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HETEROGENEITY OF MACROPHAGE POPULATIONS: ANTIBODIES DETECTING VARIOUS POPULATIONS OF MACROPHAGES

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ABSTRACT

Two groups of antibodies against mouse resident alveolar and peritoneal macrophages have been raised individually in rabbits by immunization with whole cell suspensions of lavaged alveolar and peritoneal macrophages. The whole antisera reacted positively with neutrophils and lymphocytes. However, upon extensive absorptions with these cells the activity became restricted to macrophages. The immunoglobulins were purified up to 8 fold by repeated precipitations with saturated ammonium sulphate. The antibodies were labeled with fluorescein isothiocyanate and further used. The specificity of antibodies was confirmed by direct and indirect immunofluorescence and complement-dependent cytotoxicity. In the cytolysis of target cells in the presence of complement, the antibodies could lyse more than 80% of cells at dilutions as low as 1:512.

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INTRODUCTION

Macrophages constitute a heterogenous population of cells distributed in various tissues of animals. These cells originate from a common ancestor in the bone marrow called colony forming cells-granulocyte macrophage (CFC-gm).[25] CFC-gm are progenitor cells of granulocytes as well.[1] Several steps of differentiation and proliferation of CFC-gm give rise to monocytes. Monocytes circulate in the blood and migrate to various tissues where they further differentiate into macrophages. The stimuli and processes of differentiation of monocytes at this stage are unknown.

Surface antigens of macrophages have been described by several authors.[2,10] Many laboratories have also prepared monoclonal and polyclonal antibodies against macrophages.[9-13] Macrophages from various tissues vary markedly in morphology, metabolism, surface receptors, enzyme content, and functional properties.[5,14-16] Some of the antibodies bind to all macrophages[12] and some others are directed against a specific subpopulation of macrophages.[17-19] These antibodies, which are not available commercially, pro-

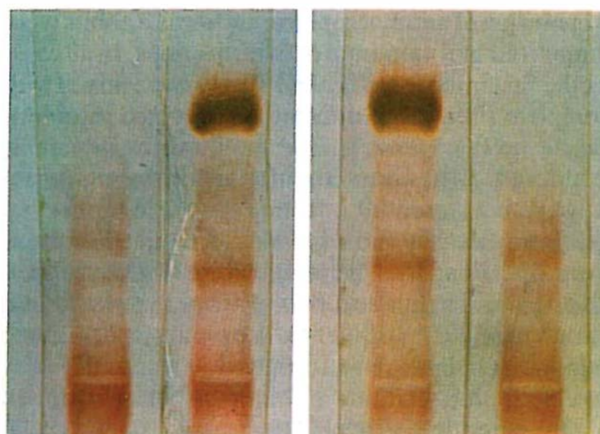

Fig. 1. Agarose gel electrophoresis of serum from the rabbit immunized against a) alveolar macrophages, and b) peritoneal macrophages.
1) Serum before ammonium sulphate precipitation.
2) Serum after third precipitation.

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vide excellent tools for studying the differentiation of various macrophage subpopulations.

In this report we describe preparation oftwo groups of polyclonal antibodies directed against mouse alveolar and peritoneal macrophages. Having been absorbed properly, these antibodies show no nonspecific binding to other blood cells.

MATERIALS AND METHODS

Animals: NIH mice of either sex were used as the source of macrophages. Antisera were raised in *Orcytalogus caniculus* rabbits.

Macrophages: Alveolar macrophages were harvested by lavaging the lung 15 times with 1 ml of saline. A total volume of 5 ml of saline was used for each mouse. Cells were pelleted down by centrifugation at 2500 g at 4°C for 20 min. Cells were resuspended in hypotonic tris-ammonium chloride buffer (0.747 g ammonium chloride; pH 7.2) for 15 min to lyse red cells. The suspension was centrifuged as above and the cells were resuspended in 1 ml saline.

Peritoneal macrophages were harvested by washing the peritoneal cavity 15 times with 3 ml saline. A total volume of 15 ml of saline was used for each mouse. Cell suspensions collected were contrifuged as above and the pellet was resuspended in 1 ml of Dulbecco's modified Eagle medium (DMEM; Gibco). Cells were incubated for 1 hr on petri dishes precoated with fetal calf serum (FCS) at 10^6 cells/cm^2 of petri dish at 37°C. Nonadhering cells were discarded. Adhering cells (about 90% macrophages) were collected, washed three times with saline and used. Cell count, Trypan blue exclusion viability test, and differential counts were performed on all cell samples.

Preparation of antimacrophage antisera: Rabbits were immunized by a minimum of five injections. In all cases for the first injections $20\pm2\times10^6$ cells were mixed with Complete Freund's Adjuvant and injected intraperitoneally (IP). The third and fourth injections ($20\pm2\times10^6$ cells) were without adjuvant and IP. Animals were boostered by injecting the same number of cells intravenously (no adjuvant). All injections were given 10 days apart. The rabbits were bled seven days after final injection and the sera were harvesed and stored individually at −20°C. A partial purification of immunoglobulins from serum proteins was achieved by repeated precipitations of antisera with saturated ammonium sulphate at pH 7.8.[20]

Gel electrophoresis of antibodies: Antisera were analyzed by electrophoresis on agarose gels with a Corning model 470 electrophoresis system. The 1% agarose gel

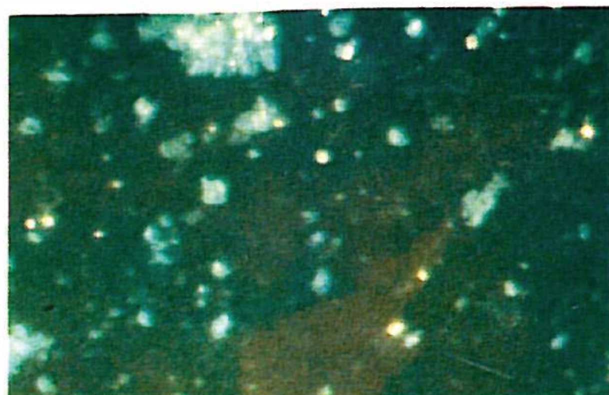

Fig.2. Nonspecific labeling of lymphocytes by antisera raised against peritoneal macrophages. 400x.

was prepared in barbital buffer pH 8.6. 1 μl of samples were applied to gel and electrophoresed for 45 min and 90 V. Gels were stained in Panceaus S for 15 min, destained and baked in 50°C oven for 20 min. Dried gels were scanned at 510 nm by Corning 470 densitometer.

Absorptions: Antisera were absorbed by thymocytes and neutrophils. Mouse thymocytes were prepared by teasing the thymus and forcing it through various gauge needles. Cells were washed three times with phosphate buffered saline (PBS). Neutrophils were collected from the peritoneal cavity 4 hrs after injecting 3 ml of sterile 1% starch in saline into the peritoneal cavity.

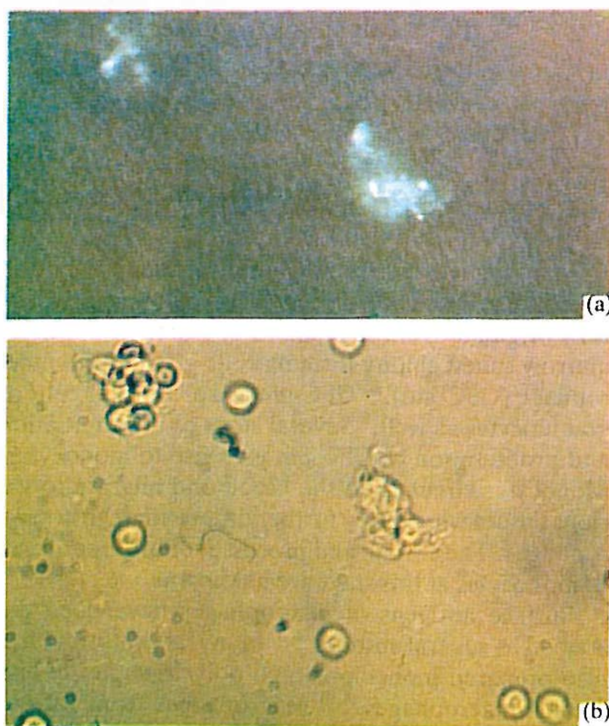

Fig.3. a) Specific immunofluorescence labeling of peritoneal macrophages by antisera raised against them and absorbed three times with lymphocytes. Notice the absence of immunofluorescence on lymphocytes. 400x. b) The same field as (a) but with ordinary light.

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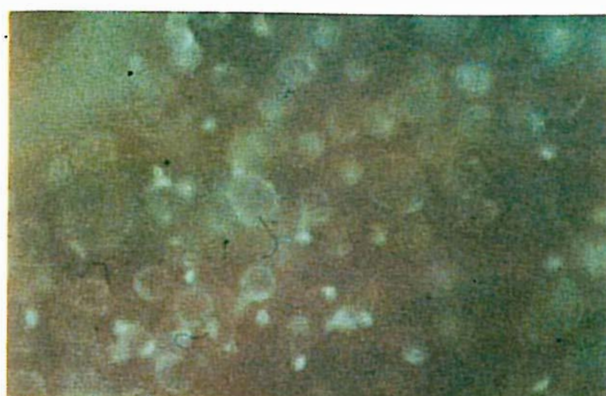
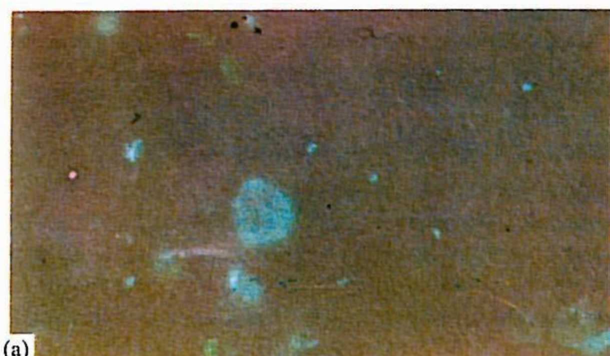


Fig.4. Nonspecific immunofluorescence labeling of neutrophils by antisera raised against alveolar macrophages. 400x.

Cells were washed three thimes in PBS. 10 ml of antisera was absorbed first, three times each with $5.5\pm2\times10^6$ thymocytes and then twice each with the same number of neutrophils. No attempts were made to lyse the red cell contamination in either thymic cells or neutrophil suspensions. Therefore, a mutual absorption with red cells was accomplished.

Complement-dependent cytotoxicity: A cytotoxic titration of antisera against peritoneal and alveolar macrophages was carried out. 5×10^5 cells were incubated 2 hrs in DMEM supplemented with 10% FCS at 37°C in four chambered dishes (NUNC). After washing the dishes adherent cells were incubated with various dilutions of antisera for 1 hr. Then the supernatant was removed and cells were washed with saline. Finally cells were incubated for 30 min with fresh human serum as the source of the complement. Cell lysis was determined by trypan blue uptake test.



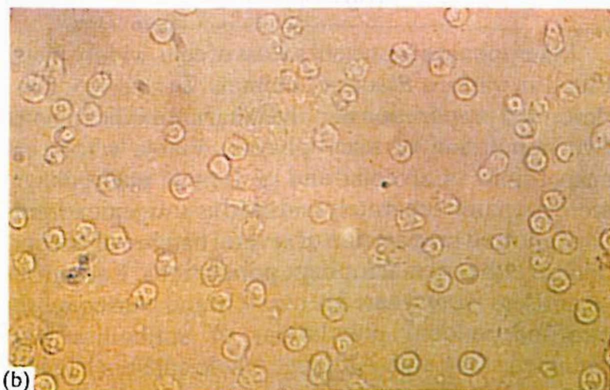


Fig.5. a) Removal of nonspecific antibodies against neutrophils by antisera raised against peritoneal macrophages after two precipitations with ammonium sulphate. Notice the absence of immunofluorescence on neutrophils. 400x. b) Same fields as a but with ordinary light.

Immunofluorescence: Binding of antibodies to target cells were analyzed by either direct or indirect immunofluorescence. For direct immunofluorescence, antibodies were labeled with fluorescein isothiocyanate (FITC), (Polysciences,Inc) dissolved in 2% solution of $NaHCo_3$ at pH 8.2.[21] To each 1 ml of antiserum 100 μl of sodium bicarbonate, pH 9 was added. Then equal volume of FITC solution containing 15 μg of FITC per mg of protein of antiserum was added slowly (within 30 min) to the antiserum. The FITC antiserum mixture was incubated for 18 hrs in dark at 4°C. Unconjugated FITC molecules were removed by filtration chromatography on Sephadex G-25 column.[12] The extent of labeling was judged by determining the molar ratio of FITC to protein (F/P molar ratio) for each labeled sample.[23] For indirect immunofluorescence FITC-conjugated anti-rabbit IgG (SIGMA) was used. For

for 45 min with target cells at 4°. Cells were then washed 3 times with PBS and incubated with FITC- conjugated anti rabbit IgG for 45 min at 4°C. Finally cells were washed with PBS and examined under fluorescence microscope.

Protein determination: The protein content of samples was determined according to the modified procedure of Lowry.[24] Bovine serum albumin was used as the standard.

RESULTS

Preparation of antisera against peritoneal and alveolar macrophages: Four rabbits were immunized against peritoneal macrophages and three were immunized against alveolar macrophages. Antiserum from each animal was treated and used separately. Antisera were collected and immunoglobulins were partially purified from serum proteins by repeated saturated ammonium sulphate precipitation. Samples from antisera before and after ammonium sulphate precipitations were analyzed by agarose gel electrophoresis. Figure 1a shows the degree of purification achieved for antiserum from one animal immunized against alveolar macrophages. Figure 1b shows similar results obtained form another animal immunized against peritoneal mac-

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rophages. In almost all cases, three precipitations with saturated ammonium sulphate decreased the serum albumin fraction from 60-70% to 2-0%, and increased the gammaglobulin fraction from 8% to 50-60%.

Agglutination studies: The titer of antibodies was determined by agglutination of target cells by corresponding antisera. All samples showed a high titre of antibodies. Up to 1:512 dilution of antisera was positive in agglutinating target cells.

Immunofluorescence: Antibodies were labeled with FITC as described in the method section. The F/P value for antibodies raised against alveolar and peritoneal macrophages is given in Table I. For both types of antibodies the F/P value was indicative of minimal non specific labeling.

Absorptions: To remove non specific antibodies, antisera were absorbed with suspensions of various cell types. First, they were absorbed with thymic cells three times, each with $5.5\pm2\times10^6$ cells. Figure 2 shows the nonspecific binding of unabsorbed antisera raised against peritoneal macrophages to thymic cells. Nonspecific antibodies were removed totally after three times of absorption with thymic cells, as indicated in Figure 3. These antisera were then absorbed twice with neutrophils collected from the peritoneal cavity of mice. Each absorption was done with $4.2\pm1.4\times10^6$ cells. Figure 4 shows the nonspecific binding of unabsorbed antisera to neutrophils. Nonspecific antibodies to neutrophils were totally removed after two times of absorption, as indicated by the absence of fluorescence on neutrophils in Figure 5. Similar results were obtained for antisera against alveolar macrophages after absorption with thymic cells and neutrophils.

The specific binding of antibodies against alveolar and peritoneal macrophages to their relevant target cells was demonstrated by indirect immunofluorescence in Figures 6 and 7.

Complement-dependent Cytotoxicity: Absorbed antisera were titrated by complement-dependent cytotoxicity against target cells. Normal serum from rabbits

Table I. The F/P ratio of various classes of antibodies raised against alveolar and peritoneal macrophages conjugated with FITC

	Anti alveolar macrophage antibody		Anti peritoneal macrophage antibody	
	Ig G	Ig M	Ig G	Ig M
F/P	2.01 ± 0.3^a	9.88 ± 1.6	2.17 ± 0.56^b	10.08 ± 2.75

a) Values are Mean ± SE of 9 samples.
b) Values are Mean ± SE of 8 samples.

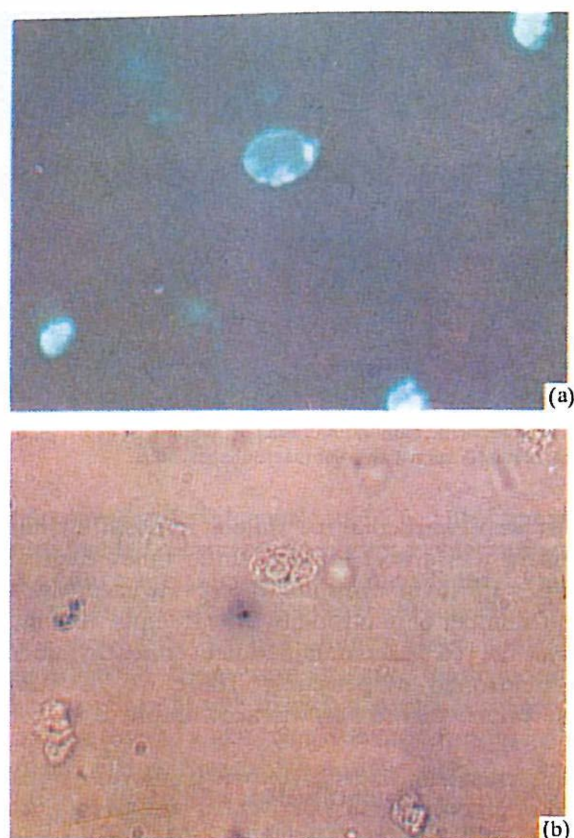

Fig.6. a) Specific labeling of alveolar macrophages by antisera raised against these cells. Indirect immunofluorescence. 400x. b) Same field as a but with ordinary light.

was used as control. The result is shown in Figure 8. Normal serum at dilutions higher than 1:128 could only lyse less than 10% of the cells. However, for absorbed antisera more than 80% of cells were lysed at dilutions as low as 1:512.

DISCUSSION

Macrophages represent a class of cells widely distributed in various tissues of animals. In this report we described preparation of polyclonal antibodies against two population of macrophages. Viable whole cell suspensions of alveolar and peritoneal macrophages were prepared separately and used as antigenic stimuli. This allowed preparation of several batches of antisera against either of macrophage populations. Each group of antisera were treated separately and consequently, two independent populations of antibodies were obtained at the end. Although whole antisera had high titer of antibody, an 8-fold concentration of immunoglobulins was achieved by repeated precipitations with saturated ammonium sulphate solution.

Final suspensions of alveolar and peritoneal macrophages used for injecting rabbits contained less than

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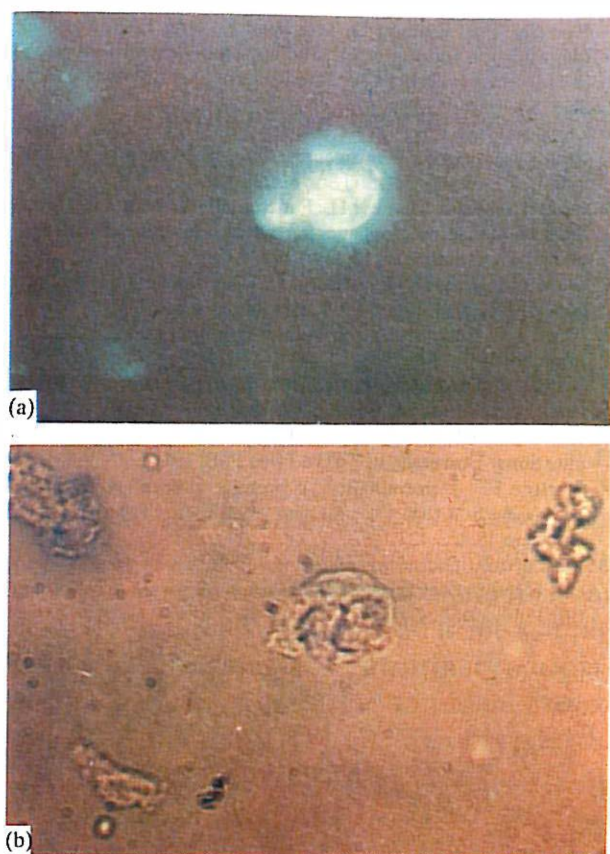

Fig.7. a) Specific indirectimmunofluorescencelabeling of peritoneal macrophages by antisera raised against these cells.400x.b)Same field as a but with ordinary light.

10% of other cell types. These cells were mainly neutrophils, lymphocytes, and erythrocytes. Direct immunofluorescence studies clearly indicated presence of nonspecific antibodies against these cell types. Therefore, the antisera were absorbed with suspensions of thymic cells and neutrophils. These cell suspensions contained enough contaminating erythrocytes in the antisera to remove nonspecific antibodies against erythrocytes. Three absorptions with lymphocytes followed by two absorptions with neutrophils removed almost all of nonspecific antibodies against neutrophils, lymphocytes, and erythrocytes.

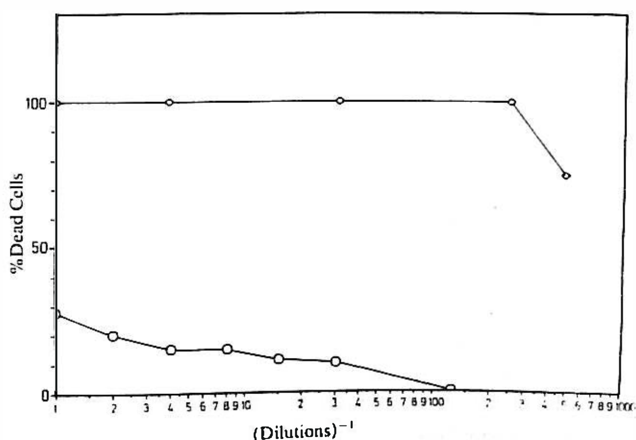

Fig.8. Complement-dependent cytolysis of target cells by antibodies raised against alveolar macrophages. Antibodies raised against peritoneal macrophages showed similar results.

Several reports have described the antigenic properties of various tissue macrophages and blood monocytes.[2-10] Also many investigators have described preparation and isolation of several monoclonal and polyclonal antibodies against macrophages.[9-13] These antibodies, which are not available commercially, are invaluable tools in studying various aspects of macrophage development and differentiation. Macrophages from various organs differ markedly in their surface antigenic properties.[6,10] However, very little information has been published on the details and mechanisms of differentiation of bone marrow cells and monocytes to specialized macrophages. The antibodies whose preparation has been described in this paper should prove as useful tools for studying differentiation of hematopoietic progenitor cells into specialized macrophages.

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