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Research Article

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Interaction of Desialated Guinea Pig Erythrocytes with the Classical and Alternative Pathways of Guinea Pig Complement In Vivo and In Vitro

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ABSTRACT We examined the fate of desialated autologous erythrocytes injected intravenously into guinea pigs (GP). Desialated GP erythrocytes (E) were lysed directly or cleared by the reticuloendothelial system in normal GP (NIH-GP) and cleared by the reticuloendothelial system in GP genetically deficient in the classical complement pathway component C4 (C4D-GP), which activate complement only via the alternative pathway. Desialated E were also cleared in cobra venom factor-treated GP (CVF-GP), which had <1% of normal C3 levels, but were not cleared at all in C4D-CVF-GP. Preinjection of asialoorosomucoid (ASOR) and ovalbumin (OVA) had no effect on the rate of E clearance. These in vivo studies indicated that complement activation is essential for clearance of desialated E and that clearance is unaffected by blockade of galactose or mannose receptors. Inhibition of complement-mediated clearance required blockade of both classical and alternative complement pathways. In vitro studies showed that lysis of desialated E could occur in NIH-GP serum (GPS) but not in C4D-GPS. Surprisingly, CVF-GPS also caused lysis of desialated E. Lysis was dependent on both natural antibody to desialated E and classical pathway activation; natural antibody was of both the IgG and IgM classes. C3 uptake studies demonstrated that almost 10 times as many C3 molecules/E were deposited by NIH-GPS as by C4D-GPS or CVF-GPS onto desialated E. Approximately equal numbers of C3 molecules were deposited by CVF-GPS, which did lyse desialated E, and by C4D-GPS, which did not. We suggest that the molecular mechanism of in vivo clearance and in vitro lysis of desialated E by CVF-GP is via classical pathway deposition of C3b into sites on the erythrocyte surface protected from inactivation by H (β1H) and I (C4b/3b inactivator). Deposition of C3b into these sites by alternative pathway activation is sufficient to cause clearance but not lysis of desialated E. CVF-GPS may not represent an adequate reagent for testing the complement dependence of various biologic phenomena, particularly if the question involves surfaces that can provide protected sites for C3b molecules.

INTRODUCTION

Loss of terminal sialic acids from the surface carbohydrates of hematopoietic cells and from the carbohydrate side chains of serum glycoproteins results in the rapid removal of both cells (1) and proteins (2) from the blood. It has been proven that desialated proteins are removed from the bloodstream in mammals via a hepatocyte receptor that recognizes a glycoprotein-bound galactose residue exposed by desialation (3). This hepatocyte cell surface receptor has also been shown to play a part in the clearance of certain types of immune complexes (4, 5). In addition, this receptor has been implicated in the clearance of certain desialated cells and particles from the blood stream (6). However, its role in normal physiology has never been definitively established. One major theory about its function is that binding to this receptor may be important in the destruction of senescent erythrocytes, which have been shown to lose sialic acid as they age (7). Indeed, Kolb et al. (8) have shown that hepatocytes will bind desialated erythrocytes via their galactose receptors.

It has been shown that cell-surface sialic acids play an important role in the control of complement activation (9, 10). Desialated sheep erythrocytes activate the human complement system via the alternative pathway as do certain mouse erythrocytes that have genetically controlled low levels of cell surface sialic

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acid (11). However, these studies were unable to demonstrate that desialated human erythrocytes activate the alternative pathway in human serum (12), so whether or not desialation of autologous cells caused activation of the alternative pathway was not established. Thus, the relevance of these observations to a potential role for complement in the in vivo clearance of desialated, senescent erythrocytes remains unknown.

We postulated that, if the alternative pathway were activated by desialated homologous erythrocytes in vivo, erythrocyte-bound C3b might play a major role in the clearance of desialated cells from the circulation in a complement-dependent interaction with the fixed mononuclear phagocytes of the reticuloendothelial system (RES).[1] In the experiments presented here, we tested this hypothesis in a well-described model of erythrocyte clearance (13, 14). We have shown that desialated guinea pig erythrocytes (E) can activate the alternative pathway in guinea pig (GP) serum (GPS), and that this complement activation is essential for clearance of desialated E from the circulation. However, activation of the alternative pathway does not lead to cell lysis. Desialated E also activate the classical complement pathway in GPS, mediated by naturally occurring antibody to the desialated cells. This classical pathway activation is very efficient and does mediate cell lysis, even after depletion of complement by cobra venom factor to levels <1% of normal. Thus, the effects of complement activation by classical and alternative pathway are dramatically different. Classical pathway activation leads to erythrocyte lysis or clearance in vivo and to lysis in vitro; alternative pathway activation deposits sufficient C3 onto desialated E to cause in vivo clearance but cannot mediate in vitro erythrocyte lysis.

METHODS

Buffers and chemicals. Veronal-buffered saline (VBS), VBS containing Ca^{++}, Mg^{++}, and gelatin ($GVBS^{++}$), VBS containing 10 mM EDTA and gelatin (EDTA-GVBS), and a dextrose containing low-ionic strength isoosmotic buffer (6 mS, 0°C) ($DGVBS^{++}$) were prepared as previously described (15). Desialation buffer was 0.13 M NaCl, 0.05 M Na acetate, pH 6.5 (9). Sterile phosphate-buffered saline (PBS) and 0.15 M NaCl (NS) were obtained from the Media Unit, National Institutes of Health. Ovalbumin (OVA) and mannan were purchased from Sigma Chemical Co., St. Louis, MO.

Complement components. Purified GP C1, C4, and C2, and cobra venom factor (*Naja naja*) were obtained from Cordis Laboratories, Miami, FL. GP C3 was prepared as previously described (16). Aliquots of GP C3 were radiolabeled with sodium borohydride (New England Nuclear, Boston, MA) to a specific activity of 6.8×10^5 dpm/μg (17) and with the Bolton Hunter reagent to a specific activity of 2×10^5 dpm/μg, both without loss of hemolytic activity.

Animals. Outbred female 300–400-g GP both complement sufficient (NIH-GP) and genetically deficient in C4 (C4D-GP) (18) were obtained from the Small Animals Section, National Institutes of Health. Some NIH-GP and C4D-GP were injected intravenously with 200 U/kg cobra venom factor (CVF-GP, C4D-CVF-GP) 16 h before an experiment. Using this regimen, these GP had C3 titers at the time of an experiment that were 0.1–1% of normal levels using a standard hemolytic assay (19).

GP serum. Serum was obtained by cardiac puncture from NIH-, C4D-, and CVF-GP. Serum from three to five animals in each group was pooled, aliquoted into 1-ml portions, and stored at −70°C until use. The CVF-GP-serum pool had a C3 titer that was 0.7% of the NIH-GPS and the C4D-GPS pools. The C4D- and CVF-GPS pools had total hemolytic complement (CH_{50}) = 0 (NIH-GPS pool, CH_{50} = 457).

Desialation of E. E, freshly drawn via cardiac puncture into 100 mM EDTA, were desialated by a modification of the method of Fearon (9). Freshly drawn E were washed twice in EDTA-DGVB and then four times in desialation buffer. Washed E were then resuspended to 2.5×10^9/ml and incubated with an appropriate amount of neuraminidase (type X, <0.017 U/mg protease, <0.006 U/mg aldolase, Sigma Chemical Co.) for 30 min at 37°C (desialated E). To control E were added either desialation buffer alone or known amounts of *N*-acetyl neuraminic acid in buffer (sham-desialated E). After incubation, the tubes were immediately chilled to 0°C and centrifuged at 1,000 *g* in the cold. Supernatants were saved for assay of free sialic acid by the method of Warren (20). A standard curve of color vs. sialic acid content was developed from the tubes to which known amounts of *N*-acetylneuraminic acid had been added without enzyme. Total cell-surface sialic acid was determined by the method of Varki and Kornfeld (21). After desialation, erythrocytes were washed four times in ice-cold NS and finally resuspended to 1×10^8/ml in NS.

E chromation. E, either desialated or sham-desialated, were incubated at 1×10^8 E/ml with 25 μCi/ml $Na_2\ ^{51}CrO_4$ (Amersham Corp., Arlington Heights, IL) for 30 min at 37°C, and then washed twice with NS.

Desialation of orosomucoid. Orosomucoid (α_1-acid glycoprotein) (OR) was the kind gift of the Plasma Derivatives Laboratory, American Red Cross, Bethesda, MD. For desialation, 250 mg OR was suspended at 50 mg/ml in 0.15 M sodium acetate pH 5.0 to which 1 U of insolubilized neuraminidase (Sigma Chemical Co.) was added. The incubation proceeded at 37°C overnight, following which the neuraminidase beads were removed by centrifugation, and the supernatant containing the desialated protein (ASOR) and free sialic acid was applied to a 45-ml Sephadex G-25 column. Fractions were assayed for free sialic acid by the method of Warren and for protein by absorption at 280 nm. Approximately 13 mol of sialic acid were removed per mole of protein. Aliquots of ASOR and mannan were radiolabeled with ^{125}I using iodobeads (Pierce Chemical Co., Rockford, IL).

[1] *Abbreviations used in this paper:* ASOR, asialo orosomucoid; C4D-GP, guinea pigs genetically deficient in C4; CVF-GP, NIH-GP injected intravenously with 200 U/kg cobra venom factor; C4D-CVF-GP, C4D-GP injected intravenously with 200 U/kg cobra venom factor; $DGVBS^{++}$, dextrose containing low ionic strength isoosmotic buffer; E, guinea pig erythrocytes; EDTA-GVBS, VBS containing 10 mM EDTA and gelatin; GP, guinea pig; GPS, guinea pig serum; $GVBS^{++}$, VBS containing Ca^{++}, MG^{++}, and gelatin; NIH-GP, complement sufficient outbread female GP; NS, normal saline; OR, orosomucoid; OVA, ovalbumin; RES, reticuloendothelial system; VBS, veronal-buffered saline.

In vivo erythrocyte clearance and destruction. GP were injected intravenously with 10^8 chromium-labeled E, desialated or sham desialated, in 1 ml NS. Samples of blood were removed into heparinized tubes at timed intervals after injection by bleeding from the retroorbital plexus. 100-μl aliquots of whole blood were then assayed for ^{51}Cr radioactivity. Samples were centrifuged at 12,000 *g* for 1 min, plasma and erythrocytes separated, and ^{51}Cr in the cell pellet and the plasma were determined separately. Whole blood radioactivity at time 0 was calculated by assuming a blood volume equal to 6% of GP body weight. This figure had been determined in preliminary experiments by injection of ^{51}Cr-labeled normal E. In some experiments GP were injected with 10 mg ASOR, 20 mg OVA, or NS 5 min before erythrocyte injection. To determine the organs by which E were removed, animals were killed by CO_2 narcosis 1 h after injection, and the ^{51}Cr in liver, lung, spleen, and blood quantitated.

In vitro lysis of E. Chromium-labeled E, desialated or sham desialated, were incubated at a concentration of 1.5 $\times$ 10^7/ml with varying amounts of GPS and GVBS^{++} in a total volume of 225 μl for 10 min at 37°C. Cells were immediately pelleted after incubation by centrifugation at 12,000 *g* for 2 min. A measured aliquot of the supernatant was removed for determination of ^{51}Cr radioactivity and used to quantitate erythrocyte lysis. For some experiments GPS was absorbed before use by two 10-min incubations at 0°C with 10^9 desialated E/ml GPS. In other experiments Mg EGTA-GPS was prepared by bringing GPS to a final concentration of 4 mM Mg^{++} and 10 mM EGTA.

Antibody to desialated E. To test for agglutinating antibody to desialated E in NIH-, C4D-, and CVF-GPS, 50 μl of desialated E at 6 $\times$ 10^7/ml were incubated with 50 μl of serial twofold serum dilutions in PBS in microtiter plates, and the inverse of the highest dilution giving 4+ agglutination was considered the serum-agglutinating antibody titer. The titers of the NIH-, C4D-, and CVF-GPS pools were each 50, and individual GP sera varied from 25 to 100. There were no differences in mean titer among NIH-GP, C4D-GP, and CVF-GP.

Separation of GP IgG and IgM. A 5–18% polyethylene glycol precipitate of 50 ml of GP plasma (polyethylene glycol 4000, J. T. Baker Co., Phillipsburg, NJ) was resuspended in PBS and applied to a 1.5-liter Biogel A 0.5-m column (Bio-Rad Laboratories, Richmond, CA). The IgG and IgM peaks from the column, as determined by double diffusion against monospecific antisera, were concentrated and reapplied to Sepharose 6B-CL (Pharmacia Fine Chemicals, Piscataway, NJ) (IgM) or Biogel A 1.5 m (IgG). The relevant fractions were again pooled and concentrated to approximately serum concentration. No cross-contamination of the IgG and IgM pools was detectable by Ouchterlony analysis. C1 fixation and transfer was performed using these antibody pools, or using rabbit IgG and IgM anti-E (13). The test was done by a modification of the traditional method of Borsos and Rapp (22) as previously described (23).

Rabbit anti-GP C3. Rabbits were immunized with 100 μg purified GP C3 in complete Freund's adjuvant. 2 wk later, they were boosted with GP C3 in incomplete Freund's adjuvant and subsequently bled via cardiac puncture. Ouchterlony analysis and immunoelectrophoresis revealed a single precipitin band vs. whole GPS, which showed complete identity with the precipitin formed with purified C3. Precipitin lines with both C3c and C3d were observed when this antibody was used in immunoelectrophoresis with aged GPS. The IgG fraction of this antiserum was prepared by octanoic acid precipitation (24) and radiolabeled with ^{125}I by iodobeads to a specific activity of 3 $\times$ 10^5 cpm/μg. Before use, ^{125}I-IgG anti-GP C3 was extensively absorbed with E and desialated E.

C3 uptake studies. In vitro C3 uptake onto desialated and sham-desialated E was assessed in the following manner: 1 $\times$ 10^8 E were pelleted, resuspended in 250 μl of GPS, and incubated at 37°C for various times. Duplicate tubes were incubated for each time point. After incubation, 1 ml of ice-cold EDTA-GVBS was added to the reaction tubes. The cells were centrifuged at 12,000 *g*, washed once with cold GVBS^{++} and once with cold DGVBS^{++}, resuspended to 1 $\times$ 10^8/ml in DGVBS^{++}, and incubated with 8.2 μg of ^{125}I-labeled IgG anti-GP C3 in DGVBS^{++}. After a 1-h incubation at room temperature, an aliquot of cells and antibody was layered onto 1 ml Versilube F-50 (General Electric Co., Waterford, NY) and centrifuged at 12,000 *g* for 2 min. The supernatant was aspirated, tubes cut, and cell pellet-associated radioactivity was determined. In initial experiments, standard curves were developed using sheep erythrocytes bearing varying amounts of ^{3}H-GP C3. Uptake of ^{125}I anti-GP C3 in this assay was compared directly with the number of [^{3}H]C3 molecules bound to cells. For inputs varying from 30 C3b/E to 1,800 C3b/E, the specific uptake of anti-C3 was always between 0.90 and 0.97 IgG/C3b. To determine the number of C3 molecules bound to E after serum incubation, uptake of ^{125}I-IgG onto the E was compared to the standard curve. As a control for nonspecific antibody binding to GPE, desialated E were incubated in GPS containing 20 mM EDTA, and sham-desialated E were incubated in normal GPS. Antibody binding to these controls was subtracted from total binding to determine specific anti-C3 binding in each experiment before comparison to the standard curve. Binding of radiolabeled anti-C3 to control cells was ~10% of the binding to desialated E, which had been incubated in GPS for 2–5 min.

RESULTS

Desialation of E. E were incubated under the described conditions with between 4 $\times$ 10^{-4} U (0.4 mU) and 5 $\times$ 10^{-2} U (50 mU) of neuraminidase. This led to a dose-dependent release of sialic acid from the cells, which was very reproducible from day-to-day (Fig. 1). Four-tenths of a milliunit of neuraminidase cleaved ~1 $\times$ 10^6 molecules of sialic acid per cell, and 50 mU cleaved ~20 $\times$ 10^6 molecules per E during the period of incubation. Total E cell surface sialic acid was determined to be ~32 $\times$ 10^6 molecules per cell (range of four GP: 25–55 $\times$ 10^6 sialic acid residues per cell).

In vivo clearance of desialated E. Chromated E, treated with various doses of neuraminidase or sham incubated, were injected intravenously into GP, and their clearance was monitored by following the disappearance of radioactivity from whole blood and plasma during a 2-h period. Treatment of E with 2 mU neuraminidase caused an initial disappearance of ~20% of the injected dose from the bloodstream. This was followed by release of the cells so that there was negligible net clearance of erythrocytes 30 min after injection (Fig. 2). Doses of 10 mU neuraminidase or higher were associated with intravascular lysis of E in NIH-GP, as judged by the appearance of significant amounts of ^{51}Cr in the plasma by 5 min after injection

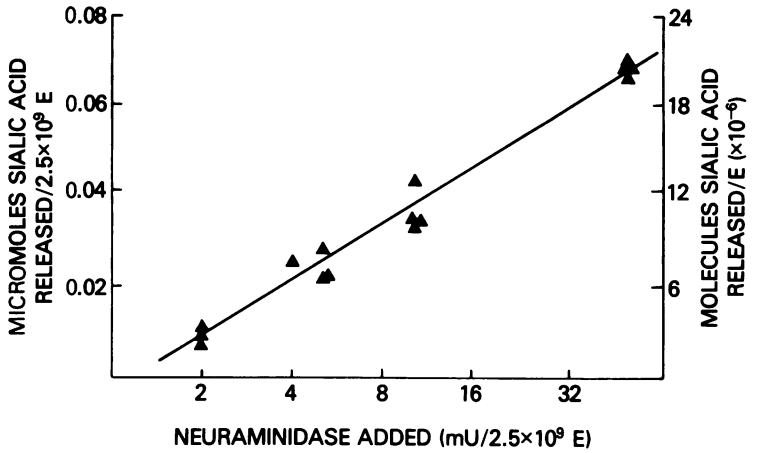

FIGURE 1 Dose response of neuraminidase incubation on removal of sialic acid from E. E were incubated at 2.5 × 10^9/ml with varying amounts of neuraminidase. Sialic acid release was quantitated by the thiobarbituric acid method (20). Incubation with 2 mU cleaved ~3 × 10^6 sialic acid molecules from each E; incubation with 50 mU cleaved ~20 × 10^6 molecules/cell. Total cell surface sialic acid averaged 32 × 10^6 molecules/E.

(insert, Fig. 2). In contrast, when C4D-GP and CVF-GP were injected with E treated with 10 mU neuraminidase, no intravascular lysis occurred. Desialated E were efficiently cleared from the bloodstream in both these groups of animals (Fig. 3 A). Sham-desialated E were not removed significantly from the blood in any group of GP. To investigate the mechanism of E clearance in C4D- and CVF-GP, some GP were injected with ASOR or OVA 5 min before injection with desialated E, and some C4D-GP were injected with CVF 16 h before the erythrocyte clearance study (two animals each group).

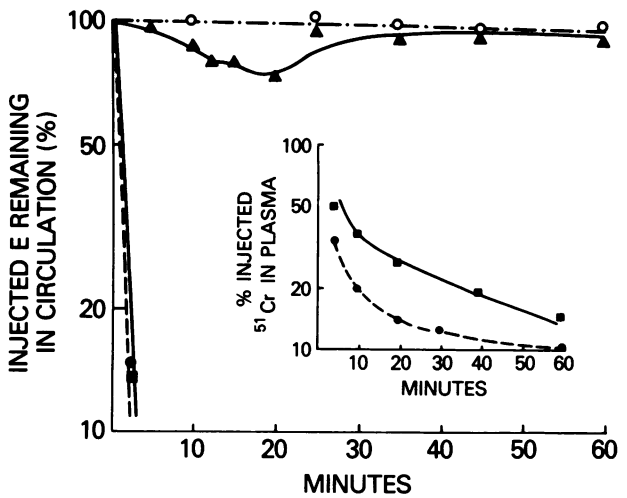

FIGURE 2 Clearance of intravenously injected desialated E in NIH-GP. NIH-GP were injected with E that had been incubated with 2 mU (▲), 10 mU (●), 50 mU (■) per 2.5 × 10^9 cells, or with buffer alone (○). The clearance of E from the bloodstream was quantitated by serial determinations of ^{51}Cr radioactivity in measured aliquots of blood. While sham-treated E were not cleared, E incubated with 2 mU neuraminidase were transiently removed from the circulation. E incubated with higher amounts of neuraminidase had a very short survival in the blood stream, but considerable ^{51}Cr was detectable in plasma (insert) indicating intravascular lysis of these cells. There were three to four GP in each group.

The doses of ASOR and OVA used were sufficient to inhibit the clearance of ^{125}I-ASOR and ^{125}I-mannan, respectively (Table I). Preinjection of ASOR increased the circulating half-life of ^{125}I-ASOR from <2 min to 48 min, but the half-life of the radiolabeled asialoglycoprotein was unaffected by preinjection of equal amounts of OR, 20 mg OVA, or 20 mg mannan. Preinjection of OVA increased the circulating half-life of ^{125}I-mannan from <2 min to 38 min, equivalent to the prolongation observed by preinjection of 20 mg mannan. There was no difference between C4D-GP and NIH-GP in the carbohydrate blockade effects of ASOR and OVA. However, neither ASOR nor OVA preinjection had any effect on the rate of clearance of desialated E in C4D or CVF-GP (Fig. 3B). In contrast, C4D-CVF-GP could not remove desialated E from the bloodstream (Fig. 3 A). Localization studies showed that of the ^{51}Cr removed from the bloodstream in CVF-GP 75.3±2.5% (SD) was in the liver, and 15.0±1.7% (SD) was in the spleen (n = 4). This was not different from the 78% of cleared ^{51}Cr E in the liver and 15% in the spleen (n = 2) of C4D-GP. There was no significant pulmonary sequestration of desialated E 1 h after injection in either CVF-GP or C4D-GP. Thus, the vast majority of desialated E were cleared by the splanchnic reticuloendothelial organs, but clearance was not dependent on the hepatocyte ASOR (galactose) receptor

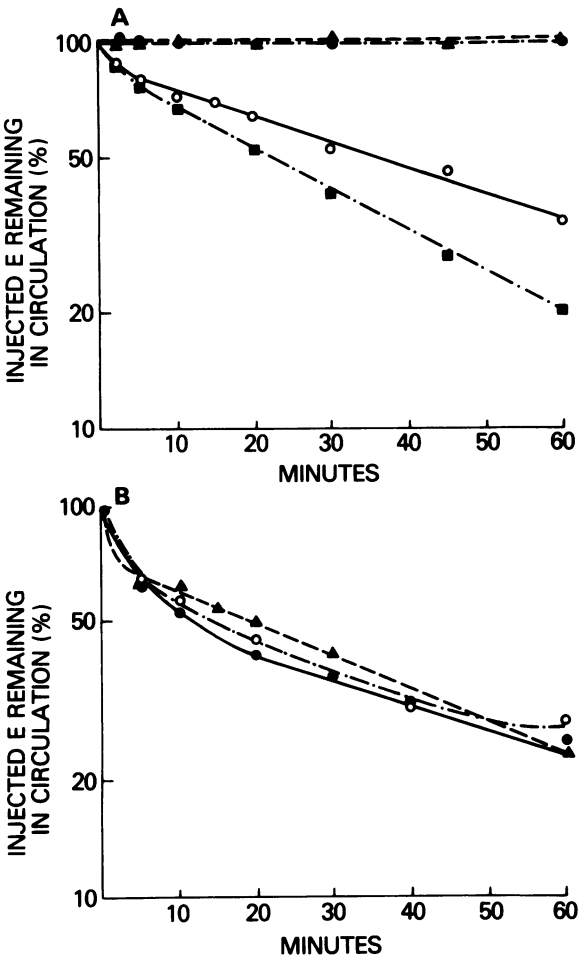

FIGURE 3 Clearance of desialated E in C4D-GP and CVF-GP. In panel A, the clearance of E from which 10×10^6 molecules of sialic acid have been removed (10 mU neuraminidase/2.5×10^9 E) is plotted vs. time. There is significant clearance of these cells in C4D-GP (■) and CVF-GP (○) but no clearance in C4D-CVF-GP (▲). The clearance of sham-desialated E in C4D-GP (●) is also shown for comparison. There were two to four animals in each group. Panel B shows the clearance of similarly desialated E in CVF-GP, which had been injected intravenously with 10 mg ASOR (○), 20 mg OVA (▲), or saline (○) 5 min before challenge with E. There are no differences in clearance in the three groups of GP ($n = 2$ for each group). Clearance in C4D-GP was similarly unaffected by OVA or ASOR.

TABLE I
*Effect of ASOR and OVA on Carbohydrate Receptor Clearance in GP**

Preinjection	Radiolabeled ligand	$t_{1/2}$
		min
Saline	^{125}I-ASOR	<2
OR, 10 mg	^{125}I-ASOR	<2
ASOR, 10 mg	^{125}I-ASOR	48
OVA, 20 mg	^{125}I-ASOR	<2
Saline	^{125}I-Mannan	<2
ASOR, 10 mg	^{125}I-Mannan	<2
OVA, 20 mg	^{125}I-Mannan	38
Mannan, 20 mg	^{125}I-Mannan	36

* GP were injected with saline or the indicated carbohydrate containing ligand 5 min before injection with ~5 μg of ^{125}I-ASOR or ^{125}I-Mannan. ASOR preinjection inhibited the asialoglycoprotein receptor. There were two to three animals in each group.

or the Kupffer cell OVA (mannose) receptor. In contrast, as shown by the failure of C4D-CVF-GP to clear these cells, complement activation was required for the sequestration of desialated E.

In vitro studies of desialated E lysis. The studies of E clearance demonstrated that desialated E were capable of activating the alternative pathway through C3 in vivo since they were sequestered in C4D-GP, but not in C4D-CVF-GP. To study this phenomenon further, experiments were carried out in vitro using serum from NIH-, C4D-, and CVF-GP. E that had ~3×10^6 molecules of sialic acid removed per cell (2 mU neuraminidase/2.5×10^9 E) were not lysed by NIH-GPS during a 10-min incubation. However, E from which 10 million or more sialic acid molecules had been cleaved (10 mU neuraminidase or more) were very efficiently lysed in NIH-GPS (Fig. 4 A). E lysis by NIH-GPS was totally inhibited by 4 mM Mg^{++} in 10 mM EGTA, which blocks classical complement pathway activation. Moreover, no E lysis occurred in C4D-GPS (Fig. 4 B). Thus, lysis of E by NIH-GPS required classical pathway activation. To investigate further the apparent inability of the alternative pathway to lyse desialated E, the incubation in C4D-GPS was extended to 1 h. E from which different amounts of sialic acid had been removed (3×10^6, 10×10^6, and 20×10^6 molecules/E using 2, 10, and 50 mU neuraminidase) were incubated in two concentrations (1.5×10^7/ml and 5×10^6/ml) with varying amounts of C4D-GPS. In no circumstance was erythrocyte lysis observed (Fig. 4 C).

The role of antibody in lysis of desialated E. To determine whether classical pathway activation leading to E lysis required antibody or if desialated E could activate the classical pathway directly (25), NIH-GPS was absorbed at 0°C with desialated E. This absorption abolished the lytic activity of the NIH-GPS for desialated E, but left undisturbed the lysis of normal or desialated E presensitized with rabbit antibody (Fig. 5 A). Thus, the ability of desialated E to activate the classical pathway was dependent on a factor absorbable at 0°C. The lytic ability of GPS for desialated E could be restored by preincubating desialated E with

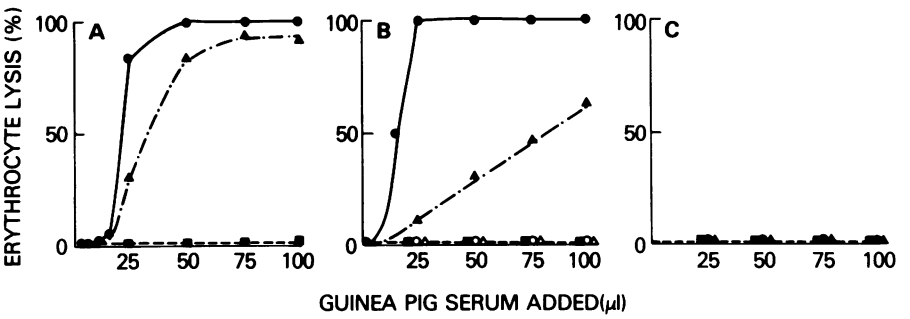

FIGURE 4 Lysis of desialated E by GPS. In panel A, the lysis of desialated E is plotted as a function of the quantity of NIH-GPS added to the incubation for cells exposed to 2 mU (■), 10 mU (▲), and 50 mU (●) per 2.5×10^9 E. In panel B, the lysis of desialated E in NIH-GPS (●) C4D-GPS (■), and CVF-GPS (▲) is again plotted as a function of serum concentration. NIH-GPS and CVF-GPS support desialated E lysis but C4D-GPS does not. Mg-EGTA blocks lysis in both NIH-GPS (○) and CVF-GPS (△). In panel C, the incubation with C4D-GPS has been extended to 1 h for E desialation by 2 mU (■), 10 mU (●), and 50 mU (▲) neuraminidase. There is no lysis in C4D-GPS even if E concentration is decreased to 5×10^6/ml.

either IgG or IgM purified from NIH-GPS (Fig. 5 B). Moreover, both the IgG fraction and the IgM fraction of GPS were shown to be capable of fixing C1 in the C1 fixation and transfer test (IgG slope = 2.0, IgM slope = 1.0) (Fig. 6 A). To ascertain whether the antibodies were directed against some component of the neuraminidase preparation that adsorbed to the E, 1 ml of 10% GPS was absorbed twice at 0°C with 0.5 ml packed neuraminidase-agarose or an equivalent amount of uncoupled agarose as a control. The absorbed sera were tested for their ability to mediate lysis of desialated E. There was no difference between the neuraminidase-absorbed and sham-absorbed GPS in either of these tests. Thus, natural antibodies to desialated E, capable of activating the classical complement pathway and present in both the IgG and IgM fractions of GPS, were required for in vitro lysis of desialated E.

Ability of xenoantisera to lyse normal and desialated E. The results of the previous experiments implied that even the small residual amount of C3 in CVF-GPS (0.7% of normal C3 levels) was sufficient to activate C5–9 and cause in vitro erythrocyte lysis and in vivo erythrocyte clearance when the classical pathway was activated by desialated E. To test whether this was because desialated E interacted differently with complement or were inherently easier to lyse than normal E, several experiments were performed. First, use of rabbit IgG and IgM anti-E in the C1 fixation and transfer test showed that the amount of C1 fixed by each dilution of antibody was the same for normal and for desialated E (Fig. 6 B). This implied that the rabbit antibodies bound equally well to normal and desialated E, and fixed complement similarly on the two surfaces. Second, when rabbit IgM was used to sensitize desialated and normal E followed by purified complement components C1, C4, C2, and ^{125}I-C3, the uptake of radiolabel was the same on normal and desialated cells. Third, lysis of rabbit antibody-sensitized E in NIH-GPS, which had been absorbed at 0°C with desialated E to remove natural antibodies, was equivalent for desialated and sham-desialated E (Fig. 5 A). These experiments demonstrated that desialated E did not interact differently with complement and were not more susceptible to complement lysis than normal E when classical pathway activation was initiated by rabbit anti-E antibodies.

C3 binding to desialated E. To test whether the lysis that occurred in NIH-GPS and CVF-GPS but not in C4D-GPS resulted simply from increased complement deposition when the classical pathway was activated, we examined C3 deposition onto desialated E after incubation in GPS. Initial experiments using ^{125}I-C3 as a trace label in serum were unsuccessful at detecting C3 on E after incubation in C4D-GPS or CVF-GPS. Because increasing the amount of ^{125}I-C3 added to the serum would significantly affect the C3 concentration and therefore potentially alter the extent of C3 deposition in CVF-GPS and C4D-GPS, an indirect assay for cell-bound C3 was used. We incubated desialated and sham-desialated E in GPS, washed them, and used ^{125}I-labeled anti-C3 to detect surface-bound C3 molecules. Molecular quantitation was achieved by comparing the amount of ^{125}I anti-C3 bound to a standard curve for which absolute quantitation of bound C3 had been determined by depositing [^{3}H]C3 onto sheep erythrocytes with purified complement components. Desialated E were incubated in NIH-, C4D-, and CVF-GPS for varying lengths of time, and C3 uptake onto the E was quantitated (Fig. 7). Maximum detectable C3 molecules were present on the desialated E incubated in NIH-GPS by 2–5 min with a decrease thereafter, probably resulting from cleavage of C3b to

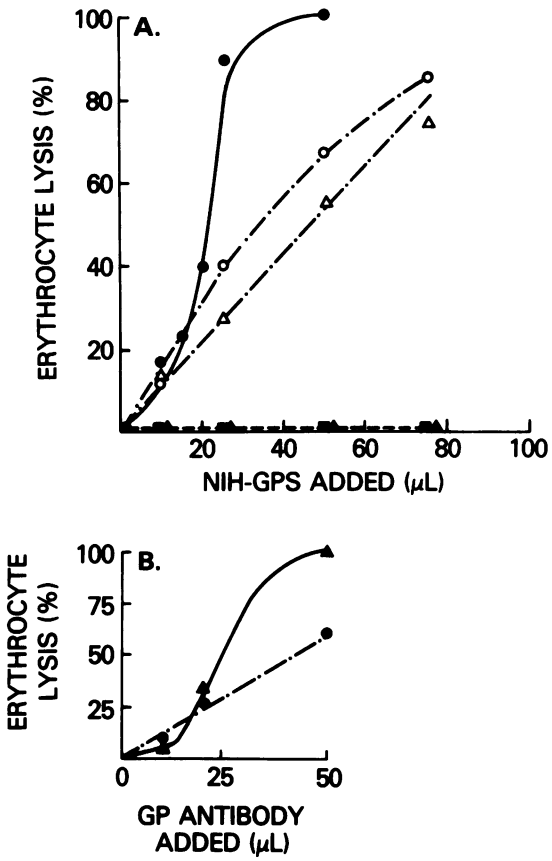

FIGURE 5 Antibody requirement for the lysis of desialated E. In panel A, the lysis of desialated E (●) and sham-desialated E (▲) in NIH-GPS are compared with the lysis of desialated E in NIH-GPS that has been absorbed at 0°C with desialated E (■). The lysis curves for desialated E (○) and sham-desialated E (△) preincubated with rabbit IgG anti-E, and then incubated in absorbed NIH-GPS are also shown. The lytic principle in NIH-GPS for desialated E can be absorbed at 0°C, and lysis can be restored to absorbed serum by rabbit antibody. In the experiment depicted in panel B, desialated E have been incubated with IgM (●, 0.93 mg/ml) or IgG (▲, 9.6 mg/ml) isolated from NIH-GPS. 50 μl of absorbed GPS have been added and lysis quantitated. Natural antibody to desialated E occurs in both the IgG and IgM fractions of GPS.

C3c and α_2d by continued incubation in plasma. While incubation in NIH-GPS led to the deposition of >1,200 C3/E, incubation in either C4D-GPS or CVF-GPS led to the deposition of <250 C3/E even after 30 min. Desialated E incubated in C4D-CVF-GPS or preabsorbed CVF-GPS (to remove antibody) did not bind more anti-C3 than did controls.

DISCUSSION

Our initial experiments were designed to explore the molecular mechanisms for removal of desialated E from the bloodstream in GP. There have been a number of models proposed for clearance of desialated cells. Some of these hypothesize that antibody, with or without complement activation, may be important in clearance (26, 27). However, no one has examined or considered a potential difference in the roles of the classical and the alternative complement pathways in clearance of desialated E. Although various heterologous sialic acid-deficient erythrocytes have been shown to activate the human alternative pathway (9, 11), it had not been demonstrated previously that erythrocyte desialation could be a signal for alternative pathway activation in an autologous system. For these reasons and others, some workers have proposed that desialated cells are cleared not by antibody or complement but by the hepatocyte galactose receptor (8), which is known to remove desialated serum glycoproteins from the bloodstream. Indeed, Kolb et al. (8) showed that desialated E will bind to isolated hepatocytes or parenchymal cells in liver slices. However, they did not perform in vivo experiments to examine the role of the hepatic glycoprotein receptor in sequestration of desialated E. Our studies led to several conclusions about the mechanism of clearance of desialated E. Erythrocytes removed from the bloodstream were all sequestered in liver and spleen, the major reticuloendothelial organs in contact with the blood, suggesting that the RES was important in clearance of desialated cells. Neither ASOR nor OVA blocked clearance, suggesting that the hepatocyte galactose receptor and the macrophage mannose receptor (28) did not participate in removal of desialated cells. Although splenic sequestration of antibody-sensitized cells may proceed in the absence of complement activation (13), this did not occur in these experiments. C4D-CVF-GP, which had normal levels of agglutinating natural antibody to desialated E, were unable to clear intravenously injected desialated cells at all. Thus, complement activation by the erythrocytes was essential for their removal from the blood stream. This complement activation could occur by either the classical or the alternative pathway since both CVF-GP, with normal levels of the early classical pathway components but minimal C3, Factor B or C5–9, and C4D-GP, which cannot activate the classical pathway, were capable of clearing desialated E. Thus, activation of either pathway could lead to the deposition of sufficient C3b on the desialated E to provide the signal for C3-dependent uptake by the fixed mononuclear cells of the RES.

Since they were cleared from the circulation in C4D-GP but not C4D-CVF-GP, desialated E are capable of activating the alternative pathway in GPS. This demonstrates that desialation of cells can be a sufficient signal for alternative pathway activation in a totally autologous system. Nonetheless, C4D-GPS was inca-

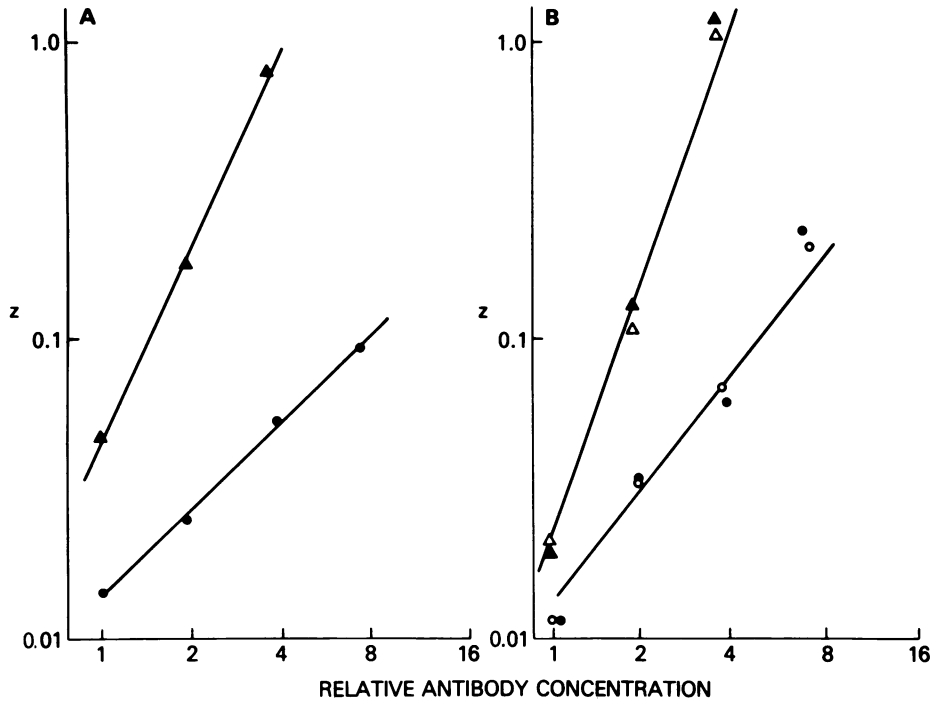

FIGURE 6 The C1 fixation and transfer test using normal and desialated E. Panel A shows C1 fixation and transfer using IgG (▲) and IgM (●) purified from NIH-GPS to sensitize desialated E. Increasing lysis with increasing antibody concentration is seen for both IgG and IgM. This is further evidence that there are both IgG and IgM natural antibodies to desialated E. Panel B shows that the C1 fixation and transfer test using rabbit IgG (▲) or IgM (●) to E is unchanged whether the antibody is used to sensitize normal (open symbols) or desialated (filled symbols) E. This implies that the binding of rabbit antibody, and its ability to fix C1 are unchanged by desialation of E.

pable of causing lysis of desialated E in vivo or in vitro. Hence, in vivo erythrocyte clearance appears to be a more sensitive assay of alternative pathway activation than is lysis. Conclusions reached by others that desialated human erythrocytes are incapable of activating the human alternative complement pathway because human serum could not lyse these cells (12) may merely have reflected the innate insensitivity of the lysis assay.

Most surprising was the discovery that desialated E were cleared at a significant rate in CVF-GP. This clearance was obviously dependent upon complement activation since C4D-CVF-GP, which had equivalent antibody titers to desialated E, could not remove desialated E from the blood stream. CVF-GP had <1% of normal C3 levels, yet they had sufficient complement to opsonize these erythrocytes for clearance. We speculate that the changes in the erythrocyte surface that enable desialated E to activate the alternative complement pathway in GPS may also be important for the clearance of these cells in CVF-GP. C3b can be deposited on alternative pathway activators in "protected sites," locations on the particle surface where the actions of the regulatory proteins H (β1H) and I (C3b/4b inactivator) are inefficient (29). Classical pathway activation by natural antibodies to desialated E, even with very little serum C3 available, may tend to deposit C3b molecules in protected sites, increasing the time available for contact between surface-bound C3b and phagocytic cell C3b receptors. C3b deposited onto nondesialated cells, not in a protected site, may be more easily inactivated and thus have less chance for successful interaction with C3b receptors.

The hypothesis that the residual C3 in CVF-GP was sufficient to play a biologically important role in vivo was supported by the in vitro studies of complement-mediated cell lysis. In these experiments, CVF-GPS clearly contained enough C3 to cause significant erythrocyte lysis. Neuraminidase-treated E were not inherently easier to lyse, since sensitization of desialated and nondesialated E with rabbit antibody led to equivalent amounts of lysis in NIH-GPS. Since CVF-GPS will not lyse normal E or sheep erythrocytes sensitized with rabbit antibody, these results supported the concept that the environment of the C3 bound to the erythrocyte very strongly influences its biological effect. C3b molecules deposited on desialated E by antibodies to

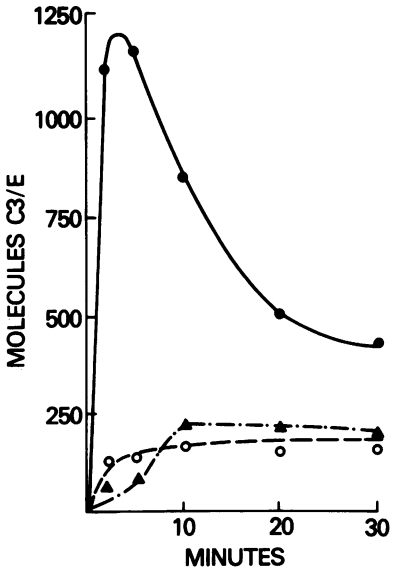

FIGURE 7 C3 uptake onto desialated E. Desialated E were incubated with NIH-GPS (●), C4D-GPS (▲), and CVF-GPS (○) for various periods of time and uptake of C3 quantitated. Uptake in NIH-GPS peaks in ~3 min at 1,200 molecules/E. Uptake in CVF-GPS and C4D-GPS never exceeds 250 molecules/E.

determinants uncovered by desialation may have been in protected sites where they are potentially able to form a C5 convertase and thus a site of assembly for a lytic membrane attack complex. Equivalent lysis of nondesialated E in NIH-GPS was associated with a 100-fold greater deposition of C3 onto the, E[2] implying that the lytic efficiency of C3 deposited in sites exposed by desialation was much greater. Hence, even the few residual C3 molecules in CVF-GPS were sufficient to cause lysis of desialated E when activated by the classical complement pathway C3 convertase.

These studies also brought out a remarkable difference between CVF-GPS and C4D-GPS. CVF-GPS supported E lysis while C4D-GPS did not, despite depositing an equal, small number of C3 molecules on the E. Presumably, in both situations, the antigenic sites exposed by desialation allowed for complement activation. In CVF-GPS, this activation occurred through the classical pathway because of antibody binding, and, in C4D-GPS, complement activation occurred via the alternative pathway, which may be initiated (30) or enhanced (31) by antibody. Whereas both classical pathway and alternative pathway activation could lead to deposition of C3 and opsonization of desialated E for in vivo clearance, only classical pathway activation could lead to in vitro lysis. Perhaps this was because the requirements for C5 cleavage differ in the two situations, since the alternative pathway C5 convertase requires two C3b molecules (32) and the classical pathway only one. In situations of limited C3b deposition, such as occur on desialated E in CVF-GPS or C4D-GPS, an effective classical pathway C5 convertase may be able to form while an alternative pathway convertase may not.

In summary, we have shown that desialated E are cleared in vivo because of complement activation by the E, and that glycoprotein receptors on hepatocytes or macrophages play no role in clearance in the absence of complement activation. It is interesting to speculate that blood-borne tumor cells (33) or hematopoetic cells infected with certain viruses (34, 35), which share antigens with desialated E and to which natural antibodies are present in mammalian sera, may localize to the liver in a complement-dependent fashion as we have described here for desialated E. Desialated E can activate complement via either the classical or the alternative pathway; classical pathway activation is initiated by natural antibodies of both the IgG and IgM classes. Although desialated E are cleared in vivo by alternative pathway activation, they cannot be lysed in vitro by this pathway; on the other hand, classical pathway activation may lead to in vivo clearance and in vitro lysis, even in CVF-GP that possess <1% of the C3 levels of NIH-GP. This is quite important since CVF is the standard reagent used for complement depletion to test the complement dependence of many in vivo phenomena (36). CVF does not affect the early components of the classical pathway, C1, C4, and C2. Hence, in CVF-treated animals, the classical pathway C3 convertase forms normally. The small amount of C3 remaining after CVF depletion would provide sufficient substrate for the conversion of a few C3 molecules if enough classical pathway C3 convertase were formed. These few surface-bound C3b molecules could continue the complement cascade if they were deposited in sites relatively protected from inactivation by H and I. Thus, when a signal for classical pathway activation is present along with protected sites for C3 deposition, CVF depletion of complement may not be adequate to test whether or not complement activation is playing an important biological role.

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REFERENCES

1. Jancik, J. M., R. Schauer, K. H. Andres, and M. von Düring. 1978. Sequestration of neuraminidase-treated erythrocytes. *Cell Tissue Res.* **186**: 209–226.
2. Ashwell, G., and A. G. Morell. 1974. The role of surface carbohydrates in the hepatic recognition and transport of circulating glycoproteins. *Adv. Enzymol.* **41**: 99–127.

[2] Brown, E. J., and M. M. Frank. Unpublished observations.

3. Hudgin, R. L., W. E. Pricer, G. Ashwell, R. J. Stockert, and A. G. Morell. 1974. The isolation and properties of a rabbit liver-binding protein specific for asialoglycoproteins. *J. Biol. Chem.* **249:** 5536–5543.
4. Thornburg, R. W., J. F. Day, J. W. Baynes, and S. R. Thorpe. 1980. Carbohydrate-mediated clearance of immune complexes from the circulation. *J. Biol. Chem.* **255:** 6820–6825.
5. Finbloom D. S., D. B. Magilavy, J. B. Harford, A. Rifai, and P. H. Plotz. 1981. The influence of antigen on immune complex behavior in mice. *J. Clin. Invest.* **68:** 214–224.
6. Kolb, H., A. Kirese, V. Kolb-Bachofen, and H. A. Kolb. 1978. Possible mechanism of entrapment of neuraminidase-treated lymphocytes in the liver. *Cell. Immunol.* **40:** 457–462.
7. Baxter, A., and J. G. Beeley. 1978. Surface carbohydrates of aged erythrocytes. *Biochem. Biophys. Res. Commun.* **83:** 466–471.
8. Kolb, H., C. Schudt, V. Kolb-Bachofen, and H. A. Kolb. 1978. Cellular recognition by rat liver cells of neuraminidase-treated erythrocytes—demonstration and analysis of cell contacts. *Exp. Cell Res.* **113:** 319–325.
9. Fearon, D. T. 1978. Recognition by membrane sialic acid of b1H-dependent delay-dissociation of amplification C3 convertase of the alternative complement pathway. *Proc. Natl. Acad. Sci. USA.* **75:** 1971–1975.
10. Pangburn, M. K., and H. J. Müller-Eberhard. 1978. Complement C3 convertase: cell surface restriction of β1H control and generation of restriction on neuraminidase-treated cells. *Proc. Natl. Acad. Sci USA.* **75:** 2416–2420.
11. Nydegger, U. E., D. T. Fearon, and K. F. Austen. 1978. Autosomal locus regulates inverse relationship between sialic acid content and capacity of mouse erythrocytes to activate human alternative complement pathway. *Proc. Natl. Acad. Sci. USA.* **75:** 6078–6082.
12. Fearon, D. T. 1979. Regulation of the amplification C3 convertase of human complement by an inhibitory protein isolated from the human erythrocyte membrane. *Proc. Natl. Acad. Sci. USA.* **76:** 5867–5871.
13. Schreiber, A. D., and M. M. Frank. 1972. Role of antibody and complement in the immune clearance and destruction of erythrocytes. I. In vivo effects of IgG and IgM complement fixing sites. *J. Clin. Invest.* **51:** 575–582.
14. Schreiber, A. D., and M. M. Frank. 1972. The role of antibody and complement in the immune destruction of erythrocytes. II. Molecular nature of IgG and IgM complement fixing sites and effects of their interaction with serum. *J. Clin. Invest.* **51:** 583–590.
15. Gaither, T. A., C. H. Hammer, and M. M. Frank. 1979. Studies of the molecular mechanisms of inactivation and a simplified assay of β1H and the C3b inactivator. *J. Immunol.* **123:** 1195–1204.
16. Brown, E. J., S. W. Hosea, C. H. Hammer, C. G. Burch, and M. M. Frank. 1982. A quantitative analysis of the interactions of antipneumococcal antibody and complement in experimental pneumococcal bacteremia. *J. Clin. Invest.* **69:** 85–98.
17. Tack, B. F., and R. L. Wilder. 1981. Tritiation of proteins to high specific activity. *Methods Enzymol.* **73:** 138–146.
18. Ellman, L., I. Green, and M. M. Frank. 1970. Genetically controlled total deficiency of the fourth component of complement in the guinea pig. *Science (Wash. DC).* **170:** 74–75.
19. Gaither, T. A., and M. M. Frank. 1979. Complement. *In* Clinical Diagnosis and Management by Laboratory Methods. J. B. Henry, editor. W. B. Saunders Co., Philadelphia. 1245–1262.
20. Warren, L. 1959. The thiobarbituric acid assay of sialic acids. *J. Biol. Chem.* **234:** 1971–1975.
21. Varki, A., and S. Kornfeld. 1980. An autosomal dominant gene regulates the extent of 9-*O*-acetylation of murine erythrocyte sialic acids. *J. Exp. Med.* **152:** 532–544.
22. Borsos, T., and H. J. Rapp. 1965. Hemolysin titration based on fixation of the activated first component of complement: evidence that one molecule of hemolysin suffices to sensitize an erythrocyte. *J. Immunol.* **95:** 559–566.
23. Brown, E. J. 1982. An improved method for performance of the C1 fixation and transfer test. *J. Immunol. Methods.* **55:** 115–122.
24. Steinbuck, M., and R. Audran. 1969. The isolation of IgG from mammalian sera with the aid of caprylic acid. *Arch. Biochem. Biophys.* **134:** 279–284.
25. Clas, F., and M. Loos. 1981. Antibody-independent binding of the first component of complement (C1) and its subcomponent C1q to the S and R forms of *Salmonella minnesota*. *Infect. Immun.* **31:** 1138–1144.
26. Steer, C. J., S. P. James, Vierling, S. W. Hosea, and E. A. Jones. 1980. The selective hepatic uptake of desialated peripheral blood mononuclear cells in rabbits. *Gastroenterology.* **79:** 917–923.
27. Pfannschmidt, G., and R. Schauer. 1980. The role of membrane sialyl and galactosyl residues in regulation of the life time of rabbit erythrocytes. *Hoppe-Seyler's Z. Physiol. Chem.* **361:** 1683–1695.
28. Bagnes, J. W., and F. Wold. 1976. Effect of glycosylation on the in vivo circulating half-life of ribonuclease. *J. Biol. Chem.* **251:** 6016–6024.
29. Fearon, D. T., and K. F. Austen. 1977. Activation of the alternative complement pathway due to resistance of zymosan-bound amplification convertase to endogenous regulatory mechanism. *Proc. Natl. Acad. Sci. USA.* **74:** 1683–1687.
30. Moore, F. D., K. F. Austen, and D. T. Fearon. 1982. Antibody restores human alternative complement pathway activation by mouse erythrocytes rendered functionally deficient by pretreatment with pronase. *J. Immunol.* **128:** 1302–1306.
31. Nelson, B., and S. Ruddy. 1979. Enhancing role of IgG in lysis of rabbit erythrocytes by the alternative pathway of human complement. *J. Immunol.* **122:** 1994–1999.
32. Daha, M. R., D. T. Fearon, and K. F. Austen. 1976. C3 requirements for formation of alternative pathway C5 convertase. *J. Immunol.* **117:** 630–634.
33. Bray, J., R. U. Lemiux, and T. A. McPherson. 1981. Use of a synthetic hapten in the demonstration of the Thomsen-Friedenreich (T) antigen on neuraminidase-treated human red blood cells and lymphocytes. *J. Immunol.* **126:** 1966–1969.
34. Lambre, C. R., M. D. Kazatchkine, F. Mailler, and M. Thibon. 1982. Guinea pig erythrocytes, after contact with influenza virus, acquire the ability to activate the human alternative complement pathway through virus-induced desialation of the cells. *J. Immunol.* **128:** 629–634.
35. Theophilopoulos, A. N., and L. H. Perrin. 1977. Lysis of cultured human lymphoblastoid cells by cell-induced activation of the properdin pathway. *Science (Wash. DC).* **195:** 878–879.
36. Müller-Eberhard, H. J. 1977. Isolation of cobra venom factor. *In* Methods in Immunology and Immunochemistry. C. A. Williams and M. W. Chase, editors. Academic Press, Inc., New York. **4:** 263.