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THE ROLE OF IMMUNOCONGLUTININ IN THE *IN VIVO* AND *IN VITRO* DESTRUCTION OF RED CELLS

F. TEDESCO, R. CORROCHER AND D. L. BROWN

Department of Haematology, Royal Postgraduate Medical School, London

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SUMMARY

Two experimental approaches were used to investigate the role of immunoconglutinin (IK) in the *in vivo* destruction of red cells in rabbits. In a first series of experiments the behaviour of EC43 (C3-coated red cells) was followed in IK-producing rabbits and in rabbits passively receiving IK, both of which had previously been complement-depleted by cobra venom factor (CoF). The red cells were sequestered to a minor degree and returned to the circulation within 20–30 min, whereas in the normal control, EC43 returned to the circulation over a period of 3–4 hr. In contrast to EC43, EC43IK (IK-coated EC43) did not form rosettes around the Kupffer cells, suggesting that IK blocks the functional activity of C3 and so interferes with the interaction between C3 and C3-receptors on fixed macrophages. In a second series of experiments EC43 and EC43IK, injected into IK-producing rabbits and normal rabbits respectively, underwent marked lysis and erythrophagocytosis. Examination of liver imprint preparations from these rabbits revealed rosette formation around the Kupffer cells, indicating the fixation of more C4 and C3 by bound IK.

In vitro experiments confirmed both the inhibitory activity and the complement-fixing capacity of IK.

The results suggest that IK normally has an amplifying effect on complement fixation *in vivo* and so potentiates the ability of complement to bring about red-cell destruction.

INTRODUCTION

Immunoconglutinins (IKs) are antibodies directed against the third (C3) and fourth (C4) components of complement fixed to immune-complexes (Lachmann, 1962; Lachmann & Coombs, 1965; Lachmann, 1966). They can be produced by a process of heterostimulation (Streng, 1930) or autostimulation (Wartiovaara, 1932; Coombs & Coombs, 1953; Henson, 1967), the latter being an indication of *in vivo* complement fixation. Once bound, IKs can

Correspondence: Dr F. Tedesco, Istituto di Clinica Medica II, Policlinico, 70124 Bari, Italy.

trigger a new complement reaction (Lachmann, 1962; Bienenstock & Block, 1966) and so fix more C3 and C4.

In vivo studies have shown that passively given or actively produced IKs can protect mice against fatal infections by clearing bacteria more rapidly from their circulation (Ingram, 1959), but the mechanism of their action is not known (Nelson, 1962).

The role of C3 fixation in non-lytic destruction of red cells *in vivo* has been extensively investigated (Brown, Lachmann & Dacie, 1970). It has been shown that red cells in the form of EC43 are sequestered following intravenous injection in rabbits, as a result of adherence to fixed macrophages in the reticulo-endothelial system (RES). The red cell bound-C3 is recognized by C3 receptors, found on macrophages and monocytes (Huber *et al.*, 1968), neutrophils and lymphocytes (Lay & Nussenzweig, 1968). The red cells may subsequently undergo phagocytosis as a result of the opsonic activity of complement (Gigli & Nelson, 1968) or return slowly to the circulation as the functional activity of C3 decays by the action of the C3 inactivator (Tamura & Nelson, 1967; Lachmann & Müller-Eberhard, 1968; Ruddy & Austen, 1969).

In order to evaluate the possible contribution of IK in the *in vivo* destruction of red cells a series of experiments was carried out both in IK-producing rabbits and in rabbits passively given IK. In addition, the action of IK was studied in an *in vitro* system.

MATERIALS AND METHODS

Animals

NZW rabbits weighing 3–4 kg were used in all the experiments. Sera from rabbits homozygous for C6-deficiency were used for the preparation of EC43.

IgM cold agglutinin antibody

IgM cold agglutinin from a patient with chronic cold haemagglutinin disease was purified as described previously (Brown, 1970).

Cobra venom factor (CoF)

Cobra venom (*Naja naja*) was partly purified by DEAE-chromatography according to the technique of Nelson (1966). The active fraction (CoF) was given daily by intravenous injection in small increasing doses until the plasma CH50 of the test rabbit fell to zero. Rabbits were used for experiments within 24 hr of complement-depletion.

Preparation of immunoconglutinin (IK)

IK was autostimulated in rabbits by Henson's technique (1967). Three rabbits received two intramuscular injection of $1{\cdot}5 \times 10^9$ formolized *E. coli*, in Freund's complete adjuvant 19 and 14 days respectively before a course of four intravenous injections of *E. coli* given every other day. The IgM fraction of the pooled sera, which had been collected at the peak of the IK response, was separated by Sephadex G-200 gel-filtration, using an 0·1 M tris-HCl buffer, pH 8·0 containing 0·5 M NaCl and 1/10,000 sodium azide. The quality of the fractionation was checked by immunoelectrophoretic analysis. When IK-producing rabbits were to be used for *in vivo* experiments, they were boosted with *E. coli* 24 hr previously.

Preparation of EC43

EC43 were prepared as described by Brown, Lachmann & Dacie (1970). For the *in vivo* experiments the red cells were labelled with ^{51}Cr prior to treatment with cold agglutinin and C6-deficient serum. The agglutinability of EC43 was checked each time with IK and anti-C3 serum.

Preparation of EC43IK

EC43 were treated with five conglutinating doses of IgM IK at 4°C for 1 hr. The cells were subsequently washed at least three times with barbitone buffer pH 7·2 containing 2 g/l gelatin (Kabat & Mayer, 1961).

IK levels

EC43 were used as indicator to estimate the IK levels, which were evaluated by a resuspension technique (Coombs, Coombs & Ingram, 1961). Controls were set up to ascertain the specificity of the reaction.

Morphological appearance of the sequestration process in the liver

Rabbits were killed by intravenous Nembutal and the liver was immediately removed. Small pieces were sliced off and the cut-surface touched onto glass microscope slides to produce imprint preparations. These were allowed to dry and stained with May-Grünwald Giemsa.

Lung macrophages

Normal alveolar macrophages were obtained from rabbits by the method of Myrvik, Leake & Fariss (1961) as described by Cohn & Wiener (1963). Rabbits were killed by injecting 50–70 ml of air into the marginal ear vein. The trachea was clamped just below the larynx, the chest was opened and the heart and lungs were rapidly dissected out *in toto*. The surfaces of the heart and lungs were washed with ice-cold saline and blood expelled from the pulmonary circulation as far as possible with cold saline. Ice-cold Hanks' solution containing heparin (100 u/100 ml) and 0·2% gelatin was then introduced by a catheter into the trachea and both lungs were filled to slight distension. The Hanks' solution was decanted from the lungs after a few seconds into cold sterile plastic containers and the lungs were filled and emptied a second time.

The containers were centrifuged at 100–200 *g* for 10 min at 4°C and macrophage pellets were obtained. The macrophages were pooled, resuspended and washed twice in heparin-free ice-cold Hanks' solution, then kept at 4°C until required. More than 80% of the cells were typical alveolar macrophages and, apart from red cells, the only major contaminants were bronchial epithelial cells and lymphocytes.

RESULTS

In vivo behaviour of EC43 in the presence of IK

(a) *Experiments in complement-depleted rabbits.* Fig. 1 illustrates the curves for circulating red-cell radioactivity following the injection of EC43 into a normal rabbit, an IK-producing rabbit and a normal rabbit which had received the IgM fraction of IK at the

same time. The rabbits, in which complement-depletion had been induced by CoF, were injected intravenously with $1{\cdot}5 \times 10^{10}$ EC43 and blood samples were taken at intervals to check the disappearance of circulating red cell radioactivity.

In the normal rabbit EC43 disappeared rapidly within 2-3 min and returned slowly to the circulation over a period of 3–4 hr. Red-cell radioactivity returned incompletely to the peripheral blood, suggesting permanent sequestration of a proportion of the EC43. In contrast in the IK-producing rabbit, EC43 underwent a briefer period of sequestration by RES and returned almost completely to the circulation within 20–30 min.

An analogous behaviour of the red cells was observed in a normal rabbit which was given IK intravenously prior to injection of EC43, suggesting that IK itself was responsible for the altered behaviour of the EC43.

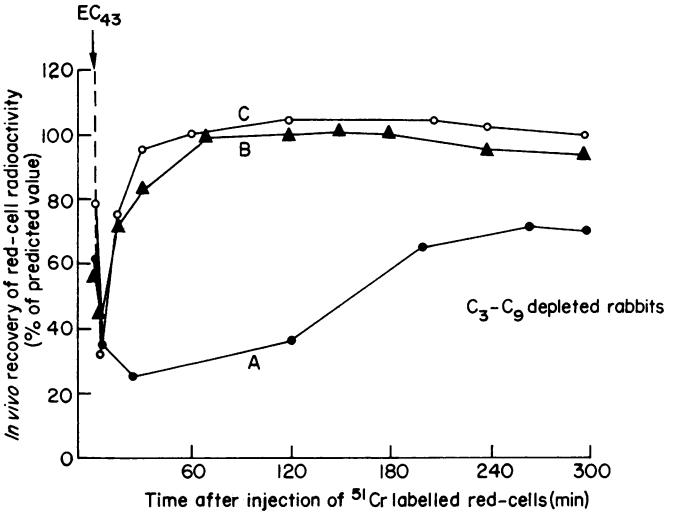

FIG. 1. Behaviour of EC43 following intravenous injection into complement-depleted rabbits A, B and C: (A) ●, receiving EC43 only; (B) ▲, receiving EC43 and IgM IK at the same time; (C) ○, IK-producing rabbit receiving EC43.

In order to find out whether IK, given at intervals after the red cells, could interfere with their sequestration, IK was injected 8 min after EC43. The red cells started returning to the circulation soon after the injection of IK, whereas in the control rabbit the circulating red-cell radioactivity reached the lowest value at 25 min (Fig. 2). No modification of the normal sequestration curve could be produced by IK given 2 hr after the injection of EC43.

Examination of the blood films of these rabbits showed agglutination of red cells up to 2 hr post-injection.

(b) *Experiments in normal rabbits.* EC43 injected into a normal rabbit underwent a minimal degree of lysis (approximately 4%) showing that there was little, if any, $C\overline{42}$ present. When the same cells were given to an IK-producing rabbit or to a normal rabbit together with IK, marked lysis occurred while the unlysed cells underwent the same process of sequestration as EC43 given alone, suggesting the fixation of more C4 and C3 by

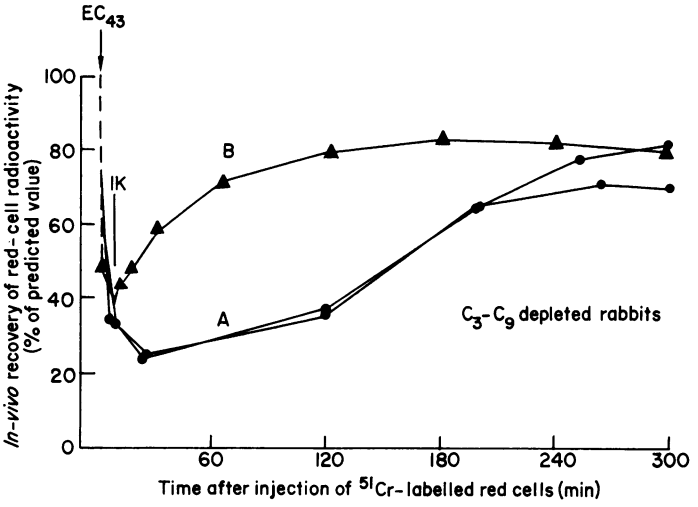

FIG. 2. Behaviour of EC43 following intravenous injection into complement-depleted rabbits A and B: (A) ●, two control rabbits receiving EC43 only; (B) ▲, receiving EC43 followed by IgM IK 8 min later.

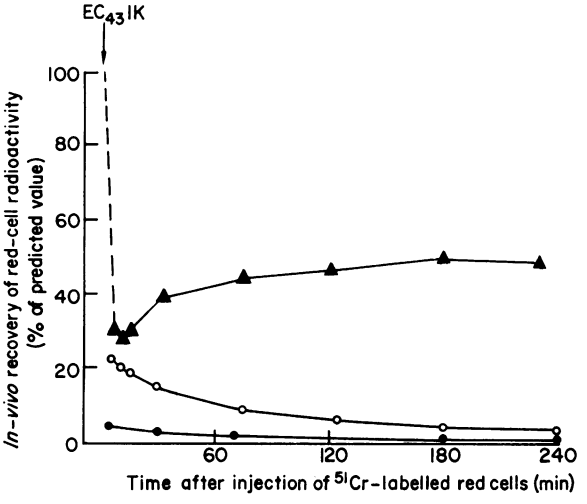

FIG. 3. Behaviour of EC43, which were sensitized with IgM IK prior to injection into a normal rabbit. ▲, Red cell radioactivity (test rabbit); ○, plasma radioactivity (test rabbit); ●, plasma radioactivity (control rabbit).

EC43IK (Figs 3 and 4). A transient fall in neutrophil and platelet counts, sometimes accompanied by an anaphylactic-like reaction, was observed immediately after the injection of EC43 and IK.

Further evidence for complement fixation by the bound IK was obtained when EC43IK were injected into a complement-depleted rabbit, followed 2 hr later by the injection of C6-deficient serum (Fig. 5). The radioactivity in the circulation dropped to a greater

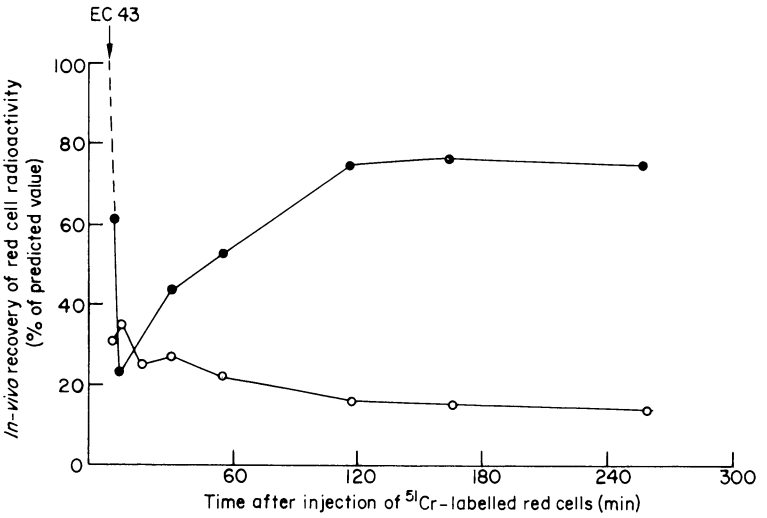

FIG. 4. Behaviour of EC43 which were injected into an IK-producing rabbit. ●, Red cell radioactivity; ○, plasma radioactivity.

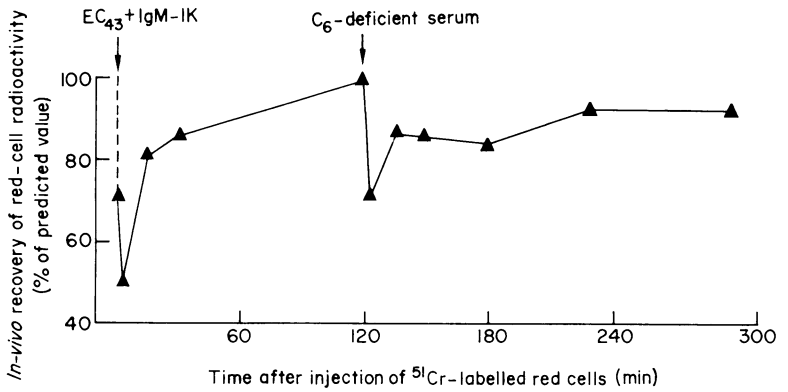

FIG. 5. Behaviour of EC43 injected into a complement-depleted rabbit at the same time as IgM IK; 20 ml of C6-deficient serum were then injected 120 min later.

extent than could be accounted for by haemodilution, suggesting that the IK had fixed more C3 to the circulating red cells.

Site of sequestration of EC43 in the presence of IK

A rabbit was injected with ^{51}Cr-labelled EC43 together with IK and killed 5 min after the injection. The radioactivity of small pieces of different organs was measured and expressed as a ratio counts/min/g of tissue. The results, presented in Table 1, show a marked uptake of the radio-labelled red cells by the liver, spleen and lungs. Liver imprint examination revealed Kupffer cells surrounded by clumps of agglutinated red cells which did not form rosettes with the macrophages. Erythrophagocytosis was virtually absent

TABLE 1. Distribution of radioactivity 5 min after injecting ^{51}Cr EC43 (expressed as a ratio $\frac{\text{counts/min/g of tissue}}{\text{counts/min/g of heart}}$)

Rabbit	Heart	Liver	Spleen	Lung	Kidney
Rabbit injected with EC43IK	1·0	18·5	17·5	11·4	2·0
IK producing rabbit	1·0	21·5	23·3	15·4	1·7
Normal control	1·0	1·5	2·1	2·8	1·0

(Fig. 6). In contrast, typical red-cell macrophage rosettes were usually seen when EC43 were injected alone (Fig. 7). Similar rosettes were formed when EC43IK were injected into normal rabbits. Platelets and neutrophils were often present in large numbers and marked erythrophagocytosis was observed (Fig. 8).

In vitro reactions of EC43 with lung macrophages in the presence of IK

Rosette formation, which developed between EC43 and rabbit lung macrophages, was markedly inhibited when EC43IK or EC43 in the presence of IK were used (Figs 9 and 10). The addition of IK 3 min, 5 min and 10 min respectively after EC43 could still result in an inhibitory effect. Treatment of EC43IK with C6-deficient serum to ensure fixation of C4 and C3 induced rosette formation and marked erythrophagocytosis.

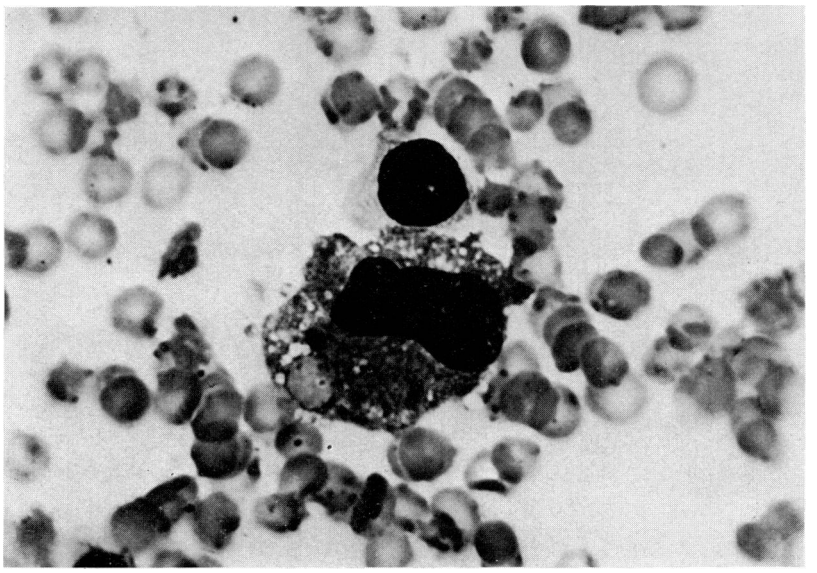

FIG. 6. Kupffer cell surrounded by agglutinated red cell not adhering to the surface; imprint preparation made 5 min after injecting EC43. (× 800).

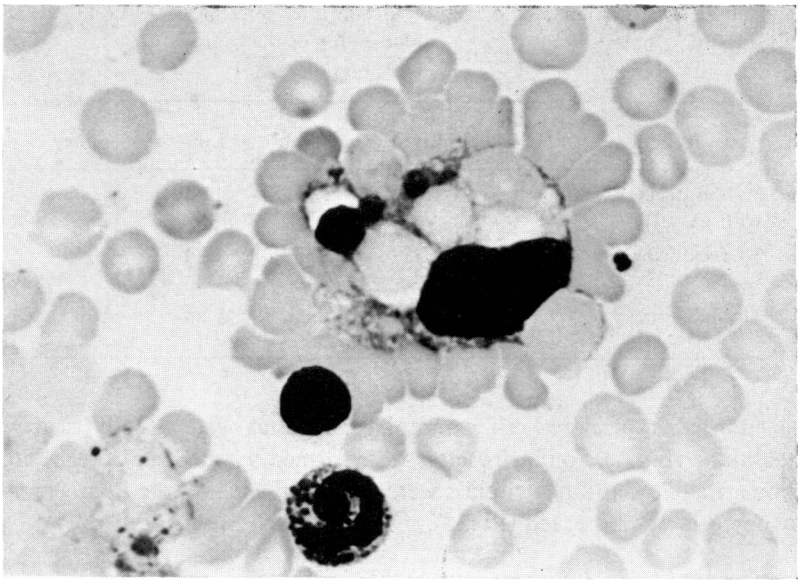

FIG. 7. Kupffer cell with contracted red cells adhering to the surface and a few ingested red cells; imprint preparation made 5 min after injecting EC43. (×1170).

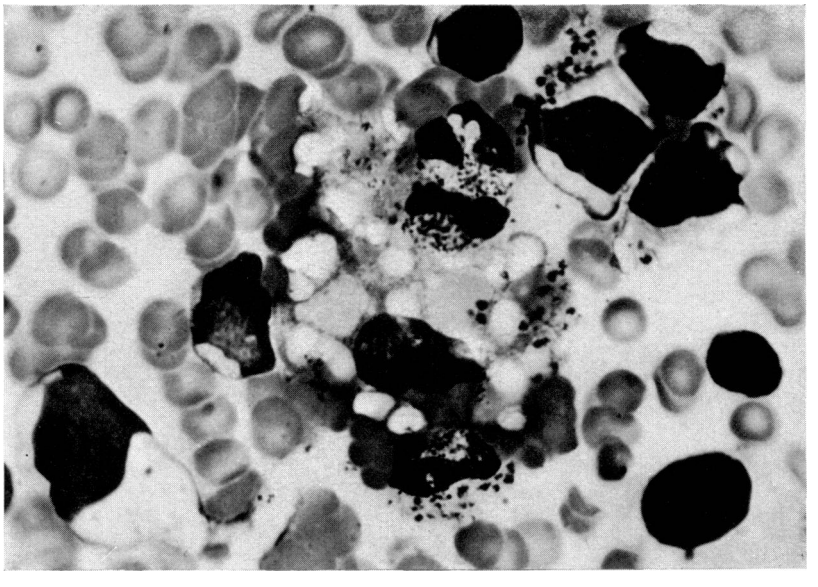

FIG. 8. Kupffer cell with several ingested red cells and agglutinated red cells adhering to the surface; imprint preparation made 5 min after injecting EC43IK. (×800).

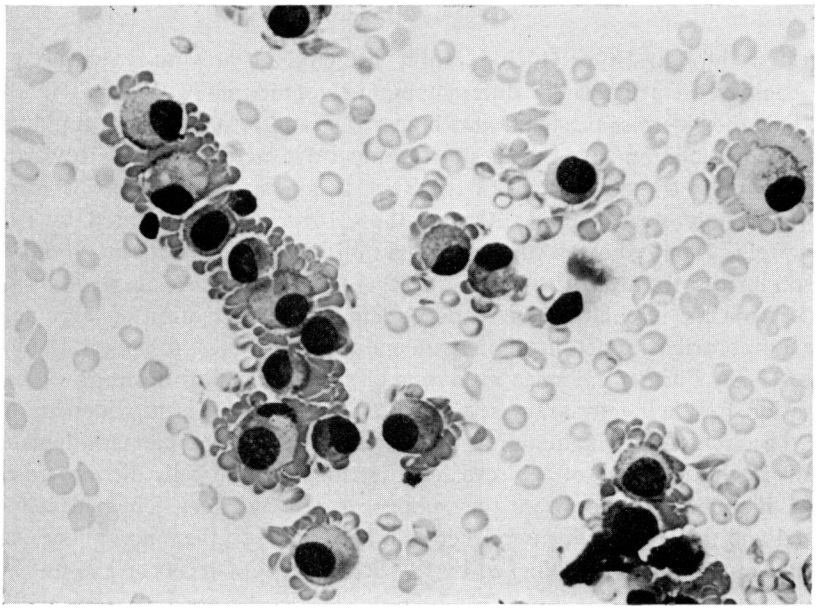

FIG. 9. Rosette formation between EC43 and rabbit lung macrophages. (*In vitro*, ×428).

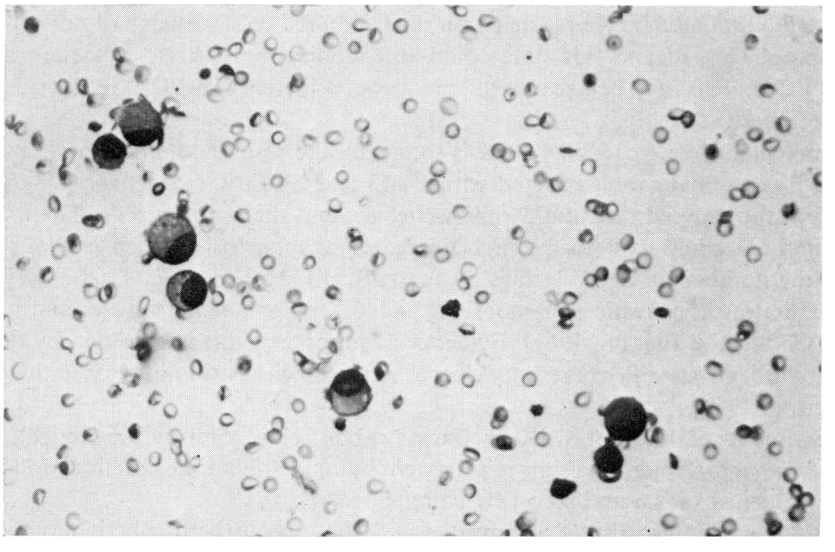

FIG. 10. Inhibition of rosette formation between EC43IK and rabbit lung macrophages. (*In vitro*, ×400)

DISCUSSION

EC43 injected into an IK-producing rabbit, which had previously been complement-depleted, behaved in a markedly different manner compared with EC43 in the control rabbit, in that they were sequestered at a higher rate and returned more rapidly and completely to the circulation. This suggests a competitive activity by IK towards the C3-receptors on the fixed macrophages of the RES. It is possible that the C3-receptor might be a cytophilic immunoconglutinin, though the C3-receptor is inhibited by EDTA (Lay & Nussenzweig, 1968) whereas immunoconglutinin is not (Lachmann, 1967). However, further experiments are needed to prove or refute this hypothesis. The rapid disappearance of EC43IK from the circulation seems dependent on the formation of large agglutinates, which are temporarily trapped in the reticuloendothelial organs, mainly in the liver (Jandl & Kaplan, 1960). Further evidence in favour of simple physical filtration of EC43IK, as an explanation for their temporary sequestration in the RES, is provided by their rapid return to the circulation. Examination of liver imprints in complement-depleted rabbits given EC43 in the presence of IK revealed that most of the cells did not form rosettes around the Kupffer cells nor were they phagocytosed by them. The red cells appeared agglutinated and the great majority of them did not adhere to the macrophages, apparently because the fixed C3 had been blocked by IK. So far an IgM-receptor had not been found on macrophages, as has been shown for IgG and C3 (Huber & Fudenberg, 1968; Huber *et al.*, 1968; Huber, Douglas & Fudenberg, 1969) and bound-IgM itself has proved to be ineffective in promoting phagocytosis (Huber & Fudenberg, 1970).

A similar inhibition of rosette formation observed in rabbits passively given the IgM fraction of IK demonstrates the specific action of IK in this respect. The IgG and IgM fractions of the rabbit sera which were the source of the IK were tested for their level of antibodies against *E. coli*. These were found mainly in the IgG fraction, so that it is unlikely that the antibodies against *E. coli* could be responsible for the results.

IK injected 8 min after EC43 reacted with the C3 on red cells, which had not yet adhered to the macrophages, and at this time could still modify the pattern of sequestration expected for EC43. This may be because the process of adherence of EC43 to the C3-receptor takes some time to develop.

The complement-fixing capacity of IK is shown by the results obtained in IK producing rabbit and in a normal rabbit injected with EC43 and EC43IK respectively. Marked lysis occurred and the unlysed cells did form rosettes around the Kupffer cells. The fixation of more C4 and C3 could explain the high degree of phagocytosis, which is thought to be related to the number of C3 molecules on the red cells (Huber *et al.*, 1968). IK probably acts by facilitating the fixation of more C4 and C3, so enhancing nonspecific immunity (Coombs, Coombs & Ingram, 1961). Evidence in support of this is provided by the observation that virulent bacteria are rapidly cleared from the circulation in mice in the presence of IK (Ingram, 1959).

The experiments carried out in IK-producing rabbits closely reflect the clinical situation in man, where complement-fixation is followed by an IK-binding reaction, followed by more complement-fixation and so on (Lachmann, 1967).

In vitro studies confirm the *in vivo* results and show both the competitive activity and the complement-fixing capacity of IK.

The significance of these experiments in relation to clinical situations in man is shown

by the finding of high levels of IK in viral infections (Marks & Coombs, 1957) and immunological disorders (Caspary & Ball, 1962) and the observation of a drop in IK level during exacerbation of SLE (Bienenstock & Block, 1967).

It is conceivable that autoimmune haemolytic anaemia, usually a chronic disease, either directly stimulates IK production or indirectly stimulates it as a result of associated infections or other autoimmune processes.

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