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The identification and characterization of antimacrophage serum (AMS) and its application to murine fibrous histiocytoma

Haruhiko Yoshida, Seishi Yamamoto, and Tokichi Yumoto
Department of Pathology, Tottori University School of Medicine, Yonago, Tottori/683, Japan

Summary. The preparation of antimacrophage serum (AMS) and its application to murine fibrous histiocytoma are reported. AMS, prepared by inoculating peritoneal macrophages from mice into rabbits, was purified by repeated absorption with kidney homogenate, red blood cells, thymus cells, non-adherent spleen cells and finally, L-cells. The molecular weight was determined as 170,000 daltons using SDS-polyacrylamide gel electrophoresis. AMS diluted to 1:32 reacted with 61.4% of peritoneal macrophages, but not red blood cells, lymphocytes, granulocytes and fibroblasts, as judged by membrane immunofluorescence.

57.1% of monoclonal B-10 macrophages and 75.6% of 28-12 macrophages transformed with SV40 reacted with AMS. B-10 and 28-12 cell tumors simulating human malignant fibrous histiocytoma gave a distinctive diffuse pattern of immunofluorescence with AMS which affected both round- and spindle-shaped cells, although positive fluorescence of spindle-shaped cells was somewhat weaker than that of round-shaped cells. However, tumor tissues from a murine fibrosarcoma showed no reaction with AMS. Based on these findings, AMS may be useful in determining the histogenesis of some soft tissue tumors of doubtful origin.

Key words: Antimacrophage serum (AMS) – Murine fibrous histiocytoma – Immunofluorescence

Introduction

Reliable macrophage (histiocyte) markers are required to identify accurately reticulo-histiocytes and their neoplastic counterparts. Although several macrophage markers have been isolated for this purpose, most of them cannot be applied to tissue sections. Another drawback is that identification often

Offprints requests to: Haruhiko Yoshida at the above address

requires a combination of two or more macrophage markers. Immunophagocytosis is a function which is specific to macrophages, but can only be recognized in cells in cell suspension or tissue culture. This treatment results, however, in structural alteration to the cells which adapt to the given tissue environment and subsequently exhibit loss or masking of some other markers.

Antimacrophage serum (AMS) has been prepared and its specificity for macrophages confirmed (Argyris and Plotkin 1969; Gallily 1971; Hirsch et al. 1969; Unanue 1968). These authors report that AMS treatment reduced the glass-adherence capacity and phagocytic activity which characterize macrophages. AMS has also been used to study the role of macrophages during infectious processes (Pearson and Osebold 1973, 1974).

Malignant fibrous histiocytoma is currently considered to be a tumor of histiocytes (Hajdu 1979; Hashimoto et al. 1979; Ozzello et al. 1963) although this is still a subject of controversy (Enjoji et al. 1980; Fu et al. 1975; Weiss and Enzinger 1978). We reported a murine fibrous histiocytoma closely resembling the human counterpart induced by inoculation of SV40-transformed macrophages (Yumoto and Morimoto 1980). The purpose of this study is to characterize the AMS prepared using mouse peritoneal macrophages, and to establish its possible use for the identification by immunofluorescence on tissue sections of the cells of murine fibrous histiocytomas.

Materials and methods

1. Preparation of antiserum (AMS)

Peritoneal macrophages were obtained four days after an intraperitoneal injection of 2 ml of 2.5% thioglycollate medium. The peritoneal cavity was washed with 2 ml of Eagle's minimum essential medium (EMEM), and the cells recovered were cultivated for one h. After the non-adherent cells were washed off three times, adherent cells were collected with a rubber policeman and used to immunize rabbits by intravenous injection into the ear vein. The cell population obtained was over 90% macrophages according to their ACPase and non-specific esterase activities. Rabbits weighing 3 kg were used for immunization. Cell suspensions (1×10^7 cells/ml) were injected three times a week and blood was obtained by cardiac puncture four weeks after confirming the presence of a narrow band in the gamma globulin region by diffusion electrophoresis. The antiserum was centrifuged at 2,000 rpm for 30 min and then incubated at 56° C for 30 min to inactivate complement.

2. Absorption

Repeated absorptions were carried out using the various tissues and cells described below to remove unwanted antibodies: (a) non-adherent spleen cells (lymphocytes): mouse spleens (NZB strain mice) were minced, suspended in EMEM and cultivated for one h at 37° C after eliminating the red blood cells by Ficoll-Conray density gradient centrifugation. The medium containing non-adherent spleen cells was then collected and centrifuged at 1,500 rpm for 10 min. (b) kidney homogenate: mouse kidneys were minced and homogenized in a suitable volume of EMEM with a glass homogenizer. This was then washed three times with EMEM. (c) thymus cells: mouse thymus was minced, broken up by pressing between two glass slides and suspended EMEM. Thymus cells were then collected after being gently washed three times. (d) L-cells: L-cells are widely accepted as a cell line originating in mouse skin fibroblasts. This cell line, maintained in our laboratory, has never shown cytoplasmic ACPase or non-

specific esterase activities. (e) red blood cells: red blood cells were collected from the orbital vein and washed three times with EMEM. The absorptions were performed by adding one tenth volume of kidney homogenate, red blood cells, thymus cells, non-adherent spleen cells and then L-cells for 30 min at 37° C and 4° C.

3. Titration assay

a) Immunofluorescence. Immunofluorescent staining with AMS was carried out as follows. 1) 1×10^7 macrophages/ml were prepared after washing three times with cold staining buffer (0.01 M, pH 7.4), 2) 0.1 ml of AMS was added and incubated for 30 min at room temperature and 3) 0.1 ml of FITC-conjugated anti-rabbit sheep IgG was added and incubated for 30 min at room temperature after washing three times.

b) Complement-dependent cytotoxicity. The cytotoxicity of AMS was determined for peritoneal macrophages and non-adherent spleen cells by the following procedures. 1) 0.1 ml of a cell suspension (1×10^7 cells/ml) was added to 0.1 ml of antibody diluted from 1:1 to 1:32 by a two-fold dilution method and incubated for 30 min at room temperature. 2) 50 µl of guinea-pig complement diluted to 1:10 was added and incubated for 60 min at 37° C, and 3) the reaction was stopped in an ice bucket and stained with 0.25 ml of 25% trypan blue.

4. Preparation of tumors from transformed macrophages (B-10 and 28-12 cells)

Originally B-10 and 28-12 cells were derived from peritoneal macrophages and bone marrow cells, respectively. These cells were infected and transformed by simian virus 40(SV40), and then cloned (Takayama 1980). Their identitiy as macrophages was determined by their displaying the property of phagocytosis, and by the demonstration of Fc and C3 receptors which were present on most of the cells. These two cell lines were kindly supplied to us and have been maintained in our laboratory. We have confirmed that each transformed cell line possess ACPase and non-specific esterase activities (Morimoto 1980). Inoculation of B-10 and 28-12 cells (3×10^7 cells) produced subcutaneous tumors in irradiated inbred nude mice after 13 to 105 days. The tumors were then successfully transplanted. The details of their biological data have been reported in other papers (Morimoto 1980; Yumoto and Morimoto 1980). Each transformed cell line and its tumor cells possess SV40 T-antigen demonstrated by anti-SV40 T-serum treatment. Tumors derived from both B-10 and 28-12 cells were excised when a few cm in diameter. They were examined by light and electron microscopy, enzyme histochemistry and immunofluorescence microsopy using AMS. Frozen sections (2 µm thickness) of the tumors were cut with a cryotome at −20° C and immediately fixed with an equal volume of acetone and alcohol for 10 min. After rinsing with cold buffer three times for 30 min, AMS diluted to 1:32 was placed on the fixed sections and incubated in a moist chamber for 30 min at room temperature. FITC-conjugated rabbit IgG (Fuji-Zoki, Nagoya, Japan, 1; 6 dilution) was then added and incubated for 30 min at room temperature.

Result

1. Characterization and identification of antimacrophage serum (AMS)

a) Characterization after Absorption. The antiserum, in which a narrow band was detected by diffusion electrophoresis, was immediately absorbed with kidney homogenate, red blood cells, thymus cells and non-adherent spleen cells. Subsequently it was further absorbed with L-cells. The antiserum was then run on a SDS-polyacrylamide gel electrophoresis column. The electrophoresis delineated a single band at 170,000 daltons molecular weight, similar to IgG (Fig. 1). The titration was then tested by complement-dependent

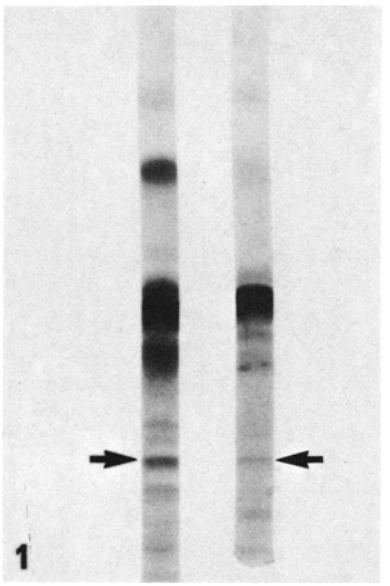

Fig. 1. Antimacrophage serum (AMS) run on SDS-polyacrylamide column (*left*: unabsorbed serum, *right*: antiserum absorbed by kidney homogenate, red blooed cells, non-adherent spleen cells, thymus cells and L-cells) at 8 mV for 3 h. *Arrows* indicate a band at molecular weight of 170,000 daltons

cytotoxicity and immunofluorescence. Peritoneal macrophages were chosen as target cells for both titration assays, and non-adherent spleen cells (lymphocytes) were chosen as controls. The data showed that the antiserum, absorbed with the appropriate tissues and cells as described above, was highly cytotoxic to peritoneal macrophages, destroying 68% using antiserum diluted to 1:16 and 61.4% at a dilution of 1:32, but not to non-adherent spleen cells (Fig. 2). The immunofluorescence test showed a positive reaction in over 70% of peritoneal macrophages using antiserum diluted to 1:16 and in 61.9% even when diluted to 1:32. However, a positive reaction occurred in only 4.5% of non-adherent spleen cells diluted to 1:32 (Fig. 3). Thus, AMS at a 1:32 dilution was chosen for the following experiments. The positive fluorescence showed preferential staining of the cell membrane giving a ring and cap appearance (Fig. 4). Ultrastructurally using an immunoperoxidase technique, reaction products were demonstrated on the cell membrane of the peritoneal macrophages (Fig. 5).

b) Antigenic property of various cell and tissue cultures

Several cell lines including tissue sections of murine lymphomas and fibrosarcomas were tested using AMS diluted to 1:32, and the cumulative results are shown in Table 1. The use of AMS without absorption by L-cells gave positive fluorescence with a minority of non-adherent spleen cells (8%), L-cells (14.3%) and fibroblasts (4.2%). When the antiserum was absorbed with L-cells, it did not react with thymus cells, granulocytes, L-cells and red blood cells, indicating it macrophage-specificity. The antiserum reacted only with macrophages giving a positive reaction with 95% from the perito-

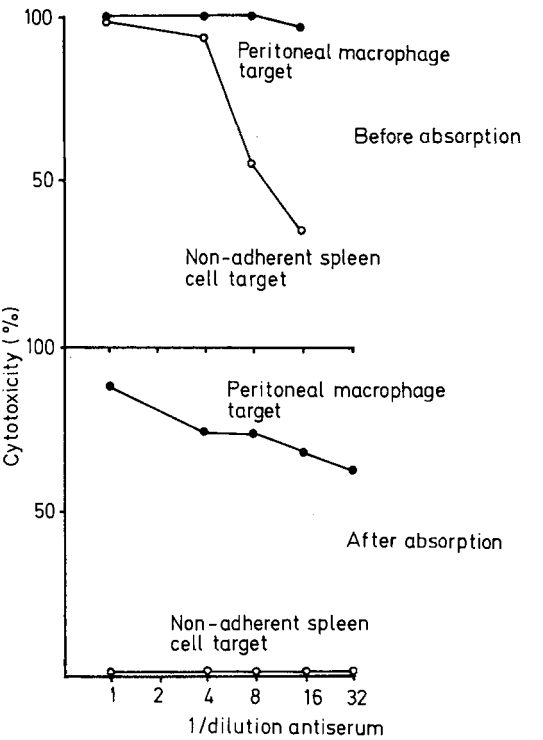

Fig. 2. Characterization of AMS by complement-dependent cytotoxicity

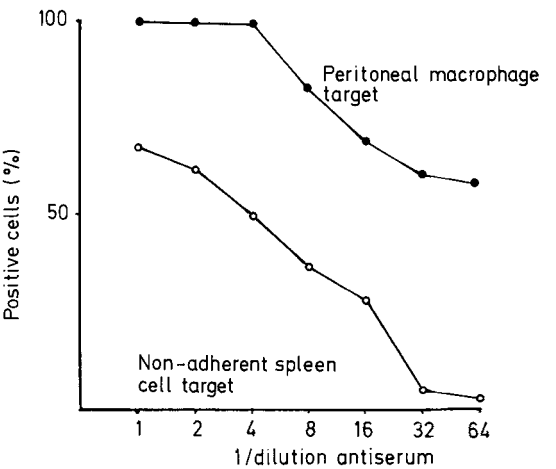

Fig. 3. Membrane immunofluorescence for peritoneal macrophages and non-adherent spleen cells

neal cavity, 76% from the spleen and 90% from the bone marrow. Positive fluorescence was obtained in only 1.4% of fibroblasts collected from newborn mouse skin and cultivated for 20 days, which was not considered to be a serious objection to its specificity for macrophages. The tissue sections from lymphomas and fibrosarcomas gave no reaction with AMS except in occasional macrophages scattered in the tumor stroma.

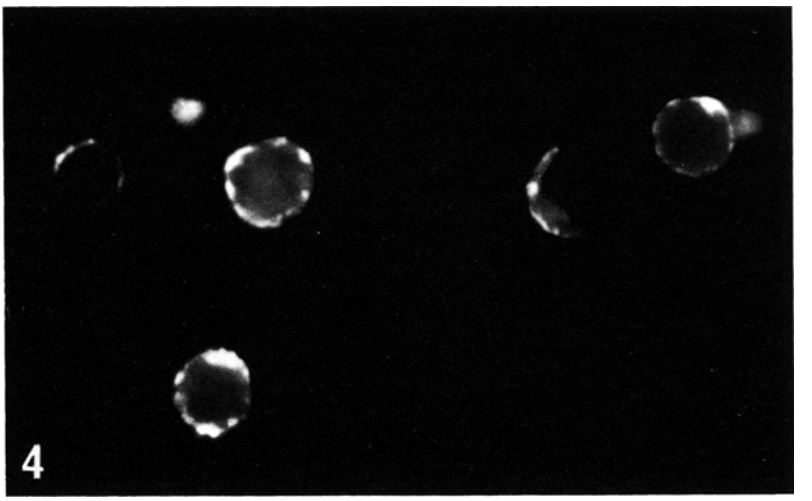

Fig. 4. Indirect immunofluorescent staining of mouse peritoneal macrophages stimulated by 2.5% thioglycollate medium, treated with absorbed AMS at a dilution of 1:32. 1×10^7 macrophages were incubated with an addition of 0.1 ml of AMS diluted to 1:32 for 30 min at room temperature. 0.1 ml of FITC-conjugated anti-rabbit sheep IgG was added and incubated for 30 min at room temperature after rinsing three times. A ring and cap appearance is illustrated. ×1320

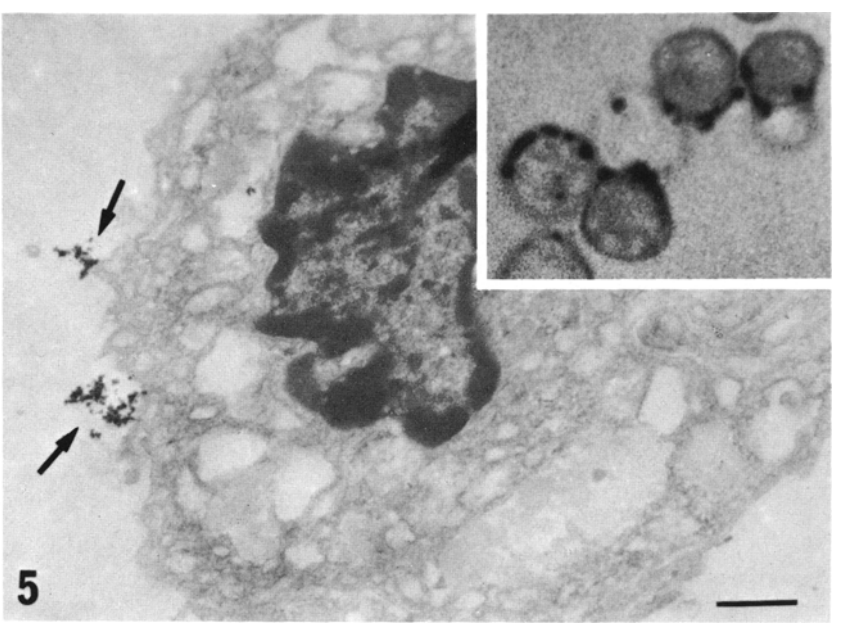

Fig. 5. Electron micrograph of mouse peritoneal macrophage using immunoperoxidase method (inset: light micrograph ×910). The reaction products are demonstrated on the cell membrane. ×11,400

Table 1. Positive fluorescence with AMS in various cells and tissues

Cells and tissues	% positive
Non-adherent spleen cells	4.5% (8.0%[a])
Thymus cells	0%
Granulocytes	0%
L-cells	0% (14.3%[a])
Fibroblasts (new-born mouse skin)	1.4% (4.2%[a])
Fibrosarcoma (mouse, subcutis)[b]	negative
Murine B-cell lymphoma (IgG_{2a})[b]	negative
Murine T-cell lymphoma ($\theta+$, Ig$-$)[b]	negative
Adherent spleen cells	76.0%
Bone marrow macrophages	over 90%

[a] AMS unabsorbed by L-cells
[b] Tissue sections from the tumors inoculated subcutaneously

Table 2. Positive fluorescence with AMS in B-10 and 28-12 cells

		B-10 Cells		28-12 Cells	
		Round and epithelioid	spindle	Round and epithelioid	spindle
Cell population in culture cells		68.6%	31.4%	95.0%	5.0%
% positive (AMS 1:32)	Attached	70.8%	27.3%	78.5%	20.0%
	Suspended	57.1%		75.6%	

2. Immunofluorescence finding in two cell lines of SV40 transformed macrophages (B-10 and 28-12 cells)

The characteristics of both cell lines have already been described; they originally possessed phagocytic activity, Fc and C3 receptors, and gave positive ACPase and non-specific esterase reactions. Moreover, a phenotypic reversible transition was observed in both cell lines; in the transformed state, the cells appear spindle-shaped and lose differentiated macrophage function (Takayama 1980). Table 2 shows the cell population in both cell lines which were cultivated with EMEM and 5% fetal calf serum in an atmosphere of 20% CO_2. Their antigenicity against AMS diluted to 1:32 was tested by immunofluorescence. Of the B-10 cells 68.6% were round or epithelioid and 31.4% were spindle-shaped. Of the cells suspended in tissue culture fluid, 57.1% showed positive immunofluorescence (Fig. 6). When attached to a dish, 70.8% of the round or epithelioid and 27.3% of the spindle-shaped cells gave positive fluorescence with AMS. On the other hand, 75.6% of the 28-12 cells showed specific fluorescence when suspended in tissue culture fluid. When attached to a dish, 78.5% of the round or epithelioid cells (95% of the 28-12 cell population), and 20% of the spindle cells (5% of the 28-12 cell population) gave positive fluorescence with AMS (Fig. 7).

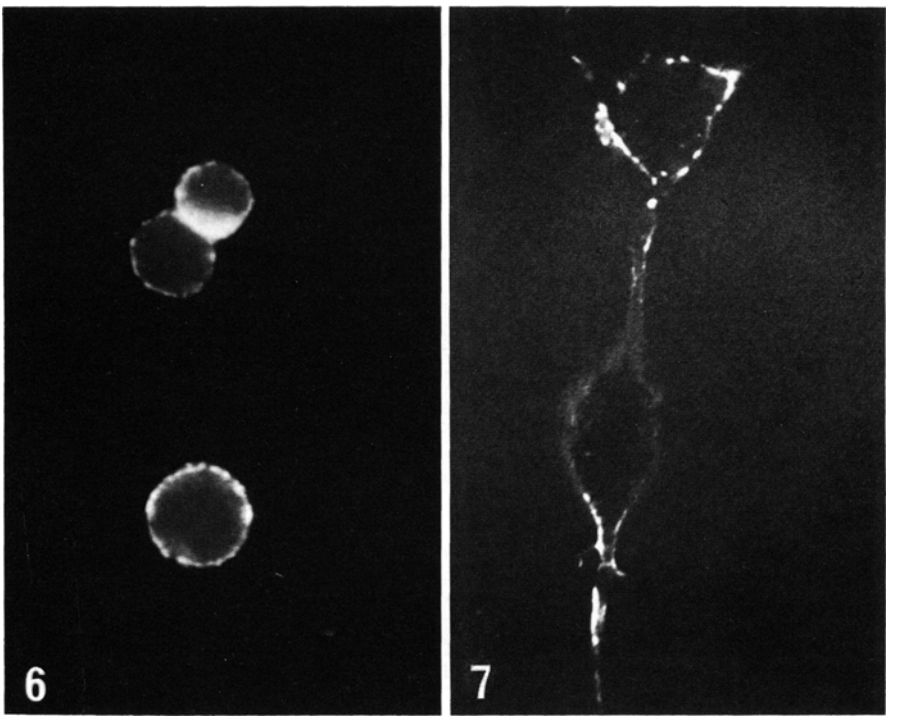

Fig. 6. Indirect immunofluorescent staining of B-10 cells which were derived from peritoneal macrophage, transformed by SV40 cloned. They were treated with AMS in the same manner as in Fig. 4. A ring and cap appearance is illustrated. × 320

Fig. 7. Indirect immunofluorescent staining of 28-12 cells which were derived from bone marrow cells, transformed by SV40 and cloned. The culture cells attached to the plastic dish are illustrated. Epithelioid and spindle-shaped cells are shown stained by fluorescent dye. × 320

3. Immunofluorescence findings on the tumors induced by transformed macrophages (B-10 and 28-12 cell tumors)

a) Histological findings. Both tumors consisted of a dense proliferation of round and plump spindle-shaped cells in a storiform arrangement (Fig. 8a). Round histiocytic cells were numerous, especially in the periphery of the tumor (Fig. 9a). Both tumors contained occasional bizarre giant cells and foam cell clusters and the only difference between them was the presence of myxoid change in the B-10 cell tumor and its absence in the 28-12 cell tumor. The myxoid change occurred in areas of loosely arranged spindle-shaped cells.

b) Immunofluorescence. Sections of tumor (2 μm thick) were cut with a cryotome and was treated with AMS diluted to 1:32 for immunofluorescent staining. B-10 and 28-12 cell tumors showed essentially similar diffuse fluo-

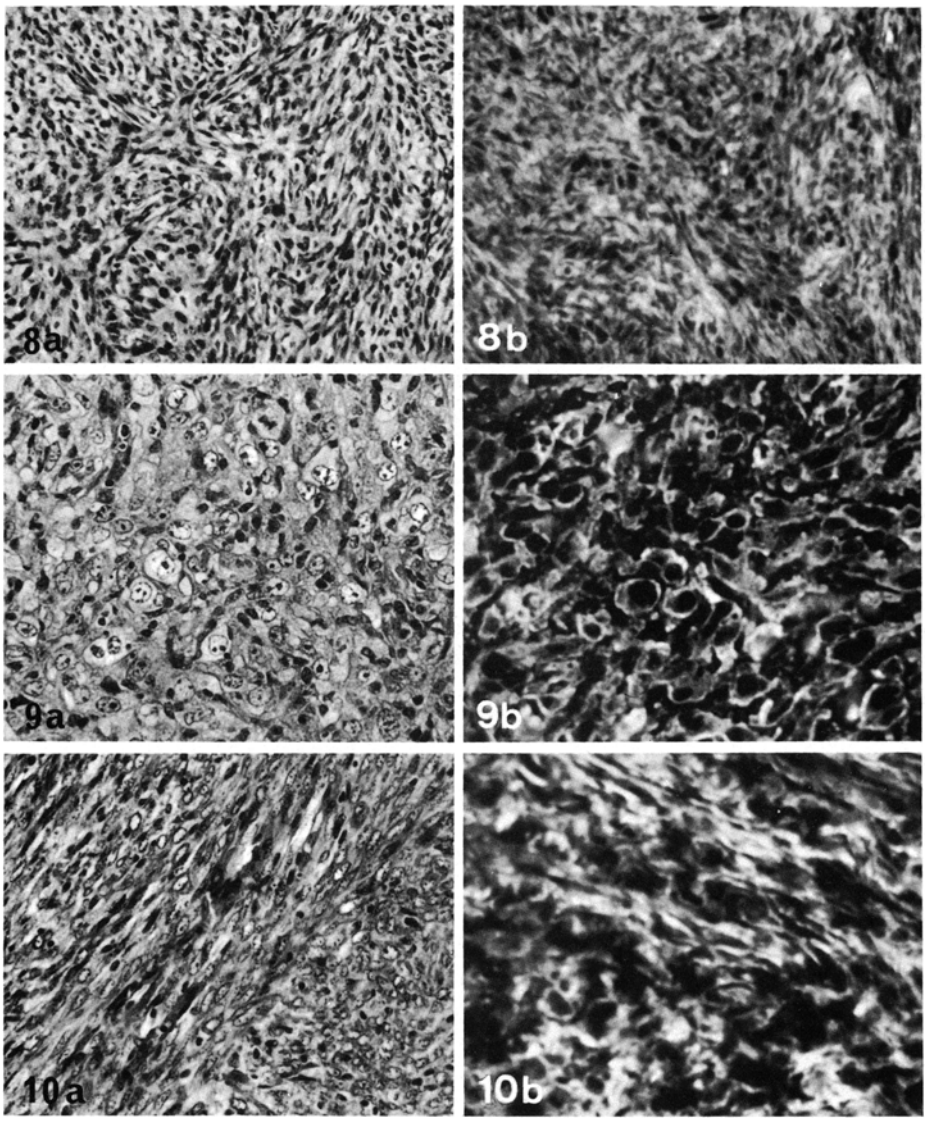

Fig. 8a, b. Transplantable B-10 cell tumor developed 60 days after subcutaneous inoculation of B-10 cells in ICR mouse. Plump spindle-shaped cells are arranged in a storiform pattern or in fascicles (**a**: H & E stain, × 180), immunofluorescence with diffuse reaction (**b**, × 180)

Fig. 9a, b. Another area of the above tumor. Large round histiocytic cells are shown (**a**: H & E stain, × 320), with specific fluorescence in the cells (**b**, × 320)

Fig. 10a, b. Transplantable 28-12 cell tumor developed 15 days after subcutaneous inoculation of 28-12 cells. Showing plump spindle-shaped cells (**a**: H & E stain, × 180) and immunofluorescence with diffuse and marginal reaction of the cells (**b**, × 320)

rescence (Fig. 8b). The myxoid areas of the B-10 cell tumor were also positive, though to a lesser extent. The intensity of fluorescence was much stronger in the round than in the spindle-shaped cells (Figs. 8b, 9b and 10b). Fluorescent staining principally affected the cell surfaces, although it also affected the cytoplasm of occasional round cells. The tumor cells showed no fluorescence when treated with normal rabbit serum.

Discussion

Antimacrophage serum (AMS) has been used previously for distinguishing macrophages from other cells (Argyris and Plotkin 1969; Gallily 1971; Gallily and Gornostansky 1971; Glenis et al. 1982; Unanue 1968; Walder et al. 1980). Glenis et al. (1973) reported that an apparently macrophage-specific antigen detected by AMS was shared with lymphocytes, granulocytes and fibroblasts. To establish an antiserum specific to macrophages, they absorbed antiserum with red blood cells, heart homogenate, lymphocytes and granulocytes. Stinnett et al. (1976) insisted that over twenty repeated absorptions with lymphocytes were necessary to obtain a specific antiserum. Our data demonstrated an antiserum specific to macrophages after repeated absorptions with kidney homogenate, red blood cells, thymus cells, non-adherent spleen cells and, finally, L-cells which were derived from mouse skin fibroblasts and then cloned. AMS eluted from these absorptions was also fixed by macrophages taken from the spleen and bone marrow as well as resident peritoneal macrophages, but not by granulocytes and fibroblasts.

With regard to the location of the antigenic determinant in macrophages, there have been a large number of reports demonstrating specific fluorescence on the cell membrane (Gallily and Gornostansky 1971; Glenis 1973; Walder et al. 1980), but others have shown positive fluorescence in the cytoplasm (Persson and Rönnbäck 1979; Thompson et al. 1982). Gallily and Gornostansky (1971) found no significant difference in cytotoxicity between regular AMS obtained by immunizing with whole macrophages and membranous AMS made by immunizing with only cell membranes. Using biochemical analysis, Stinnett et al. (1976) concluded that a macrophage-specific antigen reacting with AMS was present in the cell membrane of macrophages. They also suggested that some other cytoplasmic antigens might react with AMS. According to our immuno-electron microscopic data, the antigenic determinant of macrophages is localized exclusively in the cell membrane. In tissue sections, however, macrophage-derived tumor cells showed positive fluorescence with AMS both on their surfaces and in their cytoplasm. There are two possible explanations of this finding. Firstly the reaction may reflect diffusion of the antigenic determinant, and secondly the AMS, which was prepared by immunization with whole macrophages, may react simultaneously with an other unknown cytoplasmic antigenic determinant. Thompson et al. (1982) showed a diffuse cytoplasmic reaction with AMS using the immunoperoxidase technic on paraffin sections to locate macrophages in various murine tissues. This suggests that cytoplas-

mic fluorescence in tissue sections can be accepted as a positive reaction, even though the major antigen for AMS is in the cell membrane.

As mentioned above, there have been several reports on the identification and characterization of AMS, but none on the application of AMS to sections of tumor tissue. There is still controversy over origin of malignant fibrous histiocytoma and especially whether the spindle-shaped cells in these tumors are transformed histiocytes (Enjoji et al. 1980; Fu et al. 1975; Weiss and Enzinger 1978). In order to evaluate the histiocytic nature of this tumor, many different technics have been employed including tissue culture, immunology, electron microscopy and enzyme histochemistry. The exact histogenesis has not been determined by these methods, however, partly because of difficulties relating to the presence of non-neoplastic histiocytes within the tumor stroma. Transplantable murine tumors, resembling human malignant fibrous histiocytomas were induced by transformed macrophage lines designated as B-10 and 28-12 cells and confirmed as monoclonal macrophage-derived tumors, nevertheless consisted of a mixture of round histiocytic cells and spindle fibroblastic cells (Yumoto and Morimoto 1980). Immunofluorescent staining of these tumors with AMS was much more strongly positive in the round than in the spindle-shaped cells. This suggests that the spindle-shaped cells in these tumors may be transformed round cells, as suggested by Kouri and Ancheta (1972), and correspond to the cells called facultative fibroblasts by Hajdu (1979) and Ozzello et al. (1963). In general, fluorescence with AMS affected the cell borders, but not infrequently was present in the cytoplasm. Immunofluorescent microscopy with AMS should prove to be a useful technic for identifying macrophages and their neoplastic counterparts. Thompson et al. (1982) reported the identification of normal macrophages using AMS in paraffin sections of various murine tissues, and this paper describes the first attempt to apply AMS to tissue sections of murine fibrous histiocytomas derived from macrophages.

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