T lymphocytes from human atherosclerotic plaques recognize oxidized low density lipoprotein

(antigen/atherosclerosis/immune response/oxidation)

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ABSTRACT   Atherosclerosis, an underlying cause of myocardial infarction, stroke, and other cardiovascular diseases, consists of focal plaques characterized by cholesterol deposition, fibrosis, and inflammation. The presence of activated T lymphocytes and macrophages and high expression of HLA class II molecules are indicative of a local immunologic activation in the atherosclerotic plaque, but the antigen(s) involved has not yet been identified. We established T-cell clones from human atherosclerotic plaques using polyclonal mitogens as stimuli and exposed the clones to potential antigens in the presence of autologous monocytes as antigen-presenting cells. Four of the 27 CD4+ clones responded to oxidized low density lipoprotein (oxLDL) by proliferation and cytokine secretion; this response was dependent on autologous antigen-presenting cells and restricted by HLA-DR. All clones that responded to oxLDL secreted interferon γ upon activation, but only one produced interleukin 4, suggesting that the response to oxLDL results in immune activation and inflammation but may not be a strong stimulus to antibody production. No significant response to oxLDL could be detected in CD4+ T-cell clones derived from the peripheral blood of the same individuals. Together, the present data suggest that the inflammatory infiltrate in the atherosclerotic plaque is involved in a T-cell-dependent, autoimmune response to oxLDL.

Atherosclerosis, an underlying cause of myocardial infarction, stroke, and other cardiovascular diseases, consists of focal lesions of the arterial intima which are characterized by cholesterol deposition, fibrosis, and inflammation (1). These lesions begin as local infiltrates of monocyte-derived macrophages, T lymphocytes, and lipoproteins (1–3). Experimental data suggest that local endothelial expression of the vascular cell adhesion molecule 1 (4) and chemotactic stimulation by oxidized low density lipoprotein (oxLDL; ref. 5) may be important for the formation of this early lesion. During the subsequent progression of the lesion, macrophages are transformed into lipid-laden foam cells, presumably by uptake of oxLDL (2, 6, 7), and smooth muscle cells migrate into the lesion to form a fibrous cap around the lipid-rich core (1). Activated macrophages and T lymphocytes may, by means of their cytokine secretion, regulate foam cell transformation, smooth muscle proliferation, and the generation of free oxygen radicals (3).

Low density lipoprotein (LDL) is modified by oxidation in macrophage-rich tissues and large amounts of modified LDL can be detected in plaques (8–10). Not only does such modified LDL stimulate T-cell migration (5), but it is immunogenic and induces antibody production (9–11). In fact, the systemic, humoral immune response to oxidation-generated epitopes on LDL correlates with the progression of carotid atherosclerosis (12). Immune complexes consisting of oxLDL and antibodies

to oxLDL can be found in atherosclerotic lesions of rabbits and humans (13). We therefore speculated that the T cells and macrophages in the plaque may participate in an immune response to oxLDL.

Plaque T cells are phenotypically different from those of peripheral blood, since they represent a memory cell subset characterized by expression of the CD45R0 surface protein and by surface markers of late-stage activation (14). This phenotype has the capacity for secretion of several cytokines (15) and there is direct evidence for a local secretion of interferon γ (IFN-γ) in the plaque (16). In addition, HLA class II molecules are expressed by smooth muscle cells, endothelial cells, and macrophages of the plaque (17, 18). These proteins can be induced by IFN-γ, and the data therefore support the concept that activated plaque T cells regulate gene expression in surrounding cells by a paracrine, cytokine-mediated pathway (3, 19).

The presence of activated T lymphocytes and macrophages (14, 16, 17, 20–22) and extensive expression of HLA class II molecules (17) are indicative of a local immunologic activation in the atherosclerotic plaque, but the antigen(s) involved has not yet been identified. We have established T-cell clones from atherosclerotic plaques by using polyclonal mitogens as stimuli. Because oxLDL is present in atherosclerotic plaques in significant amounts and is immunogenic, we asked whether the isolated clones would respond to oxLDL in the presence of autologous monocytes. We now report that a significant proportion of the CD4+ clones responded to oxLDL by proliferation and cytokine secretion and that this response was dependent on autologous antigen-presenting cells and restricted by HLA-DR. These data suggest that atherosclerosis involves an autoimmune response to oxLDL.

MATERIALS AND METHODS

Isolation and Cloning of T Cells from Human Atherosclerotic Plaques. Atherosclerotic plaques were obtained from three patients undergoing carotid surgery due to transitory ischemic attacks. Cells were isolated by collagenase digestion and T lymphocytes were cloned from the cell suspension by limiting dilution (23). Mitogenic OKT3 antibodies (Ortho Diagnostics) were used to cross-link the antigen receptor complex, recombinant interleukin 2 (IL-2, Ala-125 substituted; Amersham) was used to promote growth, and irradiated peripheral blood mononuclear cells (PBMC) were used as feeder cells to maintain the clones (23). PBMC were isolated from peripheral blood by centrifugation over Ficoll-Paque (Pharmacia). A total of 105 T-cell clones were generated from the endarterectomy material of patients NN (53 clones), RB

Abbreviations: LDL, low density lipoprotein; oxLDL, oxidized low density lipoprotein; IFN-γ, interferon γ; PBMC, peripheral blood mononuclear cells; nLDL, native LDL; IL-*n*, interleukin *n*.
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(21 clones), and KB (31 clones) by using this technique. Similar clonal libraries were established from peripheral blood of the same individuals. Since polyclonal mitogens (OKT3 antibodies) rather than specific antigens were used to activate the T cells, the clonal libraries thus established had the potential of containing a multitude of different immunologic reactivities present among the T cells in the plaque (23).

Generation and Assay of Tetanus Toxoid-Specific T-Cell Clones. PBMC were isolated from the peripheral blood of a normal donor as described above. T cells were cloned by limiting dilution in Terasaki 60-well plates in the presence of 10^5 irradiated autologous PBMC and 25 μg of tetanus toxoid (SBL-Vaccin, Stockholm) per ml in RPMI 1640 culture medium with 10% fetal calf serum and 10% pooled human serum from healthy donors. Growing clones were stimulated to proliferate with OKT3 antibody and IL-2 as described (23). They were then subjected to proliferation assays in the presence of tetanus toxoid at 10–50 μg/ml, ovalbumin (Calbiochem–Novabiochem) at 25 μg/ml, and native or oxLDL at 10 μg/ml as stated in *Results*.

Immunophenotyping. T-cell clones were stained with fluorescent antibodies to CD3, CD4, and CD8 (monoclonal antibodies Leu-4, Leu-3a, and Leu-2, respectively, from Becton Dickinson) and analyzed in a FACScan flow cytometer (Becton Dickinson) by using the LYSYS II software package.

LDL Oxidation. LDL was isolated in the presence of 1 mM EDTA from the pooled sera of fasting human subjects by ultracentrifugation through KBr as described (24). KBr was removed by gel filtration on Sephadex G-25 M (PD-10; Pharmacia) and 200 μg of LDL per ml was oxidized by incubation with 5 mM $CuSO_4$ in 5 mM Hepes buffer containing 150 mM NaCl, 4 mM $CaCl_2$, and 2 mM $MgCl_2$ (pH 7.2) for 20 h at 37°C (25, 26). Oxidation was then inhibited by addition of butylated hydroxytoluene to a final concentration of 20 μM (26). The degree of oxidation was evaluated by an assay for thiobarbituric-acid–reactive substances (TBARS; refs. 25 and 27) and LDL protein was characterized by agarose electrophoresis and quantitated by Bradford's method (28). TBARS values were 16 ± 8 mmol of malondialdehyde equivalents per mg of protein for oxLDL preparations and <1 mmol/mg for native LDL (nLDL). nLDL was maintained in 20 μM butylated hydroxytoluene to prevent oxidation (11). All lipoprotein preparations were sterilized by filtration through 0.22 μm Millex GV filters before use.

Blocking Antibodies. The hybridoma L243, which produces a monoclonal antibody to human HLA-DR (29), was obtained from the American Type Culture Collection and grown in Iscove's modified Dulbecco's medium with 2 mM L-glutamine, 0.45 mM sodium pyruvate, 50 mM 2-mercaptoethanol, 0.5% Primaton RL (Sheffield Product, Kraft), and 10% fetal bovine serum. Culture in dialysis tubing (30) was used to obtain large amounts of antibody, which was purified from the medium by affinity chromatography over protein G-Sepharose (Pharmacia). The purified L243 IgG was dissolved in phosphate-buffered saline (PBS; 154 mM NaCl/10 mM sodium phosphate, pH 7.2) and used at 10 μg/ml in blocking experiments. The mouse myeloma IgG MOPC 21, which does not recognize any known determinant in human cells (31), was obtained from Sigma and used in experiments in the same way as was L243.

Antigen Presentation Assay. Venous blood samples were obtained from the patients by renewed bleedings, and PBMC were isolated as described above. CD4$^+$ T cells (10^5) were incubated with 10^5 autologous PBMC in a volume of 300 μl of RPMI 1640 medium with 10% fetal bovine serum and 10% pooled, heat-inactivated human serum in 96-well, round-bottomed, microtiter plates. The antigen-presenting PBMC were irradiated with 25 Gy from a cobalt source to eliminate their proliferative capacity before being incubated. oxLDL and/or antibodies were added to the cultures as described in *Results*, and 72 h later, 2 μCi of [^{3}H]thymidine (1 Ci = 37 GBq;

Amersham) was added to each well. Nonadherent cells were harvested on glass fiber filters with an Inotech (Wohlen, Switzerland) Cell Harvester after an additional 24 h of culture. [^{3}H]Thymidine uptake was determined by liquid scintillation.

Cytokine Secretion. IFN-γ (Biosource, Camarillo, CA) and IL-4 (R & D Systems) were measured by ELISA in 100 μl of culture medium collected from each of the T-cell clones cocultured with PBMC for 72 h after being stimulated with OKT3 and IL-2.

RESULTS

Atherosclerotic plaques were obtained from three patients undergoing carotid artery surgery due to transitory ischemic attacks. Cells were isolated by collagenase digestion, and T lymphocytes were cloned from the cell suspension by limiting dilution (23). Mitogenic OKT3 antibodies were used to cross-link the antigen receptor complex, recombinant IL-2 was added to promote growth, and irradiated, autologous PBMC were used as feeder cells to maintain the clones (23). Large numbers of T-cell clones of both CD4 and CD8 phenotypes were generated from each plaque biopsy by using this technique (Table 1). Similar clonal libraries were established from the peripheral blood of the same individuals. Since polyclonal mitogens (OKT3) rather than specific antigens were used to activate the T cells, the clonal libraries thus established had the potential of containing many of the different immunologic reactivities that had been present in the T-cell population in the plaque (23).

Southern blot analysis of the organization of the T-cell receptor genes indicated that all clones displayed different gene rearrangement patterns; they were therefore of polyclonal origin (23). A phenotypic analysis was carried out by flow cytometry. All clones expressed CD3 and either CD4 or CD8, indicating that they were mature, T-cell receptor $\alpha\beta$-type T cells (23). The majority of clones in all three libraries were of the CD4$^+$ phenotype (Table 1). Since exogenous antigens are presented by major histocompatibility complex class II molecules, which are exclusively recognized by CD4$^+$ T cells, clones of this phenotype were selected for the antigen-presentation analyses.

The hypothesis that oxLDL is an important local (auto)antigen in the atherosclerotic plaque was tested by challenging our T-cell clones with oxLDL. CD4$^+$ clones were exposed to copper-oxidized LDL in the presence of irradiated, autologous PBMC that could serve as antigen-presenting cells. CD4$^+$ T cells immunospecific for oxLDL would be expected to respond to this stimulus by proliferation. Fig. 1 shows a typical positive response. The CD4$^+$ clone RB2 responded to oxLDL by a 5-fold increase in DNA synthesis compared with the level in the absence of oxLDL (Fig. 1). The response was dependent on the presence of antigen-presenting cells, since addition of oxLDL to RB2 in the absence of PBMC did not induce proliferation (Fig. 1).

Table 1.   Patients and T-cell clones used in this study

Patient	Age, yr	Plaque-derived clones			Peripheral blood-derived clones		
		Total no.	CD4$^+$	Tested	Total no.	CD4$^+$	Tested
NN	70	53	40	10	47	39	7
RB	61	21	16	8	14	12	8
KB	57	31	26	9	—	—	—

Clones were established by limiting dilution using OKT3, recombinant IL-2, and autologous feeder cells. CD4 reactivity was analyzed by flow cytometry, and clones were randomly chosen for testing in antigen assays.
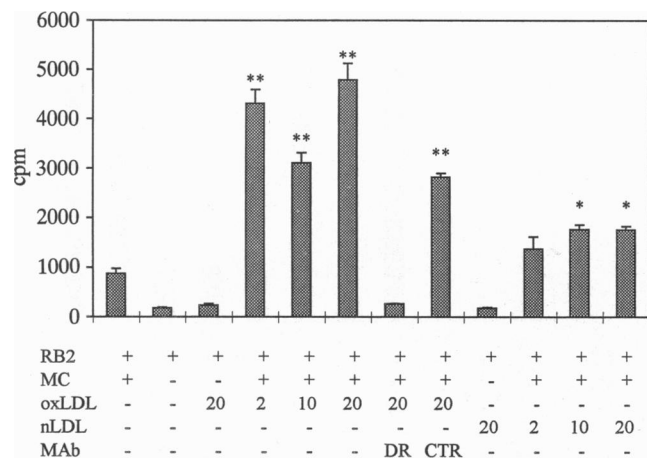
RB2	+	+	+	+	+	+	+	+	+	+	+	+
MC	+	-	-	+	+	+	+	+	-	+	+	+
oxLDL	-	-	20	2	10	20	20	20	-	-	-	-
nLDL	-	-	-	-	-	-	-	-	20	2	10	20
MAb	-	-	-	-	-	-	DR	CTR	-	-	-	-

Fig. 1. Plaque T-cell clone RB2 was activated by oxLDL at concentrations of 2–20 μg/ml but not by nLDL. Activation was dependent on the presence of autologous PBMC (MC) and inhibited by the anti-HLA-DR monoclonal antibody L243 (DR) but not by the control monoclonal antibody MOPC 21 (CTR). Activation was determined as incorporation of [^{3}H]thymidine into DNA and is presented as cpm per well (mean $\pm$ SEM) of duplicate cultures. An asterisk indicates a statistically significant difference from control (left bar) by the Mann–Whitney U test (*, $P < 0.05$; **, $P < 0.01$). MAb, monoclonal antibody.

The monocyte-dependent oxLDL response of RB2 could be blocked with the monoclonal antibody L243, which reacts with HLA-DR, but not with the control monoclonal MOPC 21 (Fig. 1). This strongly suggests that the response to oxLDL represented an immunospecific T-cell activation by an HLA class II-restricted antigen presented by autologous monocytes.

No distinctive dose–response relationship could be discerned in the pattern of reactivity of this clone to oxLDL at 2–20 μg/ml (Fig. 1), and the response was of a significantly lower magnitude than that mounted when antigen receptor complexes were cross-linked on the surface of RB2 by the anti-CD3 antibody OKT3 (data not shown). This might be partly explained by a growth-inhibitory effect of oxLDL at higher concentrations. However, no cytotoxic effects of oxLDL were observed in our experiments (see Fig. 4). A low-level proliferative response could be seen when nLDL was used as an antigen instead of oxLDL (Fig. 1). This might be due to the minimal oxidation of the LDL preparation that occurs despite precautions (11).

HLA-DR-restricted oxLDL activation was observed with several other plaque CD4$^+$ clones from the three libraries. The responses of clones NN13, KB12, and RB16 are displayed in Fig. 2. As can be seen, T-cell proliferation of these clones was also stimulated by oxLDL at 2–20 μg/ml. Again, the responses were inhibited by anti-HLA-DR but not by MOPC 21, and they were dependent on the presence of autologous monocytes in the coculture system (Fig. 2).

A summary of the proliferative responses of all CD4$^+$ clones to oxLDL is displayed in Fig. 3. In total, six different preparations of LDL were tested for activation of T-cell clones. Four of 27 tested plaque-derived clones responded with a stimulation index between 3 and 5. These clones were immunospecific for oxLDL when applying the criteria of a >3-fold increase in DNA synthesis, dependence on autologous monocytes, and inhibition by anti-HLA-DR antibodies. None of the peripheral blood CD4$^+$ clones from any of the three patients exhibited significant responses to oxLDL (Fig. 3). Interestingly, two of the plaque-derived clones showed a modest response—i.e., stimulation indices between 2 and 3—with nLDL (Figs. 1 and 3). This suggests that these clones also react with LDL that has been modified by the cells in the coculture system.

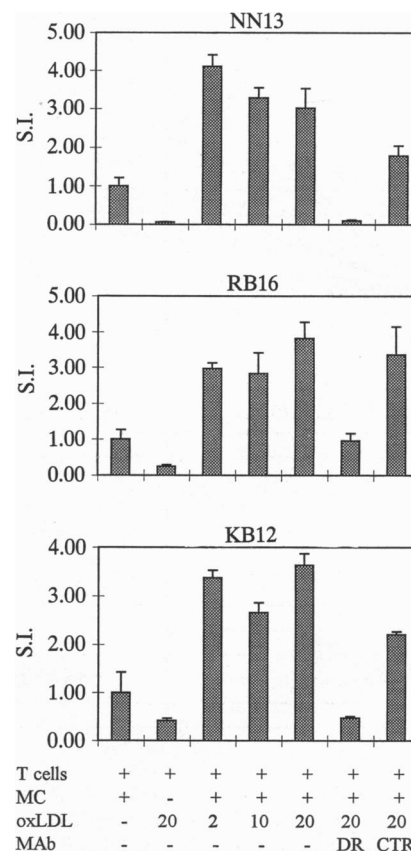| T cells	+	+	+	+	+	+	+
MC	+	-	+	+	+	+	+
oxLDL	-	20	2	10	20	20	20
MAb	-	-	-	-	-	DR	CTR

Fig. 2. Activation of three different plaque T-cell clones, NN13, RB16, and KB12, by oxLDL in the presence of autologous PBMC (MC). The response was inhibited by anti-HLA-DR (DR) but not by MOPC 21 (CTR). Data are presented as stimulation index [S.I., defined as the cpm of the actual sample divided by the cpm of the T cell/PBMC coculture in the absence of oxLDL (lane 1)]. Values shown are means $\pm$ SEM of duplicate cultures. MAb, monoclonal antibody.

To exclude the possibility that the observed proliferative response in the presence of oxLDL was due to a nonspecific enhancement by oxLDL of the immune recognition of non-LDL antigens present in the cultures, the effect of oxLDL on the antigen recognition by tetanus toxoid-specific T-cell clones was tested. Tetanus toxoid-specific T-cell clones were generated from the peripheral blood of a healthy donor. Fig. 4 shows that this clone responds to tetanus toxoid but not to oxLDL and that the rate of proliferation in response to tetanus toxoid is not affected by the simultaneous presence of oxLDL or nLDL. Two more tetanus toxoid-specific T cell clones were tested with identical results (data not shown). These results demonstrate that neither oxLDL nor nLDL enhances non-LDL-related immune recognition and support the conclusion that the oxLDL-dependent proliferative response of the plaque-derived T-cell clones is due to an immunospecific recognition of oxLDL.

The oxLDL-reactive clones secreted IFN-γ upon maximal stimulation with a CD3-crosslinking antibody (stimulation index, 629 $\pm$ 300; mean $\pm$ SD). In contrast, only one of the clones (RB16) produced significant amounts of IL-4 under the same conditions (stimulation index, 36). The responses to oxLDL stimulation under antigen-presenting conditions were generally of a lower magnitude than those exhibited towards anti-CD3 stimulation (data not shown).

DISCUSSION

The results of the present study demonstrate that human atherosclerotic plaques contain CD4$^+$ T cells immunospecific
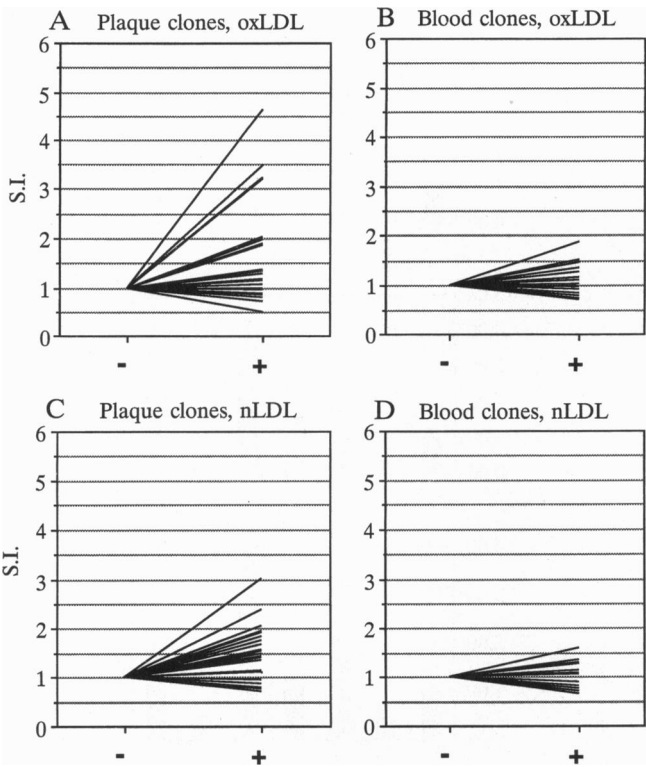
FIG. 3.   Summary of LDL-induced activation of T-cell clones isolated from either plaques or peripheral blood. (*A*) Stimulation of T-cell clones derived from plaques with oxLDL. (*B*) Stimulation of T-cell clones derived from peripheral blood with oxLDL. (*C*) Stimulation of plaque T-cell clones with nLDL. (*D*) Stimulation of blood T-cell clones with nLDL. +, Incubation of T-cell clones with oxLDL or nLDL, respectively, in the presence of antigen-presenting PBMC. −, Incubation of T-cell clones with PBMC in the absence of oxLDL or nLDL. S.I., Stimulation index, as explained in the legend to Fig. 2.
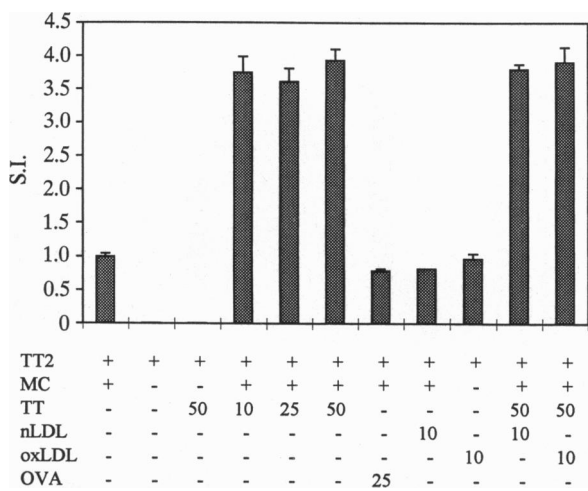

FIG. 4.   Test of the effects of oxLDL and nLDL on the proliferative response to tetanus toxoid (TT) by a tetanus toxoid-specific T-cell clone TT2. TT2 cells were activated in the presence of autologous PBMC (MC) by tetanus toxoid but not by ovalbumin (OVA), oxLDL, or nLDL. The rate of proliferation in response to tetanus toxoid was not affected by the simultaneous presence of either oxLDL or nLDL. Figures below bars indicate concentrations in μg/ml. S.L., stimulation index, as explained in the legend to Fig. 2. Values shown are means ± SEM of duplicate cultures.

for oxLDL. This report, to the best of our knowledge, is the first identification of an immunologic specificity of T lymphocytes in human atherosclerotic plaques. Our conclusion is based on the responses of three clonal libraries established from advanced atherosclerotic plaques. At least one oxLDL-reactive clone was identified among the 8–10 clones tested in each library. In contrast, no oxLDL-specific clones were identified in libraries established from the peripheral blood of the same patients. However, no certain conclusions regarding the frequency of oxLDL-specific T cells in the atherosclerotic plaque could be made by using the current approach. Instead, such data await limiting dilution assays. The clones described here should, however, be useful for characterizing the specific epitopes, receptors, and restriction elements involved in the T-cell response to oxLDL.

The response of the oxLDL-specific CD4$^+$ T cells was dependent on the presence of autologous, antigen-presenting monocytes and was blocked by antibodies to HLA-DR. This strongly suggests that the T-cell response was mounted against an HLA-DR-restricted, processed antigen derived from oxLDL.

LDL is a heterogeneous particle that contains apolipoprotein B-100, cholesteryl esters, cholesterol, triacylglycerol, and phospholipids. Oxidative modification of the lipid moieties includes degradation of polyunsaturated fatty acid residues and generation of highly reactive aldehydes and other products that can modify other lipids. The cholesterol moiety also undergoes oxidation, and apolipoprotein B undergoes nonenzymatic degradation, as well as derivatization of its lysine and other residues (32–35). Several of these modifications represent neoantigens that may elicit antibody production (11). For

example, both 4-hydroxynonenal- and malondialdehyde-modified lysine act as B-cell epitopes and are present in atherosclerotic plaques (8–11). Because oxidation of LDL represents a complex and heterogeneous series of reactions, it is likely that many structures are formed that may serve as neoantigens and give rise to many different T- and B-cell clones reactive against a large variety of epitopes.

The T-cell epitopes generated during oxidation of LDL which were recognized by these clones are at present unknown. The data of the present report allow a few predictions to be made. First, the response of the T-cell clones required the presence of antigen-presenting PBMC. This suggests that lipoprotein oxidation must be followed by receptor-mediated uptake and intracellular, lysosomal degradation to generate the mature T-cell epitopes. It is also likely that intracellular processing is necessary for the association of the T-cell epitopes of oxLDL to HLA-DR. Second, the low-magnitude, clonal T-cell response to nLDL in the presence of monocytes suggests that the latter cells generated the relevant epitopes during the coculture period—e.g., by release of oxygen radicals that generated a low level of oxidation of LDL. T-cell epitopes were therefore probably generated both by copper oxidation and by monocyte-dependent oxidation, and because the response was clonal, it is likely that the same epitopes were generated in both reactions. The rather modest degree of oxidation involved ($\approx$10–20 nmol of thiobarbituric acid reactive substances per mg of protein) suggests that the T-cell epitopes recognized in our system were generated relatively early during the oxidative modification of LDL. It will now be important to identify the individual epitopes involved and to determine whether they are native or modified peptides from the apoprotein component of LDL.

LDL is present in high concentration in atherosclerotic plaques, where oxidation may occur via several mechanisms (8, 32). Presumably, this generates large amounts of antigen during the progression of the disease (2). As mentioned, antibody responses are mounted against oxLDL (9, 11). Very few B cells are, however, present in the plaque (20). In contrast, T cells are quantitatively important cellular components of the plaque throughout its development (20). Most of these T cells express the CD45R0$^+$ phenotype (14), which is expressed by T cells that, after their first antigen encounter in

lymph nodes, migrate to peripheral tissues (36). There, they can respond to antigenic challenge and perform effector functions. The observation that oxLDL stimulates DNA synthesis in bulk cultures of peripheral-blood T cells (37) might be explained by the presence in the blood of oxLDL-reactive memory T cells en route to peripheral tissues.

The secretory pattern of CD4$^+$ cells upon activation can be functionally divided into the T_H1 and T_H2 types, although the basis for these categories in man may not be as clearcut as it is in mice (38). In the case of oxLDL-responding T cells in the plaque, our data suggest that secretion of IFN-γ is more important than IL-4 secretion. The latter induces B-cell differentiation and antibody production, whereas the former activates macrophages, resulting in inflammatory responses (38). This would be in line with data showing few B cells but large amounts of activated macrophages (3, 17, 20, 22) and secretion of both monokines (39, 40) and IFN-γ (16) in plaques. The B-cell stimulation elicited by an initial T-cell response to oxLDL may, however, not be negligible, since antibodies to oxLDL are produced in high titers in apolipoprotein E knock-out mice that develop severe atherosclerosis but not in closely related mice that do not have atherosclerosis (41). Similarly, a high titer of such antibodies predict progression of carotid atherosclerosis in humans (12).

An autoimmune response to oxLDL may be of significant pathogenetic importance in atherosclerosis. Induction of antibody production would promote the elimination of oxLDL via Fc receptors (42). Secretion of IFN-γ would be expected to promote macrophage activation (43) and down-regulate scavenger receptor expression and intracellular cholesterol accumulation (44, 45). It also inhibits smooth muscle proliferation (46), α-actin expression (47), and collagen formation (48). Together with tumor necrosis factor, IFN-γ induces production of the vessel-relaxing factor, nitric oxide (49). Several of these phenomena have been demonstrated to occur *in vivo* and are likely to inhibit the fibrotic, stenosing process. This could be beneficial for the affected individual. It is, however, also possible that macrophage activation and the loss of structural proteins could increase the risk for plaque rupture and the formation of aneurysms. In conclusion, therefore, it is reasonable to suggest that the cellular immune response in the atherosclerotic plaque plays a modulating role in the development of the disease. Further experiments will be necessary to determine whether it is essentially beneficial or destructive.

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