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The immuno-histochemical differentiation of human lymphoid cells

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INDEX OF ABBREVIATIONS

BSS	Balanced salt solution
CLL	Chronic lymphatic leukaemia
DMSO	Dimethyl sulphoxide
E	Sheep erythrocytes
EA	Erythrocytes sensitised with antibody
EAC	Erythrocytes-antibody-complement system
FITC	Fluorescein isothiocyanate
Ig	Immunoglobulin
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PTFE	Polytetrafluoroethylene
SIg	Surface bound immunoglobulin

PART ONE. INTRODUCTION

1 HISTORICAL REVIEW

Until the middle of the last decade, the lymphocyte was an enigma to cell biologists, for although its behaviour in disease conditions was familiar, its functions and origins were unrecognised. We laboured under the premise that the lymphoblast and plasmablast were stem cell precursors of separate cell lines; the one giving rise to the large and small lymphocytes of peripheral blood, the other to antibody forming, mature, plasma cells. In 1963, Porter published his Nobel prize winning theory on the structure of immunoglobulin, giving substance to current conjectures on the reactions between antibody and antigen, and the way was open for the new science of Immunology. There followed reappraisals of those diseases in which the lymphocyte was an important feature, so that Hodgkin's disease, myeloma, and agammaglobulinaemia became valuable research tools to support concurrent animal experimentation. Such models indicated that removal of the neonatal thymus led to impairment of the cellular response to antigen such as graft acceptance (reviewed by Craddock et al., 1971). Bursectomy of the chicken indicated that this organ was necessary for the development of humoral immunity (reviewed by Hansen and Good, 1974). By 1969, the immune response was seen as a heterogeneous phenomenon with the lymphocyte as the fundamental figure and so the foundations were laid for the two-cell model of modern immunology (Roitt et al., 1969; Claman and Chaperon,1969). A number of excellent reviews have since described and elaborated upon this concept, emphasising the importance of lymphocyte sub-type differentiation in the elucidation of lymphoid

diseases (Greaves et al., 1973; Möller et al., 1973). Although recent years have seen a wealth of data derived from the application of differential techniques, there are also new problems being posed that indicate that present knowledge is far from complete (Seligman et al., 1975).

2 CHARACTERISTICS OF MONONUCLEAR CELL TYPES

2.1 T Cells (Synonyms; LT; Thymus dependent or derived lymphocytes; Helper cells).

The thymus is believed to draw its primitive stem cell population from the fetal bone marrow or liver.

Development to immunologically competent thymocytes with programming for immune function takes place within and under the influence of the fetal thymus itself. Within the first week of life the cells migrate into peripheral lymphoid tissues to constitute the T cell population (Raff, 1973; Parrott, 1966). With a long life span, the T cells move in a circulation encompassing the peripheral lymphoid tissue, lymphatics, thoracic duct, and blood (Weissman, 1967). However, as resting cells they in no way differ morphologically from other lymphocytes except in a few very critical parameters such as adhesiveness (Denman, 1973; Bianco et al., 1970), or surface characteristics in the scanning electron microscope (Polliack et al., 1973). T cells comprise 40% to 90% of normal peripheral blood lymphocytes (Jondal et al., 1972; Haegert et al., 1974).

2.2 B Cells (Synonyms; LB; Bursal or Bone Marrow derived Lymphocytes; Thymus Independent Lymphocytes; Complement Receptor Lymphocytes).

Fetal haemopoetic organs also supply stem cells to the Bursa

of Fabricius, a small lymphoid organ near the cloaca of the chicken. Development into lymphocytes is accompanied after only a few weeks by immunoglobulin of IgM class on cell membranes followed shortly afterwards by IgG synthesis (Kincade, 1971; Papamichail, 1971). Although a similar sequence of immunoglobulin production in the mammalian fetus has been detected, with a switch from IgM to IgG (Lawton et al., 1974; Van Furth, 1965), there has been considerable controversy over the "Bursal equivalent" in human or rodent fetal development. It has been variously attributed to peripheral lymphoid tissues, liver or bone marrow, although recent work indicates that the latter is the most likely candidate (Howard et al., 1972). Of a life span measured only in days, B cells remain more or less localised in lymphoid tissues with minimal movement through the T cell migratory cycle under normal conditions (Craddock et al., 1971). Under scanning electron microscopy the majority of B cells show a villous surface appearance (Polliack et al., 1973), although this description is not decisive, as there may be a gradation of surface characteristics from the villous B type to the smooth T type. B cells comprise 11% to 34% normal peripheral blood lymphocytes (Haegert et al., 1974).

2.3 Macrophages (Synonyms; Histiocytes; M cells; Monocytes; Effector cells).

The identification and nomenclature of this mononuclear cell series is not clearly defined in the literature in the context of the immune response. M cells are described as blast precursers of the synonymous Macrophage-Histiocyte-Monocyte series of the cells of the reticuloendothelial system. Phagocytosis

of latex particles or finely divided iron is used as an effective basis of differentiation (Greaves et al., 1975), but not all macrophages exhibit this property (Zucker - Franklin, 1974). They may also be separated from mixed populations by their adherence to glass surfaces of petri dishes or glass or nylon columns, but this again is a non-specific phenomenon (Huber et al., 1969; Shortman et al.,1971). The origin and life span of the macrophage are also not clearly defined, although it is probable that M cells populate the adult reticulo - endothelial system from fetal haemopoetic tissues and migrate as mature macrophages through the lymphatics, blood and tissues. Their role in the immune response is recognised as being non-specific receptors of antigen or antigen-antibody complexes, with or without antigen processing, for presentation to immunocompetent cells in the peripheral lymphoid tissues. In obeying the T cell signal they function as effector cells in the cellular response to antigen (Craddock et al., 1971; Isa, 1974).

2. 4 Null Cell

This tenuous group of lymphocytes has been identified mainly in the mouse (Greenburg et al., 1973) as a small population that are lacking identifying characteristics of any of the above types yet retain antibody dependent cytotoxicity. Greenburg proposed that they were monocytic cells, but Chess et al., (1975) found that null cells produced immunoglobulin after 2 to 3 days in tissue culture, suggesting thereby that they were B cell precursors or a subset of B cells.

3 CELLULAR INVOLVEMENT IN ANTIGEN PROCESSING

The formation and nature of antigen recognition sites on

lymphocyte membranes is still a controversial subject. Surface immunoglobulin molecules on most B cells are believed to act as antigen receptors with the structural variability of the Fc portion conferring specificity (Capra and Kehoe, 1974; Cunningham et al., 1974). However, the presence of analagous material on the surface of T cells or macrophages has proved difficult to demonstrate (Crone et al., 1972; Cone and Marchalonis, 1973). Feldman et al., (1974) have indicated the presence of "IgT" on the surface of activated T cells, but its function appears to lie more in T and B cell interaction after antigen exposure. Most workers accept that the macrophage functions as an intermediary in transporting phagocytosed antigen to receptor lymphocytes where intimate cell wall contact occurs accompanied, it is assumed, by transfer of antigenic material (Huber and Fudenberg, 1968). Such recognition leads to stimulation of lymphocytes to blast cells (large lymphocytes) with clonal expansion. T cell clones then elaborate non-specific lymphokines which mediate the many facets of the cellular response. Amongst these are chemotactic factors responsible for granulocyte mobilisation, and migration inhibition factors to restrict movement of macrophages. Mitogens induce expansion of other cell populations involved in the immune response, including B cells (Craddock et al., 1971). B cell transformation to plasma cells is accompanied by immunoglobulin synthesis, initially IgM but with a switch to IgG. Some antigens termed thymus-independent antigens can stimulate B cells to a humoral response directly without

T cell help. Such antigens, however, (some bacterial pathogens and polysaccharides) achieve no more than a weak IgM response; the switch to IgG is obviously dependent on T cell activation (Claman and Chaperon, 1969).

Present data does not offer a conclusive explanation of memory cell origin. One group suggest that upon withdrawal of antigen, clone contraction leaves a residual T cell population, stimulated for specific antigen in constant circulation (Cerottini et al., 1974). The opposing school advocates that memory cells are produced as a B cell sub-type at the time of the primary clonal expansion, but they do not participate in the immediate response (Greaves et al.,1973). It is known, however, that after completion of initial antigen processing in the regional lymph nodes, stimulated T cells migrate throughout the rest of the lymphoid system activating both B and T cells (Howard et al., 1972; Sprent, 1973).

Two aspects of immunity that are worthy of consideration at this point are autoimmunity and tolerance which are very plausibly explained by the dichotomous model. The most popular explanation of tolerance postulates the existence of "Supressor T cells" which are exposed to fetal tissues, thereby recognising self-antigens and so switching off, or blocking, B cells which are coded for such antigens. Similarly, the introduction of foreign antigen in utero, perhaps in modified form, affects coding of specific suppressor T cells thus preventing the humoral response with the induction of acquired immunity. It would follow then that if there were a breakdown of self surveillance by supressor T cells then self-coded B cells would be allowed to synthesise autoantibodies (Möller,

1973). As more and more diseases are being identified with circulating autoantibodies, this aspect of immunopathology is receiving increasing attention.

4 APPLICATIONS OF THE TWO CELL MODEL

Since 1971 there has been a wealth of published material reporting the application of techniques of B and T cell differentiation to many diverse disease conditions. Some of these, notably the primary immune dyscrasias such as agammaglobulinaemias and Hodgkins disease, have served as research tools to confirm in human beings the experimental models defined in laboratory animals. Intensive investigation of the lymphoproliferative disorders has yielded data which is constantly placing currently held systems of nomenclature under review (Hansen and Good, 1974). It is generally proposed therefore that simple standardised techniques for lymphocyte subtyping will play a valuable role in diagnosis and future research (Wybran and Fudenberg, 1973; Huth, 1974).

5 TECHNIQUES FOR LYMPHOCYTE SUBTYPING

Many aspects of lymphoid function are still under intensive examination by leading research groups throughout the world, so our present state of knowledge is surely incomplete. Techniques presently available reflect an incomplete picture with many anomalies in the biological definition of mononuclear cells. There have been attempts at standardisation of laboratory methods in order to achieve some measure of conformity in results (I U I S Report, 1975; W H O - I A R C Workshop, 1974), but there remain so many variables that lymphocyte

subtyping is still fairly crude. At best a laboratory must carefully define its techniques and normals with strictest adherence to the protocol so established to gain any meaningful data. As most of the methods available at present depend on functional characteristics it is a prerequisite that the cells be viable at least in the particular function under examination. Only recently variations have been introduced that allow assessment of subtype distributions in cryostat sections, although cell suspensions have been, and still are, the main tool in lymphocyte research.

Of the techniques available, those demonstrating receptor sites on cell surfaces are most important, although in many cases the nature or even function of such reactions are but imperfectly understood. This is particularly so of the widely used E rosette test whereby T cells form fragile rosettes with sheep erythrocytes.

Receptors for the Fc portion of the IgG molecule with or without antigen modification are possessed by B cells, macrophages and polymorphonuclear leucocytes in various forms, reflecting a function in antigen processing. Receptors for bound complement using sensitised heterologous erythrocytes as a system indicator also reveal a functional characteristic of B cells and macrophages.

Immunoglobulin in B cells either as surface granules acting as antigen receptor sites (SIg) or cytoplasmic production in the stimulated plasma cell is conveniently demonstrated by fluorescent antibody or immunoperoxidase techniques. All cell types may be differentiated by their reactions to specific antisera either in fluorescent antibody tests or

cytotoxic effects. Enzyme histochemistry has shown non-specific esterase activity to be a possession of T cells (Table 1).

6 PREPARATION OF MATERIAL FOR STUDY

6.1. Peripheral Blood Lymphocytes

A pure suspension of viable lymphocytes is the most valuable and widely used tool in the field of cellular immunology but although such preparations have been in use since the inception of anti-lymphocytic serum for immunosuppression over a decade ago, attainment of purity and yield still offers difficulties. Much of the success of modern techniques must be credited to Boyum (1968). Although not the first to use density gradients, he investigated the subject so thoroughly that his recommendations have been used since with only minor modifications (Thorsby and Bratlie, 1970). Having established the factors that affect cell separation to be viscosity, density, osmolarity, and rouleaux formation, Boyum devised a reagent to give almost pure lymphocyte separations. One part was a high viscosity low density solution such as methyl cellulose, dextran, ficoll or polyvinyl pyrrolidone, with marked rouleaux inducing properties. The other part required low viscosity high density characteristics, although Boyum's "Isopaque" (Sodium N methyl 3-5 diacetamido 2-4-6 tri-iodo benzoate) has been replaced by sodium diatrizoate ("Hypaque" of urological medicine). When these are mixed to give a density of 1.077 and whole blood added as an upper layer, centrifugation yields an almost pure lymphocyte disc at the interface with minimal erythrocyte or granulocyte contamination.

Less successful techniques use gelatin, dextran or

Table 1 Differentiation of human mononuclear cells by marker systems

Cell marker	B cells	T cells	Macrophages
Sheep cell rosettes	-	+	-
Fc receptors	+	-	+
Complement receptors	+	-	+
Surface immunoglobulin	+	-	-
Non-specific esterase	-	+	+

"Plasmagel" which induce enhanced rouleaux formation with rapid erythrocyte sedimentation when added to whole blood. The leucocyte-rich supernatant must be cleared of granulocytes and monocytes by allowing them to phagocytose iron particles, followed by removal by a strong magnet. Final exposure to ammonium chloride to lyse residual red cells leaves lymphocytes in anything but their pristine condition with a marked propensity to clump.

When suspended in an adequate support medium such as serum-supplemented basal salt solution, cells remain viable for up to 48 hours. For prolonged storage, successful freezing techniques have been developed using dimethyl sulphoxide (D M S O) as a cryoprotectant. In simple reagents with glucose and serum, lymphocytes may be stored for extended periods at -80^{o}C or -170^{o}C with little loss of viability (Coombs et al., 1970; Wood et al., 1972).

6.2 Tissue Suspensions

Much experimental research on lymphocyte distributions in normal or pathological anatomy has been performed on cell suspensions derived from tissues. As the majority of techniques for typing are directed against surface markers, viable cells are usually necessary. Most workers have reported successful recovery after sieving chopped tissues into balanced salt solution followed by thorough centrifugal washing. Lymphoid material gives satisfactory suspensions by this method, but non-lymphoid tissue may require further purification on density gradients. To ensure viability, surgical material has been most extensively used, but cadaverous

specimens may be successfully used up to 6 hours post morten (Silviera et al., 1972). There is, however, some conflict of opinion on how faithful an image of in vivo cellular distribution is achieved by such separation methods.

All cell suspensions must be tested for the following;

1) Cell count by the simple expedient of direct examination in the haematological counting chamber.
2) Viability by staining dead cells with trypan blue or live cells by fluorescein.
3) Morphological differentiation by haematoxylin/eosin or romanowsky stains.
4) Monocyte contamination detected by phagocytosis of inert latex particles.

7 IMMUNOGLOBULIN; ITS RELATIONSHIP WITH IMMUNOCYTES.

Plasma cells are believed to be transformed B cells producing antibody in response to antigenic stimulation. Demonstration of immunoglobulin as a diffuse reactive material throughout the cell cytoplasm may be simply achieved by treatment of cells with antisera prepared against immunoglobulin classes or isolated chains of the Ig molecule. Fluorescent dyes (Johnson and Holborow, 1973), radio-isotopes (Wilson and Nossal, 1971), or peroxidase (Avrameas, 1970) may be used as markers in these techniques which have been in use for over a decade in a wide range of applications (Crabbé et al., 1965). However, immunoglobulin may also be demonstrated as a fine granular reaction

on the membrane surface of unstimulated lymphocytes (SIg). After exposure to antigen this material polarises to form a protein cap when studied in in-vitro experiments; a process that is inhibited by inclusion of sodium azide in the system for SIg demonstration (Taylor et al., 1971). SIg is mostly IgM (Rabellino et al., 1971; Frøland and Natvig 1972; Papamichail et al., 1971), but there is great interest at present in the finding of IgD on most B cells. This immunoglobulin is detected in only trace amounts in serum (Van Boxel et al., 1972; Seligman et al., 1975). It is believed to function as an antigen-dependent trigger for IgM production. The most critical technique for demonstration of SIg involves removal of surface globulin by trypsin from cells in culture followed later by examination for re-synthesised product by immunofluorescence (Preud'homme and Seligman, 1972). There have been scanty reports of surface Ig on a small percentage of T cells (Cone and Marchalonis, 1973), but the demonstration of surface granules by direct immunofluorescence on viable cell suspensions is still widely used as a B cell marker.

There are also receptors for immunoglobulin itself; non-specific receptors for a part of the IgG molecule known as the Fc portion after papain fragmentation (Dickler and Kunkel,1972). These Fc receptors are believed to play a part in initial antigen processing, being present on the surface of B lymphocytes and macrophages, and show greater reactivity when the immunoglobulin is bound to antigen (Basten et al., 1972). Two techniques are available for demonstration of the Fc receptor; EA rosettes (see later) reveal only macrophage activity, but both B lymphocyte and macrophage receptors may be detected

using pure immunoglobulin G, aggregated by heat at 63°C and conjugated with fluorochrome (Dickler and Kunkel, 1972). Unfortunately such aggregates of denatured protein are present in most immunofluorescent immunoglobulin reagents. As they may simulate surface immunoglobulin on lymphocyte membranes, conjugates should be clarified by centrifugation before being used for SIg testing.

The most important considerations of immunofluorescent tests lie in adequate control of the reaction. Treatment of cells or sections with properly diluted specific antisera in buffered saline for 30 minutes is a simple procedure,as is examination by ultraviolet light microscopy after selection of suitable filters. However interpretation of the results and careful exclusion of false positive or false negative reactions by absorption, blocking or control techniques offers a technical challenge of wide variability.

8 ERYTHROCYTE ROSETTE TECHNIQUES.

8.1 E Rosettes (Non-immune rosettes)

The phenomenon of viable human T cells forming rosettes with heterologous red cells, although known for some time (Brain et al., 1970; Coombs et al., 1970), is still as yet unexplained. But once procedural standardisation and reproducibility have been established the technique remains the only successful marker for T cells in extensive use (Lay et al., 1971; Jondal et al., 1972; Bach, 1973). The reaction appears to be essentially temperature dependent and optimal with sheep red cells, although variations in exposure times are claimed to define two T cell subtypes; "total" rosette forming potential with

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