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Monoclonal antibodies recognizing lipid-laden cells and extracellular regions with lipid-deposits in atherosclerotic aorta

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Summary. Monoclonal antibodies against lipid-laden cells and against extracellular regions of lipid-deposits in atherosclerotic aorta were prepared. Mice were immunized with a delipidated homogenate of atherosclerotic aorta of Watanabe-heritable hyperlipidemic rabbits. Hybridomas were obtained by fusion and cultured in hypoxanthine, aminopterin and thymidine selection medium. Specific antibodies were selected by indirect immunohistochemical staining of frozen sections of atherosclerotic aorta. Nine clones that produced antibodies that stained the atherosclerotic intima exclusively were selected and cloned by limiting dilution. Finally two clones (FCR1a/201F, FCR1b/904B) producing antibodies specific to lipid-laden cells and one clone (EMR1a/212D) producing an antibody specific to regions with lipid deposits in the extracellular matrix were established. These monoclonal antibodies may help in understanding how lipids accumulate in atherosclerosis.

Key words: Monoclonal antibody – Atherosclerosis – Lipid deposit – Foam cell – Extracellular lipid

Introduction

A striking characteristic of atherosclerosis is the accumulation of large amounts of lipids, mainly cholesterol ester, in the arterial wall. Histochemical and ultrastructural observations have shown that these lipids accumulate in both the cytoplasm of foam cells and the extracellular matrix. The foam cells have been suggested to originate from macrophages (Adams and Bayliss 1976; Brown and Goldstein 1983; Fowler et al. 1979; Gaton and Wolman 1977; Gerrity 1981; Shaffner et al. 1980; Watanabe et al. 1985), or modified smooth muscle cells (Geer and Haust 1972; Ross and Glomset 1976), while it has been suggested that the extracellular lipid deposits have originated from circulating lipoproteins or denatured lipoproteins derived from ruptured foam cells (Geer and Haust 1972; Takano et al. 1985). These extracellular lipid-deposits may be endocytized by macrophages and/or modified smooth muscle cells, resulting in formation of foam cells. To study the mechanisms of formation and destruction of foam cells, and of how cells are transformed into foam cells, we prepared monoclonal antibodies against atherosclerotic lesions using a delipidated crude homogenate of atherosclerotic aorta as a complex mixture of immunogens by the hybridoma technique established by Köhler and Milstein (1975). On selection of specific antibodies by histochemical staining, we obtained for the first time monoclonal antibodies that specifically recognized lipid-laden cells (FCR1a/201F, FCR1b/904B) and the extracellular matrix with lipid-deposits (EMR1a/212D) in atherosclerotic lesions.

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Materials and methods

Preparation of monoclonal antibodies. Female homozygous WHHL (Watanabe-heritable hyperlipidemic) rabbits of about 28 months old were anesthesized, and the intima and media of atherosclerotic aorta were removed. Pieces of these tissues were homogenized with 0.25 M SVE (0.25 M sucrose, 1 mM versene and 0.1% ethanol) in a polytron homogenizer. The homogenate was overlayed with an equal volume of 1 mM EDTA in 0.1% ethanol and centrifuged at 220 × g for 10 min. The upper layer containing a large amount of lipid was discarded, and the bottom layer was treated by 4 vol. of acetone.

The resulting precipitate was suspended in PBS (phosphate buffered saline) and used as antigen solution.

Female BALB/c mice were immunized 3 times with this antigen solution over a period of 3 months. Spleen cells from immunized mice were removed 3 days after the final injection. These spleen cells were fused with myeloma P3/U1 and cultured in HAT (hypoxanthine, aminopterin and thymidine) selection medium according to the standard procedure (Oi and Herzenberg 1980). The culture medium of hybridomas was assayed as described below, and antibody-bearing hybridomas were cloned by limiting dilution.

The generation of monoclonal antibodies was first assayed by ELISA (enzyme-linked immunosorbent assay). Homogenates treated by acetone were suspended in 100 vol. of PBS and plated in immunoplates. The immunoplates coated with the antigens were reacted with culture-medium of the hybridomas. Alkaline phosphatase-conjugated goat antimouse Ig (A + G + M) (Cappel Lab. Inc., USA) was used as second antibody.

Cells in culture wells that showed relatively high activity on ELISA were further selected by indirect immunofluorescence microscopy (Fujita et al. 1982). Immunofluorescent staining was performed on frozen sections (4–6 μm) of atherosclerotic and normal aorta, which were fixed with 10% neutral formalin immediately after autopsy. Normal aorta was prepared from a Japanese White rabbit. FITC (fluorescein-isothiocyanate)-conjugated goat antimouse IgG (H + L) (Cappel Lab. Inc., USA) was used as the second antibody. As negative controls, the frozen sections were stained with culture medium of myeloma P3/U1 and then treated with the second antibody.

Identification of regions recognized by monoclonal antibodies. Frozen sections prepared from atherosclerotic and normal aorta of female homozygous WHHL rabbits were incubated with culture medium and then subjected to immunofluorescence staining as described above. The regions recognized by the monoclonal antibodies and those stained with oil-red O were examined in alternate sections, for comparison of these regions with lipid deposits. Paraffin embedded sections prepared from atherosclerotic aorta were also stained immunohistochemically. Adjacent sections were also stained with Mayers' haematoxylin eosin in these experiments.

The classes and subclasses of monoclonal antibodies were determined by the method of Ouchterlony with antimouse IgG_1, G_{2a}, G_{2b}, G_3, M, and A (Miles Sci. Ind.).

Preparation of monoclonal antibodies specific to atherosclerotic lesions. Spleen cells prepared from BALB/c mice sensitized with delipidated homogenates of atherosclerotic aorta were fused with P3/U1 myeloma and cultured in 10 tissue culture plates containing 96 wells. The 10×98 wells containing hybridomas were examined for alkaline phosphatase activity by ELISA and 362 wells that shows relatively high activity were selected. The hybridomas out of 362 wells were selected by immunohistochemical staining: clones producing monoclonal antibodies that stained atherosclerotic lesions but not normal aorta were selected. Finally 2 clones producing antibodies against lipid-laden cells (FCR1a/201F, FCR1b/904B) and one clone producing antibody against extracellular materials (EMR1a/212D) were obtained after limiting dilution.

The antibodies from FCR1b/904B and EMR1a/212D were of the IgG_1 subclass and that from FCR1a/201F was IgM.

Results

The immunofluorescence of antibody derived from clone FCR1a/201F was observable both on paraffin sections that had been delipidated during the dehydration procedure and frozen sections presoaked in acetone for 5 min. In paraffin sections, this antibody was seen to stain the cytoplasmic area of cells filled with lipids (Fig. 1a, b). It did not stain arterial cells not containing lipids. Microscopic observation of frozen sections stained with oil-red O indicated that this antibody did not stain extracellular regions containing lipid-deposits (Fig. 2a, b).

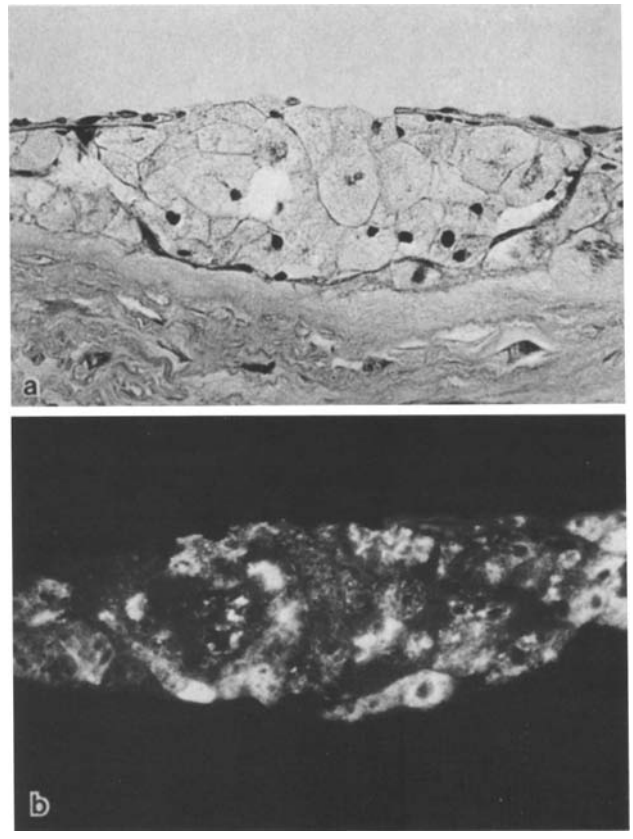

Fig. 1a, b. Typical staining pattern with FCR1a/201F antibody. **a** Foam cells are assembled beneath the endothelium. (Haematoxylin eosin staining, ×250). **b** Immunofluorescence with FCR1a/201F antibody is localized in the cytoplasm of foam cells. The nuclei of foam cells are not stained. (Indirect immunofluorescent staining of the adjacent section to that for **a**, ×250)

The specificity of antibody from clone FCR1b/904B was similar to that of antibody from FCR1a/201F: this antibody stained the cytoplasmic area of lipid-laden cells, but not extracellular regions containing lipid deposits (Figs. 3 and 4).

Two types of immunofluorescence-positive lipid-laden cells were observed: one type was round and was located beneath the endothelium (Figs. 1, 2 and 4) while the other was elongated and extended from the internal elastic lamina toward the lumen (Fig. 3). The necrotic cells located at the

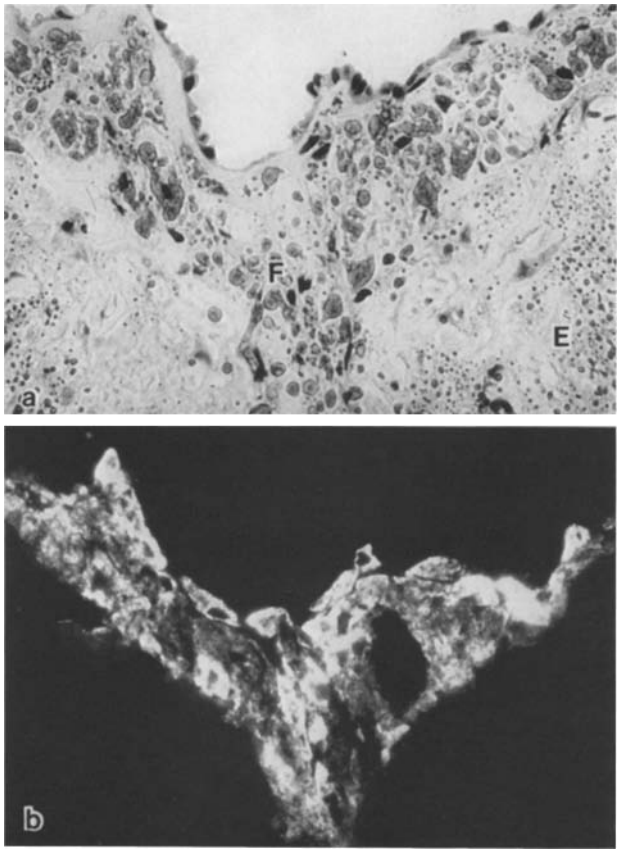

Fig. 2a, b. Correlation of immunofluorescence of FCR1a/201F antibody staining and lipid-deposits. **a** Both intracellular (F) and extracellular (E) lipid-deposits in atherosclerotic lesions are stained with oil-red O. (Oil-red O and haematoxylin staining, ×300). **b** Only intracellular lipid-deposits show immunofluorescence with FCR1a/201F antibody. (Indirect immunofluorescent staining of the adjacent section to that for **a**, ×300)

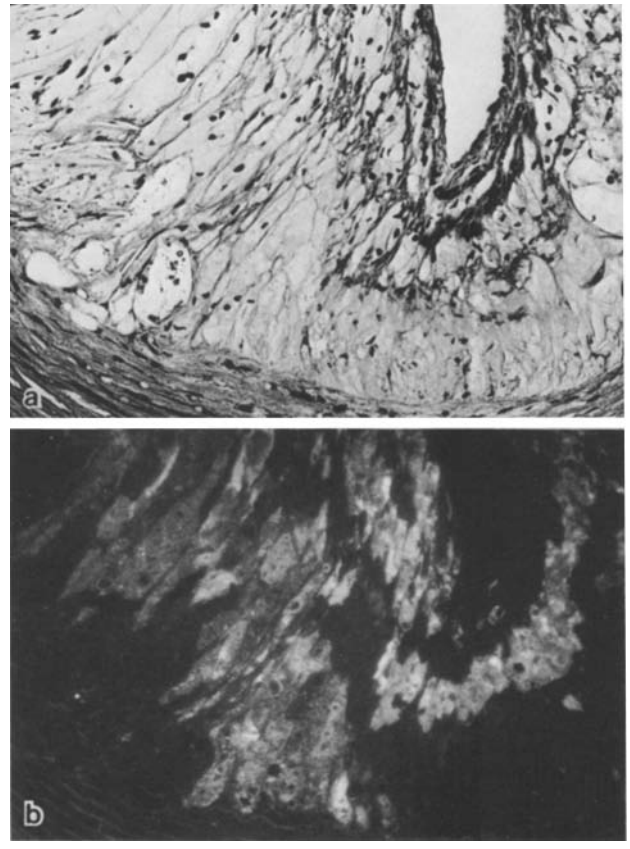

Fig. 3. Staining with FCR1b/904B antibody of foam cells extending from the internal elastic lamina toward the lumen. **a** Assembled foam cells and intimal smooth muscle cells extend from the internal elastic lamina toward the lumen. On the left, there is a necrotic area containing needle like crystals of cholesterol. (Haematoxylin eosin staining, ×150). **b** Immunofluorescence on staining with FCR1b/904B antibody is restricted to the cytoplasm of foam cells. Endothelial cells, smooth muscle cells and the necrotic area are not stained. (Indirect immunofluorescent staining of the adjacent section to that for **a**, ×150)

bottom of the intima, shown in Fig. 3a, were not stained.

The monoclonal antibody derived from clone EMR1a/212D stained both paraffin sections and frozen sections pre-soaked in acetone. Immunofluorescent staining of paraffin sections showed that this antibody stained extracellular regions of atherosclerotic intima (Fig. 5a, b). The antibody did not stain either arterial cells or the extracellular matrix of the media. Immunofluorescent staining and oil-red O staining of adjacent frozen sections were examined in parallel (Fig. 6a, b). The antibody stained extracellular regions of the intima in which there were deposits of neutral lipids, but it did not stain extracellular regions in which there were no lipid deposits. The antibody did not stain foam cells, although they contained large amounts of lipids. From these results, the antibody of EMR1a/212D was concluded to be specific for regions with lipid deposits in the extracellular matrix in the atherosclerotic intima.

Discussion

In the present investigation, 3 monoclonal antibodies against atherosclerotic aorta were prepared. The monoclonal antibodies derived from FCR1a/201F and FCR1b/904B recognized an antigen(s) localized in the cytoplasmic part of lipid-laden cells, whereas the antibody from EMR1a/212D recognized an antigen localized in extracellular deposits of lipids.

The monoclonal antibodies of FCR1a/201F and FCR1b/904B specifically stained cytoplasmic sites of lipid-laden cells, but did not stain any arterial cells without lipid (Figs. 1–4). The staining was

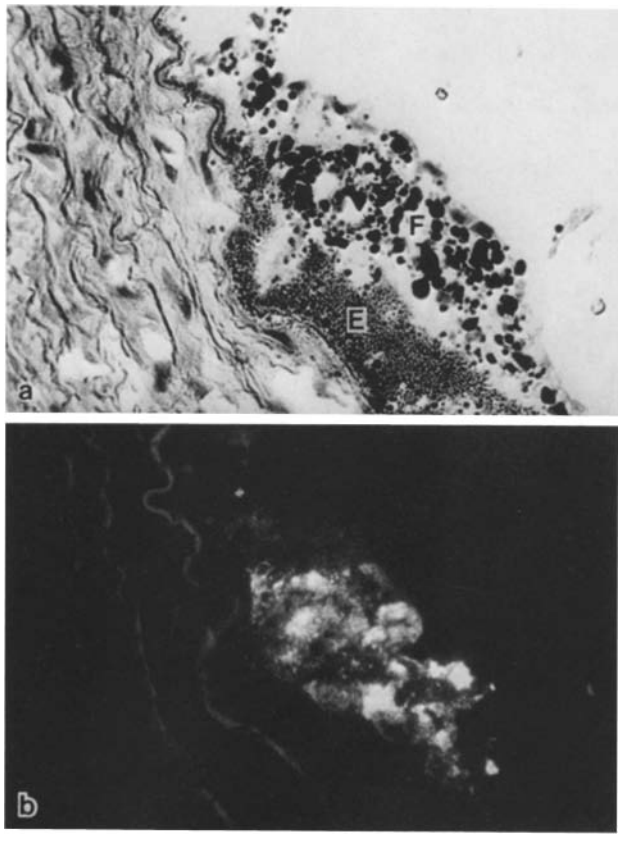

Fig. 4a, b. Correlation of immunofluorescence on FCR1b/904B antibody staining and lipid-deposits. **a** Both intracellular (F) and extracellular (E) lipid-deposits in atherosclerotic lesions are stained with oil-red O. (Oil-red O and haematoxylin staining, ×250). **b** Only intracellular lipid-deposits show immunofluorescence with FCR1b/904B antibody. (Indirect immunofluorescent staining of the adjacent section to that for **a**, ×250)

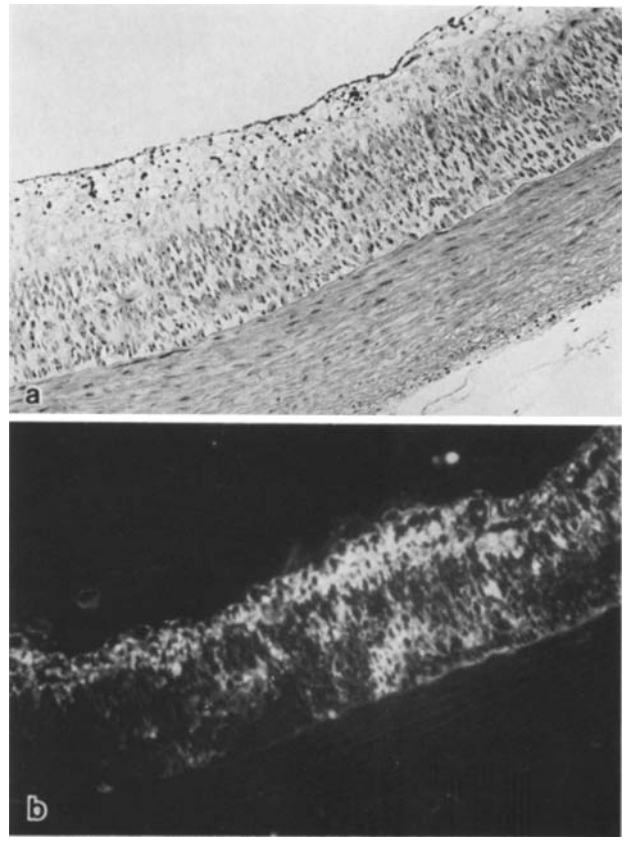

Fig. 5a, b. Comparison of immunofluorescence with EMR1a/212D antibody and haematoxylin eosin staining of atherosclerotic lesions. **a** Foam cells are assembled beneath the endothelium, and intimal smooth muscle cells oriented toward the lumen are distributed from the bottom to the middle of lesions. (Haematoxylin eosin staining, ×115). **b** Immunofluorescence with EMR1a/212D antibody is restricted to the extracellular matrix of atherosclerotic lesions, with no fluorescence in foam cell-rich areas beneath the endothelium. (Indirect immunofluorescent staining of the adjacent section to that for **a**, ×115)

not reduced when frozen sections were soaked in acetone or when paraffin sections were delipidated during the dehydration procedure, suggesting that the hapten group against these antibodies is not lipid. Two types of antibody-positive cells, round and elongated, were recognized. The round lipid-laden cells, distributed beneath the endothelium and recognized by these antibodies (Figs. 1, 2) may originate from macrophages, because their shape and the density of their cytoplasm were similar to those of macrophages. Many investigators have suggested formation of foam cells from macrophages (Adams and Bayliss 1976; Fowler et al. 1979; Gaton and Wolman 1977; Gerrity 1981; Shaffner et al. 1980; Watanabe et al. 1985). The other lipid-laden cells stained by these antibodies were elongated and extended from the internal elastic lamina toward the lumen (Fig. 3). These cells are unlikely to be derived from macrophages, because recently Watanabe et al. found that monoclonal antibody against macrophages did not stained elongated lipid-laden cells, although it stained round lipid-laden cells (Watanabe et al. 1985). These elongated cells may originate from smooth muscle cells. The existence of foam cells of smooth muscle cell origin has also been suggested by many other workers (Geer and Haust 1972; Ross and Glomset 1976). Thus, the antibodies of clones FCR1a/201F and FCR1b/904B seem to stain two types of cells of different origins, indicating that they do not stain specific cells, but cells with cytoplasmic accumulations of lipid. Thus these antibodies may recognize some materials induced as a result(s) of intracellular lipid accumulation, regardless of the species of lipid-laden cells.

The antibody of clone EMR1a/212D bound

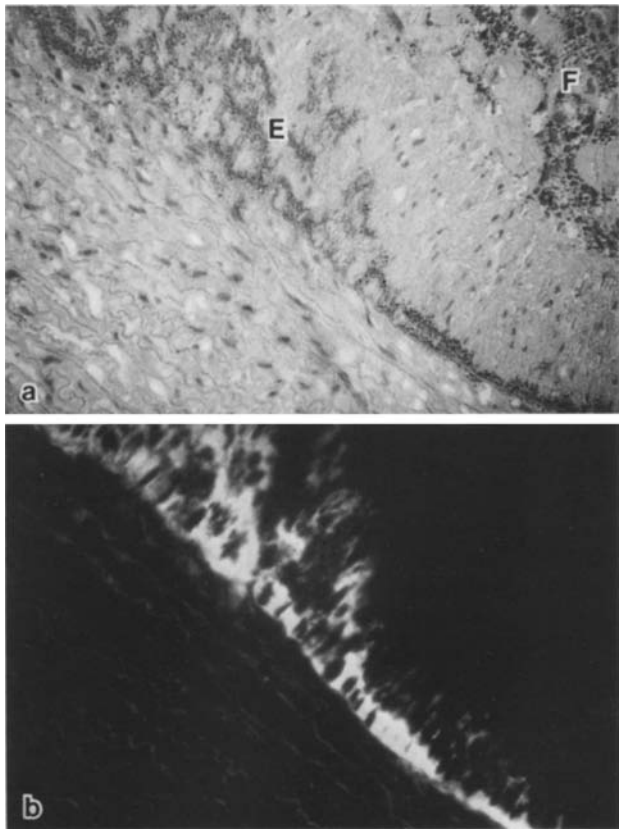

Fig. 6a, b. Correlation of EMR1a/212D antibody staining and lipid-deposits. **a** Two major areas of lipid-deposits are observed in atherosclerotic lesions by oil-red O staining. Large droplets stained with oil-red O in contact with nuclei (F) are lipids in the cytoplasm of foam cells and small scattered droplets near the elastic internal lamina (E) are lipid-deposits in the extracellular matrix. (Oil-red O and haematoxylin staining, ×150). **b** Immunofluorescence on EMR1a/212D antibody staining is restricted to extracellular regions with lipid-deposits. (Indirect immunofluorescent staining of the adjacent section to that for **a**, ×150)

specifically to intimal extracellular regions containing lipid-deposits (Figs. 5 and 6) although it is uncertain whether it stained only the intima in advanced atheromas penetrating into the media. It also stained tissue sections that had been delipidated, suggesting that its antigen in the extracellular matrix is not lipid. The antigenic material must be a protein or glycoprotein located in the extracellular matrix, since the EMR1a/212D antibody recognized 3 bands (Mw = 40,000–70,000) on immunoblots of a homogenate of atherosclerotic aorta separated by SDS-polyacrylamide gel electrophoresis (unpublished data). Possible antigenic materials that have been shown to exist in the extracellular matrix are glycosaminoglycans (Mawhinney et al. 1978; Hollander 1976) and apo-lipoproteins (Kao and Wissler 1965; Walton and Williamson 1968; Hoff et al. 1974, 1975; Yamauchi and Hoff 1984). Another possibility is that the antigen of the EMR1a/212D antibody is denatured lipoprotein deposited in the extracellular matrix. Some materials tightly bound to lipid droplets derived from ruptured foam cells could also be antigens of the EMR1a/212D antibody.

The three monoclonal antibodies obtained in this work should be very useful in studies on the mechanism of lipid accumulation in atherosclerosis. These antibodies can distinguish lipid-deposits in two regions, the cytoplasm of lipid-laden cells and extracellular regions containing lipid-deposits. Thus they should provide information on the nature or origin of lipids accumulated in these two regions. The antibodies recognizing the cytoplasmic part of lipid-laden cells should be useful for determining the cell species accumulating lipid and the mechanism of intracellular lipid accumulation, because they did not stain any arterial cells that did not contain lipid inclusions, and because they seemed to recognize different kinds of cells containing accumulated lipids. These antibodies will be helpful in selecting suitable conditions for establishing a model system of intracellular lipid accumulation *in vitro*, such as a good cell species, and also in determining the nature of the lipids deposited.

This is the first report of monoclonal antibodies recognizing lipid-laden cells and extracellular regions containing lipid-deposits in the atherosclerotic intima. These antibodies should be useful in studies on the pathogenesis of atherosclerosis in relation with intracellular and extracellular lipid accumulation, in developing effective chemotherapeutics, and in angiographic diagnosis of atherosclerosis.

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