INTERFERENCE WITH IMMUNE HEMOLYSIS BY GLYCOPROTEIN ANTIGENS*, ‡

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Hemagglutination and hemolytic titrations of antisera using red cells to which antigens have been conjugated with bis(diazo)benzidine have been used as a measure of antibody activities (1–4) in this laboratory. Hemagglutination and immune hemolysis titrations have comparable sensitivities. Immune hemolysis, however, can be modified to give a high degree of precision (5). Both methods have been used to advantage with a number of protein antigens; *i.e.*, insulin, egg albumin, bovine serum albumin, human gamma globulin, ferritin, and others (2).

This investigation is concerned with the diminished immune hemolysis and relatively elevated hemagglutination titers noted with three different immunological systems involving sialic acid–containing antigens. These glycoprotein immune systems were capable of complement fixation. Consequently, the diminished immune hemolysis could not be attributed to the lack of complement fixation. Large amounts of these three glycoproteins in solution did not interfere with immune hemolysis observed with insulin and homologous antibody. When red cell preparations were sensitized with a mixture of sialic acid–containing glycoprotein and insulin, immune hemolysis by insulin antisera was diminished. Red cells treated with neuraminidase to remove sialic acid, and then incubated with complement, hemolyzed in the absence of any known immune reaction. Furthermore, such hemolysis of neuraminidase-treated cells by complement could be interfered with by conjugating sialic acid–containing glycoproteins to the cell surface.

These observations are consistent with the postulate that during immune hemolysis a sialic acid–containing substrate is enzymatically cleaved from the cell surface prior to lysis of the red cell.

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Materials and Methods

Antigens

Insulin.—Crystalline beef insulin (Lot No. 719106)[1] was a preparation with a potency of 27.0 units per mg. Some hyperglycemic factor was present in this preparation.

Alum-precipitated insulin was prepared by suspending 50 mg insulin in 10 ml buffered saline and then adding 0.5 ml of 1 per cent alum, $AlNH_4(SO_4)_2 \cdot 12H_2O$. Alum-precipitated insulin was used for booster injections during the course of immunization. The preparation used for initial immunization contained 1.5 ml (7.5 mg) of alum-precipitated insulin plus 10 ml of complete Freund's adjuvant obtained from Hyland Laboratories, Los Angeles, California.

Human Chorionic Gonadotropin (HCG) (Lot Nos. 90259 and 90272).—This was obtained from Vitamerican Corporation, Little Falls, New Jersey. These preparations contained about 2,500 IU per mg with a carbohydrate content ranging from 20 to 30 per cent. A minimum of 8 immunological moieties could be identified by immunoelectrophoresis of the HCG preparation with homologous antiserum (6). For immunization 5 mg of HCG was suspended in 1 ml of complete Freund's adjuvant. HCG (5 mg/ml) in buffered saline was used for booster injections.

Human Transferrin (HT).—Human *apo*transferrin was obtained from Behringwerke, Marburg, Germany (Lot No. 1227/34). When assayed by titration with Fe-59, 1 mg was found to contain 0.133 μmole.[2]

For immunization, HT was suspended by sonication in Freund's adjuvant at a concentration of 4 mg/ml. HT (4 mg/ml) in buffered saline was used for booster injections.

Rabbit Transferrin (RT).—Rabbit transferrin was obtained from Pentex, Inc., Kankakee, Illinois (Lot No. 1). When assayed spectrophotometrically at 465 mμ, 1 mg was found to contain 0.13 μmole, assuming $E_{1\ \text{percent}}^{1\ \text{cm}} = 0.68$ (7) and mol wt = 67,000 (8). This preparation was contaminated with a small amount of heme-protein (less than 5 per cent) but was used without further purification.

The RT used for immunization was prepared in the same manner as the HT.

Antisera

Rabbit Antisera to the Various Antigens Used.—These were developed in adult male and female New Zealand white rabbits by injecting 0.2 ml of antigen in Freund's adjuvant into each toe pad. 3 to 4 weeks later, 2.5 to 5.0 mg of antigen in 0.5 to 1.0 ml were injected intravenously and followed by a second booster within 48 to 72 hours. A 3 to 5 ml test sample of blood was taken within 7 to 19 days following the second booster. When adequate titers of antibody were observed, 50 to 60 ml of blood were taken from the marginal ear vein. When antibody levels were low, the booster injections were repeated. Rabbits receiving insulin also received 25 ml of 50 per cent glucose subcutaneously to minimize hypoglycemic reaction.

Blood samples were allowed to stand at room temperature (25°C) for 2 hours and then stored overnight at 4°C. The following morning sera were separated by centrifugation at approximately 2500 *g* for 20 minutes. Antibody titers of the sera were determined by hemagglutination, the sera divided into aliquots, and stored at −80°C until used.

Rabbit antisera were inactivated at 56°C for 30 minutes and adsorbed with 10 mg of sheep red cell stroma/ml serum (9) at room temperature for 10 minutes.

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[1] We wish to thank Dr. Otto K. Behrens, Eli Lilly and Co., Indianapolis, Indiana, for the generous supply of cyrstalline beef insulin.

[2] Dr. Paul Saltman, Department of Biochemistry, University of Southern California, kindly performed this assay.

Guinea Pig Antisera.—Guinea pig antisera to the various antigens were developed in adult male and female mongrel guinea pigs by injecting 0.2 ml of antigen in Freund's adjuvant into each paw pad. They were desensitized 3 to 4 weeks later by the successive subcutaneous injection of 1, 10, and 100 μg of antigen at 45 minute intervals and a second 100 μg antigen injection 1 hour later. The animals were constantly observed and 0.5 ml of 1:100,000 epinephrine was injected intraperitoneally when symptoms of anaphylaxis appeared. The animals immunized with insulin received 4 ml of 50 per cent glucose following each series of inoculations.

One week following the desensitization procedure, an animal was selected at random and a 7 to 10 ml test sample of blood was taken by cardiac puncture. If an adequate antibody titer was observed, all the guinea pigs which had been immunized were anesthetized with intraperitoneal nembutal (0.5 ml of 60 mg/ml), and exsanguinated from the carotid artery within 1 to 2 days after the test sample was obtained. Guinea pig antisera were treated in the same manner as the rabbit antisera, except that the adsorption with stroma was not performed.

Solutions

Buffered saline consists of 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.4.

Cacodylate buffer contains 0.137 M sodium cacodylate and 0.007 M $CaCl_2$; pH was adjusted to 6.4 with 4 N HCl (10).

Veronal-Buffered Saline (VBS).—The diluent used for titrating complement and immune hemolysis titrations was veronal-buffered saline, pH 7.4 (11), containing 5×10^{-4} M $MgCl_2$ and 1.5×10^{-3} M $CaCl_2$.

VBS-Albumin.—The diluent used for hemagglutination titrations was VBS containing 1.5 gm bovine serum albumin (Armour Company) per liter. Prior to use, the diluent was incubated at 80°C for 1 hour and then cooled to 0°C in a water bath. Much of the non-specific agglutination observed at high dilution of antiserum was eliminated with this diluent.

Insulin.—The insulin solution was made up in a concentration of 1 mg/ml. Insulin was dissolved in 0.01 N NaOH, equal to one-half the desired final volume, and diluted to final volume with 0.22 M phosphate buffer, pH 7.4. This stock insulin solution was stored at 4°C and discarded if not used within 3 days.

Neuraminidase (Vibrio cholerae).—Neuraminidase was obtained from General Biochemicals, Chagrin Falls, Ohio (Lot No. 52632) and had a specific activity of 100 units/ml (10 units/μg N). Prior to use the neuraminidase (1.5 ml) was dialyzed against a minimum of three changes of 10 volumes each of cacodylate buffer at 0°C for 12 hours.

Complement.—Adsorbed complement (C'_a) was used for the hemolytic titration of insulin antibodies. Pools of fresh guinea pig sera were adsorbed with 10 mg/ml of the S_2 fraction of sheep erythrocyte stroma (9) at room temperature (25°C) for 10 minutes while gently agitated and then centrifuged at approximately 2000 *g* at 4°C for 15 minutes.

Titration of Complement (11–14).—Titers of C'_a were determined by 50 per cent hemolytic end points. A unit of complement is defined as the smallest amount of C'_a which will cause lysis of 50 per cent of the sensitized cells (1 ml of a 1.25 per cent suspension) in a final volume of 1.5 ml after 30 minutes at 37°C.

Erythrocyte Preparation.—Sheep red cells were stored as sheep blood collected in an equal volume of Alsever's solution. This suspension was stored at 4°C and used for a period of 14 days.

Bis(diazo)benzidine (BDB) was prepared according to Kabat and Mayer (15). The reaction was carried out in the presence of ice crystals. The final product was a pale yellow solution. This material was empirically standardized by adding 7 ml of 0.11 M phosphate buffer to 0.5 ml BDB (1:15 BDB). A deep reddish brown color developed immediately and the solution became turbid within 90 seconds ($\pm$ 5 seconds). In the event the solution did not immediately

turn deep reddish brown, the onset of turbidity was also prolonged. A few crystals of benzidine were then added and the solution retested. When the turbidity developed rapidly (less than 80 seconds), small amounts of sodium nitrite were added to the solution. When the desired preparation was obtained, the stock BDB was divided into 0.5 ml aliquots and stored at −80°C. Under these conditions BDB was active for at least 9 months.

Insulin-Conjugated Red Cells (Insulin-Cells).—Insulin-cells were prepared by conjugating insulin to washed sheep red cells with BDB as previously described (1).

HCG-Conjugated Red Cells (HCG-Cells).—Washed, packed, sheep red cells (0.1 ml) were suspended in a solution containing 1.5 mg of HCG in 3.0 ml buffered saline, and 0.6 ml of freshly diluted 1:15 BDB was added (3). The suspension was shaken gently for 10 minutes at room temperature and washed twice with veronal-buffered saline. The washed, packed, HCG-cells were resuspended in 5 ml of VBS-albumin for hemagglutination titrations and in 5 ml of VBS for immune hemolysis titrations.

Transferrin-Conjugated Red Cells (RT-Cells and HT-Cells).—Washed, packed, sheep red cells (0.1 ml) were suspended in a solution containing 1.5 ml transferrin (1 mg/ml saline) and 0.5 ml buffered saline to which 0.35 ml of freshly diluted 1:15 BDB was added. The suspension was shaken gently for 15 minutes at room temperature, washed twice, and resuspended to 5 ml with VBS. The amounts of reagents and the procedures were identical for the preparation of both HT-cells and RT-cells.

Mixed-Sensitized Cells (HT-I-Cells and RT-I-Cells).—Washed, packed, sheep red cells (0.2 ml) were suspended in a solution containing 0.5 ml of insulin (1 mg/ml), 2.5 ml of either HT or RT (1 mg/ml), and 0.7 ml of 1:15 BDB. This mixture was incubated at room temperature for 15 minutes with gentle shaking, washed twice, and resuspended to 10 ml with VBS. The presence of both transferrin and insulin on the red cell surface was demonstrated by hemagglutination (Table V).

Neuraminidase-Treated Red Cells.—These were prepared by a modification of the method of Eylar, Madoff, Brody, and Oncley (10). Sheep red cells were washed ten times in isotonic buffer, pH 7.2 (0.135 M sodium chloride and 0.02 M sodium phosphate). Cacodylate buffer (0.3 ml) and 0.2 ml neuraminidase (20 units) which had been dialyzed against cacodylate buffer were added to 0.3 ml of washed, packed, sheep red cells. This cell suspension was incubated at 37°C for 30 minutes and washed three times with approximately 100 volumes of cold (0°C) VBS and resuspended to a final volume of 30 ml in VBS.

The controls for neuraminidase-treated red cells were treated in a similar fashion except that 0.2 ml of cacodylate buffer was added to the suspension of red cells instead of neuraminidase.

Titration

Hemagglutination Titration.—Antisera were titrated by the hemagglutination method of Arquilla and Stavitsky (1), employing one of the various antigen-cell preparations and homologous antiserum. The diluent used for titrations was VBS-albumin. The solution consisted of 0.5 ml antisera, 0.05 ml for testing materials by hemagglutination inhibition, and 0.05 ml of antigen-cells. The hemagglutination titers were arbitrarily designated as the highest dilution of antiserum capable of agglutinating 0.05 ml of a 2.0 per cent suspension of antigen-cells in a final volume of 0.6 ml, and expressed as the reciprocal of the titer.

Antibody Titration by Immune Hemolysis.—This was performed by a modification of the immune hemolysis technique previously described (5). Antisera, serially diluted with VBS to a final volume of 0.5 ml, were cooled in an ice bath, and 20 units of C'_a in 0.3 ml VBS were then added. Antigen-cells (0.2 ml) were added to the solution, evenly suspended by gentle shaking, and incubated in a 37°C water bath for 30 minutes. The non-hemolyzed cells were separated by centrifugation at 4°C, at approximately 2000 *g* for 10 minutes. The supernatant was

decanted and 2 ml water added to each sample. The degree of hemolysis was measured by adsorption of each sample at 580 mμ with a Beckman model DB spectrophotometer.

Hemolysis of antigen-cells by homologous antisera in the presence of excess C'_a has been shown to be a precise measure of antibody concentration (5). Specificity of the system was controlled by inhibition of hemolysis with homologous antigen.

Complement Fixation.—This was performed in a VBS diluent in a final volume of 1 ml, consisting of 0.25 ml C'_a (10 to 25 units), 0.5 ml diluted antiserum, and 0.25 ml antigen varying between 5 and 250 μg. This mixture was incubated for about 18 hours in a 0°C water bath, and C'_a titrations were performed as previously described. The results were expressed as the number of units fixed (Tables III and IV).

TABLE I

Comparison of Hemagglutination Titers and Immune Hemolysis Titers of Insulin Antisera

Insulin antiserum	Antibody titer	
	Hemagglutination*	Immune hemolysis‡
C32d	640	320
C33d	160	320
C38c	640	640
Guinea pig pool	160	120

* Reciprocal of highest dilution of antiserum which will agglutinate insulin-conjugated erythrocytes.

‡ Reciprocal of highest dilution of antiserum which will hemolyze 50 per cent of the insulin-conjugated erythrocytes added.

RESULTS

Comparison of Hemagglutination with Immune Hemolysis Titers of Insulin Antisera and Glycoprotein Antisera.—Insulin antibody titers measured by immune hemolysis and hemagglutination of one guinea pig and three rabbit antisera were compared, using aliquots of the same insulin-cell preparation (Table I). The rabbit insulin antisera (C32d, C33d, and C38c) had hemagglutination titers (640, 160, 640) which approximated the immune hemolysis titers (320, 320, and 640). The hemagglutination titer (160) and immune hemolysis titer (120) of the guinea pig insulin antiserum were also similar (Table I). It appears, therefore, that the titer of an individual insulin antiserum measured by hemagglutination agrees favorably with the titer of the same antiserum measured by immune hemolysis.

In contrast, a marked disparity is noted between the immune hemolysis and hemagglutination titers of antisera prepared against three different glycoproteins (HCG, HT, and RT). Immune hemolysis with these three antigens is markedly suppressed and occasionally not measureable. On the other hand, the hemagglutination titers of antisera prepared against the three glycoproteins

have been consistently higher than immune hemolysis titers (Table II and Fig. 1).

The difference between hemagglutination and immune hemolysis titers of HCG antisera from three rabbits and one guinea pig is presented in Table II.

TABLE II

Comparison of Hemagglutination Titers and Immune Hemolysis Titers of HCG Antisera

HCG antiserum	Antibody titer	
	Hemagglutination*	Immune hemolysis‡
RBIL	40,000	350
RB2J	10,000	260
C12N	20,000	320
Guinea pig pool	2,560	0

* Reciprocal of highest dilution of antiserum which will agglutinate HCG-conjugated erythrocytes.

‡ Reciprocal of highest dilution of antiserum which will hemolyze 50 per cent of the HCG conjugated erythrocytes added.

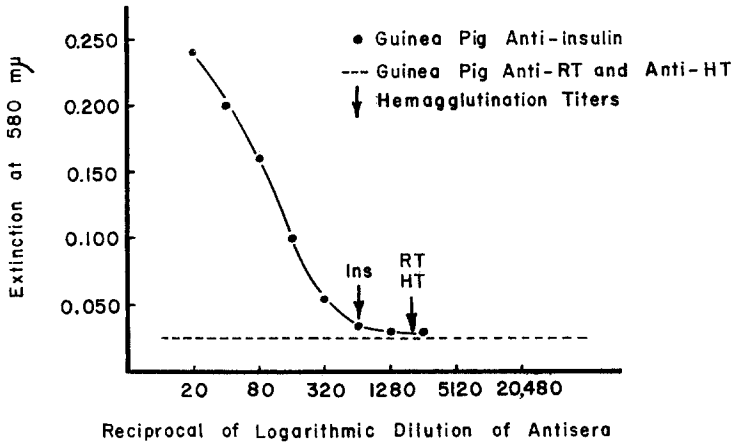

FIG. 1. Comparison of hemagglutination and immune hemolysis titers of insulin and transferrin antisera.

The three rabbit antisera (RB 1L, RB 2J, and C 12N) had elevated hemagglutination titers (40,000, 10,000, and 20,000, respectively) whereas the immune hemolysis titers of aliquots of the same antisera against the same HCG-cell preparation were much lower (350, 260, and 320). Immune hemolysis could not be demonstrated with the guinea pig antiserum which had a hemagglutination titer of 2560 (Table II).

Fig. 1 is an example of similar observations noted when human and rabbit transferrin were used as antigens. The hemagglutination titer was 1600 for both transferrin immune systems and 640 for the insulin immune system. The immune hemolysis titer in the insulin system was the same as the hemagglutination titer, whereas, no demonstrable immune hemolysis was observed with either transferrin system (Fig. 1).

TABLE III

Specificity of Complement Fixation in HCG Anti-HCG and Insulin Anti-Insulin Systems

	Units complement* fixed
HCG antibody + HCG	22
HCG Antibody + insulin	None
Insulin antibody + insulin	22
Insulin antibody + HCG	None

* Twenty-five 50 per cent hemolytic units C'_4 were used in all experiments.

TABLE IV

*Complement Fixation in HT Anti-HT and RT Anti-RT Systems**

	Units‡ complement added	Units complement fixed
HT + HT antibody	12	9.5
HT	12	None
HT antibody	12	None
RT + RT antibody	11	7
RT	11	None
RT antibody	11	None

* HT = human transferrin; RT = rabbit transferrin.

‡ 50 per cent hemolytic units.

It was not known whether reduced hemolysis in the three glycoprotein immune systems was due to poor complement fixation or to non-specific anticomplementary factors. A third possibility was interference with immune hemolysis by the carbohydrate moiety on the glycoprotein antigens.

Complement Fixation by Insulin and Glycoprotein Immune Systems.—In view of the reduced hemolysis in glycoprotein immune systems as compared to hemolysis in the insulin immune system, the complement-fixing abilities of both were tested. In addition, the presence of non-specific anticomplementary factors in the antisera and antigen preparations was investigated.

Complement is fixed in the presence of HCG antibody and HCG (Table III). This was also observed with insulin antibody and insulin. The fact that no complement was fixed in the presence of HCG antibody and insulin, and *vice versa*,

indicates that the HCG and insulin antisera and also the insulin and HCG are not anticomplementary.

In addition, complement fixation was demonstrated with both the RT and HT immune systems (Table IV). It was also noted that neither RT nor HT nor homologous antisera were anticomplementary.

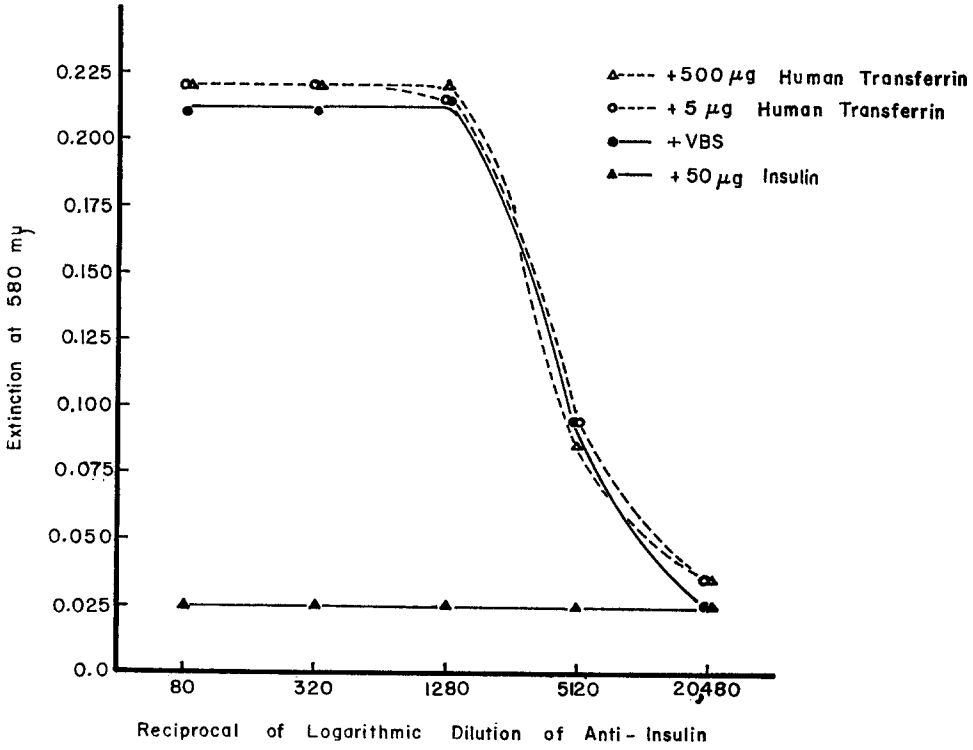

FIG. 2. Effect of soluble transferrin on insulin immune hemolysis.

Consequently, the decreased ability of glycoprotein antisera to cause immune hemolysis of glycoprotein-cells in the presence of excess complement cannot be explained on the basis of the inability of the glycoprotein immune systems to fix complement.

Effect of Soluble Glycoprotein on Insulin Immune Hemolysis.—Whether glycoproteins in solution could also inhibit immune hemolysis was tested by adding soluble glycoproteins to the insulin immune hemolysis system (Fig. 2).

The immune hemolysis of insulin-cells by homologous antisera was not interfered with when relatively large quantities (500 μg) of RT, HT, or HCG were added to the insulin immune system (Fig. 2). Master dilutions of insulin antisera were subdivided into four series. VBS was added to the first (control) series of

antiserum dilutions, the second was preincubated with 500 μg of HT, the third with 5 μg of HT, and the fourth with 50 μg of insulin. The immune hemolysis titration curves obtained when either 500 μg or 5 μg of HT was preincubated with insulin antiserum were very similar to those obtained when diluent (VBS) was preincubated with the same dilutions of antiserum. On the other hand, there was marked inhibition of hemolysis by preincubation of 50 μg of insulin with the antiserum. Therefore, glycoprotein in solution probably does not affect the immune hemolysis of antigen-coated cells by homologous antisera. Consequently, the inhibitory effect of glycoprotein on immune hemolysis may require that the carbohydrate-containing proteins be in intimate contact with the red cell surface.

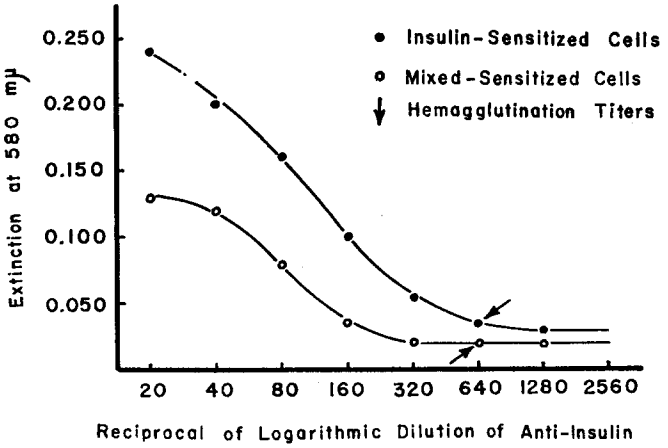

FIG. 3. Interference by transferrin with insulin immune hemolysis of mixed-sensitized cells.

Interference with Hemolysis in the Insulin Immune System by Transferrin.— Experiments were designed to test whether glycoproteins must be attached to the red cell surface to interfere with immune hemolysis. The hemolysis of red cells to which both transferrin and insulin were conjugated was compared to the hemolysis of red cells to which insulin alone was conjugated (Fig. 3 and Table VI). The presence of both transferrin and insulin on the surface of the red cells (mixed-sensitized cells) was demonstrated by hemagglutination (Table V). Anti-HT serum agglutinated these mixed-sensitized cells at a titer of 12,800. This agglutination was inhibited by HT but not by insulin. Insulin antiserum agglutinated the same cell preparation at a titer of 640 and was inhibited by insulin but not by HT. Consequently, both insulin and transferrin were attached to the cell surface and neither of these attached antigens noticeably interfered with the immunological reaction involving the heterologous immune system.

The immune hemolysis of mixed-sensitized cells was compared to that of cells sensitized with insulin alone (Fig. 3). This insulin antiserum had the same hemagglutination titer (640) against both cell preparations. The hemolysis of mixed-sensitized cells, however, was markedly less than the hemolysis of cells sensitized with insulin alone. Table VI summarizes the results of two experiments using two different guinea pig insulin antisera. In the first experiment,

TABLE V

Mixed Sensitization of Erythrocytes with Transferrin and Insulin

Antiserum	Antibody hemagglutination titer*		
	Non-inhibited	Inhibited with insulin	Inhibited with transferrin
Anti-insulin	640	0	640
Anti-transferrin	12,800	12,800	0

* All titers are expressed as the reciprocal of the highest dilution of antiserum capable of agglutinating cells sensitized with both insulin and transferrin.

TABLE VI

Interference by Transferrin with Hemolysis of Mixed-Sensitized Cells by Insulin Antisera

Insulin antisera	C 18		L 74	
Sensitized cells	HT-I	I	RT-I	I
Hemolysis* *per cent*	35.6	100	40.5	100
Hemagglutination titer	640	640	2,560	2,560

HT-I-cells are sheep red cells sensitized with both human transferrin and insulin.

RT-I-cells are sheep red cells sensitized with both rabbit transferrin and insulin.

I-cells are sheep red cells sensitized with insulin.

* The estimate of per cent lysis of mixed-sensitized cells compared to lysis of insulin cells was obtained by dividing the area under the curve of the hemolytic titration of the mixed-sensitized cells by the area under the curve of the hemolytic titration of the insulin-cells.

which has already been presented graphically (Fig. 3), guinea pig insulin antiserum (C 18) and mixed-sensitized cells prepared with HT and insulin (HT-I-cells) were used. In the second experiment guinea pig insulin antiserum (L 74) and mixed-sensitized cells prepared with RT and insulin (RT-I-cells) were used. In both of these experiments the hemagglutination titer of the antisera was the same when either the mixed-sensitized cells or the insulin-cells were used as indicators. The immune hemolysis of HT-I-cells by C 18 insulin antiserum was 65 per cent less than the immune hemolysis of insulin-cells. Similarly, the hemolysis of RT-I-cells by L 74 insulin antiserum was 60 per cent less than the hemolysis of insulin-cells. The per cent immune hemolysis was estimated by dividing the

area under the curve of the hemolytic titration obtained with mixed-sensitized cells by the area under the curve of the hemolytic titration obtained with insulin-cells.

RT, HT, and possibly HCG interfere with the hemolytic phase of immune hemolysis. These three glycoproteins in solution had no such effect (Fig. 2), whereas glycoproteins attached to the red cell surface interfered with immune hemolysis (Fig. 3 and Table VI). Sialic acid is common to HT, RT, HCG, and the surface of the red cells. It is postulated that immune hemolysis requires the enzymatic cleavage of a sialic acid–containing substrate from the cell surface.

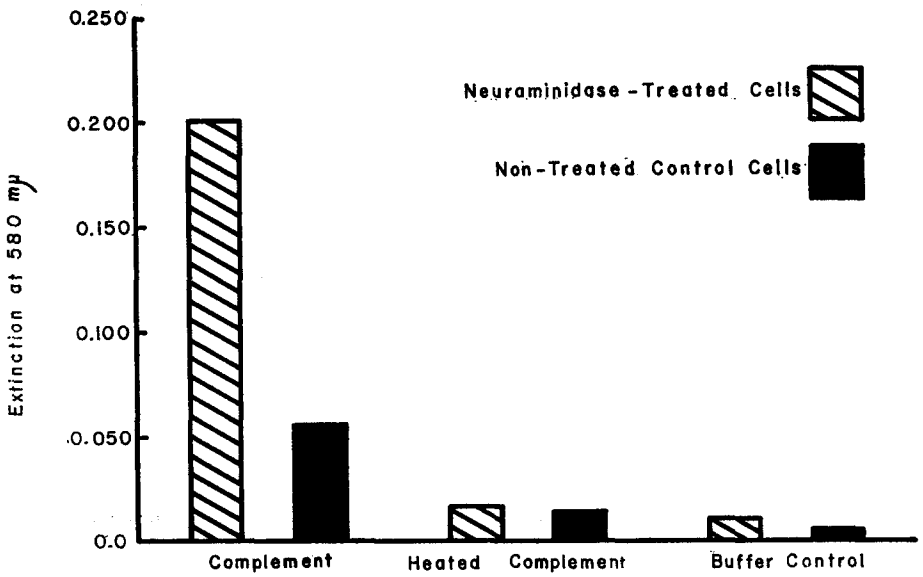

FIG. 4. Hemolysis of neuraminidase-treated erythrocytes by guinea pig complement.

When sialic acid–containing antigens are conjugated to the cell surface, such an enzymatic cleavage may be competitively inhibited and immune hemolysis by homologous antisera is suppressed.

Hemolysis of Neuraminidase-Treated Red Cells by Guinea Pig Complement.— If during immune hemolysis the enzymatic cleavage of a sialic acid–containing substrate from the cell surface precedes lysis, then the removal of sialic acid by neuraminidase should render the cell susceptible to lysis by complement in the absence of an immune reaction. In order to test this possibility, the hemolysis of neuraminidase-treated sheep red cells by C'_a was compared with the hemolysis of the same cell preparation which had *not* been treated with neuraminidase. In these experiments both cell preparations were also exposed to heat-inactivated C'_a and, as a further control, to diluent (VBS) alone (Fig. 4).

Complement caused considerably more hemolysis of neuraminidase-treated cells than non-treated cells. The increased hemolysis appears to be dependent on complement activity, since inactivated C'_a caused only trace amounts of hemolysis (Fig. 4). When the neuraminidase-treated cells were exposed to decreasing amounts of C'_a, decreasing degrees of hemolysis were observed (Fig. 5).

The amount of lysis of neuraminidase-treated cells appears to be dependent upon the amount of sialic acid removed, since more hemolysis is observed when cells are treated with 20 units of neuraminidase than with 10 units of neuraminidase (Fig. 5).

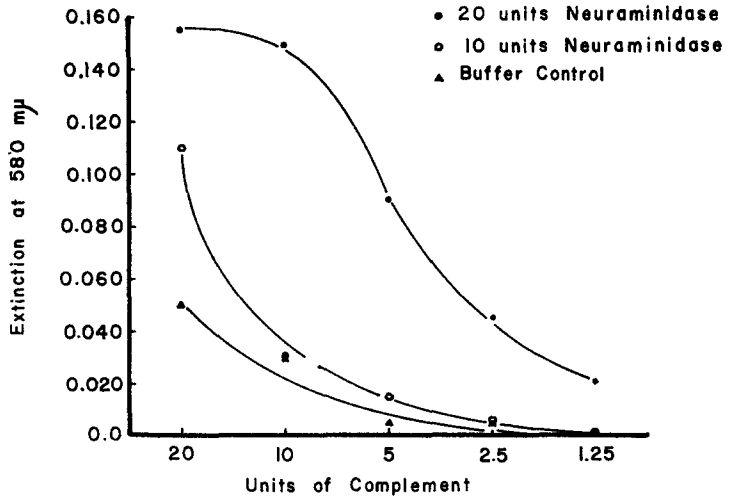

FIG. 5. Hemolytic response of neuraminidase-treated cells as a function of neuraminidase concentration.

It appears that when sialic acid is enzymatically removed from red cell surfaces, complement will cause hemolysis in the absence of any known antigen-antibody reaction.

Inhibition of Hemolysis of Neuraminidase-Treated Cells by Sialic Acid–Containing Glycoproteins.—If sialic acid inhibits the hemolytic action of complement, then a sialic acid–containing protein conjugated to the surface of neuraminidase-treated red cells should inhibit the lysis of these cells by complement (Fig. 6).

In these experiments, sheep red cells were incubated with 20 units of neuraminidase and divided into two equal aliquots. One aliquot was sensitized with RT and the second aliquot served as control. Equal amounts of each of these red cell preparations were incubated with decreasing amounts of C'_a (Fig. 6). The neuraminidase-treated cells to which RT was conjugated were lysed to a lesser degree than the cells treated with neuraminidase alone.

This observation further supports the postulate that during immune hemolysis sialic acid–containing substrate is enzymatically cleaved from the cell surface prior to lysis of the red cell.

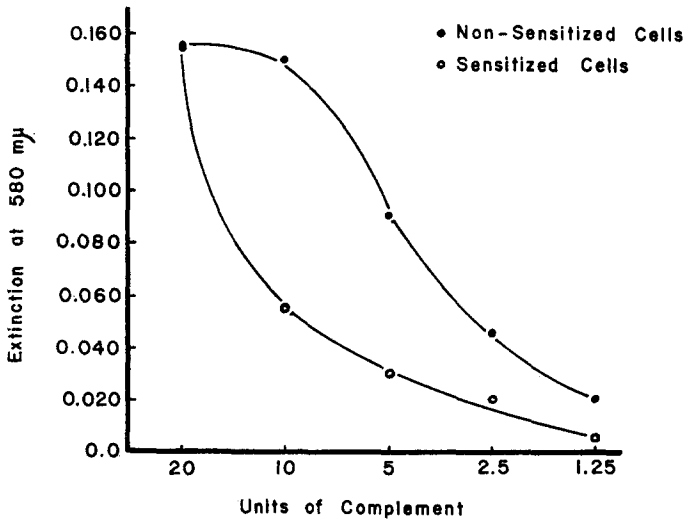

FIG. 6. Inhibition of hemolysis of neuraminidase-treated cells by sensitization with transferrin.

DISCUSSION

The agreement between hemagglutination and immune hemolysis titers of insulin antisera has also been observed with antisera directed toward other protein antigens; *e.g.*, egg albumin, bovine serum albumin, human γ-globulin, and ferritin. Occasional discrepancies between hemagglutination and immune hemolysis titers of individual guinea pig insulin antisera have been noted. This occurs early in immunization, and may possibly be related to the increased amounts of non–complement-fixing γ_1-antibodies and a relatively small concentration of complement-fixing γ_2-antibodies described by Bloch, Kourilsky, Ovary, and Benacerraf (16). Such discrepancies, however, are not as marked as the difference between the hemolytic and hemagglutination titers of the glycoprotein antisera studied in these experiments. Complement fixation was observed with the three sialic acid–containing glycoprotein immune systems which were investigated. Therefore, reduced immune hemolysis cannot be attributed to a relative absence of complement-fixing γ_2-antibodies in the antisera.

In our laboratory the immune hemolytic titers with human γ-globulin antigen are comparable to hemagglutination titers. Human γ-globulin contains at least one molecule of sialic acid per molecule of γ-globulin (17). Both human

transferrin and rabbit transferrin contain four molecules of sialic acid per molecule of transferrin.

The inability of human γ-globulin to interfere with immune hemolysis may be due either to the relative paucity of sialic acid or to the position of the sialic acid in the molecule.

Eylar estimated that the surface of a sheep (lamb) red cell contains 7.6×10^6 sialic acid molecules (10). Roberts and Haurowitz (18) estimated that the optimal sensitization of sheep red cells with antigen, using the BDB method, requires 5×10^5 molecules of antigen per cell. Transferrin contains four molecules of sialic acid. Consequently, a cell after optimal sensitization with transferrin, probably contains 2×10^6 additional sialic acid molecules. This is in good agreement with the total number of sialic acid moieties on the surface of a sheep red cell (10). This circumstantial evidence might possibly indicate that most of the sialic acid moieties on the cell surface are implicated in the process of immune hemolysis.

The hemolysis of de-sialicated red cells by guinea pig complement in the absence of any known immune reaction raises the provocative possibility that red cells deficient in sialic acid may be lysed spontaneously by complement. All the C'_8 used in these experiments had been adsorbed with relatively large quantities of sheep red cell stroma to remove materials present in complement which result in the spontaneous hemolysis of sheep red cells (2). In addition, it was assumed that any naturally occurring antibody-like materials were removed by this adsorption procedure. The possibility does exist that after treatment with neuraminidase, sites are made available on the surface of red cells which may not have been exposed in the non-treated red cells. Such newly exposed sites could possibly react with guinea pig globulins in such a way as to activate the complement system and result in a form of immune hemolysis. It is important, therefore, that the hemolysis of neuraminidase-treated cells with various complement reagents be investigated, in order to determine whether de-sialicated cells are spontaneously lysed by certain components of the complement system. These experiments are currently being performed.

The observations noted in this investigation suggest that during immune hemolysis a sialic acid–containing substrate is cleaved from the cell surface prior to hemolysis of the red cell. It is further suggested that the removal of sialic acid involves the antigen–antibody combination, in addition to one or more complement components. Hemolysis can be caused by complement in the absence of any known immune reaction when the erythrocytes are de-sialicated. The actual removal of sialic acid from the cell surface during the various stages of immune hemolysis has not been demonstrated.

When sialic acid–containing protein was conjugated to the surface of de-sialicated red cells, lysis of these cells by complement was inhibited (Fig. 6). This is probably due to a competition between the sialic acid of the RT and the

sialic acid on the surface of the red cells prior to the lytic phase of the hemolysis reaction. This substantiates the postulate that during lysis of the cell by complement, the sialic acid moiety is removed from the cell surface. It is not known at which phase during immune hemolysis the sialic acid moiety is cleaved. Methods are available which should permit such determination.

SUMMARY

1. The titer of an individual insulin antiserum measured by hemagglutination agrees favorably with the titer of the same antiserum as measured by immune hemolysis.

2. In contrast, a marked decrease is noted in the immune hemolysis titers relative to hemagglutination titers of antisera prepared against three different sialic acid–containing proteins (human chorionic gonadotropin, rabbit transferrin, and human transferrin).

3. The lower immune hemolytic titers of glycoprotein antisera are apparently not due to a lack of complement-fixing γ_2-antibodies.

4. The glycoprotein antigens in *solution* do *not* interfere with hemolysis in the insulin immune system.

5. By contrast, marked inhibition of insulin immune hemolysis occurs when cells are sensitized with *both* glycoprotein *and* insulin.

6. Cells treated with neuraminidase (to remove cell surface sialic acid) lyse in the presence of C'_s alone.

7. If neuraminidase-treated cells are sensitized with sialic acid–containing protein, the lysis of these cells by complement is inhibited.

8. It is, therefore, postulated that during some initial phase of *immune* hemolysis, a sialic acid–containing substrate is cleaved from the cell surface, rendering the cell susceptible to lysis. If this removal is interfered with, *i.e.*, by sensitization of the cell with competitive sialic acid–containing antigen, then the lytic portion of immune hemolysis cannot proceed.

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