Journal of Immunological Methods, 19 (1978) 227—234

COMPLEMENT RECEPTORS: SPECIFIC DETECTION BY MOLECULAR COMPLEXES

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(Received 3 June 1977, accepted 29 July 1977)

Immune complexes of BSA-anti BSA (IgG F(ab')2) will activate human complement by the alternate pathway. The C3 bound to these complexes binds to human peripheral blood mononuclear cells which bear complement receptors. This interaction is visualized by fluoresceinated antigen (BSA-FITC) or fluorescein conjugated antisera directed at the C3 component of complement. The assay appears to be more sensitive than the usual IgMEAC rosette technique and correlates well with the rosette method.

INTRODUCTION

Cell surface receptors for the third component of complement (C3) have been detected on B lymphocytes (Bianco, 1970), macrophages (Lay, 1968), polymorphonuclear neutrophils (Henson, 1968), eosinophils (Gupta, 1976) and renal glomeruli (Gelfand, 1975). Cells bearing C3 receptors are identified by their ability to form rosettes with sheep erythrocytes (E) sensitized with antibody (usually IgM) and complement (IgMEAC) (Lay, 1968). Complement receptors have also been identified by using bacterial organisms on which complement is fixed to the bacterial cell wall by the alternate complement pathway (Gelfand, 1976). Fc receptor binding does not appear to take place with either the bacterial (Gelfand, 1976) or IgMEAC assay (Shevach, 1973).

Alternatively, C3 receptors can be detected with soluble antigen—antibody complement complexes. These cell bound complexes may be identified

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Abbreviations used in this paper: BSA, bovine serum albumin; C, complement; C-Mo, mouse complement; C-Hu, human complement; NHS, normal human serum; C3, third component of complement; C4, fourth component of complement; BSA-F(ab')2-C, antigen—antibody—complement complex in which the antibody is rabbit anti-BSA IgG F(ab')2 fraction; AgAbC, antigen—antibody—complement complex; FITC, fluorescein isothiocyanate; SRBC, sheep erythrocyte; IgMEAC, SRBC sensitized with IgM antibody and complement.

by either indirect immunofluorescence (Theofilopoulos, 1974b) or by radiolabeling (Eden, 1973). Complement receptor cells can also be identified by incubation with complexes of heat aggregated IgG and complement (Dukor, 1973). These studies have employed whole IgG molecules to fix complement (Dukor, 1973; Eden, 1973; Theofilopoulos, 1974b). The presence of whole IgG may permit binding to both Fc and C3 receptors. Radiolabeled or fluorescein conjugated fluid-phase complement components will also detect complement receptors (Theofilopoulos, 1974a).

This paper reports the use of soluble immune complexes for the specific detection of complement receptor lymphocytes. Human complement was fixed to preformed immune complexes containing IgG F(ab')2. The binding of these complexes to complement receptors was detected by immunofluorescence.

MATERIALS AND METHODS

Sera

The intact IgG fraction of rabbit anti-BSA, the IgG F(ab')2 fraction of rabbit anti-BSA, unlabeled and fluorescein isothiocyanate (FITC)-conjugated BSA (twice crystallized), and the IgG fraction of rabbit anti-sheep erythrocyte serum were obtained from Cappel Laboratories (Cochranville, PA); the IgM fraction of rabbit anti-sheep erythrocyte serum was obtained from Cordis (Miami, FL); and rabbit anti-sheep erythrocyte serum (hemolysin) was purchased from BBL, division Becton-Dickinson (Cockeysville, MD). Goat anti-human C3 conjugated with FITC was purchased from Hyland (Costa Mesa, CA) and rabbit anti-human C4 conjugated with FITC from Behring Diagnostics (Somerville, NJ). The specificities of these anti-human C3 and C4 sera were established by immunoelectrophoresis against human serum and by immunodiffusion against the appropriate antigen.

Complement

Normal human serum (NHS) and mouse (AKR) serum were used for complement. Complement was inactivated by heating (56°C, 30 min).

Molecular complexes

Immune complexes of BSA—anti-BSA (AgAb) were prepared by the method of Reid (1971). The equivalence point was determined by mixing, in equal volumes, doubling dilutions of either rabbit anti-BSA IgG (16.4 mg/ml) or rabbit anti-BSA IgG F(ab')2 (9.6 mg/ml) in Veronal buffered saline (VBS), pH 7.5 with BSA (130 μg per ml); the mixtures were incubated at 37°C 1 h and then 4°C for 18 h. At equivalence the antibody concentration was 2.4 mg/ml for IgG F(ab')2 and 4.1 mg/ml for IgG. For labeling experiments, 4-fold antibody excess was used in order to avoid precipitation of the AgAb complexes. NHS (1 : 2 in VBS) was added to an

equal volume of AgAb complex, and incubated for 60 min at 37°C, thus forming BSA—anti-BSA-C (AgAbC).

Aggregated human IgG (AggIgG) was prepared by incubation at 63°C for 20 min and centrifugation at 1000 *g* for 30 min at 4°C prior to use.

Estimation of complement fixation

0.5 ml of NHS was added to 0.375 ml of preformed immune complexes, and incubated at 37°C for 1 h. The tubes were then centrifuged at 5000 *g* for 30 min at 4°C. The number of residual CH50 units in the supernatants was determined by the method of Kabat and Mayer (1961), and compared with NHS incubated without immune complexes.

Cells

Human peripheral blood mononuclear cells were separated from venous blood on a Ficoll—Hypaque gradient as previously described (Deegan, 1976). Sheep red blood cells (SRBC) in Alsever's solution were purchased from BBL (Cockeysville, MD).

Immunofluorescence

One hundred μl of fluorescein-conjugated complexes (480 μg of F(ab')2 complex or 820 μg of IgG complex) were added to 100 μl of mononuclear cells (2×10^5 cells) and incubated for 30 min at room temperature. The cells were washed in cold VBS and centrifuged at 200 *g*. The cells were examined with a Leitz Ortholux II microscope equipped with epifluorescence (selective FITC excitation) and phase illumination. A minimum of 200 cells was counted and the percentage of positive cells with membrane fluorescence was determined. Blocking studies were carried out by preincubating the cells with an equal volume of AggIgG (1 mg/ml) for 30 min at room temperature, washing three times in cold VBS and incubating with AgAbC as above.

The presence of cell-bound human C3 or C4 on mononuclear cells was determined. The cells were incubated with AgAbC containing unlabeled BSA, washed 3 times, and incubated for 30 min at room temperature with 100 μl of either FITC conjugated anti-human C3, or anti-human C4. The cells were washed and examined for membrane fluorescence.

Rosette assay

Sheep RBC (SRBC) sensitized with rabbit anti-SRBC (IgM fraction) and mouse complement (IgMEAC-Mo) were prepared as previously described (Deegan, 1976). NHS (1 : 100) was used to prepare IgMEAC-Hu. Equal volumes of IgMEAC (10^8 cells/ml) and mononuclear cells (2×10^5 cells) were incubated at 37°C for 30 min. After gentle resuspension, 200 cells were counted. Those cells binding 3 or more reagent red cells were recorded as positive. As a control, complement was omitted from the sensitization procedure and the reagent cells (IgMEA) were incubated with mononuclear cells as above. Binding of IgMEA to mononuclear cells was never observed.

Conditions which might have permitted T cell (E) rosette formation (i.e., centrifugation and cold incubation) were avoided.

Blocking of rosette formation by BSA-F(ab')2-C

The concentration of immune complexes required to prevent subsequent rosette (IgMEAC) formation on a standard concentration of mononuclear cells was determined. Fifty μl of mononuclear cells (10^5 cells) were added to doubling dilutions of 375 μl of the BSA-F(ab')2-C complexes and incubated for 30 min at room temperature. Unbound immune complexes were removed by 3 washes in cold VBS. The cells were incubated with 50 μl of IgMEAC (10^8 cells/ml) for 30 min at 37°C. Both IgMEAC-Mo and IgMEAC-Hu were tested.

RESULTS

Complement fixation

Experiments were carried out to confirm the ability of rabbit IgG F(ab')2 to fix human complement. The normal human serum used in these assays contained 128 CH50 units per ml. Following incubation with preformed BSA-F(ab')2 complexes the serum contained 64 CH50 units per ml, indicating 50% of the hemolytic complement had been fixed by the preformed immune complexes.

Blocking of complement receptors by BSA-F(ab')2-C

AgAbC (BSA-F(ab')2-C) inhibited rosette formation at dilutions less than 1 : 32 (>60 μg complex added) (fig. 1). This inhibitory activity declined precipitously at 1 : 32, and was not detected at lower concentrations of AgAbC. The concentration of AgAbC used in subsequent experiments was 480 μg/2 × 10^5 cells which was well within the range of maximum blocking of complement receptor sites (fig. 1).

Binding of BSA-F(ab')2-C to complement receptors

The identification of complement receptor cells with fluoresceinated AgAbC is demonstrated in fig. 2. This method is compared with the IgMEAC-Mo rosette assay using peripheral blood mononuclear cells from a number of normal volunteers (fig. 3). The straight line was determined by linear regression analysis using the least-squares method and has a slope of 1.168 and y-intercept of −0.089. Although the two methods demonstrated a relatively good correlation (r 0.78), it was noted that the percent binding with the fluorescence method was generally higher than the corresponding rosette assay. A paired t-test indicated a statistically significant difference ($P < 0.001$) between the two techniques.

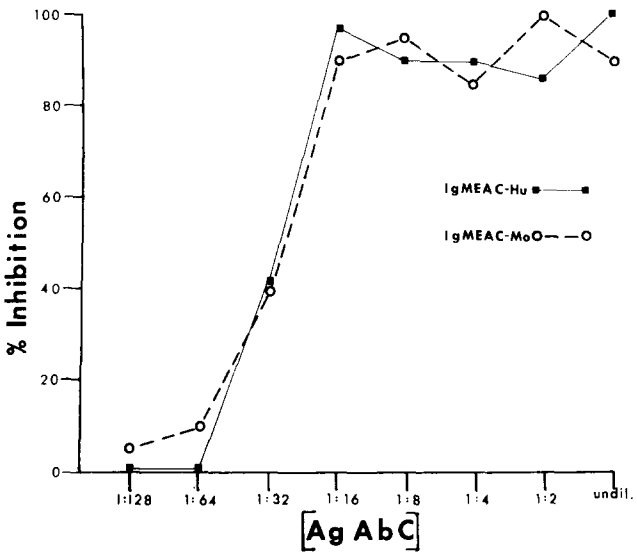

Fig. 1. Inhibition of IgMEAC rosettes by BSA-F(ab')2-C (AgAbC). Each point represents the mean of duplicates, and all are from the same donor. Control values were 12% for IgMEAC-Mo and 15% for IgMEAC-Hu. Undiluted AgAbC contained 1.80 mg of complex in 375 μl.

Specificity of BSA-F(ab')2-C for complement receptors

Studies were undertaken to demonstrate that binding observed with BSA-F(ab')2-C was specific for complement receptors and not due to Fc receptor activity. The binding of BSA-F(ab')2-C was compared with com-

TABLE 1

Specificity of immune complex detection of complement receptors *.

1st Incubation	2nd Incubation	% Positive
AgAbc (intact IgG)	None	20.0
AgAbC (F(ab')2)	None	17.5
AggIgG	AgAbC (Intact IgG)	15.0
AggIgG	AgAbC (F(ab')2)	17.0
AgAbC (intact IgG)	Anti-C3-FITC	20.0
AgAbC (intact IgG)	Anti-C4-FITC	9.5
AgAbC (F(ab')2)	Anti-C3-FITC	13.5
AgAbC (F(ab')2)	Anti-C4-FITC	0

* First incubations were performed at room temperature for 30 min. The cells were subsequently washed three times in cold VBS, incubated with the second set of reagents, washed and examined for fluorescence.

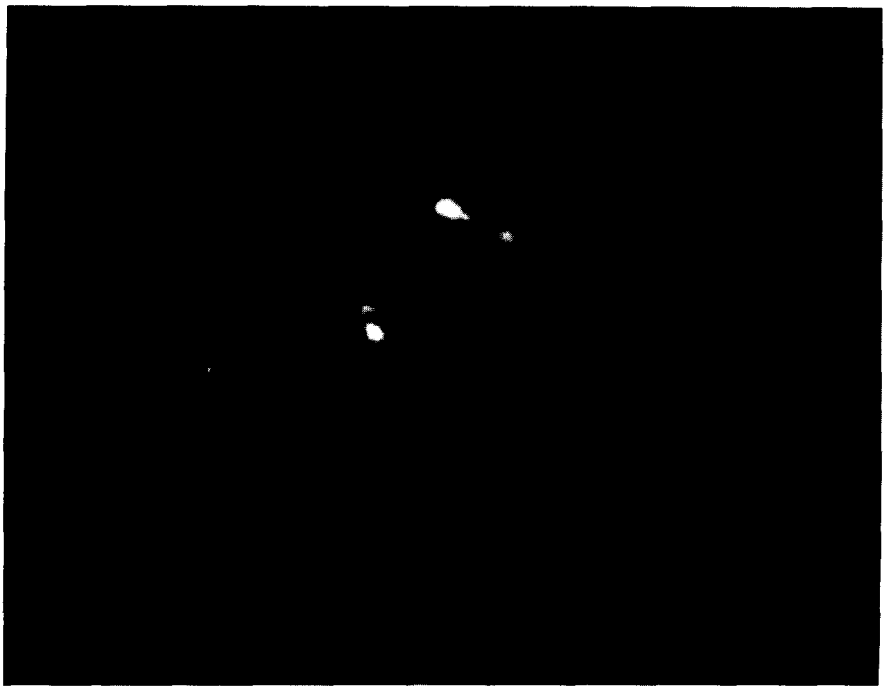

Fig. 2. Fluorescence of BSA-F(ab')2-C (AgAbC) which is bound to a human peripheral blood lymphocyte. Punctate fluorescent complexes are present at the cell membrane. Ultraviolet illumination, × 540.

plexes prepared with intact IgG. AgAbC containing intact IgG showed slightly higher binding than did BSA-F(ab')2-C (table 1). However, one-fourth of the binding seen with intact IgG could be inhibited by prior incubation of the cells with AggIgG for 30 min at room temperature (20% vs 15%). BSA-F(ab')2-C binding was not inhibited by AggIgG (table 1). If complement was not added to the complexes, or if heated complement was substituted for fresh serum, only 1% binding occurred.

Pathway of complement activation

Since C4 does not participate in the alternate pathway, whereas both C3 and C4 are activated in the classic pathway, these two pathways can be distinguished by the presence of C4 (Osler, 1976). Using immunofluorescence, C3, but not C4, was detected on the surface of cells preincubated with BSA-F(ab')2-C (table 1). Both C3 and C4 were present on those cells preincubated with AgAbC prepared with intact IgG. The percentage of cells binding complexes was comparable with anti-C3 and fluoresceinated AgAbC (table 1).

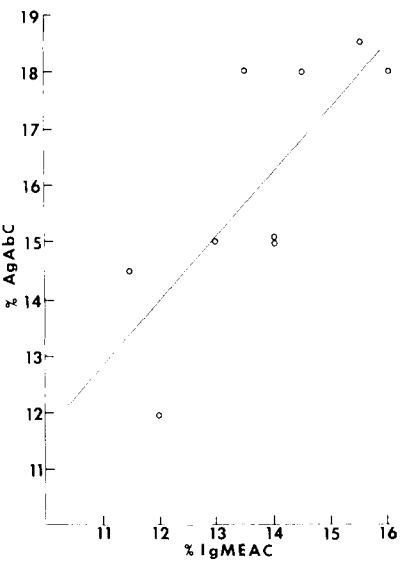

Fig. 3. Comparison of fluorescent BSA-F(ab′)2-C (AgAbC) and rosette (IgMEAC) assays on normal peripheral blood lymphocytes. The percentage of cells binding either AgAbC or IgMEAC from normal donors is indicated by the circles. Each point represents the mean of duplicates. The straight line was determined by the least-squares method for linear regression; slope = 1.168, y-intercept = —0.089, and r = 0.78. Mean ± 1 S.D. for IgMEAC = 13.78 ± 1.40 and for AgAbC = 16.0 ± 2.09, $P < 0.001$.

DISCUSSION

We have described an immunofluorescent technique for the specific identification of complement receptors on human peripheral blood mononuclear cells. Although this method correlates well with the IgMEAC rosette assay (r 0.78) the percentage of cells binding BSA-F(ab′)2-C is significantly higher than the percentage binding IgMEAC ($P < 0.001$). This difference is not due to Fc receptor binding of BSA-F(ab′)2-C, since binding was not reduced by preincubation of the cells with AggIgG, which blocks Fc receptor sites. The increased number of positive cells observed with BSA-F(ab′)2-C indicates this technique is more sensitive than the rosette assay. The smaller size of the complexes in the fluorescence method may account for the increased sensitivity. Soluble reagents have been observed to be more sensitive than particulate complexes (rosettes) in the detection of complement receptors on certain lymphoblastoid cell lines (Theofilopoulos, 1974b).

Rabbit IgG F(ab′)2 has been previously shown to fix guinea pig complement via the alternate pathway (Reid, 1971). In the present study C3 but not C4 was present in the cell bound complexes, suggesting that rabbit IgG F(ab′)2 fixes human complement by the alternate pathway.

Previously, complement receptors have been detected by soluble immune

complexes prepared with whole, intact IgG (Dukor, 1973; Eden, 1973; Theofilopoulos, 1974b). Although complement receptors are identified by these methods, there appears to be some binding to Fc receptors by IgG, since the binding can be partially inhibited by AggIgG (fig. 4). Conversely, AggIgG does not affect the binding of BSA-F(ab')2-C. In addition, only negligible binding of BSA-F(ab')2 occurred when complement was either deleted or heat inactivated, indicating that activated complement is required for binding to lymphocytes (fig. 5). This requirement for complement demonstrates: (1) that binding is specific for complement receptors and (2) attachment to mononuclear cells is *not* mediated by rabbit anti-human antibodies which might be present in the anti-serum. At high concentrations the soluble BSA-F(ab')2-C complexes were able to inhibit subsequent binding of IgMEAC to mononuclear cells, suggesting that both the soluble complexes and IgMEAC bind to the same receptor. Results from these studies indicate that the use of immune complexes containing F(ab')2 appears to be a sensitive and specific method for the detection of complement receptors on peripheral blood mononuclear cells.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Institute of General Medical Sciences (NIH-GM-15419-10) and the University of Michigan Cancer Research Institute.

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