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Comparison of the direct antiglobulin rosetting reaction with direct immunofluorescence in the detection of surface membrane immunoglobulin on human peripheral blood lymphocytes

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Summary. Comparisons were made between the Direct Antiglobulin Rosetting Reaction (DARR) and Direct Immunofluorescence (DIF) in the detection of surface membrane immunoglobulin of human peripheral lymphocytes.

The DARR was more sensitive and the results with this testing procedure (as opposed to those with the DIF) were not influenced by various treatments of the lymphocytes before testing, such as incubation in AB serum at +4°, incubation in serum-free medium at 37° or short exposure to acetate buffer at pH 4·0.

Again the DARR (as opposed to the DIF) gave essentially the same results whether the red cell-linked antiglobulin was IgG or the $F(ab')_2$ preparation.

With mixed rosetting for both T and $SmIg^+$ lymphocytes, there was only 1% or less null cells and only 5% or less lymphocytes rosetted with both marker red cells.

INTRODUCTION

Immunoglobulin (Ig) determinants are most commonly demonstrated in human B lymphocytes by direct immunofluorescence (DIF). This is reviewed by Warner (1974). Several important technical difficulties are associated with the application of fluorescent antiglobulin reagents; for example, it is claimed that lymphocyte auto-antibodies bound to T or B lymphocyte membranes may be misinterpreted as true surface membrane Ig (Winchester *et al.*, 1974). Also, positive fluorescent staining of lymphocyte membranes may occur in the absence of native surface Ig (SmIg) when experimental conditions permit binding of aggregated IgG or immune complexes to IgG (Fc) lymphocyte receptors (Winchester *et al.*, 1975; Winchester & Fu, 1976).

The Direct Antiglobulin Rosetting Reaction (DARR) has been introduced as an alternative method to immunofluorescence in the detection of Ig-bearing human lymphocytes (Giuliano *et al.*, 1974; Parish & Hayward, 1974; Haegert & Coombs, 1976; Coombs *et al.*, 1977). If this antiglobulin rosetting reaction is to have wide acceptance it must be shown to be at least as sensitive as the fluorescent technique and to have practical advantages over immunofluorescent methodology. For this reason a direct comparison was made between the DARR and DIF; the same fluorescein-conjugated reagents were used for direct immunofluorescence and for coupling to the indicator erythrocytes for the DARR.

The potential advantages of the DARR are mentioned in the Discussion and observations are made

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on the question of null cells and on the total number of Ig-bearing B lymphocytes in human peripheral blood.

MATERIALS AND METHODS

Reagents

Rabbit anti-human Fab and sheep anti-human γ Fc were prepared as previously described (Coombs *et al.*, 1977; Bright *et al.*, 1977) and their specificity established by immunodiffusion and by reverse passive haemagglutination.

Fluorescein-labelled antiglobulin reagents were purchased from Dako-Immunoglobulins (Copenhagen, Denmark). The anti-γ and anti-μ were shown to be specific by reverse passive haemagglutination.

Preparation of $F(ab')_2$

A 1 ml aliquot of the fluorescent polyvalent anti-human globulin reagent (9 mg/ml) was incubated with 2%, by weight, of pepsin (Sigma, twice crystallized) in 0·2 M glycine-HCl buffer, pH 3·2, for 2 h (see Lachmann, 1971), then further purified by affinity chromatography using Protein A-Sepharose CL-4B (Pharmacia) to remove undigested IgG (Hjelm, Hjelm & Sjöquist, 1972). A purified $F(ab')_2$ goat anti-rabbit γ Fc was prepared by the method of Lachmann (1971).

Coupling of antiglobulin reagents to indicator erythrocytes

Both fluorescein-conjugated and unconjugated antiglobulin reagents were coupled to trypsin-treated ox erythrocytes with chromic chloride as previously described (Coombs *et al.*, 1977). With the sheep anti-human γ Fc reagent, chromic chloride coupling was performed by the method of Ling, Bishop & Jefferis (1977) since for this specific reagent, the latter method produced more satisfactory coupling with less tendency for spontaneous aggregation of the indicator erythrocytes.

The efficiency of coupling was assessed by antiglobulin for actual coupling of the IgG reagent and by reverse passive haemagglutination for the effectiveness of the coupled reagent. For all tests a control preparation was included consisting of trypsin-treated ox erythrocytes coupled with normal rabbit IgG.

Preparation of lymphocytes

Human peripheral blood lymphocytes were isolated using Ficoll–Hypaque sedimentation and carbonyl iron treatment (Eremin *et al.*, 1976) then made to 5×10^6/ml in HEPES buffered Hanks's balanced salt solution containing 0·2% BSA (Hanks's BSA).

Experimental treatment of lymphocytes

Aliquots of the lymphocyte preparations were incubated for 30 min at room temperature or at 37° in Hanks's BSA or were incubated in medium containing 50% heat-inactivated human AB serum for 30 min at 4°. In other experiments sedimented lymphocytes (5×10^6) were incubated at 4° for 2 min in 1 ml 0·05 M acetate buffer, pH 4·0, containing 0·085 M NaCl, 0·005 M KCl and 0·2% BSA as described by Kumagai *et al.* (1975). All lymphocyte preparations were then washed at least three times at room temperature before fluorescent staining or rosette formation.

DARR and DIF

For the DARR, equal volumes of lymphocytes ($2000/m^3$) and antiglobulin-coated trypsin-treated ox erythrocytes (1% v/v) were mixed in RPMI 1640 containing 10% FCS then centrifuged at 100 *g* for 2 min at 4° (Coombs *et al.*, 1977). All tests were performed in duplicate and counted as previously described (Haegert & Coombs, 1976). Mixed rosettes were formed using sheep erythrocytes labelled with fluorescein and antiglobulin-coated trypsin-treated ox erythrocytes using previously described methods (Haegert & Coombs, 1976).

For DIF, lymphocytes were stained by standard techniques (Winchester & Fu, 1976) then examined using a Leitz Orthoplan microscope with Ploem illuminator. All fluorescent reagents were used at concentrations found to give a plateau of percentage positive lymphocytes.

RESULTS

B lymphocyte detection using DIF and DARR: the effects of previous incubation of lymphocytes under various conditions on the demonstration of lymphocyte surface membrane Ig

Fluorescein-conjugated anti-human μ and fluorescein-conjugated polyvalent anti-human globulin were used at the optimal concentration to stain lymphocytes directly. The reagents were also

Table 1. Comparison of DIF and DARR in the demonstration of SmIg on human peripheral lymphocytes

Antiglobulin reagent	DIF				DARR			
	4° (AB serum)	Room temperature	37°	Acid pH	4° (AB serum)	Room temperature	37°	Acid pH
1. Fluorescein-conjugated polyvalent anti-human globulin	16·7 ± 4·1	11·4 ± 2·5	6·5 ± 1·7	6·5 ± 2·6	17·3 ± 5·6	17·4 ± 5·9	17·6 ± 5·8	16·8 ± 4·1
2. Anti-human Fab	n.d.	n.d.	n.d.	n.d.	16·7 ± 4·8	18·5 ± 5·2	18·9 ± 4·7	n.d.
3. Fluorescein-conjugated anti-human μ	5·7 ± 3·2	4·4 ± 1·6	4·0 ± 2·8	4·4 ± 1·0	10·1 ± 4·8	10·0 ± 5·3	10·0 ± 5·1	9·4 ± 1·6
4. Fluorescein-conjugated anti-human γ	11·6 ± 3·2	n.d.	2·4 ± 2·4	n.d.	n.d.	n.d.	n.d.	n.d.
5. Anti-human γ Fc	n.d.	n.d.	n.d.	n.d.	1·9 ± 2·2	2·4 ± 2·2	3·1 ± 5·1	2·9 ± 2·8

Results expressed as mean percentage of lymphocytes reacting with a specific indicator system ± standard deviation. The fluorescein conjugate anti-μ and polyvalent reagents were used to stain lymphocytes directly or coupled to trypsin-treated ox erythrocytes for the DARR. For antiglobulin reagents 1. and 3. the values represent the results of tests on 8 individuals. Reagent 4. was tested on 5 individuals, reagent 2. on 4 individuals, and reagent 5. on 3 individuals.
n.d. = not done.

coupled to trypsin-treated ox erythrocytes for the DARR (Table 1). In agreement with other investigators, the number of lymphocytes staining with a polyvalent fluorescent antiglobulin reagent decreased after incubation and washing of the lymphocytes at 37° (Lobo, Westervelt & Horwitz, 1975) or at 4° in acid pH buffer (Kumagai *et al.*, 1975), whereas the number of μ-staining cells remained relatively constant. Further, the number of freshly isolated lymphocytes staining with the polyvalent reagent was increased by incubation of the lymphocytes at 4° in normal human AB serum. This suggests that under these conditions Ig may absorb onto lymphocytes from serum and provide a source for false-positive fluorescence. In contrast to these findings the number of lymphocytes forming rosettes with antiglobulin-coated erythrocytes was not influenced by the various preliminary incubations. Further with the same fluorescent anti-μ ($P < 0{\cdot}01$ by the Student's '*t*'-test) and the same fluorescent polyvalent antiglobulin reagent ($P < 0{\cdot}001$) coupled in the DARR, the number of rosetting lymphocytes was always significantly higher than the number of fluorescent staining lymphocytes even when the rosettes were formed under conditions considered to be optimal (i.e. 37°) for immunofluorescence (see Table 1).

With the specific fluorescent anti-IgG reagent the number of IgG bearing lymphocytes detected was decreased following brief incubation of the lymphocytes at 37°. A corresponding non-fluorescent anti-γ Fc reagent coupled to trypsin-treated ox indicator erythrocytes rosetted with and thus revealed only 2–3% IgG bearing lymphocytes and this number remained relatively constant under different conditions of incubation.

An anti-human Fab was coupled to trypsin-treated ox erythrocytes for comparison with the polyvalent fluorescent antiglobulin reagent since anti-Fab coated indicator erythrocytes were used in our previous experiments (Coombs *et al.*, 1977) to develop the present DARR. The number of lymphocytes rosetting with anti-Fab or polyvalent anti-Ig coupled erythrocytes may be seen to be similar (Table 1).

In control tests trypsin-treated ox erythrocytes coated with normal IgG always failed to form rosettes.

Comparison of IgG and F(ab′)$_2$ polyvalent antiglobulin reagents

In order to compare F(ab′)$_2$ and IgG polyvalent antiglobulin reagents these were brought to the same concentration (1·6 mg/ml) and then used for fluorescent staining or for the DARR. After coupling the F(ab′)$_2$ anti-globulin to the trypsin-treated ox erythrocytes, the erythrocytes (in contrast to those coupled with the undigested IgG anti-globulin) were not haemagglutinated with a F(ab′)$_2$ goat anti-rabbit γ Fc reagent. This was evidence that no intact

Table 2. DIF and DARR on human peripheral lymphocytes using either IgG or derived $F(ab')_2$ preparations of the fluorescein-conjugated polyvalent anti-human globulin reagent

	DIF		DARR	
Experiment	IgG polyvalent anti-Ig	$F(ab')_2$ polyvalent anti-Ig	IgG polyvalent anti-Ig	$F(ab')_2$ polyvalent anti-Ig
1	14·1	6·2	16·4	13·4
2	14·6	6·3	8·6	8·3
3	10·4	1·9	17·9	16·4
4	13·2	1·0	12·7	12·6

Results expressed as percentage of lymphocytes reacting with each indicator system.

IgG remained contaminating the $F(ab')_2$ anti-human globulin reagent.

Lymphocytes were incubated at room temperature (and not 37°) for 30 min and then washed before fluorescent staining and before direct antiglobulin rosette formation. In the four individuals studied (Table 2) the $F(ab')_2$ fluorescent reagent stained fewer lymphocytes than did the whole IgG fluorescent reagent. In contrast, there was no or minimal difference in the results with the DARR when the $F(ab')_2$ reagent was coupled instead of the IgG anti-human globulin.

Experiments to see whether direct antiglobulin rosetting lymphocytes also rosette with sheep erythrocytes (T marker)

Using a mixed rosette test a mean of 3·8% of lymphocytes from six donors formed mixed rosettes with sheep erythrocytes (E), a T-cell marker (Wybran, Carr & Fudenberg, 1972), and ox erythrocytes coupled with IgG anti-human Fab (Table 3). The number of lymphocytes which were both E-rosette negative, and SmIg negative, was shown to be extremely low in every case.

DISCUSSION

In the present study an average of 17% of viable human peripheral blood lymphocytes were found to be Ig-bearing using the DARR. This result is in close agreement with earlier published data from several laboratories using immunofluorescent antiglobulin staining (Warner, 1974). However, more recently several investigators (Winchester *et al.*, 1975; Lobo *et al.*, 1975; Kumagai *et al.*, 1975) have reported much lower levels of Ig-bearing lymphocytes when rigid test conditions were established to reduce supposedly non-specific staining of lymphocyte membranes. The lower levels of Ig-bearing lymphocytes have been achieved either by incubation at 37°

Table 3. Rosette formation with a mixture of trypsin-treated ox erythrocytes (non-fluorescent) coated with anti-human Fab and fluorescein-labelled sheep erythrocytes

Experiment	Rosettes with sheep (E) erythrocytes only	Direct antiglobulin rosettes only	Mixed E + Ig rosettes	Non-rosetting lymphocytes
1	78·7	17·4	3·5	0·4
2	73·3	20·8	4·9	1·0
3	77·0	18·5	4·5	0
4	77·0	19·5	3·5	0
5	83·3	15·0	1·5	0·2
6	80·1	14·4	5·0	0·5
Mean ± s.d.	78·2 ± 3·4	17·6 ± 2·5	3·8 ± 1·3	0·3 ± 0·4

For each test 500 lymphocytes were counted and scored as either rosetting with sheep erythrocytes only, ox erythrocytes only, mixed erythrocytes or non-rosetting.

aimed at elution of loosely adsorbed Ig from lymphocyte membranes before staining with fluorescent reagents (Lobo *et al.*, 1975; Kumagai *et al.*, 1975) or by use of $F(ab')_2$ conjugates (Winchester *et al.*, 1975). The latter is intended to prevent the problem of binding of immune complexes, formed between the antiglobulin reagent and residual serum Ig, to lymphocyte IgG (Fc) receptors. In view of these technical difficulties associated with direct immunofluorescence, one might have expected similar difficulties with the DARR. However, in the present study the DARR gave remarkably uniform results irrespective of the conditions of lymphocyte incubation before actual rosetting. Also, the use of $F(ab')_2$ antiglobulin reagents instead of whole IgG antiglobulin reagents did not alter the percentage of lymphocytes giving a positive DARR. These findings with the DARR conflict with previous (Winchester *et al.*, 1975; Lobo *et al.*, 1975; Kumagai *et al.*, 1975) and present immunofluorescent observations using anti-γ and polyvalent antiglobulin reagents.

These findings have several implications. First, the very strict conditions considered to be necessary for precise enumeration of SmIg-bearing lymphocytes are not required for direct antiglobulin rosetting since the DARR does not seem to be influenced by lymphocyte incubation or by use of $F(ab')_2$ reagents. Second, since there was not a parallel reduction in the number of direct antiglobulin-rosettes and of immunofluorescent staining-lymphocytes under conditions which supposedly elute adsorbed Ig from lymphocytes (Lobo *et al.*, 1975; Kumagai *et al.*, 1975), it appears that the DARR does not give rise to the situation of free antiglobulin and globulin forming complexes which may then be adsorbed on to the lymphocyte membrane (see also Bright *et al.*, 1977). Finally, since indicator erythrocytes coated with non-antiglobulin IgG did not form rosettes, it would appear that the Fc part of the Ig molecule is masked when Ig is coupled to erythrocytes by chromic chloride.

The present study indicates that there is a lymphocyte population which is immunofluorescent negative but direct antiglobulin rosette positive. Under supposedly optimal conditions for immunofluorescence (i.e. with previous 37° incubation), the difference between the two test systems was highly significant both with the anti-μ and with the polyvalent antiglobulin reagent. By mixed rosetting it is clear that at most only a small percentage of E-rosetting lymphocytes also form direct antiglobulin rosettes. In other words, few T cells are Ig positive. Also, it is evident that only very few lymphocytes are both E rosette-negative and Ig negative. Therefore, the rosetting data suggest that the previously described null cell population (Dickler, Adkinson & Terry, 1974), a percentage of which will synthesize Ig in culture (Chess *et al.*, 1975), consists of B lymphocytes with a low density of surface membrane Ig which is not readily demonstrable by immunofluorescence.

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