Auto-Sensitization of Trypsin Treated Human Red Cells by Complement.* (29682)

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That human red blood cells (RBC) may, in certain pathological conditions, become coated or sensitized with autologous complement (C′) has recently been directly demonstrated(1,2). It is now clear that such C′ sensitization is responsible for direct antiglobulin (Coombs') reactions of the "non-γ-globulin" type. Although cold agglutinins (1-4) or antibody in certain forms of drug hypersensitivity(5) have a known potential for fixing C′ to autologous human erythrocytes, such mechanisms cannot be demonstrated in many cases of autosensitization with C′ and the observed autosensitization remains unexplained(2). It was of interest, therefore, to observe that normal human RBC treated briefly with a low concentration of the proteolytic enzyme, trypsin, appear to become selectively sensitized with C′ when exposed to fresh autologous serum at 37°C(6). The serum factor responsible for this sensitization appears to be distinct from the more familiar types of human antibodies reactive with autologous RBC. The results of our early efforts to characterize this serum factor and to assess its possible relevance to clinical erythrocyte autosensitization are presented here.

Methods. A one per cent suspension of washed normal human RBC was treated for 30 minutes at 37°C with 0.01% crystalline trypsin (Worthington), usually at pH 8.0. Further purification of the trypsin was not undertaken. After thorough washing, the "trypsinized" RBC and suitable dilutions of autologous fresh serum were brought to 37°C, mixed, and incubated for one hour at that temperature and neutral pH. The RBC were then washed again and tested with a panel of antiglobulin sera of differing specificities to determine the nature of the serum proteins which had attached to the altered RBC. These antisera did not agglutinate trypsin treated RBC that had not been exposed to fresh serum. In certain experiments trypsinized homologous group O RBC were used in lieu of autologous RBC and gave comparable results. Freshly drawn RBC and RBC stored for one week in Alsever's solution at 4°C did not differ in this system. Untrypsinized autologous RBC were not detectably sensitized. When sera were serially diluted or when stored sera or serum fractions were studied, a constant excess of C′ was provided in each incubation mixture by addition of fresh cord serum or fresh compatible adult serum which had been absorbed once with packed trypsinized RBC in the cold (*v. infra*). Cold agglutinins were purified by absorption and elution as previously described(4).

Results. After exposure to either autologous or homologous fresh adult serum at 37°C the trypsin treated RBC gave positive antiglobulin reactions with a highly specific anti-C′ serum (anti-β_1A-globulin)(7) but not with antisera to human γ_2-globulin, γ_1A (β_2A)-globulin, or γ_1M (β_2M)-globulin (Table I). Negative reactions were also obtained with an antiserum (anti-E.E.) containing antibodies to numerous human serum proteins other than γ-globulins or C′. The preparation and sepcificities of these antisera have been described(2). When the fresh human serum had been previously heated at 56°C for 10 minutes or treated with 0.01 M Na_2 EDTA, hemolytic C′ activity was destroyed and sensitization of trypsinized RBC could not be detected. Fresh neonatal (cord) serum, however, despite potent C′ titers, was found to be incapable of sensitizing trypsinized RBC but could restore the sensitizing activity of a heated (10 minutes) adult serum (Table I). Fresh cord serum, however, did not restore the sensitizing activity of an adult serum which had been heated for

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TABLE I. Antiglobulin Reactions of Trypsinized RBC Sensitized by Autologous Serum.

Incubation mixture		Rabbit antisera*						
Sensitizing serum	Red cells	Anti-W.S.	Anti-γ_2	Anti-γ_1A	Anti-γ_1M	Anti-C′	Anti-E.E.	Pooled NRS†
Fresh autologous adult	Normal	—	—	—	—	—	—	—
Idem	Trypsinized	+++	—	—	—	+++	—	—
Heated‡ autologous adult	"	—	—	—	—	—	—	—
Fresh cord serum	"	—	—	—	—	—	—	—
Heated‡ adult & fresh cord	"	++	—	—	—	++	—	—

* Antisera: W.S. = whole serum (human); γ_2 = γ_2-globulin; C′ = human complement; E.E. = "everything else," *i.e.*, serum proteins other than γ-globulins or complement.
† NRS = Normal rabbit serum.
‡ 56°C for 10 min.

30 minutes. These findings suggested that sensitization of trypsinized RBC depends upon (a) a relatively heat stable factor in normal adult serum, which, for convenience, will be called "Tr. factor," and (b) hemolytically active serum C′.

To date, Tr. factor has been found in low titer (1/2-1/32; mean 1/4) in each of 13 normal adult sera, was absent in 9 cord sera and present in apparently elevated titers (1/64-1/2048 or more) in the sera of 11 of 31 patients with "autoimmune" hemolytic disease (AHD) (Table II). To be certain that these higher titers are truly abnormal, similar studies of age matched healthy persons would be desirable (see *Comment* and (10)). All 11 of the high titered sera came from patients whose RBC reacted *directly* with the anti-C′ (anti-β_1A) serum (without *in vitro* trypsin treatment of the RBC), indicating that C′ sensitization had occurred *in vivo*. Three of these 11 patients also had γ_2-globulin coating of their circulating RBC. The majority of these AHD patients had not been transfused. In 2 normal donors studied repeatedly over a 4 month period, there was no appreciable change in their low titers of Tr. factor.

Although C′ sensitization of trypsinized RBC was readily and consistently induced at 37°C, both normal and high titered AHD sera showed greater sensitizing activity at 2°C. The Tr. factor could be absorbed from such sera by trypsinized cells only at low temperature, and it was slowly and incompletely eluted again at 37°C. With high titered AHD sera, direct agglutination of trypsinized RBC was observed at 2°C but was seldom observed after the usual one hour incubation at 37°C. Direct agglutination was not observed in studies of normal sera.

Initially, the foregoing data suggested the

TABLE II. Clinical and Serological Data on Patients with High Titers of Tr. Factor.

			Patients' RBC	Patients' sera	
Patient	Age	Clinical diagnosis*	Direct antiglobulin pattern	Cold agglutinin titer	Tr. factor titer
B.P.	75	Idio. AHD	C′	512	1024
A.R.	60	" "	γ_2 + C′	80	1024
R.G.	65	" "	C′	160	512
P.F.	45	" "	C′	20	128
G.F.	56	" "	γ_2 + C′	<10	1028
M.P.	48	" "	γ_2 + C′	10	64– 128
J.S.	52	Suspected SLE	C′	80	256
F.W.	67	" "	C′	20	1024–2048
M.K.	75	SLE	C′	40	128– 256
B.F.	35	"	C′	80	64– 128
E.D.	51	Ovarian CA	C′	1024	512–1024
13 Normals (mean age 30)			—	<10	2– 32

* Idio. AHD = idiopathic "autoimmune" hemolytic disease; SLE = systemic lupus erythematosus; CA = carcinoma.

TABLE III. Studies of Cold Agglutinin and Tr. Factor in a Pathological Serum.

Incubation mixture		Hemagglutination titers			
		37°C incubation		2°C incubation	
B.P. serum or derivative	Group O red cells	Saline agglut.	Antiglobulin reaction*	Saline agglut.	Antiglobulin reaction*
1. Unabsorbed serum†	Normal	—	—	512	128
	Trypsinized	4–8	1024	512–1024	n.d.**
2. Absorbed with normal RBC at 2°	Normal	n.d.	n.d.	—	—
	Trypsinized	—	512	pos‡	n.d.
3. Absorbed with trypsinized RBC at 2°	Trypsinized	—	—	—	—
4. Eluted Tr. factor§	Normal	—	—	—	—
	Trypsinized	—	16	n.d.	n.d.

* Titers shown represent serial doubling dilutions of serum (or eluate). During the preceding incubation, excess C′ was added to each dilution, as explained in text. Antiglobulin reactions were then carried out with a constant 1/20 dilution of the antiserum.

† Separated at 37°C.

‡ Moderate direct agglutination was observed but not titered.

§ For explanation, see text.

** n.d. = not determined.

possibility that trypsin treatment of RBC might, in effect, raise the thermal amplitude of classical cold agglutinins, allowing them to fix C′ at 37°C, thus accounting for "Tr. factor" activity. The following observations, however, suggest that at least a major part of the serological activity which we have ascribed to "Tr. factor" is distinct from anti-I cold agglutinin. First, there was no consistent correlation between serum titers of cold agglutinin and Tr. factor in the 31 AHD patients as shown, in part, in Table II. Not shown are other cases with comparable cold agglutinin titers with very little Tr. factor activity. Secondly, the cold agglutinin of several cases listed in Table II was completely absorbed from the serum with unaltered I-positive RBC, with little effect on the titer of Tr. factor against the trypsinized RBC of the same donor. For example, Table III summarizes a study of one AHD serum (B.P.) with relatively high titers of both cold agglutinin and Tr. factor. The cold agglutinin in B.P. serum was capable of inducing C′ sensitization of normal RBC to a titer of 128. No sensitization occurred at 37°C. The same serum induced C′ sensitization of trypsinized RBC at 37°C to a titer of 1024. After complete absorption of cold agglutinin with normal RBC, the serum retained excellent capacity (1/512 titer) to sensitize trypsinized RBC at 37°C. The Tr. factor could then be absorbed from the serum by trypsinized RBC at 2°C, eluted at 37°C, and identified by its C′-sensitizing activity with trypsinized RBC but not with normal RBC. Finally, the apparent inactivation of Tr. factor by heating at 56°C for *30 minutes* is another point of difference from cold agglutinin.

It must be mentioned that cold agglutinins, isolated from 2 high titered sera by cold-warm cycles of absorption to and elution from normal RBC, exhibited weak but definite capacity to agglutinate trypsinized RBC at 37°C and to sensitize them with C′ at the same temperature. This is taken to mean that trypsinization of RBC does not destroy the sites at which cold agglutinins react with RBC, and, indeed, that enhanced reactivity of cold agglutinin with trypsinized RBC does occur. Such enhancement, however, cannot adequately explain the sensitizations of trypsinized RBC noted in Tables II and III, and a Tr. factor as a separate entity from cold agglutinin is therefore postulated. Trypsinized neonatal RBC, incidentally, were sensitized as strongly as trypsinized adult RBC by ABO compatible adult sera. That the Tr. factor is likewise distinct from the naturally occurring anti-H "incomplete" cold antibody is suggested by the known occurrence of the latter in cord serum(8), and by the failure of untrypsinized group O RBC to absorb the Tr. factor, even in the cold.

In work to be presented in detail elsewhere, the Tr. factor of several high titered

AHD sera behaved as a 19S globulin in sucrose density gradient ultracentrifugation and as a fast γ-globulin in starch block electrophoresis. Cold agglutinins had been thoroughly absorbed from these sera prior to fractionation. The normal sera studied to date have not had sufficient Tr. factor titers to permit physicochemical characterization.

Finally, an attempt was made to test the possibility that the Tr. factor may represent naturally acquired antibody to bovine trypsin, with C′ sensitization of trypsinized RBC resulting simply from the interaction of "anti-trypsin" antibody with membrane-bound trypsin. Trypsin, in concentrations from 0.5 to 125 μg/ml, was incubated with the Tr. factor of a normal serum for 5 minutes at 37°C prior to addition of autologous trypsinized RBC to the mixture. Inhibition of sensitization was not observed in this single experiment.

Comment and summary. The foregoing data indicate that trypsin treated human RBC become sensitized with complement (C′) during 37°C incubation with the autologous (or homologous) fresh serum of normal donors. This C′ sensitization appears to be mediated by a distinct serum factor, "Tr. factor." The greater sensitizing activity of sera from certain patients with "autoimmune" hemolytic disease (AHD) is probably attributable, in the main, to the presence in these AHD sera of a larger quantity of the same Tr. factor found in normal sera. If so, the combined evidence from studies of normal and AHD sera would suggest that Tr. factor is an acquired, normally occurring, C′-fixing 19S γ-globulin active against proteolytically altered RBC over a broad thermal range. The Tr. factor appears to be distinct from the more familiar types of C′-fixing antibodies reactive with unaltered autologous RBC, such as anti-I cold agglutinin and anti-H "incomplete" cold antibody.

Many other workers have described factors in the sera of normal donors(9-12) or of some AHD patients(13,14) capable of agglutinating, lysing or sensitizing trypsinized RBC. Indeed, quite analogous observations have been made with RBC altered by proteases other than trypsin or by other chemical treatments. To our knowledge, demonstration of the induction of C′ sensitization of the RBC in such systems has not been previously reported. The trypsin treatment employed in the present study was generally milder than that commonly used in other investigations. It seems likely, however, that our Tr. factor is the same as the factors studied by Jankovic(11) and by Yachnin and Gardner(12). The spontaneously reversing agglutination of trypsinized RBC described by Rosenthal and Schwartz(9), on the other hand, may represent a distinct phenomenon. The present observations should not be confused with the well known enhancement of antiglobulin reactions produced by treating already sensitized RBC with proteases(15).

There are a number of possible explanations for the Tr. factor. One is that it is an antibody specifically formed to antigenic determinants absent from, or "masked" in, normal RBC. Red cell senescence may normally involve gradual changes in the RBC membrane which are closely mimicked by the changes induced experimentally by trypsin. Such senescent RBC might elicit low titers of antibodies to altered or unmasked antigens, providing thereby a possible mechanism contributing to the normal removal of effete RBC. Some support for this concept may be found in the reports of augmented phagocytosis or sequestration of both trypsinized(16,17) and aged(18) RBC, by mechanisms which appear to depend upon naturally occurring, opsonin-like serum factors(17,18) and complement(17).

Whatever their mode of origin, antibody-like factors such as the Tr. factor could have pathogenetic significance if release of active proteases were to occur in certain disorders associated with *in vivo* erythrocyte autosensitization. Such released proteases might alter RBC *in vivo*. Such altered RBC in the circulation might, in turn, enhance formation of these antibody-like factors and, as a consequence, become autosensitized with C′, resulting in a positive direct antiglobulin reaction with or without manifest hemolysis. The type of RBC alteration postulated need not depend upon trypsin itself; indeed one or

more of the various tissue (lysosomal) cathepsins(19) would appear to be more likely candidates as the protease(s) involved. The original insult responsible for initiating the release or activation of proteases is not defined in this postulation.

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Effects of Hypophysectomy, Thyroidectomy and Thyroid Hormones on Steroid Metabolism in the Rat.* (28683)

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In a preliminary study(1) we found that hypophysectomized rats eliminated accumulated C^{14}-cholesterol at a markedly slower rate than normals. Inability to eliminate absorbed sterol may be the reason that such rats accumulate cholesterol of exogenous origin more easily than normals(2,3). Since the hypophysectomized animal lacks a number of target gland hormones as well as pituitary hormones, these effects may be due to one or several of these substances. Recently Wells and Ershoff(3) have observed that thyroid hormone administration largely prevents accumulation of blood cholesterol in cholesterol-fed hypophysectomized rats.

In the present experiments, we compare rates of cholesterol-bile acid metabolism and excretion in normal, thyroidectomized, hypophysectomized, and thyroid hormone-treated-hypophysectomized rats.

Methods. Twenty, 6-month-old, female albino Sprague-Dawley rats were used in these studies: 4 were normal animals; 4 had been thyroidectomized at 3 months of age by intraperitoneal injection of 900 μc(4) of carrier-free I^{131}; and 12 had been hypophysectomized† at 3 months. Following thyroidectomy or hypophysectomy, weights and CO_2 production were checked periodically for the

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