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Schistosomiasis and in vitro transdifferentiation of murine peritoneal macrophages into fibroblastic cells

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Abstract. We developed a method for avoiding contamination by fibroblasts when cultures of peritoneal cells are initiated. Macrophages were identified by immunogold detection [light microscope, transmission (TEM) and scanning (SEM) electron microscopes] of membrane antigens (Mac-1$^+$, Thy-1,2$^-$), non-specific esterase activity and ultrastructural features (TEM). As compared with controls, the yield of peritoneal macrophages was 2- and 12-fold higher, respectively, in acutely and chronically infected mice. In all, 30 "chronic", 18 "acute" and 18 control cultures were followed up. At a given cell-density seeding, the decline of control, "acute" and "chronic" cultures starts at about day 10, 15, and 27, respectively. In "chronic" cultures only, fibroblast-like cells appear from day 6 onwards; their number increases with time. Cells showing characters intermediary between macrophages and fibroblasts were observed. We suggest that fibroblast-like cells result from the in vitro transdifferentiation of a limited number of in vivo committed macrophages.

In *Schistosoma mansoni* infections, blood-dwelling trematode worm pairs are present in the mesenteric venous system. Female worms produce hundreds of eggs a day, of which a large number reach the liver via the portal vein. The eggs are trapped in the presinusoidal portal-vein tributaries, and secretion products of the eggs' larvae (miracidia) generate an immunologic granuloma that heals by fibrosis (Andrade 1963). The granulomas are an aggregation of lymphocytes, neutrophilic and eosinophilic granulocytes, macrophages and fibroblasts. They are largely due to a T-cell-mediated immune response of the host against soluble antigenic components produced by the miracidium (Bentley et al. 1982; Colley 1981; Phillips and Lammie 1986; Warren 1972). The concentrically arranged cell population in the granuloma changes with time and with the dying off of the miracidium. Granulomas gradually become more fibrotic as they grow older. Also, in chronic infections, the granulomatous reaction surrounding newly deposited eggs is modulated towards smaller granulomas (Bentley et al. 1982; Moore et al. 1977; Stocker et al. 1983).

Little is known about the mediators or promoting factors in fibrosis or about the origin of proliferating fibroblasts, although the role of granuloma cells appears to be essential to the process (Freundlich et al. 1986; Phillips and Lammie 1986). Interestingly, it has recently been proposed that rodent macrophages covering the surface of intraperitoneal blood clots gradually develop the characteristics of fibroblasts (Campbell and Ryan 1983; Mosse et al. 1985). The hypothesis that rat macrophages may transform into fibroblasts has also been suggested by Kouri and Ancheta (1972).

Mice infected with *S. mansoni* provide a valuable experimental model of the parasitic disease. The observations made by one of us (R.B.) concerning an increase in the number of peritoneal macrophages during the chronic phase of schistosomiasis are indicative of the stimulated state of these cells (Borojevic et al. 1984). It was thus interesting to check their in vitro capacity to transform into fibroblasts in a system minimizing the chances of contamination by fibroblasts on initiation of the culture of macrophages. Mature macrophages can be isolated from the peritoneal cavity in different functional states (resident, elicited, activated) and they have been used as a model of differentiation and for the in vitro study of gene expression (Gordon 1986). However, the influence of diseases

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on macrophage behaviour and the role of these phagocytic cells in disease processes are practically unknown. In the present study we took advantage of the in vitro-specific adherence of macrophages and, in a second step, of their resistance to trypsin in purifying macrophages from infected mice, with the aim of comparing their evolution in culture with that of macrophages from normal, unstimulated mice.

Materials and methods

Animals and infection

Male C3H mice, 4 weeks old, were individually infected by transcutaneous penetration of 30 *S. mansoni* cercariae (Puerto Rican strain). Animals in the acute and chronic phases of the disease were used 45 and 90 days, respectively, after cercarial penetration. Only those with a patent bisexual infection were chosen. Infection was confirmed by histological examination of liver sections, which showed periovular granulomas with fibrosis. Groups of 12 mice each were infected on different dates; 30 groups were used for studies of the chronic phase of the disease, 18 groups, for the acute phase, and 18 groups served as controls (non-infected mice).

Cell harvest and culture

Peritoneal macrophages were obtained from unstimulated control mice and infected mice by peritoneal washes with refrigerated Hanks' solution (Ca- and Mg-free). The fluids from 6–12 mice were usually pooled, but during a few experiments peritoneal adherent cells were harvested from single mice and, after purification as described below, cultivated in individual dishes.

Macrophages were purified by serum-coated plastic adherence, using the technique of Kumagai et al. (1979). The periods of incubation recommended to enable the adherence of macrophages to the substratum and their ulterior detachment with an EDTA solution were extended to 20 and 50 min, respectively. The cells harvested by this technique were seeded at a density of 10^6 cells/ml in 4-well culture dishes (Nunc) without glass coverslips when destined for transmission electron microscopy (TEM).

On the following day (15–18 h after seeding), the cultures were treated for 5 min at 37° C with a 2.5% trypsin solution (supplemented with 0.01 *M* EDTA), a concentration which, according to our tests with embryonic fibroblasts in primary culture, detaches any cell of this type. In contrast, it is well known that macrophages in culture are resistant to trypsin (Pearlstein et al. 1978). The cells were then cultivated in the presence of 5% CO_2 at 37° C in Mac Coy's medium (Gibco) with 10% foetal calf serum (FCS), 2 m*M* glutamine (Flow) and gentamycin (50 µg/ml; Boehringer). This medium was renewed every 3rd day.

As reference cultures, we used primary cultures of mouse fibroblasts and a macrophage hybrid cell line; 19-day-old C3H mouse embryos were used to initiate the fibroblast cultures (Geuskens 1986; Van Gansen et al. 1985) as previously described. The macrophage line used was hybridoma LA5-9, a generous gift from Dr. P. De Baetselier (Vrije Universiteit, Brussels); this line shows many characteristics typical for macrophages (De Baetselier and Schram 1987).

Electron microscopy (standard observations)

For TEM, cells were fixed in situ for 30 min in 2% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.2) at room temperature. After an overnight wash in buffer, the cells were again fixed for 1 h in 2% OsO_4 in the same buffer. After ethanol dehydration, the cells were embedded in situ in Epon. Ultrathin sections, made parallel to the growth plane, were stained with uranyl acetate and lead citrate and examined in an AEI EM6B electron microscope at 60 kV.

For scanning electron microscopy (SEM), the cells were seeded onto 10-mm (diameter) glass coverslips. They were fixed for 1 h in 2% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.2), thoroughly washed in buffer, dehydrated in an ascending ethanol series and critical-point-dried in liquid CO_2. The samples were sputter-coated with a thin gold layer and observed in an ISI DS-130 SEM at an accelerating voltage of 30 kV.

Cell coat and extracellular matrix contrast enhancement

To enhance the contrast of the cell-surface proteoglycans, ruthenium red (RR) (Fluka) (Luft 1971) was used. Glutaraldehyde was added at a concentration of 2.5% to a stock solution of 0.05% RR in 0.1 *M* cacodylate buffer (pH 7.2). The cells were fixed in this solution for 1 h at 4° C. After three washes in the same buffer, the cells were incubated in a dark environment at room temperature for 3 h in a freshly prepared solution made by mixing equal quantities of 4% OsO_4 in distilled water and 0.05% RR in cacodylate buffer. After three washes in the same buffer, the cells were dehydrated in ethanol and embedded in situ in Epon.

Immunocytochemistry for antigens Mac-1 and Thy-1, 2

For the immunocytochemical localization of Mac-1, a plasma membrane, complement receptor-associated, macrophage-differentiation antigen (Springer et al. 1979), cells were incubated with the antibodies in the culture wells. A rat monoclonal antibody (IgG 2b) secreted by the rat/mouse hybridoma M1/70.15.11.5 (ATCC TIB 128) (Springer et al. 1979) was used. As macrophages have Fc receptors on their surface, the supernatant of another rat/mouse hybridoma, 30-H 12 (ATCC TIB 107), was used under the same experimental conditions to confirm the specificity of the anti-Mac-1 antibody binding. The monoclonal antibody (IgG 2b) secreted is specific for a Thy-1,2 antigenic determinant and reacts with all thymocytes (Ledbetter and Herzenberg 1979). The supernatants of the hybridoma cultures were used without purification or dilution.

The cells were washed briefly in Hanks' solution and fixed for 5 min in 0.05% glutaraldehyde diluted in the same solution. After three washes in Hanks' solution, the cells were incubated with the anti-Mac-1 antibodies for 1 h at room temperature under constant agitation in Hanks' solution with 1% bovine serum albumin (BSA) (Sigma, type V) (Hanks'/BSA). After three washes in Hanks'/BSA, the cells were again incubated for 1 h at room temperature with goat anti-rat IgG antibodies adsorbed to 15-nm gold particles (GARa-G 15, Janssen Life Sciences Products, Beerse, Belgium) diluted 1:100 in Hanks'/

BSA. After three washes in Hanks' solution, the cells were fixed for 30 min at 4° C in 1% glutaraldehyde diluted in 0.1 *M* cacodylate buffer (pH 7.2).

For TEM, after an overnight wash in buffer the cells were fixed in 2% OsO_4 for 1 h at room temperature, dehydrated in an ascending ethanol series and embedded in situ in Epon. For light microscopy as well as SEM, after the washes subsequent to incubation in the gold-labelled antibodies, the cells were fixed for 15 min in 1% glutaraldehyde diluted in Hanks' solution, washed three times in distilled water and subjected to silver enlargement of the 15-nm gold particles in small glass dishes using the initiator, enhancer and fixative solution of the Intense kit (Janssen Life Sciences Products Division). For SEM, the cells were again fixed for 1 h in 2% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.2), thoroughly washed in buffer and then processed as for standard observations.

Light cytochemistry for non-specific esterases

We used the Sigma kit for non-specific esterases (kit 90) according to the instructions of the manufacturer: the cells, fixed for 30 s in citrate-acetone-methanol fixative, were incubated with α-naphtyl acetate in the presence of a stable diazonium salt (Fast Blue RR salt). Enzymatic hydrolysis of ester linkages, carried out in a dark environment, liberates free naphtol compounds that couple with the diazonium salt, forming black deposits at the sites of enzyme activity. The cells were then counterstained with a solution of Mayer's hematoxylin for 5–10 min.

Results

Cell types in initial cultures

The number of purified cells harvested from the peritoneal cavity of a control mouse was about 0.5×10^6 cells. The amount from a mouse in the acute phase of infection is about double that figure. The quantity from a chronically infected mouse is about 12-fold higher than that obtained from a control animal.

Despite this fact, all cultures looked very similar 2 days after seeding (i.e. 1 day after trypsinisation). Three types of adherent cells were observed with the light microscope (Fig. 1) and with the SEM system (Fig. 2): type A cells are spherical and look like typical macrophages; type B cells are similar but form noticeable veils on the substratum, and they are either spindle- or pancake-shaped; type C cells are very large and always multinucleate. Type A represented 10%–20% and type B, 75%–90% of the total cell populations. Type C cells were few and looked very similar to the giant multinucleate cells previously described in cultures of macrophages (Sutton and Weiss 1986) and in granulomas (Black and Epstein 1974; van der Rhee et al. 1979).

All culture cells were labelled with the anti-Mac-1 antibody before being observed with light or electron microscopes. The distribution of the Mac-1 antigen was quite homogeneous on the surface of all three types of cells. This held true for the surface folds of the spherical A cells (Fig. 3) as well as for the filopods of the more flattened B cells (Fig. 4). The uniform distribution of the Mac-1 antigen has previously been described by Scopsi et al. (1986). Embryonic fibroblasts used to

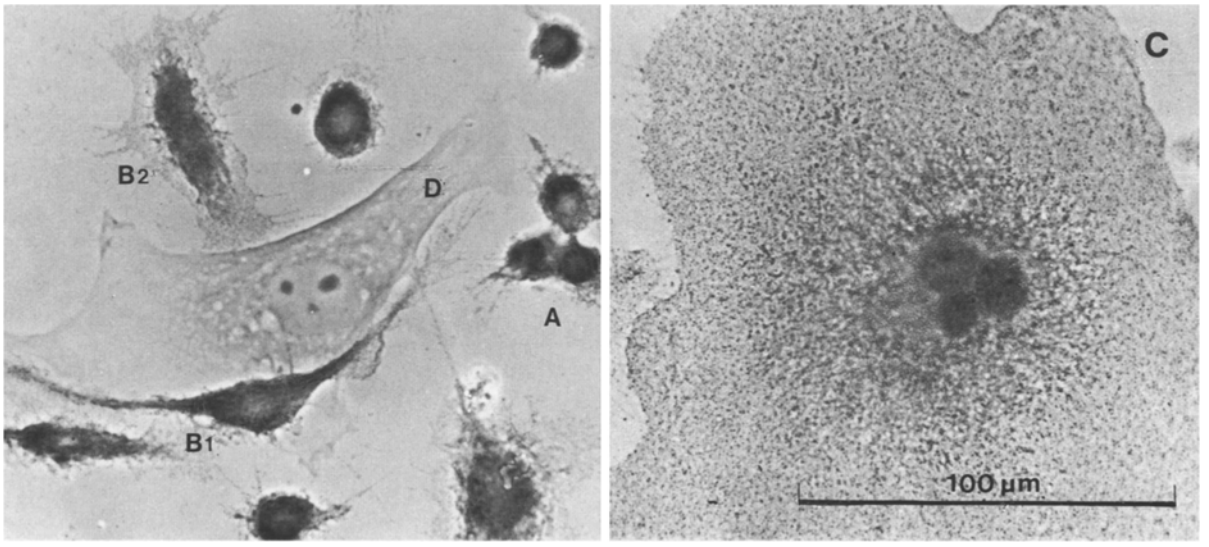

Fig. 1. A 10-day culture issued from chronically infected mice (phase-contrast light microscopy). *A*, Type A cells (spherical macrophage-like cells); *B*, type B cells (non-spherical macrophage-like cells); *B1*, spindle-shaped type B cells; *B2* pancake-like type B cells; *C*, type C cell (giant multinucleate cell); *D*, type D cell (fibroblast-like cell). At first we counted separately the percentages of B1 and B2 cells. As both populations evolve in the same manner, we pooled them in a single class B of non-spherical macrophage-like cells

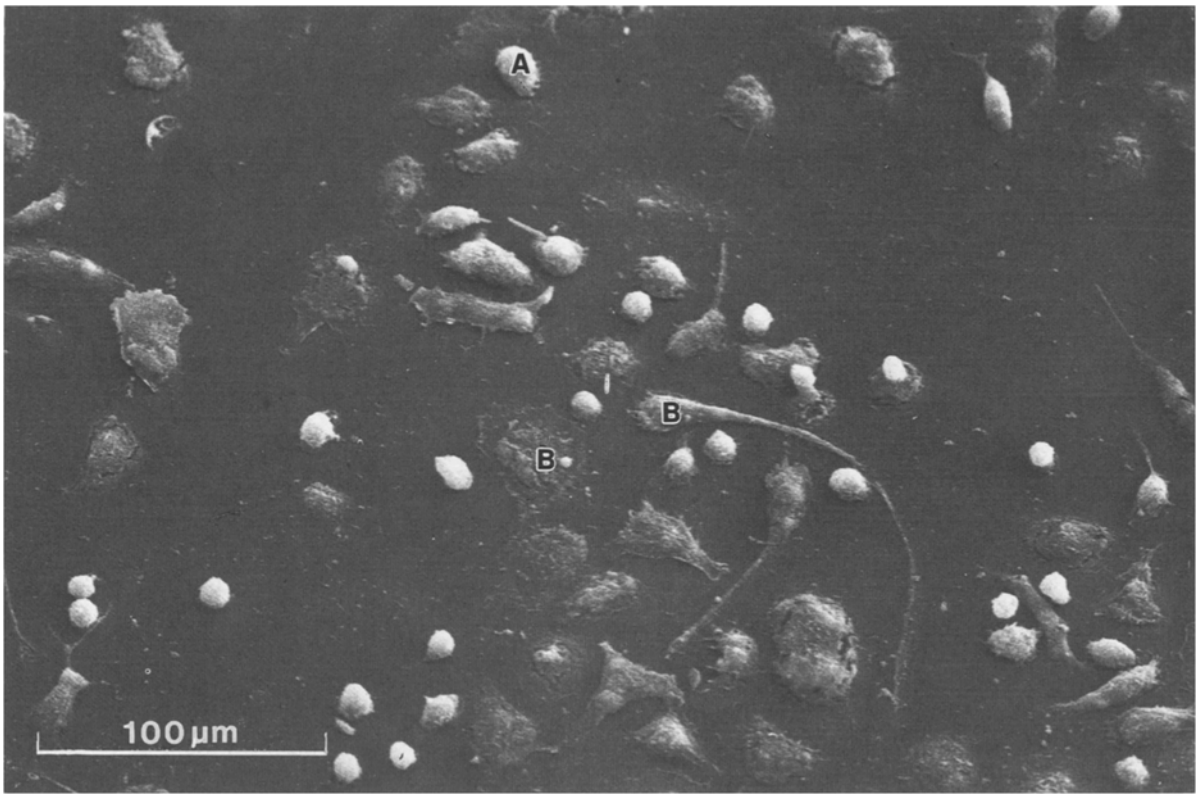

Fig. 2. A 4-day culture issued from non-infected mice (SEM). *A*, type A cells; *B*, type B cells

check the specificity of the monoclonal antibody were not labelled. Furthermore, when macrophages were used, omission of the first antibody abolished the labelling. Finally, no labelling was observed in cultures with the anti-Thy-1,2 antibodies.

All of the cells contained numerous granules positively stained for non-specific esterases. When observed with TEM, the three types of cells showed characters of mononuclear phagocytic cells: numerous cell-surface projections and invaginations bearing Mac-1 antigens, a large number of phagosomes and secondary lysosomes, and the absence of microfilament bundles. When RR was added to the fixatives, the cell sections were lined by a thin, continuous electron-dense layer that was slightly fuzzy at higher magnification.

In the present study, 30 cultures were initiated from chronically infected mice; 18, from mice in the acute phase of infection; and 18, from non-infected control mice. Fibroblastic cells were never observed at the initiation of the cultures when the latter were carried out as described in *Materials and methods* [a slight modification of Kumagai et al.'s method (1979), followed by trypsinisation 18 h after the first purification].

Evolution of the cultures

In control cultures, the cells began to detach after about 10 days; after 18 days, only a few cells (~0.2%) still adhered to the substratum. "Acute" cultures evolved in a similar way, but a little more slowly; their decline started at about 15 days. The situation was very different for "chronic" cultures, which flourished for about 27 days, after which they began to decline (thus far we could not keep them for much longer than 1 month). There was also a difference between the evolution of cell types in "chronic" cultures and that in control cultures. It mainly involved the appearance of fibroblast-like cells in the "chronic" cultures (type D cells, Fig. 1) from about the 6th day after seeding onwards; the D cells became more numerous with time.

Figure 5 compares the percentages of the different cell types determined every 2 days for a period of 18 days in control and "chronic" cultures. For each determination, a sample of 500 cells was analyzed in each culture. The percentage of type A cells (macrophage-like cells) increased in controls, making up nearly 90% of the population at 18 days, when the culture is on the border of ex-

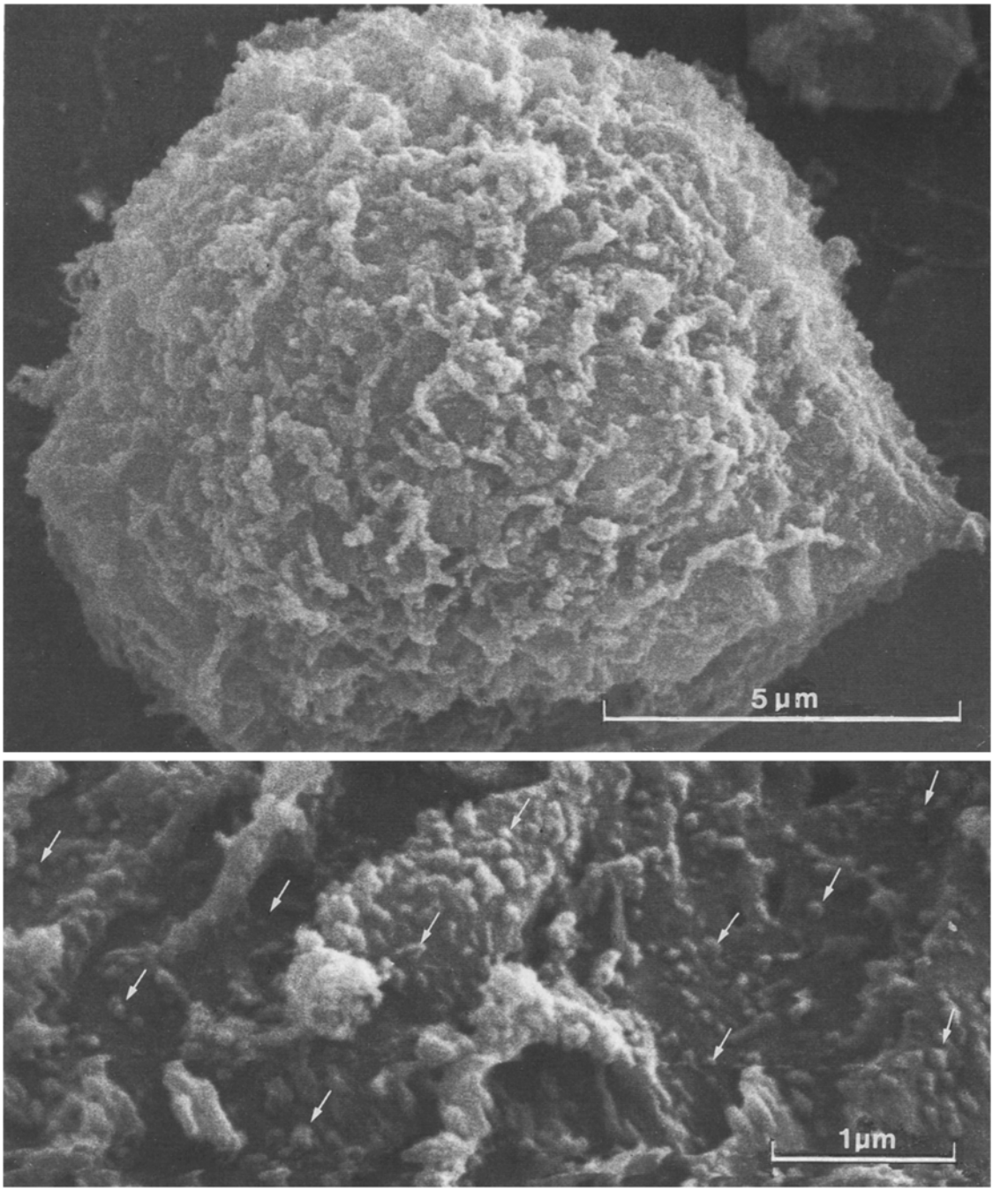

Fig. 3. Type A cell (spherical macrophage) (SEM). Silver-enhanced immunogold detection of Mac 1 antigens, showing an homogeneous distribution on the cell surface

tinction. In contrast, in the "chronic" cultures, the percentage of A cells remained fairly constant and as low as in the initial culture (about 10%). The percentage of type B cells (flattened macrophage-like cells) declined in both cultures; however, at 18 days it was still about 60% in "chronic" cultures, whereas it amounted to only about 10% in the rapidly declining controls. The percentage of type C cells (giant multinucleate cells) remained low in both cultures, but it improved slightly with time in the "chronic" cultures and declined slightly in controls. The type D cells (fibroblast-like cells) made up about 30% of the "chronic" cell population at 18 days. These cells were never observed in control cultures. If a 15-day "chronic" culture, for instance, is subjected to trypsinisation (as at

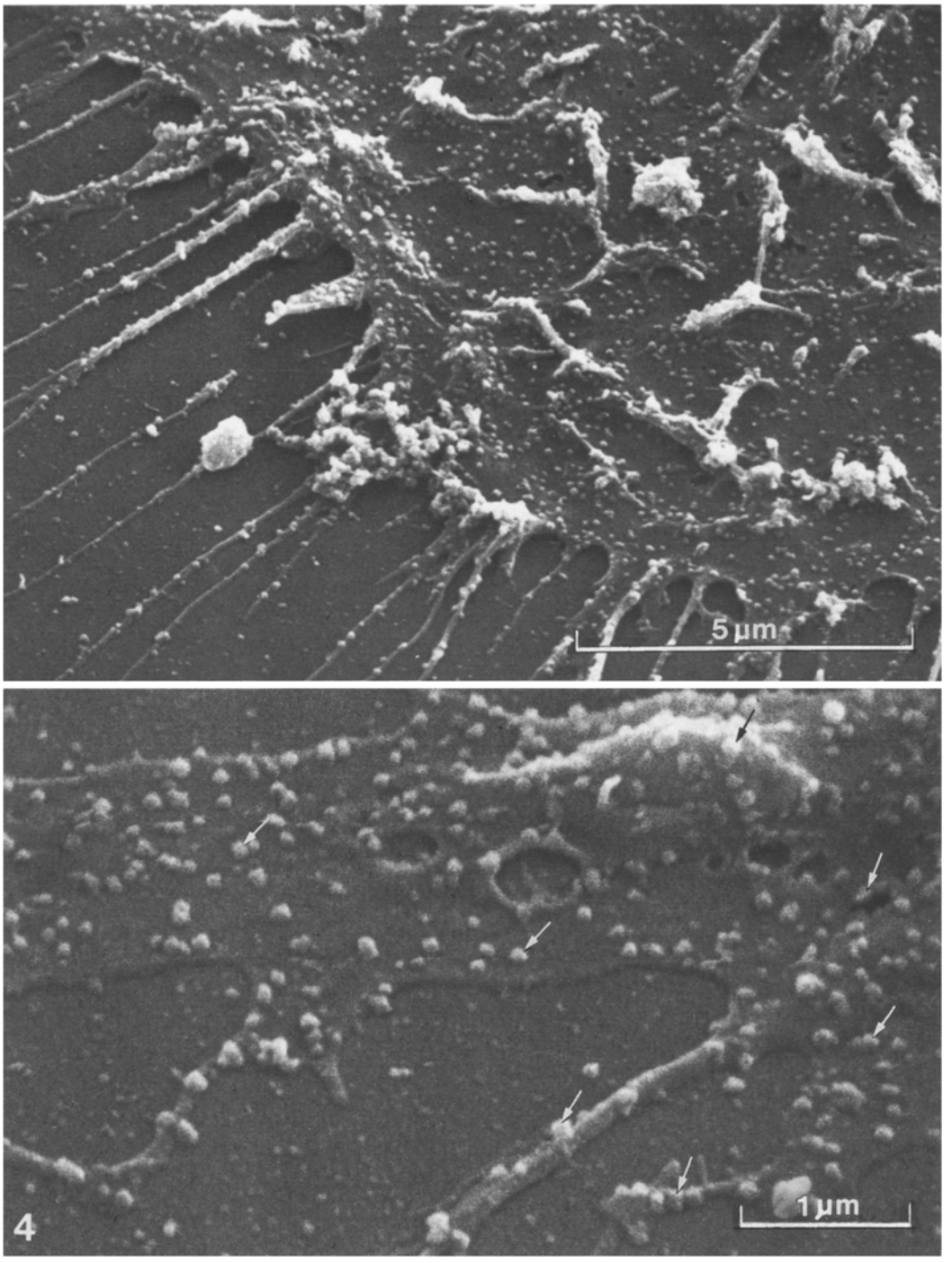

Fig. 4. Border of a type B cell (non-spherical macrophage) (SEM). Silver-enhanced immunogold detection of Mac 1 antigens evenly distributed on the cell surface, including the filopods

the initiation of the culture), all of the fibroblast-like cells are eliminated. The distribution of cell types at 2, 8 and 18 days are represented in Fig. 6. The evolution of cultures issued from mice in the acute phase of schistosomiasis was similar to that of control cultures.

The type D or fibroblast-like cells of the "chronic" cultures

The fibroblastic nature of these cells is hypothesized on the basis of morphological criteria. These large, spindle-shaped or polygonal cells were well

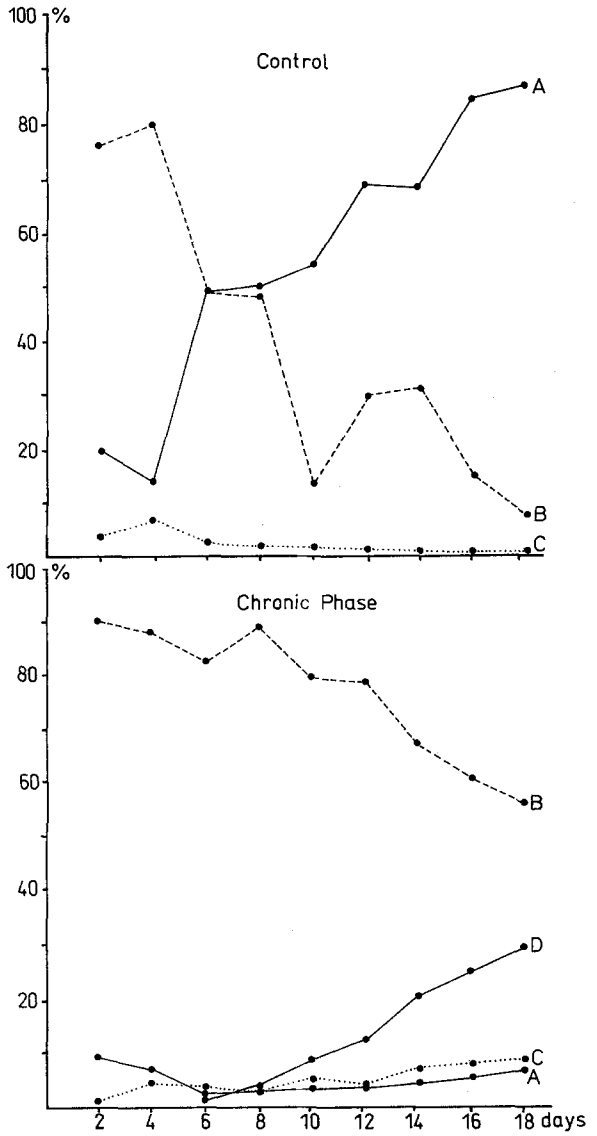

Fig. 5. Evolution of the percentages of the different cell types as compared in a control culture (issued from non-infected mice) and a culture issued from chronically infected mice. *A*, type A cells (spherical macrophages); *B*, type B cells (non-spherical macrophages); *C*, type C cells (giant multinucleate cell); *D*, type D cells (fibroblast-like cells). The *chronic* culture issued from a fraction of a pool of purified peritoneal cells harvested from six chronically infected mice. The *control* culture issued from the pool of purified peritoneal cells harvested from 12 non-infected mice

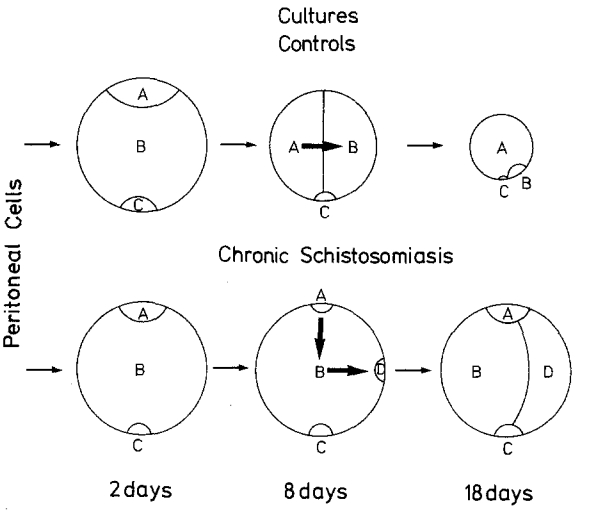

Fig. 6. Diagram of the evolution with time of the percentages of types A, B, C and D cells in cultures from non-infected mice (*controls*) and chronically infected mice (*chronic schistosomiasis*)

spread over the substratum. Some were solitary, but they generally formed small groups. They were negative for the immunogold localization of Mac-1, whereas the adjoining macrophages were positive (light microscopy, TEM, SEM). At the ultrastructural level, they showed the characters of fibroblasts: a large nucleus with dispersed chromatin and several nucleoli, several bundles of microfilaments that were often subjacent to the plasma membrane (Van Gansen et al. 1985), and large extracellular matrix at their periphery (Geuskens 1986). The type D cells were very similar to the early populations of serially cultivated mouse embryonic fibroblasts (Van Gansen and Van Lerberghe 1985).

If these cells derive from macrophages, one should observe intermediary forms; thus, we looked for such cells with the electron microscope. Indeed, some cells in the "chronic" cultures showed characters intermediary between macrophages and fibroblasts. For instance, the elongated cell shown in Fig. 7 has a nucleus with dispersed chromatin and some cytoplasmic filament bundles, which are fibroblastic characters, whereas the presence of numerous phagosomes, the folds of its surface and the light but clear Mac-1 labelling are characters of macrophages.

The fibroblast-like cells often showed very close contacts with the macrophages of the cultures (Fig. 1). Sometimes they completely engulfed the phagocytic cells, without a sign of destructive effect (Fig. 8). It must be emphasized that these close intercellular contacts are also observed in a culture issued from a single infected mouse; thus, they do not result from a non-autologous rejection reaction.

Discussion

We developed a method for avoiding contamination by fibroblastic cells when cultures of peritoneal cells are initiated. Indeed, we determined that under the trypsinisation conditions used, all em-

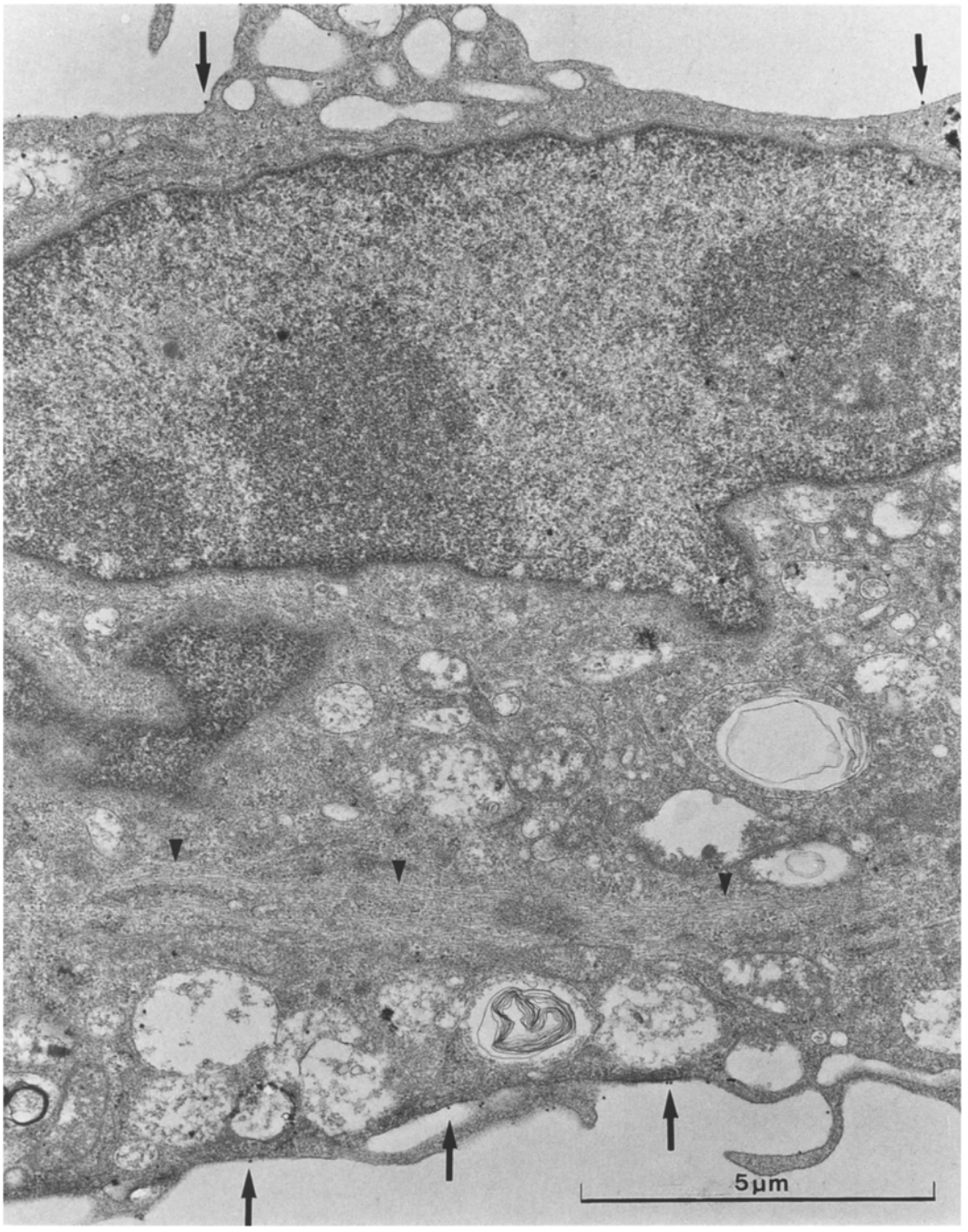

Fig. 7. Cell showing characters intermediary between macrophage and fibroblast (TEM). Immunogold detection of Mac 1 surface antigens: *Arrows*, gold dots; *arrowheads*, filament bundle (6-day "chronic" culture)

bryonic fibroblasts became detached from the substratum; in fact, during the very first days after this step we observed no fibroblastic cells in cultures of peritoneal cells.

All of the cells in the initially purified cultures were labelled with the anti-Mac-1 antibody. Moreover, these cells showed many characteristics of the mononuclear phagocytic cells, and they very probably belong to this cell lineage. The cells were not labelled with the anti-Thy-1,2 antibody, and a contamination by T lymphocytes can thus probably be excluded. However, we must keep in mind the existence of a recently described B-lymphocyte sub-population, the Ly-1 B cells (Hardy and Hayakawa 1986; Herzenberg et al. 1986a, b). These cells occur at high frequencies in the perito-

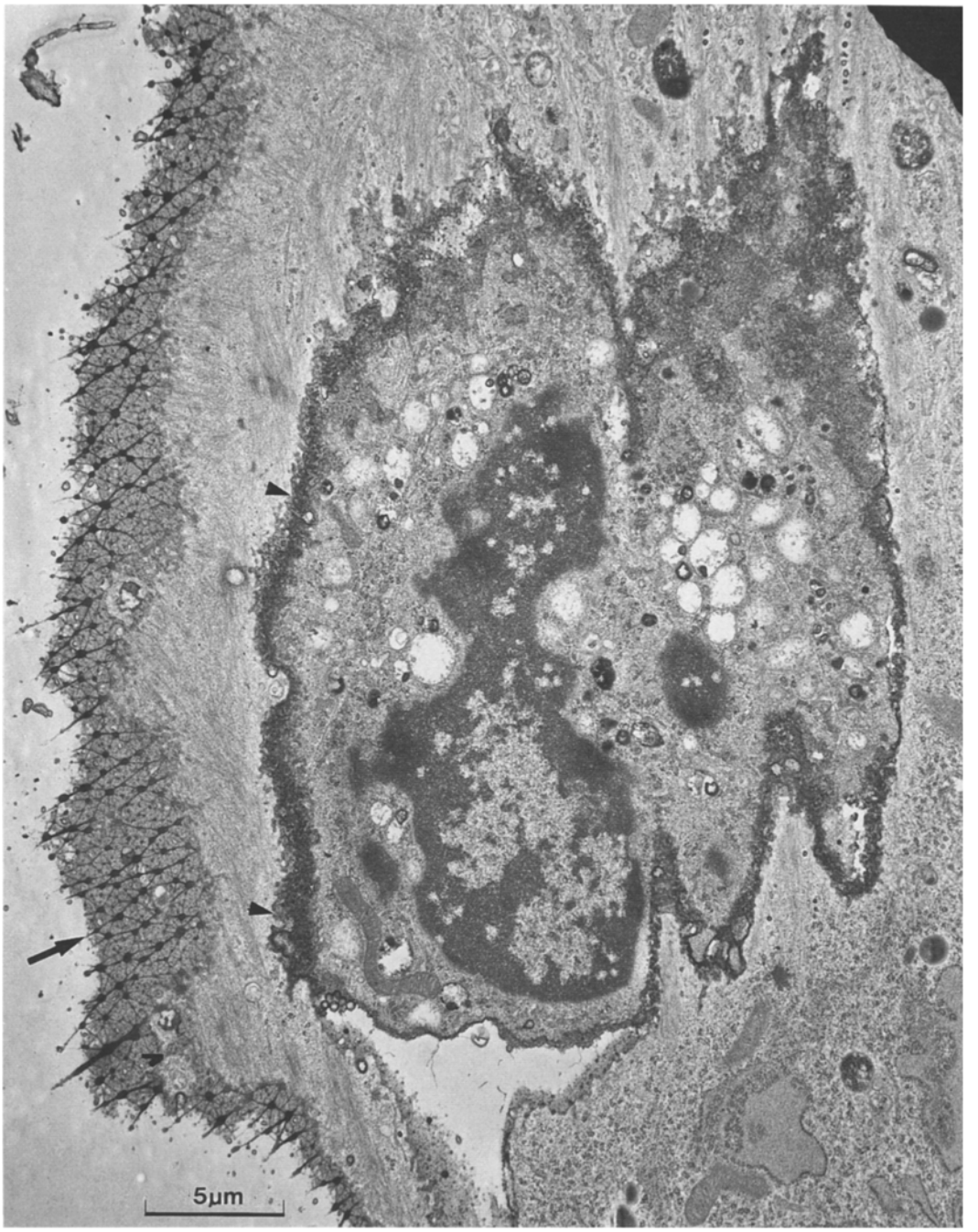

Fig. 8. Fibroblast-like cell engulfing a macrophage (TEM). Ruthenium red was added to the fixatives. *Arrow*, typical extracellular matrix of fibroblasts (reticulate aspect of the ruthenium-red-stained proteoglycans); *arrowheads*, extracellular matrix of an engulfed macrophage. Tangential section of the matrixes (7-day "chronic" culture)

neal cavity, have a long-term survival in vitro and express the Mac-1 surface antigen (Herzenberg et al. 1988).

The yield of peritoneal macrophages was 12-fold higher in chronically infected mice than in controls, and their in vitro survival was nearly twice as long (at the same cell-density seeding). The dramatic increase in the number of peritoneal macrophages in mice infected with *Schistosoma mansoni* has previously been reported by James (1986) and James et al. (1982, 1983); these macrophages, comparable with resident macrophages of normal mice by several biochemical and cytochemical criteria, exhibit an in vitro non-specific tumori-

cidal (James et al. 1982) and larvicidal (James 1986; James et al. 1983) activity associated with immunologically activated cells. This activation, which appears early in infection and is also observed after unisexual infection, is thus independent of the development of egg granulomas and is mediated in vivo by T-cell lymphokines (James 1986; James et al. 1983).

The most important observation made during the present study was the regular appearance of fibroblast-like cells in the "chronic" cultures from about the 6th day after their initiation onwards. These cells are apparently different from the "mesothelial" cells, of unknown origin, which form a feeder layer and enable the proliferation of mouse resident peritoneal macrophages when all cells collected in the washing fluid of the peritoneal cavity are seeded without purification in appropriate culture medium (Lombard et al. 1985, 1988). The fibroblastic cells that we observed appeared in extensively purified cultures, did not form confluent layers and, above all, were only seen in cultures of macrophages from chronically infected mice. All of our observations support the hypothesis that these cells could result from the transdifferentiation of a limited number of macrophages committed in vivo.

Once initiated, differentiation of macrophages from blood monocytes is generally considered to be irreversible, although adaptations to various conditions are possible under the influence of specific glycoproteins or cytokines (Defendi 1976; Gordon 1986); epithelioid and giant multinucleate cells are considered to be more advanced and final stages of the monocyte/macrophage lineage differentiation (Cohn 1968; Sutton and Weiss 1986). We use the term transdifferentiation to emphasize that fibroblast-like cells do not derive from undifferentiated cells but from conversion of differentiated macrophages in another cell type. [This term, which means the "conversion of differentiated cells into another type distinct in morphology, function and molecular constitution from the original type", is largely used in a recent volume of *Current Topics in Development Biology* (vol. 20, 1986), edited by Okada and Kondoh and dealing with this subject.]

We observed in vitro transdifferentiation of macrophages into fibroblasts only when the cells were harvested from chronically infected mice. The fact that we did not observe fibroblast-like cells in cultures of macrophages from controls or from mice in the acute phase of infection furnishes a strong supplementary argument for the absence, at the initiation of the cultures, of contaminating fibroblasts that could become stimulated to proliferate under the influence of factors released by the macrophages. However, we cannot affirm (see below) that peritoneal macrophages from uninfected, control mice would never transdifferentiate into fibroblast-like cells, as these mononuclear phagocytic cells could be stimulated to do so under the influence of an infection or tumour of which we are not aware that is unrelated to schistosomiasis.

An altered production of lymphokines, different from those responsible for the tumoricidal and larvicidal activities of macrophages observed early in infection (James 1986; James et al. 1982, 1983), soluble egg antigens and/or new cell-cell interactions could act upon the surface of macrophages and initiate the activation of quiescent genes, leading to a change of phenotype in vivo or, after in vivo commitment, in vitro. In this case, as an hypothesis consistent with our results, we suggest that the pathway for this conversion could be as follows (Fig. 6): spherical macrophages (type A cells) → flattened macrophages (type B cells) → intermediary cells (between type B cells and fibroblasts) → fibroblast-like cells (type D cells).

The ability of peritoneal macrophages to convert into fibroblasts has previously been suggested (Campbell and Ryan 1983; Kouri and Ancheta 1972; Mosse et al. 1985), but these in vivo studies are always subject to criticism. The in vitro system developed by us enables a more stringent control of the experimental conditions and more confidence concerning the absence of contaminating fibroblasts after trypsinisation, on the initiation of the cultures.

The fibroblast-like cells appearing in the cultures issued from mice in the chronic stage of schistosomiasis cannot presently be distinguished from mouse embryonic fibroblasts in primary culture by ultrastructural criteria. Studies concerning their capacity to synthesize collagen are presently in progress with the view of confirming their fibroblastic nature. We have previously observed that some macrophages are heavily stained after an anti-collagen-PAP treatment in 6-day "chronic" cultures (unpublished results).

Hepatic fibrosis and related sequelae are the major cause of clinical problems and morbidity resulting from *S. mansoni* infections. Fibrosis involves the deposition of an excess of fibrous connective tissue composed mainly of collagen in the organ and is preceded by the formation of granulomas. Collagen is extensively deposited by fibroblasts within and around the granulomas. Periportal fibrosis is generally considered to be indirectly

related to granuloma formation and diffusing soluble antigens.

The origin of the fibroblasts occurring at the onset of fibrosis is unknown. Our results support the idea that under the influence of parasitosis, a macrophage sub-population of chronically infected mice could change their phenotype into a fibroblastic one. The phenotype of a cell is determined by genes directing the synthesis of specific proteins. We must suppose that a proportion of the peritoneal macrophages could change their transcription program during the development of the disease. In vivo, these transdifferentiated macrophages with fibroblastic characteristics would proliferate and form the fibrotic tissue. The confirmation of this hypothesis would open totally new perspectives with regard to the pathogenesis of schistosomiasis and, possibly, the genesis of fibrotic tissues associated with other chronic diseases.

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