $CD4^+$ cells were very frequent and characteristically located subendothelially and above the lipid core (Figure 2, c–d). A comparison of CD4- and BM8-stained sections indicated that the staining technique did not detect any low-level CD4 expression by macrophages (Figures 1 and 2).

$CD8^+$ T cells were less frequent than $CD4^+$ cells, present in lesions of all stages and interspersed in the tissue without cluster formation (Figure 2, e–f; Table 2).

Immune activation was assessed by analyzing the CD25 subunit of the interleukin-2 receptor and the major histocompatibility (MHC) class II gene product, $I\text{-}A^b$. CD25, which is expressed by activated T cells, was detected with the same general distribution as CD4 (Figure 3, a and b) but only a minority of $CD4^+$ T cells expressed CD25. This is in line with previous findings in man.[7] $I\text{-}A^b$ is the MHC class II haplotype carried by the C57BL/6J strain. It is expressed by activated macrophages and by parenchymal cells responding to the T cell cytokine interferon-γ.[28] $I\text{-}A^b$ was found in many cells of fatty streaks and plaques (Figures 1, d and f, and 3c and Table 2). Although it was expressed in areas rich in macrophages, it stained only a small proportion of the cells (compare Figure 1, b and d), suggesting that only a subpopulation of the macrophages expressed this MHC antigen. A comparison of staining patterns for $I\text{-}A^b$ and CD4 suggested that the former was induced by some of the CD4 clusters (Figure 1, c and d). Strong $I\text{-}A^b$ expression was detected in the subendothelial region where $CD4^+$ T cells were juxtaposed with $BM8^+$ macrophages (Figure 3c). Together, the findings of $I\text{-}A^+$ cells and $CD25^+$ T cells suggest that immune activation was taking place in these lesions.

Discussion

This report shows that immunocompetent cells infiltrate fatty streaks and atherosclerotic plaques of apoE −/− mice. The pattern of localization of $CD4^+$ T cells and the expression of MHC class II antigens in the lesions strongly suggest that the infiltration of T cells and macrophages is accompanied by immune activation. These observations provide a direct link between (genetically determined) hypercholesterolemia and local immune responses in the atherosclerotic plaque.

$CD4^+$ cells were the dominating T cell type in all phases of lesion development. In fatty streaks, they were frequently found in clusters suggestive of clonal proliferation. Although not identical to $CD4^+$ cells, I-A-expressing cells were distributed in a similar pattern. One could thus envisage a situation in which fatty streak macrophages present local antigens to infiltrating $CD4^+$ T cells, which may respond by secretion of interferon-γ, which in turn induces I-A expression in surrounding cells. In support of this, interferon-γ has been detected in human atherosclerotic plaques, both by immunofluorescence and by polymerase chain reaction.[7,15]

Interferon-γ secretion could modulate the progression of the fatty streak in several ways. As a major macrophage-activating cytokine, it would not only up-regulate MHC gene expression but also increase the secretion of proteases, cytokines, and coagulation factors by the macrophage.[29] Interferon-γ also exerts direct effects on the lipid metabo-

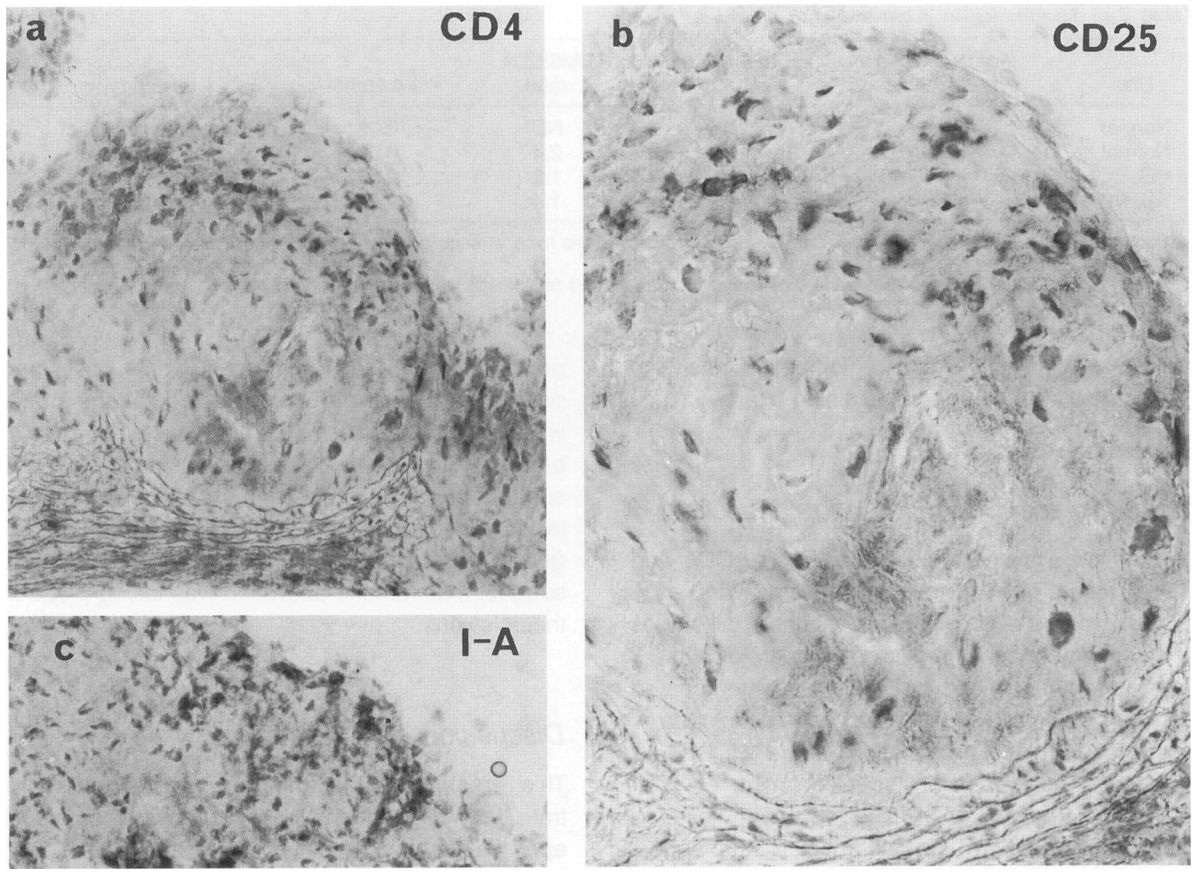

Figure 3. *Immunohistochemical analysis of immune activation markers in atherosclerotic plaques. ApoE −/− mice were analyzed at 16 weeks of age after having been fed a high-cholesterol diet for 10 weeks. Immunohistochemistry was carried out as described in the legend to Figure 1.* a: *$CD4^+$ T lymphocytes in an atherosclerotic plaque. Magnification, ×,200.* b: *CD25 staining shows expression of interleukin-2 receptors by activated T cells in the plaque. ×400.* c: *$I\text{-}A^b$ expression in the subendothelial zone and cap, where activated T cells are frequent. ×200.*

lism of the macrophage. It both reduces its capacity to oxidize lipoproteins[30] and down-regulates its expression of scavenger receptors.[31] These effects are likely to reduce foam cell formation and the intracellular accumulation of cholesterol in fatty streaks. Finally, interferon-γ inhibits smooth muscle proliferation,[19,32,33] which could reduce the formation of a fibrous cap and thus the transition from fatty streak to atherosclerotic plaque.

Although interferon-γ is likely to be a major secretory product of activated T cells, several other cytokines could be produced in a cellular immune reaction. The Th1 subtype of CD4 cells, and many CD8 cells, secrete interferon-γ, tumor necrosis factor-β, and interleukin-2, whereas the Th2 subtype mainly produces interleukin-4, -5, -6, -10, and -13 upon activation.[34] Th2 cytokines are likely to exert effects on atherosclerosis progression that are entirely different from those of Th1 products. For instance, interleukin-10 is known to inhibit the secretion of cytokines, including interferon-γ, by Th1 cells.[34] This could indirectly promote foam cell formation and smooth muscle proliferation. By activating 15-lipoxygenase, interleukin-4 could increase oxidation of lipoproteins present in the lesion,[35] and by stimulating B cells, it may increase the production of antibodies to oxidized low density lipoprotein. Our analysis of human plaque T cell clones suggests that the Th1 phenotype dominates in atherosclerotic plaques.[17] The availability of a murine model will permit longitudinal studies of the role of T cell subtypes in atherosclerosis.

The growth of atherosclerotic plaques is determined not only by cell proliferation and tissue accumulation but also by the cell death rate. Apoptotic cell death occurs frequently in human plaques.[36,37] T cell subtypes could be involved in this process as they can kill neighbor cells in an MHC-restricted manner employing both cytolytic and apoptotic mechanisms.[38] It will therefore be interesting to determine whether T-cell-dependent cytolysis and/or apoptosis occurs in these plaques.

To summarize, our immunohistochemical analysis of apoE −/− mice has demonstrated that a local

immune response dominated by $CD4^+$ T cells occurs in all phases of atherosclerosis. The presence of such a response in this model, in which the disease is induced by genetic hyperlipoproteinemia in the absence of other agents, suggests that a direct link may exist between cholesterol accumulation and local immune reaction in the arterial intima. The apoE −/− model should be suitable for dissection and manipulation of the immune response associated with atherosclerosis.

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