Immunology 1979 **37** 681

Application of human erythrocytes to a radioimmune assay of immune complexes in serum

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Received 14 December 1978; accepted for publication 17 January 1979

Summary. An immune adherence receptor exists on the surface of primate erythrocytes, and has been characterized as a receptor for the activated third component of complement (C3b). We have applied human red blood cells (RBCs, blood group O) to a sensitive determination of complement-fixing, soluble immune complexes in serum. The method involved the binding of immune complexes with RBCs in the presence of complement and the detection of cell-bound IgG molecules by radiolabelled anti-human IgG antibodies. Since the binding of RBCs with monomeric IgG was minimal, cell-bound IgG molecules were taken as representing immune complexes. When aggregated human gammaglobulin (AHG) was used as a model of immune complexes, as little as 5 μg dissolved in 1 ml of normal human serum were detected. The binding of RBCs with AHG was inhibited in EDTA solution where the classical complement pathway could not be activated. The RBC radioimmune assay was successfully applied to the determination of soluble immune complexes in pathological serum samples obtained from the patients with systemic lupus erythematosus and those with fulminant Type B hepatitis. False-positive results by autoantibodies against RBCs could be excluded by performing a Coombs test and by comparing the binding in the presence of complement with that in EDTA solution. The ubiquitous availability of RBCs coupled with a high sensitivity would allow the RBC radioimmune assay to be added to the battery of previous methods to determine immune complexes in the serum.

Abbreviations: IAHA, immune adherence haemagglutination; RBCs, human red blood cells; AHG, aggregated human gamma globulin; HB_sAg, hepatitis B surface antigen; anti-HB_s, antibody to HB_sAg.

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0019-2805/79/0700-0681$02.00

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INTRODUCTION

In 1953, Nelson observed that *Treponema pallidum*, when incubated with corresponding antibody and complement, adhered to human erythrocytes. This phenomenon, designated as immune adherence, has been defined as the specific attachment of primate erythrocytes and also non-primate platelets to antigen–antibody complexes in the presence of complement, and accepted to represent the binding of complement-fixing antigen–antibody complexes with the immune adherence receptor (receptor for C3b) on these blood corpuscles (Nishioka, 1963). By applying the appropriate number of erythrocytes in proportion to antigen–antibody complexes, immune adherence can be readily observed as haemagglutination (immune adherence haemagglutination, IAHA) (Nishioka, 1963). Owing to its high sensitivity and speci-

ficity, immune adherence has been widely applied to the determination of antigens, antibodies, and complement components (Nelson, 1953; Taverne, 1957; Brody, 1962; Woodworth, 1962; Nishioka, 1963).

Recently, immune complexes have attracted increasing attention owing to their phlogistic activities in the pathogenesis of a variety of immunological diseases highlighted by glomerulonephritis and vasculitis (Dixon, Feldman & Vasquez, 1961; Gocke, Hsu, Morgan, Bombardieri, Lockshin & Christian, 1970; Dixon, Cochrane, Koffler & Christian, 1971). A number of methods involving different principles have been developed for the determination of immune complexes in the serum. Among them are the methods based on the binding of complement-fixing immune complexes with receptors for activated C3, such as Raji cells (Theofilopoulos, Wilson & Dixon, 1976), B lymphocytes (Smith, Barratt, Hayward & Soothill, 1975) and immunoconglutinins (Eisenberg, Theofilopoulos & Dixon, 1977).

We have applied the C3b receptor on human erythrocytes to the quantitative determination of complement-fixing immune complexes in the serum utilizing a radioimmune method.

MATERIALS AND METHODS

Buffers

Isotonic Veronal-buffered saline (pH 7·6) containing 0·1% (w/v) gelatin, $1{\cdot}5\times10^{-4}$ M Ca^{2+} and 5×10^{-4} M Mg^{2+} (GVB^{2+}), and gelatin-Veronal-buffered saline (GVB) containing 0·01 M or 0·04 M EDTA, were prepared according to the methods of Nelson, Jensen, Gigli & Tamura (1966) and Mayer (1961).

Human erythrocytes

Red blood cells (RBCs) were obtained from ten laboratory personnel with blood group O. They were preserved in two volumes of modified Alsever's solution and stored at 4°. Under these conditions, IAHA reactivity of RBCs was preserved for at least 1 month. Before use they were washed twice with 0·01 M EDTA–GVB and twice with GVB^{2+}, and finally suspended in GVB^{2+} at a desired concentration. A suspension of 2×10^{8} cells, when 1 ml of which was lysed and diluted to 10 ml with distilled water, was assumed to give an optical density of 0·395 at 541 nm.

Complement

Serum samples obtained from apparently healthy individuals served as a source of complement. They were stored in small aliquots for 1 day use at −70°. They exhibited 50% units of immune adherence reactivity (C IA_{50}) of 3500–4000/ml as determined by the sheep erythrocyte–rabbit haemolysin system (Nishioka, 1963).

Immune adherence haemagglutination

The method to determine hepatitis B surface antigen (HB_sAg) was followed (Mayumi, Okochi & Nishioka, 1971). A solution of purified HB_sAg in GVB^{2+} (100 μg/ml) was serially diluted in tubes, and a 25 μl portion was delivered to the well in a microtitre plate. Then, 25 μl of a dilution of human antibody to HB_sAg in GVB^{2+} (8 IAHA units/ml) were added to each well. After incubating the plate at 37° for 1 h, 25 μl of a dilution of guinea-pig complement in GVB^{2+} (4 CH_{50} units/ml) were added, and the plate was further incubated at 37° for 40 min. Finally, 25 μl of a dithiothreitol solution (3 mg/ml in 0·01 M EDTA–GVB) and 25 μl of a suspension of RBCs (2×10^{8} cells/ml in 0·01 M EDTA–GVB) were added and the plate was incubated at 24° for 40 min. The assay was performed in duplicate and the IAHA reactivity of human RBC batches was expressed as the highest two-fold dilution (2^N)of HB_sAg that showed haemagglutination.

Aggregated human gammaglobulin

Gammaglobulin in pooled human sera was precipitated by making 1·33 M with $(NH_4)_2SO_4$ at 24°. The precipitate was washed twice with 1·33 M $(NH_4)_2SO_4$ solution, dialysed against 0·005 M sodium phosphate buffer (pH 7·5) and applied onto a column of DEAE–cellulose equilibrated with the same buffer. Fractions which passed through the column were collected, made 0·15 M with NaCl, and served as a source of human IgG. Human IgG in phosphate-buffered saline was freed of aggregates by centrifuging at 150,000 ***g*** for 90 min. The upper third of the supernate containing monomeric IgG was harvested, and diluted with phosphate-buffered saline to a desired concentration. An $E^{1\%}_{1\,cm,\,280\,nm}$ value of 1·43 was used for human IgG (Williams & Chase, 1968). The monomeric IgG preparation was stored in small aliquots at −70°, and used only once after they had been thawed. Aggregated human gammaglobulin (AHG) was prepared by heating monomeric IgG in a water bath at 63° for 30 min. After aggregation, the sample was centrifuged at 1500 ***g*** for 15 min, and only the supernate was used.

Radioiodination

Gammaglobulin fraction was obtained from rabbit

antisera containing monospecific antibodies against human IgG. It was radiolabelled with ^{125}I by the chloramine T method (Greenwood, Hunter & Glover, 1967) at a specific activity of 0·2 μCi/μg, and diluted in GVB^{2+} to a desired concentration (^{125}I-anti-IgG). Similarly, AHG, monomeric IgG and rabbit γ-globulin preparation containing anti-HB_s were radioiodinated at a specific activity of 0·2 μCi/μg (^{125}I-AHG, ^{125}I-IgG and ^{125}I-anti-HB_s).

RBC radioimmune assay for the determination of immune complexes in human serum samples

In a 12 × 75 mm glass tube, 1×10^8 RBCs in 50 μl of GVB^{2+} were mixed with 25 μl of a 1:4 dilution of the test serum in GVB^{2+} and 25 μl of a 1:4 dilution of complement in GVB^{2+}. When the complement-dependence of the reaction was studied, 25 μl of a 1:4 dilution of complement in 0·04 M EDTA–GVB were used in place of complement, which gave a final EDTA concentration of 0·0075 M in the reaction mixture. Tubes were incubated at 37° for 30 min in a moist chamber with continuous shaking. Cells were then suspended in 3 ml of GVB^{2+} and centrifuged at 1000 *g* for 1 min. After washing RBCs two additional times, they were suspended in 100 μl of GVB^{2+} containing 1·5 μg of ^{125}I-anti-IgG and further incubated with shaking at 4° for 30 min. After washing the cells with GVB^{2+} three times at 4°, the bound radioactivity was counted in a gamma counter for 1 min. A standard binding curve was drawn using normal serum containing variable amounts of AHG as a model of immune complexes. The binding by the sample was referred to the standard curve and the result was expressed as micrograms AHG equivalent per ml of the original serum.

Pathological serum samples

Serum samples were obtained from fourteen patients with systemic lupus erythematosus who had been on a daily dose of 5–30 mg of prednisolone, and nineteen patients with fulminant Type B hepatitis. They were stored at −70° until use. Serum samples were also obtained from twenty-four healthy laboratory personnel and used as controls. All of these samples were negative for antibodies against RBCs as determined by Coombs test.

Ultracentrifugation in a sucrose density gradient

A sucrose density gradient ranging from 10 to 50% (w/v) in Tris–HCl buffer (0·01 M, pH 7·6) was prepared in a Hitachi SW-65P tube (capacity 5 ml). One tenth of a millilitre of pathological serum samples containing immune complexes was overlayed onto the surface, and the tube was centrifuged at 60,000 *g* for 4 h. The tube was pierced at the bottom, and 0·3 ml fractions were collected. They were monitored for sucrose density by a refractometer and for immune complexes by the RBC radioimmune assay. They were also titrated for IgG and IgM by reversed passive haemagglutination methods utilizing glutaraldehyde-fixed sheep erythrocytes coated with monospecific rabbit antibodies against human IgG or IgM. In addition, fractions of the serum from patients with fulminant Type B hepatitis were tested as follows. Twenty-five microlitres of the sample were mixed with 1×10^8 RBCs in 50 μl of GVB^{2+} and incubated at 37° for 30 min. After washing RBCs, 1·5 μg of ^{125}I-anti-HB_s in 100 μl of GVB^{2+} was added and they were further incubated at 4° for 30 min. Finally, RBCs were washed and the bound radioactivity was counted.

RESULTS

Binding of RBCs with AHG

The reactivity of ten RBC batches with AHG was tested with appropriate controls. Table 1 gives the

Table 1. Specificity of the binding of human erythrocytes with aggregated human gammaglobulin

	Percentage of added radioactivity bound by 1×10^8 RBCs*		
	^{125}I-AHG†		^{125}I-monomeric IgG†
Erythrocyte batches	With complement	In EDTA‡	With complement
A	2·92	0·19	0·07
B	2·87	0·16	0·09
C	2·79	0·12	0·11
D	2·80	0·09	0·16
E	2·62	0·18	0·10
F	2·59	0·17	0·08
G	2·57	0·10	0·08
H	2·43	0·11	0·07
I	1·98	0·21	0·08
J	1·49	0·17	0·01
Mean ± SD	2·51 ± 0·45	0·15 ± 0·04	0·09 ± 0·04

* The binding of RBCs with radiolabelled AHG and monomeric IgG was tested according to the method described in the text.

† Ten micrograms of radiolabelled AHG or monomeric IgG were added.

‡ Human serum diluted 1:4 in 0·04 EDTA–GVB was used in place of complement, which gave a final EDTA concentration of 0·0075 M in the reaction mixture.

binding of RBCs with: (A) ^{125}I-AHG in the presence of complement, (B) ^{125}I-AHG in the presence of complement but in EDTA solution, and (C) ^{125}I-monomeric human IgG in the presence of complement. It can be seen that the binding of RBCs with AHG was specific and complement-dependent. The binding of RBCs with AHG in the presence of complement was by far the greatest among the three (mean $\pm$ SD $2{\cdot}51 \pm 0{\cdot}45\%$, range 1·49–2·92%). In EDTA solution where the classical complement pathway could not be activated, the binding of RBCs with AHG was minimal ($0{\cdot}15 \pm 0{\cdot}04\%$, range 0·09–0·21%). It is also evident that RBCs did not bind with monomeric IgG appreciably even in the presence of complement ($0{\cdot}09 \pm 0{\cdot}04\%$, range 0·01–0·16%).

Correlation between the binding of RBCs with AHG and their immune adherence reactivity

In view of the fact that immune adherence reactivity of human erythrocytes differs greatly from individual to individual (Turk, 1964), the binding of RBCs with AHG in the presence of complement was determined for ten different RBC batches and compared with their IAHA reactivity in the determination of HB_sAg (Mayumi *et al.*, 1971). As is illustrated in Fig. 1, IAHA reactivity of RBC batches varied greatly from 2^1 to 2^{10}. The binding of RBC batches with AHG was less variable, but still a double range was observed for them, and it correlated well with their IAHA reactivity. RBC batches with a high IAHA reactivity were selected and used in the subsequent experiments.

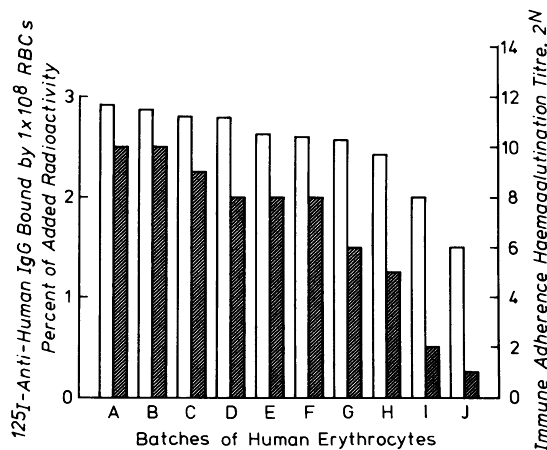

Figure 1. Correlation between the binding of aggregated human gammaglobulin and immune adherence reactivity in ten erythrocyte batches. Open columns represent percentage of ^{125}I-AHG (10 μg) bound by 1×10^8 RBCs. Shaded columns represent immune adherence reactivity of RBCs expressed by the maximal dilution of HB_sAg detectable by IAHA.

The effect of RBC numbers on the binding of AHG

The effect of varying cell numbers from 1×10^5 to 1×10^8 on the binding of ^{125}I-AHG was determined at three different doses (1 μg, 10 μg and 100 μg). The binding of AHG was remarkably enhanced by increasing cell numbers for all the doses of AHG through the range of cell numbers tested (Fig. 2). RBC numbers higher than 1×10^8 were found inappropriate due to a huge bulk of cells which made the washing incomplete, resulting in a high background binding. Accordingly, an optimal cell number of 1×10^8 was adopted and used for the assay experiments.

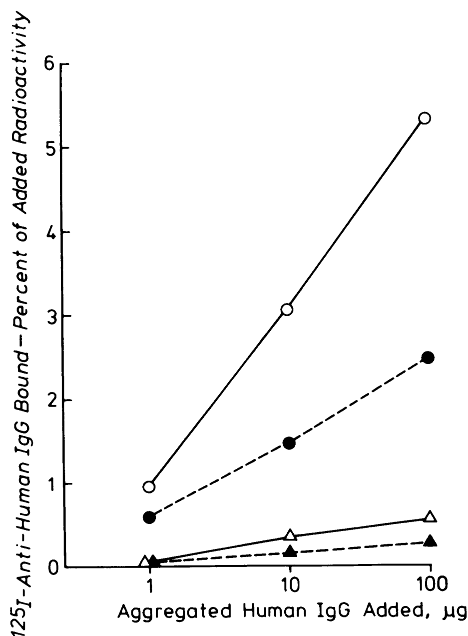

Figure 2. The effect of increasing the number of erythrocytes on the binding of aggregated human gammaglobulin. Erythrocytes were incubated with 1, 10, and 100 μg of ^{125}I-AHG in the presence of complement, and the bound radioactivity was determined. Closed triangles and dashed line represent 1×10^5, open triangles and solid line 1×10^6, closed circles and dashed line 1×10^7, and open circles and solid line 1×10^8 RBCs in the reaction medium.

Determination of AHG by RBC radioimmune assay for immune complexes

Using AHG as a model of immune complexes, the ability of RBCs to detect immune complexes in the serum was tested. RBCs (1×10^8) were incubated with varying amounts of unlabelled AHG in 25 μl of a 1:4 dilution of normal serum in the presence of complement, and the bound AHG was determined by means of the uptake of ^{125}I-anti-IgG (Fig. 3.). When 15 μg of ^{125}I-anti-IgG were used, the binding of monomeric IgG was very low, and the binding of AHG was higher than that of monomeric IgG beyond 300 ng. The effect of cutting down ^{125}I-anti-IgG to 1·5 μg was evident. Although background binding increased to about 1% of the added radioactivity, a remarkable gain in the sensitivity was achieved. Thus, 30 ng of AHG in a tube were detectable, which, after correction for the dilution factor in the test procedure, corresponded to 5 μg of AHG per ml of the original serum. The count of ^{125}I-anti-IgG taken up by RBCs was in a direct correlation with the amount of AHG up to 3000 ng per tube, and it started to bend beyond that dose. In view of a considerable elevation of background binding with 1·5 μg of ^{125}I-anti-IgG, no further reduction of labelled anti-IgG was attempted to increase the sensitivity of assay.

Determination of immune complexes in human serum samples

Figure 4 gives the results of fourteen patients with systemic lupus erythematosus and nineteen patients with fulminant Type B hepatitis. The binding of ^{125}I-anti-IgG for twenty-four apparently healthy individuals was $1{\cdot}04 \pm 0{\cdot}24\%$ (range 0·50–1·41%), and by referring to the standard curve, all of them were found to contain less than 5 μg AHG equivalent/ml. It can be seen that twelve out of fourteen serum samples from the patients with systemic lupus erythematosus (86%) contained abnormally high amounts of immune complexes. Sixteen out of nineteen samples from the patients with fulminant hepatitis (84%) revealed high values, and one of them contained much more immune complexes than any serum sample from patients with systemic lupus erythematosus. In order to see if the

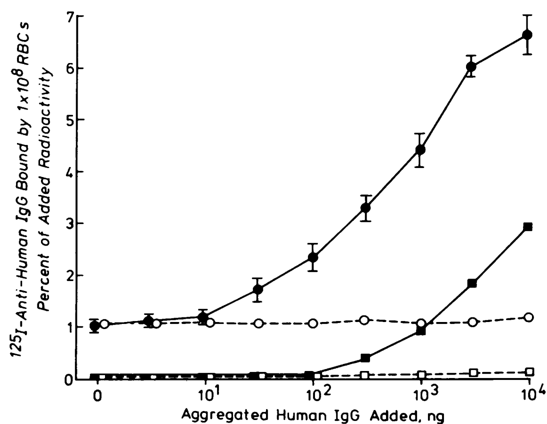

Figure 3. The uptake of ^{125}I-anti-IgG by 1×10^8 RBCs after incubation with increasing amounts of AHG. The reaction was performed in the presence of complement or EDTA-complement, and the bound radioactivity was determined by 1·5 μg or 15 μg of ^{125}I-anti-IgG. Closed circles and solid line represent the uptake in the presence of complement and open circles and dashed line the uptake in EDTA solution each detected by 1·5 μg of ^{125}I-anti-IgG. Solid squares and solid line represent the uptake in the presence of complement and open squares and dashed line the uptake in EDTA solution as detected by 15 μg of ^{125}I-anti-IgG. Each point of the binding in the presence of complement detected by 1·5 μg of ^{125}I-anti-IgG represents mean $\pm$ SD of triplicate samples tested on 3 consecutive days.

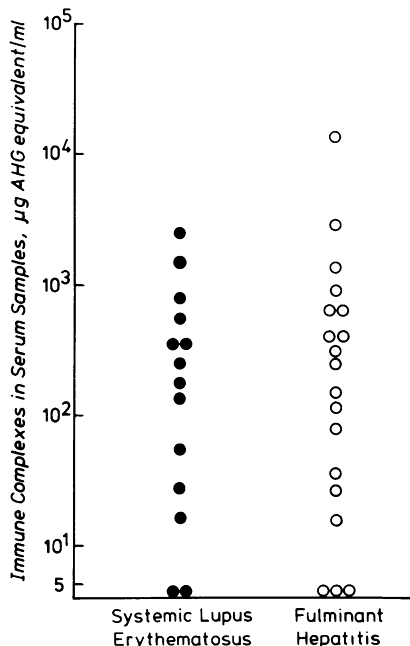

Figure 4. RBC radioimmune assay for immune complexes in serum samples from patients with systemic lupus erythematosus and those with fulminant Type B hepatitis. Closed circles represent patients with systemic lupus erythematosus, and open circles represent patients with fulminant hepatitis.

binding of pathological serum samples with RBCs was complement-dependent, the positive serum samples were incubated with RBCs in EDTA solution and then reacted with ^{125}I-anti-IgG. The bindings of ^{125}I-anti-IgG decreased by 80% or more, indicating complement-dependence of the reaction.

Characterization of immune complexes in pathological serum samples

Serum samples containing immune complexes obtained from patients with systemic lupus erythematosus and fulminant Type B hepatitis were subjected to ultracentrifugation in a sucrose density gradient, and sizes of immune complexes detectable by the RBC radioimmune assay were studied. Patterns of two representative samples are illustrated in Figs 5 and 6. Immune complexes appeared at positions corresponding to densities higher than monomeric IgG. The serum from a lupus patient revealed a peak of immune complexes between positions of IgG and IgM, and additional few peaks with densities higher than that of IgM (Fig. 5). The serum from a hepatitic patient disclosed a single peak of immune complexes at much higher density (Fig. 6). The highest binding of ^{125}I-anti-HB_s by RBCs was found at the peak of immune complexes. When the serum of the same patient was ultracentrifuged and the sediment was observed in an electron microscope, numerous immune aggregate containing HB_sAg particles were observed (not shown).

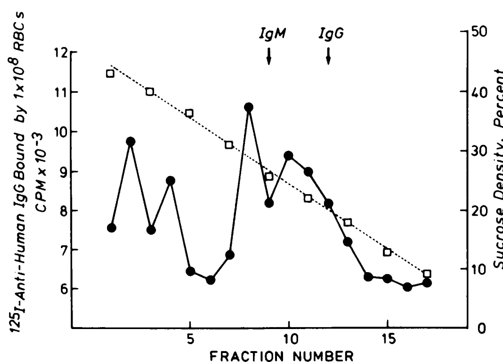

Figure 5. Ultracentrifugation of the serum from a patient with systemic lupus erythematosus in a sucrose density gradient. Closed circles and solid line represent the level of immune complexes in terms of the count of ^{125}I-anti-IgG bound by RBCs. Open squares and dotted line represent sucrose density in percentage. Positions of IgG and IgM are indicated by arrows.

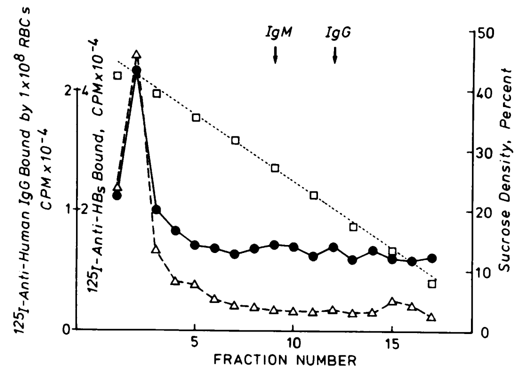

Figure 6. Ultracentrifugation of the serum from a patient with fulminant Type B hepatitis in a sucrose density gradient. Open triangles and broken line represent the binding of ^{125}I-anti-HB_s to RBCs which had been incubated with fractions of the serum. All the other symbols are as indicated in Fig. 5.

DISCUSSION

We have previously developed the methods to determine HB_sAg (Mayumi *et al.*, 1971), antibody to the core of Dane particles [presently accepted hepatitis B virus (Dane, Cameron & Briggs, 1970)] (Tsuda, Takahashi, Takahashi, Miyakawa & Mayumi, 1975) and human transplantation antigens (Miyakawa, Tanigaki, Yagi, Cohen & Pressman, 1971) by means of IAHA method. IAHA has been the method of our choice in approaching new antigens and antibodies. When we started to determine soluble immune complexes in our laboratory following the Raji cell radioimmune assay described by Theofilopoulos *et al.* (1976), it occurred to us why could we not use the immune adherence receptor (receptor for C3b) on human erythrocytes, which had been used as indicator cells in IAHA, for the detection of complement-fixing immune complexes? This idea prompted us to run experiments to evaluate human erythrocytes for this purpose. We found that human erythrocytes can be used for a sensitive determination of complement-fixing immune complexes in the serum.

RBC radioimmune assay for immune complexes involves the binding of C3b receptor on RBCs with complement fixing immune complexes. RBCs which had been prepared and washed in the manner described herein bound monomeric IgG minimally, so that an increase in the uptake of IgG over controls could be assumed to represent IgG constituting immune complexes. Subsequently, IgG bound to RBCs

was detected by the radiolabelled antibody against human IgG. The sensitivity of the present method in terms of the minimal AHG detectable was 5 μg per ml of the original test serum, comparable with that of the Raji cell radioimmune assay (Theofilopoulos *et al.*, 1976).

RBC radioimmune assay for the determination of immune complexes is a fairly straightforward technique. It has to be taken into account, however, that there is a wide range of variation in the number of immune adherence receptors present on the surface of RBC. Turk found that two out of over one hundred samples of human erythrocytes to be unreactive (1964). When we tested ten RBC batches, a wide variation was found in their binding with radiolabelled AHG *pari passu* with their immune adherence reactivity. Individuals with blood group O were selected to avoid haemagglutination by isohaemagglutinins inevitably contained in the test serum. Little care has been taken to escape Rh-dependent haemagglutination in the present assay, since Rh-negative individuals are extremely rare among Japanese population. Rh-negative donors, however, may be required in the districts where Rh-negative individuals are common. Similarly, autoantibodies to RBC may bind with RBC in the present assay, and potentially induce false positive results. These can be excluded either by performing Coombs test for the detection of anti-RBC antibodies, or by comparing the binding in the presence of complement with that in EDTA solution.

The results of density gradient ultracentrifugation of pathological serum samples indicated that the present RBC radioimmune assay was not influenced by monomeric IgG molecules in the serum. When the serum of a patient with fulminant Type B hepatitis was fractionated in a sucrose density gradient, the uptake of radio-labelled anti-IgG by RBCs paralleled the uptake of labelled anti-HB_s, lending strong support for the binding of RBCs and immune complexes involving HB_sAg and anti-HB_s gammaglobulins.

We have demonstrated the binding of erythrocytes with complement-fixing immune complexes *in vitro*. It would be natural to assume that such a binding may also occur *in vivo* in the patients with various disorders mediated by immune complexes. Nelson injected Type I pneumococci sensitized with antibody intravenously into a normal monkey and observed that essential 100% of the bacteria were associated with erythrocytes, leaving the plasma almost completely cleared (1956). Immune adherence of sensitized organisms to erythrocytes enhanced their phagocytosis by polymorphonuclear leucocytes. A cinematographic study demonstrated that polymorphs removed bacteria from the surface of erythrocytes leaving them apparently intact (Robinaux & Pinet, 1960). Indeed, erythrocytes may help dispose of immune complexes formed in circulation, in collaboration with phagocytes and reticuloendothelial systems. Nevertheless, it is certain that the binding of erythrocytes with immune complexes is much less efficient *in vivo* than *in vitro*. If erythrocytes in the bloodstream could bind with the amount of immune complexes we observed in the assay, an incredibly large amount of immune complexes would be required to saturate all C3b receptors on them and circulate free for the detection by *in vitro* assay. Probably, the binding of erythrocytes with soluble immune complexes *in vivo* would be much less tight than the binding with large particulate antigens such as micro-organisms, and could be released continuously by plasma factors such as C3 inactivator.

To date, a number of methods have been developed for the detection of immune complexes in the serum (Maini & Holborow, 1977). Apart from the methods employing physicochemical properties such as molecular size and solubility, they can be classified into three categories each involving (1) reaction with C1, (2) binding with Fc receptors, and (3) binding with receptors for activated C3. A combination of methods with different principles may be justified, since no single method can be expected to detect immune complexes of all kinds which undoubtedly are extremely heterogeneous. We should like to propose that the RBC radioimmune assay be applied in laboratories dealing with immunologically induced diseases.

ACKNOWLEDGMENTS

This work was supported in part by grants from Japanese Ministry of Health and Welfare and the Tokyo Metropolitan Government.

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