General Method for the Detection of Cells producing Antibodies against Haptens and Proteins

THE localized haemolysis-in-gel (LHG) assay[1], originally applied to the detection and enumeration of cells producing antibodies to erythrocyte cell wall antigens, has been extended to the detection of cells forming antibodies to hapten[2–7] and protein antigens[8,9]. In these experiments the antigens or haptens were bound to the erythrocyte by direct chemical reaction. From our experience, and that of others, the main drawbacks of such a chemical approach for the attachment of ligands to a living cell are as follows: (1) direct chemical modification of the erythrocyte causes damage to the cell membrane resulting in cellular fragility and tendency to haemolyse[6]; (2) because erythrocytes must be sensitized anew each day, fresh reagents and large amounts of materials (in the case of protein antigens) are required on each day of the experiment[4,8]; (3) the extent of modification may vary between preparations, and the degree of substitution is difficult to measure, resulting in irreproducibility of results.

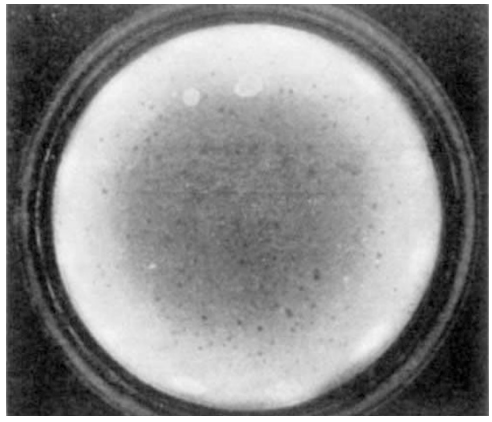

Fig. 1. Haemolytic plaques produced by cells producing anti-DNP antibodies. Direct plaques (see text) produced by 4×10^6 mouse spleen cells 7 days after immunization.

To avoid these problems we devised a general method of binding hapten and protein antigens to erythrocytes by a simple, fast, non-chemical procedure, without impairing the stability of the sensitized erythrocyte. The method makes use of the univalent fragment (Fab) of antibodies against red blood cells (RBC) as intermediates in the attachment of the hapten or protein to the RBC. Any kind of chemical modification, attachment of hapten or protein, is performed on this Fab fragment. The erythrocytes subsequently coated by the chemically modified Fab may be agglutinated or haemolysed (in the presence of complement) by antibody to the attached moiety. In the LHG assay, cells which form antibodies to the attached group give large visible plaques (Fig. 1) similar in size to those obtained by cells forming antibody to RBC. This approach is similar to that taken by Coombs *et al.*[10] for the detection of antibodies by haemagglutination using modified "incomplete" anti-RBC antibodies.

Anti-sheep red blood cell (SRBC) antibody was produced by intravenous injection of washed SRBC into rabbits as described elsewhere[11]. The Fab fragment was prepared by papain digestion of the IgG fraction of rabbit antiserum to SRBC followed by passage through CM-cellulose equilibrated with 0·01 M acetate buffer (*p*H 5·5) (ref. 12); only Fab I was used in these studies. Preparation of antigen–Fab conjugates varied according to the nature of the determinant to be coupled. Illustrative procedures are given for the preparation of conjugates with two haptens, dinitrophenyl (DNP) and penicilloyl (PEN), and with one protein, bovine serum albumin (BSA).

DNP-Fab was prepared by incubation of Fab (6 mg/ml.) with DNP-sulphonic acid (10 mg/ml.) and sodium carbonate (10 mg/ml.), final *p*H 9·3, for 24 h at 24° C. An average number of five DNP groups per Fab molecule was determined spectrophotometrically[13]. PEN-Fab was prepared by the reaction of Fab (4 mg/ml.) with penicillin G (3·3 mg/ml.) at *p*H 10·5 (0·1 M Na_2CO_3 for 12 h at 24° C). This preparation contained two or three groups of penicilloyl for each molecule Fab reacted[14]. BSA-Fab was formed by incubating Fab (3 mg/ml.) and BSA (7·5 mg/ml.) in phosphate-buffered saline (pH 7·2) with glutaraldehyde (0·1 per cent) for 1 h at 24° C, followed by termination with excess sodium bisulphite[15]. Excess reagents were removed by filtration on a 'Sephadex G-25' column in phosphate-buffered saline. The modified Fab preparations were stored at −20° C.

Modified Fab-coated SRBC were prepared by incubation of the Fab conjugates with SRBC (8 per cent) in phosphate-buffered saline for 1 h at 37° C, followed by washing with Dulbecco phosphate-buffered saline[16]. By contrast with other methods of preparing sensitized erythrocytes, the modified-Fab coated cells were as stable as unreacted washed RBC and could be used for LHG assay up to 10 days after preparation.

To show that chemical modification of the Fab did not destroy its binding capacity to SRBC, the DNP-Fab, PEN-Fab or BSA-Fab coated erythrocytes were treated with either goat anti-rabbit Fab or with antisera to the attached moiety. In all cases haemagglutination was seen, and on addition of complement, haemolysis was observed. The extent of coating with modified Fab which gave optimal haemolysis in tubes and in LHG (details below) was determined after incubation of SRBC with increasing amounts of modified Fab preparations. Table 1 shows that there is an agreement between the amount of modified Fab (0·1 mg/ml.) necessary to sensitize the erythrocytes for either haemolysis in tube or LHG. Similar results were also obtained for the PEN-Fab. In order fully to sensitize SRBC with BSA, concentrations of 1 mg/ml. BSA-Fab were necessary. At lower concentrations of BSA-Fab, comparable numbers of plaque forming cells (PFC) were observed, but the plaques were diffuse and not clear. Routinely, SRBC (8 per cent) were sensitized with 0·25 mg/ml. DNP-Fab and PEN-Fab and 1·0 mg/ml. BSA-Fab.

Table 1. CORRELATION BETWEEN NUMBER OF PFC IN LHG ASSAY AND HAEMOLYSIS IN TUBES USING SRBC COATED WITH DIFFERENT AMOUNTS OF DNP-Fab

DNP-Fab added (μg/ml. of 8 per cent SRBC)	Amount of anti-DNP antibodies required for haemolysis (μg)*	No. of direct PFC per 10^7 spleen cells†
200	0·6	161
100	0·6	158
50	10	73
25	10 (partial haemolysis)	39

* Haemolysis assay was performed using 0·1 ml. of 2 per cent coated SRBC, 0·5 ml. of serially diluted purified antibodies and 0·2 ml. of 1 : 10 diluted complement, at 37° C for 1 h.

† The spleen cells were taken from three Balb/c mice 11 days after immunization with 0·4 mg of DNP-rabbit serum albumin in 0·2 ml. of complete Freund's adjuvant.

For the assay of PFC, 0·1 ml. of the appropriate concentration of washed spleen cells suspended in Eagle's medium[17] was added to tubes containing 0·6 ml. of 0·5 per cent agarose (l'Industrie Biologique Française) in Hanks balanced salts solution (*p*H 7·4) (ref. 18) and 0·1 ml. of 8 per cent SRBC at 45° C. The tubes were quickly and thoroughly mixed and the contents poured onto 6 cm diameter Petri dishes which previously had been precoated with 2 ml. of 1 per cent agarose in Hanks balanced salts solution.

After solidification (for 5 min) the plates were incubated in a humid atmosphere at 37° C for 1 h, after which time

the plates were covered with Dulbecco phosphate-buffered saline (for direct, 19S producing plaques), or with species specific anti-γ-globulin antiserum (for indirect, 7S producing plaques)[19,20]. After an additional 90 min incubation, the plates were rinsed and covered with 1 ml. of reconstituted, lyophilized guinea-pig complement (Difco) diluted 1 : 10 in Dulbecco phosphate-buffered saline. Haemolytic plaques developed during 2 h of incubation with complement at 37° C, and were counted without magnification in direct light by two individuals.

Table 2. APPEARANCE OF ANTI-DNP FORMING CELLS IN MICE FOLLOWING PRIMARY INJECTION OF DNP-RABBIT SERUM ALBUMIN*

Day	PFC/10^7 spleen cells† Direct	Indirect‡
0	3	3
3	14	3
7	700	420
11	161	462

* Balb/c mice immunized by intraperitoneal injection of 0·4 mg DNP-rabbit serum albumin in 0·2 ml. of complete Freund's adjuvant.

† Average of three animals.

‡ Developed with goat anti-mouse IgG (Hyland Lab. lot GP 7–66) diluted 1 : 50.

This technique was applied to the assay of cells forming antibodies against two different haptens and one protein in mice, rabbits and guinea-pigs. The results (Tables 2 and 3) show rapid increase of "direct" PFC following the third day after immunization and an increase in "indirect" PFC occurring later, similar to those reported by others[3–9]. They also demonstrate that by this technique a very low background is observed and PFC of hapten (DNP) or carrier (BSA) specificity can be determined simultaneously (Table 3). The plaques detected with DNP-Fab–RBC were completely inhibited by DNP-amino caproate (10^{-5} M) and those detected with BSA-Fab–RBC were inhibited by BSA (5×10^{-7} M). In the case of rabbit spleen cells it is especially important to have highly specific anti-Fc antiserum for the elucidation of indirect PFC, otherwise non-specific lysis of the Fab coated RBC will occur. Similarly it is also not advisable to use IgG as a carrier or immunogen, for a cross-reaction of anti-IgG and the rabbit Fab used to coat the cells might confuse the results (see Table 3).

Table 3. ANTIBODY FORMING CELLS IN RABBIT SPLEEN AT DIFFERENT STAGES AFTER IMMUNIZATION*

Immunogen	Day	Erythrocytes coated with	PFC/10^7 spleen cells Direct	Indirect†
DNP-BSA	5	Fab	20	25
		DNP-Fab	70	30
		BSA-Fab	80	40
DNP-BSA	Hyperimmune	Fab	20	30
		DNP-Fab	40	13,000
		BSA-Fab	30	1,480
PEN-bovine IgG	13	Fab	37	39
		PEN-Fab	34	98

* Immunization was by multiple intradermal injections of 2 mg immunogen in complete Freund's adjuvant.

† Developed with goat anti-rabbit-Fc previously adsorbed on rabbit-Fab–'Sepharose' column.

Our technique has the following advantages: a series of modified anti-RBC-Fab preparations can be prepared and used as reagents for the assay of PFC with specificity towards the attached ligand. These reagents can be titrated, stored and subsequently used many times with high reproducibility for the coating of erythrocytes. The assay is applicable to both small and large molecules and can measure PFC towards either hapten or carrier in the same system, although the methods and optimal conditions for attachment of the required moiety should be investigated in each case. The attachment of haptens seems quite straightforward in many cases and the effect of hapten density on plaque formation can be studied by varying the number of ligands on the Fab or the number of modified Fab per RBC. The binding of proteins presents more difficulty and the use of bifunctional reagents other than glutaraldehyde is now under investigation in order to improve the assay for protein antigens. The coating of the RBC by the modified Fab is fast and simple and does not change the stability of the RBC. The background PFC is very low and the assay of different systems can be compared simultaneously, for all of them have the anti-RBC-Fab as a common denominator.

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Application of Electron Probe Microanalysis and Electron Microscopy to the Transfer of Antigenic Material

We have studied the effect of various antilymphocyte sera on lymphocytes, using the Stereoscan electron microscope[1], and more recently we described the formation of long intercellular processes between macrophages and lymphocytes in certain conditions[2]. Process formation was particularly marked if macrophages from rats sensitized to tuberculin were incubated first with PPD and then with either normal lymph node cells or lymph node cells from rats sensitized to tuberculin (Fig. 1). In the former case, many macrophages were seen to be closely surrounded by clusters of lymphocytes.

We describe here an attempt to produce evidence for the hypothesis that transfer of antigenic material from cell to cell occurs along these intercellular processes. The antigenic protein used was PPD. It was labelled with iodine and subsequent cell preparations scanned for iodine distribution by X-ray microanalysis (EMMA-4, AEI Scientific Apparatus Ltd). Previous studies of biological material using this technique have been reviewed by Robertson[3], while methods of specimen preparation have been discussed by Andersen[4], and Beaman *et al.*[5]