Transdifferentiation of macrophages into fibroblasts as a result of *Schistosoma mansoni* infection

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ABSTRACT The possibility of transdifferentiation of macrophages into fibroblasts which could be at the origin of fibrotic tissue in schistosome-infected mice was studied using immunocytochemical techniques. Macrophage cell samples extracted from the peritoneal cavity of schistosome-infected mice were fractionated on a Percoll gradient. The cultures were purified by treatment with a trypsin solution to eliminate any fibroblasts possibly collected along with the macrophages. Immunocytochemical methods were then used to characterize the cells at different points in time. The fibroblastic property of the morphologically transformed cells was confirmed by their positive labeling with the anti-procollagen antibody. However, these cells still possessed the mac-1 and mac-2 antigens which characterize the monomacrophage line.

KEY WORDS: *transdifferentiation, peritoneal macrophages, fibroblasts, schistosomiasis, immunocytochemistry*

Introduction

Schistosomiasis is a disease caused by the presence of a trematode worm of the genus *Schistosoma* in the portal and mesenteric venous system (Cox, 1987). Eggs laid by these parasites are the principal cause of pathological phenomena in man. Out of 300 eggs laid per day and per couple, only two-thirds are eliminated into the external medium. The remaining third produces multiple symptoms (Andrade, 1963): capillary embolisms, granulomas in the sinusoids of the liver and sometimes in the lungs, and development of portal fibrosis (Symmers' fibrosis), a disease that leads to the host's death.

The granulomatous reaction is a delayed hypersensitivity reaction (Smith, 1977; Epstein *et al.*, 1979). This can be seen by the fact that T lymphocytes sensitized by miracidium-produced antigens secrete various macrophage- and eosinophil-activating cytokines. The resulting granulomas are mostly composed of lymphocytes, neutrophils, eosinophils (Borojévic *et al.*, 1986), macrophages, and fibroblasts (Wyler *et al.*, 1978). The granulomas also contain an abundant extracellular matrix composed of laminin and type-I, -III, and -IV collagens. The granuloma-associated cell populations and extracellular matrix evolve over time. The granulomas become smaller and more fibrous. They become increasingly effective in destroying the miracidium (Stenger *et al.*, 1967). In man, granulomatous reactions lead to accumulation of fibrous tissues in the portal spaces, a condition called Symmers' fibrosis (Lichtenberg, 1964; Stocker *et al.*, 1983). The granulomas and Symmers' fibrosis obstruct the hepatic capillaries. This causes hepatomegaly and portal hypertension, fatal to the host. To date, the origin of this fibrotic tissue is unknown. Different hypotheses have been proposed regarding the cells responsible for the observed fibrotic phenomena.

First, there is the Ito cell hypothesis. These mesenchymatous cells located principally in the space of Disse could be responsible for the synthesis of an abundant extracellular matrix in some pathological cases (Bioulac-Sage and Balabaud, 1985; De Bleser *et al.*, 1991; Geerts *et al.*, 1991). Another hypothesis is transdifferentiation of macrophages into fibroblasts. This possibility was proposed by J. Kouri in 1971 (Kouri and Ancheta, 1972) and echoed by authors such as Campbell (Mosse *et al.*, 1985) and Borojévic (1989). It is based on the observed evolution of cultured macrophages extracted from mice in the chronic stage of schistosomiasis infection (Godoy *et al.*, 1989).

In previous work on *Schistosoma mansoni* conducted in this laboratory, the fibroblast-like cells appearing in macrophage cultures obtained from chronic-stage mice were identified on the basis of general morphological and ultrastructural criteria. Here, we have used double immunocytochemical labeling to characterize these cells. Anti-procollagen antibody was used to detect the fibroblast phenotype while antibodies raised against mac-1 (Beller *et al.*, 1982), mac-2 (May-Kinho and Springer, 1982), or mac-3 were used to specifically label monomacrophage cells.

Abbreviations used in this paper: HBSS, Hank's balanced salt solution; PBS, phosphate buffer saline; Mark-1, mouse anti rat kappa 1; GARPOD, peroxidase-coupled goat anti rabbit; EDTA, ethylene diamine tetraacetic acid; DAB, diaminobenzidine; BSA, bovine serum albumin.

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0214-6282/92/$03.00

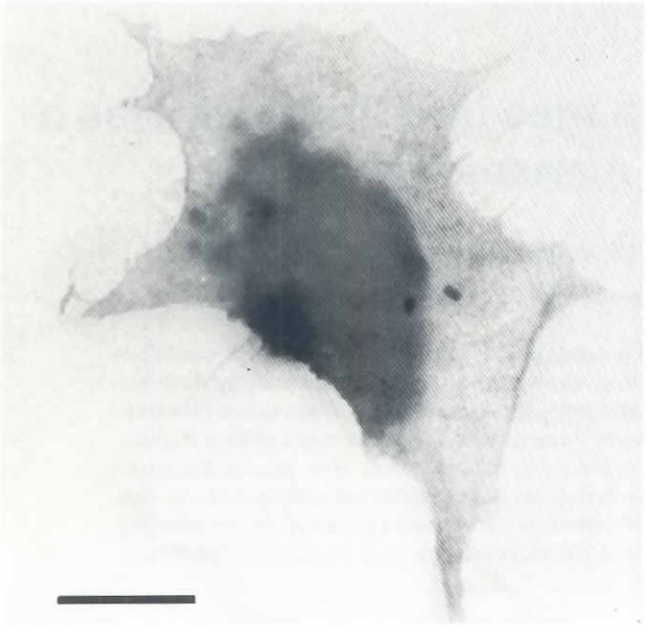

Fig. 1. Established fibroblast line (3T6) labeled with anti-procollagen antibody. *Scale bar, 1 μm.*

Results

Labeling of macrophages with anti-procollagen antibody after six days in culture

Cell samples were isolated from the peritoneal cavity of chronic-stage mice and fractionated on a discontinuous density Percoll gradient (see correspondence between cell density and fraction number in Material and Methods). Fractions 2, 3, 4 and 5 were then placed in culture. The cultures were treated with trypsin 15 to 18 hours after inoculation to eliminate any adhering non-macrophage cells (possible fibroblasts). The cells were fixed after six days in culture, labeled with anti-procollagen antibody, and the label detected by means of peroxidase-coupled goat anti-rabbit secondary antibody (GARPOD). Type-3T6 cells were used as a positive control (Fig. 1). Procollagen-positive fibroblast-like cells did appear among the various unlabeled macrophages (Fig. 2). Labeled cells with intermediate features between macrophages and fibroblasts were also seen.

Double labeling with anti-mac-1 antibody and anti-procollagen antibody

Evolution of mouse macrophage control cultures

Macrophages from the peritoneal cavity of uninfected mice were isolated and treated in the same way as the cells extracted from infected mice.

The evolution of the cell population was visualized by double labeling with anti-procollagen and anti-mac-1 antibodies over a six-day culture period.

The anti-mac-1 antibody was found evenly distributed over the surface of macrophages. On the sixth day, a few fibroblast-like cells were seen in fractions 2 and 3, but their number was very small: about one in 5000 in fraction 2 and one in 10,000 in fraction 3. These cells were mac-1-positive and very faintly procollagen-positive. No such cells were found in fractions 4 and 5.

Evolution of macrophage cultures derived from chronic-stage mice

As described above, macrophages present in fractions 2, 3, 4 and 5 of chronic-stage mice were placed in culture and trypsin-treated 18 hours later. Their evolution was observed by double labeling (mac-1, procollagen) over a six-day culture period.

The mac-1 antibody was found uniformly distributed over the surface of macrophage cells.

As early as the second day of culture, fibroblast-like cells appeared in fraction 3 (Fig. 3). These cells were mac-1-positive and faintly procollagen-positive. The proportion of fibroblast-like cells increased markedly from day 4 on (reaching 30% in fraction 3 on the fourth day of culture). They were procollagen- and mac-1-positive and distributed in spots throughout the culture. Intermediate cells with features of both macrophages and fibroblasts were also observed (Fig. 4). After six days, there were as many double labeled cells as macrophages. In fractions 4 and 5, on the other hand, the

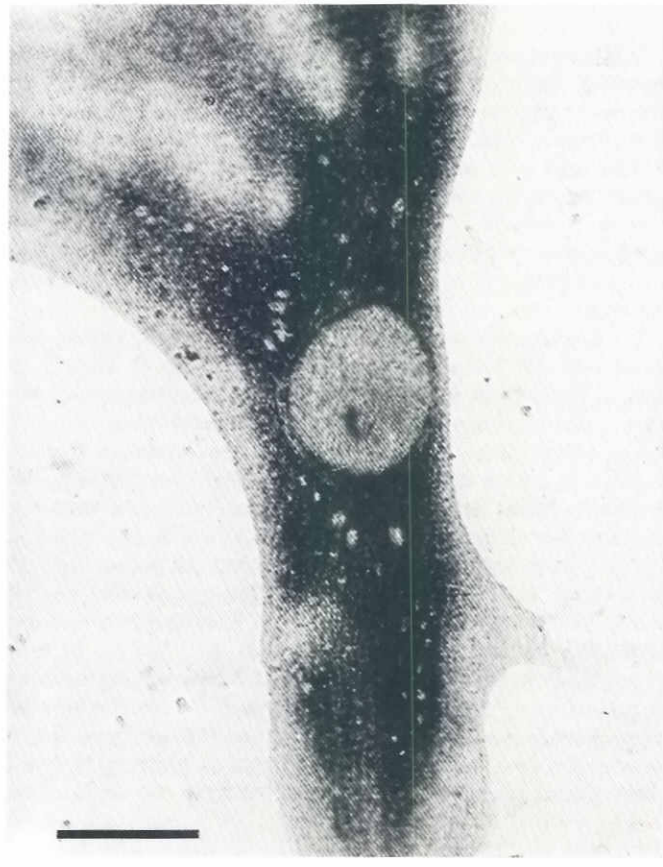

Fig. 2. Cultured macrophages from mice in the chronic stage of *Schistosoma mansoni* infection. *Fraction 3 after six days in culture. Intensely procollagen-positive fibroblast-like cell. Scale bar, 1 μm.*

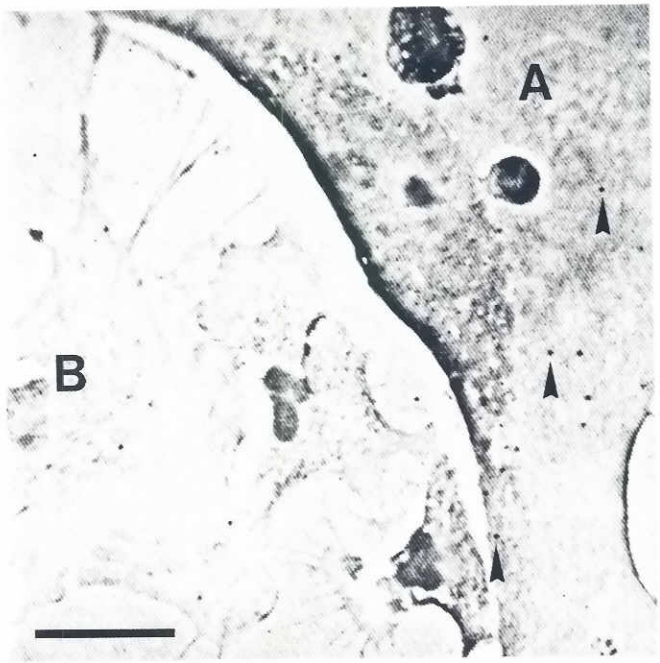

Fig. 3. Cultured macrophages from mice in the chronic stage of *Schistosoma mansoni* infection (fraction 2-day 6). *A mac-1-positive and faintly procollagen-positive giant fibroblast* **(A)** *surrounded by procollagen-negative and mac-1-positive macrophages* **(B)**. *Scale bar, 1 μm.*

proportion of procollagen-positive cells had considerably decreased and the procollagen label was still markedly fainter than in fractions 2 and 3.

Procollagen-positive cells were counted daily in 500-cell samples of each fraction of one culture. The means of 5 counts done on 100-cell subsamples are listed in Table 1, whose content is represented graphically in Fig. 5.

Labeling of mac antigens in cultured macrophages extracted from chronic-stage mice (single label, scanning microscopy)

Cell samples from the peritoneal cavity of chronic-stage mice were isolated and fractionated as described above. Cultures were trypsin-treated. The cells were fixed after six days in culture and labeled with anti-mac-1, anti-mac-2 or anti-mac-3 rat antibody. Antibody binding sites were detected by means of a biotin-coupled antibody recognizing the kappa chain of rat antibodies. Fibroblasts appearing in fractions 2, 3 and 4 (very few in the latter) were mac-1-positive (Fig. 6a, b). They also possessed the mac-2 antigen (Fig. 7b), but its distribution on their surface was markedly less dense than that of the mac-1 antigen. A few macrophages exhibited the mac-3 antigen but no fibroblasts bearing it were observed.

Scanning microscopy revealed many contacts between macrophages and fibroblasts.

Discussion

This study was undertaken in the framework of research into the etiology of fibrotic tissue in chronic-stage schistosomiasis. To complement previous studies conducted in our laboratory (Geuskens *et al.*, unpublished results; Godoy *et al.*, unpublished results), we set out to characterize the fibroblast-like cells immunocytochemically by means of antibodies targeting the procollagen, mac-1, mac-2, and mac-3 antigens.

Our findings show that the fibroblast-like cells appearing in pure macrophage cultures derived from chronic-stage mice are procollagen-positive. It is unlikely that these cells are the result of an infection with foreign fibroblasts (from the conjunctive tissue of the abdominal wall, for instance), since the cultures were trypsin-treated after 18 hours, a procedure that detaches fibroblasts from the substratum (Ackerman and Douglas, 1978). Moreover, these fibroblasts appear to bear the mac-1 antigen, a typical surface antigen of the monomacrophage line, which fibroblasts do not possess. This is a weighty argument in favor of a monocytic origin. The mac-2 antigen—typical of differentiated macrophages (May-Kinho and Springer, 1982)—is also present on the fibroblast-like cells. We conclude that these fibroblasts most probably derive from differentiated macrophages (characterized by the mac-1 and mac-2 antigens). Our observations thus reflect transdifferentiation of macrophages into fibroblasts, as defined by Okada (1991): the conversion of one differentiated cell to another, distinguishable from the first by its morphology, its function, and its molecular constituents.

The fibroblast-like cells possess no mac-3 antigen, unlike some of the macrophage cells present in fractions 4 and 5. We can draw no conclusions as to the meaning of this finding, as there have been few studies of the mac-3 antigen and its function remains obscure.

The appearance of fibroblasts in purified cultures of macrophages derived from chronic-stage mice is most obvious in fractions 2 and 3. In the other fractions, the phenomenon is transient and the proportion of fibroblast-like cells decreases rapidly after their

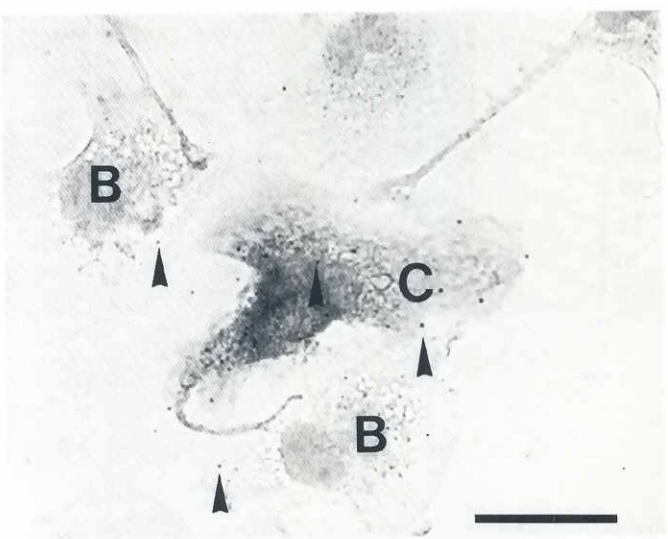

Fig. 4. Cultured macrophages from chronic-stage mice. *A cell with intermediate macrophage-fibroblast features (C). The cell is procollagen- and mac-1-positive and surrounded by procollagen-negative and mac-1-positive macrophages (B). Scale bar, 1 μm.*

TABLE 1

MEAN PERCENTAGES OF PROCOLLAGEN-POSITIVE CELLS

	Day 2	Day 3	Day 4	Day 5	Day 6
Fraction 2	0.8 ± 0.5	2.6 ± 0.5	13.4 ± 3.0	35.4 ± 4.8	29.6 ± 6.1
Fraction 3	3.2 ± 1.0	12.2 ± 2.1	32.4 ± 2.3	30.6 ± 4.1	49.2 ± 9.0
Fraction 4	0.2 ± 0.2	6.6 ± 1.3	13.2 ± 1.0	14.8 ± 2.5	5.5 ± 1.2
Fraction 5	0	2.0 ± 0.8	6.2 ± 1.3	3.8 ± 0.9	0

Mean percentages of procollagen-positive cells in each fraction of a macrophage culture from chronically diseased mice. The means were based on daily cell counts. The fractions were obtained by the Percoll gradient method.

appearance (Table 1 and Fig. 5). These observations agree with earlier autoradiographic studies carried out in this laboratory by M. Godoy and co-workers on chronic-stage mice (Godoy *et al.*, unpublished results). These authors established an undeniable correlation between macrophage division and the increase in the number of fibroblasts. In this work, we show that transdifferentiation of macrophages to fibroblasts occurs in the same fractions where incorporation of tritiated thymidine was established. These observations suggest that only a subpopulation of the macrophages present in fractions 2 and 3 divide actively and transdifferentiate into fibroblasts. Our results thus support the view that the parasitosis

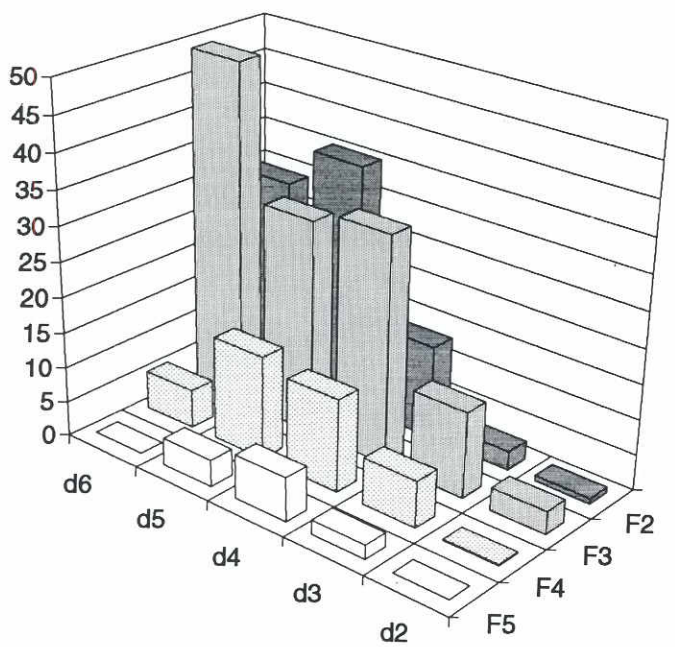

Fig. 5. Time course of the mean percentages of procollagen-positive cells in each fraction of a macrophage population extracted from mice in the chronic stage of *Schistosoma mansoni* infection. *The fractions were obtained by the Percoll gradient method.*

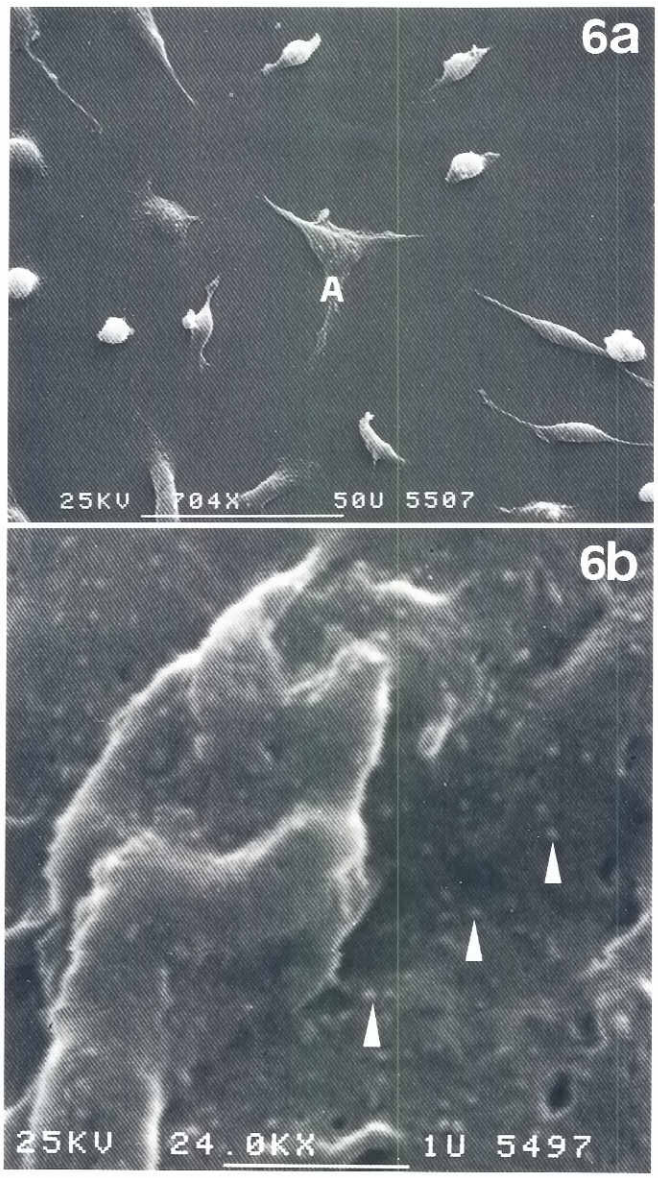

Fig. 6. Cultured macrophages from chronic-stage mice labeled with anti-mac1 antibody. (a) *General view of the culture. At center: a fibroblast-like cell (A). (SEM).* **(b)** *Detail of the membrane of the preceding fibroblast (6a: A). This fibroblast is mac-1 positive. (SEM).*

causes certain macrophage subpopulations to transdifferentiate to fibroblasts. This view by no means excludes or contradicts the Ito cell hypothesis. On the contrary, other research carried out in this laboratory (F. Zampetti, personal communication) has revealed a great many Ito cells in hepatic granulomas. It should be noted, however, that while such cells may play a role in the schistosome-infected liver, they cannot be involved in pulmonary granulomas (Warren, 1972; Edungbola and Schiller, 1979).

We conclude that, on top of the proliferation of Ito cells around the egg, the appearance of fibrotic tissue in infected mice liver could be explained by the transdifferentiation of a macrophage

subpopulation into fibroblasts. Moreover this phenomenon could be at the origin of the conjunctive tissue in pulmonary granulomas.

Materials and Methods

Animals and infection

Male and female C3H mice were individually infected by injection of thirty cercariae in the laboratory of Professor Deelder in Leyden. They were killed during the chronic stage of the disease, ninety days after the infection. Infection was monitored by histological examination of the liver, which presents hundreds of periovular granulomas when infection is successful.

Cell samples and cell cultures

Cells from the peritoneal cavity were obtained by flushing the peritoneum with Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS). The cell samples were fractionated by the discontinuous Percoll gradient method as described by Rasmussen (Rasmussen *et al.*, 1983). The densities of the various fractions were as follows: F1:1. 011; F2:1. 041; F3:1. 052; F4:1. 060; F5:1. 067; F6:1. 076; F7:1. 080; F8:1. 091; F9:1. 1. These densities were determined in a previous study done in this laboratory (Godoy *et al.*, unpublished results). The sample-containing tube was centrifuged at 2500 rpm for one hour at 4°C and the various cell types deposited at the gradient interfaces were collected. Four cell fractions (from the second to the fifth) were harvested and placed in culture. Fifteen to eighteen hours later, the cultures were treated for 5 min at 37°C with a 2.5% trypsin solution containing 0.1M EDTA (EthyleneDiamineTetraacetic Acid, SIGMA). This purification method is based on the greater resistance of macrophages to trypsin as compared to fibroblasts (Ackerman and Douglas, 1978; Schor, 1979). Unlike fibroblasts, the macrophages continue to adhere to the substratum under these conditions, so the treatment yields pure macrophage cultures.

Products for immunocytochemistry

Anti-procollagen antibody

This antibody was obtained from immunized rabbits. The procollagen used to immunize the animals was obtained by stimulating an established line of 3T6 mouse fibroblasts (ATCC CCL96) with ascorbic acid (100µg/ml). Procollagen maturation was inhibited by addition of 50µg/ml ß-aminopropionitrile (Sigma). The culture medium was collected and the procollagen precipitated by addition of 10% polyethylene glycol 400 (Ramshaw *et al.*, 1984). Freund's adjuvant in PBS was added to the procollagen-containing solutions which were then injected subcutaneously at three or four points along the rabbits' spine. Booster injections were administered 20 and 40 days after the first. The immunoglobulins were then collected by double precipitation with ammonium sulfate and caprylic acid (Lane, 1988).

Anti-mac-1 and anti-mac-2 antibodies

The anti-mac-1 antibody used in these experiments was a monoclonal rat antibody secreted by a rat-mouse hybridoma (M1/.70.15.11.5) and reacting with mouse mac-1 antigen.

The anti-mac-2 antibody was also a monoclonal antibody from a rat-mouse hybridoma (M3/38.1.2.8HL2).

Anti-mac-1 and anti-mac-2 antibodies were produced in ascites and purified by double precipitation with amonium sulfate and caprilic acid. The anti-mac-3 antibody was secreted by a rat-mouse hybridoma (M3/8.4.6.34). Hybridoma culture supernatant was used for mac-3 labeling.

Secondary antibodies

The binding sites of the anti-mac-1 and anti-mac-2 primary antibodies were revealed by means of a biotin-coupled monoclonal mouse antibody targeting the kappa chain of rat antibodies (Mark-1: Mouse anti rat kappa 1). The biotin was then visualized by means of gold-coupled extravidin (10-nm gold particles).

Polyclonal peroxidase-coupled goat anti-rabbit antibody (GARPOD, Sigma) was used to mark the sites where anti-procollagen was bound. Peroxidase activity was then revealed by means of the benzidine-peroxide reaction,

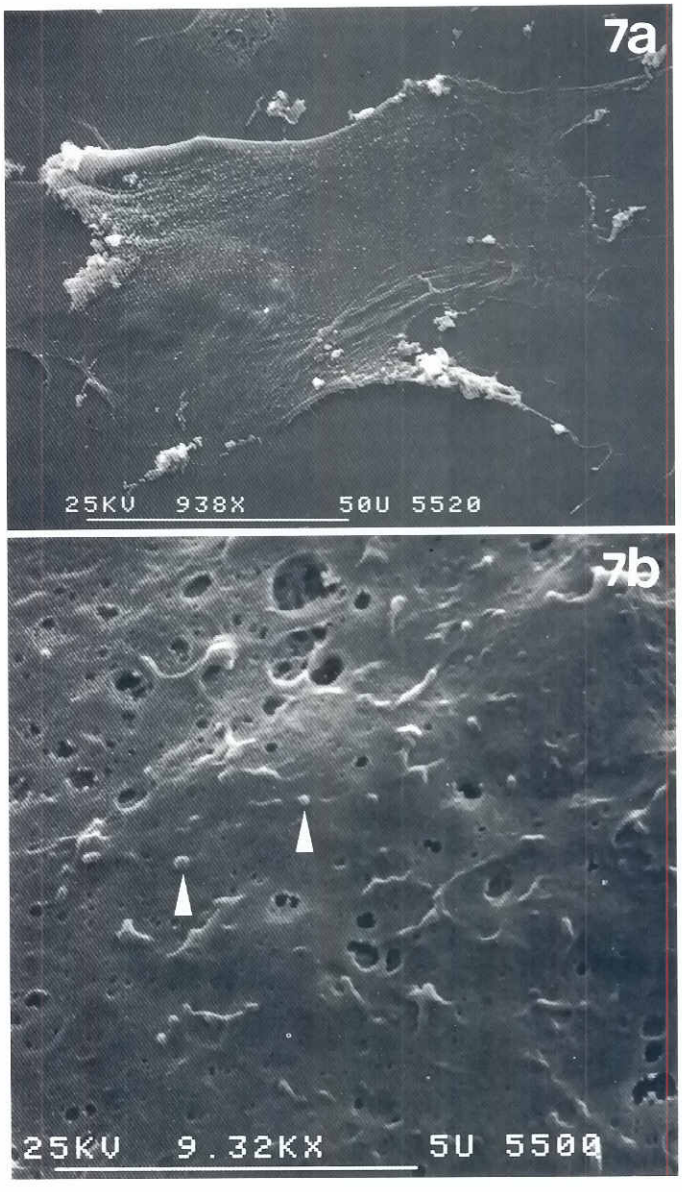

Fig. 7. Cultured macrophages from chroni-stage mice labeled with anti-mac2 antibody. (a) *Fibroblast appearing in a culture of macrophages from chronic-stage mice. (SEM).* **(b)** *Detail of the membrane of the preceding fibroblast(7a). Labeling of the mac-2 antigen. (SEM).*

nascent oxygen causing the precipitation of a fine brown diaminobenzidine (DAB) polymer.

Immunocytochemical methods

Single labeling of mac-1, mac-2, or mac-3 antigens:

Cells were fixed with 1% glutaraldehyde in PBS, washed with PBS, then incubated for 1 h at 37° with stirring in a solution containing the secondary antibody (Mark-1) diluted in PBS containing 1% bovine serum albumin (PBS-BSA 1%). The cells were then incubated in colloidal-gold-coupled extravidin. Silver-enhancement of the gold label was performed with the RPN491 Intense™ amplification kit (Amersham). The cells were then dehydrated with ethanol and critical-point-dried in CO_2. Thereafter, they were metallized by gold-spraying and observed with a 30KV ISI scanning electron microscope.

Double labeling of the mac-1 and procollagen antigens

After fixation in 1% glutaraldehyde, slides were treated with 0.5 mg/ml sodium hydroboride. The cells were then incubated for 1 h at 37°C with anti-mac-1 antibody diluted in PBS-Tween (0.05%) and permeabilized in PBS-ethanol 1:1. Non-specific sites were saturated in PBS-BSA 1% for 1 h at room temperature with stirring. Next, the cells were incubated for one hour at room temperature with anti-procollagen antibody diluted in PBS-Tween 20. They were then rinsed with PBS and incubated for one hour at 37° with stirring in a solution containing Mark-1 and GARPOD. The resulting preparations were incubated with gold-coupled extravidin and enhanced with the amplification kit. Peroxidase activity was revealed. After rinsing in distilled water, the cells were incubated in methyl green. The slides were mounted in Canada balsam after a brief rinsing in xylol. The results were observed under a light microscope.

Acknowledgments

We thank M. Geuskens and A. Sels for critical review of this manuscript. Our thanks also go to Prof. L. De Vos (Department of Animal Biology, Free University of Brussels) for his kind help in scanning electron microscopy, Prof. Bazin (Catholic University of Louvain-la-Neuve) for the Mark-1 antibody and D. Franck for assistance in preparation of figures. This work was supported by the Science and Technology for Development Program of the European Community (Contract n° TS 2-0217 B).

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