

Effects of Anti-Human Neutrophil Antibodies In Vitro. QUANTITATIVE STUDIES

Laurence A. Boxer, Thomas P. Stossel

J Clin Invest. 1974;**53**(6):1534-1545. <https://doi.org/10.1172/JCI107704>.

Research Article

Opsonic, antiphagocytic, cytotoxic, and metabolic effects of homologous and heterologous antibodies against human neutrophils were analyzed by means of quantitative assays to facilitate detection of antibody activity, and to probe membrane function of these cells. Normal human neutrophils were purified by gradient centrifugation, sensitized with heat-inactivated antineutrophil antisera, and incubated with rabbit alveolar macrophages in balanced salt solution containing nitroblue tetrazolium. The macrophages engulfed sensitized neutrophils and reduced nitroblue tetrazolium to formazan in phagocytic vacuoles. The initial rate of nitroblue tetrazolium reduction by macrophages ingesting the neutrophils was measured spectrophotometrically. Neutrophils treated with rabbit anti-human leukocyte antiserum or IgG, with sera from mothers of infants with neonatal isoimmune neutropenia, and with 27% of sera from frequently transfused patients promoted rapid rates of nitroblue tetrazolium reduction by alveolar macrophages. This indicates that antineutrophil antibodies without added complement opsonized neutrophils for ingestion by the macrophages. Some sera from frequently transfused patients with opsonic activity for certain donors' neutrophils did not agglutinate these neutrophils (44%), did not lyse them in the presence of fresh plasma (47%), and did not inhibit phagocytosis of particles by the neutrophils (26%). The reverse was not observed. The opsonic activity of antineutrophil antiserum appears to be the most sensitive and a quantitative means of detecting antibody activity in vitro.

Low concentrations of rabbit anti-human leukocyte antisera or IgG [...]

Find the latest version:

<https://jci.me/107704/pdf>



Effects of Anti-Human Neutrophil Antibodies In Vitro

QUANTITATIVE STUDIES

LAURENCE A. BOXER and THOMAS P. STOSSEL

*From the Division of Hematology-Oncology, Children's Hospital Medical Center
and the Department of Pediatrics, Harvard Medical School,
Boston, Massachusetts 02115*

ABSTRACT Opsonic, antiphagocytic, cytotoxic, and metabolic effects of homologous and heterologous antibodies against human neutrophils were analyzed by means of quantitative assays to facilitate detection of antibody activity, and to probe membrane function of these cells. Normal human neutrophils were purified by gradient centrifugation, sensitized with heat-inactivated antineutrophil antisera, and incubated with rabbit alveolar macrophages in balanced salt solution containing nitroblue tetrazolium. The macrophages engulfed sensitized neutrophils and reduced nitroblue tetrazolium to formazan in phagocytic vacuoles. The initial rate of nitroblue tetrazolium reduction by macrophages ingesting the neutrophils was measured spectrophotometrically. Neutrophils treated with rabbit anti-human leukocyte antiserum or IgG, with sera from mothers of infants with neonatal isoimmune neutropenia, and with 27% of sera from frequently transfused patients promoted rapid rates of nitroblue tetrazolium reduction by alveolar macrophages. This indicates that antineutrophil antibodies without added complement opsonized neutrophils for ingestion by the macrophages. Some sera from frequently transfused patients with opsonic activity for certain donors' neutrophils did not agglutinate these neutrophils (44%), did not lyse them in the presence of fresh plasma (47%), and did not inhibit phagocytosis of particles by the neutrophils (26%). The reverse was not observed. The opsonic activity of antineutrophil antiserum appears to be the most sensitive and a quantitative means of detecting antibody activity in vitro.

Dr. Stossel is an Established Investigator of the American Heart Association.

Received for publication 1 November 1973 and in revised form 4 February 1974.

Low concentrations of rabbit anti-human leukocyte antisera or IgG stimulated the ingestion rate of unopsonized or opsonized particles by human neutrophils, and, as previously reported by others, enhanced rates of oxidation of [1-¹⁴C]glucose by the cells. High concentrations of the antisera or IgG inhibited ingestion. All concentrations of homologous antineutrophil antisera tested only inhibited ingestion of particles by neutrophils and none altered rates of resting glucose oxidation by the cells. The findings suggest that heterologous antibodies disturb membrane antigens that trigger oxidative metabolism and enhance as well as inhibit ingestion, and that these antigens are common to all human neutrophils. In contrast to other studies with antimacrophage antibodies, antineutrophil antibodies altered phagocytic rates of both unopsonized and opsonized particles although there were differences in dose-response curves depending on the type of particle tested. Thus, antineutrophil antibodies do not merely cover selected receptor sites.

INTRODUCTION

Antibodies against polymorphonuclear leukocytes and other phagocytic cells have a number of important biological functions. Antisera against neutrophils have been employed to deplete animals of neutrophils (1-3) and to investigate the role of these cells in wound healing (4), and anticell antibodies have been used to probe receptor activity in mouse macrophages (5). Antineutrophil antibodies have been suspected in the etiology of diverse neutropenic states (6-9), and the emerging importance of granulocyte transfusion in the management of patients with bone marrow failure has increased the need to improve understanding of antineutrophil antibodies.

To gain more insight into the effects of antineutrophil antibodies, we have devised assays or utilized existing ones to analyze in vitro in as quantitative a manner as possible the action of these antibodies. We report on quantitative studies concerning opsonic, antiphagocytic, agglutinating, metabolic, and cytotoxic effects of heterologous and homologous antineutrophil antisera. We also describe the application of these quantitative assays in detecting antibody activity against neutrophils in human sera.

METHODS

Serum was collected from normal humans, from patients who required frequent transfusions for treatment of leukemia, aplastic anemia, and thalassemia and from patients with idiopathic thrombocytopenia purpura, congenital neutropenia, or Rh isoimmunization. Sera was also obtained from mothers of infants with immune neonatal neutropenia.¹ All sera, unless otherwise stated, were heat inactivated at 56°C for 30 min.

Preparation of leukocytes for assaying inhibition of phagocytes and glucose oxidation. As previously described (11), blood was collected from normal humans into acid-citrate-dextrose anticoagulant, and erythrocytes were removed by sedimentation with dextran and lysis with ammonium chloride. The leukocytes were washed with 100 vol of 0.15 M sodium chloride by centrifugation at 80 *g* for 10 min, and were suspended in Krebs-Ringer phosphate medium (KRP),² pH 7.4, approximately 1 ml/8 ml of blood originally obtained. A sample of this leukocyte suspension was removed for determination of cell number with the electronic Coulter Counter.³ These suspensions contained 80–90% neutrophils (polymorphonuclear leukocytes and band forms), 5–10% monocytes, and 5–10% lymphocytes.

Preparation of neutrophils for opsonization, chromium release, and agglutination. Human peripheral blood neutrophils essentially free of monocytes, lymphocytes, and platelets were prepared by using a sodium diatrizoate⁴-Ficoll⁵ gradient according to the technique of Böyum (12). To the individual cell pellets containing neutrophils and erythrocytes, 1 ml of AB positive plasma obtained from acid-citrate-dextrose anticoagulated blood, was added along with 0.5 ml of 6% dextran, (500,000 mol wt). The pellets were then suspended in a 50-ml plastic syringe. After sedimentation of the erythrocytes at room temperature for 45 min, the remaining erythrocytes in the supernatant plasma were lysed with a 0.87% ammonium chloride as described above, which reduced erythrocyte contamination to less than 1 red cell per neutrophil. Differential counts on the neutrophil-enriched preparations revealed the ratio of 99 neu-

trophils per mononuclear cell, 95 neutrophils per 5 eosinophils, and 10 neutrophils per platelet. Mononuclear cells were isolated from the upper layer of the gradient. These cell preparations contained on the average 1% neutrophils, 20% monocytes, and 79% lymphocytes. Approximately 1 erythrocyte and 1 platelet per 14 lymphocytes were present.

Preparation of macrophages. Suspensions of over 90% alveolar macrophages were collected by tracheal lavage (13) from rabbits treated with complete Freund's adjuvant (14). The cells were collected in 0.15 M NaCl, centrifuged at 80 *g* for 10 min and suspended in KRP.

Sensitization of leukocytes. Suspensions of mixed leukocytes or gradient-purified neutrophils in 0.15 M NaCl containing $1-2 \times 10^7$ neutrophils/ml were incubated with an equal volume of heat-inactivated (56°C, 30 min) control or test sera or sera diluted with KRP for 30 min at 25°C. The incubation was stopped by adding 10 ml of ice-cold 0.15 M sodium chloride to the leukocyte suspension, which was then centrifuged at 80 *g* for 10 min. The pellet was suspended in KRP, and samples were removed for a final determination of cell number. Heterologous antiserum tended to clump the neutrophils, but at the concentration of 100 μ l/10⁷ neutrophils or less the cells were easily dispersed.

Ingestion of sensitized neutrophils by alveolar macrophages. To siliconized glass 15-ml centrifuge tubes was added 0.4 ml of an alveolar macrophage suspension in KRP containing $1-3 \times 10^7$ macrophages/ml and 0.4 ml of nitroblue tetrazolium, which had been filtered and standardized as described previously (11). The tubes were shaken in a water bath at 37°C for 5 min and then 0.4 ml of the previously sensitized neutrophils in a concentration of $1-2 \times 10^7$ neutrophils/ml, or as described in figure legends or tables, were added. After 10 min or other time intervals described, 6 ml of ice-cold 1 mM *N*-ethylmaleimide⁶ in 0.15 M sodium chloride was added, and the suspension was centrifuged at 400 *g* for 10 min. The supernate was discarded and the cell pellet was suspended in 6 ml of 0.15 M sodium chloride. After centrifugation the tube was inverted and the cells were extracted with 1 ml of dioxane by heating at 90°C for 15 min. The quantity of nitroblue tetrazolium reduced to nitroblue tetrazolium-formazan was measured in the extracts after clarification by centrifugation spectrophotometrically as previously described (11).

Measurement of paraffin oil phagocytosis. Oil Red O was emulsified with *Escherichia coli* lipopolysaccharide 026:B6⁷ (11) or human serum albumin (15) as previously described. The lipopolysaccharide-coated and albumin-coated oil droplets were incubated with equal volumes of fresh normal human serum at 37°C for 15 min, which results in the fixation of opsonically active C3 on the particles (15, 16). In some experiments, the albumin-coated oil particles were incubated at 37°C for 15 min with goat anti-human albumin antiserum⁸ which contained 2.9 mg of antialbumin antibody protein/ml. IgG was isolated from the antialbumin antiserum by precipitation in 45% saturated ammonium sulfate and elution from DEAE cellulose with 7 mM sodium phosphate buffer, pH 6.3, and was also used to treat the particles. The paraffin oil particles, after incubation in the various reagents, were washed by centrifugation through 0.15 M sodium chloride and suspended to the original volume in KRP. Albumin-coated particles incubated with

¹ Generously provided by the National Institutes of Health Tissue Typing Bank, Bethesda, Md., and Dr. Parvis Lalezari, Montefiore Hospital, New York, N. Y. These sera agglutinate only neutrophils of certain individuals. On the basis of such reactions, neutrophil antigens have been defined: NA1, NA2, NB1, and NC1 (10).

² Abbreviation used in this paper: KRP, Krebs-Ringer phosphate medium.

³ Coulter Electronics, Inc., Hialeah, Fla.

⁴ Winthrop Laboratories, Division of Sterling Drug, Inc., New York, N. Y.

⁵ Sigma Chemical Company, St. Louis, Mo.

⁶ Nutritional Biochemical Corporation, Cleveland, Ohio.

⁷ Difco Laboratories, Detroit, Mich.

⁸ A gift of Dr. Chester A. Alper, Blood Research Institute, Boston, Mass.

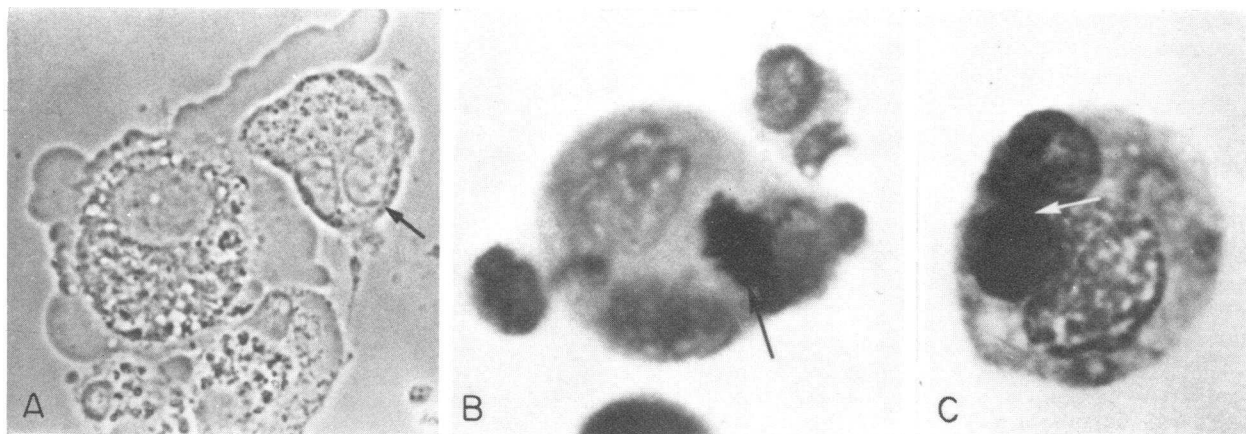


FIGURE 1 (A) Phase-contrast photomicrograph of rabbit alveolar macrophage engulfing human neutrophil indicated by the arrow. $\times 2,000$. (B and C) Human neutrophils and nitro-blue tetrazolium-formazan granules indicated by the arrow within alveolar macrophages. $\times 1,000$.

heat-inactivated antialbumin antiserum, antialbumin IgG, or fresh human serum were ingested by human leukocytes up to twofold more rapidly than unopsonized particles (incubated with heat-inactivated human serum (15-17)). Lipopolysaccharide-coated particles were essentially not ingested at all unless opsonized with fresh serum (16).

To siliconized glass 15-ml centrifuge tubes was added $2-5 \times 10^7$ sensitized leukocytes in 0.8 ml of KRP. The tubes were warmed to 37°C , 0.2 ml of opsonized paraffin oil particles were added, and the initial rate of ingestion of the particles was spectrophotometrically assayed as previously described (11).

Chromium release assay. Human neutrophils or lymphocytes were suspended in 0.8 ml of KRP and to this suspension was added 200 μCi of [^{51}Cr]sodium chromate⁹ in 0.2 ml of 0.15 M sodium chloride (18). The mixture was incubated for 30 min at 25°C . The labeled leukocytes were then washed twice with 0.15 M sodium chloride and then suspended in plasma anticoagulated with 5 mM EDTA at a final concentration of 1×10^7 cells/ml at 25,000-35,000 cpm. To 0.1 ml of the leukocyte suspension was added 0.05 ml of test sera. The reaction was initiated by adding 0.05 ml of 50 mM magnesium chloride. The mixtures were incubated for 120 min or other indicated time intervals at 37°C . 2 ml of ice-cold 0.15 M sodium chloride was added to stop the reaction, and the tubes were centrifuged at 4°C for 10 min at 500 g. The supernates were collected for determination of released radioactivity in a well type scintillation counter.¹⁰ When the leukocytes were incubated in distilled water, 75% of the [^{51}Cr]leukocyte was released in soluble form. The remaining 25% was apparently bound to membranous components and sedimented after centrifugation at 400 g for 10 min.

Preparation of anti-human leukocyte antibody. Human mixed leukocytes were separated from erythrocytes as described above and approximately 1×10^6 cells/ml in 1 ml of 0.15 M sodium chloride were injected every other day intravenously into New Zealand rabbits for a total of seven in-

⁹ 10.3 mCi/mmol. New England Nuclear Corp., Boston, Mass.

¹⁰ Gamma-Guard, Tracerlab Division, Laboratory for Electronics, Inc., Waltham, Mass.

jections. Another injection was administered after 4 wk. 7 days thereafter the animals were bled. The sera were pooled and heated (56°C for 30 min). Antileukocyte IgG was prepared from the pooled heated sera by precipitation from 45% ammonium sulfate and elution from DEAE cellulose with 7 mM sodium phosphate buffer, pH 6.3.

Glucose oxidation by human leukocytes. Human leukocytes in a concentration of 1×10^7 cells/ml were suspended in 0.8 ml of KRP containing 1 mM glucose and 0.5 μCi of [$1-^{14}\text{C}$]glucose.¹¹ Then 0.2 ml of test serum was added. The suspensions were incubated in 12×75 plastic tubes stoppered by caps fitted with cups,¹² to which 0.2 ml of hyamine hydroxide¹³ had been added. After a 30-min incubation at 37°C in a shaking water bath, 1 ml of 5 N H_2SO_4 was injected through the stopper into the incubation medium. After a 30-min equilibration period, the cups were removed and assayed for radioactivity as previously described (19).

In other experiments the rate of [$1-^{14}\text{C}$]glucose oxidation by leukocytes previously treated with test or control serum and then washed was assayed. To certain incubation tubes, 0.1 ml of either polystyrene latex particles or 0.1 ml of saline was added.

Leukoagglutination and lymphocyte cytotoxicity. Measurement of leukoagglutinins was attempted by the technique described by Payne (20). The final ratio of antiserum to neutrophils in these studies was 50 $\mu\text{l}/10^5$ cells which was considerably higher than the ratio employed in the other assays described. The micro-method of Terasaki and McClelland (21) was used to detect cytotoxic effects of test sera on lymphocytes.

RESULTS

Opsonic Effect of Antigranulocyte Antibody. Treatment of human neutrophils with antineutrophil antibodies promoted their engulfment by the larger rabbit alveolar macrophages as demonstrated by phase microscopy (Fig. 1A). During ingestion of sensitized neutro-

¹¹ 47.8 mCi/mmol. New England Nuclear Corp.

¹² Kontes Glass Company, Vineland, N. J.

¹³ Pilot Chemicals Division, New England Nuclear Corp.

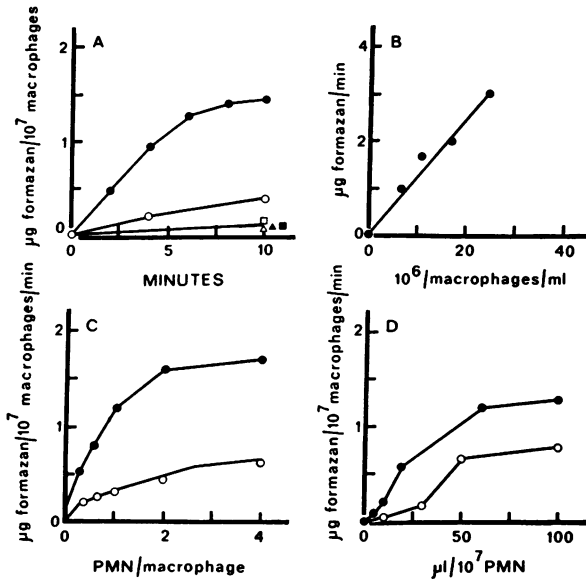


FIGURE 2 Rate of nitroblue tetrazolium reduction by rabbit alveolar macrophages incubated with human neutrophils in 0.8 ml of KRP, pH 7.4, and containing nitroblue tetrazolium, 560 $\mu\text{g}/\text{ml}$. The mean of duplicate incubations are given.

(A) Nitroblue tetrazolium reduction as a function of time. 1×10^7 alveolar macrophages were incubated with 2×10^7 human neutrophils pretreated with control rabbit serum (\circ) or rabbit anti-human leukocyte antiserum (\bullet), $10 \mu\text{l}/10^7$ neutrophils. Samples were removed at the indicated times for measurement of nitroblue tetrazolium-formazan. Some tubes were incubated at ice-bath temperature (Δ), contained 1 mM *N*-ethylmaleimide (\blacktriangle), or else contained sensitized neutrophils minus macrophages (\square), or macrophages minus neutrophils (\blacksquare).

(B) Nitroblue tetrazolium reduction as a function of macrophage concentration. Incubations containing the indicated concentration of macrophages contained human neutrophils, 2×10^7 cells/ml, sensitized with rabbit anti-human leukocyte antiserum, $10 \mu\text{l}/10^7$ cells. Samples were removed at 10 min.

(C) Nitroblue tetrazolium reduction by alveolar macrophages as a function of neutrophil (PMN) concentration. All incubations contained 1×10^7 macrophages and unsensitized neutrophils (\circ) or neutrophils pretreated with rabbit anti-human leukocyte antiserum (\bullet), $10 \mu\text{l}/10^7$ cells. Samples were removed at 10 min.

(D) Nitroblue tetrazolium reduction as a function of antiserum concentration. All incubations contained 1×10^7 alveolar macrophages and 2×10^7 human neutrophils. The neutrophils were treated with the indicated volumes of rabbit anti-human leukocyte antiserum (\bullet) or with the serum of a frequently transfused patient (\circ). The rate of nitroblue tetrazolium production in the presence of unsensitized control was $0.4 \mu\text{g}$ formazan/ 10^7 macrophages/min. Samples were removed at 10 min.

phils, alveolar macrophages reduced nitroblue tetrazolium to nitroblue tetrazolium-formazan in the vicinity of the phagocytic vacuole (Figs. 1B and 1C). A quantitative assay was devised in which the initial rate of

nitroblue tetrazolium reduction was spectrophotometrically measured as a reflection of ingestion of neutrophils by macrophages. Fig. 2 shows some features of the assay system. Nitroblue tetrazolium reduction occurred slowly unless neutrophils were previously sensitized by antiserum directed against them. The accelerated rate of nitroblue tetrazolium reduction by macrophages incubated with sensitized neutrophils was constant for 5–10 min and was proportional to the macrophage concentration (Fig. 2B). It was completely prevented by 1 mM *N*-ethylmaleimide or by incubation of the system at ice bath temperature (Fig. 2A). Minimal reduction of nitroblue tetrazolium occurred in the presence of human neutrophils or macrophages incubated with antineutrophil antiserum alone (Fig. 2A). The rate of nitroblue tetrazolium reduction was directly proportional to neutrophil concentration until saturation of the system occurred at

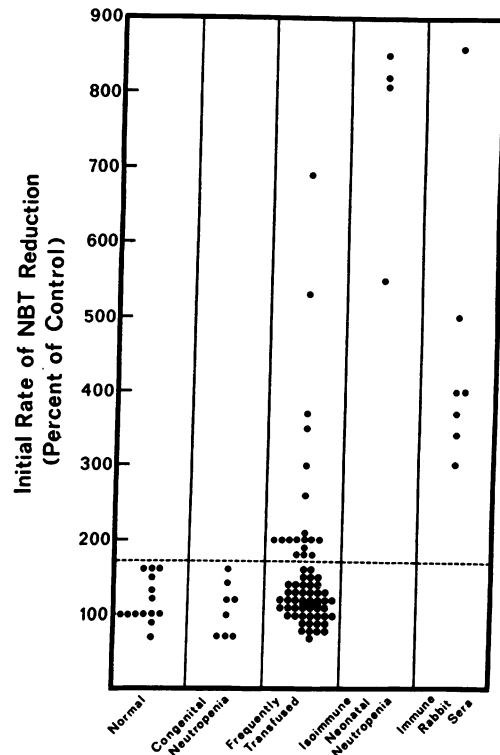


FIGURE 3 Initial rates of nitroblue tetrazolium (NBT) reduction by rabbit alveolar macrophages incubated with normal human gradient-purified neutrophils treated with various sera. The rates are expressed as percents of control rates (neutrophils treated with autologous serum, and incubated with macrophages). Test samples are considered to be abnormal if the opsonic activity exceeded the mean value of the neutrophils treated with autologous serum by more than two standard deviations as indicated by the dotted line. Homologous control sera include two patients with idiopathic thrombocytopenia purpura and one patient with a high titer anti-Rh (D) antibody.

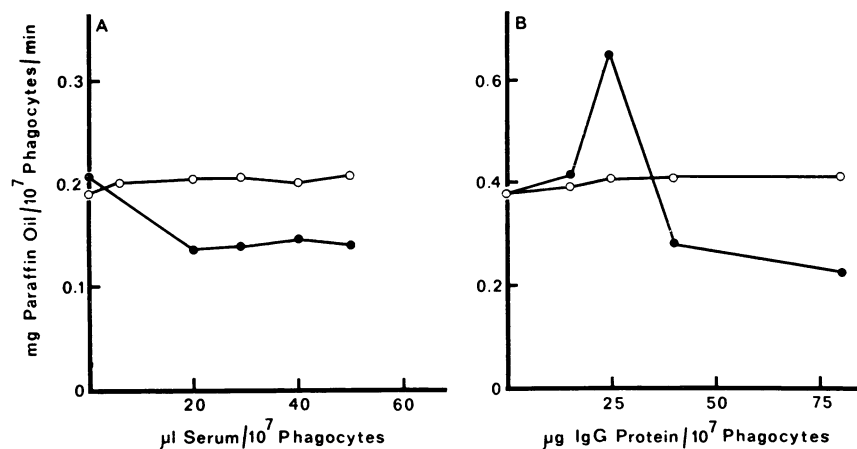


FIGURE 4 (A) Initial ingestion rate of opsonized lipopolysaccharide-paraffin oil particles by human neutrophils pretreated with varying concentrations of normal autologous serum (○) or anti-NA2 antiserum (●). (B) Initial rate of ingestion of opsonized lipopolysaccharide-paraffin oil particles by human neutrophils treated with varying concentrations of IgG prepared from rabbit anti-human leukocyte antiserum (●) or normal rabbit serum (○).

a ratio of 2–4 neutrophils per macrophage (Fig. 2C) which is representative of three experiments. The rate of nitroblue tetrazolium reduction was proportional to certain concentrations of various antisera used to sensitize the neutrophils (Fig. 2D) and the concentration of these sera which yielded maximal stimulation had the same effect in two separate experiments.

Various sera were screened for the presence of opsonic activity. The assays were performed as described in Methods and the results are shown in Fig. 3. The assay conditions were such that the initial rate of nitroblue tetrazolium reduction by macrophages in the presence of a saturating concentration of gradient-purified neutrophils was determined. Nitroblue tetrazolium reduction rates by macrophages incubated with neutrophils treated with autologous (neutrophil donor) serum were compared against reduction rates by macrophages suspended with neutrophils treated with test serum. The results are expressed as the percent change in initial reduction rates with respect to the autologous controls where the rate was $0.40 \pm 12 \mu\text{g formazan}/10^7 \text{ cells}/\text{min}$ (mean \pm SD). Nitroblue tetrazolium reduction rates by macrophages incubated with neutrophils treated with sera from normal individuals other than the neutrophil donor never exceeded that of macrophages incubated with neutrophils treated with autologous serum by more than 60% (Fig. 3). Sera from patients with congenital neutropenia or idiopathic thrombocytopenia did not opsonize any human leukocytes. Similarly, neutrophils from a donor with Rh + (D) erythrocytes were not opsonized by a high titer anti-Rh antibody. Anti-Na1, anti-NA2, anti-NB1, and anti-NC1 antisera and sera from frequently transfused patients reacted against certain donors' neutrophils. Opsonically active anti-NA2

antisera when tested on eight separate occasions and six of the sera from frequently transfused patients remained opsonic against those donor neutrophils on repeated testing. Anti-NA2 and 13 of the sera from frequently transfused patients found to be nonreactive opsonically against a given donor's neutrophils continued to be nonreactive on repeated testing. This specificity was observed in the other assays employed in this study. The opsonic activity of anti-NA2 antiserum was reduced from $0.86 \mu\text{g formazan}/10^7 \text{ cells}/\text{min}$ to 0.40 (basal rate) by incubation with neutrophils while incubation with lymphocytes did not alter opsonic activity.

Inhibition of Phagocytosis by Antineutrophil Antibodies. Certain human neutrophil preparations treated with anti-NA2 antiserum invariably exhibited reduced initial rates of ingestion of opsonized lipopolysaccharide-coated oil particles. As shown in Fig. 4A, no concentration of anti-NA2 antiserum stimulated ingestion relative to neutrophils previously incubated with autologous sera. On the other hand, as shown in Fig. 4B, low concentrations of rabbit anti-human leukocyte antisera or purified antileukocyte IgG stimulated particle uptake. An increase of 75–300% was observed in three separate experiments. Higher concentrations inhibited ingestion. Normal rabbit IgG did not demonstrate either of these effects. The effect of rabbit antileukocyte antiserum or IgG was tested on neutrophil ingested albumin-coated paraffin oil particles which are ingested more rapidly than lipopolysaccharide-coated particles without opsonization and can be opsonized either with C3 or with IgG. The biphasic effect of rabbit anti-human leukocyte antiserum was also observed on the ingestion rates of albumin particles which were not opsonized or which were treated with fresh human serum or with antialbumin

IgG. The concentrations of antileukocyte antiserum which inhibited ingestion of unopsonized albumin-coated particles were greater than those required to impair engulfment of albumin-coated particles treated with either fresh serum or with anti-albumin IgG. Rabbit antileukocyte IgG had the same effect as antileukocyte antiserum on the ingestion of unopsonized albumin-coated particles and of albumin-coated particles opsonized with anti-albumin IgG or fresh human serum (Fig. 5). Higher concentrations of antileukocyte IgG were required to produce inhibition of the ingestion rate of unopsonized particles than were needed to have these effects on opsonized particles.

Various sera were examined for the presence of anti-neutrophil activity by measuring impairment of phagocytosis (Fig 6). The results were expressed in relation to ingestion rates of opsonized lipopolysaccharide particles by neutrophils preincubated in autologous (the neutrophil donor) sera (0.32 ± 0.06 mg paraffin oil/ 10^7 cells/min) (mean \pm SD). Most homologous normal sera stimulated slightly the ingestion rate of particles by neutrophils, and none effected more than 6% inhibition. The ingestion rate of particles by neutrophils treated with sera from patients with congenital neutropenia were not different than ingestion rates of neutrophils treated with normal sera. The sera with anti-NA1, anti-NA2, anti-NB1, and anti-NC1 specificity reduced ingestion of particles by $47 \pm 8\%$ (mean \pm SD) of control values ($P < 0.001$). When anti-NA2 antiserum was absorbed with reactive neutrophils but not with lymphocytes, its inhibitory activity was completely removed. Rabbit anti-human leukocyte antisera at a ratio greater than $50 \mu\text{l}/10^7$ cells invariably inhibited phagocytosis ($38 \pm 6\%$ of control). Of sera from patients frequently

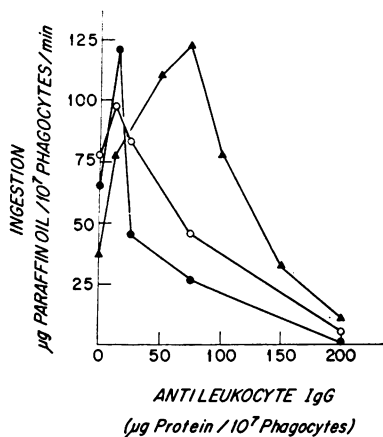


FIGURE 5 Initial ingestion rates of albumin-coated paraffin oil particles treated with heat-inactivated (▲), fresh human serum (○), or anti-human albumin IgG (●) by human leukocytes sensitized with varying concentrations of IgG prepared from rabbit anti-human leukocyte antiserum.

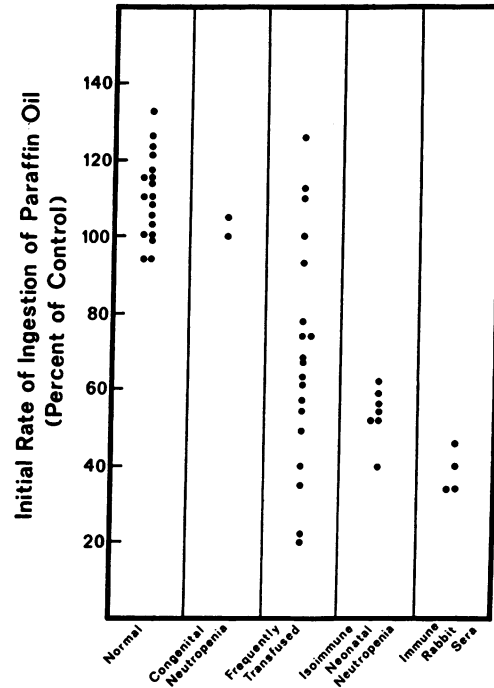


FIGURE 6 Initial rates of ingestion of opsonized lipopolysaccharide-paraffin oil particles by human leukocytes treated with various sera. The rates are expressed as percents of control rates (rates of ingestion by leukocytes treated with leukocyte donor serum equals 100%).

transfused, 26% failed to inhibit phagocytosis by neutrophils which were opsonized by these sera for ingestion by macrophages. However, as a group, the sera from frequently transfused patients did significantly ($P < 0.001$) impair ingestion of particles when compared to normal controls.

Cytotoxic Effects of Antigranulocyte Antibodies. The ability of antibody and fresh serum to lyse leukocytes was studied by measuring the release of ^{51}Cr from sensitized cells. Fresh plasma and serum were equally effective as sources of lytic activity for sensitized neutrophils provided that magnesium was added to the anticoagulated plasma. Omission of serum or plasma from the system or heating of serum or plasma for 30 min at 56°C eliminated all activity. It is likely, therefore, that the lytic property of serum in the presence of sensitized neutrophils involved and required complement components.

The kinetics of ^{51}Cr release in fresh serum and two homologous antisera is shown in Fig. 7A. The release of ^{51}Cr was constant with time for at least 60 min. It was directly proportional to the concentration of sensitized neutrophils (not shown). The amount of ^{51}Cr released was proportional to the quantity of heterologous or homologous antiserum utilized to sensitize the neutro-

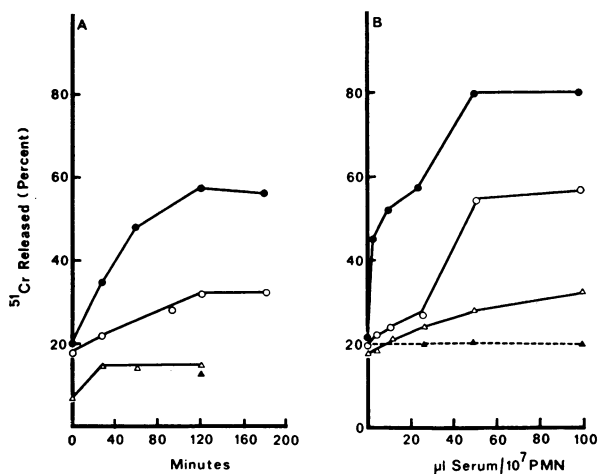


FIGURE 7 (A) Rate of ⁵¹Cr release from 10⁷ neutrophils suspended in 0.1 ml of fresh human plasma containing 5 mM EDTA to which 100 μl of anti-NA2 antiserum (●) or serum from a frequently transfused patient (○) was added. The reaction was initiated by adding MgCl₂ to a final concentration of 12.5 mM at zero time. Some incubations contained fresh plasma minus antiserum (△) or anti-NA2 antiserum and plasma minus magnesium (▲). (B) Effect of antiserum concentration on ⁵¹Cr release by human neutrophils. Neutrophils, 10⁷, suspended in fresh plasma containing 5 mM EDTA were incubated with the indicated concentrations of serum from normal humans (▲), rabbit antihuman leukocyte serum (●), anti-NA2 antiserum (○) or serum from a frequently transfused patient (△) for 120 min. The reaction was initiated by adding MgCl₂ to a final concentration of 12.5 mM at zero time.

phils (Fig. 7B). With concentrations exceeding 50 μl of antiserum/10⁷ neutrophils, there was no further effect on ⁵¹Cr release. The amount of fresh plasma employed as a complement source, 1 ml/10⁷ neutrophils, was found to be in excess of that required for maximal ⁵¹Cr-releasing activity by all antisera tested.

⁵¹Cr release by neutrophils in the presence of ten homologous normal sera and fresh plasma ranged between 1 and 6% with a mean of 2% and a SD of 5% above the autologous control mean of 19±3%. Thus, release of more than 12% of neutrophil-associated ⁵¹Cr (2 SD above the control mean which consisted of neutrophils incubated in autologous serum) was considered significant. The sera from 16 frequently transfused patients was assayed for ⁵¹Cr-releasing activity. These sera promoted phagocytosis of specific neutrophils by alveolar macrophages. The range of ⁵¹Cr released by these same neutrophils varied between 0 and 35% above the control with a mean of 15±10%, (Fig. 8); 47% of the sera from the frequently transfused patients failed to cause release of ⁵¹Cr. Anti-NA2, anti-NA1, and anti-NC1 and rabbit anti-human leukocyte antisera all lysed neutrophils in the presence of fresh plasma. The cytotoxic effect of

anti-NA2 antiserum activity against neutrophils was completely removed by absorption with neutrophils but was unaffected by absorption with lymphocytes.

Metabolic Effects of Anti-Neutrophil Antibodies. As shown in Fig. 9, sera from a patient frequently transfused and anti-NA2 antiserum had no effect on glucose oxidation by resting leukocytes. These sera promoted ingestion of the same leukocytes by alveolar macrophages. In addition, four opsonically active sera from frequently transfused patients did not influence resting [1-¹⁴C]glucose oxidation by human leukocytes. However, rabbit anti-human leukocyte antiserum stimulated the rate of [1-¹⁴C]glucose oxidation (Fig. 9). This effect was most marked with low antiserum concentrations although all concentrations of antiserum stimulated relative to untreated leukocytes. The increased rate of [1-¹⁴C]glucose oxidation with heterologous antiserum was not dependent upon the presence of the antiserum with the cells (Table

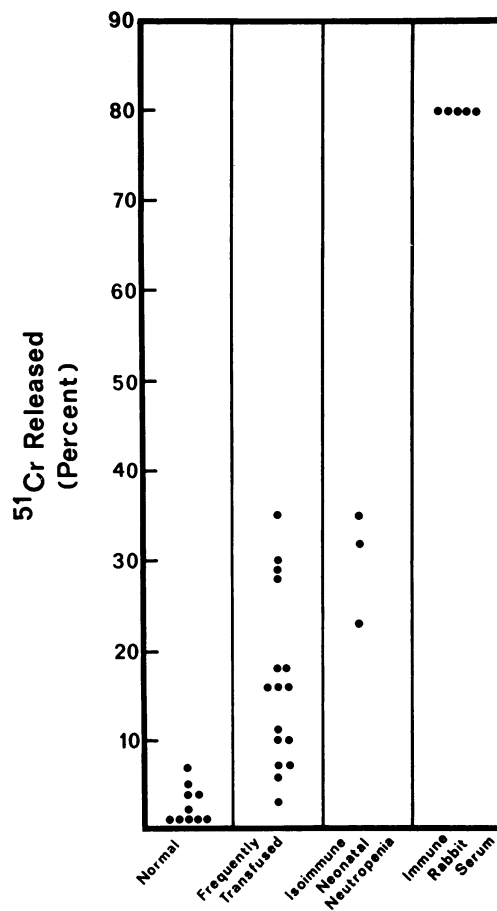


FIGURE 8 ⁵¹Cr released from human gradient purified neutrophils after 120 min treated with various sera. The amount of ⁵¹Cr released is expressed as percents above control (donor neutrophils incubated with donor serum).

I). Sensitized cells washed free of heterologous antiserum demonstrated enhanced glucose oxidation comparable to cells incubated in the presence of heterologous antiserum. Leukocytes incubated with [1-¹⁴C]glucose and 1 mM glucose and washed before addition of antiserum also manifested increased [1-¹⁴C]glucose oxidation upon the addition of rabbit anti-human leukocyte antiserum.

After incubation in autologous or normal homologous serum and washing, human leukocytes were able to increase ¹⁴CO₂ production at least tenfold upon the addition of polystyrene particles (Table I). Cells sensitized with rabbit anti-human leukocyte antiserum, anti-NA2 antiserum, and sera from two frequently transfused patients had impairment in ¹⁴CO₂ production from [1-¹⁴C]glucose after addition of polystyrene particles (Table I).

Leukoagglutination. Rabbit anti-human leukocyte antiserum or anti-NA1, anti-NA2, anti-NB1, and anti-NC1 antisera invariably agglutinated neutrophils. However, neutrophils treated with normal sera frequently clumped. Of the opsonically active sera from frequently transfused patients 44% agglutinated neutrophils. The agglutination was usually not marked and was difficult to differentiate from spontaneous aggregation of neutrophils in control sera.

Association of Lymphocytotoxic and Antineutrophil Antibodies. Sera from six frequently transfused patients were analyzed for the presence of lymphocytotoxic as well as antigranulocytic antibodies (Table II). Two

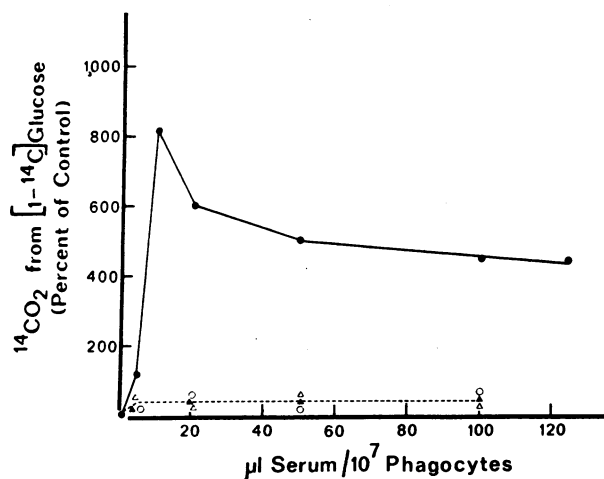


FIGURE 9 Effect of various sera on the rate of [1-¹⁴C]-glucose oxidation by human leukocytes. 1×10^7 leukocytes were suspended in 1.25 ml of KRP containing the indicated amounts of normal rabbit serum (Δ), anti-NA2 antiserum (\blacktriangle), serum from a frequently transfused patient (\circ), or rabbit anti-human leukocyte antiserum (\bullet). The sera were dialyzed against 0.13 M NaCl-15 mM sodium phosphate, pH 7.4, to remove glucose. The oxidation rates are expressed as percents relative to donor leukocytes incubated with autologous serum.

TABLE I
Effects of Various Sera on [1-¹⁴C]Glucose Oxidation by Resting Leukocytes and Leukocytes Incubated with Polystyrene Beads

	-Polystyrene	+Polystyrene
	¹⁴ CO ₂ cpm/10 ⁷ phagocytes/10 min	
Normal	166±97	2,114±216
Frequently transfused patient A	191±57	1,169±141
Frequently transfused patient B	223±39	1,205±128
Anti-NA2	197±33	928±193
Rabbit anti-human leukocyte	1,217±243	1,608±171

Leukocytes were incubated with test sera, 100 µl/10⁷ cells, and then washed as described in the text before measuring glucose oxidation. All sera were dialyzed with phosphate buffered saline, pH 7.4, to remove glucose.

sera with antineutrophil activity had no lymphocytotoxic antibodies. In two other sera, both lymphocytotoxic and antineutrophil antibodies were found. Two sera contained lymphocytotoxic antibody.

Correlation between Effects of Antineutrophil antibodies. Heterologous antileukocyte antisera opsonized all donors' neutrophils for ingestion by alveolar macrophages, inhibited ingestion of particles by all human neutrophils tested, and caused all human neutrophils to release ⁵¹Cr in the presence of fresh plasma. Anti-NA1, anti-NA2, anti-NB1, and anti-NC1 antisera either had all of these effects on neutrophils from given donors or had none of the effects. On the other hand, only 27% of sera from frequently transfused patients that were examined for antineutrophil antibodies had opsonic activity for selected donors' neutrophils. It is possible that if more donors were used that the percentage of sera with opsonic activity would have increased. Of those with opsonic activity for a given donor's neutrophils, only

TABLE II
Association of Lymphocytotoxic and Antineutrophilic Antibodies in Sera from Frequently Transfused Patients

Serum	Lymphocytes		Neutrophils		
	(Trypan blue uptake)	⁵¹ Cr release	⁵¹ Cr release	Inhibition of phagocytosis	Opsonization
1	+	+	-	-	-
2	+	+	+	+	+
3	-	-	+	+	+
4	+	+	-	-	-
5	-	-	+	+	+
6	+	+	+	-	+

74% of these sera also inhibited the ingestion of lipopolysaccharide-coated paraffin oil particles by these neutrophils. There was no correlation between the potency of opsonic activity and the ability to inhibit ingestion, although the sera with the strongest opsonic power all inhibited ingestion. A similar comparison between opsonic activity and cytotoxicity showed that half of the sera with opsonic activity failed to release ^{51}Cr from labeled neutrophils. There was no correlation between opsonic potency and the presence or absence of cytotoxic activity. All sera that had been found to inhibit ingestion by neutrophils or to release ^{51}Cr from neutrophils had also the effect of promoting phagocytosis of selected neutrophils by alveolar macrophages. On the other hand, sera from two frequently transfused patients with opsonic activity for homologous neutrophils did not promote opsonization or inhibit phagocytosis of these patients' own cells.

DISCUSSION

Lawrence, Craddock, and Campbell (1) and Simpson and Ross (2) administered rabbit anti-guinea pig neutrophil antiserum to guinea pigs and observed a prompt disappearance of circulating neutrophils. Histologic examination of the bone marrow and of lymphoid organs revealed neutrophils encased within phagocytic vacuoles in macrophages (2). These observations indicated that phagocytosis of sensitized neutrophils may have been an important mechanism responsible for the neutropenia following exposure to antineutrophil antibodies. In this study an *in vitro* system has been devised which permits quantitative analysis of immune phagocytosis of sensitized neutrophils. Rabbit alveolar macrophages, because of their large size, were found to be capable of engulfing human neutrophils coated with antineutrophil antibodies. Nitroblue tetrazolium reduction, a metabolic reaction that occurs in the phagocytic vacuole (22) and the rate of which is proportional to the rate of ingestion of particles (11) was measured to assay indirectly the initial rate of ingestion of sensitized neutrophils by the rabbit macrophages. The initial rate of nitroblue tetrazolium reduction by alveolar macrophages demonstrated first order kinetics and saturation with respect to the concentration of sensitized neutrophils. These characteristics were similar to kinetic features of other phagocytic systems which have been examined (23-26). Observation of the interaction between sensitized neutrophils and alveolar macrophages by light microscopy revealed engulfment of intact neutrophils by the macrophages, and nitroblue tetrazolium formazan was seen to accumulate in close apposition to the ingested neutrophils. Nevertheless, it is possible that some of the formazan extracted in the quantitative assays was formed due to ingestion of parts of sensitized neutrophils.

In any case, control experiments indicated that damage to or fragmentation of neutrophils possibly exerted by the sensitization procedure did not appear to cause neutrophils to be recognized by and ingested by macrophages. Conditions known to inhibit ingestion of particles by macrophages, low temperature and 1 mM *N*-ethylmaleimide (26), completely prevented reduction of nitroblue tetrazolium by macrophages incubated with sensitized neutrophils. All of these observations indicate that antineutrophil antibodies opsonized neutrophils for ingestion by macrophages and that the interactions between antibody and the neutrophil and between the sensitized neutrophil and the macrophage are amenable to quantitative analysis. The rate of nitroblue tetrazolium reduction by the macrophages was found to be proportional to the quantity of antiserum employed to sensitize neutrophils. When high concentrations of antiserum were tested, the nitroblue tetrazolium reduction rate reached a plateau suggesting that antigenic sites on the neutrophils were saturated with respect to antibody. The opsonic activity of antineutrophil sera, either from rabbits, from mothers of infants with isoimmune neonatal neutropenia or from frequently transfused patients was resistant to heating at 56°C for 30 min and was in the IgG fraction of serum suggesting that it was immunoglobulin. The opsonic activity of some sera was specific for neutrophils as evidenced by absorption studies and by failure of anti-Rh(D) or antiplatelet antibodies to opsonize neutrophils.

Ingestion of sensitized neutrophils occurs in the absence of added complement.¹⁴ Fresh serum with cytotoxic activity from some frequently transfused patients was no more effective than heated serum in promoting ingestion of neutrophils by macrophages. Thus, as suggested by Simpson and Ross (2), opsonization rather than lysis may be a major mechanism by which antineutrophil antiserum produces neutropenia *in vivo*. Similar findings have been described with red cells (28) and platelets (29).

Antimembrane antibodies are capable of modifying the phagocytic activity of neutrophils (30), of macrophages (31), and HeLa cells (32). This phenomenon was analyzed in considerable detail by Holland, Holland, and Cohn who, utilizing a morphologic assay for evaluation of ingestion, documented impaired phagocytosis of opsonized erythrocytes by mouse peritoneal macrophages treated with heterologous anti-mouse membrane antibodies (5). The availability of an assay for precise measurement of the initial rate of endocytosis permits a more quantitative analysis of the effects of anti-cell anti-

¹⁴ Macrophages have been shown to synthesize C2 and C4 (27). A contribution of complement introduced into the incubation by the alveolar macrophages has not been ruled out.

bodies on ingestion. Employing this approach, we confirmed and extended to human neutrophils the observations that heterologous anticell antisera can inhibit uptake of C3-coated or of IgG-coated particles. However, when low concentrations of heterologous anti-human leukocyte sera were used, a stimulatory effect on the ingestion rate of these particles was noted. The failure of Holland et al. to observe this phenomenon might reflect differences in species and types of phagocytes studied, or in the nature of the test particles, but most likely resulted from the difficulty in detecting enhanced ingestion with a semiquantitative assay in the setting of already efficient particle uptake. The results suggest that the effect of antisera that leads to stimulation of endocytosis involves cell surface antigens that are only recognized by heterologous antisera and implies that these antigens are common to all human neutrophils. The findings also suggest that the effects of anticell antibodies are more complex than the mere covering of phagocytic receptors.

Holland et al. concluded that the effects of anticell antibody were qualitatively selective for immunologically mediated endocytosis. Antimacrophage antisera inhibited the uptake of erythrocytes opsonized with C3 or IgG but did not appear to interfere with the uptake of formalin-treated erythrocytes, polystyrene particles, or yeast cell walls. The results were interpreted to indicate separation of recognition sites for opsonized and unopsonized particles. Such strict dissociation of receptor function was not achieved in this study of neutrophils. Heterologous antileukocyte antisera or IgG altered the initial rate of uptake of unopsonized albumin-coated paraffin oil particles by human neutrophils as well as of particles opsonized with C3 or IgG. Furthermore, the increment in $[1-^{14}\text{C}]$ glucose oxidation by neutrophils exposed to polystyrene beads has been shown by our studies and by others (9) to be blunted after treatment of the neutrophils with antineutrophil antisera. This inhibition probably reflects inhibition of ingestion of polystyrene beads, since the increment in glucose oxidation by neutrophils is directly proportional to the rate of ingestion of particles (25). The effect of antineutrophil antisera on the oxidative response to polystyrene particles was probably not secondary to impairment in glucose uptake, because the homologous antisera did not influence glucose oxidation rates in resting neutrophils, and the heterologous antisera stimulated these rates. It appears that in the case of neutrophils exposed to either homologous or heterologous antisera, ingestion of immunologically unmodified particles as well as of opsonized particles is diminished. However, a quantitative dissociation in receptor function was observed in our studies, in that higher antiserum or antibody concentrations were required to inhibit ingestion of unopsonized

particles than were needed to produce these alterations in ingestion of opsonized particles.

Rossi, Zatti, Patriarca, and Cramer reported that rabbit anti-guinea pig neutrophil antiserum stimulated oxygen consumption and hexose monophosphate shunt activity of guinea pig neutrophils (33). The increments in metabolic activity were comparable in magnitude to those occurring in neutrophils during ingestion of particles (34) or exposure to detergents (35). In this study rabbit anti-human leukocyte antiserum stimulated glucose oxidation not only in the presence of $[1-^{14}\text{C}]$ glucose but also after preincubation of the leukocytes with the labeled glucose. The source of the $^{14}\text{CO}_2$ was probably glycogen (36). Hence, the enhancement in glucose oxidation did not arise solely from increased membrane permeability. Although rabbit anti-human leukocyte antiserum stimulated glucose oxidation, homologous antineutrophil antisera with opsonic and antiphagocytic effects all failed to influence resting $[1-^{14}\text{C}]$ glucose oxidation by human leukocytes. In this respect, the increased glucose oxidation of neutrophils exposed to heterologous antisera is similar to their enhanced ability to ingest particles in the presence of small quantities of rabbit anti-human leukocyte antisera. Since heterologous antineutrophil antiserum increased glucose oxidation by the cells, whereas many potent homologous antisera did not enhance glucose oxidation, it is likely that the antigens responsible for the metabolic effect are common to all human neutrophils and distinct from those antigenic determinants (structures) recognized by the homologous antibodies.

As found for opsonic effects, the cytotoxic activity of antineutrophil sera was proportional to the quantity of antiserum added and was saturable with respect to antiserum concentration. The quantities of antiserum yielding maximal effects in the opsonic, antiphagocytic, and cytotoxic systems were roughly comparable. No chromium-releasing activity could be demonstrated by high concentrations of antineutrophil antisera in the absence of fresh serum containing free divalent cations. Therefore, the effects of these antisera on ingestibility of neutrophils by macrophages, impairment of neutrophil phagocytosis, and enhancement of neutrophil metabolic activity in the absence of added complement could not be attributed to cell damage as reflected by increased release of chromium label.

The specificity of the antibodies found in the sera from frequently transfused patients has not been defined, but from the available data certain generalizations can be made. Normal serum did not opsonize, inhibit phagocytosis of, or release ^{51}Cr from neutrophils of ABO-incompatible donors; therefore, it is unlikely that the major erythrocyte antigens have any influence on the studies presented here. Anti-Rh(D) antisera failed to op-

sonize neutrophils or inhibit phagocytosis by neutrophils from a donor with Rh(D) erythrocytes suggesting that the Rh(D) antigens were not found on the neutrophils tested. Similarly, sera from patients with idiopathic thrombocytopenia purpura failed to opsonize or inhibit phagocytosis of neutrophils. HL-A antigens have been demonstrated on neutrophils although in lower density than on lymphocytes (37). In this study and in another (38), sera from some frequently transfused patients lysed neutrophils but not lymphocytes in the presence of complement. Since HL-A antigens are usually defined by complement-mediated lysis in the presence of specific HL-A antisera, it might be concluded that certain neutrophil-cytotoxic antibodies are directed against antigens independent of the HL-A lymphocyte antigens. However, some HL-A antisera have failed to lyse lymphocytes in the presence of complement but were detectable by their ability to agglutinate the cells (39). The syndrome of neonatal neutropenia is the result of the transplacental transfer of maternal antibodies directed against neutrophils (6). The neutropenia resolves spontaneously with a time-course consistent with the rate of clearance of maternal gamma globulin (8). It is the most convincing example of a disease involving anti-neutrophil antibody. Agglutination or labeling of cells with fluorescent antibody have been used to demonstrate neutrophil specificity of the antibodies (6, 8, 10). Different antisera react only with neutrophils of certain individuals which has led to the definition of neutrophil-specific antigens designated NA1, NA2, NB1, and NC1 (10). The studies presented here analyzing other effects of sera from mothers of infants with neonatal neutropenia confirm the neutrophil specificity previously reported. It is likely that some of the sera from the frequently transfused patients reacted with the neutrophil-specific antigens. However, family studies will be needed to define the specificity and inheritance of the antigens interacting with sera from frequently transfused patients.

Although opsonically active sera from the mothers of infants with neonatal neutropenia consistently had cytotoxic effects, only half of the sera from frequently transfused patients with opsonic activity for the same neutrophils were cytotoxic. Of those sera with opsonic activity, 74% inhibited phagocytosis. Thus, measurement of the rate of phagocytosis by neutrophils in the presence of test sera might serve as a practical screening test for antineutrophil antibodies. Purification of neutrophils is not required because only neutrophils contribute significantly to the measured ingestion rates when mixed leukocytes are prepared from normal humans. Greater precision and sensitivity for detection of anti-neutrophil antibodies can be achieved by assaying ingestion of opsonized human neutrophils by rabbit alveolar macrophages.

ACKNOWLEDGMENTS

This work was supported by U. S. Public Health Service Grants AM-05581, AI-08173, and CA-13472 and a grant (1426-C-1) from the American Cancer Society Massachusetts Division, Inc.

REFERENCES

1. Lawrence, J. S., C. G. Craddock, Jr., and T. N. Campbell. 1967. Antineutrophilic serum, its use in studies of white blood cell dynamics. *J. Lab. Clin. Med.* **69**: 88.
2. Simpson, D. M., and R. Ross. 1971. Effects of heterologous antineutrophil serum in guinea pigs. Hematologic and ultrastructural observations. *Am. J. Pathol.* **65**: 79.
3. Chang, Y. H., and E. J. Gralla. 1968. Suppression of urate crystal-induced canine joint inflammation by heterologous anti-polymorphonuclear leukocyte serum. *Arthritis. Rheum.* **11**: 145.
4. Simpson, D. M., and R. Ross. 1972. The neutrophilic leukocyte in wound repair: a study with antineutrophilic serum. *J. Clin. Invest.* **51**: 2009.
5. Holland, P., N. H. Holland, and Z. A. Cohn. 1972. The selective inhibition of macrophage phagocytic receptors by anti-membrane antibodies. *J. Exp. Med.* **135**: 458.
6. Lalezari, P., M. Nussbaum, S. Gelman, and T. H. Spaet. 1960. Neonatal neutropenia due to maternal isoimmunization. *Blood J. Hematol.* **15**: 236.
7. Boxer, L. A., M. Yokoyama, and R. A. Wiebe. 1972. Autoimmune neutropenia associated with chronic active hepatitis. *Am. J. Med.* **52**: 681.
8. Boxer, L. A., M. Yokoyama, and P. Lalezari. 1972. Isoimmune neonatal neutropenia. *J. Pediatr.* **80**: 783.
9. McIntyre, P. A., Y. R. Laleli, B. A. Hodkinson, and H. N. Wagner, Jr. 1971. Evidence for anti-leukocyte antibodies as a mechanism for drug-induced agranulocytosis. *Trans. Assoc. Am. Physicians Phila.* **84**: 217.
10. Lalezari, P., G. B. Murphy, and F. H. Allen, Jr. 1971. NBI, a new neutrophil-specific antigen involved in the pathogenesis of neonatal neutropenia. *J. Clin. Invest.* **50**: 1108.
11. Stossel, T. P. 1973. Evaluation of opsonic and leukocyte function with a spectrophotometric test in patients with infection and with phagocytic disorders. *Blood J. Hematol.* **42**: 121.
12. Böyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* **21** Suppl. 97: 77.
13. Myrvik, Q. N., E. S. Leake, and B. Fariss. 1967. Studies on pulmonary alveolar macrophages from the normal rabbit: a technique to procure them in a high state of purity. *J. Immunol.* **86**: 128.
14. Moore, R. D., and M. D. Schoenberg. 1964. The response of histiocytes and macrophages in the lungs of rabbits injected with Freund's adjuvant. *Br. J. Exp. Pathol.* **45**: 488.
15. Stossel, T. P. 1973. Quantitative studies of phagocytosis. Kinetic effects of cations and heat-labile opsonins. *J. Cell Biol.* **58**: 346.
16. Stossel, T. P., C. A. Alper, and F. S. Rosen. 1973. Serum-dependent phagocytosis of paraffin oil emulsified with bacterial lipopolysaccharide. *J. Exp. Med.* **137**: 690.
17. Stossel, T. P. 1972. On the mechanism of action of heat-labile (HLD) and heat-stable (HSO) opsonins. *Blood J. Hematol.* **40**: 969. (Abstr.).

18. Aster, R. H., and S. E. Enright. 1969. A platelet and granulocyte membrane defect in paroxysmal nocturnal hemoglobinuria: Usefulness for the detection of platelet antibodies. *J. Clin. Invest.* **48**: 1199.
19. Stossel, T. P., F. Murad, R. J. Mason, and M. Vaughan. 1970. Regulation of glycogen metabolism in polymorphonuclear leukocytes. *J. Biol. Chem.* **245**: 6228.
20. Payne, R. 1965. An agglutination technique for the demonstration of leukocyte isoantigens in man. In *Histocompatibility Testing*. National Academy of Sciences-National Research Council, Publication 1229. Washington, D. C.
21. Terasaki, P. I., and J. D. McClelland. 1964. Microdrop-let assay of human serum cytotoxins. *Nature (Lond.)*. **204**: 998.
22. Nathan, D. G., R. L. Baehner, and D. K. Weaver. 1969. Failure of nitro blue tetrazolium reduction in the phagocytic vacuoles of leukocytes in chronic granulomatous disease. *J. Clin. Invest.* **48**: 1895.
23. Weisman, R. A., and E. D. Korn. 1967. Phagocytosis of latex beads by *Acanthamoeba*. I. Biochemical properties. *Biochemistry*. **6**: 485.
24. Mitchell, R. H., S. J. Pancake, J. Noseworthy, and M. L. Karnovsky. 1969. Measurement of rates of phagocytosis. The use of cellular monolayers. *J. Cell Biol.* **40**: 216.
25. Stossel, T. P., R. J. Mason, J. Hartwig, and M. Vaughan. 1972. Quantitative studies of phagocytosis by polymorphonuclear leukocytes: use of emulsions to measure the initial rate of phagocytosis. *J. Clin. Invest.* **51**: 615.
26. Mason, R. J., T. P. Stossel, and M. Vaughan. 1973. Quantitative studies of phagocytosis by alveolar macrophages. *Biochim. Biophys. Acta.* **304**: 864.
27. Colten, H. R. Synthesis and metabolism of complement proteins. *Transplant. Review*. In press.
28. Abramson, N., A. F. LoBuglio, J. H. Jandl, and R. S. Cotran. 1970. The interaction between human monocytes and red cells. Binding characteristics. *J. Exp. Med.* **132**: 1191.
29. Handin, R. I., and T. P. Stossel. 1974. Phagocytosis of antibody-coated platelets by polymorphonuclear leukocytes. *N. Engl. J. Med.* In press.
30. Stuart, A. E. 1962. Properties of antileukocyte serum. *Br. J. Exp. Pathol.* **43**: 614.
31. Jennings, J. F., and L. A. Hughes. 1969. Inhibition of phagocytosis by antimacrophage antibodies. *Nature (Lond.)*. **221**: 79.
32. Carey, F. J., and O. S. Pettengill. 1967. A time-lapse study of effects of anticellular antibody on membrane mobility and phagocytic activity of HeLa cells. *J. Cell Biol.* **33**: 709.
33. Rossi, F., M. Zatti, P. Patriarca, and R. Cramer. 1971. Effect of specific antibodies on the metabolism of guinea pig polymorphonuclear leukocytes. *J. Reticuloendothel. Soc.* **9**: 67.
34. Sbarra, A. J., and M. L. Karnovsky. 1959. The biochemical basis of phagocytosis. I. Metabolic changes during the ingestion of particles by polymorphonuclear leukocytes. *J. Biol. Chem.* **234**: 1355.
35. Graham, R. C., Jr., M. J. Karnovsky, A. W. Shafer, E. A. Glass, and M. L. Karnovsky. 1967. Metabolic and morphological observations on the effect of surface-active agents on leukocytes. *J. Cell Biol.* **32**: 629.
36. Scott, R. B. 1968. Glycogen in human peripheral blood leukocytes. I. Characteristics of the synthesis and turnover of glycogen in vitro. *J. Clin. Invest.* **47**: 344.
37. Kourilsky, F. M., D. Silvestre, J. P. Levy, J. Dausset, M. G. Nicolai, and A. Senik. 1971. Immunoferritin study of the distribution of HL-A antigens on human blood cells. *J. Immunol.* **106**: 454.
38. Hasegawa, T., R. G. Graw, and P. I. Terasaki. 1973. A microgranulocyte cytotoxicity test. *Transplantation*. **15**: 492.
39. Zmijewski, C. M., J. L. Fletcher, and W. G. Cannady. 1970. The serologic relationship of 4^a and 4^b to the HL-A system. In *Histocompatibility Testing*. Paul Terasaki, editor. The Williams and Wilkins Company, Baltimore, Md. 277.