Virchows Archiv (1995) 426:307–315

ORIGINAL ARTICLE

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Participation of T lymphocytes in atherogenesis: sequential and quantitative observation of aortic lesions of rats with diet-induced hypercholesterolaemia using en face double immunostaining

Received: 4 January 1995 / Accepted: 17 February 1995

Abstract Using en face double immunostaining coupled with electron microscopy, we studied the temporal and spatial distribution of T lymphocytes and macrophages during the development of atherosclerosis in a diet-induced rat model fed an atherogenic diet for 2–40 weeks. T lymphocytes and macrophages adhered to the aortic surface by 2 weeks on the diet, with subsequent migration under the endothelium, and formed a fatty streak-like lesion. Analysis of the cellular components revealed that infiltration of T lymphocytes was most prominent in the incipient phase of lesion formation accounting for 60%, 29% and 34% of mononuclear cells appearing in 2-week lesions of the superior thoracic, inferior thoracic and abdominal segments of the aorta, respectively. After the incipient phase, the relative number of T lymphocytes in the three segments of the aorta showed a slow decline; the proportion of T lymphocytes to macrophages was approximately 1:3 to 1:4 in 10- to 20-week lesions. An overall view of the lesional cells often demonstrated direct cellular contact between T lymphocytes and macrophages. Further, OX6/ED1 double immunostaining demonstrated that Ia antigen was expressed on most macrophages. In later stages, breakdown of foamy macrophages occurred, and the extracellular accumulation of lipids and cell debris became prominent. The results demonstrated that in the diet-induced rat model, together with macrophages, large numbers of T lymphocytes participated in all stages of aortic lesions, initially adhering to the surface at prelesional stages and later as the principal component of the atherosclerotic lesion. It is possible that the method described here will provide a good tool for examining the role of T lymphocytes in atherogenesis.

Key words T lymphocyte · Macrophage · Atherogenesis · En face double immunostaining

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Introduction

Recent advances in atherosclerotic investigation, using immunohistological techniques, have shown that large numbers of lymphocytes and macrophages are present in human atherosclerotic lesions [4, 12, 16, 27, 33]. The lymphocytes are almost exclusively CD3+ T lymphocytes that express either CD4 or CD8 molecules, and a significant number of them express activation markers such as HLA-DR, very late activation antigen (VLA)-1 and interleukin 2 receptor [13, 37, 40]. The selective accumulation of activated T lymphocytes and macrophages with abundant HLA-DR antigens on their cell surface in both fatty streaks and plaques may well imply a role for cell-mediated immune responses in atherogenesis [14, 21, 26, 39]. According to our previous observations, the T lymphocyte-macrophage interaction manifested by close cell-to-cell contact between those cells is widely recognized at the sites where atherosclerotic lesions are putatively growing or active [18, 33]. However, the relevance of these phenomena to atherogenesis remains unclear since studies have been limited to the analysis of human surgical or autopsy material.

Few experimental studies have been concerned with participation of T lymphocytes in the atherosclerotic lesion. Hansson et al. [15] examined the cholesterol-fed rabbit by immunohistological methods and observed accumulation of T lymphocytes in the intima, although their number was far smaller than that of macrophages. In a diet-induced rat model, using phagocytosis and non-specific esterase for the identification of mononuclear cells, Joris et al. [17] found adhesion of monocytes and lymphocytes to the arterial intima in the early stage of hypercholesterolaemia.

We have studied the temporal and spatial distribution of the inflammatory and immunological components of atheroslcerosis in a diet-induced rat model. Coupled with scanning and transmission electron microscopy, en face double immunostaining was adopted to identify and to quantitate the two types of immunocompetent cells, OX19-positive T lymphocytes and ED1-positive macro-

phages simultaneously. The cells exist not only on the surface of the lesion but also infiltrate throughout the intima of the aorta from the aortic arch to the iliac bifurcation. Our study demonstrates that infiltration of T lymphocytes is particularly prominent at the incipient phase of lesion development. Further, overall view of lesional cells suggests possible interactions between T lymphocytes and macrophages in all stages of atherosclerotic lesions. We are confident that the method described here will provide a good tool for the examination of the role of immunocompetent cells in the evolution and progression of atherosclerosis.

Materials and methods

Specific-pathogen free male Sprague-Dawley rats of 8 weeks of age, purchased from Charles River Japan (Tsukuba) were used. All rats were housed in 35×25×20 cm stainless wire cages (two or three rats per cage), and kept in a temperature-controlled clean room with free access to food and tap water. They were fed a standard Purina rat chow (Oriental Yeast Company, Tokyo) supplemented with 4% cholesterol, 1% cholic acid and 0.5% thiouracil (Wako Pure Chemical Industries, Osaka). Blood samples were obtained from the baseline period before the onset of the experiment and thereafter at biweekly intervals until sacrifice. Total serum cholesterol, triglyceride and phospholipid were determined by Hitachi 705 and 726 automatic analysers. Groups of eight animals were anaesthetized with sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, Ill.), and sacrificed after 2, 10, 20 and 40 weeks on the diet.

Fixation and tissue preparation for en face double immunostaining was done by perfusing the aorta at a pressure of 100–120 mmHg via the left ventricle, first with phosphate buffered saline (PBS) for 5 min and then with periodate-lysine-paraformaldehyde (PLP) solution for 5–10 min. The aorta (from aortic arch to iliac bifurcation) was dissected carefully and immersed in the PLP fixative for 3 h at 4° C. Under a dissecting microscope, the adventitia was separated from the intima and media by fine forceps and scissors. The aorta was then divided into the thoracic and abdominal segments. Both segments were slit open longitudinally in the following manner: the thoracic segment was opened along the ventral side, while the abdominal segment along the dorsal side as the branches of the coeliac and mesenteric arteries were located centrally. The aortic segments were rinsed with 10%, 15% and 20% sucrose in PBS successively.

Murine monoclonal antibodies, OX33, OX19, OX6 (Sera-lab, Crawly Down, UK) and ED1 (Chemicon International, Temecula, Calif.) were used. OX33 is a pan-B lymphocyte marker [43]. OX19 recognizes all peripheral T cells and thymocytes [2], and ED1 binds to a cytoplasmic antigen that is expressed in monocytes/macrophages [3]. OX6 reacts with the rat Ia antigens which have biochemical characteristics similar to mouse Ia antigens and human HLA-DR antigens [29]. Biotinylated horse anti-mouse IgG was purchased from Vector Laboratories, (Burlingame, Calif.). Peroxidase-conjugated streptavidin, alkaline phosphatase-conjugated streptavidin, 3-amino-9-ethylcar-bazole (AEC) substrate kit and fast blue substrate kit were from Nichirei (Tokyo).

For en face double immunostaining endogenous peroxidase was blocked with sodium azide and hydrogen peroxide in PBS for 30 min at 4° C. Each segment of the aorta was laid flat on a glass slide with the endothelium facing up and incubated successively with the following: normal horse serum for 20 min at room temperature; first antibody (OX19) overnight at 4° C; biotinylated horse anti-mouse IgG for 30 min at room temperature; peroxidase-conjugated streptavidin for 30 min at room temperature. The peroxidase reaction was developed with AEC substrate kit. After the specimen was rinsed several times with 0.1 M glycine-hydrochloric acid buffer (pH 2.2) for 2 h at 4° C, it was spread flat again on the glass slide with the endothelium facing up. The specimen was then incubated at 4° C with the second antibody (ED1) overnight, biotinylated horse antimouse IgG for 12 h and alkaline phosphatase-conjugated streptavidin for 12 h, followed by development with fast blue substrate kit. OX6 was also used as the first antibody in double immunostaining. It was mounted flat in a glycerine jelly and studied en face by light microscopy.

For electron microscopy, after perfusion fixation with 3% glutaraldehyde for 10 min, the aorta was cut into thoracic and abdominal segments and slit open longitudinally. Each segment was further divided into two parts. One portion constituted 2 mm sections taken for transmission electron microscopy, and the remaining larger portion was processed for scanning electron microscopy. All samples were immersion-fixed in cacodylate buffered 3% glutaraldehyde overnight at 4° C, and postfixed with 1% osmium tetroxide for 1.5 h. For transmission electron microscopy, samples were dehydrated in graded ethanols, treated with propylene oxide and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate, and examined with a JEM 100 CX electron microscope (JEOL, Tokyo). After dehydration in graded tertiary-butyl alcohols, specimens for scanning electron microscopy were freeze-dried by a frozen dryer ID-2 (EIKO Engineering, Ibaraki), coated with gold and viewed with a Hitachi S-700 scanning electron microscope.

Using en face double immunostained specimens, quantitative assessment of T lymphocytes and monocytes/macrophages was performed. Thoracic and abdominal segments of the aorta were scanned en face at ×200, and the total number of cells adhering to the surface and in migration below the endothelium was first counted. The thoracic segment was subdivided into two parts, superior and inferior. Then the total number of cells in a given segment was divided by the area of the segment in square millimetres. The mean number of each cell type was compared using Student's *t*-test. Two means were considered significantly different if the probability of an error was less than 0.05 ($P<0.05$).

Results

During feeding of the diet, the average cholesterol levels rose from 60–70 mg/dl to about 900 mg/dl (Table 1). Plasma triglyceride values changed but little, while plas-

Table 1 Temporal changes in serum total cholesterol, triglyceride and phospholipid (all values are means±standard deviation. Total cholesterol and phospholipid values of rats fed with cholesterol-enriched diet are significantly different from those of control groups at $P<0.05$)

Age (weeks)	Total cholesterol (mg/dl)	Triglyceride (mg/dl)	Phospholipid (mg/dl)
Control group			
8	69.5±17.1	80.0±19.7	128.5±17.3
10	61.8±4.4	109.0±43.0	119.3±22.5
12	52.8±2.9	99.3±28.1	93.3±12.0
20	60.3±10.0	159.5±12.0	123.0±25.2
28	68.5±11.7	153.8±59.5	114.3±24.7
40	67.5±13.0	140.0±53.1	115.0±15.0
Atherogenic diet group			
8+2[a]	972.0±238.8	115.6±24.4	333.8±30.4
8+4	916.8±180.1	76.1±19.1	339.4±33.4
8+12	919.5±267.3	79.9±24.4	348.5±42.0
8+20	878.2±200.7	61.5±12.0	342.7±41.9
8+32	960.8±111.6	75.3±23.5	415.3±51.8
8+40	995.6±209.6	73.4±18.2	422.0±65.2

[a] Age plus feeding period on atherogenic diet

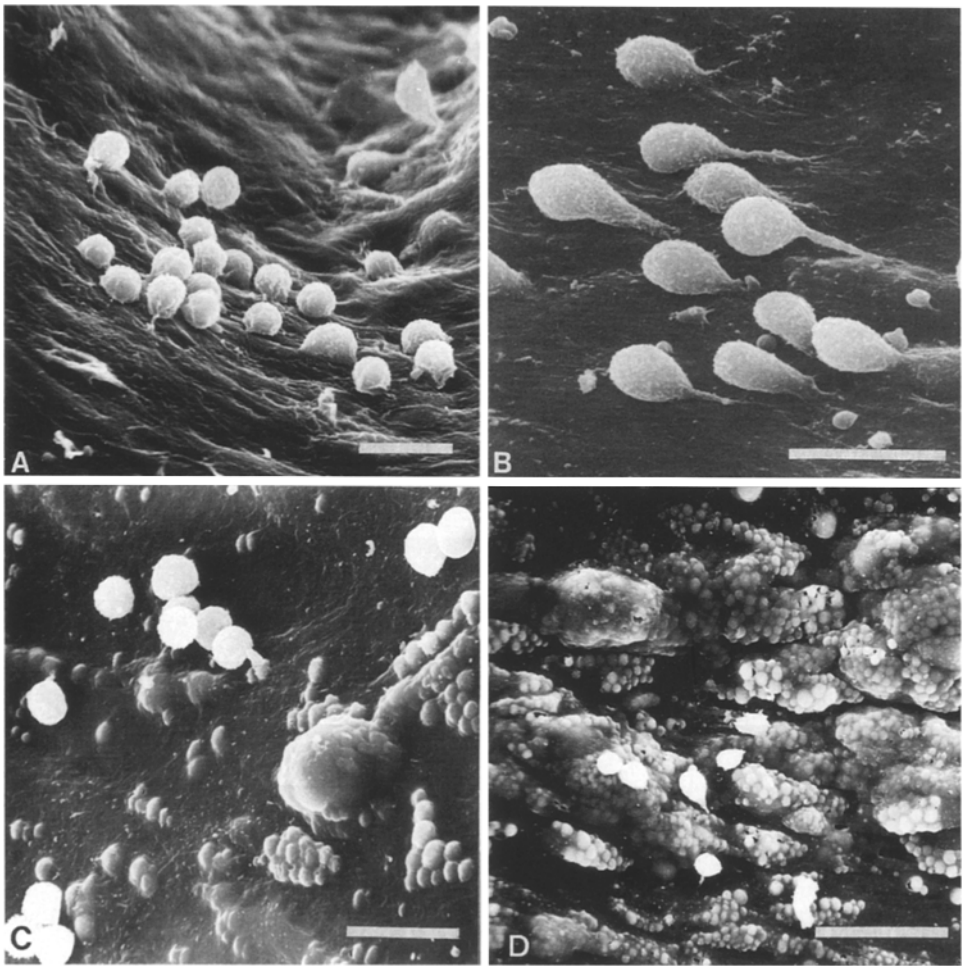

Fig. 1A–D Scanning electron micrographs of aorta from rats on atherogenic diet. **A** A cluster of leucocytes adhering to or indenting the overtly intact endothelium near the branch orifice of thoracic aorta. Two weeks on diet. *Bar*=10 μm. **B** Adherent leucocytes have tail-like protrusions and appear to be spreading over or entering the aortic intima. Two weeks on diet. *Bar*=10 μm. **C** Aortic surface demonstrates dome-like or irregularly shaped nodular bulging due to subendothelial appearance of cells. Ten weeks on diet. *Bar*=10 μm. **D** Advanced aortic lesion consisting of aggregations of nodular protrusions. The covering endothelium displays a confetti-like appearance representing accumulation of lipid vacuoles in cytoplasm. Adherent leucocytes are small in number. Forty weeks on diet. *Bar*=20 μm

ma phospholipid concentrations showed elevation exceeding the normal by about three-fold. Our previous study [5] has shown by agarose electrophoresis a profound increase of broad beta band analogous to beta-very low density lipoprotein (VLDL).

By scanning electron microscopy, adhesion of leukocytes to the endothelial cells was observed by 2 weeks into the diet. Clusters of adherent cells were located in close proximity to the ostia of branching arteries, whereas they appeared to be glued in a random fashion to other areas of the aortic intima (Fig. 1A). Many of the adherent leucocytes had tail-like protrusions, which are generally associated with cell spreading or migration (Fig. 1B). The lesion was covered by intact endothelium, and no evidence of gross endothelial damage or denudation was observed. Accumulation of mononuclear cells, singly or in clusters, was demonstrated by transmission electron microscopy in the subendothelium (Fig. 2A). Some of these cells were identifiable as monocyte-derived macrophages, while others had a round nucleus, no lysosomes, many free ribosomes, and were thus considered to be lymphocytes. We saw no polymorphonuclear cells, either adhering to the endothelium or beneath it.

At 10 weeks into the diet, the intimal surface progressively showed dome-like or irregularly shaped nodular elevations; they were potentially due to subendothelial accumulation of cells in the intima (Fig. 1C, D). By transmission electron microscopy, some of the subendothelially migrated cells accumulated lipid and took on the appearance of foam cells (Fig. 2B, C). This accumulation of subendothelial macrophages (foam cells) constituted the first stage of fatty streak formation in these animals. Lipid-laden smooth muscle cells were also observed in varying numbers in the lesion but were usually located beneath the layer of macrophages (Fig. 2C). A later stages adherent cells were present but they were not prominent in number. The extracellular matrix was increased after 40 weeks on the diet and contained numerous small vesicular structures similar to those previously described as extracellular liposomes by Simionescu et al. [34].

En face double immunostaining technique permitted the identification and quantitation of two different types of cells in a single preparation. At first we used three murine monoclonal antibodies to detect different inflammatory and immunological components of atherosclerosis. However, the cells stained by a B cell marker OX33 were scarcely noted in the lesions. The analysis was therefore focussed on the temporal observation of OX19-positive T lymphocytes and ED1-positive macrophages

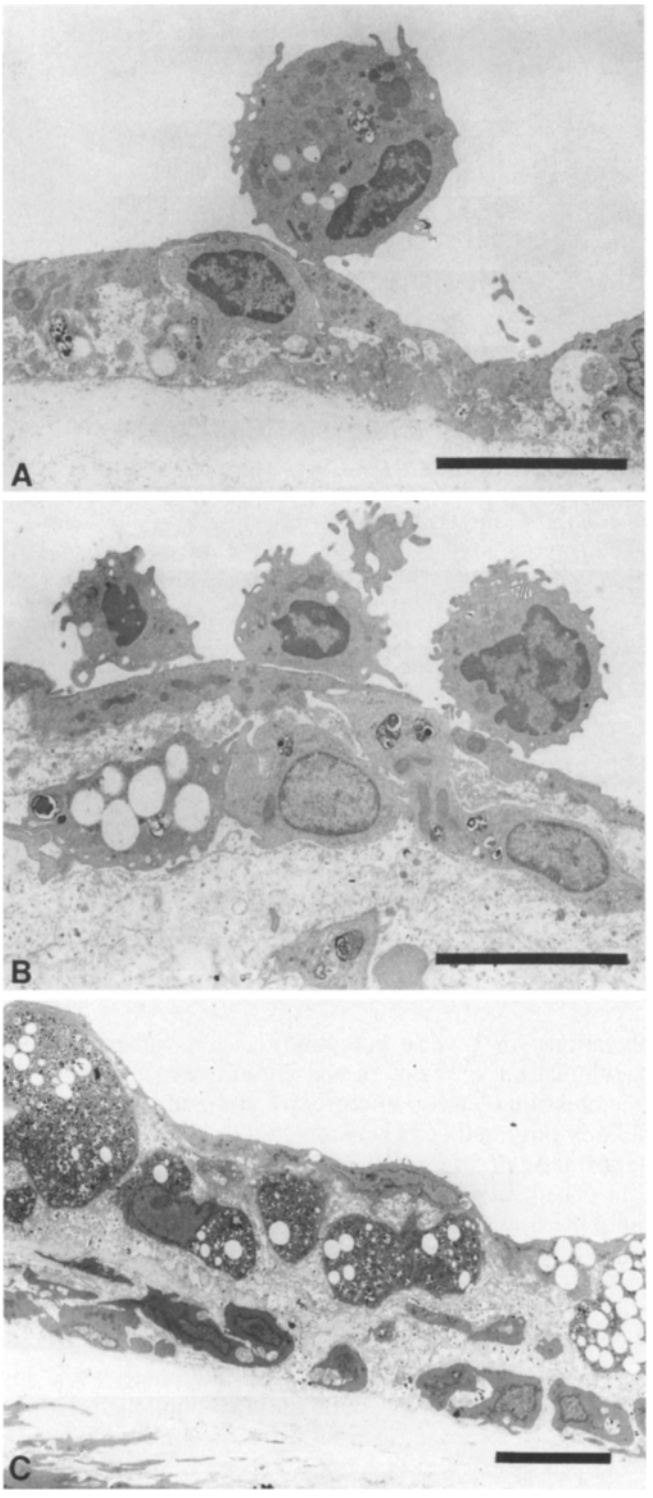

Fig. 2A–C Transmission electron micrographs of aorta from rats on atherogenic diet. **A** Aorta of rat after 2 weeks on diet shows adherent mononuclear cell on surface. Another mononuclear cell (probably a lymphocyte) is seen beneath the endothelium extending cytoplasmic processes. Note attenuation of the overlying endothelial cytoplasm. *Bar*=5 μm. **B** Aorta of rat after 10 weeks on diet. Three mononuclear cells are attached on surface. Mononuclear cells that have migrated into the intima have clear or osmiophilic lipid inclusions. These mononuclear cells contact each other with cytoplasmic processes. *Bar*=5 μm. **C** Aorta of rat after 20 weeks on diet. Lipid laden macrophages (foam cells) lie immediately beneath endothelium. Beneath the macrophages are several smooth muscle cells that are elongated to stellate in shape with short, thick extensions of cytoplasm from the cell body. There are also accumulations of extracellular matrix, lipid deposits and other dense debris around and between the cells. Notice lipid vacuoles in endothelial cell cytoplasm. *Bar*=10 μm

that participated in cellular events related to atherogenesis. We also determined the distribution of changes in a large region of the aorta because the intima and media of the vessel wall was whole-mounted to be viewed en face.

Under light microscopy, mononuclear cells adhering to the surface and in migration below the endothelium stood out clearly against the unstained background, since for T lymphocytes OX19 antibody was enhanced by the addition of peroxidase-conjugated streptavidin yielding a brown reaction while ED1-positive macrophages were blue-coloured by treatment with alkaline phosphatase reaction. In control animals, cells stained by these antibodies were usually single and widely scattered; their location bore no apparent relations to anatomical level or to branches or bifurcations. Animals on an atherogenic diet showed clusters and swarms of stained cells. Figure 3 illustrates temporal changes of lesion development schematically in the thoracic and abdominal segments of the aorta. In the thoracic aorta, lesions had a tendency to develop in the dorsal aspect of the intima often localized around branch sites. However, the ventral portion appeared more susceptible to the atherosclerotic process in the abdominal aorta where an elongated lesion developed along the axis.

Temporal changes in the number of T lymphocytes and macrophages in adhesion and migration are shown in Figure 4. After 2 weeks on the diet, the total number of infiltrating cells (the combined number of T lymphocytes and macrophages) was significantly increased in the thoracic and abdominal segments. Infiltration of T lymphocytes was most prominent in this period and they accounted for 60%, 29% and 34% of mononuclear cells that appeared in the superior thoracic, inferior thoracic and abdominal segments of the aorta, respectively. Particularly in the superior thoracic aorta, T lymphocytes outnumbered macrophages at this incipient phase of lesion formation. Thereafter, the relative number of T lymphocytes in the three segments of the aorta showed a slow decline, while infiltration of macrophages exhibited a continuous rise. Consequently, the proportion of T lymphocytes to macrophages reached approximately 1:3 to 1:4 in the thoracic and abdominal segments after 10–20 weeks on the diet. At 40 weeks into the diet, assessment of cell populations was often difficult because of break-down of foam cells and the associated accumulation of cell debris and lipids.

T lymphocytes and macrophages were observed in the area near the branches (Fig. 5A) as well as in non-branching areas (Fig. 5B) within 2 weeks on the diet. In terms of cell morphology, T lymphocytes assumed two

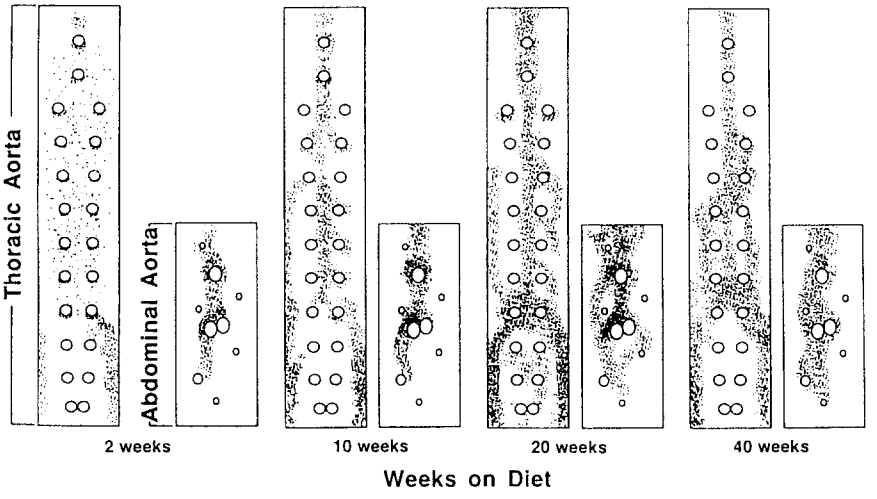

Fig. 3 This diagram demonstrates the temporal change in anatomical distribution of aortic lesions, examined en face with double immunostaining. The thoracic segment is slit open along the ventral side; abdominal segment along the dorsal side of the aorta

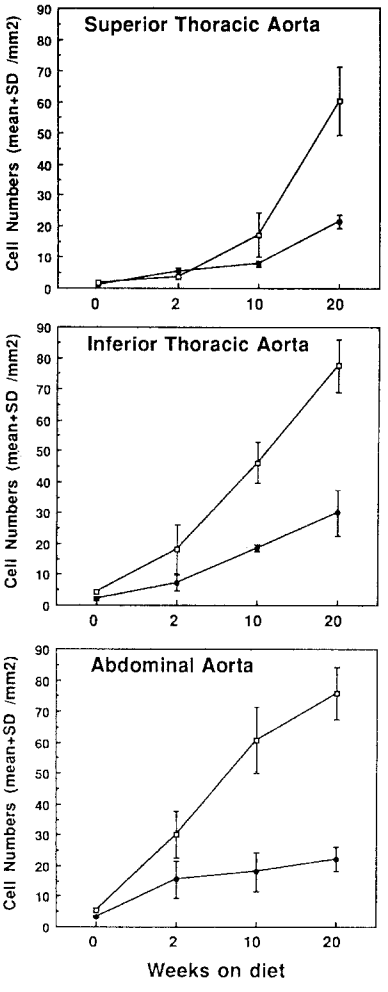

Fig. 4 Temporal changes in cell density of T lymphocytes and macrophages defined by monoclonal antibodies using en face double immunostaining. Data represent numbers of OX19-positive T lymphocytes (*solid circles*) and ED1-positive monocytes/macrophages (*open squares*) in the intima of different phases of diet-induced aortic lesions [mean±standard deviation (SD), four per group]. In all phases of lesion development, both cell numbers are significantly increased from those in week 0

distinct appearances; round to oval in one group and stellate to dendritic in the other. The cells of the latter form spread cytoplasmic processes and seemed to probe for migration in the intima (Fig. 5C). They tended to be around branch sites of the thoracic aorta. Macrophages also took on various shapes, from round or elongated to stellate or dendritic outlines, and possessed variable numbers of empty-appearing fine lipid vacuoles (Fig. 5D). Moreover, T lymphocytes and macrophages were often co-located in intimate contact (Fig. 5E).

From 10 weeks onwards, macrophages predominated beneath the endothelium and became progressively larger in size (Fig. 6A). They accumulated lipid vacuoles in their plump cytoplasm and took on an appearance of foam cells, especially in the inferior thoracic and abdominal segments of the aorta. Such clusters of foam cells could be described as fatty streaks when observed under a transmission electron microscope. At this stage most of the T lymphocytes were round to oval in shape. The direct cellular contact was often noted not only between T lymphocytes and macrophages but also between T lymphocytes or macrophages themselves. Further, OX6/ED1 double immunostaining demonstrated that Ia antigen was expressed on most macrophages (Fig. 6B). At later stages small raised lesions were formed particularly around the branches in the abdominal segment of the aorta (Fig. 6c). They were mostly composed of foam cells, and participation of T lymphocytes appeared less in those advanced lesions. The extracellular space contained cell debris or amorphous lipid material that was well observed after stepping down of the focus.

Discussion

The role of T lymphocytes in atherosclerosis has not been fully examined in animal models for the following reasons. Firstly, cell identification is difficult by conventional morphological methods even including ultrastructural study. In this context, advent of the monoclonal antibody methodology is epoch-making and its application

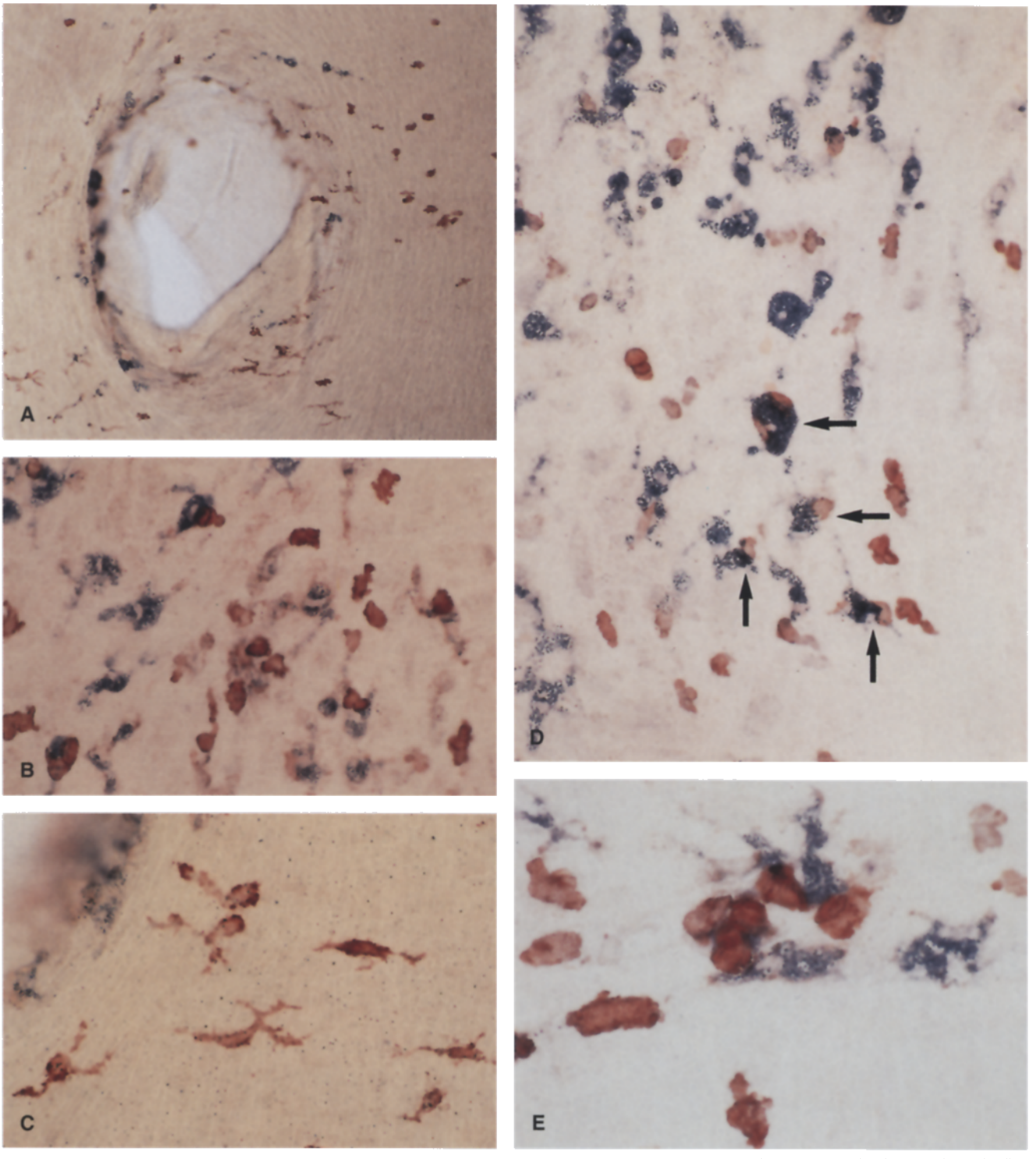

Fig. 5A–E Aortic intima of rat after 2 weeks on diet viewed en face after double immunostaining. OX19-positive T lymphocytes are brown-coloured with peroxidase method; ED1-positive macrophages are blue-coloured with alkaline phosphatase treatment. **A** Incipient lesion of thoracic segment of the aorta demonstrates infiltration of T lymphocytes and macrophages around the ostium of an arterial branch. Direction of blood stream is from right to left of the figure (×130). **B** Focal cluster of T lymphocytes and macrophages in non-branching area of thoracic segment. T lymphocytes often outnumber macrophages at incipient phase of lesion formation (×400). **C** Area of thoracic segment near branch orifice of an artery demonstrates a cluster of T lymphocytes with stellate or dendritic appearance (×400). **D** Abdominal segment of aorta demonstrates more prominent infiltration of T lymphocytes and macrophages than thoracic segment. Macrophages take on various shapes, and some of them contain empty fatty vacuoles. Note intimate cellular apposition between T lymphocytes and macrophages (*arrows*; ×400). **E** High-power view of direct cell-to-cell interaction between T lymphocytes and macrophages (×800)

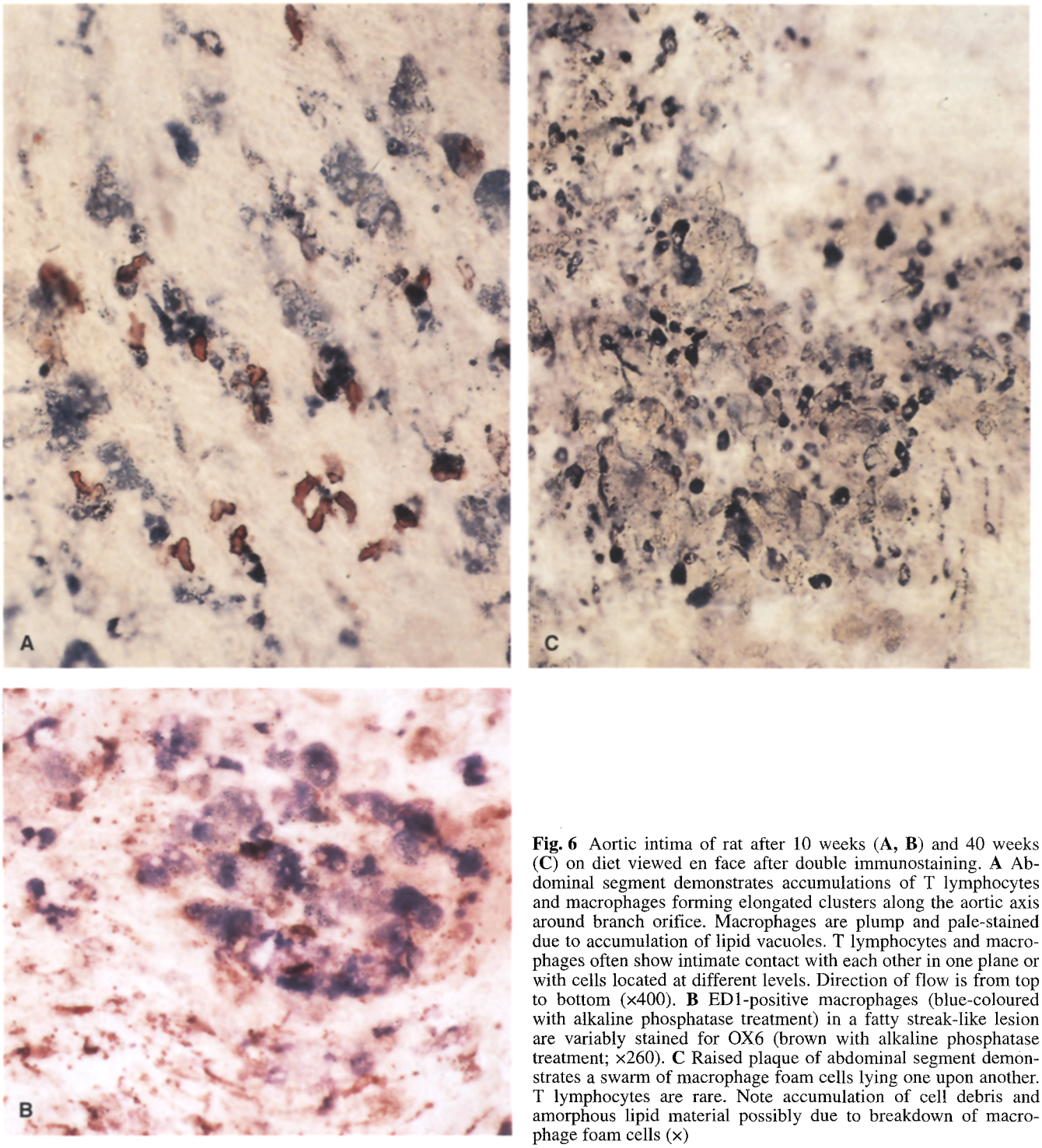

Fig. 6 Aortic intima of rat after 10 weeks (**A, B**) and 40 weeks (**C**) on diet viewed en face after double immunostaining. **A** Abdominal segment demonstrates accumulations of T lymphocytes and macrophages forming elongated clusters along the aortic axis around branch orifice. Macrophages are plump and pale-stained due to accumulation of lipid vacuoles. T lymphocytes and macrophages often show intimate contact with each other in one plane or with cells located at different levels. Direction of flow is from top to bottom (×400). **B** ED1-positive macrophages (blue-coloured with alkaline phosphatase treatment) in a fatty streak-like lesion are variably stained for OX6 (brown with alkaline phosphatase treatment; ×260). **C** Raised plaque of abdominal segment demonstrates a swarm of macrophage foam cells lying one upon another. T lymphocytes are rare. Note accumulation of cell debris and amorphous lipid material possibly due to breakdown of macrophage foam cells (×)

to immunohistological studies is indispensable for accurate identification of the cell type. Secondly, quantitative analysis of the cell is often difficult by observations in conventional tissue sections. The cell volume differs between macrophages (in particular, foam cells) and lymphocytes in the lesion area. Thus T lymphocytes appear to be many fewer than the actual number evaluated using conventional tissue sections. One might well underestimate the contribution of T lymphocytes in lesion development.

To delineate the cellular interactions between T lymphocytes and macrophages in atherogenesis, we examined the aorta of rats with diet-induced hypercholesterolaemia by en face double immunostaining coupled with scanning and transmission electron microscopy. Rats responded to an atherogenic diet by developing aortic lesions resembling human fatty streaks in their topographic distribution [35, 42]. Introduction of en face double immunostaining technique facilitated simultaneous identification and quantification of two types of immunocompe-

tent cells, T lymphocytes and macrophages, in a single preparation covering from the descending thoracic to the abdominal aorta. Of note was that infiltration of T lymphocytes was particularly prominent in the early phase of lesion formation, accounting for 60%, 29% and 34% of mononuclear cells that appeared in the superior thoracic, inferior thoracic and abdominal segments of the aorta, respectively. These figures were far greater than those observed by Hansson et al. [15] in early atherosclerotic lesions of cholesterol-fed rabbits in which T lymphocytes constituted 7.4% of the cell population as determined by counting the cells in routine histological sections.

The presence of large numbers of T lymphocytes in all stages of atherosclerosis indicates that these cells may play a biologically significant role in the development of atherosclerotic lesions. However, the mechanisms that attract T lymphocytes to the lesional area and activate them within the intima are unknown. The intimate co-localization of T lymphocytes and Ia-expressing macrophages even at the incipient phase of lesion development may imply a role for cellular immune mechanisms. T lymphocytes are activated by the complex of foreign antigens and Ia molecules expressed on the antigen-presenting cells [30]. Many experimental studies suggest that these initial interactions of T lymphocytes and macrophages require physical interaction between these two types of cells [22, 41]. Our recent observations [18] that T lymphocytes in human lesions express activation markers such as the interleukin-2 receptor further support the notion that immune response may be taking place. The nature of such a stimulus would be a key problem regarding the immunological aspects of atherosclerosis. In fact, possible candidate antigens in human atherogenesis have recently been reviewed [21], but the nature of the antigenic trigger to T lymphocytes remains unresolved.

In addition to their role in antigen recognition, activated T lymphocytes and macrophages can secrete potent biological mediators that modify vascular cell functions [1, 28]. Several T lymphocyte products such as interferon gamma are potent macrophage activators [24]. Both interleukin-1 and tumour necrosis factor are pleiotropic cytokines produced by many cell types, most notably by activated macrophages [23]. These mediators serve as prototypic inflammatory cytokines and increase the expression of adhesion molecules on the vascular endothelial cells thereby enhancing both adhesion and locomotion of monocytes [6, 20]; interferon gamma however is shown to promote the adherence of macrophages to basement membrane glycoproteins [31]. Besides, interferon gamma is a potent inhibitor of vascular smooth muscle cell growth [11, 38]. In this context, Hansson and Holm [10] have shown that interferon gamma, when administered exogenously, significantly inhibits lesion formation after arterial denudation in rats. This observation supports the notion that interferon gamma is an important regulator of tissue responses after injury.

Several pieces of evidence suggest that factors secreted by activated T lymphocytes could modulate macrophage function to accumulate modified (or oxidized) lipoproteins via scavenger receptors as macrophages differentiate into foam cells. Fogelman et al. [7] have shown that supernatants from concanavalin A-stimulated lymphocytes can suppress subsequent degradation of malondialdehyde-modified LDL cultured monocytes. Recently, Fong et al. [8] have demonstrated in a murine system that interferon gamma produced by T lymphocytes inhibits acetyl-LDL degradation by mouse peritoneal macrophages. Geng and Hansson [9] have further reported that interferon gamma blocks the formation of macrophage-derived foam cells by inhibiting expression of the scavenger receptor. Taken together, those investigators suggest that T lymphocyte-macrophage interactions may reduce intracellular cholesterol accumulation in the atherosclerotic lesion. In contrast Kotake et al. [19] have recently provided evidence that T lymphocytes activated by coculture with macrophages can produce a substance that increases the synthesis of esterified cholesterol in macrophages by activation of the scavenger pathway.

As in human atherosclerotic lesions [18, 33], the present study has demonstrated that direct cell-to-cell contact between T lymphocytes and macrophages is a common finding in the hypercholesterolaemic rat model. The intimate association between T lymphocytes and macrophages has been described in a variety of human diseases such as infections and various kinds of granulomatous lesions including temporal arteritis [32], tuberculosis [36], leprosy and sarcoidosis [25, 36]. The role played by cognate cell-to-cell interaction in these studies can only be speculated upon, however; it seems reasonable to suggest that direct cellular contact is as important as indirect spillover or influence of humoral factors in the dynamic microenvironment of the diverse pathological processes of atherosclerosis.

In conclusion, by introducing en face double immunostaining the present study has demonstrated that together with macrophages, large numbers of T lymphocytes participate in aortic lesions of the rats with diet-induced hypercholesterolaemia. Both cell types were present at all stages, initially adhering to the surface at prelesional stages and subsequently as major components of fatty streak-like lesions. These observations suggest that interactions between T lymphocytes and macrophages play a pivotal role in the formation of atherosclerotic lesions. It is clear that pathogenetic potential of immunological factors in atherosclerosis deserves further investigation.

Acknowledgements The authors are grateful to Fumihiro Muto, Shinichi Nakahara and Hisami Tabata for their technical assistance. They thank Professor K. Tokaichi for his critical reading of the manuscript. This study was supported by a grant-in-aid for scientic research from the Ministry of Education (No. 04454181), and grants from University of Tsukuba research projects and Mitsui Life Social Welfare Foundation.

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