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The immuno-histochemical differentiation of human lymphoid cells

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2015-01-27
http://hdl.handle.net/10539/16787

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A suitable substitute was found in glutaraldehyde-insolubilised proteins (Avrameas and Terynnck, 1969).

Technique No.17 The preparation of insolubilised serum proteins.

1) Normal human serum was dialysed overnight against saline buffered to pH 5.0 with acetate buffer. The significance of this step was not established, but a fluffy precipitate, possibly of albumen, had to be centrifuged off before the next step.
2) To 10.0 ml of serum, 3.0 ml of 10% glutaraldehyde in acetate buffer at pH 5.0 was added drop by drop until the material gelled.
3) The gel was broken up by forcing it through a 16 SWG needle attached to a 50 ml syringe.
4) The resulting coarse suspension was washed by sedimentation in five changes of PBS and stored at 4°C with toluene as a preservative

For use, equal volumes of serum and gel were incubated at 4°C for 30 minutes followed by centrifugation for recovery of absorbed serum.

15.4 Heterologous lymphocytes

With the risk of losing antibody activity via histocompatibility antigens shared by immunising antigen and absorbent, it was necessary to remove anti-B cell activity from anti-T cell sera and anti-T cell activity from anti-B cell sera. Lymphocyte suspensions were prepared in PBS to a concentration of 10^8

cells per ml of serum to be absorbed. (BSS was not used, to avoid contamination of serum with phenol red indicator). The suspensions were centrifuged to a pellet at 90g and the supernatant replaced by serum in which the lymphocytes were resuspended for 30 minutes at 4°C. Usually 2 or 3 such absorptions were carried out because of the small amount of absorbent. Trypan blue exclusion tests for viability of the lymphocytes showed, when applicable, that absorption was complete, by insignificant changes in the number of dead cells present after exposure to serum. Some anti-B cell sera were absorbed with dead thymocytes. It was noted that these cells showed marked clumping during absorptions.

16 CONJUGATION WITH FLUOROCHROME.

All sera were labelled with fluorescein isothiocyanate (FITC) (Miles laboratories) for use in the direct fluorescent antibody test. The performance of this procedure is simple although the theory is somewhat complex. The subject has been intensively studied by many workers over many years, the most thorough being that of The and Feltkamp (1970). The objective is to attach four molecules of FITC to each molecule of IgG containing specific antibody activity. In practice the product is a reagent of molecular heterogeneity approaching the ideal only as close as crude techniques will allow. There are three major steps involved; separation of immunoglobulins from serum (Section 13.4); labelling with fluorochrome; and purification of optimally labelled conjugate. The first stage has already been discussed. The second is basically whether to add fluorochrome to protein as a solution or a powder. The former

choice can lead to degradation of the dye before conjugation but gives a more homogeneous labelling. The latter ensures availability of active dye yet allows local foci of over-conjugated protein molecules. The author has used both techniques in the past, to arrive at the conclusion that the difference is mainly theoretical!. For this work advantage was taken of the simplicity of adding dry powder. The third stage involves removal of over and under-conjugated protein molecules by ion exchange chromatography and free dye by activated charcoal or sephadex chromatography.

16.1 Measurement of protein content of globulins.

Before proceeding with conjugation, it is necessary to know the amount of protein present in the globulin solutions in order to calculate the amount of fluorochrome that is to be added to each serum for optimum labelling. Any standard technique may be used, from simple refractometry to micro-Kjeldahl analysis. The conventional Biuret technique was used on samples in this study, estiminating total protein.

Technique No.18 Estimation of serum proteins by Biuret technique.

1) 0.2 ml of commercial protein standard of 5.5g of protein per decilitre was pipetted into 2.8 ml of water.
2) 0.2 ml of globulin solutions were treated the same way.
3) 3.0 ml of water served as a blank.
4) 5.0 ml of Biuret reagent was added to all tubes

which were incubated at 37°C for 10 minutes.

5) The optical densities were measured in an absorptiometer in green light between 540 and 560 nm using the blank as zero.

6) The protein concentrations of globulins were calculated from

$$\frac{\text{READING OF UNKNOWN}}{\text{READING OF STANDARD}} \times 5.5 \text{ g/dl}$$

7) From the measurement of the volume of globulin solution available for conjugation, the total protein content could be calculated.

Most sera showed values between 2 and 3 g/dl and were diluted to 1g/dl with PBS to give the optimum concentration of protein for conjugation.

16.2 Conjugation with fluorochrome.

Fluorescein isothiocyanate (FITC) was chosen as the fluorescent label, as it is the most popular in use at the present time for many valid reasons. The reagent is far more stable than its precursor, fluorescein isocyanate, and has superior visible light emission intensity than rhodamine or aminonaphthalene sulphonic acid derivatives. Meaningful physicochemical analysis can be done only on FITC conjugates, and the label may be obtained in reasonably pure form. Synthesis in the laboratory is a complex procedure offering no guarantee of a product suitable as a protein label, whereas a commercially produced sample, although expensive, removes a significant variable from the conjugation technique (Brandtzaeg, 1973). 100 mg of fluorescein isothiocyanate, (isomer I) was purchased from Miles laboratories, Cape Town, and stored under the

manufacturer's recommended conditions. This proved to be of exceptional quality, being immediately soluble in water, and pure FITC when separated on chromatography columns. In an alkaline environment, FITC attaches to the amino acid terminal groups of protein chains, with no recorded interference with the activity of immunoglobulin protein. Pure IgG is difficult to label (Brandtzaeg 1973) although most authors recommend that preparations should have high concentrations of this immunoglobulin for successful reagents, (The and Feltkamp, 1970). The ratio of FITC to protein in the conjugation mixture is of vital importance in avoidance of underconjugated or overconjugated protein molecules; 20 to 40 mg per gram of protein is generally recommended.

Technique No.19 Conjugation of protein with fluorescein isothiocyanate.

1) Globulin solutions, corrected to 10 mg/ml were chilled and 10% by volume of cold carbonate buffer at pH 9.5 was added.
2) FITC powder was added at a concentration of 40 ng/mg of total protein and mixed gently at 30 rpm for 1 hour at room temperature. The buffer was obviously effective as the pH of all conjugates did not drop below 9.0 and no corrections were necessary.

16.3 Purification of conjugates.

16.3.1 Dialysis.

All conjugates were dialysed against PBS at 4^{o}C for 18 hours.

One inch diameter dialysis tubing was soaked for 15 minutes in PBS to soften and dissolve out hardeners, and knotted tightly at one end. After filling with serum, a further knot was tied above an air space and the sack suspended in 1 litre of cold PBS. After 18 hours there was a variable amount of free dye at the bottom of the dialysate flask.

16.3.2 Activated charcoal.

Fothergill and Nairn (1961) discuss the use of activated charcoal for removal of free FITC. Although it effectively removes all unconjugated dye (see Sephadex G 50 chromatography, section 16.3.3) there is reputed to be some loss of protein. The author has used the technique in the past in conjunction with absorption of non-specific staining with tissue powders.

Technique No.20 Purification of conjugates with activated charcoal.

1) Activated charcoal in the proportion of 2.5 mg/mg of protein was added to conjugate in a screw capped vial.
2) The preparation was mixed at 30 rpm for 30 minutes, and centrifuged at 3 000 rpm for 10 minutes.
3) The supernatant was cleared and sterilised by passing it through a 0.45 µm Millipore filter attached to a 20 ml syringe.

16.3.3 Sephadex G50 chromatography.

As an alternative to activated charcoal for removal of free fluorochrome, chromatography on Sephadex G50 columns was

studied. Resin was washed in PBS by repeated sedimentations to remove fines and to equilibrate. A 20 ml syringe, held vertically in a clamp was filled with the slurry which was allowed to settle before a disc of filter paper was placed on top. Ten millilitres of conjugate was carefully pipetted into the column followed immediately by PBS. Unbound FITC was retained at the top of the column and the conjugated molecules were collected in the first 10.0 ml of effluent.

16.3.4 DEAE cellulose chromatography.

Fractionation and collection of optimally labelled protein molecules was effected on DE 52 (Whatman) columns (Riggs et al., 1960). The various components of the conjugates were separated by elution in steps with buffers of increasing molarity of sodium chloride. Physico-chemical analysis and assessment of antibody activity of the fractions were carried out to permit selection for optimum staining characteristics.

Technique No.21 The separation of conjugated fractions on DEAE cellulose columns.

1) 2 litres of 0.0175 molar phosphate buffer at pH 6.3 was made up and identified as "starting buffer".
2) An arbitrary amount of DE 52 requiring none of the pretreatment demanded by other cellulose preparations, was suspended in starting buffer for one hour to equilibrate.
3) A simple column was prepared by cutting down a broken 50 ml burette to contain 20 ml with a

fibreglass plug at the constriction above the tap.

4) Equilibrated DE 52 was added until the cellulose packed down to 8.0 ml (bed volume) and a disc of filter paper tamped down on top.
5) One bed volume (8.0 ml) of starting buffer was passed through the column for final equilibrium.
6) 2.0 ml of conjugate was carefully pipetted onto the filter paper and allowed to run into the bed.
7) The conjugate was followed with another 8.0 ml of starting buffer. A coloured fraction moved immediately away from the top of the column; this fall-through fraction being the first of the effluent to be collected in 2.0 ml volumes.
8) The starting buffer was replaced with 8.0 ml of the second eluate; starting buffer containing 0.125 molar NaCl (0.73 g/dl). Usually these fractions of effluent were without colour.
9) The conjugate fractions remaining on the column were eluted with step 3 eluate; starting buffer containing 0.250 molar NaCl (1.46 g/dl). A new column was prepared for each serum.
10) Thus, 11 or 12 fractions were collected in 2.0 ml volumes, among which were unconjugated proteins, heavily overconjugated molecules, and optimally labelled antibody. It is noted that all sera were subjected to activated charcoal or Sephadex treatment before chromatography so free FITC was not detected in fractions by the techniques discussed below.

16.3.5 Spectrophotometric analysis.

In addition to conjugate behaviour in the fluorescent antibody test, there are some physico-chemical parameters that further define a suitable preparation in terms of protein and fluorochrome content. Furthermore the ratio between fluorochrome and protein concentration (F/P) is used as a measure of the degree of conjugation (Brandtzaeg 1973; Johnson and Holborow 1973; Goldman, 1968). This molar F/P ratio offers a rough guide to conjugate behaviour although such preparations are essentially heterogeneous. Nevertheless preparations with molar F/P ratios below 1.0 are likely to give weak staining with high levels of antibody activity even to the extent of introducing a blocking effect. High F/P ratios, above 7.0 indicate the presence of protein molecules (not necessarily globulin) with excessive, unstable labelling from which non-specific staining may be expected. Protein concentration was measured on a Unicam SP. 1800 UV. spectrophotometer from the optical density at 280 nm, (the absorption peak of tyrosine), and 495 nm (absorption peak of FITC bound to protein). Chromatography fractions were measured untreated; finished conjugates were diluted 1:40 with PBS. The protein concentration was calculated from the published data stating that the extinction coefficient of IgG as a 1% solution in a 1 cm cuvette ($E^{1\%}$ 280 nm 1 cm) is 1.4. As the protein was conjugated to fluorochrome, a correction factor of 0.35 for FITC had to be applied (Wood et al., 1965). Thus the formula for FITC conjugated IgG was:

$$\frac{OD\ 280 - (0.35 \times OD\ 495)}{1.4}\ \text{mg/ml (Brandtzaeg, 1973)}$$

The concentration of FITC is also an important parameter which may be calculated by comparing the optical density of the conjugate with that of a reference standard at the absorption maximum of 495 nm. However the molar F/P ratio for conjugates was derived from the foregoing data by calculating the OD ratio (OD 280 nm/495 nm) which was converted to molar F/P ratio by using the logarithmic curve published by Brandtzaeg (1973) or the nomogram illustrated by Goldman (1968) which gave comparable results. With such conversion facilities available, there was no justification for using expensive fluorochrome to construct a reference curve for FITC assay.

16.4 Direct fluorescent antibody tests.

An outline of the technique, each step of which will be discussed, is given below. The procedure for cell suspensions was identical in principle except that the test was performed on live lymphocytes in 5.0 ml plastic tubes, and the cells recovered after each step by centrifuging at 90 g for 5 minutes.

Technique No.22 Direct immunofluorescence procedure.

1) Tissue was snap frozen in liquid nitrogen and stored in this refrigerant until used.
2) Sections cut at 4 µm on a cryostat for collection on coated slides.
3) After drying for 30 minutes, slides were washed for 10 minutes in PBS and dried again.
4) A drop of conjugated antiserum was placed over the section in a moist chamber for 30 minutes.
5) The sections were washed in three, five-minute

changes in PBS in a coplin jar and mounted under a coverslip with buffered glycerine.

6) The preparations were examined in the fluorescent microscope.

STEP1. Tissue preservation is a point of much contention and variability among workers in immunofluorescence. A rule-of-thumb guide is that antigen preservation is inversely proportional to morphological clarity when influenced by fixation. Some antigens will withstand paraffin wax processing, others are so labile that they are lost within a few days' deep freeze storage (Nairn 1969). A compromise is reached by brief alcohol or acetone fixation of unfixed sections but in this author's experience such exposure induces some non-specific fluorescence that is not compensated by minimal improvement in morphology. Thus the antisera produced in this study were standardised upon cryostat sections cut from tissues snap frozen in small plastic bags in liquid nitrogen. Freezing artefacts were not encountered providing blocks of tissue were smaller than 0.5 cm^3 and were not allowed to melt preparatory to sectioning.

STEP 2. Coated microscope slides were prepared by placing six drops of glycerine on one surface. A thin film of poly-tetra fluoro-ethylene (PTFE) was sprayed over the slide and allowed to dry. Washing with warm tap water and distilled water, produced six clear holes separated by non-wettable surface. A section was placed in each hole, thus affording a most convenient system for bulk examinations such as titrations.

STEP3. Experience in routine fluorescent antibody work has proven conclusively that a brief pre-wash in buffer prevents

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ugly fluorescent precipitates on sections, with less non-specific staining when studying bound, insoluble antigens.

STEP 4. Antisera were tested by double dilution external titration with and without pre-absorption with homologous cells for 30 minutes at room temperature to block specific antibody. Also with conjugated sera to block specific antigen, before exposure to conjugate. Further controls included commercially produced FITC labelled anti-immunoglobulin sera (DAKO) and a laboratory produced FITC conjugate of normal, absorbed, rabbit serum. (This latter reagent has proved to be of immense value in control of many non-specific reactions in various systems). All tests were performed in an identical manner as outlined in Technique No.20.

STEP 5. The final wash is a question of compromise; too little (a brief rinse) leaves non-specific fluorescence in the sections; too much (up to 24 hours by some authors) is inconvenient for routine diagnosis. Experience in this field has shown that three five-minute washes are adequate for removal of most unbound conjugate.

STEP 6. Experimentation in routine fluorescent antibody techniques has confirmed an observation by Nairn (1969) that an alkaline mounting medium tends to improve fluorescent emission and retard quenching. Thus preparations were mounted in glycerol, buffered to pH 8.5 with 10% by volume, phosphate buffer, under No. 1 coverslips.

STEP 7. All fluorescent work was performed on a Reichert "Binolux" microscope with an HBO 200 mercury vapour lamp. Apart from a Schott heat absorbing filter, a Schott UGI filter was used to select a transmission band of 290-410 nm for most

effective excitation of FITC at the absorption maxima of 290 and 325 nm. Illumination was dark ground with UV-light transmitting cardioid condenser, in glycerol immersion with the object slide. Secondary filtration, to give the best contrast with the apple green fluorescence of fluorescein isothiocyanate was with a yellow filter in the main light path. Photographs were taken through a split beam camera head on Kodachrome II daylight reversal colour film at standard exposures of 40 seconds.

PART 3 RESULTS

Eight sera were studied in all, four raised with thymocytes as anti-thymocyte series T1 to T4 and four raised with chronic lymphatic leukaemia lymphocytes identified at anti B1, B2, B3 and B4. Immunisation conditions varied slightly in terms of sources of cells and duration of course. (Table 3). Rabbit 5 received a second boost of thymocytes, as there was little evidence of activity at initial bleeding. Viability of immunising lymphocytes was generally high, rarely below 70%, and preparations were of satisfactory purity, with little, if any, contamination with macrophages or granulocytes on morphological criteria. Those lymphocytes recovered from EDTA anticoagulated blood samples frequently contained platelets as well. Sera were compared in their cytotoxic efficiency, their ability to inhibit lymphocyte function and their behaviour in fluorescent antibody tests. The effects of absorbents were studied and alternative techniques for conjugation and purification were compared. The most successful sera were pooled to give reagents of separate specificity against immunocyte subtypes.

No anomalies were encountered in preliminary animal work or separation of crude globulin preparations which were deep-frozen in aliquots for convenient access for various stages of investigation.

17 CYTOTOXIC EFFECTS OF CRUDE GLOBULINS.

Heterologous, homologous and peripheral blood lymphocytes were used to test the cytotoxic specificity and titre of globulin preparations (Section 14.1) Tables 5 and 6 illustrate

Table 5 Cytotoxic indices of anti-thymocyte sera.

	Target cells		
	Thymocytes	CLL cells	PBL
Anti-T 1	1.00	0.03	0.85
Anti-T 2	0.78	not tested	0.87
Anti-T 3	0.85	0.00	not tested
Anti-T 4	0.57	0.00	not tested.

relative cytotoxicity by trypan blue exclusion of anti-thymocyte and anti-CLL sera. The results are reported as cytotoxic indices, whereby complete kill is indicated by unity at one end of the activity scale, with zero reflecting no effect. The index allows for less than complete viability of the target cell population, providing for the effect of normal rabbit serum control and the presence of complement in the system (Aiuiti and Wigzell, 1973).

Anti -thymocyte preparations showed satisfactory cytotoxicity on homologous target cells, the specificity of which is confirmed by the minimal effect on CLL lymphocytes. The PBL population tested showed EAC rosette function of 14% (B cells) so the anti-thymocyte kill of 86% between the two sera tested reflects a reciprocal agreement that, for a biological system, is closer than one would hope to expect. Complement did not appear to be necessary for anti-thymocyte cytotoxicity; the addition of human AB serum in parallel systems, introduced variations well within the coefficient of variability of the method (Section 14.1). All cell suspensions were washed free of fetal calf serum preservative before use, so there was no other source of complement other than that deliberately added to the system.

In contrast, the anti-CLL sera showed no evidence of significant cytotoxicity. Serum B3 showed a poor 60% kill of homologous lymphocytes, but the specificity of this finding is suspect in view of the high cytotoxicity for thymocytes.The PBL target cells in this experiment had an EAC rosetting

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Name of thesis The Immuno-Histochemical differentiation of human lymphoid cells 1977

PUBLISHER:
University of the Witwatersrand, Johannesburg
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