PROPERTIES OF ANTIBODIES CYTOPHILIC FOR MACROPHAGES*

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Plates 21 and 22

(Received for publication 7 July 1965)

The production of an antibody cytophilic for spleen cells in rabbits immunized with human serum albumin was described by Boyden and Sorkin (1, 2, 3) several years ago. These investigators detected the presence of cytophilic antibody by the capacity of the antisera to confer upon normal cells the capability of specifically adsorbing radio-iodinated antigen. Among the deficiencies in such a system is the failure to distinguish from among the heterogeneous population of cells in the tissue suspensions that cell type which is capable of adsorbing the antigen. This deficiency was recently overcome by Boyden (4) who demonstrated in guinea pig anti-sheep red cell serum the presence of an antibody which could selectively bind to homologous peritoneal macrophages and confer upon them the ability to adsorb sheep red cells.

The present work was designed to identify some of the physical and biological properties of this antibody. In the guinea pig, 7S antibodies with the same immunological specificities may be separated into two classes (5) with different biological activities on the basis of properties contributed by their Fc fragment (6) and their H chains (7). The γ_1, fast migrating antibodies mediate anaphylactic reactions (8); the γ_2, slow migrating antibodies fix complement and are responsible for such phenomena which depend upon complement components: cell lysis, and the Arthus reaction (9). Still another biological activity of antibodies, the capacity to bind selectively to macrophages, is shown in this report to be a property of the complement-binding γ_2-class of guinea pig antibodies. This activity, which is not complement-dependent, is not easily absorbed from the antisera by guinea pig macrophages showing that cytophilic antibodies are not a small fraction of the γ_2-population. The binding site for macrophages is located on the Fc fragment and therefore on the H chains of γ_2-globulin. The effects of pH, concentration, and temperature on the binding and elution of this antibody were investigated to explore the biological significance of cytophilic antibody. The cytophilic properties of this antibody for macrophages are primarily responsible for its opsonizing activity.

* 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.

‡ Recipient of National Heart Institute Postdoctoral Fellowship 5-F2-HE-19,665-02.

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

Antibodies cytophilic for macrophages are not adsorbed by guinea pig lymphocytes, polymorphonuclear leukocytes, or fibrosarcoma cells (10).

Materials and Methods

Animals.—Hartley strain guinea pigs weighing 300 to 500 g and Swiss-Webster mice weighing 25 to 30 g were used for immunization and for lung macrophages. Rabbits weighing about 2 kg were used for lung macrophages.

Sheep Red Cells.—These were obtained weekly in equal parts of blood and Alsever's solution from Probio, Inc., Nyack, New York.

Immunization.—Guinea pigs were immunized with sheep red cell emulsions in complete or incomplete Freund's adjuvant (Difco Laboratories, Detroit) in exactly the manner described by Boyden (4). The sera were harvested approximately 3 wk later except when otherwise specified. One group of guinea pigs received a single intravenous dose of 5×10^8 sheep red cells.

Hyperimmune mouse anti-sheep erythrocyte sera have been prepared by Dr. R. Sonntag Nussenzweig. Swiss-Webster mice were injected intravenously with 10^9 red cells twice with an interval of 4 wk. The mice were bled 3 to 4 days after the second injection and the sera pooled.

Guinea pig sera to *Escherichia coli* had been prepared by Dr. K. Bloch by hyperimmunization with *E. coli* 0111.B4 in complete Freund's adjuvant.

Guinea pig antisera to 2,4-dinitrophenyl–guinea pig albumin or 2,4-dinitrophenyl–bovine gamma globulin (DNP-GPA or DNP-BGG) were prepared as previously described (11).

Rabbit anti-sheep red cell whole serum was obtained in 50% glycerine from Certified Blood Donor Service, Inc., Jamaica, New York. The sera were obtained from rabbits which had received 8 intravenous injections of sheep red cell stroma (unboiled) during a 2 wk period. They were bled 10 days after the last injection. Before use the amboceptor was dialyzed for 24 hr against phosphate-buffered saline (pH 7.6) to remove the glycerine.

Macrophages.—Peritoneal macrophages were obtained as described by Boyden (4). Lung macrophages were obtained according to the technique of Myrvik (12). Guinea pig and rabbit lungs were dissected and perfused twice with Hanks balanced salt solution with a polyethylene catheter introduced into the trachea. After each perfusion the lungs were inverted and the cell suspension allowed to pour into centrifuge tubes. Mouse lungs were perfused with Hanks with a small, drawn out Pasteur pipette inserted into the trachea. The cell suspensions were centrifuged at 1000 RPM for 5 min at 4°C. The cells were washed once and resuspended in No. 199 culture medium (Microbiological Associates, Bethesda, Maryland) at a concentration of 2×10^6 cells/ml.

Assay of Cytophilic Activity.—Wells were constructed by a modification of Boyden's technique (4). Lucite rings, such as are used for Millipore chambers were fixed onto 22 mm square, glass cover slips. The inner diameter of the rings was 12 mm and the height 2 to 3 mm. The flat surfaces of the rings were coated with thin layers of silicone grease to provide water tight adherence to glass surfaces.

0.1 ml aliquots of the macrophage suspension from normal or sensitized animals (about 200,000 cells) were added to the wells at room temperature. The macrophages were allowed to settle to the glass where they adhered. After 30 min the supernatant fluid and any floating cells were removed. When macrophages from normal animals were to be passively sensitized, 0.1 ml of the antiserum dilution was added to the well. Sensitization proceeded for 1 hr at 4°C, 37°C, or room temperature. The passively sensitized macrophages were then washed by replacing the fluid in the wells 3 to 5 times with fresh, cold No. 199 medium. 0.1 ml aliquots of a 1% suspension of washed sheep red cells in No. 199 were then added to the wells containing the actively or passively sensitized macrophages (direct technique). This procedure

was used in most of the experiments designed to investigate the nature of cytophilic antibody. It was soon realized that the same phenomenon due to the same cytophilic antibody could also be demonstrated by another more sensitive method which will be referred to as the "passive indirect technique," in which 2% sheep erythrocytes suspended in No. 199 medium were sensitized by an equal volume of serial dilutions of heat-inactivated antisera before being added to normal unsensitized lung macrophages.

In all three methods, active, passive direct, or passive 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 No. 199 medium containing 2% 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 (13). In positive preparations they were surrounded by rosettes of red cells (Figs. 1 *a* and 1 *b*). The reactions were quantitated by rating the preparations from 4+ to trace: 4+, Rosettes on almost every macrophage; 3+, red cells on almost every macrophage (rosettes on about ½ cells); 2+, red cells on about ½ macrophages (frequent rosettes); 1+, red cells on some macrophages in most fields (occasional rosettes).

Control preparations were those in which normal unsensitized macrophages, incubated only in No. 199 medium, were exposed to washed sheep red cells. These preparations almost invariably revealed no red cells attached to macrophages. Red cells older than 8 days were not used because of the possibility of nonspecific sticking (4).

Procedure for the Assay of Cytophilic Activity in Guinea Pig Antisera to Antigens other than Sheep Red Cells.—The passive, direct method was used for the sensitization of normal macrophages by antisera to *E. coli* 0111:B4 and DNP conjugates.

To demonstrate anti-*E. coli* cytophilic activity, washed sheep red cells were coated with 0111:B4 polysaccharide as previously described (14). These red cells were added to wells containing macrophages sensitized with anti-*E. coli* sera and the procedure already outlined was followed.

To demonstrate anti-DNP cytophilic activity washed rabbit red cells were coated with the antigen in the following manner: DNP-GPA with 41 hapten groups per molecule was prepared in a manner previously described (11). This antigen was conjugated to rabbit red cells with bis-diazotized benzidine (BDB) according to the method of Halpern et al. (15). At the suggestion of Dr. G. Biozzi, 0.25 ml of the BDB solution was added to 0.15 ml of a 50% red cell suspension which had been mixed with 2 mg of DNP-GPA in phosphate-buffered saline (pH 7.3). Red cells prepared in this manner did not stick nonspecifically to macrophage or glass. These red cells were then added to wells containing macrophages sensitized with anti-DNP sera, and the already outlined procedure was followed.

Hemagglutination Titration.—Serial 2-fold dilutions of the antisera were made in 0.5 ml volumes of veronal buffer (16) containing 1% normal rabbit serum. 0.1 ml of a 1% suspension of washed sheep red cells was added to each tube. The patterns of hemagglutination were read after the tubes had been left overnight at 4°C.

Hemolysis.—The scale down procedure outlined in Kabat and Mayer (16) was followed. Results were recorded in H_{50} units.

Passive Cutaneous Anaphylaxis (PCA).—PCA was performed in guinea pigs using sheep red cells as antigen as previously described (9). As many as 30 sites per animal were used.

Double Diffusion in Agar Gel.—Commercial agar plates were used (Immunoplates, Hyland Laboratories, Los Angeles). These had been prepared with Difco special Noble agar as a 2% solution in a buffer of 7.5% glycine, 1% NaCl, 0.1% sodium azide, pH 7.0–7.2, with a center-to-center distance between peripheral and central wells of 5 mm. The plates were allowed to

develop for 24 hr, washed in saline, dried, stained with amido black 10B, decolorized in an acetic acid–glycerine-water solution, soaked in 3% glycerine, and dried.

Preparation of γ_1- and γ_2-Antibody Fractions.—Purified anti-DNP-BGG antibodies were isolated (5) from two pools of antisera. One pool was harvested from guinea pigs immunized with antigen and incomplete Freund's adjuvant, and was used to isolate γ_1-antibodies. The other pool was harvested from guinea pigs immunized with the antigen and complete Freund's adjuvant and was used to isolate γ_2-antibodies. Purified γ_1- and γ_2-anti-DNP antibodies were separated by geon block electrophoresis (17) and the purity of these preparations was confirmed by immunoelectrophoresis using a rabbit antibody against whole guinea pig serum.

Starch Block Electrophoresis.—Selected guinea pig anti-sheep red cell sera were fractionated by electrophoresis on starch block as previously described (5).

Crude Gamma Globulin Fractions.—For several experiments crude gamma globulin fractions were obtained from the sera of various species by precipitation with one-third saturated ammonium sulfate. These precipitates were dissolved in sufficient isotonic saline to equal half the starting serum volume. These solutions were dialyzed overnight against phosphate-buffered saline.

Preparative Ultracentrifugation of Guinea Pig Anti-Sheep Red Cell Sera.—5 to 20% sucrose gradients, 30 ml total volume, in Lusteroid tubes were prepared according to Martin and Ames (18) except for the use of 0.05 M phosphate buffer (pH 7.6) instead of 0.05 M Tris-HCl. 0.5 ml of the crude guinea pig gamma globulin fraction was layered on the gradient. The gradients were centrifuged in a swinging bucket rotor SW 25.1 at 24,000 RPM for 21 hr. The Lusteroid tubes were punctured at the bottom. Ten 3 ml fractions were collected and dialyzed overnight against phosphate-buffered saline. The fractions were then lyophilized and resuspended in 1 ml of medium 199.

Reduction and Alkylation.—Crude gamma globulin fractions of guinea pig anti-sheep red cell sera were diluted with 2 parts isotonic saline and 1 part 0.5 M tris buffer (pH 8.2) to a protein concentration of about 7.5 mg/ml. Mercaptoethanol, diluted with 0.5 M tris buffer, was added to the protein solution to a final concentration of 0.1 M. The solutions were incubated at 37°C for 1 hr and then dialyzed against either 0.02 M iodoacetamide or buffered saline (pH 7.6) for 8 hr. Excess iodoacetamide was eliminated by dialysis overnight against buffered saline.

Pepsin digestion according to Nisonoff (19) was performed on crude gamma globulin fractions of rabbit and guinea pig anti-sheep red cell sera which had been diluted with isotonic saline to a protein concentration of 5 mg/ml.

Elution of Cytophilic Antibody from Macrophages.—

Passively sensitized cells: 8×10^7 lung macrophages in 0.1 ml No. 199 medium were sensitized by suspending them for 45 min at 37°C in 0.9 ml of anti-red cell serum from a guinea pig immunized with complete Freund's adjuvants. They were centrifuged and washed 4 times with 2 ml No. 199 medium at 4°C. The 4th supernatant fluid was saved.

The cells were then resuspended in 2 ml of No. 199 medium for 45 min at 37°C. They were centrifuged for 5 min at 1000 RPM at room temperature. The supernatant fluid was saved.

Normal cells: 8×10^7 normal lung macrophages were suspended in 2 ml of No. 199 for 45 min at 37°C. They were centrifuged for 5 min at 1000 RPM at room temperature. The supernatant fluid was saved.

All of the supernatant fluids were frozen and lyophilized. Each lyophilate was then resuspended in 0.5 ml of No. 199 medium before testing.

Labeling of Guinea Pig γ_2-Globulin with I^{131}.—Commercially prepared guinea pig γ_2-globulin (Pentex Inc., Kankakee, Illinois) was further purified by chromatography on a DEAE column as described in (20). The final preparation was shown by immunoelectrophoresis and double diffusion in agar to contain only guinea pig γ_2-globulins. This preparation was labeled with I^{131} as follows (21).

10 mg guinea pig γ_2-globulin were dissolved in 2 ml of phosphate-buffered saline, pH 7.6. 4 mc of I^{131} carrier-free without reducing agent was added to the protein. The solution was maintained at 0°C and 0.2 mg of chloramine T was added. After 5 min 0.1 mg of Na bisulfite was added. The labeled preparation was extensively dialyzed against phosphate-buffered saline before use.

Measurement of Uptake of Filtered and Unfiltered I^{131} Guinea Pig γ_2-Globulin by Macrophages.—The following technique was used throughout the experiments: 10^7 guinea pig lung macrophages were exposed at 0 or 37°C for 1 hr to varying concentrations of I^{131}-labeled guinea pig γ_2-globulin dissolved in 0.5 ml of No. 199 medium containing either 10 mg BSA/ml or fresh guinea pig serum. The cells were then centrifuged for 4 min at 0°C and washed 4 times in cold No. 199 medium. The cells were resuspended in 1 ml cold No. 199 medium in a new test tube and the radioactivity absorbed by the cells was rapidly measured in a well scintillation counter. The cells were again centrifuged at 0°C for 4 min and the last supernatant counted. The net uptake of γ_2-globulin by the macrophages was calculated by subtracting this supernatant value and was expressed in micrograms of γ_2-globulin. Elution of γ_2-globulin was studied by exposing the sensitized cells to one ml of No. 199 medium either at 0 or 37°C for 1 hr and measuring the radioactivity released by the cells. The radioactivity released which could be precipitated by 20% trichloro-acetic acid (TCA) was measured as an indication of the amount of undigested γ_2-globulin eluted. A foreign protein was added as a carrier for this procedure because of the very low concentration of protein present in the eluates. In the case of absorption experiments the supernatant from the first exposure of the labeled γ_2-globulin to the lung macrophages was added again to a fresh suspension of 10^7 lung macrophages and the procedure repeated again. The uptakes by the first and second sets of cells at various concentrations of γ_2-globulin were compared to determine whether cytophilic globulin could be easily absorbed by the macrophages.

The first experiments carried out with I^{131}-labeled γ_2-globulin dissolved in No. 199 medium containing 10 mg BSA/ml, showed a definite decrease in uptake of γ_2-globulin (nearly 50%) by the second set of macrophages after the first absorption. It was soon recognized that an important source of error in this type of experiment results from the fact that macrophages absorb very avidly aggregated or denatured proteins (22) which can thus be mistaken in these preparations for globulins with cytophilic properties. The preparations of labeled γ_2-globulins used in these experiments cannot be considered free of such aggregated or denatured molecules. To avoid this artefact, use was made of the fact that the reticuloendothelial system clears very efficiently from the blood these denatured and aggregated proteins (23). 9 mg of I^{131}-labeled guinea pig γ_2-globulin containing 1 to 2 mc of iodine were injected intravenously into a guinea pig and allowed to circulate for 1 hr. The animal was bled from the heart and the serum harvested. The γ_2-globulin concentration of the serum was measured and its specific activity calculated. This preparation will be referred to as "filtered γ_2-globulin." A suitable amount of I^{131}-labeled γ_2-globulin preparation was added to normal serum from the same guinea pig obtained before the injection of radioactive material to provide a control unfiltered preparation. Both filtered and unfiltered preparations were then used to measure the uptake of γ_2-globulin by 10^7 lung macrophages after a first and a second exposure for 1 hr at 37°C to serial serum dilutions containing decreasing amounts of γ_2-globulin.

RESULTS

Assay of Cytophilic Activity.—Confirming the findings of Boyden (4), we have observed that the sera of guinea pigs immunized with sheep red cells in complete Freund's adjuvants contain an antibody capable of binding to guinea pig peritoneal macrophages which could be demonstrated by the adherence of

TABLE I

Effect of Temperature on the Sensitization of Normal Macrophages by Cytophilic Antibody (Direct Technique)

Temperature during sensitization (1 hr)	Cytophilic activity		
	Dilution of anti-sheep red cell serum C_{48}		
	1/4	1/16	1/64
°C			
4	++	+	±
22	++++	+++	++
37	++++	++++	++++

sheep erythrocytes to the sensitized macrophages. This phenomenon could also be readily observed with lung macrophages which constitute a better defined cell population, containing generally about 90% macrophages (Figs. 1 *a* and 1 *b*). All experiments were therefore carried out with lung macrophages.

Active in vivo sensitization of lung macrophages from immunized guinea pigs was demonstrated when these cells were placed in "reaction wells" and exposed to washed sheep red cells, but the level of sensitivity of these macrophages was never as intense as could be obtained when cells from nonimmune animals were sensitized in vitro with anti-sheep red cell serum from the same sensitized guinea pigs.

Experiments were then designed to study the effect of temperature and of pH on the passive sensitization of lung macrophage by cytophilic antibodies by the direct technique. Exposure of the cells to the sensitizing antibody at 4°C results in poor sensitization. Optimal sensitization is obtained by incubation at 37°C while at 22°C the level of sensitivity obtained is less satisfactory although sufficient for many experiments (Table I). The effect of pH was investigated by diluting the sensitizing antiserum in Hanks' solution containing

TABLE II

Effect of Temperature on the Elution of Cytophilic Antibody from Sensitized Macrophages*

Temperature	Persisting cytophilic activity
°C	
4	++++
22	++++
37	±

* Macrophages were sensitized with a 1/4 dilution of serum C_{42}. The serum was then washed away and replaced by medium 199. Time of incubation: 1 hr.

phosphate buffers varying from pH 5.7–pH 8.1. No change in the level of macrophage sensitization was observed within this pH range.

In some experiments Na_2EDTA (final concentration 0.01 M) was added to the sensitizing antisera dilutions in No. 199 medium without change in the level of sensitization of the macrophages obtained showing that cytophilic antibody does not require calcium or magnesium to bind to macrophage surfaces.

The effect of temperature on the loss of sensitivity of macrophages following

TABLE III

Hemagglutinating, Hemolytic, and Cytophilic Titers after Immunization of Guinea Pigs with Sheep Red Cells and Complete or Incomplete Freund's Adjuvant*

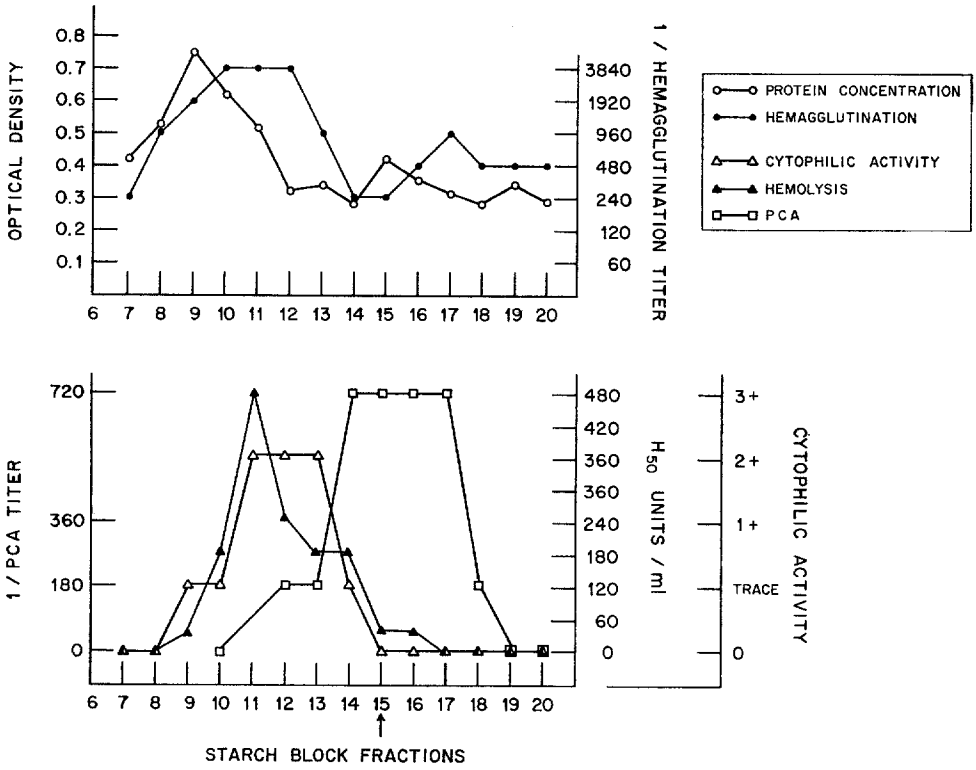
Guinea pig No.	Hemagglutinating titer	H_{50} units	Cytophilic‡ titer
	Complete Adjuvant		
C_2	1/10,240	600	1/512
C_5	1/20,480	975	1/512
C_6	1/10,240	1710	1/512
C_7	1/10,240	640	1/256
C_8	1/10,240	930	1/512
C_9	1/10,240	785	1/64
	Incomplete Adjuvant		
I_2	1/20,480	410	1/16
I_3	1/10,240	100	1/4
I_4	1/10,240	175	1/4
I_5	1/5,120	130	1/4
I_6	1/5,120	385	1/16
I_7	1/10,240	425	1/16

* Sensitization of guinea pig lung macrophages with antisera for 1 hr at room temperature.
‡ Lowest dilution with 1+ cytophilic activity (by the passive direct method).

passive sensitization was investigated. Macrophages were sensitized by the passive direct technique with a 1/4 dilution of a guinea pig anti-sheep red cell serum and then washed and incubated for 1 hr in No. 199 medium at 4°C, room temperature 22°C, or 37°C. The results presented in Table II show that loss of sensitization results from incubation in culture medium at 37°C but not at 4°C. This loss of sensitivity will be shown in other experiments with I^{131}-labeled γ_2-globulin to be due in part to the elution of cytophilic antibody and in part to the absorption of the antibody within the cell.

Cytophilic antibody specific for the *E. coli* polysaccharide or for the dinitrophenyl hapten, was demonstrated by the passive direct technique in the sera of guinea pigs immunized with heat-killed *E. coli* organisms or DNP proteins

in complete Freund's adjuvants. The reaction was much less intense than with anti-sheep red cell sera and could be clearly demonstrated at the 3 to 4+ level only with undiluted sera in spite of the high antibody content of the sera used. The lesser degree of cytophilic activity observed with antigen-coated red cells

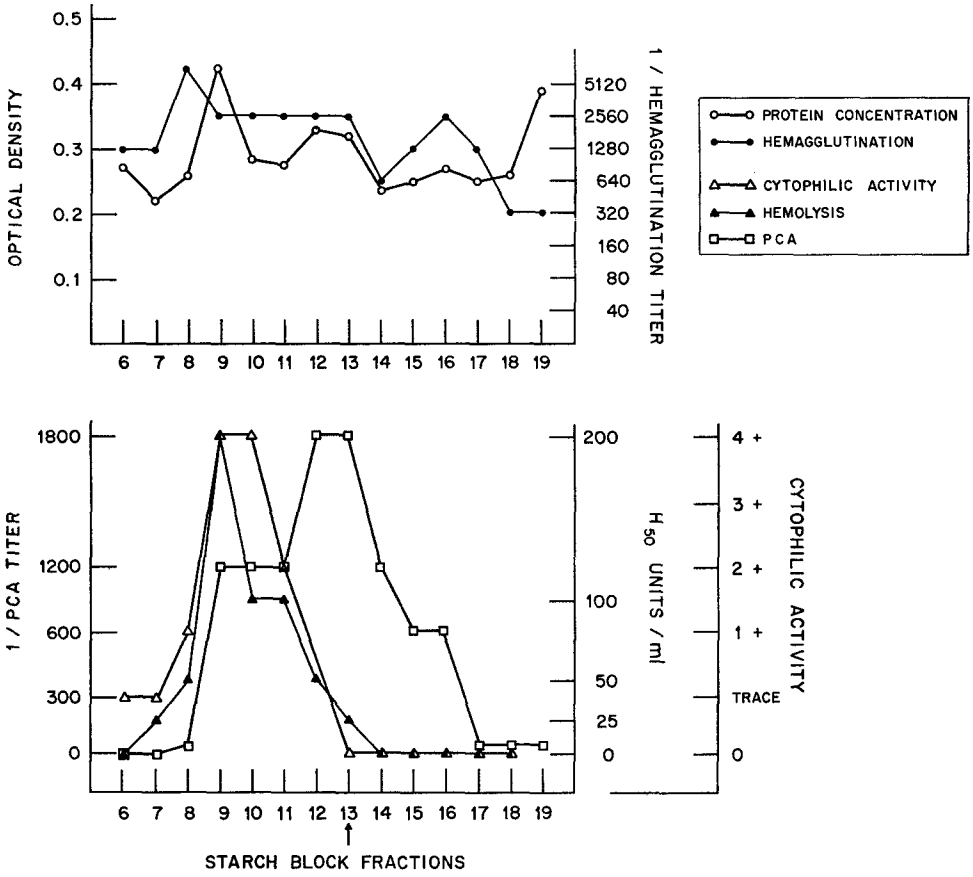


TEXT-FIG. 1. Titration of hemagglutinating, cytophilic, hemolytic, and passive cutaneous anaphylactic (PCA) activities in fractions from starch block electrophoresis of serum C_7 (5 ml) from a guinea pig immunized with sheep red cells and complete adjuvant. Protein concentration was determined by the Folin-Ciocalteu method (16) and expressed in optical density units.

in these systems may be related to the importance of coating the red cell surface with a sufficient amount of antigen for this type of reaction.

The Adjuvant Effect of Mycobacteria on the Production of Cytophilic Antibodies in Guinea Pigs.—3 wk following injection of guinea pigs with sheep red cells in complete or incomplete Freund's adjuvants, the cytophilic titers of the sera were compared by the passive direct technique and related to their hemagglutinating and hemolytic titers (Table III). Confirming Boyden's findings (4), both sets of sera had comparable hemagglutinating titers, but high titers of

cytophilic activity were present only in the sera of animals immunized with mycobacteria. It should also be stressed that high hemolytic titers were only present in the sera of guinea pigs immunized with complete adjuvants. This is not surprising since guinea pigs immunized with mycobacteria produce a high



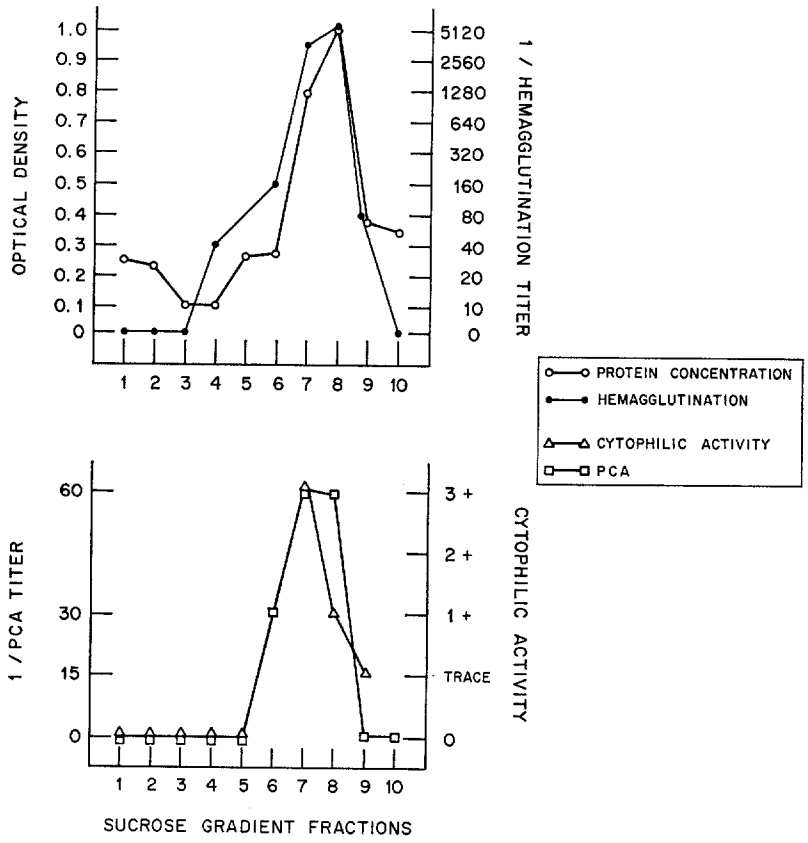
TEXT-FIG. 2. Titration of hemagglutinating, cytophilic, hemolytic, and passive cutaneous anaphylactic (PCA) activities in fractions from starch block electrophoresis of serum I_3 (9 ml) from a guinea pig immunized with sheep red cells and incomplete adjuvant. Protein concentration was determined by the Folin-Ciocalteu method (16) and expressed in optical density units.

concentration of γ_2-antibodies while animals immunized with the antigens in saline or in incomplete adjuvants synthesize predominantly γ_1-antibody globulins which do not bind complement in vitro (5).

The relative absence of cytophilic activity for macrophages in the sera of the guinea pigs immunized with sheep red cells in incomplete adjuvants strongly

suggest that γ_1-globulins do not have cytophilic activity which will be confirmed in other experiments.

Time of Appearance of Cytophilic Antibody in the Course of Immunization.— Cytophilic activity was not detected by the passive direct method in guinea



TEXT-FIG. 3. Titration of hemagglutinating, cytophilic, and passive cutaneous anaphylactic (PCA) activities in segments from a sucrose gradient ultracentrifugation of the crude gamma globulin fraction from guinea pig anti-sheep red cell sera C_8 and C_9. Protein concentration was determined by light absorption at 278 mμ and expressed in optical density units.

pig anti-sheep red cell sera until 8 days after immunization with complete adjuvants. At that time, active sensitization of lung macrophages could be observed and measurable hemolytic titers were found in the sera. Using the more sensitive indirect assay of cytophilic activity, none of 12 sera obtained 4 days after immunization with sheep red cells in complete adjuvants had cytophilic activity whereas all were strongly positive by the 8th day.

A low titer of cytophilic activity assayed by the indirect technique was also observed in the sera of guinea pigs 8 days after immunization with a single intravenous injection of 5×10^8 sheep red cells.

Identification of the Guinea Pig Antibody Type Cytophilic for Macrophages.— In order to investigate the electrophoretic properties of guinea pig cytophilic antibodies, electrophoretic separation of antisera from guinea pigs immunized with sheep red cells in complete (C) or incomplete (I) Freund's adjuvant was carried out on starch blocks. The fractions from 1/2 inch cuts concentrated to

TABLE IV

Nonspecific Inhibition by Purified γ_2-anti-DNP Antibodies of the Sensitization of Macrophages by Anti-Sheep Red Cell Serum

Concentration of competing antibody	Cytophilic activity*			
	Dilution of anti-sheep red cell serum C_{8-9}			
	1/4	1/16	1/32	1/64
No competing antibody	++++	++++	+++	++
γ_2-anti-DNP-BGG (4.5 mg/ml)	+++	0	0	0
γ_1-anti-DNP-BGG (4.5 mg/ml)	++++	++++	+++	++
	Dilution of anti-sheep red cell serum C_2			
	1/5	1/10	1/40	
No competing antibody	++++	++++	++++	
γ_2-anti-DNP-BGG (1 mg/ml)	+++	++	±	
γ_1-anti-DNP-BGG (1 mg/ml)	++++	++++	++++	
80% anti-DNP-BGG serum	±	±	0	
80% normal serum	+++	+	±	

* Assayed by the direct method.

1 ml volume were assayed for hemagglutinating, hemolytic, PCA, and cytophilic activity (Text-figs. 1 and 2).

In both separations, cytophilic activity coincides with hemolytic activity and is present only in the slow migrating fractions. Both activities can be separated from the fast migrating PCA activity. Cytophilic activity, therefore, appears to be a property of the γ_2- and not of the γ_1-guinea pig antibody class. Cytophilic activity was assayed only on undiluted fraction eluates of serum I_3 but was measurable up to 1/18 dilution of fractions from serum C_7.

Selected γ_2-fractions and γ_1-fractions from serum I_3 were also assayed for cytophilic activity by the indirect passive method and showed high titer activity in the γ_2-fractions, 1/1024, and negligible activity, 1/32, in γ_1-fractions with equal hemagglutinating activities. The small degree of activity detected

by the indirect method in the γ_1-fractions is believed to be due to slight contamination of these fractions with γ_2-antibodies.

A study was made of the sedimentation properties on a sucrose gradient of the cytophilic antibodies in 3 wk sera from guinea pigs immunized with sheep red cells in complete adjuvants (Text-fig. 3). Cytophilic activity was found only in the fractions with PCA activity. Since γ_1-guinea pig globulin, responsible for PCA sensitization, is known to be a 7S globulin, this experiment shows that gueina pig cytophilic antibody present in the serum 3 wk after immunization has approximately a 7S sedimentation coefficient, similar to both γ_1- and γ_2-guinea pig globulins (5).

TABLE V

Cytophilic Activity of Guinea Pig Anti-Sheep Red Cell Sera Measured Directly by Sensitizing Macrophages and Adding Normal Sheep Red Cells or Indirectly by Sensitizing Red Cells and Adding them to Normal Macrophages

Guinea pig No.	Direct sensitization				Indirect sensitization					
	Serum dilution				Serum dilution					
	1/2	1/8	1/32	1/128	1/200	1/400	1/800	1/1600	1/3200	1/6400
C_{35}*	++++	+++	++	++	++++	++++	+++	+++	++	++
C_{44}	++++	++++	+++	++	++++	++++	++++	++++	+++	++
C_{50}	++++	++++	+++	+	++++	++++	+++	+++	±	±
C_{51}	++++	++++	+++	++	++++	++++	++++	++++	++	+
C_{52}	++++	++++	++++	+++		++++	++++	++++	++++	++++
I_2‡	++	+	±	0	++++	++++	+++	+	+	0
I_4	+	±	0	0	++++	+++	++	0	0	0
I_5	+	±	0	0	++++	++	+	0	0	0
I_6		+	±	0	++++	+++	0	0	0	0

* C, Immunized with complete Freund's adjuvant.

‡ Immunized with incomplete Freund's adjuvant.

To confirm the γ_2-nature of cytophilic antibody the ability of nonspecific purified γ_2-antibody to inhibit the direct sensitization of macrophages by anti-sheep red cell serum was tested (Table IV). At a dilution of 1/16 the cytophilic activity of this serum was completely inhibited by 4.5 mg/ml of purified γ_2-anti-DNP antibody. γ_1-antibody at the same concentration showed no effect.

A decrease in the concentration of the competing antibody to 1 mg/ml resulted in a diminution of the inhibition. Normal serum also inhibits the binding of cytophilic antibody although it is less effective than an anti-DNP-BGG (complete adjuvant) serum. This difference probably reflects the increased γ_2-globulin concentration in the immunized animal. Thus, all sera whether from normal or immunized guinea pigs contain significant titers of nonspecific γ_2-cytophilic antibodies. The significance of the presence of these nonspecific

antibodies is evident when titrating the cytophilic activity of a serum by the indirect as well as the direct method. In the latter technique anti-red cell cytophilic antibody must compete for macrophage sites with the nonspecific cytophilic antibody present also in the serum.

The indirect technique combines, first, antibody with antigen and thus effectively eliminates competing γ_2-globulins. Table V demonstrates the manyfold increase in cytophilic activity measureable by the indirect method.

However, hyperimmune guinea pig serum can also be shown to inhibit cytophilic antibody bound to sheep red cells assayed by the passive indirect method.

This effect, which is much less marked than in the case of antibody not bound

TABLE VI

Nonspecific Inhibition by Anti-DNP-BGG Serum (1/5 Dilution) of the Binding of Sensitized Sheep Red Cells to Normal Macrophages

Serum used to sensitize red cells*	Anti-DNP serum	Cytophilic activity‡				
		Dilution anti-sheep red cell serum				
		1/400	1/800	1/1600	1/3200	1/6400
C_{51}	No	++++	++++	++++	++	+
C_{51}	Yes	++++	+++	+	±	±
C_{52}	No	++++	++++	++++	++++	++++
C_{52}	Yes	++++	++++	++		++

* Sheep red cells were sensitized with heat-inactivated antiserum at the various dilutions for 10 min at 37°C and then centrifuged for 5 min at 1000 RPM. The sensitizing serum was decanted. The red cells were resuspended in an equal volume of either No. 199 or anti-DNP serum and added to normal macrophages.

‡ Indirect technique.

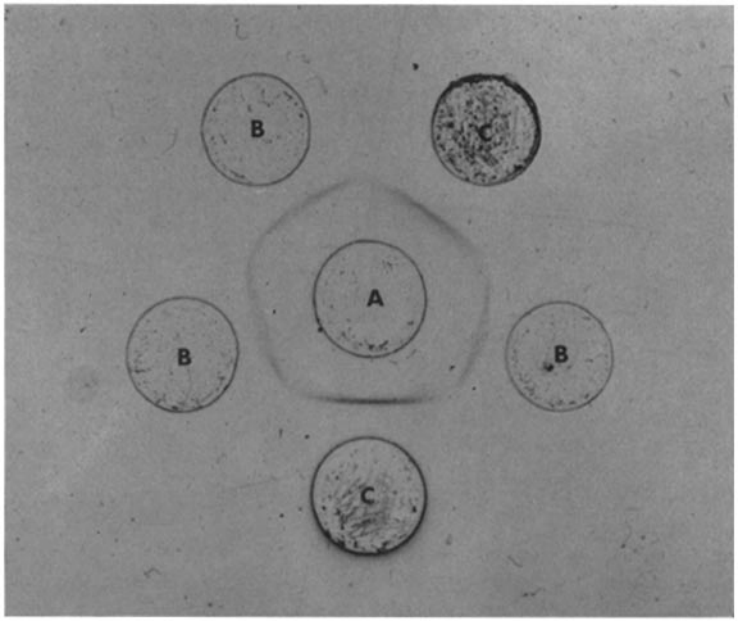
to antigen, can be demonstrated by suspending the erythrocytes sensitized by serial dilutions of the antisera in a competing serum before adding them to the macrophages (Table VI). Partial inhibition by the anti-DNP serum of the binding of sensitized red cells was observed only when the red cells were sensitized by the higher dilutions of the antisera and, thus, coated with fewer specific antibody molecules. This indicates the greatly diminished reversibility of macrophage-antibody binding once cytophilic antibody and its antigen have combined.

To establish definitely that guinea pig cytophilic antibody belongs to the γ_2-class of immunoglobulins, use was made of the fact that cytophilic antibody was shown to be easily eluted from macrophages when these passively sensitized cells are incubated in culture medium at 37°C.

Cytophilic antibody activity was demonstrated in the 37°C eluates from passively sensitized macrophages by the passive indirect technique. Dilutions

of the elutate up to 1/20 showed strong cytophilic activity. Constituents of the eluates were then identified by double diffusion in agar and by immunoelectrophoresis. Immunoelectrophoresis of the eluate from sensitized macrophages developed by a rabbit anti-guinea pig whole serum revealed only 2 lines, one in the albumin region and one in the γ_2-region. The albumin nature of the former line was confirmed by double diffusion in agar.

When the electrophoresed eluate was developed with a rabbit antiserum



TEXT-FIG. 4. Double diffusion in agar gel. (*A*), Rabbit anti-guinea pig γ_2-globulin serum $\frac{1}{16}$; (*B*), guinea pig anti-DNP γ_2-globulins, 50 μg/ml; and (*C*), eluates from guinea pig lung macrophages passively sensitized with guinea pig anti-sheep red cell serum C_{48}.

against guinea pig γ_2-globulin a single line in the γ_2-region was revealed. There was complete antigenic identity between this component of the eluate and purified anti-DNP γ_2-guinea pig antibody globulins when they were analyzed by double diffusion in agar with a rabbit antiserum to guinea pig γ_2-globulin (Text-fig. 4).

As a control for the washings of the cells before the elution procedure, immunoelectrophoresis of the last wash fluid from the passively sensitized macrophages showed no lines with either antisera against whole guinea pig serum or against guinea pig γ_2-globulin. The electrophoresed 37°C eluate from normal unsensitized lung macrophages revealed a single weaker line in the γ_2-region with both antisera.

These various experiments clearly show that the guinea pig antibody with cytophilic properties for macrophages belongs to the γ_2-family of immunoglobulins. The problems remain: (*a*) whether a small fraction of the γ_2-globulin, most of it, or all of it has cytophilic properties for macrophages; and (*b*) what is the nature of the adsorption; is it a strong or weak interaction, easily or not easily reversible? The data reported above on the ease with which this antibody

TABLE VII

Effect of Absorption with Lung Macrophages on Cytophilic Activity

	Direct method				Indirect method			
	Serum dilution				Serum dilution			
	1/60	1/100	1/200	1/300	1/200	1/600	1/1000	1/3000
Experiment I								
Control (C_{51})	++++	+++	+++					
First absorption	++++							
Second absorption	++++							
Third absorption	++++							
Experiment II								
Control (C_{51})	+++	++	+					
First absorption	++							
Second absorption	++							
Experiment III								
Control (C_{51})		+	±	±	++++		+++	
First absorption		+			++++		+++	
Second absorption		+			++++		+++	
Experiment IV								
Control (C_{51})				±		++++		++
First absorption				±		++++		++
Second absorption				±		++++		++

Absorption mixture, 10^7 lung macrophages in 0.5 ml serum dilution at 4°C for 45 min.

is eluted from macrophages at 37°C suggest that the binding of cytophilic antibody unreacted with antigen is not strong and that the reaction is easily reversible. This would be compatible with cytophilic antibody being an important fraction of the γ_2-globulin population.

Two series of experiments were carried out to explore this point. First an attempt was made to absorb cytophilic antibody activity from a guinea pig anti-sheep red cell serum by incubating the same dilution of this antiserum repeatedly with fresh lung macrophages at 4°C. The several experiments carried

out with serum C_{51} prepared by immunizing a guinea pig with sheep red cells in complete adjuvants are shown in Table VII.

The unabsorbed and absorbed antiserum dilutions were tested for cytophilic activity both by the direct and indirect passive techniques. The cytophilic activity of macrophages could not be absorbed by this procedure even when it was repeated twice. However, an inspection of the data reveals that the assay would not be able to detect an absorption of 50% of the cytophilic antibody

TABLE VIII

The Effect of the Concentration of Guinea Pig γ_2-Globulin on the Amount Bound by Lung Macrophages, Using "Filtered" and "Unfiltered" Material

Serum dilution	Amount of γ_2-globulin per 0.5 ml	Amount bound by macrophages				Filtered γ_2-globulin bound	
		Unfiltered*		Filtered‡			
		First absorption	Second absorption	First absorption	Second absorption	First absorption	Second absorption
	μg	μg	μg	μg	μg	%	%
1/2	3400	18.2	9.0	7.1	8.4	0.21	0.25
1/4	1700	8.0	5.0				
1/8	850	5.6	3.2	2.3	1.7	0.27	0.20
1/16	425	3.4	2.1	1.3	1.2	0.31	0.28
1/32	212	1.9	1.1	0.81	0.73	0.38	0.34
1/64	106	1.0	0.6	0.51	0.48	0.47	0.45
1/128	53			0.31	0.30	0.58	0.57

Time of incubation, 45 min at 37°C.

Repeated absorptions of 0.5 ml serum dilution with 10^7 fresh cells (compare first and second absorptions) did not affect the amount of "filtered" γ_2-globulin bound but substantially decreased the amount of "unfiltered" γ_2-globulin.

* Unfiltered material was similarly diluted in normal guinea pig serum.

‡ 9 mg of trace labeled I^{131} γ_2-globulin filtered by intravenous injection into a normal guinea pig bled one hr later. Radioactivity was diluted approximately 10 times in guinea pig serum by this process.

present in the original dilutions, because a sharp end point is difficult to define, as cytophilic activity decreases slowly with serial dilutions.

The problem was therefore investigated using I^{131}-labeled guinea pig γ_2-globulin. The results of these experiments are presented in Table VIII as the amount of γ_2-globulin calculated to have been absorbed by the macrophages. The uptake by the macrophages exposed to the unfiltered I^{131} γ_2-globulin is significantly larger than the uptake by the cells exposed to the biologically filtered preparation and it decreases with repeated exposure to the cells. In contrast, macrophages exposed to the filtered I^{131} globulin are not able to absorb out this protein from the serum since the uptake by repeated aliquots of cells remains constant. This was verified over a 60-fold range of γ_2-globulin concen-

trations. The absence of absorption of the filtered γ_2-globulin observed in these experiments show that the antibody with cytophilic activity is not a small fraction of the γ_2-globulin population. The net uptake by the macrophages increases with the serum concentration to reach 7 to 8 μg per 10^7 cells exposed to a 1/2 dilution of normal serum, although the per cent uptake decreases.

The data obtained with the unfiltered globulin must be considered to reflect both the phagocytosis of denatured material as well as the absorption of cytophilic globulin and, therefore, is unreliable as a measurement of uptake of cytophilic globulin. The experiments in Table VIII were carried out by sensitizing the cells at 37°C. Similar results were obtained when the cells were incubated with radioiodinated globulin at 0°C; smaller amounts were absorbed at this lower temperature.

TABLE IX

Comparison of the Binding and Elution of Guinea Pig γ_2-Globulin When Macrophages are Sensitized at 0 and 37°C

Temperature of sensitization	Amount bound	Amount eluted after 45 min at 37°C	Amount eluted and TCA precipitable
°C	μg	μg	μg
0	0.39	0.28	0.26
37	1.15	0.62*	0.39

10^7 cells in 0.5 ml 1/20 guinea pig serum containing 0.34 mg γ_2-globulin trace labeled with I^{131} and "filtered" in a guinea pig. Time of incubation, 45 min.

* A significant proportion of the labeled antibody bound at 37°C was apparently pinocytosed and degraded.

A comparison of the absorption and elution of guinea pig γ_2-globulin when macrophages are sensitized at 0 or 37°C is shown in Table IX using the filtered preparation. The uptake at 37°C is nearly three times higher than at 0°C confirming (Table I) that the cells can be more efficiently sensitized by cytophilic antibody at 37°C than 0°C. When the sensitized cells are exposed at 37°C to serum-free No. 199 medium a considerable proportion of the adsorbed globulin is eluted. Most of the eluted globulin from the cells sensitized at 0°C is TCA precipitable. In contrast, the cells sensitized at 37°C release a considerable amount of TCA nonprecipitable radioactivity indicating that some of the absorbed γ_2-globulin had been metabolized at this higher temperature.

In Table X, a study was made of the effect of temperature on the elution of cytophilic γ_2-globulin from the macrophages. Incubation of sensitized macrophages in No. 199 medium at 0°C results in very little loss of radioactivity compared to incubation at 37°C. These findings explain our earlier observations concerning the effect of temperature on the loss of sensitivity of macrophages sensitized with anti-sheep red cell serum (Table II).

Biological Properties of Cytophilic Antibody and the Role of Complement.— Complement is not required for the adsorption of cytophilic antibody by macrophages. Heating anti-sheep red cell sera for 1 hour at 56°C does not alter the cytophilic properties or titer of the sera; furthermore, passive sensitization of normal macrophages is not interfered with when the antiserum is made 0.01 M with Na_2EDTA. But cytophilic antibody can bind complement under appropriate conditions. Thus, if guinea pig complement, previously absorbed with sheep red cells, is added in a 1/50 dilution to preparations of red cell rosettes formed on macrophages sensitized by cytophilic antibodies, the rosettes are completely lysed within a 30 min period of incubation at 37°C. In Fig. 2, red cell ghosts emptied of their hemoglobin can be seen surrounding intact macrophages treated with guinea pig complement. The following control was

TABLE X

Comparison of the Elution of Guinea pig γ_2-Globulin at 0 or 37°C from Passively Sensitized Lung Macrophages

Temperature of elution (45 min)	Amount bound	Amount eluted	Amount eluted and TCA precipitable
°C	μg	μg	μg
0	0.32*	0.02	0.02
37	0.54‡	0.26	0.17

Time of incubation, 45 min.

10^7 cells sensitized in 0.5 ml of a solution of 10 mg/ml/bovine serum albumin in medium 199 containing 25 μg unfiltered I^{131}-labeled guinea pig γ_2-globulin at:

* 0°C.

‡ 37°C.

used for this experiment; tanned sheep red cells can attach to macrophages in the absence of cytophilic antibody. The addition of absorbed complement to these preparations failed to cause lysis of the red cells. These observations establish that cytophilic antibody, a γ_2-globulin, can bind complement and lyse cells, although complement is not required for its binding to macrophages.

The technique used to study anti-sheep red cell antibodies cytophilic for macrophages requires that the red cell suspension be added to the sensitized macrophages and incubated for 1 hr at room temperature before the excess red cells are washed off. Under these conditions typical rosettes are formed (Figs. 1 *a* and 1 *b*, and 3). If the rosettes are left for several hours at room temperature or overnight at 4°C, no change is observed and the preparation can be graded at any time during this period. If, however, the preparation is incubated at 37°C the macrophages become active and within 90 min they phagocytize all the red cells previously on their surface (Fig. 4). This phenomenon can be observed at 37°C in the absence of serum components when the sensitized macrophages are surrounded by red cells and suspended in No. 199 medium.

These experiments illustrate that cytophilic antibody is in fact an opsonin which causes the antigens to adhere to the macrophage surfaces to be phagocytized. The characteristic phase of red cell sticking and rosette formation is observed because the macrophages are not actively phagocytic at room temperature and allow the erythrocytes to remain adsorbed on their surface under these conditions.

Localization of the Receptor Site for Binding to Macrophages.—Cytophilic antibodies can be considered to act as opsonins. They need, therefore, to react with the antigen and to possess receptors which bind specifically with the macrophage cell membrane. An attempt was made to determine what portion

TABLE XI

Effect of Pepsin Treatment on Cytophilic Activity

Guinea pig macrophages

Guinea pig serum C_8	Hemagglutinating titer	Cytophilic activity				
		Direct undiluted	Indirect			
			1/2	1/20	1/200	1/2000
Control	1/20,480	++++	++++	+++	+++	0
Treated	1/20,480	0	+++	0	0	0

Rabbit macrophages

Rabbit amboceptor	Hemagglutinating titer	Cytophilic activity		
		Direct undiluted	1/10	1/100
Control	1/5,120	++++	+++	±
Treated	1/10,240	0	0	0

of the 7S γ_2-globulin molecule contain this receptor. Pepsin digestion of 7S mammalian globulin was shown to destroy the Fc fragment and to result in the formation of a 5S bivalent antibody capable of precipitating with antigen (19). Pepsin digestion of the gamma globulin fraction from a guinea pig anti-sheep red cell serum causes a complete loss of cytophilic activity assayed by the passive direct method. A slight degree of activity could still be detected after pepsin digestion by the much more sensitive indirect technique (Table XI), but the titer was much reduced. Similar results were obtained when rabbit anti-sheep red cell antibodies were digested with pepsin. The receptors for cytophilic attachment to macrophages are destroyed by pepsin treatment and are, therefore, probably, on the Fc fragment of the molecule. Receptors for the biological activities of various antibody globulin classes, complement fixation

TABLE XII

Effect of Reduction and Alkylation on Cytophilic Activity of Guinea Pig Serum C_{51}

Treatment	Hemagglutinating titer	Cytophilic activity				
		Direct		Indirect		
		Undiluted	1/10	1/20	1/200	1/2000
None	1/2560	++++	+	++++	+++	+
ME + IO	1/2560	+	0	++	+	0
ME		+	0	++++	+++	0
IO		++++	+	++++	+++	+

ME, mercaptoethanol, 0.1 M.
IO, iodoacetamide, 0.02 M.

(24), and anaphylactic-sensitization (25, 26), have also been shown to reside principally on the Fc fragment.

The site for complement fixation of 7S γ_2-globulin of several mammalian species has been shown to be destroyed by reduction and alkylation (27, 28).

TABLE XIII

*Affinity of Cytophilic Antibody for Heterologous Macrophages**

Serum	Serum Dilution			
	1/2	1/8	1/32	1/128
Guinea pig lung macrophages				
Mouse IV-1	++	±	0	0
Mouse IV-2	++++	+++	++	±
Guinea pig C_5	++++	++++	+	0
Guinea pig C_6	++++	+++	+	±
Guinea pig C_{52}	++++	++++	++	++
Rabbit	++++	++++	+++	++
Mouse lung macrophages				
Mouse IV-1	+++	+	0	0
Mouse IV-2	++++	++++	+++	++
Guinea pig C_5	++	0	0	0
Guinea pig C_6	++	0	0	0
Rabbit lung macrophages				
Guinea pig C_{52}	++	++	+	±
Rabbit	++++	++++	++	+

* Assayed by the passive direct technique.

A study was made of the sensitivity of the cytophilic receptor to mercaptoethanol and iodoacetamide. The results in Table XII show that reduction of the gamma globulin fraction of a guinea pig anti-sheep red cell serum by mercaptoethanol diminishes its cytophilic activity while treatment with iodoacetamide has no effect. Reduction by mercaptoethanol followed by alkylation with iodoacetamide results in still greater loss of cytophilic activity.

Cytophilic Activity of Mice and Rabbit Antibodies; Affinity of Cytophilic Antibodies for Heterologous Macrophages.—Antibody cytophilic for macrophages can be demonstrated by the same technique used to study guinea pig antibodies in sera of mice and rabbits immunized intravenously with sheep red cells. The passive direct technique was used in these experiments. Mouse and rabbit cytophilic antibodies were used to sensitize mouse or rabbit lung macrophages as well as guinea pig macrophages. As shown in Table XIII, mouse, rabbit, and guinea pig antibodies sensitize best their homologous macrophages although sensitization across species can also be obtained. Thus, mouse and rabbit antibodies can sensitize guinea pig macrophages almost as well as guinea pig antibodies but guinea pig antibodies are very inefficient sensitizers of mouse or rabbit macrophages. These results indicate some similarity but lack of identity between the receptors of cytophilic antibodies of different mammalian species.

DISCUSSION

Guinea pig antibodies cytophilic for macrophages have been identified as 7S γ_2-globulins capable of binding complement and lysing cells. 7S γ_1-antibodies do not have this property. 19S guinea pig antibodies were not investigated in this respect in this study. γ_2-Globulins bind to macrophages because of the presence of a receptor for the macrophage cell membrane on their Fc fragment. This activity of this receptor is destroyed by treatment with mercaptoethanol and iodoacetamide as is the case for the receptors for binding complement.

The antibody bond for macrophages is a weak one since large concentrations of γ_2-globulins were required to approach saturation of the available sites on the macrophages and since even at low concentrations of γ_2-globulins less than 1% was bound to but a fraction of the sites available on the macrophages. Another indication of the reversibility of the binding reaction is the ease with which cytophilic antibody is eluted at 37°C in culture medium. It is, therefore, not surprising that cytophilic activity is not easily absorbed from immune sera by repeated exposure to fresh macrophages. These results and the observation that opsonizing antibodies must be cytophilic indicate that an important portion of the γ_2-globulin fraction, if not all of it, possesses cytophilic properties for macrophages. Raising the reaction temperature from 0 to 37°C enhances passive sensitization of the macrophage by the antibody but has relatively greater effect on the elution of the antibody when the sensitizing serum is replaced by culture medium. The eluted antibody can still sensitize red cells to

adhere to fresh macrophages. At 0°C sensitization is less efficient while elution of the antibody from the sensitized cell is negligible.

When cytophilic antibody has reacted with the antigen, in this case sheep erythrocytes, the strength of the binding for macrophages is considerably increased. This is illustrated by the fact that red cells bound to macrophages by cytophilic antibody will not detach themselves spontaneously after prolonged incubation at room temperature and will not be displaced when normal or hyperimmune serum contain high concentrations of γ_2-globulin are added. Furthermore, the competition for macrophage receptors exerted by serum γ_2-globulin is less spectacular on antibodies bound to erythrocytes (indirect technique) than on free cytophilic antibody (direct technique). Several possible explanations can be proposed to account for the stabilizing effect of antigen on the binding of cytophilic antibody to macrophages: The reaction with antigen may cause allosteric changes in the antibody molecule favoring the binding of the cytophilic receptor, or the simultaneous reaction of several antibody molecules, bound stably to the red cell, with the macrophage stabilizes the binding and interferes with the spontaneous dissociation of individual cytophilic antibody molecules from the macrophages. Whatever its mechanism, this property of cytophilic antibody suggests that its primary biological usefulness resides in its capacity to opsonize soluble or particulate antigens to allow their uptake by macrophages. It has been clearly demonstrated that erythrocytes bound to macrophages by cytophilic antibody at 0 or 22°C will be rapidly phagocytized if the temperature is raised to a physiological level at 37°C. The test used to demonstrate cytophilic antibody can, therefore, be considered a process of arrested phagocytosis by a temperature too low for cellular activity, at the stage where the particle is adsorbed on the macrophage surface. The term cytophilic antibody merely reflects the fact that an opsonin must possess receptors for the phagocyte's cell membrane. Taking this into account it is not surprising that cytophilic antibody titers are found to be the highest by the indirect technique of sensitizing the antigen and allowing it to react with normal macrophages. It is probable that in most instances a similar sequence of events occurs in vivo and the antibody reacts first with the antigen before being adsorbed on the macrophages.

In these experiments complement was found not to be required for the binding of cytophilic antibody to the macrophage cell membranes, nor in this system for the phagocytosis of the bound erythrocytes. But the conditions of these experiments were not designed to demonstrate a possible enhancement of opsonic activity by complement, which has been observed by various investigators in other phagocytic systems involving macrophages and threshold amounts of antibodies (29, 30).

The observations of several groups of investigators which describe various reactions between actively sensitized macrophages and antigen suggest the possibility that the cytophilic γ_2-globulin studied in these experiments could be

responsible for such reactions. Data has been presented showing that macrophages from sensitized animals react characteristically to specific antigen. Mackaness has reported that macrophages from immunized mice proliferate in vivo when in contact with the sensitizing bacterial antigens and evolve into cells better able to suppress nonspecifically the growth of ingested microorganisms (31). Peritoneal macrophages from sensitized guinea pigs clump and adhere to peritoneal surfaces in vivo when antigen is injected (32). These same cells in vitro fail to migrate under the effect of antigen (33, 34). These responses of peritoneal macrophages were observed only in animals immunized with antigen and mycobacteria and have been considered to be cellular manifestations of delayed hypersensitivity reactions. It was, therefore, tempting, when an antibody with cytophilic properties for macrophages was described by Sorkin and Boyden and further studied by Boyden, to consider it responsible for the various reactions of actively sensitized macrophages to antigen. The data reported in this study raise some doubt concerning the responsibility for these reactions of the γ_2-globulin whose cytophilic property for macrophages was demonstrated to be an essential part of its opsonizing property. The attachment of this antibody, unreacted with antigen, for macrophages was shown to be weak and its rate of dissociation too high to effectively sensitize macrophages passively unless present in high concentration in the serum or body fluids. The antibody responsible for the behavior of sensitized macrophages described above and for delayed sensitivity reactions would be expected to bind much more effectively to macrophages even when present in very low concentration in the serum since it is well established that delayed hypersensitive animals capable of transferring delayed reactions with their peritoneal exudates, blood leukocytes, or lymph node cells showed negligible serum antibody titers (35). This discussion is not meant to argue that some cell-bound antibody is not responsible for the various reactions of sensitized macrophages which have been observed, but only to stress that the cytophilic antibody which was shown in this study to act as an opsonin is an unlikely candidate for this function. Another antibody type with considerably greater affinity for these cells may have to be considered to account for the reactions of actively sensitized macrophages to antigen.

It must also be considered whether or not macrophages can transport and effectively deliver cytophilic, γ_2, opsonic, complement-binding antibodies to sites of inflammation or tissue rejection. This possibility has to be considered if macrophages migrate from areas of high antibody concentration in serum or lymphoid organs to areas of low antibody concentration in various tissue spaces. The biological importance of such a mechanism has not been evaluated.

SUMMARY

Cytophilic activity for macrophages was shown to be a property possessed by most, if not all, of the complement binding 7S γ_2-population of guinea pig

antibodies. Cytophilic antibodies were also demonstrated in rabbit and mouse antisera to sheep red cells. While each species antibody best sensitized homologous macrophages, cross species sensitization was also observed. The binding site for macrophages resides on the Fc fragment, therefore, on the H chains, and is destroyed by pepsin hydrolysis or reduction and alkylation. The binding reaction is reversible with a high rate of dissociation at 37°C. Cytophilic activity is not complement dependent, and was shown to be that property of opsonizing antibody which provides the receptors that permit the binding of the antibody to the macrophage cell membrane in preparation for phagocytosis.

We are grateful to Dr. Pierre Vassalli for the photomicrography.

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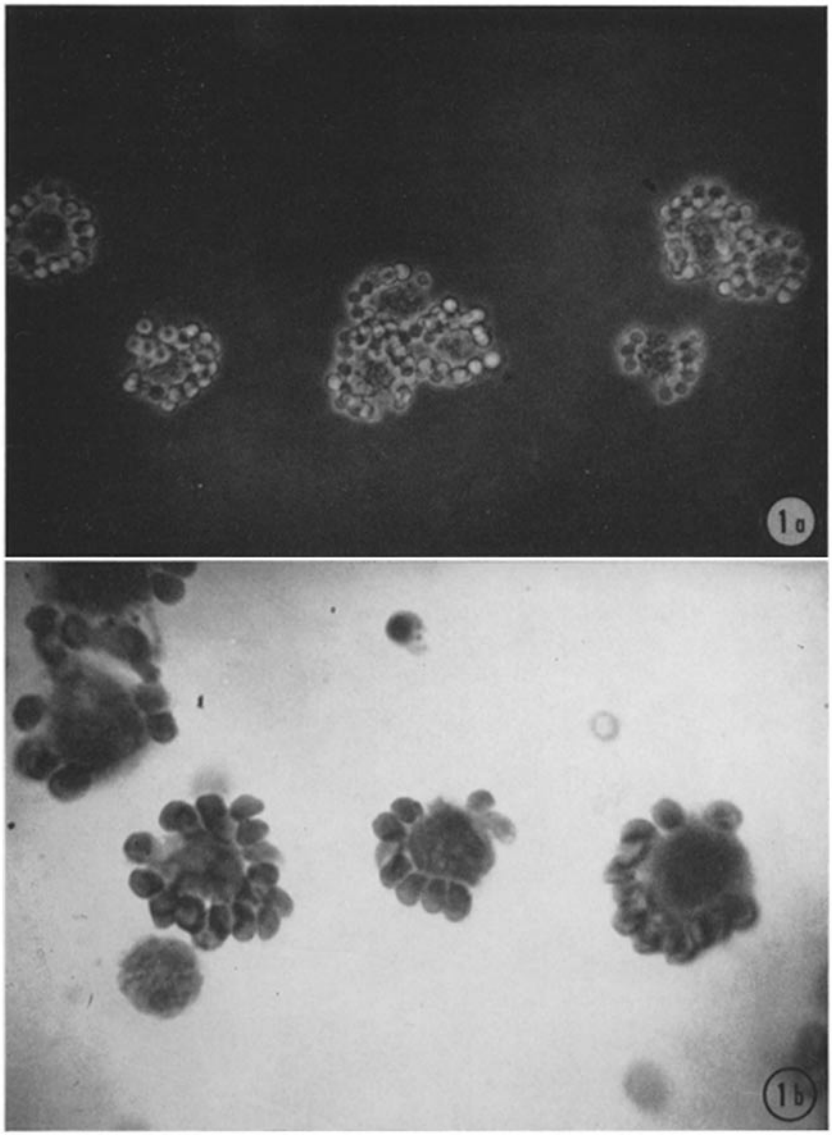
EXPLANATION OF PLATES

PLATE 21

FIG. 1 *a*. Rosettes of sheep red cells adsorbed onto the surface of guinea pig lung macrophages passively sensitized with anti-sheep red cell serum. Phase contrast, approximately × 1500.

FIG. 1 *b*. Preparation similar to that in Fig. 1 *a*. but fixed in 10% formalin and stained with hematoxylin and eosin. Light microscope, approximately × 1500.



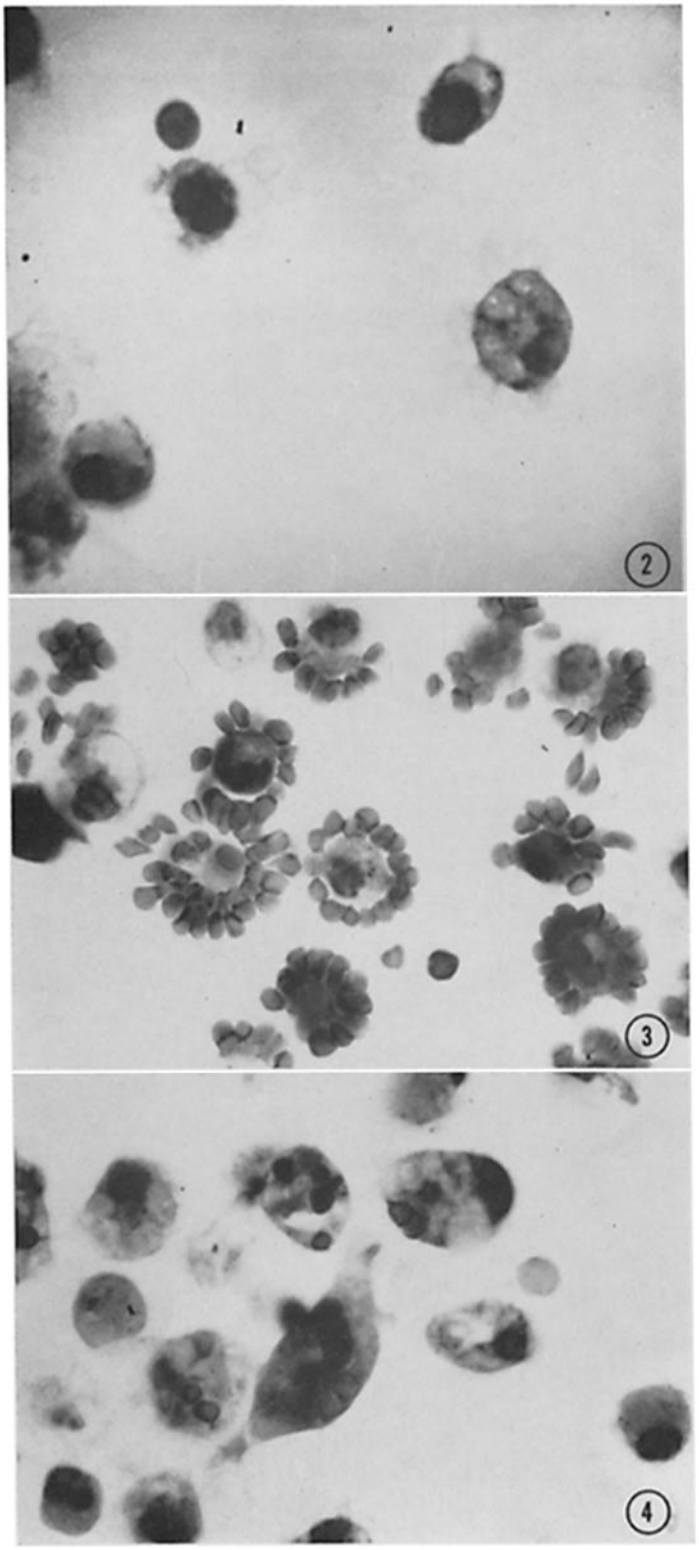
(Berken and Benacerraf: Antibodies cytophilic for macrophages)

PLATE 22

FIG. 2. Hemolysis of sheep red cells adsorbed to the surface of guinea pig lung macrophages, passively sensitized with anti-sheep red cell serum, caused by the addition of a $\frac{1}{50}$ dilution of guinea pig serum for 30 min at 37°C. Preparation was fixed with 10% formalin and stained with hematoxylin and eosin. Red cell ghosts, emptied of hemoglobin and, therefore, unstained by eosin are seen still adherent to the macrophages. A number of red cells have also been phagocytized. Light microscope, approximately × 1500.

FIG. 3. Rosettes of sheep red cells on sensitized macrophages in a preparation similar to that in Fig. 1 *b*, incubated at room temperature for 90 min in No. 199 medium. Note the absence of phagocytosis of the red cells or pseudopod formation by the macrophages. Light microscope, approximately × 1500.

FIG. 4. Preparation similar to that in Fig. 3 but which was incubated at 37°C instead of room temperature for 90 min in No. 199 medium. Note that following phagocytosis of the red cells many are seen packed within macrophages. Some red cells were being actively phagocytosed when the preparation was fixed. Light microscope, approximately × 1500.



(Berken and Benacerraf: Antibodies cytophilic for macrophages)