PROPERTIES OF MACROPHAGE RECEPTORS FOR CYTOPHILIC ANTIBODIES*

J. G. HOWARD† AND B. BENACERRAF‡

From the Departments of Medicine and Pathology New York University School of Medicine, New York City

Received for publication November 8, 1965

THE production of an antibody cytophilic for spleen cells was originally described by Boyden and Sorkin (1960, 1961). The presence of this antibody was demonstrated by the capacity of rabbit antisera against human serum albumin to confer upon normal cells the capacity of adsorbing radioiodinated antigen. Boyden (1964) later perfected a technique to detect cytophilic antibodies and showed that the sera of guinea-pigs immunized with sheep red cells in complete adjuvants contained an antibody which could selectively bind to homologous peritoneal macrophages and confer upon them the ability to adsorb sheep red cells. The properties of guinea-pig antibodies cytophilic for macrophages have been investigated by Berken and Benacerraf (1965). These antibodies were shown to belong to the γ_2 population of immunoglobulins and to be primarily responsible for the phenomenon of specific opsonization. The cytophilic properties of these antibodies depend probably upon the interaction of specific sites on the Fc fragment of γ_2 globulins with receptors on the macrophage cell membrane. The present experiments were designed (1) to investigate to what extent other cell types (lymphocytes, polymorphonuclear leucocytes, or fibroblasts) which take part in inflammatory reactions are also capable of adsorbing this cytophilic antibody and (2) to study the chemical nature of the macrophage receptors by exploring whether their capacity to react with the antibody could be destroyed by enzymes specific for various substrates or by reactive chemicals known for their characteristic reactions.

MATERIALS AND METHODS

Antisera

Guinea-pigs were immunized with sheep red cell emulsions in complete Freund's adjuvant (Difco Lab., Detroit, Mich.) in exactly the manner described by Boyden (1964). The sera were harvested approximately 3 weeks later.

Hyperimmune mouse anti-sheep erythrocyte sera were prepared as follows: Swiss-Webster mice were injected intravenously with 10^9 red cells twice with an interval of 4 weeks. The mice were bled 3–4 days after the second injection and the sera pooled.

Cell suspensions

Alveolar macrophages were obtained according to the technique of Myrvik, Leake and Oshima (1962). Guinea-pig lungs were dissected and perfused twice with Hanks' balanced

* Supported by the United States Public Health Service (Grants AI–2094 and AI–04983) and by the Health Research Council of the City of New York under Contract No. I–138.

† Visiting Career Scientist of the Health Research Council of the City of New York. Present address: Dept. of Zoology, University of Edinburgh.

‡ Career Scientist of the Health Research Council of the City of New York.

salt solution with a polyethylene catheter introduced into the trachea. After each perfusion the lungs were inverted and the cell suspensions allowed to pour into centrifuge tubes. The cells were washed once and resuspended in 199 culture medum (Microbiological Associates, Bethesda, Md.) at a concentration of 2×10^6 cells/ml.

Polymorphonuclear leucocytes.—Guinea-pigs were injected intraperitoneally with 20 mg. glycogen, and their peritoneal cavities washed out 3 hr. later with heparinized Hanks' solution. The cells were centrifuged at 900 rpm for 10 min., washed twice in Hanks' solution and suspended to a concentration of 2×10^7 cells per ml. in 199 medium. Preparations contained not less than 90 per cent polymorphonuclear leucocytes. A few macrophages and lymphocytes were also present in the preparations.

Lymphocytes.—The cervical and mesenteric nodes were dissected from guinea-pigs and dissociated in 199 medium. The cells were filtered through a fine wire mesh and then through a 2 cm. column of packed glass wool. They were centrifuged, washed, and resuspended as for the polymorphonuclear leucocyte preparation. Macrophages were totally absent from these lymphocyte suspensions as judged by the lack of cells taking up neutral red. No attempt was made to subdivide the lymphocytes into percentages according to their size.

Mouse fibroblasts (3T3) and guinea-pig fibrosarcoma cells (from a methylcholanthrene induced fibrosarcoma in a strain 13 guinea-pig) were kindly supplied to us by Dr. Howard Green and Dr. Lloyd Old respectively. Both cell types were obtained as monolayer subcultures on glass cover slips in 199 medium.

Cytophilic antibody assay

This was performed on macrophages, fibroblasts, and fibrosarcoma cells by Boyden's rosette technique. Lucite rings, such as are used for millipore chambers, were fixed on to 22 mm. square, glass cover slips. The inner diameter of the rings was 12 mm. and the height 2–3 mm. The flat surfaces of the rings were coated with thin layers of silicone grease to provide water-tight adherence to glass surfaces.

Amounts (0·1 ml.) of the macrophage suspension from normal or sensitized animals (about 2×10^5 cells) were added to the wells at room temperature. The cells were allowed to settle on the glass where they adhered. After 30 min. the supernatant fluid and any floating cells were removed. Then 0·1 ml. of the antiserum dilution was added to the well. Sensitization proceeded for 1 hr. at 4°, 32°, or room temperature. The passively sensitized cells were then washed by replacing the fluid in the wells 3–5 times with fresh, cold 199 medium. Aliquots (0·1 ml.) of a 1 per cent suspension of washed sheep red cells in 199 were then added (direct technique). In experiments using the indirect technique of passive sensitization, 0·1 ml. aliquots of a 1 per cent suspension of antibody-sensitized sheep red cells were added to wells containing the cells to be studied.

In both methods, direct or indirect, the red cells were left in the wells for 1 hr. at room temperature. The red cells not attached to macrophages were siphoned off with a Pasteur pipette. The wells were then filled with a solution of 199 medium containing 2 per cent of a neutral red solution (100 mg. neutral red dissolved in 100 ml. isotonic saline). A glass slide was then placed atop each open well. The slide was inverted so that the cover slip to which the macrophages were adherent was then on top. The preparations were examined in the light microscope. Macrophages were identifiable by the characteristic staining of their cytoplasmic granules. In positive preparations they were surrounded by rosettes of red cells. The reactions were quantitated by rating the preparations from 4+ to trace as follows. 4+ : Rosettes on almost every macrophage. 3+ : Red cells on almost every macrophage. Rosettes on about 1/2 cells. 2+ : Red cells on about 1/2 macrophages. Frequent rosettes 1+ : Red cells on some macrophages in most fields. Occasional rosettes.

For comparing the adsorption of cytophilic antibody by lymphocytes and polymorphonuclear leucocytes with that by macrophages, the percentage of cells with erythrocyte rosettes was counted in a haemacytometer instead of in the reaction wells used in the other experiments, because of poor attachment of cells to glass. For this variation, 0·25 ml. serum dilution and 0·25 ml. cell suspension ($1–2 \times 10^7$ per ml.) were incubated at 37° for 90 min. The cells were washed twice in 199 medium at 4° and resuspended in 0·2 ml. 1 per cent sheep red cells. Following incubation for 1 hr. at room temperature the mixture of test cells and sheep erythrocytes was diluted with an equal volume of 199 medium containing neutral red. The cells were gently resuspended and examined in a haemacytometer.

Enzymes

Activated papain.—Papain (Light and Co., England) was made up to a concentration of 2 mg./ml. in a mixture of equal parts of 199 medium and papain digest buffer (0·1 M phosphate buffer, pH 7·6, 0·002 M EDTA and 0·05 M sodium chloride). Cysteine was added to a concentration of 1·8 mg./ml.

Trypsin: Nutritional Biochemicals (Cleveland, Ohio), 1300.

Lipase: Nutritional Biochemicals (wheat germ type 1). Sigma 114B–8610.

Hyaluronidase: Nutritional Biochemicals (ovine testis, salt free type III), Sigma 15B–2830.

Neuraminidase (V. cholerae): General Biochemicals (Chagrin Falls, Ohio), 55604.

Lecithinase C (Cl. Welchi, Type I) : Nutritional Biochemicals Sigma 124B–1570.

Treatment of Macrophages

Enzymes.—With the exception of papain, all enzymes were dissolved in 199 medium. Monolayers of macrophages adhering to the cover slips of reaction chambers were exposed to the various enzyme preparations at concentrations indicated for 1 hr. at 37°. The cells were washed three times and compared with untreated cells from the same original preparations for their capacity to adsorb cytophilic antibody by the techniques described. The preparation was graded for cytophilic activity according to the scale indicated earlier.

Chemicals.—In a similar fashion, macrophage monolayers were treated with various chemical reactants dissolved in 199 medium at the concentrations indicated for 1 hr. at either room temperature or 37°. After this treatment the cells were washed up to 5 times with cold 199 medium and compared with untreated cells from the same source for their capacity to adsorb cytophilic antibody.

RESULTS

Lack of adsorption of cytophilic antibody by cells other than macrophages

Suspensions of lymphocytes and polymorphonuclear leucocytes were compared with lung macrophages obtained from the same non-immunized guinea-pigs for their ability to fix cytophilic antibody.

TABLE I.—*Failure of Guinea-Pig Anti-Sheep Red Cell Antibody, Cytophilic for Macrophages, to Passively Sensitize Lymphocytes and Polymorphonuclear Leucocytes*

Guinea-pig anti-sheep red cell serum no.	Cell suspension used	$\frac{\text{Rosettes}}{\text{Cells}} \times 100$ Direct method: Serum dilution 1/4	1/8	1/16	None	$\frac{\text{Rosettes}}{\text{Cells}} \times 100$ Indirect method*
C37	Lung macrophages	78	33	17	0	—
	Lymphocytes	0·025	0	0	0	—
C6	Lung macrophages	53	49	12	0	83
	Pilymorphonuclear leucocytes	0	0	0	0	2

* Sheep erythrocytes sensitized with 1/400 guinea-pig anti-sheep red cell serum C6.

The results shown in Table I demonstrate clearly that both these cell types are lacking in the necessary receptor for the antibody possessed by macrophages. The lack of cytophilic activity of the antibody for polymorphonuclear leucocytes is surprising considering that this antibody was shown to depend upon this property to act as an opsonin for macrophages and also that both cell types are similarly phagocytic and capable of taking up opsonized material. To verify this observation the more sensitive indirect test was also applied to these two cell types in parallel. The failure of the polymorphonuclear leucocytes to fix antibody was

again apparent in this test. Whereas 83 per cent of macrophages formed rosettes with sensitized sheep red cells, only 2 per cent of cells in the polymorphonuclear leucocyte suspension did so. Even this low figure could be attributed to the 5 per cent macrophage contamination in the cell suspension used here.

Guinea-pig fibrosarcoma cells and mouse fibroblasts also gave no evidence of any capacity to fix cytophilic antibody when tested as monolayers in reaction wells by both the direct and indirect techniques (Table II). Whereas guinea-pig macrophages took up sheep red cell antibody obtained from either guinea-pigs or mice, mouse fibroblasts gave equally negative results with both antisera.

TABLE II.—*Failure of Anti-Sheep Red Cell Antibodies, Cytophilic for Macrophages, to Passively Sensitize Mouse Fibroblasts and Guinea-Pig Fibrosarcoma Cells*

	Cytophilic activity		
	Direct method		
Cell monolayer used	Guinea-pig antiserum*	Mouse antiserum†	Indirect method†
Guinea-pig alveolar macrophages .	++++	+++	++++
Guinea-pig fibrosarcoma . .	0	0	0
Mouse fibroblasts . . .	0	0	0

* Guinea-pig anti-sheep red cell serum C35 diluted 1 : 2.
† Mouse anti-sheep red cell serum diluted 1 : 2.
‡ Sheep erythrocytes sensitized with 1/20 guinea-pig anti-sheep red cell serum C35.

These observations on the cellular specificity of cytophilic antibody for macrophages confirm and extend the earlier findings of Boyden (1964) that macrophages but not polymorphonuclear leucocytes or lymphocytes were able to take up cytophilic antibody from guinea-pig anti-sheep red cell sera. The present finding that two other cell types (fibroblasts and fibrosarcoma) also lack this γ_2 globulin receptor, suggests that macrophages may prove to be unique in this attribute.

Attempts to destroy the macrophage antibody receptor by treatment with enzymes and chemicals

Enzyme inhibitors.—Lung macrophages in reaction wells were incubated for 1 hr. at room temperature with the following inhibitors of energy yielding metabolic pathways which were diluted in 199 medium : sodium azide (1 per cent) ; sodium fluoride (10 mM) and dinitrophenol (4 mM). Following 3 consecutive washings, the cells were examined for uptake of cytophilic antibody by the direct method. No diminution in the intensity or frequency of rosette formation could be detected after treatment with these metabolic poisons.

Enzymes.—The results obtained after treating macrophages with various enzymes are summarized in Table III. Several experiments were carried out in each case. Neither trypsin nor activated papain were able to diminish the ability of macrophages to fix cytophilic antibody. On the contrary, rosette formation appeared to be intensified by such pre-treatment in all of 5 experiments, only one of which is included in Table III. The more sensitive indirect technique was also applied to such trypsinized macrophages, in view of the lack of agreement of these results with those reported by Sorkin (1964) who used the radioiodinated

TABLE III.—*Effect of Treatment with Various Enzymes on the Macrophage Receptors for Cytophilic Antibody*

Enzyme*	Guinea-pig anti-sheep red cell Serum No.	Cytophilic activity (direct method) serum dilution 1/4	1/16	1/64	None
0·2 per cent trypsin	C49	++++	++	+	0
0·2 per cent activated papain	,,	+++±	++++	+±	0
None	,,	++±	+±	+	0
0·2 per cent lecithinase C	C52	±	±	±	0
0·2 lecithinase C pre-heated at 80° for 10 min.	,,	+	+	±	0
0·2 per cent lipase	,,	++++	++++	++++	0
0·2 per cent hyaluronidase	,,	++++	++++	+++±	0
Neuraminidase (50 U/ml.)	,,	++++	++++	+++	0
None	,,	++++	++++	++++	0

* Macrophages incubated in enzyme solutions in 199 medium at 37° for 1 hr. previous to sensitization.

albumin technique for measuring uptake of cytophilic antibody. Rosette formation was observed to be maximal around both trypsinized and normal macrophages similarly exposed to sheep red cells sensitized with a 1/20 guinea-pig anti-sheep red cell serum.

No diminution in uptake of sheep red cell antibody could be detected with cells pre-treated with pancreatic lipase, in contrast with the 40 per cent reduction in uptake of antibody by lipase-treated macrophages which was reported by Sorkin (1964).

Hyaluronidase and neuraminidase pre-treatment was similarly without effect.

Exposure to 0·2 per cent lecithinase C for 1 hr. at 37° almost entirely inhibited the uptake of antibody by macrophages. Although the treated cells remained adherent to the glass surface and appeared superficially normal, they mostly showed diminished uptake of neutral red. The ability of this inhibitory property of the lecithinase preparation to withstand heating at 80° for 10 min., which is characteristic for lecithinase C, supports the interpretation that this action was not attributable to any contaminating enzyme.

Chemicals.—Chemical reagents characteristic for their capacity to react with sulfhydryl groups [iodoacetamide and *ρ*-chloromercurybenzoate (PCMB)], and with free amino groups (fluorescein isothiocyanate and formaldehyde) both showed definite damaging effect on the antibody receptor of the macrophages. This effect was obtained at slightly lower molar concentration with sulfhydryl reagents (Table IV). The ability of the macrophages to take up neutral red was reduced by 0·5 but not 0·05 mM iodoacetamide, or PCMB. In the case of amino reagents no effect on neutral red uptake was observed with fluorescein isothiocyanate with the concentrations used, but formaldehyde caused a reduction of uptake of the dye at 0·1, but not 0·01 per cent.

Treatment with a reducing agent (mercaptoethanol) had no effect on the adsorption of cytophilic antibody by macrophages, although it was capable of reducing the uptake of neutral red. In contrast with this, oxidizing agents at a concentration of 50 mM showed a clear effect on the antibody receptors. This effect could also be observed at 5 mM concentration, but reduced in intensity. At this level uptake of neutral red was not affected.

TABLE IV.—*Effect of Treatment with Various Reactive Chemicals on the Macrophage Receptors for Cytophilic Antibody*

Reactive chemical added*	Guinea-pig anti-sheep red cell Serum No.	Cytophilic activity (direct method) serum dilution 1/4	1/16	1/64	None
Iodoacetamide : 5 mM	C49	0			0
0·5 mM	,,	±	±	0	0
0·05 mM	,,	+±	±	0	0
Sodium metaperiodate : 50 mM	,,	+	0		0
5 mM	,,	+++	0		0
Sodium nitrite : 50 mM	,,	0	0		0
5 mM	,,	+++±	0		0
Formaldehyde : 1·0 per cent	,,	0	0		0
0·1 per cent	,,	+	0		0
0·01 per cent	,,	+±	++		0
None	,,	+++±	+++	+±	0
Mercaptoethanol : 200 mM	C52	++++	+++	++	0
50 mM	,,	++++	++++	++±	0
Fluorescein isothiocyanate :	,,				
500 μg./ml.	,,	++	±	±	0
5 μg./ml.	,,	++++	++	+±	0
None	,,	++++	+++±	++±	0
ρ-chloromercurybenzoate :					
0·5 mM	C6	+	+±	0	0
0·05 mM	,,	++++	+++	++±	0
None	,,	++++	++++	+++	0

* Macrophages were incubated with solutions of reactive chemicals in 199 medium for 1 hr. at 37° previous to sensitization.

DISCUSSION

Guinea-pig γ_2 anti-sheep red cell antibodies which can adsorb to peritoneal and alveolar macrophages and cause sheep erythrocytes to adhere to their surface as a step initiating phagocytosis are not able to be adsorbed to other cell types (lymphocytes, polymorphonuclear leucocytes, or fibroblasts) commonly found in inflammatory exudates. It would appear that none of these cells possesses the specific cell membrane receptor able to bind these cytophilic opsonins and that this antibody type interacts specifically with macrophages. These observations confirm and extend the original findings of Boyden (1964) in this respect.

However, these experiments also raise several unanswered questions concerning the mechanism of opsonization for phagocytosis by polymorphonuclear leucocytes, and suggest that this phenomenon involves opsonic factors other than cytophilic antibody which was shown to bind to macrophages in the absence of complement components (Berken and Benacerraf, 1965). Several possibilities must be considered to explain the differences observed between polymorphonuclear leucocytes and macrophages: (1) Opsonization for phagocytosis by polymorphonuclear leucocytes involves another antibody type cytophilic for these cells, which is the least likely possibility, (2) the binding of the cytophilic antibody studied in these experiments, for polymorphonuclear leucocytes is too weak to be demonstrated in our system of assay but still sufficiently strong to cause opsonization and phagocytosis, or (3) Opsonization in the case of these cells requires complement or other serum components besides antibody as observed by numerous investigators (Boyden, North and Faulkner, 1965). It may be relevant in this respect to recall

that the leucotaxic activity of immune complexes has been clearly shown to be dependent on complement components (Boyden, 1962). It is not possible to choose among these various explanations on the basis of the published data as there is a general lack of agreement by investigators using different bacterial systems on the opsonic requirements of polymorphonuclear leucocytes.

The macrophage receptors for cytophilic antibody are destroyed by agents that react specifically with free SH groups: iodoacetamide and PCMB, by oxidizing agents and by compounds which react with free NH_2 and also SH groups such as isothiocyanate or formalin (French and Edsall, 1945). They are not affected by treatment with high concentration of mercaptoethanol. These effects on the receptor are independent of the effect of some of these chemicals on the ability of the cells to take up vital dye. These results suggest that free SH groups are part of the receptor site. The binding site on cytophilic antibodies has totally different properties since it is not affected by iodoacetamide and is destroyed by reducing agents (Berken and Benacerraf, 1965).

The macrophage receptor is not altered by treatment with proteolytic enzyme, lipase or neuraminidase, but is completely destroyed by lecithinase. The effect of lecithinase may be attributed to the destruction of the integrity of the cell membrane structure by this enzyme.

The resistance to the action of proteolytic enzyme of the macrophage antibody receptor contrasts with the susceptibility of the receptors for the binding of aged erythrocytes which attach to macrophages in the absence of specific antibody (Vaughan, 1965). There are therefore different macrophage receptors for the binding of cytophilic antibody and effete red cells.

Some of our results differ markedly from those reported by Sorkin (1964), who made a similar study using the uptake of radioiodinated BSA by passively sensitized spleen cells as a measurement of cytophilic antibody. He observed that the receptors for cytophilic antibodies were destroyed by mercaptoethanol and by proteolytic enzymes, which was not verified in these experiments. Sorkin's observations were made with a more heterogeneous cell population and with an antigen which was not made from denatured or aggregated material. Such a system would appear less capable of giving reliable results than the technique used in the experiments reported here.

SUMMARY

Guinea-pig γ_2 anti-sheep red cell antibodies, cytophilic for lung macrophages, were not adsorbed by guinea-pig lymphocytes, polymorphonuclear leucocytes or fibrosarcoma cells or by mouse fibroblasts. Similar mouse antibodies were adsorbed by guinea-pig macrophages but not by mouse fibroblasts.

The antibody receptor site on guinea-pig macrophages was inhibited by treatment with reagents which react with free SH groups (iodoacetamide and ρ-chloromercurybenzoate) or with both SH and NH_2 groups (formaldehyde and isothiocyanate) as well as by oxidizing agents (periodate and nitrite) and lecithinase C. The following materials were without effect: reducing agents (mercaptoethanol), enzyme poisons (azide, fluoride, and dinitrophenol) and various enzymes (trypsin, papain, lipase, hyaluronidase, and neuraminidase).

It is concluded that free SH groups play an important part in the reactivity of the antibody receptor site peculiar to the macrophage surface membrane.

REFERENCES

BERKEN, A. AND BENACERRAF, B.—(1965) *J. exp. Med.*, in press.
BOYDEN, S. V.—(1962) *J. exp. Med.*, **115**, 453—(1964) *Immunology*, **7**, 474.
BOYDEN, S. V., NORTH, R. J. AND FAULKNER, S. M.—(1965) *In* 'Complement'. Ed. G. E. W. Wolstenholme and J. Knight. London (Churchill), p. 190.
BOYDEN, S. V. AND SORKIN, E.—(1960) *Immunology*, **3**, 272.—(1961) *Immunology*, **4**, 244.
FRENCH, D. AND EDSALL, J. T.—(1945) 'Advances in Protein Chemistry', Vol. 2 New York (Academic Press), p. 277.
MYRVIK, Q. N., LEAKE, E. S. AND OSHIMA, S.—(1962) *J. Immun.*, **89**, 745.
SORKIN, E.—(1964) *Int. Arch. Allergy*, **25**, 129.
VAUGHAN, R. B.—(1965) *Immunology*, **8**, 245.