BINDING OF SOLUBLE IMMUNE COMPLEXES TO HUMAN LYMPHOBLASTOID CELLS

II. Use of Raji Cells to Detect Circulating Immune Complexes in Animal and Human Sera*

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Circulating immune complexes are involved in the pathogenesis of several human and animal diseases (1). To date, several methods of detecting circulating immune complexes have been described (2-8) with the Clq and the monoclonal rheumatoid factor precipitation in particular being useful tools. However, no single method combines the specificity, reproducibility, and sensitivity necessary for the adequate study of most immune complex diseases. We have, therefore, attempted to develop another method employing cultured human lymphoblasts as detectors.

Human bone marrow-derived (B) lymphocytes can bind immune complexes via receptors for Fc or complement (9, 10). In an earlier work we demonstrated that Raji cells, a cultured human lymphoblastoid cell line, lack membrane-bound Ig (MBIg)[1] but have receptors for human IgG Fc, C3b, and C3d (11). In addition, it was shown that aggregated human gamma globulin (AHG), used as a model for human immune complexes, when reacted with fresh serum bound to these cells via receptors for complement (11).

This report describes the use of receptors for complement on Raji cells for in vitro detection of complement fixing immune complexes in human and animal sera.

With this method, as little as 200–300 ng AHG or immune complexes made in vitro per ml serum could be detected. The Raji cell test was used to detect complement-fixing immune complexes in sera of patients with immune complex glomerulonephritis, rabbits with acute serum sickness, and mice with acute lymphocytic choriomeningitis virus (LCM) infection.

Materials and Methods

Lymphoblastoid Cell Line. Raji cells, which are derived from a patient with Burkitt's lymphoma (12), were cultured in Eagle's minimal essential medium (MEM) as has been described (11). Cell viability was determined by trypan blue exclusion.

Preparation of AHG and 7S Human IgG. Human IgG was obtained from Cohn fraction II after fractionation on a DEAE-52 cellulose column with a 0.01 M phosphate buffer pH 7.3. Human IgG in phosphate-buffered saline (PBS) was freed from aggregates by centrifugation at 150,000 g for 90 min, and the upper one-third of the supernate (deaggregated or 7S IgG) was removed and the protein concentrations were determined by optical density at 280 mμ and extinction coefficient ($E_{1\,cm}^{1\%}$) 14.3. Fluorescein isothiocyanate (FITC)-conjugated 7S IgG and FITC AHG were prepared as in (11). Protein concentration of FITC AHG were determined by a nitrogen determination method (13).

Antisera. The IgG fraction of rabbit antiserum to human serum albumin (HSA), human IgG, bovine serum albumin (BSA), and mouse gamma globulin, as well as sheep antiserum to rabbit IgG, was isolated by DEAE-52 cellulose as above. IgG fraction of murine antiserum to LCM virus was a gift from Dr. M. B. A. Oldstone, Scripps Clinic. Pure rabbit anti-HSA antibody was obtained by solid phase immunoabsorbtion utilizing HSA-Sepharose 4B columns (14). Antiserum to human thyroglobulin (Tg) was prepared in mice, and pure antibody was obtained as described (15). Antiserum to human Tg, as well as human Tg, were a gift from Dr. J. Clagett, Scripps Clinic. The Fab' fragment of the IgG rabbit antihuman IgG was obtained by the method of Loor et al. (16) and concentrated to 5 mg/ml PBS.

Preparation of Soluble Immune Complexes. Quantitative precipitin curves were constructed for the HSA-anti-HSA and Tg-anti-Tg systems, and equivalence was determined. In a typical experiment, increasing amounts of HSA (3 μg–5 mg) were added to a constant amount of pure anti-HSA antibody (315 μg). The precipitates obtained after incubation for 1 h at 37°C and overnight at 4°C were washed twice in physiologic saline, dissolved in 0.1 N NaOH, and the protein was quantified by a nitrogen determination. Supernates obtained from each point (antibody excess to extreme antigen excess) were used in experiments in which binding of soluble immune complexes to Raji cells was studied. In order to assess the amount of antibody complexed to antigen in the supernate (in antigen excess) ^{125}I-labeled pure antibody to HSA was used.

Radioiodination of Proteins. Proteins were labeled with ^{125}I or ^{131}I according to the procedure of McConahey and Dixon (17). [^{125}I]AHG was prepared as described (11).

Sucrose Density Gradient. [^{125}I]AHG was fractionated on a linear 10–37% sucrose density gradient. 0.3 ml of [^{125}I]AHG was layered on the top of 4 ml of sucrose solution and centrifuged for 3 h at 100,000 g. 10 drop fractions were collected by piercing the tubes from below and the protein content determined in a gamma scintillation counter using the specific activity 0.3 μC/μg. Human Ig M and IgG served as 19S and 7S markers respectively.

Immunofluorescence Staining. Soluble antigen-antibody complexes obtained at threefold antigen excess for the HSA-anti-HSA system and 10-fold antigen excess for the Tg-anti-Tg system, were added to 5 × 10⁶ Raji cells in a total vol of 100 μl MEM and incubated at 37°C for 45 min. Cells were subsequently washed three times with MEM and stained at 4°C for 30 min with FITC-conjugated antiserum (18) specific for either antigen or antibody. Binding of FITC 7S IgG and FITC AHG to Raji cells was studied directly or after reacting with antihuman IgG (indirect method) (11). After the final incubation, cells were processed and observed as described in (11). To study the effect of complement in binding immune complexes or AHG to cells, immune complexes or AHG were first preincubated (37°C, 30 min) with fresh or heat-inactivated (56°C, 30 min) serum. Preliminary experiments showed that optimum Raji cell binding of immune complexes or AHG which had reacted with complement was achieved after incubation for 30 min. No difference in binding was observed at 4, 22, or 37°C.

Detection of Immune Complexes in Animal and Human Sera

Test Sera. Sera from 10 New Zealand white rabbits obtained at 0–30 days during the course of acute serum sickness induced with [^{125}I]BSA (19) were studied for presence of immune complexes by the Raji cell test. Levels of [^{125}I]BSA and [^{125}I]BSA-anti-BSA complexes were determined by precipitation with trichloroacetic acid (TCA) and ammonium sulfate respectively as described (19). Proteinuria in these rabbits was quantitated using 3% sulfosalicylic acid (19).

Murine plasma from six 4-6-wk old SWR/J mice inoculated with LCM virus intracerebrally (20) (a gift from Dr. M. B. A. Oldstone) were tested for immune complexes by the Raji cell test. The titers of infectious virus and the presence of virus-antiviral antibody complexes in the plasma of similar mice had been previously determined by diminution of viral titers in plasma after immunoprecipitation of the mouse IgG with an antimouse IgG serum (20) and are included in Table I for comparison.

42 frozen sera from glomerulonephritic patients, who had presumed immune complex type glomerulonephritis (21), were also tested for circulating immune complexes by the Raji cell test. The diagnosis of immune complex glomerulonephritis was based on the presence of deposits of IgG and complement in the glomeruli as described by Wilson and Dixon (21). The Raji cell test and the immunofluorescence test for kidney deposits were performed independently by two of us and graded semi-quantitatively, according to the intensity of staining, from negative to 3+. 30 frozen sera from normal laboratory personnel were similarly tested for circulating immune complexes by the Raji cell test and served as controls.

Raji Cell Test

RABBIT AND MOUSE SERA. 5×10^6 Raji cells were incubated (37°C, 30 min) with 50 µl of each rabbit or mouse serum to be tested. Subsequently, cells were washed three times with MEM and then stained (4°C, 30 min) with FITC sheep antirabbit IgG or FITC rabbit anti-BSA in the case of the rabbits with acute serum sickness, and with FITC rabbit antimouse gamma globulin or FITC mouse anti-LCM virus serum in the case of mice with LCM virus infection. Cells were washed three times with MEM, smeared and assayed for fluorescence positive cells.

HUMAN SERA. 5×10^6 Raji cells were incubated (37°C, 30 min) with 1 mg human IgG, washed two times with MEM and then reacted (4°C, 30 min) with 50 µl of Fab' rabbit antihuman IgG serum to diminish the background fluorescence caused by 7S IgG binding to the receptors for IgG Fc. Subsequently, cells were washed twice with MEM, and incubated (4°C, 30 min) with 25 µl of the serum from glomerulonephritic patients or the normal control subjects. Finally, cells were washed again three times with MEM, stained (4°C, 30 min) with 25 µl of FITC rabbit antihuman IgG serum, washed three times with MEM, smeared, and observed for fluorescence-positive cells.

Results

Binding of AHG and 7S Human IgG to Receptors for IgG Fc. Our eventual purpose is to develop a test in which Raji cells can be used for the detection of immune complexes in whole human serum. AHG, which possesses many properties of immune complexes (22, 23), was employed to study binding of human immune complexes to receptors for IgG Fc. Binding of 7S IgG to cell's receptors for IgG Fc interferes with the detection of the cell-bound antibody (IgG) of the immune complex; therefore, binding of 7S human IgG to Raji cells also had to be assessed. As shown in Fig. 1 a, when cells were incubated directly with FITC AHG or FITC 7S IgG, only AHG binding could be detected. However, when cells were first incubated with the fluoresceinated reagents and then reacted with an unfluoresceinated rabbit antihuman IgG (indirect method), so as to cross-link molecules possibly bound to cells, both AHG and 7S IgG binding could be detected (Fig. 1 b). The results indicated that both AHG and 7S IgG bound to receptors for IgG Fc on Raji cells with AHG more easily detectable than 7S IgG.

Concentrations of at least 250–500 μg AHG per ml medium, as shown by the indirect method, were needed to be detected on the Raji cells. The pattern of fluorescence, obtained by the indirect method, was distinct for AHG and 7S IgG. AHG was found on Raji cells in patches with many large, irregular granules on the cell surfaces (Fig. 2 a). In contrast, 7S IgG was distributed evenly with many fine granules over the entire cell surface (Fig. 2 b).
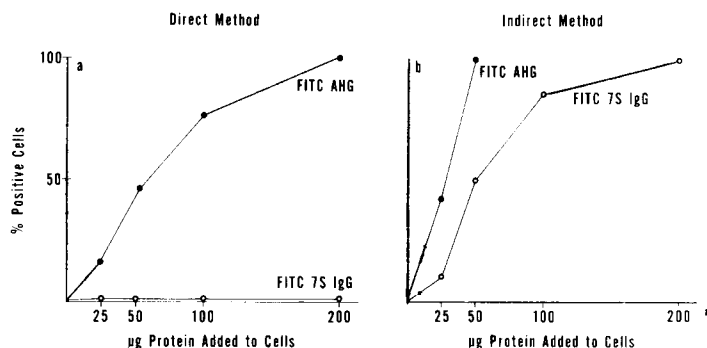

FIG. 1.   Binding of AHG and 7S human IgG to receptors for IgG Fc on Raji cells. (a) Aliquots of 5 × 10⁶ Raji cells were incubated with increasing amounts of FITC AHG or FITC 7S IgG in 100 μl MEM and observed (direct method). (b) Enhanced binding ensued when cells were treated as in the direct method, washed with MEM, and then reacted with unfluoresceinated antihuman IgG (indirect method).
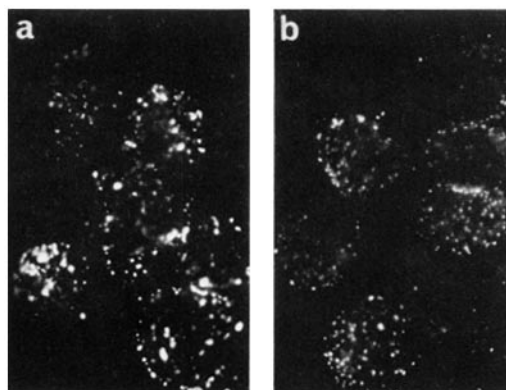

FIG. 2.   Immunofluorescence staining patterns of Raji cells carrying FITC AHG or FITC 7S IgG. (a) AHG-bearing Raji cells had many large, irregular granules of IgG on the cell surface. (b) 7S IgG-bearing Raji cells had many fine granules of IgG distributed evenly on the cell surface × 400.

Inhibition of AHG Binding to Receptors for IgG Fc by 7S IgG.   In order to determine if AHG and 7S IgG bound to the same receptors for IgG Fc on Raji cells, cells were incubated with 7S IgG and then reacted with soluble FITC AHG. No fluorescence appeared on the cell surface. This result indicated that the receptors for IgG Fc on Raji cells are the same for AHG and 7S IgG. Because both AHG and 7S IgG bound to the same receptors for Fc, the effect of excess 7S IgG on binding of AHG was analyzed. As described in Fig. 3, binding of AHG to
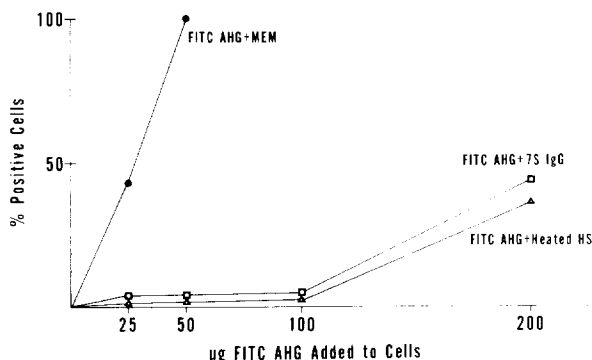
FIG. 3.   Inhibition of binding of AHG to receptors on Raji cells for IgG Fc by 7S IgG. Aliquots
of 5 × 10⁶ cells were incubated with increasing amounts of FITC AHG in 100 μl MEM, in 100 μl
MEM containing 10 mg/ml 7S human IgG, or in 100 μl heat-inactivated (56°C, 30 min) HS.
Subsequently cells were washed and reacted with unfluoresceinated antihuman IgG (indirect
method).

receptors for IgG Fc was inhibited in the presence of physiologic concentrations
of 7S IgG in heat-inactivated human serum (56°C, 30 min) or MEM. 25 μg AHG
in 100 μl MEM, as compared to at least 200 μg AHG in 100 μl heated serum or
100 μl MEM containing 10 mg/ml 7S IgG, were required to reach a concentration
sufficient for detection of immunofluorescence.

Binding of AHG and Immune Complexes Interacted with Complement.   AHG,
as well as immune complexes that have interacted with fresh serum, can bind to
cells via receptors for complement. Raji cells bind human C3 and C3b, as well as
rabbit and mouse C3, and form rosettes with sensitized red cells carrying C3b or
C3d (11). Therefore, binding of AHG in the presence and absence of human
serum, as the source of complement, was studied. As depicted in Fig. 4, when
different amounts of FITC AHG preincubated at 37°C for 30 min with fresh
human serum were added to cells, the binding of as little as 20 ng of AHG in
100 μl fresh human serum could be detected on the Raji cells, as compared to
200–300 μg of AHG in 100 μl heated serum (an increase of 10–15,000 fold).

Binding of soluble immune complexes to Raji cells was also studied in the
presence and absence of serum. No binding of HSA, Tg, anti-HSA, or anti-Tg
in serum to Raji cells was detected by immunofluorescence. HSA-anti-HSA
complexes in heated rabbit serum also did not bind to cells (Fig. 5). However,
even 20–50 ng of anti-HSA complexed with HSA and preincubated with 100 μl
fresh rabbit serum could be detected on Raji cells (Fig. 5). A similar degree of
sensitivity was obtained with the Tg-anti-Tg immune complexes in fresh mouse
serum. With both immune complexes formed in vitro antigen and antibody were
detectable on the Raji cell. The results indicated that Fc receptors on Raji cells
do not bind monomeric or aggregated (by antigen) rabbit or mouse IgG. However,
immune complexes made with rabbit or mouse antibodies and reacted with rab-
bit or mouse serum respectively, as the source of complement, can bind very
efficiently to Raji cells, presumably via receptors for complement.

*Antigen-Antibody Composition and Sedimentation Rates of AHG which Bind
to Raji Cells via Receptors for Complement.*   The efficiency of cells for binding
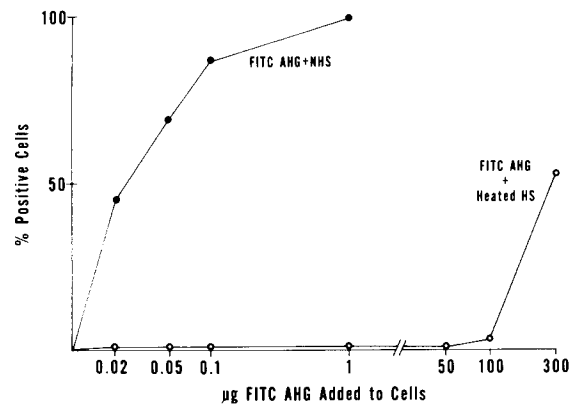
Fig. 4.   Enhanced binding of AHG to Raji cells in the presence of human serum as the source of complement. Aliquots of 5×10^6 cells were incubated with increasing amounts of FITC AHG which had reacted with 100 μl normal or heat-inactivated (56°C, 30 min) HS. Cells were washed with MEM and then reacted with unfluoresceinated antihuman IgG (indirect method).
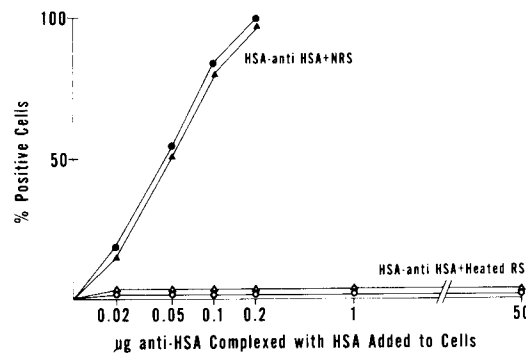

Fig. 5.   Binding of soluble HSA-rabbit anti-HSA immune complexes to Raji cells in the presence of NRS as the source of complement. Aliquots of 5×10^6 cells were incubated with increasing amounts of anti-HSA complexed at threefold antigen excess and preincubated with 100 μl normal (closed symbols) or heat-inactivated (open symbols) rabbit serum (RS). Cells were washed with MEM and then reacted with FITC antirabbit IgG (●—● and O—O) or with FITC anti HSA (▲—▲ and △—△).

immune complexes via the receptors for complement may be different at different antigen-antibody compositions. To define the conditions in which complexes bind most efficiently to cells, HSA-anti-HSA complexes from antibody excess to extreme antigen excess were reacted with fresh rabbit serum and then incubated with the cells. Subsequently, the cells were washed and stained with FITC antisera specific for the antigen or the antibody. The precipitin curve of HSA and anti-HSA and reactions of Raji cells incubated with the supernatants at various points along the curve are shown in Fig. 6. The strongest intensity of fluorescence was observed at two to six times antigen excess; no fluorescence positive cells were seen at antibody excess or beyond thirty times antigen excess. The results showed that positive cells are obtained only with supernates at antigen excess

containing soluble complexes, and that the strongest staining is seen near equivalence where presumably large complexes would be formed.

It has been previously shown that larger aggregates of γ-globulin fix complement better than smaller aggregates (22, 24). In order to estimate the sedimentation rate of aggregates bound via receptors for complement on Raji cells, uptake experiments with [125I]AHG were performed. [125I]AHG was first fractionated by sucrose density gradient to aggregates of different sedimentation rates. Fig. 7 shows a sucrose density gradient of [125I]AHG. 5 μg of each of five pools made with fractions containing aggregates of different sedimentation rates were incubated with 25 μl fresh human serum and added to aliquots of 2.5 × 10⁶ Raji cells. After an incubation period (37°C, 30 min), cells were washed three times with MEM, and the percent of radioactivity adherent to cells was determined. As

Immunofluorescence
by Raji Cells

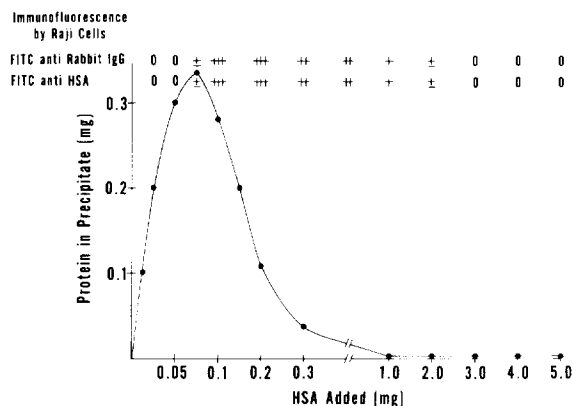|											
FITC anti Rabbit IgG	0	0	±	+++	+++	++	++	+	±	0	0	0
FITC anti HSA	0	0	±	+++	+++	++	++	+	±	0	0	0

FIG. 6.   Precipitin curve of rabbit anti HSA with HSA. 50 μl of each supernate obtained along the curve were incubated with 100 μl NRS and added to aliquots of 5 × 10⁶ Raji cells. Subsequently cells were washed and stained with FITC antiserum specific for antigen or antibody. Intensity (0 to 3+) of immunofluorescence observed with the Raji cells is shown at the top of the figure.
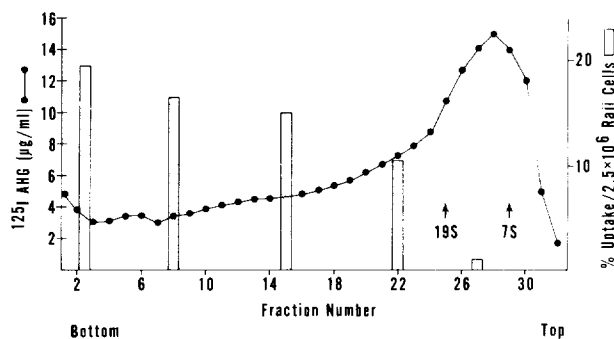
FIG. 7.   Sucrose density gradient fractionation of [125I]AHG. 32 fractions were collected. Fractions 1-4, 5-11, 12-18, 19-25 and 26-28 were pooled. 5 μg [125I]AHG from each pool were incubated (37°C, 30 min) with 25 μl NHS and added to aliquots of 2.5 × 10⁶ Raji cells. The percent uptake of each pool is shown by the shaded bars. The position of the 19S and 7S markers are indicated. Aggregates approximately 95S and smaller were retained on the gradient.

shown in Fig. 7, uptake of aggregates sedimenting more rapidly than 19S was considerably greater than that of aggregates slower than 19S.

Detection of Immune Complexes in Animal and Human Sera.   Since complement receptors on Raji cells efficiently bind soluble immune complexes as well as AHG, these cells were used in an assay system for the detection of circulating immune complexes in experimental animals and humans. In 10 rabbits with acute serum sickness, the appearance of circulating immune complexes during the course of the disease was determined by the Raji cell test. As it can be seen in Fig. 8, positivity and intensity of staining observed with the Raji cell test for BSA-anti-BSA immune complexes correlated with the amount of the same radiolabeled circulating immune complexes. 8 of 10 rabbits showed rapid elimination of the

FIG. 8.   Appearance of immune complexes in sera of a rabbit with acute serum sickness. 18 h before administration of the [^{125}I]BSA, the rabbit had been injected with anti-BSA (*arrows*). Percent injected [^{125}I]BSA in total blood volume and percent [^{125}I]BSA precipitated with SAS are indicated. Intensity of immunofluorescence (0 to 3+) by the Raji cells is shown at the top of the figure.

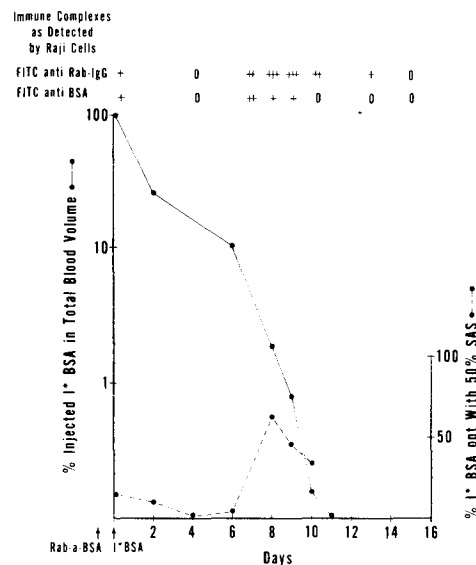antigen and presence of immune complexes at day 7–10. It should be noted that in six of the eight rabbits with circulating immune complexes, the Raji cell test remained positive for approximately 2–3 wk longer than radiolabeled immune complexes could be detected. The antigen (BSA) was not always detectable on the Raji cell surface, presumably due to masking by the antibody. Only four of the eight rabbits that developed acute serum sickness showed proteinuria (more than 20 mg/24 hr). 2 of the 10 rabbits did not eliminate the antigen rapidly nor show circulating radiolabeled immune complexes. These two rabbits were negative, or only weakly positive (in some samples), by the Raji cell test.

The presence of virus-IgG complexes in plasma of LCM virus infected mice, as detected by the Raji cell test, was compared to the presence of complexes as estimated by the diminution of viral titers in plasma after immunoprecipitation of

mouse IgG with rabbit antimouse IgG. Both tests were negative for immune complexes during the first three postinfection days. On the fourth day the immunoprecipitation method was negative but the Raji cell test was positive for four out of six animals; by the fifth and sixth postinfection day, both tests were positive for virus-IgG complexes in all six animals (Table I).

A modification of the technique was necessary to test human sera for immune complexes by the Raji cell test because fluorescence had been obtained from 7S IgG, present in normal human serum (NHS), and bound to receptors for IgG Fc. To prevent 7S IgG binding to cells, the receptors for human IgG Fc had to be blocked. This was accomplished by incubating the cells with human IgG and then with Fab' rabbit anti-human IgG. As shown in Fig. 9, when cells with nonblocked receptors for IgG Fc were reacted with NHS and then stained with FITC rabbit antihuman IgG, a background of fluorescence was observed (Fig. 9 a). When cells with blocked receptors for IgG Fc were incubated with NHS and stained with FITC rabbit antihuman IgG, they remained negative (Fig. 9 b). However, when cells with blocked receptors for IgG Fc were reacted with NHS containing AHG and stained, all cells were strongly positive (Fig. 9 c-f). The results indicated that when receptors for IgG Fc on Raji cells are blocked, human IgG is detected on these cells only if it is aggregated (immune complex) so that it can fix complement and bind to cells via the receptors for complement. As shown in Table II, when glomerulonephritic patients were studied there was a good correlation between the Raji cell test for circulating immune complexes and granular IgG deposits, presumed immune complexes, in the patients' kidneys. Only 3 out of 12 patients lacking glomerular IgG deposits had detectable amounts of circulating immune complexes as shown by the Raji cell test. The majority of patients with trace to marked glomerular immune complex deposits showed similar degrees of positivity with the Raji cell test for circulating immune complexes. 5 out of 30 patients with IgG kidney deposits were negative for circulating immune complexes by the Raji cell test (Table II). Control sera obtained from 30 healthy individuals were negative for circulating immune complexes by the Raji cell test.

TABLE I

Appearance of Virus-IgG Complexes in Plasma of 4-6-Wk Old SWR/J Mice with Acute LCM Virus Infection

Days after i.c. inoculation	Infectious titers in plasma*	Virus-IgG complexes by immunoprecipitation	Virus-IgG complexes by Raji cells
1	1.2§	0/6‡§	0/6‡
2	3.0	0/6	0/6
3	4.2	0/6	0/6
4	5.6	0/6	4/6
5	5.8	4/6	6/6
6	4.7	6/6	6/6

* Average of the reciprocal of log dilution giving a 50% lethal end point/0.03 ml i.c. inoculum.
‡ Number of mice having virus-IgG complexes in their serum per total number tested.
§ For comparison, infectious titers and virus-IgG complexes by immunoprecipitation from similar mice are included (32).
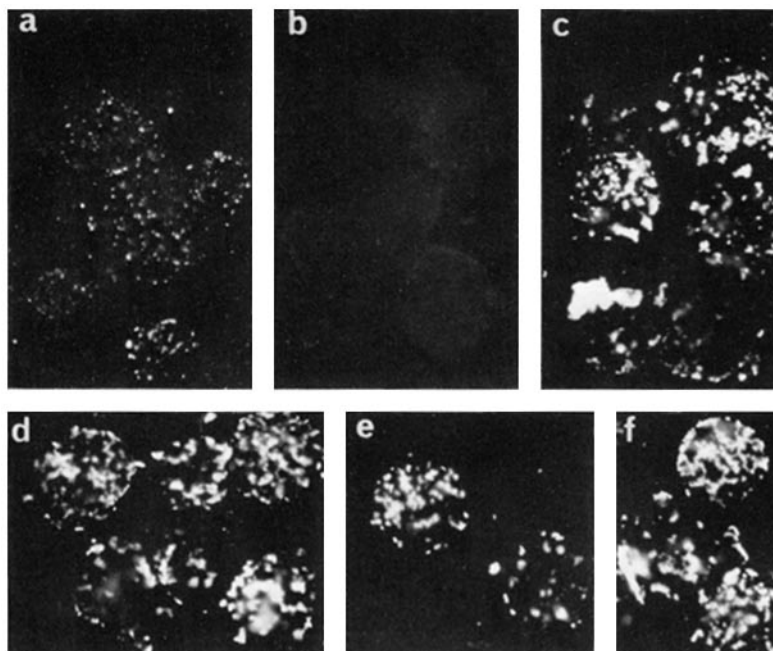
FIG. 9. Demonstration of AHG in human serum utilizing Raji cells with IgG and Fab' rabbit antihuman IgG blocked receptors for IgG Fc. Fig. 9 a shows fine granular fluorescent IgG on Raji cells with nonblocked receptors for IgG Fc incubated with NHS and stained with FITC rabbit antihuman IgG. No IgG fluorescence is seen when Raji receptors for IgG Fc are blocked, incubated with NHS and stained with FITC rabbit antihuman IgG, Fig. 9 b. Figs. 9 c-f show intense fluorescent IgG on Raji cells with blocked receptors for IgG Fc, incubated with NHS containing AHG and stained with FITC rabbit antihuman IgG. × 400.

TABLE II
Reactivity of Raji Cells with Serum from Patients with Immune Complex Glomerulonephritis

Raji cell test*	Granular glomerular IgG deposits*		
	Negative	1+	2-3+
Negative	9	3	2
1+	3	11	3
2-3+	0	4	7
Total no. of cases	12	18	12

* The Raji cell test and the immunofluorescent granular deposits (presumed immune complexes) were graded semi-quantitatively from negative to 3+.

Discussion

The data presented here showed that receptors for complement on Raji cells, a human lymphoblastoid cell with B-cell characteristics, can be used for the detection of complement fixing immune complexes in experimental animal and

presumably in human sera. Our experiments showed that binding of AHG via receptors for IgG Fc is not efficient. In fact, high concentrations of AHG were needed in order to be detected by Raji cells, and receptors for IgG Fc on these cells bound not only AHG but also 7S human IgG. Furthermore, binding of AHG to receptors for IgG Fc was inhibited in the presence of physiologic concentrations of 7S IgG. Arent and Mannik (25) inhibited adherence of soluble immune complexes to receptors for IgG Fc on rabbit alveolar macrophages by rabbit serum and rabbit or human 7S IgG. However, Dickler and Kunkel (9) found that excess human 7S IgG could not inhibit binding of AHG to receptors for IgG Fc on human B lymphocytes. It may be that our aggregates were of different size than theirs, since these authors pointed out that large aggregates have many Fc determinants and may displace 7S IgG from the cell surface. AHG binding to receptors for IgG Fc on Raji cells was demonstrated directly or after reacting with antihuman IgG (indirect method), whereas 7S IgG binding was shown only by the more sensitive indirect method. Similar methods for the detection of receptors for IgG Fc on human B lymphocytes have been employed by Dickler et al. (26), who found that binding of aggregates smaller than 200 S was demonstrable only by the indirect method. According to these authors, the anti-IgG serum cross-links and further aggregates the bound material which had been too small to be visualized by the direct method.

Binding of AHG to Raji cells was enhanced in the presence of fresh human serum. Furthermore, binding of soluble immune complexes made in vitro could not be detected by immunofluorescence in medium or in heated serum, but was easily detectable after these complexes had interacted with fresh serum. We have also found that many antigen-antibody complexes (BSA-anti-BSA, DNA-anti-DNA, KLH-anti-KLH) can be detected by immunofluorescence on the Raji cell surface if they have reacted with fresh serum as source of complement. Similarly, Eden et al. (27) have shown that soluble immune complexes in fresh mouse serum bind to mice B lymphocytes much more efficiently than the same complexes in heat-inactivated serum. However, Dickler and Kunkel (9) found that binding of AHG to human peripheral B lymphocytes was not enhanced in the presence of fresh human serum.

Enhancement of AHG binding and binding of soluble immune complexes to Raji cells in the presence of fresh serum is thought to be due to the participation of the receptors for complement in the binding. We have demonstrated that AHG reacted with fresh serum bound to Raji cells only via receptors for complement but not via receptors for IgG Fc and that both receptors for C3-C3b and for C3d were operative in the binding (11). The increased sensitivity of Raji cells in detecting AHG or immune complexes via receptors for complement indicate either that these cells have more receptors for complement than receptors for IgG Fc, or that receptors for complement are more efficient than receptors for IgG Fc in binding their ligand molecules. Another possibility is that immune complexes containing complement possess more C3 sites than Fc sites present in immune complexes without complement and therefore bind more efficiently to Raji cells.

By using HSA-anti-HSA immune complexes at different antigen-antibody compositions, we found that receptors for complement on Raji cells strongly bound complexes made at two to six times antigen excess, at which the largest soluble

complexes are formed. These results agree with the findings of Agnello, Winchester, and Kunkel (2), who reacted the same type of complexes with Clq. Eden et al. (27) also showed that binding of BSA-anti-BSA complexes to mice B lymphocytes in the presence of serum was greatly diminished at extreme antigen excess. A greater part of aggregates sedimenting faster than 19S bound to Raji cells than of the slower aggregates, presumably due to the more efficient fixation of complement. Several investigators have shown that activation and fixation of complement by aggregates of γ-globulin are directly correlated with size (2, 22, 24).

The Raji cell test was applied to detect immune complexes in sera. The method for detection of immune complexes in rabbit and mouse sera was simple because Raji cells need only to be incubated with the serum to be tested and stained with FITC antirabbit or FITC antimouse IgG serum. Since rabbit and mouse IgG do not bind to receptors for IgG Fc on Raji cells, rabbit or mouse IgG found on the cell surface was attributed to the presence in the serum of immune complexes that fixed complement and bound to cells via receptors for complement. Receptors for complement on Raji cells are known to bind C3 from sera of different species including rabbit and mouse (11). In our two animal models, circulating immune complexes were demonstrable by the Raji cell test, and results obtained by this method correlated with amount of immune complexes as detected by other methods. However, in some of the rabbits with serum sickness, positivity obtained with the Raji cell test lasted 2-3 wk after the radiolabeled complexes had disappeared. This finding may indicate that partially degraded and no longer radiolabeled immune complexes persist in the rabbit's circulation. In fact, it has been found in vitro that many antigens are not completely degraded by macrophages and that small amounts of antigen are released intact into the milieu (28).

The Raji cell test was also applied to detect immune complexes in human sera. Since receptors for IgG Fc on Raji cells bind 7S IgG as well as AHG, these receptors had to be blocked so that no fluorescence with normal human serum remained. Cells with blocked receptors for IgG Fc were able to bind aggregates in serum via their complement receptors. Sera from a majority of patients with immune complex glomerulonephritis, as evidenced by granular deposits of immunoglobulin and complement in their glomeruli, were positive for circulating immune complexes by the Raji cell technique. Such granular deposits along the glomerular filter are considered to be immune complexes made by antibodies reacted with circulating nonglomerular antigens (21). In testing human sera from glomerulonephritic patients, since the antigen was unknown, only the antibody (IgG) was sought on Raji cell surfaces. However, since Raji cells with blocked receptors for IgG Fc bind only AHG but not 7S IgG, we considered that the IgG detected on the surfaces of these cells represented antibody aggregated by antigen which was able then to fix complement and bind to blocked cells via receptors for complement. The occasional failure to find circulating complexes in patients with immune complex glomerulonephritis is not surprising since fluctuations in circulating antigen, antibody, and presumably immune complexes have been shown in patients with systemic lupus erythematosus (SLE) (29), and changes in circulating complexes are not immediately reflected in glomerular complex deposits. On the other hand, presence of circulating complexes need not necessarily be associated

with immune complex glomerulonephritis since multiple factors, including immune complex size (30), changes in vascular permeability (31) and the functional state of the reticuloendothelial system and presumably the renal mesangium (21), may influence glomerular deposition of complexes from the circulation. Therefore, even if complexes were detected in the circulation, they need not be nephritogenic at a particular point in time.

The availability of tests, such as the Raji cell test outlined in this paper, should provide a way in which to study the levels of complement fixing immune complexes in the circulation and to detect their relationship to exacerbations and quiescence of known and suspected immune complex diseases. The test should have usefulness in monitoring the effectiveness of immunosuppressive therapy so often used to treat patients with immune complex glomerulonephritis, including conditions such as SLE. It may also provide a way to determine the optimum timing for renal transplantation so as to avoid the rapid recurrence of a preceding immune complex glomerulonephritis in the transplanted kidney.

Since Raji cells bind immune complexes, these cells may also prove to be a useful tool for identifying the particular antigen(s) which initiates and serves as the target of a pathogenic immune response in humans and animals. Such identification of the antigen(s) combined with studies on the immunopathological status of the host and the activation and operation of the mediators of injury in immune complex type disease states, may provide further information as to the pathogenesis of these diseases, as well as means for their prevention. Currently, an extensive study is under way in our laboratory not only to detect but also to quantitate circulating complement fixing immune complexes in diseases of humans and animals by using the Raji cell test.

Summary

Cells from a human lymphoblastoid cell line (Raji), with B-cell characteristics, and having receptors for human IgG Fc, C3b, and C3d, were used in an immunofluorescence test as in vitro detectors of immune complexes in animal and human sera. By this test, as little as 200–300 ng aggregated human gamma globulin or immune complexes per ml serum could be detected. The receptors for IgG Fc on the Raji cells were shown to be inefficient in detecting aggregated human gamma globulin and binding of aggregates to these receptors was inhibited by physiologic concentrations of 7S human IgG. Enhancement of aggregated human gamma globulin binding and binding of immune complexes formed in vitro to Raji cells was observed when the receptors for complement on these cells were used. By using the receptors for complement on Raji cells, circulating immune complexes were detected in rabbits with acute serum sickness, in mice with acute lymphocytic choriomeningitis virus infection, and in humans with immune complex type glomerulonephritis. The Raji cell test may be useful in detecting complement fixing immune complexes in different disease states, in monitoring circulating complexes in patients with immune complex diseases and in identifying the antigen(s) responsible for the induction of pathogenic immune complexes in humans and animals.

References

1. Dixon, F. J. 1972. Pathogenesis of immunologic disease. *J. Immunol.* **109:**187.
2. Agnello, V., R. J. Winchester, and H. G. Kunkel. 1970. Precipitin reactions of the Clq component of complement with aggregated γ-globulin and immune complexes in gel diffusion. *Immunology.* **19:**909.
3. Winchester, R. J., H. G. Kunkel, and V. Agnello. 1971. Occurrence of γ-globulin complexes in serum and joint fluid of rheumatoid arthritis patients: use of monoclonal rheumatoid factors as reagents for their demonstration. *J. Exp. Med.* **134:**286s.
4. Myllylä, G. 1973. Aggregation of human blood platelets by immune complexes in the sedimentation pattern test. *Scand. J. Haematol. Suppl.* **19:**1.
5. Mowbray, J. F., A. V. Hoffbrand, E. J. Holborow, P. P. Seah, and L. Fry. 1973. Circulating immune complexes in dermatitis herpetiformis. *Lancet.* **1:**400.
6. Jewell, D. P., and I. C. M. MacLennan. 1973. Circulating immune complexes in inflammatory bowel disease. *Clin. Exp. Immunol.* **14:**219.
7. Creighton, W. D., P. H. Lambert, and P. A. Miescher. 1973. Detection of antibodies and soluble antigen-antibody complexes by precipitation with polyethylene glycol. *J. Immunol.* **111:**1219.
8. Franklin, E. C., H. R. Holman, H. J. Müller-Eberhard, and H. G. Kunkel. 1957. An unusual protein component of high molecular weight in the serum of certain patients with rheumatoid arthritis. *J. Exp. Med.* **105:**425.
9. Dickler, H. B., and H. G. Kunkel. 1972. Interaction of aggregated γ-globulin with B lymphocytes. *J. Exp. Med.* **136:**191.
10. Bianco, C., R. Patrick, and V. Nussenzweig. 1970. A population of lymphocytes bearing a membrane receptor for antigen-antibody-complement complexes. I. Separation and characterization. *J. Exp. Med.* **132:**702.
11. Theofilopoulos, A. N., F. J. Dixon, and V. A. Bokisch. 1974. Binding of soluble immune complexes to human lymphoblastoid cells. I. Characterization of receptors for IgG Fc and complement and description of the binding mechanism. *J. Exp. Med.* **140:**877.
12. Pulvertaft, R. J. V. 1965. A study of malignant tumors in Nigeria by short-term tissue culture. *J. Clin. Pathol.* **18:**261.
13. Ferrari, A. 1960. Nitrogen determination by a continuous digestion and analysis system. *Ann. N. Y. Acad. Sci.* **87:**792.
14. Cuatrecasas, P., and C. B. Anfinsen. 1971. Affinity chromatography. *Ann. Rev. Biochem.* **40:**259.
15. Clagett, J. A., and W. O. Weigle. 1974. Role of T and B lymphocytes in the termination of unresponsiveness to autologous thyroglobulin in mice. *J. Exp. Med.* **139:**643.
16. Loor, F., L. Forni, and B. Pernis. 1972. The dynamic state of the lymphocyte membrane. Factors affecting the distribution and turnover of surface immunoglobulins. *Eur. J. Immunol.* **2:**203.
17. McConahey, P. J., and F. J. Dixon. 1966. A method of trace iodination of proteins for immunologic studies. *Int. Arch. Allergy. Appl. Immunol.* **29:**185.

18. Clark, H. F., and C. C. Shepard. 1963. A dialysis technique for preparing fluorescent antibody. *Virology.* **20:**642.

19. Wilson, C. B., and F. J. Dixon. 1970. Antigen quantitation in experimental immune complex glomerulonephritis. I. Acute serum sickness. *J. Immunol.* **105:**279.

20. Oldstone, M. B. A., and F. J. Dixon. 1969. Pathogenesis of chronic disease associated with persistent lymphocytic choriomeningitis viral infection. I. Relationship of antibody production to disease in neonatally infected mice. *J. Exp. Med.* **129:**483.

21. Wilson, C. B., and F. J. Dixon. 1974. Diagnosis of immunopathologic renal disease. *Kidney Int.* **5:**389.

22. Christian, C. L. 1960. Studies of aggregated γ-globulin. I. Sedimentation, electrophoretic and anticomplementary properties. *J. Immunol.* **84:**112.

23. Ishizaka, K. 1963. Gamma globulin and molecular mechanisms of hypersensitivity reactions. *Prog. Allergy* **7:**32.

24. Ishizaka, K., T. Ishizaka, and J. Banovitz. 1965. Biological activities of aggregated γ-globulin. VII. Minimum size of aggregated γ-globulin or its piece III required for the induction of skin reactivity and complement fixation. *J. Immunol.* **94:824.**

25. Arend, W. P., and M. Mannik. 1972. In vitro adherence of soluble immune complexes to macrophages. *J. Exp. Med.* **136:**514.

26. Dickler, H. B., F. P. Siegal, Z. H. Bentwich, and H. G. Kunkel. 1972. Lymphocyte binding of aggregated IgG and surface Ig staining in chronic lymphocytic leukemia. *Clin. Exp. Immunol.* **14:**97.

27. Eden, A., C. Bianco, and V. Nussenzweig. 1973. Mechanism of binding of soluble immune complexes to lymphocytes. *Cell Immunol.* **7:**459.

28. Cruchaud, A., and E. R. Unanue. 1971. Fate and immunogenicity of antigens endocytosed by macrophages: a study using foreign red cells and immunoglobulin G. *J. Immunol.* **107:**1329.

29. Koffler, A. D., J. W. Eisenberg, R. J. Winchester, and H. G. Kunkel. 1971. Clq precipitins in the sera of patients with systemic lupus erythematosus and other hypocomplementemic states: characterization of high and low molecular weight types. *J. Exp. Med.* **134:**228s.

30. Cochrane, C. G., and D. Hawkins. 1968. Studies on circulating immune complexes. III. Factors governing the ability of circulating complexes to localize in blood vessels. *J. Exp. Med.* **127:**137.

31. Kniker, W. T., and C. G. Cochrane. 1968. The localization of circulating immune complexes in experimental serum sickness. The role of vasoactive amines and hydrodynamic forces. *J. Exp. Med.* **127:**119.

32. Oldstone, M. B. A., and F. T. Dixon. 1971. The immune response in lymphocytic choriomeningitis viral infection. Sixth International Symposium on Immunopathology. P. A. Miescher, editor. Schwabe and Co., Basel. 391.