Production of monoclonal antibodies to cells isolated from the developing rodent cerebellum and grown in culture

Thesis

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PRODUCTION OF MONOCLONAL ANTIBODIES TO CELLS ISOLATED FROM THE DEVELOPING RODENT CEREBELLUM AND GROWN IN CULTURE

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SUMMARY

Conditions previously established for the growth in culture of cells isolated from early postnatal rat cerebella, have been adapted for the growth of similarly isolated cells from mice cerebella, in the belief that such cultures may provide a useful model for studying a number of aspects of neural development. These cultures have been shown to be reproducible, both in terms of plating efficiency and cell-type composition, and survive and develop for at least three weeks in vitro.

In an effort to identify cell-type specific antigens, some of which have been implicated in the process of neural recognition, techniques have been developed for the production and detection of monoclonal antibodies to cerebellar cells in culture. The successful production of such antibodies may provide both new cell type specific markers and new experimental tools.

Preliminary efforts to characterise one antibody, produced by the methods described, indicate that it recognises an antigen present on filaments in both astrocytes and fibroblastic cells present in cultures of rat cerebellar cells.

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CONTENTS

CHAPTER 1

1.1 INTRODUCTION

The processes by which the mammalian nervous system is formed are varied and complex, many are common to histogenesis and organogenesis in general; yet neurogenesis is unique in that the final product is a diverse array of cell types, arranged in a highly ordered manner, with specific interconnections which form the basis for neuronal function. No other organ approaches the level of complexity found within the mammalian brain.

The first glimpses of this complexity were provided by early embryologists such as Ramon-y-Cajal (1960), who produced wonderfuly descriptive accounts of neurogenesis as revealed to him using classical histological techniques. Two important innovations during the 1950's served to increase the resolution possible in studying developmental events and to reveal the dynamic aspects of the processes involved. One was the application of electron microscopy to the field of neurobiology, the other the use of ^{3}H thymidine autoradiography by which patterns of cell proliferation and migration could be revealed rather than inferred (eg. Uzman, 1960).

Such studies indicated that the period of neurogenesis following the final round of neuronal cell division, could usefully be divided into three phases which, while having no clear dividing line between the end of one phase and the beginning of the next, do seem to be distinct. Recognition of their existence has helped to simplify the study of an extremely complex process. The first phase is marked by the migration of neurons from the proliferative zone in which they were formed, to their mature functional positions. This is followed by the aggregation of like cell types, or those which are functionally linked, to form the anlages of future neural centres, or into laminar layers such as those found in the hippocampus or cerebellum (for a

review see Cowan, 1979). The final phase is perhaps the most precise and involves the establishment of specific interneuronal connections.

What these studies could not reveal however were the underlying mechanisms at work in producing the final structure. The earliest attempts to elucidate these mechanisms were concerned primarily with the later stages of development, i.e. the establishment of specific connections. The reasons for this seem to have been twofold. One was that it is this phase which seems to be the most intriguing in terms of the degree of specificity involved. The second was concerned with the much more fundamental question of whether the patterning of synaptic connections between sensory, central and motor neurons was indeed specific at all. During the early 1930's Holt (1931) and several others were maintaining that the nervous system developed as an unorganised network, capable of nothing but diffuse random reaction and that neural organisation was eventually achieved through the supposed neurobioactic outgrowth of dendrites to axons excited in the course of the animals early activity.

Most of the knowledge gained in studies of this later stage of development has formed the background for studies on the preceding stages and it is not therefore for purely historical reasons that I intend to begin with a brief survey of the evidence relating to this phase.

With no experimental evidence to draw upon, the most obvious explanation to account for the manner in which growing neurites established specific connections, was to propose that the cell with which the neuron was destined to synapse acted as some form of beacon towards which it was attracted. The nature of the attracting signal was thought to be either chemical or electrical.

Ramon-y-Cajal was an early proponent of the chemical signal or "alluring substance" as he termed it. For example in his explanation for the establishment of connections of the horizontal cells of the

mouse retina, he suggested that "In the struggle with mechanical obstacles they merely project the growing expansions in the direction of least resistance, as if to explore the terrain whilst awaiting the appearance of sources of attractive substances or orienting enzymes" (Ramon-y-Cajal, 1960).

Prominent among the electrical theories to explain the structuring of integrative patterns was that of Kappers "Law of Neurobiotaxis" (1917) in which he proposed that the neuron was electrically polarised and that axons grew along the direction of a current and dendrites against it. A related hypothesis by Bok (1915) deserves a mention if only for its name of "stimulogenous fibrillation." He too had suggested that growing neurites were directed by currents emanating from stimulated fibre tracts. Such theories were originally based on rather tenuous assumptions and with the accumulation of experimental evidence during the 1930's and 1940's and the failure to detect any chemical or electrical sensitivity, they were almost entirely discredited.

It was Paul Weiss, one of the critics of the neurotrophic theories, who first proposed the theory of "contact guidance" (Weiss, 1939), the basic premise of which was that rather than being guided by some force acting at a distance, fibres were guided by interactions with their immediate contact environment. The original theory was confined to mechanical contact guidance and suggested that growing nerve fibres were directed by the orienting effects of mechanical guidelines formed in the surrounding tissue matrix by stresses on tissue ultrastructures. For example, in an irregular network of ground cells (as Weiss proposed was present in the neuropil of the central nervous system), at each intersection the growing neurite encounters it will split, but through a process of competition, all but one of the branches will be eliminated, the survivor being determined by "essentially an accident of the local situation" (Weiss, 1939). The result is a nerve course which is irregular and tortuous. The opposite extreme is one where the fibrous

matrix consists of a parallel arrangement of cells which leaves the growing nerve fibre no alternative course.

Weiss' theory is well supported by experimental evidence and it is now universally accepted that mechanical influences are ubiquitous in the grosser aspects of nervous system development. However further studies revealed examples of nervous organisation which could not be explained in these passive mechanical terms.

It was left for Roger Sperry to combine the specific aspects of the neurotrophic theories, with the local contact guidance aspects of Weiss' work, to achieve the theory of "chemical contact guidance" (Sperry, 1963). In brief the theory suggests that synaptic connections are established via highly specific cytochemical affinities amongst the different types of neuronal and non-neuronal cells with which they come into contact. Once guided to the correct target cell, such cytochemical affinities form the basis for the recognition process and lead to synaptic stabilisation.

Evidence for this came originally from a series of experiments during the 1940's on regenerating optic nerves in a number of species (Sperry, 1945). It was found that if the fibre system was disconnected and transplanted, or just scrambled, in all cases functional order was re-established. Stronger support for the theory came in 1963 when the retino-tectal connections of goldfish were manipulated in such a way that the regenerating fibres passed over a whole array of vacant tectal neurons, only to synapse with the "correct" neurons in the proper segment of the tectum (Attardi and Sperry, 1963). The volume of work relating to the establishment of retino-tectal connections is now enormous and extremely complicated, and while Sperry's original hypothesis has been shown to be less than perfect, the evidence suggests that the type of cytochemical affinities he proposed do indeed exist (for a recent review see Gaze, 1978).

The experimental evidence discussed so far has been related to the establishment of connections amongst distantly projecting neurons, not the locally projecting neurons found within the cerebral cortices, and it is reasonable to ask whether they have much in common. It would seem that any differences which do exist are quantitative rather than qualitative. Simply because neurons within the cortices do not show the same topographic organisation, it cannot be assumed that the connections which they establish are any less specific. Indeed the evidence indicates that the known connections of cell types within the cortices are, under normal circumstances, remarkably invariant and are even specific as to the region on the target neuron with which they synapse (see for example Palay and Chan Palay, 1974). Nevertheless this difference should be borne in mind.

If, as the evidence seemed to indicate, growing neurites are guided to and "recognise" their correct targets as a result of interactions of specific cell surface molecules, it seemed possible that such a mechanism could also be involved in guiding migrating neurons to their definitive positions.

All the neuronal cell types studied to date (with the exception of certain granule cells in the dentate gyrus) undergo at least one phase of migration. As yet there is no direct evidence to support any chemical contact guidance theories, largely due to the difficulties in developing suitable experimental models. Indirect evidence has come from detailed histological studies which, in at least two well documented studies, have shown an intimate relationship between migrating neurons and radially oriented glial fibres. This relationship was first described by Rakic (1971,1972) in studies on the developing cerebellum and neocortex of the Rhesus monkey. A similar relationship has been shown to exist in the developing mouse cerebellum. Evidence that it is not coincidental and appears to be essential for normal development has come from studies of two neurological mutations which affect

cerebellar development in mice. The "weaver" mutation is characterised by the degeneration of granule cells prior to migration (Sidman, 1968). It has been suggested that cell death is secondary to a failure to migrate and that this is in turn due to abnormalities in the Bergmann glia (Rakic and Sidman, 1973).

In the "reeler" mutation, the Bergmann glia appear to be structurally normal, but are arranged obliquely rather than radially. Despite this abnormal orientation, granule cells are still seen in close apposition and, apparently as a result of this association, become malpositioned (Rakic, 1976). If this relationship is functionally significant, then the specificity of neurons for radial glial fibres, rather than other similarly oriented structures such as axons or blood vessels, could be explained by cell surface mediated interactions.

That such orienting structures are not however essential for the sorting out of cell types, which is achieved by migration, has been demonstrated in vitro using cell reaggregation techniques. It has been found that cells isolated from the isocortex, hippocampus and cerebellum will, if isolated during a critical period, reaggregate to form histotypic patterns similar to those found in vivo (Garber et al., 1980; Delong and Sidman, 1970). How relevant such observations are to normal development is however unknown.

As with the other phases of development already discussed, the process of cell aggregation to form the characteristic lamellae found within the cortices, has been unyielding to in vivo experimentation. It has however proved relatively simple to develop model systems to study the aggregation of isolated cells in vitro. As early as 1943 it had been shown that cells isolated from amphibian embryos, would reaggregate to form patterns of organisation similar to those found in vivo (Holtfeter, 1943). By refining and modifying this basic system many workers have used the technique in attempts to determine the basis of the observed sorting out and, more recently, to identify

some of the macromolecules involved.

The most widely used approach has been to dissociate the tissue, or tissues, into a single cell suspension and then allow them to reaggregate whilst measuring the rate of adhesion. The "rate" of adhesion has been measured in a variety of ways, but in all cases it has been taken as a reflection of the number and types of adhesive sites present on the two cell surfaces (for a review of the methods employed see Lillien et al., 1978.

Prominent amongst the workers using these techniques has been Moscona who, between 1961-1968, conducted a series of experiments with cells isolated from chick retina and tecta. The findings of these experiments led to the proposal of the "cell ligand hypothesis" (Moscona, 1968). In what was basically a modification of Sperry's chemical contact guidance theory, it was proposed that cells from different tissues (tissue specificity) and different cell-types within tissues (cell-type specificity) sorted out and adhered due to differences in cell surface macromolecules. Cells with a high number of complimentary ligands would adhere, whereas those with fewer complimentary sites would adhere less effectively or not at all.

Moscona's original hypothesis has undergone refinement since its proposal and it now seems likely that there are quantitative, qualitative and topographical differences in the cell surface molecules involved in sorting out and adhesion.

The success of reaggregation assays in demonstrating the existence of adhesive differences prompted a search for the molecules mediating the process. Within the last few years a number of such molecules have been isolated from neural cell types. The best characterized are the retinal cell aggregating factor isolated by Lillien and Moscona (1967), which promotes adhesion amongst embryonic chick retina cells, and the cell adhesion molecule (CAM)(Thiery, J-P. et al. 1977). The use of an antiserum against CAM has however provided the only evidence for a possible role of such molecules during the histogenesis of an intact tissue.

In order to account for the large number of specific cell surface interactions, which it has been postulated occur during development, it is necessary that the molecules involved exist in a wide variety of structural variants. One such group of molecules, which appears to be well suited for the role, is that of the glycomacromolecules. They are present in large amounts on the cell surface (between 3-10% of brain proteins are glycoproteins [Brunngraber, 1969]) and have the potential for great structural variability (for a review see Barondes, 1970). Recognition between cell types could occur by a glycoprotein or glycolipid, present on the surface of one cell, binding to a complimentary protein present on the surface of another cell. Edwards (1978) has proposed that differences in cell surface glycoproteins could account for differential adhesion purely due to non-specific interactions which could explain many but not all recognition and adhesion phenomena.

The success in demonstrating the existence of such nervous system specific macromolecules, has closely paralleled the development of new techniques, and the refinement of old ones, necessary to detect them in, and ultimately isolate them from, an heterogenous mixture of cell surface components. These techniques can be divided into three groups: the fractionation of cell surface components using ion exchange and/or molecular sieve chromatography, sodium dodecyl sulphate polyacrylamide gel electrophoresis and other "traditional" biochemical techniques; the use of lectins as labels for glycoproteins and glycolipids and the use of immunological techniques.

It was using the more traditional biochemical techniques that Moore isolated the first brain-specific protein known as S-100 (Moore, 1965)*. S-100 is not located at the cell surface, and its function is still unknown, but its isolation paved the way for future attempts to isolate brain specific, cell surface macromolecules. There are now several well characterised nervous system specific membrane proteins

* Recently detected in human chondrocytes. Stefanson, K., Wollman R. L., Moore, B. W. and Aranson, B.G.W. (1981) S-100 protein in human chodrocytes. Nature, 295. 63-64.

and glycoproteins which were originally isolated by fractionation techniques (for a review see Bock, 1978).

Several workers have chosen to study the membrane composition of cells isolated from normal and neurologically mutant mice cerebella in attempts to correlate any changes, with observed anatomical alterations. Such studies have revealed membrane proteins which are specific for Purkinje and granule cells (Stuhlfauth, 1976), a protein which is absent from Purkinje cells which are anatomically abnormal (Mallet et al., 1975) and changes in the concentrations of synaptosomal proteins in three cerebellar mutants (Jørgensen, 1978). Whilst these techniques have been successful in confirming the existence of both tissue and cell-type specific macromolecules, used in isolation they offer little hope for establishing their functional role.

Lectins are a group of proteins, originally isolated from plants, which bind non-covalently to specific carbohydrate groups (for a review see Lis, 1972) and as such, when coupled to a suitable tag can be used to study the glycoprotein and glycolipid composition of tissues. Using radiolabeled lectins several groups have reported quantitative and qualitative changes in these macromolecules in neural tissues, which appears to be developmentally regulated. Nihal et al. (1979) have shown such changes in synaptic membranes isolated from the forebrain of rats of increasing postnatal age and Mintz and Glaser (1978) demonstrated similar changes in the chick retina. The latter group also found differences between the dorsal and ventral half of 8 day old retina (Mintz et al., 1981). By coupling the lectin to a marker such as horseradish peroxidase or fluorescein, the distribution of glycoproteins and glycolipids can be localised using light or electron microscopy. Using this approach Zanetta et al. (1978) found that the parallel fibres in the rat cerebellum only bound the lectin Concanavalin-A while they were growing or newly formed. Upon maturation and synapse formation the binding disappeared.

The use of lectins has added further support to the theory that glycoproteins are involved in neurogenesis. However, because they are specific for groups of carbohydrates rather than individual determinants their resolving power is limited. As such they are unlikely to be capable of detecting many of the subtle changes or differences in glycoconjugate composition during development, nor localise them to specific cell types.

An immunological approach to studying the cell surface can theoretically overcome the problems associated with other techniques. A summary of their potential uses is given below.

i) Antibodies permit the identification of specific and sometimes minor, cell surface constituents which are expressed in the developing and mature nervous system.

ii) Antibodies may serve as assay reagents to analyse the molecular nature of the corresponding antigens, which can be isolated and purified using antibody affinity chromatography.

iii) When coupled to labels which can be visualised at the light and electron microscopic level (fluorescein, ferritin, horseradish peroxidase, etc.), antibodies can be used to probe the cellular and subcellular distribution of antigens.

iv) Cell type specific antibodies can be used to isolate antigen positive and antigen negative cells from a mixed suspension using antibody affinity chromatography or fluorescence activated cell sorting.

v) Antibodies may serve as modifiers of the functional roles of the molecules with which they combine.

(Summarised from Schachner and Willinger, 1980).

Antibodies have been produced against a large number of neural antigens by immunisation with whole cells (Schachner, 1974; Mallet et al., 1979), cell homogenates (Wallenfels and Schachner, 1978) and purified proteins (Mikoshiba et al., 1979).

Such conventionally produced antisera, while having the potential to recognise specific molecules, are in fact unlikely ever to do so. Even if it were possible to isolate to purity a component of the cell surface which would serve as an immunogen (a process fraught with technical difficulties), the resulting antiserum would be a mixture of antibodies against the different determinants on the same molecule and of different antibodies against the same determinant, all with differing affinities. When cells or cell membranes are used as an immunogen, as is usually the case, the antiserum produced will contain antibodies against the many immunogenic molecules present and attempts must then be made to purify the desired antibody. One approach which has been successfully used to overcome some of these problems is that of allo-immunisation in which the antiserum is raised within a species. The advantage being that only a few determinants are recognised. The disadvantages are that well conserved and possibly important antigens are likely to go undetected and that the antisera produced are usually of a low titre (see for example Reiff and Allen, 1964). Fortunately, the lymphocyte hybridoma technique developed recently by Köhler and Milstein (1975) has revolutionised the production of specific antibodies and is now playing an important role in the study of neural cell surfaces.

The success of the technique is based in the fact that any B lymphocytes from the spleen, or other lymphoid tissue, synthesises only a single immunoglobulin species. If such a cell could be isolated and grown under conditions such that it will multiply and maintain its antibody production, it would be a source of a genuinely pure antibody - a monoclonal antibody. Unfortunately such plasma cells are short lived and have yet to be cultured in vitro.

The solution to this problem had its beginnings in an experiment performed in 1973 by Cotton and Milstein. Their area of study at that time was related to certain aspects of the genetic control of antibody

synthesis and involved the use of cells originally derived from malignant tumours of the immune system. Such myelomas, as they are known, produce large amounts of abnormal immunoglobulins (myeloma proteins) and have been successfully adapted to grow in culture. What they succeeded in doing was fusing two lines of myeloma with the result that the hybrid synthesised immunoglobulin coded for by genes from both parents, i.e. expression of antibody synthesis was co-dominant. Their success in fusing two abnormal antibody producing cells led them to an attempt to fuse myeloma cells with normal antibody producing cells from the spleens of immunised mice. The result was the same, i.e. the hybrid cells produced antibody coded for by both parents and survived and grew in culture (Köhler and Milstein, 1975).

The first specific monoclonal antibody was produced against sheep red blood cells, but the potential of the technique for studying the cell surfaces of neural tissues was quickly realised. The results obtained so far have further demonstrated that certain neural cell types can be distinguished by the presence of unique antigenic determinants (Lagenaur et al., 1980; Eisenbarth et al., 1979; Bartlett et al., 1981). In one case a monoclonal antibody produced against chick retinal cells has revealed a gradient of antigen from anteriodorsal to posterio-ventral retina and these changes in concentration can be used to identify cell position (Trisler et al., 1981). The exquisite sensitivity of monoclonal antibodies for detecting such differences, has perhaps been best demonstrated in a study of the leech nervous system where a series of antibodies were produced, some of which were specific for pairs of neurons (Zipser and McKay, 1981).

While the production and application of monoclonal antibodies was opening up new approaches to studying the development of the nervous system, work was continuing in our laboratory with an alternative and somewhat older approach - the use of tissue cultures as

models for brain development. Interestingly the very first tissue culture experiment established one of the fundamental properties of the development of the neuron that the neurite was indeed an extension of the neuronal cell body (Harrison, 1907).

The brain region selected for investigation was the rodent cerebellum, an area particularly well suited for developmental studies for a number of reasons, some of which are discussed below.

The cerebellum contains only five neuronal cell types which, in the mature cortex, are organised in a well defined geometic arrangement (Palay and Chan-Palay, 1974) consisting of three laminae. The molecular layer consists largely of the T-shaped axons from the granule neurons and dendritic arborisations, interspersed with inhibitory interneurons (the stellate and basket cells). The underlying lamina is formed by a monolayer of Purkinje neurons with both ascending and descending axons, and the ascending dendrites of the Golgi cells. The granule neurons are by far the most numerous cell type in the cerebellum and constitute the remaining laminae. Also within this layer are members of a third type of inhibitory interneuron, the Golgi cell. This relative paucity of neuronal cell types, coupled with their simple arrangement has made it possible to elucidate the circuitry within the cerebellum such that a large body of information has accumulated on the manner in which it functions (see for example Eccles, 1967).

For these same reasons the development of the cerebellum is also extremely well documented. Many of the early observations made by Ramon-y-Cajal have been confirmed and elaborated upon by the application of electron microscopy and ^{3}H thymidine autoradiography. It is not my intention to review the process here, suffice to say that the birth date and migration patterns of the cells types found within the cerebellum are well established (Miale and Sidman, 1961; Altman, 1972, a,b, c; Eccles, 1970; Llinas, 1961).

Brief mention was made earlier of several neurologically mutant mice, the study of which has given new insights as to the possible mechanisms operating during development. There is now a list of over 12 such mutations which affect the cerebellum, all of which are single gene mutations and produce major, yet precise alterations of the normal cytoarchitecture (for reviews see Mallet, 1980; Cavines and Sidman, 1978).

The three most widely studied mutations are the "weaver" (Rezai and Yoon, 1972), the "reeler" (Falconer, 1951) and the "staggerer" (Sidman and Lane, 1962).

As mentioned earlier, the "weaver" mutation is characterised by the degeneration of the vast majority of cells in the external granular layer prior to migration and that this may be related to a defect in the Bergmann glial fibres. This theory has not however gone unchallenged. Sotelo and Changeux (1974) have suggested that the primary target of the mutation is the granule cell and Trenkner et al. (1978) have shown that the effect may be mediated by the indirect action of toxic metabolites on glial and/or granule cells.

The "staggerer" mutation is marked by a small reduction in the rate of granule cell proliferation and the death of those formed, either during or after migration (Yoon, 1972). As in the case of the "weaver" the primary target of the mutation is unknown since granule cell death is preceded by the appearance of abnormalities of the Purkinje neurons (Sidman, 1978) and it has been suggested that granule cells degenerate due to the failure to establish normal synaptic contacts with Purkinje cell dendrites (Sidman, 1972).

The "reeler" mutation differs from the "weaver" and "staggerer" in that it affects all cortical structures with the exception of the olfactory bulb which is cytoarchitecturally normal. These widespread disturbances are possibly the result of a failure in the basic cell-

cell recognition process (Sidman, 1968). Whilst the cytoarchitecture of the "reeler" cerebellum is abnormal, it is nonetheless a systematic and orderly rearrangement and all classes of synapse are still found (Mariani et al., 1977).

One of the interpretations of these findings is that although the primary target of the mutations is unknown, there is evidence to suggest that the effects are mediated via changes in cell surface interactions as a result of alterations in the composition of surface macromolecules. This being the case monoclonal antibodies may prove to be ideally suited to testing this hypothesis.

The production of monoclonal antibodies and their use in tissue culture has a number of unique advantages. Specific antibodies can be used not only to identify cell types in culture, but also to isolate an homogeneous cell population from an heterogeneous suspension by the use of antibody affinity columns or fluorescence activated cell sorting. Such pure preparations can then be grown in culture and studied biochemically and morphologically in the absence of "contaminating" cell types. An alternative approach to producing such homogenous cultures is that of negative selection whereby the unwanted cells are killed by the action of specific antibodies used in conjunction with complement. The accessibility of monolayer cultures also allows for the study of the effects of antibodies against cell surface antigens, on the "normal" in vitro development including cell aggregation, migration and the formation and outgrowth of neurites.

The primary aims of this project were then two-fold. The first was to develop a system for the growth in culture of cells isolated from early postnatal normal and mutant mice cerebella and the second and major aim was to establish the techniques necessary for the production and identification of monoclonal antibodies to cerebellar antigens.

CHAPTER 2

THE CULTURE OF CELLS FROM POSTNATAL MICE CEREBELLA

2.1 INTRODUCTION

Procedures for the isolation and growth in monolayer culture of cell perikarya isolated from early postnatal rat cerebella have been developed by workers in this and other laboratories and are routinely used (Cohen et al., 1974; Currie et al., 1979; Dutton et al., 1981). Attempts to culture cells isolated from mice cerebella under similar conditions had however led to the death of the vast majority of cells within the first week in culture. We were thus deprived of a possibly important tool for studying the development of cells isolated from normal and mutant mice cerebella .

Other workers have described methods for the isolation and culture of cells from mice cerebella but, for a number of reasons, they seemed unsatisfactory. Messer (1977) has successfully cultured cells which developed over a period of several days but the culture conditions and feeding schedule were complicated. In addition the cultures were extremely variable with only 30% showing excellent morphology and survival for three or more weeks. Trenkner and Sidman (1977) have described a method for producing reaggregate cultures of cells from mice cerebella. While the method appears to be reproducible and the cultures survive for periods of up to four weeks, the vast majority of the cells are not accessible to identification using cell type specific labels, nor does the system allow for observation of anything more than gross developmental changes.

Using the established culture conditions for rat cerebellar cells, it was my intention to adapt them for the culture of similarly isolated mouse cerebellar cells. Once established, the conditions should be such that cultures could be routinely produced which have a high plating

efficiency, show survival and development of neurons for a period of three or more weeks in culture and be reproducible.

The reproducibility of the cell isolation was monitored using a Coulter Counter to measure the total cell number and their distribution within five size classes, selected because they correspond to breaks in the spectrum of cell sizes found within the intact cerebellum (Cohen et al., 1978).

In order to determine the reproducibility of the cell cultures, the plating efficiency was measured at various time points in culture and conventionally produced antibodies against cell-type specific markers were used to identify and quantify the predominant cell types believed to be present - neurons, astrocytes and oligodendrocytes. Tetanus toxin has been shown to bind specifically to neurons in cultures of cells isolated from central and peripheral nervous tissues and this binding would seem to be a general property of neurons (Dimpfel et al., 1975; Mirsky et al., 1978; Raff et al., 1979). Glial fibrilary acidic protein (GFAP) is an extensively studied protein originally isolated by Eng et al. (1971) which has been shown to be an intracellular protein associated specifically with astrocytes, both in vivo (Bignami and Dahl, 1974) and in vitro (Manthorpe et al., 1979; Kozak et al., 1978). Oligodendrocytes were identified with an antiserum against galactocerebroside (Gal C) which is present on the surface of only these cells in cultures derived from central nervous system tissue (Raff et al., 1979).

Cultures of cerebella cell perikarya, isolated from 5 day old rats, were prepared as a standard against which the growth and plating efficiency of mice cerebellar cultures could be compared. Similarly prepared cultures were also established with cells isolated from 5 day old rat olfactory bulb, hypothalamus, hippocampus and cerebral cortex, in order to determine the applicability of the technique to brain regions other than the cerebellum.

2.2 METHODS

MICE CEREBELLAR CELL CULTURES

Cell Isolation. Bb/CBA mice maintained in colonies at the Open University in England and The University of Iowa in America, were used as a source of cerebella (breeding pairs from the Open University were used to establish the colony at The University of Iowa). The method for the preparation of a single cell suspension was essentially as described by Dutton et al. (1981) which is a modification of the original procedure of Cohen et al. (1974) and Wilkin et al. (1976).

Mice between the ages of 4 and 8 days of age (where the date of birth was day 0) were decapitated and the cerebella removed. All subsequent operations were performed under sterile conditions. All solutions were sterilised by passage through a 0.22 μm filter (Millipore). The cerebella were placed on a ground glass plate, where the meninges were removed with forceps, and then transferred, in groups of four to six into a few drops of nominally calcium free Earles balanced salt solution (EBSS) containing 1.5 mM $MgSO_4$, 1.9 mM glucose and 3 mg/ml bovine serum albumin (BSA Fraction V, Sigma) (CFEBSS). This, and all other solutions used during the preparation of the cell suspension were gassed, using a mixture of 5% CO_2/95% O_2, to a pH of 7.4 and originally used at room temperature. Each group of cerebellar were chopped into blocks using a tissue chopper (Mickle Lab. Indust.) set at 400 μm and making two series of cuts, the second at right angles to the first. Tissue blocks were transferred into 50 ml siliconised conical glass centrifuge tubes containing 10 ml of CFEBSS with 250 μg/ml bovine trypsin (Type III Sigma). They were then dispersed with a spatula and the suspension quickly transferred to a 25 ml conical flask. The flasks were gassed with CO_2/O_2 and sealed prior to incubation, with gentle shaking, at 37°C. After 15 minutes the flasks were removed from the water bath and the trypsin digestion terminated by the addition of

10 ml of CFEBSS containing 1.8 mM $MgSO_4$, 80 µg/ml soybean trypsin inhibitor (SBTI Sigma) and 6.4 µg/ml deoxyribonuclease (DNase ID 4763 Sigma). The suspension was transferred back to the conical tube and the tissue pelleted by centrifuging to 200 g over a period of 10 seconds. The supernatant was discarded, 1 ml of CFEBSS containing 31 mM $MgSO_4$, 500 µg/ml SBTI and 40 µg/ml DNase, was added and the tissue disrupted by sucking the suspension in and out of a siliconised glass pasteur pipette between 10 and 12 times. The larger tissue fragments were allowed to settle out over a 10 minute period and the supernatant transferred to a plastic 12 ml conical tube (Corning 25310). This process was repeated a second time and the supernatants combined. In order to remove some of the cell fragments the cell suspension was underlain with 2 ml of a solution of CFEBSS with 400 µg/ml BSA and the cells pelleted through this by centrifugation for 5 minutes at 200 g. The supernatant was discarded and the cells resuspended in Minimum Essential Medium (Eagle) (MEM) prior to counting using a Coulter Counter ZBI (Coulter Electronics).

Cell Culture. The culture medium used throughout these experiments was MEM supplemented with a number of additional components. Those which were added as standard, to give the final concentrations shown in parentheses were: potassium chloride (23 mM), glutamine (2 mM), glucose (37 mM) and gentamycin (180 µM). The variables which were examined during the course of selecting the optimum culture conditions are set out in Table 2.1.

Cells were plated into multi-well dishes (Falcon 3008) precoated with poly-L-lysine (Sigma) using the following procedure. 400 µl of poly-L-lysine at a concentration of 15 µg/ml, was added to each well and incubated for 1 hour at 37°C. The poly-L-lysine was then removed using suction and the wells allowed to dry for at least 1 hour. All cultures were incubated at 35.5°C with an atmosphere of 100% humidity

VARIABLE	VARIATION
CELL DENSITY	$25x10^3$, $50x10^3$, $10x10^4$, $15x10^4$ CELLS/MM^2.
SERUM SOURCE	HORSE SERUM, FOETAL CALF SERUM.
SERUM CONCENTRATION	10%, 20%.
MITOTIC INHIBITOR	5-FLUORODEOXYURIDINE, CYTOSINE ARABINOSIDE.
CHICK EMBRYO EXTRACT	$\pm$ CHICK EMBRYO EXTRACT.

Table 2.1 Variatons tested during the selection of the optimum conditions for the growth in culture of early postnatal mice cerebellar cells.

and 6% CO_2. Medium was changed every 3 or 4 days.

Plating Efficiency. The plating efficiency under the culture conditions adopted as standard was determined using a total deoxyribonucleic acid (DNA) assay. DNA was extracted using a modification of the method of Schmidt and Thannhauser (1945) and assayed as described by Zamenhoff et al. (1964) which is a modification of the method developed by Burton (1956).

Single cell suspensions were prepared from 5 day old mice cerebellar as previously described. After counting, cells were resuspended in medium at a density of 1.25×10^6 cells/ml. Two ml aliquots were then either transferred to conical centrifuge tubes for immediate DNA assay, or plated into 35 mm diameter plastic tissue culture dishes (Falcon), precoated with poly-L-lysine and cultured for 1 or 7 days before assaying.

Freshly isolated cells were pelleted at 800 g for 10 minutes, the supernatant discarded and the process repeated with 10 ml of Krebs Ringer buffer (KRB) to wash the cells. Protein, DNA and RNA were precipitated by adding 1 ml of a cold solution of 5% (w/v) trichloroacetic acid. The precipitate was centrifuged at 800 g for 10 minutes and alkaline hydrolysis carried out on the pellet by adding 1 ml of 1.0 M potassium hydroxide and incubating for 2 hours at 37°C. DNA was precipitated by the addition of 1 ml of 1.0 M perchloric acid (PCA), and then pelleted by centrifugation at 800 g for 10 minutes. Final extraction of DNA was achieved by resuspending the pellet in 500 µl of 1.0 M PCA and heating for 20 minutes at 70°C. The extracted DNA was separated from the precipitate by further centrifugation for 15 minutes at 800 g. DNA content was determined by removing 400 µl of the sample supernatant, and adding 1 ml 1.0 M PCA and 1 ml of diphenylamine reagent (1.5 gm diphenylamine in 100 ml glacial acetic acid and 1.5 ml concentrated sulphuric acid) containing 5 µl of a 16 mg/ml solution of acetaldehyde. Colour was allowed to develop overnight and the

adsorption measured at 610 and 650 nm using a spectrophotometer. DNA values were determined by subtraction of the two values and comparison with calf thymus DNA (Sigma) used as a standard.

Cultured cells were washed twice with 2 ml KRB and then incubated with 2 ml of a 1 mg/ml solution of trypsin for 15 minutes at 37°C. The loosened cell sheet was then scraped off the dish using a Teflon "policeman" and transferred to a conical centrifuge tube. The assay then proceeded as for freshly isolated cells.

Cell Type Specific Labeling. Cell cultures to be labeled with antisera to cell type specific markers were grown at a density of 2.5×10^4 cells/mm^2 on 12 mm diameter glass coverslips precoated with poly-L-lysine. Cultures growing on coverslips were washed before labeling by immersing them in three successive beakers containing 50 ml of Hanks salts solution of the following composition: 1.3 mM $CaCl_2$, 5.4 mM KCl, 4.4 mM KH_2PO_4, 4.9 mM $MgCl_2$, 4.1 mM $MgSO_4$, 110 mM NaCl, 4.2 mM $NaHCO_3$, 3.3 mM Na_2HPO_4, 5.6 mM glucose and additionally buffered with 25 ml N-2-hydroxyethylenepiperazine-N'-2 ethane sulphonic acid (HEPES). To this was added 5% (v/v) of normal lamb serum (NLS GIBCO Biocult). Antisera were diluted in MEM buffered with HEPES (GIBCO Biocult) containing 20% (v/v) NLS. By placing the coverslips on 10 mm diameter 'O' rings as little as 40 µl of antiserum could be used. All incubations were at room temperature. The details for each of the labels used are given below.

Cultures to be labeled with tetanus toxin were incubated with 40 µl of a 10 µg/ml solution of tetanus toxin (gift of Dr. R.O. Thompson, Wellcombe Labs.) in MEM-HEPES 20% NLS. After 20 minutes cultures were washed and incubated for a further 20 minutes with a 1:25 dilution of an antiserum against tetanus toxoid produced in a rabbit by the following method. 100 µg of tetanus toxoid (Wellcombe Labs.) emulsified in complete Freund's adjuvant was injected subcutaneously into each of

four sites along only one flank. One month later the injections were repeated along the other flank. After a further month 400 µg of toxoid was injected intravenously followed by a second boost two weeks later. Serum was collected two weeks after the final injection. Immunoglobulins which may react with neural tissue to yield false positives were absorbed by incubating aliquots of immune sera overnight at 4°C with an homogenate of 9 day old rat cerebellum.

After removal of the primary antiserum by washing, cultures were incubated for 20 minutes in a 1:40 dilution of an antiserum to rabbit gammaglobulin produced in goats and conjugated to rhodamine (GAM TRITC, Nordic Labs.). Cultures were then washed and fixed for 10 minutes in 0.2% glutaraldehyde in phosphate buffered saline (PBS) followed by rinsing in water and dehydration in 50% and 95% ethanol. Cultures were mounted in a 1:3 dilution of glycerol in PBS.

Cultures to be labeled with an antiserum to galactocerebroside were washed and incubated for 20 minutes with a 1:20 dilution of an antiserum produced in rabbits (gift of Dr. M. Raff). The labeling and fixation then proceeded as described for tetanus toxin.

GFAP is an internal protein and it was therefore necessary to fix the cultures in a solution of 5% acetic acid in 100% ethanol (v/v) for 5 minutes at -20°C to allow penetration of antisera. After washing thoroughly cultures were incubated for 20 minutes in a 1:40 dilution of anti-GFAP antiserum produced in a rabbit (gift of Dr. R. Pruss). Labelling then proceeded as described for tetanus toxin.

Labeling was visualised using a Leitz Laborlux 12 microscope equipped with a mercury vapour lamp and optics for viewing rhodamine fluorescence. Photographs were taken using a Leitz Vario-Orthomat camera system.

Cell Counts. Cell counts were performed with the aid of a counting grid fitted into the eyepiece of the microscope. Fields were selected by

manipulating the x and y axes of the micrometer stage while not looking down the eyepieces. The only occasions when a field so selected was not counted was if it was obvious that large areas of the cell sheet had been removed during the washing procedures.

Isolation of Culture of Cells from Postnatal Rat Brain Regions. Wistar (CHFB) rats maintained in colonies at the Open University were used at 5 days. Following the removal of the olfactory bulbs, hypothalamus, hippocampus and a small portion of the frontal lobe of the cerebral cortex, the preparation of a single cell suspension proceeded as described for the mouse cerebellum.

Cell cultures were prepared as for mice cerebellar cultures. The media was that of Currie et al. (1979) in which the concentrations of the standard components were as given earlier. To this was added 10% (v/v) foetal calf serum (FCS), 2% (v/v) chick embryo extract (CEE, GIBCO) and 80 μM 5-fluorodeoxyuridine (FUDR, Sigma) as a mitotic inhibitor. Plating efficiencies were measured as described previously.

2.3 RESULTS

MICE CEREBELLAR CULTURES

Cell Isolation. The cell yield/cerebellum and the percentage of cells found in the five size ranges are shown in Table 2.2. The average cell yield/cerebellum from a 5 day mouse was $8.31 \pm 1.23 \times 10^6$. The size ranges chosen for analysis were those originally used to tentatively classify cells isolated from the developing rat cerebellum (Cohen et al., 1978). The composition of these classes was determined by light and electron microscopy of freshly isolated cells is: A. Debris (nuclei and cell membranes). B. 70% external granule cells. C. 80% external granule cells. D. A mixture of large interneurons and glial cells. E. Up to 80% Purkinje cells.

CELL ISOLATION EXPERIMENT	NUMBER OF PUPS/ LITTER	CELL YIELD/ CEREBELLUM $\times 10^6$	NUMBER OF PARTICLES IN SIZE RANGE (% OF B+C+D+E)				
			A	B	C	D	E
1	11	11.1	4.4	26.6	46.7	22.8	1.3
2	16	9.6	4.0	28.6	43.8	23.3	1.3
3	9	8.0	3.2	22.2	42.8	29.8	1.9
4	13	11.0	4.4	22.5	42.9	30.5	1.4
5	10	8.3	3.0	20.6	46.8	27.8	2.3
6	12	11.3	3.5	23.9	45.6	24.3	1.5
7	7	7.0	3.5	29.5	43.2	24.6	1.7
8	6	8.7	2.8	22.8	48.0	24.6	0.9
MEAN	10.5	8.3	3.5	24.6	45.0	25.9	1.5
±S.E.M.	1.15	1.23	0.22	1.15	0.72	1.05	0.54

Table 2.2 Data Obtained from eight cell preparations from five day old mice cerebella.

Cell preparations from 5 day old mice cerebella contained less than 4% debris. Using cerebella from older animals resulted in this figure being as high as 8.1% for a 9 day old animal, but the use of animals younger than 5 days did not significantly lower the value. Five days appeared to be the optimum age for cell isolation, since it yielded the highest number of intact cells without a significant increase in cell debris. This age was therefore selected for all further studies.

The striking feature about the cell size distribution is the remarkable reproducibility from litter to litter. In the three size classes containing the vast majority of cells the SEM is less than 5%. The seemingly large variability in the E fraction is probably a reflection of the inaccuracy of the Coulter Counter at low cell densities rather than a real variation in the number of large cells.

Cell Culture. The effects of a number of variations in the composition of the culture medium for the culture of cells isolated from 5 day old mice cerebella were examined in order to determine the optimal conditions for their growth and survival. The effects of variation in cell density was also examined.

Cell Density. In general it appeared that for any given medium composition those cultures originally plated at the higher densities (between 1 and 1.5 x $10^5/mm^2$) survived longer and showed greater growth than those at lower densities (between 2.5 and 5.0 x $10^4/mm^2$). This effect was most apparent when the medium composition was obviously sub-optimal under which conditions cells at the lower densities showed little or no growth after 24 hours *in vitro*. Although increasing the cell density did increase cell viability in culture, this did not seem to be a satisfactory solution to the problem of finding the correct growth conditions since, at higher densities it became impossible to define individual cells. For this reason 2.5 x 10^4 cells/mm^2 was adopted as the standard density (a density at which similarly prepared

cells from rat cerebella survive well in culture) and a solution to the problem sought by varying the composition of the medium.

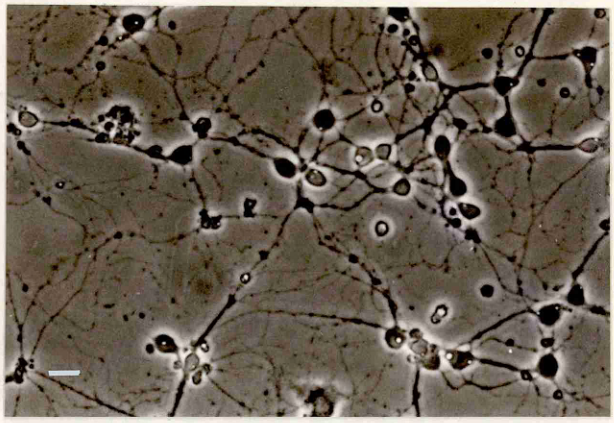
Serum Concentration. Serum concentrations of 10, 20 and 30% were tested. It was found that increasing the serum concentration could improve the viability of otherwise unhealthy cultures, but since the serum is the major source of variability in the medium I felt that other more controllable means of improving cell survival should be sought. For this reason a serum concentration of 10% was adopted as standard.

Serum Source. No obvious differences were apparent when cells were grown in medium supplemented with horse or foetal calf serum but since the latter is considerably more expensive and at times in short supply, horse serum was selected as the serum of choice.

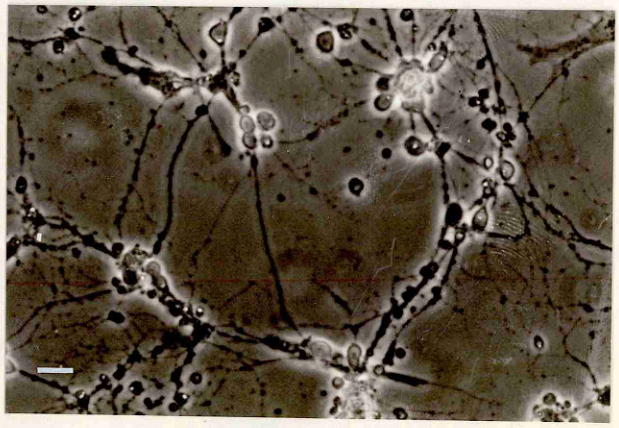
Chick Embryo Extract. Certain batches of extract appeared to be detrimental to cell survival and in no case did its addition enhance cell viability. For this reason and the fact that its inclusion in the medium introduces more uncontrolled variables, chick embryo extract was omitted from the medium.

Mitotic Inhibitors. Dramatic variations in the growth and survival of cell cultures were found when cytosine arabinoside (AraC) was added to the medium as a mitotic inhibitor instead of FudR. As early as 5 DIV (days in vitro), cultures with FudR included in the medium were underdeveloped compared to those grown in the presence of AraC. There were large numbers of dead and dying cells and the fibre bundles, which form between cell aggregates, were flimsy and thin. By 10 DIV few if any neurons were surviving in these cultures (Fig. 2.1). AraC was thus selected as the inhibitor for all future culture preparations, since it was not only less toxic to neurons than FudR but was also more effective at limiting overgrowth by non-neuronal cell types.

Fig.2.I Cell cultures produced from 5 day old rat cerebella. Cultures were grown under identical conditions to those shown in Fig.2.2 except FudR was used as the mitotic inhibitor in place of Ara C. Scale bars =20μm.



5 DIV



10 DIV

Drawing on the results of these experiments the culture medium adopted as standard for the growth of cell perikarya isolated from young mice cerebellar was: Minimal Essential Medium (Eagle Modification) containing 23 mM KCl, 180 μM gentamycin, 37 mM glucose, 10% (v/v) horse serum and 2×10^{-5} M AraC (added after 24 hours in culture). Under these conditions cell cultures can be routinely maintained for a period of three weeks during which time they show a characteristic pattern of development. The neurons develop from cell perikarya with small or non-existent projections through the stages of neurite elongation and eventually to a 'mature' stage where they form aggregates which are linked by large fibre bundles. These stages are shown in Fig. 2.2.

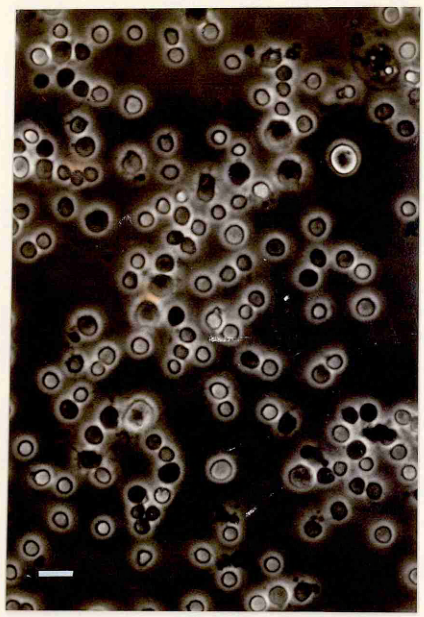
Plating Efficiency. Using a total DNA assay the plating efficiency of cells isolated in four cell preparations and grown under the conditions described above was measured. The results are shown in Table 2.3.

The largest number of cells are lost during the first 24 hours *in vitro*, at which time 63.4 ± 5.3% of the cells originally plated are firmly attached to the surface of the culture dish. After one week in culture this value had fallen to 45.6 ± 6.0% but all cultures appeared healthy and in no case was there a sudden unexplained death of cells.

Cell Type Specific Labelling. Cell cultures from four cell preparations were labeled at 1 and 7 DIV with antisera against tetanus toxin, galactocerebroside and glial fibrillary acidic protein, to identify neurons, oligodendrocytes and astrocytes respectively. A fourth set of cultures were stained with cresyl violet to aid the counting of total cell numbers. The results are shown in Table 2.4.

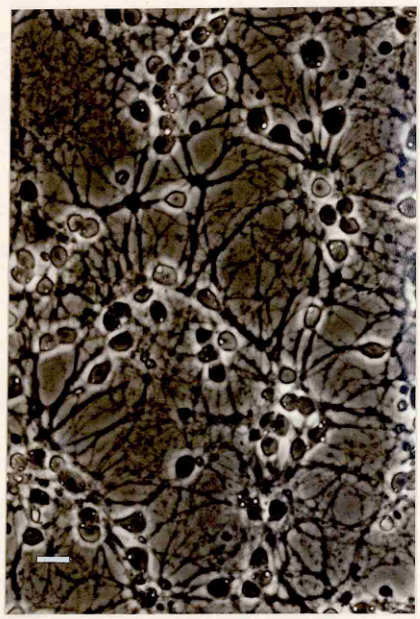
The total cell counts show considerable variability after 24 hours *in vitro* (1508 ± 211/mm^2) but by 7 DIV they are very similar

Fig.2.2 Cell cultures produced from 5 day old mice cerebella grown in Minimal Essential Medium (Eagle Modification)containing, 23mM KCL, I80μM gentamycin, I0% horse serum and $2xI0^{-5}M$ Ara C. (A) Freshly isolated cells. (B) IDIV. (C) 7DIV. (D) I4DIV. (E) 2IDIV. Scale bars = 20μm.



A

B



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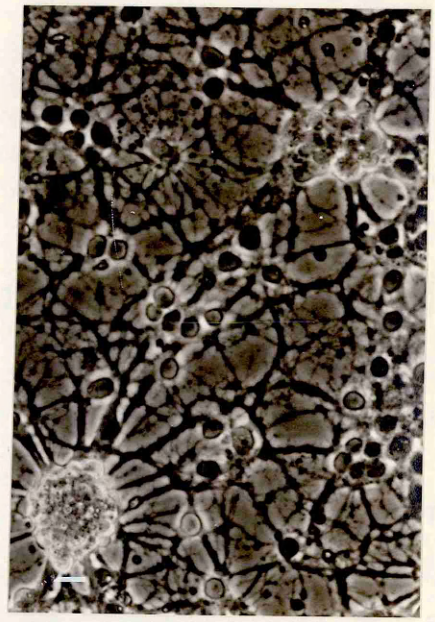
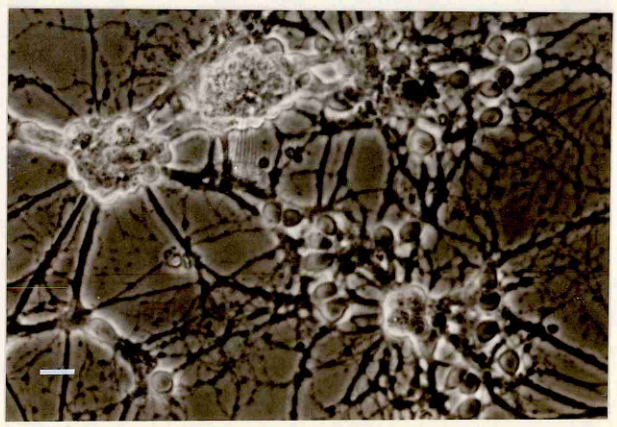


D



E

CULTURE PREPARATION		2	3	4	6
ug DNA/10^6 CELLS ORIGINALY PLATED	0 DIV	7.00 ± 0.06[6]	7.29 ± 0.05[4]	7.16 ± 0.08[5]	6.89 ± 0.09[6]
	1 DIV	4.79 ± 0.15[6]	4.19 ± 0.09[5]	4.35 ± 0.29[6]	5.07 ± 0.70[6]
	7 DIV	3.44 ± 0.09[6]	2.53 ± 0.14[5]	4.19 ± 0.24[6]	2.78 ± 0.40[6]
PLATING EFFICIENCY(%)	1 DIV	68.4	57.5	60.8	73.1
	7 DIV	49.1	34.7	58.5	40.3

Table 2.3. ug DNA recovered and the plating efficiencies, for cultures of 5 day old mice cerebellar cells from 4 isolation experiments. Numbers in parentheses are the number of cultures assayed.

CULTURE PREPARATION		2	3	4	6
CELL TOTAL/MM^2 ± S.E.M.	1DIV	1488 ± 82[10]	961 ± 96[10]	1593 ± 96[10]	1988 ± 73[10]
	7DIV	1167 + 48[10]	1005 + 39[10]	1354 + 50[10]	1334 + 61[10]
TETANUS TOXIN$^+$ CELLS/MM^2 ± S.E.M.	1DIV	1269 ± 92[10]	942 ± 47[10]	1322 ± 132[10]	1597 ± 49[10]
	7DIV	1097 + 52[10]	807 + 69[10]	——	1266 + 81[10]
%TETANUS TOXIN$^+$ CELLS	1DIV	85.3	98.0	83.0	79.9
	7DIV	92.3	80.0	——	94.9
GAL.C$^+$ CELLS/MM^2 ± S.E.M.	1DIV	8.5[20]	10.8[20]	7.8[20]	17.7[20]
	7DIV	1.0	1.0	1.0	1.0
%GAL.C.$^+$CELLS	1DIV	0.8	1.1	0.5	0.9
	7DIV	0.1	0.1	0.1	0.1
GFAP$^+$ CELLS/MM^2 ± S.E.M.	1DIV	94.6 ± 9.2[20]	45.4 ± 3.8[16]	81.5 ± 6.2[20]	69.6 ± 5.4[20]
	7DIV	78.5 ± 6.9[20]	93.1 ± 12.3[20]	63.8 ± 6.3[20]	65.8 ± 4.6[20]
%GFAP$^+$CELLS	1DIV	6.4	4.3	6.0	3.5
	7DIV	6.7	9.3	4.7	4.9

Table 2.4.Cell composition of 4 culture preparations from 5 day old mice cerebella,as determined by antibody binding to the cell-type specific labels,tetanus toxin,galactocerebrocide and glial fibrilary acidic protein.Numbers in parentheses are the number of fields counted.

(1215 ± 82/mm^2) suggesting there may be an optimal density, possibly regulated by some form of contact inhibition amongst the cells.

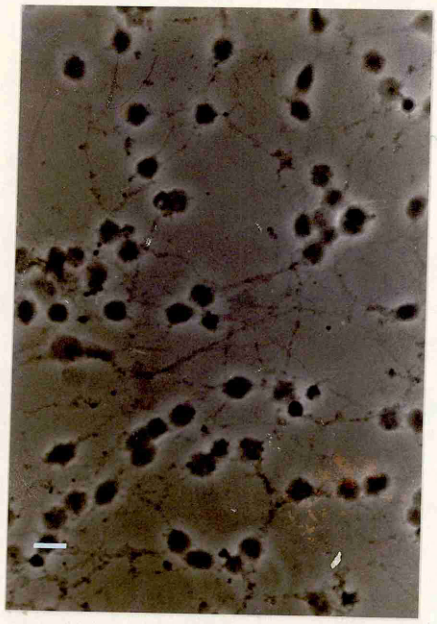
The majority of cells found in the cultures bound tetanus toxin and can therefore be identified as neurons (Mirsky et al., 1978; Raff et al., 1979). After 1 DIV the neurons were spread evenly over the coverslips and even at this early stage labeling revealed extensive neurite outgrowth as well as labeling on cell soma. By 7 DIV the neurons have aggregated into clusters which are linked by a fine network of neurites (Fig. 2.3). This sometimes made positive identification of some cells impossible, since it could not be determined whether the apparent labeling was due to a labeled neuron or a neurite growing around a non-neuronal cell. However using tetanus toxin in conjunction with morphological features, the number of errors due to this effect are probably extremely small.

The survival of neurons in these cultures was very high after 7 DIV (89 ± 5%), the overall percentage actually increasing slightly from day one - presumably a reflection of the death of non-neuronal cell types.

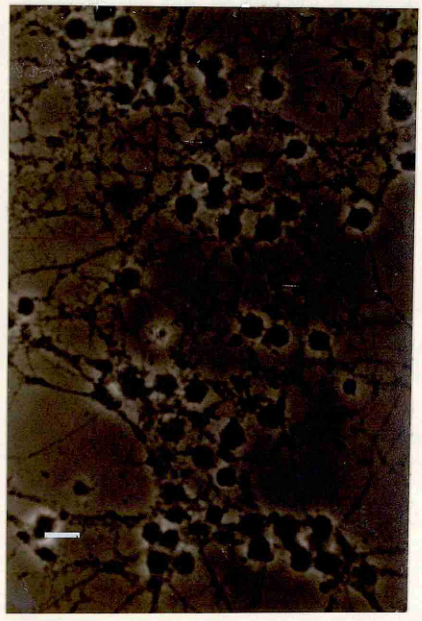
Astrocytes were identified in culture using an antiserum against GFAP. After 1 DIV GFAP positive cells constituted 5.1 ± 0.7% of the cells present and by 7 DIV this had only risen to 6.4 ± 1.1%. The vast majority of labelled cells were process bearing astrocytes with a small number of fibroblastic cells (Fig. 2.4).

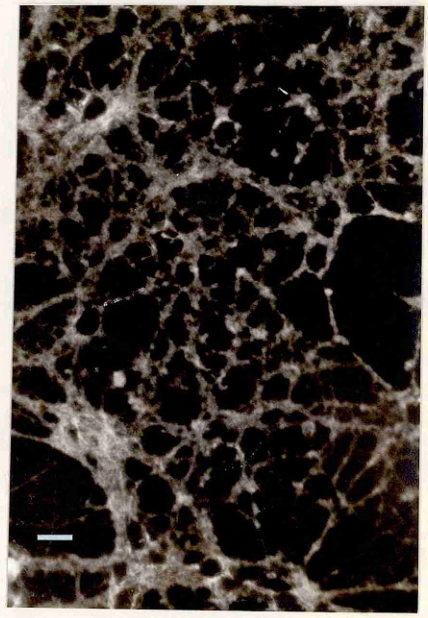
Oligodendrocytes were labeled with an antiserum against Gal. C. and constituted 0.8 ± 0.1% of the cells present. After only 1 DIV these cells showed extensive divergent processes (Fig. 2.5) but by 7 DIV the only Gal. C. positive cells were extremely necrotic and constituted less than 0.1% of the total cells. Since AraC was not added until 24 hours in culture, it seemed likely that as well as preventing oligodendrocyte proliferation, it was also preventing oligodendrocyte survival. Experiments in which AraC was added at 1 DIV and the medium

Fig.2.3 Tetanus toxin labelling of neurons. Cells in cultures produced from 5 day old mice cerebella, were labelled with tetanus toxin followed by a rabbit anti-tetanus toxin and then by goat anti-rabbit IGg conjugated to rhodamine. Photographs on the left show the image when viewed with phase contrast optics, those on the right when viewed with fluorescence optics. Scale bars =20μm.



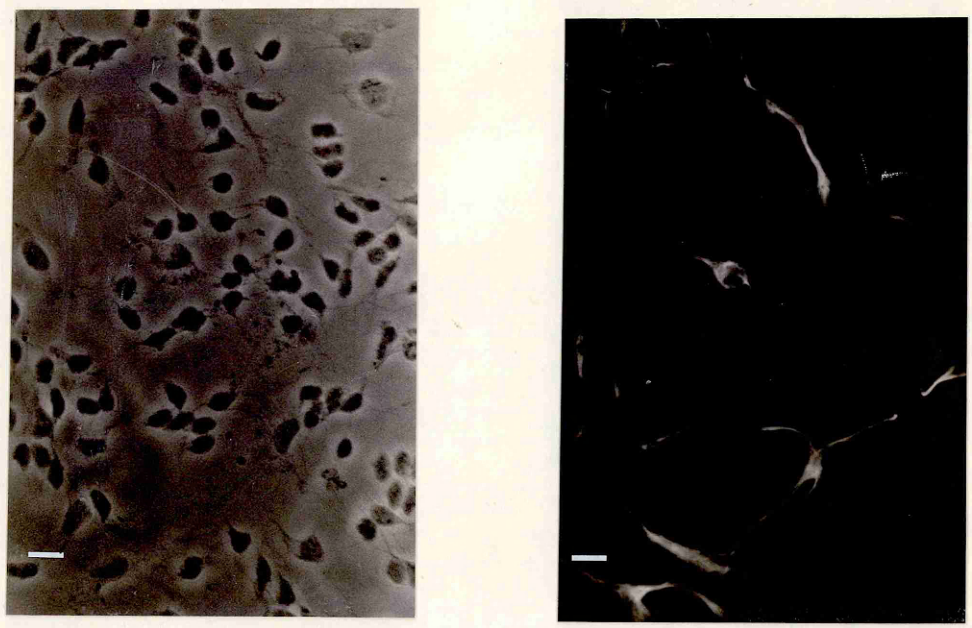
1DIV



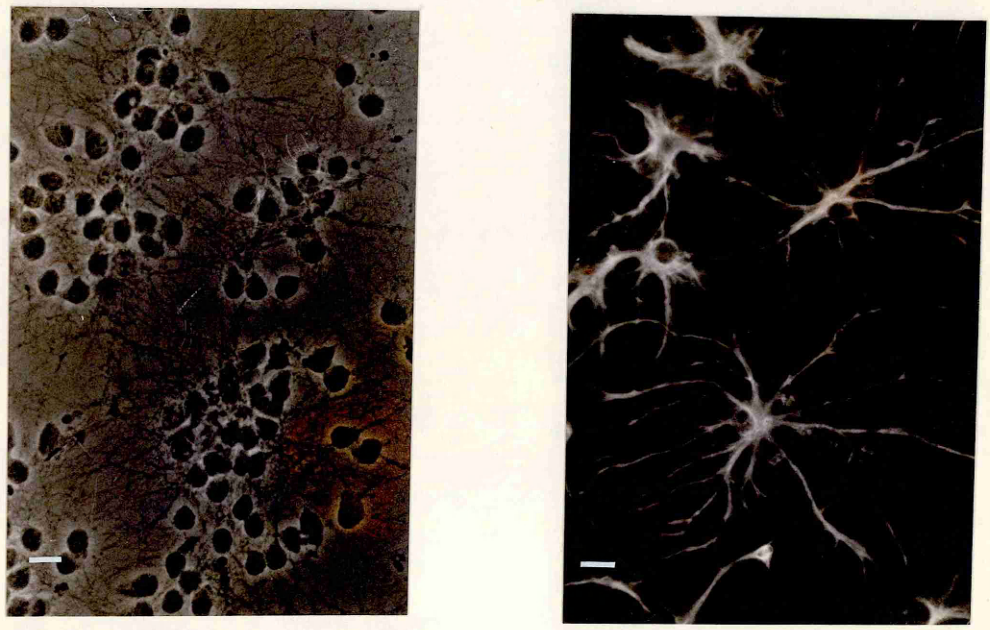


7DIV

Fig.2.4 GFAP labelling of astrocytes. Cells in cultures produced from 5 day old mice cerebella were labelled with rabbit anti-GFAP followed by goat anti-rabbit IgG conjugated to rhodamine. Photographs on the left show the image when viewed with phase contrast optics, those on the right when viewed with fluorescence optics. Scale bars = 20μm.

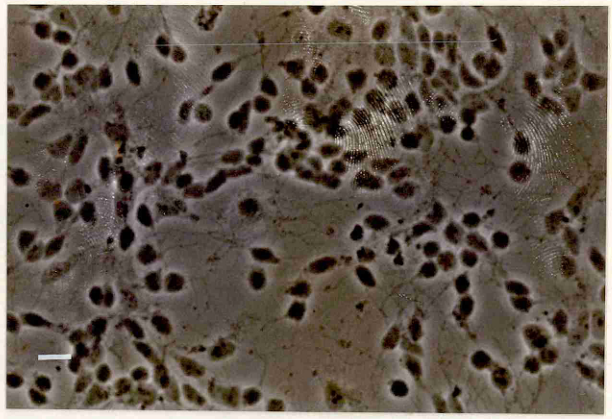


1DIV

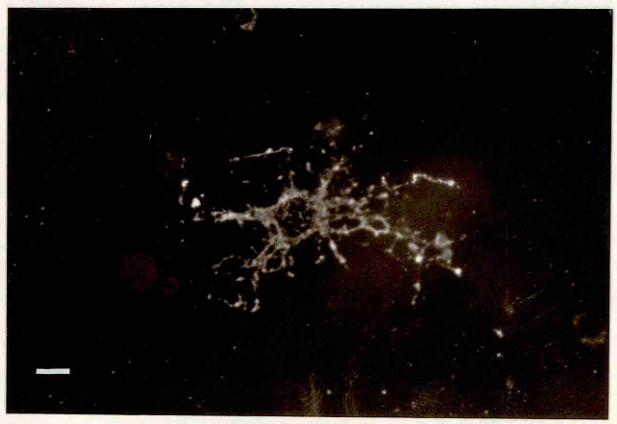


7DIV

Fig.2.5 Gal.C. labelling of oligodendrocytes. Cells in cultures produced from 5 day old mice cerebella, were labelled after IDIV with a rabbit antiserum against Gal.C.followed by goat anti-mouse IgG conjugated to rhodamine, and viewed with (A) phase contrast and (B) fluorescence optics. Scale bars = 20μm.



A



B

changed at 4 DIV, to be replaced with medium without AraC resulted in the survival of oligodendrocytes in numbers similar to those found after 1 DIV.

RAT BRAIN REGION CULTURES

Cell Isolation. The percentages of isolated rat cerebellar cell perikarya in the five size classes defined previously are shown in Table 2.5.

Plating Efficiency. The results of total DNA assays to determine the plating efficiencies of cells isolated from the five brain regions are shown in Table 2.6. While no studies of the cell type composition of these cultures has been made the plating efficiencies obtained demonstrate the success of the isolation and culture techniques as applied to regions other than the cerebellum. Cultures of the brain regions after 7 DIV are shown in Figure 2.6.

CELL ISOLATION EXPERIMENT	NUMBER OF PARTICLES IN SIZE RANGE (% OF B+ C+D+E)				
	A	B	C	D	E
1	5.4	27.7	43.3	31.8	1.4
2	4.8	28.2	41.5	30.7	0.8
3	4.5	32.4	42.8	26.3	1.2
4	4.6	25.6	42.7	25.2	1.4
5	7.9	29.4	40.4	27.7	1.4
6	4.0	30.4	43.2	32.8	1.4
MEAN	5.2	28.9	42.3	29.1	1.2
± S.E.M.	0.4	1.0	0.5	1.2	0.1

Table 2.5. Cell size distribution data obtained from five day old rat cerebella.

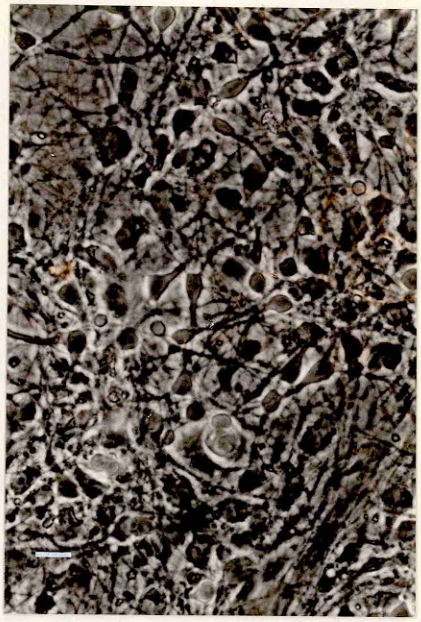
BRAIN REGION		CEREBELLUM	OLFACTORY BULB	HIPPOCAMPUS	HYPOTHALAMUS	CEREBRAL CORTEX
PLATING	1DIV	89.3 ± 0.5[3]	123.6 ± 13.8[3]	62.3 ± 2.9[3]	63.48 ±10.8[4]	48.7 ± 4.0[3]
EFFICIENCY(%)	7DIV	34.1 ± 1.8[3]	48.5 ± 4.0[3]	22.3 ± 2.8[3]	56.4 ± 9.9[3]	39.5 ± 2.7[3]

Table 2.6 Plating efficiences of cultures derived from 5 brain regions of 5 day old rats.

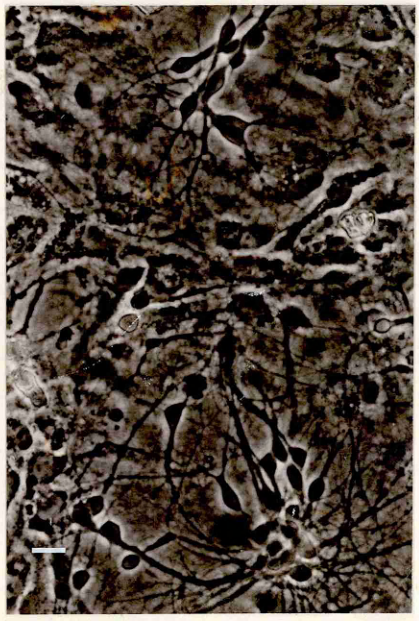
Numbers in parentheses are the number of experiments.

Fig. 2.6 Cell cultures produced from four regions of 5 day old rat brain after 7DIV. (A) Olfactory bulb. (B) Cerebral cortex. (C) Hypothalamus. (D) Hippocampus. Scale bar = 20μm.

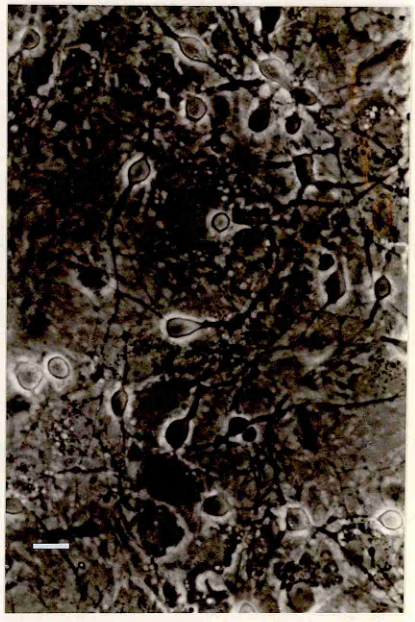
A



B



C



D

2.4 DISCUSSION

Cell Isolation. The cell isolation procedure used in these studies was as described by Dutton et al. (1981). This method was originally developed for the isolation of cell perikarya from young rat cerebella but a comparison of Table 2.3 and Table 2.5 indicates, that when applied to young mice cerebella, the method yields a cell suspension which is directly comparable in terms of cell size distribution. The similarity found between the two sets of data is particularly reassuring as to the reproducibility of the technique, since they were obtained from cell preparations in different laboratories.

Cell Culture. A number of modifications to the medium originally developed for the culture of cell perikarya from rat cerebella (Currie et al., 1979) has led to the successful long term culture of similarly isolated cells from young mice.

The modification which produced the most dramatic improvement in cell viability was a switch from the use of FudR as the mitotic inhibitor to AraC (a mitotic inhibitor is necessary to prevent over-growth of neurons - which no longer divide in culture - by non-neuronal cell types). Messer (1977) had noted that AraC appeared to be less toxic and more effective at inhibiting glial growth than FudR, but found that unless added as late as 6 or 7 days in culture, large numbers of cells, or even the entire culture, would die. Under the growth conditions described here no detrimental effects were noticed when AraC was added after 24 hours in vitro thus keeping glial proliferation to a minimum.

Why FudR is selectively toxic for mouse cerebella cells, but not rat cells, is unknown. Its mode of action as a mitotic inhibitor is via inhibition of thymidilate synthetase (Hiedelberger, 1965) but it seems likely that it may have other anti-metabolite actions to which mouse cells are more sensitive.

Few studies have been published on the possible anti-metabolite effects of AraC. At concentrations between 5 and 10^{-4} M, AraC has been shown to inhibit both RNA synthesis in chick neural retina cells (Jones and Moscona, 1974) and the incorporation of N-acetyl neuraminic acid into glycoproteins and glycolipids of hamster embryo cells (Hawtry et al., 1973). Whilst the concentrations used in these studies were 25-50 times that used in the culture medium the latter report could prove relevant considering the possible role of glycoproteins and glycolipids in cell-cell recognition phenomena. For this reason AraC was added after 24 hours in vitro and the medium changed after 4 DIV to be replaced with AraC free medium. This had no apparent effect on either the overall morphology of the cultures or on glial proliferation. An additional advantage was that oligodendrocyte survival was also ensured (see Results).

The use of horse serum, instead of foetal calf serum, as a medium supplement was suggested by Messer (1977) but she found that cells initially plated in horse serum failed to attach to the culture wells and showed little process outgrowth. In order to overcome this problem a complicated feeding schedule was adopted whereby cells were gradually "weaned off" foetal calf serum and onto horse serum. With the culture conditions developed in this laboratory this problem was not apparent - almost certainly due to the use of a poly-L-lysine coated substrate to promote cell adhesion (Yavin and Yavin, 1974; Currie et al., 1979).

Plating Efficiency. The plating efficiencies of cells derived from young mice cerebella compare satisfactorily with the values obtained for rat cerebella cultures prepared using well established techniques. The percentages of mice cerebella cells originally plated which were firmly attached to the surface of the culture dish were 63.4% and 45.6% after 1 and 7 DIV respectively. The corresponding values for rat

cerebellar cultures were 89.3% and 34.1%. It would thus seem that although the initial attachment of mice cerebella cells is lower than for rat cell perikarya, subsequent survival is improved.

The DNA values obtained for freshly isolated cells yield a DNA content per cell of $7.08 \pm 0.09 \times 10^{-12}$ g for mouse cells and $6.77 \pm 0.12 \times 10^{-12}$ g for rat cerebella cells. These values fall between the 7.6×10^{-12} g obtained from a purified preparation of mouse cerebella Purkinje cells (Cohen et al., 1973) and the 6.28×10^{-12} g (Zagon and McLaughlin, 1979) and 6.73×10^{-12} g (McEwen et al., 1972) for isolated rat cerebella nuclei.

Culture Composition. The predominant cell type present in the cultures was the granule neuron. These were identified by their presence in large numbers, small size and ability to bind tetanus toxin. The granule neuron was also the most numerous cell type present in the culture systems described by Messer (1979) and Trenkner and Sidman (1977) as shown by morphological characteristics at both the light and electron microscopic level.

In the studies to determine the reproducibility of the culture system only neurons, astrocytes and oligodendrocytes were distinguished by cell type specific labeling, since similar studies with rat cerebellar cultures had shown these to be the three major cell classes present. Also present in rat cerebella cultures in small numbers are fibroblasts [1.8% at 6 DIV (Beale, 1980)]. It seems probable that these too are present in mice cerebellar cultures and account for any discrepancies in the total percentages of cells counted.

CHAPTER 3

AN ENZYME IMMUNOASSAY FOR THE DETECTION OF ANTIBODIES AGAINST CELL SURFACE ANTIGENS OF CEREBELLAR CELLS IN CULTURE

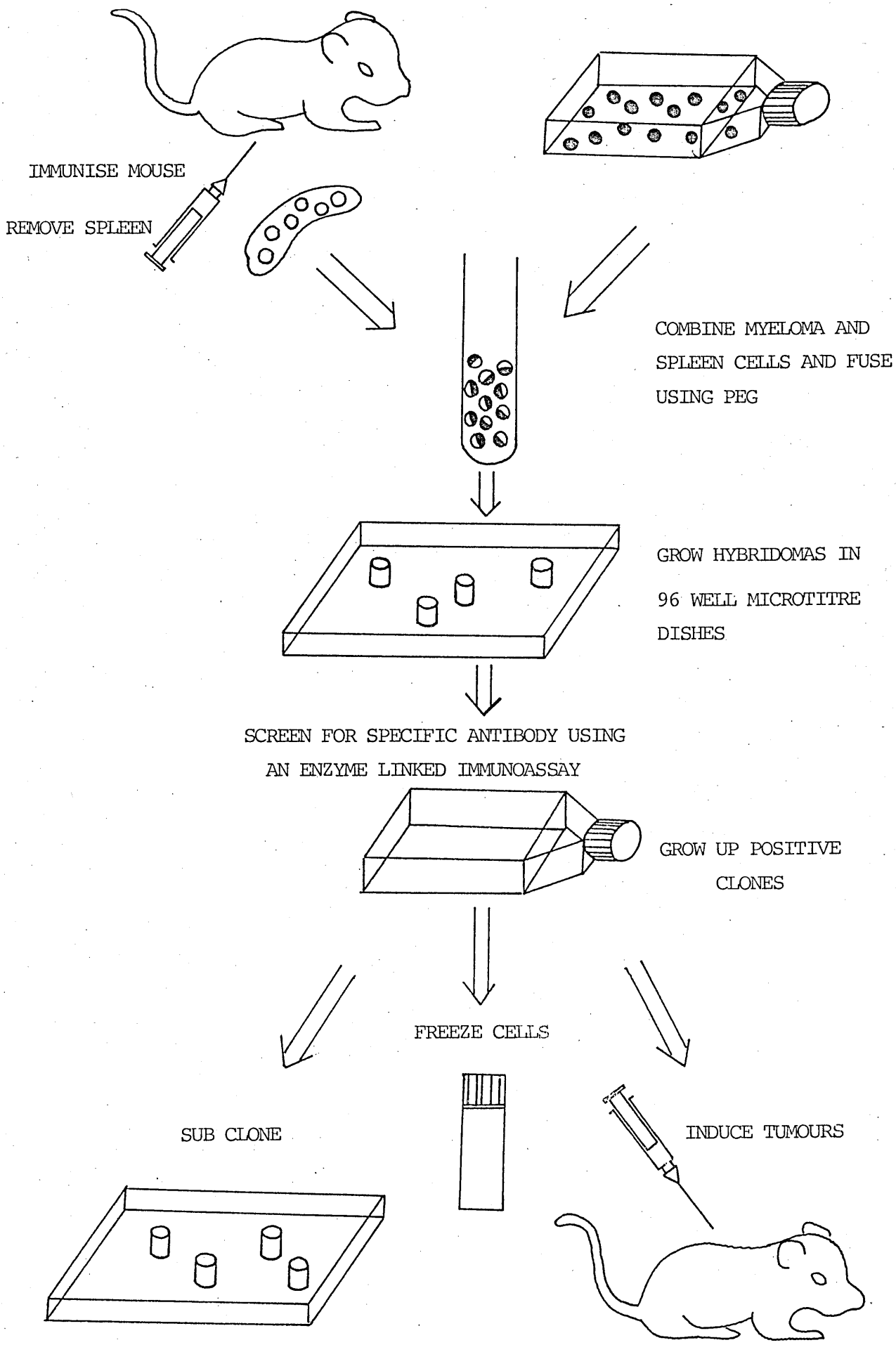
3.1 INTRODUCTION

Since the first monoclonal antibodies were produced (Kohler and Milstein, 1975), the methodology associated with their production has developed rapidly and is continuing to do so. The basic principles have however remained the same and are illustrated schematically in Fig. 3.1. The first stage involves the fusion of cells isolated from the spleen of an immunised mouse (or rat) with myeloma cells to produce hybrids. Selection for these "hybridomas" is achieved by culturing the cells in a suitable medium such that only the spleen cell-myeloma cell fusions survive and then, using a suitable assay system, the supernatants from the hybridomas are screened for antibody of the desired specificity.

The development of the assay system is one of the major methodological problems associated with the production of monoclonal antibodies. Because each fusion may require the initial screening of between 100 and 300 supernatants and this number may increase exponentially as antibody producing hybridomas are subcloned, the assay selected should be simple, inexpensive and relatively fast. There are three basic alternatives which are currently being employed to detect antibodies against cell surface antigens - radioimmunoassays, immunofluorescence assays and enzyme immunoassays.

Radioimmunoassay is currently the most widely used technique. It is well suited to large scale operation, since it can be readily automated and is extremely sensitive (Skelly et al., 1973). The disadvantages are that the detection of bound radioactivity requires sophisticated equipment and that radio-labelled ligands are expensive, have a short half-life and require special handling procedures. The

Fig.3.I A schematic outline of the stages involved in the production of a monoclonal antibody.


IMMUNISE MOUSE
REMOVE SPLEEN
COMBINE MYELOMA AND SPLEEN CELLS AND FUSE USING PEG
GROW HYBRIDOMAS IN 96 WELL MICROTITRE DISHES
SCREEN FOR SPECIFIC ANTIBODY USING AN ENZYME LINKED IMMUNOASSAY
GROW UP POSITIVE CLONES
FREEZE CELLS
SUB CLONE
INDUCE TUMOURS

technique has nonetheless been used by a large number of workers to detect monoclonal antibodies (Eisenbarth et al., 1980; Scheinder and Eisenbarth, 1980).

Immunofluorescence assays have also been successfully used to detect monoclonal antibodies (Zipser and McKay, 1981; Lagenaur et al., 1980) but they are not suited to large scale operations and are therefore tedious and time consuming.

Enzyme immunoassays are a relatively new addition to the methods available for the detection and quantification of antigen and antibody (Van Weemen and Schuurs, 1971). The technique as applied to the detection of antibodies against cell surface antigens, is basically an adaptation of a solid phase radioimmunoassay, and is dependent on the ability of an enzyme to act as a label by catalysing a reaction, the products of which can be detected and quantified. Target cells are first incubated with the primary antibody , as may be present in a hybridoma supernatant, and unbound antibody removed by washing. The enzyme is then added in a form in which it is conjugated to an antibody specific for the immunoglobulin of the species in which the primary antibody was produced. The amount of enzyme bound is quantified by the addition of a suitable substrate. The substrate is normally selected so that the reaction yields a product which can be measured colourimetrically or fluorimetrically, although thermal measurements have also been used (Mattiason, 1978).

Enzyme immunoassays can be at least as sensitive as radioimmunoassays (Douillard et al., 1980; Suter et al., 1980) and in one instance a "High Sensitivity" enzyme linked immunosorbent assay was used to detect antigen present in a concentration of 3-10 attog/ml (Shaleu et al., 1980).

It thus seemed that an enzyme immunoassay would be sensitive enough to detect antibody present in hybridoma supernatants. As well as having the required level of sensitivity the reagents used are

stable for long periods of time, are relatively inexpensive and in most, but not all cases, their use requires no special handling or precautions. Enzyme immunoassays can also be readily automated and if the enzyme-substrate reaction produces a product which can be measured colourimetrically, no expensive detection equipment is necessary.

For these reasons a decision was made to develop an enzyme immunoassay suitable for screening for monoclonal antibodies against antigens present on the surfaces of cells in primary monolayer cultures and establish the optimum conditions for detecting such binding.

The development of the assay was performed using the following antisera as a source of primary antibody: a monoclonal antibody produced against rat brain Thy 1·1 (MRC OX, Gift of Dr. A.F. Williams), a commercially available monoclonal antibody against rat T-cells, W3/13 (Sera Labs), which served as a control, and a conventional polyclonal antiserum produced in this laboratory which reacted with glial cell surfaces.

Thy 1·1 was originally detected on the surface of mouse thymus derived lymphocytes, but was also shown to be present in small amounts in neonatal mouse brain (Reif and Allen, 1964). It has been shown to bind to the cell surface of fibroblasts in cerebella cultures and to a small (<10%) population of neurons (Currie et al., 1977; Raff et al., 1979).

W3/13 was originally described by Williams et al., (1977) and is a monoclonal antibody directed against a sub-population of rat lymphocytes. It has also been shown to bind to whole brain homogenate but, unlike Thy 1·1 no activity has been shown against rat cerebella cultures (Dr. G.R. Dutton, personal communication).

The antiserum produced against mouse glial cells was shown to be directed against cell surface determinants using indirect immunofluorescence as described previously (2.2).

3.2 METHODS

Production of Anti-glial Cell Antiserum. Cell cultures were prepared from the cerebella of 5 day old mice as previously described (2.2) except AraC was omitted from the medium and the potassium concentration lowered to 5.1 mM. Under these conditions survival of non-neuronal cell types is selected for and by 14 DIV few neurons remain (<5%) as shown by tetanus toxin labeling. Cultures were grown on 60 mm diameter plastic dishes (Falcon) coated with poly-L-lysine and seeded at a density of 2×10^5 cells/mm^2. After 14 DIV cultures were washed 3x in KRB and the cells removed by scraping with a Teflon "policeman." Following centrifugation at 100 g for 5 minutes the cell pellet was resuspended in 200 µl of KRB and 100 µl injected intraperitoneally into a BALB/C mouse (Jackson Labs.). The same dose via the same route was administered 3 weeks later and the mouse sacrificed 3 days post injection. Serum was obtained by cardiac puncture.

Immunisations With Rat Cerebellar Cells: Suspensions of cells from 5 day old rat cerebella were prepared as described previously (2.5). Cells were then suspended at a density of 10^6/ml in 10 ml of medium in a sterile plastic conical tube and incubated overnight. This incubation was in the hope that the cell surface molecules cleaved off by the action of trypsin during the isolation procedure would be regenerated. The next day the cell suspension was centrifuged at 100 g for 5 minutes and the pellet washed twice in KRB. Following the second wash the cell pellet was resuspended in 200 µl of KRB and the suspension injected intraperitoneally into an adult BALB/C mouse (Jackson Labs.). The same dose via the same route was administered 3 weeks later and the mouse sacrificed 3 days later and the spleen removed for fusion.

Enzyme Immunoassay. Cell cultures to be used as targets for antibody were grown at a density of between 1 and 2 x 10^5 cells/well in 96 well flat bottomed microtitre dishes (Linbro) precoated with poly-L-lysine. Media and growth conditions for mouse and rat cerebella cultures were as previously described (2.2).

At the time cultures were to be used for an assay the plate containing the cultures was washed in phosphate buffered saline (PBS-Me) of the following composition: 137 mM NaCl, 8.2 mM Na_2HPO_4, 7 H_2O, 1.5 mM KH_2PO_4, 15 mM KCl, 1.5 mM $MgCl_2$, 3.0 mM NaN_3, 2.0 mM 2-mercaptoethanol. Each well was washed 4 times with 200 µl of PBS-Me using an 8 channel multipipette (Flow Labs) and the cells were then either fixed in PBS-Me containing 0.25% glutaraldehyde or left unfixed. Fixed cells were washed 4 times in PBS-Me before incubation for 1 hour or overnight in a solution of PBS-Me containing 1% (w/v) BSA and 100 mM glycine. Unfixed cells were incubated in PBS-Me containing 1% BSA. The addition of the glycine was a precaution taken to block any unreacted sites present on the glutaraldehyde which may not have been removed by the washing procedure. BSA served to block non-specific binding of the antisera to the wells.

Antisera were diluted in PBS-Me and after removal of the blocking buffer, 50 µl was incubated with the target cells for 2 hours at room temperature. After a further 4 washes, 50 µl of the enzyme-anti-immunoglobulin conjugate, diluted 1:200 in PBS-Me was added to each well and incubated for 2 hours at room temperature. The enzyme-anti-immunoglobulin conjugate used was β-galactosidase (E. coli) coupled to $F(ab')_2$ fragments of sheep anti-mouse IgG, specific for both heavy and light chains (Bethesda Research Labs.). A final series of washes was followed by the addition of 50 µl of substrate - in this case 1 mg/ml of para-nitrophenyl galactoside (PNPG) diluted in a 50 mM phosphate buffer containing: 137 mM NaCl, 30 mM Na_2PO_4, 7 H_2O 20 mM KH_2PO_4, 15 mM KCl, 1.5 mM $MgCl_2$, 3.0 mM NaN_3, 2.0 mM 2-mercaptoethanol.

After 1 hour at room temperature a yellow colour developed in those wells positive for antibody and at this point the reaction was quenched by addition of 50 µl of 0.5 M Na_2CO_3 to each well. The optical density of the reaction mixture was measured by reading the absorbance at 410 nm on a microtitre plate reader (Dynatech Corp.). Readings were blanked on wells containing cells incubated without the primary antibody or the enzyme anti-immunoglobulin conjugate. Background values were obtained from wells incubated without primary antiserum, or with a pre-immune serum.

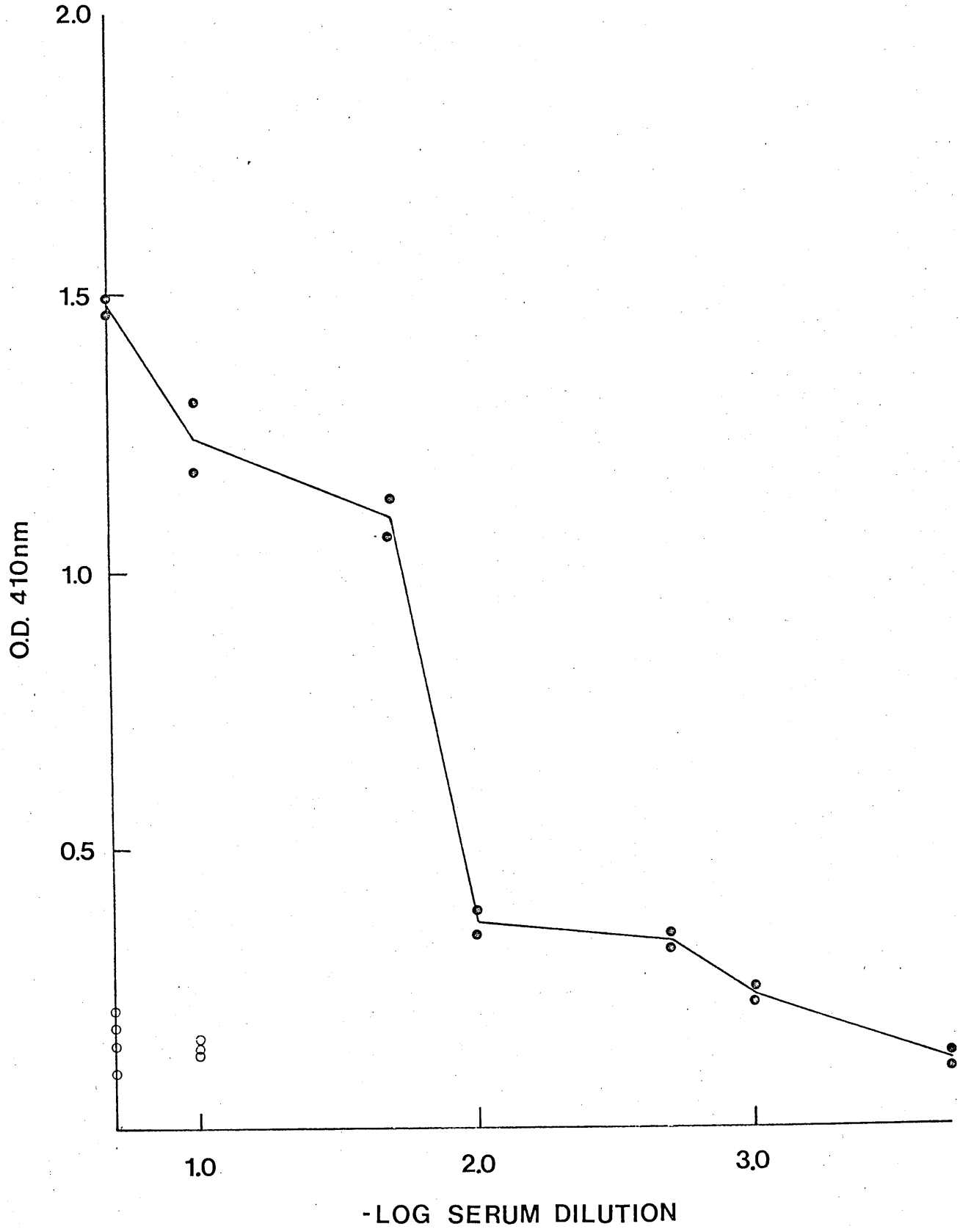
3.3 RESULTS

Effects of Cell Density. Before the enzyme immunoassay could be used to detect those wells positive for antibody, it was essential to establish whether a false reading would be obtained simply due to local changes in cell density at the point in the well through which the absorbtion was measured. In all the microtitre dishes containing cultures which were tested, the variability between wells did not exceed $\pm$ 0.01 absorbance units. This was the case even in wells where large clumps of cells were evident and this has therefore been discounted as a possible source of error.

Assays Using a Mouse Anti-glial Cell Antibody. An immunoassay was developed which was capable of detecting a conventionally produced antiserum against mouse glial cultures. This not only demonstrated the success of the short immunisation schedule in producing an immune response, but also the feasibility of using the approach to detect monoclonal antibodies directed against cell surface antigens of mice cerebella cells in culture.

The results of an assay using this antiserum are shown in Fig. 3.2. Specific antibody binding can be readily detected at a dilution of 1/1000 and is still just above background levels at 1/5000. The

Fig.3.2.Enzme immunoassay showing binding of an antiserum against glial cells, to cerebellar cells in culture. Cells from 7 day old mice were plated at a density of $1x10^5$/microtitre well and fixed after 10 DIV. Open circles show binding of the pre-immune sera.


2.0
1.5
1.0
0.5
O.D. 410nm
1.0
2.0
3.0
-LOG SERUM DILUTION

absorbance values for binding of the preimmune sera were 0.16 ± 0.02 (n=4) at a dilution of 1/5 and 0.14 ± 0.01 (n=4) at a dilution of 1/10. The absorbance measured when the primary antibody was omitted was 0.07 ± 0.01 (n=4).

The results of an assay to determine the effects of fixation are shown in Fig. 3.3. As in the previous assay specific antibody binding can be detected at a dilution of 1/1000 for both fixed and unfixed cells. It would thus seem that fixation does not disrupt antibody binding as measured by the assay. It does however increase what is apparently non-specific binding with the average absorbance values obtained with the control antibody W3/13 being 0.31 ± 0.02 (n=8) and 0.15 ± 0.01 (n=8) for fixed and unfixed cells. At the time these assays were performed glycine was not added to the blocking buffer to bind to any exposed glutaraldehyde sites still present and this probably accounts for much of the difference observed.

Despite the higher non-specific binding of antibody, fixation was chosen as the preferred method since it did not reduce the sensitivity of the assay and produced less variable results. The large variability between the duplicates of unfixed cells is almost certainly due to portions of the cell sheet being removed during the washing procedures. Fixation with glutaraldehyde greatly reduced this possibility.

Assays Using A Monoclonal Antibody. Having demonstrated that an enzyme immunoassay could be used to detect a polyclonal antiserum it was important to determine how sensitive it would be in detecting a monoclonal antibody - in this case an antibody against rat brain Thy 1·1.

The results of two assays using this antibody are shown in Fig. 3.4. Both assays were performed on cultures from rat cerebella prepared at the same time, the only difference being that the results shown in Fig. 3.4A were from cultures which had been stored in the blocking buffer for 10 days before being used in the assay. In both

Fig.3.3. Enzyme immunoassay showing binding of an antiserum against glial cells to cerebellar cells in culture. Cells from 7 day old mice were plated at a density of $2x10^5$/microtitre well and used in the assay after 10 DIV.

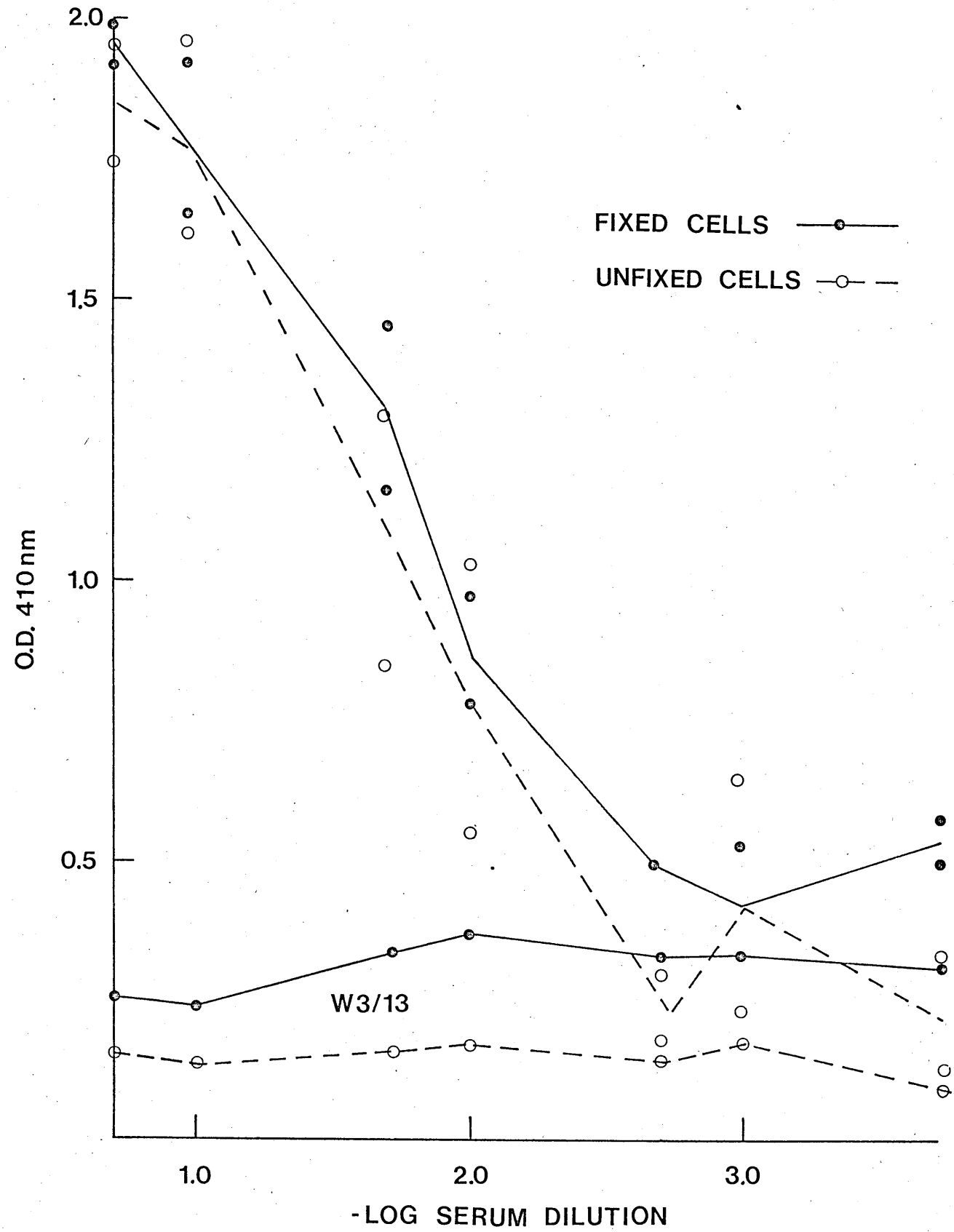
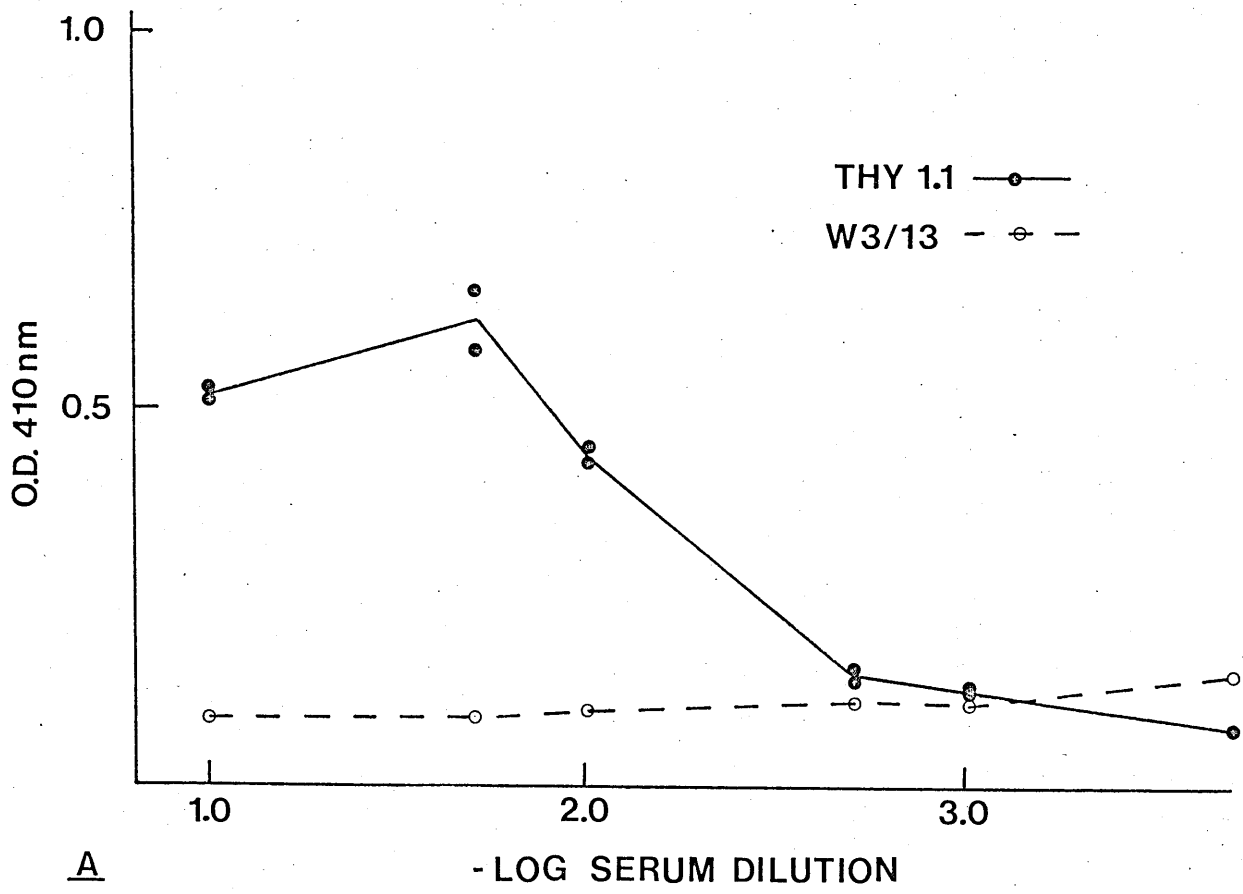
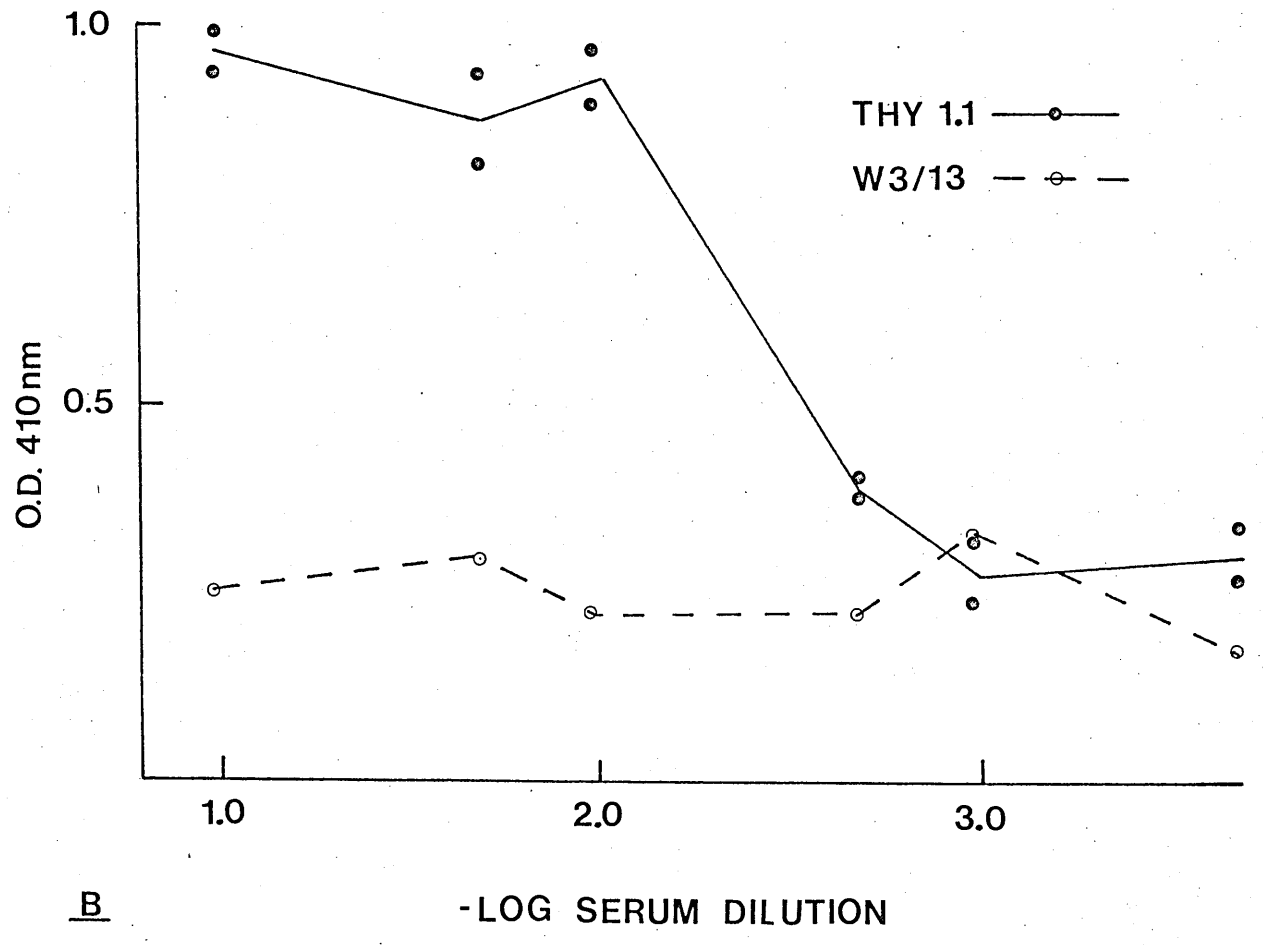

2.0
1.5
1.0
0.5
O.D. 410nm
FIXED CELLS
UNFIXED CELLS
W3/13
1.0
2.0
3.0
-LOG SERUM DILUTION

Fig.3.4. Enzyme immunoassays showing binding of a monoclonal antibody against Thy 1.1 and of W3/13, to cerebellar cells in culture. Cells from 4 day old rats were plated at a density of $2x10^5$/microtitre well and fixed after 10 DIV.

A. Binding when cultures were incubated in the blocking buffer for 10 days before the assay.

B. Binding when cultures were incubated in the blocking buffer for 1 hour.


1.0
0.5
O.D. 410nm
THY 1.1
W3/13
1.0
2.0
3.0
A
-LOG SERUM DILUTION


1.0
0.5
O.D. 410nm
THY 1.1
W3/13
1.0
2.0
3.0
B
-LOG SERUM DILUTION

cases specific binding was detectable at dilutions of 1/500. The difference between the two is in the apparent levels of non-specific binding. Cultures which had been incubated in the blocking buffer for only 1 hour and were not incubated with immune sera showed absorbance values of 0.22 ± 0.01 (n=23) whereas those cultures left in the blocking buffer for 10 days had corresponding values of 0.09 ± 0.007 (n=20). Because of this reduction in the level of non-specific binding cultures are now routinely incubated for at least 24 hours in the blocking buffer.

3.4 DISCUSSION

An enzyme immunoassay has been developed for the detection of a conventionally produced antibody and a monoclonal antibody, both directed against antigens present on the surface of cultured cerebella cells. However before the experimental work could begin two important decisions concerning the methodological approach had to be made - which enzyme anti-immunoglobulin conjugate to use, and what should be the source of target antigen.

At least eight commercially available enzyme labels have been successfully used in enzyme-immunoassays (see O'Sullivan et al., 1979). Of these only three are available linked to an anti-immunoglobulin - alkaline phosphatase, horseradish peroxidase (HRP) and β-galactosidase. The first enzyme immunoassays were performed using alkaline phosphatase (Voller and Bidwell, 1975) but were found to be lacking in sensitivity. The search for enzymes which would enhance the sensitivity of the technique led to the development of HRP conjugated anti-immunoglobulins and they are currently the most widely employed labels. They are not however without disadvantages. HRP activity is present in a wide variety of tissues which can lead to difficulties in interpreting results and many of its substrates are potentially carcinogenic (Sellakuma et al., 1969). Since one of the reasons for opting for an

enzyme immunoassay in preference to a radioimmunoassay, was to avoid some of the potential hazards involved, a safer alternative was sought.

β-Galactosidase is rarely present in serum or cells in detectable quantities (O'Sullivan et al., 1979) and its activity can be measured precisely and rapidly with a variety of non-hazardous substrates (Craven et al., 1965). It is commercially available coupled to $F(ab')_2$ fragments of sheep anti-mouse IgG (Bethesda Research Labs.). The use of $F(ab')_2$ fragments is probably responsible for a reduction in non-specific binding to the target cells via the Fc portion of the immunoglobulin molecule. At least one group of workers has however shown that the use of whole IgG will still produce the necessary level of specificity to detect monoclonal antibodies present in supernatants (Douillard et al., 1980).

While both freshly isolated cells and cell membranes have been successfully used as targets for antibodies against cell surface antigens (Stocker and Heusser, 1979; Howard et al., 1980) cell cultures were selected as the most appropriate targets for these studies for a number of reasons which are listed below.

i) The procedures for the isolation and culture of cell perikarya from young rats and mice are relatively fast, straightforward and are routinely performed in this laboratory. ii) By culturing the isolated cells for at least 24 hours, any exposed cell surface proteins which are cleaved by the action of trypsin during the isolation procedure are allowed to regenerate. iii) During growth in culture the cells appear to undergo, at least to some degree, a recapitulation of normal in vivo development with the presumed expression of cell surface macromolecules involved in the processes of cell-cell recognition and synapse formation. Such molecules would almost certainly be absent or present in very small amounts on freshly isolated cells. iv) The use of cell cultures allows for developmental studies of antigen

expression over periods of at least three weeks.

For cell cultures to be a practical target for screening for monoclonal antibody production it was important that they could, if necessary, be stored before use. In this manner the problems of co-ordinating the availability of animals, from which cerebella cultures could be produced such that they would be of a usable age at the time the supernatants were ready for screening, could be largely overcome. Successful storage was obviously dependent on fixation but the possibility then exists of modifying antibody binding sites by disruption or crosslinking. Previous work by two groups had however shown that mild glutaraldehyde fixation produced no significant changes in antibody binding. Stocker and Heusser (1979) found that lymphocytes and erythrocytes, fixed in 0.25% glutaraldehyde, bound a series of monoclonal antibodies at levels indistinguishable from that shown by unfixed cells. Similar results were shown by Suter et al. (1980) using monoclonal antibodies against human melanoma cells in cultures and fixed in 0.1% glutaraldehyde. In both cases the antibody binding capacity of the cells was unaffected for up to 6 weeks when stored at 4°C. While the risk cannot be avoided that some antibody binding sites may be disrupted - even with mild fixation - I felt that the convenience of having cells ready for use when needed would greatly increase the number of hybridoma supernatants which could be screened which would outweigh any possible disadvantages. As mentioned earlier (see Results) fixation also decreased the variability of the assay by reducing the possibility of washing cells off the culture wells.

While the absolute sensitivity of the assay has yet to be determined, there are several factors which indicated that it would be sufficiently high to detect monoclonal antibodies present in hybridoma supernatants. The most direct indication was that the monoclonal antibody used in these studies was present in a supernatant and specific

binding was detectable at a dilution of 1/500. Although the antibody titre in this sample was certainly higher than would be expected at the first screening for positive clones, the result was nonetheless encouraging. The amount of antibody present is in fact unlikely to be a major problem since the best clones yield as much as 100 µg of antibody for each ml of supernatant (Yelton and Scharff, 1980), the limiting factor is most likely to be the amount of antigen present. Tony Gard working in this laboratory and using a similar assay system has shown that specific binding of an antiserum to tetanus toxoid bound to microtitre wells, is detectable at levels below 2.33×10^{-15} g of toxoid.

CHAPTER 4

THE PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST CEREBELLAR CELLS IN CULTURE

4.1 INTRODUCTION

The methods used to produce monoclonal antibodies vary considerably between laboratories. In many instances the exact reasoning behind certain techniques is unknown but the successful production of antibodies justifies their continued use. The techniques currently employed in this laboratory have been developed over a period of 18 months and while no fundamental changes have been made a large number of details have undergone revision. These changes and the reasoning behind them will be covered in the discussion.

The results reported here were from fusions using spleen cells from two groups of mice exposed to different immunogens. Since the primary goal of the project was to produce monoclonal antibodies against cell surface components of cells from normal and mutant mice cerebella, one group was immunised with a cell suspension prepared from glial enriched cultures of cells isolated from mice. Monoclonal antibodies produced against astrocytes (the predominant cell type present in these cultures) would then be used in two ways. The first would be to examine the occurrence and distribution during development of cell surface antigens on both normal astrocytes and on astrocytes of the "weaver" mutant cerebellum. The second would be to attempt to isolate astrocytes from normal and mutant cerebella cell suspensions using an antibody affinity column. A second group of mice was immunized with a suspension of cells isolated from 5 day old rat cerebella in the hope of producing monoclonal antibodies against antigens that were either cell type specific or developmentaly regulated, or both.

4.2 METHODS

Cell Fusion. The method of cell fusion and subsequent growth of hybridomas are a combination of the methods of Fazekas de St. Groth and Scheidiggar (1980) and Kennett (1979).

On the day prior to the cell fusion a suspension of mouse peritoneal macrophages was prepared to use as a "feeder layer". Five ml of sterile 0.34 M sucrose was injected via a 16 gauge needle into the peritoneal cavity of an adult BALB/C mouse previously killed by cervical dislocation. After gently massaging the abdomen, approximately 4.5 ml of fluid could be withdrawn, which was transferred to a sterile plastic 10 ml tube and centrifuged for 5 minutes at 100 g. The resulting pellet, containing the macrophages and some red blood cells, was washed once in Dulbecco's Modified Eagles Medium (DMEM) and after centrifugation resuspended in 1 ml of DMEM and the cells counted in a haemocytometer. They were then diluted to a density of 2.5×10^4/ml in selective medium (DMEM HAT) of the following composition: DMEM with high glucose (4.5 gm/L GIBCO) to which was added 10% (v/v) heat inactivated foetal calf serum (GIBCO), 10% (v/v) NCTC 109 (Microbiological Associates), 0.15 mg/ml oxaloacetic acid (Sigma), 0.05 mg/ml sodium pyruvate (Sigma), 0.2 units/ml bovine insulin (Collaborative Research), 0.1 mg/ml gentamycin (Sigma), 2mM glutamine, 13.6 µg/ml hypoxanthine (Sigma), 0.18 µg/ml aminopterin (Sigma) and 7.6 µg/ml thymidine (Sigma). The last three components were combined in a 100x concentrate which was stored frozen. Two hundred µl aliquots of the medium containing the macrophages were distributed into 96 well microtitre dishes (Linbro) and incubated overnight at 37°C in an atmosphere of 7% CO_2 and 100% humidity.

Three days after the second injection an immunised mouse was anaesthetised with ether and a sample of serum was obtained via

cardiac puncture after which the spleen was aseptically removed and placed in a 35 mm diameter plastic culture dish containing 2 ml of fusion buffer (FB) of the following composition: 8 g NaCl, 0.4 g KCl, 1.77 g Na_2PO_4, 0.69 g Na H_2PO_4 H_2O, 2 g glucose, 0.01 g Phenol red. The cells were removed by creating a longitudinal split in the spleen and teasing out the contents with a pair of forceps. The solution containing the cells was transferred to a sterile plastic 10 ml tube and the larger tissue fragments allowed to settle out over 5 minutes. The supernatant was transferred to a second tube and diluted to 10 ml in FB. After centrifugation for 5 minutes at 100 g, the cell pellet was resuspended in 1 ml of FB. In order to determine cell number and viability a sample was diluted in a 1 part per million solution of ethidium bromide and acridine orange (Parks et al., 1979) and counted in a haemocytometer. When viewed on a microscope equipped with epifluorescence illumination, viable cells (which take up acridine orange) appear bright green, and non-viable cells (which take up ethidium bromide) orange. Red blood cells being anucleate do not fluoresc. A spleen would normally yield between 70-100 x 10^6 cells.

The myeloma used in these experiments was P3-X63.Ag8 (Gift of Dr. G. Eisenbarth) which is an IgG1 myeloma which secretes a k light chain and is resistant to 8-azaguanine. Cells were grown in 75 cm^2 plastic flasks (Falcon) containing 25 ml of DMEM with 10% FCS, 2 mM glutamine and 0.1 mg/ml gentamycin (DMEM FCS). Cell density was maintained between 1 x 10^5 and 1 x 10^6 cells/ml. With a generation time of 18 hours this necessitated splitting once every 3 days. If the cell density was allowed to exceed 1 x 10^6/ml, growth is no longer exponential and when cells in this phase are used a dramatic reduction in the number of fusions is seen.

At the time of fusion the contents of a flask were transferred to a sterile 50 ml conical tube (Corning) and centrifuged for 5 minutes at 100 g. This step was normally performed in parallel with the

preparation of the spleen cell suspension. The myeloma pellet was resuspended in 1 ml of FB and the number of viable cells determined as described for spleen cells. Myeloma and spleen cells were then combined in a 10 ml round bottomed tube at a ratio of 10:1 respectively and the volume adjusted to 10 ml with FB.

After centrifugation for 5 minutes at 100 g, the supernatant was decanted and the pellet disrupted by gentle shaking. The fusing agent used was a 50% solution of polyethylene glycol 4000 (PEG, Merck) prepared by mixing 1 gm of PEG with 1 ml of water to which was added 100 µl of dimethylsulphoxide (DMSO). The solution was sterilised by passage through a 0.22 µm filter (Millipore) and prewarmed to 37°C. One ml of this solution was then added dropwise from a Pastuer pipette over a period of 1 minute. This mixture was maintained at 37°C for a further 90 seconds with continuous gentle shaking. To stop the fusion 1 ml of FB was added over the next 30 second period, 2 ml over the following 30 seconds and a further 6 mls over 3 minutes. The contents of the tube were then left to stand at room temperature for 5 minutes. Centrifugation yielded a pellet which, after the supernatant was discarded, was very gently resuspended in sufficient DMEM HAT to give a density of 2×10^6 spleen cells/ml. Fifty µl aliquots were then added to the wells containing the macrophages prepared the previous day and the microtitre dishes returned to the incubator.

Clones normally became visible after 4-5 DIV when viewed under phase contrast optics and were readily distinguished from dead cells by their smooth surfaces and translucent appearance. After 7 DIV 100 µl of medium was removed from each well to be replaced with 100 µl of fresh DMEM HAT medium. By 14 DIV wells showing growth of hybridomas normally contained enough cells for screening (anywhere between 5×10^2 and 5×10^5). For the screening assay 100 µl samples of supernatant were transferred to wells containing cell cultures as targets, prepared as previously described. The cultures used in these assays were

routinely left in the blocking buffer for at least 24 hours and stored in the same buffer until used. The assay then proceeded as described for anti Thy 1.1 (3.2).

Control values were obtained from wells incubated with supernatant from unfused myeloma. Wells were designated positive for specific antibody production if the optical density was three standard deviations above the mean of the control values. The contents of those wells positive for specific antibody production were transferred to 2 mls of DMEMHT in a 24 well tissue culture plate (Linbro). (The aminopterin is no longer necessary at this stage since selection is complete; however hypoxanthine and thymidine are supplied to ensure that any residual aminopterin is metabolised.) When the hybridomas had expanded to form a monolayer of cells in these wells, they were once again transferred, this time into 5 ml of DMEM FCS in a tissue culture flask. Supernatants were normally monitored for antibody production once a week using the enzyme immunoassay described previously.

Localisation of Monoclonal Antibody Binding. Indirect immunofluorescence was used essentially as described previously (2.2), in an attempt to localise binding of antibodies to cerebella cells in culture. Cultures were incubated for 1 hour with clone supernatants and after washing, for 20 minutes with a 1:40 dilution of an antiserum produced in rabbits against Fab fragments of mouse IgG (RAM Fab TRITC. Nordic). The use of an anti-Fab fragment was necessary because the immunoglobulin class of the monoclonal antibodies was unknown. Although an antiserum against whole IgG would cross react with all immunoglobulins via the light chains, the degree of cross reactivity is likely to be small because most of the reactivity in an anti-IgG antiserum is directed against the class-specific Fc rather than the Fab portion of the immunoglobulin (Sternberger, 1979).

4.3 RESULTS

Figure 4.1 shows the binding of antibody present in the supernatants from a fusion, to rat cerebellar cells in culture. The immunogen in this case was a suspension of rat cerebellar cells. After the fusion the cells were distributed over two microtitre dishes at a density of 2.5×10^5 spleen cells/well and the supernatants tested 14 days after the fusion. One dish contained 5×10^3 macrophages/well, the other 2.5×10^4 non-immune spleen cells. As can be seen in Fig. 4.1A only 6 wells were positive for specific antibody production when non-immune spleen cells were used as a "feeder-layer", whereas the corresponding value for the dish containing macrophages was 40 wells from a total of 96. Examination of the wells using phase contrast microscopy, showed that the number of clones present in the two dishes was similar, but those in the dish containing non-immune spleen cells were much smaller. It thus seems likely that the level of antibody present in these wells may have been below the level of detection of the enzyme immunoassay and that screening at a later stage may have shown a larger number of wells to be positive. In both dishes the number of clones present in each well was between 3 and 5 and therefore any antibody binding detected may not have been the product of a single clone.

Figure 4.2 shows the binding of antibody, present in the supernatant from a fusion, to mice cerebellar cells in culture. The immunogen in this case was mice cerebellar glial cell cultures and the number of spleen cells was lowered to 1.25×10^5/well. A "feeder layer" of 5×10^3 macrophages was used. Almost one third of the wells showed specific antibody binding when the supernatants were tested 14 days after the fusion but the number of spleen cells/well was still too high, with between 2 and 3 clones being present in each well.

Unfortunately due to a combination of inexperience, contamination and failure to establish hybridomas in larger wells, all the contents of

Fig.4.I Binding of monoclonal antibodies to cerebellar cells in culture. Spleen cells were from a mouse immunised with a suspension of cerebellar cells isolated from 5 day old rats. Cultures to be used as targets were prepared by plating $I.5xI0^5$ cells, from 5 day old rats, into microtitre wells and fixing them after 5DIV. Dotted line indicates three standard deviations above the mean value of the controls.

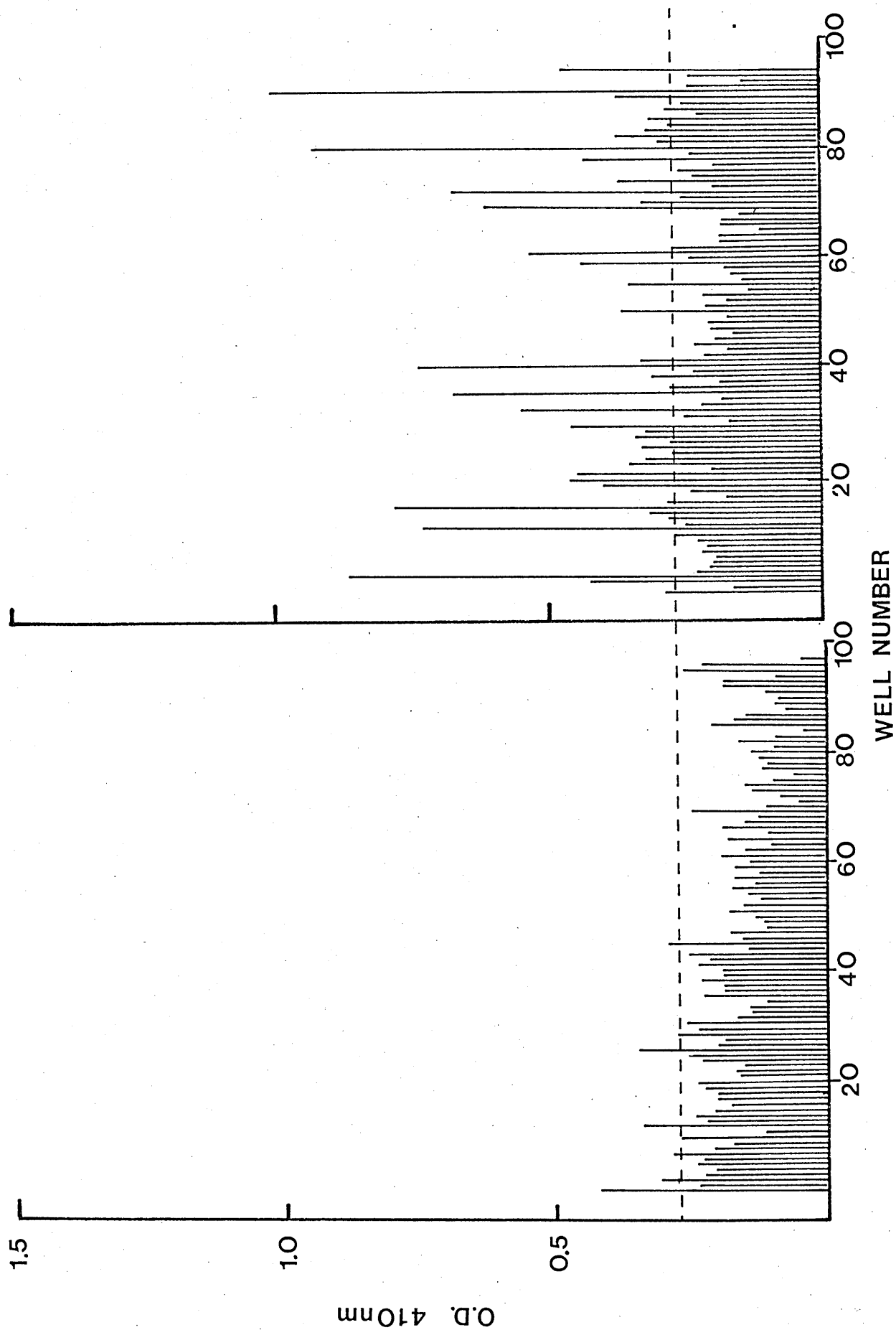
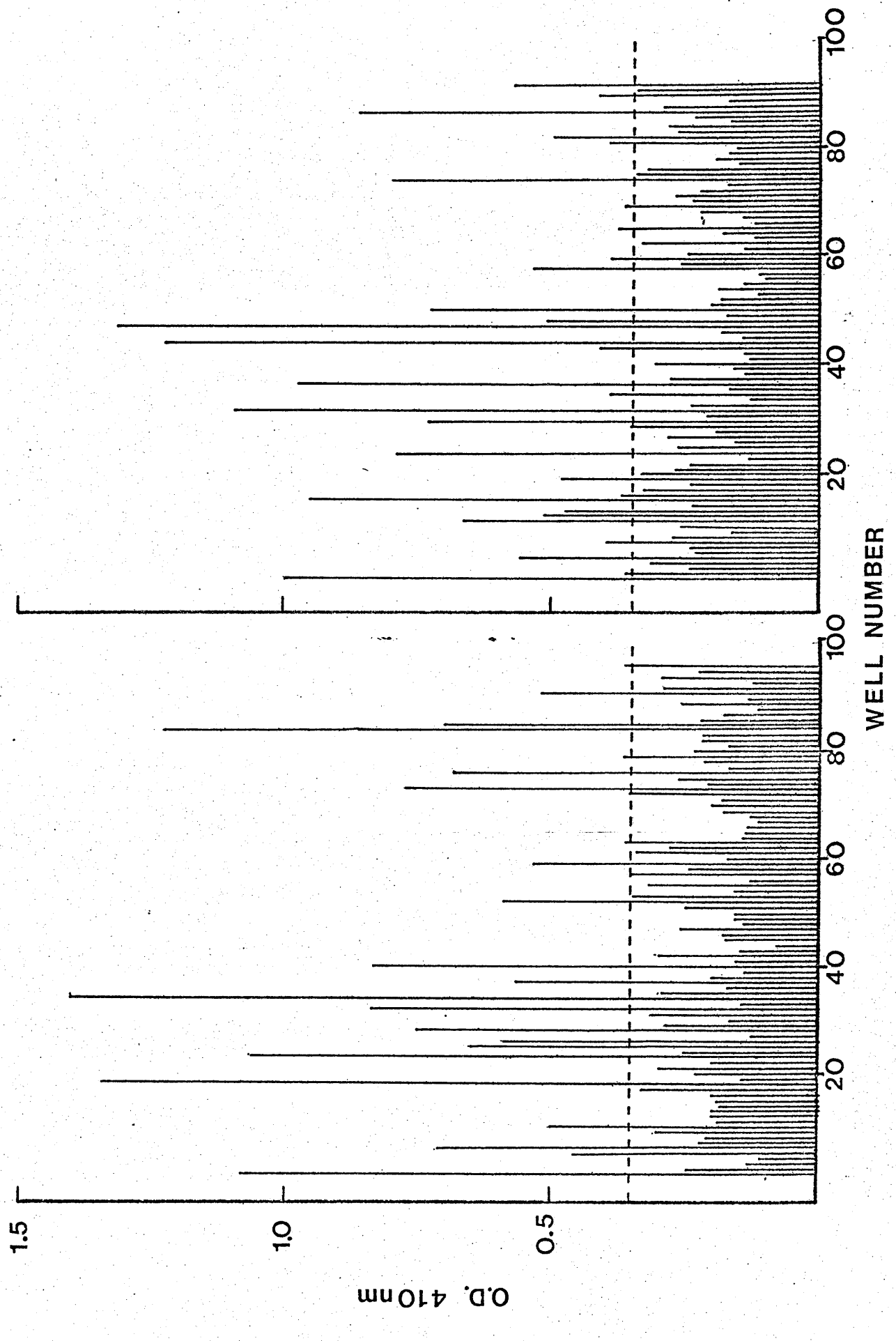

O.D. 410nm
1.5
1.0
0.5
20
40
60
80
100
WELL NUMBER
20
40
60
80
100

Fig.4.2 Binding of monoclonal antibodies to cerebellar cells in culture. Spleen cells were from a mouse immunised with glial cell cultures produced from 5 day old mice. Target cultures were prepared by plating 2.0×10^5 cells, from 5 day old mice, into microtitre wells and fixing them after 2DIV. Dotted line indicates three standard deviations abve the mean value of the controls.

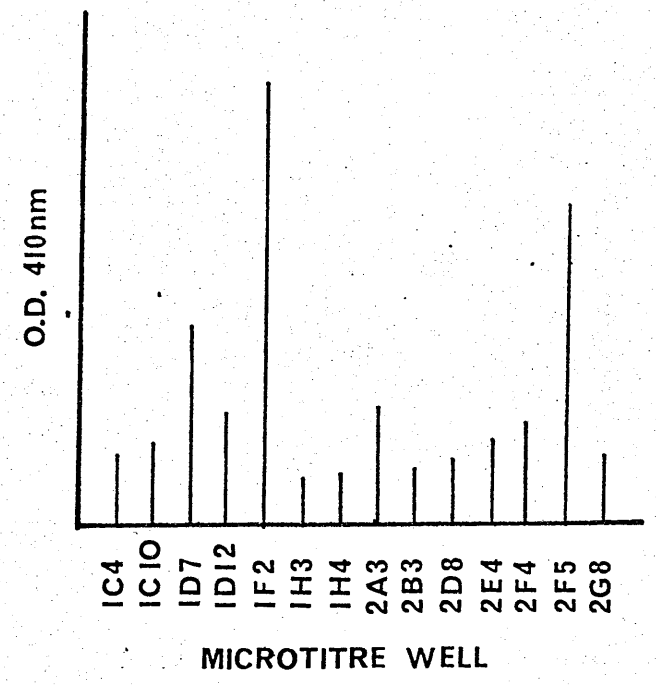

O.D. 410nm
1.5
1.0
0.5
WELL NUMBER
20
40
60
80
100
20
40
60
80
100

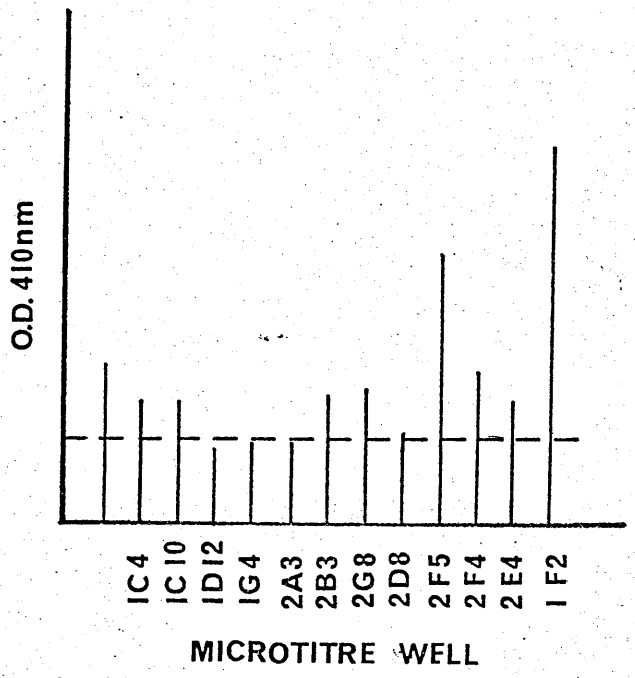
those wells originally positive for specific antibody were lost. The encouraging outcome of both these experiments was that hybrids were being produced which synthesised antibody specific for antigens of cerebellar cells in culture and that the enzyme immunoassay seemed well suited to detecting them.

The results shown in Figure 4.3 are from a second fusion in which the spleen cells were derived from a mouse immunised with a rat cerebellar cell suspension. Figure 4.3A shows the binding to cultures obtained in the original screening 14 days after the fusion. For some reason the optical densities obtained with the control supernatants were the same as the blanks incubated with buffer only, hence a value had to be selected, above which wells were assumed to be positive for specific antibody production. This was set at 0.05 absorbance units (AU). Figure 4.3B shows the binding 20 days post-fusion after the contents of those wells originally shown to be positive had been transferred to 2ml of DMEM HAT. At this stage binding of the control supernatants were 0.102 $\pm$ 0.01 AU, setting a value, above which binding was taken to be specific, of 0.13 AU. Figure 4.3 shows the results of a third assay performed 23 days post-fusion and at this point only wells 2F5 and 1F2 were still showing specific antibody production. At the time of writing only 2F5 was maintaining antibody production and has been successfully established in 75 cm^3 tissue culture flasks. As yet no attempts have been made at subcloning but this is an important next step.

Very limited attempts have been made to localise the distribution of antibody binding on rat cerebellar cells in culture using indirect immunofluorescence. The first labelling experiments were performed on unfixed cultures and showed the labelling to be confined to a small number of cells. Examination of the labelling pattern however suggested that the antibody was directed against an internal antigen and was only entering dead or dying cells. This was confirmed by fixing the cultures prior to

Fig.4.3 Binding of monoclonal antibodies to cerebellar cells in culture. Spleen cells were from a mouse immunised with a suspension of cells isolated from 5 day old rat cerebella. Target cultures were prepared by plating 2.0×10^5 cells, from 5 day old rats, into microtitre wells and fixing them after 5DIV. (A) Binding I4 days post-fusion. (B) 20 days post-fusion. (C) 23 days post-fusion. Dotted line indicates three standard deviations above the mean value of the controls.


O.D. 410nm
IC4
IC IO
ID7
ID I2
IF2
IH3
IH4
2A3
2B3
2D8
2E4
2F4
2F5
2G8
A
MICROTITRE WELL


O.D. 410nm
IC4
IC 10
ID I2
IG4
2A3
2B3
2G8
2D8
2 F5
2 F4
2 E4
I F2
B
MICROTITRE WELL

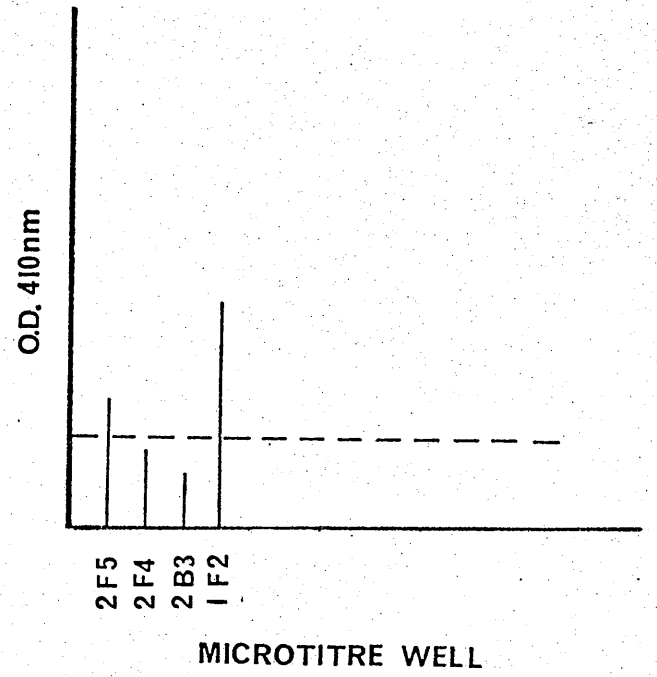
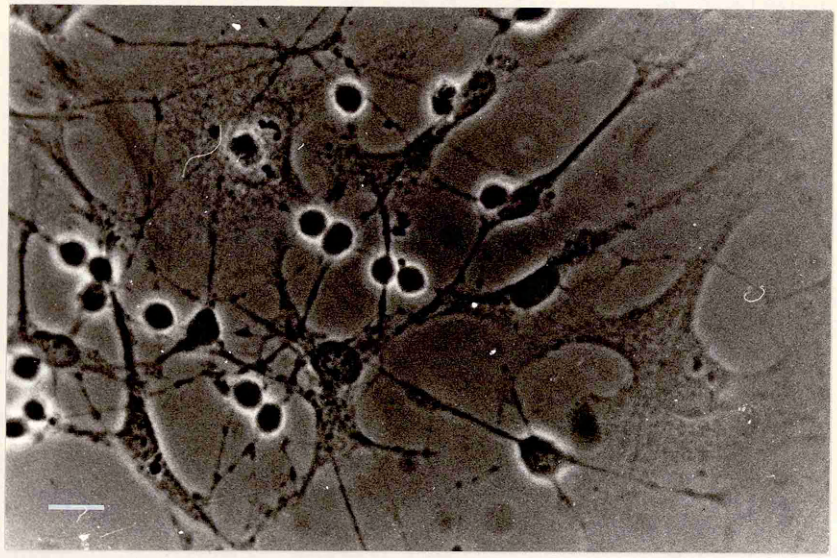
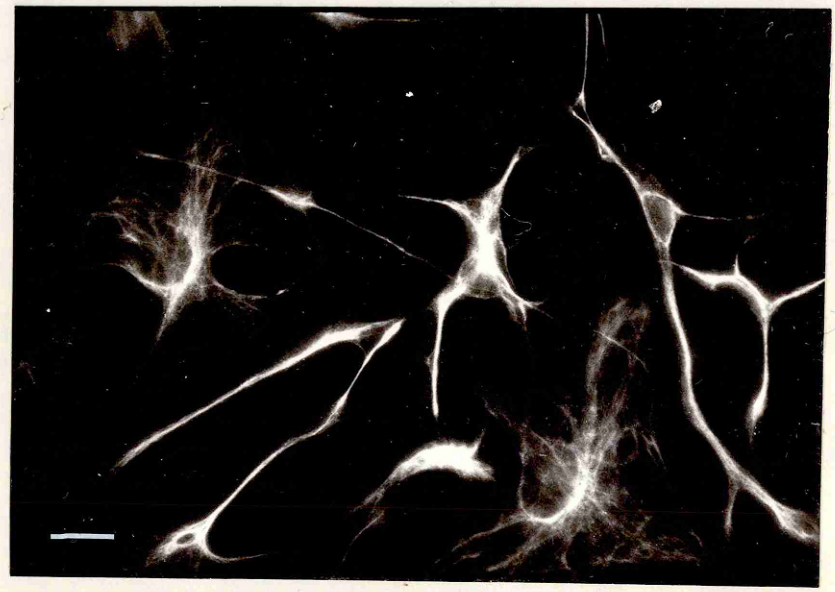

O.D. 410nm
2F5
2F4
2B3
I F2
C
MICROTITRE WELL

Fig.4.4 Labelling of cells in culture by antibody present in hybridoma supernatant 2F5. Cells from 5 day old rat cerebella were labelled, after 2 DIV, with supernatant followed by goat anti-rabbit IgG conjugated to rhodamine and viewed with (A), phase contrast and (B), fluorescence optics. Scale bars = 20μm.

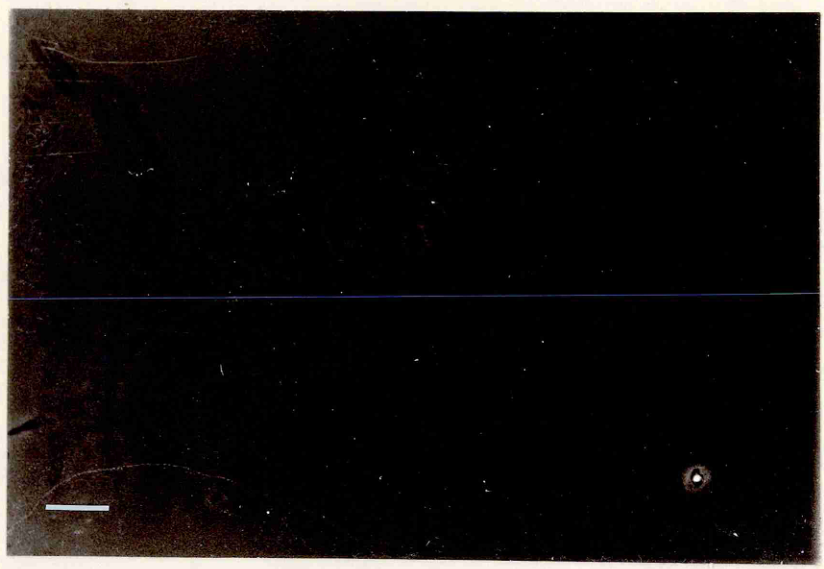


A



B

Fig 4.5 Labelling of cells in culture by antibody present in hybridoma supernatant 2F5. Cells from 5 day old rat cerebella, were labelled after 2DIV, initialy with an anti-serum against Thy I.I followed by goat anti-mouse IgG conjugated to fluorescein. The cells were then fixed and labelled with hybridoma supernatant followed goat anti-mouse IgG conjugated to rhodamine. (A) Phase contrast image. (B) Image produced under optics for viewing rhodamine fluorescence. The fine speckled labelling is surface labelling and is a combination of rhodamine and fluorescein fluorescence, since both second antibodies were against mouse immunoglobulins. The fibrous pattern of labelling is internal, and is solely rhodamine fluorescence. Scale bars = 20μm.



A

B

labelling under which conditions a large population of cells is seen to be labelled.

The general pattern of labelling produced by antibody present in supernatant 2F5, is shown in Figure 4.4 in which it appears to be binding to two cell types which are morphologically distinguishable. The most numerous type have elongated projections along which fibres appear to run. The second type are spread over the surface of the coverslip to a greater degree and do not have such clearly defined projections. In this case the pattern of labelling is much more dispersed with indications of a network of fibres radiating from a central area within the cell. Experiments in which cultures were labelled with both an antiserum to GFAP followed by an anti-immunoglobulin conjugated to fluorescein and 2F5 followed by an anti-immunoglobulin conjugated to rhodamine, indicated that the former type are astrocytes. Similar double label experiments with an antiserum to Thy 1.1 indicate that the second population of cells is fibroblastic.

Figure 4.5 shows one such cell in which the antibody to Thy 1.1 was added prior to fixation and the resulting labelling pattern is visible as a finely dispersed array of dots over the cell surface. The filamentous labelling is the result of incubating the same culture with 2F5 after fixation.

4.4 DISCUSSION

As mentioned earlier the approaches adopted in the production of monoclonal antibodies vary considerably between laboratories and a survey of the current literature revealed no particular method which appeared to utilise the most recent advances in the technology. The best approach therefore seemed to be to select and combine the most appropriate parts of a number of techniques. The reasonings behind these selections are discussed below.

Immunisations.The design of an immunisation schedule for the production of a conventional polyclonal antiserum should be such that a high titre of circulating antibody is produced. This is traditionally achieved by an initial injection of antigen, usually in complete Freunds adjuvant, which gives rise to the primary immune response. The secondary response is as a result of a later administration of antigen, usually 3 to 6 weeks after the first injection. This secondary response is characterised by a rapid rise in the total serum immunoglobulin levels over that obtained in the primary response (see for example, Uhr et al., 1962). The animal can then be hyperimmunised by repeated "booster" injections. Such schedules have been used to produce monoclonal antibodies (Sommer and Schachner, 1980; Lagenaur et al., 1980), but they would seem to be unnecessary and may even be deleterious to achieving the desired effect.

There is now good, if indirect evidence, to suggest that an immunisation schedule for the production of monoclonal antibodies should be designed, not to produce high circulating levels of antibody, but to produce large numbers of activated B lymphocytes. Such activation involves the binding of antigen to the surface of the lymphocyte, which induces blast formation and leads to clonal expansion of these cells. There are two advantages if this can be achieved. One is that

the larger the number of cells in a clone stimulated by the specific antigen, the greater the chance of obtaining a relevant hybrid when the cells are fused. Of more importance however is the evidence to suggest that those cells which fuse to form active hybrids are recently activated B lymphocytes undergoing cell division. Andersson and Melchers (1978) have shown that by separating the large cells from a suspension of spleen cells, by velocity sedimentation and using these cells in a fusion, the number of hybrids produced is higher than would normally be expected. They suggest that the reason for this is that the large cells are undergoing cell division and are in some way better suited to producing viable hybrids. Stahli et al. (1980) have drawn similar conclusions from immunisation schedules using a soluble antigen in which the level of circulating antigen was maintained at a high level prior to fusion to ensure blast cell proliferation. The evidence that traditionally hyperimmunised animals may yield a population of spleen cells ill suited for fusions has come from a study by Oi et al. (1978) in which the frequency of hybrid production was shown to be significantly higher where animals had been given only two injections prior to fusion, as opposed to a lengthy immunisation schedule.

A number of immunisation schedules have been used in this laboratory in the past but the one which has been adopted as standard for whole cells in an initial injection of 5×10^6 cells in 100 µl of PBS intraperitoneally followed by a second injection of the same dose via the same route 3 weeks later. Fusions were then performed 3 to 4 days later. Even this short schedule may be longer than is necessary. Vulliamy et al. (1981) and Cohen and Selvendran (1981) successfully used a schedule with two injections only one week apart and Trucco et al. (1978) obtained hybrids with a single injection of 3.5×10^7 cells.

Although this immunisation schedule was successful in producing a population of spleen cells which formed viable hybrids secreting specific antibody a recent paper by Fox et al. (1981) suggests that the number of potential fusion partners can be greatly increased. This can be achieved by either culturing the spleen cells from a previously immunised mouse in the presence of antigen or by transferring them to an x-irradiated mouse. Under these conditions those clones committed to antibody production multiply to a much greater extent than in the normal situation, possibly due to the removal of inhibitory circuits. Such an increase in the number of cells actively dividing and secreting specific antibody should lead to a decrease in the technical effort involved to produce a given number of hybrids.

Myeloma Cell Line. From a theoretical standpoint the ideal myeloma cell to fuse with an antibody forming cell from the spleen of an immunised mouse, should be one which has lost the ability to synthesise immunoglobulin. Such a hybrid would then secrete only immunoglobulin coded for by the spleen cell. If the myeloma parent produces either a heavy or a light chain, or worse, both chains, then only a fraction of the immunoglobulin molecules secreted will have the desired chain combination specific for the immunogen. Currently three such ideal mouse myeloma cell lines are available. P3-X63.Ag8.653 (Kearney et al., 1979) is non-immunoglobulin or sub-unit producing variant of the IgG, myeloma P3-X63.Ag8 (MOPC21) used by Köhler and Milstein (1975) in the original fusion experiments, which produces a K light chain. SP2/0-Ag14 (Shulman et al., 1978) is itself a hybrid between P3-X63.Ag8 (MOPC21) and BALB/C mouse spleen cells and FO (Fazekas de St. Groth and Scheidegger, 1980) which is a fast growing variant of SP2/0-Ag14. Another variant of P3-X63.Ag8 (MOPC21) which has been widely used to form hybrids is designated NS-1 (Köhler and Milstein, 1976). NS-1 has lost the ability to synthesise a heavy chain and the light chain is degraded internally.

Unfortunately there are other considerations when selecting a myeloma line - it should also be one which can be readily fused with spleen cells to yield hybrids producing specific antibody and such hybrids should be stable in terms of antibody production. All of the above mentioned cell lines have been used as fusion partners in this laboratory. All of them formed viable hybrids which produced specific antibody as detected by an enzyme immunoassay but only P3-X63.Ag8 (MOPC21) produced hybrids which were stable and maintained antibody production beyond two or three weeks.

Fusing Agent. The fusing agent used in the original hybridoma experiments by Köhler and Milstein (1975) was Sendai virus which, whilst producing satisfactory results, requires special knowledge for the growth and assay of the virus. For this reason its use has been largely abandoned and PEG is now the agent of choice (Gelter et al., 1977). The mechanism of action of PEG has not been extensively studied but its affects on cell membranes are likely to be complex (see Lucy, 1978). The particular PEG used in these studies was "PEG 4000 For Gas Chromatography" (Merck). Fazekas de St. Groth et al. (1980) have shown that the use of this PEG produced high numbers of hybridomas, appeared to be non-toxic and different batches produced similar results. Lower molecular weights seem to be less effective (Fazekas de St. Groth and Scheidegger, 1980) and higher molecular weights are difficult to work with because of their high viscosity. The percentage PEG used in the fusion mixture was 50. At higher percentages the toxic effects become overwhelmingly and below 30% the fusion frequency is low (Davidson et al., 1976).

Cell Input. The ratio of spleen to myeloma cell does not seem to be a critical factor with ratios between 1:1 (Oi and Herzenberg, 1979) and 10:1 (Eisenbarth et al., 1980) being used successfully. Of more importance is that when the combined cell suspension is plated into

wells after the fusion, those wells which show growth of hybrids have on average only one clone per well. The reasons behind attempting to achieve this are two fold. First, with more than one clone present in any well it is necessary to separate them to ensure that any specific antibody present in the supernatant is truly of monoclonal origin. Second, and much more of a problem, is that early on after a fusion the heterokaryons are extremely unstable and rapidly lose chromosomes. Those cells which lose the chromosomes coding for antibody production will, in all probability, grow faster than those which retain them, since the burden of producing immunoglobulin can account for 50% of the total protein synthesis (Goding, 1980). This danger of over-growth by non-producing cells is probably the major problem connected with monoclonal antibody production against which there is no guaranteed protection. Even if there is only one hybridoma present in each well, it is still possible for non-producing variants to arise at any time and overgrow the producers. The only precautions which can be taken are to ensure that wells do not become overcrowded (which favors the growth of non-producers), and to keep a check on antibody levels in hybridoma supernatants. A falling titre can almost certainly be attributed to overgrowth, in which case attempts to save the producing cells can be made by sub-cloning.

Assuming that the distribution of hybrids is random once the cells are plated out, then the number of clones in each well should follow the Poisson distribution. Fazekas de St. Groth and Scheidegger (1980) have calculated that if 60% of the wells show no growth then 1 in 4 of the wells can be expected to have two clones, 1 in 24 three and only 1 in 173 more than three. Such multiplicities seemed acceptable since any attempt to further reduce the possibility of multiple clones would increase the number of non-productive wells with an associated increase in the amount of time, effort and tissue culture supplies necessary to culture a similar number of clones.

There are two means by which this situation can be achieved. One is to alter the ratio of myeloma to spleen cells such that the number of fusions between cell types also alters, and maintain a constant number of cells per well. Such an approach would be valid if the initial cell density was a critical factor. Fazekas de St. Groth and Scheidegger (1980) suggest that the myeloma cell density should not exceed 5×10^5/ml of medium whereas Oi and Herzenberg (1979) maintain that the initial density should be 10^6/ml. If selection for hybrids is begun immediately it is difficult to see why the initial cell density is important since overgrowth will not occur even at high myeloma densities and a minimum essential density suggests that there is some sort of "feeder" or co-operative effect between cells for which I have found no evidence.

For this reason the approach I adopted was to maintain a constant ratio of 10 spleen cells to 1 myeloma cell and vary the number of cells plated into each well. At the time of writing the lowest density used was 1.25×10^5 spleen cells per microtitre well at which there were an average two or three clones in each well. As mentioned earlier for there to be a reasonable chance of wells showing hybridoma growth containing only one clone, 60% of the wells should be negative. This situation can hopefully be achieved simply by decreasing the cell density per well. The number of hybrids produced was usually one for every 4 to 6×10^4 spleen cells, which compares favorably with the value of one for every 2×10^5 spleen cells obtained by Gefter *et al.* (1977). This increase in the number of hybrids is almost certainly due to the highly immunogenic nature of whole cells. For example when using sheep red blood cells it is quite simple to immunise an animal such that one out of every one hundred spleen cells is secreting antibody (Kennet *et al.*, 1980).

Cell Fusion. The fusion protocol is essentially as described by Fazekas de St. Groth and Scheidegger (1980). While many variations in the technique have been successfully employed this particular method was selected because the combined cell pellet is only in contact with the PEG for a total of 90 seconds. Other workers have used incubation times as long as 8 minutes (Kennet and Gilbert, 1979) but since PEG is both potentially toxic and hypotonic the exposure should be kept to the minimum which produces an acceptable number of hybrids. Interestingly using this technique the cell viability as determined by acridine orange/ethidium bromide is above 90% as compared to the 32% reported by Hogg et al. (1981).

Feeder Layer. A feeder layer of macrophages was added because a number of workers have noted an increase in the number and growth rate of viable hybrids under these conditions. Andersson and Melchers (1981) have shown a ten to thirty fold increase in the number of viable hybrids when plated into medium containing thymus derived cells. Fazekas de St. Groth and Scheidegger (1980) showed that peritoneal macrophages had a similar effect and Hammerling et al. (1978) found that a layer of non-immune spleen cells enhanced the growth rate of hybridomas. How these effects are mediated is unknown. Goding (1980) has suggested that a feeder layer in some way detoxifies the plastic of the tissue culture dishes and that the same effect can be achieved simply by incubating the cells overnight in medium and then removing it before adding the suspension containing fused cells. This cannot however explain the enhancement seen when feeder cells are added immediately prior to plating. A layer of macrophages was adopted as standard because, as well as stimulating growth of hybrids, macrophages act as scavengers to clear cell debris, the accumulation of which appears to be detrimental to hybrid growth.

Hybrid Selection. Because only a small percentage of the myeloma actively fuse with spleen cells it is necessary to select for those cells to prevent overgrowth by non-fused cells. The method which seems to have been universally adopted is that of Littlefield (1964). The rationale behind the method is as follows. The myeloma used in the cell fusions are lacking in the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) and are therefore resistant to 8-azaguanine which would normally be incorporated into DNA. HGPRT is essential for survival if the normal biosynthetic pathway for purines and pyrimidines is blocked by aminopterin (a folic acid analogue), since it is required in one of the DNA salvage pathways, via which nucleotides can be synthesised if the cells are supplied with an exogenous source of hypoxanthine and thymidine. Thus in HAT selective medium, the only cells which survive are those which are fused with spleen cells which provide the HGPRT. There is no selection against either spleen cells or spleen-spleen cell fusions but, since they do not survive long in culture, this is not necessary.

The time of addition of the HAT selective medium varies between laboratories. It can either be added immediately post fusion, one day after the fusion, as originally suggested by Köhler and Milstein, or the concentration of aminopterin can be gradually increased over a period of several days (Oi and Herzenberg, 1980). The only reason for delaying the addition of HAT would be if both fused and unfused cells required the folic acid pathway during the early stages of growth. The experimental evidence does not support this (see Fazekas de St. Groth and Scheidegger, 1980) and cells are therefore routinely plated directly into HAT selective medium.

At the time of writing only two clones were still secreting antibody of the desired specificity after a period of approximately one month. The reasons for the loss of antibody production from other clones which were originally positive can probably be attributed to i) the overgrowth

of clones by non-secreting clones or variants, ii) the failure to establish clones in 2 ml wells and iii) the loss of antibody production due to chromosome loss. The problem of overgrowth can hopefully be reduced by ensuring that those wells positive for antibody production have only one clone present. There is however no way to prevent overgrowth by non-secreting variants which may arise. Greater success in establishing viable clones in 2 ml wells can possibly be achieved by culturing the hybrids in the presence of a feeder layer of thymocytes (Oi and Herzenberg, 1979). Loss of antibody production due to chromosome loss is currently a problem to be lived with, although early sub-cloning can provide some defense. The general principle behind sub-cloning is the same as used in attempts to obtain only one clone per well, i.e. the cells should be diluted to a density such that when the suspension is plated into microtitre wells, there is a reasonable probability that those wells showing growth originally contained only one cell.

Clones which are stable for antibody production can be expanded either by growing up in tissue culture flasks or by inducing tumours in mice by the intraperitoneal injection of hybrid cells. In the latter case the ascites fluid produced may contain as much as 10 mg/ml of specific antibody (Yelton and Scharff, 1980). Whichever method is employed it is important to freeze aliquots of cells known to be secreting specific antibody such that they can be recovered if for some reason antibody production ceases in the cultured cells.

Enzyme Immunoassay.The enzyme immunoassay in its present form has been successfully used to detect a monoclonal antibody against an antigen present in a population of cerebellar cells in culture. The fact that the antibody is however directed against an internal determinant, highlights one of the problems with the assay when used as the sole means of detection, in that it gives no indication of the distribution

of antigen. It is therefore important that once those clones which are producing antibody against neural cells have been detected using the enzyme immunoassay, the supernatants are screened using indirect immunofluorescence. In this way unnecessary work can be avoided in growing up and sub-cloning irrelevant hydridomas.

There are two areas in which the assay could be improved. The first is related to the reproducibility of the technique. This can hopefully be improved by standardising the age of the animals from which the target cells were isolated, the age at which the cultures are used for an assay and the cell density. The variability of the non-specific binding, as determined by incubating cultures with supernatant from unfused myeloma, can probably be reduced by using supernatant from myeloma grown at the same density for the same period of time, thus ensuring a similar concentration of myeloma IgG, present.

The second area for improvement is the time taken to complete an assay. Currently the incubation times total 5 hours, added to which is the time taken for washing between stages. Salonen and Vaheri (1981) have shown that the inclusion of 4% (w/v) PEG in the dilutent buffers greatly accelerated the reaction between the enzyme labelled anti-immunoglobulin and the primary antibody such that the incubation time could be reduced from overnight to 2 hours without any loss of sensitivity. Preliminary experiments with the assay employed in this laboratory suggest that a similar effect occurs and that the incubation time could be significantly reduced.

CHAPTER 5

CONCLUSION

The Culture of Mice Cerebellar Cells. In Chapter 2 a system was described for the culture of cells isolated from the cerebella of early postnatal mice. The most important change in the composition of the medium, previously developed for the culture of rat cerebella cells, was found to be the use of Ara C as a mitotic inhibitor rather than FudR although the reasons behind this difference are unknown. The use of cell-type specific labels showed the composition of these cultures to be consistent between preparations, as was the plating efficiency. This reproducibility, combined with survival of cultures in excess of three weeks, confirms their potential as a useful model for studying the development of cells isolated from both normal and mutant mice cerebella.

The culture of cells, isolated from the olfactory bulb, cerebral cortex, hippocampus and hypothalamus of 5 day old rats, under similar conditions, showed the applicability of the techniques to brain regions other than the cerebellum.

Enzyme Immunoassay. In Chapter 3 an enzyme immunoassay was described for the detection of antigens against cerebellar cells in culture. The assay offers a number of advantages over the more traditional radioimmunoassay and, although no absolute measurement of the sensitivity was obtained, the results indicated that it would be high enough for the detection of antibody present in hybridoma supernatants.

Production of Monoclonal Antibodies. In Chapter 4 the techniques used for the production of monoclonal antibodies to neural cells were described and preliminary results of attempts to localise the distribution of antigen detected by an antibody produced were reported.

The fusion protocol developed by combining what appeared to be the best parts of a number of previously published methods, yields numbers

of hybrids which compares well with other reported frequencies, and the enzyme immunoassay proved to be well suited to detecting those clones secreting specific antibody. Although large numbers of wells had originally been shown to contain specific antibody the vast majority of clones were lost - either due to failure to establish them in larger wells or due to problems of overgrowth by non-producing variants - or ceased specific antibody production due to chromosome loss. For these reasons at the time of writing only one clone is established which continues to produce antibody specific for an antigen present in neural cells.

While demonstrating the success of the techniques in producing a monoclonal antibody, the particular one produced is directed against an internal antigen and is therefore of no use either for studying the possible role of cell surface antigens during development, or as a "handle" for a specific cell type by which they could be isolated on an affinity column. However now that the techniques have been established for their production it should prove relatively simple to produce monoclonal antibodies with the desired specificity. This work is currently in progress as is the continuing analysis of the antibody already produced.

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