THE PRODUCTION OF MONOCLONAL ANTIBODIES TO HUMAN THYROID STIMULATING HORMONE

by

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SYNOPSIS

The application of monoclonal antibody technology is a rapidly expanding area of research, encompassing most areas of the biological sciences. The potential of the technique to provide standard but uniquely specific analytical reagents, which can be used singly or in combination is overwhelming by its ever increasing application.

In this work the main aims were to produce specific high affinity monoclonal antibodies to the human anterior pituitary hormone, Thyroid Stimulating Hormone (TSH); to characterise the monoclonal antibodies and demonstrate their application in relation to human TSH.

Two indirect enzyme-linked immunosorbent assays (ELISA), and a solid phase separation radioimmunoassay (RIA) system were developed for screening of the hybridoma cultures.

The immunogenicity of TSH in the spleen cell donor mice was monitored by standard TSH double antibody RIA.

Eight fusion experiments were done before stable positive cultures were established from the fusion experiments. Eleven positive primary cultures and 27 positive monoclonal cultures were frozen down and stored at -196°C in liquid nitrogen. Some difficulty was encountered in recovery of some of these cultures from the frozen state.

Six monoclonal antibody cultures have been characterized with regard to titre in culture supernatant and ascitic fluid, antibody content, immunoglobulin class, sub-class and light chain content. The antibody chain composition of the preparations was also investigated. Cross reactivity in relation to the other closely related anterior pituitary hormones: luteinizing hormone (LH), follicle stimulating hormone (FSH), and the placental hormone human chorionic gonadotrophin (HCG) whole molecule and the α and β subunits were investigated. The antigenic determinant specificity of these antibodies was also investigated by competitive binding between radiolabelled monoclonals and unlabelled monoclonals.

The monoclonals all had high titres and high affinity.

Only one of the monoclonal antibodies investigated displayed any recognisable cross reactivity to HCG and FSH but this was still minimal.

All six monoclonal antibodies were used in an indirect immunocytochemical immunoperoxidase procedure and demonstrated to be highly suitable for the localization of TSH in tissue. Four preparations of human anterior pituitary
were examined, in addition to human placenta, kidney, and gut. Possible cross reacting animal TSH's were also investigated using baboon, dog, rat and ferret pituitary sections.

The use of the monoclonals together with the immunoperoxidase technique offers quite distinct advantages of improved specificity, a permanent source of specific pure antibody and improved sensitivity (low observed background), and permanent stained records.

Finally one of the monoclonals was investigated for use in a two-site microtitre enzyme-linked immunosorbent assay (ELISA) with an anti-h-TSH polyclonal antisera. An enzyme-linked immunosorbent assay was developed with a detection limit of 4 mU/l based on the 95% confidence limit from zero. The assay showed good intra- and inter-assay precision over the whole range of the standard curve, and as such may be suitable for clinical application.
ACKNOWLEDGEMENTS

I wish to thank Dr. R. Hubbard and Professor V. Marks for their kind guidance, maintained interest, and encouragement throughout the course of this project. My thanks are also due to Dr. D. Teale, Dr. S. Hampton, Dr. R. Edwards, and Mr. C. Powell for their help and advice at various stages of the work, and to my colleagues in the Biochemistry Department who made working in the department pleasant and rewarding.

I also wish to thank the Science and Engineering research council, and RIA Ltd, Cardiff for financial support for this work.
DEDICATIONS

To my husband Dennis and sons Steven and Simon for their love, patience, and generous support.
ABBREVIATIONS

Ab   antibody
AF   Ascitic fluid
Ag   antigen
a/s  antisera
BSA  bovine serum albumin
CAM  cellulose acetate membrane
cm   centimetre
CPM  count per minute
DMSO Dimethyl sulfoxide
ELISA Enzyme linked immunosorbent assay
FSH  Follicle Stimulating Hormone
g    gram
H₂SO₄ Sulphuric acid
HAT  Hypoxanthine aminopterin thymidine
HCG  Human chorionic gonadotrophin
HCl  hydrochloric acid
HRPO Horse radish peroxidase
h-TSH Human thyroid stimulating hormone
Ig   immunoglobulin
LD   limiting dilution
LH   Luteinizing hormone
M    molar
mA   milliamphere
McAb monoclonal antibody
mg   milligram
ml   millilitre
NETRIA North East Thames Regional Immunoassay Unit
NIBSC National Institute Biological Standards and Control
nm   nanometres
NMS  normal mouse serum
No   number
NSB  nonspecific binding
NSI  P3 NSI/1-Ag4.1
NSS  normal sheep serum
OD  optical density
PAS  periodic acid schiff
PBS  phosphate buffered saline
PEG  polyethylene glycol
RIA  Radioimmunoassay
rpm  revolutions per minute
SD  standard deviation
SDS-PAGE  Sodium dodecyl sulphate - Polyacrylamide gel electrophoresis
SEM  standard error on mean
SN  supernatant
TSH(Barts)  TSH supplied by Dr. R. Edwards
V  volts
x  mean or average

α  alpha
β  beta
°C  degree centigrade
>  greater than
<  less than
μCi  micro curie
μg  microgram
μl  microlitre
-ve  negative
+ve  positive
%  percentage
*Ag  radiolabelled antigen

1°  primary
2°  secondary
3°  tertiary
4°  quarternary
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CHAPTER 1

Introduction
1.1 INTRODUCTION

Since the first pioneering demonstrations by Kohler and Milstein (1975 and 1976) that the specific antibody secreting function of a single cell could be immortalized, the technology has developed rapidly and has been exploited in many areas of biological research. In fact, where ever the precise identification, characterization, purification and/or quantitation of material is required, providing the material is, or can be made immunogenic, then the technique is applicable.

The production of one or more specific single specie of antibody (a monoclonal antibody) to each antigenic determinant on the immunogen is possible. They may be used singly or in subtle but defined combinations which may comprise a unique 'fingerprint' of the antigen according to the application.

Immunology, the study of the immune system and its responses encompasses and utilizes an extensive repertoire of methods and techniques (Hudson and Hay 1980, Weir 1979).

Antibodies, which are the tools of immunological methods, are exquisitely sensitive and highly specific reagents obtained conventionally from the sera of specifically immunized animals. Conventional antisera represent however, the irreproducible combination of antibody products of many antibody secreting cells or clones, containing background as well as specific antibody (Benner 1982). In practice even under strictly controlled identical conditions each animal produces a unique mixture of immunoglobulins with varying specificities and affinities. Even very simple antigens introduced in the same amounts into genetically identical animals do not elicit identical immunoglobulins in response. Serum immunoglobulin levels vary in addition with genetic background, antigenic load and age (Wostmann 1975, Van Snick and Masson 1980, Benner and Naaijman 1980 and Radl 1981).

This general lack of reproducibility of the response constitutes one of the most serious limitations of heterogeneous conventional antisera.

Monoclonal antibody production (Kohler and Milstein 1975, 1976) overcomes many of the major difficulties imposed by that limitation and even using impure antigen preparations (Secher and Burke 1980) or heterogeneous undefined antigens (Foster 1982) such as the combined antigens in whole cell preparations (Parham and Bodmer 1978, Hoffman et al 1980, Roehrig 1982, Tedder et al 1983, Schlesinger et al 1983) an unlimited supply of highly specific monoclonal antibody may be derived. Specific antisera for such antigens would be impossible under conventional antisera regimes. The special and unique properties of the immune system: namely antibody specificity, yet diversity engendered in the ability to initiate a response to an
enormous number of and including novel immunogenic stimuli, the cellular property of one plasma cell producing only one antibody, and the technology to rescue these functions in a permanent cell line have in combination produced the versatile monoclonal antibody procedure. It permits in theory the production, isolation, and maintenance of specific antibodies to any antigen or single antigenic determinant. This in turn has the potential to greatly facilitate and expand fields of study where sensitive isolation and analytical antibody procedures are desirable and applicable and in the past been impracticable.

1.2 THE IMMUNE SYSTEM

The immune system constitutes the major defense mechanism which protects against potentially deleterious invading foreign material. Material capable of evoking an immune response include stimuli from a wide range of sources such as disease promoting pathogenic microorganisms and their byproducts, cellular intrusions including foreign transplanted tissue, molecular material and chemicals from the environment.

Vertebrates have evolved in addition to the non-specific immune mechanisms possessed by lower animals (Hilderman and Reddy 1973, Marchalonis 1976) an adaptive response. The three major characteristics of memory, specificity and the recognition of non self material affords greater flexibility and a more effective response to antigenic challenge (Roitt 1980).

1.2.1 Functional Cells of the Immune System

The immune response is dependent upon populations of cells with properties of antigen recognition and processing abilities, inter-cellular communication, antigen stimulated differentiation and expression of effector function. The cells of the immune system begin as unspecialised ancestral stem cells with the intrinsic capacity for self renewal, extensive replication and differentiation (Metcalf and Moore 1971). Lymphopoiesis in the primary lymphoid organs - the Thymus and Bursar of Fabricius in birds or Bursar Equivalent probably the foetal liver and bone marrow in mammals (Miller 1966, Miller and Osolia 1967, Greaves et al 1973, Weissman et al 1974) is antigen independent. In these organs maturation yields immuno-competent small lymphocytes equipped with antigen specific receptors and specific organ homing properties.
These small lymphocytes circulate via the lymph and blood network systems but are mainly confined to discrete domains in the secondary lymphoid organs: the B cells in the primary follicles, and the T cells in the diffuse cortex (Howard 1972, Sprent 1973).

These secondary organs provide the necessary environment and accessory cells for processing and presentation of antigen, leading to the antigen dependent mature immune response. That the small lymphocytes are the functional units of, and essential for an appropriate immune response have been amply demonstrated (Gowans et al 1962, McGregor and Gowans 1963, Hunt et al 1972, Howard and Gowans 1972). Chronic depletion of lymphocytes by whatever means abolishes immunological responses while replacement therapy restores the response.

On a functional basis two antigen specific small lymphocyte populations have been defined (Miller 1962, Claman et al 1966, Miller and Mitchell 1968, Cooper et al 1966, Kiesielow et al 1975, Beverly 1977). Thymectomy grossly depleted cell-mediated reactions mediated by Thymus dependent T cells (Roitt 1980, Cooper et al 1966, Claman et al 1966), while bursectomy in chickens abolished humoral responses mediated by the B cells (Cooper et al 1966). Observations of human clinical conditions are in agreement with the functional classification of small lymphocytes. Thymic deficiencies such as Di George's and Nezelof syndromes, are immuno-deficiency syndromes characterised by the absence of T cells and cell-mediated immune function, but with near normal B lymphocyte and antibody functions. B cell insufficiency states such as Bruton's congenital agammaglobulinaemia occur on the other hand in which T cell mediated immune responses are normal but humoral immunoglobulins are greatly depleted (Herbert and Wilkinson 1977, Bergsma et al 1975).

Under the light microscope (Miller 1974), there is no apparent morphological difference between the T and B small lymphocytes. Apart from the functional distinction however, they may be identified by a variety of characteristic surface markers (Raff 1971, Loor and Roelants 1975, Kiesielow et al 1975) and reaction specificities, for example, sheep red blood cell rosette formation with human T cells (Jondal et al 1972). Table 1.1 below summarises some of the identifying markers of small lymphocyte populations.

It is the differentiated functional responses of antibody production by B lymphocytes, or the effector functions of the T lymphocyte subpopulations that are the targets for rescue in a continuously growing cell line employing hybridoma technology.
TABLE 1.1
T and B lymphocyte marker characteristics (adapted from Miller 1974)

<table>
<thead>
<tr>
<th>Identifying property</th>
<th>T cells</th>
<th>B cells</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Antigen ++ (type?)</td>
<td>++ (Ig)</td>
<td></td>
<td>Raff 1971, Marchalonis and Cone 1973, Vitella et al 1975</td>
</tr>
<tr>
<td>Receptors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fc</td>
<td>-</td>
<td>++</td>
<td>Basten et al 1972</td>
</tr>
<tr>
<td>C3</td>
<td>-</td>
<td>++</td>
<td>Dukor and Hartmann 1973</td>
</tr>
<tr>
<td>E rosettes ++</td>
<td>-</td>
<td></td>
<td>Jondal et al 1972</td>
</tr>
<tr>
<td>Differentiation cell surface antigens</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thy 1 ++</td>
<td>-</td>
<td>Schlesinger 1972</td>
<td></td>
</tr>
<tr>
<td>Ly 1 2 3 subsets ++</td>
<td>-</td>
<td>Raff 1971</td>
<td></td>
</tr>
<tr>
<td>divisions</td>
<td></td>
<td>Kiesielow et al 1975</td>
<td></td>
</tr>
<tr>
<td>Antigen stimulated release</td>
<td>Transfer factor IgG</td>
<td>AME</td>
<td>Lawrence and Landy 1969</td>
</tr>
<tr>
<td>lymphokines</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(++) effector present; (-) not present.

1.2.2 The Immune Response

A great deal of work on antibody response has involved experimentation at the single cell level. The subject has been extensively reviewed by Cunningham (1973). Of the $1-5 \times 10^7$ antibody repertoire estimated to occur in an inbred mouse (Klinman and Press 1975, Kreth and Williamson 1973, Kohler 1976) only between 1000 and 8000 seem capable of recognising any particular antigenic determinant (Kreth and Williamson 1973, Kohler 1976). Of these between 5 and 10 are observed in conventional antisera in response to each antigenic determinant (Schreier et al 1980). This indicates the probable reason for the highly heterogeneous nature of any conventional antisera and the difficulty of producing a reproducible reagent (Shulman and Kohler 1979).

According to the clonal selection theory proposed by Burnet (1959), a heterogeneous cell population exists, but each cell possesses the capacity to produce only one antibody specificity. On antigenic challenge the majority of cells remain unstimulated. They do not possess receptors which fit the
antigen. A small number however do, and these are stimulated. This theory was the first to emphasise the unit of selection was the whole cell. The receptor specificity was later confirmed by Ada (1970) and Raff (1971) demonstrated that these receptors were immunoglobulins on the B lymphocyte cell surface.

The stimulated cells were triggered to proliferate and differentiate and to produce clones of cells derived from single ancestral cells. Mitchison (1967) suggested the cell surface receptors were exact copies of the secreted antibody for B cells.

The prediction of one cell producing only one antibody specificity has been overwhelmingly demonstrated and reviewed by Cunningham (1973). The identification of multiple myeloma as a neoplasm of an antibody producing cell, the product of which occur as paraprotein in patients serum; and that such paraproteins are indistinguishable from normally secreted immunoglobulin (Weissman 1978, Potter 1978, Cebra et al 1974, Capra et al 1975, Haber et al 1977), together with the experimental induction of analogous mouse myeloma tumors producing homogeneous immunoglobulin proteins (Potter 1972) have also confirmed the essential one cell one antibody specificity prediction which forms the basis of monoclonal antibody selection.

Antigenic stimulation of B and T cells in the appropriate environment results in the following summarised cycle of events characteristic of the humoral and cell-mediated immune responses respectively.

a) **Humoral response to soluble antigen stimulus**

1. Antigen driven clonal expansion cell cooperation between B and T cells
2. Primed memory cell population for fast 2° reaction
3. Ag Accumulates in 2° lymphoid tissue
4. Neutralization, precipitation, lysis, end product etc
5. Production and release of antibodies
6. Effector plasma cells
7. 2° reaction N Effector plasma cells
b) Cell mediated immune response to cell surface or particulate antigenic determinants

Clonal expansion

in 2° lymph organs

 Primed memory cell population for fast 2° reaction

Patrolling sensitized T cells

Execution of effector function
cytolysis, suppression etc

Effector cells of appropriate type T_H T_C T_S T_D etc

2° = secondary
T_H = Helper T cell
T_C = cytotoxic T cell
T_S = Suppressor T cell
T_D = delayed hypersensitivity T cell

FIGURE 1.1

Response of the immune system:
a) 'B' lymphocyte to soluble antigens (Cunningham 1978) and
b) 'T' lymphocyte response to cellular antigens; (Munro and Bright 1976)

Further discussion will be directed towards the B lymphocyte antibody responses because it is monoclonal antibody production via hybridoma technology (Kohler and Milstein 1975 and 1976) with which we are mainly concerned, although T cell hybridomas have also been produced (Hammerling 1977 and reviewed by Beezley and Ruddle 1982).

1.2.3 Antibody Specificity and Cross Reactivity

The unique specificity of antibody antigen reactions was conclusively demonstrated by Landsteiner and Van der Scheir (1936). They demonstrated that even small changes in the antigen were detectable (figure 1.2), by the antibodies; but similar determinants were bound by the antibodies to varying degrees resulting in the cross reactivity phenomenon.
Specificity and cross reactivity of antibodies to m-aminobenzene sulphonate in reaction with various similar haptens. The effect of substitution of the different side groups scored according to their inhibitory effects on precipitation of original hapten.

+++ = strong, - = no effect. Adapted from Cummings 1978 and Roitt 1980.

It is therefore true to say that if an antibody preparation is to be a useful immunological reagent then it must be specific, binding only the antigen it was meant to recognise.

The difficulties encountered in obtaining suitably specific antisera for the clinical assay of human thyroid stimulating hormone (h-TSH) for example, serves to illustrate the importance of antibody specificity (Odell et al 1968 Odell and Utiger 1973).

Monoclonal antibodies produced by hybridoma technology should however, eliminate such problems since the antibodies are selected for their high specificity for only one antigenic determinant. Where cross reactivity is observed this will undoubtedly be due to the antigenic determinant being shared by the cross reacting molecules.

This property of strict specificity of effector function carries important implications in vivo for the animal as a whole in addition to the precise elimination of foreign material. Wider binding specificities would possibly result in disastrous cross reactions against self components in vivo. It is this particularly stringent specificity specification which is employed in vitro in immunological procedures.
Antibodies consist of four polypeptide chains (Porter 1967 and Edelman 1969, 1970); two identical heavy (H) chains and two identical light (L) chains. A covalent disulphide bridge between the H and L chains, and between the two H-L chain units constitutes the main linkage forming the whole antibody molecule.

The antibody molecule is illustrated in figure 1.3.

Suitable material for structural analysis was found to be homogeneous myeloma serum paraprotein (Seligmann and Brovet 1973). Some have been found which bind antigen and have been shown to be serologically identical to normally synthesized antibodies binding the same antigen (Potter 1978). Such myeloma protein and other homogeneous antibodies, for example, those induced against antigens such as bacterial polysaccharides (Krause 1970, Haber 1970) have been used in amino acid sequence studies (Cebra et al 1974, Capra et al 1975a, 1975b, Haber et al 1977) with concordant results.

Each heavy and light chain has two distinct regions; a variable (V) region at the amino N-terminus which contains hypervariable 'hot spots' and comprises the antibody binding domains; and a constant (C) region at the carboxyl C-terminus. This C-region of the H-chains contains the structures which confer the antibody biological activity (see Table 1.3). The structure and function of antibodies have been extensively discussed by Nisonhoff et al (1973) and Steward (1981).

Using sera prepared against the L-chain dimer Bence Jones protein and myeloma proteins (Epp et al 1974, Roitt 1980) two types of L-chains have been established and designated kappa (k) and lambda (λ) L-chains; and five classes of heavy chains have been identified. These are immunoglobulin G, A, M, D and E together with subclass variations. They all exhibit varying physical properties and biological effects which are shown in Tables 1.2, 1.3 and 1.4. Some of these biological effects and properties of the immunoglobulins and their subclasses have serious implications for the detection, selection and subsequent application of monoclonal antibodies.
**FIGURE 1.3** IgG antibody structure.
The clear areas of the H and L chain are the variable regions while the hatched area are the constant regions. The oligosaccharide groups are designated [CHO]. Papain cleaves the structure at the hinge region site (→) to yield 2 Fab + FC fragments. Pepsin cleaves below the S-S bond to yield F(ab')2. (Adapted from Lehninger 1976)
<table>
<thead>
<tr>
<th>W.H.O. designated classes</th>
<th>IgG</th>
<th>IgA</th>
<th>IgM</th>
<th>IgD</th>
<th>IgE</th>
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<tr>
<td>No of subclasses</td>
<td>4</td>
<td>2</td>
<td></td>
<td></td>
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<tr>
<td>No of basic units</td>
<td>1</td>
<td>1 or 2 (dimer)*</td>
<td>5 (Pentomer)*</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Heavy chain class</td>
<td>(\gamma, 1,2,3,4)</td>
<td>(\alpha, 2)</td>
<td>(\mu)</td>
<td>(\delta)</td>
<td>(\varepsilon)</td>
</tr>
<tr>
<td>Light chain type</td>
<td>k or (\lambda)</td>
<td>k or (\lambda)</td>
<td>k or (\lambda)</td>
<td>k or (\lambda)</td>
<td>k or (\lambda)</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>150,000</td>
<td>160,000 dimer</td>
<td>900,000</td>
<td>185,000</td>
<td>200,000</td>
</tr>
<tr>
<td>Valency for antigen binding</td>
<td>2</td>
<td>2 or 4</td>
<td>10</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Concentration range in normal serum (mg/ml)</td>
<td>8-16</td>
<td>1.4-4</td>
<td>0.5-2</td>
<td>0-0.4</td>
<td>17-450 ng/ml</td>
</tr>
<tr>
<td>% Total serum Ig</td>
<td>80</td>
<td>13</td>
<td>6</td>
<td>0-1</td>
<td>0.002</td>
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<tr>
<td>% Carbohydrate content</td>
<td>3</td>
<td>8</td>
<td>12</td>
<td>13</td>
<td>12</td>
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<td>Half-life in serum days</td>
<td>23^a</td>
<td>5.8</td>
<td>5.1</td>
<td>2.8</td>
<td>2.5^b</td>
</tr>
<tr>
<td>Occurrence</td>
<td>Serum</td>
<td>Serum</td>
<td>Serum</td>
<td>Serum</td>
<td>Serum</td>
</tr>
<tr>
<td></td>
<td>amniotic fluid</td>
<td>secretion</td>
<td>saliva and tears</td>
<td>colostrum</td>
<td></td>
</tr>
</tbody>
</table>

* IgA dimer and IgM pentomer each contain a J chain  
  a Excluding IgG\(_3\) (10 days)  
  b IgE binds basophils and mast cells and has a longer half life in tissues
<table>
<thead>
<tr>
<th>Effect</th>
<th>IgG</th>
<th>IgA</th>
<th>IgM</th>
<th>IgD</th>
<th>IgE</th>
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<tbody>
<tr>
<td><strong>Physiological role</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Most abundant Ig. Defence</td>
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<td></td>
<td></td>
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<tr>
<td>vs bacteria virus and</td>
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<tr>
<td>bacterial toxins.</td>
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<tr>
<td>Defends external body</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>surfaces.</td>
<td></td>
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<tr>
<td>Major Ig in sero-mucous</td>
<td></td>
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<tr>
<td>secretions.</td>
<td></td>
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</tr>
<tr>
<td>Produced in early primary</td>
<td></td>
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<tr>
<td>response.</td>
<td></td>
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<tr>
<td>Effective agglutinator.</td>
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</tr>
<tr>
<td>Present primarily on</td>
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<tr>
<td>lymphocyte surfaces.</td>
<td></td>
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<tr>
<td>Responsible for</td>
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<tr>
<td>immediate type allergy.</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Protection of external</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>body surfaces.</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Raised in parasitic and</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>recruits antimicrobial</td>
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<tr>
<td>agents.</td>
<td></td>
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</tr>
<tr>
<td><strong>Complement fixation</strong></td>
<td>++</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>classical</td>
<td></td>
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<tr>
<td>Alternative</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>Cross placenta</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Fix to homologous mast</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>cells and basophils</td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Binding to macrophages</td>
<td>+</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>and polymorphs</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
## TABLE 1.4
Comparison of the properties of human IgG subclasses

<table>
<thead>
<tr>
<th></th>
<th>IgG&lt;sub&gt;1&lt;/sub&gt;</th>
<th>IgG&lt;sub&gt;2&lt;/sub&gt;</th>
<th>IgG&lt;sub&gt;3&lt;/sub&gt;</th>
<th>IgG&lt;sub&gt;4&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG in serum, % of total</td>
<td>65</td>
<td>23</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Spontaneous aggregation</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Ga site reacting with rheumatoid factor</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>Combine with staphylococcal protein A</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>Crosses placenta</td>
<td>++</td>
<td>±</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Complement fixation classical</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>±</td>
</tr>
<tr>
<td>Binding monocytes</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>±</td>
</tr>
<tr>
<td>Binding heterologous skin</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Blocking Ig to binding</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

+ = gradation in effect
- = effect not present

1.2.5 Generation of Antibody Diversity

To deal with the vast array of environmental antigens, an extensive repertoire of specific antibody molecules are required. The precise mechanism by which this is achieved is somewhat still in question. Three main theories have been proposed. The germ-line theory (Hood et al 1975) advocates that all antibody genes are inherited in the usual way and are thus present in all cells of the animal. During lymphocyte differentiation, as it occurs in other cells only one complete complementary gene set is selected for expression appropriate to the differentiation type. All others are suppressed.

The somatic mutation theory on the other hand (Weigert 1970) suggests that only a very few V region genes exist at each locus in the germ line. Mutations are thought to occur in the lymphocyte V genes and prepare the cells to react adequately to challenge. Mutation is however a random event and the theory demands that during differentiation some selective mutational event occurs rapidly to make all the possible antibody types (Cotton et al 1973).

Finally rearrangement of pre-existing gene segments (Weigert et al 1978) during differentiation may be responsible for antibody diversity.

Saturation hybridization analysis (Valbuena et al 1978) and amino acid sequencing (Weigert et al 1978 and Schilling et al 1980) have supported the theory that the variable (V) region genes and constant (C) region genes of an immunoglobulin molecule are all present within the gene complement of cells. But it is the somatic recombination of these (Sakano et al 1979, Max et al 1979, Seidman and Leder 1979) during the differentiation process, together with the hypervariable regions within the V regions, which form the basis of antibody diversity we observe.

1.3 MONOCLONAL ANTIBODY

A monoclonal antibody may be defined in this context as the antigen-specific homogeneous immunoglobulin product of a B lymphocyte clone derived from one immortalized ancestral plasma cell. Monoclonal antibodies are therefore the selected product secreted from a hybrid cell line produced by fusion of a myeloma cell line with an antibody producing plasma cell (Kohler and Milstein 1975 and 1976).

Naturally occurring or experimentally induced myeloma tumors provide unlimited sources of homogeneous monoclonal antibody (Seligmann 1973, Potter 1972). Such antibodies although they have been shown to be synthesised and
assembled by processes very similar to those occurring in normal antibody production (Scharff and Laskor 1970, Kuehl 1977, Williamson 1971) and research with them has greatly advanced understanding of immunoglobulin production (Raschke 1980), their value is limited by the fact that very few react with known antigens (Potter et al 1977 and Potter 1978), and the known antigen binding myeloma proteins have only been identified by intensive effort (Cohn 1967).

Early approaches to secure a permanent supply of homogeneous antigen binding antibodies relied on the transformation of antigen sensitized cells in the presence of the antigen using a virus. Baumal et al (1971) employed Epstein-Barr (EB) virus and antigen primed peripheral blood cells. Successful specific antibody secretors were not obtained. Strosberg et al (1974) used Simian Virus 40 (SV40) with sensitized rabbit cells and obtained a cell line producing small amounts of homogeneous antibody against the antigen pneumococcal type III polysaccharide; while Steinitz et al (1977, 1979) employed Epstein-Barr virus with antigen (4-hydroxy-3, 5-dinitrophenacetic acid (NNP)) sensitized human B lymphocytes. Antigen binding antibodies were obtained which gradually lost activity with time in culture. Zurawski et al (1978, 1981) also employed Epstein-Barr virus to produce permanent specific anti-tetanus antibody secreting cell lines from antigen sensitized cells with similar results. These methods of production while having limited success were by no means satisfactory since to date the antibody secreting function was ultimately lost. The permanent production of active cell lines has to date therefore only been accomplished by the fusion techniques. Yelton et al (1981) have produced a comprehensive discussion on the development and application of hybridoma procedures.

1.3.1 Cell Fusion

Since the first observations in vitro (Barski et al 1960) of spontaneous fusion of somatic vertebrate cells, cell fusion technology has been widely applied in many areas but especially in those concerned with the investigation of gene expression and control, and the cellular and molecular mechanisms involved (Harris 1970, Ephrussi 1972, Puck 1972, Davidson and de la Cruz 1974 and Bernhard 1976).

Those early observations were unexpected because of the very nature of metazoan organisation. Somatic cells and their descendants are required, in spite of being in close permanent contact in tissues and organs, to maintain their individual status and their genetic integrity (Bernhard 1976).
Only a few exceptional cases of spontaneous fusion of eukaryotic cells are known to occur in vivo, usually representing a stage in terminal differentiation. The most notable examples include the fusion of myoblasts to form myotubules during the histogenesis of striated vertebrate muscle, fertilization, osteoclasts and foreign body giant cells formation (Ringertz and Savage 1976); although the first observations of fusion and heterokaryosis was on fungi and yeast (Fincham and Day 1971).

The earliest fusion experiments (Harris 1965, Harris and Watkins 1965) established that:

1) heterokaryons can be produced with various differentiated cell types. Fusion was not affected by any tissue incompatibility mechanism;
2) the processes of suppression of gene expression was reversible;
3) the cells of the immune system did not mount an immune response against the partner, and
4) no nuclear incompatibility was observed.

Myeloma x myeloma fusion studies including those by Milstein et al (1977) and Kohler and Milstein (1975) established conclusively that:

1) hybrids could be obtained from the fusion between different myeloma cells;
2) compatible cell partners (see phylogenetic and ontogenetic restriction) yield hybrids which exhibited codominant gene expression, producing all the immunoglobulin chains of the parent lines, and no new immunoglobulins were produced. Furthermore Kohler et al (1976) demonstrated that immunoglobulin synthesised by one parent was not extinguished by fusion with a non-producing partner and reactivation of its immunoglobulin synthesis did not occur. The hybrids continued to produce only the immunoglobulin of the active parent. Where both parents produced immunoglobulins however the immunoglobulin products of the hybrids were found to be mixed molecules containing chains from both parent cell lines (Margulies et al 1977, Kohler and Milstein 1976, Galfre and Milstein 1981);
3) Chromosome loss did occur since the total chromosome numbers in the hybrids were less than that of the sum of the parents. This loss sometimes was accompanied by the loss of immunoglobulin chains. All these observations were vitally important to the establishment and refinement of the monoclonal antibody procedure.
Phylogenetic and ontogenetic restrictions to function retention

The production of viable hybrid cells with the desired gene product (whole active immunoglobulin molecules) intact is the primary aim of monoclonal antibody technology. In the hybrid cells therefore a combination of the two parental chromosome endowment must persist so that cell division mechanisms remain functional, while retaining the required differentiated function.

Unlike normal sexual karyogamy however somatic cell fusion results in duplication of two or more diploid chromosome sets in a synokaryon. Depending on the parental combination it has been established that in the hybrids supernumary chromosome instability results in the preferential elimination of some of one of the parent cell chromosomes in an irregular and unpredictable manner (Bernhard 1976). Several examples of this phenomenon have been cited. For example, from human-mouse (Boone and Ruddle 1969, Weiss and Green 1967), human-chinese hamster (Jones et al 1972, Sun et al 1974), human-rat (Croce et al 1973b) hybrids extensive and preferential elimination of human chromosomes were reported. Chromosomal instability resulted in loss of immunoglobulin production (Kohler 1980) from human peripheral blood lymphocyte-mouse myeloma hybrids, and from frog lymphocyte-mouse myeloma hybrids (Hengartner et al 1978). Rabbit spleenocyte-mouse myeloma fusions were also unsuccessful in terms of hybrid production and rabbit immunoglobulin secretion (Shulman and Kohler 1979, Yarmush et al 1980). On the other hand syngeneic fusions (Kohler and Milstein 1976) and allogeneic fusion (Kohler et al 1977a) of mouse myeloma and spleen cells; and rat spleen cells with mouse myeloma cells (Galfre et al 1977, Howard et al 1980) have all yielded high hybrid production with stable immunoglobulin secretion. Human-mouse hybrids producing human immunoglobulins have however been produced (Koprowski et al 1978, Levy et al 1978, Schlom et al 1980). It may be suggested therefore that even with the limited interspecies success of immunoglobulin retention cited above, phylogenetic restrictions do limit the choice of fusion partners. The closer the phylogenetic relationship between the fusion partners the easier it is to obtain hybrids in which the desired function has been rescued.

Not only are there phylogenetic restrictions but, in addition the ontogenetic relationship of the fusion partners have an important influence on the success or failure of functional hybrid production. During cell fusion two different cells fuse and their nuclei come to exist in a common cytoplasmic environment. During subsequent mitosis the two nuclei fuse
giving hybrid progeny cells. Early fusion experiments were designed to answer questions concerning the expression and interaction of parental traits in inter or intra species hybrids.

Many traits, for example, tumorigenicity endowing immortality (Hobart and McConnell 1975, Harris 1970) were found to be dominant. On the other hand functional expression was shown to depend on the relationship of the cells forming the hybrid partnership. For example, fusion of mouse myeloma cells with mouse spleen cells, while supporting immunoglobulin production did not allow T cell marker Thy 1 expression (Kohler et al 1977). Fusion of thymoma cells however with spleen cells (Goldsby et al 1977, Hammerling 1977) while rescuing Thy 1 T cell marker expression did not support immunoglobulin production. The extinction of cell markers such as Thy 1 (Hyman and Kelleher 1975) and immunoglobulin production (Periman 1970, Caffino et al 1971) was also displayed by the fusion of fibroblasts and spleen cells, demonstrating the reversibility of gene expression.

Ontogenetically closely related cell lines (one of which was capable of growing in permanent culture) are therefore shown to be a specific requirement for the continued expression of the differentiated function of interest (Kohler et al 1977, Shulman and Kohler 1979).

Hybrid cell partners

As indicated above the original work by Kohler and Milstein (1976) indicated that in order to secure the rescue of the differentiated function of a normal cell by hybridization with an immortal fusion partner, then both populations of cells should be at the same state of differentiation and preferably from the same specie.

Antibody secreting hybrids are derived from antigen stimulated B lymphocytes and a myeloma cell line (Kohler and Milstein 1975, 1976). The primary source of stimulated B lymphocytes has been the secondary lymphoid organ, the spleen; where mice or rats were the antigen sensitized donors (Kohler and Milstein 1976, Galfre et al 1977), although the popliteal lymph nodes, for example, have been used as the source of B lymphocytes after foot pad immunization (Buttin et al 1978). Peripheral blood lymphocytes have also been used successfully (Levy et al 1978, Koprowski et al 1978).

A considerable number of permanent cell lines exist (Horibato and Harris 1970, Potter 1972, Melchers et al 1978). The tumor cell lines from the three main species used most frequently, in hybridoma production are listed in Table 1.5.
### TABLE 1.5
Permanent cell lines employed for hybridoma production

<table>
<thead>
<tr>
<th>Species</th>
<th>Myeloma cell line name</th>
<th>Secretes/expresses</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>SK0-007</td>
<td>IgE</td>
<td>Olsson and Kaplan, 1980</td>
</tr>
<tr>
<td></td>
<td>GH 1500.6TG-A12</td>
<td>IgG</td>
<td>Croce et al 1980</td>
</tr>
<tr>
<td></td>
<td>LICR.LON/HMy2</td>
<td>IgG</td>
<td>Edwards et al 1982</td>
</tr>
<tr>
<td>Mouse</td>
<td>P3-X63-Ag8</td>
<td>IgG1 (k)</td>
<td>Kohler and Milstein 1975</td>
</tr>
<tr>
<td></td>
<td>P3-NSI/1-Ag4-1</td>
<td>k chain only</td>
<td>Kohler and Milstein 1976</td>
</tr>
<tr>
<td></td>
<td>P3-X63-Ag8.653</td>
<td>-</td>
<td>Kearney et al 1979</td>
</tr>
<tr>
<td></td>
<td>Sp2/0.Ag14</td>
<td>-</td>
<td>Shulman et al 1978</td>
</tr>
<tr>
<td></td>
<td>45.6GT1.7</td>
<td>IgG2b (k)</td>
<td>Margulies et al 1976</td>
</tr>
<tr>
<td></td>
<td>PuBu1-On</td>
<td>IgG2a (k)</td>
<td>Kohler and Milstein 1976</td>
</tr>
<tr>
<td></td>
<td>MOPC-315</td>
<td>IgA ((\lambda^2))</td>
<td>Fazekas de St Groth and Scheidegger 1980</td>
</tr>
<tr>
<td></td>
<td>FO</td>
<td>-</td>
<td>Fazekas de St Groth and Scheidegger 1980</td>
</tr>
<tr>
<td></td>
<td>BW 5147</td>
<td>H-2k^+,Thyl.1.1^+,Fc</td>
<td>Kohler et al 1977b</td>
</tr>
<tr>
<td></td>
<td>EL4</td>
<td>Lyz^+,Thyl.2^+</td>
<td>Pauli and Pauli 1978</td>
</tr>
<tr>
<td>Rat</td>
<td>210.RCY3.Agl</td>
<td>k chain only</td>
<td>Galfre et al 1979</td>
</tr>
</tbody>
</table>

As a result of codominant expression of immunoglobulin product in hybrids the problem of mixed immunoglobulin molecules becomes apparent (Kohler and Milstein 1975, Kohler et al 1978, Galfre and Milstein 1981). Intra-cellular complementation results in immunoglobulin molecules containing specific and non-specific chains forming the antigen binding site. The result is a reduction or loss of antigen binding ability.

The problem has been alleviated however by the derivation and use of myeloma fusion partners which have lost the ability to express immunoglobulin chains (see Table 1.5). P3-NSI/1-Ag4-1 (designated NSI) is the most frequently used mouse myeloma cell line (Kohler and Schulman 1978, Kohler
and Milstein 1975), and was used for this work. It does not completely overcome the problem however since it synthesizes a light chain, but it does not secrete it.

The human myeloma lines hold great promise for the development of human monoclonal antibodies for diagnosis and therapy.

Fusogenic agents

Spontaneous cell fusion as observed by Barski et al 1960 is a very rare event, occurring at a frequency of about $1/10^6-10^7$ cells (Yelton et al 1981). Specific reagents were therefore required to facilitate and increase the fusion frequency. In early fusion experiments (Harris 1965, Cotton and Milstein 1973, Kohler and Milstein 1975, 1976, Margulies et al 1976), ultra violet (U.V.) inactivated Sendai virus was used for this purpose. A comprehensive review of virus-induced cell fusion is to be found by Post and Pasternak (1978). The frequency of viral-induced cell fusion however was found to be only marginally increased above the spontaneous rate (Yelton et al 1981). This procedure was therefore unsatisfactory from this point of view. In addition there was the persistent difficulty of obtaining reproducible standard batch preparations of virus for long term work (Pentecorvo 1975).

Polyethylene glycol (PEG) was first used to agglutinate plant protoplasts (Power et al 1978) and was found to effect extensive cell fusion on dilution with no adverse effect on viability (Kao and Michayluk 1974). PEG was however first applied in fusion of mammalian somatic cells by Pontecorvo (1975), and has since been described as a universal fusogen (Power et al 1978, Lucy 1978) because of its action on a wide range of cells.

As a consequence commercially available PEG is now the preferred fusion agent giving high fusion frequencies with little effect on viability and permitting standardization of the fusion protocol. The efficiency of PEG induced fusion has been shown to be dependent on the molecular weight, concentration and treatment time used (Davidson et al 1976, Fazekas de St Groth and Scheidegger 1980, Goding 1980, Gefter et al 1977). The inclusion in the fusion mixture of dimethyl sulphoxide (DMSO) (Norwood et al 1976), which effects fusion in its own right (Ahkong 1975), has been shown to mainly protect the cells from the effects of too rapid dilution after fusion (Fazekas de St Groth and Scheidegger 1980), with little improvement in fusion efficiency above that obtained by PEG alone.

The mechanisms of PEG induced fusion are not well understood and the possible effects are reviewed by Lucy 1978.
One final fusion promoting system must be mentioned which although in the early stages of development shows great promise for future application. This is the electric field induced fusion (Zimmermann et al 1981). This technique has been employed for the production of a variety of heterokaryons including the fusion of plant protoplast (Zimmerman and Scheurich 1980, Zimmermann et al 1981, Vienken et al 1981); sea urchin eggs (Richter et al 1981); and mammalian cells (Pilwat et al 1981, Zimmermann et al 1981) and specifically for hybridoma production involving the fusion of mouse myeloma cells and spleen cells (Vienken and Zimmermann 1982).

This fusion procedure gives high yield of viable heterokaryons which can be manually selected and cloned straight after fusion, eliminating the need for long term biochemical selection and tedious cloning procedures (Vienken and Zimmermann 1982). Further development of the procedure to allow large scale hybridoma production will no doubt precede its wider application in this field.

### 1.3.2 Selection of Hybrids

The most important requirement immediately after fusion is a selective system to allow the recovery of hybrids from the fusion mixture. Such a fusion mixture will not only contain the desirable myeloma-spleen cell hybrids, but will also contain myeloma-myeloma hybrids and spleen cell-spleen cell hybrids, in addition to unfused myeloma and spleen cells.

The hybrid spleen cells and unfused spleen cells present no problems. These cells are terminally differentiated and therefore die after a very short time in culture. The unfused myeloma cells, and the myeloma-myeloma hybrids however persist indefinitely under optimum growth conditions. It is necessary that these unwanted cell types be eliminated from the fusion products.

This is achieved using the Littlefield (1964) selection technique. Aminopterin (A), a folic acid analogue is employed to block reactions of the enzyme dihydrofolate reductase in the de novo pathway to purine and pyrimidine synthesis (figure 1.4). The alternative salvage pathway permits the rescue of normal cells by the enzymes hypoxanthine-guanine-phosphoribosyl transferase (HGPRT) (EC 2.4.2.8) or Thymidine kinase (TK) (EC 2.7.15.5) utilising supplied hypoxanthine (H) and Thymidine (T) respectively and so nucleotide synthesis continues.

The myeloma cells are selected to be HGPRT deficient and therefore die in HAT selective media containing hypoxanthine $10^{-4}$M, aminopterin $10^{-5}$M and thymidine $4 \times 10^{-5}$M.
FIGURE 1.4  Nucleotides synthetic pathways with sites of action of some inhibitors ===

HX - hypoxanthine;  G - guanine;  A - adenine;  U - uracil;  C - cytidine;  
AG - azaguanine;  TG - thioguanine;  OA orotic acid;  IMP - inosine monophosphate;  
GMP - guanosine monophosphate;  AMP - adenosine monophosphate;  
CDP, UDP, GDP,  
ADP - cytosine diphosphate etc;  dCDP etc - deoxycytosine diphosphate etc;  
dUMP deoxyuridine monophosphate;  dTMP - deoxycytidine monophosphate;  
CTP, UTP, GTP, ATP - cytosine triphosphate etc;  dCTP, dATP, dGTP, dTTP - 
deoxycytidine triphosphate etc;  T - thymidine;  B - bromodeoxyuridine;  
F - fluorodeoxyuridine;  Am - aminopterin;  AL - alanosine;  HU - hydroxyurea.  
(Adapted from Paul 1980).
The spleen cells being normal cells possess the intact enzymes in the salvage pathway. Hybrid cells of spleen and myeloma cells are therefore complemented with respect to the enzyme HGPRT and can synthesize nucleotides and therefore survive under HAT selection.

P3 NSI/1-Ag4-1 (NSI) myeloma cells are 8-azaguanine resistant and therefore are HGPRT deficient cells. This cell line was used for this work and was a kind gift from Dr Cezar Milstein, MRC Laboratories, Cambridge; via The Wellcome Foundation Ltd, Dartford, UK.

1.3.3 Hybridization

A great many detailed protocols are available for the production of monoclonal antibody secreting hybrids; examples of which include Oi and Herzenberg (1980), Flint and Hudson (1980), Hudson and Hay (1980), Schreier (1980), Galfre and Milstein (1981).

Two essential features necessary for the successful hybridization of the cell fusion partners needs to be mentioned. Firstly, it was suggested from direct observation (Kohler and Milstein 1976) that there was preferential fusion between myeloma cells and specific plaque forming cells, because of the high frequency of specific hybridomas obtained (Shulman and Kohler 1979). Rapidly dividing immunoglobulin secreting B lymphocytes which resulted from lipopolysaccharide (LPS) stimulation, have been shown to fuse more readily than unstimulated cells, and blast cells fuse better than small lymphocytes (Andersson and Melchers 1978).

Together these observations indicate that dividing cells are the preferential fusion partners. Therefore the success of the hybridoma procedure will depend upon the availability of rapidly dividing antigen-stimulated antibody producing cells. This of course will depend upon the success of the immunization regime employed with the spleen cell donor.

Secondly the status of the myeloma cells prior to fusion will also affect the success of the procedure (Galfre and Milstein 1981). In effect the same criterion for preferential fusion is expected to persist. The efficiency of fusion was greatest when the myeloma cells were maintained in logarithmic growth phase for an extensive period before fusion.

Consequently the viability of the cells to be used is critical. Greater than 90% viability of the myeloma cells is imperative (Hudson and Hay 1980).

After fusion which may be performed in suspension (Galfre et al 1977, Galfre et al 1979, Hudson and Hay 1980, Galfre and Milstein 1981, Kennett 1981) or on solid filter unit (Buttin et al 1978, Galfre and Milstein 1978) or as an adherent monolayer (McKearn 1981), the cultures are maintained by replacing most of the growth media with fresh media every two to three days.
Cloning hybrids

Cloning at an early stage is essential to prevent the loss of valuable positive clones due to overgrowth by non-producing competing clones that may be in the culture (Galfre and Milstein 1981). Cloning may be carried out in soft agar (Schreier 1980, Hudson and Hay 1980, Kohler and Milstein 1975, Caffino et al 1972, Metcalf 1977, Kennett 1981) which facilitates positive clone selection directly (Sharon 1979, Kohler and Milstein 1975, 1976, Cook and Scharff 1977).

Alternatively the cultures may be cloned by limiting dilution (Hudson and Hay 1980; Goding 1980, McKearn 1981). The cells are diluted so that they may be grown in small numbers per well. In these circumstances the growth in wells follow the Poisson distribution (Lefkovits and Waldmann 1979) such that

\[ f(0) = e^{-\lambda} \]

( where \( f(0) \) is the fraction of wells containing no cells; \( \lambda \) is the average number of clones required per well).

When \( \lambda = 1 \) then \( f(0) = 0.37 \). Therefore where one clone is required per well, then 37% of the wells should show no clones in order to obtain a reasonable probability \( P(1) = 0.6 \) that any wells with growth will contain only single clones. On this premise single clone cultures may be selected visually and positive cultures grown for further investigation. With this procedure cloning should be repeated to ensure monoclonality.

Parks et al (1979) and Dangle and Herzenberg (1982) have described cloning procedures involving the fluorescence activated cell sorter. This type of procedure, which employs fluorescent antigen to mark the specific antibody producing cells allow simultaneous selection and cloning.

The cloning strategy decided upon, however, will depend to a large extent on the number of positive cultures. Where a large number of positive cultures arise these may have to be assessed to find those of most interest prior to cloning.

Feeder layers

Many cells grown in culture are density dependent. That is why, for example, the myeloma cell line used for this work was maintained at a density of between \( 1 \times 10^5 \) cells/ml and \( 8 \times 10^5 \) cells/ml. Below or above those numbers the cells grew poorly and may die.
Consequently feeder layer cells are usually included for plating out fusion mixtures (Galfre and Milstein 1981, Goding 1980). The use of feeder cells is therefore also necessary in cloning hybrids where the cells are required to begin growth at one cell per well. Feeder cells improve the cloning efficiency which is usually low, especially in newly isolated hybrids (Caffino et al 1972, Lernhardt et al 1978). The recovery of cells from freezing is sometimes greatly improved by the inclusion of feeder cells (Edwards 1981).

Various feeder cell types have been used with good results. Some of these include thymocytes (Lernhardt et al 1978, Oi and Herzenberg 1980), peritoneal macrophages (Fazekas De St Groth and Scheidegger 1980, Hengartner et al 1978) or normal unfused spleen cells (Levy et al 1978, Hammerling et al 1978), fibroblasts (Kennett et al 1978) or contact inhibited 3T3 cells (Caffino et al 1972).

The precise function of feeder cells is by no means certain, but they are thought, in addition to providing what may be termed non-specific cell-mass effects (Foster 1982), to act as specific hybrid cell carriers during dilution, and may additionally provide undefined growth promoting factors. The successful use of cell-free conditioned cell supernatants from xenogeneic endothelial cells (Astaldi et al 1980) have suggested that the factors producing the beneficial effects were most probably soluble.

Another function of the feeder cells may be to reduce possible toxic effects of plastic culture vessels (Goding 1980). Macrophages used in this capacity have the added advantage that their phagocytic activity may assist in keeping contamination in check.

1.3.4 Screening of Hybridomas

Most workers in monoclonal hybridoma production would now agree that the success or failure of monoclonal hybridoma production depends to a very large extent on the screening procedures employed.

However, whichever procedure is decided upon it should fulfill the requirements of speed, reliability, high sensitivity and be adaptable to a high through put of samples.

The assay should also be chosen with the subsequent application of the antibodies in mind. Individual monoclonal antibodies have been shown to have some limitations in application and as such may not react as expected. The assay procedure must therefore be chosen with care from amongst the vast selection available. In Chapter 2 a description of some assay procedures used in hybridoma screening has been presented together with those developed for this work.
Monoclonal antibodies are required for use as standard reagents. It is therefore necessary to characterize them as soon as they are established. They are usually purified and characterized with respect to immunoglobulin chain class and subclass, antigen specificity and cross-reactivity, and binding affinities. Antibody sensitivity to pH or salt concentrations is sometimes important (Herrmann and Mescher 1979, Mason and Williams 1980).

1.3.5 Applications of Monoclonal Antibodies

Until the advent of monoclonal antibody technology (Kohler and Milstein 1975, 1976) the single most prominent ambition of immunologists was to find a ready source of homogeneous antigen-specific antibody (Krause 1970).

Monoclonal antibody reagents are now being described at a phenomenal rate. Their homogeneity, specificity and availability has dictated their replacement of polyclonal antisera in many areas and make them ideal standard reagents for the future in analytical/diagnostic, purification and therapeutic procedures (Sevier et al 1981).

In addition to research workers in the field, many commercial sources have predicted by their interest that monoclonal antibodies could revolutionize our perception of immunological procedures. They could for example promote new, and until now impracticable immunological procedures. But by far some of the most compelling reasons for adopting this technology include the demonstrated potential for the isolation and use of monospecific antibodies to antigenic determinants from so called 'dirty' antigenic sources like tumors comprehensively reviewed by Neville et al (1982) Kennett et al (1981) and Sevier et al (1981); and to other cell surface antigens such as the clinically important major histocompatible complex (MHC) HLA region specific antigens (Bodmer et al 1978, Brodsky et al 1979). Indeed Charion and McDevitt (1979) have suggested that the careful selection of a panel of specific monoclonal antibodies will almost certainly result in a more reliable tissue typing procedure than is at present available. This applies also to microorganisms and parasites; examples of which include Hepatitis B virus (Goodall et al 1981, Tedder et al 1983) Herpes Simplex virus (Pereira et al 1982, Showalter et al 1981); Rabies (Wiktor and Koprowski 1978, Flamand et al 1980); Epstein-Bar virus (Hoffman et al 1980, Pearson et al 1983) and amongst the parasites Malaria (Plasmodium) sporozoite stage (Yoshida et al 1980) merozoite stage (Freeman et al 1980) and to the gamete stage (Rener et al 1980). Monoclonal antibodies to rare and/or weakly immunogenic material or even to new and previously unrecognised antigens, for example, differentiation antigens (Boyse and Old 1969) have also been identified (King et al 1979, Reinherz and Schlossman 1980, Goding and Burns 1981, Tidman et al
The fine specificity of monoclonal antibodies together with unlimited, but consistent supply places the technology in the vanguard of the search for such cell specific antigens and provides the means for identification, purification and consequently allows precise biochemical comparisons to be made. Their value as analytical and diagnostic tools in this area are only now being realised.


Another important area of application is the immunoassay of soluble antigens in body fluids. High affinity non-crossreacting antibody reagents may be produced against, for example, antigens for which pure samples are difficult to obtain for the production of conventional antisera, or where structural similarities usually result in cross reactivity of the antisera. Examples include somatomedin C (Baxter et al 1981) thyroid stimulating hormone (Soos and Siddle 1982, Wada et al 1982) luteinizing hormone (Federici et al 1982) human chorionic gonadotrophin (Gupta et al 1980, Wada et al 1982) gastrin (Suddith et al 1981) substance P (Cuello et al 1979) human α interferon (Adolf et al 1982) human growth hormone (Irary and Davis 1980, Bundesen et al 1980) prostatic acid phosphatase (Wang et al 1981) carcinoembryonic antigen (CEA) (Accolla et al 1980, Kupchik et al 1981) alpha-foetaprotein (AFP) (Tsung et al 1980, Uotila et al 1980) alkaline phosphatase (Millan et al 1982, Slaughter et al 1981). These examples are by no means exhaustive, but serve to illustrate the variety of clinically important material to which monoclonal antibody technology has been applied and thereby demonstrate the versatility of the technique.

Any or all of these previously impossible, or difficult procedures may now be at the very least theoretically highly feasible. The impact of the technology is highlighted by the many excellent reviews recently published containing prodigious accounts of various aspects of the current applications of monoclonal antibodies.
Some of these include Kennett, McKearn and Bechtol (1981); Hammerling, Hammerling and Kearney (1981); Foster (1982); Edwards (1981); Staines and Lew (1980); Schroder (1980); Tse-Wen Chang (1982); Hubbard (1981); Neville et al (1982); Rawls et al (1981); McGregor (1981); McMichael and Bastin (1980); Sevier et al (1981); Melchers et al (1978); and papers are being published at an unprecedented rate.

**Human monoclonal antibodies**

The major difficulty of human therapy with xenogeneic monoclonal antibodies is that generally such antibodies induce an immune response of their own and so effectively reduce the potential worth of the therapy. Additionally a hypersensitive reaction may occur in response to repeated injection of foreign protein. These sorts of problems may be overcome using human monoclonal antibodies. The problem of the availability of myeloma cell partners may have finally been solved (Table 1.5). The antigen sensitized lymphocyte partners may however, prove more of a problem. This may eventually be solved by developing efficient in vitro lymphocyte antigen sensitization regimes.

Human-human hybridomas have however been produced. Olsson and Kaplan (1980) derived anti-2,4-dinitrophenyl (DNP) hybridomas, the hapten sensitized lymphocytes were obtained from a human spleen. Croce et al (1980) on the other hand used peripheral blood lymphocytes from a patient with subacute sclerosing panencephalitis. Hybridomas were derived which secreted human IgM antibodies specific to measles virus.

Sikora et al (1982) have also derived human-human hybridomas using malignant glioma as the permanent cell line and glioma intratumoral lymphocytes to produce hybridomas secreting antibodies which bound glioma cells.

Of course human monoclonal antibodies have also been derived by Steinitz et al (1977, 1979) by Epstein-Barr virus transformation of antigen sensitized lymphocytes and by mouse-human fusions (Kohler 1980, Koprowski et al 1978, Levy et al 1978, Schlome et al 1980), but these although successful are nevertheless restricted by the technological limitations outlined in the text.

Monoclonal antibodies have proved advantageous in a wide range of situations and as more become available will probably eventually replace conventional antisera in much of their present application. Their potential role as therapeutic agents will however remain unrealised until human monoclonal antibody generating systems are reliably and efficiently organised.
In the meantime the available antibodies may be applied to standardise procedures and as the technology matures with time, and is applied to new areas of research, will yield data which will add to our understanding of previously unyielding biological phenomena.

1.4 THYROID STIMULATING HORMONE

It has already been stated that one of the greatest potential values of monoclonal antibodies becomes apparent where specific conventional antisera is practically impossible to produce such as areas where antisera are required to distinguish between very similar molecules. High affinity non cross reacting antisera are required for the immunoassay of clinically important substances in body fluids. Among such substances is the hormone thyroid stimulating hormone (TSH).

1.4.1 TSH Structure

TSH is secreted by the anterior pituitary. It is one of a group of four glycoprotein hormones consisting of an alpha (α) and beta (β) subunit structure (Pierce and Liao 1970, Pierce 1973, Pierce et al 1971c). The α subunit is nearly identical to that of the other pituitary glycoprotein hormones: Luteinizing hormone (LH) (Liao and Pierce 1970, Stockwell-Hartree et al 1971, Pierce et al 1971a); Follicle Stimulating hormone (FSH) (Papkoff and Ekbald 1970); and the related placental glycoprotein Human Chorionic Gonadotrophin (HCG) (Canfield et al 1971). Following the separation of the subunits of bovine TSH, the subunits retain little biological activity (Liao and Pierce 1970); but the α subunits have been shown to be interchangeable and combine with the hormone specific β chains to reconstitute the specific hormone activity (Liao and Pierce 1970, Pierce et al 1971a, Pierce et al 1971b).

The β subunit conveys not only biological but also the immunological specificity (Shome et al 1968, Vaitukaitis et al 1973). However five sequence homologies have been demonstrated even between the β subunits of TSH and LH (Pierce et al 1971c). TSH has a molecular weight of about 28000 (Pierce 1974, Canfield et al 1971) similar to that of the other glycoproteins. All this together with the very low TSH content per gland (10-15 mg/1000 gland) (McLean 1981) makes complete TSH purification difficult and LH usually is found as a persistent contaminant of most purified TSH preparations (Condliffe 1973).
Electrophoretic heterogeneity (Carston and Pierce 1960, Goetinck and Pierce 1966) observed possibly results from two regions of polymorphism, one at the amino terminus and the other at the carboxyl terminus of the TSH β chain (Pierce et al 1971).

The structural similarities between the hormones and the difficulty in obtaining pure hormones for immunization for the development of specific antisera, and for labelling for assay purposes have in the past presented the greatest obstacles to the achievement of highly specific assays for TSH.

1.4.2 Immunological Characteristics Of TSH

The development of immunoassays for the specific quantitation of the hormone resulted in the increased importance of the immunological properties of TSH and the specificity of the antibodies. An understanding of the precise nature of the hormone aided in the development of specific assays and in the elimination of cross reactivity.

Shome et al (1968) demonstrated that human and bovine TSH are immunologically distinct, notwithstanding the similarities in structure and biochemical properties of the two hormones. A comparison of TSH from a variety of species has been given by (Pierce et al 1974). But perhaps the most striking and immunologically opportune observations of studies with, for example, TSH and LH, were that the antibodies to whole hormones were directed primarily at the β chain determinants (Pierce et al 1971a) which gave strong reactions with their homologous hormone antisera. The α chains on their own reacted only weakly with such antisera however. On the other hand the whole hormone reacted with antisera to either the α or the β chain (Vaitukaitis and Ross 1972). The β chain has therefore been used to derive antisera of improved specificity (Vaitukaitis et al 1973, Jacobs and Lawton 1974) for use in clinical assays.

Cross reactivity of conventional antisera is usually removed by adsorption with large amounts of HCG (Odell et al 1967) which mops up cross-reacting anti-α chain antibodies.
1.4.3  Functions of TSH

The primary function of thyroid stimulating hormone is the control of the thyroid gland's synthesis and secretion of the thyroid hormones - triiodo thyronine (T3) and thyroxine (T4). The major overt processes associated in the thyroid glands with TSH stimulation and regulation includes inorganic iodide trapping, iodide oxidation and tyrosine iodination, together with oxidative coupling of mono- and di-iodotyrosines to form T3 and T4. Also included is the colloidal uptake by follicular cells and the subsequent hydrolysis and release of stored T3 and T4 from thyroglobulin molecules. These various aspects have been discussed extensively by Van den Hove-Vandenbroucke (1980). Many of these TSH stimulated processes have been utilized in the past in bioassays of TSH (Bakke 1973).

The acute functional effects of TSH stimulation described above are accompanied by general thyroid hormone regulated physiological and biochemical effects which cover a wide variety of biological phenomena involved in general metabolism, growth and developmental control (Bernal and De Groot 1980, Dumont and Lamy 1980).

In addition to the acute effects, TSH also causes delayed trophic effects where growth and division of thyroid follicular cells are featured prominently. Chronically administered TSH results in an increase in numbers and size of thyroid follicular cells with increased vascularization. Conversely, tonic effects become evident in the absence of TSH resulting in a decline in the numbers of these cells which become flattened and vascularization is much reduced (Dumont and Lamy 1980).

TSH is therefore an important hormone with wide reaching implications in general good health. Its effects are apparent not only in its primary control of the thyroid gland function itself, but also in the capacity of the gland to function by its trophic or tonic activity.

1.4.4  Control Of TSH Secretion

The control of TSH secretion revolves around what is described as the hypothalamo-pituitary-thyroid axis (Demeester-Mirkine and Dumont 1980). TSH secretion is regulated via a negative feed back effect of the free thyroid hormones in the blood in such a way that small changes affect TSH secretion over the entire physiological range (Reichlin and Utiger 1967, Connors and Hedge 1981).
The thyrotrophs of the anterior pituitary which produce and secrete TSH display hypertrophy and hyperplasia in primary hypothyroidism (Bonnyns et al 1974), with increased serum TSH levels (Odell et al 1965, Utiger 1965, Hall et al 1971, Hersham and Pitman 1971). Hyperthyroidism on the other hand results in unchanged thyrotroph numbers but with decreased TSH content in the cells (Phifer and Spicer 1974) and in serum (Turnbridge and Hall 1976).

The thyroid hormones also exert a secondary controlling influence on TSH inhibiting the secretion of thyrotrophin releasing hormone (TRH). It has however been conclusively demonstrated that the thyroid hormones are potent inhibitors of TSH secretion in response to TRH (Ormston et al 1976), and that the inhibitory activity at the pituitary level may be due to the effective reduction in TRH binding sites (De Lean et al 1977). Figure 1.5 shows the proposed major factors controlling TSH secretion.

TSH secretion is however under the direct positive control of the neuroendocrine tripeptide TRH, secreted by the hypothalamus (Ormston et al 1971, and reviewed by Labrie et al 1979 and Demeester-Mirkine and Dumont 1980).

Additional factors such as cold in neonate and children (Woolf et al 1972) has a positive, while stress has a negative, effect (Demeester-Mirkine and Dumont 1980) on TSH secretion. Oestrogen stimulates (De Lean et al 1977) while neurotensin (Sheppard et al 1983) and somatostatin (Siler et al 1974) inhibits TSH response to TRH. The opposing dopaminergic and adrenergic effects have been reviewed by Scanlon et al (1978) and Labrie et al (1979).

TSH is therefore linked in a major self regulating system which under normal conditions maintain optimum hormone levels. Under such a regulatory system normal basal TSH levels in man fluctuate very little with basal concentrations ranging from undetectable to about 4 μU/ml with a mean of about 1 μU/ml in adults (Demeester-Mirkine and Dumont 1980) measured by RIA.

Perturbation of the system therefore leads to variation in TSH levels which may be quantified in an attempt to delineate and define precisely the various interrelationships and assess their significance in the progress of thyroid dysfunction.

1.4.5 Assays For TSH

Tests which employ TSH assays are usually undertaken in an exploratory and/or diagnostic capacity to assess primarily the functional capacity of the thyroid. The clinical evaluation of TSH is therefore usually designed to quantify TSH secretion either directly in the serum or indirectly by measurement of its biological effects in the resting state or in response to its controlling factors. The tests are therefore designed to identify the point of defect within the hypothalamus-pituitary-thyroid axis.
FIGURE 1.5 The major control pathways of the Hypothalamus-Pituitary-Thyroid axis which is linked in a feed back control system; negative (-ve) (---→), and positive (+ve) (→) links (Modified from Demeester - Mirkine and Dumont 1980).
TSH bioassays

A large variety of TSH assay methods have been described, the majority of which include the various in vivo and in vitro bioassays (Bakke 1973, Tunbridge and Hall 1976). These contain many inherent limitations. The usefulness of any assay method is judged by its sensitivity, specificity, precision and economy in application. The various bioassays have been demonstrated to be limited in many of these respects (Tunbridge and Hall 1976, Mornex and Orgiazzi 1980).

The bioassays have therefore now been superseded by other assays such as radioimmunoassays in general clinical use, but nevertheless remain essential for delineating the biological reactivity of TSH preparations as opposed to immunological reactivity measured by radioimmunoassay.

TSH radioimmunoassay

Radioimmunoassay of TSH has replaced the tedious bioassay methods has the advantages of good precision and the capacity to handle a large number of samples simultaneously.

However RIA carries its own limitations, and as described earlier, because of the similar structures and sequence homology of TSH subunits and the gonadotrophins LH and FSH and HCG, most polyclonal antisera to h-TSH cross react with these to a greater or lesser extent (Odell and Utiger 1973). Most of this cross reactivity is normally eliminated by the addition of a large amount of HCG to the assay tubes to give the required specificity.

Various modifications have been applied to the earliest TSH radioimmunoassays (Odell et al 1965, Utiger 1965) in order to improve assay sensitivity. Some of these include attempts to reduce radiolabelling damage (Marchalonis 1969, Butt 1972); the dilution of TSH standard material with TSH free serum, and the incubation of standards and test sera with the antisera prior to adding the labelled hormone, and better separation with an antiimmunoglobulin (Odell et al 1967). However TSH RIA in clinical application (Hall et al 1971, reviewed by Tunbridge and Hall 1976) are still limited by their sensitivity, not being capable of measuring TSH levels in some normal and subnormal individuals. Other persisting limitations of TSH RIA include high interassay variation, the extended assay period and the inter-centre variation in normal TSH value estimates (Tunbridge et al 1975).

Clearly because in this assay unlabelled TSH competes with radio labelled TSH for the limited amount of antibody binding sites, then the quality of the assay is dependent to a large extent upon the purity of the TSH preparations used. Pure TSH is required for immunization, for radio-labelling and as standard preparations (Bangham and Cotes 1971, Hurn and Landon 1971).
Recently reported immunoradiometric assays for TSH have solved many of the problems of TSH RIA and have approached (Sutherland et al 1982, John and Woodhead 1982) and surpassed the sensitivity (Pekary and Hershman 1984, Cobb et al 1984) obtainable by RIA.

Other assays

TSH has also been measured by other methods such as the cytochemical assay technique of Bitensky et al (1974). This assay while being very sensitive is nevertheless limited for general use by its small capacity, expense and assay time.

Enzyme-linked immunosorbent assays (ELISA) (Engvall et al 1971, Van Weeman and Sahuurs 1971, 1972, Voller et al 1979) have developed rapidly since its first inception and have now become established as an alternative immunoassay procedure to RIA. This rapid acceptance has been brought about because sensitivities can be achieved similar to RIA values and because it overcomes the major disadvantages of working with and disposing of radioactive materials. The procedure is very robust and is now fully automated which facilitates a large sample throughput.

ELISA has been described for TSH measurement (Miyai et al 1976, Tshikawa et al 1982) and commercial kits are available (Tanswell et al 1979). Recent reports of fluorometric TSH assays have also appeared (Kato et al 1980).

Even more exciting for TSH assay is the very recent report (Jones et al 1984) of a very sensitive immunochemiluminometric assay which can differentiate the lower end of the normal basal TSH level from that of the hyperthyroid state. This assay employed a monoclonal antibody instead of the usual polyclonal antisera. Sensitivities of 0.015 mU/l were achieved. This demonstrates the special facility of monoclonal antibodies to form the basis of highly specific sensitive assay systems.

1.4.6 The Clinical Application Of TSH Assays

The value of TSH assays has been established by their application in the clinical situation in the diagnosis and assessment of thyroid function and disorders of the hypothalamus-pituitary-thyroid axis.

TSH levels in the resting state

Considerable inter-centre variation have been reported for the normal TSH range (Tunbridge and Hall 1976). It is therefore necessary for each centre to establish its own normal range.
As a result of the inaccuracy of the available RIA at very low TSH levels only normal and elevated levels are recognised and are therefore clinically significant (De Visscher and Burger 1980). As a consequence the major application of TSH assay is in the evaluation of hypothyroidism where TSH levels are usually observed to be elevated.

The significance of this application is overwhelming by its importance in the diagnosis of thyroid insufficiency resulting in the condition of cretinism. The congenital neonatal frequency (Klein et al 1974, Dussault et al 1975, Walfish 1975) is greater by a factor of two or three than that of phenylketonuria (Scanlon et al 1978) for which mass screening exists and is about 1:4000 babies (Sutherland et al 1981, Hulse et al 1980, Dockeray et al 1980, Delange et al 1981, John and Woodhead 1982). This together with the good prognosis of early treatment highlights the significance of early TSH screening.

Thyroxine therapy alleviates the symptoms in overt and mild hyperthyroidism and the decrease in serum TSH levels may be used to monitor the progress of therapy. In subclinical or compensated hypothyroidism however elevated TSH levels are found in conjunction with low or normal thyroid function but without the expected hypothyroid symptoms. A comprehensive discussion of hypothyroidism, its clinical implications and treatment is given by De Visscher and Ingenbleck (1980).

Elevated TSH levels accompanied by the presence of thyroglobulin antibodies are also found in autoimmune thyroiditis (Tunbridge and Hall 1976). Elevated basal TSH levels are also found in the rare hyperthyroid condition involving oversecretion of TSH due to pituitary lesion (Mornex and Orgiazzi 1980).

Low or undetectable basal TSH levels are however the usual finding in hyperthyroidism. TSH assays are not sensitive enough to separate these levels from those of the lower end of the normal range. A rise in TSH levels however may be indicative of successful treatment with antithyroid drugs.

**TSH levels in response to TRH**

TSH response to thyrotrophin releasing hormone (TRH) has its major clinical application in the diagnosis of disorders of the hypothalamus-pituitary-thyroid axis (Ormston et al 1971b).

The suppression of TSH response to TRH is usually observed in hyperthyroidism. A normal TSH response therefore excludes this condition, and is of primary importance in diagnosing mild hyperthyroid states with absent clinical symptoms but with the suppressed TSH response to TRH still evident.
(De Visscher and Burger 1980, Scanlon et al 1978). However it must be noted that this sort of suppressed TSH response is observed in other thyroid related conditions (Scanlon et al 1978). It is therefore not a complete diagnosis of the hyperthyroid state.

In normal subjects the TSH response in the TRH test (Ormston et al 1971b) is elevated with maximum values occurring at about 20 minutes after TRH administration. The peak levels are observed to be directly related to the basal TSH levels and as such the response is exaggerated in all hypothyroid conditions.

In a few cases, congenital neonatal hypothyroidism, described above would be missed if assessed on TSH levels alone. These are the hypothalamic-pituitary hypothyroid cases. TSH response in the TRH test in secondary or tertiary hypothyroidism suggests the origin of the defect. The pituitary is indicated where there is absent or minor response to TRH. On the other hand where the defect is of hypothalamic origin, a frequently delayed but nevertheless elevated TSH response is observed (De Visscher and Burger 1980).

To summarize, the TSH assays are important when measured in the resting state to diagnose and possibly provide screening facilities for hypothyroidism. TSH levels in response to TRH exclude hyperthyroidism and in particular differentiate the mild state from an anxiety state; and delineate the point of defect in secondary or tertiary hypothyroidism while an exaggerated response confirms primary hypothyroidism.

1.5 THE MAJOR PROJECT OBJECTIVES

1) The difficulties of TSH assays especially in relation to those inherent in the use of polyclonal antisera have been outlined. We therefore set out to first immunize Balb/c mice with human TSH to produce spleen cell donors. These were to be used in fusion with NSI myeloma cells to produce anti-h-TSH antibody secreting hybridomas.

2) From these antibody secreting hybridomas we hoped to derive stable hybrids secreting specific and high affinity anti-h-TSH antibodies.

3) Characterization of the hybridomas and specific antibodies was to be in terms of cell growth and maintenance characteristics, antibody specificity and cross-reactivity, titre, affinity, antibody class and subclass.

4) It was intended to demonstrate the antibodies in specific application in immunocytochemical identification of thyrotropes in various pituitary preparations; and to attempt to utilise the antibodies in a sensitive TSH immunoassay. We chose to attempt to set up a sandwich enzyme-linked immunosorbent assay for TSH, because of the advantages of this procedure (Lancet editorial 1976, Gosling 1979).
CHAPTER 2

Development Of Assay Methods For The Detection Of Monoclonal Antibodies to h-TSH
2.1 INTRODUCTION

The range of monoclonal antibodies that can be produced is theoretically unlimited. However, in practice each antibody's detection and consequently its possible selection may be severely limited by the quality of the screening assay. Essential features of the assay chosen for screening purposes are:

1. It must be very sensitive, capable of detecting only very small amounts of antibody produced in the first few days after fusion.
2. It must be reproducible and reliable. Since it is to be used to screen very large numbers of hybridoma cultures, then detection rate must be consistent.
3. It must also be accurate. If this criterion is not met a great deal of time and effort could be wasted pursuing false positive cultures or even more disastrous, valuable positive cultures could be lost as false negatives.
4. Finally, the assay must be rapid to perform, and capable of handling a large number of samples. Speed is of the essence since slow growing hybrids which may be producing particularly important antibodies may be overgrown by faster growing non-producing hybrids or by those producing non-specific antibodies.

The success or failure of the technology then is dictated to a large extent by the screening procedures employed.

Many assays have been used to detect monoclonal antibodies some of which have been described in the study of antibody formation at the single cell level (Cunningham 1973). However, the assay must be chosen with regard to the predicted nature of monoclonal antibodies, the nature of the antigen and the uses for which the antibodies are intended (Edwards 1981, Galfre and Milstein 1981, Catty et al 1981, Foster 1982).

Monoclonal antibodies have been shown in application to exhibit important limitations which would exclude their selection by assays requiring the exploitation of any of these limiting characteristics. For example, because of the very nature of monoclonal antibody preparations, only one antibody type exists which binds only one single antigenic determinant on the molecule, (unless of course the specific epitope is a repeated unit). This is not conducive to the formation of three dimensional lattices leading to precipitation. Monoclonal antibodies can only crosslink antigens into dimers, hence cannot usually precipitate the target antigens (Milstein et al 1980). The inability of monoclonal antibody to produce immunoprecipitation was predicted by the lattice theory (Marrack 1938).
The double immunodiffusion assay of Ouchterlony (1958, 1962 and 1979), although fairly rapid and simple, is therefore not always appropriate for screening purposes. This is not to say however that monoclonal antibodies cannot be used later in these procedures. A mixing of two or more suitable monoclonal antibodies have been shown to adequately overcome the problem and produce precipitation (Jefferies et al 1980). Immune precipitation of antigen has however been used for screening by Brown et al (1980).

A similar situation has been found to exist in cytotoxic assays where some monoclonal antibodies may be missed. Monoclonal antibodies individually may be poorly cytotoxic or not at all; but, in combination are highly effective (Howard et al 1979). This is because complement mediated cytotoxicity requires for its initiation at least two molecules of IgG antibodies bound to juxtaposed antigenic determinants. In addition, complement fixing ability is dependent on the immunoglobulin class and subclasses present (see Chapter 1). Only IgG and IgM are effective; and within the IgG class, the subclasses fix complement with varying efficiencies (Roitt 1980). Modifications of such assays may however allow their use (Kennett 1981).

Mason and Williams (1980) studied the binding of monoclonal antibodies to their appropriate antigens under various assay conditions and showed also that the conformation of the antigen was important. In the solubilized state, antibody which bound strongly in the cellular configuration, for example, now exhibited rapid dissociation characteristics. Roehrig et al (1982) also demonstrated that the conformational state of the antigen also influenced the reactivity. If the antibodies are required, for example, for work with soluble membrane components, then it is appropriate that the antigens should be in the solubilized state in the selection procedure in order to obtain the best quality antibodies in terms of their binding characteristics.

It is generally possible however to adapt and modify many of the available immunological assays for the detection of monoclonal antibodies if the above considerations are taken fully into account when choosing the screening assay.

2.1.1 Screening Assays

Antibody secreting hybridomas have been detected in two main ways:
1) Indirectly, by the use of spent media from actively growing cultures; and
2) by direct detection in the microenvironment of isolated cells.
Indirect detection

The presence of antibody in spent culture media may be detected by binding assays in which specific antibody binds directly to the 'indicator' labelled antigen, and, under the appropriate assay conditions the behaviour of the complex demonstrates the antibody presence.

Such assays include the haemagglutination assay techniques described by Coombs (1981), and detailed specifically for use in hybridoma screening by Galfre and Milstein (1981) and Catty et al (1981). In these assays the antigen is coupled to red blood cells and the presence of antigen specific antibody in spent media, is indicated by agglutination.

The haemolytic spot test involving complement mediated lysis was used successfully in the pioneering experiments of Kohler and Milstein (1976). Another lytic assay which has since been used in this context was the $^{51}$Cr release assay, where the specific antibody binding was detected by the release of the radioisotope following lysis of the labelled target cells (McKearn 1981, Schreier et al 1980, Pearson et al 1977, Eisenbarth et al 1978, Galfre et al 1977).


Spent media samples were also used in indirect binding assays. This group of assays represents the largest majority of assays employed, because they do not require purified antigen in hybridoma screening. Such assays include those which employ a second labelled antiglobulin to indicate the presence of antigen binding antibodies. Several labels and binding reagents have been combined. Antiglobulins may be $^{125}$I radio-labelled according to Miles and Hales (1968), and used in the solid phase system devised originally by Catt and Tregear (1967) and reviewed by Wide (1970). The system has been adapted for use with microtitre plates and the combined solid phase assay may be used for monoclonal antibody detection as described by Goding (1980), Catty et al (1981), Galfre and Milstein (1981), Hubbard (1981) and Tsu et al (1980), Hudson and Hay (1980).

In this type of assay the antigen, which may be a cell surface component/components, or a soluble protein antigen, is adsorped onto a solid phase support. The prospective antibody solution is added to the solid phase antigen followed by the indicator antiglobulin (Bechtol 1981). The two immediate advantages of such an assay are:

1. separation of bound label from free is no longer a problem and is accomplished merely by several washing steps; and
all types of antibodies directed against the antigen will be bound and thereby detected. This type of assay therefore allows the detection of antibodies with additional specialist functions described above.

The enzyme-labelled antiglobulins reviewed comprehensively by Wisdom (1976) and Avrameas (1978), were used in enzyme-linked immunosorbent assays (ELISA) first described by Van Weeman and Schuurs (1971) and (1972), Engvall et al (1971), Engvall and Perlman (1971, 1972). The procedure has since been expanded (Bidwell et al 1976, Voller et al 1976, 1979) and has found wide application (Voller et al 1979, 1981, Voller and Bidwell 1980).

ELISA methods for the detection of monoclonal antibodies have been described by Kennett (1981), Catty et al (1981), Noat and Remmington (1981), Sundar Raj et al (1982), Ross et al (1981) and Douillard et al (1980). The antiglobulin may be substituted with Protein A which has been enzyme-labelled (Suter 1980) or radio-labelled (Brown et al 1979, Businaro et al 1981 or Nowinski et al 1981) to produce an effective assay. ELISA is performed on the same format as the solid phase RIA described above. However after excess unbound enzyme label is removed, a colourless enzyme substrate is added, which when cleaved by the enzyme, form a soluble coloured compound and the optical density of the solution is read on a simple spectrophotometer. (Hudson and Hay 1980, Catty et al 1981, Douillard et al 1980).

These enzyme-labelled antiglobulins may also be used in immunocytochemical screening (Sternberger 1979), and such an assay is analogous to the ELISA system. The main differences include antigen immobilization within the tissue or cells instead of being absorbed on to the assay well and the enzyme substrate when cleaved form an insoluble colour product at the site of the localized enzyme activity (Ross et al 1981).

Another important antiglobulin label is fluorescein. Coons et al (1941) published the first account of such labelled antibodies; and the topic is extensively reviewed by Johnson et al (1979) and Landon and Kamel (1981). This label may be used with the fluorescein-activated cell sorter (FACS) which can be adapted for simultaneous screening and cloning of hybridomas directly (Parks et al 1979, Dangl and Herzenberg 1982).

Assays based on the effect of specific antibodies on the biological activity of the antigen have also been used. These types of assays also obviate the need for pure antigen preparation for an effective and sensitive assay. Culture supernatants may be added to a biologically active antigen preparation and subsequent inhibition of the biological activity is indicative of the presence of specific antibody. The classic example of this type of assay used to good effect was in the screening of interferon monoclonal antibodies by Secher and Burke (1980).
Direct detection

In contrast to detection of antibodies in the spent media, antibody secreting hybridomas may be identified directly by detecting very small amounts of antibody in the immediate vicinity of the cells, or actually adhering to the cell surface (Galfre and Milstein 1981). As already outlined above one way of accomplishing this is with the FACS technique.

The plaque assay technique of Jerne (1965), Jerne et al (1974), has been used where the cells were grown in the semi-solid support like agar or agarose (Galfre and Milstein 1981, Kohler and Milstein 1975 and 1976). Direct visualization of specific antibody secreting cells was realised by complement dependent lysis of antigen coated erythrocytes. The cell may therefore be picked and grown up as appropriate.

Replica immunoabsorption methods may also be used to indicate specific antibody secretors in situ. These methods involve the absorption of antibody into overlaid nitrocellulose filters followed by labelled specific indicator (Sharon 1979).

All these screening assay methods outlined have their own special merits and disadvantages in terms of cost, practicability and sensitivity in specific application. Of the greatest importance to assay choice is a consideration of the numbers of cultures which may have to be screened, and even more significantly the capacity to detect antibodies to very rare and/or weakly immunogenic antigens and even the detection of antibodies to unknown antigens. A major limitation however is that the assay may have to be performed using only very small amounts of the antigen often in an impure form, eg, Secher and Burke (1980) monoclonal antibodies to interferon; or be used to detect antibodies to unknown antigenic material which is a component in cell membrane (Magnani et al 1981).

The majority of the assays described are relatively inexpensive and the reagents if not readily available are easily synthesized. Some of the assays also require very little in terms of elaborate facilities and so may be performed with ease under most laboratory conditions. Difficulty however may be encountered, for example, in the assays requiring radio labelled reagents. A specifically designated Grade C laboratory is needed for radiiodinations. The counters are also expensive to purchase specifically for this purpose unless already available in the laboratory.

Having considered the uses for which the monoclonal antibodies are intended, and the available assays which fulfill the requirements, the next immediate task was to develop and optimise the assays for screening.
In the very early stages of this work liquid phase radioimmunoassay (Hall et al 1971) using radio-labelled human thyroid stimulating hormone (h-TSH) was the assay chosen for screening. The primary reason for this choice was that an assay system with available labelled h-TSH was already in every day use by the Supraregional Assay Service Centre, Dept of Clinical Biochemistry, St Lukes Hospital, Guildford.

Later a solid phase separation RIA (Hunter 1979), and an indirect enzyme-linked immunosorbent assay (Voller 1979) (figure 2.1), was developed specifically for screening purposes.
FIGURE 2.1
Indirect ELISA for TSH specific monoclonal antibodies

STEP 1
OVERNIGHT 1 μg/ml TSH.
Ag ADSORBED TO POLYSTYRENE PLATES (DYNATECH) SENSITIZED WITH 0.1% GLUTARALDEHYDE

WASH x 3
PBS Tween + 0.1% BSA

STEP 2
ADD SPECIFIC ANTIBODY
INCUBATE 2 HRS

WASH x 3

STEP 3
ADD ENZYME LINKED SECOND ANTIBODY *
INCUBATE 2 HRS

WASH x 3

STEP 4
ADD ENZYME SUBSTRATE OPD
INCUBATE 30 MINS
THE AMOUNT OF HYDROLYSIS OF SUBSTRATE - OD Elisa 490 = AMOUNT OF SPECIFIC ANTIBODY PRESENT

* IS HORSE RADISH PEROXIDASE LINED TO GOAT ANTIMOUSE Ig (Voller) OR DONKEY ANTIMOUSE Ig (Guildhay).
2.2 MATERIALS AND METHODS

2.2.1 Preparation Of Antimouse Immunoadsorbent For RIA

Many immunoadsorbents have been used since its first conception by Catt and Tregear 1967. Some of these include the covalent coupling of the immunoglobulin fraction to sephadex or cellulose (Wide 1969, Hales and Woodhead 1980) or insoluble dextran (Wide et al 1967). Two conjugation methods are mainly used. That employing cyanogen bromide (Wide et al 1967 and Wide 1969), the various applications of which are reviewed by Wide (1970); or the diazotization method of Gurvich et al (1961). This latter diazotization method is that which was employed in the preparation of the immunoadsorbent (Addison 1971, Hales and Woodhead 1980).

Preparation of aminocellulose

A small amount of aminocellulose was obtained through the courtesy of Dr D Teale, Dept. of Clinical Biochemistry, St Lukes Hospital, Guildford. Further quantities were prepared by the method of Addison (1971). 1.4 g m-nitrobenzyloxymethyl pyridinium chloride (British Drug House, Poole, Dorset).

0.5 g anhydrous sodium acetate were dissolved in 20 ml 90% ethanol in a 100 ml beaker.

10 g cellulose powder (Whatman CC41, Whatman Ltd, Springfield Mill, Kent) was added, mixed thoroughly and dried at 80°C on a heating block in the fume cupboard.

The dried powder was then heated at 100°C for 2 hours. The nitrocellulose powder was then washed three times with 200 ml benzene and then air dried followed by washing with 3 litres deionised water. All washings were done on a sintered funnel with gentle suction applied.

The nitro groups on the nitrocellulose were then reduced to amino groups by warming to 60°C for 30 minutes in 150 ml of 20% (w/v) sodium hydrosulphite solution.

The amino cellulose (AC) was washed four times with 200 ml deionised water, three times with 100 ml of 30% (v/v) acetic acid, and four times with 200 ml deionised water until all the odour of hydrogen sulphide was removed.

The aminocellulose was dried in a desiccator and stored sealed at 4°C until required.
Suspension of aminocellulose

The aminocellulose was reprecipitated from an ammoniacal copper solution. Cupric hydroxide was obtained by dissolving 5 g copper sulphate (CuSO₄·5H₂O) in 500 ml deionised water, and 2 g sodium hydroxide (NaOH) in 75 ml deionised water and the two solutions mixed. The suspension of cupric hydroxide was washed with 2 litres deionised water on a sintered funnel. The precipitate was dissolved in 100 ml of (specific gravity 0.88) ammonia. 0.33 g sucrose and 1.0 g aminocellulose was added slowly with constant stirring for 30 minutes.

The volume was made up to approximately 400 ml with deionised water and about 300 ml of 10% H₂SO₄ is added until flocculation occurred.

The precipitate was washed by centrifugation five times with deionised water and suspended in 100 ml, ie, 10 mg/ml assuming no loss of material.

This preparation was used within 24 hours.

Diazotisation and coupling of immunoglobulin fraction

All procedures were performed at about 4°C on ice, and washes were by centrifugation.

200 ml of 2M HCl was added to the 100 ml suspension of aminocellulose - a pink colour developed. 8 ml of 1% (w/v) sodium nitrite was added and the pink colour disappeared. The mixture was allowed to stand for 30 minutes after which solid urea was added to remove excess nitrous acid which was detected with starch iodide paper.

The diazo cellulose was washed three times with 200 ml ice cold deionised water and once with 200 ml of 0.05M borate buffer pH 8.6. It was then suspended in 40 ml, (ie, at 25 mg/ml) borate buffer.

The presence of diazo groups was detected by the characteristic bright orange colour which developed when a small amount of suspension was reacted with a little saturated β-naphthol in borate buffer. The diazotized preparation is unstable and must be used immediately.

The ammonium sulphate purified donkey antimouse (Guildhay, University of Surrey) immunoglobulin 0.5 g in 35 ml PBS (= 14.28 mg/ml) or 1.5 g in 35 ml PBS (= 42.8 mg/ml) was added immediately to the 40 ml diazo cellulose suspension and mixed at 4°C for about 72 hours (immunoadsorbent A and immunoadsorbent B respectively.

The conjugates were then washed three times with 200 ml 0.05M borate buffer followed by resuspension in 40 ml of 1.0M ethanolamine in borate buffer and incubated overnight.
The immunoadsorbents were then washed with 200 ml borate buffer followed by 200 ml 0.2M acetic acid and then three times with 200 ml borate buffer.

Finally the antibody-cellulose conjugates were resuspended in 50 ml TSH assay diluent buffer which was 0.01M phosphate buffered saline pH 7.4 containing 0.2% (w/v) BSA (Miles Laboratories Ltd, Stoke Poges). The conjugate was stored at 4°C. Assuming 100% recovery the suspensions contain 20 mg/ml cellulose conjugate.

Purification of donkey antimouse antiserum

165.0 ml donkey antimouse antiserum was partially purified by ammonium sulphate precipitation (Nowatny 1979).

To donkey antimouse antiserum (D60IE 30.70.81) (kindly donated by Guildhay, University of Surrey, Guildford) was slowly added, 99.0 ml saturated ammonium sulphate to a final concentration of 37.5% (v/v), adjusting the final pH to 7.4 with NaOH. The mixture was stirred for 30 minutes at room temperature, after which the precipitate was collected by centrifugation for 15 minutes on the MSE bench centrifuge.

The precipitate was kept concentrated by redissolving in 45 ml of 0.15M phosphate buffered saline (PBS) pH 7.4. The immunoglobulins were reprecipitated by adding slowly 22.5 ml to a final concentration of 33.3% (v/v) ammonium sulphate. This was stirred for 30 minutes and then centrifuged as above. The supernatant was decanted and the precipitate redissolved to 45 ml in PBS.

The immunoglobulin preparation was dialysed against six changes of PBS at 4°C over 48 hours. The preparation was centrifuged, and the supernatant collected and stored at 4°C. The protein content of the solution was determined from absorbance values read at 280 nm (OD 280) on a Cecil CE292 spectrophotometer.

2.2.2 Assessment of Immunoadsorbent

The two essential RIA reagents required for the assessment of the immunoadsorbent were: radio-labelled hormone and specific antibodies. The assay procedure was a modification of Soos and Siddle (1982).

Labelled human Thyroid Stimulating Hormones

\[ ^{125}I \] labelled human thyroid stimulating hormone (h-TSH) was kindly supplied on a monthly basis by Dr D Teale, Dept. Clinical Biochemistry, St Lukes Hospital, Guildford.
The iodinated h-TSH was obtained as freeze dried samples which were reconstituted with 1.0 ml distilled water. This was then diluted according to the instructions 1:40 and used 100μl per assay tube. The average specific activity of the batches was 80 μCi/μg.

**Antisera**

Specific mouse anti-TSH antisera was obtained from mice immunised with h-TSH as detailed in Section 3.2.1. Both non-immune serum and TSH antiserum was obtained by bleeding the mice by retro-orbital puncture. The blood collected was left over night at 4°C then separated by centrifugation for 15 minutes at 3000 rpm in the Beckman J6 centrifuge after which the serum was decanted from the clot. The serum was then stored in 200 μl aliquotes at -20°C until required.

**Solid phase RIA**

RIA assay diluent consisted of 0.01M phosphate buffered saline pH 7.4 made up as follows:- 3.025 g Na₂HPO₄.12H₂O, 0.3 g NaH₂PO₄.2H₂O and 9gNaCl were dissolved in 500 ml distilled water. The pH was checked and the volume made up to 1.0 litre 0.2% BSA (w/v) was added immediately before use.

**Method**

The immunoadsorbent was assessed in a h-TSH assay and a methotrexate assay.

For the TSH assay, duplicate LP3 tubes (Luckham Ltd, Burgess Hill, Sussex) were set up each containing 100 μl pooled mouse anti-TSH diluted 1:8000 in assay diluent or serum from only one animal diluted 1:5000. Negative control tubes contained normal mouse serum diluted 1:8000 or 1:5000. In nonspecific binding (NSB) tubes, sera was replaced by 100 μl of assay diluent. Duplicate NSB tubes were set up for each immunoadsorbent dilution. Next 100 μl labelled h-TSH was added, the tubes were vortexed and incubated for 2 hours or 24 hours at room temperature.

At the end of the incubation period 200 μl of the solid phase second antibody diluted with assay diluent was added per tube which were vortexed. The immunoadsorbent was investigated at various dilutions 1:10, 1:5, 1:2 and undiluted. During the dispensing of the immunoadsorbent care was taken to ensure it was stirred continuously to ensure even distribution of the suspension. The tubes were incubated for a further 2 hours at room
temperature after this time, 1.0 ml assay diluent was added to dilute the contents and the tubes were spun down for 15 minutes at 3000 rpm on a Beckman J6 centrifuge. The supernatant was then aspirated. The pellets were counted (LKB Rack Gamma counter) for 100 seconds.

For the methotrexate assay, 100 µl mouse antisera diluted 1:40,000 was added to each tube followed by 100µl 125I-methotrexate. Following incubation of 4 hours at room temperature 100 µl of the diluted immunoadsorbent was added. The tubes were vortexed thoroughly then incubated for 2 hours at room temperature, followed by dilution of the contents with assay diluent (Assay diluent consisted of 0.05M Phosphate buffer pH 7.4 containing 0.1% (w/v) gelatin plus 0.5% (w/v) sodium chloride). They were then spun down at 3000 rpm on the Beckman J6 centrifuge for 15 minutes; after which the supernatants were decanted and the pellets counted. The methotrexate assay was kindly performed in the Clinical Biochemistry Lab, University of Surrey.

**TABLE 2.1**

Lowry et al (1951) Assay Protocol

<table>
<thead>
<tr>
<th>[BSA]</th>
<th>100 µg</th>
<th>80</th>
<th>60</th>
<th>40</th>
<th>20</th>
<th>10</th>
<th>5</th>
<th>0</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>ml BSA solution</td>
<td>1.0</td>
<td>0.8</td>
<td>0.6</td>
<td>0.4</td>
<td>0.2</td>
<td>0.1</td>
<td>0.05</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>ml 0.25 µNaOH</td>
<td>0.0</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
<td>0.8</td>
<td>0.9</td>
<td>0.95</td>
<td>1.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Test Sample</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.00</td>
<td>0.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Reagent 'C'ml</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.00</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>10 minutes incubation</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.00</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Reagent 'E'ml</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.50</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>30 minutes incubation</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.50</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

The absorbance at 720 nm was read on the Cecil CE 292 spectrophotometer.

**Protein content of immunoadsorbent**

The amount of protein bound to the aminocellulose was determined by the Lowry et al 1951 method.

BSA FrV (Miles Laboratories Ltd, Stoke Poges) was used as the standard protein. 1.0 mg was dissolved in 10 ml 0.25M NaOH.

The test solutions were diluted 1:25 with distilled water, then an equal volume of 0.5M NaOH was added.

The tubes were set up in duplicate as in Table 2.1.
Reagents

A - 2.0% (w/v) Na$_2$CO$_3$ in 0.1 M NaOH
B - 0.5% (w/v) CuSO$_4$·5H$_2$O in 1.0% (w/v) Potassium tartrate
C - Alkaline CuSO$_4$ - 50 ml reagent A plus 1.0 ml reagent B
    (stable for approximately one day)
E - Folin-Ciocalteau reagent - diluted 1:2 with distilled water

2.2.3 ELISA Screening Assays

A microtitre plate procedure (Voller 1979) for an indirect ELISA for the
detection and measurement of anti-human TSH antibodies was developed.

h-TSH antigen preparations

Purified human TSH was kindly supplied for the whole of this work by
Dr R Edwards NETRIA, St Bartholomews Hospital, London, in 200 µg quantities.
The TSH was dissolved in distilled water and aliquoted into vials at
10 µg/ml. These solutions were then freeze dried and stored at -20°C.
The TSH preparation potency was calibrated against the National Institute
of Biological Standard and Control (NIBSC) standard (68/38) in a standard RIA
(Dr D Teale, Biochemistry Dept., St Lukes Hospital, Guildford).
The TSH potency was found to be:- 1 µg = 10 mU NIBSC standard (68/38)
This h-TSH preparation has been designated TSH(Barts) throughout.

Antisera

The mouse anti-TSH and normal mouse sera preparations used to set up the
solid phase RIA above was also used in the development of the ELISA methods.

Enzyme conjugates

Horseradish peroxidase (HRPO) (EC 1.11.1.7) and alkaline phosphatase
(EC 3.1.3.1) are perhaps the most widely used in enzyme immunoassays (Wisdom
1976). The enzyme conjugates are usually prepared by covalent linking of the
enzyme and protein (antigen or antibody).
Conjugate Procedure

The periodate method of Wilson and Nakane (1978) and detailed by Voller (1979) was used for conjugation of donkey anti-mouse immunoglobulin and HRPO.

The donkey antimouse immunoglobulin fraction was prepared as described earlier in Section 2.2.1. The immunoglobulin concentration of the preparation was determined by assuming 1 mg/ml IgG has an optical density of 1.45 measured at 280 nm (OD$_{280}$). The protein concentration of the preparation was 42.76 mg/ml. 0.37 ml antibody preparation was diluted in 1.63 ml 0.01M carbonate bicarbonate buffer pH 9.5 immediately before use. 8.0 mg horseradish peroxidase (RZ 2.9 Sigma Chemical, Poole, Dorset) was dissolved in 2.0 ml distilled water and 400 µl of freshly prepared 0.1M sodium periodate (Na IO$_4$) (BDH Chemicals Ltd, Enfield, Middlesex) was added and the mixture stirred at room temperature for 20 minutes, followed by dialysis overnight against 0.001M sodium acetate buffer pH 4.4 at 4°C.

The following day 20 µl of 0.2M carbonate bicarbonate buffer was added and immediately the prepared antiglobulin preparation consisting of approximately 16 mg Ig in 2.0 ml carbonate bicarbonate buffer was added; and the mixture stirred for 2 hours at room temperature. 100 µl of freshly made sodium borohydride (Na BH$_4$) (4 mg/ml in distilled water) was then added and allowed to stand at 4°C for a further 2 hours. After this time the conjugate preparation was divided into two. One half approximately 2.32 ml was precipitated with an equal volume of saturated ammonium sulphate and the precipitate washed twice with 50% (v/v) ammonium sulphate followed by extensive dialysis against 0.15M PBS pH 7.4. This conjugate was then mixed with an equal volume of glycerol (BDH Chemicals Ltd) and 0.01% (w/v) thimerosal (Sigma Chemicals Ltd) was added as preservative. The conjugate was aliquoted into 0.5 ml lots and stored at 4°C. This was termed conjugate II.

The second half of the conjugate was filtered on a Sephadex G200 (Sigma Chemicals Ltd) column 35 cm x 2.5 cm equilibrated and eluted with 0.15M PBS pH7.4 containing 0.1% BSA. 2 ml factions were collected, and the OD$_{280}$ and OD$_{403}$ measured. The active fractions were pooled, an equal amount of glycerol added plus 0.01% thimerosal. The conjugate was dispensed into 0.5 ml aliquotes and stored at 4°C. This was designated conjugate III.

Conjugate I consisted of a pretitrated goat anti-mouse horseradish peroxidase preparation kindly donated by Dr A Voller, Nuffield Laboratories of Comparative Medicine, Regents Park, London. This conjugate was used diluted at 1:6000. A substrate incubation time of 30 minutes was suggested to be suitable.
Assessment of conjugates

Conjugate II

This conjugate was titrated by serial dilution between 1:100 and 1:12800 against solid phase normal mouse serum as the positive serum and normal sheep serum as the negative control. Both diluted 1:8000 in ELISA coating buffer (see below). Coating buffer alone was used as the nonspecific binding control. Conjugate I at 1:6000 dilution was included (x4 wells) as conjugate control. The assay was set up as described in Method A below. Both polyvinyl chloride and polystyrene plates were used.

Conjugate III

The absorbance at 280 nm and 403 nm of each fraction eluted from the Sephadex G200 column was determined on an SP1800 Uniscan Spectrophotometer. The fractions having the required OD$_{403}$:OD$_{280}$ ratio of between 0.3 and 0.6 (Wilson and Nakane 1978) were pooled and titrated by serial dilutions between 1:100 to 1:12800 as for conjugate II.

Testing of solid phase antigen and microtitre plates

Polyvinyl chloride and polystyrene microtitre plates (Dynatech Laboratories Ltd, Billingshurst, UK) were assessed for their suitability for use in the indirect ELISA system.

The assay used initially in the nonspecific identification of hybridomas producing antibodies was designated Method A. Next, specific anti-TSH antibody secretors were identified from amongst those antibody secretors by the assay designated Method B.

ELISA buffers

These buffers are used throughout in all ELISA assays.

Coating buffer

0.1M carbonate bicarbonate buffer pH 9.6: 1.59 g Na$_2$CO$_3$, 2.93 g NaHCO$_3$ per litre distilled water. Two litres were made up each time and stored at room temperature for not more than 2 weeks.
Washing/diluent buffer

0.15M Phosphate buffered saline pH 7.4:- 8.0 g NaCl, 0.20 g KCl, 1.15 g Na$_2$HPO$_4$ and 0.20 g KH$_2$PO$_4$ were dissolved in one litre distilled water to which was added 0.5 ml Tween 20. 3 litres were prepared each time and stored at room temperature. At the time of use 0.1% (w/v) bovine serum albumin was added.

Substrate solution

Phosphate citrate buffer pH 5.0 was prepared consisting of 24.3 ml of 0.1M citric acid and 25.7 ml of 0.2M Na$_2$HPO$_4$ plus 50 ml distilled water. In this buffer 40 mg ortho-phenylenediamine was dissolved immediately before use and 40 μl of 30% H$_2$O$_2$ was added. The solution was thoroughly mixed. (The substrate is light sensitive and so must be handled with care).

Method A

Normal mouse serum and normal sheep serum were each diluted 1:500, 1:10$^4$, 1:5 x 10$^4$, 1:5 x 10$^5$, 1:5 x 10$^6$ in ELISA coating buffer. 150 μl of the diluted sera were added to the assay wells and incubated for 2 hours or 18 hours at 37°C in a Dynatech plate incubator. Wells in which mouse serum had been omitted were developed as assay blanks.

During this time the proteins in the coating solution were passively adsorbed onto the well surfaces.

The wells were then aspirated and washed three times with ELISA washing buffer, 200 μl/well. The plates were then inverted and tapped to remove excess liquid. Carry over of loose material from one stage to the next interferes with the precision of the assay.

The Conjugate I was diluted 1:6000 in washing/assay diluent buffer and 200 μl added to each well which were incubated for 2 hours at 37°C. The wells were then aspirated and washed alternately three times. Finally the ELISA substrate solution was added at 200 μl/well and incubated for 30 minutes at 37°C. The enzyme activity was then stopped by lowering the pH with 2.5M H$_2$SO$_4$ and the wells read on a spectrophotometer at 490 nm. (Dynatech Laboratories Ltd, Billingshurst, UK mini reader).
Method B

Aliquotes of freeze dried h-TSH were dissolved in ELISA coating buffer and diluted to give 2.0, 1.0 and 0.5 μg h-TSH/ml solutions.

200 μl samples were placed in the assay wells and incubated for 18 hours at 4°C in a humidity box after which the wells were aspirated and washed three times as in Method A.

150 μl of mouse anti-h-TSH, normal mouse sera diluted as for Method A in ELISA assay diluent, or diluent buffer alone used as blank control was added and incubated for 2 hours at 37°C in a Dynatech plate incubator. Conjugate I and substrate solution was then added as in Method A.

Variability of binding to solid phase

Wells were sensitized with 200 μl 0.1% (v/v) glutaraldehyde (BDH Chemical Ltd, Enfield, Middlesex) in coating buffer incubated for 3 hours at 37°C followed by washing three times with coating buffer. 200 μl of h-TSH diluted at 1.0 μg/ml was then incubated overnight.

The other set of wells remained unsensitized and 200 μl of 1.0 μg/ml h-TSH in coating buffer was incubated overnight. The remainder of the assay was as described above for Method B.

2.3 RESULTS

2.3.1 Immunoadsorbent Solid Phase RIA

Purified donkey anti-mouse immunoglobulin

At 280 nm an optical density of 1.0 (in a 1.0 cm cuvette) = 0.69 mg/ml gamma globulin. (Hudson and Hay 1980). Therefore the protein concentration of the purified donkey anti-mouse gamma globulin fraction was determined using this conversion factor. Thus:

\[ \text{OD}_{280} \text{ of a 1:100 dilution of the dialysed preparation, ie, 0.1 ml Ig solution + 9.9 ml PBS.} \]

\[ = 0.62 \]

\[ \text{OD undiluted sample} = 62.00 \]

Since 1.0 mg/ml Ig solution gives \[ \text{OD}_{280} = 1.45 \]

Therefore [Ig] in solution = 42.76 mg/ml

There were 50 ml dialysate = 2.14 g immunoglobulins.
Protein content of the immunoadsorbent

From the standard curve which was linear over the range tested (Lowry et al 1951) it was estimated that for immunoadsorbent A (assuming no loss of aminocellulose in the immunoadsorbent preparation), the protein bound was approximately 93.75 mg protein/g aminocellulose. This was 53.13% less protein bound than obtained by Gurvich et al (1961) by the same method.

Immunoadsorbent B on the other hand contained 237.5 mg protein/g aminocellulose. This was 18.75% higher protein bound than obtained Gurvich et al (1961).

Separation of bound label from free

In order to assess the amount of immunoadsorbent to be used in the assay, a series of dilutions of the immunoadsorbent was set up with the same amount of antibody and labelled hormone per tube.

1) Immunoadsorbent A

Figure 2.2a showed that the immunoadsorbent separated <33.3% of that expected using neat immunoadsorbent. At 1:8000 dilution of the pooled mouse anti-TSH 30% bound label was expected to be separated (see figure 3.3). The nonimmune mouse sera gave correspondingly low binding <1.0%.

After 18 hour incubation (figure 2.2b), the amount of bound label separated had increased to 16.5% using undiluted immunoadsorbent - still below that expected. The binding by nonimmune sera had also increased however.

A new preparation of the immunoadsorbent was produced with 253% more donkey anti-mouse immunoglobulin protein bound, in an attempt to increase the immunoadsorptive capacity of the immunoadsorbent. The % bound was calculated as follows:

\[
\% \text{ Bound} = \frac{\bar{X} \text{ CPM} - \bar{X} \text{ NSB}}{\text{total counts}} \times 100
\]

2) Immunoadsorbent B

Figure 2.3a showed a much improved separation of bound label from free. With this immunoadsorbent B the amount of bound label separated had increased from 16.5% observed with undiluted immunoadsorbent A to 52%
FIGURE 2.2 Dilutions of immunoabsorbent separation of labelled hormone bound by Pooled mouse α TSH (●) 1:8000, single mouse anti-TSH (o) 1:5000, and normal nonimmune sera (1:5000 ■, 1:8000 △); (a) label and antisera incubated for 2 hours; and (b) label and antisera incubated overnight = 18 hours. The immunoabsorbent was incubated for 2 hours at room temperature.
FIGURE 2.3  (a) Pooled mouse anti-TSH serum diluted 1:8000 (●) and normal mouse sera 1:8000 (Δ); incubated with labelled h-TSH for 18 hours followed by 2 hours incubation with immunoadsorbent B.

(b) Mouse anti methatrexate diluted 1:4 x 10^4 (○) incubated with labelled methatrexate followed by 2 hour incubation with immunoadsorbent B. (The error bars represent SEM for 3 pairs of samples.)
using the pooled mouse anti-TSH with 18 hours first incubation. However maximally bound label was separated with the immunoadsorbent diluted 1:5 with assay diluent. The maximum bound, separated with this immunoadsorbent was 55%; greater than expected using liquid phase double antibody separation (figure 3.3). The increased anti-mouse protein bound to the aminocellulose appears however to have not only increased the specific separation properties, but also to have increased the non-specific binding (NSB) ie, binding of label in the negative controls. With neat immunoadsorbent, NSB had increased to >10%. It is to be noted that the increase in binding in the negative controls is linear, increasing with increasing immunoadsorbent added (figures 2.2 and 2.3a).

The length of time of the first incubation period however also has an effect, since an overall increase in binding in the negative tubes was observed for 18 hours incubation at all concentrations of immunoadsorbent used (figure 2.3). The binding in the negative controls with 1:5 dilution of immunoadsorbent was however only 5.5%. Figure 2.3b is the curve obtained for the methotrexate separation.

Time course of antigen antibody binding

It became apparent from figure 2.2 that a 2 hour incubation of antibody and labelled hormone was not sufficient to produce a reasonable amount of binding above the negative control to give good reliability. This was especially as the antibody content of the preparations the assay was intended to detect was initially expected to be very small. Since time is of the essence once the hybrids are detected, a 5-day incubation time (Hall, Amos and Ormston 1971) was inappropriate but it was by no means certain that a 4 hour incubation (Soos and Siddle 1982) would be adequate in our system. A realistic overall assay time was required.

Figure 2.4a and b shows that there was no difference in the amount of label bound at either temperature with immunoadsorbents B. There was also no difference in the negative controls % bound. At least 18 hour incubation of label and antibody was indicated (figure 2.4b). This gave a reasonable assay time, not too prolonged so as to lose valuable time and possibly hybrids. The indication was for incubation at 4°C for longer incubation times.

Titration of $^{125}$I-h-TSH

100 μl of a 1:40 dilution of $^{125}$I-h-TSH hormone was recommended (Dr D Teale, Dept. Clinical Biochemistry, St Lukes Hospital, Guildford). The
labelled hormone was titrated to determine whether this quantity was optimal in this system. Duplicate totals and NSB tubes were set up for each label dilution.

Figure 2.5 shows that using a greater amount of label than that recommended had little effect on the % bound, but as less label was used a greater percentage was lost in the non-specific binding, while relatively more label was extracted as bound in the pellet. The recommended amount of label was therefore satisfactory.

**Time course immunoadsorbent binding and assay reliability**

Figure 2.6 shows that after 1 hour incubation then maximum separation of bound label was achieved, further extension of the incubation time does not further increase the binding.

The determination of the inter and intra assay coefficient of variation (CV) described in the next section is presented for the optimized assay in Table 2.3 below.

### 2.3.2 Enzyme-Linked Immunosorbent Assay

**Horseradish peroxidase conjugates**

The working dilution of the conjugates was determined to produce an assay system which could be completed within one day. A long conjugate and substrate incubation time will be required with highly diluted conjugates (Voller et al 1979). On the other hand where the results are required very quickly short incubation times may be combined with the use of more concentrated conjugates. The choice of conjugate concentration had to be taken in relation to the background binding that was produced. The higher the concentration of conjugate used the higher the expected background levels.

Conjugate I was used as reference. Diluted at 1:6000 it was determined that with a solid phase preparation of 1:8000 normal mouse serum it gave very strong positive results \( OD_{490} = 1.05 \) with very low background <0.20 (see figure 2.11). Both conjugate II and III was assessed relative to conjugate I therefore.

Corrected ELISA OD for ELISA method A was calculated = Absorbance of sample x 1.05/absorbance of reference.
FIGURE 2.4  (a) Separation of bound from free with immunoabsorbent A diluted 1:5
(b) Separation of bound from free with immunoabsorbent B diluted 1:5
In each case pooled mouse anti-TSH diluted 1:8000 (●, ■) was positive sera and normal mouse sera diluted 1:8000 (○, △) was negative sera. Incubation with the labelled h-TSH was at 4°C or at room temperature (RT) and with immunoabsorbent for 2 hours at RT.
% bound label separated with immunoadsorbent B

Labelled h-TSH dilution. Reciprocal x 10^-3

FIGURE 2.5: 100 μl of 125I-h-TSH titrated with pooled mouse anti-TSH (●) and normal mouse serum (▲) both diluted at 1:8000 and used at 100 μl/tube. Immunoadsorbent B diluted 1:5 was used at 200 μl/tube. The anti-TSH and label was incubated for 4 hours at 4°C and the immunoadsorbent incubated for 2 hours at room temperature.
FIGURE 2.6 Time course of immunoabsorbent separation of antibody bound label. Immunoabsorbent B diluted 1:5 and used at 200 μl/tube. (●) pooled mouse anti-TSH; (▲) normal mouse sera diluted 1:8000 was used at 100 μl/tube. The antiserum and 100 μl 125I-h-TSH was incubated for about 18 hours followed by incubation with the immunoabsorbent at room temperature for various time periods. The immunoabsorbent bound label was separated by centrifugation and the pellet counted SEM for n = 3 pairs.
1) Conjugate II

A dilution of 1:1600 of conjugate II gave the required assay characteristics outlined above. Specifically, the optical density of 1.16 was obtained with two 2 hour incubation periods (coating and conjugate binding), and a 30 minute substrate incubation time. The background binding obtained with a negative sera (normal sheep serum) was 0.10 in the polyvinyl chloride plates (see figure 2.7).
In the polystyrene plates the dilutions of conjugate was lower 1:1040 to give the same OD\(_{490}\) = 1.16 value under the same assay conditions. The final volume of this conjugate including glycerol was 5.6 ml.

2) Conjugate III

Figure 2.8 shows the OD\(_{280}\) and OD\(_{403}\) profile of the fractions eluted from the sephadex G200 fractions. The horseradish peroxidase content of each fraction is indicated by the OD\(_{403}\) pattern, whilst the total protein content is indicated by the OD\(_{280}\) profile.

Figure 2.7 shows that a dilution of 1:560 gave an OD\(_{490}\) in the ELISA test of 1.16 in the polyvinyl chloride plates when compared to conjugates I and II. This is to be expected since the final volume of the pooled fractions including glycerol was 40 ml, that was 7.14 times more dilute than conjugate II; and, in addition, only fractions with an OD\(_{403}\):OD\(_{280}\) ratio of between 0.3 and 0.6 were included in the pooled sample. Only optimal conjugate molecules were included in conjugate III which therefore did not contain all the antibody or horseradish peroxidase contained in conjugate II. The negative controls for the sephadex filtered conjugate produced very low optical densities at all dilutions. Both conjugates were also investigated for use in immunocytochemistry in Section 5.2.2.

The major advantage of filtering the conjugate over ammonium sulphate purification in the ELISA procedure appears to be a final conjugate preparation with a much reduced background. There was very little difference between the apparent bound in either plate types when Conjugate II was used, while the difference in apparent adsorption between the plates was more marked with Conjugate III.
FIGURE 2.7  Both conjugates titrated against normal mouse sera and normal sheep sera diluted 1:8000 on solid phase. Normal mouse sera (○,■); normal sheep sera (○,▲); On polyvinyl chloride plates (○,○) and polystyrene plates (■,▲). Incubation: 2 hour coating and 2 hour conjugate binding.
FIGURE 2.8 Sephadex G200 filtered fractions of Conjugate III

$OD_{280} = (\bullet)$ and $OD_{403} = (o)$. The column was 35 x 2.5 cm. Elution buffer: PBS 0.15M pH7.4. The fractions with $OD_{403} : OD_{280}$ of between 0.3 and 0.6 were pooled, i.e., fractions 34-40.
Assessment of adsorption to microtitre plates

Conjugate I was used at 1:6000 dilution. Figures 2.9 and 2.10 show that in method A the polyvinyl chloride plates adsorbed a greater amount of specific protein than the polystyrene plates after 18 hours incubation. The inclusion of bovine serum albumin in the washing buffer greatly reduced the background binding which was higher in the polyvinyl chloride plates compared with the polystyrene plates.

After 2 hour incubation at room temperature, binding to the polystyrene plate was minimal compared with the apparent binding in the polyvinyl chloride (PVC) plates (figures 2.11 and 2.12). After such a short incubation time the apparent binding to the PVC plates was in total also reduced compared, with that observed after 18 hours incubation, and the background non-specific binding was much higher in the polystyrene plates.

Assuming on average 12.0 mg/ml mouse immunoglobulin (Hudson and Hay 1980) in the serum, then the assay using polyvinyl chloride plate incubated for 2 hours at room temperature could detect as little as 0.3 μg/ml immunoglobulin which is a $1:4 \times 10^4$ dilution; and with a 18 hour coating incubation period again in polyvinyl chloride plates the detection was as little as 0.13 μg/ml a dilution of $1:9 \times 10^4$ of mouse serum.

The lowest positive result was taken as twice the highest negative value obtained. Reference positive and negative sera were included in all assay plates.

Hybridoma cultures were expected to produce up to 10 μg/ml immunoglobulins. The assay should therefore be able to detect even the early stages of antibody production. The intra assay % CV is presented in Table 2.3.

For the specific anti-TSH method B assay polystyrene plates only were used with 18 hour incubation with coating antigen solution. Figure 2.12 showed that protein binding after 2 hour incubation was not very high as compared with 18 hour incubation, figure 2.10. The background using polyvinyl chloride plates with antigen adsorbed in the indirect assay tended to be very high (Dr A Voller personal communication). Therefore a 18 hour coating incubation period was used to investigate the optimum bound antigen concentration to be used to detect the specific antibodies.

Figure 2.13 shows 0.5 μg/ml h-TSH gave almost as good a result as 1 μg/ml or 2 μg/ml. Although more binding was observed at 1 and 2 μg/ml it was not significantly higher.

The coefficient of variation (defined below) was unacceptably high; and in some cases edge effect phenomenon (Denmark and Cheesman 1978) was observed.
FIGURE 2.9 Polyvinyl chloride plates coated with various dilutions of
normal mouse serum wash without BSA (●)
normal mouse serum wash with BSA (○)
normal sheep serum wash without BSA (△)
normal sheep serum wash with BSA (▲)

Incubation of coating solution for 18 hours at 4°C. Conjugate I diluted 1:6000
SEM for n = 4.
FIGURE 2.10  Polystyrene plates coated with various dilutions of
normal mouse serum wash without BSA (●)
normal mouse serum wash with BSA (○)
normal sheep serum wash without BSA (△)
normal sheep serum wash with BSA (▲)
Incubated with coating solution for 18 hours at 4°C.  SEM for n = 4.
FIGURE 2.11 Polyvinyl chloride plates coated with various dilutions of normal mouse serum wash without BSA (●) normal mouse serum wash with BSA (○) normal sheep serum wash without BSA (△) normal sheep serum wash with BSA (▲)

Incubated with coating solution for 2 hours at room temperature. SEM for n = 4.
Figure 2.12 Polystyrene plates coated with various dilutions of:
- normal mouse serum wash without BSA (●)
- normal mouse serum wash with BSA (○)
- normal sheep serum wash without BSA (△)
- normal sheep serum wash with BSA (▲)

Coating solution incubated for 2 hours at room temperature. SEM for n = 4.
FIGURE 2.13 Adsorption of h-TSH to microtitre wells. Mouse anti-TSH was diluted 1:8000 (●); normal mouse serum 1:8000 (○--○) negative control. Conjugate I diluted 1:6000. SEM for n = 4.
Table 2.2 shows that glutaraldehyde sensitization (Stafford and Kilgallan 1980) of the plates prior to incubation of coating protein in the wells resulted in a greatly increased adsorption to the wells, evidenced by the 44.63% increased OD over unsensitized wells. To test whether the means of the two samples differed significantly the Students t-test was applied for samples where equal population variances cannot be assumed. A $t_{84} = 7.57$ was obtained which was very highly significant at <0.1% on a 1-tail test. Thus the glutaraldehyde treatment has provided a significantly better adsorption to the wells than when no pretreatment was used.

**TABLE 2.2**

Sensitization of wells with 0.1% Glutaraldehyde compared to unsensitized assay wells prior to coating with TSH antigen at a concentration of 1 μg/ml.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ELISA OD</th>
<th>(s)</th>
<th>(s²)</th>
<th>% C.V.</th>
<th>N</th>
<th>t</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutaraldehyde</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitized wells</td>
<td>1.3860</td>
<td>0.2242</td>
<td>0.0503</td>
<td>16.17</td>
<td>50</td>
<td>7.534 ***</td>
</tr>
<tr>
<td>+ve controls (A)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unsensitized wells</td>
<td>0.9583</td>
<td>0.2803</td>
<td>0.0786</td>
<td>29.25</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>+ve controls (B)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitized wells</td>
<td>0.0842±0.013</td>
<td>15.44</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-ve control (C)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unsensitized wells</td>
<td>0.077±0.014</td>
<td>15.94</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-ve control (D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$t = \frac{\bar{x} \text{ of A} - \bar{x} \text{ of B}}{\sqrt{\frac{s_1^2}{N_1} + \frac{s_2^2}{N_2}}}$

$= \frac{1.3860 - 0.9583}{\sqrt{\frac{0.0503}{50} + \frac{0.0786}{36}}} = \frac{0.04277}{0.0565} = 7.57$ ***
The coefficient of variation (CV) was determined as follows:

\[ CV = \left( \frac{SD}{\bar{x}} \right) \times 100 \]

SD = standard deviation; \( \bar{x} \) = sample mean; N is the number of samples.
The standard error on the mean (SEM) = SD/\( \sqrt{N} \).

The %CV was reduced dramatically from 29.25% to 16.17% with sensitization of the wells. The negative controls were not affected by the treatment and NSB remained acceptably low.

As a result of this observation the assay was optimised using 0.1% glutaraldehyde sensitized plates. Figures 2.14 and 2.15 show the adsorption profiles using different amounts of h-TSH antigen and different amounts of antisera.

It was decided to use 1.0 \( \mu \)g/ml h-TSH in coating solutions for the indirect ELISA since it gave the best binding curve (figure 2.15) with maximum binding and low NSB, figure 2.14.

**TABLE 2.3**

The reliability of the optimised assay procedures was demonstrated by the calculation of the intra and inter assay %CV. (The formula for the calculation of the standard Deviation in the inter assay %CV determination is presented in Chapter 5.) Normal mouse serum and normal sheep serum; mouse anti-h-TSH and normal mouse serum used in ELISA A and in ELISA B and the RIA respectively at a dilution of 1:8000.

<table>
<thead>
<tr>
<th>Assay method</th>
<th>sample mean x OD(_{490})</th>
<th>SD</th>
<th>N</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>intra assay</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ELISA A</td>
<td>1.140</td>
<td>0.099</td>
<td>48</td>
<td>8.7</td>
</tr>
<tr>
<td>ELISA B</td>
<td>1.386</td>
<td>0.224</td>
<td>50</td>
<td>16.17</td>
</tr>
<tr>
<td><strong>inter assay</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solid phase RIA</td>
<td>48</td>
<td>2.85</td>
<td>10</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>49.5</td>
<td>6.12</td>
<td>3</td>
<td>12.4</td>
</tr>
</tbody>
</table>
FIGURE 2.14 Binding of h-TSH to 0.1% glutaraldehyde sensitized polystyrene plates: Mouse anti-TSH 1:8000 (●); 1:16000 (○) and 1:32000 (■) and corresponding normal mouse negative controls 1:8000 (●—●), 1:16000 (○—○) and 1:32000 (■—■). Conjugate I used at a dilution of 1:6000; SEM for n = 4.
FIGURE 2.15  h-TSH coating plate at 0.5 μg/ml (△)
1.0 μg/ml (●) and 2.0 μg/ml (○). Incubated with various dilutions of
mouse anti-human TSH anti sera and
normal mouse sera as negative
controls. Wells presensitised with
0.1% Glutaraldehyde.
The inter-assay variation in the ELISA methods was controlled by including reference positive samples in all assay plates (Voller 1979).

The corrected OD490 values for ELISA method B were calculated:

\[ \text{OD}_{490} = \text{Test OD value} - \text{Blank control} \times \frac{1}{\text{Absorbance of reference positive}}. \]

The reference positive sample chosen to give an \( \text{OD}_{490} = 1 \) consisted of solid phase antigen at 1 \( \mu \text{g/ml} \); mouse anti-h-TSH diluted at 1:12000 and conjugate I diluted at 1:6000 (see figure 2.15).

2.4 DISCUSSION AND CONCLUSIONS

It was necessary to develop the solid phase RIA and the ELISA for screening the hybridoma cultures. The liquid phase RIA was used to screen the mice sera after immunisation (see Chapter 3), but was found to be unsuitable for detecting low levels of hybridoma antibody produced in the early stages of growth.

2.4.1 Immunoabsorbent

With the solid phase immunoabsorbent separation system, separation of bound label from free label was achieved merely by centrifugation. Optimization of the complex precipitation conditions and such problems as prozone which may occur in the second antibody precipitation of antigen-antibody complex (Midgley 1969) was avoided.

This separation system also avoided many of the problems related to obtaining and characterization of a good precipitating second antibody. Not all antiglobulins are suitable for use as precipitating second antibodies (Morris 1981).

In addition the need for carrier serum derived from the same species as the primary antibody to assist precipitation was circumvented.

Hunter (1979) suggested that this immunoabsorbent method characteristically gave high precision possibly because the separation step was well defined therefore misclassification error was minimised. No system completely separates the free from the bound label into distinct compartments and variability in this contributes to error in the percentage bound separated compartment. By reducing the variability by clearly defining the separated phases, the assay can overcome these difficulties observed in the conventional second antibody system.
Providing the immunoadsorbent binding sites are not limited all primary antibody with or without label bound should be removable from solution. Even very minute quantities of specific antibody should therefore be detectable by bringing down bound label in a defined and precise way. Misclassification in the procedure of monoclonal antibody production can be time consuming and very costly.

One major disadvantage of the immunoadsorbent separation system however may be in the possible loss of avidity due to the coupling of the immunoadsorbent and concomitantly lead to loss of sensitivity (Arends 1971). Hales and Woodhead (1980) have however experienced no such loss of immunoreactivity.

Immunoadsorbent B is that which was chosen for use. It gave a high percentage bound with minimal non-specific adsorption at 1:5 dilution. The low non-specific adsorption appears to be characteristic of this type of immunoadsorbent (Gurevich et al 1961 and Wide 1969). Gurevich et al (1961), Addison (1972), Hales and Woodhead (1980) used 1.0 g protein antigen per 1.0 g diazotized cellulose while Wide (1969) used immunoglobulin precipitated from 0.1 ml antisera with 100-400 mg activated polymer to achieve a satisfactory immunosorbent. For this work 0.5 g and 1.5 g precipitated immunoglobulins were used. The immunosorbent B gave satisfactory results. It is important to note that Gurevich et al (1961) suggested increasing the bound protein does not necessarily increase the capacity (the amount of protein capable of uniting with 1.0 g aminocellulose) of the immunoadsorbent. Possibly due to increased steric hinderance of binding with increased protein bound to the immunoadsorbent. However, with a possibly low titre antisera, then increasing the amount of precipitated protein may also increase the amount of specific binding. The capacity of the aminocellulose is however limited. Only approximately one active site exists per 40 glucose residue (Hales and Woodhead 1980).

This assay was fairly rapid compared to a standard h-TSH RIA (Hall et al 1971) which could take up to 5 days before the results were to hand. Approximately 20 hours and this assay was complete. The procedure was greatly simplified with the use of the immunoadsorbent; and in terms of cost, while using a great deal of second antibody in the production of the immunoadsorbent (the unbound protein was saved from the washing procedures and may have been reused), this system did not require any additional sera or precipitation accelerating agents. It was therefore very cost effective.

The final assay procedure therefore was as detailed in Chapter 3.2.4. The assay was very reliable with an inter assay CV of 5.9% and an intra assay CV of 12.4%.
2.4.2 Indirect ELISA

The working dilutions of conjugates II and III were found to be 1:16000 and 1:560 respectively. Both conjugates performed satisfactorily in the microtitre plate ELISA assay. However, the sephadex G200 filtered conjugate gave consistently lower non-specific binding in the negative controls. Both ammonium sulphate and G200 purification were just as easily done. There was very little difference between them in use except for the dilution factor and the background difficulties found mainly in use in immunocytochemistry (see Chapter 5.3.1). For use in ELISA the ammonium sulphate method is quite adequate. There is less material to store and the conjugate is therefore easier to store in the more concentrated state. Both conjugates were still very active after 1.75 years stored as described at 4°C.

It must be noted here that sodium azide inhibits horseradish peroxidase and therefore should not be used as the bacteriostat (Voller et al 1979). We have found that thimerosal is very suitable for this purpose.

The primary demands on these assays were that they were quick to perform, reproducible, sensitive and amenable to large scale sample analysis preferable in a semiautomatic procedure.

With these in mind the microtitre plates were chosen for use in the ELISA assay. Washers, incubators, multipipetters and spectrophotometer equipment are available commercially for these plates therefore simplifying the procedure enormously.

McLaren et al (1981) summarizing the sources of error in the indirect ELISA have indicated that low sensitivity can be the result of any or all of the following:-

1) The choice of the solid phase support: hence the investigation of the polyvinyl chloride vs the polystyrene plates. The polyvinyl chloride plates were chosen for 2 hour coating with antibody solution for the initial non-specific screening assay. The low background non-specific binding and higher protein binding capacity evidenced by the higher OD compared to that observed in the polystyrene plates dictated their choice.

The polystyrene plates were chosen for coating with antigen over a 18 hour incubation period. This was convenient as the coating procedure was arranged for the day before the actual screening was due to be done. Polystyrene plates exhibited low non-specific binding with high specific binding over this period (figure 2.10) and were generally suggested to be better for specific antigen binding (verbal communication A Voller).

2) Antigen concentration of the coating solution is critical. Undercoating gave very low binding (figures 2.14 and 2.15). While overcoating results in the high dose hook effect (Miles and Hales 1968) (figure 2.14).
1.0 µg/ml h-TSH antigen (figure 2.15) in this work was found to be optimum giving a proportional response over the range measured (figure 2.15). Too high or too low antigen coating may result in low or very high concentrations of antibody not being accurately detected (Figure 2.14). This effect was also demonstrated by Engvall and Perlmann (1972). The optimal antigen coating concentration and adsorption times must therefore be defined for each assay (Herrmann and Collins 1976).

3) As suggested in Section 2.3.2 the effectiveness of the conjugate used is important bearing in mind the constraints on this assay, the conjugate titres were chosen therefore to yield an observable maximum response in the shortest possible time. Because the observable activity of an enzyme is affected by many factors, the chief of those, being the enzyme and substrate concentration, temperature, pH, the presence of enzyme inhibitors and incubation time, these must be strictly controlled.

4) The solubility of the enzyme substrate (Voller et al 1979) is critical. Hence orthophenylene diamine was used.

Two additional characteristics of importance in this assay were the assay specificity and the precision. The specificity would of course be affected by the quality of the antigen used on the solid phase. Highly purified h-TSH was kindly donated for this work (see Section 2.2.3). The non-specific binding effects were negligible and each step in the assay was carefully controlled.

There was difficulty with the precision of ELISA method B to begin with, and after eliminating experimenter's errors (Woodhead et al 1981) such as pipetting errors involved in dilution and filling wells and the elimination of carry over of material from one step to the next, it was decided that the poor precision observed was due to variable adsorption of protein by the polystyrene wells (Wisdom 1976, Denmark and Chessum 1978, Herrmann and Collins 1976). Glutaraldehyde treatment as described by Stafford and Kilgallan (1980) was found to alleviate the problem. A satisfactory procedure (detailed in Section 3.2.4) was therefore established within the constraints set by the technology for screening purposes.

Figure 2.16 is a photograph of a developed ELISA plate.

Both solid Phase RIA and the indirect ELISA systems are suitable for monitoring the immune response to h-TSH as well as screening for specific monoclonal antibodies. But it must be remembered that the antimouse immunoadsorbent or the antimouse enzyme label may be used as universal reagents to detect any specific antibodies raised in mice or that are mouse originated monoclonals. The assay universality of the immunoadsorbent was demonstrated in the methotrexate assay.
Conjugate II titration in row A, B and C; with conjugate I reference in rows D. Rows E, F and G contain normal sheep sera and is negative control for conjugate II. Row H is negative control for Conjugate I. Buffer blank was the final 2 wells row C.
CHAPTER 3

The Generation of Monoclonal Antibodies
Against human Thyroid Stimulating Hormone (h-TSH)
3.1 INTRODUCTION

Monoclonal antibodies are derived by the fusion of biochemically marked permanently growing myeloma cells and antigen primed spleen cells.

The myeloma cell lines available have been discussed in Chapter 1. For this work P3-NSI/1-Ag4-1 (NSI) was used.

The specific antibody secreting cells on the other hand have to be induced in appropriate animals. For this work Balb/c female mice were used as specific antibody secreting spleen cell donors.

The work described in this chapter details the induction of specific antibody secreting spleen cells and the monitoring of the immune response. The growth characteristics of NSI cells was examined under normal growth conditions; in HAT supplemented media and fungicide treated media.

Finally experiments were performed to secure specific anti-h-TSH monoclonal antibodies. Strict aseptic technique was used in all culture procedures.

3.2 MATERIALS AND METHOD

3.2.1 Preparation of Spleen Cell Donors

Twenty female Balb/c mice (supplied by Little Lions Farm, Ashley Heath, Ringwood, Herts) aged approximately 12 weeks were prepared in three groups A, B and C by repeated immunization of the spleen cell donors. The immunization schemes and inocula contents were as outlined in Table 3.1. The inocula was made up as water-in-oil emulsion and each animal was given 0.2 ml intra-peritoneally (IP).

21-28 days after each inoculation the next boost was given. The animals were marked with an indelible marker on the tails and the highest responders after the final challenge were used for fusion.

Serum collection

Blood was collected by retroorbital puncture 14-18 days after each inoculation. The blood was separated and the serum stored as described in section 2.2.2 until required.
### TABLE 3.1
Immunization schemes and immunogen content

<table>
<thead>
<tr>
<th>Immunization of Group A animals</th>
<th>Immunogen content per animal</th>
<th>Site of injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>h-TSH µg</td>
<td>Sterile H₂O µl</td>
</tr>
<tr>
<td>1°</td>
<td>10.0</td>
<td>60</td>
</tr>
<tr>
<td>2° - 4°</td>
<td>5.0</td>
<td>65</td>
</tr>
<tr>
<td>4 days prior to fusion</td>
<td>5.0</td>
<td>100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group B animals</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1°</td>
<td>10.0</td>
<td>60</td>
</tr>
<tr>
<td>2°</td>
<td>5.0</td>
<td>65</td>
</tr>
<tr>
<td>3°</td>
<td>10.0</td>
<td>65</td>
</tr>
<tr>
<td>4 days prior to fusion</td>
<td>5.0</td>
<td>100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group C animals</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1°</td>
<td>15.0</td>
<td>60</td>
</tr>
<tr>
<td>2°</td>
<td>10.0</td>
<td>65</td>
</tr>
</tbody>
</table>

**h-TSH radioimmunoassay**

The progress of the response to the immunization was monitored by the presence of anti-h-TSH antibodies in the serum by RIA. This was done with the kind assistance of Miss Jane Guthridge, West Sussex Area Health Authority, Hurstwood Park Neurological Centre, Haywards Heath.

**Reagents**

a) **Assay diluent:**

RIA assay diluent consisted of 0.01M Phosphate buffered saline pH 7.4.
b) Labelled hormone

$^{125}$I-TSH diluted with assay diluent as in section 2.2.3. The assay diluent contained human chorionic gonadotrophin (HCG) (Dr R Edwards, St Bartholomews Hospital, London) at a concentration of 150 U/ml.

c) Second antibody

Donkey anti-mouse (Guildhay, University of Surrey) was used at 1:10 dilution.

d) Antiserum dilution

The mouse antiserum and positive control Guildhay Sheep anti-TSH were diluted with assay diluent 1:500 up to 1:50,000, immediately prior to the assay.

Method

To duplicate LP3 tubes (Luckham Ltd, Burgess Hill, Sussex) was added 100 µl diluted antiserum and 100 µl labelled hormone. This was mixed thoroughly and incubated overnight at room temperature. Then 100 µl second antibody was added followed by 100 µl normal mouse serum and 200 µl 12.5% (v/v) polyethelene glycol (PEG). The tubes were then vortexed and incubated at room temperature for 30 minutes after which they were centrifuged for 10 minutes at 2000 rpm. The pellets were counted for 100 seconds.

3.2.2 Maintenance and Investigation of NSI Myeloma Cells

NSI myeloma cells were maintained routinely in tissue culture flasks (3050 or 3075 Costar via Howell Ltd UK) in standard culture media at densities of between $2 \times 10^5$ and $8 \times 10^5$/ml. They were divided every two to three days and fresh medium added. The flasks were then placed at 37°C in a humid 5% CO$_2$ in air environment.

Culture media

Both myeloma cells and hybridoma cells were maintained in liquid culture media.
The standard culture medium used throughout was made up as follows:
88 ml RPMI-1640 with L-Glutamine (Gibco Bio-cult Ltd, Paisley)
10 ml Foetal calf serum (Gibco Bio-cult Ltd) heat inactivated and
mycoplasma screened.
1.0 ml = 1 mM sodium pyruvate (Flow Laboratories Ltd, Ayrshire, UK)
1.0 ml = 50 units penicillin/50 μg streptomycin (Flow Laboratories Ltd)
For all cell manipulations the culture medium was pre warmed to 37°C.

Cell viability

Cell viability was determined generally by trypan blue exclusion test
Tennant (1964).
Trypan blue 0.4% solution was supplied by Flow Laboratories Ltd. One
part cell suspension was added to one part dye solution which was allowed to
stand for 5 minutes before the cells were examined.

\[
\text{% viability} = \frac{\text{average number of live cells counted} \times 10^4}{\text{average total cell numbers counted} \times 10^4} \times 100
\]

Cell viability was also assessed using the phase contrast microscope
(0i and Herzenberg 1980).

Freezing and recovery of cells

Freezing and recovery of NSI cells was as described by Nabholz (1980).
Cells in log phase growth were spun down gently on the MSE bench
centrifuge, 1000 rpm for 5 minutes, and resuspended at 2 x 10^6 cells/ml in
standard culture media. 20% (v/v) dimethylsulfoxide (DMSO) (supplied by BDH
Chemicals Ltd, Enfield) in standard culture medium was prepared fresh.
Into each labelled freezing screw capped plastic vial (supplied by Flow
Laboratories, Ayrshire) was placed 0.5 ml cell suspension and 0.5 ml DMSO
cryopreservation medium. The tightly closed vials were then placed in a
styroform insulating box and placed at -80°C overnight. The vials were then
transferred to liquid nitrogen storage at -196°C.

Hybridomas were treated in a similar manner except the cryopreservative
consisted of 10% (v/v) DMSO and 90% (v/v) foetal calf serum, and the cells
were resuspended directly in this mixture.
For thawing, the vials were retrieved from the liquid nitrogen store and
brought rapidly to 37°C in a water bath following which 10 ml standard medium
also warmed to 37°C was added slowly with continual agitation. The cells were then pelleted and resuspended in 2 mls fresh medium and placed in an incubator.

**NSI growth curve**

NSI growth characteristics, that is the optimum density and doubling time were determined as described by Hudson and Hay (1980). $2 \times 10^4$ NSI myeloma cells were suspended in 10 ml standard culture medium and incubated. Three such cultures were set up. The cell numbers and viability were determined each day and the cells resuspended in fresh medium. This was continued until the cultures began to decline both in viability and cell numbers. The hybridoma growth characteristics were similarly derived.

**NSI sensitivity to HAT**

$2 \times 10^5$ cells/ml were suspended in standard media containing HAT. (HAT was supplied as x50 strength solution by Flow Laboratories Ltd, Ayrshire and was therefore diluted 1.0 ml HAT in 50 ml standard medium to give x1 normal strength HAT.) The cell numbers and viability was determined every 2-3 days when the medium was renewed. NSI cells in HAT medium was also set up with each fusion experiment.

**NSI sensitivity to a fungistat agent**

Unfortunately even with the most rigorous aseptic technique in long term culture, fungal and mould contamination can prove a major and persistent problem. The spores are airborne and are very resistant to heat, alcohol and the usual cleansing agents (Goding 1980). Although badly contaminated cultures are usually discarded, occasionally a culture is too valuable to lose, or it becomes necessary to protect other cultures where contamination is a persistent problem. Any antifungal agent must therefore of necessity exert only minimal toxic effects on the cells in culture while at the same time exerting the maximum antimycotic effects. NSI is one of the proposed fusion partners and therefore represents a suitable model cell system on which to test the agent.

Econazole was chosen as the antimycotic agent because of its reported advantages of broad antifungal, antibacterial activity and broader margin between cytotoxic and inhibitory concentrations, its higher stability and solubility, when compared to the popularly used fungizone (Wyler et al 1979).
NSI viability, cell proliferation and DNA synthesis are excellent indicators of the condition of the culture and as such these parameters were examined under the influence of econazole dose recommended for use at 1 μg/ml (Wyler 1979).

a) Viability in econazole

2 x 10^5 NSI cells/ml were cultured in 5 ml of standard culture media in 25 ml tissue culture flasks (Costar via Howell Ltd). Two cultures were set up:-

1) The test culture, maintained in standard growth medium supplemented with 1 μg/ml = 10 μl econazole solution/100 ml medium; (econazole lactic; econazole base 1-(2,4-dichloro-β-(p-chlorobenzyloxy)-phenetyl)-imidazole (10 mg/ml) lactic acid (20 mg/ml) in aqueous solution; 20 ml was kindly donated by Cilag-Chemic Ltd, Scheffhausen, Switzerland).  

2) The control culture was grown in unsupplemented standard culture medium.

The Cells were maintained for 7 days. Cell viability was assessed as stated above and the % survival was also determined by:-

\[
\text{% survival} = \frac{\text{Number of live cells in test}}{\text{Number of live cells in control}} \times 100
\]

The viability of the cultures was also determined after the cells had been cultured in various concentrations 0-1 μg/ml of econazole for 48 hours. The cells were incubated in 1.0 ml cultures in lymphocyte culture tubes (Nunc).

b) DNA synthesis in econazole

2 x 10^5 NSI cells were cultured in 1.0 ml volumes in lymphocyte culture tubes (Nunc). The cultures were incubated in standard culture medium supplemented with various concentrations of econazole 0-1 μg/ml. One set of duplicate cultures was incubated with econazole and 5 μCi ³H-thymidine (Radiochemical Centre, Amersham International PLC, UK) per tube for 48 hours, while the second set was incubated with econazole alone for 46 hours then 5 μCi ³H-thymidine was added per assay tube for the final 2 hours. The cells were then spun down and 2.0 ml 10% (w/v) trichloracetic acid (TCA) added to each pellet. The cells were then harvested on 2.5 cm Whitman glass fibre papers. These were washed twice with 10% TCA and then air dried before counting for 10 minutes in 8 ml scintillant.
Scintillation Fluid:

The scintillant was made up as follows:

- Toluene: 3.33 l
- Metapol HC 100: 1.67 ml
- 2,2'-p-Phenylene-bis (5-phenyloxazole) (PoPoP): 1.00 g
- 2,5-Diphenyloxazole (PPO): 25.00 g

Each powder was mixed thoroughly in the toluene before the next was added. When all the ingredients were finally added the mixture was stirred for about 24 hours before use. The preparation was stored in a dark bottle.

3.2.3 Hybridoma Production

A great many protocols exist. However they are all very similar with only minor variations in the PEG fusion regime described. The most important of these include the addition of DMSO investigated by Fazekas De St Groth and Scheidegger (1980), and variations in the molecular weight of the PEG used and the fusion incubation time (Davidson et al 1976, Gefter et al 1977, Pontecorvo 1977).

Preparation of NSI for fusion

NSI cells were maintained routinely as described above so that they were kept in exponential growth continually. On the day before they were required for fusion (day -1), the cells were counted and the viability determined. Below 90% viability the culture was usually not used. The cells were split to $1 \times 10^5$/ml in 50 ml standard culture media. (This allowed on average one complete division of the cells in the culture by the time they were required. About $10^7$ cells were required for fusion.) On the day of fusion (day 0) the viability was rechecked and the cells resuspended in fresh medium in a conical Falcon centrifuge tube. They were then kept in the incubator until required.

Preparation of immune spleen cells/feeder layer cells

The hyperimmunized female Balb/c mouse/control mouse (for feeder layer) was killed by cervical dislocation. It was placed on a dissecting board right side down and drenched with 70% (v/v) alcohol. The spleen - a dark red bean shaped organ was removed using sterile procedure. The spleen was transferred to a sterile petri-dish containing 10 ml standard medium. The
A dish containing the spleen was placed in the laminar flow cabinet in which all further work was done. The spleen was cut into pieces and teased apart through a wire sieve to release the spleen cells. These were collected in a Sterilin centrifuge tube and the suspension allowed to stand for 5 minutes to allow the large lumps to settle out. The supernatant containing the suspended cells was decanted into another tube and centrifuged for 5 minutes. The cells were then resuspended in 5.0 ml fresh standard medium.

0.5 ml spleen cell suspension was diluted to 10 ml in Tuerks solution. The number of cells in this preparation was then counted. The feeder layer spleen cells were prepared on day-1 and diluted such that 2 mls of $1.5 \times 10^5$ cells/ml or 200 μl of $1.5 \times 10^5$ cells/ml were plated into 24 or 96 well culture plates respectively and incubated. The hyperimmune cells for fusion were prepared on day 0. The cell numbers were determined as follows:

Average cells counted = $\bar{x}$

Number of cells/ml = $\bar{x} \times 10^4$

Cell dilution = 1/20

hence $\bar{x} \times 10^4 \times 20$ cells/ml

5 ml spleen cell = $\bar{x} \times 10^4 \times 20 \times 5$ total cells/spleen

Tuerk solution: - 1.0% (v/v) glacial acetic acid which destroys erythrocytes, and a tinge of gentian violet which stains leucocytes.

Fusion solutions

1) 50% (w/v) polyethelene glycol (PEG) molecular weight 1500 (supplied by BDH Chemicals Ltd, Enfield, Middlesex) in serum free medium (Oi and Herzenberg 1980). This was made up as follows: 12.5g PEG was autoclaved and kept hot. To this was added 12.5 ml warmed serum free medium.

2) The fusion solution was later modified as follows:

a) 41.6% PEG 1500 containing 15% DMSO in serum free medium. This was made up so that 50 g PEG 1500 was sterilized with 15 ml DMSO to which was added 55 ml serum free medium;

b) the second solution consisted of 25% PEG 1500 in serum free medium made up of 12.5 g autoclaved PEG 1500 plus 37.5 ml serum free medium (Sikora et al 1982).
3) This fusion solution was made up of 12.5 g PEG 4000 (BDH Chemicals Ltd) which was autoclaved with 11.5 ml normal saline (Normal saline 0.14M consisted of 8.5 g sodium chloride per litre of distilled water), and 1.25 ml DMSO (BDH Chemicals Ltd). This constituted a 50% PEG solution containing 5% DMSO (Fazekas de St Groth and Scheidegger 1980).

Fusion and plating out

All solutions were used at 37°C. The two cell suspensions - NSI and the hyperimmune spleen cells were mixed thoroughly in a large 50 ml Falcon centrifuge tube and the supernatant thoroughly drained off after the cells were pelleted.

a) TSH fusion 1 and 2

The pellet was loosened by tapping the container and to the cell mixture was added 0.8 ml fusion solution 1 whilst stirring continuously. The PEG solution was added over 1 minute. Then 10 ml serum free medium was added very slowly over a period of 5 minutes. The fusion mixture was then pelleted gently and resuspended in 10 ml standard medium. This was made up to 100 ml so that 1 ml of this fusion mixture was distributed to each culture well containing feeder cells from which 1 ml of supernatant had been removed.

b) TSH fusion 3 - 6

To the loosened mixed cells 0.5 ml of fusion solution 2a was added. The cells were gently agitated for no more than one minute. Next 0.5 ml fusion solution 2b was added to the cells with gentle agitation for 2-3 minutes. 4 ml medium containing 20% (v/v) foetal calf serum was then added followed by 20 ml medium containing 20% (v/v) foetal calf serum. 1.5 ml supernatant was removed from the wells containing feeder cells and replaced with 0.25 ml fusion mixture topped up finally to 2 ml with medium containing 20% (v/v) foetal calf serum.

c) TSH fusion 7 and 8

The procedure followed was essentially the same as in TSH fusion 1 and 2 with the exception that fusion solution 3 was used. For fusion 7 the fusion mixture was suspended in a total volume of 60 ml and plated out 100 μl per well after 100 μl supernatant had been removed from the wells containing feeder cells.
The plates were then incubated at 37°C in a humidified 5% CO₂ in air incubator. The cell content of each fusion has been tabulated in Table 3.2, and figure 3.1 summarises the fusion protocol and hybrid maintenance used.

**HAT selection**

On day 1, one half the culture medium volume was removed from each well and replaced with twice strength (HAT x2) HAT in standard culture media.

(HAT x2 = 2 ml HAT x50 concentrate in 50 ml standard medium)

The plates were incubated as described and every 2 days thereafter one half of the medium was replaced with x1 HAT in standard medium.

On day 12 the HAT was replaced by x1 HT solution in standard medium. (HT was also supplied by Flow Laboratories Ltd as a x50 solution.) HT supplement was continued for about 8 weeks in continuous culture. When early fusion products were recovered from frozen stocks this was done in HT supplemented medium which was maintained for a similar period.

**Expanding positive cultures/clones**

As soon as positive wells were identified the cells were split and placed with feeder cells into progressively larger cultures, ie from 200 μl cultures to 1-2 ml cultures, then on to 5-10 ml cell cultures and finally to 50 ml culture volumes. The master wells were maintained for comparison in future assays. The medium was replaced every 2 days as previously described.

**3.2.4 Screening Assays**

Fusions 1-6 were screened by the standard RIA described above and used for monitoring the antibody response to h-TSH. The only exception was that HCG was omitted and the samples were counted in the first instance for 10 seconds, and later for 100 seconds. The double antibody assay precipitation was checked by reassaying some culture supernatants neat or diluted 1:50. Finally the assay was set up as described; but the phase separation was accomplished by the addition of 1 ml 20% (w/v) PEG and 100 μl normal non-immune mouse serum. The tubes were mixed and incubated at room temperature for 10 minutes after which they were centrifuged for 20 minutes at 2000 revolutions per minute (rpm). The pellets were counted for 300 seconds.
Day 0

**Feeder layer in culture wells** $3 \times 10^5$ non-immune spleen cells per well

**FUSION**

- 0.8 ml warmed 50% PEG 4000 in normal saline + 5% DMSO.
- Cells allowed to agglutinate for no more than 1 minute.
- 10 mls serum-free medium added slowly over 5 minutes.

Centrifuge mixture setting 2.
MSE bench centrifuge for 5 minutes.

Resuspend fusion mixture carefully.
Plate out into chosen number of wells.

Day 1

Replace $\frac{1}{2}$ culture medium with HAT x 2.

Day 2 until Day 11

- Replace $\frac{1}{2}$ culture medium with HAT x 1.
- Renew $\frac{1}{2}$ culture medium every 2 days thereafter.

Day 12

Replace HAT x 1 medium with HT x 1 medium at first cell expansion.
Renew medium every 2 days.
The positive control in the RIA was mouse anti-TSH at 1:500 dilution. Normal nonimmune mouse sera diluted 1:500 and NSI spent culture media was the negative control.

Fusion 7 and 8 were screened by the ELISA and solid phase RIA developed specifically for this purpose.

**Enzyme-linked immunosorbent assay (ELISA)**

The ELISA methods utilized were:-
Method A - for the detection of any antibody secreting hybridomas.
Method B - for the identification of those hybridomas secreting specific anti h-TSH antibodies.

In method A duplicate polyvinyl chloride microtitre wells were coated with hybridoma supernatant diluted 3:1 in ELISA coating buffer, and duplicate positive control mouse anti-TSH and negative control normal sheep sera diluted 1:8000 in coating buffer were incubated for 2 hours at 37°C.

**TABLE 3.2**

<table>
<thead>
<tr>
<th>Fusion No.</th>
<th>× number of cells/fusion</th>
<th>NSI myeloma</th>
<th>Spleen cells</th>
<th>% Viability</th>
<th>Ratio NSI: Spleen</th>
<th>No. wells plated</th>
<th>Fusion solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSH 1</td>
<td>1.0 x 10^7</td>
<td>1.05 x 10^8</td>
<td>93.7</td>
<td>1:10.5</td>
<td>96</td>
<td>50% PEG 1500 in serum free medium.</td>
<td></td>
</tr>
<tr>
<td>TSH 2</td>
<td>1.0 x 10^7</td>
<td>9.6 x 10^7</td>
<td>90.4</td>
<td>1:9.6</td>
<td>96</td>
<td>41.6% PEG 1500 + 15% DMSO in serum free medium and PEG.</td>
<td></td>
</tr>
<tr>
<td>TSH 3</td>
<td>1.7 x 10^7</td>
<td>1.74 x 10^8</td>
<td>93.14</td>
<td>1:10.2</td>
<td>96</td>
<td>50% PEG 1500 in serum free medium.</td>
<td></td>
</tr>
<tr>
<td>TSH 4</td>
<td>1.0 x 10^7</td>
<td>9.9 x 10^7</td>
<td>93.3</td>
<td>1:9.9</td>
<td>96</td>
<td>50% PEG 1500 in serum free medium.</td>
<td></td>
</tr>
<tr>
<td>TSH 5</td>
<td>1.5 x 10^7</td>
<td>1.5 x 10^8</td>
<td>82.9</td>
<td>1:10</td>
<td>96</td>
<td>50% PEG 1500 in serum free medium.</td>
<td></td>
</tr>
<tr>
<td>TSH 6</td>
<td>1.3 x 10^7</td>
<td>1.3 x 10^8</td>
<td>87.6</td>
<td>1:10</td>
<td>48</td>
<td>50% PEG 1500 in serum free medium.</td>
<td></td>
</tr>
<tr>
<td>(Picked up fresh NSI from Frozen store)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSH 7</td>
<td>2.2 x 10^7</td>
<td>2.3 x 10^8</td>
<td>93.1</td>
<td>1:10.5</td>
<td>576</td>
<td>5% DMSO in normal</td>
<td></td>
</tr>
<tr>
<td>TSH 8</td>
<td>1 x 10^7</td>
<td>1.04 x 10^8</td>
<td>94.2</td>
<td>1:10</td>
<td>96</td>
<td>5% DMSO in normal</td>
<td></td>
</tr>
</tbody>
</table>
In method B glutaraldehyde sensitized polystyrene microtitre wells were coated overnight with a solution of 1.0 μg h-TSH/ml in coating buffer. The plates were incubated in a humidity box in the cold room (about 4°C).

The wells were washed as described in Chapter 2. 100-150 μl hybridoma supernatant was added to duplicate wells according to the arrangement below in Table 3.3 and incubated for 2 hours at 37°C.

**TABLE 3.3**
Microtitre plate layout for ELISA method B

<table>
<thead>
<tr>
<th>Well number</th>
<th>Test antigen</th>
<th>Antibody</th>
<th>Antimouse HRPO conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 - 88</td>
<td>+</td>
<td>hybrid supernatant</td>
<td>+</td>
</tr>
<tr>
<td>89 - 90</td>
<td>+</td>
<td>(a) positive control sera</td>
<td>+</td>
</tr>
<tr>
<td>91 - 92</td>
<td>+</td>
<td>(b) negative control sera</td>
<td>+</td>
</tr>
<tr>
<td>93 - 94</td>
<td>+</td>
<td>(b) negative control</td>
<td>+</td>
</tr>
<tr>
<td>95 - 96</td>
<td>-</td>
<td>positive control sera</td>
<td>+</td>
</tr>
</tbody>
</table>

a = mouse anti-h-TSH diluted 1:12000  
b = normal nonimmune mouse sera diluted 1:12000 or NSI supernatant

Following this stage the methods were identical. The wells were washed as before and 200 μl goat antimouse-horseradish peroxidase diluted 1:6000 or donkey antimouse-horseradish peroxidase diluted 1:1600 in ELISA assay diluent was added to the wells which were incubated for 2 hours at 37°C. Following washing 200 μl substrate solution (section 2.2.3) was added to the wells and incubated for 30 minutes at 37°C. The reaction was stopped by the addition of 50 μl 2.5M H₂SO₄ and the optical density read at 490 nm on a Dynatech mini reader. The reader was zeroed on a well containing 200 μl of substrate solution plus 50 μl 2.5M H₂SO₄.

**Solid phase RIA**

125I-TSH was as described in section 2.2.2 and the immunoadsorbent B was diluted 1:5 with assay diluent (section 2.2.2). The positive mouse anti-h-TSH and the negative mouse control sera have been described above in ELISA methods.
LP3 tubes were set up for the incubation which were done in duplicate and incubated according to the protocol set out in Table 3.4.

**TABLE 3.4**
Solid phase RIA protocol used for hybridoma screening

<table>
<thead>
<tr>
<th>Additions</th>
<th>Test Tubes</th>
<th>Control Tubes</th>
<th>NSB Tubes</th>
<th>Total Tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay diluent</td>
<td>-</td>
<td>-</td>
<td>100 µl</td>
<td>-</td>
</tr>
<tr>
<td>Hybridoma supernatant</td>
<td>100 µl</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Positive mouse anti-TSH or negative mouse sera diluted 1:8000 in assay diluent/NSI supernatant</td>
<td>-</td>
<td>100 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$^{125\text{I}}$-TSH label</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>incubate 18 hours at 4°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunoadsorbent B 1:5 dilution</td>
<td>200 µl</td>
<td>200 µl</td>
<td>200 µl</td>
<td>-</td>
</tr>
<tr>
<td>incubate for 1 hour at room temperature</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Assay diluent</td>
<td>1 ml</td>
<td>1 ml</td>
<td>1 ml</td>
<td>-</td>
</tr>
</tbody>
</table>

The tubes were then centrifuged at 3000 rpm on the Beckman J-6 for 15 minutes. The supernatants were aspirated and the pellets counted for 100 seconds.

### 3.2.5 Cloning

Positive primary cultures were cloned by limiting dilution. Feeder layers of nonimmune mouse spleen cells were prepared as described above. The hybrid cells were suspended by pipetting up and down with a sterile Pasteur pipette and then counted. The cultures were cloned using HT supplemented medium. The cells of each positive hybrid culture were diluted to approximately 50 cells/ml, 10 cells/ml and 5 cells/ml (Oi and Herzenberg 1980). 100 µl/well was dispensed into 36 wells, 36 wells and 24 wells respectively per 96 well Costar microculture plate. One 96 well plate was used per positive culture. The cultures were maintained by replacement of the culture media as described before for the primary cultures. At about day 12-14 wells containing clones when examined under the phase contrast microscope which appeared to be monoclonal were screened for specific antibody production by the ELISA method B or the solid phase RIA.
Two or three positive clones were expanded up to 2 ml, 5 ml then 10 ml cultures and the supernatants were screened at each stage. Finally aliquots of positive hybrid clones were frozen down and stored at -196°C as described.

3.2.6 Antibody Production

Monoclonal antibody preparations were obtained either from spent culture supernatant which contained minimal extraneous immunoglobulins, or in larger amounts from ascites fluid but this preparation contains a small amount of other mouse immunoglobulins.

For ascites production each female Balb/c mouse was primed by intraperitoneal injection with 0.5 ml pristane (2, 6, 10, 14 tetramethyl pentadecane supplied by Aldrich Chemical Co, Gillingham, Dorset), 10 days later 1 x 10⁷ hybridoma cells were injected also intraperitoneally in 0.2 ml serum free culture medium. Each animal was carefully marked for later identification. 10-14 days later when it was evident by the distended abdomen that fluid had collected the mice were killed and the fluid aspirated using a syringe and a 19 g needle. The fluid was then clarified by centrifugation at 2000 rpm on the MSE bench centrifuge for 15 minutes after which the ascitic fluid was aliquoted in 0.25 - 0.5 ml amounts and stored at -20°C. Samples from each mouse were assayed for specific antibody.

Immunoglobulin content of antibody preparations

The ascitic fluid obtained from the mice after ascertaining the positive antibody content by ELISA and or solid phase RIA, were assayed by cellulose acetate electrophoresis (Kohn 1976). The technique is used routinely to characterize serum monoclonal immunoglobulin (Keshgegian and Peiffer 1981).

a) Cellulose acetate electrophoresis

Electrophoretic buffer - Barbitone buffer of 0.05 - 0.07M, pH 8.6 was made up as follows:-

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diethylbarbituric acid</td>
<td>7.36 g</td>
</tr>
<tr>
<td>Sodium diethylbarbiturate</td>
<td>41.20 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>4.0 litres</td>
</tr>
<tr>
<td>Thimerosal</td>
<td>0.01 % w/v</td>
</tr>
</tbody>
</table>
Stain - Ponceau S stain solution consisted of 0.2% (w/v) in 3% (w/v) aqueous trichloracetic acid. The strips were cleared by washing in three changes of 5% (v/v) aqueous acetic acid.

Nigrosin stain solution consisted of 0.001% Nigrosin in 2% (v/v) aqueous acetic acid. The strips were cleared by washing in three changes of 2% (v/v) aqueous acetic acid.

Method

Electrophoretic buffer was placed in the tank (Shandon Southern Products Ltd, Cheshire) covering the electrodes. Thoroughly soaked filter paper wicks formed the connection between the buffer in the electrode compartment and the shoulder pieces on which the cellulose acetate membranes (CAM) rested.

The CAM strips were marked with the sample number and then thoroughly soaked in buffer. The membranes were blotted with filter paper to remove excess buffer. The samples diluted 1:10 with electrophoresis buffer were then applied to the sample applicator bed and the applicator (Shandon Southern Products Ltd) was dipped into the samples. Sample solutions taken up by the applicator were then pressed firmly onto the CAM at the midpoint of the strip. With this application about 5 µl of each sample was applied uniformly and simultaneously to the strip.

The CAM with the applied samples was then placed in the electrophoresis tank resting on the two filter paper wicks. Care was taken not to handle the CAM with fingers and forceps were used throughout the procedure when handling the strips. The tank lid was firmly closed and the power supply was then connected and switched on. The voltage was adjusted to about 160 V at a constant current of 10 mA. The samples were electrophoresed for 30 minutes. The CAM was then removed from the tank and stained for 5 minutes in the Ponceau S staining solution, or in nigrosin staining solution for 10 minutes. These were then destained in several changes of washing solution. The wet Ponceau S stained CAM strip was then sandwiched between two pieces of glass and scanned on the Vitatron TLD 100 densitometer.

The total protein content of the ascitic fluid samples was determined by the Lowry et al method (1951) (see Chapter 2), and from the integrated densitometer scan of the CAM electrophoresis strips the percentage immunoglobulin in the sample was obtained. This was used to quantitate the absolute amount of immunoglobulin in the total protein.
A human IgG serum monoclonal sample and antihuman IgG was kindly supplied by Dr J Kohn, Protein Unit University of Surrey, Guildford. The human para-protein sample was electrophoresed as described following which the separated protein was immuno-fixed.

Immunofixation

The method described by Ritchie and Smith (1976) was used. After electrophoresis the CAM strip was left in position in the tank. The antiserum was diluted 1:3 in immunofixation buffer. This consisted of 0.5M phosphate buffer pH7.2 containing 2% (w/v) PEG 6000. Cellulose acetate strips were cut which fitted exactly over the electrophoresed proteins. The strips were impregnated with the diluted antiserum and was then carefully laid over the electrophoresed proteins. The tank lid was replaced and the preparation incubated for 10 minutes. The CAM was then washed under gently running tap water to remove excess antiserum solution. Finally they were washed 3 x 30 minutes in 3% (w/v) saline containing 0.05% (v/v) tween 20. The CAM strips were stained in nigrosin as described above.

Single radial immunodiffusion

Single radial immunodiffusion was used to determine the amount of mouse immunoglobulin in the supernatant samples after the hybridomas had been allowed to grow to stationary phase.

Square glass slides were precoated with a solution of 0.5 g agar in 100 ml distilled water and were allowed to dry at room temperature.

The top agar was made up as follows:-

3% (w/v) PEG 6000 (BDH Chemicals Ltd, Enfield) was dissolved in 0.15M phosphate buffered saline pH 7.4. To this was added Noble agar (Difco Laboratories, East Molesey, Surrey) 1.5% (w/v), in a boiling water bath until the agar was completely dissolved. 12 ml aliquots were stored at 4°C until required.

When required the aliquots were melted in a boiling water bath then transferred to a 56°C water bath.

256 µl sheep antimouse immunoglobulin IgG2a (supplied by Serotec Ltd, Oxon) was added to the 12 ml dissolved agar and thoroughly mixed. This gave an antisera distribution of 4 µl per square cm of plate. The agar was poured onto the levelled plate. The mouse reference sera MFI (supplied also by Serotec Ltd) was double diluted 1:20 - 1:1280 in phosphate buffered saline and 5 µl samples of this reference sera and 5 µl of the supernatants were
placed in duplicate wells cut in the agar. The plates were placed in a humidified box and incubated at room temperature for 48 hours. The plates were rinsed in 0.15M phosphate buffered saline overnight then stained. The plates were immersed in staining solution for 5 minutes. The staining solution consisted of 1.25 g coomassie brilliant blue R250 (BDH Chemicals Ltd, Enfield) in 235 ml of 0.02% (v/v) glacial acetic acid. They were then destained in several changes of the 0.02% (v/v) glacial acetic acid.

3.3 RESULTS

3.3.1 Anti-TSH Response

All 10 mice in group A gave an antibody response to the immunogen h-TSH. Some however gave a higher response observed in the antiserum titres, reflecting the individual variability of the immune response to the same immunogen. The antiserum dilution curves obtained after the primary and quaternary challenge are presented in figures 3.2 and 3.3. All the 5 animals in group B also showed high antibody response determined only after the tertiary challenge. The antiserum dilution curves are presented in figure 3.4 which also include the curve for a pooled mouse anti-h-TSH preparation.

It was necessary to obtain sufficient positive antisera to use as control in the assays. The pooled anti-TSH sera was used for this purpose.

The increased immunogen content in the inoculum in the third challenge of group B animals did not appear to increase the response to any great extent since % bound even at the lower dilutions were similar to that obtained after the primary challenge in group A. Group C sera were not assayed although the sera were collected and stored. The animals giving the highest response were used in fusion.

3.3.2 Investigations of NSI Myeloma Cells

NSI growth characteristics

The growth characteristics of NSI myeloma cells and the density dependence was demonstrated in figure 3.5. The cultures were maintained under conditions where nutrients were not limiting. The numbers of live cells in the culture and hence the cell viability rapidly declined when the cell density was in excess of 7.9 x 10^5 cells/ml.
FIGURE 3.2 Antiserum dilution curve from 10 mice immunised with TSH after 1\textsuperscript{st} immunisation.
**FIGURE 3.3** Antiserum dilution curves from 10 mice immunised as above after 4° boost.
FIGURE 3.4 Antibody responses of the 5 group B animals after 3º inoculation. Pooled mouse anti-TSH (△--△) positive control and negative control sera (□--□) and (△--△).
FIGURE 3.5 Starting cultures $2 \times 10^4$ cells/ml, growth media was renewed every day therefore nutrients were not limiting.

($\Delta-\Delta$) = total cells/ml in culture

($\bullet-\bullet$) = number of viable cells in culture

($O-O$) = % viability of the culture.

The average cell numbers from 3 cultures were plotted.
Cultures maintained between these limits of $2 \times 10^5$ and $8 \times 10^5$/ml therefore and providing other factors such as nutrient and environment were optimally maintained should give consistently healthy cultures. Figure 3.6 is a logarithmic growth plot demonstrating the precise exponential growth phase during which the cell population was doubling and the asymptote which defines the density limit.

The generation time was calculated by:-

\[
T = \frac{0.3010t}{\log_{10}N - \log_{10}N_0}
\]

where 
- $T =$ mean cell generation time
- $t =$ anytime interval ($t_2-t_1$)
- $N =$ final cell density at $t_2$
- $N_0 =$ starting cell density at $t_1$

$T$ was found to be 20.93 hours. See Table 3.6.

The cell densities at which the NSI cultures were maintained were therefore ideal for the survival of actively growing viable cultures. Figure 3.7 is a photograph of actively growing NSI cells taken under phase contrast x200 magnification.

**NSI growth in HAT**

Since the selection procedure was intended to ensure only hybrids survived in culture it was necessary to check the myeloma cells performance in HAT. Figure 3.8 shows that at day 0 NSI viability was >90%. By day 5 however, the viability of the cells in the test cultures was reduced to 0 while the viability of the control cultures remained >90% and these cultures had to be split on day 3. No cell proliferation was observed in the HAT cultures.

**The effects of econazole on NSI growth**

The viability of NSI cells in the control cultures was found to vary only marginally over the seven day culture period (figure 3.9). Such variability as was observed was due mainly to density effects. The test cultures however showed a very dramatic fall in viability. On day 3 the test cultures had a viability lower by a factor of 2.7 compared to the control. By the 7th day the viability of the test cultures was reduced to zero. Cell proliferation was observed to be greatly inhibited in the test situation (figure 3.10).
FIGURE 3.6 Logarithmic growth plot of NSI (■—■) and a hybridoma SY/T8/2 (▲—▲) growth data.
FIGURE 3.7
NSI cells in suspension culture at x200 magnification.
FIGURE 3.8 $2 \times 10^5$ cells/ml set up per culture, the test culture (o—o) was grown in HAT x 1 media while the control culture (●—●) was grown in unsupplemented standard media. The control culture was split to $2 \times 10^5$ cells/ml on day 3. The mean of three cultures were plotted.
FIGURE 3.9 Viability of NSI control culture (○—○) grown in standard media, and test culture (O—O) grown in standard media supplemented with 1 μg/ml econazole.
FIGURE 3.10 Survival and proliferation of NSI cells in the control culture (●—●) and in the test culture (○—○), grown in medium supplemented with 1 μg/ml econazole. % survival expressed with respect to the culture at day 0.
Indeed by day 3 the viable cell numbers in the test cultures was 84% below that in the controls. In addition under phase contrast microscopy the cell morphology was observed to be altered in the presence of the econazole.

All concentrations of econazole tested were observed to have a marked and unacceptably damaging effect on the NSI cells. After the initial sharp decline in cell viability (figure 3.11), and the percentage survival (figure 3.12) when the cells were grown in 0.125 µg/ml, the test cultures appeared to stabilize. Above 0.5 µg/ml econazole in the culture media the decline was again dramatic although some 4 times slower than the initial fall. The dose of econazole which reduced the test cell populations by 50% (ED$_{50}$) was 0.97 µg/ml less than the recommended antifungal effective dose (Wyler et al 1979).

The apparent immediate decline in viability and survival of the test cultures in 0.125 µg/ml econazole was also demonstrated by $^3$H-thymidine incorporation by the cells (see figure 3.13). However as econazole concentration was increased to 0.5 µg/ml, the amount of DNA synthesis was observed to be greatly enhanced as was shown by the increased $^3$H-thymidine incorporation. In this concentration range it may be postulated that the increased DNA synthesis was intrinsically linked to, and was responsible for the observed culture tolerance and the culture stabilization observed.

Above 0.5 µg/ml econazole, DNA synthesis began to decline as did viability and percentage cell survival.

### 3.3 Fusion Experiments

The results of fusions 1-6 were very disappointing in terms of stable positive hybrids derived. At the time of the initial screening the number of wells with hybrids was variable with a greater percentage of wells observed to contain hybrids when fewer wells were plated initially. Table 3.5 summarises the results of fusions 1 to 8.

For fusion 1-3 the numbers of wells with hybrids were not counted but all wells were assayed. However the wells which contained actively growing hybrids were indicated by the change in colour of the culture medium from salmon pink to pale yellow.

All wells were nevertheless examined under the phase contrast microscope, since new hybrids were sometimes too small to effect this obvious pH change. The numbers of wells with hybrids were counted in the other fusions.
FIGURE 3.11 The concentration dependent effect of econazole on the 48 hour viability of NSI myeloma cells
FIGURE 3.12 The dose response curve plotted on a log linear scale showing survival of NSI cells after 48 hours incubation in econazole. The survival is expressed as a percentage of the control cultures ($ED_{50} = 0.97 \mu g/ml$).
FIGURE 3.13  The concentration dependent effects of econazole on the incorporation of $^3$H thymidine by NSI myeloma cells. Test cultures (■) incubated with econazole 0-1 µg/ml and 5 µCi $^3$H thymidine for 48 hours (left hand scale). Test cultures (○) incubated with econazole 0-1 µg/ml for 46 hours plus $^3$H thymidine for the final 2 hours (right hand scale).
TABLE 3.5
Summary of fusion experiments

<table>
<thead>
<tr>
<th>TSH Fusion Experiments</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of fusion walls</td>
<td>96</td>
<td>96</td>
<td>96</td>
<td>96</td>
<td>48</td>
<td>576</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>No. of wells with hybrids</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>69</td>
<td>18</td>
<td>45</td>
<td>199</td>
<td>86</td>
</tr>
<tr>
<td>% wells with hybrids</td>
<td>-</td>
<td>-</td>
<td>71.9</td>
<td>18.75</td>
<td>93.75</td>
<td>34.55</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td>No. positive primary cultures detected by liquid phase RIA</td>
<td>5</td>
<td>10</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ig Secretors detected by ELISA A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>70</td>
<td>29</td>
</tr>
<tr>
<td>Anti-h-TSH secretors Detected by ELISA B or solid phase RIA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>13</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>No of stable cultures cloned by limiting dilution</td>
<td>-</td>
<td>-</td>
<td>8</td>
<td>-</td>
<td>-</td>
<td>7</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>No. monoclonal cultures frozen</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>13</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>

In both RIA methods used for screening, positive cultures were arbitrarily taken to be those binding greater than 1.5 times the amount of radiolabelled hormone bound by the negative controls. In the ELISA methods positive cultures were taken to be those having an optical density of twice that observed in the negative controls.

No positive monoclonal cultures were obtained from fusion 3 cloning and the primary cultures all lost positive activity. Attempts were made to recover positive cultures from frozen stocks but these were found to have also lost positive antibody production.

Positive antibody activity was found to be lost in all primary cultures of fusions 1 and 2 by the second assay, therefore no frozen stocks were obtained. It must be noted that all the positive cultures identified by liquid phase RIA were only weakly positive by the criteria for positivity. No positive cultures were detected in fusions 4-6. However 7 primary positive cultures and 13 monoclonal cultures were isolated from fusion 7 and similarly 4 primary positive cultures and 14 monoclonal cultures were obtained from fusion 8.
Frozen cells stored in liquid nitrogen

1) 80 vials each containing 1-2 x 10^6 NSI myeloma cells were frozen.
2) 8 primary cultures from fusion 3 were frozen.
3) 6 positive primary cultures from fusion 7 were frozen (5 vials/culture).
4) The 13 monoclonal hybrids from fusion 7 were also frozen (5 vials/culture).
5) 4 positive primary cultures from fusion 8 were frozen (10 vials/culture).
6) 14 monoclonal hybrids from fusion 8 were also frozen (5 vials/culture).
7) 6 monoclonal cultures recloned from one of the 14 from fusion 8.

The monoclonal and primary cultures from fusion 7 proved difficult to recover from the frozen stocks and where such cultures were eventually revived antibody production was found to be absent.

No such difficulty was observed with the positive products of fusion 8.

The 14 monoclonal cultures from fusion 8 was designated SY/T8/1-14 of which SY/T8/1-6 were selected for further study and characterization. Table 3.6 presents the growth characteristics of these 6 cell lines and figure 3.6 contains the logarithmic growth curve of one cell line. The binding of radiolabel TSH and optical densities observed for the positive cultures are given in Table 3.7. Figure 3.14 is a photograph of a monoclonal culture.

**TABLE 3.6**

Growth characteristics of hybridoma lines and NSI myeloma cells. All hybridomas were grown in 10% foetal calf serum supplemented RPMI 1640.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Doubling time (hours)</th>
<th>Density limit (cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSI</td>
<td>20.93</td>
<td>7.9 x 10^5</td>
</tr>
<tr>
<td>SY/T8/1</td>
<td>24.08</td>
<td>1.0 x 10^6</td>
</tr>
<tr>
<td>SY/T8/2</td>
<td>17.20</td>
<td>3.6 x 10^5</td>
</tr>
<tr>
<td>SY/T8/3</td>
<td>17.20</td>
<td>5.0 x 10^5</td>
</tr>
<tr>
<td>SY/T8/4</td>
<td>20.64</td>
<td>6.3 x 10^5</td>
</tr>
<tr>
<td>SY/T8/5</td>
<td>18.06</td>
<td>4.5 x 10^5</td>
</tr>
<tr>
<td>SY/T8/6</td>
<td>28.89</td>
<td>4.8 x 10^5</td>
</tr>
</tbody>
</table>
FIGURE 3.14
A Hybridoma Clone at x200 magnification.
TABLE 3.7

Binding of radio-labelled h-TSH by monoclonal antibodies and enzyme-labelled anti-mouse in specific ELISA. Reflecting the presence of anti-TSH antibodies.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Solid Phase RIA</th>
<th>ELISA B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X CPM</td>
<td>% Bound</td>
</tr>
<tr>
<td>Total</td>
<td>11906</td>
<td>-</td>
</tr>
<tr>
<td>Positive control one primary culture supernatant</td>
<td>3073</td>
<td>25.8</td>
</tr>
<tr>
<td>Positive control mouse anti-h-TSH (pooled)</td>
<td>3595</td>
<td>30.2</td>
</tr>
<tr>
<td>Negative control NSI supernatant</td>
<td>283</td>
<td>2.4</td>
</tr>
<tr>
<td>Supernatant SY/T8/1</td>
<td>915</td>
<td>7.7</td>
</tr>
<tr>
<td>Supernatant SY/T8/2</td>
<td>978</td>
<td>8.2</td>
</tr>
<tr>
<td>Supernatant SY/T8/3</td>
<td>859</td>
<td>7.2</td>
</tr>
<tr>
<td>Supernatant SY/T8/4</td>
<td>1110</td>
<td>9.3</td>
</tr>
<tr>
<td>Supernatant SY/T8/5</td>
<td>1098</td>
<td>9.2</td>
</tr>
<tr>
<td>Supernatant SY/T8/6</td>
<td>1183</td>
<td>9.9</td>
</tr>
<tr>
<td>Supernatant SY/T8/7</td>
<td>976</td>
<td>8.2</td>
</tr>
<tr>
<td>Supernatant SY/T8/8</td>
<td>1190</td>
<td>9.9</td>
</tr>
<tr>
<td>Supernatant SY/T8/9</td>
<td>689</td>
<td>5.8</td>
</tr>
<tr>
<td>Supernatant SY/T8/10</td>
<td>907</td>
<td>7.6</td>
</tr>
<tr>
<td>Supernatant SY/T8/11</td>
<td>1348</td>
<td>11.3</td>
</tr>
<tr>
<td>Supernatant SY/T8/12</td>
<td>921</td>
<td>7.7</td>
</tr>
<tr>
<td>Supernatant SY/T8/13</td>
<td>831</td>
<td>6.9</td>
</tr>
<tr>
<td>Supernatant SY/T8/14</td>
<td>942</td>
<td>7.9</td>
</tr>
</tbody>
</table>
Ascitic fluid collection

Various amounts of ascitic fluid (table 3.8) was collected from the cell primed animals. None of the animals developed solid tumors although 2 mice died before the ascitic fluid could be collected.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. animals</th>
<th>Ascitic fluid (ml)</th>
<th>Days after injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>SY/T8/1</td>
<td>4</td>
<td>9</td>
<td>11-12</td>
</tr>
<tr>
<td>SY/T8/2</td>
<td>4</td>
<td>6.3</td>
<td>11-12</td>
</tr>
<tr>
<td>SY/T8/3</td>
<td>4</td>
<td>7.8</td>
<td>11-12</td>
</tr>
<tr>
<td>SY/T8/4</td>
<td>3</td>
<td>4.5</td>
<td>12-14</td>
</tr>
<tr>
<td>SY/T8/5</td>
<td>4</td>
<td>9</td>
<td>12-14</td>
</tr>
<tr>
<td>SY/T8/6</td>
<td>2</td>
<td>4.5</td>
<td>12</td>
</tr>
</tbody>
</table>

3.4 Antibody Content

a) Ascitic fluid

Figure 3.15 is a photograph of the cellulose acetate electrophoresed samples showing the monoclonal immunoglobulin peaks prominently. From these, integrated densitometer readings allowed the resolution of the percentage monoclonal immunoglobulin in the total protein sample electrophoresed.

Figure 3.16-18 depicts the densitometer traces of the 6 ascitic fluid samples. The amount of protein in the monoclonal immunoglobulin peaks was very high in some cases (see table 3.9). The control used was a known human IgG paraproteinaemia sample (kindly supplied by Dr Kohn, Protein Unit, University of Surrey, Guildford).

The corrected % paraprotein in the electrophoresed sample was calculated thus:

\[
\% \text{ Ig} = \frac{\text{Integrator No of Ig peak}}{\text{Total Integrator No of sample}} \times \frac{\text{Weight (mg) of Ig portion of peak}}{\text{Total weight (mg) peak}}
\]

b) Supernatant

From the linear calibration curve for the single radial immunodiffusion measurement of supernatant immunoglobulin, the immunoglobulin concentrations were determined (see table 3.9).
FIGURE 3.15  Cellulose acetate electrophoresed monoclonal antibody samples. The ascitic fluid samples were diluted 1:10 and the supernatant samples (1', 2', 3' etc) were used neat. The control human Ig samples were included for identification of the monoclonal immunoglobulin peaks.
FIGURE 3.16 Integrated Densitometer traces of cellulose acetate electrophoresed ascitic fluid antibody preparation.

a) Ascitic fluid antibody SY/T8/1
b) Ascitic fluid antibody SY/T8/2

Total peak (-----)
Ig peak (---)
FIGURE 3.17  Integrated Densitometer traces of cellulose acetate electrophoresed ascitic fluid antibody preparation.
a) Ascitic fluid antibody SY/T8/3
b) Ascitic fluid antibody SY/T8/4
Total peak (- - -)
Ig peak (- - -)
FIGURE 3.18  Integrated Densitometer traces of cellulose acetate electrophoresed ascitic fluid antibody preparation.

a) Ascitic fluid antibody SY/T8/5
b) Ascitic fluid antibody SY/T8/6
Total peak (-- --)
Ig peak  (-- --)
<table>
<thead>
<tr>
<th>Sample/determination</th>
<th>Total protein (mg/ml)</th>
<th>% Ig by integrated densitometer (b)</th>
<th>Total Ig mg/ml by (a and b)</th>
<th>No. Ig bands on CAM</th>
<th>Ig µg/ml (single radial immunodiffusion)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SY/T8/1</td>
<td>52.75 - 22.25</td>
<td>34.5</td>
<td>1</td>
<td>31.62</td>
<td>10.96</td>
</tr>
<tr>
<td>SY/T8/2</td>
<td>35.5 - 25.39</td>
<td>38.34</td>
<td>2</td>
<td>24.55</td>
<td>16.60</td>
</tr>
<tr>
<td>SY/T8/3</td>
<td>35.5 - 25.39</td>
<td>38.34</td>
<td>2</td>
<td>24.55</td>
<td>16.60</td>
</tr>
<tr>
<td>SY/T8/4</td>
<td>35.5 - 25.39</td>
<td>38.34</td>
<td>2</td>
<td>24.55</td>
<td>16.60</td>
</tr>
</tbody>
</table>

TABLE 3.9
Ig content of monoclonal antibody preparations from supernatant (SN) and ascitic fluid (AF).
DISCUSSION AND CONCLUSIONS

3.4 Spleen Cell Donor Response to Immunization

Immunization serves two purposes:

1) to effect the expansion of antigen specific clones within the animals and in the spleens in particular;
2) to produce antigen stimulated actively dividing B lymphocytes for fusion.

Although the nature of cells which fuse best is not precisely known, it has been implied in Chapter 1 that the specific antibody content of a serum reflects the antigen dependent stimulation, proliferation and maturation of antigen binding cells. Thus the antibody content of the immunized mice indicated the enrichment of the antigen binding clones required for fusion (Siddle and Soos 1981). Additionally Andersson and Melchers (1978) indicated that fusion occurs preferentially with rapidly dividing blast cells. For this reason fusion is done 3-4 days after the final boost in order to obtain cells in rapid mitosis (Davis et al 1969, Nossel 1975) rather than at the height of antibody production which would be expected to occur at about day 7-8. By this time mitosis would have ceased, and the majority of cells would be small mature lymphocytes producing antibody. The route of introduction is also thought to influence the site of maximum antigen localization and processing. This, together with the preferential antigen independent spleen seeking activity of B cells (Weissman et al 1974) indicated the final intravenous challenge, and the spleen as the most appropriate donor organ.

Hyperimmunization by multiple challenge has been suggested to be inappropriate (Oi et al 1978). However it has also been suggested that the quality of the antibody in terms of affinity matures with time as the immune response matures (Eisen and Siskind 1964, Nossal 1975, Odell et al 1969) especially when low concentrations of antigen are used. In explanation it was suggested that as the response progressed and antigen levels decrease so only cells with high affinity receptors were able to capture sufficient antigen to produce further cell divisions (Steiner and Eisen 1967). By this process progressive immunization would be expected to result in higher and higher affinity cells available for stimulation. Since our objective was to produce high affinity monoclonals we concluded that an extended immunization schedule would probably yield the desired high affinity product. On these premises the described protocol was adopted for immunization to maximise the specific high affinity clone types available for fusion.
4.2 Characteristics of NSI myeloma cells

The NSI myeloma cells were found to grow optimally up to a density of 8 x 10^5/ml after which the culture rapidly declined both in viability and cell numbers. Indeed this was also the case with the hybridomas examined. These grew to a maximum density which was not maintained for long and the cultures rapidly declined thereafter. Five of the hybridomas were very intolerant of high density. Hybridoma SY/T8/1 on the other hand grew up to a very high density under the same growth conditions.

The central importance of the NSI sensitivity to HAT selection (Littlefield 1964) was described in Chapter 1; and such sensitivity was clearly demonstrated in figure 3.8. Under this selection regime NSI myeloma cells which did not contain the intact HGPRT enzyme was effectively eliminated from the culture. It was therefore vital that the NSI cultures were examined under HAT selection regularly in case normal revertants appeared spontaneously within the cell population. Such normal revertants would be distinguished by their ability to grow in HAT media. Revertant NSI cells used in fusion would endanger any positive hybrids by overgrowing these cells and using up vital culture resources. Many hybrid cells are slower growing than the NSI cells (table 3.6).

Hybrid instability described in Chapter 1 also appeared to be exacerbated by the trauma of freezing and thawing. Many primary and monoclonal cultures with continued antibody production could not be resuscitated.

We found however that extended culture time after fusion and cloning alleviated the problem. This however greatly increased the risk of hybrid loss due to contamination. Early hybrid products in addition to the longer growing cultures were therefore stored.

4.3 Contamination control

The problems of fungal contamination control with econazole was not resolved. Strict aseptic techniques were relied upon to maintain the sterility of the cultures. This was accomplished successfully, with only very minor outbreaks of contamination over the whole period of this work. Contrary to expectations (Wyler 1979), econazole reduced NSI viability dramatically, impaired cell growth and proliferation and induced observable and undesirable membrane changes. These findings were similar to the reported adverse effects of the most widely used antimycotic agent fungizone on chick embryo fibroblast (Dolberg and Bissell 1974) and murine cells.
Because of its reported advantages (Wyler et al 1979) econazole was tested to be used instead of fungizone, but was found to be unsuitable.

4.4 Fusions

The first six fusions were unsuccessful in terms of stable positive hybrids derived. This was possibly due primarily to the unsuitability of the screening RIA used for detecting the small quantities of antibodies that were initially expected. The quantity of second antibody used for precipitation of bound from free was difficult to optimise when the limits to be tested were unknown. The solid phase RIA and ELISA systems overcame such problems and simplified the screening protocols, reduced the assay times and increased the handling capacity. With the ELISA system the advantages were very obvious. A large number of samples could be easily handled in the microplate system and a rapid visual positive/negative assessment was possible. The conclusion that the RIA screening procedure was responsible for the lack of identification of positive hybrids from the many viable cultures observed was reinforced by the observations that PEG separation procedure alone was as good as the second antibody separation procedure. A similar amount of bound label was observed in the positive controls as when the second antibody was used. This suggested the assay procedure was adequate at these antibody concentrations but the major question remained open with this assay at the very low antibody concentrations. The over-riding indications were therefore for the development of the special screening assays described in Chapter 2 and used successfully in fusions 7 and 8.

Three fusion solutions were used. Solution 1 was abandoned because it was suggested (Fazekas de St Groth and Scheidegger 1980) that low molecular weight PEG was toxic to cells. Fusion solution 2 was used successfully by (Aldersson and Sikora 1982) but the fusion procedure was made more complicated with no apparent added advantage in terms of increased hybrid numbers. We therefore finally decided to use fusion solution 3 (Fazekas de St Groth and Scheidegger 1980) which was very successful in terms of primary cultures secreting immunoglobulins and specifically anti-TSH antibodies derived, and the simplified fusion procedure. The results confirmed that the success of the fusion procedure was dependent on the viability of the NSI cells used in the fusion. In situations where <90% viable cells were used the number of wells with viable hybrid cultures were drastically reduced.
The probability that each positive well would contain only one clone was greatly improved with the microculture (96 well) procedure and was a major attraction when compared to larger (2 ml) cultures. The cloning procedure was therefore simplified. However the microculture procedure entailed two major disadvantages:

1) The large numbers of cultures which had to be handled greatly increased the risk of contamination and;

2) Concomitantly increased the work load as was the case in fusion 7. It was therefore concluded that it was preferable to work with fewer larger cultures and expend the effort at the later screening and cloning stages rather than maintaining large numbers of primary cultures the majority of which were negative.

4.5 The monoclonal antibody products

The monoclonal cultures SY/T8/1-6 which were chosen for investigation were shown to be high antibody producers. In the literature (Goding 1980, Galfre and Milstein 1981, Oi and Herzenberg 1980) 10-100 μg/ml specific antibody in the supernatants and up to 15 mg/ml antibody in ascitic fluid was reported. The derived monoclonal antibodies produced exceeded that range in most cases.

Two monoclonal bands were observed in the CAM electrophoresed ascitic fluid samples of SY/T8/2 and 5.

The peaks were still apparent even after recloned cell lines were used to produce the ascitic fluid. It was concluded therefore that the second peak was probably extraneous mouse immunoglobulins secreted in an immune response to the injected cells and the antibody products they produced. It must be remembered however that the monoclonal antibody and cells are of mouse origin. The immune response should therefore be minimal. The monoclonal antibodies were characterized and assessed in specific application.
CHAPTER 4

The Characterisation Of Monoclonal
anti-human-TSH Antibodies
4.1 INTRODUCTION

One of the primary advantages of monoclonal antibodies over polyclonal antisera is that the precise chemical nature of the antibody is defined. This according to Milstein et al (1979) is perhaps the major attraction of hybridoma technology.

One of the implications of monoclonal antibodies because of the unique properties and the limitless supply, is that the derived antibody may be distributed and become established as standard reagents in specific application. For this to become possible, or even in local application they must be precisely characterized and described. Not only must the overt antigen binding characteristics be defined, but specification of other properties such as avidity, titre and cross reactivity must be outlined. Such empirical descriptions allow the quantitative application of antibodies not previously possible.

4.2 MATERIALS AND METHOD

4.2.1 Assessment Of Antibody Preparations

a) Monoclonal antibody titre

The six selected monoclonal antibodies SY/T8/1-6 were titred by three methods:-

1) The indirect ELISA method B described in Chapter 2. Both ascitic fluid and supernatants were titred by this method. The antibody preparations were diluted in ELISA assay diluent. The supernatant was taken from cultures which had grown to stationary phase.

2) The solid phase RIA method was also described in Chapter 2. Only the ascitic fluids were titred by this method.

3) The ascitic fluids were also titred by the standard RIA method by the kind courtesy of Dr D Teale, Biochemistry Dept, St Lukes Hospital, Guildford.

Displacement curves

In addition to the above titration and displacement curves were obtained using the supernatant antibody preparations from cultures which had grown to stationary phase. The wells were previously coated with 1 µg TSH/ml as described in ELISA method B.
The supernatants were diluted in ELISA diluent and 100 μl diluted supernatant plus 100 μl of 500 mU/l TSH (Barts) or National Institute of Biological standards and control - (NIBSC) TSH (68/38) standard was added to quadruplicate wells for the displacement curves. For the titration curves the TSH solution was replaced by 100 μl assay diluent.

The wells were further incubated for 2 hours at 37°C and the assay completed as described for ELISA method B.

NIBSC TSH dilution

TSH ampoule contained 150 mU TSH. This was dissolved in 50 ml distilled water to give 3.0 mU/ml solution of TSH. 0.5 ml aliquots of this solution were freeze dried and stored at -20°C.

To get a 500 mU/l solution of TSH one aliquot was dissolved in 0.5 ml distilled water. This was made up to a final volume of 3.0 ml with distilled water to give the final TSH solution.

b) Monoclonal antibody cross reactivity

The cross reactivity of each monoclonal antibody preparation was determined by the inhibition of binding of the antibody to the specific antigen by various concentrations of the possible crossreacting antigen. 100 μl of the supernatant diluted as in table 4.4 was placed in h-TSH antigen adsorbed wells as described for ELISA method B. Then 100 μl of the various dilution of the possible crossreacting antigen was added to quadruplicate wells. Wells which contained 100 μl of the appropriate antibody dilution and 100 μl of assay diluent were taken as 100 percent (%) binding wells.

The binding in the other wells was scored in relation to these 100% binding wells.

The results were calculated and plotted as relative percentage (%) binding vs antigen concentration:

\[
\text{Relative } \% \text{ binding } = \frac{a}{b} \times 100
\]

where 'a' is the mean binding (optical density) of wells with the crossreacting hormone and antibody; and 'b' is the mean binding (optical density) of wells containing only the antibody.

The hormones tested for crossreactivity with the monoclonal antibodies were those supplied by the National Institute for Biological Standards and Control (NIBSC) Holly Hill, Hampstead, London NW3 6RB.
1) 1st International reference preparation of human pituitary LH (68/40);
2) 2nd International reference preparation of human pituitary FSH/LH (78/549);
3) 1st International reference preparation of human Chorionic Gonadotrophin (HCG) (75/537);
4) 1st International reference preparation of HCG α subunit (75/569);
5) 1st International reference preparation of HCG β subunit (75/551);
6) In addition purified HCG kindly supplied by Dr R Edwards, St Bartholomews Hospital, London EC1 and designated HCG(Barts) was tested. The hormone preparations were initially dissolved as outlined in table 4.1 after which they were diluted 1:10 in ELISA diluent.

**TABLE 4.1**

Hormones tested for crossreactivity with monoclonal antibody to h-TSH, concentrations used.

<table>
<thead>
<tr>
<th>Hormones</th>
<th>HCG(Barts) 75/537</th>
<th>HCG 78/549</th>
<th>LH 68/40</th>
<th>FSH/LH 78/549</th>
<th>HCG α sub-Unit 75/569</th>
<th>HCG β sub-Unit 75/551</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assigned unitage in hormone preparation</td>
<td>3000.0 IU</td>
<td>650.0 IU= 77.0 IU</td>
<td>25 IU LH</td>
<td>70.0 IU</td>
<td>70.0 IU= 70μg</td>
<td>70μg</td>
</tr>
<tr>
<td>Dissolved initially in distilled water</td>
<td>3.0 ml</td>
<td>6.5 ml</td>
<td>10.0 ml</td>
<td>10.0 ml</td>
<td>7.0 ml</td>
<td>7.0 ml</td>
</tr>
<tr>
<td>Initial concentration</td>
<td>1000.0 IU</td>
<td>100.0 IU= 10.77μg</td>
<td>770.0 mU</td>
<td>2500 mU LH</td>
<td>10.0 IU= 10μg</td>
<td>10μg</td>
</tr>
</tbody>
</table>

**c) Monoclonal antibody affinity**

A modification of the procedure for the rapid determination of binding constants with special reference to monoclonal antibodies described by Frankel and Gerhard (1979) was used. The major modification involved the use of the goat anti-mouse-HRPO conjugate I instead of a radiolabelled antimouse immunoglobulin as the observed bound signal.
The indirect ELISA method B was used to obtain the data for the determination of the affinity binding constants. The experiments were run in triplicate and culture supernatant was used as the antibody preparation.

First each supernatant was titrated by serial dilution against 1 µg/ml solid phase TSH. A standard curve was derived from culture supernatant 1 for which the antibody content had been determined previously by radial immunodiffusion (Chapter 3).

Finally increasing antigen concentration 1, 2.5, 5, 10 and 20 µg/ml TSH was coated to a series of wells and one dilution of each supernatant (Table 4.2) was titrated against the series of increasing antigen coated wells.

**TABLE 4.2**

Supernatant dilutions used with increasing antigen coating concentrations in affinity constant determination. These were arbitrarily chosen to give high OD readings under the assay conditions.

<table>
<thead>
<tr>
<th>Dilutions</th>
<th>SN1</th>
<th>SN2</th>
<th>SN3</th>
<th>SN4</th>
<th>SN5</th>
<th>SN6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:200</td>
<td>1:200</td>
<td>1:180</td>
<td>1:120</td>
<td>1:60</td>
<td>1:120</td>
</tr>
</tbody>
</table>

d) **Immunoglobulin class, subclass and light chain composition**

The immunoglobulin class, subclass and light chain composition secreted by the monoclonal antibody cell lines were determined by the Ouchterlony double immunodiffusion technique (Ouchterlony and Nilsson 1979).

The plates were subbed as described for single radial immunodiffusion in Chapter 3. 1.5% (w/v) Noble agar dissolved in phosphate buffered saline containing 3.0% (v/v) PEG 6000 was poured hot onto the subbed plates on a levelling table. The cooled plates were then stored in a humidified box. Immediately before they were to be used the wells were cut and the agar plugs aspirated. The well sizes were such that they held 5 µl sample volume. The antimouse immunoglobulins antisera (supplied by Serotec Ltd, Bicester, Oxon), anti IgA, IgM, anti IgG, IgG2a, IgG2b, IgG3; anti-K and anti-λ, were placed in the central wells. They were used at three concentrations neat, diluted 1:5 and 1:10. Neat supernatant (SNa) and ammonium sulphate precipitated supernatant antibody (SNb) and 1:50 dilution of ascitic fluid (AF) were placed in the individual wells arranged around the central well (see figure 4.1).
The plates were then incubated in a humidified box at 4°C for 48 hours, after which they were washed gently in phosphate buffered saline for about 2 hours. They were then stained, destained and pressed dry by placing several layers of filter paper on top of the gel and placing an even weight on top. They were left overnight after which the plates were examined for precipitation lines.

**Staining and destaining**

Staining solution consisted of 1.25 g Coomassie Brilliant blue R250 (BDH Chemicals Ltd, Enfield, Middlesex), dissolved in a solution of 21.3% (v/v) glacial acetic acid. After mixing and leaving overnight the solution was filtered before using. The plates were immersed in staining solution for 5 minutes after which they were differentiated in the acetic acid solution. The plates were washed until the background was clear.
Pattern of wells cut in agar and the distribution of samples for
Ouchterlony double immunodiffusion. (O) Plate identification mark. Well
No.1 contains antihuman antisera. Wells 2, 3 and 4 = one antibody prepara-
tion SNa, SNb and AF respectively. Similarly wells 5, 6 and 7 = another
monoclonal antibody SNa, SNb and AF antibody preparation respectively.
4.2.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoretic Analysis Of Monoclonal Antibody Preparations

The method of Laemmli (1970) was used to analyse reduced and unreduced preparations of internally labelled antibodies. One of the important advantages of monoclonal antibodies is the facility for easy radiolabelling by biosynthetic incorporation of $^{14}\text{C}$, $^{3}\text{H}$ or $^{35}\text{S}$ into the immunoglobulin molecules.

This is usually accomplished by substitution of a radiolabelled essential amino acid in the culture media. The method described by Galfre and Milstein (1981) was used to internally label the monoclonal antibodies.

Preparation of internally labelled antibodies

The incorporation media consisted of 9.0 ml (-lys) RPMI 1640: RPMI 1640 lysine free medium (supplied by Gibco Bio-cult Ltd, Washington Road, Paisley). 1.0 ml of $[14\text{C}]$ lys: L-$[U-14\text{C}]$ lysine monohydrochloride (Cat.No. CFB.69) 50 μCi/ml (supplied by The Radiochemical Centre, Amersham, International PLC, UK) and 1.0 ml foetal calf serum which has been dialysed against double distilled water after which balanced salt solution (Flow laboratories) was added to 1/9th the volume of the dialysed serum.

$2 \times 10^6$ cells from each exponentially growing monoclonal culture with cell viabilities of between 90.8 and 94.5% were pelleted on a bench MSE centrifuge for 5 minutes. The cells were washed with 10 ml lysine free RPMI 1640. After pelleting the cells of each culture were resuspended in 1.0 ml incorporation media and plated into 24 well Costar tissue culture plates. These were then incubated for 37°C in 5.0% CO$_2$ in air humidified environment.

After 8 hours incubation a further $2 \times 10^6$ cells, washed and resuspended in 1.0 ml lysine free medium were added to each radioactive culture. Following a further 15 hours incubation the supernatants were collected and stored at -20°C.

Intracellular labelling of myeloma k chain

The method described by Cotton et al (1973) was used for the intracellular incorporation of radiolabel into the immunoglobulin kappa light chain synthesized by the myeloma cells (Kohler and Milstein 1975). The cells were lysed and the immunoglobulin chain preparation obtained according to the method of Awdeh et al 1970. It was necessary to release the labelled
immunoglobulin L-chains because they are synthesized but not secreted by NSI myeloma cells.

$5 \times 10^6$ log phase growth NSI cells were washed in 10 ml RPMI 1640 lysine free medium and then pelleted and resuspended in 10 ml fresh RPMI 1640 lysine free medium in which they were incubated for 15 minutes at 37°C.

The cells were then recovered by centrifugation on the MSE bench centrifuge. Following this they were resuspended and incubated for 15 minutes in 0.8 ml RPMI 1640 lysine free medium containing 10% (v/v) dialysed foetal calf serum and supplemented with sodium pyruvate and penicillin/streptomycin as described in Chapter 3. To this was added 200 µl = 10 µCi L-[U-$^{14}$C] lysine monohydrochloride (Amersham Int UK). The cells were then pelleted. The supernatants were collected and stored. These represented the extra-cellular fluid.

Cell lysis

The intracellular soluble proteins were extracted by resuspension of the cells in 0.8 ml of solution E consisting of 0.01 M Tris (hydromethyl) amino methane pH7.5, 0.01M potassium chloride (KCl) and 0.001M Magnesium chloride (MgCl$_2$).

Cell lysis was accomplished by the addition of 0.2 ml of 5% (w/v) sodium deoxycholate.

The lysed cell material was layered on to sucrose gradient consisting of 1.0 ml 4% (w/v) sucrose in solution E, 1.0 ml 10% (w/v) sucrose and 8.0 ml 30% (w/v) sucrose. The material centrifuged for 1 hour in a Beckman 60 Ti Rotor at 39,000 RPM. The top 3 ml was then removed and stored at -20°C until required.

Preparation of Gel

Stock solutions

Gel Stock: 30% (w/v) acrylamide containing 0.8% (w/v) N,N-methylenebis-acrylamide in distilled water.
Buffers A: consisted of 1.5 M Tris-HCl pH8.0 containing 0.4% (w/v) SDS.
B: 500mM Tris-HCl pH6.8 containing 0.4% (w/v) SDS.
C: 25mM Tris-HCl pH8.3 containing 192 mM glycine and 0.1% (w/v) SDS.
D: 62.5mM Tris-HCl pH6.8 containing 2.3% (w/v) SDS, 15% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.001% bromophenol blue.
The cuvettes were each made up of two glass plates 120 mm x 100 mm x 1.5 mm; one of which had a 20 mm deep x 100 mm long notch cut out from one long edge. The two clean dry plates were placed together, with the notched top edge open and 5 mm wide plastic spacers between the plates along the two sides and bottom edge. The plates were then clamped securely together and the sides and bottom of the cuvette sealed with molten 1.5% (w/v) agar. The assembled cuvettes were secured in a vertical position. Great care was taken to ensure that the cuvettes were water tight. The lower running gel consisting of 10.0 ml of buffer A, 13.3 ml gel stock, 16.6 ml distilled water was mixed immediately before required, and polymerization was initiated by the addition of 20 μl tetra-methylene diamine (TEMED) and 240 μl of a freshly prepared 10% (w/v) solution of ammonium persulphate. The mixture was then poured into the cuvettes to a height of 80 mm. A layer of distilled water was gently overlaid on top of the gel mixture. After polymerization was completed (about 30 minutes) the water layer was carefully aspirated. The stacking gel consisting of 2.5 ml buffer B, 1.0 ml gel stock, 6.5 ml distilled water mixed with 60 μl of 10% (w/v) ammonium persulphate solution and 20 μl TEMED was poured into the cuvettes on top of the running gel.

Before polymerization, a perspex comb was introduced into the stacking gel to form sample wells. After polymerization the comb was carefully removed so as not to distort the wells.

Buffer C was poured into the wells of the stacking gel to keep them patent. The bottom spacer was carefully removed from the cuvette which was then placed into the electrophoresis tank, the upper and lower reservoirs of which were filled with buffer C. It was important to remove any trapped air bubbles from the bottom edge of the gel in the cuvette.

Sample preparations

Two types of radioactive samples were introduced into the wells. The first were the reduced monoclonal antibody samples. These samples were diluted in buffer D in a ratio of 1:3 buffer: sample. The samples were placed in a boiling water bath for 3 minutes. They were then cooled and 30 μl were applied with an Eppendorf pipette carefully to wells in the stacking gel.

The second sample type was the unreduced samples. These were also diluted 1:3 buffer: sample. In buffer D used here 2-mercaptoethanol was replaced by 4.0% (w/v) iodo-acetamide (Hudson and Hay 1981) after which they were treated similarly to the reduced samples.
Standard proteins

Standard proteins were also included in the sample wells. 1 mg of each standard was mixed together in 1 ml buffer D for reduced or unreduced samples and treated as described above. 15 μl of the standard samples were applied to the standard wells. The standard proteins included those in Table 4.3 below.

### TABLE 4.3

Standard proteins used at 1 mg/ml in SDS-PAGE analysis of monoclonal antibodies and their molecular weights

<table>
<thead>
<tr>
<th>Standard Proteins</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep IgG</td>
<td>150,000</td>
</tr>
<tr>
<td>Albumin (BSA)</td>
<td>68,000</td>
</tr>
<tr>
<td>Albumin (Hen egg)</td>
<td>45,000</td>
</tr>
<tr>
<td>Aldolase</td>
<td>40,000</td>
</tr>
<tr>
<td>Chemotrypsinogen</td>
<td>25,000</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>12,500</td>
</tr>
</tbody>
</table>

**Electrophoresis**

The electrodes were finally connected to the power supply with the anode to the lower chamber and the cathode in the upper. A constant current of 20 mA was applied until the bromophenol blue just entered the running gel at which stage the constant current was increased to 40 mA until the bromophenol blue marker is about 5 cm from the bottom end of the gel. The power was switched off. The cuvette was removed from the tank and the plates carefully separated. The stacking gel was cut off and the gels stained.

**Staining**

Staining solutions consisted of 0.25% (w/v) Coomassie Brilliant Blue dissolved in methanol:acetic acid:water, 5:1:5. The gels were placed in this solution for about 45 minutes after which they were destained in several changes of destaining solution (methanol:acetic acid:distilled water 5:1:5) until the background was clear.
Drying

The cleared gels were placed on to a clean piece of clear plastic and was overlaid with damp filter paper. These were then transferred to drying equipment - the paper towards the porous plate. The gels were then dried by applying a vacuum. The gels were reduced to a thin film attached to the filter paper.

Autoradiography

The gels were stored under a weight to keep them flat. These were then placed flat onto a Kodak No . screen film NS-2T (Kodak, USA) held firmly between two glass plates. The gel and film were kept in contact for 5 days after which the films were processed. All manipulations were done in a photographic dark room.

4.2.3 Radioiodination Of Monoclonal Antibodies

As indicated earlier one advantage of monoclonal antibody is the facility for biosynthetic labelling. However for some purposes eg, immunoradiometric assay (Miles and Hales 1968) gamma emitting label may be preferred.

Antibodies required for radioiodination or for other purposes for example as the solid phase capture antibody in the sandwich ELISA (see Chapter 5) need to be highly purified.

The antibody binding characteristics and differential elution profile of various antibody subclasses on protein A, together with its simplicity in application (Ey et al 1978 and reviewed by Goding 1978) dictates its use for affinity chromatography in these circumstances.

Protein A purification

The procedure outlined in Hudson and Hay (1980) was used.
Buffers: (1) 0.15M Phosphate buffered saline pH 7.2 containing 0.01% (w/v) thimerosal.
(2) 0.1M Phosphate buffer pH8.0.
(3) 0.1M Citrate-phosphate buffers pH6.0, 5.5, 4.5 and 3.5.
(4) 1.0M Tris-HCl buffers pH8.5 and 9.0.

1.5g Protein A-Sepharose CL-4B (supplied by Pharmacia (GB) Ltd, Hounslow, Middlesex) was swollen in 10.0 ml phosphate buffered saline for 1 hour at
The swollen gel was packed with phosphate buffered saline into a 5 ml syringe which contained a small scinter disc in the end. This column was stored and run at 4°C.

The column was equilibrated by running through 30 ml of 0.1M phosphate buffer pH8.0. The ascitic fluid or supernatant was centrifuged gently for 5 minutes on the MSE bench centrifuge. 0.5-1.0 ml ascitic fluid was diluted with an equal volume of 0.1M phosphate buffer pH8.0, and the pH of the samples ascitic fluid or supernatant adjusted to pH8.1 with 1.0M Tris-HCl pH9.0.

The sample was then applied to the top of the column and wash through with 0.1M phosphate buffer pH8.0 until no protein was detectable in the eluate. The bound immunoglobulins were then eluted as follows:

- IgG_I was eluted with 30 ml 0.1M citrate phosphate buffer pH6.0. This was followed by 30 ml 0.1M citrate phosphate buffer pH5.5.
- IgG_2a and IgG_2b were eluted at very low pH, therefore 2.5 ml Tris HCl buffer pH8.5 was added to collecting tubes prior to collecting the fractions. IgG_2a was eluted with 30 ml of 0.1M citrate-phosphate buffer pH4.5. Finally IgG_2b was eluted with 30 ml of 0.1M citrate phosphate buffer pH3.5. The column was then re-equilibrated to pH8.0. The optical density of the fractions were checked at 280 nm. The fractions containing the monoclonal antibody were pooled and the OD_{280} determined and used to calculate the antibody content of the pooled fraction as described in Chapter 2.

Radioiodination procedure

The monoclonal antibodies were radioiodinated by the chloramin T method of Hunter and Greenwood (1962) and described in detail by Hunter (1979). The radioiodination was carried out in the Grade C laboratory, Biochemistry Dept, University of Surrey.

Reagents

Na_{125}I was supplied by The Radiochemical Centre, Amersham International PLC, UK.

- 0.5M sodium phosphate buffer pH7.5 (iodination buffer).
- 50 μg purified antibody protein in 50 μl.
- Sodium metabisulphite 0.05% (w/v) in distilled water.
- 0.15M phosphate buffered saline pH7.4 containing 0.1% (w/v) bovine serum albumin (BSA) (elution buffer).
- Chloramin-T 2.6% (w/v) in phosphate buffered saline freshly prepared.
The iodination was carried out in small V-bottomed iodination vial on a magnetic stirrer, a small teflon coated triangular magnetic flea (Pierce and Warner (UK) Ltd, Chester, Cheshire) was used to stir the iodination mixture. Stop clock was also essential.

A chromatograph column 30 cm x 1.5 cm was packed with Sephadex 150 (supplied by Pharmacia (GB) Ltd, Houslow, Middlesex) swollen overnight in 0.15M phosphate buffered saline pH7.4 and equilibrated with 50 ml elution buffer.

Procedure

50 μl containing 50 μg monoclonal antibody protein was put into the iodination vial containing the magnetic flea.

10 μl of iodination buffer was added followed by 10 μl Na $^{125}$I (10 μl = 1 mCi), with continual stirring.

10 μl freshly prepared chloramin-T was added to the iodination vial and its contents. Precisely 30 seconds later 200 μl sodium metabisulphite was added.

The iodination mixture was then put onto the prepared gel filtration column. The iodination vial was carefully rinsed with 200 μl elution buffer and this was also applied to the top of the column which was eluted with the elution buffer. 10 μl samples of each fraction was counted.

4.2.4 Distinction Of Epitope Specificity

Frequently it is important to determine whether two monoclonal antibodies are directed at precisely the same antigenic determinant. This is particularly relevant when they are required for use in, for example, two site binding type assays (Hales and Woodhead 1980, Wisdom 1976) where the antigen is required to be bound by a solid phase antibody at the bottom of the sandwich and by another antibody at the top. If both antibodies used in such an assay was directed at the same epitope then one would be expected to considerably hinder the binding of the other especially where the epitope was a single non-repeating unit.

Epitope specificity may be distinguished by examining the competitive inhibition of binding of a labelled antibody to the solid phase antigen by the unlabelled competitor antibody.
1 µg/ml h-TSH was coated to polyvinyl chloride wells as already described. 100 µl of each unlabelled protein-A purified monoclonal antibody at 4 µg/ml was added to a series of wells.

This was followed by the addition of 100 µl (about 100,000 CPM) of each 125I antibody in a checker board sequence such that each unlabelled monoclonal antibody is in combination with each labelled antibody. The positive control was the competition between the labelled and unlabelled preparations of the same antibody. Total binding was derived from binding in wells which contained labelled antibody but the unlabelled antibody was replaced by ELISA diluent buffer. The wells were incubated at room temperature for 2 hours after which the radioactive waste was aspirated and the wells washed three times with ELISA washing buffer. The wells were then air dried separated and counted. Four replicates were done for each antibody mix.

4.3 RESULTS

4.3.1 Characteristics Of Monoclonal Antibody Preparations

a) Antibody titre

The dilution curves obtained by the indirect ELISA method B and solid phase RIA for the monoclonal antibodies ascitic fluid preparations are presented in figures 4.2 and 4.3 respectively and for the supernatants in figure 4.4.

The monoclonal antibody titres determined by these methods and standard liquid phase RIA are tabulated in Table 4.4. The titres were defined as follows:

ELISA titres were taken as the dilutions of the antibody preparations which gave optical densities OD greater than the highest negative observed for a series of negative samples examined. OD values greater than 0.5 were therefore regarded as the titres. The RIA titres were taken as the dilutions which bound 50% of the maximum labelled hormone bound. The monoclonal antibodies have been demonstrated to be applicable in all three assay systems. The apparent titre by ELISA was higher than that observed by the solid phase by a factor of between 2 and 8, and similarly the titres were higher by the solid phase method than by the standard liquid phase RIA by a factor of between 8 and 80. With the two assay methods developed specifically for detecting the monoclonal antibodies the antibody could be used at much greater dilution.
FIGURE 4.2 ELISA Titration curves for 6 Monoclonal Antibodies from TSH8. These cell lines were named SY/T8/1-6. AF1 ● ● ● ●; AF2 □ □ □ □; AF3 O ○ ○ ○; AF4 ■ ■ ■ ■; AF5 △ △ △ △; AF6 △ △ △ △. The titres (ie OD values >0.5 is regarded as the positive titre) range between $3.3 \times 10^5$ and $1.4 \times 10^5$. △—△ is a pooled mouse Anti TSH antiserum positive; △—△ is a non-immune mouse serum (negative control).
FIGURE 4.3 Solid phase RIA titration curves.
Ascetic fluids 1 ● — ● ; 2 □ — □ ; 3 ○ — ○ ;
4 ■ — ■ ; 5 △ — △ ; 6 △ — △ .
RIGHT HAND ordinate △ — △ = positive control
pooled mouse Anti TSH antiserum; △ — △ — △ = negative
mouse serum.
FIGURE 4.4 ELISA titration curves of supernatants, 1 •••• 2 □□□;
3 O---O; 4 ■■■; 5 △△△; 6 △△△. Titre taken as the
dilution with OD ≥1.0. △-----△ NSI supernatant.
TABLE 4.4

Antibody titres observed by ELISA, Solid Phase RIA and standard liquid phase RIA for ascitic fluids (AF) and supernatants (SN). For the ascitic fluid, the ELISA titre was taken dilution with OD\textsubscript{490} greater than the highest negative (ie, >0.5). For the SN the ELISA titre was taken as the dilution with OD\textsubscript{490} = 1.0. The RIA titre was taken as the dilution which bound 50% of the maximum bound label. % inhibition is calculated from the displacement of dilution curves by TSH, see figures 4.5 - 4.7.

<table>
<thead>
<tr>
<th>Sample</th>
<th>ELISA titre (AF)</th>
<th>Solid Phase titre (AF)</th>
<th>Standard RIA titre (AF)</th>
<th>ELISA titre (SN)</th>
<th>% Inhibition with TSH in SN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:1.8 x 10\textsuperscript{5}</td>
<td>1:6.4 x 10\textsuperscript{4}</td>
<td>1:1.6 x 10\textsuperscript{3}</td>
<td>1:290</td>
<td>76.0%</td>
</tr>
<tr>
<td>2</td>
<td>1:1.0 x 10\textsuperscript{5}</td>
<td>1:3.5 x 10\textsuperscript{4}</td>
<td>1:0.6 x 10\textsuperscript{3}</td>
<td>1:200</td>
<td>79.0%</td>
</tr>
<tr>
<td>3</td>
<td>1:1.2 x 10\textsuperscript{5}</td>
<td>1:4.8 x 10\textsuperscript{4}</td>
<td>1:0.6 x 10\textsuperscript{3}</td>
<td>1:230</td>
<td>65.0%</td>
</tr>
<tr>
<td>4</td>
<td>1:3.0 x 10\textsuperscript{5}</td>
<td>1:3.5 x 10\textsuperscript{4}</td>
<td>1:4.0 x 10\textsuperscript{3}</td>
<td>1:200</td>
<td>66.7%</td>
</tr>
<tr>
<td>5</td>
<td>1:1.9 x 10\textsuperscript{5}</td>
<td>1:5.6 x 10\textsuperscript{4}</td>
<td>1:2.5 x 10\textsuperscript{3}</td>
<td>1:100</td>
<td>65.0%</td>
</tr>
<tr>
<td>6</td>
<td>1:6.4 x 10\textsuperscript{4}</td>
<td>1:5.2 x 10\textsuperscript{4}</td>
<td>1:1.2 x 10\textsuperscript{3}</td>
<td>1:200</td>
<td>85.0%</td>
</tr>
</tbody>
</table>

Displacement curves

In addition to dilution curves displacement curves are usually set up. These give useful information about the affinity of the antiserum. The degree of displacement is an indirect measure of the affinity of the antibody preparation (Morris 1981, Hunter 1979) for the antigen.

The displacement curves obtained are presented in figures 4.5 - 4.7. No displacement was observed with the NIBSC TSH preparation and any of the monoclonal antibody preparations. A similar result was obtained by Dr D Teale in the standard liquid RIA performed using ascitic fluid antibody preparations.

Preincubation of the antibody and the NIBSC TSH for 2 hours at 37°C did not result in improved displacement. At the supernatant titres in Table 4.4 the percentage inhibition or displacement with TSH(Barts) was calculated thus:

\[
\text{% Inhibition} = \left(1 - \frac{x \text{ ELISA OD}_{490} \text{ displacement wells}}{x \text{ ELISA OD}_{490} \text{ antibody dilution wells}}\right) \times 100
\]
FIGURE 4.5  Supernatant dilution curves (●—●); dilution curve displaced with 500 mIU/l TSH(Barts) (○—○); or displaced with 500 mIU/l NIBSCTSH (■—■).
Supernatant dilution curves (O-O): TSH (Barts) 

or displaced with 500 μl/l NIBSC TSH ( ).
FIGURE 4.7. Supernatant dilution curves ( ● --- ● );
dilution curve displaced with 500 mU/l TSH (Barts) ( ○ --- ○ );
or displaced with 500 mU/l NIBSCTSH ( ■ --- ■ ).
The results are contained in Table 4.4. The results suggest SN6 should be the highest affinity antibody since it displayed the greatest displacement under these conditions.

b) Antibody cross reactivity

The results of these specificity studies are presented in figures 4.8 - 4.12. Antibody SY/T8/4 showed the most cross reactivity, evidenced by the apparent inhibition of binding relative to that observed in the control wells which was taken as 100% binding. The inhibition was most marked with HCG β subunit, HCG(Barts) preparation, and FSH at 100 mU/well. The inhibition of binding although apparent, occurred to a lesser extent with HCG α subunit.

The mouse anti-TSH antiserum, however, displayed the greatest inhibition of binding in the presence of HCG α subunit (figure 4.12). Antibody No4 on the other hand did not show any cross reactivity with the NIBSC HCG (figure 4.10).

A particularly notable phenomenon was the overt enhancement of binding by antibody SY/T8/1 observed in the presence of HCG β subunit (figure 4.9). TSH(Barts) inhibited the binding of the monoclonal antibodies to the solid phase antigen to the greatest extent as expected (figure 4.8). The cross reactivity results are summarized in Table 4.5.

<table>
<thead>
<tr>
<th>Antibody SY/T8/-</th>
<th>HORMONES</th>
<th>NIBSC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HCG(Barts)</td>
<td>HCG</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**TABLE 4.5**

Summary of antibody hormone specificity. Binding of the antibody to solid phase TSH was inhibited by various concentrations of hormones.

+ = cross reactivity - = no cross reactivity
FIGURE 4.8 Binding of monoclonal antibodies to solid phase TSH in the presence of TSH (Barts).

SN1 (●—●), SN2 (□—□),
SN3 (○—○), SN4 (■—■),
SN5 (▲—▲), SN6 (△—△),
FIGURE 4.9 Relative binding of monoclonal antibodies to TSH in the presence of NIBSC HCG α and β subunits. SN1 (●●●); SN2 (□□□); SN3 (○○○); SN4 (■■■); SN5 (▲▲▲); SN6 (△△△).
FIGURE 4.10  Relative binding of monoclonal antibodies to TSH in the presence of NIBSC supplied whole HCG and HCG from the same source as the solid phase TSH. SN1 ●●; SN2 □□; SN3 ○○; SN4 ■■; SN5 ▲▲; SN6 △△.
FIGURE 4.11  Relative binding of monoclonal antibodies to TSH in the presence of NIBSC supplied LH and FSH.
SN1 ●—●; SN2 □—□; SN3 ○—○; SN4 ■—■; SN5 ▲—▲; SN6 △—△.
FIGURE 4.12 Relative binding of mouse anti TSH to solid phase TSH in the presence of HCG •—•; and α subunit Δ—Δ; and β subunit ○—○.
c) **Antibody affinity**

The antibody antigen binding is a reversible reaction which obeys the law of Mass action:

\[
\text{Ab} + \text{Ag} \xrightleftharpoons[k_2]{k_1} \text{AbAg}
\]  

\(\text{Ab} = \) antibody, \(\text{Ag} = \) antigen, \(k_1\) and \(k_2\) = forward and reverse reaction rates respectively.

At equilibrium therefore:

\[K = \frac{k_1}{k_2} = \frac{[\text{AbAg}]}{[\text{Ab}][\text{Ag}]} \]  

The rationale of the experimental procedure was based on the Scatchard (1949) transformation of the above relationship to give the following equation.

\[
\frac{[\text{Ab} \text{bound}]}{[\text{Ab} \text{free}][\text{Ag} \text{total}]} = sK_i - (\frac{[\text{Ab} \text{bound}]}{[\text{Ag} \text{total}]}) K_i n
\]  

where \(K = \) the equilibrium binding constant; while \(s\) and \(n\) are the valency of the antigen and antibody respectively.

The expression was rewritten:

\[
\frac{[\text{Ab} \text{bound}]}{[\text{Ab} \text{total}]} - [\text{A} \text{bound}] = (sK_i[\text{Ag} \text{total}]) - ([\text{Ab} \text{bound} K_i n)
\]  

This expression formed the basis of the determination of the binding constant described by Frankel and Gerhard (1979). The equation requires that only antibody bound and antibody total quantities be known and providing the antigen concentration is kept constant which it is in the solid phase, then the absolute concentration is not required to be known.

The amount of antibody bound ([Ab]bound) at each dilution was derived by extrapolation to the linear standard calibration curve of known antibody concentration. The antibody total ([Ab]total) was taken as the maximum antibody bound at maximum antigen concentration derived for one antibody dilution. At this increased antigen concentration in the assay wells 99% of antibodies with equilibrium constants greater than \(4 \times 10^7 M^{-1}\) and 95% with equilibrium constants greater than \(9 \times 10^6 M^{-1}\) will be bound (Frankel and Gerhard 1979). The total antibody in the other dilutions was then calculated in relation to that antibody dilution.
The results of the binding experiments are presented in figure 4.13 and 4.14. These figures show that at between 300 and 500 ng antibody added per well, the antigenic sites were saturated under the experimental conditions.

A plot of [Ab]bound/[Ab]total-[Ab]bound vs [Ab]bound yield a slope of -2K for a bivalent antibody. Figure 4.15 is the Scatchard plot of the data, linear regression analysis of which yields the equilibrium constant values outlined in Table 4.6.

**TABLE 4.6**

Linear regression analysis of Scatchard plots data allows determination of slopes = -2K and hence equilibrium binding constants K.

<table>
<thead>
<tr>
<th>Hybridoma Antibody Supernatant</th>
<th>Equilibrium Constant K(M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SY/T8/1</td>
<td>7.31 ± 0.23 x 10¹⁰</td>
</tr>
<tr>
<td>SY/T8/2</td>
<td>2.37 ± 0.29 x 10¹¹</td>
</tr>
<tr>
<td>SY/T8/3</td>
<td>8.15 ± 0.31 x 10¹⁰</td>
</tr>
<tr>
<td>SY/T8/4</td>
<td>5.25 ± 0.13 x 10¹⁰</td>
</tr>
<tr>
<td>SY/T8/5</td>
<td>5.04 ± 0.19 x 10¹⁰</td>
</tr>
<tr>
<td>SY/T8/6</td>
<td>1.13 ± 0.41 x 10¹¹</td>
</tr>
</tbody>
</table>

d) *Antibody classes*

Precipitation lines were observed with all of the ascitic fluid preparations and the neat anti-IgG₂a and anti-k antisera.

Precipitation reactions were also found with the ammonium sulphate precipitated and concentrated supernatant and the neat IgG₂a and k antisera. The results are presented in Table 4.7.

The antibody class IgG was confirmed in a newly published procedure (Kohn 1984).
FIGURE 4.13 ELISA of supernatant from monoclonal antibody cultures. 200 µl of each supernatant dilution was added to a series of wells coated with 1 µg/ml TSH. SN1 (●●); SN2 (□□); SN3 (○○).
The antibody total was obtained in relation to that bound at maximum antigen concentration.
FIGURE 4.14 ELISA of supernatant from monoclonal antibody cultures. 200 µl of each supernatant dilution was added to a series of wells coated with 1 µg/ml TSH. SN4 (■■); SN5 (▲▲); SN6 (▲▲). The antibody total was obtained in relation to that bound at maximum antigen concentration.
Figure 4.15 ELISA binding data from monoclonal antibody supernatants 1-6. Data from figure 4.15 and 4.16 replotted in the modified Scatchard form. Linear regression analysis of the data yields slopes $= 2k$.

- SN1 (•-•); SN2 (□-□); SN3 (○-○); SN4 (■-■); SN5 (▲-▲); SN6 (△-△).
### Table 4.7

Ouchterlony immunodiffusion analysis of the monoclonal antibodies SY/T8/1-6.  
+ = precipitation lines;  - = no precipitation lines formed

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Antimouse immunoglobulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μ</td>
</tr>
<tr>
<td>SY/T8/1</td>
<td>-</td>
</tr>
<tr>
<td>SY/T8/2</td>
<td>-</td>
</tr>
<tr>
<td>SY/T8/3</td>
<td>-</td>
</tr>
<tr>
<td>SY/T8/4</td>
<td>-</td>
</tr>
<tr>
<td>SY/T8/5</td>
<td>-</td>
</tr>
<tr>
<td>SY/T8/6</td>
<td>-</td>
</tr>
</tbody>
</table>

#### 4.3.2 Electrophoretic Analysis Of Monoclonal Antibody Protein

Figure 4.16 and 4.17 are photographic records of Coomassie Blue stained SDS-PAGE electrophoresed reduced and unreduced supernatant samples; and autoradiographic development of the biosynthetically radiolabelled antibodies respectively.

No heavy chain was observed to be secreted by NSI in Figure 4.17 as no radiolabelled protein tracks were observed in the extracellular fluid. The internally labelled protein solution however showed several bands one of which coincided with the L-chain band in the reduced sample of the purified sheep IgG sample track, figures 4.16 and 4.17. This also identified the L and H chain bands of the monoclonal antibodies reduced sample. The specific antibody binding activity may therefore be assigned to the occurrence of new H and L chains. These band positions were also confirmed by the mobility of the samples in relation to the standards (see figure 4.16).

No myeloma H chains were expected or observed so all H chains must be of spleen cell origin the only contaminant chain expected was the NSI synthesized k chain which was shown to be minimal. A lengthy incubation period was required for these bands to show up by autoradiography.
**FIGURE 4.16**

10% SDS-Page analysis of reduced and unreduced supernatant samples. Gel stained with coomassie blue and vacuum dried onto filter paper.
FIGURE 4.17

 Autoradiography of polyacrylamide gel after electrophoresis. Biosynthetically labelled monoclonal antibody and NSI intra-cellular and extra-cellular fluids.
## 4.3.3 Antigenic Determinant Specificity

In order to radioiodinate the monoclonal antibodies the various preparations were purified by Protein A chromatography. The elution profiles obtained are presented in figures 4.18 - 4.20 and the elution of the peak protein in each case at pH4.5 is consistent with the antibody classes being IgG2a.

There was some additional protein eluted at pH5.5 from ascitic fluid 1-4 this was probably contaminant mouse immunoglobulin against the hybridoma cells. This was however not confirmed. The specific antibody was confirmed by the indirect ELISA to reside in the pH4.5 eluted peaks.

The monoclonal antibodies so purified were each radioiodinated. Radioiodination of the antibodies did not appear to interfere with their binding to the antigen.

The mean of the wells containing labelled antibody in which the unlabelled antibody was replaced by assay diluent buffer was taken as the 100% total binding. The other test activities were then expressed relative to these thus:-

\[
\text{Relative \% Inhibition in the presence of unlabelled antibody} = \frac{(1 - \frac{\text{CPM bound in test wells}}{\text{CPM in total binding wells}})}{100}
\]

The results presented in table 4.8 suggest that there is some interfering interaction between all the labelled antibody binding the solid phase antigen and the unlabelled antibodies. The main inference may be that they all may bind the same antigenic determinant resulting in the reciprocal inhibition observed although the degree of inhibition is variable. This however is by no means entirely substantiated and the results may have any of several other interpretations.

For instance the binding of any of the unlabelled antibody may cause the alteration of the configuration of other antigenic determinants thereby resulting in reduced binding by the labelled antibody. On the other hand the binding of the unlabelled antibody may cause steric hinderance to the binding of the labelled antibody because the antigenic determinants are not identical but very close. One distinct feature of these competitive binding results is that the labelled SY/T8/4 is inhibited to a lesser extent by the other unlabelled antibodies. Unlabelled SY/T8/2 however inhibited SY/T8/6 by 93.3% much greater than the inhibition of labelled SY/T8/2 by the unlabelled
FIGURE 4.18 Elution profile of ascitic fluid antibody from Sepharose - protein A chromatography column by stepped pH gradient system (---).

a) SY/T8/1     b) SY/T8/2
FIGURE 4.19 Elution profile of ascitic fluid antibody from Sepharose - protein A by stepped pH system (---).

a) SY/T8/3       b) SY/T8/4
FIGURE 4.20  Elution profile of ascitic fluid antibody from Sepharose - protein A by stepped pH system

a) SY/T8/5  
b) SY/T8/6
SY/T8/2. This would suggest either that 6 bound the same antigenic determinant as 2 but with a greater affinity or the labelling procedure has altered the binding site of 6 sufficiently to decrease binding efficiency to the antigenic determinant. The nonspecific binding in all cases was very low <3.0%.

**TABLE 4.8**

Epitope binding specificities determined by competition of labelled and unlabelled monoclonal antibodies. The results were expressed as percentage (%) inhibition compared to the binding in wells in which the unlabelled antibodies were replaced with assay diluent. These were taken as 100% total binding.

<table>
<thead>
<tr>
<th>Unlabelled monoclonal antibodies SY/T8/-</th>
<th>125I-monoclonal antibodies SY/T8/-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1  2  3  4  5  6</td>
</tr>
<tr>
<td>Relative % inhibition</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>66.0 63.4 63.7 20.5 44.9 64.8</td>
</tr>
<tr>
<td>2</td>
<td>57.3 63.2 60.4 21.4 55.6 74.2</td>
</tr>
<tr>
<td>3</td>
<td>66.7 75.9 74.75 35.2 62.2 27.0</td>
</tr>
<tr>
<td>4</td>
<td>23.9 32.4 39.8 62.3 49.6 29.4</td>
</tr>
<tr>
<td>5</td>
<td>54.2 62.8 61.0 51.4 66.9 32.1</td>
</tr>
<tr>
<td>6</td>
<td>62.4 93.3 21.4 23.4 35.4 74.2</td>
</tr>
</tbody>
</table>
DISCUSSION AND CONCLUSIONS

All the monoclonal antibody producing hybridomas gave high titre antibody preparations as measured by ELISA solid phase RIA and standard liquid phases RIA.

The antibodies were shown to be highly specific for binding with TSH(Barts) antigen. No crossreactivity was observed with SY/T8/1, 2, 3 or 6. Antibody SY/T8/1 however did show an unexplained enhancement of binding to the antigen in the presence of HCG β subunit (figure 4.8). SY/T8/4 showed the greatest inhibition of binding to the solid phase antigen in the presence of all the hormones tested except the NIBSC HCG preparation where no cross-reactivity was observed.

One major observation was that none of the monoclonal antibodies could be displaced by the NIBSC TSH 68/38 standard (see figures 4.5 - 4.7) even when very large amounts were used. This situation was also observed in the liquid phase RIA kindly performed by Dr Teale, Clinical Biochemistry, St Lukes Hospital, Guildford, Surrey. He reported no displacement of the radio-labelled TSH by unlabelled TSH 68/38 standard in assays set up using diluted ascitic fluid.

Because this characteristic was observed in both the ELISA and RIA using cell supernatants and ascitic fluid as the antibody preparations respectively, it was concluded that the results could not be due to the assay conditions which prevented the antibodies binding TSH 68/38. It was concluded therefore that the antigenic determinants to which the antibodies were specifically directed could not be present at least in sufficient numbers in TSH 68/38 standard to be detected above the background.

TSH 68/38 could not therefore be used to set up an assay using these monoclonal antibodies. The TSH(Barts) antigen which was used to prepare the spleen cell, calibrated against TSH 68/38 in a liquid phase RIA (see Chapter 2) was therefore used as standard in the ELISA (Chapter 5).

All the monoclonal antibodies were found to be IgG2a with very high affinities of between $5 \times 10^{10}$ and $2.37 \times 10^{11}$. The high affinities meant they could possibly be useful in immunocytochemical procedures where high affinity specific antibodies are required.

High affinity antibodies are not so critical in immunometric type assay as was proposed. The antibody in such assays are usually added in excess thereby ensuring complex formation. Conditions influencing the equilibrium of reaction therefore have very limited effects in such assay systems (Ekins 1981).
There was no evidence of antibody contamination by the myeloma k light chain. But the case of internal labelling of the monoclonal antibodies was demonstrated. On the other hand they could also be radiolabelled with relative ease and still retained antigen binding capacity.

Although the antibody protein in supernatants was contaminated by only a minimal amount of foetal calf immunoglobulin and was therefore very pure, the preparation nevertheless contained serum proteins. For some purpose such as radioiodination the antibody needed to be purified. The ease and efficiency of protein A purification was demonstrated, and the antibody binding capacity did not appear to be diminished by the treatment.

The antibodies all appeared to bind the same epitope with the possible exception of SY/T8/4 (table 4.8). If this epitope is absent or diminished on the TSH 68/38, this then would explain the lack of binding of any of the antibodies with that TSH preparation.

From the results of characterization SY/T8/2 with the highest affinity was chosen to set up the ELISA; and all the monoclonal antibodies were demonstrated in the immunocytochemistry procedure.
CHAPTER 5

Applications of Monoclonal anti-human-TSH Antibodies
5.1 INTRODUCTION

Antibodies specific to h-TSH have been derived and used in two main areas of TSH investigations. They have been used to describe by immunocytochemical procedures the localization of the particular hormone secreting cells in the pituitary gland (Phifer and Spicer 1973, Nakane 1970, Tangard et al 1980). All six monoclonal antibodies which have been characterized have been used in an indirect immunoperoxidase technique to demonstrate the occurrence of the antibody specific determinants in various pituitary and other tissue sections. The second important application has been their use in specific quantitation of h-TSH in body fluids (Odell and Utiger 1973, Hall et al 1971, Miyai et al 1976, John and Woodhead 1982). One monoclonal antibody was therefore selected and used to develop an enzyme linked immunosorbent assay for h-TSH.

5.1.1 Immunocytochemical Demonstration Of Thyrotropic Cells

The hypophysis is located at the base of the brain. It is a small gland weighing about 0.5g. It is linked in a feedback system of actions with the endocrine organs it controls, and the neurosecretions by which it is controlled (Labrie et al 1979).

The unequivocal identification of the individual hormone secreting cell types is the primary objective of pituitary immunocytochemistry. The clarification of the functional classification and nomenclature of the various cell types have only been achieved with immunocytochemical techniques. The correlation of the tinctorial cell types (Bloom and Fawcett 1975) with immunocytochemical identification is well documented (Halmi and Morianty 1977, Nakane 1970 and 1975, Kovacs et al 1981).

The thyrotropic cells

Histologically these cells were described as basophils which were preferentially distinguished from acidophils by their pink staining with the periodic acid-schiff (PAS) reaction. Thyrotropes however have been distinguished from the other basophils: the gonadotropes, by the selective staining of their granules with aldehyde fuchsin (Bloom and Fawcett 1975).
Immunocytochemical identification of thyrotropes have to the present time been performed exclusively with polyclonal antisera to thyroid stimulating hormone (Phifer and Spicer 1973). These antisera have of course carried the inherent disadvantages of cross reactivity to the gonadotrophic glycoproteins described in Chapter 1. Much of the difficulty was however eliminated with the use of antisera to the β-subunit of the hormone (Tougard et al 1980, Baker and Yu 1971). These procedures together with electron microscopic studies (Nakane 1975) have confirmed the polygonal or stellate shape of thyrotropes arranged in clusters in the center of the cord. Their glandular distribution is depicted in figure 5.1. Although most descriptions of these cells have been derived primarily from studies on rat pituitaries (Nakane 1970, 1975, Baker 1974) they are similar to those of other species. Human thyrotropes have however been described (Phifer and Spicer 1973, Pelletier et al 1978, Moriarty and Tobin 1976).

A comprehensive description of pituitary immunocytochemistry is given by (Petrusz and Ordronneau 1983) and immunocytochemistry generally by Sternberger (1979), Polak and Van Noorden (1983).

A well characterized monoclonal antibody in terms of specificity and affinity provides the means of overcoming the persistent cross reactivity problems of a polyclonal antisera. Low affinity monoclonal antibodies may however limit their application here. Since only a single species of antibody is contained in the antibody preparation it may be easily eluted during the procedure and so specific binding may be lost. This is unlike the situation occurring in a heterogeneous polyclonal antibody preparation where accidental elution of low affinity antibodies would not affect the specific binding of the remaining high affinity antibodies. High affinity monoclonal antibodies are therefore a priority for this purpose.

The importance of pituitary immunocytochemistry is by no means limited to characterizations in the normal gland, but are of critical importance in identifying pituitary tumors (Heitz 1983) and in the localization of pituitary hormones as non pituitary tumor markers (Heyderman 1983).
5.1.2 ELISA

Several modifications of ELISA procedures have been described for the detection and measurement of antigens (Voller 1979). These include as for RIA procedure, the competitive type of assay (Avrameas and Guilbert 1971, Engvall and Perlmann 1971, Van Weeman and Schuurs 1971 and 1972) and the non-competitive binding assays (Engvall and Perlmann 1972, Maiolini and Masseyeff 1975, Guesdon and Avrameas 1977).

Avrameas (1981) suggested that for the measurement of multivalent antigens they found the non-competitive assays more sensitive. The additional advantages of such assays were that they were more robust, less expensive in terms of precious antigen and as such were more suitable for routine work. This type of assay also requires labelled antibodies which were universal reagents described in Chapter 2, instead of the labelled antigen of the competitive system.

Consequently we attempted to set up double antibody sandwich non-competitive ELISA for measuring h-TSH. This was described diagramatically in figure 5.2.
FIGURE 5.2
Double antibody sandwich ELISA for measuring antigens

STEP 1
Wash x 3
PBS Tween + 0.1% BSA

STEP 2
2 hour incubation
Antibody A adsorbed to polyvinyl chloride plates (monoclonal Ab).

STEP 3
Test antigen sample added. Incubated overnight

STEP 4
Add specific antibody B. Incubate 2 hours

STEP 5
Add enzyme labelled anti-B antibody. Incubate 2 hours

Add enzyme substrate OPD.
Incubate 30 mins.

5.2  MATERIALS AND METHODS

5.2.1 Tissue Preparation

Four human pituitary autopsy specimen preparations, and other tissue preparations including human kidney, placenta and gut were all kindly supplied by Professor Gibbs, Pathology Dept., Royal Surrey County Hospital, Guildford. The tissue preparations were all supplied as formalin fixed, paraffin embedded blocks and some mounted sections.

The baboon pituitary sections were kindly supplied by Dr J Drake, University of Surrey, Guildford; as Bouins fixed mounted sections. They were derived from young females.

The dog pituitary sections were kindly supplied by Dr J Hooson, Wyeth Laboratories, Burnham, Bucks; as formalin fixed, paraffin embedded mounted sections.

The rat and ferret pituitary preparations were kindly supplied by Miss J Howarth, Pathology Lab., University of Surrey, Guildford; as formalin fixed paraffin embedded sections. These sections were cut and mounted as described below.

Cutting sections

Additional human pituitary and other tissue sections were cut from the blocks at a thickness of 6 μ using an American Optical Spencer 820 rotary microtome. The sections were floated on distilled water kept at 50°C until the creases disappeared. The sections were then mounted by dipping the clean slide into the water. By withdrawing it the thin section was drawn onto the slide. The slides were then dried on a hot plate after which they were stored overnight in 37°C incubator. The slides were then packed and stored at room temperature until required. The mounted sections supplied by Professor Gibbs Dept were cut and mounted as described above (Verbal communication: Mr P Jenkins, Pathology Dept. Royal Surrey County Hospital, Guildford).

Histological staining

Periodic Acid Schiff (PAS) stained sections of the 4 human pituitary preparations and haematoxylin eosin stained sections were kindly supplied (Mr P Jenkins, Royal Surrey County Hospital, Guildford). In addition PAS sections and haematoxylin eosin stained sections of the rat and ferret pituitaries were prepared by Miss J Howarth, Pathology Lab, University of Surrey).
5.2.2 Immunocytochemical Procedure

Since neither the monoclonal antibodies nor the enzyme-linked second antibodies had previously been used in this procedure, then a range of dilutions of the two antibody preparations had to be investigated to find the most effective combinations. A variety of techniques have been described (Van Noorden and Polak 1983, Sternberger 1979). The technique used in this study is the indirect method. This is regarded as more sensitive than the direct method and the second enzyme-labelled antibody can be used to locate a variety of antigens provided the primary antibodies are obtained from the same species.

Reagents

1) Buffers: 0.025M Tris saline buffer pH7.6

9.1g Tris (hydroxymethyl) aminomethane (Sigma London Chemical Co Ltd, Poole, Dorset) was dissolved in 750 ml distilled water;
25.5g Sodium Chloride was added and mixed thoroughly.
The tris saline solution was titrated to pH7.5 with 1.0M HCl. Then the volume was made up to 3.0 litres with distilled water. Finally the pH was adjusted to pH7.6. (Buffer may be kept for up to one month stored at 4°C.)

2) Citrate Acetate buffer pH5.0

5.3g citric acid (BDH Chemicals Ltd, Enfield) was dissolved in 500 ml distilled water
12.0g Ammonium acetate (BDH Chemicals Ltd, Enfield) was dissolved in 3 litres distilled water. The citric acid solution was added to the ammonium acetate solution until the pH was 5.0. (This buffer was also kept at 4°C for one month.)

Peroxidase blocking solution

This was made up of 120 ml hydrogen peroxide (H₂O₂) (30% w/v) (BDH Chemicals Ltd, Enfield), and 200 ml methanol (BDH Chemical Ltd, Enfield). This peroxide blocking solution was made up fresh every two days. It was used to block endogenous peroxidase activity in the tissue sections which could cause non specific staining effects.
Chromogen

The chromogen solution consisted of 340 ml citrate acetate buffer pH 5.0 in which was dissolved 125 mg 3.3-diaminobenzidine tetrahydrochloride (DAB) (supplied by BDH Chemical Ltd, Enfield), and finally 200 µl hydrogen peroxide was added. (Note: DAB is said to have possible carcinogenic properties therefore extra care was taken in handling the material.)

Sera

1) 1% (v/v) normal goat serum (Guildhay) in Tris-saline.
2) 1% (v/v) normal donkey serum (Guildhay) in Tris-saline.
3) Monoclonal antibodies as ascitic fluid preparations were diluted 1:50 up to 1:500,000 in Tris saline buffer containing 1.0% normal goat or donkey serum. (Normal goat or normal donkey serum was used with the corresponding goat or donkey HRPO conjugate.)
4) Goat anti-mouse HRPO conjugate I was diluted 1:20 to 1:100 in tris saline buffer.
5) Donkey anti-mouse HRPO conjugate II and III were diluted 1:4 up to 1:64.

Protocol

The preliminary experiments were set out after the protocol outlined below in Table 5.1.

<table>
<thead>
<tr>
<th>Goat anti-mouse HRPO Conjugate I</th>
<th>Monoclonal antibody dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a 1:50 b 1:250 c 1:500 d 1:1000 e Buffer control</td>
</tr>
<tr>
<td>f. 1:20</td>
<td>af bf cf df ef</td>
</tr>
<tr>
<td>g. 1:100</td>
<td>ag bg cg dg eg</td>
</tr>
</tbody>
</table>

The sections were dewaxed by soaking for 10 minutes in xylene followed by progressive rinsing in alcohol 100%, 75% and 50% (v/v) in distilled water for 1 minute each, and finally to distilled water for 1 minute.
The dewaxed sections were then soaked for 10 minutes in gently running tap water, followed by soaking for 10 minutes in the peroxidase blocking reagent.

The sections were washed for a further 10 minutes in gently running tap water. Care must be taken not to dislodge the sections from the slides.

The excess water was removed by tapping the slides on clean tissue paper. Then 200 µl of 1.0% normal goat serum or normal donkey serum was spread carefully over the tissue sections with a bent Pasteur pipette. These were incubated at room temperature for 30 minutes.

The excess liquid was then removed by tapping the slides on edge. 200 µl of the appropriate monoclonal antibody dilution was then spread evenly over the sections which were incubated overnight in a humidified slide box in the cold room at approximately 4°C.

The slides were then washed twice by squirting tris saline buffer onto them and leaving for 10 minutes between each wash.

The tissue free areas of the slides were dried and 200 µl of the appropriate dilution of the second antibody, goat or donkey anti-mouse HRP0 conjugate was evenly spread over the sections. These were incubated at room temperature for 2 hours in the humidified slide box.

Following 2 x 10 minutes washes with tris saline buffer the slides were immersed in the chromogen solution for exactly 3 minutes at room temperature.

They were washed for 10 minutes in gently running tap water, followed by counter staining with haematoxylin. At each successive stage, care was taken to ensure that the tissue sections did not dry out since this would increase the background.

**Haematoxylin staining and mounting**

The sections were placed in Ehrlich's acid haematoxylin (supplied by R A Lamb Ltd) for 15 minutes. They were then gently washed in running tap water for 5 minutes, followed by differentiation in acid alcohol (consisting of 1% (v/v), hydrochloric acid in 70% (v/v) alcohol) with gentle agitation for about 5 seconds.

The sections were again rinsed in running tap water for 2 minutes and examined by low power microscopy to ensure sufficient differentiation.

The sections were rinsed finally for 15 minutes in tap water and progressively dehydrated by passing through 70% (v/v) alcohol followed by 100% alcohol twice for 30 seconds each time. They were then cleared in two changes of xylene for 30 seconds. The sections were mounted by placing one drop of DPX (BDH Chemicals Ltd, Enfield) mountant onto a clean coverslip and
inverting a slide taken directly from the xylene on to the coverslip and pressing down so that the mountant spread evenly over the coverslip and tissue section and excluded all air bubbles.

The slides were then ready for microscopic examination. In all succeeding batches a human pituitary section processed with 1:1000 dilution of the monoclonal antibody and 1:20 dilution of the goat anti-mouse conjugate I or 1:8 dilution of conjugate III was included as positive reliability control of the method. In addition, the negative control sections were prepared in which normal nonimmune mouse serum was used at the same dilution as the positive sera or positive sera was replaced with buffer solution, and binding indicated with 1:20 dilution or 1:8 dilution of the appropriate conjugate.

Adsorption controls

Two adsorption procedures were tried.

1) Liquid phase adsorption was attempted by diluting the monoclonal antibody 1:32000 and incubating with 10 μg TSH at 4°C for 24 hours the solution was then used in the indirect immunochemical staining procedure (Van Noorden and Polak 1983, Wahlstrom et al 1981).

2) Solid Phase adsorption. This was kindly done by Dr R Edwards, NETRIA St Bartholomew's Hospital, London. They prepared the purified h-TSH used in the course of the work; and have available a TSH immunoadsorbent column. The ascitic fluid preparations were returned as freeze dried samples derived from 10 ml eluate in 0.05M phosphate buffer. This was a 1:20 dilution of the original sample. These immunoadsorbed preparations were used for staining controls (Van Noorden and Polak 1983).

5.2.3 Two Site ELISA I

For this ELISA procedure it was proposed to use a sheep anti-h-TSH antisera as one side of the sandwich and the selected specific monoclonal antibody on the other. The high specificity of the sheep antisera was not critical since the specificity of the assay system was expected to be provided by the monoclonal antibody. An enzyme-linked immunosorbent assay has been described by Wada et al 1982 in which anti-α-subunit monoclonal antibodies were used as solid phase capture antibody for three of the glycoprotein hormones TSH, LH and HCG. The enzyme conjugated monoclonal anti-β-subunit antibody specific to each hormone provided the required specificity for the assay.
Sheep anti-h-TSH

Sheep anti-h-TSH batches (HP/S/1001-18.11.81) and (HP/S/1001-11.12.81) were kindly supplied by Guildhay, University of Surrey. The antisera had been stored at 4°C with 1.0% sodium azide added as preservative.

Purification of sheep anti-h-TSH

Both antisera preparations were purified by diethylaminoethyl (DEAE) cellulose ion exchange chromatography after ammonium sulphate fractionation.

Buffers:–

1) 0.3M Phosphate buffer pH7.3 made up as follows:

160g - di Sodium hydrogen phosphate (Na$_2$HPO$_4$);
51g - Potassium di-hydrogen phosphate (KH$_2$PO$_4$) and
18.6g - Ethylenediaminetetra-acetic Acid di-sodium salt (Na$_2$EDTA.2H$_2$O) were dissolved in 5 litres of distilled water.

2) 0.03M Phosphate buffer pH7.3 consisted of a 1:10 dilution of 0.3M Phosphate buffer.

a) Ammonium sulphate purification

100 ml of each antisera was precipitated with 60 ml saturated ammonium sulphate as described previously section 2.2.1. Each precipitate was redissolved to a volume of 40 ml in 0.03M Phosphate buffer pH7.3 followed by extensive dialysis against several changes of 0.03M Phosphate buffer. Finally the preparations were spun down on the bench MSE centrifuge for 5 minutes, and the supernatant collected and stored at 4°C until required.

b) DEAE cellulose ion-exchange chromatography

A column 25 cm x 2.5 cm packed with DEAE cellulose was available by the kind courtesy of Guildhay, University of Surrey, Guildford.

The column was extensively eluted with several volumes of 0.3M phosphate buffer pH7.3. The pH of the eluate was checked and the column was equilibrated until the eluate pH was equal to the loading buffer.

Two volumes of 0.03M Phosphate buffer was then washed through the column. The ammonium sulphate prepared fraction was loaded carefully on top of the column, which was then eluted with 0.03M phosphate buffer pH7.3. The eluate was collected in 8 ml fractions.
Regeneration of the column was achieved by elution with 0.3M phosphate buffer pH7.3 followed by two volumes of 0.1M sodium hydroxide to remove any bound lipoproteins. Finally the column was again eluted with 0.3M phosphate buffer to return to the starting pH of 7.3.

The protein content of the fractions was monitored on the Cecil CE292 spectrophotometer at 280 nm. The protein peak fractions were pooled, dialysed against 0.15M phosphate buffered saline (PBS) pH7.4. The volume was made up to that of the original serum. An aliquote was taken to assess the immunoreactivity. The protein content was determined by the Lowry et al 1951 method (Chapter 2) and then diluted to give 10 mg/ml. This was stored at 4°C with 0.01% (w/v) thimerosol as preservative.

Assessment of immunoreactivity of sera

A liquid phase RIA was use to determine the immunoreactivity of the DEAE purified antibody preparation compared to the neat unpurified antisera. This was set up according to the following protocol (Table 5.1). (Dr S Hampton, Guildhay, University of Surrey, personal communication).

**TABLE 5.2**

Dilutions of the sera used in the assessment by RIA of DEAE-purified sheep anti-h-TSH antibody compared to the neat antiserum

<table>
<thead>
<tr>
<th>1st Antibody Sheep anti-h-TSH</th>
<th>Initial normal sheep serum dilution</th>
<th>Initial 2nd antibody Donkey anti-sheep</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1k</td>
<td>1:1200</td>
<td>1:8 (100 μl)</td>
</tr>
<tr>
<td>1:2k</td>
<td>(100 μl)</td>
<td>1:16</td>
</tr>
<tr>
<td>1:4k</td>
<td>(100 μl)</td>
<td>1:32</td>
</tr>
<tr>
<td>1:8k</td>
<td>(100 μl)</td>
<td>1:64</td>
</tr>
<tr>
<td>1:16k</td>
<td>(100 μl)</td>
<td>1:128</td>
</tr>
<tr>
<td>1:32k</td>
<td>(100 μl)</td>
<td>1:256</td>
</tr>
<tr>
<td>1:64k</td>
<td>(100 μl)</td>
<td>1:512</td>
</tr>
<tr>
<td>1:128k</td>
<td>(100 μl)</td>
<td>1:1024</td>
</tr>
</tbody>
</table>
Each first (1st) antibody preparations were assayed in duplicate at each dilution. 125I-TSH (kindly supplied by Dr D Teale, Biochemistry Lab, St Lukes Hospital, Guildford) was diluted 1:40 in distilled water and 100 μl of this containing approximately 110 pg was added to each LP3 assay tube. The specific activity of the preparation was 80 μCi/μg. The normal sheep serum and precipitating donkey anti-sheep antisera were both kindly supplied by Guildhay, University of Surrey, Guildford. Two non specific binding tubes containing 100 μl RIA assay diluent and hormone label were set up and the 1:8 dilution of the second antibody was used in the nonspecific binding tubes.

This procedure was used as a speedy assessment of the binding of the purified antibody preparations as compared to the unpurified antisera.

Assessment of solid phase adsorption of sheep anti-h-TSH

The solid phase adsorption of purified sheep anti-h-TSH to polyvinyl chloride microtitre plates (Dynatech Ltd) was assessed.

Sheep anti-h-TSH preparations DEAE-cellulose purified (batch HP/S/1001-11.12.81) and diluted in 0.15M PBS pH7.4 to 10 mg/ml protein stock solution.

This solution was serially diluted 0,2.5,5,10,20,40 and 80 μg/ml with ELISA coating buffer, and 200 μl of each dilution was added to four wells. The 'O' wells contained 200 μl coating buffer alone. These were incubated for 2 hours at 37°C after which the wells were washed three times with ELISA washing buffer and 100 μl 125I-TSH consisting of about 100 pg TSH with specific activity of 80 μCi/μg was added to each well and incubated for 2 hours at 37°C. The wells were then alternately aspirated and washed carefully three times. The wells were then air dried.

Negative binding wells were set up with normal sheep serum diluted as for the sheep anti-TSH antisera.

The optimum adsorption time was determined by incubating 200 μl/ml purified sheep anti-h-TSH or normal sheep sera for various time intervals between 2 and 24 hours. The wells were then washed three times as described and 100 μl of the 125I-TSH was added and incubated for 2 hours at 37°C. Finally the radioactive contents were aspirated from the wells which were washed carefully three times.

The wells were then air dried and separated using a plate cutter designed in the University workshop. The cut out wells were placed in LP4 tubes (Luckham Ltd, Burgess Hill, Sussex) and counted for 100 seconds on 1260 LKB multi Gamma II.
Titration of sheep anti-TSH and monoclonal antibody

Polyvinyl chloride micro-ELISA wells were coated with 0, 2.5, 5, 10, 20 and 40 μg/ml DEAE-cellulose purified sheep anti-TSH as described, above. After a 2 hour incubation at 37°C the wells were washed three times. 150 μl of 125.0 μl/l TSH(Barts) was added to each well and incubated overnight at 4°C. After washing, 200 μl of ascitic fluid SY/T8/2 or normal mouse sera diluted between 1:200 to 1:51200 was added to appropriate wells in a cross titration pattern (see table 5.3). Each dilution was assayed in quadruplicate. These were incubated for 2 hours at 37°C after which 200 μl of 1:1600 dilution of donkey anti-mouse horseradish peroxidase conjugate II was added and incubated for 2 hours at 37°C. After careful washing three times the chromogen solution was added as in Chapter 2. This was incubated for 30 minutes after which the enzyme-activity was stopped and the optical density read.

Table 5.3
Cross titration of sheep anti-TSH and the monoclonal antibody
NSB = nonspecific binding, these contained normal sheep serum

<table>
<thead>
<tr>
<th>Sheep anti-TSH antibody μg/ml</th>
<th>Monoclonal antibody ascitic fluid/normal mouse serum (NMS) dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 1:200 1:400 1:800 1:1600 etc.</td>
</tr>
<tr>
<td>NSB</td>
<td>150 μl of 125.0 μl/l TSH(Barts) in each well</td>
</tr>
<tr>
<td>2.5</td>
<td>200 μl Donkey anti-mouse HRP conjugate in each well</td>
</tr>
<tr>
<td>NSB</td>
<td>2.5</td>
</tr>
<tr>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>NSB</td>
<td>5.0</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>NSB</td>
<td>10 etc.</td>
</tr>
</tbody>
</table>

Titration of monoclonal antibody and enzyme conjugate

200 μl of 40 μg/ml sheep anti-TSH in coating buffer was incubated in all wells of polyvinyl chloride micro ELISA plates. After 2 hour incubation at 37°C the wells were washed and 150 μl of 125.0 μl/l TSH(Barts) was added to each well which were incubated overnight at 4°C followed by washing three times after which various dilutions 1:200 to 1:51200 of ascitic fluid SY/T8/2 was added to the wells. These were incubated for 2 hours at 37°C followed by washing and 200 μl donkey anti-mouse HRP conjugate II was added to the wells as appropriate (see table 5.4).
TABLE 5.4

Cross titration of monoclonal antibody SY/T8/2 and donkey antimouse HRPO Conjugate II.

<table>
<thead>
<tr>
<th>Donkey anti-mouse HRPO Conjugate dilution</th>
<th>Monoclonal Antibody Ascitic Fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1:400</td>
<td></td>
</tr>
<tr>
<td>1:800</td>
<td>40</td>
</tr>
<tr>
<td>1:1600</td>
<td>150</td>
</tr>
<tr>
<td>1:3200</td>
<td></td>
</tr>
</tbody>
</table>

A 1:200 dilution of normal mouse sera was incubated simultaneously with each conjugates dilution for non specific binding assessment.

The wells were finally developed with chromogen as previously described and the optical density read.

Immunoradiometric investigation of monoclonal antibody binding

This method was a modification of Hales and Woodhead 1980.

40 µg/ml sheep anti-TSH was coated to polyvinyl chloride wells as described above. 150 µl of various dilutions of TSH (Barts) (125 mU/l to 0.48mU/l) was added to quadruplicate wells and incubated overnight at 4°C. This was followed by washing and 200 µl ¹²⁵I labelled monoclonal antibody (McAb) (see Chapter 4) was added according to the following:-

\[
¹²⁵I \text{ Mc Ab} = 50 \text{ µg labelled}
\]

assuming 80% recovery = 40000ng in 2 ml

stored as 200 µl aliquots = 4000 ng/aliquote

400 µl labelled antibody was diluted in 20 ml assay diluent

200 µl diluted label per well = 80.0 ng/well

= 3.0 x 10¹¹ antibody molecules/well

The labelled antibody was added in excess of the amount of TSH added per well:-

\[
12.5 \text{ ng/ml} = 125\text{mU TSH/litre}
\]

200 µl per well = 2.5 ng/well

= 5.4 x 10¹⁰ TSH molecules/well
The amount of labelled antibody: the highest amount of TSH added per well was therefore 5.6:1.
Additionally the TSH and labelled antibody were preincubated overnight before incubation in the wells containing the solid phased sheep anti-TSH antibody to allow prior binding of monoclonal antibody.

5.2.4 Two Site ELISA II

A suitable assay could not be derived with the sheep anti-TSH antibody as the solid phase antibody we therefore decided to reverse the antibody sandwich for the reasons which are discussed in Section 5.4.

Assessment of solid phase adsorption of monoclonal antibody

Protein A purified monoclonal antibody (see Chapter 4) was double diluted 1.25, 2.5, 5, 10, 20, 40, and 80 µg/ml with ELISA coating buffer. The procedure was as described for sheep anti-TSH in the preceding section. 125I-TSH added was approximately 110 pg/well with a specific activity of 100μCi/µg. Alternatively, to each well containing solid phase monoclonal antibody was added 200 µl of a 1:1600 dilution donkey anti-mouse HRPO conjugate II, and the assay performed according to ELISA method A, Chapter 2.

ELISA II

The enzyme conjugate was a donkey anti-sheep HRPO conjugate kindly supplied by Guildhay, University of Surrey, Guildford.

The sheep anti-TSH and the donkey anti-sheep HRPO conjugate were cross titrated with 40 µg/ml protein A purified ascitic fluid adsorbed to the wells and 125 mU/l TSH(Barts) added to each well as described above. Sheep anti-TSH was diluted between 1:1000 to 1:8000 and the donkey anti-sheep HRPO was diluted between 1:250 to 1:32000. The procedure was as described for the 'Two-site' ELISA I above.

Time optimization studies

To investigate the influence of time on the binding of the various parts of the ELISA sandwich the following studies were undertaken:
1) Optimum time for monoclonal antibody adsorption on to microtitre plates.
2) Optimum time for antigen binding to solid phase antibodies.
3) Optimum time for sheep anti-TSH binding to the captured antigen.
4) Optimum time for donkey anti-sheep HRPO binding.
**Reagents**

Protein A purified antibody SY/T8/2 at 40 μg/ml, 200 μl per well; 125mU/l TSH(Barts) was used at 150 μl per well; and sheep anti-TSH at 1:8000 dilution used at 200 μl per well; with donkey anti-sheep HRPO diluted 1:2000 and used at 200 μl/well for this series of experiments.

**Procedure**

1) The Monoclonal antibody solution was allowed to adsorb for 30 minutes, 1 hour, 2, 15.5, 17.5 and 19.5 hours on to microtitre wells. The TSH antigen was then added and incubated for 2 hours followed by sheep anti-TSH incubated for 2 hours and donkey anti-sheep incubated for 2 hours and finally the chromogen was incubated for 30 minutes. The ELISA OD₄₉₀ was then read.

2) The solid phase antibody was incubated for 2 hours followed by the addition of h-TSH at various times allowing 30 minute, 1 hour, 2, 4, 15.5, 17.5 hours incubation. The remaining procedure was as described above (1).

3) The solid phase antibody was incubated for 2 hours followed by the addition of TSH antigen and incubated overnight. The sheep anti-TSH was then added at various times to allow 30 minutes, 1 hour, 1.5, 2, 3.5, 5.5 hours incubation. The procedure was then completed as described above.

4) The solid phase antibody was incubated for 2 hours followed by addition and incubation of h-TSH overnight, then sheep anti-TSH was incubated for 2 hours. Finally donkey anti-sheep HRPO was added at various times to give 30 minutes, 1 hour, 2, 3, 4 and 5.5 hours incubation times. Chromogen was then added and incubated for 30 minutes.

It must be noted that careful washing procedure was always performed between each addition at each stage of the procedure (see figure 5.2).

**Standard curve**

Standard curves were set up according to the following procedure:-

200 μl of a 40 μg/ml solution of protein A purified SY/T8/2 in ELISA coating buffer was incubated for 2 hours in each well. The wells were emptied and washed carefully with ELISA washing buffer. Then various dilutions between 0 and 250 mU/l h-TSH(Barts) was added at 150 μl/well. These were incubated overnight at approximately 4°C. Three human TSH control samples were included in the assay:

Sample I low concentration = 1.9 - 2.5 mU/l
Sample II medium concentration = 7.1 - 9.1 mU/l
and Sample III high concentration = 16.0 - 20.0 mU/l
The control samples were supplied by Dr. D. Teale, Biochem. Dept., St. Lukes Hospital, Guildford. The samples were originally obtained from Environmental Chemical Specialists Inc., 3700 East Micaloma, Ca 92807.

The contents of each vial was reconstituted as recommended in 5 ml distilled water.

After incubation, the wells were emptied and washed, then 200 μl of a 1:8000 dilution of sheep anti-TSH was added and incubated for 2 hours at 37°C. The wells were again washed, 200 μl donkey anti-sheep HRP O was added diluted at 1:2000 and incubated for 2 hours at 37°C. Finally after washing the wells 200 μl of chromogen solution was added to each well and incubated for 30 minutes at 37°C following which the optical densities were read at 490 nm.

A standard curve was also set up with this double antibody sandwich system using the standard TSH preparations and control TSH samples supplied as a part of the Dac-Cel TSH kit for TSH Radioimmunoassay (Wellcome Diagnostics, Wellcome Foundations Ltd, Dartford). The control sample TSH value was given as 14.5 ± 2.5 mU/l.

The standard curves were set up with the h-TSH standard diluted in ELISA diluent buffer and in Horse Serum 3 (HS38 supplied by Wellcome Reagents Ltd, Wellcome Foundation Ltd, Dartford). Horse Serum was recommended for use in place of TSH free human serum (Wood et al 1975) (personal communication Dr. D. Teale, Biochem. Dept, St. Lukes Hospital, Guildford).

5.3 RESULTS

5.3.1 Immunocytochemistry

Histological indications

The haematoxylin eosin stained sections were prepared for general microscopic examination of the tissue. These showed typically blue staining nuclei and bright pink staining cytoplasm. The integrity of the tissue preparations was demonstrated. The human pituitary sections showed some post mortem deterioration exhibiting typical pycnotic nuclei. This was not apparent in the rat or ferret preparations since they were fixed as fresh preparations. PAS positive cells were however clearly evident in the human preparations demonstrating the presence of basophilic cell types. These were demonstrated in figure 5.3. PAS and Haematoxylin eosin stained preparations of both the rat and ferret pituitaries were prepared and results for the rat are presented in figure 5.4.
FIGURE 5.3
Human Anterior Pituitary Sections

a) Haematoxylin eosin stained
b) Periodic Acid Schiff stained
at x600 magnification
FIGURE 5.4
Rat Pituitary Sections

a) Haematoxylin eosin stained

b) Periodic Acid Schiff stained

at x 79 magnification
Indirect immunoperoxidase staining

All six ascitic fluid monoclonal antibody preparations (SY/T8/1-6) gave strong staining reactions with the human pituitaries, at all dilutions up to 1:500,000 with conjugate I diluted 1:20. The conjugate I was too dilute at 1:100, and gave very little specific staining at any dilution of the primary monoclonal antibody. At 1:500,000 dilution the apparent intensity began to diminish slightly, primarily for SY/T8/2.

A striking observation was that as the primary antibodies were diluted out so the intensity of staining appeared to increase. No non-specific background staining was observed with any of the monoclonals and conjugate I (except when the preparations were allowed for whatever reason to dry out; care must be taken therefore to prevent this occurring during the procedure).

Conjugate II was diluted up to 1:64 but excessive background was observed at each primary antibody dilution although strong positive staining was also observed.

Conjugate III on the other hand was found to give good positive staining at 1:8 dilution with all concentrations of the primary antibodies with no background staining.

The human pituitary preparation No. 4 was found to give the most abundant TSH positive reactive cells. It was also the best preserved preparation. The distribution of the positive staining observed in this preparation is shown in figure 5.5a at x79 magnification. Figure 5.5b demonstrates the distribution of the positive staining obtained in the baboon pituitary also at x79 magnification. It was noted that the specific staining was more intense in the baboon sections with all the monoclonal antibodies. This is clearly demonstrated by a comparison of 5.5a and 5.5b. There also appeared to be a higher number of positive cells in the baboon section. It was thought this was not the result of overt differences in the areas from which the sections were derived, although this was not controlled, but was probably due to differences in the ages of the specimens. The cell density in the baboon section was very much greater than that in the human sections.

Figures 5.6 and 5.7 shows the human and baboon positive sections at magnifications of x313 and x800 respectively.

These demonstrate the individual cellular staining of the preparations. The staining is therefore not a background artifact but confined within discrete cellular boundaries.

All the sections photographed were processed with SY/T8/1 ascitic fluid diluted at 1:32000 and the conjugate I diluted 1:20.
FIGURE 5.5
Positive Anterior Pituitary Sections

a) Distribution of TSH positive cells - Human

b) Distribution of TSH positive cells - Baboon

(both at x79 magnification). Sections were processed with 1:32000 monoclonal antibody and 1:20 enzyme labelled anti-immunoglobulin.
FIGURE 5.6

Positive Anterior Pituitary Sections

a) Human section

b) Baboon section

at x313 magnification showing the cellular nature of staining. Sections were processed with 1:32000 monoclonal antibody and 1:20 enzyme labelled anti-immunoglobulin.
FIGURE 5.7
Positive Anterior Pituitary Sections

a) Human section

b) Baboon section

at x800 magnification showing the cellular staining. Sections were processed with 1:32000 monoclonal antibody and 1:20 enzyme labelled anti-immunoglobulin.
The negative control human pituitary sections (figure 5.8); the rat, ferret and dog pituitary sections (figures 5.9 and 5.10a) showed no positively stained cells. Additionally all other tissues, ie, human placenta (figure 5.10b), human gut and kidney (figure 5.11) showed no positively stained cells.

Adsorbed controls

The liquid phase adsorption was found to be inappropriate. Under the conditions of the procedures the entire section was stained. There was very little apparent difference between previously positive cells and the background.

However the solid phase adsorption removed the positive staining property in four of the ascitic fluids except SY/T8/3-4 where the intensity was greatly reduced but not completely extinguished. There was also residual binding of radiolabelled h-TSH in these two which although greatly reduced was not completely removed. (Verbal communication Dr Ray Edwards NETRIA, St Bartholomews Hospital, London.)

Summary

The immunocytochemical results are summarized below.

TABLE 5.5
Immunocytochemical staining using monoclonal antibodies to human-TSH

<table>
<thead>
<tr>
<th>POSITIVE</th>
<th>NEGATIVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human anterior pituitary sections</td>
<td>Dog rat and fettet anterior pituitary sections</td>
</tr>
<tr>
<td>Baboon anterior pituitary sections</td>
<td>Human kidney gut and placenta sections</td>
</tr>
</tbody>
</table>
FIGURE 5.8

Human anterior pituitary negative control section processed with 1:32000 normal non immune mouse sera and 1:20 enzyme labelled anti-immunoglobulin (x79 magnification).
FIGURE 5.9

Negative Pituitary Sections

a) Rat pituitary

b) Ferret pituitary

Section processed with 1:32000 dilution of monoclonal antibody and 1:20 dilution enzyme labelled anti-immunoglobulin (x79 magnification).
FIGURE 5.10
Negatively stained tissue sections

a) Dog pituitary
b) Placenta section

Sections were processed with 1:32000 diluted monoclonal antibody and 1:20 enzyme labelled anti-immunoglobulin (x79 magnification).
FIGURE 5.11

Negatively stained tissue sections

a) Human gut

b) Human kidney

Sections were processed with 1:32000 diluted monoclonal antibody and 1:20 enzyme labelled anti-immunoglobulin (x79 magnification).
### 5.3.2 Double Antibody ELISA 1

**Investigation of sheep anti-TSH antibody as solid phase antibody**

The immunoreactivity of the purified sheep anti-TSH was nearly identical to that observed in the unpurified antiserum as demonstrated in figure 5.12. The greatest difference was apparent at the highest dilutions of the two preparations. The majority of the protein eluted from the DEAE-column was in the first peak eluted and by the observed immunoreactivity we may assume that the majority of sheep immunoglobulins was the protein in this peak. No binding of $^{125}$I-TSH was observed in the fractions with low protein content or in the second small peak eluted. Table 5.6 summarizes the results.

**TABLE 5.6**

Protein determination by Lowry et al 1951 method of DEAE-cellulose eluted pooled fractions.

<table>
<thead>
<tr>
<th>OD720nm</th>
<th>µg/ml Protein at 1:250 dilution</th>
<th>Undiluted Protein content</th>
<th>Diluted to 10 mg/ml (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.135</td>
<td>64 µg/ml</td>
<td>16 mg/ml</td>
<td>160 ml</td>
</tr>
</tbody>
</table>

The purification procedures did not greatly diminish the antigen binding capacity of the purified sheep anti-TSH preparation (figure 5.12), while removing a great deal of irrelevant serum protein, and sodium azide which inhibits peroxidase activity.

**Solid phased adsorbed antibodies**

Both the purified sheep anti-TSH (figure 5.13) and the protein A purified monoclonal anti-TSH (figures 5.16 and 5.17) adsorbed maximally onto polyvinyl chloride plates at a coating concentration of 40 µg/ml. A higher $^{125}$I-TSH background binding was observed with the normal mouse sera used as negative control. But this was perhaps to be expected because this control sera was not purified and the label was nearing the end of its shelf life.

Two hour coating of the sheep anti-TSH was found to be sufficient to give maximum adsorption on to the wells (figure 5.14).
FIGURE 5.12 Antiserum (A/S) dilution binding of $^{125}$I-TSH before purification (△-△); after DEAE-cellulose purification the pooled fractions (▲-▲). (A/S batch 1001-11.12.81)
FIGURE 5.13 The binding of $^{125}$I-TSH by solid phase sheep anti-TSH (▲-▲) and normal sheep sera (Δ-Δ) adsorbed onto polyvinyl chloride plates. Error bar represents the standard error on the mean (SEM for n = 4).
FIGURE 5.14  Sheep anti-TSH (●-●) diluted at 40 μg/ml adsorbed on to polyvinyl chloride plates for various times. Negative control normal sheep sera (○-○). (SEM for n = 4).
FIGURE 5.15 Immunoradiometric standard curve with 40 μg/ml solid phase sheep anti-TSH and ¹²⁵I-monoclonal anti-TSH.
(The NSB was 104 CPM and total count was 227561 CPM.)
(SEM for n = 4)
FIGURE 5.16 Binding of $^{125}$TSH by solid phase protein A purified ascitic fluid (■-■) and normal mouse serum (□-□) onto polyvinyl chloride plates. Solid phase adsorption and antigen incubation times 2 hours each. (SEM for n = 4)
FIGURE 5.17 Ascitic fluid monoclonal antibody adsorbed by polyvinyl chloride plates indicated by Donkey antimouse HRPO conjugate II and ELISA substrate. (SEM for \( n = 4 \))
ELISA I

125mU/l TSH was used as the top standard. None of the combinations of sheep anti-TSH, monoclonal antibody and donkey anti-mouse HRP conjugate resulted in a sufficiently wide difference between the positive and negative control sera (table 5.7) or between wells containing TSH and those not containing TSH (table 5.8) to be useful in an ELISA assay.

**Table 5.7**

<table>
<thead>
<tr>
<th>Sheep anti-TSH ( \mu g/ml )</th>
<th>Monoclonal antibody dilution/normal mouse serum negative control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:200</td>
</tr>
<tr>
<td>0 +ve</td>
<td>0.35</td>
</tr>
<tr>
<td>0 -ve</td>
<td>0.26</td>
</tr>
<tr>
<td>2.5</td>
<td>0.42</td>
</tr>
<tr>
<td>2.5</td>
<td>0.36</td>
</tr>
<tr>
<td>5.0</td>
<td>0.44</td>
</tr>
<tr>
<td>5.0</td>
<td>0.41</td>
</tr>
<tr>
<td>10.0</td>
<td>0.44</td>
</tr>
<tr>
<td>10.0</td>
<td>0.43</td>
</tr>
<tr>
<td>20.0</td>
<td>0.52</td>
</tr>
<tr>
<td>20.0</td>
<td>0.49</td>
</tr>
<tr>
<td>40.0</td>
<td>0.57</td>
</tr>
<tr>
<td>40.0</td>
<td>0.50</td>
</tr>
</tbody>
</table>
**TABLE 5.8**

Titration of monoclonal antibody and donkey anti-mouse HRP O. The OD was measured at 490 nm. (NMS = normal mouse sera)

<table>
<thead>
<tr>
<th>Donkey antimouse dilutions</th>
<th>TSH per well µU/l</th>
<th>Monoclonal antibody dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1:200 1:400 1:800 1:1600 1:3200 1:6400 1:200</td>
</tr>
<tr>
<td>1:400</td>
<td>125</td>
<td>0.52 0.49 0.44 0.40 0.33 0.24 0.25</td>
</tr>
<tr>
<td>1:400</td>
<td>0</td>
<td>0.26 0.22 0.25 0.21 0.24 0.21 0.25</td>
</tr>
<tr>
<td>1:800</td>
<td>125</td>
<td>0.51 0.45 0.40 0.37 0.26 0.21 0.21</td>
</tr>
<tr>
<td>1:800</td>
<td>0</td>
<td>0.21 0.25 0.26 0.21 0.21 0.20 0.21</td>
</tr>
<tr>
<td>1:1600</td>
<td>125</td>
<td>0.54 0.41 0.37 0.34 0.23 0.22 0.26</td>
</tr>
<tr>
<td>1:1600</td>
<td>0</td>
<td>0.23 0.23 0.21 0.21 0.20 0.20 0.23</td>
</tr>
<tr>
<td>1:3200</td>
<td>125</td>
<td>0.52 0.36 0.31 0.29 0.22 0.22 0.24</td>
</tr>
<tr>
<td>1:3200</td>
<td>0</td>
<td>0.24 0.21 0.25 0.19 0.21 0.21 0.21</td>
</tr>
</tbody>
</table>

**125I-Monoclonal antibody binding**

The amount of labelled monoclonal antibody bound by the captured TSH was very low. This was demonstrated in figure 5.15 although the total count added was very high and the amount of antibody added was in excess. Preincubation of the label antibodies and TSH before addition to the sheep anti-TSH coated wells did not improve the observed amount of bound label.

This low binding was not the result of failure of the sheep anti-TSH to adsorb onto the wells or a failure of the adsorbed antibody to bind TSH since both these parameters were shown to give good resolution (figure 5.13). It was also not the result of failure of the monoclonal antibody to bind TSH, since it bound sufficient TSH in the solid phase (see Chapter 3); and also when the monoclonal antibody was itself solid phase adsorbed (figure 5.15).

**5.3.3 Double Antibody ELISA II**

The monoclonal antibodies was adsorbed maximally on to the polyvinylchloride plates when coated at a concentration of 40 µg/ml (figure 5.16). This was confirmed in the indirect ELISA (figure 5.17).
Cross titration of Sheep anti-TSH and Donkey anti-sheep HRPO

Very good resolution was obtained between the TSH (125 mU/l) containing wells, for example, OD$_{490}$ = 1.651, and the corresponding '0' TSH wells OD$_{490}$ = 0.123 (see Table 5.9) this was in contrast to the results in Table 5.6. The conjugate titration curve is given in Figure 5.18.

**TABLE 5.9**

<table>
<thead>
<tr>
<th>Sheep anti-TSH dilutions</th>
<th>TSH per well mU/l</th>
<th>Donkey anti-sheep HRPO Dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1:250</td>
</tr>
<tr>
<td>1:1000</td>
<td>125</td>
<td>&gt;2.0</td>
</tr>
<tr>
<td>1:1000</td>
<td>0</td>
<td>0.27</td>
</tr>
<tr>
<td>1:2000</td>
<td>125</td>
<td>&gt;2.0</td>
</tr>
<tr>
<td>1:2000</td>
<td>0</td>
<td>0.26</td>
</tr>
<tr>
<td>1:4000</td>
<td>125</td>
<td>&gt;2.0</td>
</tr>
<tr>
<td>1:4000</td>
<td>0</td>
<td>0.26</td>
</tr>
<tr>
<td>1:8000</td>
<td>125</td>
<td>&gt;2.0</td>
</tr>
<tr>
<td>1:8000</td>
<td>0</td>
<td>0.25</td>
</tr>
</tbody>
</table>

A 1:8000 dilution of sheep anti-TSH and 1:2000 dilution of donkey anti-sheep HRPO was selected to be used with 40 μg/ml monoclonal antibody as the solid phase support in the ELISA sandwich. The observed background binding was very low at these concentrations.

**Optimization studies**

The influence of incubation times on the adsorption and binding of the various items in the ELISA sandwich was investigated.

The following optimum incubation times were found. Two hour coating of solid phase monoclonal antibody and 4 hour incubation for the second layer TSH gave maximal optical density readings (Figures 5.19 and 5.20) respectively. Above 4 hour incubation and the amount of solid phase antibody
FIGURE 5.18 Donkey anti-sheep HRPO. Enzyme conjugate titrated against sheep anti-TSH diluted 1:8000 and TSH 128 mU/l (■-■) and 0 mU/l (□-□).
FIGURE 5.19 Monoclonal anti-TSH (protein A purified) coated at 40 μg/ml and allowed to adsorb onto polyvinyl chloride plates for various times. Wells then incubated with 125 mU/l TSH for 2 hours; followed by sheep anti-TSH incubated for 2 hours and then Donkey anti-sheep HRPO incubated for 2 hours. Substrated incubated for 30 minutes. (SEM for n = 4)
Solid phase monoclonal antibody coated at 40 μg/ml and incubated for 2 hours followed by addition of TSH at various times. Sheep anti-TSH diluted 1:8000 and donkey anti-sheep HRP0 diluted 1:2000 were each incubated for 2 hours followed by 30 minute substrate incubation. (SEM for n = 4)
Incubation time (hrs) for sheep anti-TSH binding

**FIGURE 5.21** 40 μg/ml monoclonal antibody coating on polyvinyl chloride plate for 2 hours followed by 125 mU/l TSH incubation overnight. Finally sheep anti-TSH added at various times. Donkey anti-sheep HRPO was incubated for 2 hours followed by 30 minute substrate incubation. (SEM for n = 4)
adsorbed rapidly declined, possible due to de-adsorption or steric hinderance of the antibody molecules. The binding of the sheep anti-TSH in the sandwich had not reached maximum in the period investigated (figure 5.21). However over the period the recorded optical density was sufficiently high to give a good assay, and total assay time is an important consideration. Two hour incubation was therefore considered to be long enough to provide enough binding for a suitable assay.

A 2 hour incubation of the HRP conjugate was demonstrated (figure 5.22) to be optimum. For practical purposes however 2 hour incubation of solid phase antibody was employed followed by overnight incubation of TSH samples, 2 hour incubation of the sheep anti-TSH and HRP conjugate with a final 30 minute chromogen incubation.

5.3.4 Assessment Of The ELISA II Assay

The standard curve obtained with TSH_{Barts} was shown in figure 5.23. Dissolving the TSH standard in horse serum depressed the observed binding, but to a greater extent at the higher concentrations of TSH.

Figure 5.24 demonstrates the standard curve obtained in this sandwich ELISA system using Wellcome TSH standard preparations.

Assay sensitivity

The sensitivity may be described as the ability of an analytical method to detect small quantities of the measured component (International Federation of Clinical Chemistry 1975).

This in turn suggest the practical detection limit of the assay system is the smallest amount of substance which is distinguishable with confidence from Zero concentration.

The definition of sensitivity used here was that suggested by Borth 1959, and denotes the 95% confidence limit from zero. It was therefore given as the mean (\bar{x}) of several zeros plus twice the standard deviation (SD).

The Detection Limit = \bar{x} + 2.SD.
FIGURE 5.22 Solid phase monoclonal antibody coated at 40 µg/ml for 2 hours followed by TSH overnight and sheep anti-TSH for 2 hours. HRPO conjugate was added at various times, followed by substrate incubated for 30 minutes. (SEM for n = 4)
FIGURE 5.23  •-• TSH standard dissolved in assay diluent
     o-o TSH standard dissolved in horse serum
FIGURE 5.24 Standard curve with Wellcome Foundation standard TSH preparations. (n = 6)

Standard preparations were taken from the Dac-Cel TSH kit RD48.
Table 5.10 tabulates the sensitivities observed.

**TABLE 5.10**

Detection limit of the assays were defined as $\bar{x} + 2SD$

<table>
<thead>
<tr>
<th>Assay standard curve</th>
<th>Number of Zero samples (n)</th>
<th>Mean x OD/ hour of Zero sample</th>
<th>SD</th>
<th>Sensitivity mU/l TSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSH in assay diluent</td>
<td>12</td>
<td>0.2824</td>
<td>0.0302</td>
<td>1.8</td>
</tr>
<tr>
<td>TSH in serum</td>
<td>32</td>
<td>0.2652</td>
<td>0.0304</td>
<td>4.0</td>
</tr>
<tr>
<td>Wellcome TSH</td>
<td>25</td>
<td>0.2540</td>
<td>0.0220</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Assay precision

The within batch precision was calculated from determinations of the three control sera and the Wellcome control sera which were assayed 10 times, and 6 times respectively.

The between assay precision demonstrating the reproduceability of the assay was also calculated from the values obtained for the three control sera assayed on 8 different occasions.

The standard deviation was calculated from 8 pairs of estimations using the formula:

$$SD = \sqrt{\frac{\sum(X_2 - X_1)^2}{2n}}$$

where SD = standard deviation

$X_2$ = observation 2 ) of each pair

$X_1$ = observation 1 )

n = number of pairs
The precision estimates obtained are shown in Table 5.11 below. All the TSH values observed for the three controls were well above that expected.

**TABLE 5.11**

Precision estimates within and between assays

<table>
<thead>
<tr>
<th>TSH samples</th>
<th>Mean TSH mU/l</th>
<th>SD</th>
<th>Within Assay C.V.%</th>
<th>Mean TSH mU/l</th>
<th>SD</th>
<th>Between Assay C.V.%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample I (low)</td>
<td>5.8</td>
<td>0.8</td>
<td>13.8</td>
<td>6.6</td>
<td>1.6</td>
<td>24.2</td>
</tr>
<tr>
<td>Sample II (medium)</td>
<td>15.5</td>
<td>1.5</td>
<td>9.7</td>
<td>15.5</td>
<td>2.5</td>
<td>16.1</td>
</tr>
<tr>
<td>Sample III (high)</td>
<td>23.5</td>
<td>2.0</td>
<td>8.5</td>
<td>23.5</td>
<td>3.5</td>
<td>14.9</td>
</tr>
<tr>
<td>Wellcome control</td>
<td>14.0</td>
<td>1.0</td>
<td>7.04</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 5.25 gives the intra and interassay precision profiles obtained from the serum based standard curves assayed 10 and 8 times respectively. The intra assay precision was fairly good with the interassay precision, rising dramatically at the lower end of the TSH dose.
FIGURE 5.25 (o-o) intra assay precision profile (•-•) inter assay precision profile

on serum diluted TSH standards curve

n = 10 and n = 8 respectively.
5.4 DISCUSSION AND CONCLUSIONS

5.4.1 Immunocytochemistry

The major consideration in immunocytochemistry is the positive identification of immunoreactive substances within the tissue or cell preparations.

Many peptides have sequence homologies; and those of the pituitary glycoproteins have already been described in Chapter 1. Since each antibody within a polyclonal antiserum population may be regarded as a site specific rather than an antigen specific reagent (Larsson 1981), this results in the many cross reactions observed with polyclonal antisera.

Monoclonal antibodies on the other hand are also site specific, but the preparations contain antibodies specific to only one site. Any observed cross reactivity cannot be removed by diluting it out as may be possible with polyclonal antisera where the cross reacting antibodies may represent only a small part of the antibody population. Dilution therefore seriously diminishes its proportion in the antibody preparation and therefore its prominence. Observed cross reactions with monoclonal antibodies indicate truly homologous shared structures.

Almost all immunocytochemical localizations up to the present time have been performed with polyclonal antisera (De May 1983). But the critical criteria is that the specificity of the antisera whether poly or monoclonal be firmly established. Swaab (1982) has suggested that the usual RIA specificity characterization is not adequate since by RIA there may be no cross reactivity which may later become apparent in immunocytochemistry possibly as a result of the high dilutions at which the antibody preparations are used in RIA. However RIA is usually the most convenient available assay procedure. For this work the specificity in an ELISA system which in vitro mimics immunocytochemistry procedure was established using highly purified h-TSH and the other glycoprotein preparations (see Chapter 4). This in combination with the adsorption studies suggests that the specific staining was indeed human TSH, with similar antigenic determinants occurring in the baboon TSH. However the determinants were absent in the dog rat and ferret preparations.

Although the disappearance of staining after adsorption of a polyclonal antibody merely suggests that the antigen bound the antibodies, it does not exclude staining due to contaminating antibodies induced by an impurity in the antigen (Swaab 1982). In the case of monoclonal antibody disappearance of staining does not preclude the possibility that the selected antibody was directed against an antigen contaminant also present in the immunoabsorbent.
However a successful adsorption is usually suggested as a primary indication of specificity (Van Noorden and Polak 1983). In the liquid phase adsorption attempted in this work we concluded that nonspecific adsorption of the antigen-antibody complex onto the entire surface of the tissue section was responsible for the widespread staining observed in these sections, since the control sections gave the expected reactions.

The antigenic determinants bound by these monoclonal antibodies were absent in the other tissues - gut, kidney and particularly the placenta. The placenta results were especially significant since they confirm the antibodies specificity in terms of HCG cross reactivity. The placenta is a rich source of HCG demonstrated with a monoclonal immunoperoxidase technique (Wahlstrom et al 1981). No placenta staining was observed, although SY/T8/4 was found to cross react minimally with HCG, α and β-subunits. In addition, human placentas have been found to produce a material with thyrotropic activity (Hershman and Starnes 1969), which did not react in the immunochromic procedure used, possibly indicating the absence of the particular pituitary TSH antigenic determinant in the placental material.

The antibodies have therefore been demonstrated to be highly specific in terms of their binding to h-TSH secreting cells with negligible background nonspecific binding. Remarkable primate and tissue specificity was also clearly demonstrated. These antibodies with the exception of 3 and 4, which require further adsorption studies, were found to be eminently suitable for use in immunocytochemical investigations. The universality of the antimouse HRPO conjugate was also demonstrated where it was used in this and the ELISA procedures.

5.4.2 ELISA

Monoclonal antibodies are specific to only one antigenic determinant. Only one molecule of antibody therefore will bind an antigen unless of course the antigenic determinant is a repeating unit on the antigen. We therefore decided to use an available polyclonal sheep anti-TSH antiserum as the solid phase capture antibody. This antiserum was purified because the majority of irrelevant serum protein would have taken up adsorption sites which were required for the anti-TSH antibodies. Very little immunoreactivity was lost in the purification procedure as demonstrated in figure 5.12. The sensitivity of sandwich ELISA for TSH has been shown to be improved dramatically using purified adsorbed antibodies (Tshikawa et al 1982).
The purified sheep anti-TSH was adsorbed onto the well of the micro ELISA plates and bound TSH maximally at 40 μg/ml. However no satisfactory resolution of top TSH standard and 'O' TSH could be obtained using 125I-labelled monoclonal antibody (figure 5.15), or in the ELISA system (tables 5.7 and 5.8). Even though the ELISA system carried the potential for appreciable signal amplification, unsatisfactory signal detection in terms of magnitude was found. It was reasoned that the low observed signal was probably due to the singular specificity of the monoclonal antibody binding possibly only one or a very few antigenic determinant units per antigen molecule. Only a very limited number of bound monoclonal antibodies would therefore be available for binding by the enzyme labelled anti-immunoglobulins. Hence the limited signal intensity observed.

If this was the case then turning the assay upside down it was reasoned should provide a better signal amplification. The monoclonal antibody captured a precise amount of antigen. All other antigenic determinants would be available for binding by the polyclonal antisera, containing as it does antibodies to several antigenic determinants. By this procedure many more antibody molecules should be available for binding by the enzyme labelled anti-immunoglobulin, resulting in an increased signal.

This was convincingly demonstrated to be the case and a standard curve (figure 5.23) was derived. Indeed Ehrlich and Moyle (1983) have shown that mixtures of monoclonal antibodies act in a cooperative way to yield sensitive immunoassays not derived using the same antibodies singly. Ehrlich et al (1982) and Moyle et al (1983) have also reported increased antibody affinities when more than one monoclonal antibodies were mixed in the assay of HCG.

The specificity of the assay relies on that endowed by the monoclonal antibody in the sandwich, although the crossreactivity of HCG with the sheep anti-TSH was given to be <0.1% (Guildhay, University of Surrey), and as such the assay should not suffer from crossreactivity interference as experienced by Wada et al (1982).

The sensitivity of the assay of 4 mU/l in the serum response curve was not as good as that observe by Wada et al (1982) or in RIA (Hall et al 1971) or more recently the highly sensitive chemiluminescent assay reported by Jones et al (1984). However the possibility exists to provide a more sensitive assay probably of the chemiluminescence type reported by Weeks et al (1983) for measuring α foetaprotein or of course that for measuring TSH reported by Jones et al (1984) since our assay system already incorporates the peroxidase enzyme then an assay based on luminol mechanism is feasible (Olsson and Thore 1981). Alternatively the assay sensitivity may be improved
using a combination of monoclonal antibodies as described by Ehrlich and Moyle (1983), or even using monoclonal antibody Fab fragments to increase antigen binding potential of the adsorbed antibody (Tshikawa et al. 1982).

The theoretical sensitivity of this type of immunometric assay is probably as little as one molecule of analyte (Ekins 1981). However in practice the detection limit is determined to a great extent by the background noise levels against which the minimum signal must be perceived. Consequently such very high sensitivities can only be achieved in situations where a low background is combined with an amplified positive signal. The background in this assay is very low but the signal at low analyte concentrations is also small hence the low sensitivity. A signal detection system including those already suggested capable of resolving and amplifying such small signal differences would be more practical. Wada et al. 1982 pointed out that the sensitivity of the assay could be improved by increasing the amount of enzyme conjugate added. But it must also be noted that the background also increases with increasing conjugate concentration (figure 5.18).

This does not therefore remedy the sensitivity problem if the increased signal at low hormone concentration is obscured by the increased background. Tshikawa et al. (1982) therefore recommended the use of the lowest conjugate concentration to give a good calibration curve.

The within assay precision of between 8.5 and 13.8 and the between assay precision of 14.9 and 24.2 for the three control samples was good although the observed TSH values were all high. The precision may however have been improved with glutaraldehyde pretreatment of wells (see Chapter 2), but this was not attempted. Between assay precision up to 20.0 per cent were reported as common in TSH RIA (Tunbridge and Hall 1976). The precision profile shows good assay precision over the entire range therefore.

The Wellcome TSH was substituted in our assay to demonstrate the binding of the monoclonal antibody to purified TSH other than our specific antigen. This was because of the difficulties observed with the binding to the NIBSC TSH preparation. Figure 5.24 was the standard curve obtained.

The total assay time was approximately 26 hours and could be as little as 10.5 hours if the antigen was incubated with solid phase antibody for 2 hours rather than overnight which is done for convenience. However this may also be reduced by storing the antibody precoated plates until required (Voller et al. 1979). However monoclonal antibodies, especially in the purified state are sometimes unstable. Therefore storage conditions would need to be investigated.
The monoclonal antibody investigated was therefore shown to be suitable for use in this ELISA assay. However the results demonstrated some of the difficulties which may be encountered using monoclonal antibodies. The first assay ELISA I indicated that a suitable assay was not feasible under the conditions used. Had we used a solid phase monoclonal antibody with a top monoclonal antibody in the assay, the system may have been abandoned altogether. It demonstrates once again that all aspects of monoclonal antibodies must be investigated to define their appropriate terms of uses, and it must be emphasized that their behaviour is not equivalent in many respects to polyclonal antibodies.

Presented here is the basis of an assay which shows tremendous potential for a sensitive reliable and speedy assay for TSH and which carries great promise for clinical application in possibly neonatal hypothyroid screening.
CHAPTER 6

Discussion and Conclusions
Conventional antisera unlike monoclonal antibody suffers from the considerable disadvantages listed below in Table 6.1. In addition, in general greater than 90% of antibodies in a hyperimmune sera are irrelevant immunoglobulins which do not bind the antigen (Klinman and Press 1975, Kreth and Williamson 1973, Kohler 1976), and the specific antibodies in a conventional antisera, bind most of the antigenic determinants present on the immunogen. As a consequence conventional antisera display negative properties such as cross reactions, and batch variations.

Monoclonal antibodies on the other hand circumvent these major limitations of conventional antisera. Table 6.2 summarises the many advantages of monoclonal antibody preparations.

**TABLE 6.1**
Properties and disadvantages of conventional antisera

1) Heterogeneous mixture of antibodies.
2) A pure immunogen is necessary and/or
3) Purification of antisera for specificity.
4) Cross reactions may not be genuine.
5) Antisera is not exactly reproduceable in terms of specificity or binding characteristics.
6) Physical properties, such as avidity, are an average.

**TABLE 6.2**
The advantages of monoclonal antibodies summarized

1) Antibody product is pure - only one molecular species.
2) Impure immunogen may be used.
3) Specificity for one antigenic determinant.
4) Cross reactions usually mean a shared antigenic determinant.
5) High titre antibody samples may be obtained.
6) High or low affinity antibodies may be selected depending on the proposed application.
7) Antibody quality is reproduceable in terms of specificity and binding characteristics.
8) Large animals/farms are not required.
9) Unlimited amounts of specific antibody are produced in vivo and in vitro.
10) Immortal cell lines for continuous production.
11) Carries the potential to provide world wide standard reagents.
Monoclonal antibody production although possessing all these advantages is by no means a simple technique. In addition to the fact that the technique is expensive and time consuming, the many inherent problems of the technology such as the control of contamination, the rarity of the fusion event, hybrid instability and storage and recovery difficulties all combine to complicate the procedure.

We suggest here however, that even with these major difficulties the technique is well worth while when viewed in conjunction with the many advantages and its wide applications. Perhaps the most immediately striking feature of this technology for immunochemists is that a meaningful measure of specific antibody quantity is possible. As a result antibody techniques can now incorporate precise quantitative methodologies which allow definitive comparisons in routine immunochemistry.

The immunization of mice with highly purified human thyroid stimulating hormone permitted the successful generation of the high affinity, highly specific monoclonal antibodies. Their suitability in two specific areas of application have been successfully and clearly demonstrated.

6.2.1 Immunoassay screening

The successful conclusion of the technique - the rescue of the specific antibody secreting function, as suggested earlier was largely dependent upon the integrity of the screening assay. A liquid phase radioimmunoassay (Hall et al 1971) was used for detecting the monoclonal antibodies in the first 6 fusions.

This assay was demonstrated (Chapter 3) to be eminently suitable for monitoring the antibody responses of the TSH immunized spleen cell donor mouse and as a result was considered to be adequate to detect the mouse anti-TSH monoclonal antibodies in culture supernatant. After 6 fusions however with 528 primary cultures screened and 46 possible positives from fusions 1-3 rescreened, together with 8 primary cultures cloned from fusion number 3 consisting of 768 wells, with no stable positive culture derived, it was concluded that the screening assay was probably deficient. The possible positives carried forward to the next stage in fusion 1-3 were only marginally positive by the criteria set up for describing positive cultures.

At the earliest stages when the first assays were performed only minute quantities of specific antibody was expected to be present to bind the label. Therefore only a small amount of bound label would be available for
detection. In addition to this, the double antibody separation system depends on the precise balance of the various reagents to allow sufficient complex formation incorporating the first antibody and labelled antigen in quantities which permit the precipitation of perceptable labelled material. The amount of such precipitate even in normal RIA procedures is usually very small and significant loss of material arises during supernatant aspiration. Practically, normal carrier sera from the same species as that in which the first antibody was produced is added to increase the bulk of the precipitate and therefore to allow a more complete precipitation (Hunter 1979, Morris 1981), with concomitant proportionally less loss of material. But even with this modification, because of the small quantities involved in the first instance in monoclonal antibody screening then the detection level must be low. Additionally the effect of prozone which is the resolubilization of the complex in (in this case) second antibody excess may also be a prominent factor in the failure of this screening procedure.

However the use of PEG as the precipitating agent (Desbuquois and Aurbach 1971) did not improve the results for positive cultures. This would suggest therefore that the difficulty arose with the minute amount of specific antibody available initially in the cultures. It must be noted that the monoclonal antibodies, once they were established, performed satisfactorily in the liquid phase RIA procedures, and was used to derive the titres of the ascitic fluid monoclonal antibody preparations (see table 4.4). There was therefore no intrinsic hindrance to the use of monoclonal antibodies in the RIA procedure.

On the above considerations therefore it was decided to develop the indirect ELISA and solid phase RIA screening procedures described in Chapter 2. In the indirect ELISA system the solid phase absorbed antigen acted as an immunoadsorbent surface effectively extracting, concentrating and purifying the specific antibody from the culture supernatants. The binding of the enzyme labelled anti-mouse immunoglobulins also carrying the intrinsic facility of signal amplification suggested a more sensitive assay system for screening purposes. This was indeed the case and the developed assay was used to good effect in the next fusion No.7, and a gradation in the intensity of positive cultures was observed with this assay by which 7 stable cultures were derived and finally stored.

On the same premise it was thought that the cellulose bound solid phase second antibody would immuno-extract any first antibody present in the supernatant, those binding labelled antigen and those not binding antigen. This method circumvented the need for carrier protein and micelle formation accelerators.
This system however suffered from one major difficulty demonstrated in figure 4.3. Where there was excess first antibody then the hook effect phenomenon (Miles and Hales 1968) was apparent. This was possible due to the fact that the second antibody bound the first antibody labelled and unlabelled antibody species in the proportion in which they occurred in the assay mixture. Therefore where there was a larger excess of first antibody then a lower quantity of labelled antibody will be evident. This consideration was however not expected to present difficulties in screening as enough first antibody was not expected to be present to cause this effect.

The precision of the ELISA method B was however not very good. This was possibly due to the differential adsorption of protein to the plastic wells (Denmark and Chessum 1976, Voller A, personal communication). Pretreatment of the wells with 0.1% (v/v) glutaraldehyde (Stafford and Kilgallan 1980) improved the within assay precision. There have been various verbal reports that prewashing with distilled water, ELISA diluent without the nonspecific protein, or even decon improves the precision of the assay. For control of the between assay precision the results were corrected in terms of the reference positive wells to account for day to day minor variations in assay conditions.

No such difficulty was observed for the solid phase RIA. Both screening assays developed during the course of this work specifically for the screening of hybridoma cultures have been shown to be particularly suitable for this type of work, and has been used to good effect. The assays are particularly relevant because theoretically they may be adapted for use in the detection of any monoclonal antibodies of murine origin. The versatility of the solid phase RIA for example was demonstrated in the methotrexate assay (figure 2.4).

Both the enzyme labelled anti-immunoglobulins and the cellulose bound anti-immunoglobulins were relatively cheap to produce and were stable for long periods >1.75 years and 1 year respectively with no apparent deterioration.

Both assays were very suitable for screening at all stages of hybridoma production and were particularly appropriate because of the capacity to process a large number of samples simultaneously. The ELISA method was semi-automated while the solid phase RIA required less pipetting of material and so the whole procedure was simplified. Where there is an objection to the use of radiolabelled material or visual distinction of positive from negative alone is required and a quick assay is indicated then the ELISA method is preferable. However on the basis of precision, the solid phase RIA was preferred. The importance of the correct choice of screening assay has been clearly demonstrated. Perhaps one small drawback (if it may be so termed) to
the ELISA assay is that it is likely to result in the selection of only high affinity antibodies (Catty et al 1981) ie, those that can withstand the washing stages. This, of course, is a major objective of this work, and the proposed application in immunocytochemistry in particular demands the use of high affinity antibodies (Sternberger 1979).

The back up screening with the solid phase RIA allowed for the selection of those antibodies which may have been of lower affinity.

6.2.2 Contamination Control

The cell culture environment provides an ideal situation for the growth of not only the intended culture cells, but also for many microorganisms. All the essential nutrients are present in addition to favourable temperature and gaseous concentrations. As a consequence exceptional aseptic culture practices are essential for routine long term culture. Bacterial contamination was usually controlled with a penicillin/streptomycin preparation. Fungal and yeast contamination which in long term cultures may prove more of a problem than bacterial contamination may be inhibited by fungistatic agents such as fungizone or nystatin (Perlman et al 1961, Paul 1980). However where widespread contamination persists, it is more feasible to discard the contamination cultures than to try to save them. Fungal spores are airborne and as such spread rapidly if not swiftly eliminated (Paul 1980).

One other major and persistant contaminant of tissue culture cells consists of the pleuropneumonia-like organisms (PPLO) or mycoplasma (Carski and Shepard 1961, Macpherson 1966, Paul 1980). These organisms live intracellularly and lead to the slow but eventual death of the culture.

Again it is sometimes necessary to attempt the rescue of valuable cultures. Kanamyran (Kannasyn) has been suggested as the most effective treatment (Paul 1980). However the cells may be laundered so to speak by passaging them interperitoneally in syngeneic mice as for ascites production (Hudson and Hay 1980) and the cells, free from contamination, recovered from the ascites tumor (Edwards 1981, Marcus et al 1980 and Schimmelpfeng et al 1980). Antimycoplasma monoclonal antibodies have now been derived (Buck et al 1982) which should speed up the identification of contaminated cultures before they are damaged irretrievably.

With the control of mould and fungal outbreaks in mind the effects of econazole using NSI cells as the cell model system were investigated. Unfortunately econazole was found to exert dramatic cytotoxic effects on the cells (see Chapter 3). It was therefore demonstrated to be unsuitable for use in this hybridoma production work. Rather than using fungizone with
similar reported adverse effects (Dolberg and Bissell 1974, Hackett et al 1972) a strict aseptic routine was followed very successfully to the control of such contamination.

6.2.3 Fusion Promotion and Hybrid Stability

All the fusion solutions used gave good hybrid production with the majority of wells bearing actively growing cells in most cases. However because of the possible toxic effects of low molecular weight PEG on the cells (Fazekas de de St Groth and Scheidegger 1980), and the risk of contamination with the more complicated fusion procedure (Sikora et al 1982) with no obvious beneficial effects in terms of greater hybrid numbers obtained, these fusion systems were abandoned in favour of fusion solution no.3 described in Chapter 3. In addition it is thought PEG effects membrane changes of a variety of possible origins (Poste and Nicolson 1978), therefore the fusion system causing as little perturbation to the hybrids as possible was used while effecting fusion.

Hybrid instability leading to the loss of antibody activity is one of the main causes of loss of valuable cultures. This may be manifest by the unexplained death of the entire culture or of course as the loss of specific antigen binding activity, while neither of these manifestations are directly under the control of the experimenter nevertheless unnecessary manipulations are avoided since these may exacerbate the situation (Pontecorvo et al 1977). For example immediately after fusion too rapid dilution of the cells may result in extensive cell lysis (Fazekas de St Groth and Scheidegger 1980). Culture-stable antigen positive hybrids were successfully derived with fusion protocol 3 while none were derived with protocols 1 and 2 (see Chapter 3). This was not to say the fusion protocols were necessarily faulty as the screening assay was probably not effective.

6.2.4 Hybrid instability - freezing and recovery.

For several reasons the preservation and storage of living cells are necessary in the long term. As soon as positive cultures become established aliquots must be frozen to ensure against contamination losses.

Cell hybrids tend also to lose chromosomes (Ringertz and Savage 1976), it is therefore prudent to store positive cultures at each stage of the procedure so that if losses occur at any stage, the cell line is not lost but may be resurrected at the previous stage. Positive cell lines are also lost due to overgrowth by unimportant faster growing hybrids, or even due to dilution effects (Yelton et al 1981). In addition cells kept for extended
periods during expansion or cloning in a rapid state of proliferation tend to change characteristics (genetic drift) leading to the production of variants (Cotton et al. 1973, Galfré, Milstein 1981) and such change may lead to a changed product or loss of production altogether (Goding 1980).

Positive cells were therefore frozen at each stage of the procedure and thawed rapidly when required.

This recovery sometimes presents a problem as was discovered for the cell lines frozen in fusion 7. The cultures could not be thawed satisfactorily. On some occasions there was no growth at all on thawing of the cultures. On other occasions the cells grew slowly at first then eventually died. It is possible the freezing procedure used resulted in ice crystal formation which resulted in the death of some cultures. But all cell lines were not frozen down in the same batch, and the same procedure was used for the cell lines of fusion 8 with no difficulty experienced upon recovery of these cells. The conclusion was that the hybrid instability due to the freezing and/or thawing process was one unexplained difficulty of the technique. Other workers have verbally reported similar hybrid recovery problems. This problem was specifically linked to the hybrid cells as no difficulty was experienced upon recovery of the NSI cells. The use of feeder layer cells did not improve the recovery of the affected cultures. This was therefore not a dilution cell density effect.

6.3 CELL LINES AND MONOCLONAL ANTIBOYDIES

It has been clearly demonstrated that the hybrid cells growth like the parent NSI cells, was density dependent. This is an important relationship to identify and is critical to the continued good health of the cultures. These findings indicate that for good culture management they should be allowed to grow to no greater density than the maximum empirically derived (see table 3.6).

In general however the cultures may be routinely split every two to three days. With experience microscopic examination of the cultures was sufficient to indicate that the maximum density had been reached. The first indications of overgrowth was the budding up of the cell membranes from being smoothly rounded. In addition the fresh culture media which contained a pH indicator was salmon pink but the spent media was pale yellow. These observations indicated the cultures required immediate attention. With the appearance of these indicators the cultures die off very rapidly if ignored. In figure 3.4 the culture death was somewhat inhibited by the fact that the nutrient was not limiting. But routinely the death of the culture would be more rapid because in addition to the density effect the nutrients were being rapidly depleted.
Spent culture supernatant was used as a source of the antibody. In such cases a separate smaller culture was usually maintained to continue the culture without the need to perhaps resuscitate the overgrown cells. The second source of monoclonal antibody was from induced ascites (Wright 1980).

The monoclonal cell lines were selected visually from cloning wells, and only wells with a single small culture was selected. However cellulose acetate electrophoretic analysis of the ascites antibody preparation in two cases, SY/T8/2 and 5, showed a double immunoglobulin band. The less intense band could possibly be mouse immunoglobulins secreted in response to the introduced cell line. This phenomenon has been suggested (verbal discussion with other workers) to be sometimes responsible for the diminished quality and content of ascites-produced monoclonal antibody. The injected mouse produces antibodies not only against the injected cells but also against their products - the monoclonal antibodies. This reaction is not always manifest and is a response of the individual mouse. Because of this contamination by other such mouse antibodies some workers prefer to use the supernatant antibodies which contain only minimal foetal immunoglobulins.

Protein A purification of the antibody preparations (figures 4.18-4.20) however yielded a very pure antibody preparation since the subclasses are differentially eluted (Ey et al 1978, Kohler et al 1978, Goding 1978). Such affinity purified antibodies were suitable for purposes where a pure preparation free from contaminating serum proteins was required.

Purified antibody preparations are a primary requirement, for enzyme or radiolabelling or where they are required for use in solid phase systems, all of which were required in this work.

6.4 MONOCLONAL ANTIBODY PRODUCTS

A dominant facet of monoclonal antibody technology is the considerable potential for the production of unlimited amounts of antibody with constant properties.

In this work the monoclonal antibodies were produced in vitro in culture supernatant or in vivo in cell primed mice. A large-scale cytostat method (Fazekas et al St Groth 1983) for in vitro production and immediate purification has recently been reported. It serves to demonstrate the ease with which the monoclonal antibody may be harvested once the stable hybridoma cell line has been established.

All the monoclonal antibodies characterized were found to be IgG2a, k-light chain and this was confirmed by the protein A elution profiles of the antibodies. Both chain type composition and antigen binding character was
stable in the six cell lines examined, persisting even after continuous culture for six months. During this period both properties were examined frequently. The contamination of the monoclonal antibody product by the k-light chain synthesized by the NSI cells was not demonstrated. Only minimal k-light chain was shown to be synthesized by the NSI cells as demonstrated by SDS PAGE electrophoresis of the intracellular NSI protein. A NSI k-light chain band was not observed in the reduced monoclonal antibody product.

Because NSI synthesize k-light chain but do not secrete it (Cowan et al 1974) intracellular heavy and light chain complementation was nevertheless expected to result in three types of immunoglobulin molecules (Milstein and Kohler 1977) see figure 6.1. It is possible that such complementation did occur but that the NSI k-light chain was present in only very small amounts. The k-chain in the NSI is degraded as fast as it is synthesized, and the rate of the antibody secretion is equal to that of synthesis in the secreting partner (Cowan et al 1974). There is no evidence to suggest that in the hybrid the two rates of synthesis - that of the NSI partner and the spleen cell partner becomes synchronized. If therefore the rate of synthesis/secretion of the spleen cell derived immunoglobulin is faster than the rate of synthesis of NSI k-chain then the amount of NSI k-light chain contamination observed should be minimal.

6.5 MONOCLONAL ANTIBODY PROPERTIES

The affinity of all six monoclonal antibodies was found to be very high. This therefore, it may be argued, justified the rationale behind the immunization procedure used to prepare the spleen cell donors. This was that the affinity and specificity of the antibodies increases with time (Odell et al 1969). However Wada et al (1982) reported anti-β-TSH monoclonal antibody with affinity of $3.5 \times 10^{10} \text{M}^{-1}$ while Soos and Siddle (1982) reported monoclonal antibody affinities of between $2 \times 10^{8}$ and $5 \times 10^{10} \text{M}^{-1}$ with two immunizations. Moderately high affinity antibodies may therefore be derived with less intense immunization schedules. We maintain however that consistently high affinities were the result of the more intense antigen challenge employed in this work. The immunization regime was undertaken with two primary objectives therefore. The first was the production of high responding spleen cell donors, the antibody titre of which was indicative of the numbers of antigen positive cells available for fusion (Soos and Siddle 1982). The response was monitored by TSH radioimmunoassay of serum (figures 3.1 - 3.3). The second objective was to make available for fusion, cells with high affinity antigen receptors, corresponding to the antibody quality (Mitchison 1967).
FIGURE 6.1

Immunoglobulin molecules expected to be secreted by hybrids formed from P3-NSI-1/Ag4-1 myeloma cells and antigen primed spleen cells.
The six monoclonal antibodies investigated were demonstrated to be highly specific to the h-TSH antigen.

No appreciable cross reactivity was demonstrated to human FSH, LH, HCG or the α and β subunits of HCG.

HCG preparations from two sources were investigated ie, the MRC standard 75/537 and purified HCG(Barts) preparation (see Chapter 4).

Additionally and perhaps more convincingly were the concordant results obtained by the two independent procedures used to investigate HCG cross-reactivity. The first was as indicated above, the indirect ELISA competitive assay, in which HCG competed with solid phase TSH for the limited number of monoclonal antibodies, while in the second procedure the monoclonal antibody was used in an indirect immunocytochemical procedure (Chapter 5) to demonstrate the localization of specific antigenic determinants in the placenta. The placenta in addition to being a rich source of HCG (Wahlström et al 1981) also contain a thyrotrophic material (Hershman and Starnes 1969). No specific staining was observed (figure 5.10b). Contrast this with the positive staining of the human pituitary section (figure 5.5a).

There was no cross reactivity with HCG in the placental tissue but also any thyrotropic material contained did not possess the TSH antigenic determinants to which the monoclonal antibodies were directed.

A major difficulty however arose from this study. None of the monoclonal antibodies bound the MRC TSH standard 68/38 in the displacement experiments figures 4.5 - 4.7. This presented difficulties for establishing an assay where TSH 68/38 was to be used as the standard. The assay was however established using TSH(Barts) as the standard and was calibrated against TSH 68/38.

Two major differences are known to exist between TSH 68/38 and TSH(Barts).

1) TSH 68/38 was a standard preparation established in 1974, while TSH(Barts) was prepared fresh on a fortnightly basis (McLean et al 1981).

2) TSH 68/38 was prepared by harsh procedures which used acetone-dried pituitaries or pituitary powders as the starting material (Concliffe 1973), while TSH(Barts) preparations were extracted into solution from deep frozen glands with buffers of near normal pH (McLean et al 1981).

It is the contention that the age of the TSH 68/38 and/or the harsh treatment of the starting material together with the extraction procedures (Pierce et al 1971) resulted in the loss or gross deterioration of the antigenic determinants on this TSH preparation so they were not recognised by the monoclonal antibodies. This view was supported by McLean et al (1981) in discussing growth hormone derived from acetone dried glands as opposed to
Deep frozen glands (growth hormone is the first anterior pituitary hormone extracted from such glands and from the remaining product the gonadotrophins and TSH are then differentially extracted). They described many disadvantages of the acetone prepared material in clinical application. For example the immunogenicity was altered from that found in the frozen gland preparations, and the harsh procedure caused major denaturation including aggregation of the extracted material. Children whose growth after long term treatment with the acetone prepared material went into a refractory phase upon further treatment with this material became responsive again when treated with the frozen gland prepared material. This would suggest that the method of preparation of the material not only governs its immunogenicity and structural integrity but also its biological activity. As a result of this difficulty, TSH standard from another source was used in the two site ELISA with the monoclonal antibody to confirm its specificity for TSH. A standard curve was derived (figure 5.24) with the Wellcome TSH standards.

The specificity of the monoclonal antibodies for TSH was however also confirmed overwhelmingly in the immunocytochemical procedure, the positive cells were located as expected for thyrotropes. In addition to the clustered distribution a few were to be found scattered throughout the section. In addition when compared to the periodic acid schiff (PAS) stained sections it is clear that not all the PAS positive cells were positive by the specific immunoperoxidase procedure. The numbers, location, and distribution of TSH positive cells, described in Chapter 5, were found to be entirely consistent with that expected. Additionally, species specificity of the monoclonal antibodies for primate derived TSH was demonstrated. The monoclonal antibodies were demonstrated to be suitable for immunocytochemical work and the ascitic fluids could be used up to a dilution of 1:500,000 without loss of specific colour intensity. It must be noted that the Sephadex purified and selected conjugate III fractions were found to be better in this procedure in terms of the low background observed as compared to the ammonium sulphate purified conjugate II.

The difficulties observed with TSH 68/38 were by no means confined to the ELISA procedure but were also found in the RIA procedure (verbal communication Dr D Teale, Clinical Biochemistry Dept, St Lukes Hospital, Guildford, Surrey). No displacement of $^{125}\text{I}}$-TSH was observed when unlabelled TSH 68/38 was added in competition.

The results obtained for antigenic determinant specificity were consistent with all but one of the monoclonal antibodies binding the same determinant. They were all mutually inhibitory but to varying degrees. This was unlike the clear cut situation observed by Soos and Siddle (1982) where
some were reciprocally inhibitory while the binding of other to the antigen was unaffected. However this is not the sole explanation of the results and perhaps as likely an explanation is that the binding of one monoclonal antibody prevented the binding of the second not because they both bind the same determinant, but because the binding of one caused conformational changes in the molecule and hence the other antigenic site, then binding is inhibited. Steric hindrance of the second antibody binding by the first may also arise if the two antigenic sites are juxtaposed or even overlapping. It is to be remembered that in Chapter 5 it was concluded that only one or a very few SY/T8/2 monoclonal antibody specific epitopes were expressed per TSH molecule and therefore only minimal inhibition would be required to give a large effect. The same could be true for the other monoclonal antibodies. However having said that, it is not unreasonable that all the monoclonal antibodies could be directed to the same determinant which may have been an immunogenically dominant structure.

Antibody preparations which bind distinct epitopes are essential for the derivation of two site immunoassays (Hale and Woodhead 1980). Where only one antigenic determinant is present on the antigen or the available antibody preparations bind only one determinant then this type of assay is not feasible. This information therefore establishes the limits to the application of the monoclonal antibodies and suggests modifications which may be required for the successful use of the antibody preparations.

6.6 MONOClonAL ANTIBODIES IN ASSAY

Ekins (1981) observed that the primary obstacle to the widespread use of the theoretically more sensitive labelled antibody technique over the labelled antigen procedures in immunoassays has been the limited availability of large amounts of pure antibody. Indeed up to recent times such labelled antibody techniques have only been instituted with affinity purified conventional antisera (John and Woodhead 1982, Sutherland et al 1982).

The advent of monoclonal antibody technology (Kohler and Milstein 1975, 1976) has however provided the solution to the availability problem. In this work monoclonal antibodies have been derived in large quantities in vitro and in vivo. The antibody preparations in culture supernatant are relatively free from contaminating immunoglobulins, containing only minimal amounts of foetal calf immunoglobulin and other serum proteins.

The ascitic fluid on the other hand contains extraneous mouse immunoglobulins in significant quantities. These preparations of antibody however have been shown to be easily purified (figures 4.18 - 4.20) and to be very
versatile in their labelling capacity. Not only have they been successfully radiiodinated but they have been shown to be easily radiolabelled by the biosynthetic incorporation of radioactively labelled essential amino acids. There have been reports that some monoclonal antibodies are unstable following radioiodination (Mason and Williams 1980). Where such difficulties arise then biosynthetically labelled monoclonal antibodies may be a practicable alternative.

The monoclonal antibody used in the development of the two site ELISA method in this work was never enzyme labelled directly but its binding was indicated by an enzyme labelled anti-mouse horse radish peroxidase conjugate. Nevertheless monoclonal antibodies have been used directly labelled as has been demonstrated (Wada et al 1982, Weeks et al 1983, John et al 1984).

The additional layer of labelled anti-immunoglobulin was used in the ELISA developed here for two main reasons. First and most importantly this additional layer provided more scope for the signal amplification. Not only can each enzyme molecule catalyse the conversion of many molecules of substrate but since the antigen bound antibody molecules presented many more binding sites for the second antibody to bind to, then more enzyme would be made available (see figure 6.2). The second reason was that the enzyme conjugates were available and were known to be immunologically and enzymatically active. One of the critical factors of enzyme labelling is to effect the joining of antibody and enzyme whilst retaining antigen binding and substrate cleaving activity.

The two site ELISA developed for the measurement of human TSH was derived using a monoclonal antibody and a polyclonal antibody. From the results of the determinant binding data it became obvious that using two of the monoclonal antibodies may not provide a satisfactory assay if they both bound the same antigenic determinant and especially if only a very few of such determinants were present on the antigen. It was concluded therefore that the use of a monoclonal antibody which bound to specific determinants in a two site sandwich assay leaving the remaining determinants free to be bound by the polyclonal antibodies would constitute a suitable assay system.

The ELISA assay as described in this work shows good sensitivity with a detection limit of 4 mU/l and shows good intra- and inter-assay precision over the working range (see figure 5.23). The sensitivity was however not as good as has been reported for immunoradiometric assays (IRMA) using polyclonal antisera. Sutherland et al (1982) reported IRMA sensitivity of <2 mU/l while John and Woodhead (1982) reported 3.4 mU/l, while Jones et al (1984) reported for a chemiluminometric assay incorporating a monoclonal antibody a sensitivity of 0.015 mU/l.
FIGURE 6.2 Signal amplification.

(a) Enzyme labelled antibodies directed against the antigen.
(b) Anti-immunoglobulin enzyme labelled.

In system (b) more binding sites are available for enzyme labelled antibody, resulting in extensive signal amplification.
The theoretical sensitivity of these labelled antibody type of assays according to Ekins (1981) is one molecule of analyte which is realised as the antibody quantity is increased towards infinity. Under such conditions of antibody excess the analyte-antibody complex formation is kinetically favoured. The antibody affinity therefore has little effect in these systems.

Of greater importance to the sensitivity of the assay in these circumstances according to Ekins (1981) is the signal detection, and this is dictated by the ability to differentiate a minimum positive signal from any inherent background noise. This of course is dependent upon the specific activity of the label which is a measure of the amount of enzyme bound per antibody molecule in the conjugate preparation. This on the other hand is indicated by the number of substrate molecules transformed per unit time per molecule of antibody. The $\text{OD}_{403\text{ nm}}/\text{OD}_{280\text{ nm}}$ describing the ratio of horseradish peroxidase to immunoglobulin molecules in the conjugate was suggested as optimal between 0.3 to 0.6, representing an average of 1 to 2 horseradish peroxidase molecules bound per antibody molecule (Wilson and Nakane 1978). Ratios greater than this represented antibody molecules which were over conjugated and lead to diminished antibody activity in addition to high background. This suggests that there is a limit to the number of enzyme molecules per antibody molecule giving the required assay characteristics. Additionally where the enzyme antibody conjugate was increased the background similarly increased (figure 5.18). Above a certain level of enzyme content therefore the results are self-defeating because as background increases so the detection at lower antigen levels decreases. In practice therefore even with the inbuilt signal amplification the sensitivity obtainable with the enzyme conjugate is limited by this factor. Other factors which affect the sensitivity and precision of the ELISA system were described in Chapter 2. This is a complex assay system incorporating many variables all of which must be controlled to yield a serviceable assay system.

The assay achieved by comparison to RIA (Hall et al 1971) is reliable, time saving, the enzyme conjugate is cheap and stable for very long periods (>1.75 years) compared with radiolabelled material (radiolabelled material is usable for up to 1 month at the most. The half life of $^{125}\text{I}$ is only 57 days). These together with the many advantages of the monoclonal antibody already stated constitutes a highly suitable assay for TSH measurement. The constraint of polyclonal antisera still exists for the second antibody used in the system however, but because this is a two site assay the monoclonal antibody provides the precise specificity for the assay and any anti-TSH antisera providing only attachment points for the enzyme labelled antibodies.
The testing of such antisera therefore need not be as rigorous as would be required otherwise. The automated microtitre plate procedure facilitates a high sample handling capacity and is therefore suitable for large scale screening purposes.

The successful generation and application of these anti-TSH monoclonal antibodies establishes them as very useful reagents in both immunocytochemistry and the ELISA procedures. The use of the alternative enzyme label not only avoids the hazzards associated with the use of the most frequently used radiolabel, but in some countries where the law severely restricts the application and use of radioisotopic procedures then the enzyme-labelled procedures come into their own. The use of radiolabelled procedures require expensive counting equipment and special disposal facilities which are not always available in small laboratories or again in some countries. In these circumstances the ELISA procedures may be substituted (Voller 1979). The automated ELISA equipment is relatively inexpensive and requires no special facilities except those available in even the most basic laboratories.

As the procedures develop, and the sensitivities and reliability of the enzyme and other non-isotopic labelling procedures approach or surpass those obtainable in the established assay systems they will no doubt gain greater acceptance in the wider clinical setting.

6.7 SUGGESTED FUTURE WORK

Additional work is required to further evaluate the ELISA developed for measuring TSH. This includes the correlation of observed serum assay values as measured by the two site ELISA method and the established RIA method. This describes the new assays performance in relation to the established method. The two site ELISA therefore requires further validation prior to its application in a clinical environment.

During the course of the work several issues arose which merited further investigation but time and finance did not permit further elaboration of these points. Some of these were of critical importance to the hybridoma production scene. For example, there is still the urgent need to find a suitable antifungal agent which is compatible with normal cell growth in culture. A great deal of work now involves long term cell and tissue cultures which are affected by contamination. A safe universal antifungal agent would greatly assist in this type of work.
The investigation of hybridoma stability under various freezing and recovery regimes may suggest ways of avoiding the disastrous affects encountered in fusion 7 where the cells could not be resuscitated following freezing.

Another important issue was the identification of the particular antigenic difference between the TSH 68/38 and the TSH(Barts) which gave rise to the differential binding observed with the two preparations. The antigenic difference may be as small as a charge difference which may be investigated by the binding of the antibody at different pH, or as large as the disruption of a unit structure in which case they may have to be identified from protein fragment analysis for example. Such investigations would be useful for providing specific information about the immunogenic/antigenic structures of TSH.

The Characterization of the remaining monoclonal antibodies is required and it may be that their combination with others from different sources may improve the performance in application.

With these final points remaining to be settled, the anti-h-TSH monoclonal antibodies have been shown to be highly suitable for immunocytochemical work and their potential to establish a highly specific and sensitive assay has been demonstrated.
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