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Studies on immune adherence (C3b) receptor activity of human erythrocytes: relationship between receptor activity and presence of immune complexes in serum

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SUMMARY

Human erythrocytes (E) have surface receptors for the third component of complement (C3b-IA receptors) which mediate immune adherence haemagglutination (IAHA). We have observed that E from patients with systemic lupus erythematosus had impaired or defective C3b receptor (C3b-R) activity when circulating immune complexes (CIC) were found in serum. This phenomenon has been investigated by a newly developed method involving competitive inhibition of IAHA in patients with immune complex diseases. IAHA involving sheep E coated with antibody and complement (EAC), and indicator human E was inhibited by lysates of E with normal C3b-R activity from healthy donors and a monkey. In contrast, the lysates of E from 95% of patients bearing CIC did not inhibit IAHA, which indicated such E had defective or impaired C3b-R activity. This phenomenon was supported by control studies in which IAHA was not inhibited by lysates of E with absent, inactivated or occupied C3b-R. In those patients, in whom CIC disappeared during immunosuppressive therapy, C3b-R activity slowly returned to normal levels. Moreover, it was observed that C3b-R activity of patients' E decreased with the reappearance of CIC during exacerbations of disease. These observations suggest that CIC are carried *in vivo* by the C3b-R of E as well as those of the mononuclear phagocyte system, and that the E C3b-R may also contribute to the clearance of CIC.

INTRODUCTION

In systemic lupus erythematosus (SLE), circulating immune complexes (CIC) are detected during active disease (Nydegger *et al.*, 1974) and are believed to cause tissue damage when deposited with activated complement. The mechanisms that govern the fate of CIC in man and their predilection for certain tissues are not clear.

There have been only a few studies of kinetics in the clearance of immune complexes from the circulation (Mannik *et al.*, 1971; Mannik & Arend, 1971; Mannik, 1980; Arend & Mannik, 1971; Scornik & Drewinko, 1975). Functions of the mononuclear phagocyte system have been explored, measuring the functional capacity of receptors for complement and IgG-Fc (Frank *et al.*, 1979; Haakenstad & Mannik, 1974; Jaffe *et al.*, 1978; Lockwood *et al.*, 1979).

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Although human erythrocytes (E) also possess complement receptors (C3b-R) little attention (Siegel, Lin & Gleicher, 1981; Nelson, 1955) has been directed to the role of E in immune complex diseases.

This paper reports studies of levels of C3b-R activity of E from normal healthy donors and from patients with immune complex diseases in relation to the presence of immune complexes in serum. We observed that E from patients with active disease have defective or impaired C3b-R activity when CIC were present.

MATERIALS AND METHODS

Erythrocyte (E) specimens. One hundred and three E specimens from 82 such patients during various stages of therapy and 10 healthy human donors were tested for C3b-R activity. Also tested were samples of E from the following animal species: hamster, rabbit, dog, guinea-pig, sheep and monkey.

Heparinized blood was washed five times with 0·01 M EDTA-GVB. To 60 μl of packed E, 660 μl of water were added. The mixture was kept at room temperature for 5 min. Appropriate amounts of a five times concentrate veronal buffer and 0·1 M EDTA solution and gelatin were added to constitute 1 ml of a 0·01 M EDTA-GVB solution. In this manner, a lysate of 6% E was achieved and serially diluted with 0·01 M EDTA-GVB for lysate concentrations of 3, 1·5 and 0·75%.

To inactivate C3b-R activity, 1·5% human E suspension in 0·01 M EDTA-GVB was incubated at 37°C for 30 min with an equal volume of 40 mM DTT or 0·25% trypsin in EDTA-GVB.

One percent E (blood type O, Rh positive) suspension in EDTA-GVB was incubated with a 1:10 dilution of anti-Rh antibody (A. Ripley serum) at 37°C for 30 min to observe effects of incomplete antibody on C3b-R activity.

Either heat aggregated human IgG (16 μg/ml) or antigen–antibody complexes between human IgG (128 ng/ml) and rabbit anti-human IgG serum (1:400) were made to adhere to C3b-R of normal human E in the presence of complement and DTT using the microtitre plate method for IAHA. E showing 4+ haemagglutination were collected from each well with a pipette.

The E, pre-treated as described above, were washed five times and lysed with water and reconstituted to 0·01 M EDTA-GVB as above.

Measurement of C3b-R activity of erythrocytes. To determine C3b-R activity of E, an adaptation involving competitive inhibition of immune adherence haemagglutination (IAHA) was applied as follows;

(a) preparation of antigen–antibody complexes (EA): a sheep E suspension (1×10^9 cells/ml of GVB^{++}) was incubated with an equal volume of optimally diluted rabbit anti-sheep E antibody at 37°C for 30 min with agitation. After incubation, EA was washed three times with GVB^{++} and adjusted to an appropriate concentration showing 4+ haemagglutination pattern at the highest dilution of EA in the IAHA microtitre method.

(b) measurement of C3b-R activity: an appropriate concentration of EA (25 μl, approximately 3×10^7 cells/ml of GVB^{++}) was incubated with 25 μl of guinea-pig complement (2·5 CH_{50} units/ml of GVB^{++}) in a microtitre plate at 37°C for 40 min. Twenty-five microlitres of DTT solution (20 mM in EDTA-GVB) were then added to the reaction mixture. Subsequently, 25 μl of an aqueous lysate of test E samples which had been reconstituted on 0·01 M EDTA-GVB were added to the reactant. After 30 min incubation at room temperature, 25 μl of the standardized indicator human E suspension (1·5% in EDTA-GVB) were added. As summarized in Fig. 1, IAHA between EAC and C3b-R of indicator E was competitively inhibited by C3b-R of test E lysates depending on their C3b-R activity and on the concentration of the lysed E. Thus positive IAHA patterns (no inhibition of IAHA) were interpreted as indicative of absence of C3b-R activity of test E samples. Negative IAHA patterns (inhibition of IAHA) were interpreted as indicative of high C3b-R activity of test E samples.

Detection of immune complexes in serum (CIC). The IAHA procedure for detecting CIC has been described (Inada *et al.*, 1981). In summary, each serially diluted serum (heat-inactivated at 56°C for 30 min) was incubated with guinea-pig complement (2·5 CH_{50} units/ml) at 37°C for 40 min

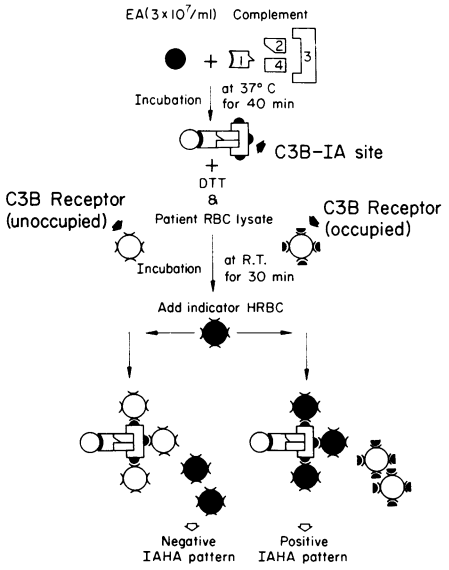

Fig. 1. Schematic drawing of the reaction sequence for competitive inhibition of the IAHA test by the erythrocyte lysates with and without C3b receptor activity. Complement lysed sheep E (EAC) are used to agglutinate unlysed indicator human E via C3b-IA sites on the EAC and the C3b receptors on the human E. If water lysed normal human E are mixed with EAC, then EAC are bound to the lysed human E and thereby inhibited from agglutinating unlysed indicator human E. Water lysed E from patients with CIC, because their C3b receptors might be blocked by bound CIC, do not inhibit agglutination of unlysed indicator human E. Activation of a complement cascade from 5 through 9 is not shown.

in a permanent type U bottom microtitre plate (Nihon Microtec, Tokyo, Japan). Dithiothreitol solution (DTT, 20 mM) and a standardized indicator human E suspension (1·5%) was added. After 60 min at room temperature, the extent of haemagglutination was visually estimated on a scale of 0 to 4+. The titre was expressed as the highest dilution of serum yielding a 2+ haemagglutination pattern. A 2+ haemagglutination pattern represented the equivalent of 2 μg/ml of heat-aggregated human IgG in an IAHA assay system. A final concentration of DTT at 5 mM stabilized the binding of C3b to C3b-R (Takahashi, 1972) and had no effect on C3b-R activity of human E (Dierich *et al.*, 1974).

Direct Coombs' test. Patients' E suspension (1% in EDTA-GVB) were mixed with serially diluted antisera; goat anti-human gamma chain (Kallestad, Laboratories, Cheska, Maine, USA), rabbit anti-human C3c or C4 (Miles Laboratories, Elkhart, Indiana, USA) in a microtitre plate, to confirm the presence of immunoglobulin and complement components on E surfaces. The presence of the above proteins was estimated by the degree of the haemagglutination pattern rated 0 to 4+.

Measurement of total haemolytic complement and C3 component of serum. Titrations of total haemolytic complement (CH_{50} units/ml) of serum samples were performed by Mayer's method (Mayer, 1961). The third complement component of serum was assayed by single radial immunodiffusion kit (Calbiochem-Behring Corp., La Jolla, California, USA).

RESULTS

Inhibition of IAHA by non-treated or treated normal human E lysates

As shown in Table 1, a control assay mixture consisting EA, complement, DTT and the buffer instead of an E lysate showed a 4+ haemagglutination pattern in the inhibition assay system. With the addition of 1·5% E lysates from normal healthy donors, the positive IAHA patterns were reduced from 4+ to 1+. Furthermore, the addition of 3 or 6% lysates resulted in negative IAHA pattern.

Table 1. Inhibition of IAHA test by erythrocyte lysates

Inhibitors (water lysates)	IAHA pattern				
	% of erythrocyte lysate added				
	6·0%	3·0%	1·5%	0·75%	Buffer
Human E	−	−	+	+++	++++
Human E coated with anti-Rh antibody*	−	−	+	++/+++	++++
Human E bearing *in vitro* formed immune complexes	+++	++++	++++	++++	++++
Human E pre-treated with DTT	++++	++++	++++	++++	++++
Human E pre-treated with trypsin	++++	++++	++++	++++	++++
Hamster E	++++	++++	++++	++++	++++
Rabbit E	++++	++++	++++	++++	++++
Dog E	++++	++++	++++	++++	++++
Guinea-pig E	++++	++++	++++	++++	++++
Sheep E	++++	++++	++++	++++	++++
Monkey E	−	−	+	+++	++++

* DTT was omitted in this system.
(++++)=No inhibition in IAHA system, i.e. no C3b receptor activity.
(−)=Inhibition in IAHA system, i.e. high C3b receptor activity.

The lysates of normal human E pre-treated with anti-Rh antibodies also inhibited the IAHA system to the same extent as non-treated human E lysates. DTT was omitted in these experiments, because non-specific aggregation between DTT and indicator E had been observed. We had found that DTT induced non-specific aggregating activity with E highly coated with anti-Rh antibody. It was suspected that antibody might have been released from anti-Rh antibody coated E by aqueous lysis which then reacted with Rh positive indicator E in the reaction mixture. This phenomenon would have resulted in aggregation of indicator E in the presence of DTT. Accordingly, non-specific aggregation was avoided by using type O, Rh negative E as indicator cells in the presence of DTT or type O, Rh positive E as indicator cells in the absence of DTT.

Lysates of normal E bearing *in vitro* formed complexes via C3b-R did not inhibit IAHA even in 6% concentrations. Likewise, lysates of normal human E, pre-treated with DTT or trypsin, did not inhibit IAHA. Thus it was confirmed that IAHA patterns were competitively inhibited by lysates of E with free or unoccupied C3b-R.

Inhibition of IAHA by non-primate and primate E lysates

It is well known that C3b-R exist on both human and primate E, but not E from other mammals such as hamsters, rabbits, dogs, guinea-pigs and sheep.

E lysates from the above non-primates failed to inhibit the IAHA patterns even in higher concentrations (Table 1). On the other hand, lysates of monkey E inhibited IAHA patterns similarly to normal human E.

Inhibition of IAHA by the lysates of E from patients with immune complex diseases

One hundred and three E samples from 82 patients with immune complex diseases were assayed for C3b-R activity (Fig. 2). Concomitant serum specimens from the E donors were also tested for the presence of CIC.

It was observed that 55 serum specimens from 40 patients contained CIC ranging from 4 μg/ml

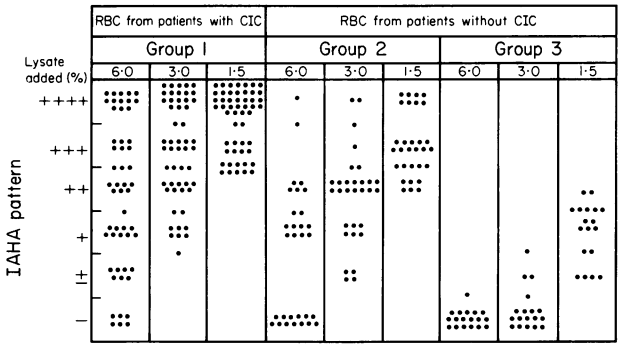

Fig. 2. Inhibition of the IAHA test by patients' E lysates. Various haemagglutination patterns were observed by adding E lysates depending on their C3b receptor activity and on the concentration of E in the lysates.

to 512 μg/ml (Group 1), while 48 serum specimens from the remainder of the patients had no CIC (Groups 2 and 3).

As shown in Fig. 2, 52 lysates from 39 patients having CIC (Group 1) did not inhibit IAHA patterns when the lysate concentration was 1·5%. When 3 or 6% concentrations of E lysates were added to the system, various degrees of inhibition occurred depending on the clinical status of the patients. It was concluded that C3b-R activity was absent in E from 22 of the above 52 samples. Since their lysates did not inhibit IAHA in this system even at higher concentrations (IAHA patterns of + +/+ + + or greater with 6% E lysates). It should be noted that three E samples from the one remaining patient having CIC did inhibit the IAHA (negative pattern) as did the E from normal donors (not shown).

Forty-eight E samples from 42 patients without CIC (Groups 2 and 3) were also tested for C3b-R activity. These 48 samples were classified by their C3b-R activity. Group 2 represents patients with low receptor activity and Group 3 patients with normal receptor activity. As shown in Group 2 (Fig. 2), 30 E samples from 25 patients were found to partially inhibit the IAHA, but to a greater degree than the samples from Group 1 patients and to a lesser degree than the samples from normal donors. Eighteen of the E samples in Group 3 are from the remainder of the patients without CIC. These inhibited the IAHA to the same extent as normal E. When the inhibitory activity of C3b-R of E from patients with and without CIC was compared using 1·5% E lysates, a significant difference was observed. Thirty-four of 55 samples from patients with CIC had no inhibitory activity, while only eight of 48 samples from patients without CIC showed no inhibitory activity.

Serial E and serum samples from four SLE patients were studied in regard to the levels of CIC,

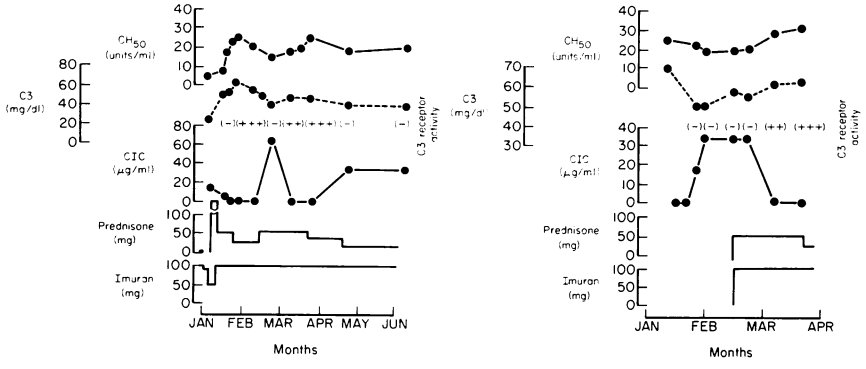

Fig. 3. The levels of CIC and receptor activity of serial blood samples from SLE patients. C3b receptor activity abbreviated as (+ + + +) to (−) was measured with 6% E lysates. Impaired receptor activity was observed and continued for a certain period after the disappearance of CIC.

Table 2. Inhibition of IAHA test by patients erythrocyte lysates

Sample date		IAHA pattern					CIC level (μg/ml)
		% of erythrocyte lysate added					
		6·0%	3·0%	1·5%	0·75%	Buffer	
Case 1	Feb 20	+++	+++/++++	++++	++++	++++	64
	Mar 5	+++	+++/++++	++++	++++	++++	(−)
	Apr 16	−	−	+/++	+++	++++	(−)
	Sep 4	++	+++	++++	++++	++++	8
	Oct 28	++	+++	++++	++++	++++	4
Case 2	Mar 13	+++	++++	++++	++++	++++	32
	Apr 10	++	++/+++	++++	++++	++++	32
	Apr 17	++	++	+++	++++	++++	(−)
	May 22	−	±	++	+++	++++	(−)
	June 5	++	+++	++++	++++	++++	8
	Sep 4	++	++/+++	++++	++++	++++	8

CH_{50} and C3 of serum and C3b-R activity of E. The C3b-R activity was measured with 6% E lysates. Fig. 3 shows a reverse correlation between levels of total haemolytic complement (the first diagrams from the top) or the third complement component (the second diagrams from the top) and the presence of CIC (the third diagrams from the top). Moreover, a reverse correlation was observed between the presence of CIC and E C3b-R activity indicated by a score of (−) to (++++). Patients' E had defective or impaired C3b-R activity when CIC were detected in the sera. With the disappearance of CIC during therapy, C3b-R activity gradually returned to normal. C3b-R activity continued to be impaired for a certain period subsequent to the disappearance of CIC. Also C3b-R activity decreased with the reappearance of CIC during exacerbations of diseases (Fig. 3 left, Table 2). It should be noted that CIC as determined by the IAHA method disappeared with suppression of disease activity by corticosteroids.

Presence of immunoglobulin and complement components

None of the patients' E having normal C3b-R activity were positive for Coombs' antibodies. In contrast, 47 of the 82 E samples that showed impaired C3b-R activity had positive Coombs' tests. In 18 of these samples, both IgG and complement component(s) (C3c and/or C4) were detected. In the remaining E samples, only IgG (12 samples) or a complement component (17 samples) was detected. A relatively higher frequency of positive Coombs' tests was found in E with defective C3b-R activity (2+ or greater IAHA pattern with 6% E lysates in Groups 1 and 2; 36 samples) than in E with impaired C3b-R activity (less than 2+ IAHA pattern with 6% E lysates in Groups 1 and 2; 46 samples). Twenty-eight of 36 E samples (78%) with defective C3b-R activity reacted with Coombs' antibodies, compared to 19 of 46 E samples (41%) with impaired C3b-R activity.

DISCUSSION

Although the IAHA method is used for identifying C3b molecules on both soluble and particulate antigens, little is reported concerning variations in reactivity of C3b-R among indicator cells. Recently, Turk (1964) and Rothman *et al.* (1975) reported that a screening of E from over 100 normal donors showed adequate receptor activity with little variation in the degree of haemagglutination patterns. However, there is only one report of C3b-R activity of E from donors with immune complex disease (Miyakawa *et al.*, 1981). In this report, the method for E C3b-R activity was a qualitative system using a fixed number of E to be tested with varied amounts of *in vitro* formed complexes. Thus they only observed the presence or absence of C3b-R activity. Since

haemagglutination patterns are influenced by amounts of diluted complexes as well as availability of C3b-R, their method did not estimate relative numbers of available receptors. To study E C3b-R activity quantitatively, a method was developed based on competitive inhibition of IAHA by C3b-R with fixed amounts of *in vitro* formed complexes and varying concentrations of E to be tested. If the inhibitory activity of E lysates is related to the E concentrations in lysates, it is suggested that C3b-R activity can thus be quantified by their total binding capacities.

The data in Table 1 confirm that positive IAHA reaction between EAC and indicator E is inhibited in a competitive manner by C3b-R of E lysates. Nelson (1963) and Dierich *et al.* (1974) reported that pre-treatment of E with proteolytic enzymes or reducing agents led to a loss of C3b-R activity. Thus no competition occurred when the lysates of E without C3b-R activity were added as inhibitors. The experiments using the lysates of E coated with incomplete antibodies confirmed that these antibodies did not interfere with C3b-R activity. In experiments using the lysates of E carrying *in vitro* formed complexes, the presence of human IgG, rabbit immunoglobulin, guinea-pig C3 and C4 was demonstrated by direct Coombs' testing on such E, which should have no inhibitory activity in our assay system. Further confirmation of the inhibition phenomenon by C3b-R was obtained from the results of experiments using the lysates of non-primates' E possessing no C3b-R (Nelson, 1953).

The inhibitory activity by lysates of E from patients with CIC (Group 1) varied from 4+ to 0 at lysate concentration of 6%. Whereas no inhibitory activity occurred at lysate concentration of 1·5%. These observations suggest that either more available lysate C3b-R are added or there are variations in the affinity of the lysate receptors to EAC. It is not known whether both active and impaired receptors can co-exist on a single E, or if there are two E populations: one with active receptors and another with impaired receptors.

Three E samples from one patient (polyarteritis nodosa associated with hepatitis B antigenemia) showed normal inhibitory activity, although immune complexes were present in the patient's sera. Since total haemolytic complement titres in these serum samples ranged from 10 to 15 CH_{50} units/ml, there may have been insufficient complement production for *in vivo* binding of CIC, because of his hepatic disease. The fact that lower C3b-R activity was observed in Group 2 patients even without CIC is considered as varying stages of total C3b-R activity during the recovery to normal levels. When CIC have disappeared, the C3b-R activity of E was gradually restored to normal levels either because of the disappearance of E bearing CIC or the reappearance of E with normal C3b-R activity. This may be explained by a shortened life span *in vivo* of E bearing CIC.

From the results of four SLE patients, a reverse correlation between C3b-R activity and the presence of CIC suggests that the difference of C3b-R activity in each E specimen is not because of genetically controlled affinity of C3b-R, but rather the number of C3b-R available, and also suggests blocking of receptor sites by immune complexes. If the binding of CIC by circulating cells with C3b-R augments removal of CIC by phagocytosis (Ross *et al.*, 1978; Scribner & Fahrney, 1975; Ehlenberger & Nussenzweig, 1977), human E would have a greater capacity to bind CIC than other circulating C3b-R bearing cells, because of the preponderance of E. Nakai *et al.* (1977) and Gluckman, Beaufils & Sanchez (1976) have reported that circulating lymphocytes with Fc receptors are decreased, while those with complement receptors are within normal ranges in RA and SLE patients with nephritis. If CIC initiate a complement cascade in the vicinity of circulating cell surfaces, it is possible that CIC–complement complexes adhere to E C3b-R. If so, gammaglobulin and/or complement components might be demonstrated on E surfaces.

In our studies, defective or impaired C3b-R activity could not be explained by the variations in the amount of C3c, C4 or IgG on these patients' E as measured by the Coombs' test. It appeared that small amounts of immune complexes or complement components, undetectable by conventional serological techniques, were responsible for the results in our Coombs' testing. Several investigators (Mongan *et al.*, 1967; Gilliland, Leddy & Vaughan, 1970; Finkelstein *et al.*, 1961) have suggested that the frequency of a positive direct Coombs' test to E from SLE and RA patients using anti-IgG and anti-complement antibodies was not high enough. However, they suggested that the presence of immunoglobulins and complement components on E surfaces might have resulted from secondary antibodies to E and the presence of complement components activated by the reaction between E and its antibodies (Mongan *et al.*, 1967; Gilliland *et al.*, 1970). If true, the C3b activated by the

reaction between E and its antibodies should bind to a C3b acceptor rather than C3b-R on the E surfaces (Dierich & Bokisch, 1977). If C3b-R of E are occupied and C3c is detected on E surfaces, it could be concluded that the C3b was derived from the activation of a complement cascade by an antigen–antibody reaction not involving E as the antigen. Nelson (1955) reported that *in vitro* formed immune complexes injected into primates adhered to circulating E surfaces by C3b-R. Therefore, the presence of immunoglobulins and complement components on E surfaces does not always indicate the presence of antibodies to E and of complement activated by the reaction between E and its antibodies. This possibility strongly supports our hypothesis concerning a reaction between C3b-R and CIC.

In summary, our observations suggest that human E can bind complement-bearing immune complexes *in vivo* by their C3b-R, and thus may have a role in the clearance of CIC. On the basis of Nelson's observations (1953, 1955), it is further suggested that such E may function as carrier cells of CIC with opsonic activity and assist in the sequestration of CIC by the fixed mononuclear phagocyte system. Also, it is possible that binding of CIC by C3b-R of E may inhibit immune complex deposition in tissues. If bound C3b (with immune complexes) is inactivated by C3 inactivator, β1H and proteolytic enzyme and if consequently C3d bearing immune complexes are released from E surfaces, such immune complexes will not bind to C3b-R of tissue constituents. Moreover, such E bearing C3c (no C3b-R activity) will be sequestered in the liver. To support this concept, Fearon (1978) suggested that a possible mechanism for recognition and clearance of aging or altered E could be membrane deposition of C3b molecules.

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