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BIOCHEMICAL STUDIES OF REGULATORY
MOLECULES IN THE IMMUNE RESPONSE

A THESIS

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ABSTRACT

Human T cell hybrids were produced following the polyethylene glycol-mediated fusion of concanavalin-A-activated peripheral blood lymphocytes with a 6-thioguanine-resistant subline of the human T cell acute lymphocytic leukemia line CCRF-CEM.

Four T cell hybrid lines were established by hypoxanthine-aminopterin-thymidine selection and flow cytometric selection of the OKT3⁺ hybrids by sterile cell sorting on a FACS IV. Cell surface phenotype and the acquisition of functional hypoxanthine guanine phosphoribosyl transferase activity following hybridisation was used to confirm true human T cell hybrids were produced.

Two assay systems, enabling the measurement of human in vitro antibody responses were developed and validated. Using one of these assays, a mitogen-dependent system, it was demonstrated that hybrid line TT-4 constitutively produced an activity which suppressed pokeweed mitogen-induced antibody production. In addition, it was demonstrated that the TT-4-derived activity was capable of suppressing the proliferative response of normal lectin-activated T cells to exogenous sources of interleukin-2.

Preliminary biochemical studies of the hybrid-derived activity were undertaken. Sephacryl S-200 chromatography produced three major peaks of suppressor activity (>250, 120-140, 60-80 Kdal). The activity was partially acid and alkali labile, dithiothreitol sensitive, affected by the monosaccharides α -methyl mannoside, L-fucose and N-acetyl glucosamine but not by L-rhamnose.

These studies confirm that somatic cell hybridisation techniques can be successfully applied for the production of human regulatory lymphokines.

BIOCHEMICAL STUDIES OF REGULATORY MOLECULES IN THE IMMUNE RESPONSE

by

P.R. Macdonald

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LIST OF ABBREVIATIONS

AEF	allogeneic effect factor
APC	antigen presenting cell
8AG	8-azaguanine
AH	azaserine-hypoxanthine selection medium
B cell	B lymphocyte
BCGF	B cell growth factor
cDNA	cloned deoxyribonucleic acid
CM	conditioned medium
CMI	cell mediated immunity
ConA	Concanavalin A
CSF	colony stimulating factor
CTL	cytotoxic T lymphocyte
DMSO	dimethyl sulphoxide
DNase	deoxyribonuclease
DNP-BSA	dinitrophenyl linked to bovine serum albumin
DNP-KLH	dinitrophenyl linked to keyhole limpet hemocyanin
DTT	dithiothreitol
ELISA	enzyme linked immunoabsorbent assay
EMS	ethyl methane sulphonate
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
FITC	fluorescein isothiocyanate
HAT	hypoxanthine-aminopterin-thymidine selection medium
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid
HGPRT	hypoxanthine guanine phosphoribosyl transferase
³ H-HX	tritiated hypoxanthine
³ H-TdR	tritiated thymidine
IFN	interferon
Ig	immunoglobulin
IL-1	interleukin 1
IL-2	interleukin 2
IL-3	interleukin 3
Kd	dissociation constant
Kdal	kilodalton
KLH	keyhole limpet hemocyanin
LCL	lymphoblastoid cell line
McAb	monoclonal antibody

MHC	major histocompatibility complex
MLC	mixed lymphocyte culture
MW	molecular weight
PBL	peripheral blood lymphocyte
PBS	phosphate buffered saline
PEG	polyethylene glycol
PHA	phytohemagglutinin
PWM	pokeweed mitogen
RIA	radioimmunoassay
RPMI	Rosewell Park Memorial Institute tissue culture medium
SRBC	sheep red blood cell
T-ALL	T cell acute lymphocytic leukemia
T cell	T lymphocyte
TCGF	T cell growth factor
TdT	terminal deoxynucleotidyl transferase
Th	helper T cell
ThF	T helper factor
TK	thymidine kinase
TRF	T cell replacing factor
TsF	T suppressor factor
6TG	6-thioguanine
V _H	Ig heavy chain variable region
20 α SDH	20 α -steroid dehydrogenase

BACKGROUND

The work presented in this thesis forms part of a larger study being undertaken in the Department of Medicine, Christchurch Clinical School of Medicine investigating human immune response regulation. Studies undertaken in this laboratory have centered on the regulation of human cytotoxic T lymphocyte activation by suppressor lymphocytes and by a mixed lymphocyte culture-derived suppressor factor (Crosier & Broom 1981a,b).

During the course of these studies it became apparent that alternative, stable sources of suppressor lymphokines would be necessary before detailed studies of the biochemical nature of these lymphokines could be undertaken.

My brief was composed of two parts :

- 1) to develop somatic cell hybrids as alternative sources of human suppressor lymphokines, and
- 2) to determine if these suppressor lymphokines affected other types of human immune responses.

It was decided to investigate the role, if any, of hybrid-derived lymphokines in the regulation of human antibody responses. This necessitated the development of assay systems capable of measuring human in vitro antibody responses.

CHAPTER 1.

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

"The immune system is an infinitely complex and finely tuned network of cells, receptors and molecules which interact with one another in a genetically controlled manner that is manifested ultimately in the process known as differentiation" (Katz, 1977).

The essential function of an immune system is defence against pathogens and tumors. Lower animals possess innate (non specific) immune mechanisms, for example phagocytosis of bacteria by specialised cells. In addition higher animals have evolved an adaptive or acquired immune response. This type of immune response is flexible, specific and provides a more effective protection against infection. Adaptive immune responses display three important characteristics; memory, specificity and the ability to recognise "non-self". Initial contact with antigen imparts some memory which allows a rapid secondary response if the same antigen is encountered at some later stage. The establishment of memory or immunity by one antigen does not confer protection against another unrelated antigen, hence the specificity of immune responses. The ability to recognise one antigen and to distinguish it from another goes further to enable the recognition of "foreignness" and the discrimination of "self" antigens from "non self" antigens. A failure of this important discrimination can lead to autoimmune disease.

On theoretical grounds, what would be the most efficient way to organise an immune system which could adequately protect an animal from infection and display the ideal characteristics of memory, specificity and the recognition of "non self" ?

The endocrine and nervous systems have evolved in such a way that all the specialised organs and tissues can work in a fully integrated manner. Integration is achieved by two systems; firstly by means of electrical impulses and secondly by chemical substances, or hormones, which are produced in certain tissues and glands and are distributed throughout the body by means of the circulatory system. Hormones therefore produce their effects

by influencing the activities of their target organs, which in most cases are spatially separated from the cells producing them. Hormones are therefore the chemical messengers which integrate the endocrine system. The immune system has a number of parallels to the endocrine and nervous systems. To enable a fully integrated response to foreign "antigens", the immune system is organised as sets of clones of cells and each clone expresses on its surface receptors for a particular antigenic structure. A foreign antigen, if it crosses the external barriers of the body, under normal circumstances will be recognised by a particular antigen-reactive clone of cells. Specific antigens therefore select a responding clone and cause its activation. This clone of antigen-reactive cells can then act to eliminate the threat via its effector mechanisms. In a manner analogous to the endocrine system, the immune system can integrate this cellular response to antigen by secreting molecules ("immunological hormones") which can either recruit other immunocompetent cells or can amplify the response by expanding the numbers of antigen-reactive cells. Organising an immune system in such a way that utilises specific antigen-reactive cells and "immunological hormones" enables a faster, more integrated response to antigen than would be possible with a generalised mechanism of recirculating cells of multiple specificity and reactivity. This form of regulation also enables the down-regulation of immune responses when the invading antigen is no longer a threat.

1.2 ORGANISATION OF THE IMMUNE SYSTEM

Tissue culture techniques have permitted the closer investigation of this concept of integration of immune responses by selected populations of cells under conditions which can be controlled to a greater extent than is possible in intact animals. Murine and human studies have established that an effective immune response involves complex interactions between effector and regulatory cells of the haematolymphoid system.

To understand these interactions one must first consider, in general terms, the organisation of the immune system. Lymphoid cells of the haematolymphoid system are divided into two major classes, T lymphocytes (T cells) and B lymphocytes (B cells). Immune molecules associated with, or secreted by these two classes of cells, have two important roles in immune responses. One is protective and the other regulatory. This protective role requires two classes of effector molecules to deal with the major classes of infectious disease. The

regulatory functions of the immune cells determines self-nonsel self discrimination as well as the magnitude of the responses.

Humoral antibody (an effector molecule) is immunoglobulin secreted by activated B cells and has a major protective role in the defence against extra-cellular pathogens e.g. bacteria, fungi, protozoa. Cell mediated effector molecules are non-immunoglobulin in nature and act as antigen receptors on thymus-derived effector T cells. Effector T cells are concerned with combatting intracellular pathogens e.g. viruses, tumorigenic cells, rickettsia. Humoral antibody plays a regulatory role via antigen-specific feedback and can either enhance or inhibit responsiveness. There is a fundamental asymmetry in the immune system i.e. T cells are required for the induction of B cells and not vice versa. Regulatory T cells are seen as the controllers of immune responsiveness. The function of T cells as regulators or effectors results from their interaction in mediating precisely controlled cell-cell signalling interactions.

Different T cell subpopulations constitute the effectors or regulators e.g. delayed type hypersensitivity T cells can mediate effector functions in vivo. Cytotoxic T cells also act as effectors by lysing virally infected target cells. In contrast, regulatory T cells can act to augment (T helper cells, Th) or suppress (T suppressor cells, Ts) immune responses. The first important concept of the immune system therefore is the organisation of lymphocytes as sets of clones of effector and regulatory cells.

The second concept is that an immune response (and its regulation) results from the interaction of these clones of cells. T cells are known to interact with B cells and macrophages as well as other T cells (Katz, 1977). These interactions result in the modification of the immune response. As stated above this modification may be either positive (help), negative (suppression), or more likely some combination of both. This overall effect is referred to as immunoregulation. Every type of immune response has now been shown to be affected by immunoregulation. These include antibody production (Rowley & Fitch, 1964), changes in immunoglobulin isotype expression (Kishimoto et al, 1978), changes in antibody specificity and binding avidity (Gershon & Paul, 1971), termination of antibody responses (Taylor & Bastan, 1976), T and B cell proliferation (Rich & Rich, 1974), activation of cytotoxic T cells (Altman et al, 1979), and even the regulatory mechanisms themselves (Durum et al, 1980).

The concept of cellular interaction in immunoregulation was first

demonstrated in the studies by Claman et al (1966), Davies et al (1967) and Miller & Mitchell (1968). These groups established that Th cells interact with B cells in the development of antibody responses. These observations lead to the third important feature of the immune system, the idea that soluble factors secreted by lymphocytes mediate these interactions by acting as communication signals between the cells producing them and other cells carrying the relevant acceptor/receptor sites for the factors. The first report by Dutton et al (1971), that a mediator present in the supernatant of allogeneic mixed leukocyte cultures could augment antibody responses against sheep red blood cells in vitro paved the way for the identification of numerous other immunoregulatory factors.

These studies have shown that soluble mediators produced predominantly by activated T cells can have profound regulatory effects on the immune system, as well as other systems of the multicellular organism.

This network of immunoregulatory factors is exceedingly complex when one considers that numerous soluble factors (lymphokines) can be generated by stimulating T cells with specific heterologous antigens, alloantigens or mitogens. These lymphokines can suppress or help immune responses, are antigen-specific or nonspecific, bear or lack antigenic determinants encoded by the major histocompatibility complex and are either major histocompatibility-restricted or unrestricted in their action on target cells.

1.3 CONVENTIONAL LYMPHOKINE SOURCES

1.3.1 Limitations of conventional lymphokine sources

Even though a large number of publications have described T cell-derived lymphokines and their effects on biological responses, there are still major difficulties in determining the structure-function relationships and biological significance of these lymphokines. These difficulties result from the shortcomings of conventionally derived lymphokine preparations;

- (1) the heterogeneous producer cell population and
- (2) the limited amounts available for detailed biochemical analysis.

T cell subpopulations or clones can be distinguished by function or on the basis of cell surface receptor expression. Highly purified T cell subpopulations have become possible through the use of monoclonal antibodies to cell surface

receptors (Reinherz et al, 1979), but since macrophages (or other types of antigen processing cells) and their secreted products are necessary for T cell activation in conventional cultures, it is difficult to exclude the contribution of non-T cells to the activities detected in the culture. It is likely therefore that conventional lymphokine preparations will contain factors produced by different T cell subpopulations as well as those arising from non-T cells. Workers have reported culture supernatants containing mixtures of a) antigen-specific or non-specific helper and suppressor factors (Kilburn et al, 1979; Tokuhisa et al, 1978; Kishimoto & Ishizaka, 1974) or b) antigen-specific suppressor factors produced by at least two different Ts subpopulations (Hirai & Nisonoff, 1980). This heterogeneity could lead to incorrect interpretation of the biological role of the factors; if for example, a suppressor factor is masking the effect of a helper factor.

Normal lymphocytes have a finite and usually short lifetime in culture, therefore it is in general difficult to obtain uniform and homogeneous lymphokine preparations in large enough quantities to allow detailed biochemical purifications and structural studies to be undertaken. As the biological assays available to identify these lymphokines have expanded so have the numbers of lymphokine activities reported increased. This had led to confusion in some cases in deciding whether two distinct biological activities are mediated by the same or by different molecules (Watson et al, 1979; Farrar et al, 1980a; Aarden et al, 1979).

1.3.2 New approaches

Lymphokine biologists in the last five years have attempted to overcome these problems by establishing stable and homogeneous T cell lines secreting distinct lymphokines. For these two important criteria to be met, the lymphokine-producing T cell line should be monoclonal, or at least highly enriched, and have a long lifespan in culture. Enrichment of the desired T cell subpopulation is now possible. Monoclonal antibody staining and positive selection utilising fluorescence activated cell sorting, negative cell depletions using monoclonal antibody and complement-mediated lysis or conventional limiting dilution cloning are now widely used. Techniques allowing the immortalisation of the derived T cell lines have also been refined in recent years (Fathman & Fitch, 1982).

1.4 ALTERNATIVE LYMPHOKINE SOURCES

1.4.1 Introduction

Techniques allowing the immortalisation of lymphokine producing cell

lines have been developed for normal as well as neoplastic T cells. Neoplastic T cells have been selected for constitutive or induced lymphokine secretion or they have been fused with lymphokine-secreting normal T cells using somatic cell hybridisation techniques. Normal T cells can now be maintained in culture by the addition of "growth factors" to the culture medium, or in some instances in the addition to "growth factors", by repeated stimulation of the cells with antigen. These three techniques all have advantages over the conventional production of lymphokine-containing "conditioned-medium" which involves stimulating a mixed population of cells with antigen or mitogen to produce a diverse population of lymphokines (see Sections 1.4.2 - 1.4.4).

1.4.2 Neoplastic T cell lines

During the last ten years investigators have attempted to develop model systems which fulfill the requirements of clonality and immortality by lymphokine-secreting lymphocytes. The basic assumption that tumors are derived from a single transformed cell have prompted workers to try adapting human and murine T cell leukemias and lymphomas to long-term growth in culture (Minowada et al, 1972). This inherent clonality probably represents normal lymphocytes which have been "locked" in a certain stage of differentiation and which are unable to proceed further along their differentiation pathway.

Plasmacytomas (neoplastic mature immunoglobulin-secreting B cells) have been useful in the study of antibody diversity and the B cell receptor for antigen. B cell tumors as well as secreting homogeneous immunoglobulin molecules have also been shown in some cases to retain other functions characteristic of normal B cells such as responsiveness to activation and regulatory signals (Moller, 1977).

Even though a number of human and murine T cell leukemias and lymphomas can now be successfully grown in culture (Gillis & Watson, 1980a; Ruscetti & Gallo, 1981; Farrar et al, 1980b; Gillis et al, 1980b), few definitive methods for reproducibly culturing neoplastic T cells are available. Little is known regarding the capacity of these lines to perform particular immunological functions.

Few neoplastic T cells have been described which exhibit antigen-specific functions. Glade & Hirschorn (1970) though, reported that a number of human lymphoid cell lines could secrete various antigen-nonspecific lymphokines.

As new techniques have become available to define more precisely both the cell surface phenotype and functional characteristics of murine and human neoplastic T cell lines, so have a number of antigen-nonspecific lymphokine activities been identified. In some instances these lymphokines have been characterised (discussed later).

Antigen-specific neoplastic T cell clones have been developed in mice (Finn et al, 1979) by priming T cells with keyhole limpet hemocyanin and infecting these in vitro with radiation leukemia virus. These infected cells were propagated by injecting them into the thymus of syngeneic recipients. Some antigen-specific cells derived in this way were shown to serve as carrier-specific Th cells in both in vivo and in vitro responses to dinitrophenyl-KLH conjugates (DNP-KLH). Later studies confirmed that these techniques could be used to create neoplastic murine T cell lines which secrete antigen-specific factors (Eshhar, 1982).

Similar approaches using human T cells primed and transformed in vitro have not been reported. Human B cell transformations using Epstein-Barr Virus have been widely used to generate lymphoblastoid cell lines (Baumal et al, 1971). Additional human neoplastic T cell lines will undoubtedly be established in the future as the techniques enabling functional and phenotypic studies of neoplastic T cells are now widely used.

1.4.3 Normal T cell lines

Studies by MacDonald et al (1974), demonstrated that antigen-specific T cells could be grown in culture for extended periods if they were periodically restimulated with alloantigen. In general though, these lines eventually stopped growing and ceased secreting biological factors.

In 1976, Morgan et al reported that conditioned-medium derived from phytohemagglutinin (PHA)-stimulated human lymphocytes could support the growth of other activated human lymphocytes. Surface phenotype and functional studies demonstrated that the proliferating cells were in fact normal T cells (Ruscetti et al, 1977). Gillis and Smith (1977) named the factor responsible for this proliferation, T cell growth factor (TCGF). TCGF was later renamed interleukin 2 (IL-2), to ease the difficulties relating to a number of lymphokines described by different groups on the basis of function, but which probably represented the same molecular entity (Aarden et al, 1979). The terms TCGF

and IL-2 are used interchangeably, but strictly, crude TCGF refers to a soluble lymphokine defined by its ability to support the long-term growth of mitogen or antigen-activated lymphocytes. Both murine and human IL-2 have been biochemically characterised (Watson et al, 1979; Gillis et al, 1980c). A cDNA coding for human IL-2 has also been cloned. The DNA sequence corresponding to the putative human IL-2 gene has also been determined (Taniguchi et al, 1983). IL-2, in some cases in association with repeated antigen stimulation, has enabled the establishment of T cell lines with cytotoxic functions (Gillis & Smith, 1977; Baker et al, 1979). Improvements in technology have assisted the progression from T cell lines to T cell clones to the extent that antigen-reactive clones specific for alloantigens, particulate antigens and now soluble protein antigens have been reported (reviewed in Fathman & Fitch, 1982). It has not been established though to what extent these "IL-2-addicted" T cells truly represent normal T cells. Chromosomal aberrations have been found in certain IL-2-dependent murine cytotoxic T cell lines, but these do not appear to be malignant in vivo (Nabholz et al, 1980).

Attempts to create monoclonal T cell lines usually rely on the repeated stimulation in vitro with antigen plus antigen presenting cells in the presence of IL-2 (Apte et al, 1981). These approaches create several potential problems, for example a biased selection of a T cell subpopulation which grows well in culture, selective stimulation of T cells responsive to IL-2 but not antigen-specific, and the selection of a T cell subpopulation with abnormal specificities for antigen.

1.4.4 Somatic cell hybrids

A third approach to the problem of developing stable sources of regulatory lymphokines has taken advantage of the techniques of somatic cell hybridisation as refined by Kohler & Milstein (1975) for the production of B cell hybridomas secreting antibodies of defined specificity. This monoclonal antibody (McAb) technology has revolutionised modern immunology (reviewed by Katz, 1982). T cell somatic cell hybridisation allows the fusion of a single lymphokine-secreting normal or activated T cell to a selected neoplastic T cell. The resulting hybrid is immortalised due to the property of the neoplastic cell to grow indefinitely, thereby preserving the secretory function of the normal lymphocyte. This approach was initially applied to murine T cells (Hammerling, 1977; Goldsby et al, 1977; Kohler et al, 1977) and was adapted by Grillot-Courvalin et al (1981) to enable the isolation of human T cell hybridomas with specific T cell functions.

This first described human hybridoma was prepared from the fusion of a bromodeoxyuridine-resistant (thymidine kinase negative) derivative of the human T cell acute lymphocytic leukemia (T-ALL) line KE37 with peripheral blood T cells from a patient with agammaglobulinemia. One of the hybrid lines isolated constitutively secreted a factor which suppressed B cell differentiation induced by pokeweed mitogen (PWM).

The biochemical rationale enabling the construction of human T cell hybridomas will be discussed in Section 1.8.2. A number of functional human T cell hybridomas constructed by other workers will be discussed in Section 1.8.

1.5 SELECTION OF T CELLS FOR IMMORTALISATION

Selecting and enriching the desired activated T cells is vital, especially in the case of lines expressing antigen-specific functions. The proportion of cells specific for antigen, following primary antigenic stimulation, is often very low, therefore it is desirable to enrich the antigen-specific population before they are immortalised.

Antigen-specific human Th or cytotoxic T cells are usually enriched by repeated stimulation *in vitro* (Bach et al, 1979). This treatment eliminates most of the irrelevant T cells and highly enriches for antigen-specific cells.

Selective activation and enrichment of Ts cells has been possible with high doses of soluble antigens or high doses of T cell specific mitogens (Rich & Pierce, 1973; Uytdehaag et al, 1979; Taniguchi & Miller, 1978; Peavy & Pierce, 1974).

The advent of monoclonal antibodies and fluorescence activated cell sorting has enabled the selection of T cell subpopulations depending on their specific membrane phenotype expression (Reinherz et al, 1979).

1.6 BIOLOGICAL ASSAYS FOR LYMPHOKINE ACTIVITY

Assuming one is able to generate stable, homogeneous and immortal cell lines the next step is screening the derived lines for constitutive or inducible lymphokine activity. As only a very limited number of lymphokines have been purified to homogeneity, the assays for these lymphokines are still primarily

biological in nature. For this reason it is often difficult to determine if two distinct lymphokines possess the same biological activity or whether one lymphokine species may mediate different biological effects.

Most of the assays currently available have two major limitations. Firstly, since most preparations have more than one activity present, it is difficult to ascribe a particular activity to a given lymphokine. Secondly, the target cells used to assay lymphokine activities are often heterogeneous in nature.

Cloned cell lines with an absolute requirement for a particular lymphokine, such as the IL-2-dependent CTLL-2 line (Gillis & Smith, 1977), will help to overcome these difficulties in assigning biological activity to a particular molecule. In the case mentioned above, IL-2 activity is measured by its ability to promote the growth of this IL-2-dependent cytotoxic T cell line. By using a cloned T cell line, it is clear that IL-2 acts directly on a T cell. Similarly the interleukin-1 (IL-1) bioassay developed by Gillis & Mizel (1981) relies on a cloned line, in this instance derived from a murine T cell lymphoma. This cloned line responds to Concanavalin A (ConA)-stimulation by producing IL-2, but only when it is costimulated with the macrophage-derived lymphokine IL-1. This therefore provides a definitive assay for IL-1 activity.

The development of antibodies, in particular McAbs, to specific factors will greatly advance lymphokine biology by assisting the development of binding assays for lymphokines.

Somatic cell hybridisation techniques have enabled the production of McAbs recognising human IL-2 (Gillis & Henney, 1981) and alpha interferon (IFN- α) (Secher & Burke, 1980).

Lymphokine research in the future should allow the closer investigation of the mechanism of action and physiological role of lymphokines. The techniques being developed include; cloned lymphokine-secreting T cell lines, anti-lymphokine McAbs and cloned populations of target cells for use in bioassays.

Even though clonal assays are being developed to measure lymphokine activity, less specific assays reported over the last 10 years have still enabled the identification of various lymphokines.

The first assays for regulatory lymphokines generally measured their effects

on antibody responses in vitro, specifically their ability to augment unfractionated spleen cell antibody production, or Th-replacing factor activities in cultures of T cell-depleted spleen cells (Rubin & Coons, 1972). Later, other assays measuring the polyclonal activating properties of lymphokines were developed. This was assessed by the proliferation of responding B cells or total Ig secretion (Geha et al, 1977). Other bioassays were developed to measure the effects of T cell-derived lymphokines on macrophages, for example the T cell derived lymphokine responsible for the activation of macrophages, macrophage activating factor (Nathan et al, 1980). Other T cell derived mediators, which act on target cells not directly involved in the immune response have been assayed in nonimmunological systems. These include:

- 1) factors which promote the differentiation of hematopoietic progenitor cells i.e. colony stimulating factor,
- 2) mast cell growth factor, which mediates mast cell growth in culture and,
- 3) gamma interferon, which was originally assayed by its antiviral activity, but which is known to have potent immunoregulatory activity.

1.7 REGULATORY LYMPHOKINES IDENTIFIED TO DATE

In the last ten years a large number of biologically active mediators have been described. Although relatively few studies of human T cell-derived immunoregulatory factors have been performed it is now clear that many of the findings and rules established in murine studies apply equally well to human lymphokines. Regulatory lymphokines have been derived from normal or neoplastic T cells, or more recently from T cell hybridomas.

It is convenient to classify T cell-derived immunoregulatory factors into those which are either antigen-specific or antigen-nonspecific in their action. Antigen-specific factors regulate immune responses exclusively to the antigen which induced their formation. These factors bind the respective antigen and bear determinants encoded by the MHC. These factors have been reviewed by Tada & Okumura, (1979) and Altman & Katz, (1980).

In contrast, antigen-nonspecific factors act in a polyclonal manner, and many, although not all, do not bear detectable MHC encoded products.

1.7.1 Antigen-specific lymphokines

Depending on biological activity, antigen-specific lymphokines are divided into two major groups i.e. helper factors (ThF) or suppressor factors (TsF). ThF act to replace Th cells and provide help to antigen-primed effector cell precursors, whether T or B cells whereas TsF act to inhibit or suppress the immune response.

1.7.1 (a) Antigen-specific helper factors

Table 1.1 describes a number of recently identified antigen-specific factors displaying helper activity. In the main, the derivation of T cell lines secreting antigen-specific immunoregulatory factors has involved in vitro or in vivo immunisation of the T cells with the relevant antigen. An extensive review of previously described monoclonal, antigen-specific helper factors has been presented by Altman & Katz (1982).

1.7.1 (b) Antigen-specific suppressor factors

Suppressor factors are soluble inhibitors of the immune response which have been shown to actively participate in the regulation of the immune system. Four major classes of antigen-specific T cell suppressor factors have been described. These are:

- 1) carrier-specific suppressor factors,
- 2) polypeptide-specific factors,
- 3) factors which cause tolerance and suppression of delayed type hypersensitivity and,
- 4) factors which contribute to the failure of host immune responses to reject certain tumors.

Table 1.2 describes a number of selected antigen-specific suppressor factors which have been reported. There have been a number of contradictory reports concerning the expression of MHC and V_H determinants, MHC restriction, antigen versus idiotype specificity and the target cells for antigen-specific factors. Krakauer & Clough (1981) have published an extensive review of suppressor cells and their factors and discuss many of these questions.

The availability of monoclonal sources of antigen-specific lymphokines will allow the closer investigation of a number of immune mechanisms, for example: 1) using monoclonal TsF or ThF and cloned target cell populations

TABLE 1.1

ANTIGEN - SPECIFIC HELPER FACTORS

Antigen	Derivation	Assay system	Comments	References
<u>Human</u>				
Influenza virus strain A/3X1	7d in vitro priming, TCGF medium	2° Ab response to virus	Factor genetic restriction associated with HLA-DR	1
(T,G)-A-L	In vitro priming, (T,G)-A-L primed mφ & TCGF	ThF activity in murine spleen cell Ab production	Factor binds antisera to HLA-DR & V _H determinants	2
(T,G)-A-L, GAT	4 d in vitro priming	IgM response to hapten-carrier conjugates	Response segregates with HLA complex	3
Tetanus toxoid	T cell hybridoma : Jurkat 6TG-3 X TeT-specific Th line	IL-2 production after antigenic stimulation in presence autogenous irradiated mφ	Hybridoma retains helper activity of parent	4
Ovalbumin	120 hr in vitro priming	Initiation of T-dependent IgM response	Acts in early stages antigen-induced B cell activation & later stages B cell differentiation	5
<u>Murine</u>				
KLH, ovalbumin or human IgG	T cell hybridoma : Antigen primed T cells X tumor lines	IL-2 production following antigen stimulation, anti-TNP plaque response	Stimulatory B cell signals independent of Ig receptors by T cell recognition of antigen and/or I region	6

(T,G)-A-L	T cell hybridoma: adoptively primed (T,G)-A-L specific thymocytes X BW 5147 thymoma line	Spleen cell Ab production	Binds heterologous rabbit antisera to V _H region of murine Ig	7
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Ab	: antibody	(T,G)-A-L	: Tyrosine, glutamic acid, alanine, lysine polymer
ThF	: T helper factor	GAT	: glutamic acid, alanine, tyrosine polymer
mφ	: macrophage	KLH	: Keyhole limpet hemocyanin

References:

- | | |
|--------------------------|------------------------|
| 1. Fisher et al, 1981 | 5. Heijnen et al, 1981 |
| 2. Apte et al, 1981 | 6. Roehm et al, 1982 |
| 3. Woody et al, 1979 | 7. Essher et al, 1980 |
| 4. DeFreitas et al, 1982 | |

TABLE 1.2

ANTIGEN - SPECIFIC SUPPRESSOR FACTORS

Antigen	Derivation	Assay system	Comments	References
<u>Human</u> ovalbumin	120 hr in vitro priming with high doses ovalbumin	Suppression Antigen-induced PFC formation	Effector Ts product, acts on Th cells in antigen-induced PFC response	1
<u>Murine</u> KLH	T cell hybridoma : in vivo primed & enriched Ts cells x BW 5147 line	Suppression anti-DNP IgM & IgG spleen cell response	Antigen binding & I-J coded molecules on TsF indep. synthesised in cytoplasm, secreted in active associated form	2
SRBC	T cell hybridoma : 5d SRBC primed murine spleen cells x BW 5147 line	Suppression IgM response to SRBC	85 & 25 Kdal peptides, 200 Kdal native molecule. Heavy chain binds SRBC	3,4
Sheep erythrocyte glycophorin	Ts clone : SRBC primed spleen cells	Inhibition anti-SRBC helper-function	70 Kdal suppressor glycoprotein, 45 & 24 Kdal subunits	5
GAT	T cell hybridoma : GAT primed Ts cells x BW 5147 line	Suppression GAT-MBSA response	24 Kdal hydrophobic glycoprotein	6
SRBC : sheep red blood cell Th : T helper cells PFC : plaque forming cells MBSA : methyl bovine serum albumin Ts : T suppressor cells TsF: T suppressor factor				

References:

- | | | |
|--------------------------|-----------------------------|-----------------------|
| 1. Uytdehaag, 1981 | 3. Taussig et al, 1979 | 5. Fresno et al, 1981 |
| 2. Taniguchi et al, 1980 | 4. Taussig & Holliman, 1979 | 6. Webb et al, 1982 |

it should be possible to use reductionist studies to reconstruct the events of help or suppression and the targets of a given factor in the control of various immune responses,

- 2) this approach should allow the definition of the sites of MHC restriction in an immunoregulatory pathway,
- 3) sufficient quantities of monoclonal antigen-specific factors will become available for purification and sequencing, as well as preparing cDNA probes,
- 4) the therapeutic potential of these factors in allowing specific immune intervention are considerable.

1.7.2 Antigen-nonspecific lymphokines

T cell-derived antigen-nonspecific lymphokines are a heterogeneous population of molecules in a number of respects. They vary enormously in structure and function e.g. some have immunoregulatory effects and others act on targets outside the immune system. These lymphokines can act on T or B cells, macrophages, or hematopoietic progenitor cells. Even though these factors act in an antigen-nonspecific manner, their production can be induced by specific antigens (soluble, heterologous or alloreactive). In some cases normal T cells have been induced to secrete lymphokines by the action of polyclonal T cell mitogens. T cell hybridomas and lymphoma lines have been shown to secrete lymphokines constitutively or following induction.

A selected review of a number of important antigen-nonspecific lymphokines is presented below. This list is by no means complete and extensive reviews by Altman & Katz (1982), and Immunological Reviews 51 (1980) cover these and other factors in more depth.

1.7.2 (a) Antigen-nonspecific suppressive lymphokines

Table 1.3 describes a number of human and murine T cell factors which have been demonstrated to have suppressive effects on a variety of immune responses. Human T cell hybridomas elaborating suppressor molecules will be dealt with in Section 1.8.

It is important to note that a number of the suppressive factors presented in Table 1.3 appear to have effects on B cell responses. In many cases it has not been determined if these factors act on B cells directly or if they mediate their

TABLE 1.3

ANTIGEN - NONSPECIFIC SUPPRESSIVE FACTORS

Antigen		Derivation	Assay system	Comments	References
<u>Human</u>	IsF	T cell hybridoma : KE37 line X agammaglobulinaemic T cells	Suppression PWM-induced B cell differentiation	No suppression T-indep. mitogenesis, target probably T cell, 45 Kdal	1,2
	Soluble immune suppressor of T cell proliferation (SISS-T)	ConA-treated human PBL supernatants	Inhibition T cell proliferative response to mitogens & antigens	30-45 Kdal, NAG inhibits activity, unstable at 56°C	3
	Soluble immune suppressor of Ig production (CTC-SISS-B)	IL-2 dependent human CTL line	Inhibition polyclonal B cell Ig production	60-90 Kdal, binds L- rhamnose, T cell product	4,5

<u>Murine</u>	IgE-selective suppressor factor (IgE-TsF)	Released in DNP-mycobacterium cultures of primed spleen cells, T cell hybridoma	Suppression IgE biosynthesis	IgE & I region binding sites, binds IgE-specific B cells	6,7
	Immunoglobulin binding factor (IBF)	In vivo alloactivated T cells, T cell hybridoma	Suppression 1 ^o response to T-dependent & independent mitogens	38-40 Kdal	8,9

NAG : N-acetyl glucosamine

References:

- | | |
|----------------------------------|--------------------------------|
| 1. Grillot-Courvalin et al, 1981 | 6. Kishimoto et al, 1978 |
| 2. Grillot-Courvalin et al, 1982 | 7. Suemura et al, 1982 |
| 3. Greene et al, 1981 | 8. Gisler & Fridman, 1975 |
| 4. Greene et al, 1982 | 9. Neauport-Sautes et al, 1979 |
| 5. Fleisher et al, 1981 | |

effects via other cells. In some instances assays were performed on enriched B cell populations, but the possible contribution of residual T cells or macrophages must not be overlooked.

1.7.2 (b) Antigen-nonspecific T cell helper or replacing lymphokines

T cell-derived lymphokines which augment or facilitate various kinds of immune responses have been induced by a variety of methods and have been shown to effect both humoral and cell mediated immune responses. T cell helper or replacing factors can be divided into a number of groups. The first group are closely related molecules which act specifically on T cell targets. IL-2 is the best characterised lymphokine in this group. The second group are factors which appear to act on B cell targets, either directly or via other cells such as macrophages. These have been termed T cell replacing factors. As the name implies these lymphokines can replace Th cells in antibody responses. Another important mediator termed allogeneic effect factor is distinct from the two former groups in that it displays Ia determinants.

1.7.2 (c) Factors acting on T cells

Models explaining the role of antigen-nonspecific lymphokines in T and B cell activation have been proposed (Farrar et al, 1982). Figure 1.1 describes one such model and the role of the lymphokine cascade in the amplification of antigen-specific T and B cell responses.

T cell activation requires two signals:

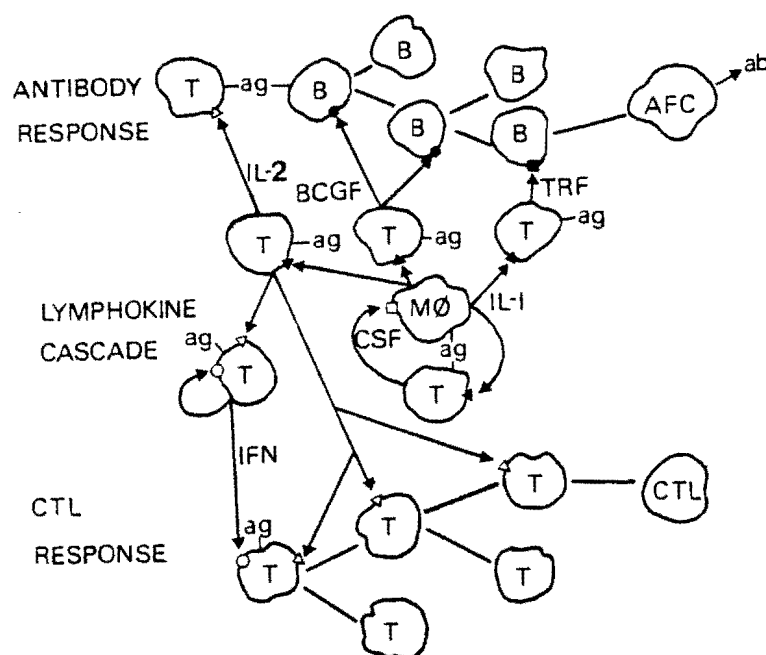
- 1) antigen selects the appropriate clone and induces the expression of receptors for growth factors and
- 2) growth factors clonally expand the receptor-expressing antigen-selected clones of effector T cells.

B cell activation involves an analogous process:

- 1) the recognition and selection of specific B cell clones by antigen, and
- 2) the clonal expansion and maturation of these clones into antibody forming cells.

FIGURE 1.1

ROLE OF ANTIGEN - NONSPECIFIC LYMPHOKINES IN T AND B CELL ACTIVATION



(based on Farrar et al, 1982)

Abbreviations:

AFC	: antibody forming cell	ab	: antibody	ag	: antigen
CTL	: cytotoxic T lymphocyte	mØ	: macrophage	T	: T cell
B	: B cell	CSF	: colony stimulating factor		
IFN	: interferon	TRF	: T cell replacing factor		
BCGF	: B cell growth factor				

The lymphokine cascade described in the centre section results from macrophages interacting with T cells to produce IL-1 and subsequently IL-2, BCGF and TRF. The induction of IFN- γ production by IL-2-activated T cells is also shown. The upper section describes the sequential roles IL-2, BCGF and TRF in the development of antibody responses. The lower section indicates the role of IFN- γ in the development of CTL responses.

Interleukin 2 (IL-2)

IL-2 is a genetically unrestricted, soluble immunoenhancing factor produced by Th cells which have been stimulated with either T cell mitogens or alloantigens (Aarden et al, 1979). A definitive assay for IL-2 measures the ability of the factor to maintain the growth of "IL-2-addicted" cytotoxic T cell lines. A number of other biological activities have been ascribed to IL-2; for example, the induction of thymocyte proliferation, augmenting the proliferation of alloantigen-stimulated T cell populations and inducing the synthesis of gamma interferon (Farrar et al, 1981). IL-2 has also been shown to contribute a second signal to the activation of antigen-specific Th cells and maintaining these cells in long-term culture (reviewed by Farrar et al, 1982). IL-2 has been shown to augment natural killer cell activity (Henney et al, 1981). Extensive characterisation of IL-2 has been undertaken and human IL-2 has an apparent MW of 15 Kdal compared to 25-30 Kdal for murine IL-2, as assessed by AcA 54 chromatography. Human IL-2 has an isoelectric point of 6.5, is sensitive to protease, insensitive to DNase and RNase and neuraminidase and is not reduced by 2-mercaptoethanol or dithiothreitol. The physical properties of human and murine IL-2 have been investigated by Gillis and his coworkers (Gillis et al, 1982).

By utilising monoclonal sources of IL-2 and cloned target cells it has been clearly established that IL-2 is a T cell product, which acts on activated T cells (Watson et al, 1979). IL-2 constitutes a class of closely related factors which serve as growth (and perhaps differentiation) factors for T cells. This appears to result from its binding to specific receptors on the cell surface (Smith, 1980). Hilfiker & Farrar (1981) suggested that the process by which IL-2 signals the target cell to proliferate is via receptor-mediated endocytosis. Their studies concluded that "absorption" may be a two-step process: temperature-independent reversible binding to the receptor followed by temperature-dependent internalisation by receptor-mediated endocytosis. The affinity of the receptor has been estimated ($K_d = 5 - 25 \times 10^{-12} \text{M}$) (Robb et al, 1981).

1.7.2 (d) Factors acting on B cells

(i) T cell replacing factors (TRFs)

Dutton et al (1971) and Schimpl & Wecker (1972) first demonstrated that the response of T cell-depleted spleens could be restored by

T cell-derived mediators. This initial work utilised mixed lymphocyte cultures and it was soon shown that other culture supernatants of ConA or antigen-activated T cells contained a similar factor. Functional studies of TRF led to the idea that it provides a late differentiation signal to antigen-stimulated, proliferating B cells, which causes them to mature into antibody-secreting cells. A number of monoclonally-derived human and murine TRF preparations have recently been shown to act in a similar manner (Takatsu et al, 1980; Kaieda et al, 1982; Elkins & Cambier, 1983). TRF and TRF-like molecules therefore comprise a heterogeneous class of B cell-specific immunoregulatory molecules which provide differentiation and proliferation signals to B cells at distinct differentiation stages. These molecules probably have a fundamental role in T-B cell collaboration by either turning on or shutting off B cell responses.

(ii) B cell growth factor(s) (BCGFs)

BCGFs have only recently been described (Howard et al, 1982) and probably function to deliver a proliferation signal to specific B cell clones which have responded to antigen and have initiated their response. BCGFs may therefore be involved, in combination with other activating factors, in the amplification of B cell antibody responses. It has been suggested that IL-2, BCGF, and TRF may function in a three-way synergism to augment the anti-SRBC plaque-forming cell response to highly purified B cells (Farrar et al, 1982). The concept of a lymphokine cascade being involved in the induction of antibody synthesis is an attractive one and may help to explain the interrelationships between the various antigen-nonspecific lymphokines described here. IL-2 is involved in the early initiation of B cell responses indirectly, by activating some form of linked recognition helper function from residual T cells. Subsequently B cell proliferative responses begin and are amplified by BCGF. Proliferating B cells may then develop receptors for, and be stimulated with TRF to differentiate into antibody-forming cells.

(iii) Allogeneic effect factor (AEF)

AEF is a 45 Kdal glycoprotein secreted into the supernatants of short-term mixed lymphocyte cultures of in vivo alloantigen-activated T cells (Armerding et al, 1974). AEF-containing supernatants have also been demonstrated to induce naive T cells, in the absence of other exogenous stimuli, to proliferate and differentiate. This activity is therefore distinct from that of IL-2, which is also present in AEF supernatants and which provides a proliferative

stimulus for activated T cells. Altman et al, (1982), described the production of a murine T cell hybridoma which secreted high levels of AEF activity.

1.7.2 (e) Other antigen-nonspecific lymphokines

(i) Interleukin-1 (IL-1)

The macrophage-derived factor IL-1 acts in association with macrophages in the activation of antigen-specific T cells. Antigen-specific T-cell activation requires at least two distinct but interdependent macrophage signals. Macrophages bind, process and present antigens in an immunogenic form to T cells (Schwartz et al, 1978) and the second activating signal is IL-1. IL-1 appears to act as a maturational signal, preparing T cells to respond to antigen or secondary signals. IL-1 also induces the synthesis and secretion of IL-2 (Smith et al, 1980). The link between IL-1 and IL-2 is essential in T cell activation because it is the conversion of a primary macrophage-derived signal into a secondary T cell-derived proliferative signal that results in the amplification of specific immune responses. Gery et al (1972) first described IL-1, at that time termed lymphocyte activation factor, which was mitogenic for thymocytes. IL-1 has also been demonstrated to have effects on non-T cells. Schrader (1973) showed that macrophages produced a soluble factor which could restore the in vitro antibody responses of spleen cells from congenitally athymic nude/nude mice. Wood & Gaul (1974) used an experimental system of T cell-depleted (anti-Thy 1 treated) spleen cells from normal mice and demonstrated a similar effect. In both studies the authors concluded that the macrophage product was acting directly on the antibody producing B cell. Wood et al (1976) termed this activity B cell activating factor. The nomenclature for IL-1 was clarified in 1979 (Aarden et al), and IL-1 is now considered to be synonymous with lymphocyte activating factor, B cell activating factor, thymocyte proliferation factor and B cell differentiation factor.

Murine IL-1 has been purified to apparent homogeneity (Mizel & Mizel, 1981a) and has been demonstrated to be a 12-15 Kdal protein with high affinity for hydrophobic resins that exhibits resistance to several proteases. This unusual stability of IL-1 indicates that it probably exists in a compact form in its native configuration. This may have physiological significance as there is considerable evidence that IL-1 may be involved in inflammatory responses, which are known to cause the release of a number of proteases into the inflammatory site (Mizel et al, 1981b). The biochemical and immunological effects of IL-1 have been

reviewed (Mizel, 1982).

(ii) Interleukin 3 (IL-3)

The term IL-3 was first introduced by Ihle et al, (1981) to describe a factor responsible for the induction of the enzyme 20 α -steroid dehydrogenase (20 α -SDH) in cultures of murine nude/nude splenocytes. Unlike IL-2, IL-3 primarily influences a stage or stages of immature lymphocyte differentiation. This view is disputed by Watson & Prestige (1983), who feel IL-3 is not merely an activated T cell product as it has been shown to be produced by the myelomonocytic leukemia line WEHI-3 among others (Ihle et al, 1982). A variety of colony-stimulating activities have been associated with preparations of IL-3 and the question has been raised whether IL-3 and a class of colony-stimulating factors are in fact the same. Sequential purification of IL-3 using gel chromatography, ion exchange chromatography and reverse phase high performance chromatography appears to copurify these activities. The apparent MW of IL-3/CSF is 41Kdal on sodium dodecyl sulphate electrophoresis and the apparent dissociation constant of the molecule in inducing 20 α -SDH activity has been estimated at 10^{-11} M. The debate whether IL-3 and multi-CSF (WEHI-3-derived IL-3) are the same, and whether IL-3 is predominantly a regulator of hematopoietic differentiation or a mediator of immune modulation, will continue and may be answered when detailed biochemical analyses of these molecules are performed.

(iii) Interferons (IFNs)

The interferons are a heterogeneous protein family produced by a variety of cell types. Originally the IFNs were defined as proteins which exert virus-nonspecific antiviral activity, but they are now regarded as important mediators of the immune response as they can induce both enhancing and suppressive effects. The IFNs have been categorised into two groups. Type 1 (IFN- α and IFN- β) consists of virus-induced IFN and those induced by specific agents. Type 1 or "classical" IFN has been observed to suppress both primary and secondary antibody responses to T-dependent and T-independent mitogens and antigens. IFN- α has also been shown to suppress the proliferative phase of a human mixed lymphocyte reaction. Type 2 IFN (IFN- γ) is produced by a population of T cells which are macrophage-dependent. Because IFN- γ is produced during antigen or mitogen stimulation of T cells (which also causes the production of other lymphokines) it has been difficult to

ascribe a given immunoregulatory activity to IFN- γ . A number of T cell lines and hybridomas have been shown to produce IFN- γ (reviewed by Krakauer & Clough, 1981).

A summary of the various antigen-nonspecific factors discussed in the preceding section, as well as factors acting on macrophages, are presented in Table 1.4.

1.8 HUMAN T CELL HYBRIDS

1.8.1 General introduction

As mentioned, and evidenced by the preceding review, the closer investigation of these lymphokines has been hampered by the presence of heterogeneous lymphokine species in conventional culture supernatants. Cloned T cell lines therefore have the advantage that such heterogeneity can be reduced to an extent. This problem of multiple secretion of lymphokines is not as great with T cell lines which secrete antigen-nonspecific lymphokines. These lines do not appear to produce specific lymphokines, and secondly, if they did, it would be relatively simple to purify the antigen-specific lymphokine by affinity chromatography on immunoadsorbents coated with the relevant antigen. It may be more difficult, in the case of antigen-nonspecific lymphokine-producing cells, to achieve separation between distinct molecular species. Methods therefore which favour a more restricted pattern of lymphokine production would be valuable.

The work presented in this thesis addresses this problem by utilising somatic cell hybridisation techniques for the production of human regulatory lymphokines. Table 1.5 presents a number of human T cell hybridomas recently described in the literature. It is evident from this summary that little biochemical analysis of the hybridoma-derived factors have been undertaken. These deficiencies will no doubt be rectified in the near future and a better understanding of the physical and chemical nature of these mediators will result.

TABLE 1.4
OTHER ANTIGEN - NONSPECIFIC FACTORS

Factor(s)	Derivation	Target cell(s)	Comments	References
Interleukin 2 (IL-2)	Stimulated Th cells	T cells	Activated-T cell growth factor, Murine 25-30 Kdal, Human 15 Kdal	1
T cell replacing factor (TRF)	MLC cultures	B cells	Late differentiation signal to antigen-stimulated, proliferat- ing T cells	2,3
B cell growth factors (BCGFs)	Activated T cells	B cells	Proliferation signal to specific activated B cell clones	4
Allogeneic effect factor (AEF)	Short-term MLC cultures	Induces naive T cell proliferation	45 Kdal glycoprotein	5
Interleukin 1 (IL-1)	mφ	T cells in association with mφ	T cell maturational signal, induces IL-2 secretion, murine IL-1 12-15 Kdal, hydrophobic	6,7
Interleukin 3 (IL-3)	Activated T cells, WEHI-3 line	T cells, hematopoietic progenitor cells	Induces 200x SDH activity	8,9

Interferons (IFNs)	Variety cell types	T cells, other cell types?	Antiviral activity, induces other LK production	10
Macrophage activating factor (MAF)	Activated T cells	mØ	Induces mØ-mediated tumoricidal activity, 50-55 Kdal, pH 4 inactivation, 56°C inactivation, pI = 4.5	11,12
Macrophage inhibitory factor (MIF)	Activated T cells	Inhibits mØ migration	0.1M L-fucose inactivation	13

MLC : mixed lymphocyte culture
 20α SDH : 20-alpha steroid dehydrogenase
 pI : isoelectric point
 LK : lymphokine

References:

- | | | | |
|---------------------------|--------------------------|------------------------|-----------------------------|
| 1. Gillis et al, 1982 | 4. Farrar et al, 1982 | 7. Mizel & Mizel, 1981 | 10. Krakauer & Clough, 1981 |
| 2. Dutton et al, 1971 | 5. Armerding et al, 1974 | 8. Ihle et al, 1981 | 11. Screiber et al, 1982 |
| 3. Schimpl & Wecker, 1972 | 6. Gery et al, 1972 | 9. Ihle et al, 1982 | 12. Screiber et al, 1983 |
| | | | 13. Pick et al, 1979 |

TABLE 1.5

HUMAN T CELL HYBRIDS AND DERIVED FACTORS

Functional properties of Hybrids and (or) factors	Assay systems	Parental tumor line	T cell partner & activation regime	Comments	References
Suppression B cell differentiation	PWM induced B cell differentiation	KE37 (TK ⁻)	Unstimulated agammaglobulinaemic T cells	No suppression to I-independent mitogens, 45 Kdal	1,2
MIF & LT	MIF & LT	Emetine & actinomycin D treated CEM	Normal PBL, 40 hr PHA-P (5µg/ml)	Producer clone phenotype only, no biochem. analysis	3,4
Inhibition PWM-induced Ig production, no inhibition lectin or antigen-induced T cell proliferation	PWM-induced Ig, lectin & antigen induced T cell proliferation	CEM (HGPRT ⁻)	Normal PBL, 48 hr ConA (10µg/ml)	70-85 Kdal, B cell or mØ target	5
IgA-specific help, PC B cell activation, B cell proliferation	Induction Ig, B cell proliferation	J3 (HGPRT ⁻) KE37 (HGPRT ⁻)	90-95% OKT4 ⁺ , 72 hr ConA (10µg/ml)	No biochem. analysis	6
Constitutive BCGF production in absence IL-2	IL-2 : CTC line proliferation, BCGF : purified & activated B cell proliferation	CEM-6 (HGPRT ⁻)	AET-rosetted normal T cells, 24 hr ConA (10µg/ml)	18-20 Kdal	7

Constitutive IL-2, MGF & IFN- γ production	IL-2 : PHA-stimulated PBL proliferation	HUT 102-B2 (HGPRT ⁻)	Normal PBL, 48 hr ConA (10 μ g/ml)	All producing hybridomas expressed Th phenotype	8
Constitutive IL-2 secreting clones, also after $\pi\phi$ or PHA treatment, KHF clones	IL-2 : CTC line proliferation, KHF : helper activity of culture supernatant in CTC differentiation	CEM (HGPRT ⁻)	E-rosetted normal T cells, ConA (10 μ g/ml) or MLC T cells	No biochemical analysis	9
Tetanus toxoid-specific hybrids produce IL-2 after mitogen or antigen stimulation	IL-2 : CTLL proliferation Helper activity : T hybrid binding to prepulsed $\pi\phi$	Jurkat 6TG-3	TeT-specific T cell lines	Parental activity retained by hybrid	10,11
Several Th clones	Hybrid cell induction or regulation of PWM-induced Ab production	CEM (HGPRT ⁻)	E-rosetted normal T cells, 96 hr PHA (10 μ g/ml), 192 hr PWM (5 μ g/ml), 48 hr ConA (2 μ g/ml)	Functional activity recovered after cloning	12

IL-2 production by	48 hr stimulation	Jurkat	EBV-immune PBLs, 9 d	No biochemical	13
EBV-reactive hybrids			LCL stimulation	analysis	

MIF : macrophage inhibitory factor	KHF : killer helper factor
LT : lymphotoxin	CILL : murine IL-2-dependent cytotoxic T cell line
CTC : IL-2-dependent cytotoxic T cell line	E rosetted : SRBC receptor positive T cells
AET : 2-aminoethylisothiuronium	EBV : Epstein-Barr Virus
MGF : mast cell growth factor	LCL : lymphoblastoid cell line

References:

- | | |
|----------------------------------|----------------------------|
| 1. Grillot-Courvalin et al, 1981 | 8. Le et al, 1983 |
| 2. Grillot-Courvalin et al, 1982 | 9. Okada et al, 1981 |
| 3. Kobayashi et al, 1982 | 10. DeFreitas et al, 1982a |
| 4. Asada et al, 1983 | 11. DeFreitas et al, 1982b |
| 5. Greene et al, 1982 | 12. Irigoyen et al, 1981 |
| 6. Mayer et al, 1982 | 13. Lakow et al, 1983 |
| 7. Butler et al, 1983 | |

1.8.2 Techniques for the production of human T cell hybrids

1.8.2 (a) Introduction

The successful production of hybridomas secreting monoclonal antibodies has had a dramatic effect on modern immunology. Okada (1962) first described the phenomenon of cell fusion mediated by Sendai Virus. Modifications of these early techniques are now widely used to produce highly specific immunological reagents.

A similar rationale is used in producing T cell hybrids with specific functions deriving from the parental T cells. The expectation therefore, is that specific T cell functions will continue to be expressed within the background of the proliferating T cell lymphoma.

T cell hybrids have applicability in a number of areas of immunology. One application has been analysis of the antigen-recognising structures on T cells i.e. T cell receptors (Rajewsky & Eichman, 1977). The assumption here is that T cell hybrids will be obtained that secrete homogeneous factors which bind membrane antigens specifically.

T cell hybridisation has also been applied to the analysis of the origin of genetic restrictions in cell interactions. It is widely believed that T cells recognise antigen in "association" with self cell surface components. Presumably, T cell hybrids exhibiting antigen restricted recognition should allow a more elegant analysis of this problem than by using heterogeneous T cells.

The third important use of T cell hybrids is in the study of T cell secretory activity. Recently, several laboratories have been successful in constructing human T cell hybrids expressing T cell function of the nonlymphoma partner as evidenced by the secretion of soluble mediators of immune responses (Table 1.5).

Spontaneous fusions of cells in culture are infrequent, but if this does occur, it is still difficult to identify the hybrids arising from these fusions.

Improved fusion techniques and selection systems for cell hybrids have overcome many of these early difficulties. In the case of human T cell hybrids, the hybridisation technique involves mixing a suspension of antigen-or mitogen-activated T cells with a selected T lymphoma line. The T cells are fused with

polyethylene glycol. A selection system is used to eliminate the parental cells and to permit growth of the hybrids. The primary non-neoplastic parent dies after a limited period in culture. Elimination of the lymphoma parent is accomplished usually by a drug selection system. The most frequently used drug selection system is tissue culture medium supplemented with hypoxanthine, aminopterin and thymidine (HAT) as devised by Szybalski et al (1962). This selection system relies upon the fact that cells deficient in either hypoxanthine guanine phosphoribosyl transferase (HGPRT) or thymidine kinase (TK) die in the presence of aminopterin. Hybrids that have inherited complementary unaltered HGPRT and TK genes from two parents are able to grow (Littlefield, 1964).

1.8.2 (b) Selection of purine and pyrimidine analogue resistance in mammalian cells

Somatic cell hybridisation takes advantage of purine and pyrimidine analogue resistance. Variant-resistance to these analogues initially represented important and technically useful markers which were used to characterise permanent cultured cell lines. The usefulness of these variants results from several properties:

- 1) 8-azaguanine (AG) and 6-thioguanine (TG) in many cases selects for the loss of the enzyme HGPRT, which is controlled by an X-linked hemizygous gene (Shows & Brown, 1975),
- 2) purine and pyrimidine nucleosides are relatively stable substances which occur in limited concentrations in the serum and are not liberated by the breakdown of serum components. Competition between analogues and their natural correlates is therefore not a significant problem (Peterson et al, 1976),
- 3) the mutation rates in these variants is low enough (in either direction) to allow their study (Morrow, 1970),
- 4) mammalian cells are not usually prototrophic for purines and pyrimidines and their addition to the medium is not required for cell survival.

Drug resistant cells arise spontaneously in culture, but the use of X-rays

(Albertini & DeMars, 1972) or chemical mutagens such as ethyl methane sulphonate (EMS), greatly increases the mutation rate. After exposure to mutagens, drugs such as azaguanine (Sato et al, 1972) and thioguanine (Van Zeeland et al, 1974) kill normal cells because the drugs are metabolised and interfere with nucleotide and nucleic acid synthesis. This effect is mediated by the enzyme HGPRT which converts the drugs to abnormal nucleotides. Mutants which lack the HGPRT enzyme (HGPRT^-) are resistant to AG or TG and therefore survive the treatment. A number of purine and pyrimidine nucleoside analogues can be used in the selection of drug-resistant variants. Some of these are presented in Table 1.6. There appears to be a large variation in the ease with which drug-resistant mutants can be isolated from different cell lines. This variation may result from differences in the number of copies of the gene present in the cell line, or could be due to suppression or inactivation of the gene (Morrow, 1977).

1.8.2 (c) Principle of HAT selection system

Purine and pyrimidine nucleotide biosynthesis in mammalian cells is achieved via endogenous (de novo) pathways or via salvage pathways in times of stress or starvation in the cell. When the de novo biosynthetic pathways for purine and pyrimidine nucleotides are blocked by the folic acid analogue aminopterin (Figure 1.2), normal cells (HGPRT^+ , TK^+) can bypass this block and utilise preformed hypoxanthine and thymidine to synthesise nucleic acid precursors via their respective salvage pathways. Somatic cell variants lacking these salvage enzymes (HGPRT^- , TK^-) cannot survive because they are unable to synthesise nucleotides from hypoxanthine (HGPRT^- cells) or from thymidine (TK^- cells) (Littlefield, 1964).

Aminopterin acts as a stoichiometric inhibitor of folic acid reductase (Littlefield, 1969). Aminopterin has also been shown to block the endogenous synthesis of purines, one-carbon-transfer reactions, and the synthesis of certain amino acids. Littlefield (1964), demonstrated that the HAT medium as well as being able to screen for rare revertants of HGPRT^- mutants to HGPRT^+ cells, was also extremely useful if selecting for somatic cell hybrids derived from parents lacking HGPRT and TK activity. While parental (HGPRT^-) tumor cells are killed by HAT, only hybrids that have inherited complementary unaltered HGPRT and TK genes from two parents are able to grow. This selection system has been used almost exclusively for the selection of B cell hybridomas as well as human T cell hybridomas. Several modifications of this system have been

TABLE 1.6

PURINE AND PYRIMIDINE NUCLEOSIDE ANALOGUES USED IN
THE SELECTION OF DRUG-RESISTANT VARIANTS

Analogue	Natural correlate	Enzyme involved in resistance	References
6-thioguanine (6TG)	Guanine	HGPRT	1
8-azaguanine (8AG)	Guanine	HGPRT	2
5-bromodeoxyuridine (BUdR)	Thymidine	TK	3
2,6-diaminopurine	Adenine	APRT	4
6-azauridine	Uridine	UK	5

HGPRT :	hypoxanthine guanine phosphoribosyl transferase
TK :	thymidine kinase
APRT :	adenine phosphoribosyl transferase
UK :	uridine kinase

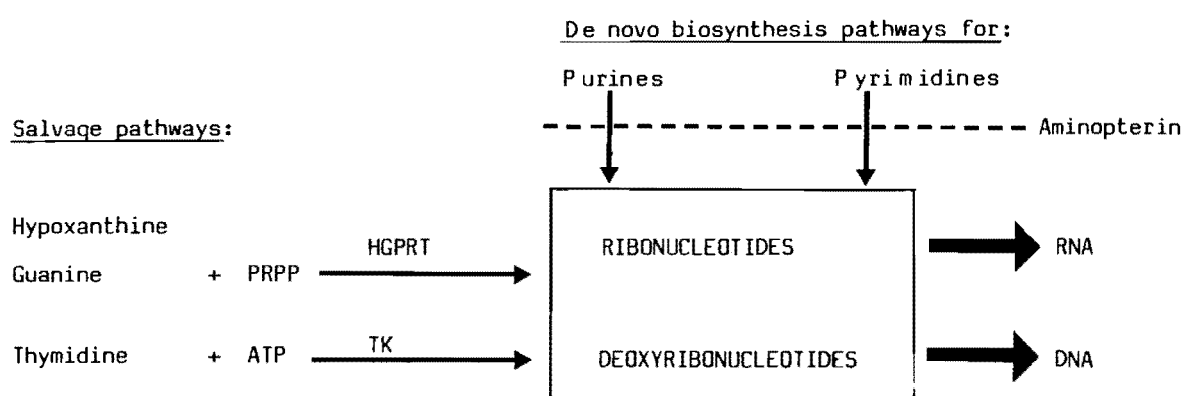
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2. Morrow J. (1970) Genetics 65, 279.
3. Kit. S., Dubbs D.R., Piekarski L & Hsu T.C. (1963) Exp. Cell. Res. 31, 297.
4. Chasin L.A. (1974) Cell 2, 37.
5. Vesely J. & Cihak A. (1973) Oncology 28, 204.

used to overcome a number of problems e.g. aminopterin toxicity, and cellular variations in nucleic acid metabolism, which can all affect the efficiency of HAT selection. Azaserine, an inhibitor of several amido transferases, has been used with hypoxanthine to block endogenous purine synthesis in the selection of hybrids (Siniscalco et al, 1969).

FIGURE 1.2

PRINCIPLE OF HAT SELECTION SYSTEM



Purine and pyrimidine biosynthesis is achieved via de novo pathways, or via salvage pathways in times of stress. If the de novo biosynthetic pathways are blocked by aminopterin, normal cells (HGPRT^+ , TK^+) are able to bypass this and utilise preformed hypoxanthine and thymidine to synthesise nucleic precursors via their respective salvage pathways. Cells lacking these salvage enzymes (HGPRT^- , TK^-) do not survive as they are unable to synthesise nucleic acid precursors.

1.8.2 (d) Phenotypic selection of hybrids

As the HAT selection system can only be used with mutant tumor lines deficient in certain enzymes, it would be of value to have other selection systems to ensure true hybrids are produced. Taniguichi & Miller (1978) devised a selection method using the fluorescence activated cell sorter which enabled non-mutant T cell lines to be used in T cell hybridisation programmes. This allowed the separation of cells stained with fluorescent antibodies directed against cell-surface components present only on normal T cells, and, presumably, also expressed by any hybrid cells resulting from

the fusion of normal and tumor cells. Dangi & Herzenberg (1982) discussed the use of fluorescence activated cell sorting for B cell hybridomas which substantially eased the process of isolating stable hybridomas, or selecting hybridoma variants with desired structural and functional properties.

1.9 CONCLUSIONS

From this literature review it is evident that little of the structural nature of human regulatory lymphokines is known despite their extensive characterisation in biological assay systems. These shortcomings in our knowledge are due primarily to the lack of homogeneous T cell lines producing restricted numbers of T cell regulatory lymphokines. The following sections of this thesis are directed to the problem of developing in vitro techniques for the production and assaying of human lymphokines which affect antibody responses. Somatic cell hybrids have been used as a source of these lymphokines.

CHAPTER 2.

MATERIALS AND METHODS

2.1 CELL CULTURES AND CRYOPRESERVATION OF CELL LINES

2.1.1 Peripheral blood lymphocyte (PBL) isolation

Heparinised (Upjohn, U.K.) venous blood samples from healthy hospital workers were diluted 1:2 with sterile phosphate buffered saline (PBS) pH 7.4 and centrifuged on Ficoll-Hypaque (Boyum, 1968), 25ml 1:2 diluted blood was layered onto 15ml Ficoll-Hypaque ($\delta = 1.077\text{g/ml}$) and centrifuged at 400g for 25 minutes. Mononuclear cells from the interface were recovered with a sterile pasteur pipette, washed twice in serum-free tissue culture medium, and counted using a hemacytometer by dye exclusion (0.1% eosin). Lymphocyte isolations and cell cultures, were in all cases performed using standard "sterile techniques" in either a Laminar-Flow (Clemco, Aust) or Biohazard (Clemco) tissue culture hood.

2.1.2 Tissue culture medium

Cell cultures were performed in RPMI 1640 medium (GIBCO, Cat. No. 430-1800) containing 25mM HEPES (Research Organics, Ohio) supplemented with 100 U/ml penicillin (Glaxo, U.K.), 100 $\mu\text{g/ml}$ streptomycin (streptomycin sulphate, Glaxo, U.K.), 4mM additional L-glutamine (Sigma) and heat-inactivated (56°C, 60min) foetal calf serum (FCS) (GIBCO) at 10,15 or 20% as indicated. Cell cultures were incubated at 37°C in a 5% CO₂ atmosphere in a water jacketed incubator (Forma Scientific) with 100% relative humidity. Disposable plastic tissue culture ware was used as described.

2.1.3 Cryopreservation of cell lines

At regular intervals, stocks of the cell lines were frozen in case contamination or chromosomal instability, leading to the death of the line occurred. Cells to be frozen were chilled on ice for 30mins (approx. 10×10^6 cells in 0.5ml RPMI, 20% FCS) in 2ml cryopreservation tubes (Nunc). 0.5ml ice cold FCS, 20% dimethyl sulphoxide (v/v) (Sigma) was then added dropwise with mixing. The cryopreservation tubes were frozen overnight in a -70°C Revco

freezer in an upright position. Next day the ampoules were transferred to a Linde (LR-40, Union Carbide, USA) liquid nitrogen cryopreservation container.

When required, cells were rapidly thawed in a 37°C water bath and diluted dropwise with ice cold RPMI (20 volumes) over approximately 5 mins. These thawed cells were washed twice with RPMI and resuspended in tissue culture medium with the relevant supplements. If low cell viabilities were evident (by dye exclusion) the thawed cells were centrifuged over Ficoll-Hypaque to remove the non-viable cells, and then cultured.

2.2 CONSTRUCTION AND SELECTION OF HUMAN T CELL HYBRIDS

2.2.1 Introduction

Human T cell hybrids were constructed between normal lectin-activated peripheral blood lymphocytes and a 6-thioguanine resistant (TG^R) subline of the human T cell acute lymphocytic leukemia (T-ALL) line CCRF-CEM. Polyethylene glycol (PEG) was used as the fusigen and T cell hybrids were subsequently selected using HAT-supplemented tissue culture medium and flow cytometric selection by sterile cell sorting on a Fluorescence Activated Cell Sorter.

2.2.2 Drug-resistant parental T cells

A 6-thioguanine resistant (HGPRT deficient) subline of the CCRF-CEM (Foley et al, 1965) T-ALL line was established after mutagenesis with ethyl methane sulphonate (EMS) and subsequent selection in 6-thioguanine supplemented tissue culture medium.

CCRF-CEM was established in 1965, from the peripheral blood of a three year old female child with acute lymphocytic leukemia. This line has been extensively characterised (Foley et al, 1965; McCarthy et al, 1965; Schachtschabel et al, 1966; Suomalainen et al, 1980) and various drug-resistant subclones of this line have been used as a parental tumor line for the construction of human T cell hybrids (Table 1.5).

Exponentially growing CCRF-CEM cells (5×10^5 /ml) were mutagenised with varying concentrations of EMS (Sigma) (1,5,10 or 50 µg/ml) for 24 hrs at

37°C in a 5% CO₂ atmosphere (based on the methods of Sato et al, 1972; Epstein et al, 1977). Surviving cells were plated directly into 96-well round-bottomed microtitre plates (Nunc), 1000 cells/well, in supplemented RPMI 1640, 15% FCS and 0.1 µg/ml 6-thioguanine (6TG) (Sigma). Wells exhibiting growth were expanded into tissue culture flasks (Nunc) and the 6TG concentration was increased stepwise up to 18 µg/ml over the next 6 weeks. The cultures were fed every 3-4 days by aspirating approximately half the spent medium and replacing it with fresh 6TG-supplemented tissue culture medium. A line of rapidly growing, mutagenised CCRF-CEM cells, (CEM-6TG^R), resistant to 18 µg/ml 6TG was established in this manner and maintained in 6TG-supplemented tissue culture medium, 15% FCS. At regular intervals aliquots of cells were frozen and stored in liquid nitrogen (Section 2.1.3).

A number of functional and phenotypic characteristics of the CCRF-CEM and mutagenised CEM-6TG^R lines were assessed. These studies included:

1) Growth rate	Section 2.2.3
2) Terminal deoxynucleotidyl transferase staining	Section 2.2.4
3) HAT sensitivity	Section 2.2.5
4) Cell surface phenotype	Section 2.3
5) Karyotype	Section 2.4
6) Functional HGPRT activity	Section 2.5

2.2.3 Growth rate

Replicate 10ml cultures of CCRF-CEM or CEM-6TG^R cells were seeded at 1×10^4 /ml or 1×10^5 /ml (> 90% viable) in RPMI, 15% FCS (CEM-6TG^R supplemented with 18 µg/ml 6TG) at time 0. At various intervals a replicate cell culture was harvested by centrifugation and a total and viable cell count was performed. In general, twelve hemacytometer fields were counted and the average viable numbers/ml was calculated. The doubling times of the cell lines was estimated from a plot of log viable cell number/ml versus time in culture.

2.2.4 Terminal deoxynucleotidyl transferase (TdT) staining

TdT is a DNA polymerase which is recognised as a "marker"

for primitive lymphocytes in the hematopoietic system (Janossy et al, 1979). Fixed cells were treated with a specific rabbit anti-bovine TdT antibody. Fluorescent staining of the immune complex with FITC labelled goat anti-rabbit IgG was used to visualise the percentage TdT⁺ cells. The technique used was that recommended by the manufacturer (Biotech Research Laboratories, Inc, Rockville, MD. Product No. 9331SA).

2.2.5 HAT sensitivity

Growth of the CEM-6TG^R line in 6TG-supplemented medium suggested it was deficient in the salvage enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT, EC 2.4.2.8) (Sato et al, 1972; Van Zeeland et al, 1974). Cell lines deficient in HGPRT activity are killed in HAT medium (Szybalski et al, 1962) and this was assessed directly by culturing cells (5×10^5 /ml, >90% viable) in hypoxanthine (0.1mM), aminopterin (0.4 μ M), thymidine (16 μ M) (HAT)-supplemented RPMI, 15% FCS. Every 2 days the cells were counted and recultured at a viable density of 5×10^5 /ml.

2.2.6 Other parental lines

An 8-azaguanine resistant subline of CCRF-CEM (CEM-AG^R), kindly provided by Professor T. Kishimoto (Dept. Medicine, Osaka Univ. Hosp, Osaka, Japan) was also used in some fusion experiments. This line has been used to generate functional human T cell hybrids (Okada et al, 1981). CEM-AG^R was assessed for growth rate (Section 2.2.3) and cell surface phenotype (Section 2.3).

2.2.7 Activation of normal T cells

Freshly isolated human PBLs (Section 2.1.1) were activated for either 2 or 5 days with 10 μ g/ml Concanavalin A (ConA) (Sigma) at 1×10^6 /ml in supplemented RPMI, 10% FCS. Samples of these activated cells were stained by indirect immunofluorescence to determine the percentage T and B cells (Section 2.3) and the remaining cells were fused with the CEM-6TG^R line.

2.2.8 Fusion procedure

The method used was based on that of Grillot-Courvalin et al, (1981). Equal numbers of ConA-activated PBLs and CEM-6TG^R cells

(8×10^6 to 15×10^6 of each, in 4 separate experiments) were mixed and centrifuged at 1000g for 5 minutes, washed once in warm RPMI (37°C) and a second time in warm RPMI containing 5% (v/v) dimethyl sulphoxide (DMSO) (Sigma); to enhance the polyethylene glycol (PEG) mediated cell fusion (Norwood et al, 1976). The pellet was gently resuspended in 1ml warm 50% (v/v) PEG 4000 (Sigma) which was added dropwise over approximately one minute. After gentle mixing for a further minute the cells were centrifuged at 500g for 3 minutes, the supernatant was removed and 20mls warm RPMI was added dropwise. The cells were centrifuged and again washed. These fused cells were suspended in RPMI supplemented with HAT, 20% FCS and plated at a density of 1×10^6 /ml in 96 well flat-bottomed microtitre plates (Nunc) in a volume of 200 μ l, cultured at 37°C and fed every 2-3 days.

2.2.9 Drug selection of hybrids

Though the standard HAT medium is widely used to select for successful hybrids it has been reported that the thymidine component causes growth inhibition of the CCRF-CEM line (Schachtschabel et al, 1966; Fox et al, 1981). An alternative selection medium utilising azaserine and hypoxanthine (AH) (Siniscalco et al, 1969) was used to select for putative hybrids from a fusion experiment. 15×10^6 48 hr ConA-activated PBLs were fused with 15×10^6 CEM-AG^R cells as described above. These fused cells were plated at 1×10^6 /ml in 96 well flat-bottomed microtitre plates in either HAT medium, 20% FCS or azaserine (Sigma) (1 μ g/ml) and hypoxanthine (0.1mM), 20% FCS and fed every 2-3 days. After 14 days the cells were conditioned through HT medium (no aminopterin), 20% FCS or H medium (no azaserine), 20% FCS for the respective plates, for 14 days, then into tissue culture medium lacking anti-metabolites. At 30 days post-fusion the number of wells containing viable cells, as assessed by direct microscopic examination, were counted. This estimate of the numbers of wells containing viable cells was used to compare the 2 drug selection systems.

2.3 SURFACE MEMBRANE STAINING AND FLUORESCENCE ACTIVATED CELL SORTING

2.3.1 Introduction

The fluorescence activated cell sorter (FACS) (Becton-Dickinson FACS Systems, Sunnyvale, California) is an instrument which enables both the

rapid analysis of cell populations and the physical separation of selected cell subpopulations. The FACS allows large numbers of cells to be analysed on a cell-by-cell basis, is highly sensitive to fluorescence signals (e.g. fluorochrome-labelled cell populations), allows the objective interpretation of cell population data, and in addition, allows the sterile collection of specific cell subpopulations.

Analysis of individual cells results from the sample passing down a sterile saline stream which is intersected, at right angles, by a focussed argon laser. When the laser strikes a cell in the stream, the cell emits a number of signals, each of which is characteristic of that cell. These signals can be:

- 1) fluorescence; resulting from either cell autofluorescence or fluorochrome-labelled antibodies which have been bound to specific cell surface receptors, or
- 2) light scatter; resulting from reflection, refraction and diffraction of laser light striking the cell. The magnitude of the scatter signal is determined primarily by cell size i.e. larger cells in general scatter more light.

To physically sort cells, the operator sets ranges for each signal into which a cell must fall to be sorted. By vibrating the fluid stream at high frequency it breaks into well defined drops. By charging the stream just as the required cell is about to become part of a drop it is given a charge which causes it to be deflected further on as it passes through a constant electric field. Depending on the charge given, it is deflected to either the left or right, and the desired cell can be collected.

2.3.2 Analysis of data

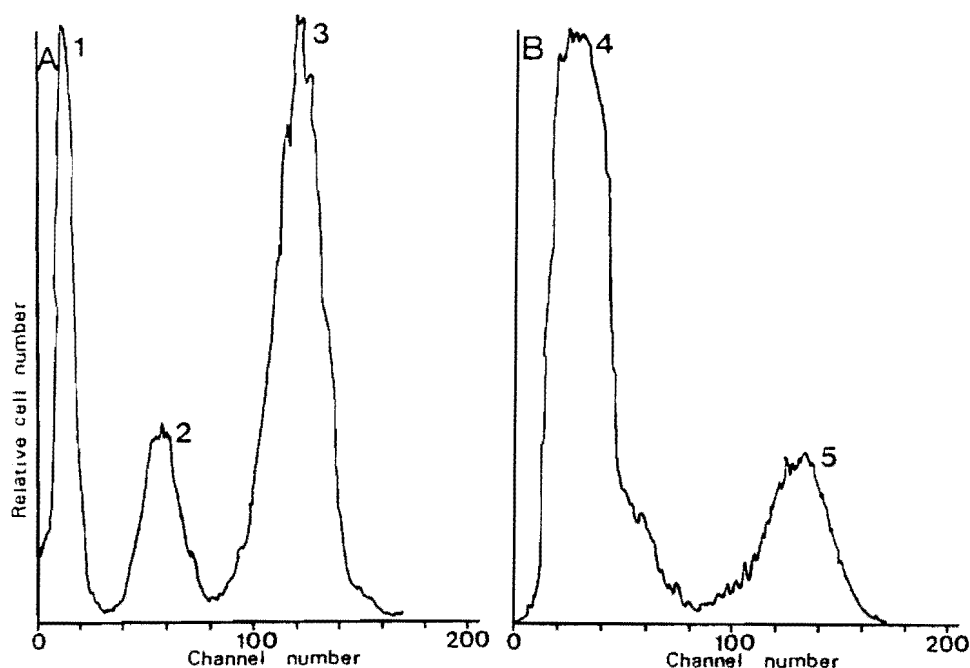
As each cell is analysed, four separate signals are generated, one for scatter, two for fluorescence and one representing a ratio of any two signals.

2.3.2 (a) Single parameter display of data

This method of display consists of histograms of two selected parameters, in this case scatter and fluorescence (Figure 2.1). The horizontal axis is the intensity of the signal and the vertical axis is the relative number of cells at each intensity of signal.

FIGURE 2.1

SINGLE PARAMETER DISPLAY OF FACS DATA



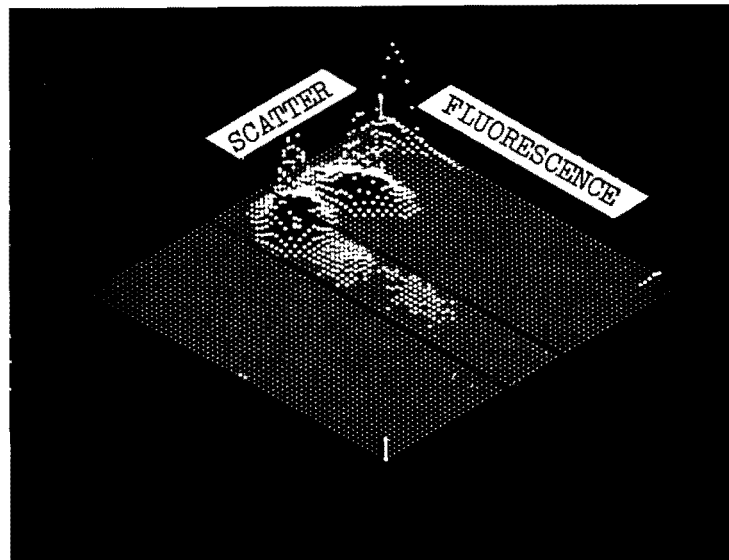
- A. Histogram of light scatter (horizontal axis) versus relative number of cells at each intensity of signal (vertical axis). Peak 1 : debris, cell fragments etc; Peak 2 : small cells; Peak 3 : large cells.
- B. Histogram of log fluorescence (horizontal axis) versus relative number of cells at each intensity of signal (vertical axis). Peak 4 : non fluorescing cells; Peak 5 : fluorescent cells.

2.3.2 (b) Dual parameter display of data

Single parameter histograms unfortunately do not allow the correlation between parameters to be expressed i.e. one cannot infer what peak (s) on the scatter histograms belong with what peak(s) on the fluorescence histogram. Dual parameter or three dimensional displays overcome this problem as each horizontal axis represents a signal strength of one of the selected parameters and the vertical scale gives the number of cells with that combination of signal amplitudes. Figure 2.2 represents a typical display using dual parameter analysis.

FIGURE 2.2

DUAL PARAMETER DISPLAY OF FACS DATA



Dual parameter display of peripheral blood lymphocytes stained with a reagent directed against surface membrane immunoglobulin (SmIg); causing B cells to fluoresce. The profiles of scatter versus log fluorescence are shown. The height of each peak indicates the relative number of cells at each intensity of signal.

2.3.2 (c) Gated analysis

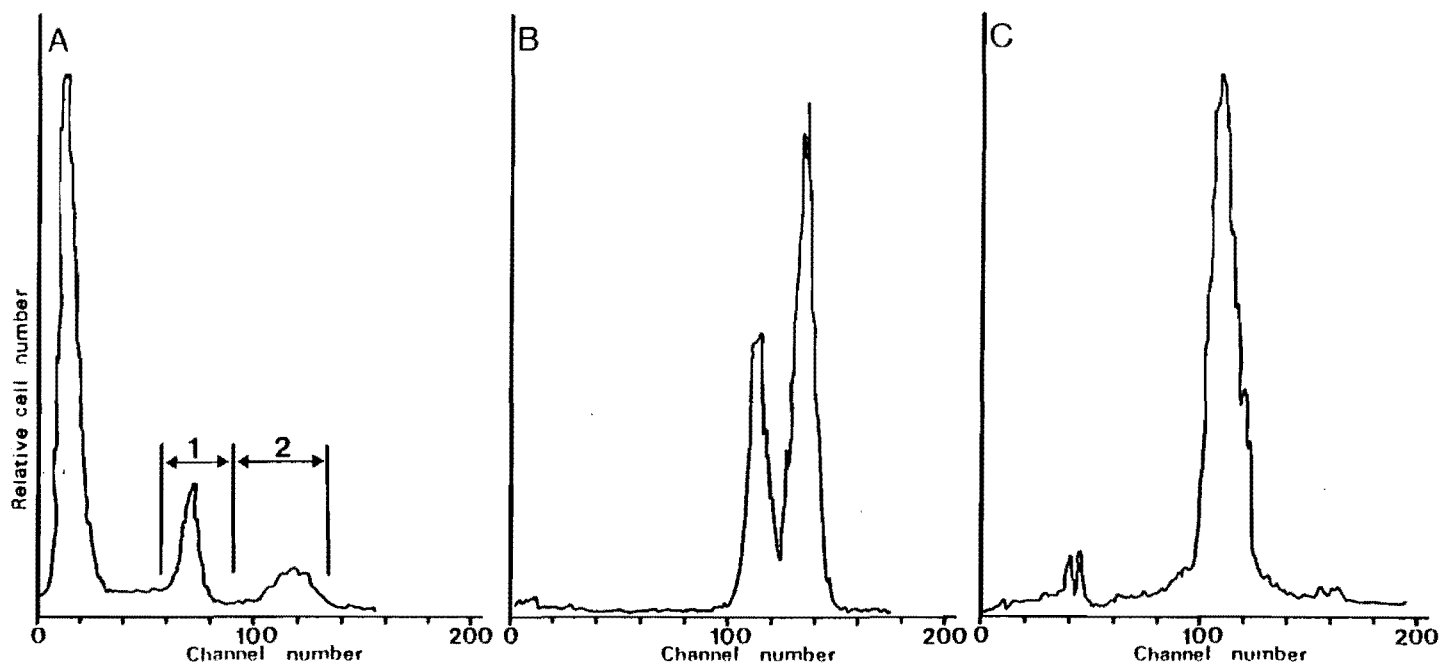
This analysis method overcomes many of the problems inherent in single parameter analysis. Two ranges are set, into which a cell must fall, before it is counted and displayed on a histogram. For example, if one wishes to investigate the fluorescence distribution of lymphocytes this is achieved by gating on the part of the scatter histogram which corresponds to the lymphocytes and then display the resulting fluorescence histogram (Figure 2.3).

The use of highly specific, fluorochrome-labelled, monoclonal antibodies to human cell surface receptors, in conjunction with FACS analysis and sorting, are powerful techniques which can be used for the quantitation and isolation of specific T cell subpopulations depending on the particular antigen array they

express on their cell membranes.

FIGURE 2.3

GATED DISPLAY OF FACS DATA



This figure describes the use of gating for the analysis of cell distributions.

- A. Ungated fluorescence profile of peripheral blood mononuclear cells stained with a monoclonal antibody which identifies : Peak 1 : lymphocytes;
Peak 2 : monocytes.
- B. Scatter distribution of cells falling in the low positive fluorescence peak (Histogram A, peak 1).
- C. Scatter distribution of cells falling in the high positive fluorescence peak (Histogram A, peak 2).

2.3.3 Monoclonal antibodies (McAbs) to human cell surface receptors

The membrane phenotype of the parental tumor lines (CEM-6TG^R, CEM-AG^R) was determined by using a wide range of McAbs to human cell surface receptors. This approach aimed to use the membrane phenotype as a method to select hybrids in conjunction with the usual drug selection. The McAbs used, and the cell antigens or subpopulations they define are presented in Table 2.1. The indirect immunofluorescence staining technique is presented in Section 2.3.4.

2.3.4 Indirect immunofluorescence staining using monoclonal antibodies

Indirect immunofluorescence staining using murine monoclonal antibodies directed against human cell surface antigens was performed according to the manufacturers methods (Bectin-Dickinson, Ortho Diagnostics). Cell suspensions were washed twice with PBS-5% FCS and adjusted to a concentration of $10 \times 10^6/\text{ml}$. 100 μl of this suspension (1×10^6 cells) was added to RTU tubes (Becton-Dickinson) or round-bottomed microtitre plates containing the requisite amount of McAb (Appendix 1) and PBS-5% FCS to a final volume of 200 μl . The cell and McAb mixture was incubated on ice for 30 mins with the occasional gentle mixing. The tubes/plates were centrifuged for 10 mins at 400g, at 4°C, and the supernatants were carefully aspirated from the cell pellet. Cells were resuspended and washed twice with PBS-5% FCS to remove excess unbound McAb. The second layer antibody was affinity purified Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Tago). The McAb labelled cell pellet was gently resuspended by tapping the tube/plate, 100 μl of the second layer antibody was added ($5\mu\text{g}/1 \times 10^6$ cells) and the cells were incubated for 30 mins on ice. The stained cells were washed twice with cold PBS-5% FCS and resuspended in 100 μl of the same buffer for flow cytometric analysis.

Counting of B cells also used an indirect immunofluorescence technique. The first layer antibody was affinity purified goat anti-human Ig ($3.7\mu\text{g}/1 \times 10^6$ cells) (Tago), 30 mins on ice. After 2 washes with PBS-5% FCS, bound antibody was detected with FITC-conjugated swine anti-goat Ig ($5\mu\text{g}/1 \times 10^6$ cells) (Tago). After washing, labelled cells were resuspended in 100 μl PBS-5% FCS for analysis.

Controls of cells stained with the second layer antibody alone were used in

TABLE 2.1

MONOCLONAL ANTIBODIES TO HUMAN CELL SURFACE RECEPTORS

Antigen				
Name	MW (Kdal)	Cellular distribution	Population defined	References
Leu-1 *	67	95% thymocytes, > 85% E ⁺ T cells, 72% normal PBL	Mature T cells	1,2
Leu-2a/OKT8 ^Δ	32 & 43	20-40% peripheral T cells, 60-85% thymocytes	T suppressor/ cytotoxic	2,3,4
Leu-3a	55	35-55% PBLs, 80-95% thymocytes	T helper/inducer	2,3,5
Leu-3a + 3b/ OKT4	55 62	"	"	6,7,8
Leu-4/OKT3	20 - 30	68-82% PBLs, 80-85% E ⁺ T cells, 65-85% thymocytes	Pan T cell	2,8
Leu-5	50	78-88% PBLs, 95-100% E ⁺ T cells, 96-99% thymocytes	E rosette	9, 10
HLA-DR/FMC4	28 & 34	7-15% PBLs	B cells, activated T cells	11, 12
β ₂ M	12	All human cells	β ₂ microglobulin	11
HTA1	45	85% thymocytes	Thymocyte	13
W6/32	28 & 34	-	HLA-ABC framework	14
TG-1	-	-	Granulocyte	15
2D-1	-	-	Common leucocyte	15
3A-1	40	65% T cells	Activated T cell	16
OKT6	-	70% thymocytes	Cortical thymocyte	17
OKM1	-	78% adherent mononuclear cells, 18% null cells	Monocyte	18

* Leu series McAbs : Becton-Dickinson, Sunnyvale, California.

Δ OK series McAbs : Ortho Diagnostic Systems Inc., Raritan, New Jersey.

References

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all experiments.

Cell analysis was on a FACS IV (Becton-Dickinson, Sunnyvale, CA) with a 2 watt argon laser (Spectra Physics model 164-06) tuned at 488nm with 180mV power.

2.3.5 Sterile sorting of hybrids

At 14 days post fusion, the plates containing CEM-6TG^R X ConA PBL putative hybrids were assessed microscopically for evidence of cell growth. Two wells from fusion experiment 1 and one well from fusion experiment 2 were selected because of viable cell growth and these individual wells were expanded into tissue culture flasks (Nunc) for 1 week in RPMI, HAT, 20% FCS. The expanded cultures were then stained with the OKT3 McAb (Ortho Diagnostics, pan T cell McAb) and the OKT3⁺ staining population was sterile-sorted directly into a 2054 tube (B-D) containing 1ml RPMI. Dual parameter gated analysis was used in setting up narrow selection windows to exclude non-viable and OKT3⁻ hybrids. These sorted cells were cultured and conditioned through HT (no aminopterin) supplemented media into RPMI, 15% FCS over the next 12 days. Four hybrid lines were established in this manner. TT-1 and TT-2 were established by separate sterile sorts of cells from fusion experiment 1, TT-3 resulted from a second OKT3⁺ sort of TT-2 and TT-4 was established from fusion experiment 2. These lines were maintained in culture in RPMI, 15% FCS and samples were frozen at regular intervals and stored in liquid nitrogen. The surface phenotype of the lines was assessed from 10-140 days post fusion.

2.4 CHROMOSOMAL ANALYSIS

Chromosomal analysis of parental and hybrid cells used standard procedures. Exponentially growing cell cultures were treated with colchicine (0.2µg/ml) for 1-2 hrs, centrifuged and washed with 0.075 M KCl. Cells were fixed with methanol : acetic acid (3:1, v/v) and metaphase slides were prepared by airdrying. Banding was performed using trypsin.

2.5 FUNCTIONAL HYPOXANTHINE GUANINE PHOSPHORIBOSYL TRANSFERASE (HGPRT) ACTIVITY

The ability of parental and hybrid cells to incorporate preformed hypox-

anthine via the salvage enzyme HGPRT, was assessed directly by utilising ^3H -Hypoxanthine. The method used was based on that of Morley et al (1983).

Twelve replicates of 5×10^4 cells per well were grown in 96 well round-bottomed microtitre plates for 48 hrs in a total volume of 200 μl . Six replicates were pulsed with 2.0 μCi [^3H (G)]-Hypoxanthine (NEM, spec act 9.1 Ci/mmol) and the other six replicates with 2.0 μCi [^3H]-Thymidine (Amersham, spec act 5 Ci/mmol) for 21 hrs. The cells were then harvested onto glass fibre filters using an automated cell harvester (Skatron, Norway), dried at 60°C for at least 1 hr and counted by liquid scintillation (Appendix 2).

2.6 FUNCTIONAL ASSAYS

2.6.1 Introduction

Functional assays were developed prior to the construction of human T cell hybrids to enable the screening of hybrid-derived supernatants for possible effects on in vitro T and B cell responses. Two B cell antibody systems were developed and validated. The first, utilised a relatively simple radioimmunoassay to measure total and specific antibody production to the hapten-linked proteins dinitrophenyl-bovine serum albumin (DNP-BSA) and dinitrophenyl-keyhole limpet hemocyanin (DNP-KLH) in the presence of the polyclonal activator pokeweed mitogen (PWM). A number of methods were exploited to circumvent the need for PWM to trigger B cell differentiation and immunoglobulin secretion. This assay system was used to screen for hybrid-derived lymphokine effects on the polyclonal activation of normal human B cells in vitro. A second antibody assay, a hapten-specific system, was developed which enabled the generation and quantitation of hapten-specific antibody responses in the absence of polyclonal activators or other exogenous stimuli. This system will be of use in the future for the development and assaying of hybrids secreting lymphokines with specificity for hapten-carrier conjugates. The effects of hybrid-derived lymphokines on T cell responses were measured using a short term phytohemagglutinin (PHA)-stimulated T cell proliferation assay.

2.6.2 Human antibody assay (pokeweed mitogen-dependent)

2.6.2 (a) Antibody tracers

Direct binding assays were performed to quantitate total

and specific IgG or IgM antibody production using ^{125}I labelled goat anti-human IgG or IgM as the radioactive tracer.

The chloramine T method of Hunter (1974) was used to radioiodinate the tracer antibody to a relatively high specific activity.

All tracer iodinations were performed in a modified fume hood using biogamma (Ansell) gloves. Standard "hot laboratory" procedures were strictly applied.

5 μl (0.5mCi) Na ^{125}I (Amersham, IMS.30, pH 7-11, 100mCi/ml) was mixed with 20 μl 0.5M Na₂ PO₄ buffer pH 7.4 and cooled on ice. 50 μl (50 μg) affinity purified goat anti-human IgG or IgM (Tago) was then added and cooled on ice for 30 secs. 20 μl chloramine T (Sigma) (1mg/ml) was added, to oxidase the Na ^{125}I , and mixed for 60 secs on ice. 20 μl sodium metabisulphite (2mg/ml), to reduce any unreacted ^{125}I back to ^{125}I iodide, was added and stirred on ice for 120 secs. 50 μl potassium iodide (10mg/ml) was added to stabilise the labelled ^{125}I . The iodinated protein mixture was applied to a Sephadex G50 (Pharmacia Fine Chemicals) column (1.5 x 12cm) and eluted with PBS pH 7.4 at a flow rate of 0.5ml/min to separate the free and bound tracer. 0.5ml fractions were collected and 5 μl aliquots were gamma counted (Beckman Biogamma) to determine the positions of the labelled antibody and free tracer. A pool of the most active fractions was made and the specific activity calculated from the d.p.m and protein concentration.

2.6.2 (b) Antigens

DNP-BSA (Calbiochem) 34.6 DNP residues/BSA residue, stock solution prepared in deionised H₂O. DNP-KLH (Calbiochem) 625 moles DNP/mole hemocyanin; based on a MW of 2×10^6 daltons, stock solution prepared in ethanol. PWM (Sigma) stock solution prepared in RPMI. Antigen and mitogen stocks were filter sterilised using a 0.2 μm disposable filter (Acrodisc, Gelman) and frozen at -20°C until used.

2.6.2 (c) Culture conditions

Optimal cell and antigen concentrations were determined for both DNP-BSA and DNP-KLH in the presence of PWM (1 $\mu\text{g}/\text{ml}$) (mitogenic dose). 2ml cultures were performed in supplemented RPMI 1640, 10% FCS in 24 well

macro-plates (Nunc) for 6-7 days at 37°C in a 5%CO₂ atmosphere. At the end of this period cell free supernatants were collected by centrifugation, assayed immediately for total IgG/IgM or anti-DNP IgG/IgM antibodies, or stored at -20°C for subsequent antibody determinations.

2.6.2 (d) Antigen concentration

Freshly isolated PBLs (1×10^6 /ml) were cultured with varying concentrations of DNP-BSA or DNP-KLH and PWM (1µg/ml). The antigen concentrations were 0.01, 0.1, 1.0 and 10.0µg/ml. Controls utilised cells cultured in the absence of antigen to determine the level of specific antibody production in response to PWM stimulation alone.

2.6.2 (e) Cell concentration

Varying numbers of PBLs (0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0 and 5.0×10^6) were cultured with DNP-BSA (0.1µg/ml) and PWM (1µg/ml). Controls utilised varying PBL numbers cultured with PWM or PBLs alone.

2.6.2 (f) Alternatives to polyclonal activation with pokeweed mitogen

Cultures were performed using hydrocortisone (10^{-5} M) (Lobo & Spencer, 1979) to inhibit T suppressors without affecting the T helpers, or IL-2 (1:10) to expand the antigen-activated T cells (Aarden et al, 1979).

PBLs (1×10^6 /ml) were cultured with either DNP-KLH (0.1µg/ml) + IL-2 (1:10) (BRL), Hydrocortisone (10^{-5} M) + DNP-KLH (0.1µg/ml) or DNP-KLH alone. All cultures were performed in the presence and absence of PWM (1µg/ml). Controls utilised PBLs cultured with and without PWM.

2.6.2 (g) Radioimmunoassay

A solid phase RIA, based on the method of Nonaka et al (1981) was developed. 96 well round-bottomed flexible polyvinyl plates (Cooke) were coated with goat anti-human IgG or IgM (2µg/ml), to measure total IgG or IgM antibodies, or DNP-carrier (100µg/ml) to measure DNP-specific antibodies, in PBS pH 7.4 at 4°C overnight. The plates were washed with PBS and non-specific binding sites were blocked with 3% BSA, 0.2% azide in PBS for 30 mins

at room temperature. After extensive washing, 50 μ l samples (triplicate determinations) were incubated at 4°C for 4 hrs. After washing, 50 μ l 125 I goat anti-human IgG or IgM (spec act 8-12 μ Ci/ μ g at time of preparation, diluted to give approximately 8×10^4 cpm/50 μ l) in 3% BSA-PBS was added and incubated at 4°C overnight. The wells were aspirated and extensively washed with PBS. Individual wells were cut out and the bound radioactivity was counted in a gamma counter (Beckman Biogamma). Results of triplicate determinations were expressed as cpm-background \pm SD of sample means. Background binding in each assay was assessed using FCS at the same concentration as the samples, and was generally 100-200 cpm. Using this RIA one was able to measure total or DNP-specific antibody production.

2.6.2 (h) Validation of the radioimmunoassay

Three methods were used to validate the RIA.

- 1) culture supernatants from a PBL culture (1×10^6 /ml) with DNP-KLH (0.1 μ g/ml), PWM (1 μ g/ml) were serially diluted with RPMI, 10% FCS and assayed to ensure a "classical" dose response curve for total IgG and IgM antibodies was produced.
- 2) Supernatants from a PBL (1×10^6 /ml), IL-2 (1:10) DNP-KLH (0.1 μ g/ml), PWM (1 μ g/ml) culture were titrated with increasing amounts of ovalbumin, DNP-BSA and KLH. The amount of free antibody left in the supernatant was determined after an overnight incubation at 4°C and centrifugation to remove antigen-antibody complexes.
- 3) An IgG standard curve was constructed using a serum standard quantitated by the Biochem. Dept., Christchurch Hospital, in the range of 0.1ng-100 μ g/ml. A standard curve of IgG concentration versus 125 I G α Hu IgG cpm bound was constructed from this.

2.6.2 (i) Effect of hybrid-derived supernatants on a PWM-induced polyclonal antibody response

Cell free supernatants derived from the hybrid lines,

TT-1 to TT-4, were screened for possible regulatory effects on the polyclonal antibody response of normal PBLs stimulated with PWM. Hybrids were tested for both constitutive and induced (ConA-treated) production of lymphokines.

Preliminary experiments indicated that supernatants derived from the 4 lines had a suppressive effect on the generation of an in vitro PWM-induced polyclonal antibody response. One line (TT-4), had the most potent effect and this was chosen for more detailed study.

i) Constitutive lymphokine production

Cells from the TT-4 line were grown at three densities (0.5, 1.0, $5.0 \times 10^6/\text{ml}$) for 5 days and cell free supernatants derived from these hybrid cells were added at the commencement of a PWM-induced antibody culture at final dilutions of 1:2, 1:10 and 1:50. The assay was that described above and the culture was for 7 days. The cell free supernatants from this PWM culture were then assayed for total IgG antibodies by the solid phase RIA described in Section 2.6.2 (g).

ii) Hybrid induction

TT-4 cells were seeded at two densities (0.5 and $1.0 \times 10^6/\text{ml}$) and each cell culture was stimulated for 48 hrs with 1,5 and $10\mu\text{g}/\text{ml}$ ConA. At the end of this period, cell free supernatants were harvested and added at the commencement of a PWM-induced antibody assay at a final dilution of 1:2. After 7 days the cell free supernatants from this PWM culture were assayed for total IgG antibodies as described.

2.6.3 Human hapten-specific antibody responses

2.6.3 (a) Introduction

A microculture and RIA was developed which was capable of generating and detecting very small levels of human anti-hapten antibody produced in a primary response without the addition of high concentrations of serum, mitogens or the prior immunisation of the donor with antigen. This assay was optimised for cell density and antigen concentration. In addition, the kinetics, T cell dependence and specificity of the anti-hapten response were investigated.

2.6.3 (b) Antigens

DNP-BSA and DNP-KLH hapten-carrier conjugates were the same as described in Section 2.6.2 (b).

2.6.3 (c) T and B cell purifications

i) Preparation of antibody coated plates

Petri plates (Falcon, 100mm diameter) were incubated overnight at 4°C with 5ml rabbit anti-human IgG/Fab (Behring) at 232µg/ml in Tris buffered saline pH 7.4. Coated dishes were washed 4 times with PBS.

ii) Cell separations

The panning technique of Mage et al (1977) was used. PBL suspensions ($5 \times 10^7/5\text{ml}$), were added to the coated plates and incubated for 2 hrs at room temperature. Half-way through the incubation period, the non-adherent cells were resuspended by manually swirling the plate and allowing to resettle. Non-adherent cells were then removed by gentle decantation and the plates were gently washed 5 times with 5-10ml PBS-1% FCS. Adherent cells were removed by vigorously pipetting with 10-15ml PBS-1% FCS with a pasteur pipette followed by scraping with a rubber policeman.

The non-adherent cells were further depleted of contaminating T cells by complement-dependent lysis using a monoclonal antibody and complement. Adherent cells were incubated with OKT3 (5µg/ 1×10^6 cells) in a volume of 200µl in deficient RPMI 1640 (Irvine Scientific), 5% FCS for 30 min on ice, then washed twice with deficient RPMI. The cell pellet was incubated with rabbit complement (GIBCO) (100µl/ 1×10^6 cells), at 37°C for 1 hr and washed twice with RPMI.

iii) T and B cell quantitation

T cells were quantitated in the final adherent and non-adherent populations by indirect staining with the Leu-1 McAb as described in Section 2.3.4. B cell quantitation utilised an immunofluorescence technique as described in the same section and cell analysis was on a FACS IV.

2.6.3 (d) Culture conditions

The optimal antigen concentration, cell density and kinetics for the generation of anti-hapten antibodies were determined. Human PBLs or purified B or T cell populations were cultured for 5-7 days with either DNP-BSA, DNP-KLH or in the absence of antigen in a total volume of 200 μ l in 96 well flat-bottomed microtitre plates. The culture medium was supplemented RPMI 1640, 5×10^{-5} M 2-mercaptoethanol, 10% FCS. At the end of the culture period the cells were washed 3 times with fresh medium and resuspended in medium without antigen. After a further 4 days in culture, the supernatants were immediately assayed or stored at -20°C .

2.6.3 (e) Antigen concentration

1×10^6 cells/200 μ l were cultured in the range of 0.1-50 μ g of antigen as described and the level of anti-hapten antibodies was then determined.

2.6.3 (f) Cell concentration

Varying numbers of human PBLs ($0.5-20.0 \times 10^5$) were cultured with DNP-BSA, DNP-KLH (1.0 μ g/ml) or without antigen for 5 days. After washing and a further 4 days in culture, supernatants were assayed for the presence of anti-hapten antibodies.

2.6.3 (g) Kinetics of anti-hapten response

The kinetics of the anti-hapten response was studied by setting up cell cultures of PBL (1×10^6) with DNP-BSA, DNP-KLH (1.0 μ g) or without antigen. At the times indicated (Figure 3.18) the cells were washed 3 times with fresh medium and cultured for a further 4 days in the absence of antigen. Culture supernatants were harvested and stored at -20°C until assayed as one batch.

2.6.3 (h) T cell dependence of anti-hapten response

To determine if this antigen-driven culture system was T cell dependent, varying numbers of purified T cells were mixed with a constant number of B cells (5×10^5) and cultured with DNP-BSA (0.1 μ g) in the

microculture.

2.6.3 (i) Hapten-specific radioimmunoassay

The assay procedure was essentially that described (Morimoto et al, 1981). 96 well round-bottomed flexible polyvinyl plates were coated with DNP-BSA (1mg/ml in PBS) at 4°C overnight. After blocking and washing as previously described, 50µl aliquots (triplicate determinations) of culture supernatants were assayed. Again the tracer was affinity purified (μ chain specific) ^{125}I goat anti-human IgM (8×10^4 cpm) in 1% BSA-PBS. After overnight incubation and washing individual wells were cut out and gamma counted. Background binding was determined using 10% FCS with the results being expressed as cpm - background \pm SD of sample means.

2.6.3 (j) Specificity of radioimmunoassay

The specificity of the RIA was confirmed by titrating DNP-BSA or ovalbumin into anti-hapten antibody culture supernatants as described in Section 2.6.2 (h).

2.6.4 T cell proliferation assay

TT-4 supernatants were added at the commencement of a T cell proliferative response assay to determine if the hybrid-derived supernatants contained lymphokines which could in some way modify this response. Purified PBLs were stimulated with 1% PHA (M form) (GIBCO) for 48 hrs, washed twice to remove residual lectin, and plated in a 96 well round-bottomed microtitre plates (5×10^4 /well). Cells were cultured in RPMI, 15% FCS with partially purified human IL-2 at a final dilution of 1:10. Dilutions of hybrid supernatants were titrated in with the cells and IL-2. Cells were cultured for a further 48 hrs followed by a 16 hr pulse with ^3H -Thymidine (2.0µCi/ well). Cells were harvested onto glass fibre filters, dried and counted by liquid scintillation.

2.7 PRELIMINARY BIOCHEMICAL STUDIES OF HYBRID-DERIVED FACTORS

2.7.1 Gel filtration chromatography

Concentrated TT-4 supernatant was chromatographed on a

Sephacryl S-200 column which had been calibrated using protein standards of known molecular weights.

2.7.1 (a) Column calibration

A LKB K26 column (90 x 2.6cm) was packed with Sephacryl S-200 (Pharmacia Fine Chemicals). The flow rate was 15ml/hr and the column was eluted with PBS pH 7.4. A 5ml cocktail of the following protein standards was pumped onto the equilibrated column and eluted at 15ml/hr. The protein standards were: 1mg each of catalase (210,000), phosphorylase b (97,000), BSA (67,000), ovalbumin (43,000) and myoglobin (16,700). Blue dextran was added to the cocktail to estimate the void volume ($V_0 > 2.5 \times 10^5$ daltons). A standard curve of K_{av} versus log MW was constructed from the elution volumes of the protein standards and blue dextran.

2.7.1 (b) TT-4 chromatography

90ml of TT-4 supernatant was concentrated to 6.5ml by Amicon ultrafiltration over a PSAC-10³ MW membrane (Millipore Corporation). 5ml of concentrated supernatant was chromatographed on the calibrated S-200 column at 15ml/hr with 5ml fractions being collected. Tubes corresponding to various MW ranges were pooled, concentrated to 3ml over a PSAC-10³ membrane, filter sterilised and assayed in the short-term T cell proliferation assay.

2.7.2 Physicochemical characterisation

Certain physicochemical characteristics of the TT-4 supernatants were investigated. Supernatants were dialysed against 0.2M glycine buffer pH 2.0 or 0.2M Tris buffer pH 9.0 for 24 hrs at 4°C. Supernatants were also treated with 50mM dithiothreitol (DTT) (Research Organics). These treated supernatants were dialysed extensively against RPMI and filter sterilised before being tested in the short-term T cell proliferation assay. Supernatants were also cocultured with the monosaccharides L-rhamnose, α -methyl mannoside, L-fucose and N-acetyl glucosamine (Sigma) (all 50mM) in the T cell proliferation assay. Controls for all samples utilised RPMI, 15% FCS treated in an identical manner to the TT-4 supernatants.

CHAPTER 3.

RESULTS

3.1 INTRODUCTION

The aim of this work was to develop somatic cell hybridisation techniques as a means of immortalising normal T cell function. The basic assumption made with cell hybridisation studies such as these is that the secretory activity of the normal lectin-activated T cell will be immortalised on the continuously proliferating background of the parental leukemic cell.

3.2 CHARACTERISTICS OF PARENTAL CELL LINES

This section of the thesis describes a number of growth and phenotypic characteristics of the parental leukemic and derived T cell hybrid lines. Sections 3.2 - 3.4 present the results of these studies.

3.2.1 Derivation of drug-resistant parental T cells

A 6-thioguanine-resistant ($6TG^R$) leukemic T cell line was established as the parental line for the construction of human T cell hybrids.

Table 3.1 describes the efficiency of recovery of CCRF-CEM cells following an initial mutagenesis with varying concentrations of EMS. 38-56% (50-1 μ g/ml EMS respectively) of the mutagenised cells were still viable after 24 hrs compared to 85% in the non-mutagenised controls.

TABLE 3.1

EMS MUTAGENESIS OF CCRF-CEM LINE

EMS concentration ($\mu\text{g/ml}$)	0	1	5	10	50
Viable cells seeded ($\times 10^{-6}/\text{ml}$)	1.0	1.0	1.0	1.0	1.0
Viable cells recovered after 24 hrs ($\times 10^{-6}/\text{ml}$)	0.85	0.56	0.53	0.42	0.38
Efficiency of recovery (%)	85	56	53	42	38

Exponentially growing CCRF-CEM cells were mutagenised with varying concentrations of EMS (0-50 $\mu\text{g/ml}$). The efficiency of recovery of the EMS-treated cells after 24 hrs is shown.

Table 3.2 (a) describes the recovery of cells following an increase in the 6TG concentration from 0.1 $\mu\text{g/ml}$ ($5.5 \times 10^{-7}\text{M}$) to 1.0 $\mu\text{g/ml}$ ($5.5 \times 10^{-6}\text{M}$). Recoveries of 54-76% were achieved after 5 days.

TABLE 3.2 (a)

6TG SELECTION OF EMS-TREATED CCRF-CEM LINE

EMS concentration ($\mu\text{g/ml}$)	1	5	10	50
6TG concentration ($\mu\text{g/ml}$)	1.0	1.0	1.0	1.0
Viable cells seeded ($\times 10^{-6}/\text{ml}$)	0.5	0.5	0.5	0.5
Viable cells recovered after 5 days ($\times 10^{-6}/\text{ml}$)	0.27	0.38	0.30	0.28
Efficiency of recovery (%)	54	76	60	56

EMS-treated CCRF-CEM cells were cultured for 7 days in RPMI, 15% FCS supplemented with 0.1 $\mu\text{g/ml}$ 6TG. Cells exhibiting growth were expanded into tissue culture flasks at the viable cell concentrations shown and treated with 1.0 $\mu\text{g/ml}$ 6TG. The efficiency of recovery after 5 days is shown.

These cultures were then reseeded at the densities indicated in Table 3.2 (b) and the 6TG concentration was increased to 1.36 μ g/ml (7.5×10^{-6} M). Recoveries of 48-79% were achieved after 3 days.

TABLE 3.2 (b)

6TG SELECTION OF EMS-TREATED CCRF-CEM LINE

EMS concentration (μ g/ml)	1	1	5	5	10	10	50	50
6TG concentration (μ g/ml)	1.0	1.36	1.0	1.36	1.0	1.36	1.0	1.36
Viable cells seeded ($\times 10^{-6}$ /ml)	0.85	0.85	0.95	0.95	0.60	0.60	0.28	0.28
Viable cells recovered after 3 days ($\times 10^{-6}$ /ml)	0.51	0.41	0.50	0.48	0.44	0.43	0.22	0.14
Efficiency of recovery (%)	60	48	52	51	73	72	79	50

EMS-treated cells were further treated with increasing concentrations of 6TG. Cells were seeded at the viable cell concentrations shown and treated with 1.0 and 1.36 μ g/ml 6TG. The efficiency of recovery after 3 days is shown.

The EMS-mutagenised CCRF-CEM line which exhibited the best growth characteristics (10 μ g/ml EMS) was selected with increasing concentrations of 6TG. Table 3.2 (c) describes the viable cell recovery when this line was treated with 1-5.44 μ g/ml 6TG (5.5×10^{-6} - 3.0×10^{-5} M).

A line resistant to 5.44 μ g/ml 6TG continued to grow well in culture, and was further selected with 6TG at 18 μ g/ml (1.0×10^{-4} M). This line, CEM-6TG^R, was maintained in 6TG-supplemented tissue culture medium, 15% FCS and was used as the drug-resistant parental hybrid line.

TABLE 3.2 (c)

6TG SELECTION OF EMS-TREATED CCRF-CEM LINE

EMS concentration ($\mu\text{g/ml}$)	10	10	10
6TG concentration ($\mu\text{g/ml}$)	1.0	1.36	5.44
Viable cells seeded ($\times 10^{-6}/\text{ml}$)	0.34	0.33	0.18
Viable cells recovered after 4 days ($\times 10^{-6}/\text{ml}$)	0.40	0.40	0.36
Efficiency of recovery (%)	118	121	200

A line of EMS-mutagenised CCRF-CEM cells ($10\mu\text{g/ml}$ EMS), resistant to $1.0\mu\text{g/ml}$ 6TG, was seeded at the viable cell concentrations shown and selected with 1.0, 1.36 and $5.44\mu\text{g/ml}$ 6TG. The efficiency of recovery after 4 days is shown. A line resistant to $5.44\mu\text{g/ml}$ 6TG was selected with $18\mu\text{g/ml}$ 6TG and maintained as the CEM-6TG^R parental hybrid line.

3.2.2 Growth rates

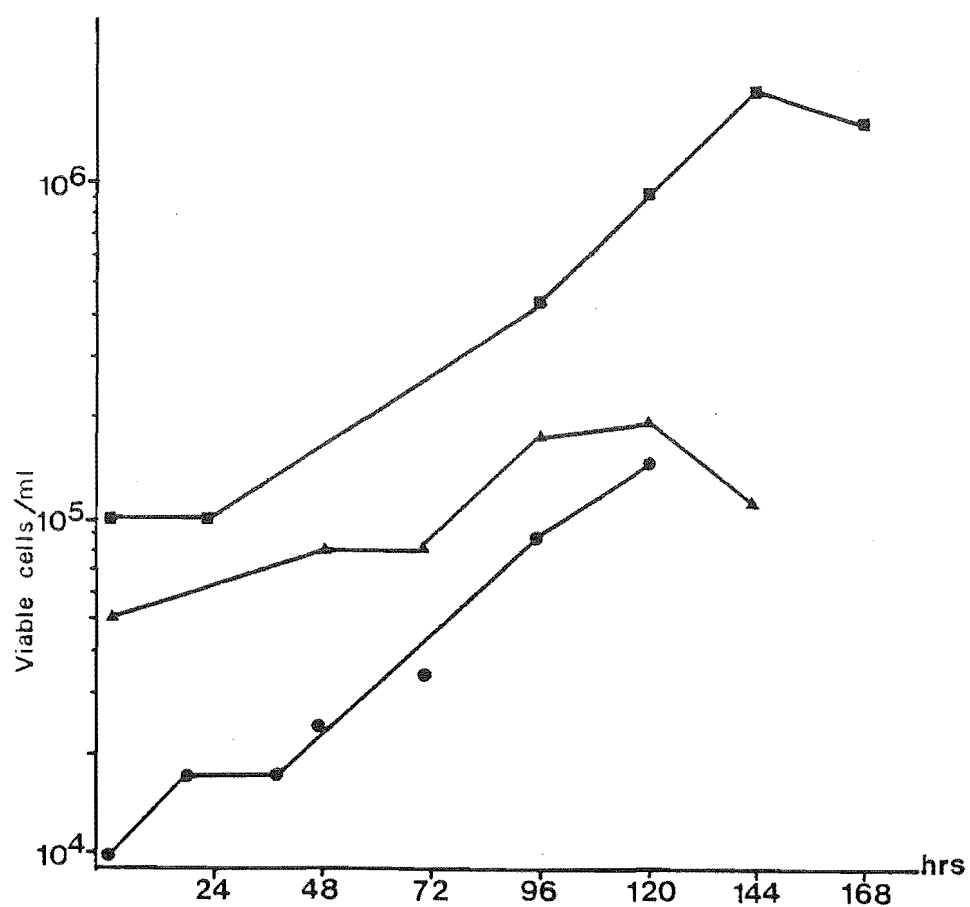
Figure 3.1 describes the growth characteristics for the CCRF-CEM, CEM-6TG^R and CEM-AG^R lines. Estimates of the doubling times of these lines were:

- 1) CCRF-CEM 26 hr,
- 2) CEM-6TG^R 30 hr,
- 3) CEM-AG^R 28 hr.

Inhibition of growth was observed after 120 hrs in culture ($2 \times 10^5/\text{ml}$) for the CCRF-CEM line and 140 hrs ($2 \times 10^6/\text{ml}$) for the CEM-AG^R line.

FIGURE 3.1

GROWTH RATES OF PARENTAL CELL LINES



Graph of log viable cell number/ml versus hours in culture. Estimates of doubling times for the parental lines were made while the cells were in exponential growth (48-96 hrs in culture).

- CEM - AG^R
- ▲—▲ CCRF - CEM
- CEM - 6TG^R

3.2.3 Terminal deoxynucleotidyl transferase staining

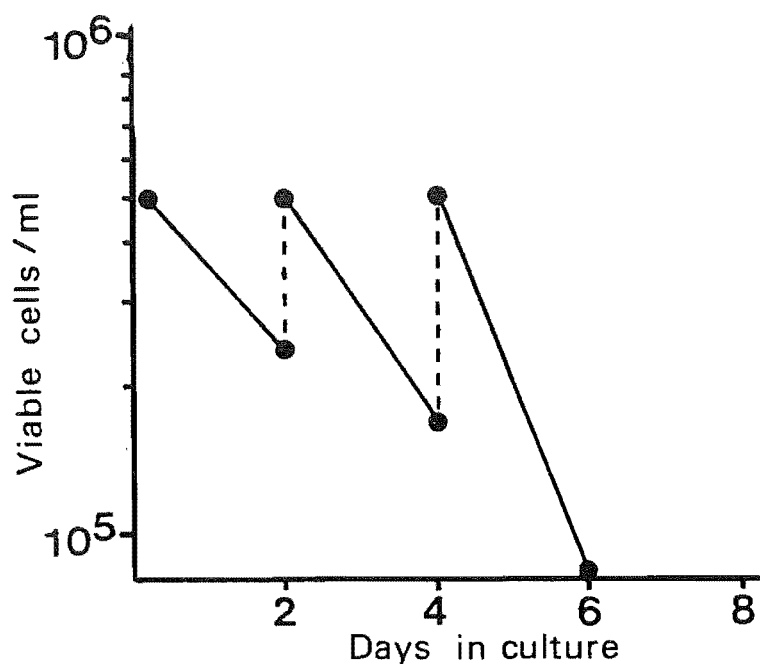
Both CCRF-CEM and CEM-6TG^R were TdT negative as assessed by the fluorescent antibody staining technique described. Mr M. Southern, Dept. Pathology, Christchurch Clinical School of Medicine, kindly performed the TdT staining.

3.2.4 HAT sensitivity

HAT sensitivity experiments were performed by culturing CEM-6TG^R cells directly in HAT-supplemented tissue culture medium. Every two days the cells were counted and recultured at a viable density of 5×10^5 /ml. Figure 3.2 indicates that no viable cells were present after 7 days in culture.

FIGURE 3.2

HAT SENSITIVITY OF CEM-6TG^R LINE



CEM-6TG^R cells were seeded in HAT medium at 5×10^5 /ml (Day 0). Every 2 days cells were counted and reseeded at a viable density of 5×10^5 /ml in fresh HAT medium. The graph shows the number of viable cells/ml at the times indicated.

3.3 HUMAN T CELL HYBRIDS : CRITERIA FOR HYBRIDISATION

To confirm that true human T cell hybrids had been produced from the drug and phenotypic selection programme adopted, three independent techniques were used as indicators of hybridisation. The three criteria investigated were:

- | | | |
|----|--|---------------|
| 1. | cell surface phenotype | Section 3.3.1 |
| 2. | acquisition of functional HGPRT activity | Section 3.3.2 |
| 3. | chromosomal analysis | Section 3.3.3 |

3.3.1 Surface phenotype of parental and hybrid cells

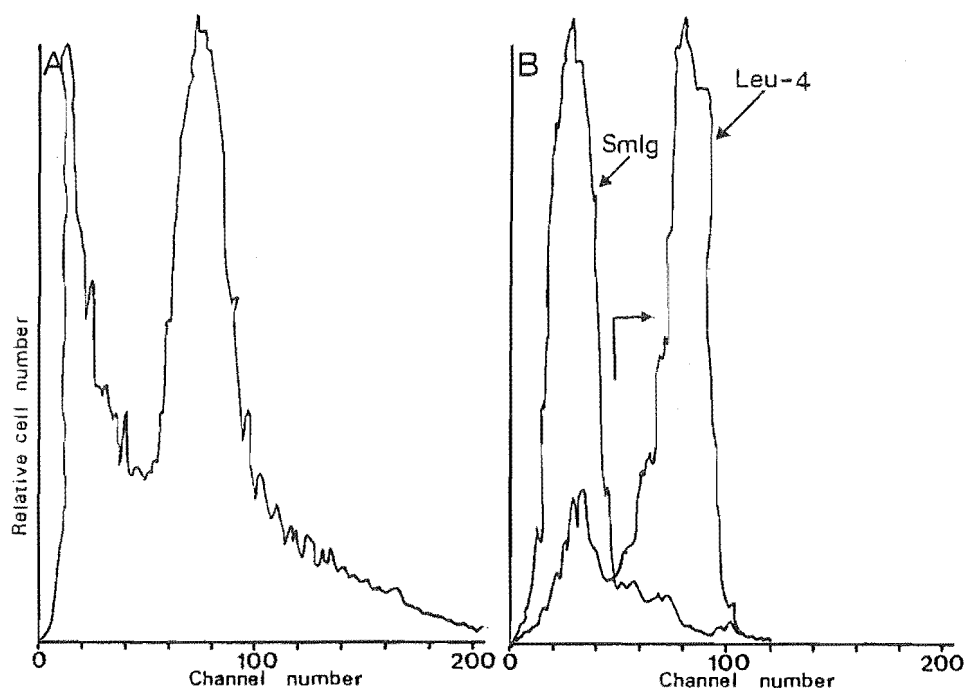
Cell surface phenotype studies were performed on both the parental and hybrid cells to determine whether particular cell surface receptors, absent on the parental tumor line but present on normal T cells, were subsequently expressed on the HAT - selected putative hybrid cells.

Indirect immunofluorescence staining indicated that the 5 day ConA-activated PBLs contained greater than 85% Leu-4 positive (T cells) and less than 7% surface membrane immunoglobulin (SmIg) positive cells (B cells). FACS profiles for the T and B cell staining are in Figure 3.3.

CEM-6TG^R and CEM-AG^R cells were screened with a range of monoclonal antibodies to the human cell surface receptors described in Table 2.1.

FIGURE 3.3

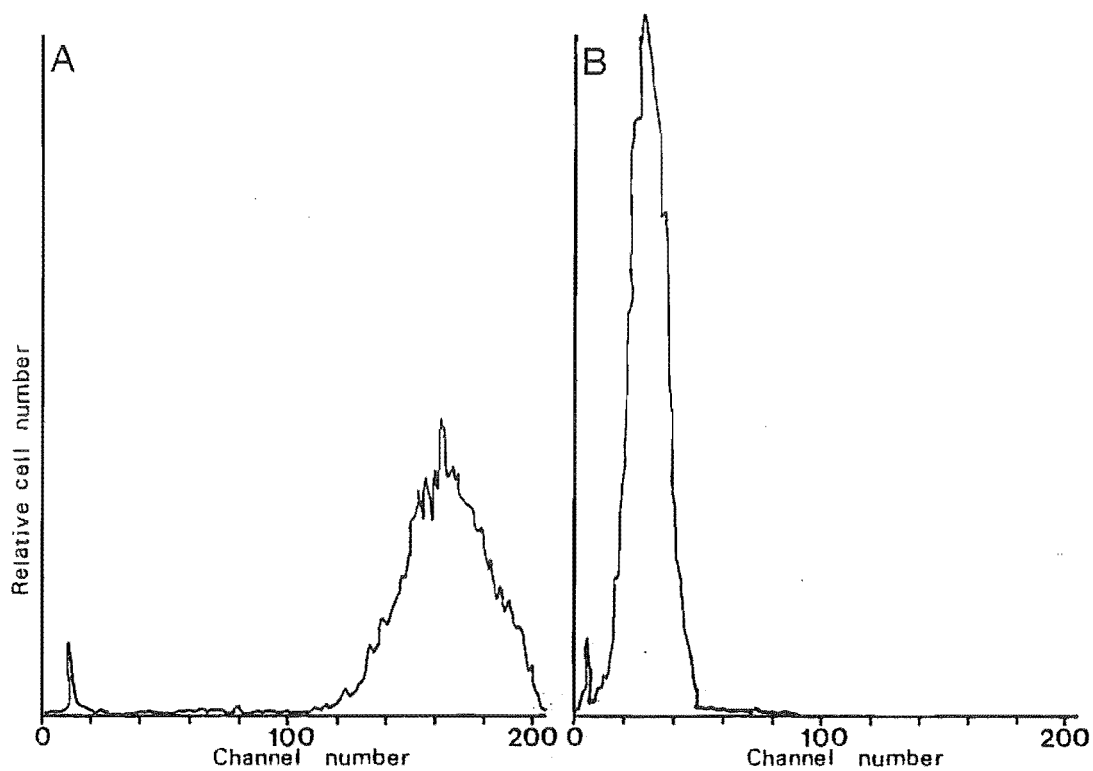
FACS PROFILE OF 5 DAY ConA-ACTIVATED PBLs



- A. Histogram of light scatter (horizontal axis) versus relative cell number for the 5 day ConA-activated PBLs.
- B. Composite histogram of log fluorescence (horizontal axis) versus relative cell number for the 5 day ConA-activated PBLs stained with SmIg (B cells) and Leu-4 (T cells). Percentages of cells positive staining were calculated to the right of the cursor line indicated. The cursor position was determined after comparison with unstained control cells.

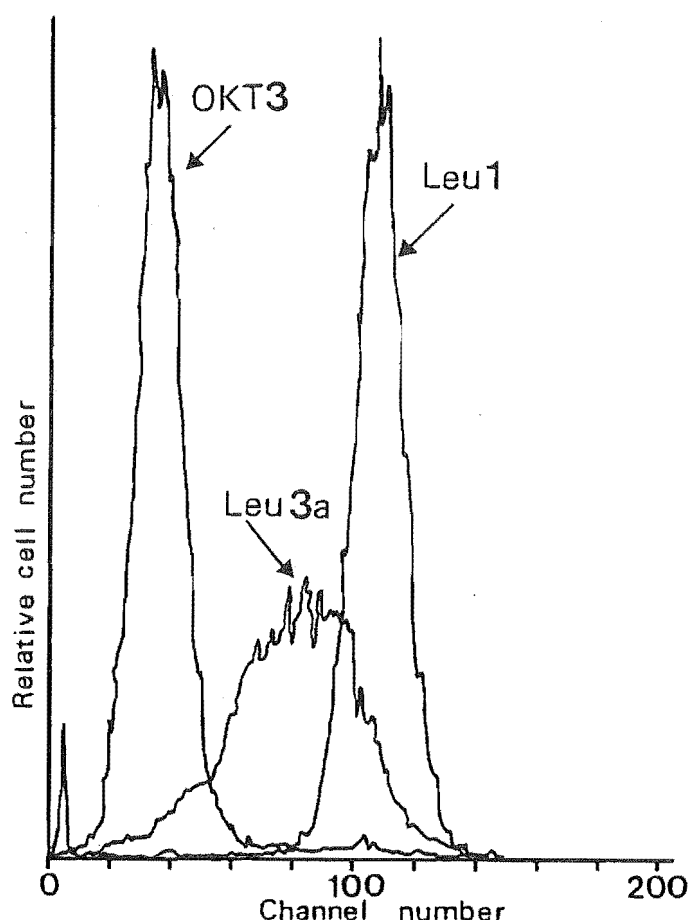
Figure 3.4 (a) shows the scatter and fluorescence profiles for the unstained CEM-6TG^R line. Figure 3.4 (b) shows the fluorescence profiles for the CEM-6TG^R line stained with the indicated McAbs.

FIGURE 3.4 (a)

FACS PROFILE OF UNLABELLED CEM-6TG^R LINE

- A. Histogram of scatter versus relative cell number for CEM-6TG^R cells.
- B. Histogram of log fluorescence versus relative cell number for CEM-6TG^R cells.
The channel number of the peak indicates that unlabelled CEM-6TG^R cells do not exhibit appreciable levels of autofluorescence.

FIGURE 3.4 (b)

FACS PROFILE OF McAb - LABELLED CEM-6TG^R LINE

Histogram of log fluorescence versus relative cell number for CEM-6TG^R cells labelled with the indicated McAbs. Gated analysis indicated that > 95% of cells were Leu-1 and Leu-3a positive. < 5% of cells were OKT3 positive.

Table 3.3 presents the cell surface phenotype data for the CEM-6TG^R and CEM-AG^R lines. Interestingly, neither expressed the receptor recognised by the OKT3 or Leu-4 McAbs. As normal T cells express this receptor, its presence (following fusion and HAT selection) was used to set sorting windows on the FACS to facilitate sterile sorting of the OKT3 receptor positive hybrid cell population.

Both lines failed to express the E rosette receptor recognised by the Leu-5 McAb. This marker is usually expressed on 95-100% human T cells.

TABLE 3.3

CELL SURFACE PHENOTYPE OF CEM-6TG^R & CEM-AG^R LINES

McAb	Antigen Recognised	CEM-6TG ^R	CEM-AG ^R
Leu-1	Mature T cell	+	+
Leu-2a / OKT8	Ts/c	±	±
Leu-3a	Th/i	+	+
Leu-3a / OKT4	Th/i	+	+
Leu-4 / OKT3	T cell	-	-
Leu-5	E rosette	-	-
HLA-DR / FMC4	HLA-DR framework	-	-
β ₂ M	β ₂ microglobulin	+	+
HTA1	Human thymocyte	-	-
W6/32	HLA-ABC framework	+	NT
TG-1	Granulocyte	-	-
2D-1	Common leucocyte	+	+
3A-1	Activated T cell	±	±
OKT6	Cortical thymocyte	-	NT
OKM1	Monocyte	-	NT
PNA	Peanut agglutinin receptor	NT	+

NT	:	not tested
+ staining	:	> 95% cells positive
- staining	:	< 5% cells positive
± weak staining	:	5-15% cells positive

Indirect immunofluorescence staining was performed on the CEM-6TG^R and CEM-AG^R lines using the monoclonal antibodies described in the table. The method used was that described in Section 2.3.4 and cell analysis was on a FACS IV.

Simplified membrane phenotypes of the T cell hybrid lines, TT-1 to TT-4, are shown in Table 3.4. This table shows that 2.6-3.0% of the putative hybrid cells were expressing the OKT3 marker 10 days post fusion. The four lines demonstrated relatively stable OKT3⁺ percentages or an enrichment of the OKT3⁺ cells when stained at 76-90 days post fusion. By 140 days post fusion, a significant loss of expression of the OKT3 marker had occurred in all the four lines (0-6.0% OKT3⁺).

TABLE 3.4

OKT3 McAb STAINING OF HUMAN T CELL HYBRID LINES

Days post fusion	Hybrid line Δ			
	TT-1	TT-2	TT-3	TT-4
10	2.6	2.6	2.6	3.0
14	27	27	28	27
26	NT	NT	NT	37
56	NT	27	28	40
76	26	40	20	66
90	46	66	48	27
140	0	2	6	5

Δ : % of cells expressing OKT3 marker

NT : Not tested

Hybrid cells from the 4 lines were stained with the OKT3 McAb from 10-140 days post fusion using the indirect immunofluorescence staining technique. Cell analysis was on a FACS IV.

Table 3.5 shows that at 90 days post fusion, all four lines exhibited dual expression of both the Leu-2a (T suppressor/cytotoxic) and Leu-3a (T helper/inducer) markers. Interestingly, the levels of expression of either marker were comparable within each of the hybrid lines, but a large variation in staining was evident between the lines (31-80% Leu-2a⁺, 33-72% Leu-3a⁺).

TABLE 3.5

SIMPLIFIED CELL SURFACE PHENOTYPE OF HUMAN T CELL HYBRID LINES

McAb	Hybrid line ^Δ			
	TT-1	TT-2	TT-3	TT-4
Leu-1	95	100	100	100
Leu-2a	44	72	31	80
Leu-3a	47	72	33	72

Δ : % of cells expressing the indicated markers

Leu-1 : mature T cell;

Leu-2a: T suppressor / cytotoxic;

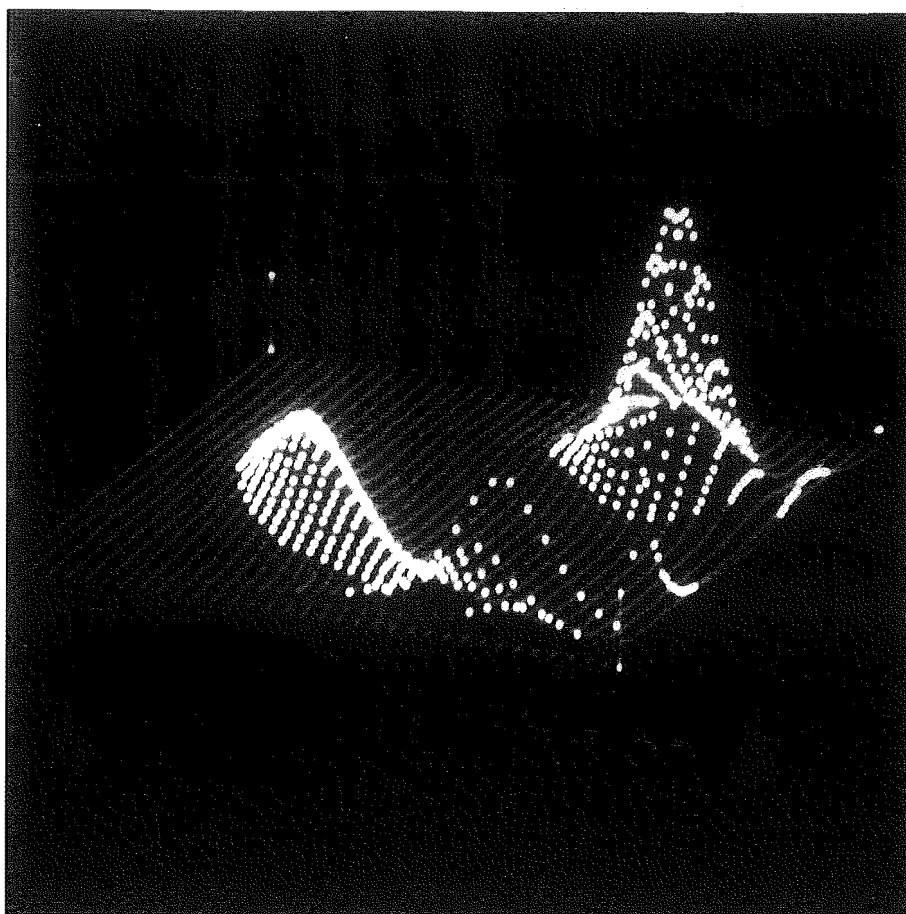
Leu-3a: T helper / inducer.

Hybrid cells were stained with the indicated McAbs at 90 days post fusion using the indirect immunofluorescence staining technique. Cell analysis was on a FACS IV.

Figure 3.5 (a) describes the 3 dimensional display of the hybrid cells sorted to establish the OKT3⁺ TT-1 hybrid line. Figure 3.5 (b) is a line diagram indicating the scatter and fluorescence axes and the sorting windows used to isolate these hybrid cells. It is evident from Figs. 3.5 (a), (b) that the OKT3⁺ cells formed a discrete population on the scatter versus fluorescence profile. This separation enabled the positive selection of the OKT3⁺ hybrid cells as well as the exclusion of non-viable and OKT3⁻ cells.

FIGURE 3.5 (a)

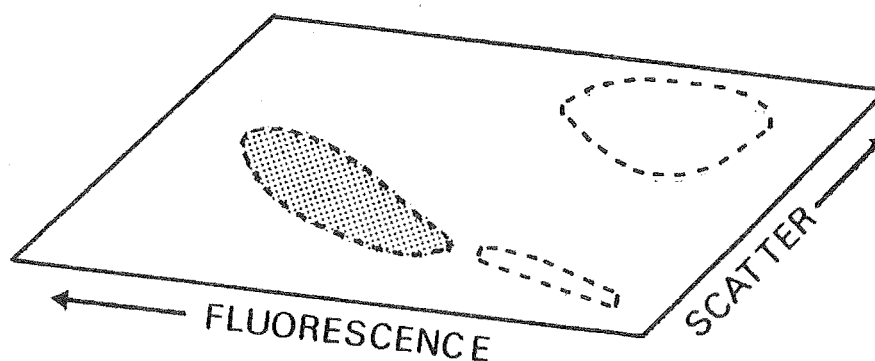
THREE DIMENSIONAL DISPLAY OF OKT3 LABELLED HYBRID CELLS



Three dimensional display of OKT3 labelled putative hybrid cells. Fig. 3.5 (b) describes the scatter and fluorescence axes and sorting windows used to isolate the OKT3⁺ hybrid cells identified in Fig. 3.5 (a).

FIGURE 3.5 (b)

LINE DIAGRAM OF OKT3 LABELLED HYBRID CELLS



Line diagram indicating the scatter and fluorescence axes of the OKT3-stained hybrid cells described in Figure 3.5 (a). The highlighted area describes the OKT3⁺ hybrid cell population which was sterile sorted to establish the TT-1 line.

3.3.2 Functional hypoxanthine guanine phosphoribosyl transferase (HGPRT) activity

Resistance of the mutagenised CCRF-CEM line to 6TG suggested that it was deficient in HGPRT activity.

To confirm that the CEM-6TG^R line was in fact deficient in HGPRT activity, the ability of the line to incorporate preformed exogenous hypoxanthine was assessed with ³H-Hypoxanthine. A ratio of ³H-Hypoxanthine to ³H-Thymidine (³H-HX / ³H-TdR) incorporation is shown in Table 3.6. This ratio relates cell growth rate to the ability to incorporate ³H-HX via the HGPRT enzyme. Table 3.6 shows the uptake of ³H-HX, ³H-TdR and the incorporation ratios for the control cells (lines 1-4) and hybrid lines (lines 5-8).

A reduced ³H-HX / ³H-TdR incorporation ratio for the CEM-6TG^R fusion partner results from a significantly reduced ability to incorporate hypoxanthine compared to the non-mutagenised CEM line (0.006 vs. 0.49 respectively). T cell hybrid lines 1-4 had ratios approaching those of the non-mutagenised line; which suggested they received functional HGPRT activity from fusion with the ConA-activated PBLs. This is therefore evidence for hybrid formation.

TABLE 3.6

INCORPORATION OF ^3H -HYPOXANTHINE & ^3H -THYMIDINE BY
PARENTAL CELLS AND T CELL HYBRID LINES

Sample	^3H -HX cpm (\pm SD)	^3H -TdR cpm (\pm SD)	^3H -HX / ^3H -TdR ratio
1. CEM (non mutagenised)	275638 (1.2)	557067 (7.7)	0.49
2. CEM (mutagenised, hybrid parental cells)	1498 (8.9)	249739 (9.4)	0.006
3. PBL	1367 (15.1)	299 (15.3)	5.97
4. PBL & ConA (hybrid parental cells)	6397 (10.8)	7878 (17.1)	0.81
5. TT-1	288432 (13.0)	553229 (11.0)	0.52
6. TT-2	315360 (4.3)	656673 (8.3)	0.48
7. TT-3	316379 (6.3)	693173 (2.5)	0.46
8. TT-4	327937 (4.7)	756222 (7.3)	0.44

12 replicates (5×10^4 cells/200 μl) were cultured for 48 hrs. 6 replicates were pulsed with 2.0 μCi ^3H -HX and 6 with 2.0 μCi ^3H -TdR for a further 21 hrs. Cells were harvested onto glass fibre filters and counted by liquid scintillation.

3.3.3 Chromosomal analysis

The CEM-6TG^R line had a mean chromosome number of 92 (range 89-93); indicating tetraploidy. Analysis of TT-4 cells 120 days post fusion gave a range of chromosome number from 89-93 also. Some chromosome instability was evident in this line.

Figure 3.6 describes a representative screen from the CCRF-CEM parental line.

CHROMOSOME SCREEN OF CCRF-CEM LINE

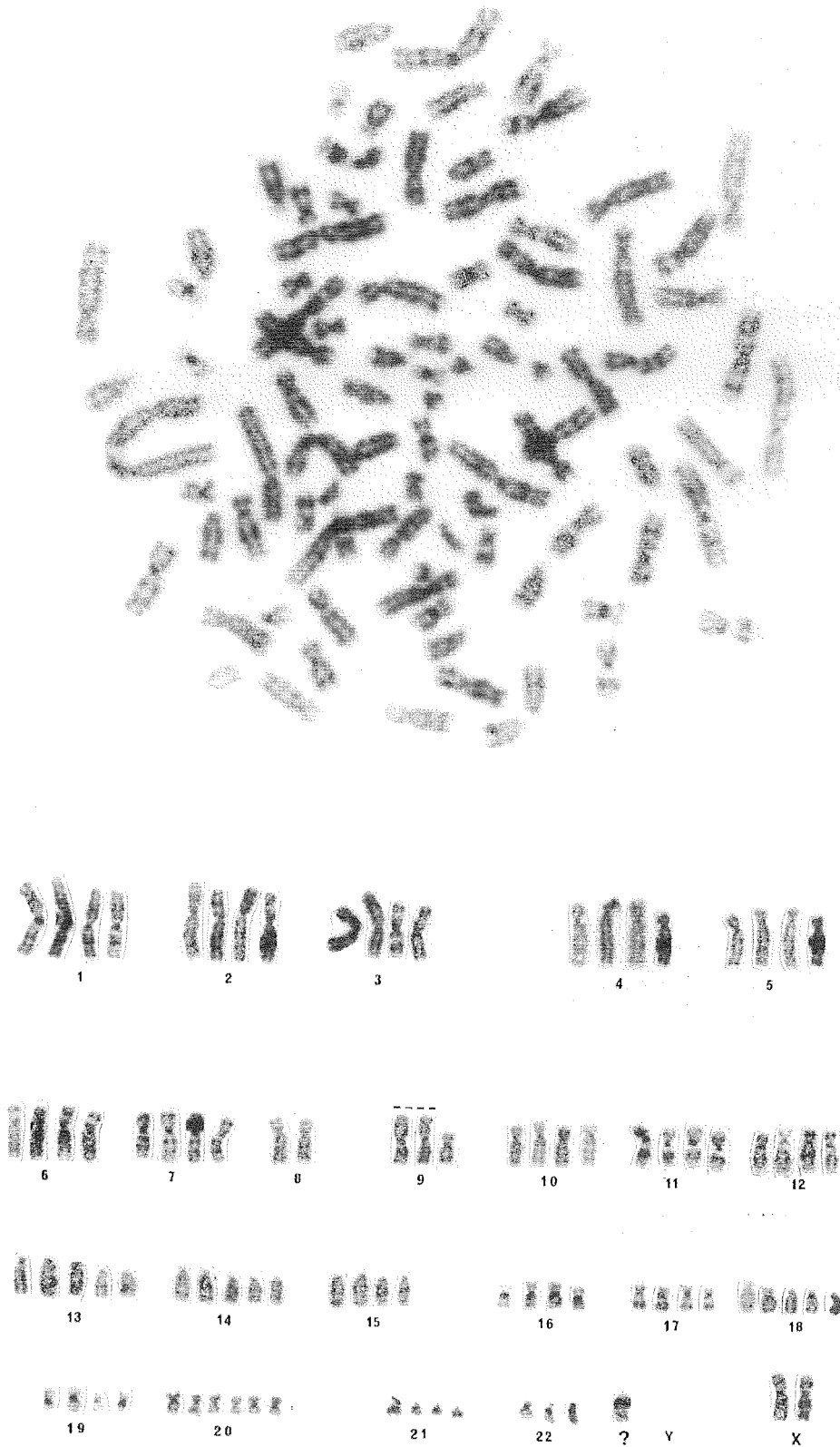


FIGURE 3.7

BANDING PATTERN AND CHROMOSOME NUMBERING OF
CCRF-CEM LINE

Figure 3.7 describes the banding pattern and chromosome numbering for the CCRF-CEM line. Two abnormal number nine chromosomes are present as well as one unidentified chromosome.

The karyotyping was kindly performed by Dr P. Hollings, Cytogenetics Unit, Christchurch Hospital.

3.4 ALTERNATIVE DRUG SELECTION SYSTEMS

A direct comparison between the hypoxanthine, aminopterin, thymidine (HAT) and azaserine, hypoxanthine (AH) drug selection systems was made by culturing the putative hybrid cells from a fusion experiment in either medium and screening for visible cell growth at 30 days post fusion.

Table 3.7 shows that of 144 wells seeded in either selective media, 14 wells (9.7%) showed evidence of viable cells in the standard HAT media compared to 25 wells (17.4%) in the alternative AH media.

TABLE 3.7

NUMBERS OF T CELL HYBRIDS SELECTED IN EITHER HAT
OR AH MEDIUM

Number of wells seeded	Medium	Number of wells with viable cells	% wells positive
144	HAT	14	9.7
144	AH	25	17.4

Putative hybrids from a fusion experiment were selected in HAT or AH medium. Numbers of wells containing viable cells were quantitated by direct microscopic examination 30 days post fusion.

3.5 FUNCTIONAL ASSAYS : DEVELOPMENT AND VALIDATION

Assay systems, capable of quantitating human PBL antibody responses were developed and validated. Hybrid-derived supernatants were screened in these assays to determine if the hybrids were secreting regulatory factors which could in any way modify a normal human antibody response.

3.5.1 Human antibody assay (pokeweed mitogen-dependent)

(a) Antibody tracers

The chloramine T method of Hunter (1974) was used to iodinate the tracer antibodies (Goat anti-human IgG or IgM) used in the RIAs. Immediately following the iodination, free and bound ^{125}I label was separated on a G50 gel filtration column. Figure 3.8 describes a typical separation profile obtained for the iodination of 50 μg GaHu IgG using 0.5mCi Na ^{125}I . Peak 1 contained the labelled antibody and a pool of these active fractions was made. The protein content was estimated from the A_{280} using an IgG or IgM standard curve.

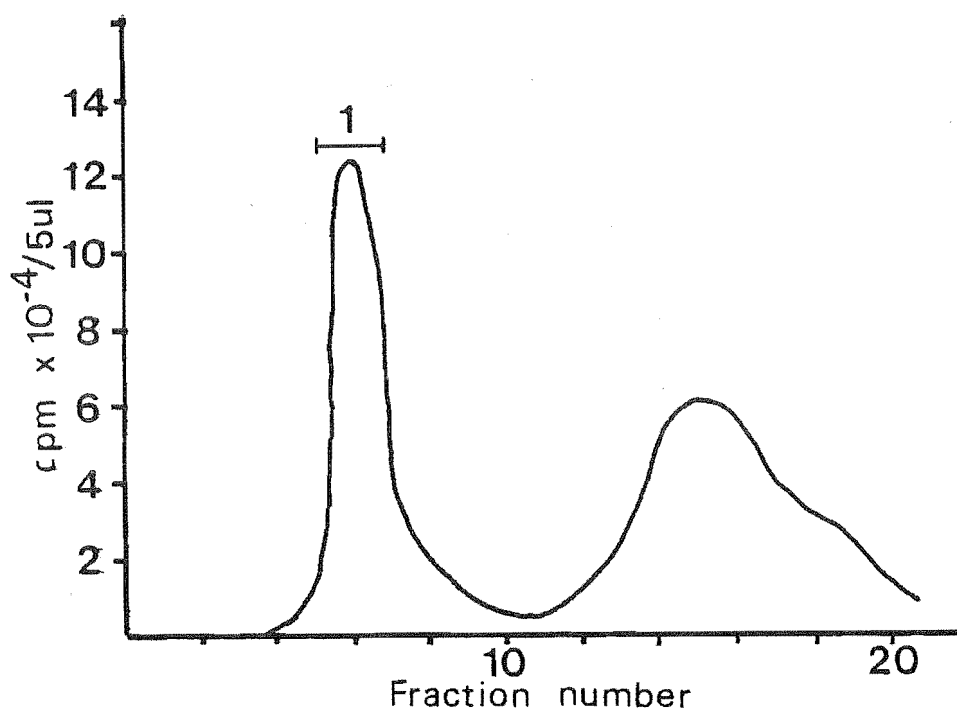
Specific activities of the radioisotope preparations were calculated from the following relationships:

$$\begin{aligned} (1) \quad \text{radioactivity (dpm)} &= \frac{\text{observed count rate (cpm)}}{\text{efficiency}} \\ (2) \quad \text{specific activity} &= \frac{\text{total dpm } (\mu\text{Ci})}{\text{total protein } (\mu\text{g})} \end{aligned}$$

Specific activities, at the time of preparation, were routinely between 8-12 $\mu\text{Ci}/\mu\text{g}$ with a total protein recovery of 30-55% (6 experiments).

FIGURE 3.8

RADIOACTIVITY PROFILE OF G50 PURIFIED ^{125}I -GOAT
ANTI - HUMAN IgG



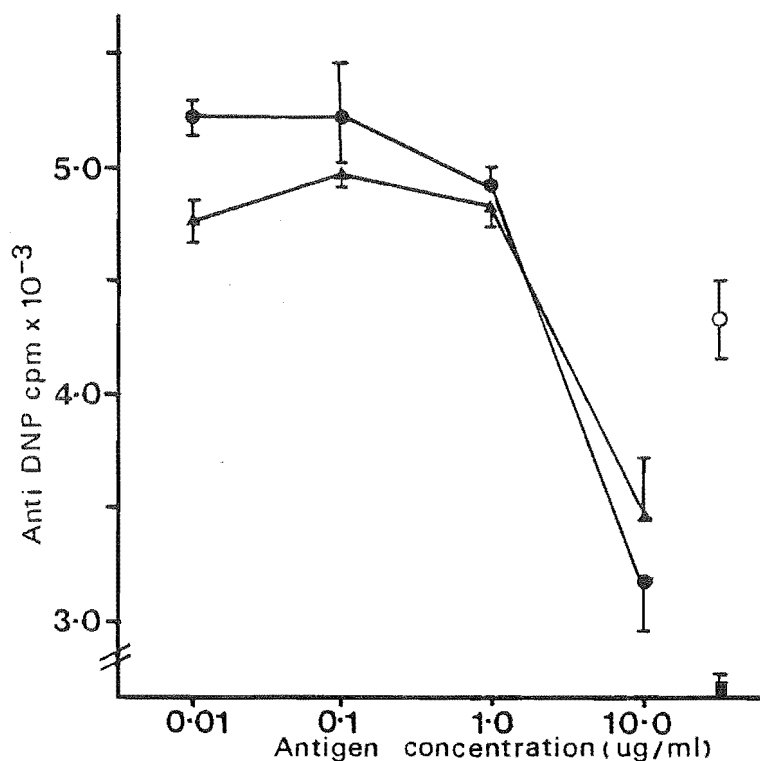
Typical separation profile of $50\mu\text{g } ^{125}\text{I-G} \propto \text{Hu IgG}$ chromatographed on a Sephadex G50 column (1.5 x 12cm). A pool of the most active fractions was made (Peak 1) and the protein concentration determined to enable the calculation of the specific activity of the preparation.

(b) Antigen concentration

Figure 3.9 describes the anti-hapten responses obtained when varying concentrations of DNP-BSA or DNP-KLH were cultured with PBLs in the presence of PWM. The levels of specific anti-hapten antibody produced in response to PWM alone is also shown. A broad response peak was evident, with the optimal response being at $0.1\mu\text{g/ml}$. Significant levels of anti-hapten antibody were produced in response to PWM alone.

FIGURE 3.9

ANTIGEN CONCENTRATION USED FOR OPTIMAL HUMAN ANTIBODY RESPONSE



Anti-hapten antibody responses obtained with PBLs (1×10^6 /ml) cultured in the presence of PWM and varying concentrations of antigen.

Antigens used were:

- | | |
|-------------|--------------|
| ●—● DNP-BSA | ○ PBLs + PWM |
| ▲—▲ DNP-KLH | ■ PBLs alone |

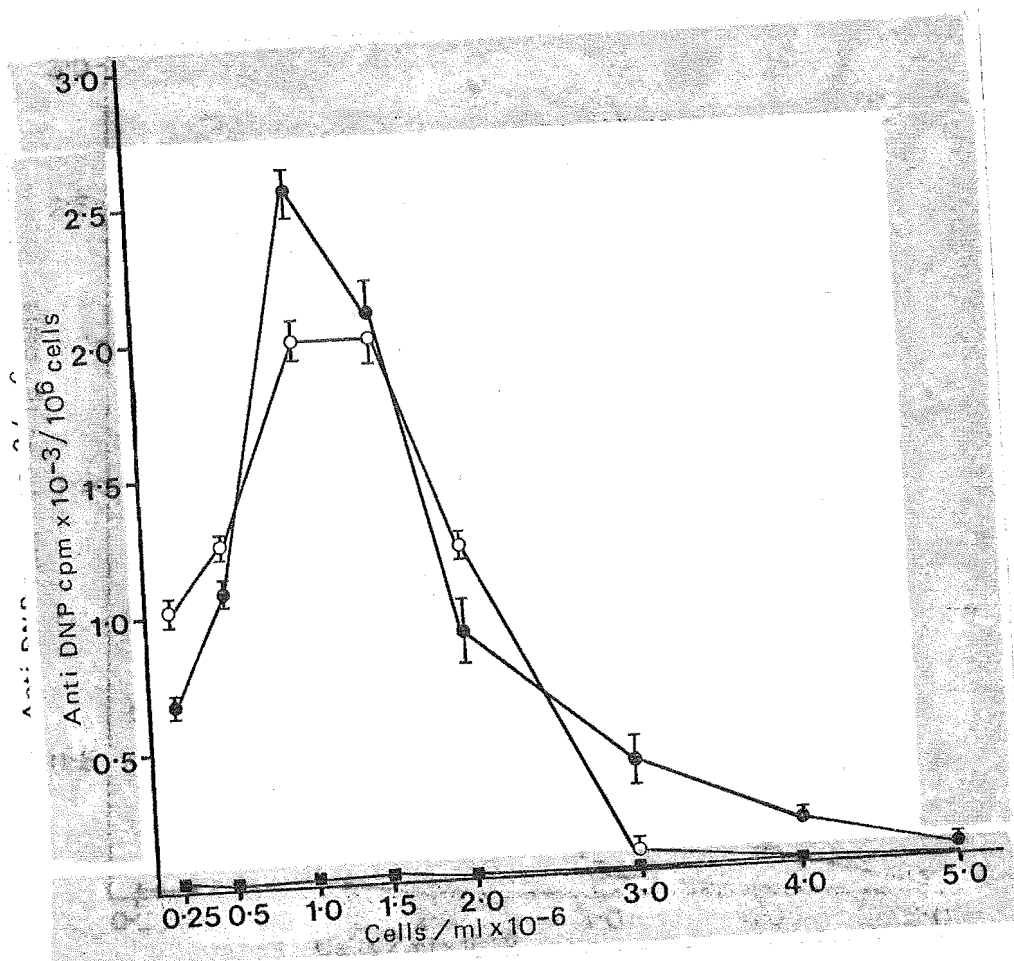
Standard deviations from triplicate determinations are shown.

(c) Cell concentration

Varying numbers of PBL ($0.25 - 5.0 \times 10^6$) were cultured with DNP-BSA and PWM. Figure 3.10 shows that the peak of the response was at a cell concentration of 1.0×10^6 /ml. Culturing PBLs in the absence of antigen or PWM failed to produce significant levels of antibody. Culturing PBLs with PWM, again produced significant amounts of hapten-specific antibody, with the peak of the response being at cell concentration between 1.0 and 1.5×10^6 /ml.

FIGURE 3.10

CELL CONCENTRATION USED FOR OPTIMAL HUMAN
ANTIBODY RESPONSE



Anti-hapten antibody responses obtained / 10^6 cells with varying numbers of PBL cultured in the presence of DNP-BSA and PWM or PWM alone.

●—● DNP-BSA + PWM

■ PBLs alone

○—○ PWM alone

Standard deviations from triplicate determinations are shown.

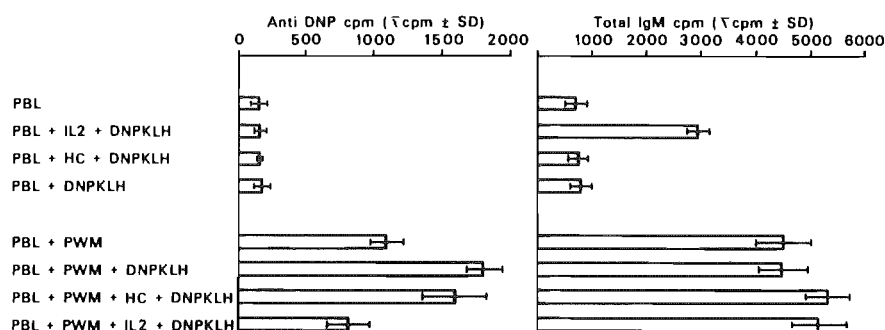
(d) Alternatives to polyclonal activation with pokeweed mitogen

PBLs were cultured with IL-2, hydrocortisone and DNP-KLH or with DNP-KLH alone. All cultures were performed with and without PWM and control cultures utilised PBLs cultured with and without PWM. Figure 3.11 indicates that both IL-2 and hydrocortisone enhanced hapten-specific and total IgM antibody production in the presence of PWM, but antigen plus PWM still

gave the best hapten-specific IgM antibody response. Interestingly, PBLs cultured with DNP-KLH and IL-2 alone produced significantly higher levels of total IgM antibodies compared with the other cultures, even though this regime produced only comparable levels of hapten-specific IgM antibodies.

FIGURE 3.11

IL-2 & HYDROCORTISONE EFFECTS ON HUMAN ANTIBODY RESPONSES



Anti-hapten and total IgM antibody responses of PBLs ($1 \times 10^6/\text{ml}$) cultured under the conditions indicated in the diagram.

IL-2 : (1:10)
 DNP-KLH : ($0.1 \mu\text{g}/\text{ml}$)
 HC : hydrocortisone (10^{-5}M)

Standard deviations from triplicate determinations are shown.

The optimal cell culture conditions chosen were therefore at a cell concentration of $1 \times 10^6/\text{ml}$, antigen concentration of $0.1 \mu\text{g}/\text{ml}$ with PWM at $1 \mu\text{g}/\text{ml}$ for 6-7 days.

(e) Validation of the radioimmunoassay

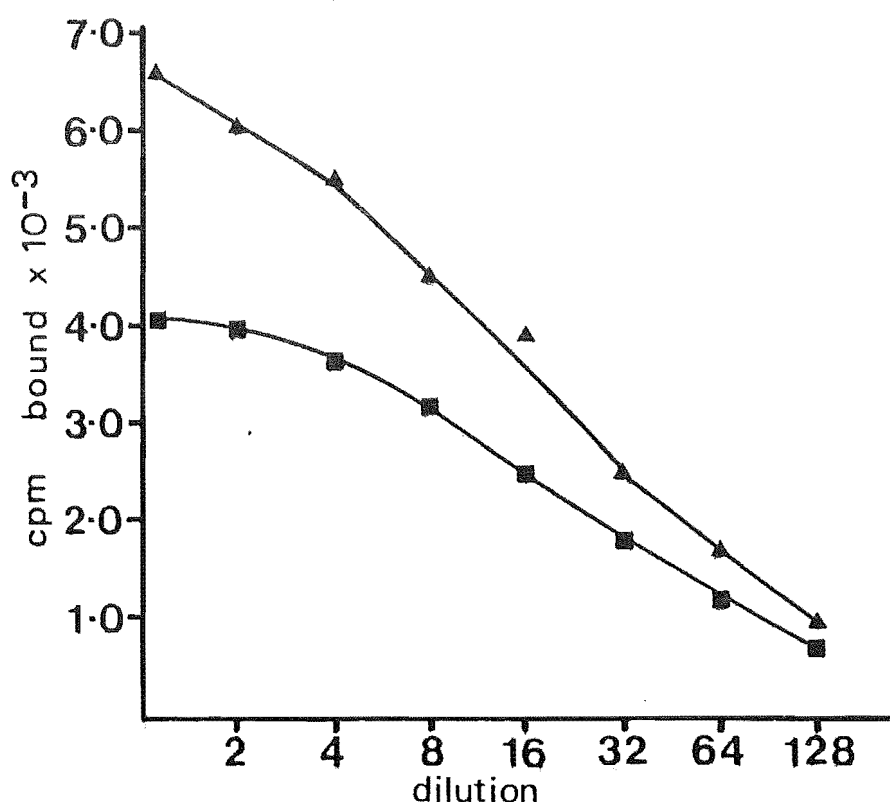
Three methods were used to validate the PWM-driven culture system and RIA.

(i) Dose response curve

Figure 3.12 describes the dose response curve obtained when supernatants from PBLs cultured with DNP-KLH and PWM were serially diluted and assayed for total IgG or IgM antibodies using the RIA.

FIGURE 3.12

RIA DOSE RESPONSE CURVES



Dose response curves of serially diluted supernatants from PBLs ($1 \times 10^6/\text{ml}$) cultured with DNP-KLH ($0.1\mu\text{g}/\text{ml}$) and PWM.

▲—▲ Total IgM antibodies
■—■ Total IgG antibodies

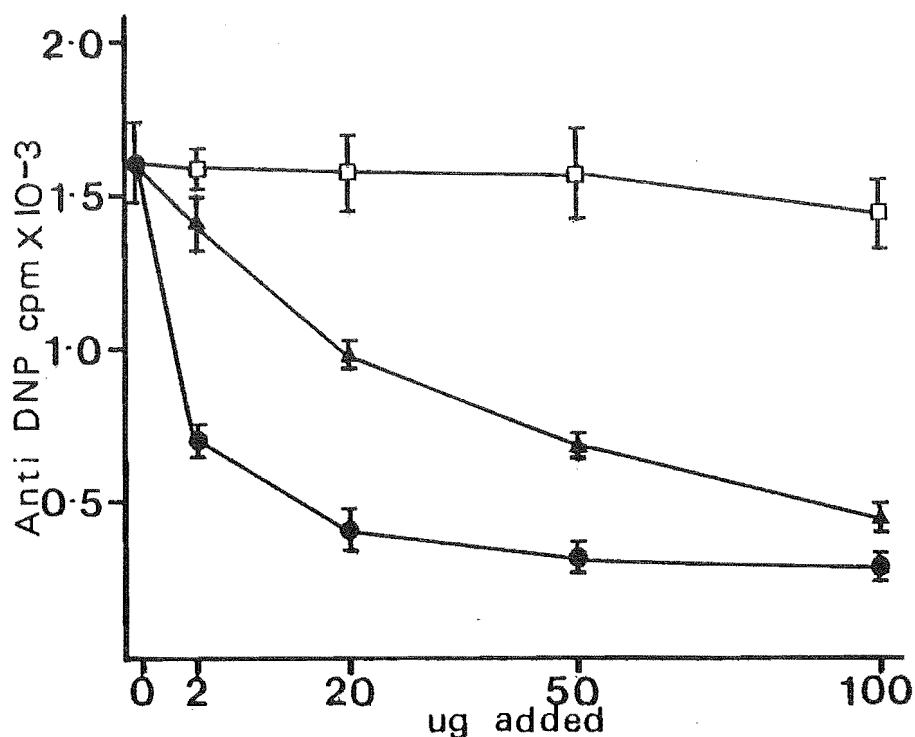
(ii) Inhibition curve

As Figure 3.13 shows, good inhibition of binding occurred as increasing amounts of hapten-carrier (DNP-BSA) were titrated into the supernatants. Significant levels of inhibition of binding occurred when carrier alone (KLH), was titrated into the supernatants; indicating that the PWM had

induced the production of both carrier-specific and hapten-specific antibodies. An unrelated, non-haptenated protein, ovalbumin, had little effect on binding.

FIGURE 3.13

COMPETITIVE INHIBITION STUDIES OF CULTURE
SUPERNATANTS



Inhibition of binding of supernatants from PBLs ($1 \times 10^6/\text{ml}$) cultured with IL-2 (1:10), DNP-KLH (0.1 $\mu\text{g}/\text{ml}$) and PWM which had been titrated with increasing amounts of :

- ovalbumin
- ▲—▲ KLH
- DNP-BSA

Anti-hapten antibody responses shown. Standard deviations from triplicate determinations are shown.

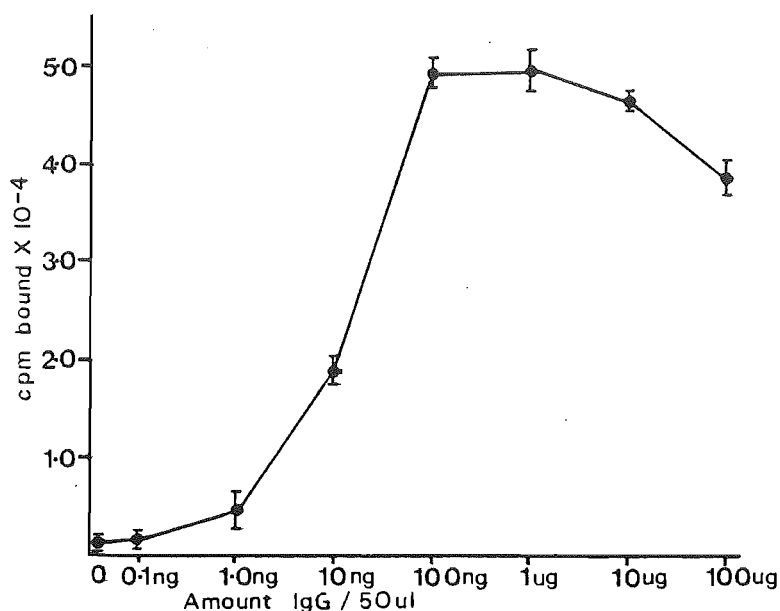
(iii) IgG standard curve

Dilutions of a known serum IgG standard were assayed in the RIA using polyvinyl plates coated with goat anti-human IgG at a concentration of 2 $\mu\text{g}/\text{ml}$ (100ng IgG/well). As Figure 3.14 describes, the maximal binding of the test IgG standard occurred at equivalence (100ng IgG added). Above or

below the equivalence point decreased amounts of the standardised serum IgG were bound to the coated plates as detected by the ^{125}I -labelled secondary antibody.

FIGURE 3.14

RIA IgG STANDARD CURVE



Standard curve of IgG concentration versus ^{125}I G α Hu IgG cpm bound. Polyvinyl plates were coated with 2 $\mu\text{g}/\text{ml}$ (100ng/well) G α Hu IgG.

Standard deviations from triplicate determinations are shown.

3.5.2 Human hapten-specific antibody responses

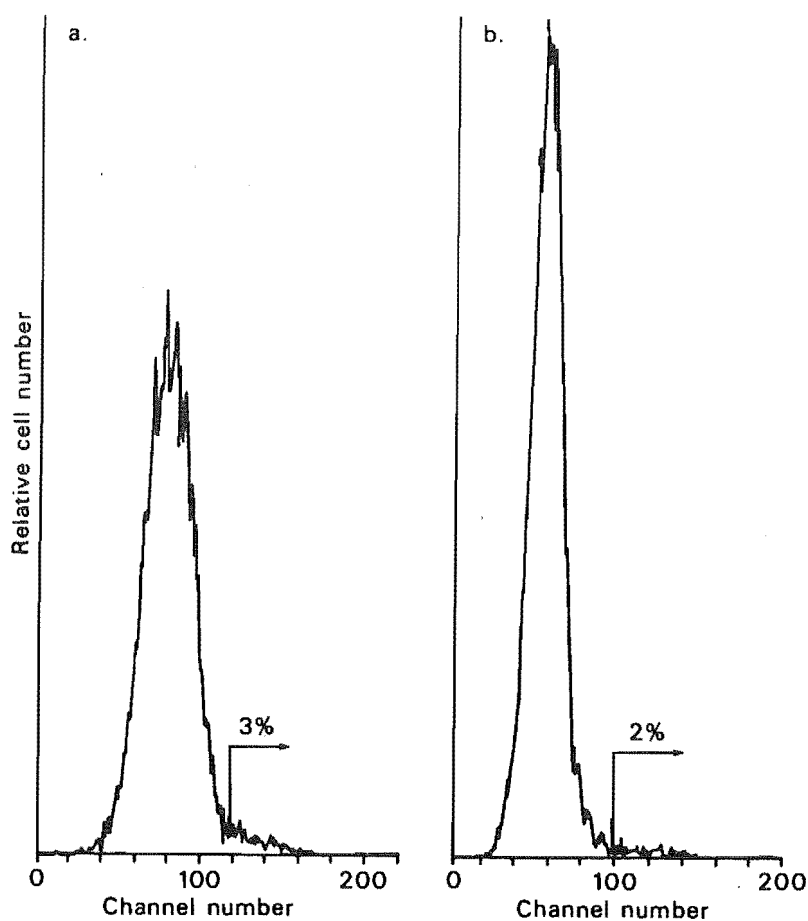
A second, mitogen-independent, antibody assay and RIA system was developed. This hapten-specific antibody culture system was optimised and the specificity of the RIA was confirmed.

(a) T and B cell purifications

Figure 3.15a shows that a maximum of 3% T cells were present in the purified B cell population. Figure 3.15b indicates that less than 2% B cells were present in the purified T cell population.

FIGURE 3.15

FACS PROFILES OF T & B CELL PREPARATIONS
USED IN THE ANTI -HAPTEN ANTIBODY
ASSAY



- a. Fluorescence profile of purified B cells stained with anti-T cell McAb Leu-1.
- b. Fluorescence profile of purified T cells stained with goat anti-human Ig.

(b) In vitro anti-hapten responses

The optimal antigen concentration, cell concentration and kinetics for the generation of anti-hapten antibodies were determined.

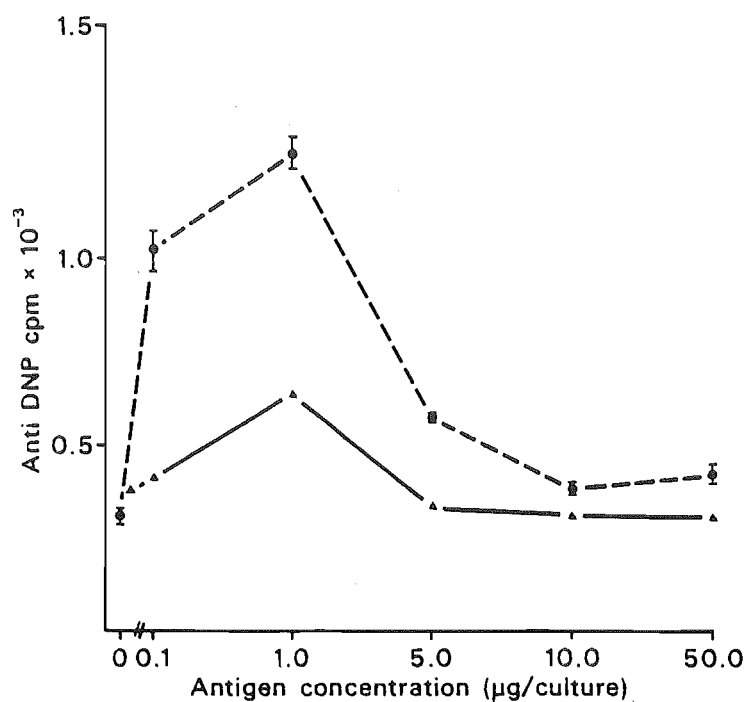
(i) Antigen concentration

One million cells/0.2ml were cultured in the range of 0.1-50 μ g of antigen. Figure 3.16 shows the optimal anti-hapten response was at an antigen concentration of 1.0 μ g/0.2ml culture. At higher antigen concen-

trations a marked reduction in anti-hapten antibody production was observed.

FIGURE 3.16

ANTIGEN CONCENTRATION USED FOR OPTIMAL ANTI-HAPTEN
ANTIBODY RESPONSE



Anti-hapten antibody responses obtained with PBLs ($1 \times 10^6/0.2\text{ml}$) cultured in the presence of varying concentrations of antigen.

Antigens used were:

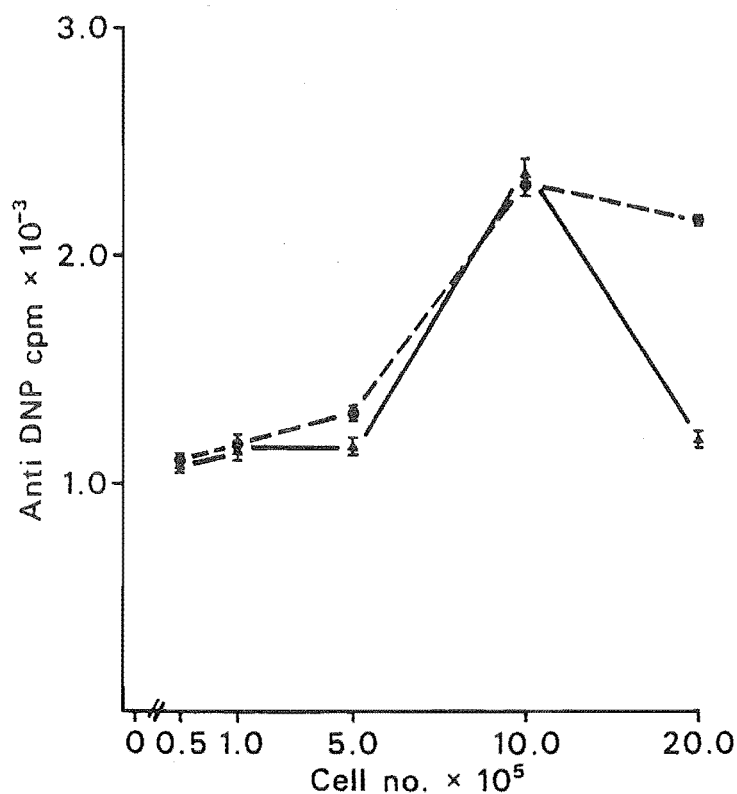
- — ● DNP-BSA
- ▲ — ▲ DNP-KLH

Standard deviations from triplicate determinations are shown.

(ii) Cell concentration

Varying numbers of PBLs ($0.5 - 20.0 \times 10^5$) were cultured with DNP-BSA, DNP-KLH ($1.0\mu\text{g/ml}$) or without antigen as described. Figure 3.17 shows that the optimal cell density was at 1.0×10^6 cells/ 0.2ml culture. Above or below this density decreased amounts of specific antibody was produced.

FIGURE 3.17

CELL CONCENTRATION USED FOR OPTIMAL ANTI-HAPTEN
ANTIBODY RESPONSE

Anti-hapten antibody responses obtained with varying numbers of PBLs cultured in the presence of DNP-BSA, DNP-KLH (1.0 μg/ml).

● -- ● DNP-BSA

▲ — ▲ DNP-KLH

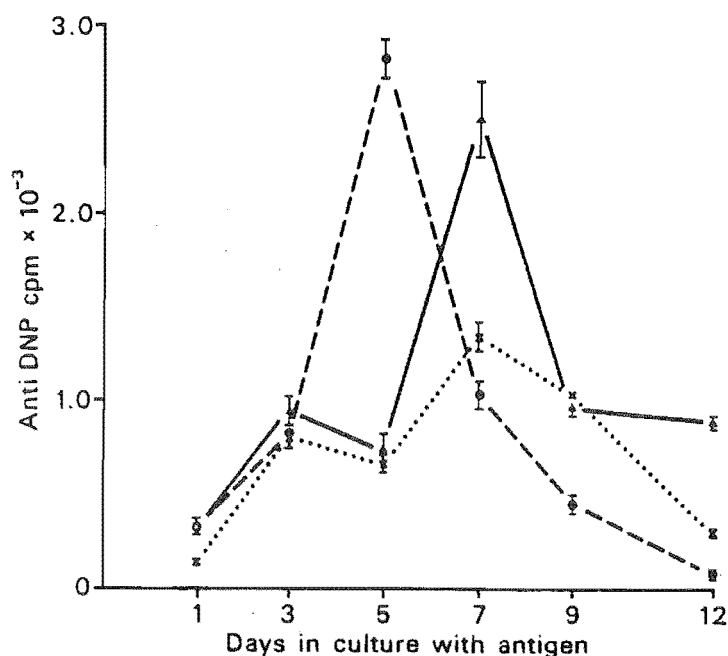
Standard deviations from triplicate determinations are shown.

(iii) Kinetics

The kinetics of the anti-hapten response was studied by determining the levels of anti-hapten antibodies in the culture supernatants at the times indicated in Figure 3.18. The supernatants were harvested at the relevant times and frozen at -20°C until assayed as one batch in the same RIA. Figure 3.18 shows that the peak of the anti-hapten response was between days 5 and 7.

FIGURE 3.18

KINETICS OF ANTI-HAPTEN ANTIBODY RESPONSE



PBLs ($1 \times 10^6/0.2\text{ml}$) were cultured with DNP-BSA, DNP-KLH ($1.0\mu\text{g}/0.2\text{ml}$) for various intervals. On days 1, 3, 5, 7, 9 and 12 culture cells were washed 3 times with fresh medium and cultured for a further 4 days in the absence of antigen. These culture supernatants were stored at -20°C until assayed in the anti-hapten antibody RIA.

- - - ● DNP-BSA
- ▲ — ▲ DNP-KLH
- X ····· X PBLs alone

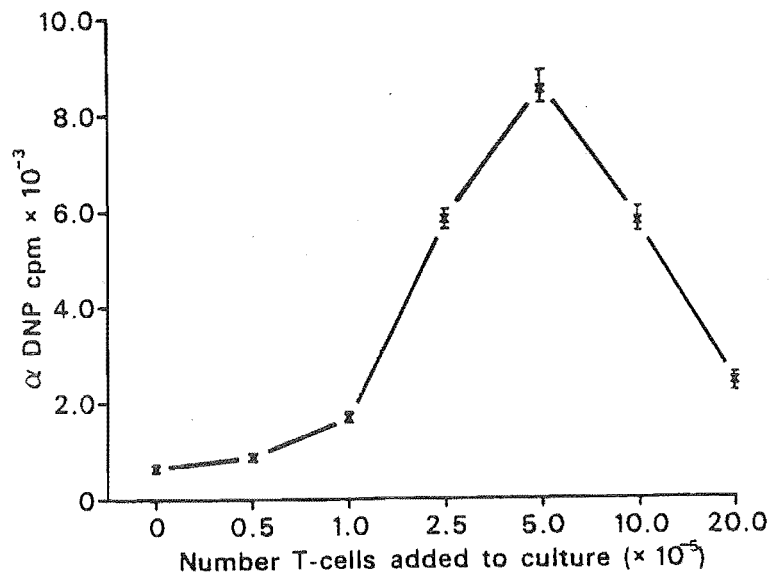
The optimal culture conditions chosen therefore were at a cell concentration of $1 \times 10^6/0.2\text{ml}$, antigen concentration of $1.0\mu\text{g}/0.2\text{ml}$ and 5 days in culture with antigen followed by 4 days without antigen.

(c) T cell dependence of anti-hapten response

Figure 3.19 shows the maximal response was obtained when equal numbers of B and T cells were present in the culture (5×10^5 of each). B or T cells alone gave a minimal response to antigen. Increasing the numbers of T cells up to equivalence had an amplifying effect on the anti-hapten response whereas the addition of extra T cells, greater than 5×10^5 , markedly reduced the anti-hapten response.

FIGURE 3.19

T CELL DEPENDENCE OF ANTI-HAPTEN RESPONSE



Varying numbers of purified T cells were cultured with a constant number of purified B cells (5×10^5) and DNP-BSA ($1.0 \mu\text{g}/0.2\text{ml}$). The anti-hapten antibody responses obtained for the various T and B cell combinations are shown.

Standard deviations from triplicate determinations are shown.

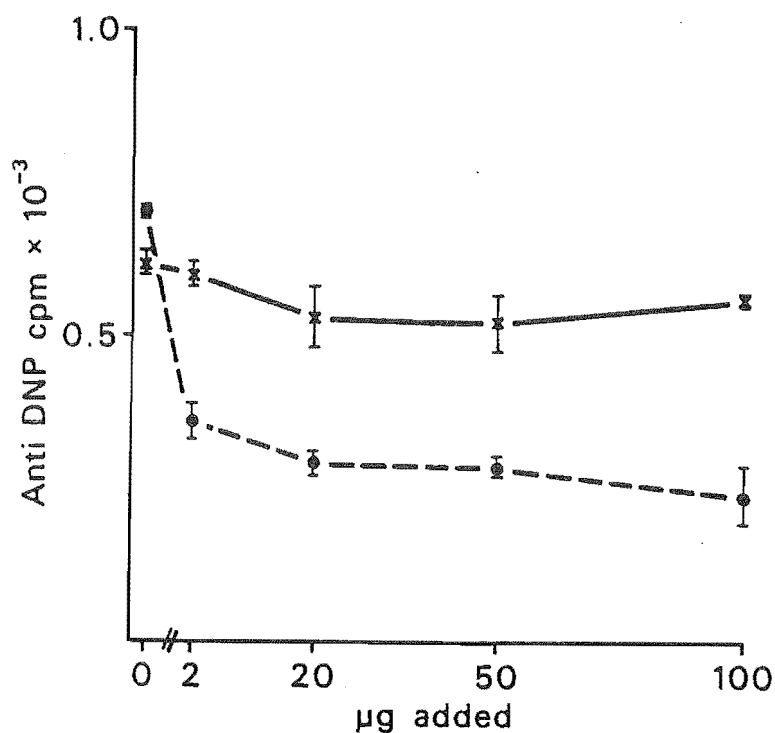
(d) Specificity of the radioimmunoassay

The specificity of the anti-hapten antibody RIA was confirmed by titration of anti-hapten culture supernatants with hapten-carrier conjugate or ovalbumin.

As Figure 3.20 shows, good inhibition of binding occurred as increasing amounts of DNP-BSA were titrated into the supernatants. A non-haptenated protein, in this case ovalbumin, had little effect on binding.

FIGURE 3.20

SPECIFICITY OF ANTI-HAPTEN ANTIBODY RIA



Inhibition of anti-hapten antibody binding to DNP-BSA-coated plates. Supernatants from PBLs ($1 \times 10^6/0.2\text{ml}$) cultured with DNP-BSA ($1.0\mu\text{g}/0.2\text{ml}$) were titrated with increasing amounts of :

x—x ovalbumin

●--● DNP-BSA

Standard deviations from triplicate determinations are shown.

3.6 HUMAN T CELL HYBRID FUNCTIONAL STUDIES

3.6.1 Effect of hybrid-derived supernatants on a PWM-induced polyclonal antibody response

Preliminary experiments indicated that hybrid-derived supernatants from all four lines were capable of suppressing, to varying extents, the PWM-induced antibody response of the normal indicator lymphocytes. Hybrid line TT-4 had the most potent suppressive effect on the generation of polyclonal IgG antibody, therefore it was this line which was investigated in depth.

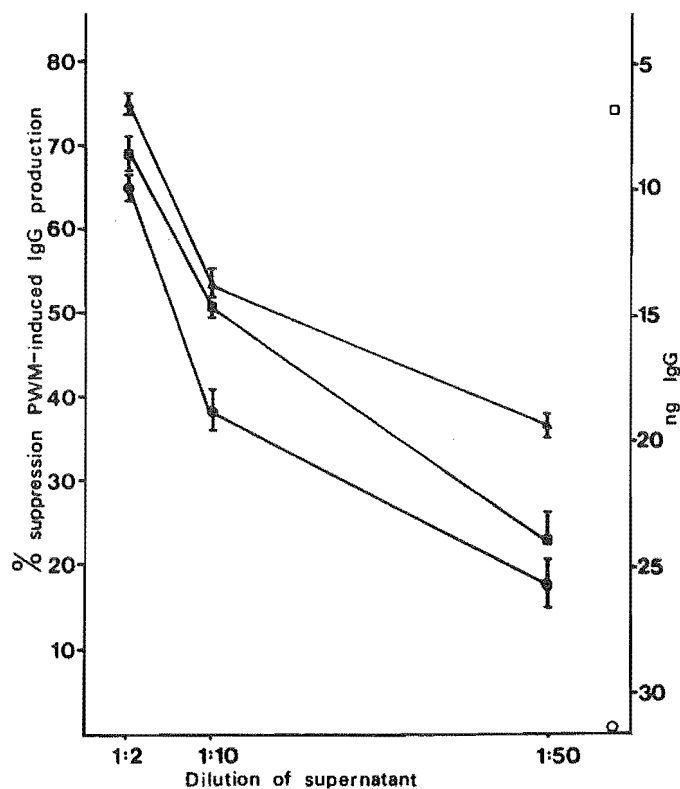
(i) Constitutive lymphokine production

TT-4-derived supernatants were assayed for their ability to suppress the PWM-induced antibody assay.

Figure 3.21 describes the curve of % suppression of the PWM-induced IgG production versus dilution of supernatant for the three cell concentrations tested. Figure 3.21 shows a dose and producer cell concentration dependent suppressive effect of the TT-4 supernatants on the generation of an in vitro PWM-induced polyclonal IgG antibody response. Suppression of between 19% ($0.5 \times 10^6/\text{ml}$, 1:50 dilution) and 75% ($5.0 \times 10^6/\text{ml}$, 1:2 dilution) was achieved. This suppression corresponds, in ng IgG, to a reduction from 32ng IgG produced in response to the mitogen down to 7ng at $5.0 \times 10^6/\text{ml}$ (1:2) and 26ng at $0.5 \times 10^6/\text{ml}$ (1:50)

The level of stimulation of the indicator PBLs, from $6.8 \pm 0.2\text{ng}$ when cultured in the absence of PWM, to $32 \pm 1.5\text{ng}$ IgG in the presence of PWM, indicated that a significant level of mitogenesis of the indicator PBLs had occurred. A suppression of this response, by up to 75%, indicated that hybrid-derived supernatants from the TT-4 line could exert a potent regulatory effect on the generation of an in vitro PWM-induced antibody response.

FIGURE 3.21

EFFECT OF TT-4 SUPERNATANT ON A PWM-INDUCED
ANTIBODY RESPONSE

Results expressed as % suppression \pm SD (triplicate determinations) of the response obtained with PWM alone - background (PBLs + PWM; 32 ± 1.5 ng IgG, PBLs alone; 6.8 ± 0.2 ng IgG).

Control supernatants derived from CCRF-CEM cells or normal PBL cultures had only a minimal effect on the PWM-induced antibody response.

Cell concentrations of hybrid-derived supernatants added to assay:

- | | |
|---------------------------|--------------|
| ●—● 0.5 $\times 10^6$ /ml | ○ PBLs + PWM |
| ■—■ 1.0 $\times 10^6$ /ml | □ PBLs alone |
| ▲—▲ 5.0 $\times 10^6$ /ml | |

(ii) Hybrid induction

Supernatants, derived from TT-4 hybrid cells which had been pretreated with ConA were screened in the human antibody assay.

Figure 3.22 indicates that the addition of supernatants derived from

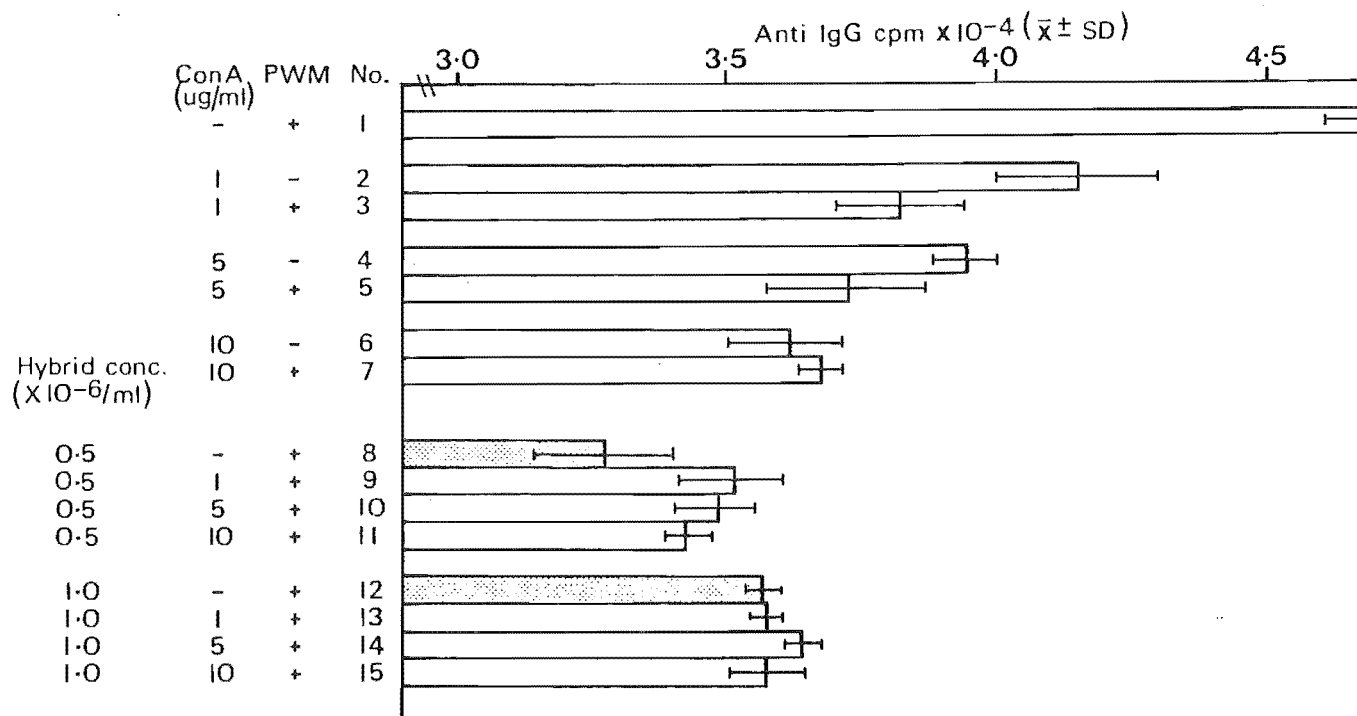
from unstimulated hybrid cells (lines 8 & 12) resulted in a significant reduction in the levels of IgG antibody produced in response to PWM (28 & 24% suppression respectively). Control cultures (lines 2-7) utilising PBLs cultured with varying concentrations of ConA, in the presence and absence of PWM, led to a reduction in the levels of IgG produced by the indicator PBLs (11 to 23% suppression, lines 2 & 6).

Induction of the hybrid cells with increasing amounts of ConA (1, 5 or 10 $\mu\text{g/ml}$) did not result in increased levels of suppression of the PWM-induced IgG response compared to supernatants derived from non-induced hybrid cells. At a hybrid cell concentration of $0.5 \times 10^6/\text{ml}$ (lines 8-11) stimulation with ConA resulted in marginally less suppression of the PWM-induced antibody response (25-27% suppression cf 28% for non-induced hybrids). For the hybrid cells grown at $1.0 \times 10^6/\text{ml}$ (lines 12-15), induction with ConA had no significant effect on the ability of the hybrid-derived supernatants to suppress the PWM-induced antibody response of the indicator PBLs.

The experimental results presented in Section 3.6.1 indicated therefore that the TT-4 hybrid line was able to exert a potent suppressive effect on the generation of antibody by normal indicator B cells in response to the polyclonal activator pokeweed mitogen. This suppressive effect of the hybrid-derived supernatants was not augmented if the hybrid cells were stimulated with concanavalin A prior to the collection of the hybrid supernatants.

FIGURE 3.22

ConA-INDUCTION OF TT-4 HYBRID CELLS



Effect of supernatants derived from ConA-treated TT-4 hybrid cells on a PWM-induced antibody response. Results expressed as anti-IgG cpm x 10⁻⁴ (± SD of triplicate determinations).

The effects of varying concentrations of ConA on the normal PWM-induced antibody response (No. 1) are shown in Nos. 2-7. The effects of supernatants derived from hybrid cells grown at 2 cell concentrations and pre-induced with 1-10 μg/ml ConA on the PWM-induced antibody response (No. 8-15) are shown.

3.6.2 T cell proliferative responses

Supernatants, derived from TT-4 hybrid cells were screened in the T cell proliferation assay.

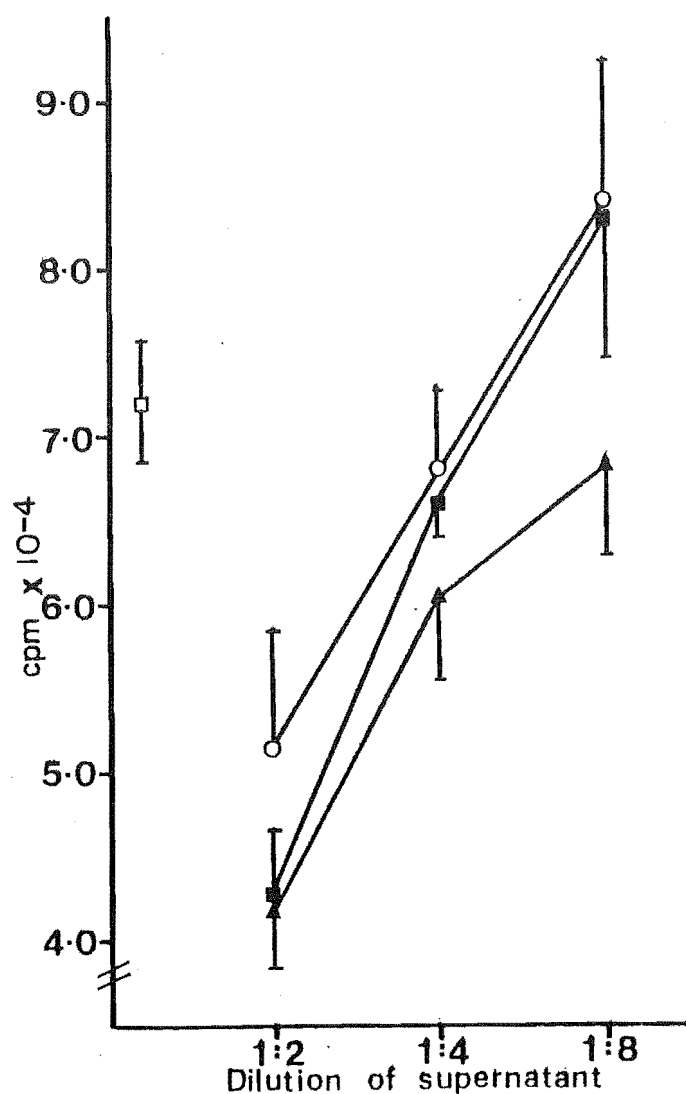
The indicator cells were 48 hr PHA-stimulated PBLs cultured for a further 48 hrs in the presence of hybrid supernatant, with and without exogenous IL-2 (1:10 dilution).

Figure 3.23 indicates that significant suppression of this IL-2 driven T cell proliferative response occurred at dilutions of 1:2 and 1:4. Suppression of the

response was obtained at 1:8 for hybrid cells grown at $5.0 \times 10^6/\text{ml}$. The suppression was therefore dose and cell concentration dependent.

FIGURE 3.23

EFFECT OF TT-4 SUPERNATANTS ON T CELL PROLIFERATION
IN THE PRESENCE OF IL-2



Effect of TT-4 supernatants on a T cell proliferative response.

Results expressed as :

^3H -TdR uptake ($\text{cpm} \times 10^{-4}$) by 48 hr PHA-stimulated PBLs in the presence of IL-2 (1:10) and dilutions of TT-4-derived supernatants. PHA-stimulated PBLs were cocultured with the supernatants and IL-2 for a further 48 hrs, then pulsed for 16 hr with $2.0\mu\text{Ci}$ ^3H -TdR. Cells were harvested onto glass fibre filters and counted by liquid scintillation.

Cell concentrations of hybrid-derived supernatants added to assay:

- $0.5 \times 10^6/\text{ml}$
- $1.0 \times 10^6/\text{ml}$
- ▲—▲ $5.0 \times 10^6/\text{ml}$
- 48 hr PHA-PBLs + IL-2 (1:10)

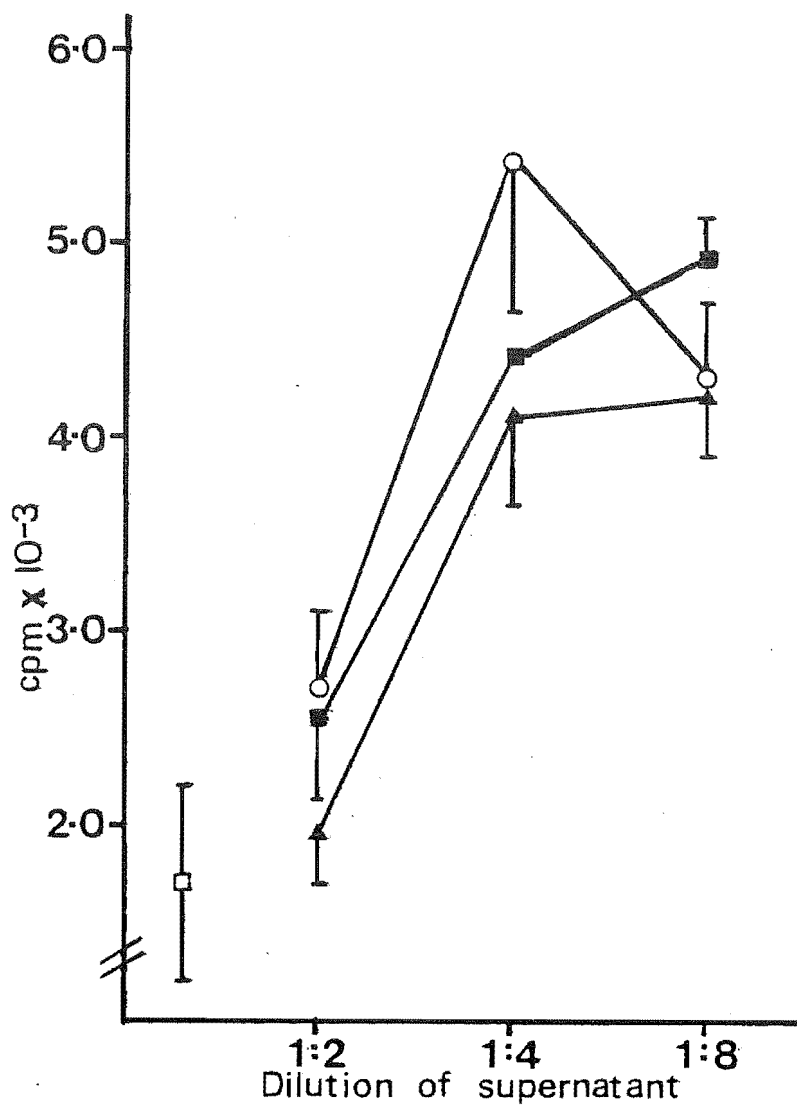
Standard deviations from quadruplicate determinations are shown.

Figure 3.24 describes the response obtained when 48 hr PHA-stimulated PBLs were cocultured with hybrid supernatant in the absence of exogenous IL-2. The level of proliferation, again assessed by ^3H -TdR uptake, was significantly reduced in the absence of exogenous IL-2 (1735 ± 500 cpm cf 72000 ± 5000 cpm in the presence of IL-2). At hybrid supernatant dilutions of 1:4 and 1:8 the ^3H -TdR uptake increased 2-3 fold. Some amplification of the response was therefore obtained, but this was only minimal when compared to the effect of exogenous IL-2.

The results presented in Section 3.6.2 indicated that TT-4 supernatants could suppress the proliferative response of lectin-activated T cells to exogenous IL-2. Several possibilities exist as to the mode and site of action of the hybrid-derived activity in suppressing this T cell response and these will be discussed in Chapter 4.

FIGURE 3.24

EFFECT OF TT-4 SUPERNATANTS ON T CELL PROLIFERATION
IN THE ABSENCE OF IL-2



Results expressed as :

³H-TdR uptake ($\text{cpm} \times 10^{-3}$) by 48 hr PHA-stimulated PBLs in the presence of TT-4 supernatants without exogenous IL-2. PHA-stimulated PBLs were cocultured with the supernatants for a further 48 hrs, pulsed with $2.0 \mu\text{Ci}$ ³H-TdR for 16 hrs and counted by liquid scintillation.

Cell concentrations of hybrid-derived supernatants added to assay:

- $0.5 \times 10^6/\text{ml}$
- $1.0 \times 10^6/\text{ml}$
- ▲—▲ $5.0 \times 10^6/\text{ml}$
- 48 hr PHA-PBLs alone

Standard deviations from quadruplicate determinations are shown.

3.7 PRELIMINARY BIOCHEMICAL STUDIES OF HYBRID-DERIVED FACTORS

It was possible that the biological effects of the TT-4 line were due to more than one regulatory molecule present in the hybrid supernatant. Preliminary biochemical characterisation of the TT-4 supernatants was therefore undertaken. Gel filtration chromatography was performed to determine:

- 1) the apparent MW of the suppressor activity, and
- 2) to investigate whether one or more activities were present in the hybrid supernatants.

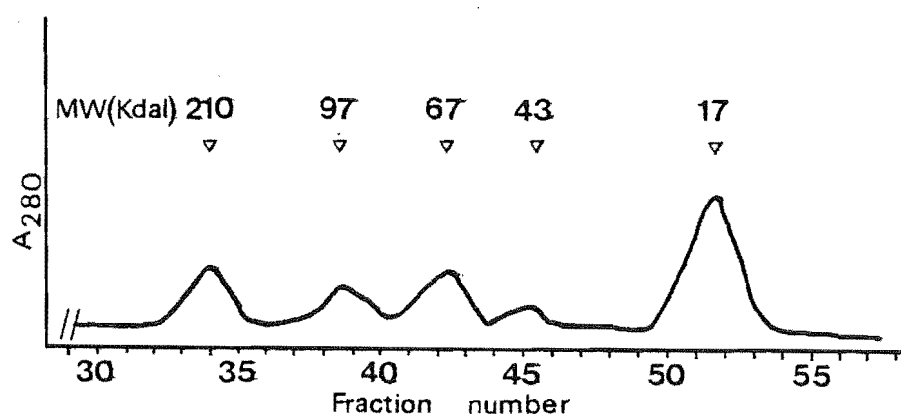
3.7.1 Apparent Molecular Weight

A Sephacryl S-200 column packed and equilibrated with PBS pH 7.4 was calibrated using known MW protein standards. Blue dextran was used to estimate the void volume.

A selectivity (or calibration) curve was constructed from a plot of K_{av} versus log MW. Figure 3.25 describes the elution profile for the MW standards used and Figure 3.26 is the calibration curve of the K_{av} versus log MW for the protein standards.

FIGURE 3.25

ELUTION PROFILE OF S-200 CHROMATOGRAPHED MW STANDARDS

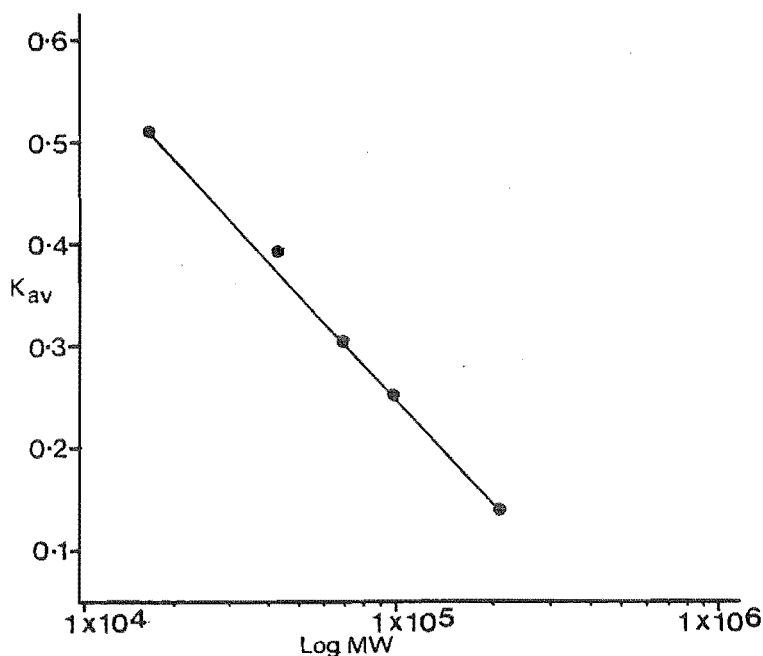


A 90 x 2.6cm column packed with Sephacryl S-200 was calibrated with known MW protein standards. Flow rate = 15ml/hr. Buffer = PBS pH 7.4.

Protein standards were: 1mg each of catalase (210 Kdal), phosphorylase b (97), BSA (67), ovalbumin (43) & myoglobin (17). The void volume was estimated with blue dextran ($> 2.5 \times 10^5$ Kdal).

FIGURE 3.26

S-200 MW CALIBRATION CURVE



Calibration curve of K_{av} versus $\log MW$ constructed from the elution volumes of the protein standards and blue dextran. K_{av} calculated from the following relationships :

$$K_{av} = \frac{V_e - V_o}{V_t - V_o} \quad \text{where;}$$

K_{av} = fraction of stationary gel volume which is available for diffusion of a given solute species.

V_o = void volume, the elution volume of molecules which are only distributed in the mobile phase because they are larger than the largest pores in the gel.

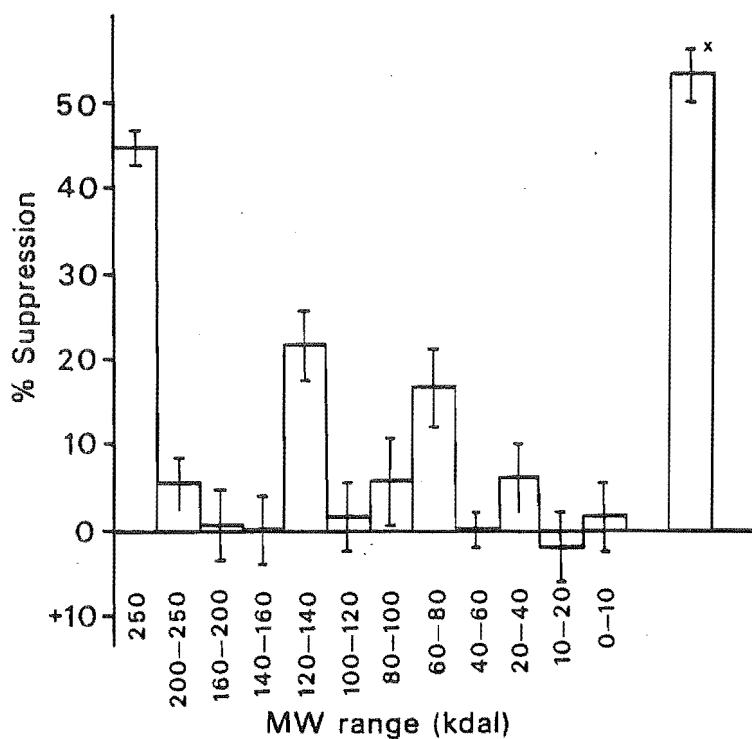
V_e = elution volume of a particular molecular species.

V_t = total volume of the gel bed.

Pooled concentrated fractions (corresponding to different MW ranges) from S-200 chromatographed TT-4 supernatant were assayed in the T cell proliferation assay at a final dilution of 1:4 and produced 3 major peaks of suppressive activity (Figure 3.27). These corresponded to greater than 250 Kdal (V_o), 120-140 Kdal and 60-80 Kdal. Concentrated precolumn TT-4 supernatant gave $53 \pm 3\%$ suppression at a final dilution of 1:4.

FIGURE 3.27

EFFECT OF CHROMATOGRAPHED TT-4 SUPERNATANT FRACTIONS
ON A T CELL PROLIFERATIVE RESPONSE



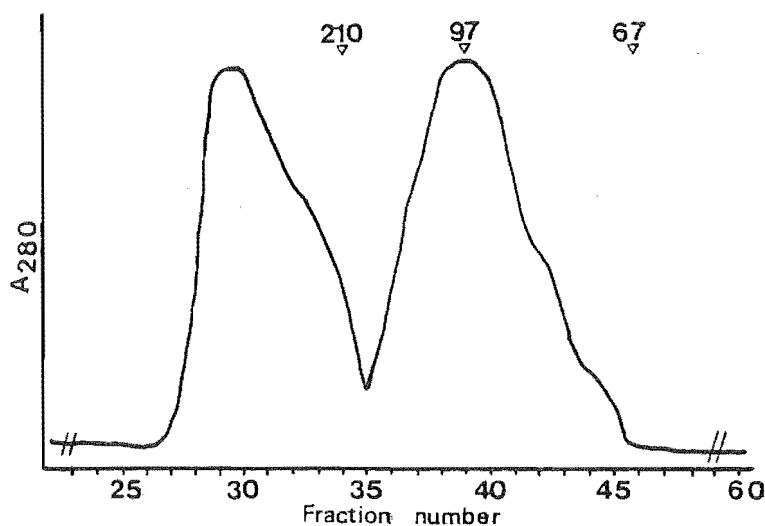
Results expressed as % suppression \pm SD (triplicate determinations)

$$= \frac{(\text{Column fraction cpm}) - (\text{PHA-PBL alone cpm})^1}{(\text{PHA-PBL} + \text{IL-2 cpm})^2 - (\text{PHA-PBL alone cpm})^1}$$

1. 48 hr PHA-stimulated PBLs, no exogenous IL-2 (PHA-PBL alone
= 17000 \pm 750 cpm)
 2. 48 hr PHA-stimulated PBLs + 1:10 partially purified IL-2
(PHA-PBL + IL-2 = 167000 \pm 8900 cpm)
- X Precolumn TT-4 supernatant, 1:4 final dilution.

Figure 3.28 describes the elution profile of the chromatographed TT-4 supernatant before the fractions were pooled, concentrated and assayed in the T cell proliferation assay.

FIGURE 3.28

ELUTION PROFILE OF S-200 CHROMATOGRAPHED TT-4
SUPERNATANT

90ml of TT-4 supernatant was concentrated to 6.5ml by ultra-filtration over a PSAC - 10^3 MW membrane. 5ml of this concentrated supernatant was chromatographed on the calibrated S-200 column. Flow rate = 15ml/hr, 5ml fractions were collected.

3.7.2 Physicochemical characterisation

Several physicochemical characteristics of the hybrid-derived activity were investigated.

Table 3.8 describes the physicochemical characteristics of the TT-4 supernatants. TT-4 was partially labile at both pH 2.0 and pH 9.0 (9.4 ± 2.8 and 4.0 ± 3.0 % suppression respectively cf 33.0 ± 3.0 % suppression of the T cell proliferative response by untreated supernatants). 50mM dithiothreitol reduced the suppression from 33.0% down to 8.4 ± 4.0 %. α - methyl mannoside, L-fucose and N-acetyl glucosamine decreased the suppression from 33.0% in the controls to 6.9 ± 6.5 , 10.5 ± 7.5 and 10.2 ± 6.1 % respectively. Interestingly, L-rhamnose had no significant effect on the TT-4 suppression of T cell proliferation (24.8 ± 6.4 cf 33.0 ± 3.0 %).

TABLE 3.8
BIOCHEMICAL CHARACTERISATION OF TT-4 SUPERNATANT
ACTIVITY

Treatment of supernatant ψ	Control cpm δ (\pm SD)	Experimental cpm (\pm SD)	% suppression (\pm SD) Δ
Untreated	194439 (10000)	130325 (5895)	33.0 (3.0)
pH 2.0	126866 (9719)	115031 (3595)	9.4 (2.8)
pH 9.0	222939 (3797)	213540 (6874)	4.0 (3.0)
DTT	190555 (7903)	175000 (7731)	8.4 (4.0)
L-rhamnose	201754 (10571)	152162 (13034)	24.8 (6.4)
α -methyl mannoside	187889 (15754)	174689 (12322)	6.9 (6.5)
L-fucose	208992 (15885)	187389 (15754)	10.5 (7.5)
N-acetyl glucosamine	215419 (14603)	193770 (13210)	10.2 (6.1)

The assay utilised 48 hr PHA-stimulated PBLs + 1:10 partially purified IL-2
+ control or test supernatant.

ψ see Section 2.7.2 for treatment of TT-4 supernatant.

δ all controls utilised RPMI, 15% FCS treated in an identical manner to the TT-4 supernatant.

Δ data expressed as:

$$\% \text{ suppression } \pm \text{ SD} = \frac{(\text{Experimental TT-4 supernatant cpm}) - (\text{background cpm})^1}{(\text{Control Supernatant cpm}) - (\text{background cpm})^1}$$

¹ Background cpm; 48 hr PHA-stimulated PBLs, no exogenous IL-2 = 430 ± 35 cpm.

CHAPTER 4.

DISCUSSION

The significance of the experimental techniques developed and data presented in this thesis will be discussed under the following sections :

- 4.1 Introduction and aims of experimental work.
- 4.2 Characterisation of mutagenised hybrid parental lines.
- 4.3 Human T cell hybrids : establishment, selection and criteria for hybridisation.
- 4.4 Functional assays : development and validation.
- 4.5 Functional studies of human T cell hybrid lines.
- 4.6 Biochemical characterisation of hybrid-derived factors.
- 4.7 Biological significance of T cell hybrid-derived suppressor factor.
- 4.8 Future studies.

4.1 INTRODUCTION AND AIMS

Detailed investigations of the structure-function relationships and the biological significance of lymphokine regulation of human immune responses have until recently been difficult. Many of the biological effects, as well as an appreciation of the pivotal role T cell-derived lymphokines play in immune regulation are known (see Immunological Reviews 63), but the biochemical nature and mechanism of action of these "immunological hormones" are still largely unknown. These deficiencies in our knowledge result, in part, from the limitations imposed by conventional lymphokine preparations i.e. the heterogeneous nature of the producer cell population and the limited amounts available for detailed biochemical analysis. These shortcomings will only be overcome when stable and homogeneous T cell lines secreting distinct, or restricted numbers of lymphokines are established.

Three major approaches are currently being used to create homogeneous and stable lymphokine-secreting T cell lines. These techniques are :

- 1) the adaptation of T cell leukemias and lymphomas to growth in culture,

- 2) the maintenance of normal T cells in culture by the addition of "growth factors" and,
- 3) immortalising T cell function by fusing normal lymphokine-secreting T cells with a neoplastic T cell line.

This thesis has addressed the problem of creating stable sources of human T cell-derived regulatory lymphokines by utilising the third of these approaches. The techniques of somatic cell hybridisation, as refined by Kohler & Milstein (1975) for the construction of murine B cell hybridomas secreting monoclonal antibodies, have been adapted for the production of human T cell hybridomas (Grillot-Courvalin et al, 1981; Irigoyen et al, 1981). Fusion of lectin-activated T cells with a drug-resistant leukemic T cell line aims to immortalise normal T cell secretory activity on a background of continued proliferation by the leukemic T cell.

Human T cell hybrid lines were constructed between a 6TG-resistant human T-ALL line and ConA-activated normal human PBLs using PEG as the fusigen. Selection of T cell hybrids was achieved by a combined approach which utilised both drug and phenotype selection (Sections 2.2, 2.3). Selection systems are necessary to ensure that "true" hybrids, arising from only drug-resistant tumor cells and that lectin-activated T cells are produced. In contrast to B cell hybrids, where the success of the fusion can be assessed with relative ease by screening the derived lines for the production of antibodies of unique specificity, T cell hybrids pose special problems. Short-term RIA or ELISA techniques can be used to assess if the drug-selected B cell hybrids are producing monoclonal antibodies not secreted by the parental tumor line and which therefore must be derived from the genetic contribution of the normal B cell to the hybrid. Short-term assay systems such as these enable the selection of hybrid clones based on functional activity. Similar short-term assays confirming functional hybridisation in human T cell hybrid lines are not currently available. For this reason long-term assays are required to screen T cell lines for biological activity (Section 2.6.2). To increase the frequency of true human T cell hybrids arising from the fusions, and therefore the likelihood of establishing lines with immunoregulatory activity, two selection systems were used. The HAT drug system was used to select for hybrids resulting from the fusion of the HGPRT⁻ parental tumor line X HGPRT⁺ normal T cells (rationale explained in Section 1.8.2). A second approach utilising the cell surface phenotype of the resultant hybrids was also adopted. Acquisition of a marker, absent on the parental tumor line and

subsequently expressed on the putative hybrid cells, allowed the isolation of true hybrid cells. This double selection approach was successful in establishing human T cell hybrid lines possessing immunoregulatory activity.

Extensive characterisation of the human T cell lines was undertaken and one hybrid line, TT-4, was shown to elaborate a soluble factor which suppressed both B cell antibody and T cell proliferative responses. Preliminary characterisation of this hybrid-derived suppressive activity was undertaken.

The following sections of this chapter discuss the significance of these studies.

4.2 CHARACTERISTICS OF MUTAGENISED HYBRID PARENTAL LINES

A 6-thioguanine-resistant line, CEM-6TG^R, derived from the human T-ALL line CCRF-CEM was produced. CEM-6TG^R was used as the primary hybrid parental tumor line for the construction of human T cell hybrids. Cell lines resistant to 6TG have been shown to be deficient in the salvage enzyme HGPRT (Sato et al, 1972) and are therefore killed in the HAT selective media devised by Szybalski et al (1962). The HAT system was used to select for putative hybrid cells arising from the PEG-induced fusion of ConA-activated normal human PBLs with the CEM-6TG^R line.

CEM-6TG^R was established by treating CCRF-CEM cells with the mutagen EMS, and subsequent selection in 6TG-supplemented tissue culture medium. EMS is a guanine base-specific alkylating agent which results in the formation of a quarternary nitrogen which destabilises the deoxyriboside link thus releasing the deoxyriboside (Conn & Stumpf, 1972). Loss of a base can lead to its replacement by any of the four bases or even rupture of the DNA, hence its powerful mutagenic properties. Albertini & De Mars (1970) and Sato et al (1972) have shown that mutagenised cells resistant to 8AG or 6TG are deficient in HGPRT activity. Normal wild type (HGPRT⁺) cells are killed because these drugs when metabolised interfere with normal nucleotide and nucleic acid synthesis. The uptake in metabolism of these nucleotide analogues is mediated by the HGPRT enzyme which converts the drugs to abnormal metabolites. Mutant cells which lack the HGPRT enzyme are resistant to 6TG and therefore survive the treatment.

Following EMS mutagenesis of CCRF-CEM cells, the 6TG supplement added

to the culture medium was added stepwise from 0.1-18 μ g/ml over approximately six weeks. The data presented in Table 3.1 indicate that a maximum of 56% of the cells survived the initial EMS treatment (1 μ g/ml) compared to 85% in control cultures. These results compare favourably with the data described by other workers also studying mammalian cells.

Kao and Puck (1968) using Chinese hamster ovary cells, demonstrated that the survival curve for EMS treatment has a large initial shoulder. At concentrations of up to 100 μ g/ml, reasonably high levels of cell survival were achieved (up to 78%). In contrast, Sato et al (1972), mutagenised a human lymphoblastoid cell line with varying concentrations of EMS and demonstrated that at 50 μ g/ml, approximately 30% of cells survived a 24 hr treatment (cf 38% in this study). Sato et al also estimated the plating efficiency of EMS-treated and untreated cells in the presence of varying concentrations of 6TG. In the absence of EMS mutagenesis, 6TG concentrations greater than 1 μ g/ml resulted in no 6TG-resistant colonies arising in culture. Following EMS mutagenesis though, the frequency of 6TG-resistant mutants obtained was increased 109-fold (to approx. 2×10^{-4} /cell/generation), over the spontaneous frequency of 6TG-resistant cells arising. EMS was therefore shown to be of value in the induction of 6TG-resistance in these human cells.

Twenty-four hour EMS-mutagenesis followed by 6TG-selection (0.1 μ g/ml) was shown in this study to enable the construction of the CEM-6TG^R line. This drug-resistance was subsequently demonstrated to be due to a significantly reduced level of functional HGPRT activity when compared to the parental CCRF-CEM line (Section 2.5).

Thioguanine was observed to have a significant inhibitory effect on the growth of the EMS-treated cells until some 20 days post mutagenesis. One mutagenised line, (10 μ g/ml EMS) regained its normal growth characteristics in the presence of 6TG after the initial drug "shock", and it was this line which was further selected with increasing concentrations of 6TG up to a maximum of 18 μ g/ml. This line was termed CEM-6TG^R and was used as the drug-resistant hybrid parental line for the construction of human T cell hybrids.

CEM-6TG^R cells were maintained in the presence of 6TG to ensure that any rare revertants to HGPRT⁺ cells did not arise in culture. Revertant cells survive in HAT-supplemented selective media and could possibly overgrow any true hybrids. Fortunately the frequency of revertants arising in culture is very

low ($<10^{-7}$) (Morrow et al, 1982), and does not therefore constitute a major limitation of somatic cell hybridisation studies.

CEM-AG^R, an 8-azaguanine-resistant derivative of CCRF-CEM, kindly provided by Professor T. Kishimoto (Osaka), was used for some fusion experiments.

Growth rates of CEM-6TG^R, CEM-AG^R and the non mutagenised CCRF-CEM line were determined. The estimated doubling times for these lines were 30, 28 and 26 hrs respectively. Both drug-resistant lines therefore exhibited doubling times comparable to that of the parental CCRF-CEM line. Doubling times for CCRF-CEM (26 hrs) are comparable to that quoted by Greene et al (1982) (24 hrs) but considerably less than that described by Foley et al (1965) (46 hrs). It is important to note that the study by Foley et al related to a diploid clone of the CCRF-CEM line at a time when it was probably still adapting to growth in vitro. Growth inhibition after 120 hrs (2×10^5 /ml) for CCRF-CEM and 140 hrs (2×10^6 /ml) for CEM-AG^R was observed.

Terminal deoxynucleotidyl transferase (TdT) is a DNA polymerase which is regarded as a marker for primitive lymphocytes in the hematopoietic system (Janossy et al, 1979). Both the CCRF-CEM and CEM-6TG^R lines were TdT⁻ as assessed by the fluorescent antibody technique described. TdT⁺ control cell lines stained simultaneously were TdT⁺, minimising the possibility of a technical failure. Subsequent staining of the lines has confirmed these observations. CCRF-CEM is regarded as an early T-ALL and has been previously demonstrated to express the TdT marker (Janossy et al, 1979). Failure to detect the TdT marker may be consistent with the observations made by Suomalainen et al (1980), that many established long-term leukemia lines have acquired profound aberrations in their chromosome sets as well as their phenotype. These observations could relate in this instance to the chromosome carrying the gene coding for TdT expression. The chromosomal analysis and phenotype studies performed on the CEM-6TG^R line may confirm this hypothesis (Sections 3.2.1, 3.2.3).

Growth of the CEM-6TG^R line in 6TG-supplemented media suggested it was deficient in HGPRT activity and should therefore be killed in HAT media (Littlefield, 1964). HAT sensitivity experiments performed by culturing CEM-6TG^R cells directly in this media showed that no viable cells were present after 7 days in culture. HAT sensitivity confirmed the CEM-6TG^R line would be of use in the construction of human T cell hybrids. The biochemical rationale enabling the use of HAT media for the selection of somatic cell hybrids has been described

(Section 1.8.2) but briefly, parental (HGPRT⁻) tumor cells are killed in HAT and only hybrids which have inherited a complementary HGPRT gene from a wild type (HGPRT⁺) normal T cell are able to survive.

4.3 HUMAN T CELL HYBRIDS : ESTABLISHMENT, SELECTION AND CRITERIA FOR HYBRIDISATION

Human T cell hybrids were established following the PEG-induced fusion of CEM-6TG^R cells with ConA-activated normal human PBLs. PEG is almost universally adopted for the construction of hybrids. DMSO was added to the PEG fusion solution as it has been reported to decrease the hyper-osmotic effects of the PEG solution and enhance the PEG-mediated fusion of mammalian cells (Norwood et al, 1976).

Conventional HAT selection followed by phenotypic selection of the OKT3 expressing hybrids by sterile cell sorting on a FACS IV was used to establish 4 human T cell hybrid lines (TT-1 to TT-4). Phenotypic selection of the HAT-selected hybrid cells was possible because the CEM-6TG^R parental line did not express the antigen recognised by the OKT3 McAb and its presence on the hybrid cells must have therefore resulted from the contribution of the activated normal T cells. Normal T cells die in culture without the addition of exogenous growth factors and therefore do not contribute to the HAT selected hybrid population. As stated, selection systems were also necessary due to the lack of short-term screening assays capable of detecting low levels of lymphokine secretion by the hybrid lines.

Three techniques were used to confirm that "true" human T cell hybrids had been produced :

4.3.1 Surface phenotype studies of parental and hybrid cells

Surface phenotype studies were performed on both the parental and hybrid cells to determine if the expression of receptors, absent on the parental CEM-6TG^R line but present on normal T cells, were subsequently expressed on the HAT-selected putative hybrid cells. The acquisition of a particular cell surface receptor from the normal T cell and its expression on the resulting T cell hybrid line would therefore be indicative of true hybrid formation. Furthermore, the capacity of the FACS to sort, under sterile conditions, cell populations

possessing defined phenotypic characteristics was exploited in the establishment of the hybrid lines.

Indirect immunofluorescence staining and FACS analysis of the ConA-activated PBL fusion partners indicated that greater than 85% were Leu-4⁺ (T cells) and less than 7% were SmIg⁺ (B cells). Though specific enrichments of the normal ConA-activated T cells e.g. E rosetting, were not performed prior to fusion a significant level of enrichment in T cell percentages resulted from the lectin-activation.

CEM-6TG^R and CEM-AG^R cells were screened with a wide range of monoclonal antibodies to human cell surface receptors. The results presented in Table 3.3 indicated that neither line expressed the receptor recognised by the OKT3 or Leu-4 McAbs. Absence of the OKT3 receptor on the CEM-6TG^R line and its subsequent expression on the HAT-selected hybrid cells was used to set sorting windows on the FACS to facilitate sterile sorting of the OKT3⁺ hybrid population. OKT3 expression on the HAT-selected hybrids was deemed indicative of true hybrid formation between OKT3⁻ parental tumor cells and OKT3⁺ normal lectin-activated T cells.

Dual parameter gated analysis was used to sort the OKT3⁺ population whilst excluding non-viable and OKT3⁻ cells (tumor x tumor hybrids and hybrids not expressing the receptor).

Taniguchi & Miller (1978) have described a similar approach which allowed non-mutant T cell lines to be used in murine T cell hybridisation programmes. The selection techniques adopted in this thesis, if used in conjunction with dual colour fluorescence, have the potential to allow hybrid selection based on the expression of multiple receptors. This approach will be used in conjunction with specific receptor deletions (by chemical mutagenesis and selection) to investigate the relationship between receptor expression and lymphokine secretion by human T cell hybrids.

The frequency of OKT3⁺ hybrids arising from the fusions or the frequency of revertants to HAT resistance has not been addressed. The lack of expression of the OKT3 receptor by the CEM-6TG^R line coupled with positive selection of the OKT3⁺ hybrids was used to exclude the possible contribution of CEM x CEM revertants to the hybrid cell population. Few studies have considered the reversion rates of HAT-sensitive cells to HAT-resistance, but these rates have

been shown to be very low (Morrow et al, 1970).

Minowada et al (1977) reported that CCRF-CEM had lost its ability to form sheep rosettes. This was confirmed for both CEM-6TG^R and CEM-AG^R by a lack of reactivity with the Leu-5 McAb. Howard et al (1981) showed that the Leu-5 receptor is usually expressed on 95-100% of human T cells.

Membrane phenotype studies were performed on the hybrid lines from 10-140 days post fusion. These studies showed that 2.6 - 3.0% of the putative hybrid cells were expressing the OKT3 marker 10 days post fusion. Relatively stable percentages of OKT3⁺ cells or an enrichment of this population had occurred when staining was performed 76-90 days post fusion. OKT3 staining beyond this time (up to 140 days) indicated that a significant loss of OKT3 receptor expression had occurred in all the 4 lines. Even though a loss of OKT3 receptor expression occurred over 3-4 months in culture, hybrid-derived supernatants still produced significant levels of suppression of a PWM-induced antibody response (discussed in Section 4.5). The expression, or lack of expression, of the OKT3 receptor by the lines cannot therefore be correlated directly with biological activity. OKT3 expression must therefore be viewed as only an indicator of "true" hybrid formation in the early stages following fusion and drug selection.

Phenotyping hybrid cells 90 days post fusion indicated that all 4 lines exhibited, to varying extents, expression of both the Leu-2a and Leu-3a markers. The levels of expression of these markers were comparable within each line, but a wide variation in staining (31-80% Leu-2a⁺, 33-73% Leu-3a⁺) was apparent between hybrid lines. This apparent discordant expression of receptors has been reported in other human T cell hybridoma studies (DeFreitas et al, 1982). The Leu-2a (Ts/c) and Leu-3a (Th/i) defined antigens are usually regarded as mutually exclusive markers of normal T cell subpopulations therefore their apparent dual expression on the hybrid lines suggests that the expression of these antigens is not regulated in T cell hybrids as they are in normal T cells. For this reason one must conclude that surface phenotype need not necessarily correlate with functional activity in human T cell hybrids. An inability to detect significant levels of helper activity for T cell proliferation in chromatographed hybrid supernatants (Section 3.6.1 (b)) perhaps reinforces this hypothesis.

4.3.2 Functional HGPRT activity

Cells resistant to 6TG have been shown to be deficient in functional

HGPRT activity (Sato et al, 1972) and are therefore killed in HAT-selective media (Littlefield, 1964). Hybrids between HGPRT⁻ parental tumor cells and HGPRT⁺ normal T cells survive the HAT-selective media because they are able to bypass the aminopterin block of de novo nucleotide biosynthesis by utilising their restored salvage pathways.

Deficiency in functional HGPRT activity by the parental tumor line and its subsequent acquisition following hybridisation would be further evidence for true hybrid formation.

Comparison of the in vitro ³H-HX / ³H-TdR incorporation ratios for the CCRF-CEM, CEM-6TG^R and T cell hybrid lines confirmed that:

- 1) the parental CEM-6TG^R cells were HGPRT deficient and,
- 2) that HAT and phenotype selected hybrids did receive functional HGPRT activity following fusion with activated T cells.

This provided strong evidence that true human T cell hybrids were produced.

4.3.3 Chromosomal analysis

CEM-6TG^R cells exhibited a modal distribution of chromosome number (mean = 92); indicating tetraploidy. Suomalainen et al (1980) have reported a different observation for the CCRF-CEM line. Their study indicated that the CCRF-CEM line consisted of two clones; a near-tetraploid clone with a modal chromosome number of 85 (70% of cells in line), and a near-diploid clone with a modal chromosome number of 44 (30% of cells). The CCRF-CEM line used in this study does not appear to have the 44 or 85 chromosome clone present, and this may simply result from selective enrichment of the tetraploid clone in culture or from some form of karyotype evolution. Karyotyping of the CCRF-CEM line shortly after its establishment (McCarthy et al, 1965), indicated the presence of an unidentified chromosome "marker". This may correspond to the unidentified chromosome shown in Figure 3.7 (Section 3.2.3). Two abnormal number nine chromosomes are present in the screen shown.

The studies by Suomalainen et al (1980) of CCRF-CEM (and 5 other ALL lines studied) concluded, that during continuous culture the lines have acquired profound aberrations in their chromosome sets as well as in their phenotypes

(e.g. CCRF-CEM loss of E rosette receptor). The CCRF-CEM line used in this study appears to be another example, to add to those described by Suomalainen et al, of lines with both altered chromosomes and altered phenotypes.

TT-4 cells, 120 days post fusion, had a modal distribution of chromosome number with a mean of 92 and showed some evidence of chromosomal instability. This could reflect the observations made in the surface phenotype studies (Table 3.4). Because of the chromosomal instability evident when the karyotyping was performed, cell surface phenotype and HGPRT activity were used as the main criteria confirming the success of the hybridisation.

The development of other techniques confirming hybridisation e.g. isoenzyme studies, HLA typing of parental and hybrid cells (Okada et al, 1981), or short-term screening assays for constitutive lymphokine secretion were beyond the scope of this investigation but may be warranted if somatic cell hybrids are to be used as stable sources of human regulatory lymphokines in the future.

4.3.4 Alternative selective media

HAT selection is widely used to select for successful somatic cell hybrids. Reports (Schachtschabel et al, 1966; Fox et al, 1981) have stated that the thymidine component of this medium causes growth inhibition of the CEM line. An alternative drug selection system using the amido transferase inhibitor azaserine combined with hypoxanthine (AH) (Siniscalco et al, 1969) was utilised to select for putative hybrids arising from a fusion experiment. 48 hr ConA-activated PBLs PEG-fused with the CEM-AG^R line were selected in either HAT or AH media for 14 days, then conditioned through media lacking anti-metabolites (no aminopterin or azaserine respectively) into standard tissue culture medium supplemented with 20% FCS. Direct microscopic examination, 30 days post fusion, was used to determine the numbers of wells containing viable cells. 9.7% of the cells seeded in HAT medium showed evidence of viable cells compared to 17.4% of wells in AH medium (144 wells of each). This direct comparison suggested the AH selection system was enabling more hybrids to arise from the fusions and this system will be investigated further to determine if this is the case. Screening of the hybrid lines using the hybridisation criteria adopted will also be performed.

4.4 FUNCTIONAL ASSAYS : DEVELOPMENT AND VALIDATION

Assay systems, capable of quantitating human antibody responses in vitro to hapten-carrier conjugates were developed. These developmental studies were undertaken as part of a continuing project in this laboratory investigating the role of a mixed lymphocyte culture-derived suppressor factor (MLC-SF) in regulating the activation of cytotoxic T cells (Crosier & Broom, 1981b). It was of interest to this group to determine whether the MLC-SF could also regulate B cell responses, hence the necessity for a human antibody assay. My role in the project was to develop human antibody assays as well as somatic cell hybrids as alternative sources of human regulatory lymphokines (especially SFs). The antibody assay was therefore used to screen the T cell hybrid lines as well as the studies of the MLC-SF; which do not form part of this thesis material.

One human antibody assay, a mitogen-dependent system, was used to screen T cell hybrid-derived supernatants for possible immunoregulatory effects on normal human PBL antibody responses. A second assay, a hapten-specific antibody system, was also developed. This assay was not used to screen the T cell hybrid lines but will be of general use in the future for the investigation of lymphokine regulation of antigen or hapten-specific antibody responses.

4.4.1 Human antibody assay (mitogen-dependent)

This antibody assay system utilised a RIA to measure total and specific antibody production by human PBLs in response to the hapten-carrier conjugates DNP-BSA or DNP-KLH in the presence of the polyclonal activator PWM (Section 2.6.2).

As other groups have demonstrated (Volkman et al, 1981; Clifford-Lane et al, 1981) the polyclonal activator PWM was required to enhance the antigen-specific antibody response. Culturing PBLs in the absence of antigen or PWM failed to produce significant levels of antibody. PBLs cultured with PWM alone produced significant levels of anti-hapten antibody (Figs. 3.9, 3.10).

PWM, in this assay system, was therefore required to trigger hapten-specific antibody production as part of the total polyclonal response induced by the mitogen. PWM however, has limitations, as it has been shown to "bypass" the requirement for histocompatibility in T-B collaboration in specific murine antibody responses (Hirano et al, 1977). Two methods were utilised to circumvent

the need for PWM to trigger B cell differentiation and Ig secretion. Hydrocortisone (HC) was used in an attempt to inhibit Ts cells without affecting Th cells (Lobo & Spencer, 1979), and IL-2 was used to expand the antigen-activated Th cells (Aarden et al, 1979).

Both HC and IL-2 were demonstrated to enhance hapten-specific and total IgM antibody responses in the presence of PWM, but antigen plus PWM still produced the largest hapten-specific IgM response. This suggested that PWM was enhancing the production of a hapten or antigen-specific amplifying factor.

Figure 3.11 (Section 3.4.1 (a)) showed that PBLs cultured with DNP-KLH and PWM alone produced significantly higher levels of total IgM antibodies compared with the other non-PWM-stimulated cultures even though comparable levels of hapten-specific antibodies were produced. These observations could have two explanations:

- 1) the indicator PBLs respond poorly, if at all to DNP-KLH (in the absence of PWM); hence the low levels of hapten-specific antibody produced. The amplified total IgM response (in the presence of DNP-KLH and IL-2 alone) could result from a small number of in vivo-activated T cells being expanded and stimulated in vitro to produce non-specific B cell differentiation factors. B cell growth and differentiation factors are known to augment in vitro B cell responses (Howard et al, 1982). The minimal response of the indicator PBLs to DNP-KLH alone suggest that only low levels of antigen-responsive B cells are present in the culture.
- 2) A second possibility is that factors present in the IL-2 containing conditioned-medium, or produced by IL-2 responsive T cells, are suppressing the anti-hapten response.

Hapten-specific antibody production was enhanced by PWM but DNP-KLH plus PWM gave the largest response (Figure 3.11). HC was not observed to amplify the response (i.e. by inhibiting Ts cells) suggesting resistance or merely

suboptimal levels in the culture. Reduction of the hapten-specific response of the indicator PBLs in the presence of IL-2 to less than that of PBL + PWM points to active immune suppression or non-specific suppression by factors present in the IL-2 medium.

Studying this culture system has, in addition to aiding the development of a simple assay for the quantitation of human antibody responses provided some interesting information about human in vitro B cell responses. These include:

- 1) specific antibody production is associated with polyclonal activation in this system,
- 2) antigen-specific B cells can be triggered to produce antibody by PWM provided the relevant B cells are present in the culture,
- 3) at high antigen concentrations antibody responses can be suppressed. Eardley et al (1976), demonstrated that the adjustment of experimental conditions in assay cultures significantly affected the regulatory response produced by spleen cells educated with SRBC. To summarise their study, spleen cells could be educated in vitro to induce T help or T suppression of the antibody response to SRBC. Low doses of SRBC induced Th cells whereas high doses induced Ts cells in culture. The observations made in this human antibody assay may be due to analogous effects resulting from the in vitro culture conditions adopted.
- 4) cell density and vessel geometry are extremely important for in vitro B cell responses. Clifford-Lane et al (1981) suggested this could be explained by the need for enhanced cell-to-cell contact for the generation of non-specific helper factors. ThF have been shown to act by bypassing the need for linked recognition in immune responses (Katz, 1977, p482).

Three approaches were used to validate the human antibody RIA.

- 1) the IgG and IgM dose response curves described in Figure 3.12 are characteristics of ideal binding in RIAs (Kalmakoff et al, 1977).
- 2) Figure 3.13 showed good inhibition of binding occurred when supernatants from a DNP-KLH antibody culture were titrated with increasing amounts of DNP-BSA and assayed for the levels of hapten-specific antibody remaining in solution. Titration with ovalbumin had little effect on binding whereas with carrier alone (KLH) the curve indicates that both carrier and hapten-specific antibodies were produced in culture though hapten-carrier conjugates were more effective in inhibiting binding.
- 3) the IgG dose response curve shown in Figure 3.14 indicated that maximum binding of the test IgG to the goat anti-human IgG coated plates occurred at equivalence (100ng IgG) confirming the quantitative nature of the RIA.

These three methods confirmed that the RIA was measuring specific antibody production and furthermore that the culture system could produce antigen or hapten-specific antibody in the presence of PWM.

4.4.2 Human hapten-specific antibody responses (mitogen-independent)

A second antibody assay, a mitogen-independent hapten-specific system, was developed and validated. Even though this system was not used to screen the T cell hybrid lines, it has a number of desirable characteristics which will enable the assay to be used in the investigation of human B cell responses.

The optimal culture conditions for the generation of hapten-specific antibody were demonstrated to be at a cell density of $1.0 \times 10^6/0.2\text{ml}$, antigen concentration of $1.0\mu\text{g}/0.2\text{ml}$ and 5 days with antigen followed by 4 days without antigen. The peak of the T cell dependent response was between days 5 and 7 (Section 3.4.2).

This assay system overcomes many of the shortcomings of conventional human in vitro antibody studies e.g. their reliance on the addition of helper factors from activated cells (Luzzati et al, 1976), PWM to provide polyclonal activation (Volkman et al, 1981), allogeneic cells or a high concentration of serum supplements to stimulate antibody production (Morimoto et al, 1981). Preimmunisation of the donor with antigen has also been used (Clifford-Lane et al, 1981). As this microculture system circumvents, in particular, the need for PWM to stimulate B cell responses to hapten-carrier conjugates, it will aid in the investigation of human primary in vitro antibody responses. These studies could include PBL responses to regulatory lymphokines, human cell-to-cell interactions during immune responses, or the closer investigation of T and B cell interactions in disease.

4.5 FUNCTIONAL STUDIES OF HUMAN T CELL HYBRID LINES

The human antibody assay (Section 2.6.2) was used to screen supernatants derived from the T cell hybrid lines. These experiments aimed to determine whether hybrid supernatants contained regulatory molecules which could in any way modify the response of normal indicator PBLs to PWM. From the studies undertaken in developing the assay it was apparent that significant levels of total and hapten-specific antibodies were produced by the indicator PBLs in response to PWM alone. As the assay was to be used merely to screen hybrid-derived supernatants for any gross immunoregulatory effects on normal PBL antibody responses, PBLs stimulated with PWM alone were used as the indicator cells. Only total PWM-induced antibody and not hapten-specific antibody levels were therefore determined.

Studies by Grillo-Courvalin et al (1981) and Greene et al (1982), also using assay systems measuring PBL responses to PWM, have shown their value in screening human T cell hybrids for regulatory effects on human B cell antibody responses.

Hybrid cells were screened for constitutive or inducible (following ConA-activation) production of factors which could modify the antibody response of indicator PBLs to PWM.

4.5.1 Constitutive lymphokine production

The primary aim of this work was to establish stable sources of human immunoregulatory lymphokines, and if possible lymphokines possessing

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suppressive characteristics. Preliminary experiments indicated that hybrid-derived supernatants from all four lines were capable of suppressing, to varying extents, a PWM-induced antibody response. Hybrid line TT-4, which exhibited the most potent suppressive effect, was used for more detailed studies.

Supernatants derived from TT-4 hybrid cells were demonstrated to cause a dose and producer cell concentration-dependent suppressive effect on the generation of an in vitro PWM-induced IgG response (up to 75% for the 3 cell concentrations and dilutions tested). Stimulation of the indicator PBLs with PWM resulted in a greater than four-fold increase in IgG secretion. This confirmed that the assay was working at near optimal mitogenic levels. If this is the case, these levels of suppression caused by the hybrid supernatants are therefore significant.

4.5.2 Hybrid induction

Induction of cultured TT-4 hybrid cells with ConA prior to assaying the hybrid-derived supernatants for possible effects on a PWM-induced antibody response was undertaken. These experiments aimed to determine if stimulation of the hybrids resulted in the enhanced secretion of suppressive, or other regulatory lymphokines.

The results of these studies indicated that ConA pretreatment of the hybrid cells (1-10 μ g/ml ConA) did not alter to any significant extent the ability of hybrid-derived supernatants to suppress the IgG response of the PWM-stimulated indicator PBLs.

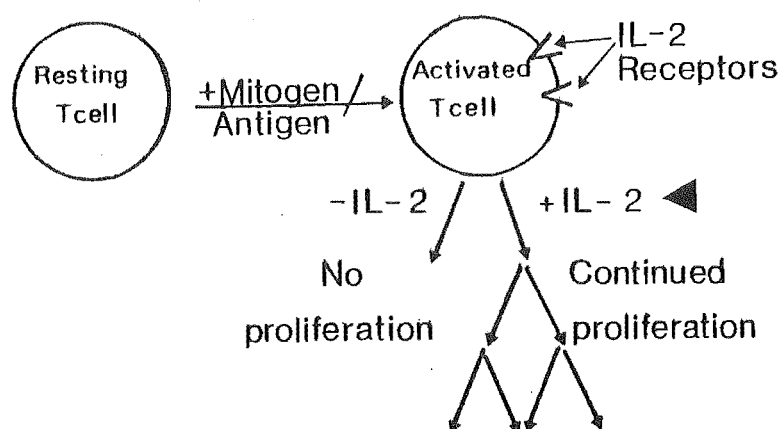
4.5.3 T cell proliferative responses

The constitutive ability of the hybrid-derived supernatants to suppress a normal B cell antibody response in vitro could be due to a number of possible effects. The hybrid activity could be acting on the B cells directly, or alternatively via some other immunoregulatory T cell or antigen presenting cell in the indicator culture. The experiments discussed here aimed to investigate if the suppression of antibody production was due to a direct effect on T cells in the culture. The effect of TT-4 supernatants on the continued proliferation of lectin-activated T cells in response to exogenous IL-2 was investigated in an attempt to answer this question.

Lectin-activation of normal T cells results in their expression of receptors for IL-2. Exogenous sources of IL-2 cause the continued proliferation of these activated cells. In the absence of IL-2 they fail to proliferate (Gillis et al, 1978). Figure 4.1 describes this phenomenon of IL-2-dependent T cell proliferation.

FIGURE 4.1

IL-2-DEPENDENT T CELL PROLIFERATION



Mitogen or lectin-activation of normal T cells leads to expression of receptors for IL-2. In the presence of exogenous sources of IL-2 activated T cells continue to proliferate, whereas in the absence of IL-2 they fail to proliferate.

TT-4 supernatants were demonstrated to suppress the proliferative response of lectin-activated T cells to exogenous IL-2 in a manner dependent on dose and concentration of producer cells. In the absence of exogenous sources of IL-2 the activated indicator T cells failed to proliferate. TT-4 supernatants enhanced their proliferation marginally, but this was not significant when compared to the effects of IL-2. This could be due merely to the addition of extra FCS to the culture. The ability of TT-4 supernatants to suppress the IL-2-dependent proliferation of activated T cells suggests that the activity is in some way interfering with the response to IL-2. The possible mechanisms of this effect are discussed in Section 4.7.

4.6 BIOCHEMICAL CHARACTERISATION OF HYBRID-DERIVED FACTORS

The ability of TT-4 supernatants to suppress both B cell antibody and T cell proliferative responses suggested that more than one substance present in the

supernatant could be causing these effects. Preliminary biochemical characterisation of the hybrid activity was therefore undertaken.

Gel filtration of TT-4 supernatants indicated that 3 major fractions; corresponding to >250 Kdal (Vo), 120-140 Kdal and 60-80 Kdal respectively, were capable of suppressing a T cell proliferative response. This suggested a 60-80 Kdal suppressive activity and possibly an 120-140 Kdal oligomer were present in the supernatant. The Vo activity could be due to either high MW oligomers or from activity being carried through with the excluded material. Importantly the levels of suppression for each MW range did not merely reflect differences in the total protein concentration of the fractions (data not shown).

No fractions provided significant levels of helper activity for T cell proliferation, perhaps reinforcing the belief that receptor expression by human T cell hybrids need not correlate with functional activity (DeFreitas et al, 1982).

Further experiments will need to be performed to determine if the different MW suppressive activities do belong to one or a number of species of molecules.

Preliminary physicochemical characterisation of TT-4 supernatants indicated the suppressive effect on T cell proliferation was acid, alkali and DTT sensitive; α methyl mannoside, L-fucose and N-acetyl glucosamine affected its activity; but interestingly L-rhamnose had only minimal effects on the suppression of T cell proliferation. Particular care was taken in treating the control supernatants in an identical manner to the test supernatants. This was to exclude the possibility that any changes in biological activity of the hybrid supernatants following the treatment were due to effects on the FCS supplement, leading to enhancement or increased suppression of T cell proliferation. Experiments involving protease treatment of control and test supernatants produced inconsistent results in the T cell proliferation assay. As the validity of the test data could not be assured, due to a wide variability in the control responses, these results have not been included.

Several workers have identified human regulatory factors which have similar properties and actions to the TT-4 activity described here. Greene et al (1981), partially characterised soluble immune suppressor supernatants (SISS) derived from ConA-activated normal peripheral blood mononuclear cells. These supernatants have been shown to elaborate 2 suppressor activities; one SISS-T (30-45 Kdal) inhibits both mitogen and antigen-induced T cell proliferation and is inactivated

by N-acetyl glucosamine. The second, SISS-B (60-90 Kdal) inhibits B cell Ig production and is inactivated by L-rhamnose.

The TT-4 activity may be related to these activities as the stimulation regime (10µg/ml ConA, 2-5 days) used to activate the normal PBLs could be expected to induce the production and secretion of these factors. Greene et al (1982) have also described a human suppressor T cell hybrid-derived factor (70-85 Kdal) that produced reversible non-cytotoxic inhibition of lectin-activated Ig production. Grillo-Courvalin et al (1982) partially characterised a 45 Kdal hybridoma-derived factor which suppressed PWM-induced Ig production but not Nocardia-induced (T-independent) Ig production. The ability of TT-4 supernatants to inhibit both T cell proliferative and B cell antibody responses, differing apparent MWs, inactivation by NAG but not by L-rhamnose suggests the activity is related to, but still distinct from the previously described factors.

Importantly it must be determined if the biological effects of TT-4 supernatants on both T and B cell responses results from a single molecule or a number of molecules. The preliminary biochemical studies suggest its biological effects cannot be completely explained by the presence of IFN-γ in the supernatants (labile at both pH 2 and 9). Further definitive testing will be necessary to confirm these observations. Ethidium bromide - acridine orange staining of TT-4 cells confirmed that they were not mycoplasma contaminated suggesting the suppressive activity is not mycoplasma-derived.

4.7 BIOLOGICAL SIGNIFICANCE OF T CELL HYBRID-DERIVED SUPPRESSOR FACTOR

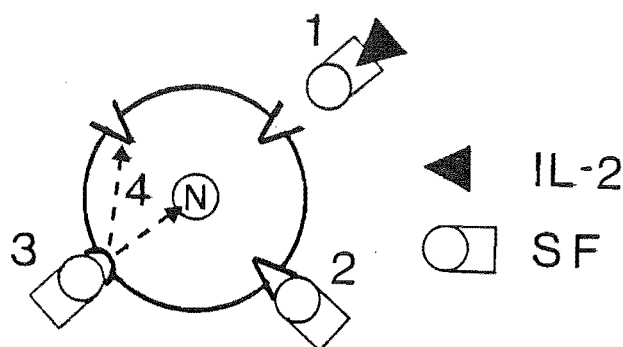
What could be the mechanism and site of action of the suppressor factor (s)? The hybridoma TsF of Greene et al (1982) is thought to act directly on B cells or monocytes by a non-cytotoxic mechanism rather than indirectly modulating the activity of other immunoregulatory T cells. Grillo-Courvalin et al (1982) believe their factor is acting directly on T cells; again by a non-cytotoxic mechanism.

Studies undertaken in this laboratory by other workers suggest the TT-4 activity may act by inhibiting T cell proliferation of both T helper/inducer and T suppressor/cytotoxic subpopulations, again not via any apparent cytotoxic mechanism (Bissell, Macdonald & Crosier, 1983). As the activity also affects the T cell-dependent B cell response to PWM, it is tempting to speculate that

it may act by interfering with the delivery of or response to the T cell proliferation signal of IL-2. Malkovsky et al (1982,1983) have described a murine non-specific suppressor factor (ns INH), which suppressed IL-2 production by stimulated lymphocytes. This effect resulted from the non-specific inhibition of DNA synthesis. The TT-4 factor may act in an analagous non-specific manner or via an IL-2-specific mechanism. The IL-2-specific mechanisms could include; binding IL-2 in solution or blocking IL-2 receptor sites, alternatively suppression may be mediated within the target cell by blocking the IL-2 signal specifically or by non-specific effects on DNA synthesis. It is intended to pursue these possible mechanisms by using partially purified IL-2, hybrid suppressor factor(s) and cloned IL-2-dependent T cell lines as targets. The possible sites of action of the hybrid-derived suppressor activity are summarised in Figure 4.2.

FIGURE 4.2

POSSIBLE SITES OF ACTION OF THE TT-4-DERIVED SUPPRESSOR
FACTOR



Several possibilities exist as to how the TT-4-derived SF could be inhibiting the IL-2-dependent proliferative response of lectin-activated T cells:

1. The SF could bind IL-2 in solution,
2. Could block IL-2 receptor sites,
3. The SF could bind to specific receptors and block the IL-2 signal specifically within the target cell or,
4. Could non-specifically affect DNA synthesis (N).

4.8 FUTURE STUDIES

Somatic cell hybridisation techniques have been used to construct T cell hybrids between an HGPRT deficient human T-ALL line and ConA-activated normal human PBLs. One hybrid line, TT-4 was demonstrated to produce constitutively an activity which suppressed both B cell antibody and T cell proliferative responses.

This study has shown that somatic cell hybrids are viable sources for the production of human T cell-derived lymphokines. The major shortcoming of the technique is the problem of chromosomal instability, presumably leading to the loss of phenotypic markers and biological activity. The CCRF-CEM line, while permitting the construction of human T cell hybrids is limited by its failure to permanently retain parental chromosomes. Recent studies of the TT-4 line have indicated that a loss of biological activity has occurred (>6 months in culture). This may be as a direct result of the chromosomal instability of the line. Repeated early cloning or sterile cell sorting using the FACS may help to overcome this instability problem and aid in the establishment of hybrids which retain their phenotypic, chromosomal and biological characteristics for longer periods.

A number of biochemical studies need to be performed on the TT-4-derived suppressor activity. Are the different MW species identified by gel chromatography oligomers of one molecule or a number of molecules? Is one activity mediating both the T cell and B cell suppressive effects? What is the precise mode of action of the suppressive activity?

In conclusion, future biochemical studies of this and other hybrid-derived activities will enable a better understanding of the fundamental role lymphokines play in the regulation of human immune responses in both normal and disease states.

APPENDICES

1. Amounts of McAbs used to stain human lymphocytes

<u>Antibody</u>	<u>Supplied</u>	<u>Amount/10⁶ cells</u>
Leu-1,2a,3 5, HLA-DR	0.2mg/ml	1 μ g
Leu-3a + 3b	0.3mg/ml	1.5 μ g
Leu-4	0.2mg/ml	2 μ g
β_2 -micro- globulin	0.6mg/ml	3 μ g
OKT3, T4, T6, T8, M1	1mg/ml	5 μ g
W6/32	culture supernatant	1:10
HTA1	ascites	1:100
PNA	5mg/ml	0.5mg

2. Liquid scintillation counting

2,5-diphenyloxazole (PPO) 6g/l

(1,4-bis[² (4-methyl-5-phenyl-oxazolyl)] benzene) (POPOP) 0.01g/l
in Toluene.

5mls scintillation fluid added to dried filter discs in counting vials.

Vials counted in Packard Tri-carb liquid scintillation spectrometer

Settings (³H) window : 50-1000

gain : 50%

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