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T Lymphocytes in Aortic and Coronary Intimas

Their Potential Role in Atherogenesis

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In order to investigate the role of mononuclear cells in infiltrates during the initial stages of atherogenesis, the authors have studied by immunohistochemical methods the aortas and coronary vessels of children and young adults (ages 15–34) dying of acute trauma. Eccentric intimal thickening often accompanied by intimal mononuclear cell infiltration was commonly observed in sections of the lower thoracic aorta. These changes were usually related to intercostal branching sites and thus greater in the dorsal (posterior) than on the ventral aspect of the aorta in 64 of 75 cases examined. In some of these samples the authors were able to demonstrate the presence of T lymphocytes and monocyte-macrophages (mono/macs) by the use of the monoclonal antibodies T11 and Leu-M5, respectively. Many of the T lymphocytes were T8-positive and thus of the cytotoxic/suppressor subtype. T4-positive cells of the inducer/helper subtype were seen occasionally. T cells of both T4 and T8 subsets and mono/macs were also demonstrated in areas of eccentric intimal thickening in coronary arteries and in raised coronary lesions. In both the aortas and the coronary lesions the T cells and mono/macs were often closely associated with one another. This finding is of interest in view of the well-known cell-regulatory and cytotoxic potential of these cells. Extrapolating from findings in nonhuman primates, the authors suggest a potential role for mononuclear cells in human atherogenesis. (Am J Pathol 1988, 130:369–376)

MONONUCLEAR CELLS, including both monocytes and lymphocytes, have been shown to be among the first participants in early experimental and spontaneous atherogenesis. In the experimental animal these cells have been demonstrated to adhere to the vessel wall as early as 7 days and to penetrate and lie within the intima as early as 1 month after the induction of diet-induced hypercholesterolemia.[1–8] As far back as 1958, Duff et al[9] were able to demonstrate subintimal mononuclear cells in the aortas of hypercholesterolemic rabbits. More recently, Joris et al[10] provided evidence in abstract form that there are subendothelial monocytes and lymphocytes in human coronary arteries with diffuse intimal thickening or calcified plaques. Additional evidence that points to the monocyte-macrophage (mono/mac) as a principal participant in atherogenesis includes its role as a precursor of the foam cell,[4,11–14] its role in arterial cell lipid metabolism,[15,16] and, more recently, its role in secreting factors that stimulate the growth of smooth muscle cells.[17–23]

Although the presence of lymphocytes have long been recognized in the adventitia of advanced atheromatous lesions,[24] very little has been written about the presence and role of lymphocytes in the intimas of early human lesions. The presence of T cells could be important because of their potential ability to secrete factors that are chemotactic for mono/macs[25,26] and to regulate B cells and mono/macs and their ability to regulate lipoprotein uptake by macrophages.[16] At the same time, B cells or plasma cells might secrete antibodies resulting in immune complex formation and activation of complement. The potential role of immune complexes and activated complement in inducing acute inflammation is well known.[27] Complement components have also been shown to have regulatory effects on lymphocytes and macrophages.[28]

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To investigate further the possible role of mononuclear cells in atherogenesis, we have studied by immunohistochemical methods the aortas and coronary vessels of children and young adults (ages 15–34) dying of acute trauma.

Materials and Methods

Specimen Collection

Aortic segments were obtained from the Office of the Chief Medical Examiner of Cook County, Illinois, courtesy of Dr. R. Stein and staff. Donors were of both sexes, and limited to trauma victims, ranging in age from 15 to 34 years. Cases with major systemic diseases such as diabetes, hypertension, collagen-vascular diseases and cancer were excluded. Two adjacent aortic sections were taken transversely through the eleventh intercostal ostia in a manner enabling us to distinguish the dorsal and ventral aspects of the aortic wall. One was snap-frozen in isopentane cooled in liquid nitrogen and stored at −80 C until further studies. The second section was fixed in 10% Ringer's formalin, embedded in paraffin, and sectioned at 6 μ for hematoxylin and eosin (H&E), Masson trichrome, and van Gieson staining. The coronary sections included the proximal portions of the left anterior descending, left circumflex, and right coronary arteries. These samples were also snap-frozen and stored at −80 C. The time interval between death and processing of tissues varied from 7 to 32 hours.

Inmunohistochemical Techniques

Frozen specimens were embedded in O.C.T. (Miles Laboratories Inc., Lab-Tek Div., Naperville, Ill), and sections were cut at 6 μ, placed on glass slides coated with chrome alum-gelatin adhesive, and air-dried at room temperature for two hours. Sections were fixed in acetone (reagent grade) for 10 minutes at room temperature and washed for 1 minute in phosphate-buffered saline, pH 7.3 (K_2HPO_4, 7.52 g; NaH_2, PO_4, 1.32 g; and NaCl, 7.20 g/l) with 1% bovine serum albumin (Fraction V, Sigma Chemical Co., St. Louis, Mo) (PBS–BSA). The sections were stained with murine-derived monoclonal antibodies and a Vectastain ABC Kit (mouse IgG) (Vector Laboratories Inc., Burlingame, Calif). They were incubated at room temperature with 1) blocking solution consisting of normal horse serum diluted 1 : 20 in PBS-BSA (Vectastain ABC Kit) for 15 minutes and 2) monoclonal antibodies for 30 minutes; 3) washed with PBS-BSA, 1 minute; 4) incubated with biotinylated horse anti-mouse IgG or IgM, diluted 1 : 50 in PBS (Vectastain ABC Kit), for 30 minutes; 5) washed in PBS-BSA for 1 minute; 6) incubated with avidin–biotin complex (ABC) (Vectastain ABC Kit) prepared according to the manufacturer's directions for 30 minutes; 7) washed with PBS-BSA for 1 minute; 8) incubated with peroxidase substrate solution consisting of 0.05% diamino-benzidine tetrahydrochloride (DAB), 0.01% H_2O_2 in PBS for 5 minutes; and 9) rinsed in distilled H_2O, 5 minutes. Slides were then counterstained with Harris' hematoxylin, dehydrated, and mounted in Permount (Fisher Scientific, Fairlawn, NJ).

Sections were examined by conventional light microscopy using low-power magnification (×40) for locating areas of intimal thickening and mononuclear infiltration. Intimal thickening and mononuclear infiltration of the posterolateral aspects of the aorta (both lateral thirds) were compared with the ventral aspect (middle third). High-power magnification (×400) was then used for identifying and enumerating positively stained cells. In this regard, it is important to point out that it was not possible to perform any type of quantitative analysis of the T cells or their subsets because of the extreme variability of surface membrane antigen preservation. Because our study made use of postmortem tissue, it was not possible to avoid postmortem deterioration of tissue antigens. Moreover, it was not even possible to correlate the degree of postmortem changes with the interval between death and the processing of tissues. Positive cells in their most characteristic forms were identified by the presence of a distinct rim of brown granularity. In many of the specimens (approximately 50%), the brownish staining was somewhat less distinct and diffuse, but definitely associated with specific cells. In a few sections, small clusters of cells were diffusely stained, indicating that at least a few of the cells within the cluster possessed the specific antigen. We believe that the somewhat less distinct staining of cells seen in some of our sections is real and probably due to postmortem diffusion of surface membrane antigen.

Monoclonal Antibodies

The monoclonal antibodies used and their isotypes, specificities, and sources are summarized in Table 1. All antibodies except Leu-3a + 3b and Leu-M5 were used diluted 1 : 10 in PBS-BSA. The Leu-3a + 3b and Leu-M5 were used undiluted. The horse anti-mouse IgG was purchased from Vector and used diluted 1 : 50 in PBS-BSA. The above dilutions were found by checkerboard analysis to be optimal for the staining of postmortem aortic tissues obtained in this study. These antibody concentrations are somewhat higher than those generally used for fresh tissues.

Table 1—Specificity of Monoclonal Antibodies Used

Monoclonal antibody	Ig Chain composition	CD cluster	Specificity
1. T11*	IgG1,K	CD2	E rosette receptor-associated (Pan T)
2. T3	IgG1,K	CD3	T cell
3. T4	IgG1,K	CD4	T helper/inducer
4. T6	IgG1,K	CD1	Common thymocyte, dendritic cell
5. T8	IgG1,K	CD8	T suppressor/cytotoxic
6. B1	IgG2a,K	CD20	B cell
7. Mo2	IgM,K	CDw14	Monocyte/macrophage
8. Leu3a,3b†	IgG1,K	CD4	T helper/inducer
9. Leu-9	IgG2a,K	CD7	T cell
10. Leu-14	IgG2b,K	CD22	B cell
11. Leu-11b	IgM,K	CD16	Natural killer/killer cell (Large granular lymphocyte)
12. Leu-M5	IgG2b,K	—	Monocyte/macrophage
13. HLA-DR	IgG2a,K	—	B cells, monocyte/macrophage, activated T cell
14. IL-2 receptor	IgG1,K	CD25	Interleukin-2 receptor
15. Desmin‡	IgG	—	Muscle cells (SMC in vascular wall)

*Antibodies 1–7, Coulter Immunology, Hialeah, Florida.
†Antibodies 8–14, Becton Dickinson Monoclonal Center, Mountain View, California.
‡Lab Systems, Morton Grove, Illinois.

Results

Aortic samples from 75 cases were studied. All displayed a variable degree of eccentric intimal thickening. Most of the thickened areas were infiltrated by mononuclear cells. In some, there were but a few mononuclear cells; in others the infiltrate was extensive (Figure 1A). Mononuclear infiltration and/or intimal thickening was greater in the dorsal aspects of the aorta than in the ventral aspect in 64 of 75 cases. Of the 11 remaining cases the dorsal and ventral alterations did not differ, whereas in 2 cases the changes were more extensive in the ventral portions. This is of potential significance because of the known predilection of the dorsal region for the development of spontaneous atheromas.[29] In adjacent frozen sections we were able to demonstrate that many of the small mononuclear cells were reactive with T11 monoclonal antibodies. Some of these cells displayed a very distinct rim of brown granularity (Figure 1B); other T cells were surrounded by a narrow (1–5-μ) margin of diffuse staining. Many of these same or similar cells in adjacent sections stained similarly with T8 antibody (Figure 1C). Evidence that the positive cells are T cells includes the fact that similar or adjacent cells were not stained in control slides in which PBS-BSA or irrelevant third-party monoclonal antibody of identical isotype (IgG1) was substituted for the primary antibody. The negative results with the latter antibody indicate that the T11 and T8 antibodies were not reactive with the cells by means of their Fc receptors. Only a few cells were reactive with T4 antibody. Although these findings suggest that cytoxic/suppressor cells predominate in these areas, we cannot be certain that our inability to stain more T-helper/inducer cells is not an artifact due to postmortem alterations of surface membrane antigens.[30]

In many cases we could also demonstrate scattered larger mononuclear cells and nests of foam cells reactive with Leu-M5 and HLA-DR antibodies, indicating that both are of mono/mac origin. Both surface membrane and cytoplasmic staining was observed with both of these antibodies (Figure 1D and E). These cells also generally displayed cytoplasmic staining of somewhat lesser degree with T4 or Leu-3a + 3b antibodies, a finding consistent with those of Wood et al.[31]

There was no consistent anatomic relationship between the location of lymphocytes and mono/macs in these studies. In some areas the lymphocytes and mono/macs were closely intermixed (Figure 1F), whereas in others such associations could not be demonstrated. Other monoclonal antibodies tested, including T3, Mo2, B1, Leu-9, Leu-14, and IL-2, were nonreactive in all sections studied.

We have also studied the coronary arteries in 75 cases. All displayed a variable degree of diffuse or eccentric intimal thickening. In 10 of the arteries, small nonocclusive raised lesions were examined. Scattered T cells and mono/macs were occasionally observed individually and in clusters within the thickened intimas of coronary arteries exhibiting eccentric intimal thickening. These cells were only rarely seen in the thickened intimas of coronary arteries with diffuse intimal thickening. In the raised lesions, the T cells and mono/macs were more plenti-

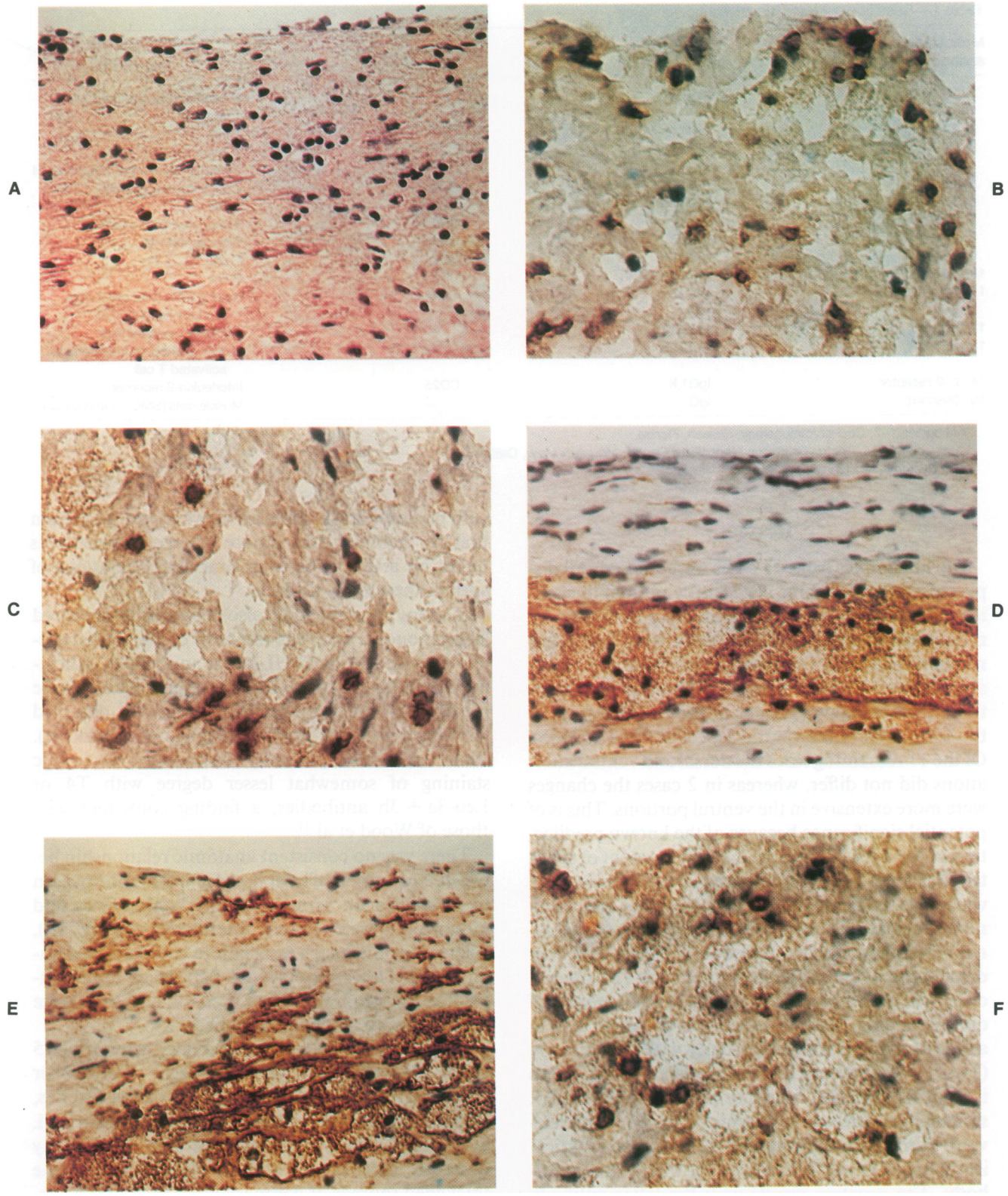

Figure 1—Section of aortic intima with intimal thickening and mononuclear infiltration. **A**—H&E, ×535. **B**—ABC immunoperoxidase stain of thickened aortic intima showing scattered T11 positive T cells. (×775) **C**—Same as **B**, showing T8-positive cells. (×775) **D**—Thickened aortic intima showing elongated nest of Leu-M5-positive foam cells. (ABC immunoperoxidase, ×535) **E**—Same as **D**, showing nests of foam cells also positive for HLA-DR antigen. Note that many of the cells above the foam cells also react with the HLA-DR antibody. (×535) **F**—Same as **B** and **C**, showing T11-positive cells intermixed with poorly preserved, slightly stained foam cells. (×775)

ful and often observed in clusters in close association with one another. T cells were seen around the entire circumference of the lesion, including the fibrous cap and base, but predominated along the lateral edges (Figure 2A and B). Both cytotoxic/suppressor (T8) (Figure 2C) and helper/inducer (T4) (Figure 2D) T cells could be identified within the T-cell population. Mono/macs were also most abundant along the lateral edges of the fibrous core intimately associated with T cells (Figure 2E). Most of the mono/macs in these areas were foams cells, as evidenced by their plump configuration and foamy cytoplasm, which stained positively with oil red O. Mono/macs were only rarely seen in the fibrous cap. The mono/macs in these coronary artery lesions were also strongly positive for HLA-DR antigen (Figure 2F).

Discussion

The most important observation made in this study is that T cells may be present in the subendothelial intimal space of human arteries. They were found in areas of the thoracic aorta with an increased predilection for developing atherosclerosis, in coronary arteries with eccentric intimal thickening, and in coronary artery raised lesions. These studies provide no evidence, however, that the observed T cells are activated and contribute to lesion progression. We are currently investigating the question of activation, by determining whether the T cells coexpress activation antigens. The latter question is a far more complex one and might be best approached by the use of experimental animal models. Our findings are compatible with and extend those of Jonasson et al,[32] who demonstrated T cells, macrophages, and smooth muscle cells (SMCs) in the atherosclerotic plaques of surgically removed internal carotid arteries.

A second important observation is the frequent association of these T cells with mono/macs. Mono/macs have been identified in atherosclerotic lesions by many investigators.[4,11-14] Their involvement in atherogenesis has been established in several animal models in which experimental atherosclerotic lesions were induced by hypercholesterolemic diets.[1-8] In these studies monocytes were seen to adhere to and infiltrate the aortic vessel wall shortly after the induction of hypercholesterolemia. They could then demonstrate the transition of such lesions to the fatty streak and ultimately the fibrous plaque. The factors responsible for mono/mac recruitment are still unknown and remain one of the intriguing problems of atherosclerosis research. Proposed mechanisms include alterations in the surface of endothelial cells and/or macrophages (including surface charge, receptor, or antigen expression),[6-8] chemotactic factors,[33-37] endothelial associated IgG,[38] and endothelial cell-modified low-density lipoprotein.[39] The close association of T cells with mono/macs in the intima is not surprising in view of the well-known regulatory interactions between these two cell types. Consider, for example, a possible role for the T lymphocyte in regulating the attachment and penetration stages of the monocytes. Activated T lymphocytes can elaborate a monocyte chemotactic factor.[25,26] Of interest in this regard are the studies of Hunninghake et al[40] suggesting that the accumulation of monocytes in the lungs of patients with sarcoidosis is mediated by the local production of monocyte chemotactic factor by activated lung T lymphocytes. Similarly, it is conceivable that T lymphocytes within the subendothelial intimal space play a significant role in establishing and maintaining the presence of mono/macs in atherosclerotic lesions. Also of interest are the recent studies of Pober et al[41] suggesting that lymphotoxin, a secretory product of activated T cells, may activate local endothelial cells. These endothelial alterations could then, in turn, amplify an intimal cell-mediated immune response by enhancing the local adhesion and subendothelial migration of circulating mononuclear cells.

Other regulatory effects of activated T cells may be relevant to atherogenesis. Lymphokines secreted by activated T cells can activate macrophages and regulate their lipoprotein uptake.[16] Activated macrophages, in turn, can secrete a wide variety of potentially atherogenic agents, including mono/mac-derived growth factors,[17-23] lipoprotein lipase,[42] cytotoxic substance,[43,44] plasminogen activator,[45] and substances that regulate low-density lipoprotein in metabolism.[45]

How the T cells get into the vessel wall and elevated atherosclerotic lesions is not presently known. Possible mechanisms include those of selective recruitment of lymphocytes with specific reactivity to vessel wall components (autoimmune mechanisms) and chemotaxis induced by macrophage secreted interleukin-1.[47] Viral infection of the vessel wall represents another potential mechanism for attracting T lymphocytes. Possible mechanisms involved in viral-induced T-cell recruitment have been recently reviewed.[48] Of interest in this regard are recent studies demonstraing herpes simplex virus or cytomegalovirus in human aortic or carotid tissues.[49-52] The studies of Yamashiroya et al[52] were performed on aortic and coronary samples from a similar autopsy population from the Office of the Chief Medical Examiner of Cook County, Illinois.

We were unable to identify B cells unequivocally among the intimal mononuclear cells, despite the use

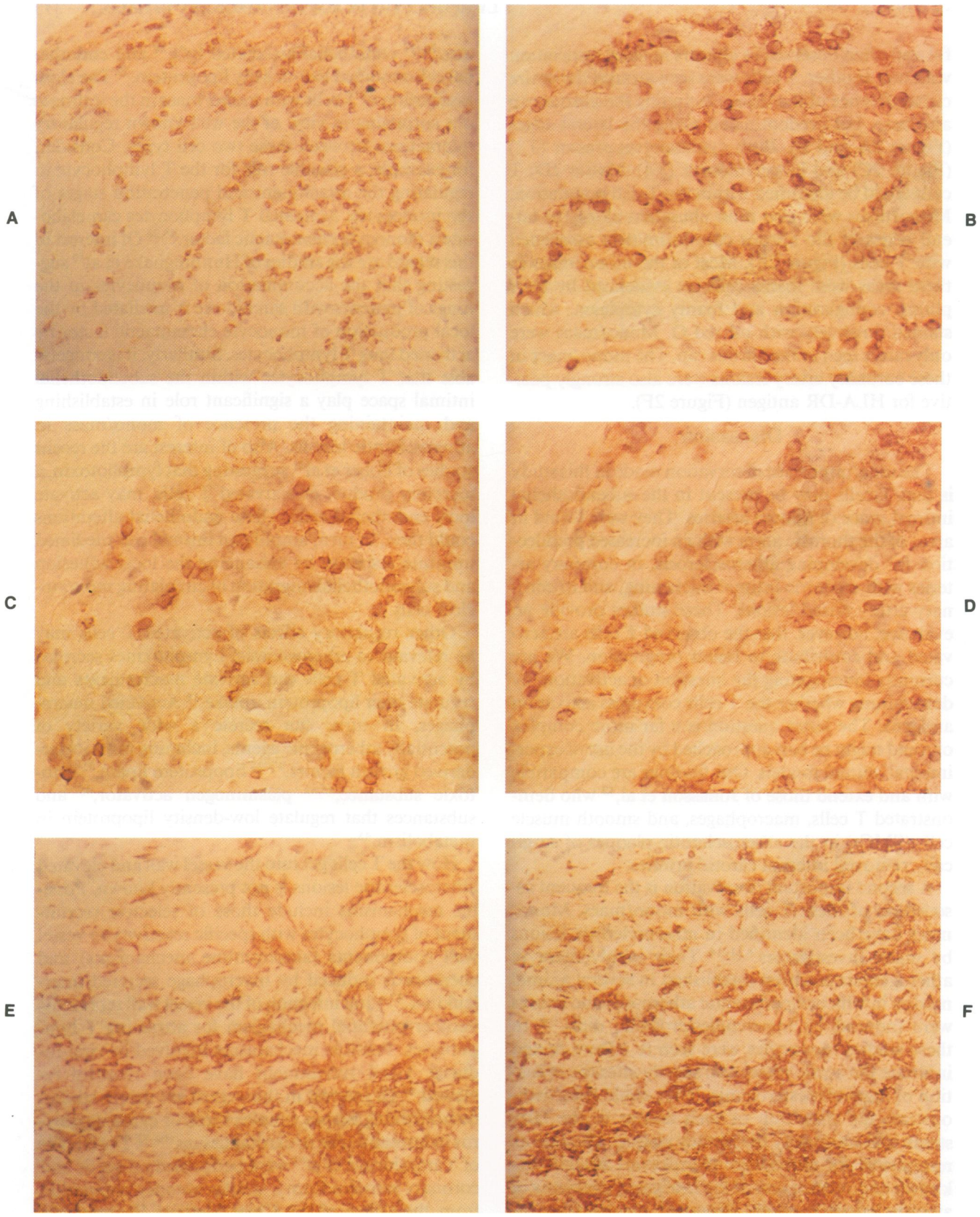

Figure 2—Sections taken through the lateral edge of a typical elevated lesion of the left coronary artery. **A**—Low-power view showing extensive infiltration of the lateral margin of the lesion by T11-positive T cells. The vessel lumen is seen in the left upper corner, and the necrotic core of the lesion lies just beyond the left lower corner. (ABC immunoperoxidase, ×210) **B**—High-power view of **A**, showing more detailed surface membrane staining of most of the individual mononuclear cells present. (ABC immunoperoxidase, ×715) **C**—Serial section from the same lesion stained with T8 antibody, showing surface membrane staining of approximately 50% of the cells. (ABC immunoperoxidase, ×715) **D**—Serial section from the lesion above stained with T4 antibody, also showing membrane staining of approximately 50% of the cells. (ABC immunoperoxidase, ×715) **E**—Serial section from the lesion above stained with Leu-M5 antibody. There is extensive staining of cell membranes and membrane fragments in the same area as the lymphocytes demonstrated above. (ABC immunoperoxidase, ×210) **F**—Serial section from the lesion above stained with HLA-DR antibody. There is extensive staining of cell membranes and membrane fragments again in the same area as the above-demonstrated lymphocytes and mono/macs (ABC immunoperoxidase, ×210)

of antibodies to two B-cell-specific surface membrane markers (B1 and Leu-14) and polyclonal and monoclonal IgG and IgM antibodies. In the previously cited study of Jonasson et al,[32] they were able to identify very few B cells (<1.0%) within the lesions they studied. Although it is possible that B cells were not a component of the mononuclear infiltrates, a more plausible explanation is that the B-cell markers we attempted to identify were altered *post mortem*.[30] B-cell effector mechanisms, ie, antibody production, with potential relevance to atherogenesis include the formation of immune complexes, complement-mediated cytotoxicity (CMC), and antibody dependent cell-mediated cytotoxicity (ADCMC). Immune complexes and complement have been identified in atherosclerotic lesions.[53,54] Components of activated complement are chemotactic for mononuclear cells, can activate mono/macs and polymorphonuclear leukocytes, and have recently been shown to have profound regulatory effects on both T and B lymphocytes.[28,55] CMC and ADCMC mechanisms could contribute to the core of necrotic debris seen in advanced complicated atherosclerotic lesions.[56,57]

T lymphocytes occupy an important pivotal position in the pathogenesis of a wide variety of human diseases. The concept that the T lymphocytes within the subendothelial intimal space occupy a similar position with regard to atherosclerosis and are not merely passive onlookers is both appealing and reasonable. T cells within the subendothelial space have the potential ability to directly modify the functions of neighboring endothelial cells, mono/macs, and SMCs. Their effects on endothelial cells and mono/macs may secondarily effect the SMCs. The possible interplay of these four cell types provides numerous potential mechanisms for mediating the inflammatory, reparative, and degenerative changes seen in atherosclerotic lesions. This hypothesis raises many unanswered questions, which are currently under investigation.

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