C3d antiglobulin haemagglutination of human red blood cells

A demonstration of two types of cell-bound C3d by means of trypsin digestion

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SUMMARY Washed human red blood cells from blood collected in EDTA were tested by Auto-Analyzer for a percentage of maximum antiglobulin haemagglutination (AH) using monospecific antisera to human C3d and C3c.

The cells from normal persons were found to be agglutinated by anti-C3d but not by anti-C3c. To a fixed dilution of antiserum, the normal C3d AH values ($\bar{X} \pm 2$ SD) were $34 \pm 19\%$ for adult cells ($n = 29$) and $14 \pm 19\%$ for cord cells ($n = 19$); the difference was significant ($P < 0.0001$). By pretreatment of these cells with trypsin the C3d AH was either completely abolished or markedly reduced. Its difference between the adult and cord cells was eliminated as the observed values were $4 \pm 7\%$ and $3 \pm 4\%$ respectively ($P = 0.15$). The supernatant fluid of a cell-trypsin mixture, treated with trypsin inhibitors, was found to be inhibitory to C3d AH but not to C3c AH. In contrast, the AH of C3d-coated red blood cells resulting from complement fixation *in vivo* (ie, cold agglutinin disease) or *in vitro* (eg, sucrose water reaction) was resistant to trypsin treatment.

The difference between the trypsin-sensitive and trypsin-resistant cell-bound C3d is postulated to be at its attachment mechanism to the cell membranes. In addition, both the advantage and limitation of using trypsinised cells for C3d antiglobulin tests are demonstrated.

Following the identification of the C3c and C3d fragments of activated C3 (C3b) (West *et al.*, 1966; Bokisch *et al.*, 1969) and the characterisation of human C3b inactivator (INA) (Ruddy and Austin, 1971) or conglutininogen-activating factor (Lachmann and Müller-Eberhard, 1968), it has been clearly demonstrated that red blood cells (RBC) coated with complement components by *in vivo* complement fixation, as those from patients with cold agglutinin disease (CAD) and warm autoimmune haemolytic anaemia (AIHA), carry determinants for C3d but not for C3c (Engelfriet *et al.*, 1970; Moore and Chaplin, 1974). This is presumably due to the action of serum C3b INA which cleaves C3c off the bound C3b, leaving the cells coated with C3d. Burkart *et al.* (1974) have reported that washed

normal human RBC are agglutinable by anti-C3 serum; such reactions have been shown to be due to the presence of C3d on the cell surfaces (Graham *et al.*, 1976; Rosenfield and Jagathambal, 1978). This is a study of haemagglutination of human RBC by anti-C3 sera which demonstrates two types of cell-bound C3d by means of trypsin digestion.

Material and methods

BLOOD COMPONENTS

RBC from normal persons and patients were prepared from blood collected in EDTA by removal of the buffy coat and washing with 8.5 g/l NaCl. Fresh sera as a source of complement were prepared daily. A human serum lacking C3 (ARL C-3 serum) was kindly provided by Dr C. A. Alper, Center for Blood Research, Boston, Mass, USA. Human C3 and C3 INA were obtained from Cordis Laboratories, Miami, Florida, USA.

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TREATMENT OF RBC WITH ENZYMES

Reagents prepared were trypsin (0.1%, unless otherwise indicated), *Vibrio cholerae* neuraminidase (50 U/ml), thrombin (50 U/ml), and plasmin (1 U/ml) diluted respectively in phosphate buffered saline (pH 7.4). Washed packed RBC were incubated with 4 vol of the solution at 37°C for 30 minutes and washed with 8.5 g/l NaCl.

COMPLEMENT FIXATION TO RBC IN VITRO

The low-ionic-strength sucrose water reaction (SWR) was performed according to Hartmann and Jenkins (1966), and cold agglutinin reaction was performed by incubating washed packed RBC with 4 vol of a fresh anti-I serum (titre 128) at 4°C for 30 minutes.

INACTIVATION OF CELL-BOUND C3

This was carried out by the incubation of washed packed RBC with 4 vol of C3 INA (250 U/ml) at 37°C for 30 minutes.

ANTIGLOBULIN TESTS (AT) BY AUTOANALYZER

These were performed as described (Burkart *et al.*, 1974; Hsu *et al.*, 1974). Polyvinylpyrrolidone (PVP) used was 1%, but the incubation coil temperature was 22°C rather than 37°C. Monospecific antisera to human C3d and C3c, kindly provided by Dr H. J. Müller-Eberhard, Scripps Clinic and Foundation, La Jolla, Calif., USA, were heated at 56°C for 30 minutes, adsorbed with trypsinised normal RBC, and diluted to 1:100 for use. The test results were expressed as a percentage of the maximum degree of antiglobulin haemagglutination (AH) in accordance with changes in optical density (OD 550 nm) of the residual 'unagglutinated' cells. The equation for this computation was:

$$\% \text{ AH} = \frac{\text{OD (PVP)} - \text{OD (PVP + antibody)}}{\text{OD (PVP)}} \times 100$$

The AH inhibition by serum or protein solutions was carried out as described (Burkart *et al.*, 1974). The serum was diluted 1:10 for use.

Patients were classified according to the clinical and serological findings into four groups: (1) those with warm AIHA; (2) those with positive direct AT associated with Aldomet (α -methyldopa) therapy; (3) those with CAD; and (4) those with haemolytic disease of the newborn (HDN). Four patients, found to have both warm-reactive and abnormal cold-reactive RBC autoantibodies, were excluded.

Serological studies, including manual tube AT with commercial reagents, cold agglutinin titrations, and antibody identifications, were performed by the standard methods.

Results

C3d AH VALUES

RBC from normal persons were found to be agglutinated by anti-C3d to varying degrees but not by anti-C3c. This C3d AH was inhibitable by normal serum or human C3 but not by ARL C-3 serum. As shown by the results in Fig. 1, to the fixed dilution of anti-C3d, normal C3d AH values ($\bar{X} \pm 2 \text{ SD}$) were $34 \pm 19\%$ for adult cells ($n = 29$) and $14 \pm 19\%$ for cord cells ($n = 19$). The difference was significant ($P < 0.0001$). In patients, cells from 20/39 (51%) adults with warm AIHA, 4/25 (16%) adults with positive direct AT associated with Aldomet therapy, and 18/20 (90%) adults with CAD had C3d AH values above the normal range; the values for cord cells from all eight infants with either ABO or Rh HDN were within the normal limit. One infant was found to have HDN due to anti-K antibodies. The cord cells showed an abnormally high C3d AH value but were not agglutinated by a commercial anti-C3d serum in manual testing. The anti-K antibody from the serum of the mother was found to be IgG capable of coating K-positive RBC with complement in the presence of fresh compatible serum. None of the cells from these patients was agglutinated by anti-C3c.

Fig. 1 C3d antiglobulin haemagglutination of normal persons and patients.

EFFECT OF TRYPSIN ON C3d AH OF NORMAL RBC
By pretreatment of the above normal RBC with trypsin, the C3d AH was either completely abolished or markedly reduced. The difference between the adult and cord cells was eliminated as the observed values were $4 \pm 7\%$ and $3 \pm 4\%$ respectively

Trypsin effect on human RBC-bound C3d

($P = 0.15$). When 1% trypsin was used, no augmentation of the enzyme effect was observed ($n = 6$). The C3d AH of normal RBC was enhanced by pretreatment with neuraminidase (eg, 36% as compared to a control of 29%) but was not affected by pretreatment with thrombin, plasmin, or C3 INA. The supernatant fluid of a cell-trypsin mixture, treated with soybean trypsin inhibitors and concentrated fivefold, was found to be inhibitory to C3d AH but not to C3c AH when tested using cells coated with complement by the SWR. In addition, normal RBC, treated with trypsin or neuraminidase, were not agglutinated by anti-C3c. One experiment with normal RBC from a single donor revealed that whether the cells were prepared from blood collected in EDTA, heparin, ACD, or CPD, or from clotted or defibrinated blood, the observed C3d AH and its sensitivity to trypsin were similar.

EFFECT OF TRYPSIN ON C3d AH OF ABNORMAL RBC

In contrast to the above, the cell-bound C3d resulting from both *in vitro* and *in vivo* complement fixation was found to be resistant to trypsin treatment. Normal RBC at first were trypsinised and then coated with C3d by the SWR or cold agglutinin reaction followed by C3 inactivation with C3 INA. The C3d AH of these cells was not affected by subsequent trypsin treatment even when 1% trypsin was used. The results of an experiment illustrating a trypsin-sensitive v a trypsin-resistant C3d AH are shown in Table 1.

The C3d AH values of RBC from 10 adult patients with CAD along with normal controls are shown in Table 2. The C3d AH values of untreated and trypsinised cells were termed respectively as total and trypsin-resistant. The trypsin-sensitive C3d AH value was the total value minus the trypsin-resistant value. It was observed that the total C3d AH values for cases 1 to 8 were abnormally high and those for cases 9 and 10 were normal. In contrast, the trypsin-resistant C3d AH values for all 10 patients were above the normal range. In addition, it was noticed that the trypsin-sensitive C3d AH values for cases 1 to 6 were below the normal limit. One sample of

Table 1 Trypsin-sensitive v trypsin-resistant C3d antiglobulin haemagglutination of human red blood cells

Treatment in sequence	% Haemagglutination by anti-C3d
Normal RBC	23
+ trypsin	0
+ SWR* + C3 INA	28
+ trypsin	29

*Sucrose water reaction with 0.5 serum volume

Table 2 C3d antiglobulin haemagglutination of adult patients with cold agglutinin disease

Case	% Haemagglutination by anti-C3d		
	Total (a)	Trypsin resistant (b)	Trypsin-sensitive (a-b)
Normal ($\bar{X} \pm 2$ SD; $n = 29$)	34 ± 19	4 ± 7	29 ± 16
1	75	74	1
2	74	73	1
3	70	66	4
4	64	60	4
5	62	53	9
6	62	50	12
7	60	47	13
8	58	28	30
9	51	28	23
10	44	27	17

RBC from a patient with paroxysmal nocturnal haemoglobinuria was found to have a trypsin-resistant C3d AH (81%) greater than its total C3d AH (77%).

To simulate an '*in vivo* complement fixation to RBC', aliquots of normal RBC were at first subjected to the SWR with varying amounts of fresh serum followed by C3 inactivation. These cells were then tested for both total and trypsin-resistant C3d AH values. The results (Fig. 2) revealed that the trypsin-resistant C3d AH curve was a better indicator for complement fixation to RBC than was the total C3d AH curve, as comparing the portions of the curves where the C3d AH values were above the corresponding controls. As indicated by the stippled area, there was a progressive decrease of trypsin-sensitive C3d AH value associated with an increasing serum volume for the SWR.

All RBC from those patients with warm AIHA or with Aldomet therapy were agglutinated by anti-IgG. Trypsinisation of these cells resulted in varying degrees of spontaneous haemagglutination by PVP; this made the test results uninterpretable as to their trypsin-resistant C3d AH values.

Discussion

In this study we demonstrated the presence of two types of bound C3d on the surfaces of human RBC; one was sensitive to cleavage by trypsin and the other was not. Normal RBC carried two populations of bound C3d, predominantly of trypsin-sensitive type, whereas bound C3d resulting from complement fixation to RBC was of trypsin-resistant type.

It is well known that activation and inactivation of human C3 by C3-convertase plus C3 INA or by trypsin result in the formation of C3a, C3d, and C3c fragments (Bokisch *et al.*, 1969; Ruddy and Austin, 1971; Molenaar *et al.*, 1974). There is no

Fig. 2 Total and trypsin-resistant C3d antiglobulin haemagglutination reactions of normal RBC coated with C3d by the sucrose water reaction (SWR) and C3 inactivation with C3 INA.

known enzyme capable of cleaving C3 to generate a C3a-C3d fragment. Therefore, the absence of a demonstrable C3c AH from normal RBC indicates that bound C3d on the cell surfaces is without an attached C3a. According to Müller-Eberhard (1975), human C3d carries at least two functional binding sites: (1) a labile binding site at the N-terminal end of the molecule exposed by C3-convertase is responsible for the attachment of activated C3 (C3b) to the target surfaces; and (2) a stable binding site at the C-terminal end exposed by C3 INA for the C3d receptors of certain lymphocytes (Ross *et al.*, 1973). There is evidence that the C3d is resistant to destruction by trypsin digestion (Bokisch *et al.*, 1969; Molenaar *et al.*, 1974). Thus, it is most likely that the difference between these two types of bound C3d is at their attachment mechanisms to the cell membranes rather than their structures. It may be postulated that the presence of two populations of bound C3d on normal RBC is the result of an *in vivo* C3 activation in the fluid phase followed by C3b inactivation. The attachment of C3 to RBC through the labile binding site of C3d is resistant to tryptic attack, and the C3 attachment through a C3d site other than the labile binding site or mediated by a plasma protein forming a cell-

protein-C3d linkage is trypsin-sensitive. Under this hypothesis, the cleavage of bound C3d by the proteolytic effect of trypsin is due presumably to a destruction of either the membrane 'receptors' or the linking proteins. The basis for the difference in C3d AH between the adult and cord cells remains to be defined.

Supported by the study results (Figs 1 and 2; Table 2), the wide variation of normal C3d AH values clearly affects the accuracy of C3d AT, especially of the direct tests where autologous cells as controls are not available. Thus, the incidence of positive C3d AT in the patients studied (Fig. 1) may be expected to be higher than that observed. The advantage of using trypsinised RBC for C3d AT is illustrated in Table 2 and Figure 2. However, the spontaneous haemagglutination of PVP of warm-antibody-coated RBC associated with trypsinisation of the cells limits its clinical application. This may be overcome by the isotope method with the use of rabbit ^{125}I -IgG anti-C3d containing human IgG in excess.

The enhancement of C3d AH of normal RBC by neuraminidase treatment is consistent with the observation on the enzyme effect to the blood group antigen-antibody interactions (Greenwalt and Steane, 1973). The decrease of trypsin-sensitive C3d AH values associated with an increasing complement fixation to RBC (Table 2; Fig. 2) is likely to be factitious due to the non-parallelism between the total and trypsin-resistant C3d AH curves where the AH is enhanced by trypsinisation of the cells.

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