Proceedings of the Society for Experimental Biology and Medicine

VOL. 127 FEBRUARY 1968 No. 2

SECTION MEETINGS

Effect of Antiserum on the Attachment of Modified Erythrocytes to Normal or to Trypsinized Macrophages* (32688)

M. RABINOVITCH (Introduced by J. G. Hirsch)

The Rockefeller University, New York, New York 10021

Little information is available on the cell-surface receptors with which particles interact during the initial stages of phagocytosis. Some particles require no serum factors for uptake by phagocytes, whereas others must be coated with heat-labile complement-like materials or with specific antibody (1,2). It is possible that different receptors exist at the cell surface for these various types of particles. Erythrocytes treated with glutaraldehyde (GRC) attach themselves to the surface of macrophages in serum-free medium and this interaction is reduced by treatment of the phagocytes with trypsin (3). This paper presents the results of work examining the effects of antierythrocyte serum and of purified IgG on the attachment of GRC to untreated or to trypsinized macrophages.

Materials and Methods. Media. Buffered saline (PBS) was prepared by adding one volume of .07 *M* phosphate buffer pH 7.3 to 10 volumes of 0.85% sodium chloride. Tissue culture Medium 199 was purchased from Microbiological Associates, Bethesda, Maryland.

Animals. Mice of the NCS strain, The Rockefeller University colony, of both sexes, weighing 20–25 gm were used as a source of macrophages. New Zealand rabbits, 3–4 kg body weight, of both sexes, provided normal or immune serum.

Red cells. Horse and sheep erythrocytes were obtained from Animal Blood Centre, Syracuse, New York, and washed three times with PBS before use.

Preparation of antiserum. Rabbits were injected intravenously with 1 ml of 10% fresh horse cells in PBS on days 1, 3, 5, and again on days 31, 33, and 35, and bled 7 days after the last injection. The titer of the pooled serum was 1:32,000 as determined by macroscopic agglutination of fresh horse red cells. Fresh red cells were used as the antigen since glutaraldehyde fixed cells (GRC) were found to be poor immunogens. Normal rabbit se-

*This work was supported by grant AI 07012 from the United States Public Health Service, National Institutes of Health, Bethesda, Maryland.

rum diluted 1:6 did not agglutinate the horse cells. Sera were inactivated at 56° C for 30 min and stored at —15°C. In some experiments antiserum was absorbed with one-fourth its volume of packed horse or sheep fresh erythrocytes. Whereas absorption with the sheep cells did not appreciably influence the titer, absorption with horse cells reduced the titer markedly to 1:500.

Purification of IgG. The globulin fraction obtained by precipitation of immune serum with sodium sulfate was dialysed against 0.01 *M* phosphate buffer pH 7.5 and applied to a 3.0 × 9.0 cm column of diethylaminoethyl cellulose previously equilibrated with the same buffer (4). IgG was eluted with the 0.01 *M* buffer and the first eight fractions containing protein and agglutinating activity were pooled (40 ml), concentrated and stored at —15° C. The preparation, henceforth called "immune IgG," had a titer of 1:6, 300 per milligram protein per milliter. "Nonimmune IgG" was similarly prepared.

Treatment of horse red cells with glutaraldehyde was as previously described (3, 5).

Treatment of GRC with serum or with IgG. GRC (30,000/μl) were treated with serum or with IgG diluted in PBS for 16 hours at 5° C. The cells were washed three times with PBS and adjusted to 10,000 per μl.

Collection of peritoneal macrophages was as described elsewhere (3, 5).

Treatment of macrophages with trypsin. Macrophages attached to coverglasses were treated with 500 μg/ml crystalline trypsin (Sigma Chemical Co., St. Louis, Missouri) in Medium 199 for 45 min at 34° C in 95% air–5% CO_2 atmosphere. Controls were incubated in Medium 199 alone. Macrophages were rinsed with PBS prior to the attachment of GRC. Macrophages treated with trypsin showed more spreading than the control macrophages.

Attachment assay (5). GRC suspensions were applied for 10 min at 31°C. Non-attached GRC were removed by rinsing in PBS and the macrophages were fixed with glutaraldehyde and stained with Giemsa. The number of GRC attached to 200 macrophages was determined with the light microscope and the GRC/macrophage ratios were calculated. Three of five replicate coverglasses were used for each treatment. The spread in the counts was such that no significance was attached to variations up to 10% around the control means within the same experiments.

Results. Attachment to untreated macrophages of GRC treated with antiserum or with purified IgG. Figure 1 shows the results of

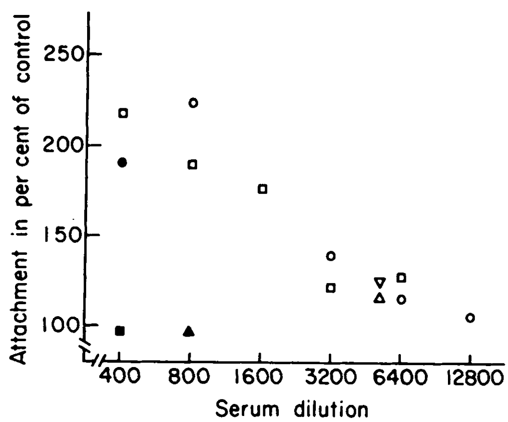

FIG. 1. Attachment of horse GRC treated with anti-horse red cell serum, to mouse peritoneal macrophages. Ordinates: attachment of treated GRC as the percentage of the attachment to untreated GRC. Abscissae: reciprocal of serum dilutions. Different open symbols are results of separate experiments. Closed symbols are: circle = antiserum absorbed with sheep red cells; square = antiserum absorbed with horse red cells; triangle = normal rabbit serum.

several experiments in which GRC were treated with different dilutions of antiserum, washed, and attached to macrophages. The phagocytes were incubated in Medium 199 for 45 min at 34°C before challenge with GRC. Preliminary experiments showed that similar results were obtained when the preincubation was omitted. Results are expressed in the percentage of the attachment of untreated GRC. Treatment of GRC with antiserum increased their attachment to macrophages and the effect was still detectable when the serum was diluted to 1:3,200. Attachment was not increased when GRC were treated with normal rabbit serum or with immune serum absorbed with horse red cells. Absorption of the immune serum with sheep red cells did not affect its ability to increase GRC attachment. Treatment of GRC with immune IgG increased attachment in a fashion similar to that seen with whole immune serum; in contrast, treatment of GRC with nonimmune IgG did not promote their attachment to macrophages. The results, shown in Table I, are given in GRC/macrophage ratios.

TABLE I. Attachment to Macrophages of Horse GRC Treated with IgG from Normal Serum or from Anti-Horse Red Cell Rabbit Serum.

Treatment of GRC	Attachment[a]
None	1.03 ± .065 (8)
IgG, nonimmune, 8 μg/ml	1.14 ± .078 (5)
IgG, immune, 2 μg/ml	1.47 ± .106 (5)
IgG, immune, 4 μg/ml	1.59 ± .023 (5)
IgG, immune, 8 μg/ml	1.73 ± .077 (5)

[a] GRC/macrophage ± SE (n° samples).

Attachment to trypsinized macrophages of GRC treated with antiserum or with purified IgG. Attachment to macrophages of untreated GRC or of GRC treated with normal rabbit serum was markedly reduced by preliminary trypsinization of the macrophages (Fig. 2).

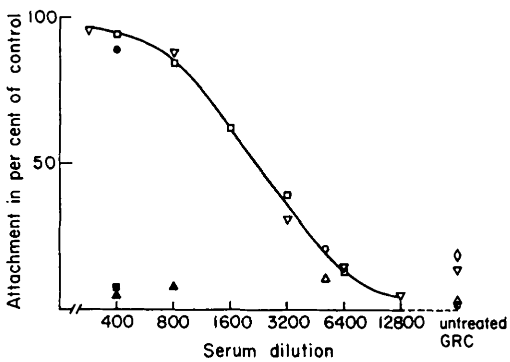

FIG. 2. Attachment of horse GRC, untreated or treated with anti-horse red cell serum, to trypsinized macrophages. Ordinates: attachment in percent of the attachment to control macrophages. Abscissae: reciprocal of serum dilutions. Different open symbols are results of separate experiments. Closed symbols are: circle = antiserum absorbed with sheep red cells; square = antiserum absorbed with horse red cells; triangle = normal rabbit serum.

In four experiments it ranged from 4 to 19% when expressed in percentage of the attachment to untreated macrophages. In contrast, GRC treated with immune serum attached themselves quite well to these trypsinized macrophages. The effect of the antiserum was evident to a dilution of 1:3,200. At higher doses of antiserum, attachment to trypsinized macrophages approached that seen with GRC and untreated macrophages. The antiserum effect was primarily one of increasing the proportion of trypsinized macrophages bearing attached GRC. Thus, antibody did not increase GRC/macrophage ratios by increasing markedly the number of GRC attached to a few trypsinized macrophages. Stimulation of the attachment of GRC disappeared following absorption of the serum with horse but not with sheep red cells. In other experiments, not included in Fig. 2, GRC were treated with immune or with nonimmune IgG prior to attachment. In a typical experiment, the attachment to trypsinized macrophages of GRC treated with 4 μg/ml nonimmune IgG was only 8.7% of their attachment to untreated macrophages, whereas when GRC were treated with 4 or with 8 μg/ml immune IgG, their attachment to trypsinized macrophages was, respectively, 35.1 and 48.2% of the attachment to untreated macrophages (the figures are averages of triplicates).

Discussion. The attachment of GRC to macrophages was increased by exposure of the red cells to immune (anti-red cell) serum or to immune IgG. Furthermore, GRC treated with antiserum or with immune IgG attached themselves to trypsinized macrophages under conditions where the attachment of untreated GRC was low. That the action of the antiserum is exerted through its content of specific antibody is indicated by the ineffectiveness of nonimmune serum, of nonimmune IgG, and by the reduction in the activity of antiserum that followed absorption with homologous but not with heterologous erythrocytes. In all experiments the red cells were washed three times in saline after the serum treatment and prior to their addition to the macrophage test system. Thus, effects of the serum, when observed, were primarily reflection of absorption to and modification of the red cell surface. The mechanism underlying attachment of GRC to macrophages is not understood at present. We have previously suggested that the reduced attachment of GRC after trypsinization of the macrophages could be due to the removal of a surface receptor (3).

The difference in pattern of attachment of untreated and of antibody coated GRC to control and to trypsinized macrophages could mean that different receptors are involved. According to this hypothesis, attachment to macrophages of GRC coated with antibody could be thought of as consisting of two components: one, due to the interaction of the GRC surface proper with a receptor on the macrophage surface which is markedly susceptible to trypsinization; the other, mediated through antibody bound to the red cell antigens, an interaction with a different receptor on the macrophages, relatively unaffected by trypsinization. Therefore, the amount of antibody bound to the red cells would govern the relative participation of the two postulated interactions in the overall attachment. The data seem consistent with this interpretation.

Alternatively, receptors of one kind could be involved in the attachment of GRC that have or have not been treated with antibody. This hypothesis would require that the affinity of the receptor be higher for antibody-coated than for untreated GRC, which is a plausible assumption. However, this hypothesis would not be consistent with a model in which trypsin would destroy an appreciable number of macrophage receptors. If such were the case, a similar ratio of attachment of antibody treated/untreated GRC for control and for trypsinized macrophages would be expected, but this it not borne out by the data. In addition, a reduction in the number of receptors would lead to the improbable conclusion that trypsin increases the affinity of the remaining receptors for antibody coated GRC over and above that of the receptors of untreated macrophages. Thus, a single receptor alternative theory would necessitate that the number of receptors not be markedly affected by trypsinization but that some change be induced in the receptors leading to differential attachment of untreated and/or antibody-treated GRC. While such change is conceivable, this case would be difficult to distinguish from a two-receptor hypothesis. More information is required before a more definitive choice may be made between the two alternatives.

It should also be noted that since trypsinized macrophages tend to spread more than control cells, the surface area of the former made available for GRC attachment may exceed that of the latter (see 5). It is thus possible that attachment to trypsinized macrophages is somewhat overestimated by the GRC/macrophage counts.

We have shown elsewhere (5) that the ingestion of untreated GRC that were attached to macrophages occurred at low rate when, after attachment, the macrophages were further incubated in Medium 199. The rate of ingestion was markedly increased by specific anti-red cell sera (6). In order to obtain information on the relative sensitivity of the increase in attachment and of the stimulation of ingestion induced by antiserum, the same pool of antiserum used in the present experiments was assayed for "ingestion stimulatory activity" (6). Significant stimulation was obtained when the GRC were treated with as little as 1:25,600 antiserum (Rabinovitch, unpublished results).

Proteolytic enzyme activity has been thought to be increased in inflammatory foci (7). The attachment of particles coated with antibody to macrophages treated with trypsin may thus have relevance to the phagocytic phenomena occurring *in vivo*.

Summary. Exposure of glutaraldehyde-treated red cells (GRC) to anti-red cell serum or to immune IgG increased their attachment to mouse peritoneal macrophages in saline medium. GRC treated with antibody also attached to trypsinized macrophages under conditions where the attachment of untreated GRC was very low. Use of nonimmune serum or of nonimmune IgG, as well as absorption experiments showed that the effect of antiserum was due to antibody. The results are consistent with the hypothesis that different receptors are involved in the attachment to macrophages of GRC that have or have not been treated with antibody.

The author is thankful to Dr. James G. Hirsch for his criticism and advice, and to Mrs. P. A. Gary for her technical assistance.

1. Hirsch, J. G. and Strauss, B., J. Immunol., 92, 145 (1964).

2. Boyden, S. V. and North, R. J., *In* "Complement," (G. E. W. Wolstenholme and J. Knight, eds.), p190. Little, Boston (1965).

3. Rabinovitch, M., Proc. Soc. Exptl. Biol. Med., 124, 396 (1967).

4. Levy, H. B. and Sober, H. A., Proc. Soc. Exptl. Biol. Med., 103, 250 (1960).
5. Rabinovitch, M., Exptl. Cell Res., 46, 19 (1967).
6. Rabinovitch, M., J. Immunol., 99, 232 (1967).
7. Wilhelm, D. L., *In* "The Inflammatory Process," (B. Z. Zweifach, L. Grant, and R. T. McCluskey, eds.), p389. Academic Press (1965).

Received Sept. 5, 1967. P.S.E.B.M., 1968, Vol. 127.

Humoral Antibodies in Sera of Patients with Bullous Skin Diseases* (32689)

K. KANO,[1] E. H. BEUTNER, AND F. MILGROM
Department of Bacteriology and Immunology, State University of New York at Buffalo, School of Medicine, Buffalo, New York

It has been shown that sera of patients with pemphigus vulgaris contain antibodies to an intercellular antigen of stratified squamous epithelium demonstrable by indirect immunofluorescent (IF) staining (1). More recently, evidence was presented that these antibodies combine with the patient's own skin and may be justifiably called autoantibodies (2).

This study shows that sera of some patients suffering from bullous skin diseases react with established human cell lines such as HEp-2 cells, and that some of the sera also contain antihuman gamma globulin antibodies resembling the rheumatoid factor.

Materials and Methods. The study included 11 sera of patients with bullous skin diseases, all of which were previously examined by IF staining (1,2). Monolayer cell cultures of heteroploid human cell lines: HeLa, HEp-2, and AV-3 were prepared in test tubes, as descrribed previously (3). Cell cultures of diploid-cell lines originating from human embryonic lung, WI-26, WI-38, and MA, as well as from human embryonic skin and muscle, MAF, were purchased from Microbiological Associates, Inc., Bethesda, Md. Primary cell cultures of kidney and lung of rhesus monkey and guinea pig origin were prepared following the procedure described by Karzon *et al.* (4).

In order to detect antibodies combining with cell-surface antigens, the mixed-agglutination test was employed. Similar procedure has been used in this laboratory for demonstration of human transplantation antibodies (5–7). Briefly, monolayer cell cultures were incubated with the tested serum at various dilutions. Thereafter, indicator erythrocytes were added to the cell cultures. For most experiments these were sheep erythrocytes sensitized by human antiserum to sheep erythrocytes and agglutinated by monospecific rabbit antiserum to human IgG. For some experiments, instead of anti-IgG serum, a monospecific anti-IgM serum or a multispecific antiserum against human globulins were used to prepare the indicator erythrocytes (7). Adherence of the indicator erythrocytes to the cell cultures was interpreted as a positive reaction.

To demonstrate anti-human gamma globulin factors, the hemagglutination test with human O Rh-positive erythrocytes sensitized by a human anti-CD serum "Ripley" was used (8). The serum "Ripley" was kindly supplied by Dr. M. Waller of the Medical College of Virginia, Richmond, Va. The procedure of the test was described previously (9).

Results. Table I lists results of a representative mixed-agglutination study with sera of four patients suffering from bullous skin diseases and HEp-2 cell cultures. It may be noted that in this particular experiment three sera gave positive results. Interestingly, some positive sera showed prozones which in Table I are exemplified by reactions of sera M.C. and S.M. Serum of patient A.T. who received multiple transfusions served as a positive con-

* Supported by USPHS Research grant CA-02357 from The National Cancer Institute and the Western New York Chapter of the Arthritis and Rheumatism Foundation.

[1] Henry C. and Bertha H. Buswell Research Fellow.