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Comparison of various tests for Fc receptors on different human lymphocyte sub populations

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SUMMARY

Six different immune complex test systems for the detection of IgG Fc receptors were applied to the study of various human lymphocyte populations. The extent of binding varied widely according to the system and the cell type employed. Two systems bound preferentially to a high proportion of B lymphocytes from peripheral blood or tonsils, one of which bound with only a very few T cells. In contrast, four other test systems which bound well with the Fc receptors on T lymphocytes gave weaker reactions with Fc receptors on B cells. The reactivity of Fc receptors on null or third population lymphocytes was similar to that of the Fc-positive T cells. Pronase digestion experiments showed a graded selective loss of reactivity with the different Fc reagents. No one system was optimal for all of the lymphocyte populations, although aggregated IgG exhibited the broadest spectrum of reactivity. A pronounced effect of temperature was evident on the binding reactions, and native IgG showed strong binding at 4°C, particularly to the Fc receptors on T cells.

INTRODUCTION

Surface Ig and Ia determinants are characteristic of B lymphocytes, but the IgG Fc receptor has been reported to be present on B cells (Dickler & Kunkel, 1972), a minor population of T cells (Grey, Kubo & Cerrotini, 1972; Winchester *et al.*, 1975; Ferrarini *et al.*, 1975) and on lymphocytes found in a residual or 'third' population that lack specific B or T markers (Frøland & Natvig, 1973). But the literature on Fc receptors is confusing. For example, although Fc receptors were demonstrated by means of aggregated gammaglobulins on the vast majority of B cells in the initial report (Dickler & Kunkel, 1972), others have concluded that B cells entirely lack them (Frøland, Natvig & Michaelsen, 1974) or have only low levels of weakly reactive receptors (Lobo & Horowitz, 1976; Gergely *et al.*, 1977; Winfield, Lobo & Hamilton, 1977; Samarut, Brochier & Revillard, 1976). Similarly, Fc receptors on T cells and on the residual or 'third' lymphocyte population have been the subject of divergent opinion.

We have, therefore, studied Fc receptors on B lymphocytes and other lymphocytes in blood and tonsils, and on leukaemic B lymphocytes. Six methods were used, including aggregated IgG (Dickler & Kunkel, 1972) soluble immune complexes (Dickler, 1974) or antibody coated erythrocytes (Hallberg, Gurner & Coombs, 1973). Extensive use was made of the Ia antigen because of its value as a B lymphocyte marker Hoffman *et al.*, 1977; Winchester *et al.*, 1976; Humphreys *et al.*, 1976; Ross *et al.*, 1978) that permitted a clearer definition of the other lymphocyte populations.

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MATERIALS AND METHODS

Antigens. Keyhole limpet haemocyanin (KLH) and crystalline ovalbumin (OA) were dissolved in phosphate-buffered saline (PBS) at 2 mg/ml. Rabbit-pooled IgG (fraction II) was stored at 10 mg/ml. $F(ab')_2$ fragments of pooled rabbit IgG IgG were prepared by pepsin digestion purified by gel filtration (Winchester, 1976) and used to immunize a sheep. A rabbit was immunized with freshly washed ox red cells. Molecules bearing Ia determinants were isolated from membranes of a B lymphoid cell line by detergent solubilization and used to immunize rabbits (Hoffman *et al.*, 1977). Pooled human IgG was obtained from Lederle Co., Pearl River, New York, as fraction II (Lot No.C-961). After dissolving in 0·15 M sodium chloride at 10 mg/ml, the solution was heated at 63°C for 10 min and the resulting aggregates partially purified by slowly adding sodium sulphate to a final molarity of 0·62. The precipitate was harvested by centrifugation and dialysed against PBS containing 0·02% sodium azide. The final concentration was adjusted to 0·5 mg/ml, 2% bovine serum albumin (BSA) was added, and it was stored at 4°C. Human serum 'Ripley' was obtained from Dr M. Waller of Richmond, Virginia. Fc fragments were prepared by papain digestion, gel filtration and electrophoresis.

Antisera processing. $F(ab')_2$ fragments of antibodies were prepared and whole IgG fraction were conjugated with fluorochromes as described previously.

The fluorescent reagents were absorbed with insolubilized proteins and cells as follows: Anti-OA, anti-KLH and anti-rabbit IgG with pooled human IgG, IgM, human O+ erythrocytes and pooled human B and T lymphoid line cells; anti-Ia with pooled IgG, IgM and T-cell lymphoid line cells; anti-IgG_1K with IgMK and $F(ab')_2$ of pooled human IgG. $F(ab')_2$ fluorescent reagents separately specific for IgD and IgM were used as a mixture (Winchester, 1976).

Cell preparation. The mononuclear cells isolated by Ficoll–Hypaque from blood or teased tonsils were labelled with latex to identify monocytes. E-rosette depleted and enriched fractions were prepared using sheep erythrocytes treated with vibrio cholera neuraminidase (En) and were dissociated by incubation at 37°C and vigorous resuspension, followed by Ficoll–Hypaque centrifugation (Moretta, Ferrarini & Cooper, 1978). Hypotonic lysis was avoided.

Rosettes. All rosette assays were performed by mixing 0·025 ml of cells at 2×10^6/ml with 0·025 ml of the erythrocyte suspension at 0·5% v/v, centrifuging at 50 *g* for 5 min and incubating 18 hr at 4°C. Suspension and enumeration was as described previously (Forni & Pernis, 1975). E_{OX} and E_h were sensitized according to Hallberg *et al.* (1973) and Frøland *et al.* (1974). E_h sensitized with Ripley serum were designated EA_H and the E_{OX} sensitized with rabbit antiserum EA_R.

Immunofluorescence. Cell-surface immunofluorescence was performed at 4°C using 5×10^5 mononuclear cells in 0·025 ml of PBS containing 2% BSA (PBS-BSA) (Winchester, 1976).

Lymphocytes binding aggregated IgG were detected by incubating them with 0·025 ml volumes of doubling dilutions of the IgG for 30 min. The cells were washed three times with 2 ml of ice-cold PBS-BSA and all but approximately 0·025 ml of the last wash discarded. The cells were suspended and 0·025 ml of the fluorescent anti-IgG reagent was added. This system was designated agg IgG. The anti-Ia reagent labelled with a contrasting fluorochome was added at this time in certain experiments.

Immune complex binding by lymphocytes was detected, using complexes of varying antigen–antibody ratios by adding 0·025 ml of an appropriate concentration of either whole anti-OA, anti-KLH, or anti-rabbit IgG and incubating this with the cells for 5 min at 4°C. The appropriate antigen was added and incubation continued at 4°C for 30 min. The samples were washed three times with 2 ml volumes of PBS-BSA at room temperature and examined by fluorescent microscopy. When double label experiments were preformed using $F(ab')_2$ anti-Ia reagents labelled with a contrasting fluorochrome, the anti-Ia reagent was added shortly after the addition of the antigen. Specificity controls were performed using $F(ab')_2$ fragments of anti-OA, anti-KLH, or anti-rabbit IgG. These systems are designated KLHR~KLH, OA-R~OA, and RIgG-S~RIgG.

Microprecipitin curves. Microprecipitin curves were constructed in 0·4 ml plastic microfuge tubes using the same proportions of fluorescent antibody and antigen in various dilutions. These were incubated at 4°C for 1 hr and centrifuged for 5 min. 0·005 ml of the supernate was removed by a lang-levy pipette and diluted ten-fold. 0·005 ml of the diluted supernate was mixed with 50–200 Sepharose 4B beads conjugated with the same antigen in order to determine the zone of antibody excess. The presence of fluorescence on the beads indicated unbound antibodies. Specificity controls included the use of an antigen unrelated to the fluorescent antibodies.

Pronase digestion. 1×10^7 mononuclear cells from patients with chronic lymphatic leukaemia or purified T cells from selected normal subjects were washed with RPMI 1640 and adjusted to a volume of 1 ml. Each sample was digested with 0, 0·5, 1·5, or 3·0 mg/ml pronase (Sigma Biochemical Co.) for 15 min at 37°C. The cells were washed three times with RPMI 1640 containing 20% foetal calf serum. Viabilities exceeded 95%.

RESULTS

Characterization of different immune complex systems

The influence of the proportion of antigen to a constant amount of antibody labelled with fluorochrome on the number of lymphocytes stained with immune complexes is illustrated in Table 1 for OA-R~OA immune complexes. Immune complexes formed in the presence of the lymphocytes bound

TABLE 1. Effect of varying amounts of ovalbumin antigen on demonstration of Fc receptors by a constant amount of fluorescent rabbit anti-ovalbumin antibody

Amount OA added (ng*)	Free R~OA detected in supernate†	Lymphocyte staining in separated populations from a single normal person (%)	
		Non-E-rosette	E-rosette-forming
500	0	0	4
250	tr	2	5
100	tr	12	10
50	+	25	10
25	+	22	14
12	+	9	7
6	+	3	1
0	+	0	0

* 50 μg of rabbit Ig containing anti-ovalbumin antibodies conjugated to fluorochrome (R~OA) were added to the lymphocytes in each tube prior to the addition of the OA.

† Determined in a parallel quantitative precipitin curve.

with a discrete zone of reactivity to both T cells (E-rosette-forming) and the non-E-rosette-forming cells that included B cells and those of the third population.

A parallel microprecipitin curve revealed that optimum reactivity occurred where the amount of OA was approximately one-tenth of that required for equivalence. At equivalence and slight antibody excess, gross precipitate was present among the cells, but very few cells bound these complexes. Addition of larger amounts of antigen, not illustrated in Table 1, did not result in improved binding. The pattern of staining progressively changed with further antibody excess to numerous fine particles. With antigen at one-hundredth of equivalence, the staining became weaker. No staining was obtained with antibody alone. $F(ab)_2$ fragments of antibody did not stain cells at up to three times the concentration of whole antibody, even with optimal antigen concentrations. In subsequent experiments, three concentrations of

TABLE 2. Comparison of Fc receptor reactivity of different lymphocyte populations with three different IgG-complex systems. Effect of varying reagent concentrations

	Detection system and separated lymphocyte population (% positive)						
	SLH-R~KLH		RIgG-S~IgG		Agg IgG		
Amount of antigen added (ng)	Non-E-rosette	E-rosette	Non-E-rosette	E-rosette	Non-E-rosette	E-rosette	Amount of IgG added (ng)
200	8	4	4	3	3	3	1000
100	12	2	9	1	14	6	500
50	13	3	24	1	39	9	250
25	30	5	36	1	78	10	125
12	32	9	66	1	89	10	60
6	29	10	71	1	47	9	30
3	20	5	68	1	38	8	15
0	1	1	1	1	1	1	0

TABLE 3. Detection of Fc receptors by different methods on E-RFC-enriched or depleted lymphocyte populations

Individual	Blood lymphocyte population	Agg IgG	RIgG-S~RIgG	KLH-R~KLH	OA-R~OA	EA_R	EA_{HU}
A	Non-E rosette	64	40	38	34	40	20
	E rosette	7	0	5	6	5	6
B	Non-E rosette	70	66	49	28	37	25
	E rosette	9	1	10	10	8	6
C	Non-E rosette	80	68	34	43	48	30
	E rosette	16	3	14	15	17	10
D	Non-E rosette	83	62	49	55	52	28
	E rosette	12	0	10	13	15	12
E	Non-E rosette	74	56	34	30	39	25
	E rosette	9	1	9	16	16	12
F	Non-E rosette	70	57	35	20	50	20
	E rosette	6	2	5	4	5	5
Average	Non-E rosette	74	58	34	33	46	25
	E rosette	10	1	9	11	11	10

antigen were used and the highest percentage obtained among these was taken as the measure of the number of cells with Fc receptors.

Contrasting reactivities for different lymphocyte populations

The proportion of T cells that bound KLH-R~KLH or agg IgG was comparable to that obtained with OA-R~OA as shown in Tables 2 and 3. In contrast, the RIgG-S~RIgG bound to significantly fewer T cells in the form of a few large agglomerates only at the highest concentrations of antigen used. The findings with the non-E-rosette-forming cells did not parallel those obtained with the T cells. Both RIgG~RIgG and agg IgG gave the highest levels of binding with the non-E-rosette-forming cells, detecting over twice as many cells in this fraction as did OA-R~OA and KLH-R~KLH. Thus, RIgG-S~RIgG was distinctive in that it yielded primary reactivity with the non-E-rosette-forming cell fraction, while OA-R~OA and KLH-R~KLH reacted relatively better with the T lymphocytes. Agg IgG bound effectively to both lymphocyte populations.

In order to define whether the rabbit IgG or sheep IgG Fc regions were the primary determinants of Fc-receptor binding, $F(ab)_2$ fragments of both constituents were used in different combinations. Less intense, but quantitatively similar, reaction was obtained with $F(ab)_2$ fragments of rabbit IgG used with whole sheep antibodies to rabbit IgG. No complexes bound when the sheep antibodies were used as $F(ab)_2$ fragments.

The region of optimum reactivity for the agg IgG is illustrated in Table 2. Higher quantities of agg IgG resulted in a reduction in the percentage of stained cells; further dilution yielded fewer staining particles per cell but maintained a similar percentage of positive cells.

Unlike the immune complex systems which depend on antigen–antibody proportion, the rosette assays with EA_h and EA_R varied little over a 100-fold range of sensitizing reagents. The results obtained resembled those of the OA and KLH immune-complex systems in that greater reactivity was found among the T cells. This was especially apparent with the EA_h system that uniformly gave the lowest level of Fc receptors detected on the non-E-rosette-forming population, in proportion to those detected on T cells.

Variation in Fc receptors on Ia positive and negative non-E-rosetting lymphocytes

Non-E-rosette-forming cells from six individuals were labelled with Ia antibodies at concentrations that did not inhibit and tested for Fc receptors (Table 4). The agg IgG system bound to 90% of the Ia-bearing cells and 54% of the Ia-negative cells. In contrast, the KLH and the OA systems bound to fewer cells with Ia determinants but to similar numbers of cells without. Because of interference, RIgG-S~

TABLE 4. Results of five Fc receptor tests on non-E-rosette-forming lymphocytes classified by Ia determinants into B cells and third population cells

	Percentage of peripheral blood lymphocytes in different subpopulations reacting with different Fc receptor systems			
	Non-E-rosette forming			
Test	Total cells detected	Ia-positive cells	Ia-negative cells	E-rosette-forming
Agg IgG	74	90	54	10
KLH-R~KLH	34	40	42	9
OA-R~OA	33	32	73	11
EA_R	46	38	58	11
EA_H	25	3	51	10

RIgG was not used. The EA_R rosette system was positive for 38% of both Ia-bearing lymphocytes, and Ia-negative cells. The EA_H rosette assay detected Fc receptors on only 3% of Ia-bearing lymphocytes and reacted principally with Fc receptors on cells lacking Ia determinants.

Temperature-dependent binding of 7S IgG to Fc receptors

Fresh serum, isolated normal and myeloma IgG bound to Fc receptors as shown by staining with a fluorescent anti-IgG reagent. This binding was markedly temperature-dependent and was obtained only when washing and incubation were done at 4°C. The number of positive T cells was equal to those obtained by EA_H or OA-R~OA. Ia-negative, non-E-rosette-forming cells also reacted strongly, while Ia-bearing B cells were stained weakly or not at all. Six chronic lymphatic leukaemic cell preparations containing over 90% of B lymphocytes also stained weakly at 4°C. Controls using BSA-PBS in place of the added IgG were entirely negative.

The same results were obtained using highly purified IgG Fc obtained from pooled human IgG. In studies using whole IgG incubated at 4°C but with washes done at 22°C the number of T cells binding IgG varied from essentially none to half those obtained with washing at 4°C. In contrast, the various immune-complex systems gave similar results under both conditions. Binding of aggregated IgG, EA_R and EA_h was only slightly less at 37°C than at 4°C, while no binding of 7S IgG or purified Fc was detected.

Detection of Fc receptors on tonsils and chronic lymphatic leukaemic lymphocytes

From 98 to 100% of tonsil cells either have Ia or form sheep E-rosettes (Table 5) and thus lack a 'third' population. More T-cell depleted tonsil lymphocytes bind, agg IgG and RIgG-S~RIgG than EA_H and EA_R rosette systems. (Table 5). However, staining was less bright than that of peripheral blood.

TABLE 5. Comparison of EA rosette and fluorescent assay detection of Fc receptors on tonsil lymphocytes depleted on E-rosette-forming cells

		Fc receptors detected (%)			
Tonsil	Ia-bearing cells (%)	Agg IgG	RIgG-S~RIgG	EA_R	EA_H
A	98	95	96	13	0
B	98	93	92	11	1
C	100	96	98	12	0
D	99	93	91	7	0
E	99	92	95	9	1

TABLE 6. Comparison of EA rosette and fluorescent assay detection of Fc receptors on lymphocytes from patients with chronic lymphatic leukaemia. Comparison of different methods for the detection of IgG Fc receptor on CLL

	Fc receptors detected (%)						
Patient	Ia-bearing cells (%)	Agg IgG	RIgG-S~IgG	KLH-R~KLH	OA-R~OA	EA_R	EA_H
A	99	94	89	3	5	93	3
B	89	86	90	83	85	88	4
C	86	82	84	15	5	90	4
D	95	96	91	5	15	96	1
E	96	94	95	96	86	95	95

Staining with OA-R~OA and KLH-R~KLH was similar to that obtained with EA_R. One to 7% mean 4%) of isolated tonsil T lymphocytes bound EA_R. Essentially similar results were obtained by KLH-R~KLH, OA-R~OA and EA_H. RIgG-S~RIgG and agg IgG gave from 0 to 2% staining.

The Fc receptors of lymphocytes from patients with chronic lymphatic leukaemia illustrated varied properties (Table 6). RIgG-S~RIgG, agg IgG and EA_R were positive on most of the lymphocytes from all the patients. The lymphocytes of two patients bound high percentages of KLH and OA immune complexes. The lymphcoytes of patient E formed a high percentage of rosettes with the EA_H system. Controls, including unsensitized RH+ eyrthrocytes were negative. In patients A, B and E over 75% of the lymphocytes stained brightly for IgM, while in patients C and D a few cells stained weakly.

Pronase digestion experiments

Because of the contrast in findings among B lymphocytes from different sources, the effects of pronase digestion on receptor reactivity were studied.

Digestion with graded concentrations of pronase revealed that EA_R and KLH-R~KLH binding was more susceptible to pronase digestion than was RIgG-S~RIgG binding using lymphocytes from a patient with lymphatic leukaemia similar to that of case B. The larger complexes produced by IgG antigen in moderate antibody excess bound more readily than did the smaller complexes produced in more extreme antibody excess (Table 7). The staining became less intense with increasing pronase

TABLE 7. Differential effects of pronase digestion on binding of different Fc receptor detection systems using chronic lymphatic leukaemic cells

	Digestion with pronase mg/ml (% positive cells)			
Fc detection system	0	0·5	1·5	3·0
RIgG-S~RIgG				
ng RIgG 250	99	96	89	70
100	99	92	88	41
50	99	90	81	20
25	98	82	67	3
KLH-R~KLH				
ng KLH 250	61	9	3	4
125	19	10	5	2
EA_R	95	25	8	4

concentration. Evidence was obtained that the residual 2–5% of cells with Fc receptors that resisted pronase digestion largely were monocytes. Similar results were obtained with lymphocytes from two other leukaemic patients.

There was no difference between the effect of pronase on the Fc receptor of T cells and B cells from two non-leukaemic individuals who had 35 and 38% of T cells with Fc receptors demonstrable by EA_H and EA_R.

DISCUSSION

There was wide, reproducible variation in the results of different tests for Fc receptors under different conditions. According to the method used, B cells positive for Fc receptors varied from 3 to 90%, T cells from 1 to 11% and third population from 30 to 73%. Moreover, some tests for Fc receptors reacted preferentially with B cells and others with T cells. Qualitative factors in a given system and temperature importantly influenced the binding. For instance, native serum IgG and Fc fragments bound only in the cold and primarily to T cells; and despite the presence of a constant amount of antibody only antibody excess complexes bound well to the Fc receptors under the standard conditions. Antibody–antigen proportion was also critical in a rabbit allotype–anti-llotype system (Forni & Pernis, 1975). The findings in the agg IgG system suggest that only a fraction of aggregated IgG molecules bind effectively. These results emphasize the importance of subtle aspects of technique and could explain the conflicting reports in the literature.

The use of the Ia antigen as a marker for B cells (Hoffman *et al.*, 1977; Winchester *et al.*, 1976; Humphreys *et al.*, 1976), permitted recognition of B cells on which surface Ig was difficult or impossible to demonstrate (Winchester *et al.*, 1976; Humphreys *et al.*, 1976; Ross *et al.*, 1978), and which otherwise would be considered in the third population. The different results of these different Fc receptor tests on the three different lymphocyte populations defined by these markers shows that no single test is optimal for the demonstration of all Fc receptors. The four test systems (EA_H, EA_R, KLH-R~KLH and OAR~OA), that best demonstrated T cell Fc receptors, reacted less well with B lymphocytes. Thus, the Fc receptor of the B cell appears weaker then that of T cells, as has been reported (Winfield *et al.*, 1977; Samarut *et al.*, 1976; Arbeit, Henkart & Dickler, 1976). However, with agg IgG and particularly RIgG-S~RIgG, the reverse conclusion may be drawn and the B cell Fc receptor considered the more avid. These experiments on B cells also showed that the binding of RIgG-S~RIgG required the Fc portion of the IgG molecule, and that primary binding by other portions of the IgG molecule, as has been postulated (Frøland *et al.*, 1974), doesnot occur under these circumstances.

The similar results of the different test systems with T cells and with lymphocytes of the third population suggests that the two cell populations are related. The finding of effector cells for antibody-dependent cell-mediated cytotoxic in both these populations supports this (Van Oers, Zeijlmaker & Schellenkens, 1977; Pape, Troye & Perlmann, 1977; Kay *et al.*, 1977), as does the observations of Bentwich *et al.* (1973) that treatment of Fc-binding lymphocytes resulted in new E rosette-binding cells, presumably from the third population. Also consistent is the lack of the third population in tonsillar lymphocytes in association with the very low frequency of T cells with Fc receptors and the essentially normal proportion of third population lymphocytes among patients with X-linked agammaglobulinaemia (Hoffman *et al.*, 1977).

All B lymphocytes displayed a considerable heterogeneity in the binding of EA_R, EA_H, OA-R~OA and KLH-R~KLH but shared strong binding of agg IgG and RIgG-S~RIgG. Tonsillar B lymphocytes bound poorly with all reagents but agg IgG and RIgG-S~RIgG. However, in contrast even EA_H, which bound best to T cells, bound to few B cells and one B type lymphatic leukaemia. Pronase digestion of leukaemic lymphocytes removed the high degree of binding to EA_R and the related KLH system, resulting in a pattern of receptor reactivity that simulated tonsil B cells.The pronase digestion experiments of Winfield *et al.* (1977) also provided evidence for a range of Fc receptors. The simplest interpretation of these findings is that a high density of Fc receptors are required to react with EA_R and related systems and that Fc receptors are progressively removed by digestion.

The difference in Fc receptors on B cells as compared to T and third cell populations raises the question of whether two distinct Fc receptors are involved because receptor density alone cannot account for them. Such an explanation might account for the different binding of agg IgG and EA_H, both of which contain human IgG. The present studies were not directed to an examination of these possibilities, and further studies are required.

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