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The immuno-histochemical differentiation of human lymphoid cells

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overnight incubation and "active" rosettes after only 1 to 2 hours (Wybran et al., 1973). Lymphocyte suspensions and sheep erythrocytes in the proportion of 1 : 100 are lightly centrifuged to a pellet and incubated at 4°C. The resultant extremely fragile rosettes are either examined directly, stabilised with 0.4% gluteraldehyde, stained in fixed films or put through a density gradient (Wybran et al., 1973). The effects of enhancement of the test are as empirical as the phenomenon itself; Papain (Wilson et al., 1975) and 2 amino-ethyl-isothiourium bromide (AEB) (Pellegrino et al., 1975) are reputed to increase test sensitivity. Neuraminidase (Weiner et al., 1973), and fetal calf serum overcome fragility in the formed rosettes, thus ensuring more reproducible results. Some normal sera (Pellegrino et al., 1975), cyanide radicle and sodium azide (Kiszkiss et al., 1973) inhibit the reaction. E rosette formation is not dependent on complement (Wortis et al., 1973). Using sheep red cells as the indicator system, human peripheral blood lymphocytes show 47% - 82% E rosette-forming cells (Jondal et al., 1972). Rat, monkey and baboon red cells give a much reduced rosetting effect (Brain et al., 1970). Human and ox erythrocytes do not work at all (Coombs et al., 1970). Recent developments using Protein A of Staphylococcus aureus cell envelope as an indicator system offers a more sensitive technique for investigating and applying the rosette phenomenon.

8.2 EA Rosettes (Opsonic adherence)

As observed earlier, some populations of mononuclear cells have a membrane receptor for the Fc portion of the IgG molecule.

Demonstration of such receptors, however, depends very much on technical methodology and the state of the immunoglobulin molecule. Although the Fc portion of class-specific heavy chains of immunoglobulin do not actually take part in antigen complexing, it is believed that structural changes are subsequent to the reaction (Roitt, 1972). Heat aggregation of the molecule must induce similar changes, as the Fc receptor of mononuclear cells does not appear to function against unaltered IgG. Whereas heat aggregated IgG is detected by both B cells and macrophages, IgG altered by antigen is only accepted by macrophages in vitro (Hansen and Good, 1974), in a position corresponding to that for cytophilic antibody. Such Fc receptor sites were elegantly demonstrated by isotope labelled antigen-antibody complexes by Basten et al., (1972), but the method of Huber et al., (1969) has been accepted into more general use (Shevach et al., 1974; Frøland and Natvig, 1973; Tønder et al., 1975). Sheep or ox erythrocytes are sensitised with heterologous specific antibody of IgG class (Hallberg et al., 1973; Ross et al., 1973), and thereafter applied to cell suspensions or fresh frozen sections. Opsonification by macrophages gives adherence in rosette formation of the indicator red cells. Exposure of substrate cells to EA suspensions for 30 minutes at room temperature or 37°C appears adequate for sturdy rosette formation revealing 10-26% rosette forming cells in peripheral blood lymphocyte suspensions (IUIS Report, 1975). There are no receptors for the IgM Fc portion on human lymphocytes so IgM EA reagents offer a valuable control in the EA testing system (Jaffe et al., 1974).

8.3 EAC Rosettes (Complement receptors)

This most widely used lymphocyte marker technique revealing complement receptors on the surface of B cells and macrophages has been most thoroughly investigated since its significance was first reported by Bianco et al. in 1970. The method, applied to cell suspensions, monolayers and sections is relatively simple, involving adherence of sensitised indicator cells in rosettes on target lymphocytes (Dukor et al., 1970; Chused et al., 1974)

Indicator System.

Although ox red cells are most successful, sheep cells have been most extensively used (Bianco et al., 1970). Human and monkey erythrocytes have their own surface receptors for complement and are not recommended (IUIS Workshop 1974). Guinea pig cells, by virtue of their large size have been applied very ingeniously with sheep EA reagent at the same time (Shevach et al., 1974). Gelfand et al. (1975) reports the use of fluorescent bacteria as indicator cells in a more sensitive technique. The red cells may be stored in glucose-citrate for approximately one week, but prepared as a washed 5% suspension for use. However, blood from individual animals may vary in susceptibility to sensitising antibodies.

Antibody.

Specific antisera are required to sensitise or coat the indicator cell system as an intermediary for complement binding. These may be specifically prepared in rabbits or goats by immunising with red cells or their stroma (Mayer, 1961), or they may be obtained from other sources such as incomplete anti-Rhesus (D) or anti-Forssman antibodies (Mendes et al., 1974).

The sera should be subjected to DEAE cellulose chromatography to obtain the 19s IgM fraction.

Complement.

The role of complement in antibody-antigen reactions is extremely complex, but fortunately only the early stages of the activated complement cascade are involved in the immune adherence phenomenon of EAC rosettes (Roitt, 1972). The structural changes in the immunoglobulin molecular chains in the Fc portion after antigen attachment activates the complement sequence. This is strong in IgM but weak in IgG, and proceeds in the sequence of conversions of C_1 to C_4 to C_2. Complement factor C_2 activates C_3 to form cleavage products C_{3a} and C_{3b} (Ross et al., 1973). Activated C_3 is the main target of mononuclear cell surface receptors, although work with artificial or naturally occuring complement-deficient sera indicates that factors C_1, C_4 and C_5 may have specific membrane receptors (Seligman et al., 1975). Many sources of complement have been tried in the EAC system with the guinea pig and mouse serum being most widely used (Bianco et al., 1970). Human serum of blood group AB possesses very strong haemolytic properties. Rabbit serum is not used as it seems to show, on occasions, anti-lymphocytic activity (Pellegrino et al., 1975). Sera should be collected and frozen as quickly as possible to preserve labile complement components and absorbed with target erythrocytes before use to remove naturally occurring antibodies that may interfere (WHO/IARC Workshop, 1974).

EAC Reagent

Erythrocyte suspension is exposed to antibody for an hour at 37°C to form an EA reagent. This is further treated with

dilution of complement for another hour to yield an EAC suspension of red cells with surface bound complement. At the inception of the test, and at any point when reagents are changed, the optimum sub-agglutinating and sub-haemolytic dilutions of antiserum and complement may be assessed by a checkerboard titration, performed under the general conditions of reagent preparation.

EAC Rosette Techniques

Lymphocytes and EAC reagent are mixed in the proportions of between 1:50 and 1:100 for incubation at 37°C as a centrifuged pellet. After resuspension the preparation is examined for rosettes of more that 3 red cells to a lymphocyte although they can be further processed as outlined for E rosettes. Tissue sections are treated in a similar manner followed by fixation and staining with haematoxylin and eosin (Silviera et al., 1972; Dukor et al., 1970) or amido black (Allison et al., 1976). E rosettes do not form at 37°C and do not form on frozen sections, so do not interfere in the test (Mendes et al., 1974), although EA rosettes may be a complication if the sensitising antibody was not pure IgM. The addition of EDTA to the indicator system inhibits EAC adherence to macrophages in the mouse (Dukor et al., 1970), but this has been shown not to be successful in human cell systems (Lay et al., 1971). EDTA may prevent erythrocyte clumping if sheep cells are being used (Ross et al., 1973). EAC rosettes detect B cells in peripheral blood lymphocytes between 10% and 19% (WHO Workshop, 1974).

9 CELLULAR ENZYMES (Non specific Esterase)

Meuller (in press) reports that T cells exhibit non-specific

esterase activity as a differential characteristic from B cells which do not. They propose that the criterion may be of value in lymphocyte sub-typing of cell suspensions and sections of lymph node. A most comprehensive, earlier paper by Lam and Yam (1973) reports that the property is by no means unique to T cells; a variety of substrates at two pH values were tested against human leukocytes which all showed some level of esterase activity so it would appear that the technique would have applications only in testing purified lymphocyte suspensions. Dried, fixed films are exposed to a buffered substrate of α-naphthyl acetate with simultaneous coupling with either hexazonium pararosanalin or one of the stabilised diazonium compounds. T cells show submarginal granules indicating sites of enzyme activity.

10 ANTI-LYMPHOCYTE ANTIBODIES.

For over a decade the immunosuppressive effects of antisera prepared against lymphocytes have been utilised in both clinical and research medicine. As it happens, these reagents were usually prepared from peripheral blood lymphocytes with a predominance of T cells. The result in many cases was a crude anti-T cell antiserum exercising its greatest effect on the cellular immune response in graft or transplant rejection and cancer therapy. It is surprising, therefore, that there have been so few reports of the use of specific anti-immunocyte sera in human B and T cell differentiation. There have been a number of reports of successful preparation of anti-T cell sera (Martin and Miller, 1968; Toben and Cooper, 1972; Chin et al., 1973; Aisenberg et al., 1973; Wortis et al., 1973;

Touraine et al., 1973; Goodfellow et al., 1976, and Brochier et al., 1976). It is of interest that in contrast, there are so few reports of anti-B cell sera. Williams et al., (1973) admit failure to produce a successful reagent, but Greaves and Brown (1973) report an antiserum with cytotoxic activity against B cells and another against CLL cells later in 1975. Many authors have reported incidental use of such sera in control of specificity of functional tests such as E and EAC rosettes (Wortiss et al., 1973), or as reagents for small animal research where cytotoxicity has been of major interest (Martin and Miller, 1968). Such techniques measured by ^{51}Cr release from cells killed from a labelled population, or by trypan blue exclusion of dead cells have been extensively used as a check on antibody specificity (Iverson, 1970), on cell identification in suspensions (Aiuti and Wigzell, 1973; Smith et al., 1973), or in histocompatibility testing (Takahashi, 1970). Rolland et al . (1971) first introduced T and B cell identification by specific antisera in the indirect fluorescent antibody test, but confined their work to cell suspensions, so it was not until very recently that antisera have been directly conjugated with fluorochromes for use in immunocyte differentiation or tissue localisation (Goldschneider and Mcgregor, 1973; Husby et al., 1975).

The preparation of conjugated antisera involves a diversity of technical procedures starting with the need for a pure antigen for inoculation into rabbits or other suitable host such as goats or horses. After a suitable immunising course the resultant sera must be fractionated to recover the immunoglobulins which are examined for titre and specificity by in vitro

tests. Any anomalies are corrected by absorption procedures before or after conjugation with fluorochromes, isotopes or horse radish peroxidase. Finally the product must be exhaustively tested against control material before acceptance for use. The results in most cases justify the means as such antisera have been shown to be particularly suitable for tissue localisation of mononuclear cells participating in the immune response.

The techniques outlined above have been standard procedures for preparation of most immunofluorescent reagents (Nairn 1969), so only those problems pertinent to anti-T and B cell sera will be discussed.

Most authors have used either thymocytes from fresh fetal thymus as T cell antigen or peripheral blood lymphocytes from patients with chronic lymphatic leukaemia as a source of B cells. Although it is recognised that the preparations do not represent complete antigenic homogeneity with normal lymphocytes (Albin and Morris, 1973) the latter have not been widely used because of difficulties in specificity and supply. Mouse and dog brain have been suggested as a source of T cell antigen (Greaves and Brown, 1974), but there would appear to be some controversy over the efficacy of human brain for this procedure (Kongshavn, et al., 1974).

There have been various immunisation schedules used for antibody production. The most popular is the "two pulse" technique of Levey and Medawar, (1966), with two intravenous doses of live lymphocytes two weeks apart. The animals were bled at the peak of antibody production on the 21st day after the initial dose. The part played by adjuvants and the duration of

immunisation are of some importance as there are five factors to be considered in planning antiserum production (Gozzo et al., 1971). Clinical immunosuppression, rosette inhibition and specificity are enhanced by adjuvants and by short dose schedules. Antibody titre and cytotoxicity on the other hand are increased by repeated doses of antigen over longer periods. The two pulse method offers a happy compromise.

All sera must be absorbed with at least human red cells of the group AB Rh. (D) positive to remove some histo-compatibility antibodies. Tissue homogenates, usually liver, remove non-specific connective tissue antibodies. Antibody to complementary lymphocytes (T or B cells) which may have contaminated the immunising antigens are removed by absorption with live lymphocytes (Goldschneider and McGregor, 1973 ; Touraine et al., 1974). Some workers have used macrophages, bone marrow suspensions and glutaraldehyde-insolubilised serum proteins, but in practice these refinements may not be necessary.

To visualize antibody-antigen sites, antisera may be conjugated with a variety of agents, although the choice really depends on availability of equipment. Horse radish peroxidase is conjugated with relative ease and visualised with benzidine histochemical techniques for conventional light microscope examination of permanent preparations (Avrameas 1970). Fluorescent dyes such as fluorescein isothiocyanate (FITC), lissamine rhodamine B (RB 200) or dimethylamino naphthalene-5-sulphonic acid (DANS) may be used for labelling antiglobulin (Nairn, 1969), although ultra-violet light microscopy is a necessity and permanent preparations are not possible. Radio-isotopes have also been used as antibody labels in this context, the techn-

iques utilising autoradiographic methods for ultimate light microscopy. ^{131}Iodine (Speafico, 1970) has been used very little, but ^{125}Iodine (Unanue et al., 1971), with its weaker, less hazardous radiation has been successful, particularly for localisation of antibody by electron microscopy (Basten et al., 1972).

There have been a number of commissions whose mandate was standardisation of labelled antibody techniques, especially immunofluorescence (MRC report, 1971). They have established clear guidelines for conjugate definition in terms of protein and fluorochrome content, titre and specificity, the attainment of which should be greatly helped by developments such as Defined Antigen Substrate Spheres (DASS system) for reagent evaluation (Knapp et al., 1974). Improved ultraviolet light microscopy and photometric techniques are permitting more critical interpretation of the immunofluorescent preparations.

Laboratories are now receiving requests from surgeons and pathologists for identification of lymphoid cells in tumours, inflammatory infiltrates and immune phenomena. Of the techniques outlined above, the immunofluorescent method would appear to offer the most convenient system for subtyping lymphocytes in tissues, particularly as it has already been successfully applied to the animal model. The work reported in subsequent pages reflects an attempt to prepare specific antisera that will be capable of describing the distribution of T and B lymphocytes in human lymph nodes.

PART TWO; MATERIALS AND METHODS

11 CELL SUSPENSIONS

The viability of this project depended entirely on the availability of sources of suitable lymphocytes for immunisation, testing and absorptions of projected antisera. Therefore, every opportunity was taken to acquire such cells which were preserved in a frozen state. As samples were often available at awkward times, full characterisation was only done at the time of use.

11.1 Peripheral blood lymphocytes (PBL)

Suspensions of lymphocytes from normal donors were required as substrates in defining lymphocyte parameters for use in initial characterisation of crude antisera. In any sample, there should be reciprocity between the T and B cells making up the total lymphocytes present. Fresh whole blood was the usual source of PBL, with Heparin initially used as anticoagulant (10 units/ml of blood), although it was later found that Sequestrene (EDTA) was equally satisfactory, Twenty millilitres of blood were usually obtained from either laboratory staff, or regular blood transfusion donors. Samples were processed immediately after collection. A later, more rewarding yield of lymphocytes came from regular venesections by the Department of Haematology for collection of plasma. The buffycoats from centrifuged cellular deposits were collected separately in physiological saline and thereafter treated as whole blood.

11.1.1 Separation of lymphocytes

Hypaque-Ficoll gradients (Boyum, 1969) were used most

successfully to recover lymphocytes from whole blood. Hypaque was obtained from Winthrop Laboratories as a 65%, sterile solution in 20 ml ampoules. It was prepared for use by mixing the contents of one ampoule with 19.0 ml of distilled water. Ficoll reagent was prepared by dissolving 9.0 g in 100 ml of distilled water on a magnetic mixer for 30 minutes.

Technique No.1 The use of Hypaque-ficoll gradients to prepare lymphocyte suspensions.

1) The gradient was prepared by mixing 30 ml of dilute Hypaque with 72 ml of 9% ficoll.
2) Specific gravity of the mixture was measured with a hydrometer (1.000 to 2.000) and corrected if necessary to between 1.077 and 1.080 at approximately 15°C. Distilled water was used to lower the specific gravity, and undiluted Hypaque used to raise the specific gravity.
3) The reagent was sterilised by autoclaving and dispensed into 4.0 ml volumes in sterile 15.0 ml screw-capped centrifuge tubes.
4) With a 10.0 ml calibrated pipette, approximately 11.0 ml of whole blood or buffycoat suspension was layered gently onto the gradient mixture.
5) The tubes were centrifuged in a swing-out head machine at 2 300 rpm (90g), for 45 minutes.
6) The disc of cells at the gradient-plasma interface was recovered with a pasteur pipette and pooled into Hank's balanced salt solution.(Small volumes of sterile BSS were obtained from the

Department of Immunology when required).

7) The cells were washed twice by centrifuging suspensions at 90 g, decanting the supernatant and resuspending the pellet in fresh BSS.

8) The final suspension was made up to contain approximately 10^6 to 10^7 lymphocytes/ml (Section 11.1.2).

11.1.2 Assessment of lymphocyte suspensions

One drop of suspension and one drop of 1% Trypan Blue in physiological saline were mixed and pipetted into a Bürker haematological counting chamber. Viability was measured by counting the blue, dead lymphocytes in 100 cells. Concentration was calculated by conventional haematological cell counting techniques. Preparations at this stage showing greater than 70% viability, were recentrifuged and the pellet suspended in 50% fetal calf serum in BSS to give a final concentration of 10^7 cells/ml in preparation for freezing.

11.2 B cells

The selection of a suitable source of B cell antigens was given careful consideration. The ideal of B cells separated from PBL suspensions is logical, but the anticipated consumption made this unsuitable. Assuming approximately 50% recovery from normal blood lymphocyte count of 3 000/mm^3, 100 ml of whole blood would yield only 10^8 cells. Greater yield could be obtained by using peripheral blood lymphocytes from patients in leukaemic phase of chronic lymphatic leukaemia (CLL). Such cells have been shown to share many B cell characteristics:

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complement receptors, surface immunoglobulin and lack of T cell features. (IUIS Report, 1974; Greaves and Brown, 1973; Touraine et al., 1974). Furthermore, CLL cells from mice and men have been used to prepare antisera that were cytotoxic for B cells (Goldschneider and McGregor 1973). As blood samples from chronic lymphatic leukaemia patients were available from the leukaemia clinic of Johannesburg General Hospital via the auspices of the Department of Haematology, it was decided to use this source of B cells throughout this work. With the co-operation of the clinical haematologists, bottles of heparinised blood were collected when required, and processed exactly as for PBL (Sections 11.1.1, 11.1.2).

11.3 T cells

Of the sources of T cells discussed in the literature, all are difficult of access. Lymphocytes from patients with agammaglobulinaemia (B cell deficiency) could not be considered in view of the rarity of the condition (Touraine et al., 1974). Peripheral blood T cells are difficult to recover in any measure of purity (Denman, 1973), but some workers have reported success using infant thymocytes in antisera production. (Smith et al., 1973; Husby et al., 1975). Touraine et al., (1974) suggest that thymic T cells exhibit a lower density of histocompatibility antigens, which would indicate an ideal source for immunisation purposes. Fortunately recent legislation had made potentially useful material available through the Department of Obstetrics and Gynaecology although more problems were encountered than was anticipated. Therapeutic abortions were induced by Prostaglandin, usually on morning

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