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Demonstration of Antibodies in Acquired Hemolytic Anemia With Anti-Human Globulin Serum.

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(Introduced by A. L. Bloomfield.)

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The technic described by Coombs, Mourant and Race[1] for detecting the presence of the heat stable or "blocking" Rh antibody by agglutinating the sensitized cells with an antiglobulin rabbit serum should be applicable to the investigation of other types of hemolytic disease in which the presence of an immune body is suspected. Agglutination of the sensitized cells depends on the interaction of the attached globulin of the blocking antibody and the antiglobulin rabbit antibody. It has been demonstrated by Boorman, Dodd and Loutit[2] that washed erythrocytes from patients with acquired hemolytic anemia are agglutinated in the antiglobulin rabbit serum whereas those from patients with familial hemolytic jaundice are not, indicating that only the former have a globulin antibody attached to the cell. Observations are reported here which indicate that the erythrocytes of 2 patients with acquired hemolytic anemia were coated with an immune body which could be released and transferred to normal cells *in vitro*.

Methods. The immune rabbit serum was produced by the intracutaneous and subcutaneous injection of 1 cc of group O human serum at weekly intervals for 2 courses of 4 injections each. The cell antibodies in the serum were absorbed by incubation with an equal volume of a 50% saline suspension of pooled A and B cells at 37°C for one hour. After 2 absorptions the serum did not agglutinate normal cells of any group or type, but when further diluted 1-10 with isotonic saline it produced agglutination of Rh_1 cells after they had been exposed to a 1-128 saline dilution of antiRh_0 serum containing only the heat stable or "blocking" antibody. The agglutinating titer of the antiRh_0 serum when diluted with 30% beef albumin was 1-128. Serial dilutions of the immune rabbit serum produced a visible precipitin reaction with a 1-10,000 dilution of commercial preparation of human immune globulin.

Erythrocytes were obtained by collecting capillary blood in normal saline or by defibrinating venous blood. The cells were washed 3 times with normal saline before suspending in the antiglobulin serum. The capillary tube technic[3] as well as the usual test tube method followed by centrifugation were used to detect agglutination and results were checked by observation of the suspensions on a glass slide.

The elution technic of Landsteiner and Miller[4] was used in the transference of the erythrocyte antibody as follows: Washed cells from the patient and from a normal group O individual were each suspended in twice their volume of normal saline and kept in a 56° water bath for 5 minutes with gentle agitation. The suspensions were then centrifuged quickly in heated cups and the supernatant fluid removed. The degree of hemolysis was observed to be roughly equal in the 2 samples. The eluate was divided into 1 cc amounts and 0.1 cc of a concentrated suspension of normal group O cells was added. The suspensions were kept over night at varying temperatures with intermittent agitation. At the end of this period samples were removed and the cells washed before suspending in the antiglobulin serum.

Results. Observations were begun with a patient who has been shown to hemolyze transfused cells as well as her own at an

1 Coombs, R. R. A., Mourant, A. E., and Race, R. R., *Brit. J. Exp. Path.*, 1945, **26**, 255.

2 Boorman, K. E., Dodd, B. E., and Loutit, J. F., *Lancet*, 1946, **1**, 812.

3 Chown, B., *Am. J. Clin. Path.*, 1944, **14**, 114.

4 Landsteiner, K., and Miller, C. P., *J. Exp. Med.*, 1925, **42**, 853.

TABLE I.
Agglutination of Normal Erythrocytes by Anti-human Globulin Serum After Exposure to Eluate from Erythrocytes of a Patient with Acquired Hemolytic Anemia. Positive results were obtained only from suspensions which stood at ice box temperature. Agglutination of samples of the same suspension was increased by subsequent incubation at 37°C.

	Temp. incubation, °C	Agglutination in anti-globulin serum
Eluate from patient's cells	37	0
plus	20	0
0.1 volume normal O cells	5	+ (37°C 2 hr) ++
Eluate from normal cells	37	0
plus	20	0
0.1 volume normal O cells	5	0 (37°C 2 hr) 0

accelerated rate.[5] A hemolysin is presumed to be the cause of the hemolytic anemia which has continued without improvement. Her erythrocytes have shown a consistent agglutinability in all dilutions of the anti-globulin serum as high as 1-80 in contrast to the absence of agglutination in any dilution of normal erythrocytes or cells from patients with congenital hemolytic jaundice. It was noted in preparation of the eluate that exposure of the patient's cells to 56°C for 5 minutes removed or destroyed this agglutinable property.

When normal group O cells were suspended in the eluates prepared in the manner described above only those cells which had been suspended in the eluate from the patient's cells and kept at ice box temperature showed a tendency to agglutinate in the anti-globulin serum, while the cells suspended in the eluate from the control showed no tendency to agglutinate. Cell suspensions kept at room temperature and 37°C gave negative results. It was observed that agglutinability of normal O cells after exposure to the eluate from the patient's cells became more pronounced if, after removal from the ice box, a sample of the suspension was allowed to stand at 37°C for 1 or 2 hours before the cells were washed and suspended in the antiglobulin serum shown in Table I. These observations were repeated with freshly prepared eluate and cells of various types with the same results.

A second patient with an acute hemolytic anemia of the acquired type was studied and observations of similar nature have been made. She was 23 years of age and had no past or family history suggestive of hemolytic disease. For 3 weeks she had noted increasing weakness, nausea and yellow color. She was pale and jaundiced and the spleen was palpable below the costal margin. The hematocrit was 10, and Hb. 3.4 g/100 cc. Reticulocytes were 10% and the icterus index 50. Red cell fragility in hypotonic saline was greatly increased and spherocytosis was evident in the blood smear. She was group O, Rh_1. Repeated multiple transfusions had a temporary effect in elevating the erythrocyte count and hemoglobin and it seemed certain without more exact determinations that transfused cells were involved in the hemolytic process. Cold agglutinins were demonstrated in a titer of 1-32 and an atypical agglutinin active at 37°C was demonstrable for about 30% of O cells but had no Rh or MN specificity. An atypical hemolysin was not demonstrated.

This patient's erythrocytes showed marked agglutination in a 1-160 dilution of the anti-globulin rabbit serum even when observations were carried out at 37°C to eliminate the cold agglutinin as a factor. Her cells failed to show agglutination after exposure to 56°C for 5 minutes. Normal O cells from 3 individuals showed no agglutination in her serum, but when incubated in the eluate from her cells acquired a susceptibility to agglutination in the antiglobulin serum as shown in Table II.

Discussion. It is evident that the erythrocytes from the 2 patients with acquired hemolytic anemia had a property uniquely

[5] Evans, R. S., *Arch. Int. Med.*, 1946, **77**, 544.

TABLE II.

Demonstration of an Immune Body in a Saline Eluate from the Cells of a Second Patient with Acquired Hemolytic Anemia. After 18 hr at 5°C the cells showed more intense agglutination in the anti-globulin serum after an additional incubation at 37°C.

	Group O cells 0.1 cc	Anti-globulin rabbit serum	
		18 hr ice box	+ 2 hr 37°C
Eluate from patient's cells 1.0 cc	1	±	++
	2	±	++
	3	±	++
Eluate from normal cells 1.0 cc	1	0	0
	2	0	0
	3	0	0

different from that of cells of normal individuals in being readily agglutinated by the antiglobulin rabbit serum. This susceptibility to agglutination has been observed so far only in cells that have been exposed to the Rh "blocking" antibody and presumably coated with an immune globulin which attaches to the cell but does not produce agglutination in saline suspension. This suggests that there is an immune body present in acquired hemolytic anemias which attaches to the cell but does not produce agglutination under conditions in which the cells are usually observed. This analogy was further borne out when it was observed that the washed erythrocytes from these patients became agglutinated when placed in 30% beef albumin and 2.0% acacia as are cells known to be sensitized with the Rh "blocking" antibody.[6,7] However, the patient's cells did not agglutinate when suspended in human serum as do cells sensitized by the Rh "blocking" antibody.[8]

The failure to remove the factor responsible for the agglutination of the cells by repeated washings and the ready removal by heating to 56°C again suggests a cell antibody combination.[4] In addition, the demonstration that a substance was released at 56°C which was then capable of attaching to normal cells and withstanding repeated washing further identifies the substance as an erythrocyte antibody. The transfer of the Rh blocking antibody has been carried out by this method.[9,10]

Only tentative explanation can be offered for the temperature effects which seemed to be important in the transfer of the antibodies to normal cells. It is possible that adsorption of the immune globulin on the cells proceeded better in the cold and that subsequent incubation at higher temperatures was necessary for fixation so as to prevent removal by repeated washing.

Although an antibody could be detected on the surface of the erythrocyte from the 2 patients it could not be demonstrated in the serum. The first patient has never shown evidence of an atypical agglutinin or hemolysin in the serum, and moreover group O cells which were suspended in her serum at 37°C failed to agglutinate when washed and suspended in the antiglobulin serum. The second patient exhibited a relatively low titer of cold agglutinins and an atypical agglutinin of an undetermined specificity active at 37°C. The origin of this atypical agglutinin is not clear since the patient had not had transfusions prior to the onset of her illness and a single pregnancy had terminated at the end of 4 months. While the specificity of this agglutinin remained to be determined there is evidence that it was not responsible for the hemolytic process. The group O cells which were not agglutinated by the patient's serum and judged to be compatible were washed and tested with the antiglobulin

[6] Diamond, L. K., and Denton, R. L., *J. Lab. and Clin. Med.*, 1945, **30**, 821.

[7] Levine, P., *Am. J. Clin. Path.*, 1946, **16**, 597.

[8] Wiener, A. S., *J. Lab. and Clin. Med.*, 1945, **30**, 662.

[9] Haberman, S., and Hill, J. M., *Texas State J. Med.*, 1944, **40**, 182.

[10] Carter, B. B., and Loughrey, J., *Am. J. Clin. Path.*, 1945, **15**, 575.

serum for the attachment of an incomplete antibody with negative results. Although these cells failed to show any evidence of sensitization after exposure to her serum *in vitro* at 37°C it was evident after repeated transfusions that these "compatible" cells shared in the rapid hemolysis *in vivo*. In other words, they were being destroyed by an immune body that could not be demonstrated in the patient's serum. It is probable that the immune body which caused the accelerated hemolysis in both patients was not demonstrable in the serum because it is adsorbed as it is produced by the large amount of cell antigen present.

It is not certain, however, that the particular body which was attached to the erythrocytes of both patients could not produce hemolysis or agglutination if present in sufficient concentration. Even if it is passive in this regard, the adherence of a protein to the cell surface probably exerts a detrimental effect by interfering with osmotic equilibrium. The spherocytosis observed may be produced by the accumulation of metabolites which raise the internal osmotic pressure and cause the absorption of fluid, thereby increasing osmotic and mechanical fragility of the erythrocyte and hastening its destruction. The failure to obtain agglutination of erythrocytes from patients with congenital hemolytic jaundice is compatible with the observations of Dacie and Mollison[11] that transfused cells have a normal longevity in this disease which suggests a cell defect rather than a hemolytic agent as the underlying cause.

Summary and Conclusions. The erythrocytes of 2 patients with acquired hemolytic anemia and spherocytosis have been shown to be readily agglutinated by an immune rabbit serum containing an antihuman globulin antibody. Since both patients have exhibited abnormal hemolytic activity for transfused cells this finding is compatible with the existence of an immune body type of hemolytic agent as the cause of the accelerated hemolysis.

Evidence has been presented to show that the immune substance can be separated from patients' cells by heating the cells to 56°C for 5 minutes and can then be combined with normal cells. The similarity of this immune body to the Rh blocking antibody is discussed. The demonstration of the presence or absence of an immune body attached to the erythrocytes should become a routine procedure in the study of hemolytic anemias.

11 Dacie, J. V., and Mollison, P. L., *Lancet*, 1943, **1**, 550.

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Role of Adrenalin in Recovery of Inhibited Conditioned Reactions.

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It was shown in earlier papers from this laboratory[1,2] that insulin coma and chemically- or electrically-induced convulsions cause the reappearance of previously inhibited conditioned reactions (c.r.). No distinct effect was exerted on positive c.r.'s by these procedures.[3,4] Since it had been shown previously[5,6] that the action which these proce-

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1 Gellhorn, E., and Minatoya, H. J., *J. Neurophysiol.*, 1943, **6**, 161.

2 Kessler, M., and Gellhorn, E., *Am. J. Psychiat.*, 1943, **99**, 687.

3 Gellhorn, E., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 155.

4 Gellhorn, E., *Arch. Neurol. and Psychiat.*, 1946, **56**, 216.

5 Gellhorn, E., *Arch. Neurol. and Psychiat.*, 1938, **40**, 125.