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Functional Heterogeneity of Macrophages: Subclasses of Peritoneal Macrophages with Different Antigen-binding Activities and Immune Complex Receptors

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Summary. Cell suspensions prepared from 24-hour cultures of adherent peritoneal exudate cells from rabbits were separated into density subclasses on discontinuous gradients of Ficoll. Of the five subclasses obtained, macrophages comprised over 95 per cent of the cells in the two least dense subclasses and over 90 per cent of the cells in the subclasses of intermediate density. The most dense subclass contained approximately 80 per cent macrophages and 20 per cent lymphocytes.

The antigen-binding properties of the subclasses were studied with a selected number of ^{125}I-labelled antigens in the presence and absence of added antibodies. In the absence of antibody the subclasses differed from one another in antigen-binding activities; these differences were independent of both the test antigens and the state of antigen aggregation. In the presence of specific antibody, two macrophage subclasses bound substantially more antigen than the other subclasses. The enhanced responses of these subclasses were confirmed by studying the cells' capacity to form rosettes when incubated with sensitized chicken erythrocytes. The results indicated that macrophages in these two subclasses differed quantitatively and/or qualitatively from other macrophage subclasses with regard to immunoglobulin receptor sites.

These studies demonstrate the feasibility of using gradient separation procedures to obtain functionally distinct subclasses of cells rich in macrophages. The availability of well-defined macrophage populations should permit more precise studies of macrophage functions in immunity.

INTRODUCTION

The many diverse roles assigned to macrophages during the inductive phase of antibody formation, and the observation that these cells form two species of immunogenic RNA, each with distinct chemical and biological properties (Adler and Fishman, 1969), suggested that the peritoneal macrophage population is functionally heterogeneous.

Direct support for this hypothesis came with the demonstration that oil-induced peritoneal exudate cells (PEC) rich in macrophages could be separated into density

subclasses that differed in antigen-binding properties and ability to form immunogenic RNA species (Walker, 1971; Rice and Fishman, 1974). Because the exudate cells in these experiments consisted of lymphocytes as well as macrophages, the observed binding heterogeneity might have resulted from lymphocyte contamination of the PEC density subclasses. The lymphocytes might also have been the source of RNA molecules responsible for the induction of IgM antibodies with the allotype markers of the macrophage donor animals (Adler, Fishman, and Dray, 1966).

The presence of lymphocytes in PEC subclasses has complicated attempts to define macrophage functions precisely, and has necessitated the development of methods that produce essentially lymphocyte-free subclasses. Described in this report are the results of density gradient separations of cell suspensions prepared from the surface-adherent population of rabbit PEC. The purpose of the study was to determine (a) the conditions under which the adherent PEC population could be separated into density subclasses, (b) the degree of macrophage purity within the various subclasses, (c) the antigen-binding properties of the various subclasses as measured with a selected number of antigens in the presence and absence of specific antibody, and (d) the distribution of surface immunoglobulin receptor sites on macrophages from the density subclasses.

MATERIALS AND METHODS

Reagents

Tissue culture medium RPMI 1640 (Associated Biomedic Systems, Incorporated, Buffalo, New York, U.S.A.) contained in final concentration 10 per cent heat-inactivated (56°, 30 minutes) foetal calf serum (FCS), and 100 units of penicillin/ml and 100 μg of streptomycin/ml. Medium 199H (Grand Island Biological Company, Grand Island, New York) contained in final concentration 25 mM HEPES buffer and 10 per cent FCS. Ficoll diluent (solution F) contained, per litre, 8·0 grams NaCl, 0·40 grams KCl, 1·072 grams $Na_2HPO_4.7H_2O$, 0·095 grams KH_2PO_4, 0·020 grams Phenol Red, and 1 per cent FCS. The pH of the diluent was adjusted to $7{\cdot}40 \pm 0{\cdot}05$ with 0·3 M NaOH or HCl, and the osmolarity to 285–295 milliosmolar (freezing point depression, Model 3W osmometer, Advanced Instruments, Newton Highlands, Massachusetts). Solution B, a phosphate-buffered saline, pH 7·8, was prepared as previously described (Fishman, 1961).

Peritoneal exudate cells

Peritoneal exudates were induced in 1–1½ kg albino rabbits of either sex by intraperitoneal injection of 30 ml of sterile (autoclaved) Klearol mineral oil (Ruger Chemical Company, Irvington, New Jersey). Animals were killed 4 days later with intravenous pentobarbital and each exudate was collected separately by washing out the peritoneal cavity with 1 litre of sterile solution B. Aseptic techniques were used throughout and only the exudates with no visible red cell contamination were pooled for use. The yields were $5–8 \times 10^8$ PEC per animal, of which 60–80 per cent were macrophages.

Peritoneal adherent cells (macrophages)

Pooled PEC were held at room temperature for 10 minutes in a separatory funnel. The cells in the aqueous phase were harvested, washed once in solution B, once in medium 1640 and then resuspended to 1×10^7 cells/ml. Ten-millitre quantities of the cell suspension were plated in 100-mm plastic culture plates (Falcon number 3003) and in-

cubated 60–90 minutes at 37° in an humidified 7 per cent CO_2–air incubator. The nonadherent cells were then removed after vigorous rotation of the plates and 10 ml of fresh medium was added. After 24 hours of further incubation, the adherent cells were washed with solution F to remove additional nonadherent cells. The cells were then removed with a rubber policeman, washed once in solution F and counted. Recovery of adherent cells ranged from 30 to 60 per cent of the plated cells and consisted primarily of macrophages (>90 per cent), of which 85–95 per cent were viable as estimated by Trypan Blue dye exclusion.

Ficoll gradients

Ficoll (Pharmacia) was dissolved in and extensively dialysed against deionized water, lyophilized, redissolved in solution F to approximately 20 per cent (w/v) and stored at −20°. Before use, the stock was diluted in solution F to the appropriate concentration as determined by refractometry (RI) at 24°. For convenience, the Ficoll solutions are referred to by their approximate percentages: 5 per cent, RI = 1·3412; 8 per cent, RI = 1·3452; 9 per cent, RI = 1·3472; and 10 per cent, RI = 1·3482. All solutions were tested for their osmolarity, with 290±5 milliosmolar considered acceptable for gradient use.

Discontinuous gradients were formed in 15-ml plastic centrifuge tubes (Falcon number 2095) by overlayering 2·0 ml 10 per cent Ficoll with 3·0 ml each of 9 per cent, 8 per cent, and 5 per cent Ficoll. All solutions and tubes were maintained in an ice bath.

Antigens and radiolabelling

Antigens were labelled with carrier-free ^{125}I (New England Nuclear, Boston, Massachusetts) by the chloramine T procedure (McConahey and Dixon, 1966). Labelled antigen preparations were >90 per cent precipitable in 10 per cent TCA, and nitrogen was estimated with Nesseler's Reagent.

(a) *Bacteriophage T2.* T2 was grown, harvested and purified as previously described (Walker, Liu and Adler, 1969), and was labelled with 4 mCi ^{125}I/200 μg N. Free iodine was removed by dialysis and gel filtration. After labelling, the preparations had specific activities of $4–8 \times 10^5$ cpm/μg N.

(b) *Aggregated and nonaggregated bovine serum albumin.* Heat-aggregated bovine serum albumin (Agg-BSA) was prepared by heating a solution of crystalline BSA (Nutritional Biochemicals Corporation, Cleveland, Ohio) at 20 mg protein/ml in phosphate-buffered saline, pH 6·5, for 10 minutes at 70° (Wolberg, Liu and Adler, 1969). Agg-BSA was separated from the nonaggregated BSA component (Mon-BSA) by gel filtration on Sephadex G-200 columns. A portion of the Agg-BSA fraction was labelled with 4 mCi ^{125}I/100 μg N to a specific activity of $2·5 \times 10^5$ cpm/μg N, while the Mon-BSA was labelled with 2 mCi ^{125}I/100 μg N to a specific activity of 3×10^6 cpm/μg N. After labelling, the Agg-BSA and Mon-BSA fractions were retested for their appropriate distribution on Sephadex G-200.

(c) *Polymerized flagellin.* Flagella were isolated from *Salmonella adelaide* (ATCC 10718, 35:F, g) and polymerized flagellin (POL) was prepared as described by Ada, Nossal, Pye and Abbot (1964). Preparations were labelled with 2 mCi ^{125}I/75 μg N to a specific activity of approximately $3–5 \times 10^3$ cpm/μg N.

(d) *Chicken erythrocytes.* Red blood cells from White Leghorn chickens (CRBC) were used for both immunization and experimental purposes within 3 days of isolation.

Antisera

(a) *Rabbit anti-BSA.* Anti-BSA antisera were raised by immunizing albino rabbits with 10 mg crystalline BSA in Freund's incomplete adjuvant (Difco). Animals were bled 30 days later and the sera were harvested, pooled, and heat-inactivated prior to use.

(b) *Rabbit anti-CRBC.* Anti-CRBC were obtained from albino rabbits that had received a series of 1·0-ml intravenous injections of a 10 per cent suspension of CRBC in saline over a 3-week period. The animals were bled 10 days after the last injection. The pooled heat-inactivated antisera used in this study had a haemagglutination titre of 6400. The antibody was predominantly of the 7S class as determined on linear sucrose gradients.

Antigen binding

Radiolabelled antigens (5 μl containing 5–20 μg N) were added to pellets of 6×10^7 PEC or adherent macrophages in the presence or absence of specific antibody. Solution B (5 μl) was added to separate cell pellets which were used to determine the distribution of cell numbers and morphological types in the gradients. A series of preliminary experiments revealed no effect of antigen on the gradient distribution of the cells. After incubation for 30 minutes at 37°, the cells were washed twice and resuspended to 1×10^7/ml in ice-chilled solution F, and 2-ml portions were overlaid onto duplicate gradients. All gradients in an experiment were centrifuged simultaneously at 2000 rev/min for 15 minutes at 4° in a PR-6 centrifuge (number 253 head, International Equipment Company, Needham Heights, Massachusetts). The cells banded at the several Ficoll interfaces and were harvested with the aid of a suction device. Previous studies (Walker, 1971) had shown that these gradients yielded true density subclasses. The subclasses are designated by their location at the gradient interfaces; e.g. cells of the 5/8 subclass banded at the interface of 5 per cent and 8 per cent Ficoll solutions. After harvesting, the cells in each subclass were counted electronically (Model S, Coulter Electronics, Incorporated, Hialeah, Florida) and their morphological types determined (Giemsa stain). Cell-associated radioactivity was determined in an Autogamma Counter (Model 5220, Packard Instrument Company, Incorporated, Downers Grove, Illinois, U.S.A.).

A relative binding activity (RBA) was determined for each subclass so that antigen-binding activities could be compared in a single experiment and between separate experiments. The RBAs were estimated from the following formula (Zembala and Asherson, 1970):

$$\frac{\text{cpm in subclass/total cpm recovered in gradient}}{\text{number of cells in subclass/total recovered cells in gradient}}.$$

Enhanced binding activity by a subclass results in an RBA greater than unity; the opposite is observed in subclasses with reduced activity.

Macrophage rosettes

Culture wells were constructed from coverslips and Lucite rings as described by Berken and Benacerraf (1966). Ficoll density subclasses isolated from suspensions of adherent macrophages were washed free of Ficoll and resuspended to 1×10^6 cells/ml in TC 199H containing 10 per cent FCS. Cell suspensions (0·2 ml) were plated into culture wells which were then incubated for 30–60 minutes at 37° to allow the macrophages to attach to the glass surfaces. Culture medium and nonadherent cells were removed with a Pasteur pipette and the adherent cells were washed three to four times in TC 199H containing no

serum. Suspensions of CRBC (0·2 ml of a 0·1 per cent suspension) sensitized at various concentrations of rabbit anti-CRBC antibody were added and the incubation was continued for 30 minutes at room temperature. Unattached CRBC were then removed by repeated gentle washings of the culture wells with TC199H. The wells were then filled with TC 199H containing 0·004 per cent Neutral Red (Berken and Benacerraf, 1966), sealed with a glass slide and examined for rosettes. A macrophage surrounded by four or more erythrocytes was scored as a rosette. At least 300 cells were counted in duplicate culture wells.

Chicken RBC were sensitized by resuspending washed cell pellets to 10 per cent in the appropriately diluted heat-inactivated antisera. The mixtures were incubated for 30 minutes at 37° followed by additional incubation for 90 minutes at room temperature. The cells were then diluted twenty-fold in TC 199H, pelleted, washed twice, and resuspended to a 0·1 per cent suspension in 199H without serum.

RESULTS

CELL DENSITY AND ANTIGEN-BINDING OF RABBIT PERITONEAL EXUDATE CELLS

Since T2 bacteriophage was the only antigen used in earlier demonstrations of antigen-binding heterogeneity among rabbit PEC (Walker, 1971), it became important to determine whether the binding activities of the PEC subclasses were independent of the test antigen. Table 1 illustrates a typical separation experiment in which PEC were incubated with either radiolabelled T2 or Agg-BSA and banded in Ficoll gradients. The least dense subclass (0/5), which contained 19 per cent of the recovered cells, bound 35 per cent of the T2 and 34 per cent of the Agg-BSA, resulting in relative binding activities (RBA) of 1·8 for both antigens. The remaining subclasses had reduced binding activities, with the 9/10 cells binding the least amount of either antigen. In addition, the percentages of bound T2 and Agg-BSA were similar in each subclass, indicating that the binding activities of the subclasses were independent of the test antigens.

Oil-induced peritoneal exudates contained a large proportion of cells which ingested oil. Although most of the oil-containing cells located in the oil-phase in a separatory

TABLE 1

CELL DENSITY AND ANTIGEN-BINDING HETEROGENEITY OF PERITONEAL EXUDATE CELLS SEPARATED ON DISCONTINUOUS FICOLL GRADIENTS

PEC subclass*	Percentage of cells recovered†	^{125}I-T2		^{125}I-labelled Agg-BSA	
		Percentage bound	RBA‡	Percentage bound	RBA‡
0/5	19	35	1·8	34	1·8
5/8	26	28	1·1	27	1·0
8/9	20	10	0·5	11	0·5
9/10	14	4	0·3	4	0·3
10-P	21	23	1·1	24	1·1

* Expressed as location of cells at interfaces of Ficoll solutions.
† Total cell recovery from gradient was 68 per cent.
‡ Relative binding activity—see Materials and Methods section.
Rabbit peritoneal exudate cells (PEC) were incubated with the indicated antigens for 30 minutes at 37°, washed and separated on Ficoll gradients. Total labelled T2 and Agg-BSA bound was 9 and 11 per cent, respectively.

funnel, some of these cells were in the aqueous phase. Such cells were phagocytically very active as evidenced by their uptake of oil. These cells located primarily at the 0/5 interface of the gradients which suggested that this subclass was an artifact of the oil induction procedure. This was confirmed by the gradient separation of cells obtained from peritoneal washings of unstimulated rabbits. Such cell suspensions yielded all the described density subclasses except the 0/5 (Walker, unpublished observations; Rice and Fishman, 1974).

SEPARATION OF ADHERENT PERITONEAL CELLS (MACROPHAGES) INTO DENSITY SUBCLASSES

In the previously reported study (Walker, 1971) PEC subclasses were not morphologically homogeneous. Likewise, in the present study, macrophages comprised 90–100 per cent of the cells in the 0/5 subclass, 85–95 per cent of the 5/8 and 8/9 subclasses, 60–70 per cent of the 9/10 subclass, and generally 50 per cent of the 10-P or pellet population. The remainder of the nucleated cells were lymphocytes and, in the 10-P population, polymorphonuclear leucocytes.

Cell suspensions prepared from 24-hour cultures of adherent PEC consisted mainly of macrophages (90 to 95 per cent). The remaining cells had the morphological characteristics of lymphocytes. These cell suspensions will be referred to as adherent macrophages. Presented in Fig. 1 is the distribution of adherent macrophages according to their density in Ficoll gradients. A distinct pattern of cell distribution is apparent in the figure with the 5/8 and 10-P subclasses being most variable.

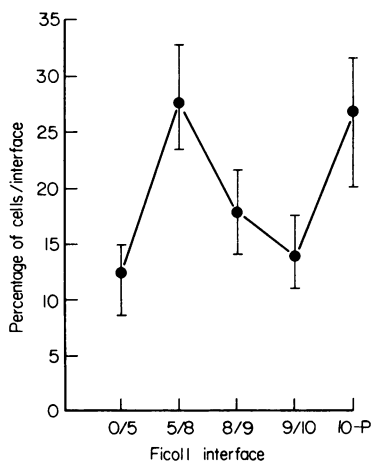

FIG. 1. The density subclass distribution of rabbit adherent peritoneal macrophages on discontinuous Ficoll gradients. The mean value and ranges from five separate experiments are given.

Morphological examination of the subclasses from these and additional experiments revealed that macrophages made up 95 per cent or more of the 0/5, 5/8 and 8/9 subclasses, and over 90 per cent of the 9/10 subclass. The contaminating cells in both the 8/9 and 9/10 subclasses were exclusively lymphocytes. In the 10-P subclass 70 to 80 per cent of the cells were macrophages and the remainder were lymphocytes. Approximately 50 per cent

of the cells in the 0/5 subclasses contained engulfed oil. These data demonstrate the feasibility of using adherent macrophage suspensions for the separation of density subclasses highly enriched in macrophages.

ANTIGEN-BINDING PROPERTIES OF ADHERENT MACROPHAGE SUBCLASSES

The reproducible separation of adherent macrophage cell suspensions into density subclasses with a high degree of morphological homogeneity permitted testing of the antigen-binding activities of these subclasses in the absence of gross lymphocyte contamination. Aliquots of adherent macrophages were incubated with ^{125}I-labelled T2 or ^{125}I-labelled Agg-BSA and separated on Ficoll gradients. The combined results of three experiments (Table 2) provide evidence of binding heterogeneity within the subclasses toward both antigens.

TABLE 2

RELATIVE BINDING ACTIVITIES OF RABBIT ADHERENT MACROPHAGE SUBCLASSES INCUBATED WITH ^{125}I-LABELLED T-2 AND ^{125}I-LABELLED AGG-BSA

Macrophage subclass	Mean percentage distribution of cells* (range)	Percentage of macrophages	^{125}I-labelled T2 bound		^{125}I-labelled Agg-BSA bound	
			Mean percentage‡ (range)	Mean RBA† (range)	Mean percentage‡ (range)	Mean RBA† (range)
0/5	11 (9–14)	>95	22 (19–27)	2·1 (1·9–2·3)	19 (15–22)	1·8 (1·1–2·5)
5/8	26 (24–29)	>95	21 (19–27)	0·9 (0·7–1·2)	25 (23–29)	1·1 (1·0–1·2)
8/9	19 (17–22)	95	15 (13–17)	0·7 (0·6–0·7)	14 (12–18)	0·7 (0·7–0·8)
9/10	15 (14–18)	90–95	11 (11–13)	0·7 (0·6–0·9)	10 (9–11)	0·7 (0·6–0·8)
10-P	29 (20–34)	70–80	32 (31–33)	1·1 (1·0–1·5)	32 (28–34)	1·0 (0·9–1·1)

* Mean percentage distribution of cells recovered from the gradients; total recoveries ranged from 80 to 105 per cent.

† Relative binding activity—see Materials and Methods section. Mean values from three experiments.

‡ Mean percentage distribution of bound antigen.

The data represent cell and binding activity distributions from three experiments in which adherent macrophages were incubated with ^{125}I-labelled T2 or ^{125}I-labelled Agg-BSA for 30 minutes at 37° and separated on gradients. Total uptake of antigens in each experiment was approximately 10 per cent.

The 0/5 subclass, representing about 10 per cent of the recovered cells, bound similar amounts of each antigen (T2, 22 per cent; Agg-BSA, 19 per cent) as did the 5/8 subclass, which consisted of over twice as many recovered cells (26 per cent). Comparison of the RBA values for both antigens (Table 2) revealed that the 0/5 subclass (>95 per cent macrophages, RBA = 2·1 and 1·8) was more active than the 5/8 (95 per cent macrophages, RBA = 0·9 and 1·1), which was moderately more active than either the 8/9 (95 per cent macrophages, RBA = 0·7) or 9/10 subclass (90–95 per cent macrophages, RBA = 0·7). The 10-P subclass, containing approximately 20 per cent lymphocytes, exhibited binding activities similar to the 5/8 subclass (RBA approximately 1·0).

In addition to confirming density and binding heterogeneity, these data showed that the binding activities of the adherent macrophage subclasses were independent of the test antigens, since nearly identical proportions of T2 and Agg-BSA were bound within each subclass. This observation was tested with polymerized flaggellin (POL) as antigen (Table 3), and the results agree with those in Table 2.

TABLE 3

RELATIVE BINDING ACTIVITIES OF RABBIT ADHERENT MACROPHAGE SUBCLASSES INCUBATED WITH ^{125}I-LABELLED POLYMERIZED FLAGELLIN (POL)

Macrophage subclass	Percentage of recovered cells	^{125}I-labelled POL	
		Percentage bound	RBA*
0/5	13	32	2·5
5/8	22	30	1·4
8/9	17	8	0·5
9/10	14	7	0·5
10-P	35	24	0·7

* Relative binding activity—see Materials and Methods section.
Adherent macrophages were incubated with ^{125}I-labelled POL and separated on gradients. POL uptake was 3·4 per cent, and cell recovery from the gradient was 88 per cent.

Aggregation of serum protein antigens enhances both their immunogenicity and uptake by macrophages (Weissmann and Dukor, 1970). This property prompted a comparison of the uptake of Agg-BSA and Mon-BSA by cells of the various subclasses (Table 4). Typically, the relative distributions of the two proteins were similar in the subclasses, even though the total amount of Mon-BSA bound was less than that of Agg-BSA (2 per cent and 12 per cent, respectively). These results confirm the observations in Tables 2 and 3 and suggest that the relative binding activities of the subclasses are independent not only of test antigen but also of the state of antigen aggregation.

TABLE 4

EFFECT OF ANTIGEN AGGREGATION ON RELATIVE BINDING ACTIVITIES OF RABBIT ADHERENT MACROPHAGE DENSITY SUBCLASSES

Subclass	Percentage of cells recovered*	^{125}I-labelled Mon-BSA		^{125}I-labelled Agg-BSA	
		Percentage bound	RBA†	Percentage bound	RBA†
0/5	9	23	2·6	19	2·1
5/8	26	26	1·0	27	1·0
8/9	21	15	0·7	16	0·8
9/10	16	12	0·8	13	0·8
10-P	28	23	0·8	25	0·9

* Total recovery was 100 per cent of cells placed on the gradients.
† Relative binding activity—see Materials and Methods section.
Antigen-binding activities of macrophages incubated with either ^{125}I-labelled Mon-BSA or ^{125}I-labelled Agg-BSA. Percentage uptake of labelled Mon-BSA and Agg-BSA was 2 per cent and 12 per cent, respectively.

THE EFFECT OF ANTIBODY

The effect of specific antibodies on the uptake of antigens was studied by incubating 24-hour adherent macrophages with antigens in the presence of increasing amounts of specific antibody followed by gradient cell separation. In a typical experiment (Table 5) cells of the 9/10 subclass responded strikingly to the addition of antibody, binding 10·6 times more Agg-BSA in the presence of 10 μl of anti-BSA antisera than in the absence of

TABLE 5

THE EFFECT OF ANTIBODY ON THE BINDING OF ^{125}I-LABELLED Agg-BSA BY RABBIT ADHERENT MACROPHAGE SUBCLASSES

Macrophage subclass	Percentage of recovered cells	Antigen-binding ratio* per volume of antiserum added		
		10 μl	20 μl	40 μl
0/5	12	1·7	3·4	5·7
5/8	21	1·7	4·1	9·5
8/9	20	2·4	7·6	19·5
9/10	13	10·6	28·3	47·5
10-P	34	2·1	4·8	7·9

* Antigen-binding ratio = cpm bound in presence of antibody/cpm bound in absence of antibody. Pellets containing 6×10^7 adherent macrophages were incubated with 5 μl of ^{125}I-labelled Agg-BSA in the presence of the indicated amounts of rabbit anti-BSA antiserum or in the presence of 10 μl normal rabbit serum. After 30 minutes at 37°, the cells were washed and separated on Ficoll gradients. Macrophages bound 3 per cent of the antigen in the absence of added antibody and 5, 9, and 20 per cent of the added antigen in the presence of 10, 20, and 40 μl of antibody, respectively. The percentage of recovered cells from the gradients was 89 per cent.

added antibody. The other subclasses were much less sensitive, binding only 1·7 to 2·9 times more opsonized BSA than non-opsonized antigen under identical conditions.

The subclasses bound increasing levels of Agg-BSA in the presence of increasing amounts of antisera. For example, the 5/8 subclass bound 1·7, 3·4, and 5·7 times the amount of Agg-BSA in the presence of 10, 20 and 40 μl of antisera, respectively, while the 9/10 subclass bound 10·6, 28·3, and 47·5 times more antigen in the presence of similar amounts of antibody. These increases in antigen-binding activity were roughly proportional to the amounts of antibody added. The 9/10 subclass, though more sensitive initially to added antibody, responded the same as other subclasses after the first addition.

Results similar to those in Table 5 were obtained with the other antigens tested (T2 and Mon-BSA). It should be noted, however, that in many experiments both the 8/9 and 9/10 subclasses responded similarly, with marked uptake of antigens in the presence of specific antibody. This indicated that cells located in the 8 to 10 per cent region of the gradients differed in their capacity to respond to opsonized antigen from cells in the other subclasses.

IgG RECEPTOR SITES

To characterize the cells further with regard to the binding of immune complexes, subclasses of macrophages were tested for their rosette-forming capacity using CRBC coated with varying amounts of antibody. Adherent macrophage suspensions were separated into density subclasses which were then re-adhered to the glass surfaces of the culture wells. This procedure yielded pure adherent macrophage populations as judged by morphological criteria, the rapid uptake of Neutral Red dye (Berken and Benacerraf, 1966), and the fact that essentially all the cells formed rosettes (Huber, Douglas and Fudenberg, 1969) when incubated with CRBC coated with a sufficiently high concentration (1:20 dilution) of rabbit anti-CRBC antisera (Table 6).

TABLE 6

ROSETTE FORMATION BY ADHERENT MACROPHAGE SUBCLASS INCUBATED WITH SENSITIZED CRBC

Macrophage subclass	Percentage of recovered cells*	Percentage of macrophage rosettes per dilution of anti-CRBC antibody in coating mixture				
		1:20	1:200	1:2000	1:20,000	1:200 NRbS†
0/5	7	98	30	15	3	0
5/8	26	99	35	15	0	1
8/9	17	99	61	16	0	0
9/10	9	99	91	52	0	0
10-P	41	98	31	10	2	0
Adherent‡	—	98	54	31	0	0

* Total recovery of cells from gradient was 97 per cent.
† Heat-inactivated normal rabbit serum.
‡ Adherent macrophage suspension re-adhered in culture wells.
Ficoll density subclasses were re-adhered in culture wells and incubated with suspensions (0·1 per cent) of CRBC coated with rabbit anti-CRBC antibody at the indicated dilutions. After removal of unbound erythrocytes, the chambers were filled with medium containing Neutral Red dye and examined for rosettes (>4 CRBC/macrophage). Haemagglutination titre of the rabbit anti-CRBC was 6400.

These results (Table 6) further showed that the intermediate density macrophages (subclasses 8/9 and 9/10), when incubated with CRBC coated at a 1:200 dilution of antiserum, formed two to three times more rosettes (61 per cent and 91 per cent, respectively) than macrophages from the other subclasses. Incubation of the subclasses with CRBC coated with antiserum diluted 1:2000 revealed that 52 per cent of the cells in the 9/10 subclass formed rosettes which was three times greater than the number formed by the other subclasses (10–16 per cent).

These results indicate that the enhanced responses of the intermediate density macrophages to opsonized antigens (Table 5) may be due to qualitative and/or quantitative differences in the immunoglobulin receptor sites on their surfaces.

DISCUSSION

The primary objective of this study, the separation of rabbit peritoneal exudate adherent cells into distinct density subclasses highly enriched in macrophages, was achieved (Fig. 1, Table 2). These studies, the results of which are summarized in Table 7, further demonstrate that gradient procedures yield macrophage subclasses that are functionally enriched (Walker, 1971).

With regard to functional heterogeneity, the macrophage subclasses differed in their antigen-binding activities in the absence of opsonizing antibody. These differences were independent not only of the test antigens (Tables 2, 3) but of the degree of antigen aggregation as well (Table 4). In the presence of specific antibody, the uptake of antigen by subclasses 8/9 and 9/10 was markedly enhanced compared to that by the 0/5, 5/8 and 10-P subclasses. The former two subclasses were very sensitive to the addition of antisera, but all subclasses responded to increases in antibody in approximate proportion to the amount of antisera added (Table 5).

Experiments designed to detect IgG receptor sites on the surfaces of macrophages (Huber *et al.*, 1966) revealed that the 8/9 and 9/10 subclasses contained a higher proportion of macrophages capable of forming rosettes than did the other subclasses (Table 6). While

TABLE 7

SUMMARY OF ANTIGEN-BINDING ACTIVITIES OF ADHERENT MACROPHAGE DENSITY SUBCLASSES*

Macrophage subclass	Antigen binding† no antibody	Enhanced uptake‡ with antibody	Ig receptor sites§ (macrophage rosettes)
0/5	4+	1+	1+
5/8	2+	1+	1+
8/9	1+	2–3+	3+
9/10	1+	4+	4+
10-P	2+	1+	1+

* Data summarized on an increasing scale of 1+ to 4+ for the indicated activity.
† Data from Tables 2, 3, and 4.
‡ Data from Table 5.
§ Data from Table 6.

these data are consistent with the view that macrophages possess immunoglobulin receptor sites, they also indicate that the sites differ between peritoneal macrophages, possibly in terms of their number, density, or avidity for sensitized erythrocytes (Arend and Mannik, 1973).

Others have shown that macrophages are functionally heterogeneous (Blanden, 1968) and, when isolated from various tissues, differ in morphology (Hirsch and Fedorko, 1970), lysosomal enzyme content (Weissman and Dukor, 1970), phagocytic activity and membrane receptors for migration inhibition factor (MIF) (Leu, Eddleston, Hadden and Good, 1972). In addition, Fishman and Adler (1967) found that while peritoneal exudate macrophages were a source of the immunogenic RNA species, alveolar macrophages were not, an observation confirmed by Gottlieb (1968). Preliminary evidence further illustrating macrophage functional heterogeneity has been obtained by us from experiments similar in design to those of Kolsch and Mitchison (1968), who described subcellular antigen 'compartments' in mouse peritoneal macrophages. In our experiments the subclasses of adherent rabbit macrophages differed in subcellular distribution and compartmentalization of antigen. The relationship between such antigen compartments and the immunological function(s) of the subclasses is currently under investigation.

Recent studies by Rice and Fishman (1974) have confirmed that the functionally distinct immunogenic RNA species are derived from separate PEC subclasses (Walker, 1971). Moreover, their studies revealed that in those subclasses yielding immunogenic RNA there was an enrichment of a distinct type of mononuclear cell which they termed 'A-cells'. The presence of morphologically atypical mononuclear cells in the lymphoid tissues of mice has been recently reported by Steinman and Cohn (1973). The immunological functions of such cells remain undetermined, as does the question of whether the density and functional heterogeneity of macrophages reported here and elsewhere are related to stages of cellular differentiation (maturation) (van Furth and Cohn, 1968) or to possible differences in precursor cells (Howard, Christie, Boak and Kinsky, 1969).

The roles of macrophages in antibody formation are complex and controversial. Evidence suggests that these cells can function as nonspecific helper cells which may serve to bring thymus-independent lymphocytes (B-lymphocytes) and thymus-dependent lymphocytes (T lymphocytes) together (Feldmann, 1972a) or to protect (Unanue and Cerottini, 1970), or present (Mitchison, 1969) antigen determinates in a manner conducive to lymphocyte immunization. Feldmann (1972b) has suggested that complexes

between certain antigens and T lymphocyte-derived immunoglobulin (IgT, possibly monomeric IgM) become bound to the surfaces of macrophages and may be responsible for the immunization of B lymphocytes. Consistent with this notion is the recent demonstration by Rhodes (1973) that splenic macrophages possess receptor sites for monomeric IgM.

An additional role for macrophages is suggested by studies showing that these cells are processors of antigen, an interaction considered by some to be an essential step in the induction of antibody synthesis to some antigens (Adler and Fishman, 1969). It may be that at least two functionally distinct macrophage subclasses participate directly in the induction of antibody synthesis. One type could process antigen and the other could bind antigen–IgT complexes to their surfaces. It seems reasonable to propose that the latter type of macrophage would possess receptor sites for IgT immunoglobulin, while the former type, after endocytosis and processing of antigen, may exocytose antigen possibly in the form of RNA–antigen complexes, which would then interact with lymphocytes.

Experiments are presently in progress using isolated macrophage subclasses to test these and other possibilities.

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