THE PRODUCTION OF POLYCLONAL AND MONOCLONAL ANTIBODIES AGAINST MORPHINE

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ABSTRACT

In the 1970s it was demonstrated that opiate-like receptors were present in the brain of vertebrate animals. This finding led to the discovery of a long series of endogenous opioid peptides, which include the enkephalins, the endorphins and the dynorphins. With these discoveries, a whole new era of narcotic research began. Hundreds of scientific papers have been produced regarding the properties, distribution and pharmacology of these opioid peptides. In this study, we have attempted to examine the possible structural and conformational similarities between morphine and the opioid peptides by using antibodies raised against morphine. After 6 trials, we were unsuccessful in producing a monoclonal antibody against morphine. As a result, the proposed correlation was studied by using polyclonal antisera against morphine. The specificities of the resulting antisera were determined by competition experiments against I-125 morphine binding using a series of selected opiate ligands. Our results demonstrated that the anti-morphine sera bound morphine with high affinity but showed no recognition for the opioid peptides or their

analogues. However, their partial cross-reactivity with the two opiate drugs, naloxone and etorphine, suggests that they were most probably directed at a region near the C6 of the morphine structure. The fact that they showed no cross-reaction with the opioid peptides indicates that the binding sites for morphine in the antisera may be different from those of the opioid receptor to which both morphine and the active peptides will bind selectively.

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CHAPTER I GENERAL INTRODUCTION

1.1 Historical aspects of morphine

Opium has been used as a drug since classical Greek times , not only because it relieves pain but also because it gives rise to euphoria. In 1803, the German pharmacist Setürner achieved the isolation of morphine as one of the active ingredients of opium . He named the compound after Morpheus, the Greek god of dreams. Among other derivatives of opium are codeine, thebaine, narceine, narcotine and papaverine. From the isolation of pure morphine to the discovery of its structure by first Gulland and Robinson (1925) and later Schöpf (1927) took another 120 years. The total synthesis of morphine by Gates and Tschudi (1952, 1956) confirmed the structure of this molecule in the early 1950s. However, the benefits derived from the pain-killing effects of morphine and its derivatives have been tempered by their toxicity and addictiveness. These undesirable side-effects have led to an intensive search for other opiates with the good properties of morphine but without the bad ones.

1.2 The discovery of the opiate receptor

It has been known for a long time that the opiates exert their effects at a very limited number of sites in animals. This fact indicated the presence of specific opiate receptors located at these sites. In 1973, following a stereospecific binding technique developed by Goldstein et al.(1973), three groups of investigators (Pert and Snyder; Simon,Hiller and Edelman; and Terenius; 1973) independently described saturable stereospecific binding sites for the opiates in the animal's nervous system. It was felt at that time that these receptors were not provided by the animal just to react with exogenous opiates; rather there should exist an endogenous compound which interacts with these receptors to carry out a physiological function. Thus, the years following 1973 were marked by a most intensive search for the endogenous ligands. And within the next three years, a good number of endogenous ligands were isolated from various tissues and subsequently identified.

1.3 The discovery of endogenous opioids

In 1975, Hughes and Kosterlitz et al.(1975) described the isolation from pig brain two pentapeptides which competed in opiate receptor assays and showed morphine-like activity in in-vitro smooth-muscle bioassays on guinea-pig ileum (GPI) and mouse vas deferens (MVD). They named the two peptides met-enkephalin and leu-enkephalin.

Fig. 1.1

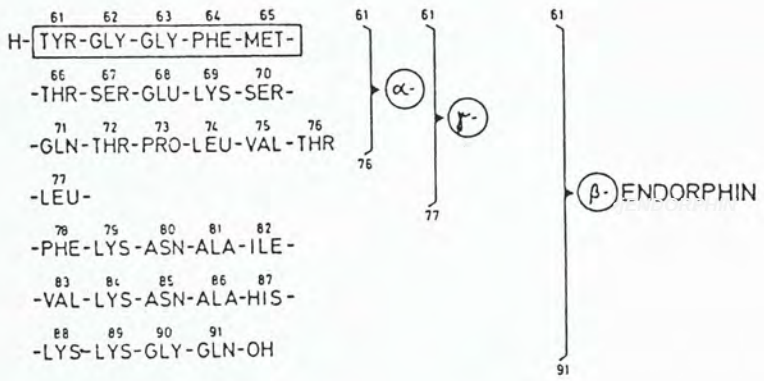
H-TYR-GLY-GLY-PHE-LEU-OH	LEUCINE-ENKEPHALIN
H-TYR-GLY-GLY-PHE-MET-OH	METHIONINE-ENKEPHALIN

As shown above, the two peptides shared a common tetrapeptide sequence, Tyr-Gly-Gly-Phe-, varying only in the C-terminal. Perhaps even more exciting than this discovery was the realization that met-enkephalin is contained within a 91 amino acid pituitary hormone, β-lipotropin (β-LPH), and thus the pituitary gland was recognized to be an important source of opioid peptides. This was correct as many opioid peptides isolated from the pituitary were proved to be different fragments of β-LPH.

In 1976, C.H.Li (and later Bradbury et al.) isolated a peptide which he named β-endorphin (β-LPH 61-91; also called "C-fragment") from the pituitary gland of a camel. It was found that the first five residues of β-endorphin are identical with met-enkephalin, and that this peptide

is much more potent than met-enkephalin in both in vivo and in vitro tests. This was followed by the finding of two additional peptides: α- endorphin (β-LPH 61-76) and γ-endorphin (β-LPH 61-77) by other research groups in the same year (Guillemin et al. 1976; Ling et al. 1976).

Fig. 1.2



But β-LPH is not the only source of endogenous opioids in the pituitary gland. In 1979, dynorphin was characterized by Goldstein et al.(1979). This peptide differs from β-endorphin (lower MW, more basic, more potent in GPI assay, resistant to CNBr) and its thirteen residues start with leu-enkephalin at the N-terminal.

Fig. 1.3

dynorphin(1-13)

H-Tyr-Gly-Gly-Phe-Leu^{5}-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys^{13}-OH

and dynorphin (with four extra residues:Trp-Asp-Asn-Gln^{17})

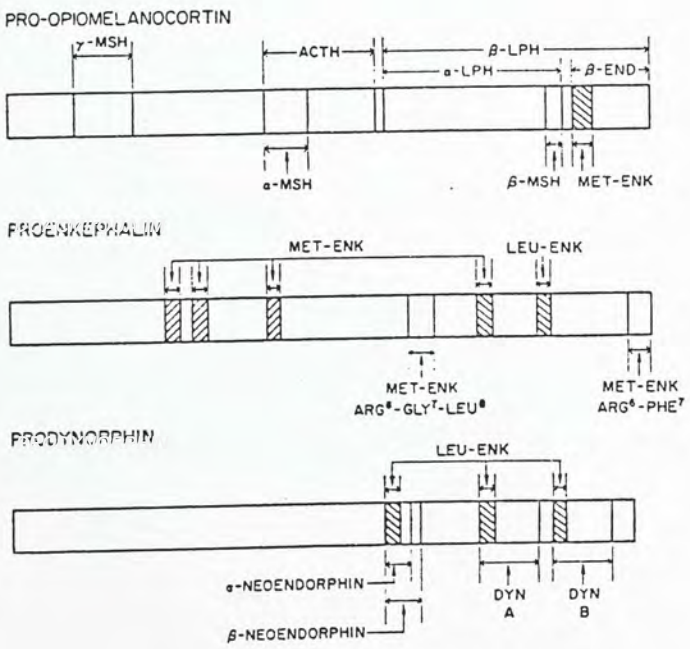
Since then, other opioid peptides of less importance were also isolated by various research groups (Igarishi 1979; Couri 1981; Udenfriend 1982).

1.4 Families of opioid peptides

With the aid of modern nucleic acid technology, the opioid peptides can be classified into three different families: the enkephalins, the endorphins and the dynorphins. Each family is derived from a genetically distinct precursor polypeptide and has a typical anatomical distribution. These precursors are proenkephalin, proopiomelanocortin (POMC), and prodynorphin , as shown below.

Fig. 1.4

Schematic representation of the structures of the protein precursors of the three families of opioid peptides



Each of these precursors contains a number of biologically active peptides, both opioid and non-opioid, which have been found in blood and various tissues. The POMC contains the amino acid sequence for melanocortin (γ-MSH), adrenocorticotropin (ACTH) and β-lipotropin (β-LPH). Within the β-LPH sequence β-endorphin and β-MSH are found. Although β-endorphin contains met-enkephalin at its N-terminal, it is not converted to this peptide; instead, met-enkephalin comes from proenkephalin. Leu-enkephalin is also derived from proenkephalin. Prodynorphin yields five peptides which contain leu-enkephalin: (i) Dynorphin A(1-17) which can be cleaved to (ii) dynorphin A(1-8); (iii) dynorphin (1-13); and (iv) α- and (v) β-neoendorphins. The peptides from POMC is mainly in the pituitary and those from proenkephalin and prodynorphin are spread widely throughout the CNS, where they are often found in the same region but in different groups of neurons.

1.5 Multiple opioid receptors

Studies of the binding of opiates and opioid peptides to specific sites in the brain and other organs have shown the existence of as many as 8 types of opioid

receptors. In the CNS, there are four major types of receptors:

- μ (mu) : morphine and morphine-like drugs are agonists.
- δ (delta) : receptor for enkephalins.
- κ (kappa) : the drug ketocyclazocine and dynorphin are preferential agonists.
- σ (sigma) : N-allylnormetazocine is the agonist.

;and there may be sub-types of each of these receptors. Analgesia has been related to both μ and k receptors, while psychotomimetic effects have been connected with σ receptors. δ receptors are thought to be involved in affective behaviour. With the exception of some types of σ receptors, the antagonist naloxone is found to bind to all receptors .but its affinity for μ receptors is the highest.

The actions of opiate drugs that are now available are found to react with μ, k and σ receptors, where they may act as an agonist, a partial agonist, or an antagonist.

The opioid peptides show a variety of relative affinities for different types of receptors. For example, met-enkephalin-arg^6-gly^7-leu^8 has equal affinity for μ and

δ sites, but other peptides from proenkephalin show a preference for δ sites. All the peptides from the prodynorphin series bind predominantly to k sites, whereas dynorphin B and dynorphin A(1-8) also bind to μ and δ sites respectively. In the CNS and peripheral tissues, β-endorphin binds to both μ and δ receptors.

1.6 Structure and function of morphine and its analogues

The structure of morphine was first revealed by Gulland and Robinson in 1925. Since then, many semisynthetic derivatives have been made by simple modifications of the morphine or thebaine molecule. Codeine, with a methyl substitution on the 3-OH, has about 20% of the potency of morphine. Thebaine differs from morphine in that both OH groups are methylated (3-OMe, 6-OMe). It has little opioid activity but is a precursor of several important 14-OH compounds such as oxycodone (3-OMe, 6-O, 14-OH), and naloxone (3-OH, 6-O, 14-OH, 17-allyl group). Other derivatives of thebaine such as etorphine are more than 1000X as potent as morphine. Heroin, which is 2X morphine in potency, is prepared by acetylation (-OAc) at C3 and C6 positions. Hydromorphone (6-O), oxymorphone (6-O, 14-OH), hydrocodone (3-OMe, 6-O) and oxycodone (please see above) are also made by modifying

the morphine molecule in different ways. In addition, there are a number of other structurally distinct groups of drugs with morphine-like activity, and these include the morphinans, benzomorphans, methadones and phenylpiperidines . All the compounds mentioned above have shown common features of possessing the phenolic OH at C3, the benzene ring and the piperidine ring N atom. It is believed that these functional groups are very important for eliciting analgesic actions.

The masking of 3-OH of morphine, as in codeine (3-OMe) results in several fold reduction in potency. In contrast, the 6-OH group does not seem too important for opioid activity. Levorphanol, which lacks the alcohol group at C6, is a more potent drug than morphine. Moreover, strong acid rearrangement of the C-ring double bond in morphine or codeine results in the production of the ketone (6-O): hydromorphone or hydrocodone. Both are several times more active than morphine and codeine in opioid activity. Profound effects on analgesic actions occur when the substituent on N atom varies. For example, substitution of an allyl group for the methyl group on N converts the agonist morphine into the antagonist nalorphine. In general, a 14-OH tends to increase opioid potencies particularly in antagonist series. Substitution of

a 6-ketone in 14-OH series produces molecules with high opioid activities eg. oxymorphone and oxycodone. Finally, it is found that the benzene ring, although not essential for opioid activity, is necessary in most opiates and its binding at the opioid receptor is augmented by a free 3-OH group.

1.7 Conformational studies of opioid peptides

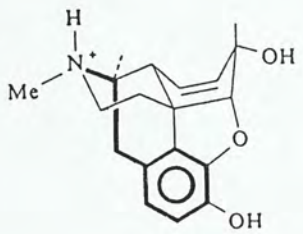
One significant point should be made at this stage: at least two characteristics are found common to the opioid peptides mentioned so far. They all act like opiates and they all contain the sequence Tyr-Gly-Gly-Phe-Met or Tyr-Gly-Gly-Phe-Leu. It is clear from the above that these are the sequences which display opioid activities, either as pentapeptides, or as N-terminal fragments of larger peptides.

After the discovery of the enkephalins, various attempts were made to explain their steric conformations using opiate structures as models. Most investigations were based on the assumption that all five residues of the peptide might contribute to its steric conformation. In 1976, Horn and Rodgers(1976) proposed that the "tyramine"

moiety of the Tyr residue, involving the phenolic hydroxyl group (at C3 position), the benzene ring and the basic N atom, is the essential feature for opioid activity of the enkephalin molecule.

Fig. 1.5

Morphine showing the Tyr moiety (the highlighted portion)

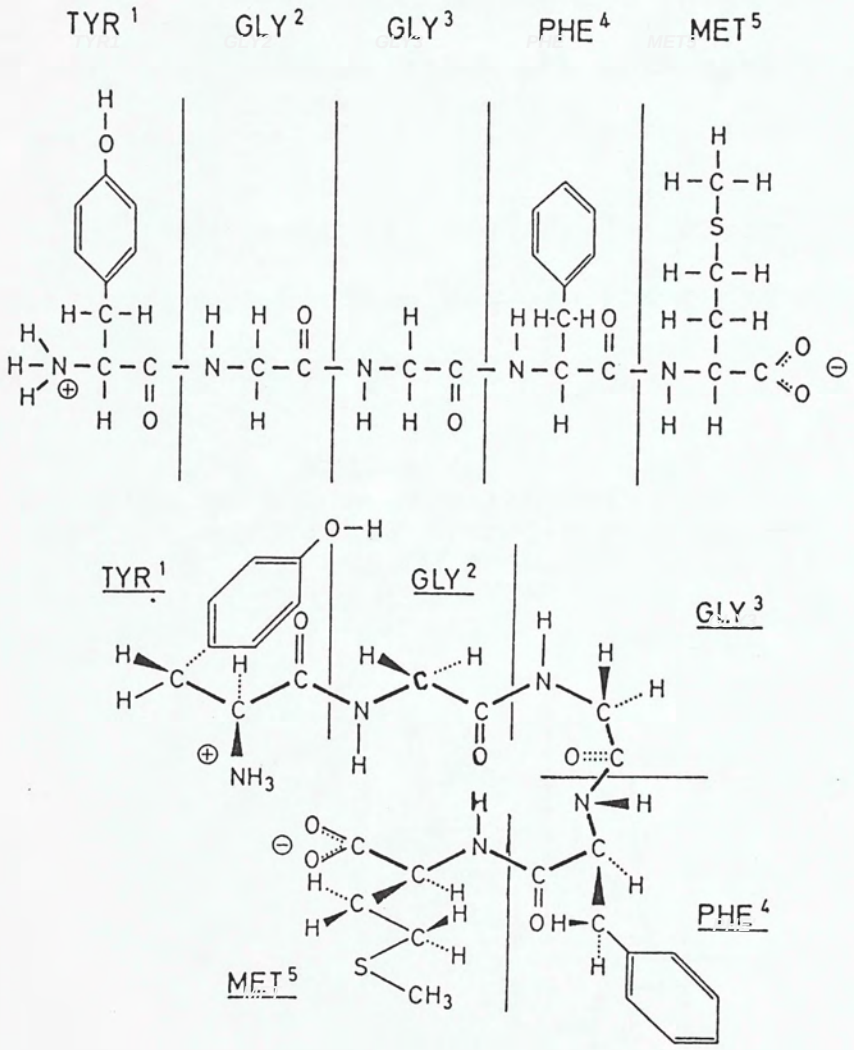


The importance of this moiety was experimentally confirmed by Schiller et al. (1977). On the basis of structure-activity studies, they came to conclusion that the configuration of met-enkephalin had to be equivalent to that of morphine in order to bind to same receptors.

High-resolution NMR spectra carried out by two groups (Roques et al. 1976; Jones et al. 1976) reported a solution conformation of the enkephalin molecule characterized by a β-bend between Gly^3-Phe^4 stabilized by a Gly^2 (carbonyl)-Met^5/ or Leu^5 (amide) hydrogen bonding.

Fig. 1.6

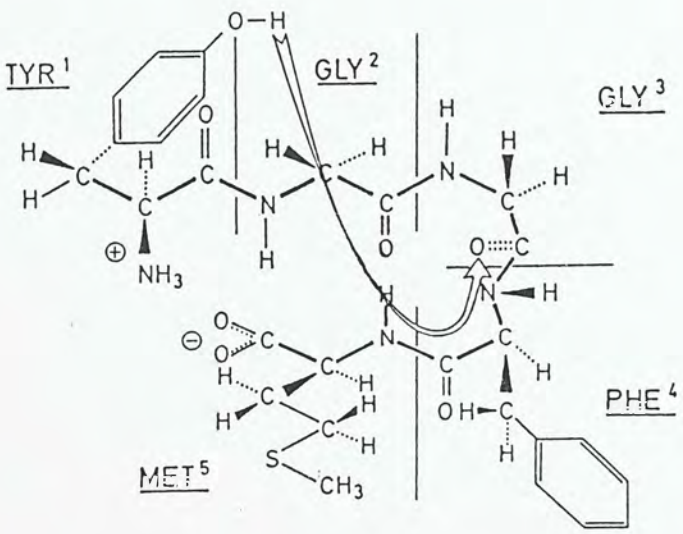
The possible conformation of the enkephalin molecule



However, Bleich and co-workers (1977) found no basis for such an intramolecular hydrogen bonding. Some of these conflicting results were explained by Khaled et al. (1977), who indicated by NMR, UV and circular dichroism studies that the spectral properties of enkephalin differed at different concentrations. These authors then proposed two conformations which are both consistent with the NMR data:

A structure with a Gly^3-Phe^4 β bend, with an additional hydrogen bonding between the hydroxyl group of Tyr^1 and the carbonyl group of Gly^3,

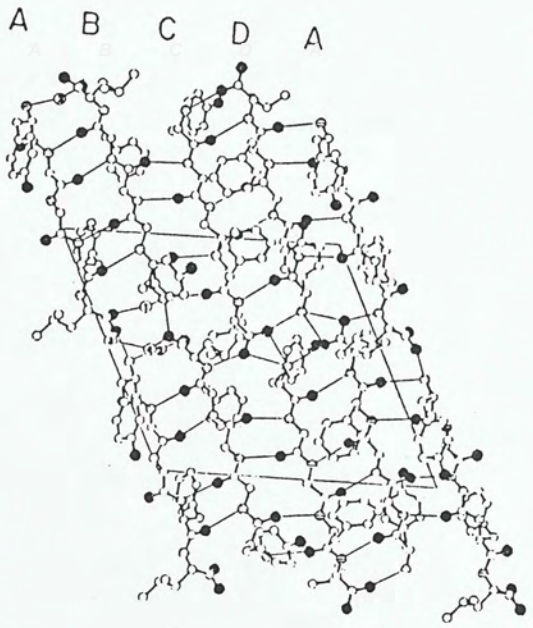
Fig. 1.7
Conformation of the enkephalin molecule.The arrow indicates an additional hydrogen bond between the CO group of GLY^3 and the OH group of TYR^1 stabilizing a conformation in which the TYR^1 residue is folded over the molecule.



and extended conformation molecules associated by intermolecular NH-CO hydrogen bonds to form an antiparallel β-pleated sheet structure, as shown below:

Fig. 1.8

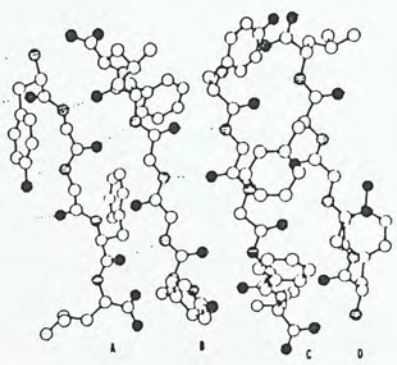
A two-dimensional antiparallel β-pleated sheet of the enkephalin molecule.



One explanation for different enkephalin conformations shown by various spectroscopic and theoretical analyses is that these pentapeptides are highly flexible and capable of having more than one conformation in solution. In 1983, Camerman et al.(1983) confirmed the extended conformation by reporting a solid-state study of leu-enkephalin by X-ray diffraction. He indicated that the crystal structure of leu-enkephalin contains 4 independent enkephalin molecules and much water and dimethylformamide solvent in the asymmetric unit. The four enkephalins have extended backbones with the amino acid side chains above and below the backbone.

Fig. 1.9

Conformations of the four independent pentapeptides comprising the Leu-enkephalin crystal structure asymmetric unit.



Of particular interest is that in the same crystal lattice, the Tyr, Phe and Leu side chains are found to have different orientations and that the peptide backbone can assume different conformations depending on environmental factors.

There is so far only limited information available about the steric conformation of the larger endorphins. Hollosi et al. (1977) and Bayley et al. (1977) independently found that the peptide can be present with an α helix conformation of the sequence between residues 73-89 of β-LPH, depending on the environment. These authors assume a spatial array of the helical stretch and the non-helical NH_2-terminal portion of the molecule. It was reported that this arrangement might be responsible for the high resistance of the peptide against enzymatic degradation.

1.8 Aim of the present thesis

After the discovery of endogenous opioid peptides, much time has been given to the conformational analysis of these peptides in a search for structural similarities between the active opioids and the rigid molecule of morphine. As the two groups are known to compete for the same receptors and give a similar pharmacological

response, it is reasonable to suggest that an opioid peptide may mimic the spatial array of active groups of morphine during binding interactions. However, this problem is a very difficult one because of the flexible nature of opioid peptides and their many conformational forms, especially in solution.

In order to better understand the proposed correlation, the Hybridoma technique was used so as to generate a series of monospecific antibodies against different determinants on the opiate. It was hoped that with the aid of these specific analysing agents, we would be able to investigate fully the likely conformation of opioid peptides at specific antibody binding sites, and to detect any structural and conformational similarities that may exist between the two groups. Morphine was used as a model for the generation of monoclonal antibodies because it has a rigid skeleton and its conformation has been well established.

The main advantage of applying Monoclonal Technology to such studies is that the method offers the availability of large amounts of homogenous antibodies which can be standardized and quality-controlled. In addition, the distinctive specificity of these reagents makes it

possible to analyse precisely any molecule on an epitope-to-epitope basis. Their ability to detect even single residue changes at active binding sites provides valuable information on the fine antigenic structure of the molecule.

However, it was most unfortunate that in spite of numerous attempts, there have been no apparent success in obtaining these highly specific reagents against morphine. The procedures employed and experimental data will be given in chapter II of this thesis. Consequently, a comparison of the structural conformation between opioid peptides and their analogues and morphine was made with the use of polyclonal antisera. The results of such studies will be duly presented in Chapter III.

CHAPTER II PREPARATION OF MONOCLONAL ANTIBODIES TO MORPHINE

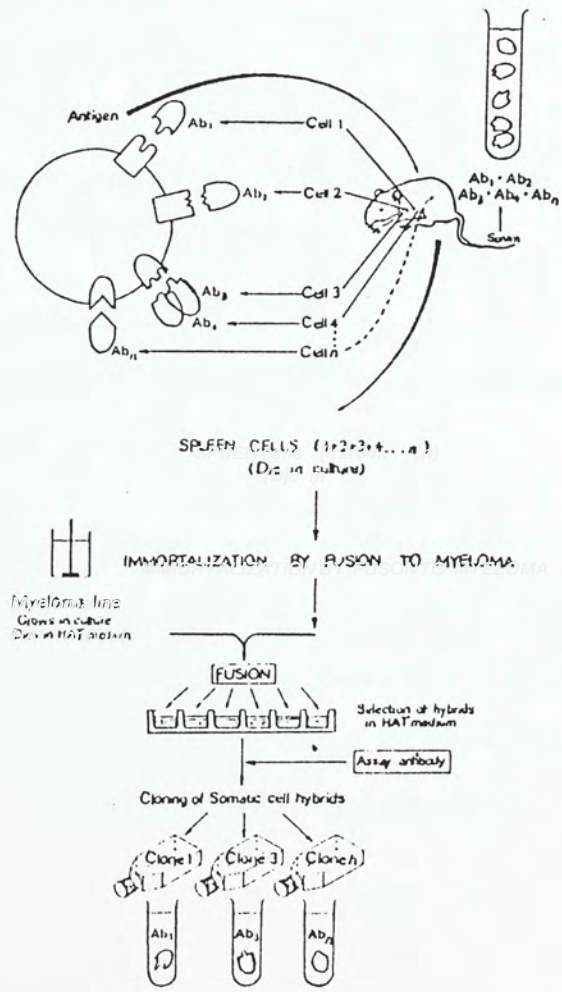
This chapter will be mainly concerned with the attempts to produce monoclonal antibodies specific for morphine. In the first part of the chapter, experimental procedures such as the preparation of immunogen and the establishment of the specific screening assay will be described in detail. The second part of the chapter will consist of experimental data produced by each of the six subsequent fusions and end with a discussion.

2.1 Principle of Hybridoma technology

When an animal is injected with an immunogen, the animal responds by producing an enormous diversity of antibody structures directed against different determinants of the immunogen (Fig. 2.1). Once these are produced, they are released into the circulation and it is almost impossible to separate all the individual clones present in serum. As each antibody is made by individual cells, the immortalization of these cells by somatic cell fusion followed by cloning of the appropriate hybrids allows permanent production of each of the antibodies in separate

culture vessels. The cells can then be injected into animal to develop myeloma - like tumours. The serum of the tumour - bearing animals contain large amounts of these monoclonal antibodies.

Fig. 2.1 Protocol for generation of hybrid cells producing monoclonal antibodies.



2.2 Experimental procedures for the production of hybridomas

2.2.1 Preparation of immunogen

Because of its low molecular weight, morphine (MOR) was coupled to a carrier molecule to increase its immunogenicity. In our studies, two types of carriers were used for the conjugation: hemocyanin (hem) and bovine serum albumin (BSA).

2.2.1.1 Preparation of free base

The free base of morphine was crystallized by precipitation of MOR-HCl (15 gm) with 2 N NaOH. The suspension was filtered and dried under vacuum.

2.2.1.2 Synthesis of 6-succinylmorphine

Free MOR was succinylated at the 6-hydroxyl group by the method of Simon et al. (1972).

3.4 gm of MOR - base and 3.4 gm of succinic anhydride in 100 ml benzene were placed in a 250-ml flask fitted with a condenser and heating mantle. After 2 hours of heat at reflux temperature (80°C), 3.4 gm of succinic anhydride was added and reflux was continued for 1 hour.

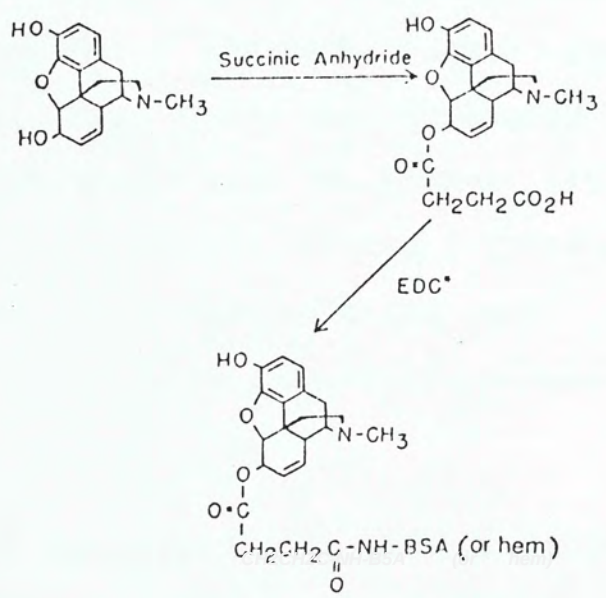
The reaction mixture was then cooled to room temperature, the benzene was decanted and discarded, and the residual benzene was evaporated in a stream of nitrogen. The residue was dissolved in 40 ml of water and adjusted to pH 2 with 2 N HCl. After filtration to remove a small amount of acid-insoluble material, the pH was raised to 9 with 2.5 N NaOH and the solution was filtered to remove unreacted morphine. The pH was adjusted to 5 with HCl, and 6-succinyl morphine crystallized on standing at 4°C overnight. Crystals were harvested by filtration and dried over calcium chloride under reduced pressure. The yield of 6-succinyl morphine was about 40-50%.

2.2.1.3 Conjugation to a carrier

100 mg of succinyl morphine was dissolved in 0.5 ml of 50% ethylene glycol and 50 mg of hemocyanin or BSA in 0.5 ml of water was added. 200 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide in 0.5 ml of 50% ethylene glycol was then added to the mixture and incubated at room temperature overnight (Fig. 2.2). The reaction mixture was dialysed at 4°C against 1 litre of phosphate buffered saline (PBS , pH 7.4), for two days (three changes). The dialysed protein solution was filtered (0.8 um filter) and freeze-dried. The dry MOR-

conjugate was then weighed, dissolved in PBS, aliquoted and stored at -20°C. The degree of conjugation was traced by using I-125 labelled succinyl-morphine. It was found that an average of 16 molecules of morphine was conjugated to one molecule of hemocyanin; in the case of BSA, it was an average of 6 molecules of morphine to a molecule of BSA.

Fig. 2.2 Preparation of the succinylmorphine conjugate.



•EDC• 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride

2.2.2 Immunization of mice

Morphine-hemocyanin conjugate (4 mg/ml) in PBS was mixed with an equal volume of complete Freund's adjuvant and vortexed to prepare a water-in-oil emulsion. The emulsion (0.2 ml., approx. 400 ug conjugate) was injected into each of the eight Balb/c mice. Subsequent injections using a similar emulsion prepared with incomplete Freund's adjuvant were given at two week intervals. After a total of four injections (one time "Complete" and 3 times "Incomplete"), blood samples were taken from the tail vein, and anti-MOR activity was detected in the sera by I-125 morphine RIA method (the same procedure would be used later for the screening of culture supernatants). The mouse with the highest titre (i.e. with antibody activity in serum diluted 1:1000) was given a final injection without adjuvant (50 ug of immunogen in 0.2 ml PBS, pH 7.4) at 3-4 days before fusion.

2.2.3 Screening assays for monoclonal antibody activity

The whole process of producing monoclonal antibodies requires a quick, sensitive and simple assay system to detect the desired clones. In our studies, four different immunoassay techniques were examined and it was found that radioimmunoassay (RIA) with the use of a solid-phase

second antibody ("Sac-Cel") provided the greatest sensitivity and reliability in the measurement of specific antibodies. These test systems will be described in detail in the following sections.

2.2.3.1 RIA with iodine,- labelled morphine

(a) Materials:

1. I-125 morphine tracer.
2. Anti-morphine serum (for screening the clones, the culture supernatants would be used instead).
3. Donkey anti-mouse immunoglobulins coupled to cellulose beads (Sac-Cel, Wellcome, U.K.).
4. RIA buffer : 0.15 M phosphate buffer, pH 7.4, containing 0.1% BSA (RIA grade, Sigma) and 0.1% Triton X-100.

(b) Iodination technique :

1 mg of Iodogen was dissolved in 1 ml of dichloromethane (CH_2Cl_2) and the solution was further diluted 1:30 with CH_2Cl_2 . 30ul of the diluted Iodogen solution was pipetted into a 1.5 ml microfuge tube and incubated at 60° C for 1-2 hour to evaporate the solvent. A morphine solution of 0.2 mg/ml in 0.1 M phosphate buffer, pH 7.4, was pre-

pared . To the Iodogen-coated tube, 1 mCi of I-125 (carrier free) was added, immediately followed by 10 ul of the morphine solution. The reaction was allowed to continue for 11 minutes at room temperature. At the end of the reaction time, 0.5 ml of 0.05 M phosphate buffer, pH 7.4, containing 0.5% Triton and 0.1% sodium meta-bisulfate, was added. The reaction mixture was transferred to a clean microfuge tube and the content was charged into a prewashed Seppak C-18 cartridge (Waters Associates, USA) with a 1 ml syringe. After the radioactive solution had been pushed through, the syringe was discarded. The cartridge was then washed ten times with 2 ml of PBS (pH 7.4) using a clean 5 ml syringe. I-125 morphine was eluted by five washings of 2 ml absolute methanol. The highest radioactivity was collected in the first fraction. The I-125 morphine prepared in this manner was tested and was stored at 4°c until use. For RIA, the tracer for binding to either mouse or rabbit antimorphine sera was diluted in RIA buffer to give 10^4 cpm/100ul. Table 2.1 shows the result of an iodination procedure performed on 6/4/88.

Table 2.1 Iodination of morphine

Fraction no.	Radioactivity(cpm/5ul)	Time(min.)
Unabsorbed material	409692	0.5
Methanol eluted materials		
1	976082	0.5
2	443318	0.5
3	18368	0.5
4	9564	0.5
5	4860	0.5

(c) Preparation procedure:

1. The serum taken from an immune mouse was diluted to 1:1000 , 1:2000 and 1:4000 in RIA buffer.
2. 3 dilutions of 1:100, 1:500 and 1:1000 were prepared from a previously characterized pooled antiserum as positive controls.
3. Normal mouse serum at a 1:100 dilution was prepared. This sample together with a blank buffer were included as negative controls.
4. During the screening of hybridoma clones, a sample of the medium or culture fluid, would be used in place of the Blank.

(d) Assay procedure:

100 ul of antisera and controls were pipetted into round-bottomed plastic tubes (12x75 mm),

followed by 100 ul of I-125 morphine tracer. The mixture was vortexed and incubated at 4° C overnight. 100 ul of Sac-Cel was added. The samples were mixed and further incubated at room temperature for 30 minutes. At the end of incubation, 1 ml of cold water was added and the precipitated immunocomplex was centrifuged at 3000 cpm for 15 minutes at 4° C. Supernatants were discarded and the pellets were counted at 2 minutes each in a gamma-counter.

The results obtained from a "test-bleed" experiment dated 3/3/1988 using the Sac-Cel method are shown in the following table:

Table 2.2 Radioimmunoassay using Sac-Cel

	Radioactivity (cpm)	Time(min.)
Positive control	5158 (1:100)	2.00
	2349 (1:500)	2.00
	1660 (1:1000)	2.00
Normal mouse serum	955 (1:100)	2.00
	853 (1:100)	2.00
Blank	846	2.00
	825	2.00
Immune mouse no.1	1998 (1:1000)	2.00
	1225 (1:2000)	2.00
	1062 (1:4000)	2.00
Immune mouse no.2	1613 (1:1000)	2.00
	1136 (1:2000)	2.00
	966 (1:4000)	2.00
Immune mouse no.3	1385 (1:1000)	2.00
	1027 (1:2000)	2.00
	957 (1:4000)	2.00

2.2.3.2 ELISA (Enzyme-linked immunosorbent assay)

(a) Materials:

1. Carbonate-bicarbonate coupling buffer, pH 9.6.
2. Washing buffer: phosphate buffered saline (PBS), pH 7.4.
3. PBS/T : PBS containing 0.05% Tween-20.
4. PBS/T/milk : PBS containing 0.05% Tween-20 and 3% dry milk.
5. Enzyme-labelled second antibody: goat anti-mouse IgG - peroxidase conjugate (Bio-Rad) diluted in PBS with 0.05% Tween-20 and 0.1% BSA.
6. Substrate solution: O-phenylenediamine (OPD) 0.8 mg/ml plus 0.04% H_2O_2 , in citrate - phosphate buffer. (pH 5.0) prepared by 24.3ml 0.1 M citric acid + 25.7 ml 0.2 M Na_2HPO_4 + 50 ml water + 80 mg OPD +40 ul H_2O_2 .
7. 96-well microtitre plates (NUNC): flat-bottom.

(b) Assay procedure:

200 ul of MOR-BSA conjugate solution (25 ug/ml in coupling buffer) was added to each well, and the plate was stored overnight at 4°C. Unbound antigen was removed by washing the plate 3X with PBS/T/milk. Excess protein binding sites were blocked by incubating with 200ul of PBS with 1% BSA

at 37°C for 30 minutes. Following this incubation, the plate was washed 3X with PBS/T, and 100 ul of appropriately diluted antibody solution (or culture supernatant) was added. The samples were allowed to incubate at room temperature for 2 hr. At the end of incubation, the plate was washed 5X with PBS/T/milk, and 100 ul of a 1:3000 dilution of enzyme-labelled second antibody was added. This was followed by a further incubation of 1 hr at room temperature. The plate was then washed 3X with PBS/T/milk and 2X with PBS/T, and finally 100 ul of freshly made substrate solution was applied into the well. After 30 minutes at room temperature, the reaction was terminated and the absorbance was measured on an automated ELISA reader at 492 nm.

(c) ELISA vs RIA in sensitivity

In order to compare the sensitivity of the ELISA to that of the RIA described in the previous section, the following experiments were performed.

ELISA

(1) 25 ug/ml of MOR-BSA conjugate was applied to the 96-well microtitre plate at 0.2 ml per well.

(2) The serum from an immune mouse served as a source of antibodies. It was initially diluted to 1:100 and further diluted to give a series of eight serial doubling dilutions. The antiserum was then applied onto the plate in a vertical direction. At the same time, normal mouse serum at a 1:100 dilution was added to the last row as a negative control.

After washing away the serum, the plate was treated with second antibody and colour development solution. The results obtained are shown in Table 2.3.

RIA(Sac-Cel)

(1) A 1:100 dilution of a previously pooled mouse antiserum was prepared in RIA buffer as a positive control.

(2) Normal mouse serum at a 1:100 dilution was prepared. This sample together with a blank buffer were used as negative controls.

(3) The serum from the immune mouse was diluted to 1:100 and was prepared in the same way as above (please see ELISA (2)).

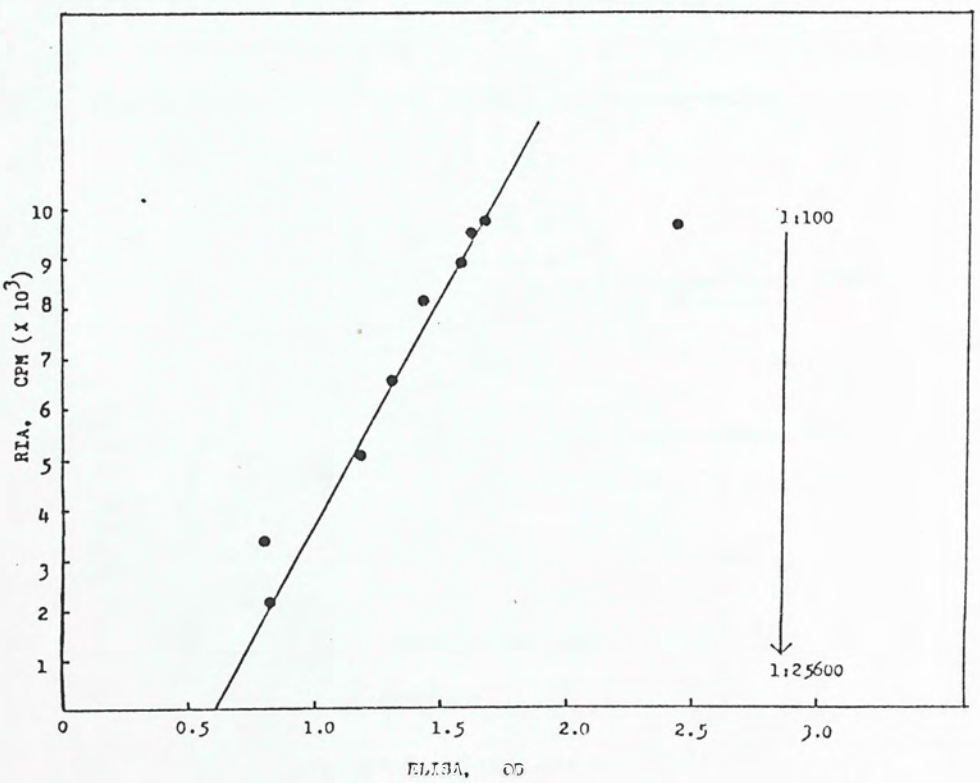
All the samples were prepared in duplicate. Sac-Cel separation was performed, and the resulting counts are given in Table 2.3.

Table 2.3 Radioimmunoassay vs ELISA in sensitivity

	RIA		ELISA	
Antibody dilution	CPM	Average CPM	OD	Average OD
100X	9685	9559	2.500	2.466
	9434		2.500	
			2.432	
200X	9652	9737	1.651	1.656
	9821		1.668	
			1.648	
400X	9399	9443	1.639	1.616
	9486		1.641	
			1.569	
800X	8586	8821	1.587	1.567
	9055		1.605	
			1.510	
1600X	7823	8066	1.473	1.414
	8309		1.326	
			1.443	
3200X	6255	6503	1.290	1.300
	6750		1.301	
			1.308	
6400X	4789	5035	1.038	1.086
	5280		1.089	
			1.130	
12800X	3316	3360	0.822	0.798
	3404		0.770	
			0.801	
25600X	2084	2122	0.773	0.834
	2160		0.791	
			0.937	
Positive control	10467			
	10607			
Negative control-normal mouse serum	687		0.085	0.032
	702		0.012	
			0.000	
Blank	715			
	696			

A graph of absorbance (OD) plotted as a function of radioactivity bound (cpm) was produced (Fig. 2.3). The data suggested that there was a good correlation between the two assay systems. The correlation coefficient (r) was 0.85.

Fig. 2.3 A plot showing the correlation of ELISA with RIA



2.2.3.3 The dot immunobinding assay

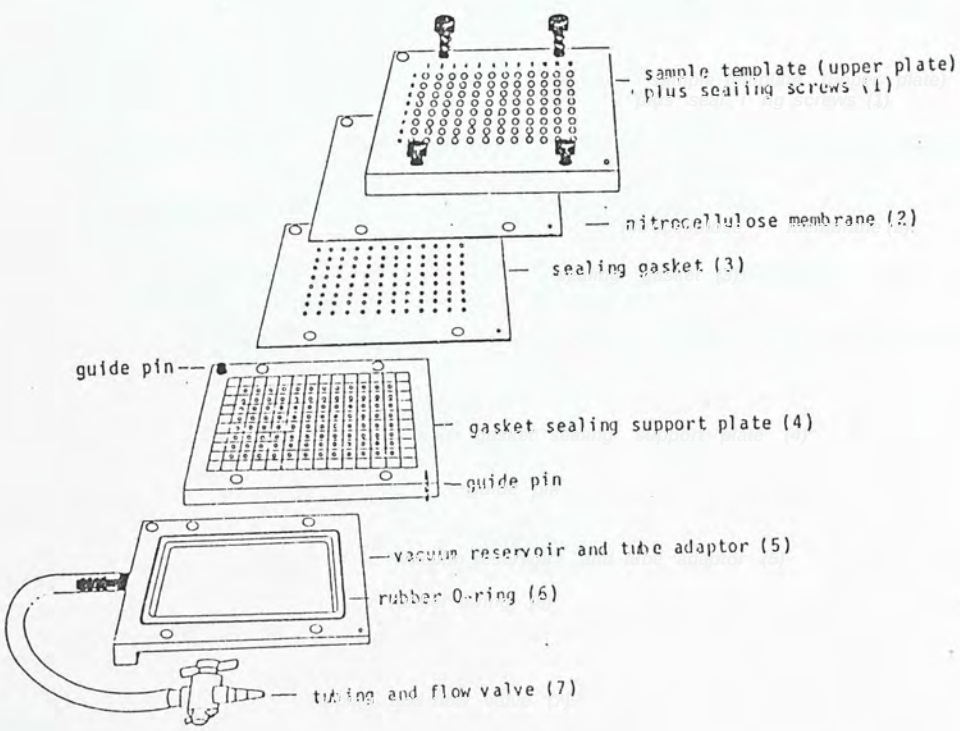
(a) Materials:

1. Mouse anti-morphine serum
2. Working buffer : phosphate buffered saline (PBS), pH 7.4
3. I-125 morphine solution: PBS_5 containing I-125 morphine (10^5 cpm/ml), 1% Triton X-100, 0.5% Deoxycholic acid (DOC), 0.1% Sodium dodecyl sulfate (SDS) and 1% dry milk.
4. Bovine serum albumin (BSA).

(b) Assay procedure:

A bio-dot apparatus designed in the format of a 96-well microtitre plate, was used (Fig.2.4).

Fig. 2.4 The assembly of a bio-dot apparatus



Samples are applied into the well and drawn through the nitrocellulose membrane passively. Wells are washed repeatedly by applying a vacuum. Afterwards, the membrane is removed and processed for autoradiography.

In our assay, the antiserum was tested at two-fold fold decreasing concentrations from 1:100 dilution to 1:1600 dilution. Normal mouse serum was prepared in the same way as control. The samples were applied into the wells and allowed to drain through the filter under gravity (approximately 1 hr). The nitrocellulose membrane was then removed and the excess binding sites on it were blocked with a PBS solution containing 1% BSA for 1 hr at room temperature. After blocking, the membrane was transferred to a large petri dish containing I-125 morphine solution and incubated overnight at $4^{\circ}C$. At the end of incubation, the membrane was removed and washed 6X in PBS containing 1% Triton, 0.5% DOC and 0.1% SDS (30 minutes per wash). Finally the membrane was dried and autoradiographed at $-70^{\circ}C$ for 24 hr using an intensifying screen.

(c) Results:

Several recent reports (Hawkes et al. 1982; Yeoman et al. 1983) have shown that the dot blotting technique

has been successfully used in the screening of hybridoma clones. On one nitrocellulose membrane, 96 supernatants may be rapidly screened, and the assay is said to offer an equal or greater sensitivity than the ELISA. However, in our studies, we detected no apparent binding of I-125 morphine to the antiserum on the nitrocellulose membrane. To solve the problem, we doubled the amount of radioactivity used (i.e. from 10^5 cpm/ml to 2 x 10^5 cpm/ml), and also extended the exposure time to 48 hr. The result again proved to be disappointing and it was thus decided that we should consider other screening methods as better alternatives.

2.2.3.4 Radioimmunoassay using a microharvester

During the production of monoclonal antibodies, it is necessary to assay large numbers of wells for specific antibodies. Ideally the assay should be quick, sensitive and simple. ELISA fulfils many of these requirements but it is well known that its sensitivity cannot be compared to that of the RIA. The experiment procedure described here is a modification of the Sac-Cel method which can process up to 96 samples on one microtitre plate while offering a greater sensitivity than the ELISA.

(a) Materials:

1. I-125 morphine tracer (10^5cpm/ml).
2. Anti-MOR serum (or culture supernatant).
3. Sac-Cel second antibody.
4. RIA buffer (please see section 2.2.3.1).
5. 96-well PVC plates ("used" plates).
6. Micro-harvester (Titertek).

(b) Assay procedure:

The mouse antiserum was diluted to a 1:1000 dilution and further diluted to give a series of doubling dilutions down to 1:32000. The antiserum (100 ul) was applied onto the 96-well plate in a vertical direction. I-125 morphine (50 ul) was added to each well. After mixing gently, the plate was incubated overnight at 4°C. Sac-Cel (50 ul) was then added and the samples were further incubated at room temperature for 30 minutes. At the end of incubation, the wells were washed with PBS (pH 7.4) on a microharvester, and the contents harvested by suction and filtration . The pellets collected on the filter paper (harvester filter) were dried and the radioactivity from each well was measured in a gamma-counter.

(c) Results:

The results obtained are given in Table 2.4 as below:

Table 2.4 Radioimmunoassay using a microharvester.

Antibody dilution	Radioactivity (cpm)	Time (min.)
1:1000	1074	0.5
	928	
	1000	
	1690	
	1100	
	916	
1:2000	732	0.5
	2946	
	1092	
	744	
	796	
	672	
1:4000	500	0.5
	560	
	622	
	666	
	614	
	594	
1:8000	582	0.5
	600	
	572	
	1062	
	520	
	604	
1:16000	498	0.5
	686	
	456	
	438	
	524	
	438	
1:32000	574	0.5
	598	
	554	
	426	
	1286	
	538	
Normal mouse serum 1:100	384	0.5
	460	
	376	
Blank	478	0.5
	496	
	370	

It was hoped that with the modified Sac-Cel method, we would be able to avoid the time-consuming steps of sample manipulations and centrifugations. However, our data suggested that the sensitivity of this assay was somewhat lower than expected. The experiment was repeated twice and there was only a slight improvement in the results. The reason for this could be due to the uneven washing of the wells by the microharvester. It was difficult to maintain a constant speed and control during the washes, and this could have affected the sensitivity of the assay considerably.

2.2.3.5 Summary of screening assays

The initial screenings of hybridomas are usually performed with hundreds and even thousands of assay samples that contain low amounts of antibodies. The main factors to be considered during the screening process are sensitivity, simplicity, speed and reliability. In our studies, it was found that while ELISA offered speed and simplicity, it was not very sensitive, with a high non-specific assay background. In contrast, the Sac-Cel method was sensitive, fast, easy to perform, and the data obtained was consistent as compared to those produced by other screening systems. Thus the Sac-Cel RIA was

selected for the detection of monoclonal antibody activity as it better satisfied the main factors mentioned above.

2.2.4 Cell lines and culture media

NS-1 myeloma cells were kindly supplied by Department of Microbiology, University of Hong Kong. The cells were maintained in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS) (both from GIBCO). The growth medium also contained penicillin (50 units/ml) and streptomycin (50 ug/ml). The cells were grown at 37°C in a humidified atmosphere with 7% CO_2. They were maintained in optimal conditions (i.e. in logarithmic phase of growth) for at least one week before fusion was performed.

2.2.5 Cell fusion and culturing

The fusion procedure follows the basic principles of Köhler and Milstein (1976). Spleen was removed from the immune mouse and splenocytes were fused with NS-1 myeloma cells using 50% polyethylene glycol (PEG 4000, Merck). Fused cells were suspended in 30 ml culture medium (RPMI-1640 supplemented with 10% FCS, penicillin and streptomycin) and incubated in a large culture dish (90 mm) at 37°C for eight hours. A 100 x HAT stock selective

medium containing 10^{-4}M hypoxanthine, 4 x 10^{-7}M aminopterin and 1.6 x 10^{-5}M thymidine was prepared. At the end of incubation, the fused cells were dispensed into 3 x 96-well tissue culture plates (NUNC) containing 0.1 ml of 2 x HAT medium per well. Approximately 0.5 x 10^{6} fused cells were placed in each well. When hybridoma growth had reached about one third of the well, the culture supernatant was taken and tested by the Sac-Cel RIA method (Section 2.2.3.1). This usually occurred between 10-14 days after fusion.

2.2.6 Cloning of hybridomas by Limiting Dilution

Hybridomas producing antibody which bound I-125 morphine were cloned by limiting dilution. Cells were plated in a 96 well plate at the following concentrations: 36 wells with an average of 5 cells/ well, 36 wells with an average of 1 cell/well and 24 wells with an average of 0.5 cell/well. Approximately 0.5 x 10^{6} splenocytes were added into each well as feeders.

2.3 Experimental data for the production of hybridomas

Altogether six fusions were performed in this study. A total of 749 wells containing growing hybridoma cells were observed. 49 of these scored positive upon initial screening by the Sac-Cel RIA method. The results, together with other relevant information, are presented in the following sections:

Fusion experiment A: Twenty-five million NS-1 cells were mixed with 188 x 10^6 immune spleen cells in a 50 ml conical tube, and plain medium (RPMI 1640, GIBCO, supplemented with penicillin and streptomycin) was added to a volume of 30 ml. The cell mixture was centrifuged at 1100 rpm for 5 minutes and was washed once more in plain medium. After centrifugation, the supernatant was carefully removed and the cell pellet was loosened by gentle tapping of the tube. 1 ml of pre-warmed 50% PEG solution was added dropwise in 1 minute, with occasional gentle shaking. Two ml of warm plain medium was then added dropwise over a 2 minute period, followed by a further 10 ml over the next 5 minute period. The cell suspension was centrifuged at 1200 rpm for 10 minutes. After removal of

the supernatant, the cell pellet was gently suspended in 30 ml plain medium containing 10% FCS and incubated for 8 hours. At the end of incubation, the fused cells were dispensed into 384 microtiter plate wells at 0.5 x 10^6 cells/well in HAT medium (see Section 2.2.5). The cultures were fed on day 7 and every 3-4 days afterwards. Small colonies of hybrid cells were microscopically visible between 7-10 days. As soon as cell colonies grew to about one third of the well, 0.1 ml supernatant was taken and screened for antibody activity.

Table 2.5 shows that in this experiment, 8 out of 143 wells (0.06%) containing hybrid colonies produced antibodies which bound I-125 morphine with an affinity of about 1.2x background reactivity. Hybridomas from 5 of these wells with the highest antibody activity were chosen and cloned by limiting dilution (for details of the method, please see Section 2.2.6). From these, 2 wells were assayed positive for antibody production. They were subsequently reclóned, and in each case the hybrid colonies obtained were found to be totally negative.

Table 2.5 Results of fusion experiment A

	N	W/hybrids	W/+ve	Specific antibody activity	W/-ve
Primary culture	384	143	8	1.2x background	135
First limiting dilution	I:96	52	1	2.5x "	51
	II:96	4	0	-	4
	III:96	17	1	1.8x "	16
	IV:96	34	0	-	34
	V:96	29	0	-	29
Second limiting dilution	I :96	47	0	-	47
	III(a):96	23	0	-	23
	III(b):96	30	0	-	30

N = total number of wells seeded
W/hybrids= well with hybridoma growth
W/+ve = wells with antibody-secreting hybrid cells
W/-ve = wells with non-producers
Specific antibody activity = positive signal secreted by hybrid cells (please see Section 2.2.3.1 for assay procedure)

Fusion experiment B: Twenty-five million NS-1 cells were fused with 125×10^6 immune spleen cells. The fusion mixture was distributed into 384 wells at 0.4×10^6 cells/well in HAT selective medium containing 10^5 splenocytes as feeder cells. The data obtained from the first screening on day 10 were found to be totally negative, as shown in Table 2.6. Meanwhile, it was observed that the growth of the hybrid colonies was very slow, with a large number of fibroblasts and macrophage-like cells spreading at the bottom of the wells. A second screening 4 days later again resulted in a negative finding. The attempt was finally abandoned after 19 days of culture.

Table 2.6 Results of fusion experiment B

	N	W/hybrids	W/+ve	Specific anti-body activity	W/-ve
Primary culture	384	91	0	-	91

Please see footnotes of Table 2.5 for explanation.

Fusion experiment C: Thirty-four million NS-1 cells were fused with $175x10^6$ immune spleen cells using 50% PEG. The resulting cell mixture was dispensed into 288 wells at $0.7x10^6$ cells/well in HAT medium. Table 2.7 shows that 8 out of 136 wells (0.06%) with hybrid growth were found positive with an affinity of 1.2x background reactivity. One well showing the highest antibody secretion (i.e. positive by 2 successive screenings) was selected for subcloning. Thirty wells with hybrid colonies were obtained. Six of these (0.2%) were positive for antibody activity, from which 2 wells were chosen for further subcloning: I(a) and I(b). In the case of I(a), 3 out of 9 wells were initially identified positive (1.4x background reactivity), but these 3 wells lost antibody production on second screening. In the case of I(b), 6 out of 29 wells scored positive (1.3x background reactivity) but again all yielded negative results on the second screening.

Table 2.7 Results of fusion experiment C

	N	W/hybrids	W/+ve	Specific anti-body activity	W/-ve
Primary culture	288	136	8	1.2x background	128
First limiting dilution	I:96	30	6	1.5x "	24
Second limiting dilution	I(a):96	9	3---*--->0	1.4x ---*--->0	6
	I(b):96	29	6---*--->0	1.3x ---*--->0	23

Please see footnotes of Table 2.5 for explanation.
* "------>0" indicates a loss of signal on rescreening by RIA.

Fusion experiment D: Seventeen million NS-1 cells were fused with $110x10^6$ immune spleen cells. The fused cells were distributed into 320 wells at $0.4x10^6$ cells/well in HAT medium. As indicated in Table 2.8, 12 out of 81 wells (0.15%) with hybrid growth were positive for antibody activity(1.4x background reactivity). Hybridomas from 7 of these wells were chosen for subcloning by limiting dilution: I-VIII. Wells I and V were subcloned in duplicate. Subsequently, in I(b) 5 of 43 wells with hybridoma growth were positive(1.3x background reactivity), however, they all lost antibody activity on second screening. The same was observed in subclones II and III; in the former case, 3 out of 54 wells scored positive (1.3x background reactivity) on initial screening, and in the latter, 2 out of 51 wells were positive(1.4x background reactivity). During the course of the experiment, cultures IV, VI and VII were overgrown by macrophage and fibroblast cells, thus resulting in no hybrid growth. On the other hand, although hybrid colonies were obtained from I(a), V(a) and V(b), they were found to be negative for antibody production.

Table 2.8 Results of fusion experiment D

	N	W/hybrids	W/+ve	Specific antibody activity	W/-ve
Primary culture	320	81	12	1.4x background	69
First limiting dilution	I(a): 96	36	0	-	36
	I(b): 96	43	5---*--->0	1.3x ---*---->0	38
	II : 96	54	3---*--->0	1.3x ---*---->0	51
	III: 96	51	2---*--->0	1.4x ---*---->0	49
	IV: 96	0	-	-	-
	V(a):96	7	0	-	7
	V(b):96.	52	0	-	52
	VI :96	0	-	-	-
	VII :96	0	-	-	-

Please see footnotes of Table 2.5 for explanation.
* Please see Table 2.7.

Fusion experiment E: Forty-four million NS-1 cells were fused with $176x10^6$ immune spleen cells. The cell mixture was dispensed into 480 wells at $0.45x10^6$ cells/well in HAT medium. As indicated in Table 2.9, 6 out of 76 hybrid cultures (0.07%) were shown positive. From these, 3 wells were chosen for subcloning by limiting dilution; I-III. In I, 58 wells with hybrid growth were obtained. 9 of these wells(0.15%) were found to be positive for antibody activity, from which 5 wells were selected for further subcloning; I(a) - I(e). Since II yielded no hybrid growth, the plate was discarded after 2 weeks. In III, 10 out of 27 wells (0.4%) had antibody activity, from which 3 wells were chosen for further subcloning; III(a)- III(c). However, all of the hybrid colonies subsequently generated from the second limiting dilution were found to be totally negative.

Table 2.9 Results of fusion experiment E

	N	W/hybrids	W/+ve	Specific anti-body activity	W/-ve
Primary culture	480	76	6	1.2x background	70
First limiting dilution	I:96	58	9	1.4x "	49
	II:96	0	-	-	-
	III:96	27	10	1.3x "	17
Second limiting dilution	I(a):96	8	0	-	8
	I(b):96	2	0	-	2
	I(c):96	4	0	-	4
	I(d):96	0	-	-	-
	I(e):96	2	0	-	2
	III(a):96	17	0	-	17
	III(b):96	2	0	-	2
	III(c):96	18	0	-	18

Please see footnotes of Table 2.5 for explanation.

Fusion experiment F: Twenty-eight million NS-1 cells were fused with $150x10^6$ immune spleen cells. The cell mixture was distributed into 336 wells at $0.5x10^6$ cells/well in HAT medium. Table 2.10 shows that 15 of the 222 wells (0.06%) with hybrid growth were identified positive for antibody production. From these, 6 wells were chosen for subcloning by limiting dilution. On initial screening of the subcloned cultures, only one well from IV was positive(1.3x background reactivity). However, the hybrids in this well lost all antibody activity on second screening.

Table 2.10 Results of fusion experiment F

	N	W/hybrids	W/+ve	Specific antibody activity	W/-ve
Primary culture	336	222	15	1.2x background	207
First limiting dilution	I:96	12	0	-	12
	II:96	8	0	-	8
	III:96	19	0	-	19
	IV:96	47	1---*--->0	1.3x---*--->0	46
	V:96	19	0	-	19
	VI:96	23	0	-	23

Please see footnotes of Table 2.5 for explanation.
* Please see Table 2.7.

2.4 Discussion

Six fusions were performed during the preparation of monoclonal antibodies against morphine. In our studies, a positive culture was screened twice before it was subcloned by limiting dilution. As can be observed from our results, a good number of clones which were initially positive lost antibody activity after the first screening. This problem continued to exist even after we had subcloned the cultures which were confirmed to be positive by two successive screenings. It was found that of the many hybrid clones generated after limiting dilution, a majority gave negative results, with only a few yielding weakly positive signals. Moreover, the initial weakly positive hybrid cell lines soon lost all antibbody production and became non-producers. This mostly occurred before the second screening of the subcloned hybrid cells. The situation was not helped by a further subcloning of these cultures. In short, although we managed to obtain a good number of positive clones after a fusion, we somehow found it difficult to generate a stable cell line out of these hybrid cultures.

To overcome this difficulty, we are at present considering some other strategies which may be helpful in obtaining successful results. It has been estimated that the splenocytes needed for a fusion contain only a small subset of the heterogeneous spleen cell populations, approximately 1%. Thus for a mouse spleen of 10^8 cells, only 10^6 cells will be actually secreting the desired antibody. According to a published study (Scharff et al., 1980), one successful hybrid can be formed per $2x10^5$ of these splenocytes. Therefore if 1% of the 10^8 spleen cells can secrete the right antibody, five desirable hybridomas will be generated from one spleen. This is correct when applied to highly immunogenic molecules, but for less immunogenic antigens such as morphine conjugates, the number of hybridomas formed from a mouse spleen will be much less.

Based on this theoretical extrapolation , we propose that in the future, splenocytes from 3-4 immune mice may be fused at the same time and plated out in a large number of 96-well tissue culture plates. In order to speed up processing so many cultures at a time, the screening will be done in two steps; firstly by ELISA then followed by RIA. Basically the procedure involves a quick ELISA to select potential hybrid cultures. Then these will

be rescreened by RIA at a later stage. Moreover, the high assay background inherently associated with ELISA can be minimised by running two sets of ELISA's. For the determination of antibodies to morphine, MOR-hemocyanin coated plates are used and for the determination of antibodies to hemocyanin, hemocyanin coated plates are used. By comparison of the results from the two assays, antibodies recognizing the hemocyanin moiety of the MOR-hemocyanin conjugate can be eliminated. The positive cultures obtained in this manner can then be confirmed by the more specific RIA (using Sac-Cel) procedure.

Another possible method to successfully obtain hybridomas may be to use a different myeloma cell line for fusion. It is well known that NS-1 synthesizes k light chain and that NS-1 fusion hybrids will secrete molecules incorporating this light chain (Hurrell, 1982). As a result, only one immunoglobulin molecule in four will be actively coded for by the spleen parent, the other three will have low or no binding affinity for the antigen. On the other hand, a non-producing myeloma line synthesizes neither heavy nor light chains and therefore produces hybridomas which will make only the spleen cell antibody.

The most common non-producing myeloma lines are listed in Table 2.11 below, together with the NS-1 line for the comparative purpose.

Table 2.11 Myeloma lines which have been successfully used in hybridoma production

Cell line	Derived from	Ref.	Fusion efficiency	Hybridoma stability
NS1 - Ag4/1	X63	Köhler and Milstein 1976	+++	+++
X63 - Ag8.653	X63 - Ag8	Kearney et al. 1979	++	+++
Sp2/0 - Ag14	X63 - Ag8x Balb/c	Shulman et al. 1978	++	+++
FO	Clone of Sp2/0	Fazekas de St. Groth & Scheidegger 1980	+++	+++
NSO/1	NS1 -Ag4/1	Gaefrè and Milstein 1981	++	+++

Another good approach to increase the probability of generating a successful hybridoma, is to have morphine coupled to hemocyanin in different ways. The advantage of 6-succinylmorphine is the availability of all functional groups essential for pharmacological activity. Coupling is done via the 6-OH group, which is unnecessary for opioid activity. In a recent paper, Findlay et al. (1981) has reported that the attachment of the MOR molecule to a carrier at a site close to, but distinct from the N atom (i.e. at C2 or C8 positions), may lead to antiserum with the best overall specificity characteristics. The resulting antiserum is said to be very sensitive to changes in structure at C3 or C6 positions, as well as at the N atom. It will certainly be of great interest to see whether a different morphine conjugate may enhance the possibility of obtaining successful monoclonal antibodies.

CHAPTER III The production and use of polyclonal antibodies specific for morphine

3.1 Introduction

As has already mentioned, morphine and opioid peptides are found to bind to the same opioid receptors in the brain. Surely there must exist some kinds of structural similarities between the opiates and their endogenous peptides. It was originally hoped that this investigation would be carried out by using a panel of monoclonal antibodies directed against different determinants of the morphine molecule. However, since we had no success so far in producing these highly specific reagents, polyclonal antisera specific for morphine from rabbits and mice were used as alternatives to test our original hypothesis. The specificity of each of these antisera was characterized by its ability to bind to a range of related opiate ligands in the radioimmunoassay system.

3.2 Materials and Methods

3.2.1 Antiserum preparation.

Rabbits were injected subcutaneously at multiple sites in the back with morphine-hemocyanin conjugate (1 mg/rabbit) in 1 ml phosphate buffered saline (PBS, 0.15M NaCl, 0.01M phosphate buffer, pH 7.4) emulsified with an equal volume of complete Freund's adjuvant. Five to six additional booster injections in incomplete Freund's adjuvant were given similarly at 2 week intervals. Blood was collected from the median artery of the ear after one week. Two samples of rabbit anti-morphine antiserum were obtained as a result of immunization and these were designated as RB 3 and RB 4. For the preparation of mouse anti-morphine antiserum, please see Section 2.2.2 of Chapter II. Six sera were produced and numbered MOS 1- MOS 6.

3.2.2 RIA procedure.

(a) Materials.

I-125 morphine tracer (10^5 cpm/ml).

Donkey anti-mouse antisera coupled to cellulose beads (Sac-Cel, Wellcome, U.K.).

Rabbit IgG, concentration= 30 mg/ml. For working, dilute 100x with RIA buffer.

Goat anti-rabbit serum (P_3). One ml will precipitate 3 mg IgG. For working, dilute 10x in RIA buffer.

RIA buffer: 0.15M phosphate buffer, pH 7.4, containing 0.1% BSA (RIA grade) and 0.1% Triton X-100.

(b) Assay method.

Antiserum titres were determined by incubating 0.1 ml of various dilutions of antiserum in RIA buffer with 0.1 ml of I-125 morphine tracer, and a further aliquot of 0.1 ml RIA buffer. Incubation was carried out at 4 °C overnight. For the mouse antiserum, antibody-bound radiotracer was precipitated by incubation for 30 minutes at room temperature with 0.1 ml Sac-Cel. After the addition of 1 ml distilled water, the tubes were centrifuged at 3000 rpm for 15 minutes. The supernatants were removed and the pellets were finally counted at 2 minutes each in a gamma counter. The optimal titre chosen was the dilution which bound 20-40% of the total radioactive tracer added. Fig. 3.1 summarizes the titres of each of the mouse anti-morphine serum. The optimal dilutions chosen are summarized in Table 3.1. For the rabbit antiserum, the samples were prepared and incubated in the same way as described above, except that on the next day, 0.1 ml rabbit IgG and 0.1 ml goat anti-rabbit antiserum were added to each sample, and incubation was carried on for another night at 4°C. On the third day, 1 ml cold PBS, pH 7.4, was added, and the

tubes were then centrifuged at 5200 rpm for 45 minutes. The pellets eventually collected were counted at 2 minutes each in a gamma counter. The results of the rabbit samples are shown in Fig. 3.2. The optimal dilutions chosen for RB 3 and RB 4 were 1:1600 and 1:3200, respectively.

3.2.3 Selection of opiate ligands.

(a) Rationale

A group of opiate-like compounds was selected and used as unlabelled ligands in the competition experiments against I-125 labelled morphine for binding sites in the antibodies. Studies on the binding of the various opioids have shown that morphine, which is the standard drug for producing analgesia, is a potent agonist for the μ-binding receptor (Martin, 1976). On the other hand, naloxone is a competitive antagonist at the μ-, δ-, and k- sites but its binding to the μ-site is considered to be the most selective (Lord, 1977). Etorphine, being 700x more potent than morphine in human and 1000x more potent in animals (Lister, 1967), has the highest affinity for the k-site (Robson, 1982). Apart from the 3 drugs mentioned above, some of the endogenous peptides and their synthetic

analogues were also included in this study. β-endorphin is found to be equipotent in displacing the binding of tritiated μ- or δ-ligands (Kosterlitz, 1982). Both met-enkephalin and its stable analogue, [D-Ala^2, D-Leu^5]-enkephalin (DADLE), bind predominantly to the δ-site (Kosterlitz, 1982). A C-terminus extension of leu-enkephalin results in the formation of leu-enkephalin-arg. However, this active peptide loses its δ-selectivity which is typical of the enkephalin, and shows a marked affinity for the k-site (Corbett, 1982). Both dynorphin$_{1-13}$ and dynorphin B interact preferentially with the k-site (Corbett, 1982), while [D-Ala^2, MePhe^4, Gly-ol^5]-enkephalin (DAGO) is a μ-selective ligand, being 220 times more active at the μ-than at the δ-binding site (Kosterlitz, 1981). Table 3.2 summarizes the properties of the opiate ligands selected for our study. It is expected that these ligands would provide a good spectrum of structural features which will enable us to dissect the similarity of morphine with them in the binding to distinct sub-types of the opioid receptors.

Table 3.2 List of opiate ligands used in this study.

Ligand	Molecular weight	Receptor type	Relative affinity* at the μ-site	δ-site	κ-site
DADLE	569	δ	0.1	1	<0.01
Met-enkephalin	573	δ	0.1	1	<0.01
Leu-enkephalin-arg	711	κ	NA**	NA	NA
Dynorphin A_{1-13}	1604	κ	0.15	0.05	1
Dynorphin B	1571	κ	0.07	0.06	1
β-endorphin 1-31	3500	μ, δ	1	0.9	0.02
DAGO	513	μ	1	<0.01	<0.01
Etorphine	411	κ	0.2	0.4	1
Naloxone	327	μ	1	0.07	0.1
Morphine	285	μ	1	0.02	<0.01

* The affinity of unlabelled opioids was estimated as the inhibition constant (Ki) from their ability to displace the binding of labelled ligands of known characteristics. For each ligand the potency at the site for which it had highest affinity was taken to be 1.
(Ref. : Hughes, J. (1983) ed. Opioid peptides, Churchill Livingstone, pp.32-34).

** NA : data not available.

(b) Method of assay

The affinity of anti-morphine antisera to recognize different opiate ligands was determined in competition studies in which varying concentrations of the cold ligands were used to displace I-125 morphine from the

antibodies. Serial dilutions of the unlabelled ligands were prepared by a 3-fold dilution from 10^4 nM to 0.056 nM in RIA buffer. The appropriately diluted opiate ligand (0.1 ml) was incubated with 0.1 ml I-125 morphine and 0.1 ml antiserum of the chosen titre. The tube that contained I-125 morphine and antiserum but no unlabelled ligand served as a measure of the maximum antibody-bound radioactivity (B_{max}). A control containing no antibody was used as the blank. Incubation conditions and separation of bound and free radioactivity were as described in 3.2.2. All the binding data are expressed as B/Bo in which B is equal to activity bound minus blank and Bo is the maximum activity bound minus blank.

3.3 Results

(a) Optimization of antiserum titres.

Antisera were used at optimal dilutions required to bind about 20-40% of I-125 morphine in the absence of a competing ligand. The results are shown in Fig. 3.1 and Fig. 3.2 respectively. Table 3.1 summarizes the optimal antibody dilutions of the mouse samples.

Fig. 3.1 Determination of antibody titres in mouse samples

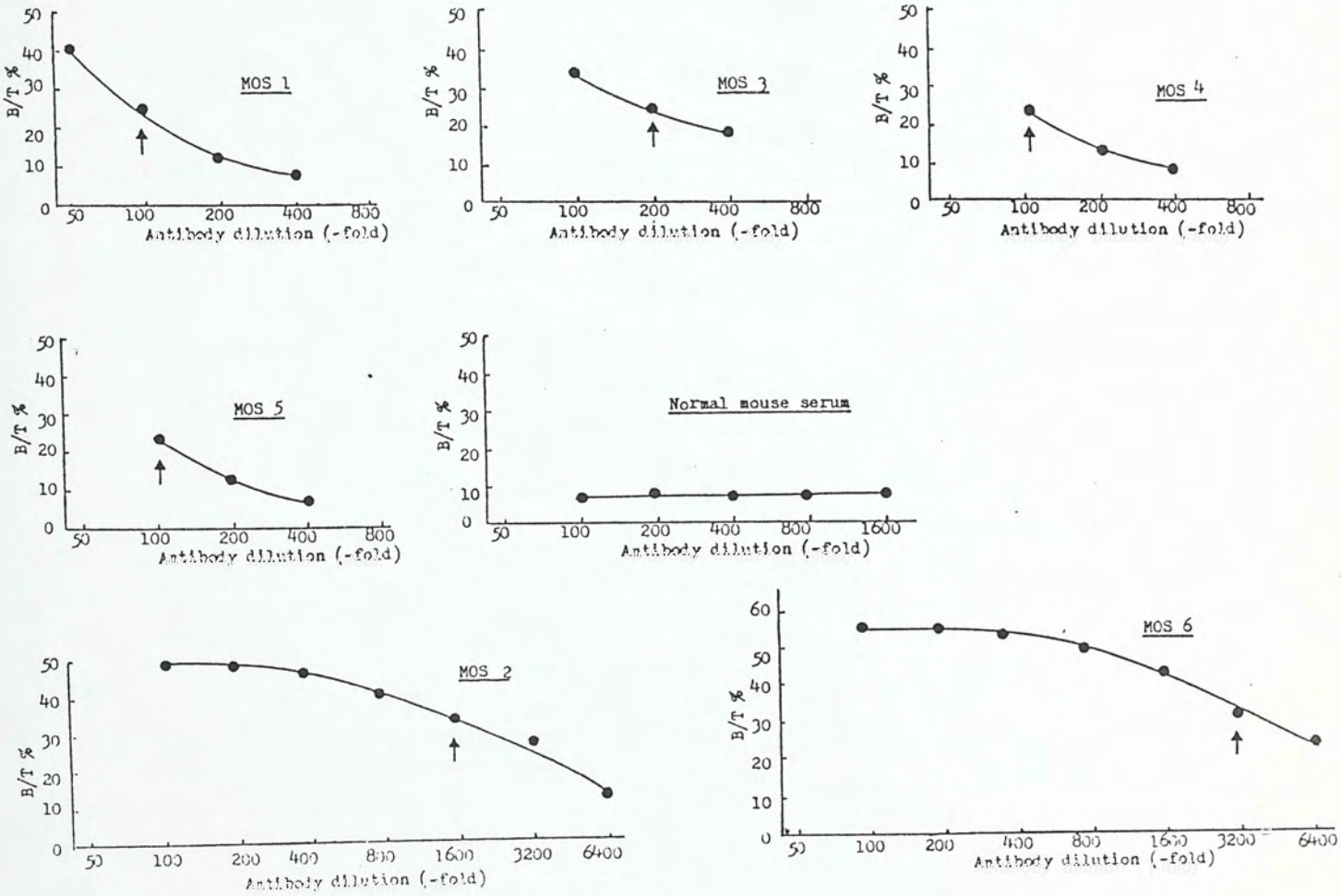
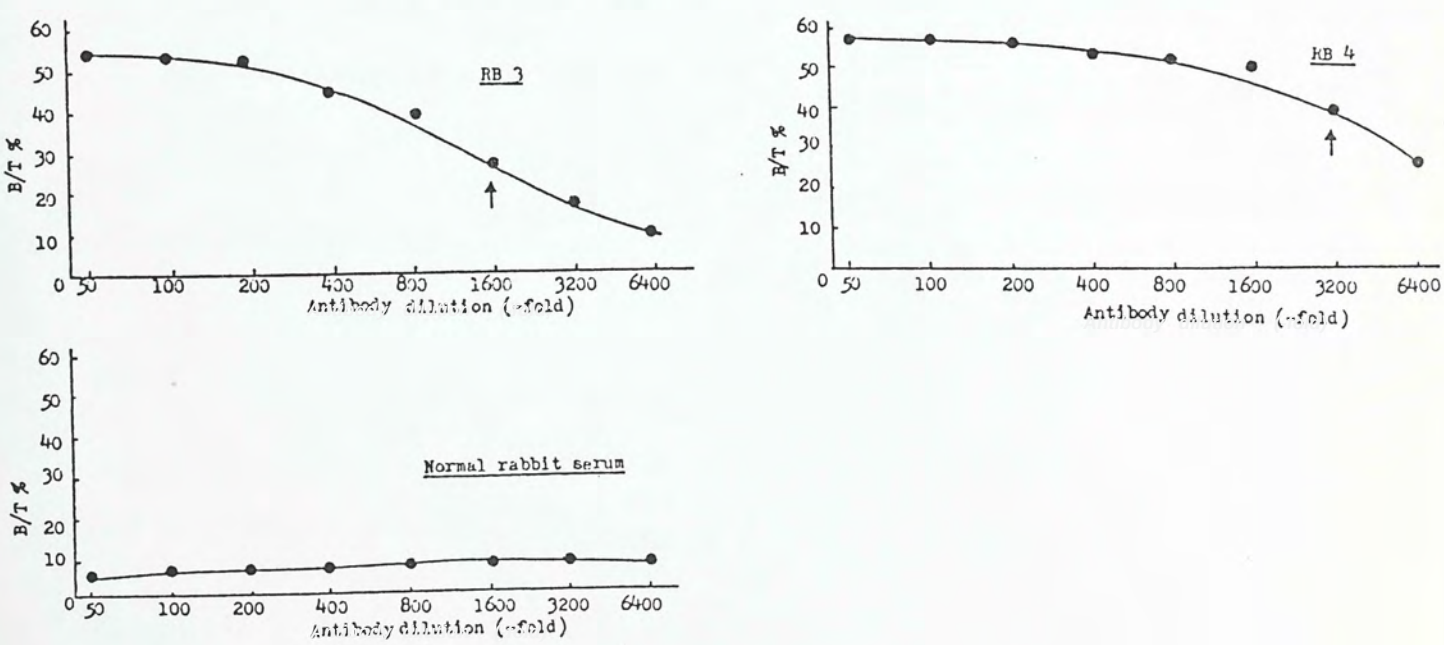


Table 3.1 Optimal antibody dilutions of mouse samples.

Sample No.	Optimal antibody dilution
MOS 1	1:100
MOS 2	1:1600
MOS 3	1:200
MOS 4	1:100
MOS 5	1:100
MOS 6	1:3200

Fig. 3.2 Determination of antibody titres in rabbit samples



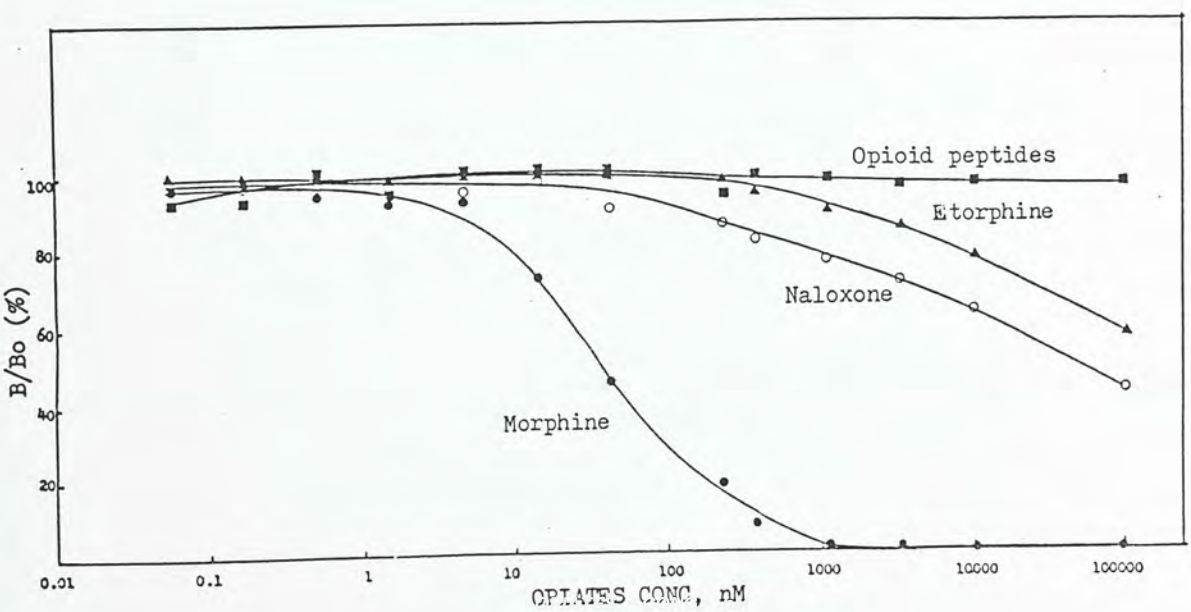
(b) Competition experiments.

In our studies, 2 rabbit antisera together with 6 mouse antisera were produced. The specificity of the antibodies was examined by competitive radioimmunoassay with I-125 morphine against a series of opiate ligands. Our results demonstrated that these antisera were quite similar to each other in that they all bound morphine with high affinity. Some recognition was also observed with high concentrations of the opiate antagonist, naloxone, and the opiate agonist, etorphine. However, there was no measurable cross-reactivity with the endogenous opioid peptides or their synthetic analogues such as DADLE and DAGO. The results of these assays are presented individually in the following sections and summarized in Tables 3.3 and 3.4.

(i) Mouse anti-morphine serum: MOS 1

Both naloxone and etorphine showed a very small degree of cross-reactivity with antiserum MOS 1. No inhibition was obtained with all the opioid peptides or their analogues even at the highest ligand concentrations tested. The IC_{50} with standard morphine was at 33.6 nM, and with naloxone and etorphine were at 49 uM and 230 uM, respectively.

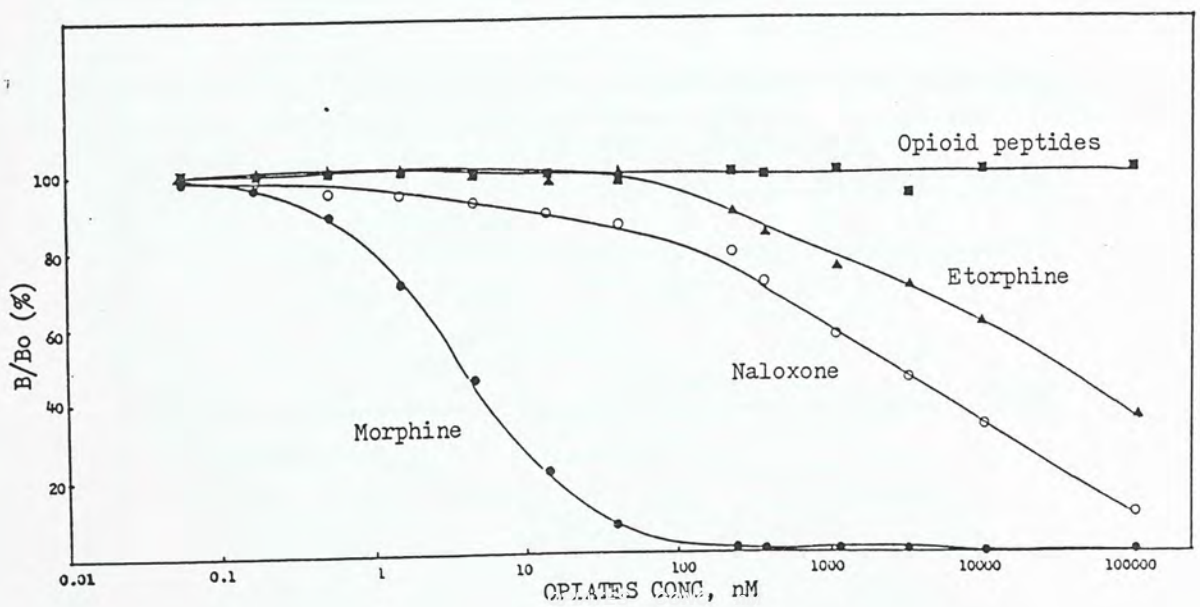
Fig.3.3 Inhibition of I-125 morphine binding to antiserum MOS 1 by various opiate ligands. The optimal dilution of the antiserum used was 1:100. B/Bo was defined as the percentage of specific radioactivity bound to the antiserum in the presence of sample to the specific radioactivity bound in the absence of the opiate ligand. I-125 morphine bound was determined in the presence of different concentrations of morphine (●), naloxone (o), etorphine (▲), and all opioid peptides & analogues (■).



(ii) Mouse anti-morphine serum: MOS 2

This antiserum was more sensitive and only 3.8 nM of morphine was required to inhibit 50% of the I-125 morphine binding. The IC_{50} of naloxone and etorphine were at 2 uM and 23 uM, respectively. Again, there was no observable cross-reactivity with the endogenous opioid peptides or their analogues.

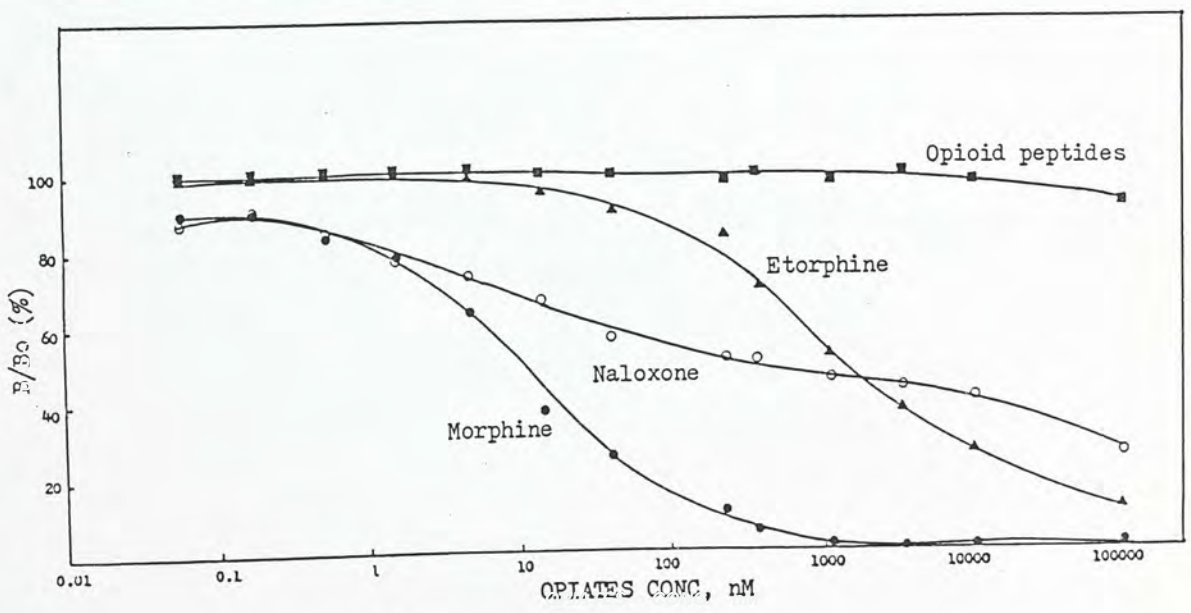
Fig. 3.4 Inhibition of I-125 morphine binding to antiserum MOS 2 by various opiate ligands. The optimal dilution of the antiserum used was 1:1600. Please see Fig. 3.3 for details of symbols used.



(iii) Mouse anti-morphine serum: MOS 3

One very interesting feature in this antiserum is that it bound etorphine with higher affinity than the previous 2 antisera. The slope of the binding inhibition curve was parallel with that of the standard morphine, indicating that this antiserum may possess certain binding sites which recognize similar determinants on morphine and etorphine. The IC_{50} with standard morphine was at 4.9 nM, and with etorphine was at 1439 nM. This antiserum also demonstrated significant cross-reactivity with naloxone, with IC_{50} value at 450 nM. However, the inhibition curves obtained with morphine and naloxone had quite distinctive slopes.

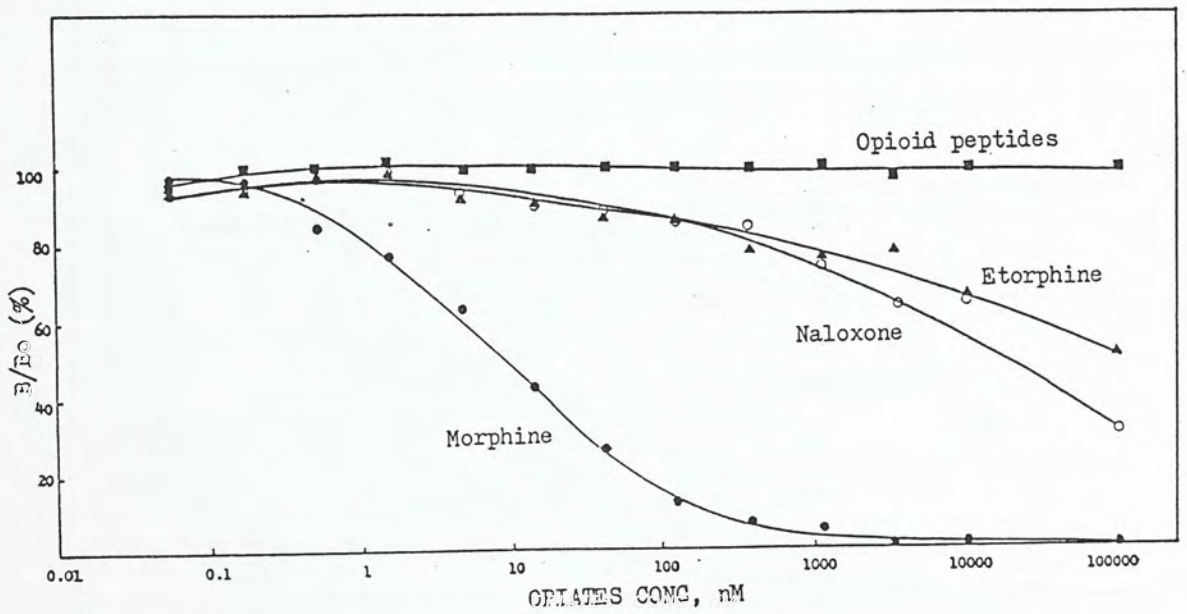
Fig. 3.5 Inhibition of I-125 morphine binding to antiserum MOS 3 by various opiate ligands. The optimal dilution of the antiserum used was 1:200. Please see Fig. 3.3 for details of symbols used.



(iv) Mouse anti-morphine serum: MOS 4

This antiserum showed only slight cross-reactivity with naloxone and etorphine. The IC_{50} with standard morphine was at 9.2 nM, and with naloxone and etorphine were at 10 uM and 235 uM, respectively.

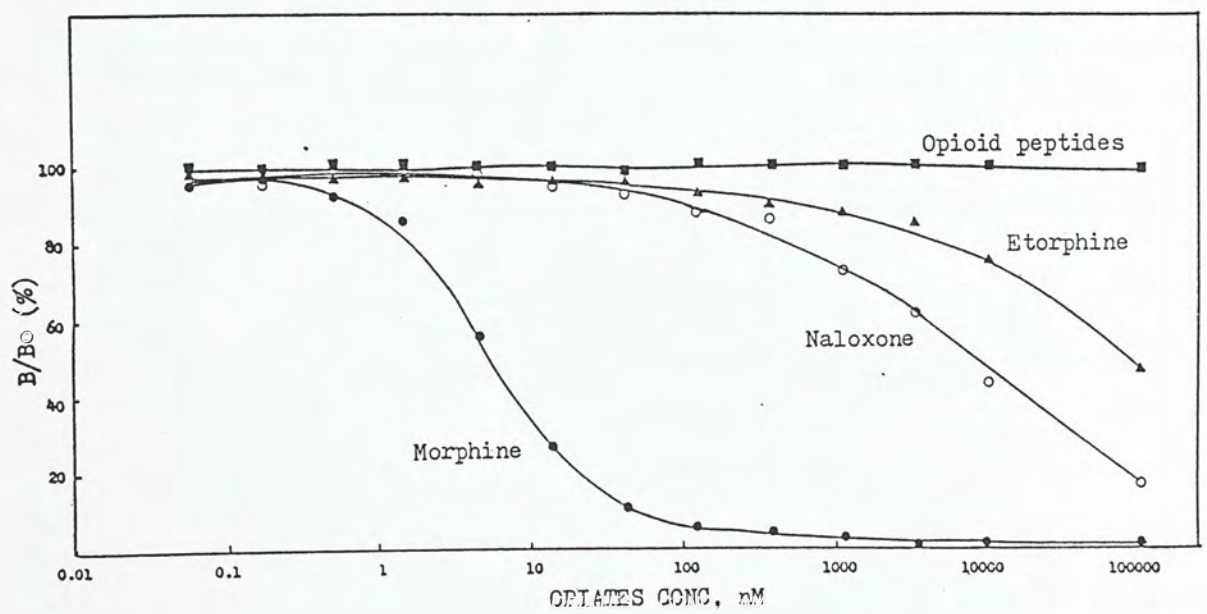
Fig. 3.6 Inhibition of I-125 morphine binding to antiserum MOS 4 by various opiate ligands. The optimal dilution of the antiserum used was 1:100. Please see Fig.3.3 for details of symbols used.



(v) Mouse anti-morphine serum: MOS 5

The binding of this antiserum to naloxone was relatively similar to that of antiserum MOS 2 in Fig. 3.4 . The IC_{50} with morphine was at 5.8 nM, and with naloxone and etorphine were at 6 uM and 77 uM, respectively.

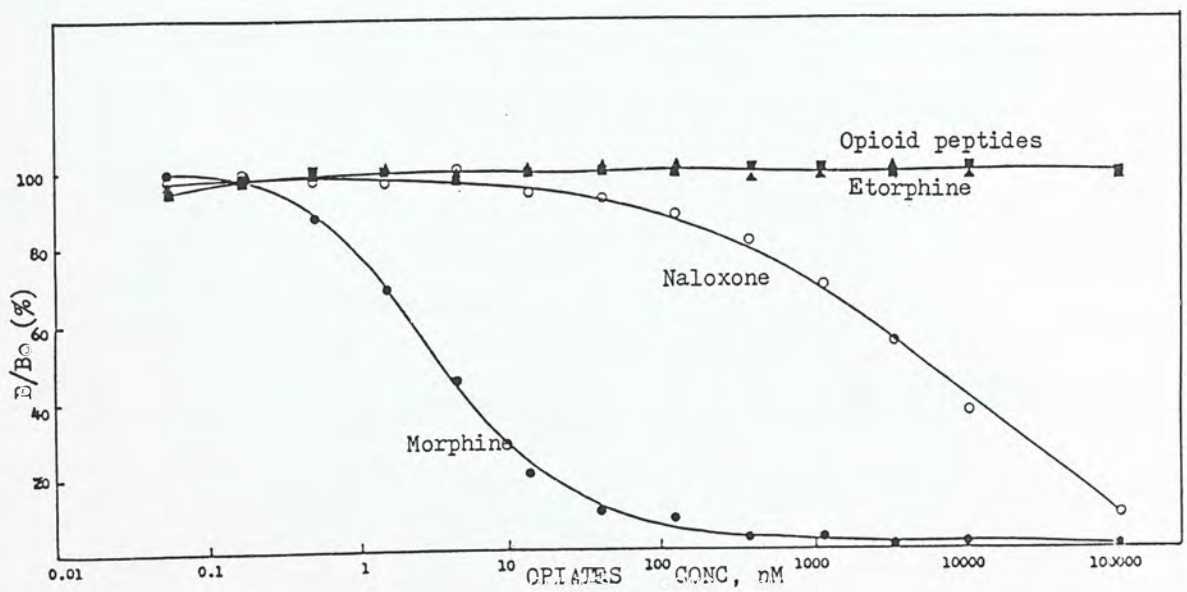
Fig. 3.7 Inhibition of I-125 morphine binding to antiserum MOS 5 by various ligands. The optimal dilution of the antiserum was 1:100. Please see Fig. 3.3 for details of symbols used.



(vi) Mouse anti-morphine serum: MOS 6

Our results showed that the binding of this antiserum to naloxone was similar to that of antiserum MOS 2 in Fig. 3.4 and of antiserum MOS 5 in Fig. 3.7. In contrast to the previous 5 antisera, no measurable inhibition was observed with etorphine. As before, no binding with other ligands was detected. The IC_{50} values with standard morphine was 3.2 nM and with naloxone was 4 uM.

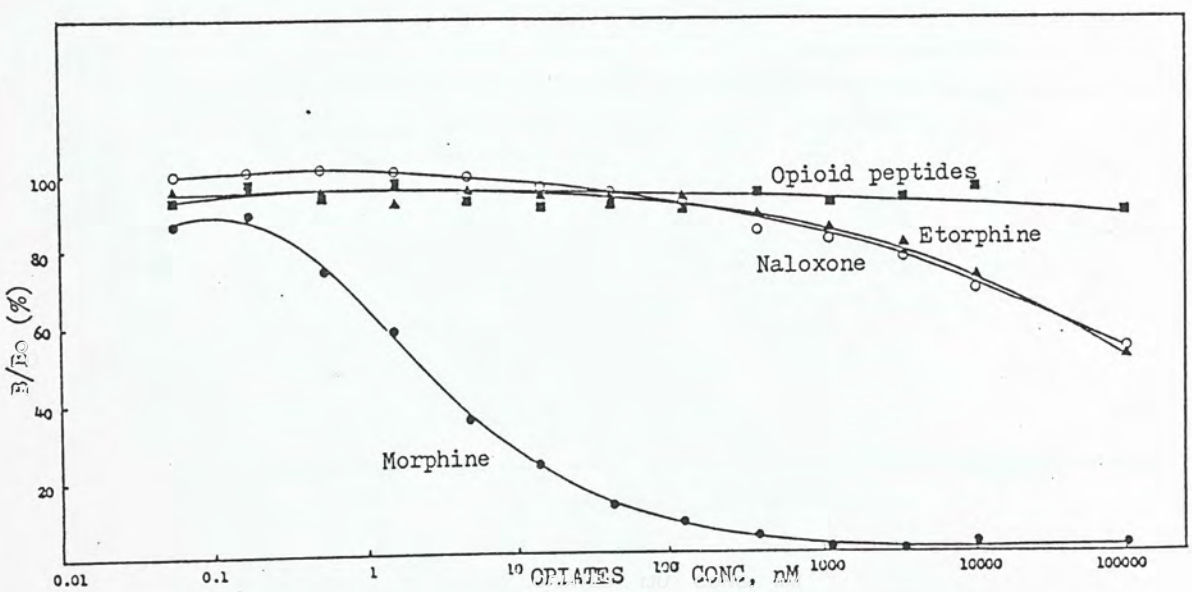
Fig. 3.8 Inhibition of I-125 morphine binding to antiserum MOS 6 by various ligands. The optimal dilution of antiserum used was 1:3200. Please see Fig. 3.3 for details of symbols used.



(vii) Rabbit anti-morphine serum: RB 3

The cross-reactivity of the antiserum to naloxone, etorphine and opioid peptides was almost negligible. The IC_{50} of morphine was at 1.9 nM which indicated that the antiserum was by far the most sensitive in binding to morphine.

Fig. 3.9 Inhibition of I-125 morphine binding to antiserum RB 3 by various ligands. The optimal dilution of the antiserum used was 1:1600. Please see Fig. 3.3 for details of symbols used.



(viii) Rabbit anti-morphine serum: RB 4

There was hardly any inhibition of I-125 morphine binding to this antiserum by naloxone, etorphine and other opioid peptides. The IC_{50} with morphine was 4.6 nM.

Fig. 3.10 Inhibition of I-125 morphine binding to antiserum RB 4 by various ligands. The optimal dilution of the antiserum used was 1:3200. Please see Fig. 3.3 for details of symbols used.

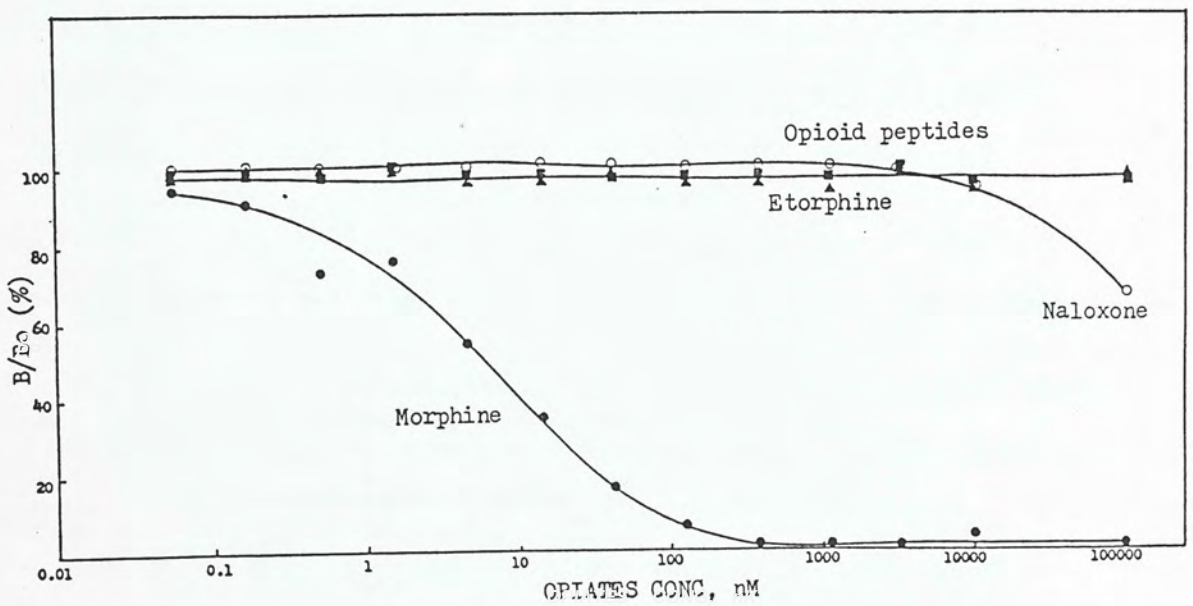


Table 3.3 IC_{50}* (nM) of opiate ligands for inhibition of I-125 morphine binding to various antisera.

Antisera	Morphine	Naloxone	Etorphine	Opioid peptides & analogues
MOS 1	33.6	49000	231000	NI**
MOS 2	3.8	2096	23000	NI
MOS 3	4.9	450	1400	NI
MOS 4	9.2	10160	235000	NI
MOS 5	5.8	6000	77200	NI
MOS 6	3.3	3967	NI	NI
RB 3	1.9	272100	64200000	NI
RB 4	4.6	NI	NI	NI

* IC_{50}: ligand concentration required to inhibit 50% of I-125 morphine binding.

** NI : no inhibition at the highest opiate ligand concentration tested (i.e., 0.1 mM).

Table 3.4 Cross-reactivities* (%) of opiate ligands with various anti-morphine sera.

Antisera	Morphine	Naloxone	Etorphine	Opioid peptides & analogues
MOS 1	100	0.07	0.01	NI**
MOS 2	100	0.2	0.02	NI
MOS 3	100	1.1	0.34	NI
MOS 4	100	0.09	0	NI
MOS 5	100	0.10	0	NI
MOS 6	100	0.08	0	NI
RB 3	100	0	0	NI
RB 4	100	0	0	NI

*Cross-reactivities were determined as the percentage ratio of the IC_{50} of morphine and the IC_{50} of the opiate ligand tested.

**NI : Please see Table 3.3.

3.4 Discussion

Polyclonal antisera were raised to morphine-hemocyanin conjugate with the aim of producing a recognition site which may interact with the opioid receptor binding determinant of the opioid peptides. In the present study, an attempt was made to identify these binding sites for various opiate ligands in the anti-morphine sera by competitive binding with I-125 morphine. In order to investigate the specificity of the binding, the ability of these opiate ligands to compete with the radio-ligand for binding to the antibody recognition sites was compared. Our results indicated that the antisera raised in rabbits had a high specificity for the parent drug, morphine. On the other hand, the antibodies obtained from the mice demonstrated a wider spectrum of specificity. In addition to binding morphine, these antisera also cross-reacted partially with the opiate antagonist naloxone, and to a lesser degree, with the opiate agonist etorphine.

It has been shown in previous reports (Martin, 1976) that morphine is a potent μ-selective ligand for the brain opioid receptor. This follows that the antiserum raised against morphine will most probably possess binding sites

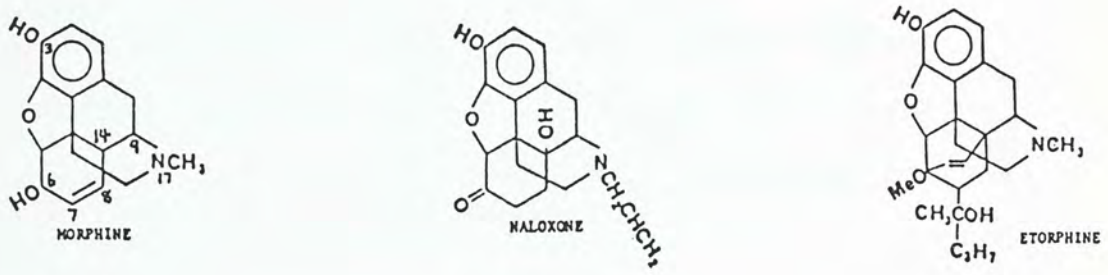
which closely resemble the μ-receptor. In turn this antiserum will be very likely to interact with other potent μ-ligands such as DAGO, β-endorphin and naloxone in the RIA binding assays. However, our results suggested that this is not the case. It was shown that the antiserum generated from mice, apart from binding morphine, only exhibited a limited cross-reactivity with naloxone and etorphine; in the order of morphine > naloxone >etorphine. Contrary to our reasoning, all the pharmacologically active opioid peptides and their stable analogues had no displacement of I-125 morphine in the antiserum. In order to better understand the interaction of the opiate ligands with the receptor, it is necessary to carefully examine the binding characteristics of different types of opioid receptors (please also see Table 3.2). It is shown that at the μ -binding site of the receptor, where DAGO is a highly selective agonist, the relative binding affinities for the 3 alkaloid opiates are in the order of morphine= naloxone > etorphine. At the δ-site, where DADLE is the δ-specific agonist, the order of selectivity is etorphine > naloxone > morphine. At the k-binding site, where the dynorphins are reckoned to be the selective ligands, the order for the inhibition of binding is etorphine > naloxone > morphine. From these data, it can be seen that

the binding properties of the anti-morphine sera used in our competition experiments were not identical with those shown by the 3 types of opioid receptors. This is different from our expectation, which stipulates that the antiserum would resemble the μ-receptor in its binding behaviour. Thus we could conclude at this stage that the antibody recognition sites for morphine in our antisera may be quite different from those of the opioid receptor to which the active endogenous peptides bind with high affinity. It is highly probable that the opioid receptor may possess an extensive binding site, of which only one part will interact with morphine and other structurally similar opiates. Perhaps, it is this lack of similarity in the conformation of the recognition sites which accounts for the inability of the antiserum to cross-react with the opioid peptides.

The fact that the anti-morphine sera cross-reacted partially with the 2 opiate drugs indicate that, in general, these antibodies recognized ligands containing a basic morphine structure. As shown in Fig. 3.11 below, naloxone only differs from morphine in the possession of a hydroxyl group at the C14 position, a carbonyl group at the C6 position, as well as the substitution of an N-methyl group by an allyl group. On the other hand,

etorphine is synthesized from morphine by having its C6 and C7 positions substituted by different functional groups.

Fig. 3.11 The molecular structures of morphine, naloxone and etorphine.



The results of our experiments suggested that the antibodies showed no recognition for the region around the tyramine fragment, i.e., the part containing the phenolic hydroxyl group and the benzene ring. If this is not the case, there should be a marked cross-reactivity between the three opiate drugs tested. It is apparent that these antibodies were perhaps more selective for the region in the lower half of the morphine molecule, i.e., at the C6, C7 & N17 sites (Fig. 3.11). Here the molecular structures

between morphine, naloxone and etorphine are considerably different from each other. It is very likely that the lack of structural similarity in this area had contributed to the small degree of cross-reactivities obtained in our studies. Finally since we were interested in antibodies which mimic the receptor binding site, it may be worthwhile to try to produce additional anti-morphine antisera through different conjugation procedures, and to compare their specificities for various opiate ligands. It would certainly be very interesting to see this outcome, but until then, it would be premature to draw any further conclusion based on our present findings.

CHAPTER IV SUMMARY AND CONCLUSIONS

Opium is probably one of the oldest known medication and morphine has been known since the nineteenth century to be the major alkaloid responsible for most of its pharmacological and medicinal effects. Since then, large-scale efforts were made in many laboratories to synthesize a nonaddictive analgesic. As a result, thousands of compounds structurally related to morphine had been made and a large amount of information on the structural requirements for analgesic action came out of this work. It was realized that the analgesic action exists in only one of the enantiomers of a racemic mixture, usually the levorotatory isomer. Furthermore, it became apparent that parts of the morphine molecule could be altered without major changes in pharmacological potency. On the other hand, even small changes in certain parts of the molecule resulted in profound effects in itspotency. The example of such change is the substitution of the methyl group on the N atom by an allyl group, which causes the resulting molecule to become a specific antagonist. Some of these antagonists retain part of their "agonist" properties (e.g. nalorphine), while others are "pure" antagonists (e.g. naloxone).

The recognition of the remarkable stereospecificity and structural constraints placed in the opiates led to the receptor hypothesis. This hypothesis states that the opiates must bind to specific sites on brain cells and that this binding triggers the pharmacological responses observed. These sites, or receptors, would permit only drugs with suitable structures and stereochemistry to bind. Evidence for the existence of stereospecific opiate binding came in 1973, from 3 separate research groups (Simon; Pert & Snyder; Terenius; 1973). The discovery of the opioid receptors in discrete localizations of the brain sparked off an intense search for the endogenous ligands for these receptors. Within the next 3 years, 5 endogenous compounds with opiate-like activities were isolated from various mammalian organs, especially from the brain and the pituitary gland. These include the 2 enkephalins (Hughes and Kosterlitz, 1975) and the 3 endorphins (Li; Guillemin ;Ling; 1976). They are all peptides, consisting between 5 to 31 amino acid residues. The 2 enkephalins and α- and γ- endorphins are indistinguishable in their activity from morphine, whereas β-endorphin is 5-10 times more potent than morphine in several opiate-binding assays (Guillemin, 1977). In addition, there exists another class of opioid peptides, the dynorphins, which were identified in 1979 by Goldstein and

coworkers. Being 50x more active than β-endorphin, this peptide is by far the most potent opioid known. The discovery of opioid receptors and of endogenous opioid peptides, has caused much excitement in laboratories all over the world. This was followed by a subsequent finding that there are 3 distinct families of opioid peptide gene products (Terenius, 1978; Bloom, 1983; Akil, 1984) and multiple categories of opioid receptors (Martin, 1976; Kosterlitz, 1977; Chang & Cuatrecasas 1979). Since then, much information has been gathered about the properties, distribution and the pharmacology of the opioid peptides.

The fact that the opioid peptides mimic the opiates almost perfectly in their effects and behave like opiates in opiate receptor binding assays has stimulated active research on the structural relationships between the 2 classes of compounds. It is believed that if the opiates and opioid peptides are competing for the same receptors in the brain, they are very likely to have some structural and conformational similarities. Based on this hypothesis, an investigation was carried out by using antibodies to morphine as a model of a specific opioid receptor. First,

antibodies were produced to a receptor-selective ligand with the aim of producing an antigen binding site that resembles the binding site of the opioid receptor for the ligand. Thus these antibodies can be considered equivalent to the receptor. It seems most probable that an opioid peptide which binds to the receptor with high affinity will cross-react with the antibodies. The first attempt of this thesis was to generate a panel of monoclonal antibodies for different active regions of the morphine molecule. It was hoped that the binding specificity of these antibodies would be similar to the opioid receptor in that the antibodies would also recognize the peptides. However, since we were unsuccessful in raising these specific antibodies, the proposed structural study was carried out instead by using polyclonal antisera against morphine. Chapter II of this thesis provides a comprehensive account of the experimental procedures related to the production of monoclonal antibodies.

As discussed earlier in our studies, the problem of our failure to produce monospecific antibodies for morphine seems to lie in the instability of our hybridoma lines. A puzzling feature observed during our work is that a majority of the initially positive clones lost antibody

activity readily and became non-producers. Moreover, subcloning of the positive cultures did not seem to improve the chance of maintaining the positive clones. Up to now, we are still unable to determine the cause of this rather uncharacteristic behaviour (we have successfully used a similar protocol to raise monoclonal antibodies against low density lipoproteins and alcohol dehydrogenase). In a recent paper, Zagon(1987) have demonstrated that both exogenous and endogenous opioid substances can inhibit neoplasia growth, and that this antitumour action can be completely blocked by the use of the opiate antagonist, naloxone. Their results also reveal that opioid agonists can exert a growth inhibitory effect on tumor cells in culture and that this effect is dose dependent, stereospecific and blocked by naloxone. Perhaps, this finding may provide a probable explanation for our present difficulties in raising a monoclonal antibody against morphine. It is apparent that the NS-1 cell line is derived from MOPC-21, a Balc/c myeloma cell line. When this cell line is used as the parent myeloma, the resulting hybridomas will carry half of the characteristics possessed by the parent cancerous cells. It is rationalized that there may be morphine-like factors present in the culture medium and that they can bind to

the morphine-specific immunoglobulins on the surface of the hybridoma cell resulting in the inhibition of growth. Because of this, the morphine-antibody carrying hybridomas may be selectively killed and thus preventing further cloning. The proposed hypothesis can be tested by concommitant administration of naloxone or morphine into the culture medium to determine if they have any modulating activities in hybridoma growth.

It has been suggested in a recent paper by Strange (1987) that the interaction between an antigen and its antibody is similar to the interaction between a neurotransmitter and its receptor. It is therefore of great interest to compare the specificity of a receptor and an antibody directed against the same ligand. In chapter III, polyclonal antisera were generated by the immunization of laboratory animals with a morphine-6-succinyl-hemocyanin conjugate. The specificities of the resulting antisera were analysed by competitive displacement assays with a series of opiate ligands. Our investigation was based on the hypothesis that if the binding sites of these antibodies resemble the conformation of the opioid receptor, it is highly likely that they will also interact with the opioid peptides which are known to bind to the same type of receptors. In

particular, since morphine is a potent μ-selective ligand, we would expect that the antibodies may possess binding sites similar to μ-receptor and interact with other μ-ligands such as DAGO and β-endorphin. However, this is not the case. Apart from binding to morphine with high affinity, our antisera exhibited no significant interaction with the opioid peptides or their analogues. On the other hand, some partial cross-reactivities were observed with high concentrations of the opiate antagonist, naloxone, and with the opiate agonist, etorphine. Thus in short, although we were unsuccessful in using monoclonal antibodies to evaluate our hypothesis, the investigation carried out by polyclonal antisera revealed that antibodies generated against morphine behave differently from the opioid receptors in binding specificities.

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