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Receptors for IgM on human lymphocytes

III. SPECIFICITY OF RECEPTORS

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SUMMARY

Receptors for the Fc portion of IgM (RFcμ) can be detected on a large percentage of human peripheral lymphocytes. To approach an understanding of the function of these receptors we have examined the relative efficiencies of different preparations of human IgM and their $F(c)_5\mu$ fragments in binding to RFcμ. This was measured by blocking rosette formation between lymphocytes and IgM-sensitized ox erythrocytes (EAμ). IgM from pooled normal human serum completed blocked formation of IgM rosettes (EAμ-RFC) at much lower concentrations than IgM preparations from individual Waldenström's macroglobulinaemic patients (WM). The differences in effectiveness of these IgM preparations in inhibiting EAμ-RFC suggested the existence of receptors for subclasses of IgM on different subpopulations of human lymphocytes. $F(c)_5\mu$ fragments of IgM were more effective in blocking EAμ-RFC than the parent molecule, which was in turn more efficient than the 8S subunit. These results indicate that $F(c)_5\mu$ may have more Fc regions exposed per molecule for binding to the matrix of RFcμ than pentameric IgM and that Fc multivalency may be important for the stable binding of IgM to RFcμ.

INTRODUCTION

A large percentage of human peripheral lymphocytes display receptors for the Fc portion of IgM (RFcμ) (Moretta *et al.*, 1975; McConnell & Hurd, 1976; Fanger & Lydyard, 1979). Although primarily associated with a subpopulation of T cells in human peripheral blood, RFcμ have also been found on a small subpopulation of non-T cells (Fanger & Lydyard, 1979; Pickler & Knapp, 1977; Ferrarini *et al.*, 1977). Human T lymphocytes expressing this receptor have been implicated as helper cells for pokeweed mitogen (PWM) induced differentiation of B cells into plasma cells (Moretta *et al.*, 1977). Other data on the modulation of this receptor by antigen *in vivo* (Birch, Bernier & Fanger, 1979) and by T cell mitogens *in vitro* (Lydyard & Fanger, 1979) seem consistent with a regulatory role for RFcμ-bearing lymphocytes in the immune response.

Previous workers have found that detection of RFcμ required overnight incubation of the lymphocytes under rather stringent conditions (Moretta *et al.*, 1975). Using a slightly different assay procedure, however, it has been possible to detect RFcμ on freshly isolated lymphocytes (Fanger & Lydyard, 1979). Studies on the specificity of these receptors (Ferrarini *et al.*, 1976) have primarily used pentameric IgM, although monomeric IgM from a patient with an 8S monoclonal spike has been reported to bind RFcμ with the same efficiency as the pentameric molecule (Preud'homme *et al.*, 1977). It has also been reported that IgM binds to RFcμ through sites in its C4 domain (Conradie & Bubb, 1977).

Some evidence has been obtained which suggests that the RFcμ-bearing subpopulation may be further divided on the basis of the different properties expressed by cells within this group (Lydyard & Fanger, 1979; Hayward *et al.*, 1978). Although no discriminatory cell surface markers have so far been noted, it is

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possible that differences within the RFcμ-bearing cells may be related to the specificity of their receptors for IgM. At present, it is unclear whether there is more than one class of receptor for IgM. Moreover, it would seem important for an understanding of the function of this receptor to determine the relative efficiencies of pentameric IgM, its 8S subunit and its $F(c)_5\mu$ fragment in binding RFcμ. Our approach has been to compare IgM preparations isolated from normal human serum and from several different Waldenström sera for their ability to interfere with the interaction of IgM-sensitized indicator cells (EAμ) and lymphocytes with RFcμ. Using this approach we have obtained evidence which suggests the existence of different classes of receptor for IgM on human peripheral lymphocytes and thus the existence of distinct RFcμ phenotypes potentially related to functionally distinct subpopulations.

MATERIALS AND METHODS

Lymphocyte preparation. Peripheral blood from healthy adults was defibrinated and the lymphocytes obtained by gradient centrifugation on Ficoll–Hypaque (Fanger & Lydyard, 1979). Cells were washed in RPMI 1640 containing 10% foetal calf serum (FCS) and used after overnight incubation at 37°C in medium 199 containing 20% FCS.

Blocking of IgM rosette formation by IgM and its derivatives. The IgM fraction of rabbit antisera raised against ox red cells (ORBC) was used in sub-agglutinating concentrations to coat ORBC (Fanger & Lydyard, 1979). Rosettes were made by mixing lymphocytes with IgM-sensitized ORBC at a ratio of 50–100 to 1, and centrifuging at 150 ***g*** for 7 min. For blocking studies, IgM (25 μl) was mixed with the lymphocytes (25×10^3 cells, 25 μl) and incubated for 30 min at 37°C. This mixture was then cooled to 4°C prior to the addition of IgM-sensitized ORBC and centrifugation (at 150***g*** for 7 min). Rosettes were gently resuspended and counted 60 min after incubation on ice. Lymphocytes with more than three attached ORBC were counted as positive.

Preparation of human IgM and its $F(c)_5\mu$ and 8S derivatives. IgM was isolated from the sera of five patients with Waldenström's macroglobulinaemia (WM, Mo-, Br-, Ca-, Cat- and Go-) and a pool of normal serum, as previously described (Fanger & Lydyard, 1979). Briefly, euglobulin fractions were subjected to Pevicon block electrophoresis and separated on a Sepharose CL-6B column equilibrated in 0·1 M Tris-HCl buffer, pH 8·6, containing 0·25 M NcCl (TSB). The peak IgM containing fractions were pooled, concentrated and dialysed against phosphate-buffered saline (PBS). IgM preparations were shown to be free of IgG by double diffusion analysis using rabbit anti-human heavy chain specific antiserum (Wellcome Reagents Ltd., Beckenham, Kent). Determination of IgM concentration was by quantitative radial immunodiffusion (Miles Yeda, Indiana), using standards provided by the WHO.

Heat-aggregated IgM(agg-IgM) was prepared by incubating IgM (8 mg/ml) for 5 min at 63°C. That significant aggregation had occurred was shown by the elution profile of these preparations on Sepharose CL-6B. A large percentage of the agg-IgM was found to elute at the void volume of the column, in contrast to the IgM before aggregation which was free of such material.

$F(c)_5\mu$ fragments of IgM were prepared by the method of Zikan & Bennett (1973) as described previously (Fanger & Lydyard, 1979). Briefly, IgM was dialysed against 0·005 M Tris-HCl, 0·005 M $CaCl_2$, pH 8·0 and pre-warmed to 56°C. Trypsin (Sigma, St. Louis, Missouri) was added to give a final substrate to enzyme ratio of 50 : 1 and the mixture was incubated at 56°C. An equivalent amount of soybean trypsin inhibitor (Sigma) was added after 1 hr and the sample cooled to 4°C. The digests were separated on Sepharose CL-6B equilibrated in TSB. All of the eluted fractions reactive with anti-μ by Ouchterlony analysis were pooled, concentrated and dialysed against PBS. The concentration of $F(c)_5\mu$ fragments was determined by calculation from quantitative amino acid analysis on a Beckman Model 199 amino acid analyser assuming a molecular weight of 340,000 for $F(c)_5\mu$ (Zikan & Bennett, 1973).

The 8S monomeric subunit of IgM was prepared by reduction of the pentamer with 0·03 M cysteine, pH 8·2 at 37°C. After 90 min, a two-fold molar excess of iodoacetamide was added (British Drug House, Poole, Dorset). The mixture was incubated for a further 60 min at 37°C and fractionated on Sepharose CL-6B equilibrated with TSB. Using double diffusion analysis with anti-μ antiserum, reduced and alkylated IgM protein was found to have eluted as a single symmetrical peak at a volume slightly in excess of that for IgG. The concentration of 8S IgM was determined assuming the same extinction coefficient as for the parent IgM from which it was derived.

RESULTS

Blocking of IgM rosettes by different preparations of human IgM

The ability of IgM preparations obtained from normal human serum and from five different patients with macroglobulinaemia to inhibit the formation of rosettes between EAμ and human peripheral lymphocytes is shown in Fig. 1. Although the same procedure was used in the isolation of each of these IgMs, the curves of the percentage of rosette-forming cells (RFC) as a function of the concentration of IgM used for blocking are clearly not all identical. In particular, IgM obtained from normal human serum

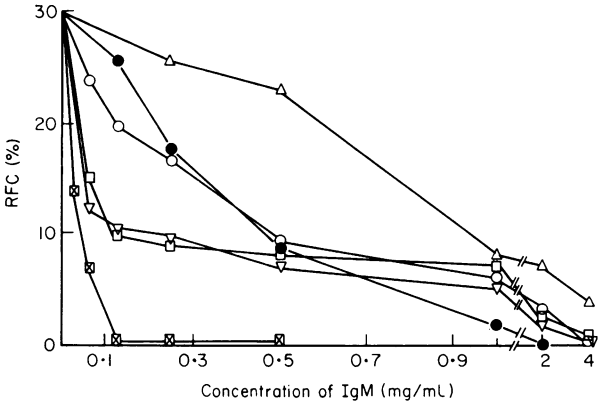

FIG. 1. The percentage of lymphocytes forming rosettes with EAμ in the presence of various concentrations of different human IgM preparations. IgM (25 μl) was incubated with lymphocytes (25×10^3, in 25 μl) for 30 min at 37°C. The mixture was cooled to 4°C and EAμ indicator cells (25 μl) were added. The cells were centrifuged in the cold for 7 min at 150 *g* and then incubated for 1 hr at 4°C before counting. Each point represents the average percentage of lymphocytes forming rosettes with EAμ in duplicate tubes. Deviation from the mean was generally less than ±3%. (△) Br-IgM; (●) Cat-IgM; (○) Mo-IgM; (□) Ca-IgM; (▽) Go-IgM; (⊠) NHu-IgM.

(NHu-IgM) completely blocked IgM rosette formation at concentrations less than one tenth of those required of IgMs from patients with macroglobulinaemia. Moreover, there were considerable differences in the ability of IgM from different WM patients to block rosette formation even at concentrations as high as 4 mg/ml. At 0·5 mg/ml, for example, NHu-IgM completely blocked EAμ-RFcμ interactions whereas Br-IgM decreased rosette formation by about 20%, and all the other WMIgM preparations gave 65% inhibition.

Although the data presented in Fig. 1 represent information on the blocking of IgM rosette formation using lymphocytes from one individual, similar patterns of blocking were obtained using these same preparations and lymphocytes from other individuals. The consistent features of these patterns are that: (1) NHu-IgM always blocked rosettes at very much lower concentrations than those required for IgM from patients with macroglobulinaemia; (2) IgM from some patients with WM are more efficient inhibitors than others; (3) for certain IgM preparations (in Fig. 1, Ca-, Cat- and Go-IgM), about 30% of the lymphocytes bearing receptors for IgM can only be blocked at very high concentrations of IgM (2–4 mg/ml), whereas approximately 60% can be blocked by low concentrations of these same preparations (0·1 mg/ml).

It seemed possible that differences in the ability of the various IgM preparations to block IgM rosettes may have been related to differences in their quantities of IgM aggregates, i.e. preparations of IgM with more aggregates might be more or less avid in their interaction with RFcμ than pentameric IgM. Major contamination by IgM aggregates seemed unlikely since care was taken to obtain IgM fractions from each of the sera which eluted at the same volume from Sepharose CL-6B. These fractions were well separated from the larger molecular weight fractions eluted at the void volume of the column and which probably did contain IgM aggregates. Moreover, these fractions were refiltered on Sepharose CL-6B to ensure removal of material eluting at the void volume of the column. Even so, it seemed important to determine to what extent non-specific aggregation could influence the ability of a macroglobulinaemic IgM to inhibit IgM rosettes. IgM preparations were therefore heat-aggregated and compared with their non-aggregated counterparts in their ability to block RFcμ. Fig. 2 shows a representative experiment in which Mo-IgM and agg-Mo-IgM were compared. Clearly, heat aggregation neither enhanced nor decreased the ability of this IgM (or the others tested, data not shown) to block IgM rosettes.

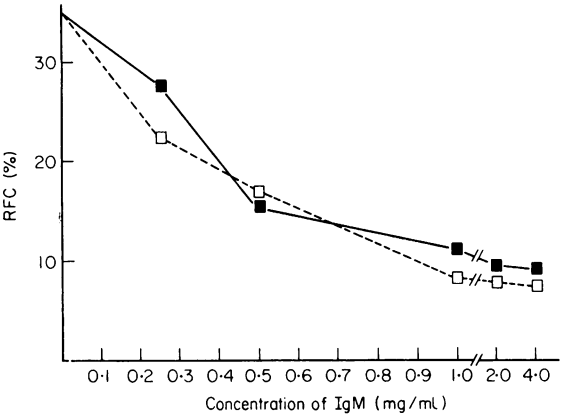

FIG. 2. Comparison of the ability of IgM and its heat-aggregated derivative to inhibit rosette formation between EAμ and lymphocytes. The percentage of lymphocytes forming IgM rosettes in the presence of Mo-IgM (□——□) or heat-aggregated Mo-IgM (■——■) was determined as described in Fig. 1 and in the Materials and Methods section.

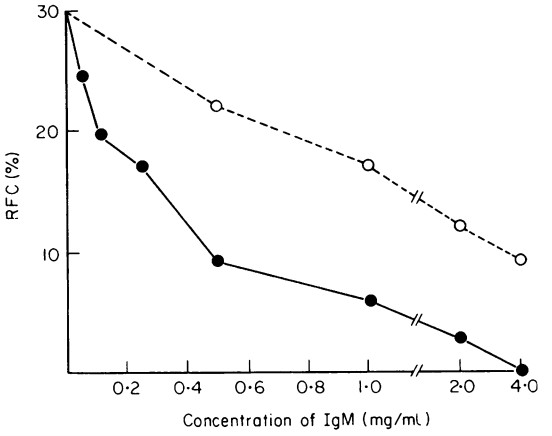

FIG. 3. The percentage of lymphocytes forming IgM rosettes in the presence of Mo-IgM or its 8S subunit. Conditions for blocking and assay were the same as those described in Fig. 1 and in the Materials and Methods section. Mo-IgM (●——●); 8S subunit of Mo-IgM (○ — — ○).

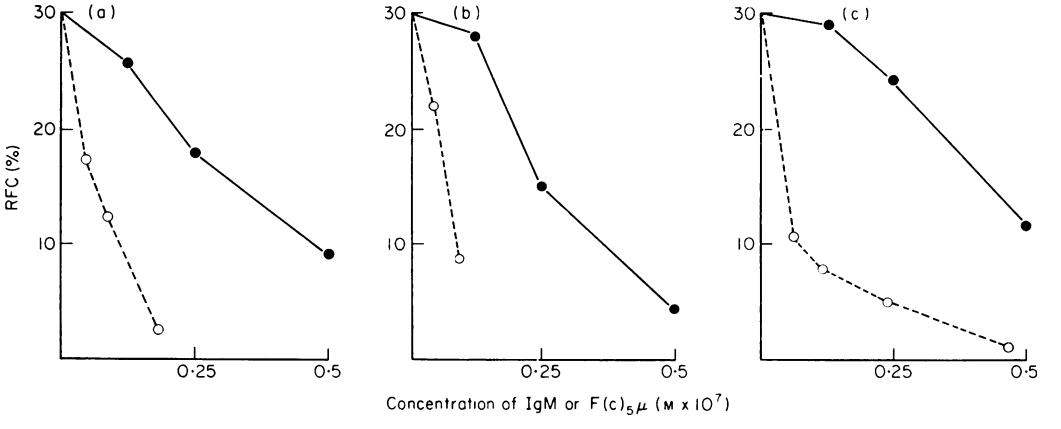

FIG. 4. The percentage of lymphocytes forming IgM rosettes in the presence of various IgMs and their respective F(c)$_5$μ fragments. Conditions for blocking and assay were the same as those described in Fig. 1. (a), Cat-IgM (●——●), Cat-IgM F(c)$_5$μ (○ — — ○); (b), Go-IgM (●——●), Go-IgM F(c)$_5$μ (○ — — ○); (c), Mo-IgM (●——●), Mo-IgM F(c)$_5$μ (○ — — ○). Molar concentrations have been used to permit a direct comparison on a per molecule basis of the IgM rosette blocking ability of IgM and its F(c)$_5$μ derivative.

Blocking of IgM rosettes by 8S and $F(c)_5\mu$ derivatives of IgM

IgM was found to be much more efficient per mg in blocking rosette formation than its reduced and alkylated 8S derivative (Fig. 3). Nevertheless, interference with IgM rosetting by the 8S subunit of IgM indicates that it can bind to RFcμ, although less well than its pentameric parent molecule.

Fig. 4 shows the relative abilities of WM IgMs and their $F(c)_5\mu$ fragments to decrease rosette formation. All of the $F(c)_5\mu$ fragments blocked IgM rosette formation at much lower concentrations than the parent WM IgMs from which they were derived. It would seem, therefore, that $F(c)_5\mu$ had a higher avidity per molecule for RFcμ than IgM

DISCUSSION

Distinct differences were noted in the ability of IgM preparations from different patients with macroglobulinaemia to block IgM rosette formation. Furthermore, NHu-IgM was a much better inhibitor of IgM rosette formation than any of the WM IgM preparations studied. On the basis of the percentages of RFC obtained in the presence of IgM at 0·2 mg/ml, it is possible to distinguish three different groups of IgM: NHu-IgM (no rosettes), Go- and Ca-IgM (9% RFC) and Cat-, Mo- and Br-IgM (18–26% RFC). Perhaps more interesting was the finding of a plateau value of RFC, corresponding to approximately 30% of the total IgM-rosetting cells, which could only be blocked at very high concentration of WM IgM (Ca-, Cat- and Go-IgM). Although it is possible that the differences observed might have been the result of different degrees of contamination of these preparations by aggregates, a great deal of effort was directed toward obtaining IgM preparations free of aggregated IgM. Moreover, studies to determine the effect of non-specific aggregation on blocking ability of the IgMs (Fig. 2) suggested that contamination of these preparations by aggregates would not readily explain the differences. Even so, it was possible that small percentages of antigen–IgM antibody complexes were present in different quantities in these preparations, and that these complexes were more efficient at blocking IgM rosettes than pentameric or heat-aggregated IgM. Such a possibility is difficult to evaluate since as little as 1% contamination by IgM complexes, a level difficult to detect by our procedures, might be important. Were this the case, however, it would be difficult to explain why NHu-IgM is ten to twenty times a better inhibitor than WMIgM and yet, as indicated by filtration on Sepharose CL-6B, clearly does not contain 20% contamination by aggregates or complexes. Although this evidence does not rule out the possibility that contamination by aggregated IgM may partly explain the results presented in Fig. 1, it does cast some doubt on this possibility and permits a consideration of some other more interesting explanations.

One explanation consistent with these results is that there is more than one kind of RFcμ expressed on human peripheral lymphocytes and that these RFcμ differ in their specificity and/or avidity for IgM. This possibility also implies that there are different subclasses of IgM all of which are present in normal human serum but are present in much smaller quantities (relative to the total IgM) in the IgM fraction of serum from patients with macroglobulinaemia. Considering that the IgM concentration in the serum of patients with WM was twenty to fifty times that in normal serum and that complete inhibition of IgM rosettes (Fig. 1) required rather high concentrations of this IgM (2–4 mg/ml), it is possible that inhibition at these concentrations is mediated by small quantities of the much more efficient normal IgM contaminating these WM IgM preparations.

On this basis, and interpreting the data in the most straightforward way, the existence of two different RFcμ would seem likely, each present on a different subpopulation of cells. In this interpretation, one third of RFcμ cells have an RFcμ different from the others in specificity and/or avidity. Blocking of these receptors is accomplished by small quantities of NHu-IgM, but very much larger quantities of WM IgM (perhaps contaminating NHu-IgM). The other two-thirds of the cells appear to have RFcμ that are inhibited almost equally well by NHu-IgM and at least some preparations of IgM obtained from patients with macroglobulinaemia. In this context, it seems relevant to note that other workers have suggested that there is some degree of heterogeneity both in terms of subclass specificity and avidity among Fc receptors for IgG on different cell types (Heusser, Anderson & Gray, 1977, Arbeit, Henkart & Dickler, 1977).

Considering the range of effectiveness of the various IgM preparations, it is also possible to interpret the data as suggesting more than two different RFcμ-bearing populations. This seems premature, however, without further characterization of the various IgM preparations, especially regarding their classification into subclasses. In this context, there has been a report on the existence of two types of human IgM differing in their ability to fix complement (Mackenzie *et al.*, 1969). Nevertheless, it does appear that RFcμ bearing cells can be classified into at least two groups on the basis of the inhibition of their formation of IgM rosettes by NHu-IgM and WM IgM. It should be emphasized that IgG does not inhibit these rosettes and that the NHu-IgM preparation is free of IgG (Fanger & Lydyard, 1979).

It has previously been shown that IgM binds to RFcμ through its Fc region (Playfair, 1974) and, in particular, through its C4 domain (Conradie & Bubb, 1977). In the present study, the relative ability of IgM and its $F(c)_5\mu$ fragment to bind RFcμ was evaluated. Our results suggest that, per molecule, the $F(c)_5\mu$ fragment of IgM is much more efficient in blocking rosettes than the IgM from which it was derived. Why this should be the case is not clear, but it is consistent with the observation that Fc fragments of IgM fix complement more efficiently than intact IgM (Plant, Cohen & Tomasi, 1972). One possibility which might explain this difference is that $F(c)_5\mu$ may have more Fc regions per molecule available for binding to the matrix of RFcμ on a lymphocyte than pentameric IgM. Stereochemical blocking of access to Fc domains in pentameric IgM may be produced by $F(ab)_2$ arms which are normally in the plane of the $F(c)_5\mu$ dish. It is tempting to imagine that under appropriate conditions antigen–IgM antibody complexes may form in which the $F(ab)_2$ arms fold out of the plane of the central dish and so permit an exposure of Fc regions analogous to that suggested for $F(c)_5\mu$ fragments of IgM. In contrast to our findings that $F(c)_5\mu$ was more effective than pentameric IgM in blocking IgM rosettes, a recent study (Bubb & Conradie, 1978) has suggested that the reverse is true. It is difficult to make a direct comparison with their results, since these workers used only one WM IgM preparation for the whole study and employed a different method of blocking IgM rosettes.

Monomeric Fcμ might be expected to bind less avidly to RFcμ than the pentameric unit, since the avidity of $F(c)_5\mu$ may be enhanced by its multivalent interactions with RFcμ. This is confirmed by the data on the IgM rosette blocking ability of the 8S subunit of IgM prepared by mild reduction and alkylation. This subunit is much less efficient at blocking IgM rosettes than the IgM from which it was derived. The relative effectiveness of the IgM monomer and pentameric IgM in blocking IgM rosette formation is consistent with our previous findings on the importance of the degree of sensitization on detection of RFcμ-bearing lymphocytes (Fanger & Lydyard, 1979). These studies suggested that the more IgM (and thus Fc region) on the indicator cell, the greater is its avidity for cells bearing receptors for IgM. In contrast, others have found that native IgM monomer obtained from the serum of a patient with WM was as good, or better, than pentameric IgM in blocking rosettes (Preud'homme *et al.*, 1977). Although this seems inconsistent with our data on reduced and alkylated IgM monomer, it may be that there is a conformation difference between native IgM monomer and monomer produced by reduction and alkylation.

It would be of value to be able to define subpopulations of RFcμ-bearing cells and to develop method for their isolation and study. The data presented in this paper suggest that such subpopulations exist and may be obtained by rosetting in the presence of certain concentrations of WM IgM. With these isolated subpopulations we may be able to approach a better understanding of their functions, especially as they relate to help and suppression in the humoral immune response.

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