RECEPTORS FOR COMPLEMENT ON LEUKOCYTES*

By WALTRAUT H. LAY,‡ M.D., and VICTOR NUSSENZWEIG, M.D.

(From the Department of Pathology, New York University School of Medicine, New York 10016)

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Several types of cells participate in the immune response and in related inflammatory processes: lymphocytes, macrophages, monocytes, granulocytes, and mast cells. It is to be expected that some of them should possess recognition mechanisms or specific receptors for substances which are produced, released, or modified after antigenic stimulation or antigen-antibody interaction. The most important of these substances are the immunoglobulins and the complement factors. A few specific cell receptors of this kind have been previously described or their presence postulated: for example, the receptors for cytophilic antibodies on macrophages (1–3), and the receptors for antibodies capable of mediating the release of histamine on mast cells (review in reference 4). It is the purpose of this paper to describe the presence and some of the properties of receptor sites for complement factor(s) on macrophages, polymorphonuclear cells (PMN), and lymphocytes.

Materials and Methods

Reagents.—Rabbit gamma globulin (γG) and bovine serum albumin (BSA) were obtained from Mann Research Laboratories, N.Y. Trypsin (2X crystallized) was from Nutritional Biochemical Corporation, Cleveland, Ohio. Sodium salt of heparin "Liquaemin Sodium 50" was purchased from Organon, Inc., West Orange, N.J. Actinomycin D was a gift from Dr. Howard Green. Puromycin dihydrochloride was from Sigma Chemical Co., St. Louis, Mo. *N*-acetylglucosamine and *N*-acetylgalactosamine were obtained from Nutritional Biochemical Corporation. Dextran, Clinical grade H, average molecular weight 186,000 was from the Development Department of Pharmachem Corp., Bethlehem. Pa. MacNeal Tetrachrome Stain was from National Aniline Division, Allied Chemical, N.Y. Neutral red was obtained from National Aniline Division, Allied Chemical, N.Y., in a solution of 1 mg/ml in 0.85 M NaCl, and trypan blue stain, in Hanks' balanced salt solution, from the Grand Island Biological Co., Grand Island, N.Y. Na_3HEDTA stock solution (0.1 M): ethylenediaminotetracetic acid, disodium salt, was brought to pH 7.8 with NaOH. Na Azide was purchased from Amend Drug and Chemical Co., Inc., N.Y., and Merthiolate, from Eli Lilly and Co., Indianapolis, Ind. Sephadex G-200 was from Pharmacia, Uppsala, Sweden, and DEAE-cellulose (Selectacel) from Carl Shleicher and Schuell Co., Keene, N.H.

Diluents.—Hanks' balanced salt solution and medium 199 with sodium bicarbonate were obtained from Microbiological Associates, Inc., Bethesda, Md. Tris buffer: 0.12 M NaCl,

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‡ Present address: Department of Microbiology, Faculty of Medicine, Sao Paulo, Brazil.

0.025 M trishydroxymethylaminomethane, 0.005 M KCl, pH 7.35. Veronal buffer was as described in reference 5.

Animals.—Mice of the CF1 strain (Carworth Lab Cages, Rockland County, N.Y.) or of the AKR strain (Jackson Laboratories, Bar Harbor, Me.), weighing 20-30 g, were used as donors of peritoneal macrophages, blood leukocytes, and lymph node or thymus lymphocytes.

Sheep red blood cells (E), were obtained weekly from Certified Blood Donor Service, Inc., Woodbury, N.Y., and washed three times in saline solution (0.85% NaCl).

Amboceptor was obtained from Certified Blood Donor Service, Inc., and produced by immunization of rabbits with boiled E stroma (anti-Forssman antibodies).

Mouse Complement.—Blood was collected from anesthetized CF1 or AKR mice by cutting the axillary vessels. It was allowed to clot at room temperature for 5 min, and then centrifuged at 4°C to separate the serum. Sera from 5–10 mice were pooled and aliquots of 0.5 ml were stored at −20°C, and used as source of C′ for up to 1 month.

Sensitization of Sheep Red Blood Cells.—Equal volumes of a 5% suspension of washed E and an appropriate amboceptor dilution were incubated for 30 min at 37°C. The dose of amboceptor used contained twice the concentration necessary for maximum sensitization of E (5). Sensitized cells were washed once and resuspended to 5% in veronal buffer (EA). For reaction with C′, equal volumes of EA and $\frac{1}{10}$ or $\frac{1}{30}$ dilutions of mouse complement were again incubated at 37°C for 30 min. Under these conditions, the red cells lysed only slightly, and after three washings in veronal buffer, a final suspension (EAC′) was made in medium 199. Whenever necessary a suspension of EA was also made in medium 199.

Harvesting of Leukocytes.—

Macrophages: Mice were killed with ether inhalation. 4 ml of medium 199 containing heparin (10 units/ml) was injected into the peritoneal cavity. The liquid was collected after gentle massage of the abdomen. The cell population consisted of 60–70% of macrophages as indicated by their uptake of neutral red solution (6) and the rest were other types of mononuclear cells. Very few polymorphonuclear cells and mast cells were found in the peritoneal fluid.

Blood leukocytes: Blood was collected by cutting the axillary vessels of anesthetized mice and it was made incoagulable either with heparin or by shaking it with glass beads for 5 min. 1 volume of blood was then mixed with 2 volumes of 3% dextran and left at room temperature for 20–30 min. After the sedimentation of the red blood cells the leukocyte-rich plasma was withdrawn.

Lymphocytes: Cervical, axillary, and inguinal lymph nodes of mice were dissected, washed in Hanks' solution, teased with sharp scissors in medium 199, and filtered through stainless steel wire mesh (United Foundation wire mesh, No. 200). The same procedure was used for thymus lymphocytes. The cells were then washed twice with medium 199 by centrifugation at 1000 rpm for 10 min (200 *g*), counted in a hemacytometer and brought to a concentration of 2×10^3 cells/mm^3 in medium 199. Less than 1% of these cells took the neutral red dye (6) and could be considered macrophages. The rest appeared to be lymphocytes. Most of the cells were not damaged by the procedure and 90% or more excluded the trypan blue dye (7).

Assays for Adherence of EA and EAC′ to Leukocytes.—

Slide method: The method to be described was used for macrophages, blood monocytes, and polymorphonuclear cells (PMN), all of which adhere readily to glass. A slight modification of Berken and Benacerraf's technique (3) was used. Lucite rings with inner diameter of 12 mm and height of 3 mm were affixed to microscope slides with the aid of silicone stopcock grease. The wells so formed were filled with the leukocyte suspension: 0.5 ml of a macrophage suspension containing 100 cells/mm^3, or 0.5 ml of leukocyte-rich plasma. When macrophages were used, one drop of fresh mouse serum was added. The slides were incubated for 20 min (macrophages) or 1 hr (blood leukocytes) at 37°C to allow adherence of cells to the glass surface. After removal of the supernatant the slides were washed by immersion in warm (37°C) Hanks'

solution (three times for 2–3 min, volumes of 30 ml). The wells were then filled with 0.5 ml of the proper red blood cell suspension (E, EA, or EAC') at a concentration of 5×10^4 cells/mm^3, and incubated for 10–30 min at 37°C. The supernatant was removed, the ring carefully detached, and the slides washed in Hanks' solution until freed from all nonadherent red blood cells. Then the slide was dipped into a mixture containing 40% Hanks', 10% mouse serum, and 50% of water and quickly dried on a heated surface under an air stream. Cells were stained with MacNeal tetrachrome stain and examined under the light microscope. Estimation of the number of "rosettes," that is, of leukocytes surrounded by three or more red blood cells, was done by scoring more than 200 consecutive leukocytes. Duplicate slides of each preparation were made. Among blood leukocytes, only monocytes and PMN adhered to the glass slide. Even after rosette formation it was frequently possible to identify correctly the cell involved, but some leukocytes were so deformed or covered with erythrocytes that a precise identification was impossible. For this reason when blood leukocytes were involved, the percentage of rosette formation was estimated among all white cells sticking to the glass slide.

Tube method: This method was always used for lymphocytes, which do not adhere readily to glass. Disposable plastic tubes with caps (Falcon Plastics, Los Angeles, Calif.), originally 12×75 mm, were cut 12 mm above the bottom in order to hold 1 ml of liquid and allow for only a small air bubble when closed with caps. Such tubes were filled with the mixture of lymphocytes and proper red cell suspension (final concentration of 10^3 lymphocytes/mm^3 and 5×10^4 erythrocytes/mm^3) and were sealed with paraffin. These tubes were rotated in a water bath at 37°C for 30 min. A rotator with a horizontal axis was used, turning at approximately 20 rpm. Then a sample was taken and both the number of rosettes and of free lymphocytes were counted in a hemacytometer. Results were expressed as a percentage of lymphocytes forming rosettes.

Preparation of Conjugated Protein.—A dinitrophenylated (DNP) preparation of BSA was obtained as described in reference 8 by reacting 1-fluoro-2,4-dinitrobenzene with the protein in alkaline conditions, followed by extensive dialysis. It contained 40 groups of DNP per molecule of BSA.

Preparation of Purified Anti-DNP Antibodies.—These were obtained from the serum of guinea pigs immunized with DNP-BSA, as described in references 8 and 9.

Separation of 19S and 7S Antibodies from Rabbit Anti-Sheep Red Blood Cell Antiserum.—Rabbit 7S and 19S anti-sheep red blood cell antibodies were separated by filtration through a Sephadex G-200 column (2.5×33 cm) equilibrated with 0.1 M tris buffer, pH 8.0, containing 0.5 M NaCl. The rabbit antiserum was dialyzed against saline, and a 3 ml sample, to which 200 mg of sucrose had been added, was then passed through the column. The 19S and 7S fractions were collected and pooled separately.

The 19S fraction was purified by passage through the same column after concentration to a volume of 3 ml by ultrafiltration. This time only the 19S fraction was collected.

The 7S fraction from the first Sephadex column was concentrated, dialyzed against 0.01 M Na phosphate buffer, pH 7.6, and chromatographed on a DEAE-cellulose column. The protein contained in the break-through fraction was concentrated and consisted exclusively of 7S γG-globulin, as determined by immunoelectrophoresis using specific antisera against components of rabbit serum.

EA prepared with these purified antibody fractions will be designated as EA (7S) or EA (19S).

Electron Microscopy.—The same chamber-slides previously described were used for electron microscopy after washing off the nonadherent cells. The fixative employed was a mixture of 2% gluteraldehyde, 1% osmic acid, in medium 199 brought to pH 7.4 with $NaHCO_3$. After fixation and ethanol dehydration, embedding was accomplished by inverting gelatin capsules of epon directly on the slides still damp with ethanol. The epon was removed and sectioned.

The grids were stained with lead citrate or with both uranyl acetate and lead citrate. They were then examined using a Zeiss model 2a electron microscope.

Treatment of Leukocytes with Trypsin.—

Lymphocytes: Washed lymph node cells were suspended in a 0.1% solution of trypsin in medium 199, and incubated at 37°C for 15 min. The tube containing the cells was then brought to a temperature of 0°C by immersion in an ice bath, and washed by centrifuging twice at 1000 rpm (200 *g*) for 10 min. The cells were then counted in a hemacytometer and brought to a concentration of 2×10^3 cells/mm^3 in cold medium 199.

Macrophages, PMN, and monocytes: These cells were treated with trypsin after they adhered to a glass slide as described previously. The trypsin solution was added to the chamber and the slide was incubated for 15 min at 37°C. The cells were then washed with an excess of Hanks' solution.

RESULTS

Effect of C′ on the Adherence of EA(7S) and EA(19S) to Mouse Leukocytes.— Sheep red blood cells sensitized with rabbit 19S or 7S anti-Forssman antibodies differ in their ability to adhere to mouse leukocytes. EA(7S) readily form rosettes on mouse peritoneal cells, and to a lesser degree on blood monocytes and PMN, but they do not adhere to lymphocytes (Table I). 7S anti-Forssman antibodies can be shown to display intense rosette forming activity in vitro in a medium lacking serum proteins (medium 199). This activity roughly parallels the agglutinating capacity of the antibodies for the red cells, and the end points for agglutination and rosette formation usually coincide in a given 7S fraction. 19S anti-Forssman antibodies, however, fail to opsonize sheep red cells even when a concentration 30 times higher than the minimum agglutinating dose is used to sensitize the erythrocytes (10). Higher concentrations cannot be employed because the agglutination is too intense and the red cells cannot be resuspended. However, if EA(19S) cells are first incubated with fresh mouse serum for 30 min at 37°C, washed three times, and then incubated with the leukocytes, their properties change radically. The complex which is formed (EAC′) adheres readily to most peritoneal cells (macrophages) and PMN, and to a few monocytes. The evidence for the involvement of C′ components in this reaction will be discussed in another section of this paper. As shown in Table I, receptor sites for EAC′ are also found on a minority of the lymphocytes obtained from lymph nodes (10–25%). EAC′ prepared with 7S anti-Forssman antibodies showed very similar properties (Table I). In this case, as already mentioned, EA can form rosettes on most peritoneal macrophages, on some blood monocytes, but only on a few PMN. After addition of C′ factors there is a substantial increase in the percentage of rosettes formed on blood leukocytes. This increase is due almost entirely to the enhanced adherence of EAC′ (7S) to PMN. Also, EAC′ (7S) forms rosettes on 10–25% of lymph node lymphocytes but not on thymus lymphocytes.

The aspect of these rosettes is shown in Fig. 1. The majority of peritoneal cells found adherent to the microscope slide are large phagocytic cells, and all

were found to possess receptors for EAC'. Fewer rosettes were detected among other adherent mononuclear cells, morphologically similar to small or medium sized lymphocytes. As for lymphocytes, no morphological difference was found between those showing or not showing rosettes. Electron microscope studies were made of rosettes on monocytes and PMN (Fig. 2). These cells showed a remarkable increase in membrane activity. Finger-like cytoplasmic extensions were seen on the cells, some enveloping the erythrocytes. The red cells showed several points or zones of adhesion to the leukocytes, and very frequently they

TABLE I

Adherence to Mouse Leukocytes of Sheep Erythrocytes Sensitized by Rabbit 19S or 7S Anti-Forssman Antibodies and Mouse Complement

Target cells	Nature of antibodies	Cells showing rosette formation			
		Blood monocytes and PMN	Peritoneal cells	Lymphocytes from	
				Lymph node	Thymus
		%	%	%	%
E	—	0	0	0	0
E + Mouse* serum	—	0	0	0	0
EA	19S	0	0	0	0
EAC'	19S	87.3‡	74.3	18.9	0
EA	7S	9.0§	80.0	0	—
EAC'	7S	62.0‖	—	12.8	0

* SRBC incubated with a 1/10 dilution of normal mouse serum and washed twice in saline.

‡ Rosettes on most PMN and on few monocytes.

§ Rosettes mostly on monocytes and fewer on PMN. Phagocytosis observed frequently.

‖ The increase over EA (7S) mainly due to the formation of rosettes on PMN.

appeared deformed. Complete phagocytosis was infrequent, but many times small fragments were seen coming out of the red cells. Some of these fragments could be shown to be inside the cytoplasm of the leukocytes. However, in view of the complexity of the membrane processes, it is difficult to exclude the possibility that some of this apparent fragmentation is artefactual.

Evidence for the Participation of C' in the Adherence Phenomenon.—The results of several experiments indicating the participation of C' components in the binding of sensitized red cells to leukocytes are shown in Table II and can be summarized as follows: (*a*) The heating of mouse serum destroys its activity. Heat-inactivated serum does not induce EA(19S) to acquire the leukocyte adherence property. (*b*) The presence of Na_3H EDTA (0.01 M) during the incubation of EA with fresh mouse serum inhibits the formation of the active complex EAC'. (*c*) Fresh mouse serum absorbed with an unrelated antigen-

antibody complex is inactive. This experiment was performed as follows: 1.5 ml of serum from a guinea pig immunized with DNP-BSA was precipitated with 0.25 mg of DNP-BSA in the presence of 0.01 M EDTA. This serum contained 0.300 mg of precipitating antibody per milliliter. The precipitate was washed three times with saline and was used to absorb 1 ml of a 1/10 dilution of normal mouse serum. The absorption was carried out at 37°C for 15 min and the mixture was then centrifuged at 3000 rpm (1800 *g*) for 30 min. After centrifugation, the supernatant was used to sensitize the EA complex, and the results showed that the mouse serum thus absorbed had lost its rosette-inducing property. The following controls were used in this experiment: (*a*) Preincuba-

TABLE II

Role of Complement Factors in the Adherence to Mouse Leukocytes of Sheep Erythrocytes Sensitized by 19S Anti-Forssman Antibodies

Incubation of EA with mouse serum			Rosette formation on	
Nature of serum	Time	Temperature	Peritoneal cells	Lymph node lymphocytes
	min	°C	%	%
No serum	—	—	0	0
CF1, fresh	60	0	0	0
CF1, fresh	30	37	41.5	17.3
AKR, fresh	30	37	50.5	18.0
CF1, heat inactivated	30	37	0	0
CF1 + 0.01 M Na_3H EDTA	30	37	0	0
CF1 absorbed with AgAb complexes	30	37	0	0

tion of the same dilution of mouse serum with the antigen (DNP-BSA) at the concentration that was present in the experimental tube. (*b*) Incubation of this dilution of mouse serum with purified anti-DNP antibodies, also at a concentration identical to that present in the experimental tube. None of these procedures altered the subsequent sensitization of the EA cells. Therefore, the active components were still present in the mouse serum after treatment with antigen or antibody alone, while they were removed by antigen-antibody complexes.

The role of C′ components in the adherence phenomenon is also strongly suggested by the demonstration that human red cells coated by nonimmunological means with complement components (11) form rosettes on human or mouse PMN (10).

The precise C′ components which participate in the adherence phenomenon have not been identified. The active components are formed or bound to the red cell membrane only when the incubation of EA with fresh serum is carried

out at 37°C. When the temperature is 4°C, EA does not acquire the leukocyte-binding properties even after 1 hr of incubation. Serum from AKR mice, which are deficient in C'5, is a good source of C' for adherence. This indicates that only the first four components are necessary to induce the formation of active EAC'. Also, serum from rabbits deficient in C'6[1] can be used as a source of complement for the adherence reaction on rabbit PMN and monocytes.

The percentage of rosette formation on macrophages is directly dependent on the concentration of fresh serum used to sensitize EA (Table III). It is also

TABLE III

Percentage of Rosette Formation after Incubation of Mouse Peritoneal Cells with EA Sensitized with Various Dilutions of Mouse Complement*

Dilution of C' used to sensitize EA	Rosettes
	%
1/10	43.5
1/20	41.6
1/40	28.8
1/80	18.2
1/160	1.9
1/320	0.3

* EA was prepared, as indicated in the Methods section, with a dilution of rabbit anti-Forssman antiserum which contained a high proportion of 19S antibodies. EA cells, in the conditions of the test, did not adhere to leukocytes.

clear that mouse serum, which lyses EA poorly, can nevertheless induce effective leukocyte adherence, even at relatively high dilutions.

The EAC' complex is quite stable, and it can be maintained for a minimum of 1 hr at 37°C or 24 hr at 4°C without change in its leukocyte-adherence properties. Also, treatment of EAC' with Na_3H EDTA for 1 hr at 37°C does not remove or alter the C' components responsible for the reaction with macrophages, blood leukocytes, or lymphocytes.

Conditions for the Reaction of EAC' with Leukocytes.—

Influence of temperature (Table IV): The speed of interaction of the leukocytes with EAC' is very much decreased at 4°C. After 30 min of incubation of EAC' with peritoneal cells or lymphocytes at 4°C, only 2% of leukocytes showed rosette formation. Even after 60 min of incubation the number of rosettes was still very low. If the temperature of incubation was raised to 36°C, the proportion of rosettes rose sharply during the first 15 min and a plateau was reached after 30 min.

[1] Kindly given by Dr. K. Rother.

Influence of the presence of γG-globulin: As indicated before by the results of other investigators (3, 12), the binding of EA(7S) to macrophages should be inhibited by normal γG-globulin. The basis of this inhibition would be a competition between the specific and nonspecific 7S molecules for the receptor sites on the membrane of the macrophages. As seen in Table V, the inhibition took place, and its degree depends on the intensity of sensitization of the red cell by the 7S antibody and on the concentration of γG-globulin in the incubation medium. Bovine serum albumin (10 mg/ml) does not inhibit the binding of EA(7S) to macrophages.

TABLE IV

Influence of the Temperature and Time of Incubation on the Adherence of EA and EAC'* to Mouse Leukocytes*

Condition of incubation		Rosette formation on			
		Peritoneal cells incubated with		Lymph node lymphocytes incubated with	
Temp.	Time	EA	EAC'	EA	EAC'
°C	*min*	%	%	%	%
5	30	0	2.7	0	2.4
25	30	0.7	21.4	—	—
36	30	0	39.3	0	18.4
5	60	0	4.2	—	—
25	60	0.8	24.1	—	—

* EA and EAC' were prepared, as indicated in the Methods section, with a dilution of rabbit anti-Forssman antiserum which contained a high proportion of 19S antibodies.

Contrasting with these results, the adherence of EAC' to leukocytes is not inhibited by the presence of γG-globulin in the incubation medium. Also, the presence of up to 50% of serum in the medium does not decrease the proportion of PMN or macrophages which bind EAC'. This result suggests that the C' component taking part in the adherence is not present as such in the serum, and could be a C' component modified by interaction with the antigen-antibody complex.

Influence of divalent cations on rosette formation by EAC' on PMN and macrophages: Adherence of EAC' to PMN and macrophages depends on the presence of divalent cations in the incubation medium. Na_3H EDTA completely inhibits rosette formation of EAC' on PMN and macrophages. The effect of Na_3H EDTA is equally apparent using EAC' prepared either with 7S or 19S antibodies, but it does not influence the adherence of EA(7S) to macrophages. This inhibitory effect is not due to an irreversible damaging effect of Na_3H EDTA on the leukocytes, because if the incubation medium containing Na_3H

EDTA is removed and replaced with medium 199, rosette formation can again be demonstrated. Also, Na_3H EDTA does not alter the EAC′ complex, as mentioned before and as shown in Table VI. EAC′ cells pretreated with Na_3H EDTA or with medium 199 for 30 min at 37°C, and then washed, show similar leukocyte-adherence properties.

The important role of divalent cations in the binding is further demonstrated by the ability to elute EAC′ from the macrophages by treatment with Na_3H EDTA, as shown in the following experiment. Mouse peritoneal macrophages were allowed to adhere to the glass at the bottom of four chambers, as described in the Methods section. After careful washing of the macrophages, a suspension of EAC′ (10^5 in medium 199) was added to each chamber and allowed to settle for 3 min at 37°C. Then the rings were removed and the slides

TABLE V

*Inhibition of Rosette Formation by Rabbit γG-Globulin**

Dilution of 7S antibody used to sensitize erythrocytes	% of rosettes when the concentration of γG in the medium is (mg/ml):		
	0	1	10
1/30	80.0	64.5	3.3
1/50	71.3	21.5	5.6

* Rosette formation on mouse peritoneal macrophages by sheep erythrocytes sensitized by rabbit 7S anti-Forssman antibodies.

washed. Two of them were washed for 60 sec in medium 199 at 0°C. Two others were washed simultaneously in medium 199 containing 0.01 M Na_3H EDTA. After drying, the slides were stained and the proportion of rosettes on large macrophages counted under the microscope. Whereas 22.6% of these large macrophages displayed rosettes when they had been washed in medium 199, only 1% showed rosettes when the washing had been done in the presence of Na_3H EDTA. It is thus possible to reverse the reaction of macrophages with EAC′ through the binding to divalent cations. However, the elution of EAC′ from the macrophages can be accomplished only when the time of contact between the macrophages and the red cells has been quite short. No reversion of adherence was obtained when EAC′ had been in contact with the macrophages for more than 5 min. It is likely that the adherence of the red cells to the receptors on the membrane of the macrophages triggers other processes which lead to the engulfment and apprehension of the target cell, as shown in Fig. 2.

The essential ion necessary for the binding of EAC′ to PMN and macrophages is Mg^{++}, as demonstrated in the experiment shown in Table VII. In this experiment the incubation medium used was a simple salt solution (NaCl,

TABLE VI

*Influence of Na_3H EDTA on the Adherence of EA and EAC′ to Mouse Leukocytes**

Pretreatment of target cells	Nature of antibodies	Incubation medium	Rosettes on		
			Blood monocytes and PMN	Peritoneal cells	Lymph node lymphocytes
			%	%	%
EA + 199	19S	199	0	0	1.3
EA + 199	19S	199 + EDTA	0	0	0
EAC′ + 199	19S	199	28.3	37.3	14.6
EAC′ + 199	19S	199 + EDTA	0	0	16.0
EAC′ + 199	19S	199 + EDTA, followed by 199‡	10.7	42.6	—
EAC′ + EDTA	19S	199	28.5	42.4	—
EA + 199	7S	199 + EDTA	18.5	—	0
EAC′ + 199	7S	199	62.0	—	12.8
EAC′ + 199	7S	199 + EDTA	22.5	—	—

* The erythrocytes were first incubated for 30 min at 37°C as indicated in the first column, then washed twice in medium 199. The time of incubation between the red cells and the leukocytes was 10 min, except in the case of the lymphocytes, when the incubation period was 30 min.

‡ The leukocytes were first exposed for 10 min to EAC′ in medium 199 containing 0.01 M Na_3H EDTA. These erythrocytes and the medium containing EDTA were then removed and the leukocytes reincubated for 10 min with a suspension of EAC′ in medium 199.

TABLE VII

Influence of the Presence of Ca^{++} and Mg^{++} in the Incubation Medium on the Adherence of EA and EAC′* to Mouse Peritoneal Cells‡*

Target cells	Molarity of divalent cations in the incubation medium		Percentage of rosettes
	Mg^{++}	Ca^{++}	
EA	10^{-2}	—	0
	—	10^{-2}	0
EAC′	10^{-2}	—	75.8
	10^{-3}	—	77.3
	10^{-4}	—	12.9
	—	10^{-2}	8.5
	—	10^{-3}	7.6
	—	10^{-4}	2.1
	—	—	7.1

* EA and EAC′ were prepared with a dilution of a rabbit anti-Forssman antiserum as indicated in the Methods section. This antiserum contained a high proportion of 19S antibodies anti-SRBC.

‡ In these experiments, tris buffer was used as a diluent, to which were added increasing amounts of Ca^{++} and Mg^{++}.

KCl, and tris buffer) to which Mg^{++} and Ca^{++} ions ($MgSO_4$ and $CaCl_2$) were added in increasing amounts. It can be seen that Mg^{++} but not Ca^{++} is necessary for the adherence. A concentration between 10^{-3} and 10^{-4} M of Mg^{++} cations seems to be needed for the maximum intensity of rosette formation under the conditions of the test.

Influence of divalent cations on rosette formation by EAC′ on lymphocytes: Contrasting with the previous results, rosette formation on lymphocytes is not inhibited in the absence of Mg^{++} or Ca^{++} and takes place in the presence of 0.01 M Na_3H EDTA (see Tables VI and VII). These results indicate that at least two different kinds of receptors are involved in the adherence of EAC′ to leukocytes.

Other potential inhibitors: No effect on rosette formation was observed when heparin (500 units/ml), *N*-acetylglucosamine or *N*-acetylgalactosamine (0.03 M) were incorporated in the incubation medium. Also, the presence of Na Azide (0.01 M) or Na merthiolate (0.1%) in the incubation mixture for the reaction between lymphocytes and EAC′ did not influence rosette formation. Lymphocytes could also be pretreated with 0.1% Na merthiolate in medium 199 for 2 hr at 37°C and they still were able to bind EAC′, as before treatment. After the treatment with merthiolate, virtually all lymphocytes were damaged, as judged by their uptake of the trypan blue dye. The presence of actinomycin D (2.5 μg/ml) or puromycin (10 μg/ml) in the incubation medium did not influence rosette formation of EAC′ on lymphocytes or macrophages. However, when macrophages were in addition preincubated for 20 min with puromycin or actinomycin D in medium 199 before the addition of EAC′, a small but definite inhibition of rosette formation was noticed. While the control macrophages preincubated with medium 199 showed 39.5% of rosettes, the percentage of rosettes on the macrophages preincubated with puromycin and actinomycin D was 26.0% and 30.7% respectively.

Trypsin digestion of the receptor sites for C′ present on mouse leukocytes: The receptor sites for EAC′ on macrophages, PMN, monocytes, and lymphocytes are destroyed by treatment of these cells with 0.1% trypsin for 15 min at 37°C (Table VIII). In contrast, the receptor sites on macrophages for EA(7S) are not destroyed by the same treatment similarly to what has been reported by Rabinovitch (13) and for cytophilic antibodies (14, 15).

DISCUSSION

The results obtained demonstrate the presence of distinct receptor sites for rabbit γG and mouse C′ component(s) on mouse leukocytes. Some of the properties of these different types of receptors are summarized in Table IX.

Receptors for Rabbit γG.—The receptor sites for rabbit γG which are found on mouse macrophages, monocytes, and PMN share many properties with the receptor sites for cytophilic antibodies (3, 14, 16, 17). Both react readily with 7S, neither is digested by trypsin, and the binding is independent of divalent

cations. Moreover, the receptor sites for rabbit γG and for cytophilic antibodies are found predominantly on macrophages, and, in agreement with the findings of Uhr (15), in a few PMN. Thus, it is probable that these receptors are the same and that in the rabbit, as in the guinea pig (3), cytophilia is an exclusive property of some kinds of 7S antibodies. However, the alternative possibility that γM or other classes of antibodies may also display opsonizing properties

TABLE VIII

Effect of Trypsin Treatment on the Receptor Site of Leukocytes for EA (7S) and EAC'

Target cells	Trypsin treatment of leukocytes	Rosette formation on		
		Peritoneal cells	Blood monocytes and PMN	Lymphocytes
		%	%	%
EA (7S)	No	45.9	14.0	—
EA (7S)	Yes	45.6	6.5	—
EA (19S)	No	0	0	0
EAC' (19S)	No	42.7	27.0	17.3
EAC' (19S)	Yes	0	0	0

TABLE IX

Receptors for IγG and Complement Factor(s) in Mouse Leukocytes

Receptor for	Cells in which receptor has been detected					Digestion by trypsin	Inhibition of binding by EDTA
	Lymphocytes		Macrophages	Monocytes	PMN		
	Lymph node	Thymus					
IγG	No	No	Yes	Yes	Yes	No	No
Complement (A)	No	No	Yes	Yes	Yes	Yes	Yes
Complement (B)	Yes	No	No	No	No	Yes	No

cannot be excluded. In our studies, for example, the lack of adhesion of EA (19S) to macrophages may be a consequence of the experimental model chosen, in which antibodies and cells are not obtained from the same animal species. This possibility is currently under investigation.

Receptors for C'.—Two different receptors for C' component(s) were detected on mouse leukocytes.

1. One of these receptors was found on 10–25% of lymphocytes from lymph nodes, but not on lymphocytes from the thymus, macrophages, monocytes, or PMN. The same receptor was also present on a similar proportion of lym-

phocytes obtained from the thoracic duct of the rat.[2] This receptor is destroyed when the lymphocytes are incubated with a 0.1% trypsin solution for 15 min.

The binding of the EAC′ complex to the receptor does not depend on the presence of divalent cations in the incubation medium, and takes place in the presence of Na_3H EDTA. Receptors of a possibly similar nature were previously detected by Uhr (15, 16) on the surface of guinea pig lymphocytes, but the participation of C′ in the binding was not unequivocably demonstrated.

It would be of great interest to determine the effect of the adherence of these complexes on lymphocytes. Bloch-Shtacher et al.[3] have recently shown that antigen-antibody complexes in the presence of fresh serum, but not of aged serum, can effectively stimulate the proliferation of lymphocytes from nonimmunized humans. The fate of these cells is, however, still unknown.

2. Another type of receptor for C′ was found in macrophages, monocytes, and PMN, but not on lymphocytes or mast cells. The binding to this kind of receptor is dependent on Mg^{++} ions. This was demonstrated by (*a*) using an incubation medium without divalent cations to which these ions were added; (*b*) by adding Na_3H EDTA to the incubation medium; and (*c*) by reversing the binding of EAC′ to macrophages with Na_3H EDTA. The mechanism through which Mg^{++} ions determines the binding of antigen-antibody-C′ complexes is unknown. Our data are compatible with the idea that these ions either participate directly in the binding to the leukocyte membrane or influence it indirectly through, for example, an allosteric mechanism.

Our results confirm and extend some observations made by Nelson (18) during his investigations on the immunoadherence phenomenon. Immunoadherence (IA) has been defined as the binding of antigen-antibody-C′ complexes to primate red blood cells and to platelets of some non-primates. Nelson also noticed that these complexes, prepared with human or guinea pig C′, can adhere to macrophages and PMN from guinea pigs, and suggested that the occurrence of the IA receptor on the red cells and platelets may indeed not have any biological significance, except as a tool for the experimental immunologist. Actually the IA receptors for red cells and platelets have never unequivocally been shown to be the same, and one observation would rather point to the contrary: IA to human red cells is not inhibited by EDTA (18) while IA to rabbit platelets does not take place in the absence of divalent cations (19). If similarities between the receptors for IA and for C′ on leukocytes do exist, it is probable that, in view of the different sensitivities to EDTA which have been pointed out, the IA receptor on red cells would correspond to the C′ receptor on

[2] Dr. B. Gesner gave us the thoracic duct lymphocytes.

[3] Bloch-Shtacher, M., K. Hirschorn, and J. W. Uhr. The response of lymphocytes from nonimmunized humans to antigen-antibody complexes. Manuscript submitted for publication.

lymphocytes, while the receptors for C′ on rabbit platelets and mouse macrophages or PMN would be analogous.

As shown in the previous section, the fixation of C′ components is extremely important for the binding of antigen-antibody complexes to leukocytes. The possibility could be raised that the actual binding to the cells is done through the antibody which would be modified in some way after C′ fixation to the complex. The data showing that EAC′ (7S) and EAC′ (19S) are taken up equally by the leukocytes render this hypothesis unlikely because it would be necessary to admit that C′ fixation modifies both 19S and 7S antibodies in a similar or identical way, so that they can combine with the receptor sites.

As for the physiopathological role of these C′ receptors on PMN and macrophages, they are probably of primary importance for the clearance and phagocytosis of opsonized particles. It has been shown by others (3, 12, 20) and ourselves (10) that 7S antibodies alone, without the participation of C′ factors, can be opsonizing. It is also remarkable the similarity between the electron micrographs of rosettes obtained by Lo-Buglio et al. (12) in a system involving red cells sensitized by 7S antibodies and those shown in Fig. 2, in which the binding is mediated by C′ component(s). The basic mechanism for clearance in vivo probably does not depend on the direct interaction between 7S receptors on cells and 7S antibodies for the following reasons: (*a*) The cytophilic property is a property mainly or exclusively of γG-antibodies and there is evidence that particles sensitized by 19S antibodies are sometimes cleared more rapidly from the circulation than those sensitized by 7S antibodies (21). (*b*) The uptake of particles sensitized with 7S antibodies is only marked among macrophages. Some uptake by PMN also takes place but it is much less prominent. PMN cells, however, actively participate in the phagocytic process in vivo. (*c*) Finally, the presence of serum or "nonspecific" 7S γG markedly inhibits the adherence of the 7S antigen-antibody complex to the macrophages. For these reasons it seems more likely that only in special circumstances could the opsonization by 7S antibodies play a decisive role in the adherence to leukocytes in vivo. LoBuglio et al. (12), for example, propose that in the splenic red pulp, where the red cell concentration is very high, there could be a direct entrapment of antibody-sensitized erythrocytes by macrophages without C′ participation. On the other hand, several experiments have already indicated the importance of C′ factors for the clearance of sensitized particles (21, 23), but it has never been established clearly through which mechanism it operates. Our results demonstrate that C′ factors can mediate the *binding* of the antigen-antibody complexes to the leukocytes. Consequently, phagocytosis should be viewed as a multistep process: the first is the adherence of antigen-antibody-C′ complex to the membrane of the phagocytic cell. According to our results, only the first four components of C′ are necessary for this step. The precise requirements for the engulfment stage are not known. It is clear from our observations that the

adherence of a sensitized particle to the membrane of a leukocyte is not necessarily followed by actual phagocytosis. It is possible that 7S antibodies, as demonstrated by Rabinovitch (20), can specifically stimulate the ingestion phase. Intense phagocytosis of red cells sensitized with guinea pig cytophilic (γ_2) antibodies was also noticed by Berken and Benacerraf (3).

The presence of C′-receptor sites on PMN can also account for the trapping of these cells by antigen-antibody-C′ complexes, particularly in those cases where the antigen is bound to tissue structures, as in some types of glomerulonephritis. It is known that the release of lysosomal enzymes from PMN plays an important role in the pathogenesis of lesions found in some inflammatory processes (review in reference 24). The first step for the release of these enzymes could be the attachment of PMN to the antigen-antibody-C′ complexes.

Finally, the uptake of antigen-antibody-C′ complexes by macrophages is obviously one way through which antigens could reach these cells during the immune response. Even during the primary response, the presence in the circulation of small amounts of "natural" antibodies, particularly of the 19S type, could lead to the formation of antigen-antibody-C′ complexes and adherence to macrophages. In this respect, the findings of Nossal et al. (25) of antigen bound to the membrane of reticular cells of lymph node follicles may be an in vivo expression of the phenomenon which we describe in vitro.

The understanding of the function of the binding of antigen-antibody-C′ complexes to part of the population of lymphocytes is much less clear. It is remarkable that the receptor site involved differs from that found on macrophages and PMN, and also that it is absent from the thymus lymphocytes. Before any useful speculation on the role of this receptor is possible, some work on the nature and origin of the cells involved is necessary, as well as further information on the effect of the contact between the antigen-antibody-C′ complexes on these lymphocytes.

SUMMARY

Sheep red blood cells sensitized by 7S, but not by 19S rabbit anti-Forssman antibodies, adhere and form rosettes on mouse macrophages and on a few monocytes and polymorphonuclear cells (PMN). When, however, C′ factors from mouse serum are added to the antigen-19S antibody complex (EAC′), rosettes are formed on most mouse peritoneal macrophages and PMN and on a few monocytes. In addition EAC′ also adheres to 10–25% of lymph node lymphocytes but not to thymus lymphocytes. EAC′ prepared with 7S anti-Forssman antibodies has identical properties. The adherence of red cells induces an increase in the membrane activity of the leukocytes and causes injury to the red cells which rapidly become deformed and fragmented.

Adherence of EAC′ occurs at 37°C and is minimal at 4°C. Probably only the

first four C′ components are involved in this phenomenon as mouse serum deficient in C′5 or rabbit serum deficient in C′6 can be used as a source of C′ components. Treatment of EAC′ with EDTA does not modify its leukocyte-adherence properties. The adherence of EAC′ to the leukocytes is not inhibited in the presence of serum.

The receptors for C′ on macrophages, PMN, and monocytes differ from those found on lymphocytes. Rosette formation by EAC′ on macrophages, PMN, and monocytes depends on divalent cations (Mg^{++}) and can be reversed by Na_3H EDTA, while adherence to lymphocytes is independent of these ions and occurs in the presence of 0.01 M Na_3H EDTA. Both types of receptors for C′ components are destroyed by trypsin treatment of the leukocytes, in contrast with the receptors for 7S antibodies on the same cells which persist after enzyme treatment.

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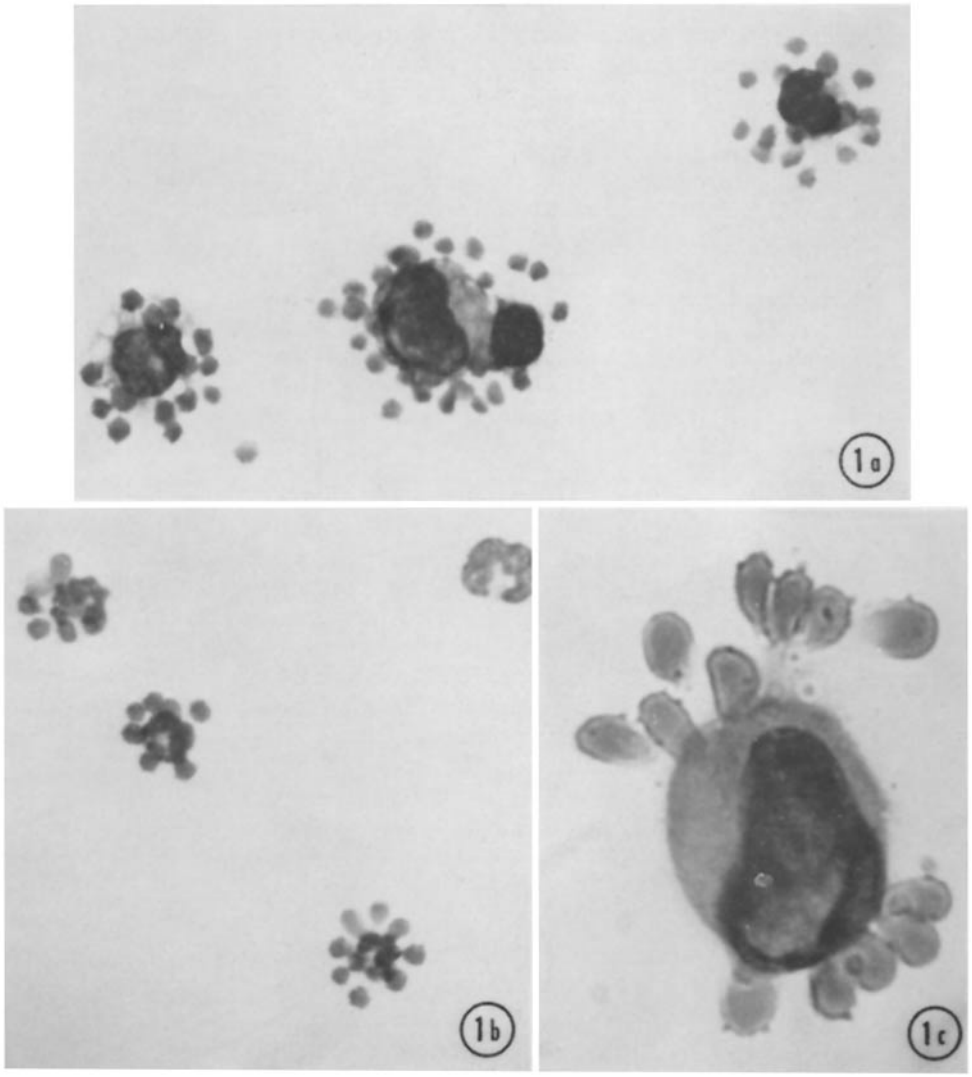

FIG. 1. Rosettes on (*a*) macrophages (× 1000), (*b*) PMN cells (× 1000), a lymphoid cell (× 2500).

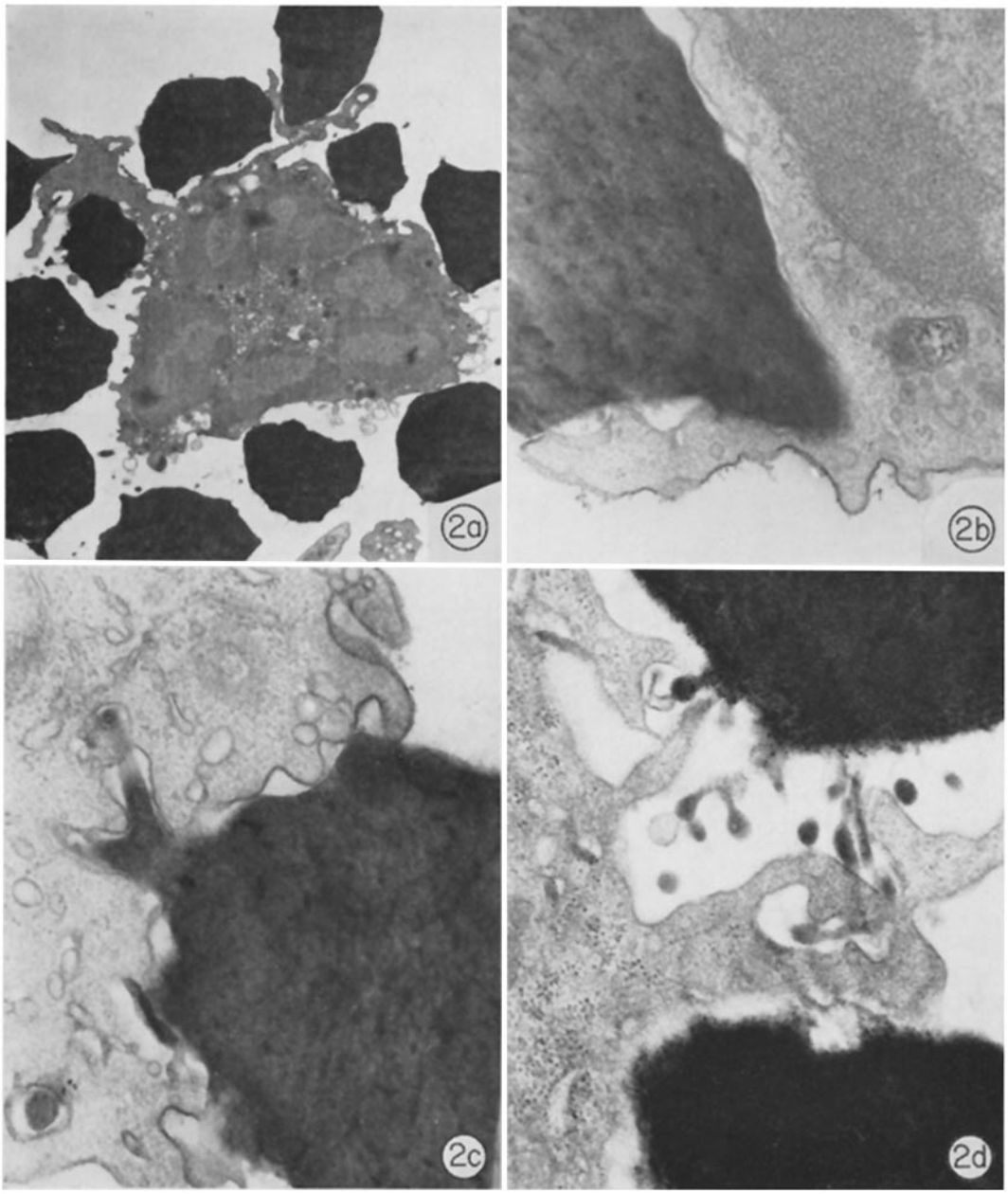

FIG. 2. (*a*) Rosette on a PMN cell (× 10,800); (*b*), (*c*), and (*d*) higher magnification (× 25,000) showing the sites of attachment of the red cell to the membrane of the leukocyte. Also conspicuous are the finger-like processes surrounding the red cell, which appears deformed and fragmented.