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Monoclonal antibody development to a new opiate receptor

Jeffrey Fein
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MONOCLONAL ANTIBODY DEVELOPMENT TO A NEW OPIATE RECEPTOR

A Thesis

Presented to

The Faculty of the Department of Chemistry

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Jeffrey Fein

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ABSTRACT

MONOCLONAL ANTIBODY DEVELOPMENT TO A NEW OPIATE RECEPTOR

by Jeffrey Fein

A new member of the opiate receptor family, ORL1, has recently been cloned from a human brain stem cDNA library using a murine delta opiate receptor probe. ORL1 appears to contain seven hydrophobic helical transmembrane sequences characteristic of the opiate receptor family. Furthermore, like all of the opiate receptors, ORL1 inhibits adenylate cyclase in the presence of the universal opiate agonist, etorphine. However, other pharmacological studies, as well as the presence of unique amino acid residues, N-terminal sequences, and C-terminal sequences suggest that ORL1 is different from established opiate receptors. It is, therefore, the goal of this project to develop monoclonal antibodies to both the N- and C- terminal receptor sequences, characterize the produced antibodies, and to probe for this epitope in order to deduce ORL1 distribution in rat brain slices.

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LIST OF ABBREVIATIONS

ABTS	2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
Ag	Antigen
AON	Anterior Olfactory Nuclei
AOV	Anterior Olfactory Nucleus, pars ventral
AU	Absorbance Units
BLOTTO	Bovine Lacto Transfer Technique Optimizer
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary DNA
CFM	Cell Freezing Medium
CNS	Central Nervous System
COS	Monkey Originating Cells
CSHL	Cold Spring Harbor Laboratory
C/W	Cells/Well
DG	Dentate Gyrus
dH ₂ O	Distilled Water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DOR	Delta Opiate Receptor
DCC	Dicyclohexylcarbodiimide
E	Enzyme
ELISA	Enzyme-Linked Immunosorbent Assay
FCA	Freund's Complete Adjuvant
FCS	Fetal Calf Serum
FIA	Freund's Incomplete Adjuvant
GDP	Guanine Diphosphate
G _i	Inhibitory G-Protein
G _s	Stimulatory G-Protein
GTP	Guanine Triphosphate
HAT	Hypoxanthine-Aminopterin-Thymidine
Hil	Hilus Dentate
HGPRT	Hypoxanthine-Guanine Phosphoribosyl Transferase
hORL1	Human Opiate Receptor Like 1
HRP	Horseradish Peroxidase
HT	Hypoxanthine-Thymidine
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHC	Immunohistochemistry
kDa	Kilodaltons
KLH	Keyhole Limpet Hemocyanin
KOR	Kappa Opiate Receptor

LMol	Lacunosum Moleculare
MAb	Monoclonal Antibody
MAPS	Multiple Antigen Presentation System
mg	milligram
mL	milliliter
MOR	Mu Opiate Receptor
MW	Molecular Weight
NFM	Non-Fat Dried Milk
OR	Oriens
ORL1	Opiate Receptor Like 1
ORL2	Opiate Receptor Like 2
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PEG	Polyethylene Glycol
Pi	Primary Olfactory Cortex
POMC	Proopiomelanocortin
Py	Pyramidal
PS	Penicillin–Streptomycin
Rad	Radiatum
SDS	Sodium Dodecyl Sulfate
SCM	Sterile Conditioned Medium
t-Boc	N-(tert-butoxycarbonyl)
TMC	Thymocyte Conditioned Medium
TMS	Transmembrane Segment
UCLA	University of California at Los Angeles
XOR1L	Opiate Receptor Long
XOR1S	Opiate Receptor Short

INTRODUCTION

(I) OVERVIEW: OPIATES AND OPIATE RECEPTORS

Opiates are nitrogen containing compounds derived from the opium poppy. These compounds effect pain mediation, neuroendocrine secretion, emotional behavior, learning, memory, immune response, and motor control. One of the unfortunate side effects of these drugs is their ability to induce a strong physiological dependence. As a result of interest in the opiate drug known as morphine in the early 1970s, opiate receptors were first identified in mammalian brain tissue (1). The discovery of the receptor led to the isolation of endogenous opiate peptides.

Opiate Peptides

Though the opiate alkaloids are a class of organic compounds distinct from the endogenous opiate peptides, specific functional groups are held in similar secondary structure orientations. For example, the tyrosine side chain and amino end of Met-enkephalin may be oriented spatially to correspond to the phenolic-like ring and tertiary amino group of morphine (2). This similarity in structure presumably results in both classes of compounds having receptor binding ability. Furthermore, the phenylalanine group of methionine enkephalin is suggested to be responsible for enhanced receptor binding ability over morphine which lacks any similar lipophilic group (ibid.). To date, three types of endogenous opiate peptides have been extracted from brain tissue: enkephalins,

endorphins, and dynorphins. The enkephalins are small pentapeptides, whereas endorphins and dynorphins are larger peptides, ranging from 16 to 31 amino acids in length. This family of neuroactive peptides are derived from three precursor molecules: proenkephalin, proopiomelanocortin (POMC), and prodynorphin (3). Six of these neuroactive peptides, along with their associated precursor molecule and sequence, are illustrated below in Figure 1.

<u>Precursor</u>	<u>Peptide</u>	<u>Peptide Sequence</u>
Proendorphin	Met-enkephalin	Tyr-Gly-Gly-Phe-Met-(OH)
	Leu-enkephalin	Tyr-Gly-Gly-Phe-Leu-(OH)
POMC	β -endorphin	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Val-Lys-Asn-Ala-His-Lys-Gly-Gln-(OH)
Prodynorphin	Neoendorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys-(OH)
	Dynorphin-A	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Glu-(OH)
	Dynorphin-B	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gly-Phe-Lys-Val-Val-Thr-(OH)

Figure 1. Endogenous precursor molecules, peptides, and peptide sequences. Note that (OH) represents the carboxyl end of the peptide. POMC represents the precursor molecule proopiomelanocortin. (Adapted from reference 4).

Opiate Receptors

Pharmacological studies have defined three major types of opiate receptors designated μ (mu), δ (delta), and κ (kappa) (4,5). There are also two controversial minor types known as sigma and epsilon (6) that have not been extensively characterized. Though definitive roles for the receptors have not been completely established, some generalities have been substantiated (for review, see (7)). Mu receptors are involved in pain regulation and sensorimotor integration, whereas delta receptors are involved in motor, olfaction, and cognitive functions. Furthermore, kappa receptors are also involved in pain perception as well as food intake, neuroendocrine function, and water balance regulation (ibid.).

Receptor Structure and Function Relationships

With the advent of DNA cloning techniques came the means to isolate the receptor-encoding cDNA and compare amino acid sequence homology. Expression of fully functioning cDNA-derived receptors in transfected cells has allowed researchers to study ligand specificity among the various receptors. To date, cDNAs coding for rodent δ -(3, 8), μ -(9, 10, 11), and κ -(12, 13) opiate receptors have been cloned and characterized. Furthermore, two rat cDNAs, named XOR1S and XOR1L with unique C-terminal and N-terminal sequences, were transfected into COS cells resulted in expression of two unique receptors (14). They were considered part of the opiate receptor family based on the amino acid sequences predicted from their respective cDNAs displaying 67 – 72% homology with mu, delta, and kappa receptors. They are considered unique based on weak, nonspecific opiate ligand binding assays.

Recently, a Human Opiate Receptor Like protein (hORL-1) has been cloned by two different investigators (15, 16). Like all known opiate receptors, this receptor also has seven hydrophobic sequences homologous to the transmembrane regions of rhodopsin. All such receptors, known as TMS-type (for transmembrane segments), are coupled to the G-protein second messenger system (17). The TMS family includes the following: muscarinic, rhodopsin, angiotensin, opiate, and serotonin receptors.

In addition to establishing that opiate family receptors inhibit adenylate cyclase through the G-protein second messenger system, expression of cloned receptor genes has served to demonstrate that these receptors activate outward K^+ channel currents and inhibit Ca^{2+} entry. This results in a hyperpolarized membrane and the blocking of action potentials. This may provide an explanation for the relief of pain (3, 14).

In addition, subtle differences in ligand binding have been observed. All three major receptor types have differing degrees of affinity towards opiates and endogenous ligands. The μ receptor has highest affinity with morphine and related alkaloid analogs, the δ receptor has highest affinity for enkephalins, and the κ receptor has highest affinity for the endorphin protein dynorphin (18). A set of common opiate ligands for all three include diprenorphine (antagonistic) and etorphine (agonistic). Both have been recently referred to as the universal opiate receptor family antagonist and agonist (15).

Distribution of Opiate Receptors

Differences have been observed in the distribution of opiate receptors throughout the rat brain. Thus, mu opiate receptors are more abundant than are

delta opiate receptors in the hippocampus. In each case, where overlap between receptors does exist, there are discrete patterns within each receptor type distribution (19). For example, in the neocortex, both mu and delta opiate receptors are dense but in alternating layers. The distribution seems somewhat complementary, with layers I, III, and IV containing mu, and layers II, III, V, and VI containing delta opiate receptors. A similar pattern is seen in the olfactory bulb. In this case, mu opiate receptors are most prominent in the glomerular region while delta receptors are abundant in the external plexiform region. Mu opiate receptors also appear to be abundant in the hippocampus, especially in the pyramidal cell body layer and stratum moleculare. The delta and kappa opiate receptors on the other hand, are relatively lighter in this structure.

In a more general sense, both the mu and delta opiate receptors seem to be more prominent than kappa opiate receptors in the telencephalon. Examination of structures from the diencephalon reveals a predominance of kappa opiate receptors. The delta opiate receptor appears to have a rather low distribution in all regions besides the telencephalon, whereas the mu opiate receptor appears to be distributed throughout all central nervous system regions. A summary of important general loci in rat brain anatomy illustrating the major receptor types is shown below in Table 1. This table is broken down into the five major central nervous system subdivisions and includes several of their substructures. Distributions are based on autoradiographic studies and represent a relative measure within a receptor distribution. As indicated by the table, opiate receptors vary throughout major regions as well as in specific structures. Though this table seems to infer that opiate receptors are everywhere, the cerebellum is completely void of any of the opiate receptor types.

Opiate Receptor Loci of the Rat CNS			
<u>Brain Section</u>	<u>Opiate Receptor Type</u>		
	μ	δ	κ
<u>Telencephalon</u>			
Frontal Cortex	+++	++	+
Pyriform Cortex	++	++	++
Neo Cortex	+++	+++	+
Amygdala	+++	+++	+++
Hippocampus	+++	++	+
Dentate Gyrus	+++	+	+
Anterior Olfactory Nucleus	++	+++	++
Caudate Putamen	+++	+++	+++
<u>Diencephalon</u>			
Hypothalamus	+	+	++
Thalamus	+++	+	++
<u>Mesencephalon</u>			
Midbrain	+++	+	++
<u>Metencephalon</u>			
Pons/Medulla	+++	+	++
<u>Spinal Cord</u>			
Ventral horns	+++	++	++
Dorsal Horns	+++	++	++

Table 1. Opiate receptor loci of the rat central nervous system (CNS) for mu, delta, and kappa receptor types.

Low (+), Moderate (++), Dense (+++). (Adapted from reference 7)

Note that, receptor distribution varies greatly between species, especially in the telencephalon regions. For example, in the hippocampus and hypothalamus, delta receptors are relatively dense in monkey compared to the rat (20). In contrast, some areas of the metencephalon and spinal cord show great similarities in receptor distribution (ibid.). From the viewpoint of brain evolution, greater divergence is expected in late brain structures (e.g. cortex regions), whereas greater similarity is expected in ancient brain structures (e.g. metencephalon and spinal cord).

Receptor Mediation of Adenylate Cyclase Activity

All three major opiate receptor types are coupled to adenylate cyclase through a G-protein mediated process. Furthermore, they have all been shown to inhibit the cyclase enzyme. Thus, the class of G-protein bound by opiate receptors is the inhibitory G-protein, G_i . Evidence for rat delta receptor inhibition came from studies by P.Y. Law (21) and D.M.F. Cooper (22). Evidence for the mouse mu receptor came from H. Chneiweiss (23). Evidence for the rat κ receptor came from B. Attali (24).

The receptor mediated mechanism of adenylate cyclase inhibition has been clearly elucidated (25, 26). The receptor-agonist complex binds to the inhibitor G-protein, effecting GTP for GDP exchange by the alpha subunit of the G-protein (see Figure 2). The GTP binding to the alpha subunit results in two different events. Firstly, the G-protein dissociates from the receptor-agonist complex. Secondly, the alpha subunit dissociates from the beta/gamma subunit complex of G protein. Inhibition of adenylate cyclase may occur by one or both of the following mechanisms: (1) the inhibitory alpha subunit directly binds and inhibits adenylate cyclase, or (2) the inhibitory beta/gamma subunit complex acts as a competitive inhibitor for GTP activated G_s (stimulatory G-protein) of a stimulatory receptor. In both mechanisms, the result is the inhibition of adenylate cyclase from producing cAMP. The net result is the opiate-induced down regulation of a cAMP-mediated cascade. Thus, the opiate agonist inhibits the action of a stimulatory agonist binding to a stimulatory receptor (e.g., pain receptor).

Figure 2. The opioid receptor inhibits adenylate cyclase via an inhibitory Gi - protein comprised of three subunits (alpha, beta, gamma). Inhibition can occur either through direct interaction of "Gi alpha" with adenylate cyclase or by "Gi beta/gamma" unit binding to the stimulatory "Gs alpha/GTP" associated with the stimulatory receptor (adopted from reference 26)

Opiate Receptor Structure

Another important similarity amongst the opiate receptors is their structural arrangement in the membrane. As mentioned earlier, one of the features common to many G-protein coupled receptors is the set of seven hydrophobic transmembrane segments (3). For example, J. Kyte and R. Doolittle (27) have predicted that membrane-spanning sequences contain a high content of hydrophobic amino acids. The hydrophobic regions of the opiate receptors are

highly homologous, suggesting that similar secondary structure, as well as perhaps similar functions and potential binding sites may occur. Furthermore, seven regions of conserved hydrophobic sequences are also found in a variety of other G-protein coupled receptors including the rhodopsin receptor (28). The three-dimensional structure of rhodopsin has been solved by optical reconstruction of electron diffraction patterns of the tightly packed and ordered regions of rhodopsin in the purple membranes of Halobacteria (29, 30). From comparison of the hydrophobic regions of opiate receptors to rhodopsin, opiate receptors are predicted to have seven transmembrane helical domains (3, 15).

In addition, comparing sequences of opiate receptors can indicate essential residues that are always present. These residues are good candidates for site mutagenesis in order to determine their importance in receptor function (31). For example, Evans refers to point mutation studies concerning the histidine residue of the sixth transmembrane segment which is postulated to be critical for ligand binding (16).

Our collaborator, Chris Evans has successfully identified a cDNA that encodes a delta opiate receptor (3). The cloning method used by Evans was as follows : cDNA library from mouse delta opiate receptor expressing cells (NG108-15 cells) was transfected into COS cells. These cells were then screened with a radioactive iodine (^{125}I)-labeled agonist (D-Ala², D-Leu⁵ - enkephalin) resulting in identifying the positive cells. The DNA was extracted from these cells, purified and retransfected twice. The final cDNA transfected COS cells were used in selective delta opiate antagonist binding studies to determine if the transfections had been successful. Similar ligand specificity values observed for the NG108-15 cells supported their conviction that the

identified cDNA did encode for a delta opiate receptor. This receptor will be termed as “mDOR–1”.

The cloned cDNA of mDOR–1 was used to analyze the protein structure of the receptor. An amino acid sequence was predicted from the nucleotide sequence that was homologous with the seven transmembrane regions of the opiate receptor family. In addition to the histidine residue described above, two asparagine residues were identified as potential N-linked glycosylation sites. Such glycosylation sites are considered universal extracellular markers (32, 33). The Evans model also indicates a disulfide bridge between the second and third extracellular domains, six intracellular sites for phosphorylation, two amphiphilic domains in the third intracellular loop and C-terminal tail, and aspartate residues in the second and third membrane regions.

The aspartate at D₉₇ is suggested to be involved in the Na⁺ ion effect. High concentrations of Na⁺ ions induce high affinity states and disrupt agonist binding (34, 35). The D₁₃₀ residue functions as a part of the agonist binding site (36).

Palmitoylation of cysteine residues found directly after the seventh TMS in the cytoplasmic C–terminal is a common feature of the G–protein coupled receptors (37). Point mutations of such a residue of the human adrenergic receptor (at Cys³⁴¹) to glycine resulted in the loss of adenylate cyclase stimulation (38). It was suggested that palmitoylated cysteine acts as an anchor for the C–terminal end of the receptor and aids in the creation of secondary structure critical for receptor coupling to its G–protein.

Phosphorylation occurs via protein kinase A and protein kinase C at the cytoplasmic side of the membrane (32). Two different labs (39, 40) showed that

phosphorylation of adrenergic receptors resulted in a loss of receptor interaction with the G-protein system and therefore, an important aspect of receptor regulation.

The extracellular disulfide bond homology with other G-protein receptors, such as opiate receptors, suggests that these cysteines may also be critical in structure and ligand binding (see studies on effects of extracellular cysteine mutations in rhodopsin in reference 41).

A proposed model of mDOR-1 illustrating these sites is shown in Figure 3 which was adapted from Garrett and Grisham (26).

Figure 3 Model structure for mDOR1. There are potential glycosylated (branched structures) asparagines in the postulated extracellular N-terminal sequence and a disulfide bridge between the second and third extracellular loops. Cytoplasmic serine and threonine residues are potential phosphorylation sites. Membrane residues aspartate and histidine are indicated and discussed in text.

Human Opiate Receptor Like Protein (hORL-1)

This work by Evans, et al. established a COS cell line expressing functional delta opiate receptors. Its significance was that it provided Catherine Mollereau with crucial structural characteristics which she used in the isolation of a new opiate receptor (15). A pair of oligonucleotides encoding highly conserved transmembrane domains of the delta opiate receptor and somatostatin receptor were applied in PCR amplification of a human DNA probe (hOP01, 761bp). Application of the probe was used in screening a human brainstem cDNA library. The result was the isolation and characterization of a cDNA clone that encodes a human Opiate Receptor-Like (hORL1) protein. The protein was stabilized in transfected CHO-K1 cells and established to be a G protein coupled receptor containing seven transmembrane domains. The protein consists of 370 amino acids having a molecular weight of 40,690. It is a member of the opiate family based on structural and functional similarities, and it is unique based on a set of unpredicted pharmacological interactions.

As mentioned, the structure of this new receptor protein is very similar to the G protein–coupled opiate receptors. Key structural features of hORL1 common to other opiate receptors include: two aspartate residues (D₉₇ and D₁₃₀) found respectively in the second and third transmembrane domains; palmitoylated cysteines (C₃₂₉, C₃₃₄, and C₃₃₅); three glycosylated asparagine residues in the N-terminal domain; multiple intracellular protein kinase C and protein kinase A sites in the third looped domain; and cytoplasmic phosphorylation sites at various serines and threonines. Two aspects that make this receptor distinct are an absent sixth transmembrane histidine and several

amino acids commonly conserved in other opiate receptors that are not found in ORL1 (16).

Mollereau has calculated that the hORL1 has 50% protein sequence homology with murine μ , δ , and κ opiate receptors. In addition, (^3H)etorphine, a universal opiate antagonist, binds to hORL1 and inhibits adenylate cyclase via a G-protein messenger system (15). However, hORL1 is a unique receptor of the opiate family in that the endogenous agonists (β -endorphin, enkephalin, and dynorphin) do not appear to affect the receptor adenylate cyclase activity. In addition, higher than normal concentrations of (^3H)etorphine and its antagonist diprenorphine were required for activation and deactivation .

Mollereau has also compared sequences of the five known rat somatostatin and rat opiate (δ , μ , and κ) receptors to the hORL1 sequence and found a 67% homology in the putative transmembrane helices. In particular, the highest homologies were found in the second (82%), third (82%), and the seventh (80%) transmembrane segments. Mollereau also identified a region of the receptor having 75% homology with the first twelve residues in the C-terminal domain adjacent to the seventh TMS.

Recently, while screening for DNA sequences homologous to the mDOR-1 clone (3), the Evans group isolated two "orphan" clones, including hORL1, as well as a second opiate receptor ORL2 (16). Both clones were isolated from a human brain stem cDNA library using murine DOR-1 as the probe. Comparison of amino acid sequences shows that ORL1 had a higher homology to the murine μ - opiate receptor (44%) than ORL2 (25%). Conversely, ORL-2 had a high homology to the angiotensin receptor family. In addition, the ORL1 cDNA was identical to the cDNA isolated by Mollereau(15).

Evans raises an important distinction of the hORL1. As discussed above, in the sixth transmembrane segment of all opiate receptors is a histidine residue that is missing in hORL1. Results from point mutations of this specific histidine in the tachykinin receptor family suggests that it plays a role in ligand interaction and therefore may be critical for opiate binding (16).

The hORL1, not drawn to scale, is shown in Figure 4. A comparison of amino acid sequences between hORL1, hORL2, human μ -opiate receptor, murine κ -opiate receptor, and human δ -opiate receptor is illustrated in Figure 5. Hybridoma technology focuses on the uniqueness of both the N-terminal and C-terminal sequences.

Figure 4. A model of hORL1. It is a seven transmembrane receptor with three proposed glycosylated asparagine residues on the extracellular side of the membrane. Note that several amino acids commonly conserved in other opiate receptors are not present in ORL1.

Figure 5. Illustrated on the following page is an amino acid sequence comparison between the opiate like receptors, human μ -receptor, human δ -receptor, and murine κ -receptor. All sequences begin with the N-terminal at the top of the page. The regions overscored by solid circles represent the predicted transmembrane segments. Letters in capitals indicate an existing homology, while lower case letters represent amino acid uniqueness. The protein kinase sites ("PKA" and "PKC") are underlined and glycosylated extracellular Asn residues are represented by the "N" sign. Lower case letters preceded by arrows represent alternative amino acids. (Figure adapted from Evans (16))

10 20 30 40 50 60
 hMOR HDSSAAPTYSYCTDALAYSSCSPAPSPGSMVYLSHLQGLSDPCGPYRTNLGGRDSLCPPTGSP
 70
 hDOR mePAPSoGaelq-ppLfaYaSDaypsacpsaGaYaSgpParsas
 mKOR mesplqifrgdpgptcspSaC-lIPYssSWfp-nmaesdsYgsvGsedqqLes-ahI-sP-aIP
 80 90 100 110 120
 ORL1 neplfPaP-fXlygSHLqGVLsll-sPYhslLpphllLYashGal
 ORL2 meeggdfdnyygadYqSeCeyTawk
 130
 hMOR SHITAITIMALYSIVCVVGLFGNFLVMYIVRYTQKKTATNIYIFNLALADALATSTLPFQSVNY
 hDOR SlolAIaItaLYSaVcavGLIGHVLVHfgIVRYTKMKTATNIYIFNLALADALATSTLPFQSAKY
 mKOR -vI--IT--AvYSWfVVGVLvGHsLVHfVIRYTKMKTATNIYIFNLALADALvTtTmPFQSAvY
 ORL1 plglkvTIvgLYlaVcVGLIGHCLVMYIIRhTKMKTATNIYIFNLALADtLVlITLPFQgtdI
 140 150 160 170 180 190
 ORL2 S-sgal-IpaIymIVfllGttGNGLVlntvFRsrrkrrsodIFlasLavADltfwTLPlatyTY
 200 210 220 230 240 250 260
 hMOR LSSAIGLPVHFHATTXYRQGSIDCTLTFSHPTMYHENVKICVFIFAFIMPVLIITVCYGLMLR
 hDOR LaSgvGvPiMvHAvTrpRdGavvCmLqFpsPSMYWdtvtKICVFIFAFvWPILIIITVCYGLMLR
 270 280 290 300 310 320
 mKOR LaSsvGisaivlggTKvRedvIeCsLqFpddex-WdlfmKICVFvFAFviPVLIIIVCYtLMILR
 hORL1 LaSvvGvPVaiMgsaqvedeeIeClveiptPqdYWgpvfaICiFIIsFivPVLvIsVCYSLMIR
 330 340 350 360 370 380 390
 hMOR LGYTHSCLNPVLYAFLDENFKRCFRFCIPTSSNIEQQNSTRIQNIIRDHPSTANTVDRTHQLE
 hDOR LGYhNSsLNPVLYAFLDENFKRCFRqCrkpcgrpdssfsRaReaIarervTactpsdggpggga
 mKOR LGYTHSsLNPVLYAFLDENFKRCFRdFCFPIkmmErQsInRvR-NTvqdPasmdvggmnpv
 ORL1 LGYvNSCLNPiLYAFLDENFKaCFRkFCcasalrrdvQvSdRvRgiakDvalackTsetvprpa
 400
 hMOR NLEAETAPLP
 h-DOR aa
 ORL2 eqmheksipysqetlvwd

(II). HYBRIDOMA TECHNOLOGY

The purpose of hybridoma development is to create an immortal, monoclonal antibody producing cell line. A hybridoma cell is a fused myeloma cell and a B-cell harvested from an immunized animal.

Success is dependent upon the fusion, selection media, growth media, sub-cloning, and screening. These topics will be delineated below.

Polyclonal vs. Monoclonal Antibodies:

An immunological response to an antigen results in an antiserum that is termed polyclonal. This means that the antiserum contains a large population of antibodies recognizing different antigenic determinants that may have varying degrees of specificity and cross-reacting tendencies. This is advantageous for an in vivo immune response, but not so much for research applications.

If, however, a person could pick out the one cell that was producing the single desired antibody and grow it up in culture, the result would be a monoclonal antibody source. This source of cells would produce identical antibodies having the same specificity against a single antigenic determinant. Unfortunately, antibody producing cells (B-cells) do not survive for very long in culture medium. This problem was solved by a fusion technique known as hybridoma technology (42).

The need to develop a monoclonal antibody was apparent from immunohistochemical results conducted with polyclonal serum raised against the unique N- and C- terminal peptides of hORL1 by Dr. Evans' lab. Polyclonal rabbit serum gave high background, possibly due to anti-poly lysine activity (see

below, "MAPS") or to other low affinity cross-reactive antibodies present in the serum.

Monoclonal Antibody Production:

The theory of monoclonal antibody (MAb) production builds on our knowledge of the immune system. An immune response stimulates proliferation and differentiation of the B lymphocytes. All of the cells that originated from the same parent cell are known as clones and produce antibodies recognizing the same antigenic determinant. The cells then mature into antibody secreting plasma cells. Unfortunately, plasma cells do not divide and eventually die. This problem has been solved by fusing the antibody secreting plasma cell with an immortal cell of similar origin. The immortal cells were isolated from lymphocyte tumors called myelomas. Fusion of the myeloma and plasma cell produced a hybridoma which would continually divide and produce a specific antibody.

Prior to cell fusion, there are several aspects that need to be considered. These include: choice of myeloma partner, fusion reagent, polyploid nature of the hybridoma, hybrid selection medium, screening method, and subcloning.

Choice of Myeloma Partner

Prior to the fusion, another important consideration of hybridoma development is the choice of the tumor cell partner. One of the most important aspects to be determined, is whether the partner is compatible with the plasma cell. In practice, myeloma cells are used as B lymphocyte partners, as both originate via the B cell developmental pathway of the immune system. In

addition, cross–species hybrids tend to be unstable; therefore, both cell partners must originate from the same species.

Secondly, it must be recognized that many myelomas produce their own immunoglobulin chains. The advantage of these cells is that they produce a greater number of hybridomas. The disadvantage is that these chains can become incorporated into the hybridoma antibody, and result in the risk of losing the specificity that had been attained from B cell/antigen interaction. On the other hand, there are myeloma cell lines that do not produce their own immunoglobulin chains. The resulting hybridoma antibodies reflect only the immunoglobulin chains of the plasma cell.

A third consideration in the choice of myeloma partner is the fusion efficiency (43). The myeloma line P3.X63.Ag8.653 was chosen due to its good efficiency as a fusion partner and also because it does not produce its own immunoglobulin chain which could be incorporated into the hybridoma's antibodies.

Fusagen

To create a hybridoma, the myeloma and plasma cells are mixed in the presence of a fusion agent and then transferred into hybrid selection medium. Although Kohler and Milstein initially used Sendai virus to promote cell fusion, a successful agent that promotes the fusion of cell membranes was subsequently identified as polyethylene glycol (PEG) (44). The plasma cells that do not fuse with a myeloma cell simply die out over time and do not divide. The unfused myelomas die due to the toxic nature of the hybrid selection medium as described below.

Fused Cell Selection

The selection medium, known as HAT, contains a mixture of hypoxanthine, aminopterin, and thymidine. The selection mechanism is based on an alternate nucleotide synthesis pathway and the fact that the chosen myeloma cell line lacks the HGPRT enzyme (Hypoxanthine–Guanine–PhosphoRibosylTransferase) needed by the alternate pathway. This alternate pathway in nucleotide production is called the salvage metabolic pathway, in contrast to de novo synthesis. The salvage pathway generates purine nucleotides as long as the cell is provided with hypoxanthine and thymidine. The function of aminopterin is to inhibit tetrahydrofolate production. In the absence of tetrahydrofolate, as a carrier molecule in the de novo pathway, the cell is forced to use the salvage pathway. Since the myeloma lacks the HGPRT enzyme, in the presence of aminopterin, it can no longer produce nucleotides by either pathway and eventually dies. The plasma cells on the other hand do possess the enzyme. The hybridoma therefore receives plasma cell chromosomes for HGPRT and myeloma chromosomes for continuous cell division. The result is that plasma cells die due to their inability to divide or survive in culture, myelomas die because they lack HGPRT which is necessary for the salvage pathway, and only the hybridomas survive.

ELISA Screening Method

In order to keep up with hundreds of cell colonies produced, a sensitive, rapid, and high volume method for antibody screening is required for post–fusion events. The double–antibody Enzyme–Linked Immunosorbent Assay (ELISA) method fulfilled these prerequisites.

ELISA is a rapid method that combines antibody specificity with a sensitive spectrophotometric enzyme assay. Supernatant is incubated in the wells of a 96-well plate that has been coated with antigen. The specific antibody binds to the antigen while all other materials are washed away. The wells are then exposed to an anti-immunoglobulin antibody that has been labelled with horseradish peroxidase (HRP). Once again, a series of washes are applied to the plate in order to remove any of the unbound secondary antibody. Hydrogen peroxide and a substrate are added and allowed to incubate. The presence of the desired antibody is indicated by visualization of the enzymatic reaction with the substrate. This step by step procedure is illustrated below in Figure 6 (45) for antibody presence in supernatant from mouse hybridoma cultures. The same procedure is also used for testing antibody levels in mouse serum.

The labelled antibody acts as an indicator of supernatant antibody presence. Visualization results from the oxidation of the chromogenic substrate, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) known as ABTS. The first step involves a two electron reduction of hydrogen peroxide by HRP. Two electrons are removed from the horseradish peroxidase, resulting in cleavage of hydrogen peroxide and the production of a cation radical enzyme. The oxidized HRP is then reduced via oxidation of two ABTS molecules to stable green cation radicals. The ABTS cation radicals are stable at slightly acidic conditions, absorb strongly, and can be detected with a spectrophotometer at 405 nm (46). A schematic representation of this reaction, as well as ABTS and its stable cation radical, are shown below in Figure 7.

The HRP labelled antibody used in the ELISA assays is a goat antibody specific for mouse immunoglobulin light and heavy chains.

Figure 6. An illustration of a general ELISA procedure. Antigen (Ag) coated wells are exposed to supernatant antibody followed by a wash and then incubation with an enzyme (E) labelled antibody that binds with the supernatant antibody. Visualization occurs with the addition of hydrogen peroxide and a substrate. (Adapted with permission from reference 45).

Figure 7. HRP oxidation of ABTS to a stable green radical cation whose absorption is detected at 405nm. There are two stable radicals produced from one HRP enzyme.

Rapid Subcloning

The polyploid nature of the hybridoma is also of concern. The term polyploid means that there are several copies of chromosomes. That is, a hybridoma not only receives B cell chromosomes, but also myeloma chromosomes. One of the inherent problems encountered in the propagation of a tetraploid line is that daughter cells often contain slightly more or slightly less than the $4n$ content of chromosomes. A consequent frequent observation is that in the beginning stages of hybridoma proliferation, chromosomes are rapidly lost. The loss of chromosomes can result in daughter cells with complete loss of antibody production.

Once hybridoma colonies begin to develop and grow, not only is it imperative to determine which cultures contain the desired antibodies, but it is equally important to sub-clone and select the desired hybridomas before they are overgrown by variants which no longer produce antibody. Such overgrowth by non-productive variants is a consequence of the growth advantage conferred upon cells which are no longer burdened by the need to produce massive amounts of antibody. Such commonly observed loss of a productive clone is termed "hybridoma heartbreak."

The goal of subcloning is to have single colonies (monoclonals) grow up in each well. Those colonies exhibiting positive ELISA signals are expanded, subcloned, and re-analyzed again by ELISA. This series is repeated a third time to ensure that the cell line is stable and monoclonal.

IMMUNOGENS AND IMMUNIZATION SCHEDULES

Two unique peptides were chosen as immunogens: the N-terminal and C-terminal of hORL1. The first priority was to obtain an anti-N-terminal MAb that could be used to 1) characterize receptor distribution in the brain, and 2) to isolate a transfected cell line expressing receptor to be used to screen endogenous ligands. The second priority was to obtain an anti-C-terminal MAb to be used to characterize G-protein interactions of this receptor.

N-terminal Peptide

The MAPS immunogen carrier is a branching matrix of seven lysines having eight exposed amino groups as shown in Figure 8. The MAPS structure is constructed using a step-wise solid-phase method on an insoluble support matrix (47). The matrix is a cross-linked polystyrene resin that has a chloromethyl substituent at the para position of the benzene ring. Synthesis uses tert-butyloxycarbonyl (t-Boc) derivatives of lysine, in which the alpha and epsilon amino groups of the lysine are chemically protected with t-Boc prior to coupling to the matrix. Synthesis begins by coupling the carboxyl group of t-Boc lysine #1 to the polystyrene matrix. Subsequent synthesis proceeds bidirectionally from lysine #1. The amino groups of the lysine are deprotected with trifluoroacetic acid, which does not affect the lysine – matrix ester bond. The next step is addition of t-Boc protected lysine, again both alpha and epsilon amino groups, in the presence of dicyclohexylcarbodiimide (DCC) to the unprotected amino groups of the matrix linked lysine. Another round of deprotection, lysine addition, and deprotection resulted in the final heptalysine MAPS structure having eight exposed amino groups used as peptide attachment sites as shown in Figure 8.

The N-terminal peptides were coupled by the same method followed by cleavage from the support matrix with liquid hydrogen fluoride. This step also removed the t-Boc moieties. The synthesis was carried out by Chris Evans' lab at UCLA.

Figure 8. A schematic illustration of the heptalysine MAPS core. The circles represent the alpha and carbonyl carbons of each lysine. Conjugated circles are joined by amide bonds. The eight free amino groups serve as sites for peptide attachment.

This synthesis of heptalysine matrix with peptide is commonly referred to as a multiple antigen peptide system (MAPS). According to Jean-Paul Briand, et al. (48), there are several advantages to using the MAPS. Firstly, it enhances immunogenicity of a peptide, and secondly it presents all the peptides in a similar orientation. Work by Tam and Zavala (47), concluded that the heptalysine matrix showed the highest immunogenic activity and that peptide attachment to the matrix does not alter the antigenic recognition properties of the induced

antibodies (47). The one limitation of the MAPS is the possibility of anti-peptide antibodies cross-reacting with the lysine backbone.

The sequence of the selected N-terminal amino acid chain is $^+_3\text{HN-M-E-P-L-F-P-A-P-F-W-I-Y-G-S-H-L-Q-G-COO}^-$. The reasons for the choice of this peptide are two-fold. Firstly, this is the end that is presumably exposed outside the cell and would therefore make the receptor accessible to probing. Secondly, this sequence is unique compared to the other members of the opiate receptor family (see Figure 4).

C-terminal Peptide

For the C-terminal peptide, the MAP system was not used. Instead, the peptide was conjugated via its carboxyl end to the carrier protein, keyhole limpet hemocyanin (KLH). The sequence was the last eighteen amino acids ($^+\text{H}_2\text{N-A-K-D-V-A-L-A-C-K-T-S-E-T-V-P-R-P-A-KLH}$). Again, the conjugation of peptide to carrier was done at UCLA by Evans' lab.

PROJECT GOALS

MONOCLONAL ANTIBODY DEVELOPMENT

An eighteen amino acid sequence of the unique N- and C- terminal ends of the receptor will be used as immunogens in order to elicit an immune response. The resulting antibody producing cells will be isolated and used to create hybridomas.

HYBRIDOMA STABILIZATION

The hybridomas will be screened to the point that the chosen hybridomas are stable and producing monoclonal antibodies to the terminal ends of the receptor .

ANTIBODY CHARACTERIZATION

The produced antibodies will be characterized in terms of their immunoglobulin class and whether they have any cross-reactivity with other proteins.

ANTIGEN DISTRIBUTION IN THE BRAIN

The monoclonal antibodies will be used to detect the antigen in order to deduce ORL1 distribution in rat brain slices.

MATERIALS / METHODS

(I). IMMUNOGEN PREPARATION

The immunogens were: 1) the first N-terminal 18 amino acids of hORL1 bound to a polyvalent MAPS carrier and 2) the last 18 amino acids of the hORL1 C-terminus coupled to KLH. The final concentrations of both the N-terminal peptide-MAPS and C-terminal peptide-KLH were estimated by Dr. Evans to be approximately 1 mg peptide/mL matrix. Using these values, the final immunogens used for immunizations were prepared as follows :

Materials

MAP or C-terminal peptide

Dulbecco's Phosphate Buffered Saline (1X) (PBS)

GIBCO Laboratories; Cat# 310-4190AK; Control# 11P8791

Final buffer concentrations: 0.01 M phosphate and 0.15 M NaCl

Complete Freund's Adjuvant (CFA)

GIBCO Laboratories; Cat# 660-5721AD; Control# 17P3481

1 mg of heat killed and dried mycobacterium tuberculosis is added to 1 mL of IFA

Incomplete Freund's Adjuvant (IFA)

Sigma; Cat# F-5506; Lot# 014H-8800

Each mL contains 0.85 mL of paraffin oil and 0.15 mL of mannide monooleate

Pair of glass syringes, 5 cc

3-way valved teflon stopcock (Baxter; Cat# K75)

Procedure

Immunogens for both C-terminal and N-terminal peptide antigens were prepared in the same manner as described in the protocols below.

Immunogen Preparation Protocols

Primary Immunogen

A 135 μ L sample of antigen was mixed with 365 μ L of PBS and then take up into one of two glass syringes. A 500 μ L sample of CFA was taken up into the other glass syringe. The contents of both syringes were then mixed together as described below.

Subsequent Immunogens

A 67.5 μ L sample of antigen was mixed with 432.5 μ L of PBS and then take up into one of two glass syringes. A 500 μ L sample of IFA was taken

up into the other glass syringe. The contents of both syringes were then mixed together as described below.

The immunogen was prepared as an emulsion between the Freund's Adjuvant and antigen using a pair of glass syringes connected with a 3-way valved teflon stopcock. The two components were mixed repeatedly back and forth through each syringe until forming a thick ("good"), white emulsion. The emulsion was then tested by depositing a drop into a Petri dish filled with distilled water, and the amount of dispersion noted. Poor emulsions disperse rapidly (within seconds) on the water's surface. It was later noted that temperature was a factor in time required to achieve a good emulsion, with the use of ice to lower temperature of the adjuvant decreasing the time necessary. Good emulsions maintained the majority of the white suspension droplet intact for one half hour or longer.

(II). IMMUNIZATIONS

Six, 8-9 week old female BALB/c mice were chosen for each peptide immunization protocol. A general guideline for immunization, as shown in Table 2, was set forth by S. Hockfield, et al.(43). Similar immunization schedules were followed for mice immunized with the N-terminal sequence and mice immunized with the C-terminal sequence as described below. Deviations from the schedule shown in Table 2 were minor, and did not present any problems in terms of a successful immunization. The slight deviations involved only the times of administering the immunogens as described below.

Immunization Schedule

<u>Time</u>	<u>Injection Type</u>	<u>Immunogen in</u>
Day 1	I.P.	CFA Emulsion
Day 14	I.P.	IFA Emulsion
1-3 Mo. Interval	I.P.	IFA Emulsion
3 Days prior to fusion	I.V.	Saline

Table 2. A general schedule for mouse immunization. I.P. is an injection into the peritoneal cavity, whereas I.V. is an injection into the main tail vein of the mouse.

For each peptide, three mice received I.P. injections of 0.2 mL immunogen, and another three mice received I.P. injections of 0.1 mL immunogen on the first immunization. The immunization schedules below in Tables 3 and 4 show data for mice immunized to N-terminal and C-terminal peptide antigens. The concentration of antigen given were calculated by assuming that the original antigens were 1 mg/mL :

N –terminal MAPS Peptide

<u>Mouse</u>	<u>1' (6-2-94)</u>	<u>2' (6-11-94)</u>	<u>Pre-fusion</u>
109	13.5 µg	6.75 µg	----
110	13.5 µg	6.75 µg	20 µg
112	13.5 µg	6.75 µg	30 µg
129	27.0 µg	13.5 µg	----
114	27.0 µg	13.5 µg	40 µg
115	27.0 µg	13.5 µg	40 µg

Table 3. The schedule for N-terminal peptide immunizations. The 1' immunization was IP, given as an emulsion in CFA. The 2' immunization was IP, given as an emulsion in IFA. The pre-fusion injections were IV (tail vein), done without any adjuvant, and only in PBS. A three day interval existed between pre-fusion and fusion. Pre-fusion injections for mice 110 and 112 were done on the same day (6-27-94). Pre-fusions for mice 114 and 115 were also done on the same day (7-12-94).

C –terminal Peptide

<u>Mouse</u>	<u>1' m.</u>	<u>2' im.</u>	<u>3' im.</u>	<u>Pre-Fusion</u>
103	13.5 µg	6.75 µg	3.4 µg	----
104	13.5 µg	6.75 µg	3.4 µg	50 µg
106	13.5 µg	6.75 µg	3.4 µg	----
135	27 µg	13.5 µg	6.8 µg	----
136	27 µg	13.5 µg	6.8 µg	----
137	27 µg	13.5 µg	6.8 µg	50 µg

Table 4. The schedule for C-terminal peptide immunizations. The 1' (06-02-94) immunizations were IP emulsions in CFA. The 2' (06-11-94) and 3' (07-27-94) immunizations were IP emulsions in IFA. The pre-fusion injections (01-07-95) and fusions (01-12-95) were conducted at UCLA by myself and Benito Anton in the laboratory of Dr. Chris Evans.

(III). ELISA PROTOCOL

The ELISA protocol below was used for both serum titrations and MAb screenings.

Materials

96-Well ELISA Plates (1 X 12 Strip Wells)
Corning - Cat# 24121-12, Lot# 18292002
Flat-bottom polystyrene, tissue culture treated

ELISA Coating Buffer
50 mM $\text{Na}_2\text{H}_2\text{CO}_3$ pH9.6
Sigma Cat# S-4019 Lot# 50H-01358

pH adjustment using 50mM Na_2CO_3 (Mallinckrodt Cat# 7528)

High Salt PBS
1.25 L 4 M NaCl (Baker Lot#22364)
8.65 L dH_2O (distilled water)
0.10 L NaPi, pH 7.4
19 mL 1M NaH_2PO_4 (Fisher Lot# 926276)
81 mL 1M Na_2HPO_4 (Fisher Lot#922624A)
pH adjustment with 0.1 M HCl

Final Concentrations :

0.4 M NaCl
0.01 M NaPi

Non-Fat Milk(NFM) (Mixed at room temperature)
25 g Non-Fat Dried Milk (Carnation)
180 mL dH₂O

Final Concentration : 0.125 g/mL

ELISA Wash Buffer (PBS-BLOTTO)
95% High Salt PBS
5% NFM

Peptide Antigens
MAPS–N–terminal and KLH–C–terminal
Each assumed to be 1 mg/mL in PBS

HRP-conjugated Goat Antimouse Antibody
(TAGO Inc., Cat# 6440, Lot# 1703)
1/1000 dilution in PBS-BLOTTO

Sodium Citrate, 0.1 M pH4.2
14.7 g Sodium Citrate • 2H₂O (MW = 294.1)
(Baker Cat# 3646-1, Lot# 402346)
Dissolve in approximately 300 mL distilled H₂O
Adjust to pH 4.2 using 0.2 N HCl (approximately 100 mL)
Adjust final volume to 500 mL with distilled H₂O

Substrate Solution
10.0 µL 30% H₂O₂ (CMS Lot# M156 KHML)
0.5 mL 20 mg/mL ABTS* (Sigma Lot# A1888)
(Stock solutions stored at –20° C)
10.0 mL 0.1 M Sodium Citrate, pH 4.2

*ABTS : 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)

**H₂O₂ should be added last and just prior to use.

Note : All procedures done at room temperature unless otherwise indicated

Coating Procedure

Dilute antigen in coating buffer to 10 or 20 µg/mL (see Results), and then add 50 µL to each well of the 96 well plate. Seal plate with sealing tape from Baxter Healthcare (Cat# 430454). Incubate overnight at 4° C. Coated plates can be stored frozen after 24 hours.

Assay Procedure

- 1) Wash 2X with PBS-BLOTTO
- 2) Add 200 µL PBS-BLOTTO to each well
- 3) Incubate 2 hours at room temperature (RT)
- 4) Remove PBS-BLOTTO and wash 3X with PBS-BLOTTO
- 5) Add 50 µL cell culture supernatant or diluted serum to each well
- 6) Include 50 µL positive and negative controls in separate wells on the same plate
- 7) Seal and incubate plate overnight at 4° C
- 8) Remove antibody solutions and wash 3X with PBS-BLOTTO
- 9) Add 50 µL goat anti-mouse antibody to each well
- 10) Incubate 2 hours at RT
- 11) Remove antibody solutions and wash 3X with PBS-BLOTTO
- 12) Add 100 µL substrate solution to each well
- 13) Incubate 15 - 45 min. at RT (protect from light)
- 14) Record absorption at 405 nm using a Molecular Devices microplate reader

(IV). HYBRIDOMA DEVELOPMENT

Hybridoma development includes protocols for myeloma thaw, cell count and viability, thymocyte preparation, fusion, supernatant collecting, cell expansion, and well scoring. The fusion protocol was carried out according to the procedure described by Hockfield, et al. (43), except for the addition of a thymocyte feeder layer. Its purpose was to support hybridoma growth by lymphokine secretion. Another alternative would be the use of a macrophage feeder layer, which is also very effective in supporting cell growth. All protocols are described below.

Myeloma Thaw

Materials

Laminar Flow Hood
Sterile 15 mL centrifuge tubes
Sterile wide-bore pipette
Sterile 24-well(flat bottom) tissue culture plates
Falcon Cat# 3047
Dry Ice and styrofoam container
Sterile Complete Medium (SCM)* :
89% DMEM (Sigma Cat# 05036, Lot# 14H4617)
5% Fetal Calf Serum (Sigma Cat# F4010, Lot# 11H-0749)†
5% Fetal Clone (Hyclone Cat# A6165L, Lot# 61652012)†
1% L-Glutamine (Sigma Cat# G7513, Lot# 83H4685)

*Add fresh L-Glutamine every 14 days to unused medium

†Some formulations used lower percent of Hyclone product and higher percent of FCS (See Results).

Procedure (Sterile technique must be followed)

- 1) Remove vial from liquid nitrogen tank and transfer in dry ice to Tissue Culture Lab
- 2) Immediately thaw by swirling in a 37° C water bath
- 3) Once thawed, gently transfer cells into centrifuge tube and add 10 mL of 37° C SCM
- 4) Gently pipet up and down twice, in order to thoroughly dilute the cell freezing medium.
- 5) Pellet cells for 10 minutes at approximately 1000 rpm and resuspend in 1.0 mL of fresh SCM and tube to keep cells in suspension.
- 6) Immediately remove a 50 µL sample into a microcentrifuge tube for a cell count.
- 7) Adjust the concentration to approximately 5×10^5 cells/mL and plate. lower cell densities, such as 1×10^5 cells/mL or 2×10^5 cells/mL can be used for very stable cell lines. Place plate into incubator and check in 24 hours.

** SCM should be tested for contamination by pipeting 6-8 mL into a T25 test flask and then placing the flask and its contents into the incubator for a minimum of 24 hours.

Cell Count and Viability

Materials

Eppendorf Pipet
Sterile Pipet Tips
Light Microscope
Hemocytometer
0.4% Trypan Blue Stain(Sigma Cat#T-8154, Lot# 22H-4646)
96 Well Plate
Cell Suspension in a microfuge tube

Procedure

- 1) Cell suspension should be well dispersed. With the Eppendorf, pipet 20 μ L into one well of the 96 well plate.
- 2) With a new pipet tip, add 20 μ L of the trypan blue to the well containing the cell suspension aliquot and gently mix several times with pipet. Note: dilution factor in this case is 2.
- 3) Fill each side of the hemocytometer (cover slip in place) with approximately 10 μ L of the mixture. Use a dry, fresh pipet tip for each side when doing quantitative work.
- 4) Adjust the microscope and count both live(clear) and dead(blue) cells in all four corner grids. Note: volume of each grid is 10^{-4} mL.
- 5) Calculate cell concentration (cells/mL) with the following equation:

$$\frac{(\text{Number of Live Cells}) (\text{Dilution Factor})}{(\text{Number of Grids}) (\text{Volume of each Grid})}$$

- 6) Calculate percent cell viability with the following equation :

$$\frac{(\text{Number of Live Cells}) (100\%)}{(\text{Live Cells}) + (\text{Dead Cells})}$$

*See reference (33) for hemocytometer use.

Myeloma cells were thawed as described above and kept in a log growth phase ($2-5 \times 10^5$ cells/mL) for three days prior to fusion. Four T-75 flasks, each containing approximately 40 mL of cell suspension, were retained for the fusion.

The live cell count was 4.1×10^5 cells/mL in a total of 160 mL. Cell viability was calculated to be 96%.

Thymocyte and Thymocyte Conditioned Medium (TCM) Preparation

Materials

- SCM (equilibrated in a 5% CO₂ atmosphere at 37° C)
- 100X Penicillin-Streptomycin Medium (100X PS)
(Sigma Cat# P7539 Lot# 93H4644)
- Sterile PBS (1X)
(GIBCO Cat# 310-4190AK, Control# 11P8791)
- 96-Well tissue culture plates (20)
- Sterile petri dishes (12) (Falcon Cat# 1005)
- Sterile dissection tools
 - Scissors dedicated to skin
 - Scissors dedicated to intraperitoneal cavity
 - Two pairs of fine forceps
 - Dissection board and pins
- Sterile 50 mL centrifuge tubes(2) filled with 70% ethanol
- Sterile 50 mL centrifuge tubes(2) for thymus collection
- Sterile frosted-end glass microscope slides
- Sterile tea strainer
- Hemocytometer
- Two BALB/c mice, 4 - 6 weeks old
- Sterile 50% PEG solution
 - 5.0 g PEG-1500 (BDH Cat# 29575, Lot# 9160470D)
 - PBS - equal volume
- Autoclave all sterile materials. Liquids for 15 minutes (wet cycle) and instruments for 20 minutes (dry cycle).

For each preparation, two BALB/c mice were sacrificed and thymuses removed. Each thymus was washed in a series of five petri dishes containing 20 mL PBS. In the sixth petri dish also containing 20 mL PBS, the thymus capsule was broken open by pressing between the frosted ends of sterile microscope slides. The contents were transferred through the tea strainer into a 50 mL tube. An additional 10 mL PBS rinse of the sixth dish was also passed through the strainer and into the tube. The tubes were then spun at 1000 rpm for 10 minutes.

The pellet was resuspended in 10 mL SCM. For the fusion, the cell count was 2.2×10^8 cells/mL having a viability of 99%. A 3.0 mL aliquot of the cell suspension was diluted with 193 mL of 2XPS SCM that resulted in a final suspension of 3.3×10^6 cells/mL. This cell suspension was evenly distributed in 0.1 mL aliquots to twenty 96-well plates (thymocyte plates). Cells from the fusion were then plated together with the thymocyte cells by adding 0.1 mL/well of the fusion suspension. The final concentration of thymocytes was thus approximately 1.6×10^6 cells/mL.

The remaining cell suspension was used to make thymocyte conditioned media. Thymocytes were cultured at a concentration 4×10^6 cells/mL in SCM in sterile tissue culture flasks. The cells were incubated for 48 hours at 37° C with a CO₂ level of 5% and 100% humidity. Cells were removed by centrifugation at 1000 g for 10 minutes and followed by filtration through 0.2 µm sterile filters. The clarified TCM was stored at “-80° C” until needed for future feedings.

Fusion

N-terminal MAPS Peptide Antigen

Fusions for both N-terminal MAPS peptide immunized mice (114 and 115) were done side by side on the same day according to the protocol by Hockfield et al.,(49). Mice were sacrificed and their spleens removed and separately washed twice in DMEM. Splenocytes were released from spleens using frosted glass microscope slides as before (cf. thymocyte prep.), strained through a tea strainer, and transferred to individual sterile 50 mL centrifuge tubes. The volume of each tube was brought up to 20 mL using SCM and cell counts were determined. The cell count for 160 mL myeloma cell suspension was also determined. The results are shown below in Table 5.

Fusion Cell Counts

Mouse 114 Splenocytes	1.53×10^7 cells/mL
Mouse 115 Splenocytes	2.28×10^7 cells/mL
Myeloma Cells	4.10×10^5 cells/mL

Table 5. Cell concentrations for mouse splenocytes and myelomas prior to the fusion procedures.

The desired splenocyte to myeloma ratio is approximately 5:1 according to Hockfield, et al.,(50). Given the above cell counts, the following combinations of splenocytes and myelomas shown in Table 6 were prepared for the fusion.

Cells for Fusion

MOUSE (Number)	SPLENOCYTES (Cells)	MYELOMAS (Cells)
114	1.65×10^8	3.3×10^7
115	1.65×10^8	3.3×10^7

Table 6. Number of splenocytes and myelomas used in each fusion.

For each preparation, splenocytes and myelomas were combined to a volume of 50 mL in a new centrifuge tube using SCM. The cells were then washed three times in DMEM (37° C) by centrifuging and then resuspending. The purpose of washing is to remove serum which inhibits cell fusion. After the third wash, cells were not resuspended, but gently dislodged from the tube bottom by tapping the tube on the bench top in the hood. Cells were then fused by slowly adding (over a 30 second period) 1.0 mL of 37° C 50% PEG 1500 while gently agitating the tube. Immediately after PEG addition, 12 mL of 37° C of sterile PBS was added

over the time schedule below in Table 7 (50). Again, the tube was gently agitated to facilitate PEG dilution, however, care was taken not to disrupt the cell pellet.

After the PBS additions, the cell suspension was centrifuged at 1000 rpm for 5 minutes and the supernatant was removed without disturbing the pellet. Carefully, 10 mL of 37 °C SCM was layered on top of the pellet which was then incubated at 37 °C for ten minutes in an atmosphere of 5% CO₂. The elapsed time from removal of spleens from mice to incubation of hybridomas was approximately 140 minutes.

Time Schedule for PBS Addition

<u>Amount</u>	<u>Time</u>
1 mL	1 min
2 mL	1 min
3 mL	1 min
6 mL	1 min

Table 7. A total of 12 mL PBS was added to the fused cells over 4 minutes as outlined.

The pellet was then gently resuspended and split to two new 50 mL centrifuge tubes. An addition of 45 mL of 2X HAT (hypoxanthine, aminopterin, and thymidine) (with 2X Penicillin–Streptomycin (PS)) Medium (prepared using 2 mL 50X HAT (Sigma Cat# HO262, Lot# 13H9404) and 48 mL SCM) was made to each tube and gently mixed. Hybridoma suspensions from each tube (2 tubes/fusion X 2 fusions) were evenly distributed over 10 thymocyte plates at approximately 0.1 mL per well. Each well had final concentrations of 1X HAT, 1X PS, and approximately 1.6×10^6 thymocytes. All 20 plates were then transferred

to the incubator set at 5% CO₂ and 37° C. The elapsed time for hybridoma plating and transfer to the incubator was approximately 30 minutes.

One week post-fusion, the wells were fed with thymocyte conditioned medium (TCM) as follows: (1) half of the supernatants were removed from each well; (2) each well was then fed with 50% TCM (prepared by mixing equal volumes of clarified TCM with 2X HAT in sterile conditioned medium containing penicillin and streptomycin, each at 1X). Final concentrations of medium additives in hybridoma cultures were as follows: 25% fresh medium, 25% clarified TCM, 1X HAT, and 1X PS.

Two weeks post-fusion, the feeding medium was changed from (1X) HAT to (1X) HT (hypoxanthine and thymidine) (Stock 50X Sigma Cat# H0137, Lot# 112H9400). Final concentrations in the hybridoma cultures were as follows: 25% fresh medium, 25% clarified TCM, 1X PS, 1X HT, and aminopterin at (1/2)X. Each subsequent feeding dropped the aminopterin concentration 2-fold, while maintaining HT and PS at 1X and TCM at 50%.

Four weeks post-fusion, the medium was slowly changed over to straight SCM. That is, concentrations of HAT, PS, and TCM were diluted 2-fold with each feeding of fresh sterile conditioned medium containing no extra additions (SCM). Again, the procedure consisted of aspirating off 0.1 mL and then replenishing with 0.1 mL of new medium.

C-terminal Peptide Antigen

The C-terminal fusions were done at UCLA with Benito Anton in the lab of Dr. Chris Evans. The same protocol as described above was used, with the following exceptions: a macrophage feeder layer was used instead of the

thymocyte feeder layer. In addition, splenocyte and myeloma counts were omitted.

Collecting Supernatant for ELISA

Approximately one week post-fusion, hybridoma colonies began to appear in the wells. When the cells from each colony filled greater than 33% of the well, the supernatant was sampled for anti-peptide activity by ELISA. Sampling was done by removing an aliquot of 100 μ L of supernatant from the well. Care was taken to stay near the surface and away from the growing colony. In addition, a new pipet tip was used for each well in order to avoid any cross contamination. This aliquot was replaced with the same amount of fresh SCM.

Cell Expansion

Hybridoma colonies that had positive ELISA scores and covered at least 60% of the well, were expanded into a new well on a separate 96-well plate and fed with 50% TCM in SCM. Approximately half of the cells from the original well were transferred to the new plate. Cells were then split 1:1, meaning that to every 1 mL of cell suspension, 1 mL of fresh SCM was added every 1-3 days thereafter. Once 3-4 wells had been created, these wells could then be pooled and transferred to one well of a 24-well plate. Such expansion wells could be fed with an equal volume of SCM. Expansion cultures could be split 1:1 every 1-3 days thereafter. At the time of transfer, a back-up culture was maintained on the original 96-well transfer plate. An orange to yellow color medium indicated that cells were growing very fast. Neglecting immediate expansion resulted in cell death.

As soon as one culture well (on the 24-well plate) of the original antibody-secreting colony had been created, the culture was ready for subcloning (see below). Furthermore, as soon as 6 culture wells (on the 24-well plate) with 60% - 80% confluency were obtained, the colony was ready to freeze for storage (see below). The importance of subcloning and freezing as soon as possible cannot be over-emphasized (see Discussion).

Scoring Wells

Approximately 5–10 days after a fusion or subcloning, the plates were checked for colony formation as follows: plates were set up on an inverted light microscope that had been wiped down with 70% alcohol. Under low power, each plate was quickly scanned for colonies. The number of colonies per well was recorded in the notebook on an 8 X 12 matrix grid plate map as either mono (one colony), bi (two colonies), or poly (three or more colonies). This information was also recorded directly on the plate above each well using a dot coding system (e.g. one dot = monoclonal, etc.). When colonies filled greater than 33% of the well, they were numbered consecutively and assayed by ELISA.

ELISA readings indicating positive and negative clones were scored and recorded. On plate maps and actual cell plate covers, the numbers of the ELISA positive colonies were circled. Conversely, an "X" inscribed over wells indicated ELISA negative colonies.

(V). SUBCLONING

Cells were sub-cloned using thymocytes or macrophage as feeder layers. In most cases, parallel subclonings were done without feeder layers as a back-up in case of contamination in the primary cell cultures.

Macrophage Feeder Layer Preparation

Materials

Same as for thymocyte preparation except for the following additions:

Sterile 0.34 M sucrose (ice-cold)
Sterile 10 mL syringe (Becton Dickinson Cat# 309604)
22 gauge needles (Becton Dickinson Cat# 305155)

Procedure

The macrophage preparations were carried out according to the Cold Springs Harbor Laboratory manual (51). All aspects of the preparation were carried out under sterile conditions. Animals were dissected in one hood dedicated for animal work, and the rest of the preparation done in a second hood dedicated for sterile cell work. A dead-space hood was used for the former, whereas laminar flow filtered hoods were used for the latter. The protocol described below was used.

Each 3 to 6 month old BALB/c mouse is killed by cervical dislocation, pinned on a dissection board, and thoroughly washed with 70% alcohol. A midline cut is made through the skin that is pinned back to expose the peritoneum. Care must be taken not to puncture this thin, transparent muscle layer. Inject 5–8 mL, depending on size of mouse, of an ice-cold 0.34 M sucrose solution into the peritoneal cavity just below the hypophysis using a sterile 22 gauge needle. Hold the syringe perpendicular to body axis and puncture the peritoneum midline and just below the ribcage. The needle should be inserted

with the bevel pointing downward towards the base of the abdomen so that the sucrose can flow freely throughout the peritoneal cavity. The sucrose solution should be injected slowly.

Gently jiggle the expanded peritoneal cavity for about 20 seconds and then, with the needle bevel facing down near the level of the liver, slowly withdraw fluid. Transfer the 4–6 mL of collected fluid into a sterile 50 mL centrifuge tube, count the cells using a hemocytometer, and centrifuge at 1200 rpm for 5 minutes. Aspirate the supernatant being careful not to remove the transparent cell pellet. Dilute and resuspend in the appropriate ice–cold medium (SCM $\pm$ HAT or HT) to a volume of 20 mL per sacrificed mouse. This should be enough to evenly plate two 96–well plates. Plate a 96–well plate with approximately 0.1 mL of the cell suspension per well. The final macrophage concentration should be approximately $2\text{--}5 \times 10^4$ cells/well. It is noted that ice cold solutions should be used to prevent the macrophage from adhering to the tubes. In addition, cells should be plated within fifteen minutes of isolation from the animal, or significant loss via cell adhesion will occur. Feeder layers can be prepared, plated, and maintained at 37 °C in the cell culture incubator (5% CO₂ and 100% humidity) 1–2 days prior to their use. For best results, pool cell pellets from three or more mice prior to plating.

Subcloning Protocol

As mentioned in the introduction, the purpose of subcloning is to stabilize and maintain a hybridoma cell line as monoclonal. Subcloning was done by limited dilution as described in greater detail by the CSHL manual (52), and in abbreviated form below.

Procedure

Cells to be subcloned should be in log growth phase (2×10^5 to 5×10^5 cells/mL) as determined using a hemocytometer. A 1.0 mL stock solution in SCM was prepared at a concentration of 1×10^4 cells/mL. Using the stock solution, three sterile centrifuge tubes were prepared containing 10 mL SCM and cells at concentrations of 10, 50, and 100 cells/mL. One tube per 96-well macrophage plate (preparation described above) was plated with cells such that each well received approximately 0.1 mL of the diluted cell suspension. This resulted in three subcloning plates at densities of 1, 5, and 10 hybridoma cells/well. Each plate also contained 0.1 mL of either macrophage (at approximately 2×10^4 cells/well) or thymocytes (at approximately 2×10^6 cells/well). The plates were then transferred to a 37 °C incubator with a 5% CO₂ level and 100% humidity. Plates were checked for colony formation (see scoring monoclonal wells section) 5 days later. The clones were ready for assay 10–14 days after plating. All plates were checked for growth and plating density (see below) to ensure effective subcloning. Wells containing monoclonal cultures with high ELISA absorption readings were chosen for expansion and further subcloning. In order to increase the probability that each colony is monoclonal, an attempt was made to ensure that at least one plate of each round of subcloning had $\leq 30\%$ of the wells containing colonies. Thus, subsequent subclonings might be plated at 0.5 cells/well, 1.0 cells/well, and 2.0 cells/well. In addition, three rounds of subcloning were performed to further ensure that the cell line was monoclonal.

(VI). SUB-CLASS ELISA

In order to determine the type of Ig immunoglobulins produced by the hybridomas, sub-class analysis was conducted. The analysis screened for IgG₁, IgG_{2a}, IgG_{2b}, IgG₃, IgM, and IgA. The sub-class ELISA was carried out just as the previously described ELISA but with the addition of sub-class specific rabbit antibodies, followed by HRP-Goat anti-rabbit antibodies. After monoclonal supernatants from cell lines of interest were applied to the ELISA plate, and the plate washed as usual, 50 µL of rabbit anti-mouse secondary antibody was applied to the appropriate well and incubated at room temperature for two hours. The secondary rabbit anti-mouse antibodies used are listed below in Table 8. The plate was then washed three times with PBS-BLOTTO. The third antibody, HRP conjugated Goat anti-rabbit antibody (BIORAD Laboratories Cat# 172-1019, Control# 72248) was diluted 1:1000 in PBS-BLOTTO to a volume such that every well received 50 µL. The plate was then incubated at room temperature for two hours. The substrate solution and method of visualization were the same as the ELISA protocol described above.

Rabbit Anti-Mouse Antibody	
Antibody	BIORAD Catalogue#
IgG ₁	113564 A
IgG _{2a}	113571 A
IgG _{2b}	113572 A
IgG ₃	113573 A
IgM	113574 A
IgA	113575 A

Table 8. Secondary antibodies used to determine type of Ig immunoglobulins produced by the hybridomas.

(VII). BSA ELISA

The ELISA for BSA (bovine serum albumin) affinity was run according to the peptide ELISA protocol described previously, with the exception that the coating antigen was BSA. The BSA (Bovine Albumin Fraction V; Metrix Lot# M46105) was prepared in ELISA Coating Buffer as a 20 µg/mL solution and plated at 50 µL per well.

(VIII). MYCOPLASMA TESTING

Each cell line was tested for the presence of mycoplasmal contamination. The mycoplasma assay used was the Hoechst 328 fluorochrome DNA staining test conducted by Bionique Testing Laboratories (phone : 518-891-2356; fax : 518-891-5753) in Saranac Lake, New York. Cells from each cell line were grown for 48 hours in the absence of antibiotics, harvested, and then fixed to special slides (provided by Bionique) using Carnoy's fixative (1 part glacial acetic acid plus 3 parts absolute methanol) as described below.

A 2 mL cell suspension was incubated at room temperature for one hour on the provided Bionique microscope slide. The Bionique slide had been pretreated to promote cell adhesion. The medium was removed, and the slide completely submerged in Carnoy's fixative (approximately 20 mL). The slide incubated at room temperature for 5 minutes. The fixative was removed and replaced with fresh fixative and allowed to incubate with the slide for an additional ten minutes. The slide was removed, allowed to air dry, and sent to Bionique for

analysis. Samples were processed within 48 hours of receipt. Mycoplasma positive cultures would be revealed by the presence of fluorescent dots of genetic material in the cell cytoplasm under 100X oil immersion fluorescence microscopy.

(IX). CELL FREEZING AND THAWING

The materials used in both freezing and thawing were similar to those used in myeloma thaw as described above. The only additional component was the Cell Freezing Medium which is a mixture of 90% fetal calf serum (FCS) and 10% dimethyl sulfoxide (DMSO).

Freezing Protocol

Four to six culture wells of one cell line from a 24–well plate were harvested when such cultures were greater than or equal to 60% confluency. Cells were harvested either by gentle resuspension with a polished tip glass pipet or mini cell scraper, collecting 50% to 80% of the cell suspension from each well. Cells were centrifuged at 1000 rpm for 10 minutes, and the supernatant saved for ELISA analysis and immunohistochemical analysis. The cell pellet was resuspended in 0.1 to 0.2 mL cold Cell Freezing Medium at a density greater than or equal to 10^6 cells/mL. The cell suspension was then immediately transferred to a pre-labelled cryogenic vial. The vials were immediately packed on ice, and then transferred within 15 minutes to an insulated container (styrofoam vial rack) to a -80°C freezer. After 2–3 days, vials were transferred over dry ice to a liquid nitrogen cell bank and maintained in vapor phase of the tank.

Thawing Protocol

The cell vial was removed from liquid nitrogen tank, transferred over dry ice to the cell culture laboratory, and immediately thawed by swirling in a 37° C water bath. Once thawed, the cells are transferred into a sterile 15 mL centrifuge tube containing 10 mL SCM (equilibrated to 5% CO₂ in an incubator). The cells are then centrifuged at 1000 rpm for 10 minutes and then resuspended in 0.5 mL fresh SCM. An aliquot is removed for a viable cell count. The cells are then diluted with SCM to an approximate concentration of 5×10^5 live cells/mL and plated in a sterile 24–well plate at 0.5, 1.0, and 2 mL of cell suspension per well. The cultures are topped up with fresh SCM to a total volume of 2.0 mL/well, resulting in cell densities of 1.25, 2.5, and 5×10^5 cells/mL. If insufficient cells are present for plating at all three densities in a 24–well plate, a 96–well plate should be used to plate cultures with 50 µL, 100 µL, and 200 µL/well, again topping up each well with fresh SCM to 0.2 mL/well. If possible, thymocytes and/or macrophage should be used as helper cells, especially if hybridoma viability is low ($\leq 50\%$ viable). The plate is then placed in the incubator and checked the next day for growth. Cultures are expanded by 1:1 dilution with fresh SCM every 1–3 days.

(X). IMMUNOHISTOCHEMISTRY

All immunohistochemical studies were performed by Dr. Evans' lab at UCLA. Free floating unfixed rat brain cryostat sections were washed in 1X PBS (pH 7.4) and then blocked with 10% goat serum in 1X PBS (pH 7.4). Sections were then incubated in monoclonal antibody supernatant for 24 hours at 4° C with constant rotation. After three washes in 3X PBS (pH 7.4), sections were

incubated with the secondary antibody in 5% goat serum and 1X PBS (pH 7.4) for one hour at room temperature. The secondary antibody was a goat anti-mouse IgG (H + L) HRP antibody used at a 1:1500 concentration. The sections were washed four times in 1X PBS (pH 7.4) and then stained with 0.03% 3,3'-diaminobenzidine tetrahydrochloride (Sigma, Cat# D5637) and 0.3% nickel sulfate (Sigma, Cat# N4882) for three minutes followed by the addition of 0.001% hydrogen peroxide for five minutes. Sections were incubated in dH₂O for five minutes to stop the enzyme reaction. Sections were then mounted on slides, air dried, and observed under a light microscope.

RESULTS

(I). ELISA PLATE COATING CONCENTRATION

Initially, immune sera were used to determine that a 20 µg/mL coating of MAPS antigen was sufficient to obtain good signal : background ratios (See Figures 10a and 10b). Subsequently, monoclonal antibody supernatant was used to determine the minimal amount of antigen required to obtain a satisfactory signal. The antibody used came from hybridoma colony M1.24 obtained in the MAPS fusion. As illustrated by Figure 9, a decrease in the antigen coating concentration produced a subsequent decrease in ELISA sensitivity. Due to having a low amount of antigen, we were forced to coat plates at 10 µg / mL rather than the previously used 20 µg / mL. This resulted in an approximate 25% loss in signal.

Figure 9. Determination of ELISA sensitivity as a function of antigen concentration.

(II). ANIMAL SELECTION FOR FUSION

N-terminal MAPS Immune Sera :

All mice immunized with the N terminal-MAPS were screened for antibody titer using ELISA techniques. Ultimately, mice #114 and #115 were chosen for fusion since a yeast contamination developed in #110 and #112 fusions. The data is tabulated in Table 9 and graphed in Figures 10 and 11. Pre-immune sera was collected, pooled, and used as negative controls in duplicate wells. It was discovered that the negative controls along the edge of the plate were unusually high. That is, early assays had high levels of color development in negative control wells placed on the borders ("edge") of the 96-well ELISA plate as shown in Table 9. The corner well had the highest value (0.771 AU), the next highest

were the edge wells (0.413 ± 0.70 AU), and the smallest negative control values came from the interior wells (0.259 ± 0.022 AU, where the data point 0.466 AU was thrown out). Note that the last column of numbers (column 6 in Table 9) represent wells running down the center of the ELISA plate. The edge effect may have been due to excessive evaporation of 50 μ L samples during 2 hour, room temperature incubation of secondary antibody, resulting in sticking of protein to side walls. Alternatively, excessive light hitting edge wells may have increased spontaneous conversion of ABTS substrate to colored reaction product. The problem was corrected by: 1) placing test samples in interior wells, 2) filling edge wells with water, 3) sealing the plate tightly, 4) incubating in a moistened tupperware box, and 5) developing away from direct light (e.g in a drawer or covered with tinfoil).

N-terminal Mice Titer ELISA: Raw Data							
Plate Column :		1	2	3	4	5	6
Plate Row	Mouse#	DILUTION FACTORS					
		250	750	2250	6750	20250	60750
Absorbance Units (AU)							
A	109	0.329	0.378	0.493	0.382	0.208	0.168
B	110	0.916	0.412	0.231	0.205	0.219	0.166
C	112	0.890	0.552	0.312	0.204	0.220	0.174
D	129	0.710	0.283	0.361	0.158	0.262	0.269
E	114	0.476	0.315	0.332	0.286	0.208	0.150
F	115	0.499	0.333	0.278	0.302	0.177	0.208
G	Neg.Cntrl	0.403	0.283	0.231	0.466	0.254	0.229
H	Neg.Cntrl	0.771	0.450	0.418	0.358	0.533	0.315

Table 9. ELISA absorbance data on N-terminal immunized mice sera. A high absorbance response indicates antibody presence. Negative controls are pooled pre-immune sera.

Figure 10. ELISA absorbance on antibody titer for mice : 110, 112, 114, 115, and pre-immune. Antigen was N-terminal MAP peptide.

Figure 11. ELISA absorbance on antibody titer for mice# 109, 129, and pre-immune Note: Signal from #109 sera may be edge effect. (See Table 9 and text for explanation).

C-terminal KLH Immune Sera

The ELISA data for the C-terminal immunized mice is tabulated below in Table 10 and graphed in Figures 12 and 13. This data comes from the same plate as N-terminal data, except C-terminal wells were in plate columns 7–12. This section of the plate was coated with 50 μ L of the peptide coupled to KLH at 20 μ g/mL. The absorbance value of the corner well in this case was much lower than the that of N-terminal assay. The edge wells of the negative control had an average value of 0.362 ± 0.087 and the interior negative control wells had an average value of 0.257 ± 0.065 .

C-terminal Mice Titer ELISA: Raw Data

Plate Column :								7	8	9	10	11	12
Plate Row	Mouse#	DILUTION FACTORS											
		250	750	2250	6750	20250	60750						
		Absorbance (AU)											
A	103	0.374	0.334	0.230	0.238	0.271	0.271						
B	104	1.830	0.726	0.380	0.243	0.360	0.336						
C	106	0.661	0.278	0.301	0.192	0.338	0.204						
D	135	0.476	0.258	0.266	0.212	0.215	0.252						
E	136	1.482	0.636	0.246	0.201	0.191	0.257						
F	137	1.645	0.638	0.348	0.214	0.270	0.248						
G	Neg.Cntrl	0.198	0.382	0.259	0.251	0.222	0.230						
H	Neg.Cntrl	0.354	0.429	0.494	0.376	0.287	0.377						

Table 10. ELISA absorbance data on C-terminal immunized mice sera. A high absorbance response indicates antibody presence. Negative controls are pooled pre-immune sera.

Figure 12. ELISA absorbance on antibody titer for C-terminal peptide immunized mice# 103, 104, and 106 (low dose).

Figure 13. ELISA absorbance on antibody titer for C-terminal mice# 135, 136, and 137 (high dose).

(III). MAb POSITIVE HYBRIDOMA CLONES

Following the cell fusion for mice #114 and #115, a total of 162 different hybridoma colonies were obtained and tested for antibody to the N-terminal peptide by ELISA. Over 95% (A total of 156 out of 162 colonies) of these colonies were from mouse #115. This result is consistent with the larger spleen size of mouse #115, and the relatively large splenocyte count (5×10^8 cells versus 3×10^8 cells) (cf. pg. 39). A normal mouse (non-immunized) splenocyte content is approximately 10^8 cells. The supernatants from 21 out of the possible 162 hybridoma colonies were positive in ELISA. All of these anti-peptide positive colonies were from mouse #115. Since a total of ten 96-well plates were used for each mouse fusion, approximately 15% of the wells (156/960) from mouse #115 fusion produced hybridoma colonies. Approximately 2% of the wells (21/960) from mouse #115 resulted in hybridomas that were producing anti-peptide antibodies.

The ELISA results, summarized below in Figure 14, show the positive colonies, averaged negative colonies, and negative controls for all three assays. The negative controls for each ELISA were 1XHAT in SCM. Table 11 summarizes the positive clones and their associated negative controls. Though negative controls were run in only one well for each plate (therefore averages and standard deviations could not be calculated), a significant observation was noted. That is the negative control for assay #1 was in an edge well, while negative controls for assays #2 and #3 were in the interior and surrounded by wells filled with water. The negative control for assay #1 had an absorbance of

more than twice those found in assays #2 and #3. Furthermore, the average background signals for assays #2 and #3 were lower than for assay #1. Table 12 summarizes data for assays #1, 2, and 3 concerning ELISA negative colonies. Many of the positive cultures were expanded and subcloned. The supernatants were retested in subsequent peptide ELISA, BSA ELISA, and sub-typing ELISA. Also, aliquots were sent to UCLA for immunohistochemical analysis.

Figure 14. Raw ELISA results for all positive colonies, negative controls, and negative colonies. ELISA positive colonies are numbered and shown as individual scores. ELISA negative colonies' scores are shown as the average for each assay ("neg. clone 1, neg. clone 2, and neg. clone 3). The negative controls are shown as the individual score for each assay. Plates were coated with antigen at a concentration of 10 μ g/mL. Negative controls were 1X HAT in SCM in each assay. **Note:** See text for explanation of higher assay #1 negative control.

Assay and Positive Colony Code Number

Assay Number		
1 (08 - 14 - 94)	2 (08 - 14 - 94)	3 (08 - 18 - 94)
4	104	123
21	108	139
44	110	140
49	111	142
61	112	144
85	113	
89	117	
90	121	

Table 11. The three assays done for N-terminal peptide are tabulated with their corresponding well numbers that produced positive ELISA signals, and therefore have colonies producing antibody. All plates were coated with antigen at 10 µg/mL.

Average Absorbance for Negative Colonies of all Assays

Negative Colonies	Assay #1	Assay #2	Assay #3
	Absorbance (AU)		
All	0.179 ± 0.028	0.105 ± 0.008	0.090 ± 0.005
Border	0.183 ± 0.030	0.103 ± 0.002	0.090 ± 0.005
Interior	0.176 ± 0.027	0.105 ± 0.008	0.090 ± 0.005

Table 12. Average absorbances for colonies not producing antibody based on very weak ELISA responses of all three assays. **Note:** "Border" on assay #1 is true edge of plate. For assays #2 and #3, "borders" are adjacent to true edge wells filled with water.

Only assays #2 and #3 had water in wells surrounding the sample wells. Assay #1 was the only assay done that had samples placed along the true edge of the plate. Comparing assay #1 data to assays #2 and #3, it is apparent that surrounding sample wells with water and keeping everything tightly sealed, not

only lowers negative wells but also decreases the standard deviation magnitudes. In either case, values were not significantly different between border and interior wells. It is noted that all three assay plates were coated at 10 $\mu\text{g/mL}$.

Figure 15 shows corrected data (signal of positive colonies minus negative control average signal) for each assay (#1, #2, and #3).

Figure 15. First ELISA results for corrected positive colonies. Negative controls were subtracted from raw data as shown in Figure 14.

(IV). CLONE CHARACTERIZATION

Immunohistochemistry

Supernatants from all colonies that were consistently found to have positive ELISA absorbance signals, were sent to the UCLA lab of Dr. Evans for immunohistochemical (IHC) analysis. The tissue samples used in the analysis were rat brain sections. Out of a total of 17 different cell lines showing

consistently strong ELISA positives, only seven cell lines showed varying staining levels in IHC analysis. The three levels were relative to background staining and done by eye. This background staining was simply the brain sections in the presence of all reagents with the exception that supernatant was replaced with straight sterile conditioned medium. In addition to clone characterization, the results of IHC analysis were used as a criterion in determining which clones were to be further pursued. Supernatants found positive in IHC analysis, along with their determined immunoglobulin subclass as described below, are summarized in Table 13.

Clone Characterization		
CLONE	SUBCLASS	IHC *
M1.144	IgG1	+++
M1.44	IgG1	+++
M1.108	IgG1	++
M1.123	IgG1, IgG2b	++
M1.49	IgM	++
M1.89	N.D.	+
M1.85	IgG1, IgA	+

Table 13. Clones that were found positive in IHC analysis were further characterized by determining their immunoglobulin subclass.

IHC* : ImmunoHistoChemistry

+++ HIGH
 ++ MODERATE
 + LOW
 N.D. NOT DONE

The ten colonies that were found positive by ELISA, but negative in the immunohistochemical assays, are shown in Table 14. These cultures were subsequently not tested for immunoglobulin subclass.

Negative Immunohistochemical Clones			
M1.23	M1.90	M1.112	M1.140
M1.24	M1.104	M1.113	
M1.61	M1.111	M1.139	

Table 14. Tabulated clones that were found positive by ELISA, but negative by IHC. These colonies were not tested for immunoglobulin subclass.

The use of immunohistochemical analysis was found to be an important aspect of the screening process. The results identified ten clones producing antibodies that did not recognize the native conformation of the epitope. Therefore, only additional supernatants from M1.144 and M1.44 were collected and sent to UCLA for further IHC analysis. The best signal to noise ratio was seen with clone M1.144.X (see subcloning section for its derivation). Consequently, all future work was done with this isolate. Supernatants from subsequent rounds of subcloning were then sent to UCLA. Identical staining patterns were seen with M1.144, M1.144.X, M1.144.35 (original isolate and two independent derivatives; see subcloning section for descriptions of derivatives). The results of IHC analysis are presented in Figures 16–18. These figures are a small sample of the staining patterns found in rat brain sections. The darkened regions are the areas recognized by the antibody. Note the heavy staining patterns located mainly in long processes and dendritic fibers. See figure heads for greater descriptions.

Figure 16A. Distribution of ORL1 immunoreactive material in olfactory areas from rat brain slices. ORL1 is highly expressed in the anterior olfactory nuclear complex (A) in most of its major subdivisions such as the pars ventral (B). AON = anterior olfactory nuclei; AOV = anterior olfactory nucleus, pars ventral.

Figure 16B. In the primary olfactory cortex (pyriform cortex) (C), ORL1 is distributed in a dense network of highly reactive fibers in the superficial layers of this cortical area. High magnification is used for frame (D). Pi = primary olfactory cortex.

Figure 17A. Immunohistochemistry for ORL1 in the rat hippocampus. There are three frames presented on this and the following page. Note the high expression of ORL1 throughout the hippocampal fields CA1–CA4 and dentate gyrus (A). The distribution of ORL1 (B) is predominantly segregated in many dendritic puncta and long fiber processes through hippocampal layers of the stratum oriens (OR), stratum pyramidal (Py), stratum radiatum (Rad) and stratum lacunosum moleculare (L Mol) of the CA1–CA4 fields. In the dentate gyrus area (DG, see high magnification in C), ORL1 is highly expressed in many fine dendritic reactive puncta and fiber processes in the stratum granulosum and stratum moleculare (Mol) as well as in scattered pyramidal shaped cell bodies in the hilus dentate (hil).

Figure 17B. High magnification of the hippocampus sections. See figure head on previous page for further description.

Figure 18. Immunohistochemistry for ORL1 in the rat parietal cortex. Note the very dense network of heavily stained long fiber processes throughout the cortical layers II-V. Less abundant stained fibers are observed in layer VI.

The ORL1 is highly expressed in the CNS and is compared to the distribution of μ , δ , and κ opiate receptors in Table 15.

Opiate Receptor Loci of the Rat Central Nervous System				
<u>Brain Section</u>	<u>Opiate Receptor Type</u>			
	μ	δ	κ	ORL1
<u>Telencephalon</u>				
Frontal Cortex	+++	++	+	XXX
Pyriform Cortex	++	++	++	XXX
Neo Cortex	+++	+++	+	XXX
Amygdala	+++	+++	+++	XX
Hippocampus	+++	++	+	XXX
Dentate Gyrus	+++	+	+	XXX
Anterior Olfactory Nucleus	++	+++	++	XXX
Caudate Putamen	+++	+++	+++	XX
<u>Diencephalon</u>				
Hypothalamus	+	+	++	XXX
Thalamus	+++	+	++	XXX
<u>Mesencephalon</u>				
Midbrain	+++	+	++	XXX
<u>Metencephalon</u>				
Pons/Medulla	+++	+	++	XXX
<u>Spinal Cord</u>				
Ventral Horns	+++	++	++	XXX
Dorsal Horns	+++	++	++	XXX

Table 15. Comparison of μ , δ , κ , and ORL1 opiate receptor distributions in rat central nervous system structures. Note that μ , δ , and κ opiate receptor distribution is based on autoradiography studies, whereas ORL1 distribution is based on IHC.

Low (+), Moderate (++), Dense (+++). (Adapted from reference 7).
Moderate (XX), Dense (XXX).

Ig Sub-Class and BSA Binding

Supernatants from positive IHC assay cultures were then tested for immunoglobulin subclass, as shown in Table 16. SCM background was

subtracted and graphed in Figure 19. Supernatants were also assayed for non-specific binding to generic protein (BSA binding). All BSA and sub-class ELISA tests were done on supernatants originating from the same hybridoma colony in parallel assays on the same day (10-24-94). In other words, a sufficient amount of supernatant was collected at one time such that both assays could be done in parallel. It is clear that there is low level of anti-BSA activity present in the immune serum from mouse #112. Certain hybridoma cultures also show low levels of BSA reactivity. Perhaps significantly, the lowest level of BSA binding was seen with the hybridoma that gave the best IHC signal to noise ratio (M1.144).

ELISA Data for Subclassed Clones

Clone #	BSA	MAP Peptide			
		IgG1	IgG2b	IgM	IgA
	Absorbance (AU)				
M1.144	0.084	0.125	0.084	0.102	0.090
M1.44	0.099	0.138	0.092	0.089	0.095
M1.108	0.101	0.108	0.087	0.096	0.089
M1.123	0.114	0.148	0.103	0.123	0.100
M1.49	0.087	0.110	0.090	0.147	0.107
M1.85	0.141	0.211	0.103	0.115	0.133
S-112	0.132	3.068	0.190	1.468	0.138
SCM	0.080	0.088	0.094	0.094	0.115

Table 16. Immunoglobulin subclassing of cultures that tested positive in IHC assays. Supernatants were also tested for non-specific binding to BSA (bovine serum albumin). Absorbance signals were taken at 405 nm. The positive control is sera taken from immunized mouse #112 (S-112).

Figure 19. Antibody isotyping for positive immunohistochemical assays. The positive control (S-112) came from immunized mouse #112. Note that some of the data for S-112 goes off-scale.

In order to conclusively distinguish between specific and non-specific binding, MAPS peptide and BSA ELISAs were run in parallel using supernatant from a set of M1.144.X subclones. The MAPS peptide ELISA results shown in Figures 20 and 21 indicate high binding for 45 subclone samples from M1.144.X. In fact, some of the samples were found to be off-scale and out of the linear range. These samples were also run on a plate coated with BSA at a concentration of 20 $\mu\text{g/mL}$. The results of BSA analysis shown in Figures 22 and 23 indicate very low reactivity for the same 45 subclone samples from M1.144.X. All samples run on BSA plates show absorbances at background levels. All ELISA data was collected on the same day (9/29/94) and assayed under similar conditions.

Figure 20. ELISA for MAPS peptide in M1.144 subclone samples #1–23 to determine specific versus non-specific binding. Absorbances for samples #6, 13, 18, and 23 are off-scale and out of the linear range.

Figure 21. ELISA for MAPS peptide in M1.144 subclone samples #24–45, S-112 (sera from mouse #112), and SCM to determine specific versus non-specific binding. S-112 is a positive control and SCM is the negative control. Absorbances for samples #24, 27, 30, 32, 33, and S-112 are off-scale and out of the linear range.

Figure 22. ELISA absorbances for BSA in samples #1–23 for the purpose of determining specific versus non-specific binding. ELISA was conducted on 9/29/94. All samples are different subclones originating from M1.144.

Figure 23. ELISA absorbances for BSA in samples #24–45, S-123 (sera from mouse #123), and SCM for the purpose of determining specific versus non-specific binding. S-123 is a positive control and SCM is the negative control. ELISA was conducted on 9/29/94. All samples are different subclones originating from M1.144.

Mycoplasma Testing

Contamination with mycoplasma is a relatively common occurrence. This organism produces large amounts of nucleoside phosphorylase which can alter nucleic acid metabolism in the cultured cells and lead to cell death (51). Bionique Laboratories reported that all cell lines were free of mycoplasma.

(V). SUBCLONING

The method of subcloning used was by limited dilution (52). The goal is to dilute the cells into wells of a 96-well plate at a density of 0.1 to 0.3 cells/well, and to have a high probability that each cell will live and multiply (53). Thus, for each 96 wells plated, some 10 to 32 clones would grow. According to Poisson analysis, the observation of 70% of the wells (or more) showing no clones (the null set) would indicate a high probability that the thirty wells which did contain clones were single hit events, that is, monoclonal (see Appendix) (54). Since we did not know what to expect in terms of how well a single cell would survive and multiply (plating efficiency), a range of dilutions were plated. In addition, macrophage cells and/or thymocytes were added as a feeder layer as a source of lymphokines.

Three hybridoma colonies (M1.144, M1.44, and M1.49) were subcloned once each (See Table 23). Based on clone characterization data (see above), clone M1.144 was chosen for further extensive subcloning.

The first subcloning (08 - 30 - 94) of M1.144 consisted of three 96-well plates having densities of 2.5, 5, and 10 cells/well. All three plates had a

thymocyte feeder layer at a density of 1×10^6 cells/well. Nine days later, the plates were examined under dissection microscope for number of wells containing single colonies (monoclonal). Plates were also examined for wells containing two or more colonies (polyclonal). The results of plate analysis are shown below in Table 17. Note that the Poisson predicted value for number of monoclonal cultures is 34 (see Table 17 legend), which is reasonably close to the observed value of 37.

At the time of subcloning, the original M1.144 colony had been in culture for 43 days since fusion date (07 - 18 - 94). Theoretically, it should have been subcloned several weeks earlier, but we were too busy assaying, feeding, and freezing primary cultures.

M1.144.X Plate Data and Analysis

Plate Density (cells/well)	Mono- clonal (#)	Poly- Clonal (#)	Null Set (#)	P _i
2.5	37	23	36	35%
5	20	≥ 8	N.A.	N.A.
10	5	91	0	0

Table 17. Plate density refers to cells plated per well (theoretical). P_i = probability that any well is monoclonal. N.A. = not analyzed. Thus, for 96 wells, 35% = 34 wells should contain monoclonal cultures.

Samples were collected for ELISA. The SCM negative control was run only once and had an absorbance value of 0.108 AU. The background due to the negative control was subtracted from the resulting samples which are shown below in Figures 24, 25, and 26. All monoclonals are shown in Figures 24 and 25, while polyclonals are shown in Figure 26. The percent antibody-positive clones was estimated by dividing the number of monoclonals with ELISA absorbance signals greater than 0.25 AU by the total number of monoclonals

assayed. Thus, percent positive putative monoclonal MAb clones on this first round of subcloning was 88%, indicating that the line was fairly stable from the beginning. The SCM used for this round of subcloning was 5% fetal calf serum with 5% Hyclone Fetalclone growth factor (see below for discussion of Hyclone effects). Supernatant was collected from the hybridoma colonies and then assayed by ELISA 10 days after plating. Four of the strongest absorbance readings were associated with the M1.144.X subclones labeled as 29, 40, 41, and 42. The subclones were all monoclonal except for number 42 which was observed as being polyclonal. Rather than expanding and subcloning each one individually, all subclones were pooled (in error) together. This pooled collection of clones were expanded and frozen as M1.144.X. See Table 23 on page 86 for a complete record of frozen clones.

Figure 24. M1.144.X subcloned ELISA absorptions from 2.5 cell/well plate (monoclonals).

Figure 25. M1.144.X subcloned ELISA absorptions from 5 and 10 cell/well plates (monoclonals).

Figure 26. M1.144.X polyclonal cultures.

In a repeated attempt to isolate a true monoclonal derivative culture, two sets of M1.144 subclonings were done from a thawed vial of frozen cells made from the original M1.144 culture. Thawing was done on 11/10/94 and subcloned on 11/15/94. Again, dilutions used were 2.5, 5, and 10 cells/well. In both sets, macrophage feeder layers were used. Seven days after subcloning, the plates were checked for the presence of hybridoma cell colonies. Wells were described as either monoclonal (one colony present), biclonal (two colonies present), or polyclonal (three or more colonies present). Plating efficiency was calculated from the total number of monoclonals plus 2X biclonals plus 3X polyclonals divided by the number of cells plated. The calculated plating efficiency is therefore only an approximation. The data is summarized below in Table 18. Note that the large number of colonies found in the polyclonal wells at the higher plating densities prevent accurate estimation of plating efficiency. However, the high number of colonies present suggested the need to plate future subclonings at lower cell densities to further ensure the probability of isolation of a true monoclonal derivative.

M1.144.X Second Set of Plate Data and Analysis

Set #	Plate Density (cells/well)	Mono-clonal (#)	Bi-clonal (#)	Poly-clonal (#)	Plating Efficiency (%)	Null Set (#)
1	2.5	36	19	6	38%	35
	5.0	32	17	9	19%	38
	10.0	26	15	52	22%	3
2	2.5	32	23	9	44%	32
	5.0	21	35	16	29%	24
	10.0	2	8	81	27%	5

Table 18. M1.144.X second set of data for the types of colonies found (mono, bi, and poly) in each well, plating efficiency, and null set values. Note that the plate density at 2.5 cells/well in set #1 has a null set value of 35 which corresponds to a 37% probability that any well is monoclonal. Thus, for 96 wells, 37% = 36 wells should contain monoclonal cultures.

A total of 80 putative monoclonal colonies were sampled from the 2.5 and 5.0 cells/well plates of both sets. Both sets of monoclonals were numbered #1–40 and from either set #1 or 2. Both sets were transferred to one ELISA plate and therefore analyzed together. Figures 27 and 28 are ELISA data with the background subtracted. The negative control (SCM) was run only once and had an absorbance value of 0.088 AU. Again, the percent of positive MAb clones was fairly high (91%), and therefore indicative of stability retention. The subclones (11/15/94) were plated in 5% fetal calf serum with 5% Hyclone Fetalclone growth factor, and assayed by ELISA (11/15/94) 10 days after plating.

Figure 27. ELISA data for (#1-40) monoclonals chosen from the first set of M1.144.X subclone plates.

Figure 28. ELISA data for (#1-40) monoclonals chosen from the second set of M1.144.X subclone plates.

Clone number 35 from the second set was chosen for further expansion and subcloning after twentyseven days in continuous culture from single cell stage. This clone is named M1.144.35. Any subcloning of this cell line is named M1.144.35.X. At the time of subcloning, the culture of M1.144.35 had a cell count of 1.8×10^5 c/mL and 85% viability. Appropriate dilutions were made for two sets of plates at densities of 0.5 and 2 cells/well. Each plate had a macrophage feeder layer. Eight days later, the plates were checked for monoclonal colonies. The results displayed below in Table 19 show an average plating efficiency of 11%. In addition, it was noted that few biconals, and even fewer polyclonals were present on the low density plates. This result suggests that all monoclonal colonies were, in fact, probably true monoclonals. This conclusion is further supported by

Poisson analysis (see Appendix). The results also indicate that lowered plate densities may have been the cause of low plating efficiencies.

M1.144.35.X Plate Data and Analysis

Set (#)	Plate Density (cells/well)	Colonies (Total #)	Mono-clonal (#)	Plating Efficiency (%)	Null Set (#)
1	0.5	5	3	10%	92
	2.0	27	23	14%	71
2	0.5	6	6	12%	90
	2.0	17	15	9%	80

Table 19. The two subcloning sets of M1.144.35. The total number of colonies, monoclonal colonies, plating efficiency, and null set values are included. Note that the predicted number of monoclonal cultures by Poisson analysis agrees well with the observed frequency. See Appendix for explanation of Null Set significance.

A total of 51 clones were assayed using ELISA. This number was a combination of all monoclonals found in both sets. The remaining biclonal colonies were sampled from the first set. All polyclonal colonies were excluded from ELISA analysis. The results of monoclonal analysis is shown below in Figure 29. The SCM negative control (0.148 AU) was subtracted from the collected data. Note that the percent of strong positive MAb clones is lower than previously calculated (40% vs. 89% average of previous two assays). In this round of subcloning, cells were plated in 7% fetal calf serum and 3% Hyclone Fetalclone growth factor, and assayed 13 days after plating. The lower percentage of growth factor and the length of time between the assay date and plating date, have both been suggested as possible sources that influence cell growth, and therefore antibody production. Clone #5 was chosen for further

subcloning because it was from a monoclonal colony and had the second highest ELISA response.

Figure 29. ELISA data (1/17/95) for M1.144.35.X monoclonals. In this round of subcloning it is important to note that the amount of growth factor in the medium was 3%. The subcloning was done 1/4/95.

The third and final round of subcloning was conducted on M1.144.35.5 at cell densities of 1 and 2 cells/well. Again a macrophage feeder layer was included. Prior to subcloning, cells had a viability of 72% and a density of 1.4×10^5 cells/mL. Twelve days later the two plates were examined for colony formation. This data shown in Table 20 had an average plating efficiency of 33% which was a marked improvement from the previous subcloning. It is suggested that this is a result of increased plate densities. Clones #17 and #28 being

monoclonal colonies and having a high ELISA response, were selected for expansion and freezing.

M1.144.35.5.X Plate Data and Analysis

Plate Density (c/w)	Colonies (Total #)	Mono-Clonal (#)	Plating Efficiency (%)	Null Set (#)
1.0	35	19	36%	77
2.0	60	24	31%	72

Table 20. A single subcloning set of M1.144.35.5.X. The total number of colonies, monoclonal colonies, plating efficiency, and null set values are included. Note that the predicted number of monoclonal cultures by Poisson analysis agrees well with the observed frequency. See Appendix for explanation of Null Set significance.

The final round of subcloning ELISA data is shown below in Figures 30 and 31. The percentage of positive MAb clones (59%) slightly higher in comparison to the previous round of subcloning. In this round of subcloning (1/29/95), plating medium was 7% fetal calf serum and 3% Hyclone Fetalclone growth factor, and ELISA scoring was done 12 days after plating.

Figure 30. ELISA absorbance values (2/10/95) for M1.144.35.5.X monoclonals.

Figure 31. ELISA absorbance values (2/10/95) for M1.144.35.5.X biclonals.

(VI). CELL LINE STABILITY

The final step in hybridoma technology was to determine the stability of the hybridoma cell line. Due to variation in percent positive monoclonal cultures (by ELISA) in successive rounds of subcloning, the stability of the cell line was questioned. To determine if this variation was due to poor precision in the assay, samples were run in triplicate. The raw triplicate data was averaged together and shown in Table 21. Clearly, the increase in the number of negative ELISA clones is not due to increased precision error in the assay, but more likely from the antibody titer as described in greater detail below. The data is also shown below in Figure 32. In addition, to determine how sensitive the assay was to variations

in MAb concentrations, dilution series of the parent cell culture supernatants were tested.

ELISA Precision and Dilution Effects

DILUTION	M1.144	M1.144.35	M1.144.35.5	SCM
Absorbance (AU)				
NEAT	0.382 ± 0.016	0.350 ± 0.005	0.750 ± 0.024	0.084
1/2	0.305 ± 0.058	0.211 ± 0.041	0.467 ± 0.054	0.130
1/4	0.334 ± 0.022	0.235 ± 0.013	0.335 ± 0.074	0.134
1/8	0.314 ± 0.080	0.160 ± 0.027	0.257 ± 0.061	0.106
1/16	0.236 ± 0.009	0.161 ± 0.028	0.198 ± 0.040	0.105
1/32	0.247 ± 0.021	0.154 ± 0.022	0.155 ± 0.013	0.181

Table 21. The average of triplicate absorbances for serial dilutions of M1.144, M1.144.35, and M1.144.35.5. ELISA conducted on all three clones and the SCM negative control was performed on the same plate.

Figure 32. ELISA dilution effects. Note that a dilution factor of 2 means the sample was diluted by half and a dilution factor of 4 means the sample was diluted by a fourth, etc. (144=M1.144; 35=M1.144.35; 5=M1.144.35.5).

The data from the M1.144.35 assay indicated that a mere 2-fold dilution of MAb served to drop the ELISA absorbance signal below the 0.25 AU cut-off used to score negative MAb clones. A similar effect is seen with the M1.144.35.5 supernatant, in that an 8-fold dilution dropped signal into the noise and background range of the negative controls. It is quite likely, therefore, that many of our observed negative assay signals may have come from clones which were sampled before a sufficient cell density had been reached. It was noted that early rounds of subcloning used 5% Hyclone Fetalclone growth factor, whereas subsequent rounds used 3% Hyclone Fetalclone growth factor. Earlier work in our lab had established that increased concentrations of Hyclone (up to 5%) decreased cell doubling time dramatically (55). In fact, there appears to be a correlation between percent Hyclone used in subcloning and percent monoclonal cultures resulting in positive absorbance signals in ELISA (see Table 22). This hypothesis can be tested by sampling all monoclonals at different cell densities and noting the point at which the cut-off level is reached. The expected observation is an increase in absorbance signal as cell density increases over time. Once past the cut-off level, the absorbance signal should level off as wells become overgrown and cells begin to die.

Hyclone Effects on ELISA Scores.

Subclone Date	8/30/94	11/15/94	1/4/95	1/29/95
%Hyclone	5	5	3	3
%MAb colonies positive in ELISA	88	91	40	59

Table 22. The effect of %Hyclone on ELISA assays. A decrease in Hyclone lowered the %MAb colonies that had positive signals in ELISA analysis. A decrease in Hyclone also resulted in decreased ELISA absorbance readings.

(VII). CELL STORAGE

As cells were expanded and subcloned, they were also collected for storage. Table 23 is a record of all cells frozen for storage in the liquid nitrogen tank. These cell lines can be easily accessed whenever MAb is requested.

Cells Frozen for Storage

Cell Line	Date	Vials	Cell Line	Date	Vials
M1.04	08/94	1	M1.144	09/94	12
M1.44	09/94	8	M1.144	11/94	5
M1.44.2	09/94	4	M1.144.X	09/94	5
M1.44.7	10/94	2	M1.144.7	11/94	2
M1.44.7	11/94	2	M1.144.19	12/94	1
M1.44.14	09/94	3	M1.144.27	12/94	3
M1.44.38	09/94	2	M1.144.35	12/94	2
M1.49	09/94	3	M1.144.35	01/95	3
M1.49.XX	09/94	7	M1.144.38	12/94	4
M1.85	08/94	2	M1.144.35.5	01/95	2
M1.85	10/94	1	M1.144.35.5	02/95	6
M1.89	08/94	2	M1.144.35.8	01/95	2
M1.90	08/94	2	M1.144.35.10	02/95	5
M1.104	08/94	4	M1.144.35.51	01/95	2
M1.108	10/94	2	M1.144.35.5.17	03/95	4
M1.123	09/94	1	M1.144.35.5.28	03/95	4

Table 23. Cell storage record for all cell lines frozen and kept in the liquid nitrogen tank. M1.X refers to anti-hORL-1 N-terminal peptide hybridoma colonies produced from mouse #115, where X is a number between 04 and 144. M1.49.XX is a pool of several subclones of M1.49. M1.44.2, .7, .14, .38 are individual subclones of M1.44. M1.144.X is a pool of subclones of M1.144 (see p 76). M1.144.7, .19, .27, .35, and .38 are individual subclones of M1.144. M1.144.35.5.17 and .18 are individual subclones of M1.144.35.5.

(VIII). EPI TOPE MAPPING

Peptide ELISAs were run at the UCLA lab to determine the fine specificity of clone M1.144.35. It was shown that binding is localized to the N-terminal

sequence NH₂–SLFPAP–COOH (56). In future reference, this clone will be known as the murine monoclonal antibody raised against the N–terminal 18 amino acids of ORL1, code name “MN18–ORL1”.

(IX). C–TERMINAL FUSIONS

Fusions for mice #104 and #137 were done at UCLA with Benito Anton (01–12–95). Several hundred colonies were observed by the UCLA team by early February and screened by ELISA. The few positive clones were frozen and await to be subcloned at a later date.

DISCUSSION

(I). IMMUNOBLOTTING AND IMMUNOPRECIPITATION

The applications of monoclonal antibodies go beyond just identifying the existence of an antigenic site on a receptor or a receptor’s distribution. One common problem confronted by investigators is the determination of molecular weight. Several groups have reported molecular weight values in the range of 58–65 kDa for purified receptors (57). Molecular weight variations may be associated with coupled membrane bound proteins. Immunoprecipitation can therefore be useful in determining apparent molecular weight of the epitope with its associated proteins glycosylations.

The initial step in receptor characterization is the use of SDS-PAGE. The goal is to electrophoretically separate proteins on the basis of mass under denaturing conditions. The negatively charged SDS disrupts noncovalent interactions in the protein and binds in proportion to the protein's mass. When an electric field is applied to the sample in a gel, the proteins migrate towards the anionic electrode at rates according to mass. Once separated, Western Blots are used to indicate the location of specific proteins of interest on the gel. The result is the determination of their molecular weights (58).

The Western Blot is used to expose the immunoreactive protein. The one problem which can occur is that the epitope can be destroyed by SDS exposure. However, this is less likely in the case of a monoclonal antibody raised against a small peptide, since little tertiary structure is likely to be present from the immunogen stage onwards. Once the proteins are transferred to nitrocellulose, the monoclonal antibody can be applied followed by a second antibody that is used in the visualization procedure. Only the proteins with the antigenic epitope will be detected.

In immunoprecipitation, protein-protein interactions remain intact and therefore reveal the existence of a larger protein complex. The monoclonal antibody is linked to an insoluble matrix such as polyacrylamide beads or protein G coupled to agarose beads and exposed to the antisera. This complex is then incubated with the antigen followed by SDS-PAGE and then visualized (59).

For example, Kobilka (60) deglycosylated adrenergic receptors with an endoglycosidase treatment. The result was the removal asparagine linked oligosaccharides and the determination of the protein's true molecular weight.

These oligosaccharides are commonly found on many proteins and are considered to act as recognition markers for the cell (61).

(II). MULTIPLE ANTIGEN PEPTIDE SYSTEM

The use of this system provided an excellent approach to eliciting an immunologic response. Our peptide was anchored, and therefore exposed in a very specific orientation which limited the number of ways it could be recognized. Therefore, we were able to influence the development of the antibody - antigen interaction.

In addition, eight peptides could be associated with one lysine matrix. The advantage is that multiple copies of the desired antigen were introduced, and therefore increased the chances of an immunological response. An appropriate experiment to test this claim would be a comparison of the octameric peptide with a monomeric peptide MAPS. James Tam and Fidel Zavala (47) did such a study and found no reactivity with the monomeric system and strong reactivity with the octameric system.

Another advantage of this system was the decrease of cross-reactivity with a carrier protein. Since the goal of the project was the development of monoclonal antibodies, it was necessary to limit this problem. One result of cross-reactivity is lack of antigen specificity. Therefore, by decreasing the number and availability of possible epitopes, the chance of pulling out splenocytes producing antibody to the desired epitope is greatly enhanced. This was accomplished by the fact that there is a high density of peptide epitopes at the surface in comparison to the lysine MAPS which comprises the interior core.

(III). STERILE TECHNIQUE

Probably the most critical aspect of hybridoma success is sterile technique. Without it, the risk of contamination is high and the result is the loss of a cell line. As mentioned earlier, contamination was introduced into the first fusion and resulted in the loss of cells from mice that had produced sera with strong ELISA signals. The source of this contamination is thought to have originated from the collection of macrophages for feeder layers, possibly from the dead space animal dissection hood used or from the mouse itself.

The condition of the hood was not very good. For one thing, there was a crack in the plexiglass hood directly above the sterile field. This was corrected by using a new hood and wiping the entire inside with 70% ethanol. The mouse itself may have been the source of contamination. For example, during the first macrophage collection, a 10 mL injection of the sucrose solution resulted in the development of leaks. This in turn resulted in contact between the sucrose solution and the animal's skin. To be safe, only 5 -7 mL were used for later sets of macrophage collection where leaks were no longer observed.

(IV). ELISA

One of the biggest problems encountered in the ELISA was edge effect. This caused absorbance readings to be excessively high. This problem was solved by sealing the plates with tape, using a sealed plastic container for storage, keeping all reagents cold in order to prevent evaporation, and incubating

in a dark location during the enzyme/substrate reaction. With these modifications, it was no longer necessary to avoid the use of the outer wells for test samples.

Once these problems were corrected, the ELISA proved to be a very effective method of rapidly screening for stable clones producing monoclonal antibodies.

(V). EXPANSION AND FEEDING

The survival and health of hybridomas is directly related to feeding and expansion. An easy method of determining when a colony required attention was through the color of the phenol red indicator in the medium. Colonies that were not fed on a regular basis had a decreased cell viability and eventually died. Cells that were approximately 50% confluent in pinkish medium seemed to survive and grow best. A well containing medium that had turned yellow indicated an acidic environment and required immediate attention. Overgrowth and/or contamination were both associated with this situation.

An additional concern was glutamine levels. Sigma has done stability studies on glutamine in medium. Their findings were that glutamine was most stable at 4° C, though degradation did occur. After 2 weeks, approximately 20% had degraded. The solution was a 1% glutamine addition every two weeks. Unfortunately, in this study, cells were incubated at 37° C. Sigma's studies at 35° C indicates that glutamine deteriorates rapidly. By day nine, 50% of the glutamine had degraded. This was a concern especially after fusions and subclones when plates were to be left undisturbed for a period of 5 -7 days.

An interesting observation was noted during expansion. Cells were collected from one well and transferred to two or three new wells. It was observed that the new wells which had a confluency around 30% - 50% grew faster than the original well having a new confluency of 20% or lower. It was then proposed that cells grow best when there are other cells present. This hypothesis is consistent with the fact that cells secrete trophic factors that enhance growth. On the other hand, a large colony can quickly deplete food sources such as glutamine.

Another aspect of the feeding medium is the use of 5% Hyclone Fetalclone growth factor with 5% fetal calf serum (FCS). Based on personal observations, the speed of cell growth was enhanced when 5% Hyclone was present. The negative aspect of using this Hyclone growth factor was that cell growth rapidly deteriorated if cells were in this environment for a long period of time ($\geq$ several weeks). It was best to start cultures in the presence of 5% Hyclone with 5% FCS, and then ween them off into just 10% FCS. This was evident in sets of cells which were growing fine in 5% Hyclone and then mysteriously began dying. They were saved by expanding into medium having increasingly relative amounts of serum versus Hyclone Fetalclone growth factor.

(VI). MACROPHAGE FEEDER LAYERS

The use of a macrophage feeder layer greatly increased growth and survival rates of cells. The macrophage provided the cells with two functions. Firstly, they degrade cell debris and secondly, they secrete growth factors such as cytokines.

Supporting the conclusion that macrophages are an important part of having a strong growth response, two sub-clone plates at 5 cells/well, one with macrophage feeder layer and the other without, showed dramatically different results. The plate with macrophage had hybridomas in every well, whereas the plate without macrophage was approximately 50% full .

This enhancement was also witnessed in a recent fusion done at UCLA in Dr. Evans' laboratory. Fusion colony formation observed previously in the UCLA laboratory had been in the range of 20 - 40 colonies per fusion. These colonies were weak and did not survive. Upon the use of the macrophage feeder layer, the lab observed 200 - 300 strong colonies per fusion. Of course, it may have been the immunization protocol (we used the C-terminal immunogen mice and two other sets of immunogens not described herein). Unfortunately, the previous UCLA work was done with different opiate receptor peptides as immunogens, so a direct comparison is not possible. Though the use of a macrophage feeder layer is highly recommended, there is the danger of cells becoming dependent on the secreted growth factors.

(VII). IMMUNOHISTOCHEMISTRY

Staining appears to predominate in the long processes and dendritic fibers. Comparing Table 1 with Table 15, it is evident that ORL1 shares many regions of the brain with the other receptor types. This may suggest that ORL1 is involved in a variety of functions. For example, the frontal cortex is involved with cognitive thinking, while hippocampus is part of the limbic system and the

hypothalamus is involved in neuroendocrine control. In any case, it is quite apparent that ORL1 is highly populated in all three of these regions.

CONCLUSIONS

There are several successful aspects of this project. Most importantly, a stable cell line has been isolated producing a highly specific monoclonal antibody to the Nterminal of the hORL1 receptor. It has been determined that the antibody selectively binds to the N-terminal sequence NH₂-SLFPAP-COOH.

Secondly, antibodies from several cell lines have been characterized in terms of their sub-classes. These antibodies were also tested for non-specific protein binding and displayed low levels of anti-BSA activity. The cell line (M1.144) chosen for further expansion and subcloning, displayed the lowest anti-BSA activity out of all six analyzed cell lines.

Thirdly, several aspects of hybridoma production proved to be critical for success. These included: an enhanced antigenic immunogen via the MAPS structure, a highly specific ELISA screening method, sterile tissue culture techniques, immediate subcloning, and the use of macrophage feeder layers.

Fourthly, immunohistochemical analysis on rat brain slices provided specific staining patterns. The significance of this specificity led to the localization and relative density of this antigen in the rat central nervous system as shown in Table 15.

The accomplishments of this research work have resulted in the development of a valuable tool for probing this novel receptor. It is hoped that

future efforts will be made in applying this monoclonal antibody to further characterization. Such efforts are currently being pursued in the areas of affinity column development, immunoprecipitation, and transfected cell line identification.

APPENDIX

POISSON ANALYSIS

The probability (P_x) of X clones/well is calculated from the following equation:

$$P_x = [m^x (e^{-m})]/X!$$

where $m = \ln$ [total number of wells/number of null wells]. Since $N = 96$ (number of wells in the plate) and $n_0 = 67$ (number of null wells) then $m = 0.3597$, where $n_0 = Ne^{-m}$. Thus, Table 24 displays the number of monoclonal wells (n_1), biconal wells (n_2), and triconal wells (n_3) for null sets of 70%, 75%, 80%, 85%, 90%, and 95% of the wells of a 96-well plate:

	$n_0 =$	70%	75%	80%	85%	90%	95%	Poisson equation
n_0	67	72	77	82	86	91		Ne^{-m}
n_1	24	21	17	13	10	5		$n_0(m)$
n_2	4	3	2	1	0	0		$n_1(m/2)$
n_3	0.5	0.3	0.15	0.1	0	0		$n_2(m/3)$

Table 24. Poisson analysis for the number of monoclonal wells (n_1), biconal wells (n_2), and triconal wells (n_3) from null sets of 70%, 75%, 80%, 85%, 90%, and 95% of the wells of a 96-well plate.

Thus, if only 10 colonies grow on a 96–well plate, they are probably all monoclonal. However, if 14 colonies grow (null set = 82/96), the probability that any one is monoclonal is $(13/14) = 93\%$. At the other end of the range, where no = 70%, the probability that any one colony is monoclonal is 83%. Thus, the overall probability that, on three rounds of subcloning yielding null sets of $\geq 70\%$, of a clone not being monoclonal is less than 0.5%.

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