

Monocyte-Mediated Destruction in the Presence of Serum of Red Cells Coated With Antibody

By R. J. Kurlander and W. F. Rosse

In order to determine the conditions under which monocytes are able to destroy red cells coated with antibody in vitro in the presence of fluid-phase IgG, human peripheral blood monocytes and red cells coated with IgG anti-D antibody were incubated in media containing IgG (neat serum or medium containing 1000 $\mu\text{g}/\text{ml}$ of purified IgG). Although phagocytosis was markedly inhibited in media containing IgG, significant amounts of erythrophagocytosis were seen in these media. In neat serum, erythrophagocytosis was proportional to the concentration of red cells to 20 vol % and was not further increased at higher red cell volume. Phagocytosis was proportional to the concentration of RBC-bound antibody and was observed with as little as

1000–2000 molecules of antibody/RBC. Ingestion was markedly augmented by concurrent coating of the red cell with complement components; complement coating alone induced very little ingestion. Statistically significant extracellular red cell lysis, quantitated by measuring radioactive chromium release from ^{51}Cr -labeled red cells, occurred in the presence of serum if the red-cell–monocyte mixture was centrifuged to a pellet prior to incubation. Lysis under these conditions was augmented by increasing red cell concentration. These studies demonstrate that destruction by monocytes of antibody-coated red cells can occur under conditions that obtain commonly in the body.

MONOCYTES,¹ macrophages,¹ and neutrophils² interact in vitro with red cells coated with antibody to produce immune adherence and, in some cases, destruction of red cells by phagocytosis with or without spherocyte formation.^{1,3} Since spherocytes and erythrophagocytosis are observed during the course of immune hemolysis in vivo,⁴ antibody-dependent, cell-mediated destruction by monocytes and macrophages is thought to be important in the pathophysiology of hemolysis by warm-reactive IgG antibodies, particularly by those antibodies directed against antigens of the Rh locus, since these antibodies are unable to lyse red cells by direct complement-mediated lysis.⁵

Many previous studies, however, have shown that small amounts of unbound fluid-phase IgG, equivalent to 1/10,000 that present in normal human serum, markedly inhibit red cell destruction mediated by these effector cells in vitro.^{1,6–8} This has raised questions about the relevance of the in vitro reaction to in vivo hemolysis.⁹ In these experiments, we demonstrate that under appropriate conditions, monocyte-mediated destruction of red cells coated with small amounts of anti-Rh₀ (anti-D) antibody can be detected in vitro even in the presence of neat serum.

From the Department of Medicine, Durham Veterans Administration Hospital, and the Laboratories of the Howard Hughes Medical Institute, Duke University Medical Center, Durham, N.C.

Supported in part by research and education funds from the Durham Veterans Administration Hospital and by NIH grant RD1-CA-10267-10.

Submitted December 19, 1978; accepted July 24, 1979.

Address reprint requests to Roger J. Kurlander, M.D., Hematology Section, Durham Veterans Administration Hospital, Durham, N.C. 27705.

© 1979 by Grune & Stratton, Inc. 0006-4971/79/5405-0018\$01.00/0

MATERIALS AND METHODS

Buffers and Reagents

Veronal-buffered saline (VBS), Alsever's solution A, 0.015 M EDTA in distilled water (0.015 M EDTA), and 0.015 M EDTA in VBS (0.015 M EDTA + VBS) were prepared as previously described.¹⁰ RPMI 1640 and fetal calf serum were obtained from Grand Island Biologicals, Grand Island, N.Y. RPMI 1640 with 5% fetal calf serum and 25 mM/liter of HEPES (Sigma, St. Louis, Mo.) adjusted to pH 7.2 was the medium used in these experiments.

Chromium Labeling of Human Red Cells

Blood group O, Rh-positive red cells were obtained from a normal volunteer after informed consent. Blood was collected under sterile conditions and stored in Alsever's solution for up to 4 wk prior to use. The procedure for chromium labeling and antibody sensitization has been described in detail previously.⁸ In brief, 100 μ l of washed red cells were incubated with 200 μ Ci suspension of sodium chromate solution (New England Nuclear, Boston, Mass.) for 1 hr on the day of each experiment. Cells were washed free of radionuclide, and the radioactivity per 10^5 red cells was determined. Chromium-labeled or unlabeled red cells, at a concentration of $2-4 \times 10^8$ /cc in VBS, were incubated with an equal volume of purified IgG fraction with high titer of anti-D(Rh₀) activity obtained from hyperimmune human volunteers (Rhogam, Ortho Pharmaceuticals Corp., Raritan, N.J.) at a dilution of from 1:50 to 1:1600 for 1 hr at 37°C. Unbound antiserum was then removed by washing the cells with VBS four times, and the cells were resuspended at the desired cell concentration in medium.

Quantitation of IgG Antibody Affixed to Red Cells

Washed red cells coated with antibody were lysed using hypotonic 0.015 M EDTA solution or alternate freezing and thawing. The stromata were washed twice and centrifuged at 12,000 g for 10 min in a Sorval fixed-head centrifuge (Sorval, Inc., Newton, Conn.) to recover stromata quantitatively following each wash. The amount of IgG affixed to stromata was measured using a modification of the quantitative antiglobulin consumption technique described previously.⁸ The mean number of molecules of IgG fixed per red cell was calculated assuming a molecular weight of IgG of 160,000 daltons. The IgG bound to O Rh-negative red cells after incubation with antiserum was measured simultaneously, and this nonspecific adherence of IgG was deducted from the total amount of cell-associated IgG to determine specific IgG anti-D fixed per red cell.

In parallel experiments, it was determined that the conversion of red cells to stromata did not affect the amount of specific antibody fixed. In addition, the use of stromata permitted the freezing of samples so that the samples from several experiments could be assayed simultaneously.

Preparation of Fresh Human Serum

After obtaining informed consent, 30-100 cc of venous blood was collected from normal donors. The blood was rapidly transferred to Silicon centrifuge tubes and centrifuged at 12,000 g at 0°C for 2 min in a Sorval fixed-head centrifuge. The supernatant plasma was removed, permitted to clot, and the serum was separated from the clot. The serum was either used immediately or stored at -70°C for up to 2 wk. Prior to use, 25 mM/liter of HEPES was added and the pH adjusted to 7.2.

Preparation of Mixed Mononuclear and Monocyte Suspensions

The red-cell-leukocyte suspension obtained after removal of plasma, as described above, was rapidly diluted with 0.015 M EDTA + VBS and carefully layered over 20 cc of Ficoll-Hypaque solution¹¹ with a specific gravity of 1.078 (Pharmacia Fine Chemicals, Piscataway, N.J.) in 50-cc plastic conical tubes. The tubes were centrifuged at 500 g for 10 min and then at 1000 g for an additional 20 min. The leukocyte layer formed at the interface of Ficoll-Hypaque with the suspension was removed and washed 3 times in medium. Following washing, the resultant mononuclear cell suspension was either suspended in medium or in undiluted autologous serum. Purified monocyte preparations were prepared by incubating mononuclear leukocyte suspension at a concentration of 5×10^6 /cc in plastic Petri dishes (Scientific Products, Atlanta, Ga.), and after removal of nonadherent cells by 2 washes, the remaining adherent leukocytes were then removed from the Petri dishes mechanically by gently scraping the plate

with a rubber "policeman." The cells were washed once in medium and vigorously resuspended in medium or in autologous serum.

Evaluation of the Composition of Leukocyte Suspensions

The cellular composition of leukocyte suspensions was evaluated by direct microscopic examination of Giemsa-stained slides prepared by centrifugation of leukocyte suspension mixed with equal volume of 22% albumin in a cytocentrifuge (Cytospin, Shandem Southern, Camberley, Surrey, England). Mixed mononuclear cell suspensions contained 15%–25% monocytes, 0%–2% neutrophils, and 65%–85% lymphocytes. Purified monocyte preparations contained 70%–95% monocytes and 5%–30% lymphocytes.

Quantitation of Phagocytosis

Ten-microliter samples of mononuclear cell suspension at a concentration of 2×10^8 /cc and equal volume of red cell suspension in medium or serum were mixed in 12×75 mm plastic tubes (Fisher Scientific, Pittsburgh, Pa.) and incubated in a moisturized 5% CO₂ incubator for 1 hr at 37°C. The samples then were agitated vigorously, and 5- μ l portions were placed between 2 coverslips, which were then gently drawn apart to produce thin films that were air-dried, treated with Giemsa stain, and mounted on slides. Three-hundred monocytes from each pair of cover slips were examined under a microscope for phagocytosis at a magnification of 970. The mean and standard error of triplicate samples was calculated. In experiments in which medium alone was used to suspend the cells, 20 μ l of 22% albumin (Dade, Miami, Fla.) was added to each sample before making slides to prevent traumatic lysis of cells while producing thin films on the cover slips. The standard t test was used to assess the significance of differences between sets of samples.

Antibody-Dependent, Monocyte-Mediated Cr Release

This was measured after incubation of labeled red cells with or without antibody coating with monocytes. Antibody-sensitized washed red cells suspended in medium or in serum (50 μ l of a suspension at a concentration of $2\text{--}500 \times 10^5$ /cc) and purified monocytes (100 μ l with 1×10^5 cells/cc) were mixed in 12×75 mm plastic tubes in triplicate (Fisher Scientific, Pittsburgh, Pa.) and incubated in a moisturized 5% CO₂ incubator at 37°C for 2 hr. In some experiments, the cell suspension was pelleted by centrifugation for 30 sec at 1000 g prior to incubation. Following incubation, 0.85 ml of EDTA + VBS were added, and each tube was centrifuged at 1000 g for 5 min to sediment unlysed cells. Five-hundred microliters of supernatant fluid was then carefully removed from each tube and the radioactivity determined using a gamma emission spectrometer. Chromium release from antibody-coated red cells incubated without leukocytes, and chromium release from uncoated red cells incubated with leukocytes, were measured in each experiment. Since monocyte-mediated chromium release from uncoated red cells was consistently absent, antibody-dependent, monocyte-mediated red cell lysis was calculated using the formula:

$$\text{Antibody-dependent monocyte-mediated red cell lysis (cells lysed/tube} \times 10^{-5}) = \frac{{}^{51}\text{Cr release from antibody-coated red cells with monocytes} - {}^{51}\text{Cr release from antibody-coated red cells without monocytes} \times 2}{\text{Radioactivity}/10^5 \text{ red cells}}$$

The standard error in red cells lysed $\times 10^5$ /tube was calculated dividing the standard error of the mean¹² of the numerator of this equation by the denominator, and the standard t test was used to determine the significance of the difference between chromium release in the presence and absence of monocytes.

Preparation of IgG and Determination of Inhibition of Lysis or Phagocytosis by IgG1

Purified IgG1 was prepared by DEAE cellulose purification¹³ in 0.005 M phosphate buffer (pH 8.0) of serum obtained from a patient with multiple myeloma, which contains more than 12 g/100 ml of monoclonal IgG1. The IgG-containing effluent from a DEAE column was concentrated by vacuum dialysis against 0.15 M NaCl and frozen in small lots for future use. In experiments measuring

inhibition of lysis or phagocytosis, red cell and monocyte suspensions were prepared in medium with and without 1000 mg/ml of IgG1 and were then assayed for monocyte-mediated red cell destruction using the methods detailed above.

Complement Coating of Red Cells

Human red cells were coated with complement using a high-titer cold agglutinin and fresh human serum and methods described previously.⁸ Cells treated in this manner are coated with large amounts of C3b, C3d, C4b, and C4d.

RESULTS

When red cells, coated with about 5000 molecules of IgG anti-D per cell, were incubated at graded concentrations (0.8%–95+) with monocytes in medium, phagocytosis (measured as the percent of monocytes that had ingested one or more red cells) decreased with increased red cell concentration (Fig. 1). Phagocytosis was significantly greater in the presence of a 0.8% red cell suspension than in a 20% or a 95+ % suspension ($p < 0.01$). Ingestion in the presence of serum was greatly diminished but was nonetheless detectable at all red cell concentrations. Ingestion under these conditions was significantly greater ($p < 0.01$) at 4%, 20%, or 95+ % red cell concentration than at 0.8%.

Phagocytosis in the presence of culture medium, serum, or culture medium plus 1000 μ g IgG1 was compared using leukocytes from 5 normal donors and a packed red cell concentration of 20% (Fig. 2). Phagocytosis in the presence of serum or medium + IgG1 was significantly lower than ingestion in medium ($p < 0.01$) but was significantly greater than ingestion of unsensitized red cells, which were usually ingested by less than 0.5% of monocytes ($p < 0.01$). The amount of phagocytosis occurring in the presence of serum varied directly with the concentration of cell-bound antibody (Fig. 3). The presence of complement in addition to antibody markedly increased the amount of phagocytosis occurring in the presence

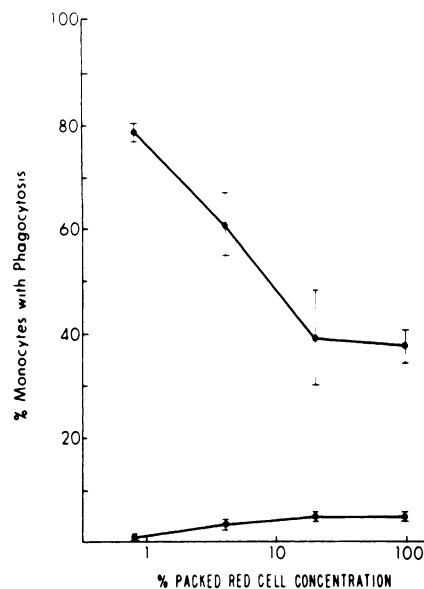


Fig. 1. The effect of red cell concentration on ingestion of red cells coated with anti-D by monocytes. Almost 80% of monocytes incubated with 0.8 vol % suspension of red cells in medium ingested red cells. However, the frequency of ingestion decreased as red cell concentration increased (upper curve). In contrast, less than 1% of monocytes ingested red cells in the presence of 0.8 vol % red cell suspension in undiluted human serum, but ingestion increased 5–10-fold when monocytes were incubated in suspensions containing red cells at 20 vol % or higher red cell concentration (lower curve).

Fig. 2. A comparison of the effect of serum and IgG₁ on ingestion of red cells coated with anti-D by monocytes from 5 normal donors. Phagocytosis was markedly and equally inhibited by neat serum and by 1000 µg/ml of IgG₁, compared to lysis in medium alone. Nonetheless, statistically significant ($p < 0.01$) ingestion still could be detected by monocytes from all 5 donors, despite the high concentration of unbound IgG.

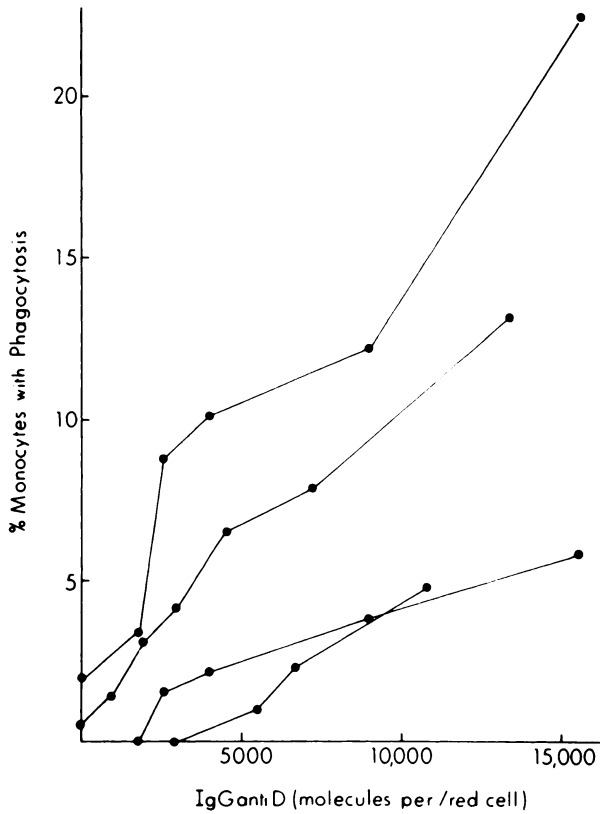
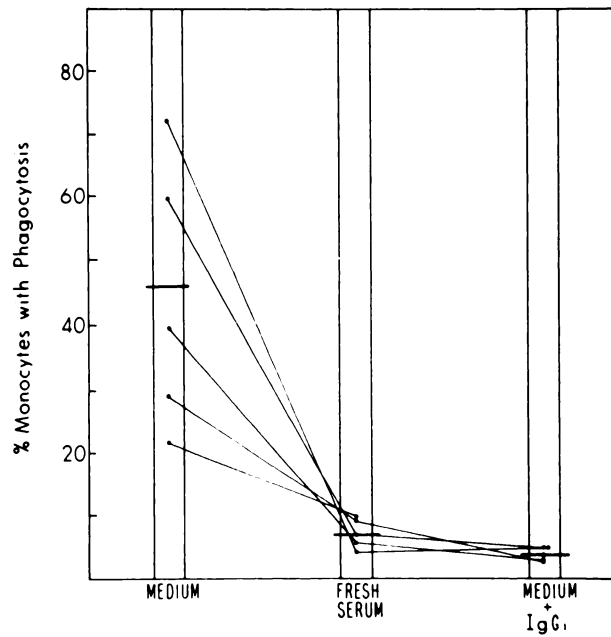


Fig. 3. The quantitative relationship between phagocytosis by monocytes and the amount of red cell antibody coating. Phagocytosis by monocytes from 4 normal donors was measured following incubation with red cells coated with known amounts of IgG anti-D in undiluted human serum. Monocytes from some donors ingested red cells coated with less than 1000 molecules/cell of IgG anti-D.

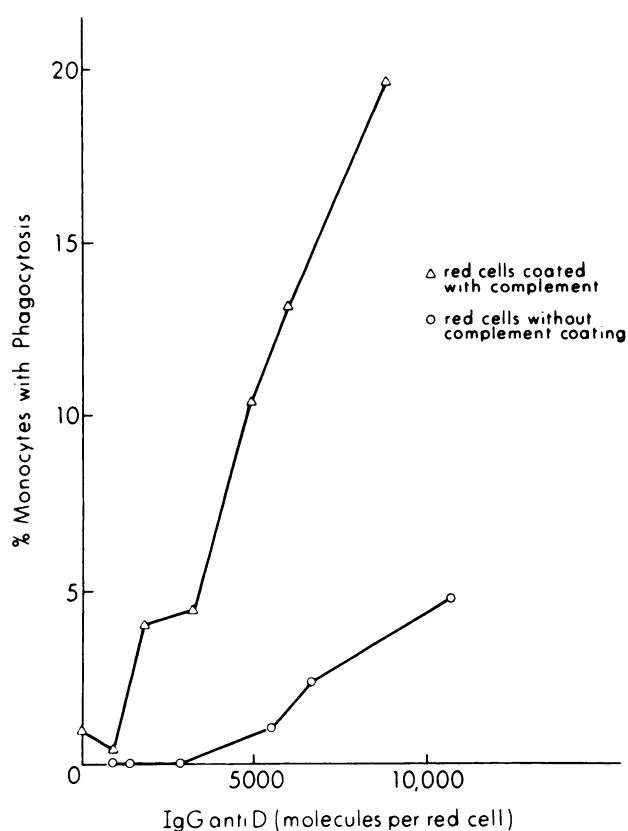


Fig. 4. The effect of complement on phagocytosis by monocytes of red cells coated with IgG anti-D in the presence of undiluted human serum. At equal levels of antibody coating, many more monocytes ingested complement-coated red cells (open triangles) than cells without complement coating (open circles). Complement coating in the absence of antibody, however, produced very little phagocytosis.

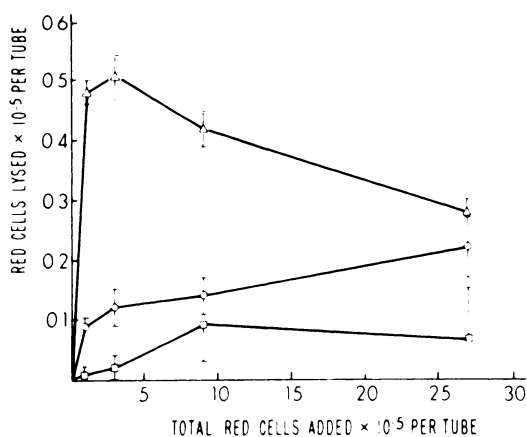
of serum (Fig. 4). Complement coating alone induced ingestion in only 1% of monocytes.

To confirm these observations using another common *in vitro* measure of antibody-dependent, monocyte-mediated red cell destruction, ^{51}Cr release from labeled red cells coated with antibody was measured in medium and in undiluted serum at several red-cell-monocyte ratios. Monocyte-mediated ^{51}Cr release from antibody-coated red cells in serum exceeded spontaneous release, though this difference did not attain statistical significance ($p > 0.05$). If the red-cell-leukocyte mixture was centrifuged to a pellet prior to incubation to enhance cell-cell contact, however, statistically significant antibody-dependent monocyte-mediated lysis was noted at all red-cell-leukocyte ratios ($p < 0.01$) (Fig. 5). Lysis was nonetheless much smaller than that observed under identical conditions in medium free of unbound IgG. The absolute number of red cells lysed increased as the red-cell-leukocyte ratio was increased above 1:1.

DISCUSSION

The demonstration of antibody-dependent, cell-mediated destruction of red cells *in vitro* has suggested that this mechanism may account for much of the hemolysis due to IgG observed *in vivo*.¹ However, the ease with which this *in vitro* phenomenon can be inhibited by unbound IgG in concentrations much lower than those encountered in the serum^{1,6-8} has engendered doubt concerning the importance of

Fig. 5. Comparison of antibody-dependent, monocyte-mediated lysis of red cells coated with anti-D incubated in medium without added IgG or undiluted human serum. Lysis in medium was detected at all red cell concentrations from 1 to 27×10^6 red cells/tube (open triangles). Monocyte-mediated lysis in neat serum (open squares) was noted at high red cell concentrations but did not achieve statistical significance ($p > 0.05$). If the red-cell-monocyte suspension in serum was centrifuged to enhance red-cell-leukocyte contact prior to incubation, monocyte-mediated lysis increased and was statistically significant at all red cell concentrations ($p < 0.01$).



this mechanism in producing hemolysis *in vivo*.⁹ The inhibition by fluid-phase IgG is presumed to be the result of competition between it and membrane-bound IgG antibody for binding to a limited number of receptors on the membrane of monocytes (the so-called Fc receptors), which interact with a portion of the Fc fragment of IgG. The complex formed between membrane-bound IgG and the Fc receptor is the basis for immune adherence and, thus, is probably a prerequisite for the destruction of target red cells by effector cells of the immune system. Hence, competitive displacement of membrane-bound IgG by fluid-phase IgG results in inhibition of both adherence and destruction.¹⁴

When large amounts of antibody are present on the red cell surface, inhibition by unbound IgG in the fluid phase is decreased and may be undetectable when more than 20,000 molecules of antibody are affixed to the red cell.^{8,15} Such concentrations of bound IgG molecules appear to be able to compete successfully with fluid-phase IgG for the Fc receptors. These concentrations of membrane-bound antibody cannot be achieved by antibodies against antigens of the Rh system, including anti-D antibody (the antibody used in the present experiments), since the number of antigen sites is limited;¹⁶ hence, under most conditions, adherence or destruction of red cells even maximally coated with anti-D is virtually completely inhibited by small amounts of unbound IgG.⁶⁻⁸

In this study we have shown that monocyte-mediated phagocytosis and lysis of red cells coated with as few as 1000–2000 molecules of IgG anti-D per cell by monocytes from some donors can occur *in vitro* in the presence of neat serum when the concentration of red cells is increased or when the effector and target cells are concentrated by centrifugation. Lo Buglio et al.¹ previously demonstrated that antibody-dependent adherence of red cells to monocytes *in vitro* in the presence of serum increases as the concentration of red cells increases, and in experiments similar to these, Fleer et al.¹⁵ showed that monocyte-mediated lysis in the presence of unbound IgG also increases as the concentration of target cells is increased, however, the concentration of unbound IgG used in those experiments was $1/200$ that in serum. Paradoxically, phagocytosis of red cells coated with antibody in the absence of unbound IgG decreases as the red cell concentration is increased. Presumably, the presence of too many red cells adherent to the monocyte membrane inhibits the phagocytosis of any single cell.

Red cells coated with complement adhere to monocytes, macrophages, neutrophils, and lymphocytes,^{17,18} but this adherence usually does not result in phagocytosis or lysis.^{3,8,18} When complement and IgG antibody are present together, however, the amount of destruction for a given amount of antibody is increased and the minimal quantity of antibody inducing lysis is decreased.^{8,19} In these experiments, we have shown that complement coating augments monocyte-mediated phagocytosis in the presence of serum as well as in its absence. These results are consistent with observations *in vivo* that the clearance of red cells coated with antibody is augmented by concurrent complement coating.²⁰

Our data were obtained using peripheral blood monocytes, whereas reticuloendothelial macrophages may be more important effectors in producing hemolysis *in vivo*. Though monocytes and macrophages share similar properties, macrophages are thought to be more "mature" cells,²¹ since they are larger, contain larger amounts of proteolytic enzymes,²¹ and may possess larger numbers of surface Fc receptors.²² Therefore, macrophages may be capable of lysing greater numbers of target cells and may be less sensitive to the inhibitory influence of unbound IgG. Comparative studies of reticuloendothelial and monocytic effectors are currently in progress in our laboratory.

In these studies, the amount of destruction of red cells by monocytes in the presence of fluid-phase IgG was small. However, monocyte-mediated destruction *in vivo* is a cumulative process, and the rate of destruction per monocyte observed in these experiments might be consistent with the production of significant hemolysis under conditions such as those *in vivo*, in which phagocytosis continued at a steady rate over a longer period of time.

ACKNOWLEDGMENT

We are deeply indebted to Judy P. Adams for technical assistance in these experiments and to Norma Martell for help in preparation of the manuscript. Dr. Rosse is a Medical Investigator for the Howard Hughes Medical Institute.

REFERENCES

- LoBuglio AF, Cotran RS, Jandl JH: Red cells coated with immunoglobulin G: Binding and sphering by mononuclear cells in man. *Science* 118:1582, 1967
- Henson PM: The adherence of leukocytes and platelets induced by fixed IgG antibody or complement. *Immunology* 16:107, 1969
- Rosse WF, de Boisfleury A, Bessis M: Interaction of phagocytic cells and red cells modified by immune reactions. Comparison of antibody and complement coated red cells. *Blood Cells* 1:345, 1975
- Dacie JV: The Haemolytic Anaemias, Congenital and Acquired. Part II. The Autoimmune Haemolytic Anaemias (ed 2). London, Churchill, 1962, pp 354-360
- Mollison PL: The role of complement in antibody mediated red cell destruction. *Br J Haematol* 18:249, 1970
- Holm GE, Engwall S, Hammarström S, Natwig JB: Antibody induced hemolytic activity of human monocytes. *Scand J Immunol* 3:173, 1974
- Huber J, Douglas SD, Fudenberg HH: The IgG receptor: An immunologic marker for the characterization of mononuclear cells. *Immunology* 17:7, 1969
- Kurlander RJ, Rosse WF, Logue GL: Quantitative influence of antibody and complement coating of red cells on monocyte-mediated cell lysis. *J Clin Invest* 61:1309, 1978
- Logue G, Rosse WF: Immunologic mechanisms in autoimmune hemolytic disease. *Semin Hematol* 13:277, 1976
- Kabat EA, Mayer MM: *Experimental Immunochemistry* (ed 2). Springfield, Ill, Charles C Thomas, 1961, pp 135-139
- Bøyum A: Isolation of mononuclear cells and granulocytes from human blood. *Scand J Clin Lab Invest* 21(Suppl 97):77, 1968

12. Mainland D: *Elementary Medical Statistics* (ed 2). Philadelphia, WB Saunders, 1963, p 290
13. Sober HA, Peterson EA: Protein chromatography on ion exchange cellulose. *Fed Proc* 17:1116, 1958
14. Brown DL: The behavior of phagocytic cell receptors in relation to allergic red cell destruction. *Ser Haematol* 7:348, 1974
15. Fleer A, van de Meulen FW, Linthout FLs, von dem Borne AFG Kr, Engelfriet CP: Destruction of IgG sensitized erythrocytes by human blood monocytes: Modulation of inhibition by IgG. *Br J Haematol* 39:425, 1978
16. Rochner E, Hughes-Jones NC: The use of purified ¹²⁵I labelled antigammaglobulin in the determination of the number of D antigen sites on red cells of different phenotypes. *Vox Sang* 10:675, 1965
17. Ross GD, Polley MJ: Specificity of human lymphocyte complement receptors. *J Exp Med* 141:1163, 1975
18. Mantovani B, Rabinovitch BM, Nussenzweig V: Phagocytosis of immune complexes by macrophages: Different roles of the macrophage receptor sites for complement (C3) and immunoglobulin (IgG). *J Exp Med* 135:790, 1972
19. Ehlenberger AG, Nussenzweig V: The role of membrane receptors for C3b and C3d in phagocytosis. *J Exp Med* 145:357, 1977
20. Schreiber AD, Frank MM: The role of antibody and complement in the immune clearance of erythrocytes. II. Molecular nature of IgG and IgM complement fixing sites and effects of their interaction with serum. *J Clin Invest* 51:583, 1972
21. Cline MJ: Monocytes and macrophage: Differentiation and function, in Greenwalt TJ, Jamieson GA (eds): *Formation and Destruction of Blood Cells* (ed 3). Philadelphia, JB Lippincott, 1970, pp 222-239
22. van Loghem JJ, van der Meulen FW, Fleer A, Van der Hart M, von dem Borne AEG Kr, Engelfriet CP: The importance of the Fc receptor for red cell destruction under the influence of non-complement-binding antibodies in, Mohn JF, Plunkett RW, Cunningham RK, Lambart RM (eds): *Human Blood Groups. Fifth International Convocation on Immunology*, Buffalo, N.Y. (ed 4). Basel, S Karger, 1977, pp 75-84