MONOCLONAL ANTIBODIES TO VASOPRESSIN AND ATRIAL NATRIURETIC PEPTIDE:

Preparation, Characterization and Use in Two-Site Immunometric Assays.

Shirley Carolyn Smith

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DECLARATION

I hereby declare that the following thesis is of my own composition, that it describes my own work, and that no part of it has been submitted for any other degree.

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ABSTRACT

Arginine vasopressin (AVP) and atrial natriuretic peptide (ANP) are small peptide hormones consisting of 9 and 28 amino acids, respectively, which control water balance in the body. Small and sick premature infants often have problems with water balance but the current assays for AVP and ANP are unsuitable for assessing the day-to-day clinical management of these individuals because most assays require pre-extraction of the peptides from large plasma samples (up to 10 ml) followed by long incubation periods (3-5 days). Clinically-useful assays would measure AVP and ANP in small volumes of unextracted plasma within a few hours of sampling. Two-site immunometric assays have the required speed because they use excess antibody reagents and therefore reach equilibrium rapidly. Monoclonal antibodies (MAbs) were the preferred reagents because they are highly specific and can be produced in bulk quantities.

However, MAb production was difficult because AVP and ANP were poor immunogens, even after conjugation to thyroglobulin, so immunization produced low mouse serum antibody titres and subsequent cell fusions generated few antigen-specific hybridomas. Culture supernatant screening for anti-ANP MAbs was relatively simple, but screening for anti-AVP MAbs was complicated by a lack of suitable positive control samples. Immune serum was initially the only positive control available but it was later shown that early immunizations had failed to generate anti-AVP IgG in the mice. An AVP-capture ELISA bound anti-AVP antibodies from high titre immune serum but could not detect anti-AVP MAbs in culture supernatants, and produced false positive results with the anti-immunoglobulin and anti-BSA antibodies which were generated in response to contaminants in the thyroglobulin-conjugated immunogen. An AVP-coated-plate ELISA also failed to detect anti-AVP MAbs under conditions in which immune serum antibodies bound strongly, because higher AVP-coating concentrations were required for efficient MAb detection, while RIA screening could not detect MAbs which bound close to the tyrosine residue of AVP. Despite these

difficulties, four anti-AVP MAbs (two IgG1, one IgM and one IgA) and four anti-ANP MAbs (one IgG2a and three IgG1) were produced.

Two-site immunometric assays for AVP were possible with a range of antibody combinations despite the small size of the peptide, although steric hindrance severely limited antibody binding and none of the assays were sufficiently sensitive to measure normal physiological AVP concentrations (1-5 pg/ml plasma). The two IgG1 MAbs, ESVP 1 and ESVP 2, recognized the AVP ring and the tail tripeptide, respectively, and could bind simultaneously to AVP although their relatively low binding affinities (2.8×10^8 and 2.2×10^8 l/mole, respectively) limited the sensitivity of a two-site ELISA to 200 pg AVP/ml and that of a direct IRMA to 1 ng AVP/ml. An immunofiltration assay with these MAbs could be carried out in only 1 hour, but its detection limit was 10 ng AVP/ml. The most sensitive assay was an IRMA that used ^{125}I-ESVP 1 with a solid phase-bound affinity-purified polyclonal anti-tail antibody, and that had a detection limit of 130 pg AVP/ml after a 4 hour incubation period. A two-site ELISA that used ESVP 1 MAb-coated wells and biotinylated affinity-purified polyclonal antibody was less sensitive because of high non-specific binding but was used to analyse the separation of ^{125}I-AVP from unlabelled AVP on a Sephadex G-25 column and, therefore, to determine the specific activity of the purified ^{125}I-AVP.

The four anti-ANP MAbs which were produced in this project (with binding affinities of 8.3×10^7 to 2.4×10^9 l/mole), and three other anti-ANP MAbs, were tested for compatibility in two-site assays but there were no pairs of MAbs which could bind to ANP simultaneously. Two-site assays could only be carried out using ^{125}I-MAbs with a polyclonal antibody preparation in the IRMA format, although steric hindrance limited antibody binding. The detection limit of the most sensitive assay was 1 ng ANP/ml, whereas normal physiological ANP concentrations are in the range 5-70 pg/ml plasma. The ELISA format was unsuitable for two-site ANP assays because ANP adhered non-specifically to coated ELISA wells, irrespective of the composition of the coating material or the assay buffer.

ABBREVIATIONS

Ag	Antigen
ANP	Atrial natriuretic peptide
AVP	Arginine vasopressin
AVT	Arginine vasotocin
BSA	Bovine serum albumin
dCDAVP	1-Desamino-6-monocarba-[8-D-Arg]-vasopressin
dDAVP	Desmopressin
DMF	N,N-dimethylformamide
DMSO	Dimethyl sulphoxide
dNMeAVP	1-Desamino-[8-Me-Arg]-vasopressin
EDC	1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride
EDTA	Ethylenediaminetetraacetic acid, disodium salt
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
HAT	Hypoxanthine, aminopterin and thymidine
Hep	Heptapeptide
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HT	Hypoxanthine and thymidine
IRMA	Immunoradiometric assay
KLH	Keyhole limpet haemocyanin
LVP	Lysine vasopressin
MAb	Monoclonal antibody
MBS	m-Maleimidobenzoic acid N-hydroxysuccinimide ester
MTM	Mixed-thymocyte culture-conditioned medium
NBCS	Bovine (new-born calf) serum

NRS	Normal rabbit serum
OT	Oxytocin
PAG	NH_2-Pro-Arg-Gly-NH_2
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
PPD	Purified protein derivative
PTH	Parathyroid hormone
RIA	Radioimmunoassay
SAM	Sheep anti-mouse
tGLVP	Glypressin
Tris	Tris-(hydroxymethyl)-methylamine

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CHAPTER 1

INTRODUCTION

1.1 ARGININE VASOPRESSIN

AVP, the antidiuretic hormone of most mammals, is a nonapeptide which is derived from a 166-amino acid precursor molecule (Russell et al., 1980; Land et al., 1982) that is synthesized by the magnocellular neurones in the supraoptic and paraventricular nuclei of the hypothalamus. The precursor is packaged into secretory granules, then processed to produce AVP, neurophysin II and a glycopeptide of unknown function as the granules are transported along the axons to the posterior pituitary (Baylis, 1989) where they are stored at the nerve endings (Brownstein et al., 1980). In the Brattleboro rat, a single base deletion in the gene coding for the precursor molecule produces a protein which contains the AVP sequence but is not recognized by the post-translational processing system (Schmale and Richter, 1984; Ivell et al., 1986) so the AVP molecule is not released and the animal suffers diabetes insipidus. Similar mutations might also occur in humans although central diabetes insipidus is usually caused by damage to the hypothalamus or posterior pituitary (Zerbe et al., 1981; Kaplowitz et al., 1982). AVP synthesis was also reported in peripheral tissues such as the thymus, adrenal gland, ovary and testis (Rehbein et al., 1986), and in foetal and neonatal skeletal muscle (Smith et al., 1992), although its function at these sites is uncertain, and the peptide was detected in various parts of the central nervous system where it may act as a neurotransmitter (Zimmerman et al., 1984; Burbach and Liu, 1989).

AVP controls water reabsorption in the kidney and so maintains the osmolality of the extracellular fluid within narrow limits. An osmoreceptor, located within the circumventricular organs of the anterior hypothalamus (Baylis, 1989), detects the hyperosmolality that is caused by solutes, such as sodium, which do not readily penetrate cell membranes (Robertson, 1977; Schrier et al., 1979) and responds by increasing AVP secretion from the pituitary. The osmotic threshold for AVP secretion varies between individuals (Robertson, 1977; Schrier et al., 1979) but above this point there is a close relationship between plasma osmolality and AVP

concentration, although there is disagreement about the precise nature of this relationship (Robertson, 1977; Weitzman and Fisher, 1977; Hammer et al., 1979; Baylis et al., 1986). Once in the kidney, AVP binds reversibly to the V2 receptors on the basolateral surface of the collecting duct epithelial cells, where it activates adenylate cyclase (Valtin, 1987) and leads to the insertion of water channels into the apical membrane (Harris et al., 1991). This produces a dramatic increase in the water permeability of the collecting duct so that water, but not small non-electrolytes such as urea, can flow along an osmotic gradient from the tubule lumen to the medullary interstitium (Valtin, 1987; Harris et al., 1991). AVP also increases the urea permeability of the inner medullary collecting duct, stimulates NaCl reabsorption from the thick ascending limb of Henle's loop, and raises the glomerular filtration rate of the juxtamedullary nephrons. These actions all increase the osmotic gradient and therefore assist the reabsorption of water and the production of a concentrated urine (Valtin, 1987). However, some individuals suffer renal insensitivity to AVP, which causes nephrogenic diabetes insipidus (Zerbe et al., 1981).

AVP is also secreted in response to various non-osmotic stimuli, such as hypovolaemia, hypoxia, hypoglycaemia, nausea (Schrier et al., 1979; Baylis, 1989) and pain (Kendler et al., 1978), which all seem to exert their influence via the baroregulatory system (Robertson, 1977; Schrier et al., 1979). Baroreceptors in the aortic arch and carotid arteries detect changes in blood pressure, while receptors in the left atrium detect decreased volume (Miller, 1984; Elijovich and Krakoff, 1985; Baylis, 1989). This system is much more sensitive to changes in pressure than in volume but is considerably less sensitive than the osmoregulatory system (Dunn et al., 1973; Miller, 1984; Baylis, 1989).

AVP is a potent vasoconstrictor (Elijovich and Krakoff, 1985) which was originally detected because pituitary extracts raised the blood pressure of experimental animals (Oliver and Schäfer, 1895). It binds to the vascular V1 receptors, producing a calcium flux which causes smooth muscle contraction (Elijovich and Krakoff, 1985; Vallotton et al., 1990). At normal physiological concentrations, AVP

can produce significant vasoconstriction without raising blood pressure because the increase in total peripheral resistance is balanced by decreased cardiac output (Elijovich and Krakoff, 1985). Increased blood pressure is apparent only at the very high AVP concentrations that are secreted in response to severe haemorrhage or hypotension (Cowley et al., 1980; Miller, 1984), under which conditions AVP makes a significant contribution towards the restoration of arterial pressure.

AVP secretion occurs in a pulsatile manner, the amplitude of the secretory bursts depending on the degree of stimulation, while their frequency and duration vary between individuals (Weitzman et al., 1977; Sadler et al., 1983). Plasma AVP concentrations also fluctuate diurnally, with the highest concentrations tending to occur at night independently of any change in plasma osmolality (George et al., 1975; Uhlich et al., 1975). Consequently, the AVP concentration of a single plasma sample may not seem to correlate with the subject's water balance status.

In contrast, measurements of urinary AVP are thought to provide integrated values for AVP secretion over a period of time (Robertson, 1977; Thibonnier et al., 1981; Miller, 1984; Moses and Steciak, 1986) and therefore to sensitively detect sustained changes in secretion (Seckl et al., 1986). However, AVP excretion is only a small fraction of the total renal clearance because most of the filtered AVP is reabsorbed in the proximal tubule (Pruszczynski et al., 1984; Moses and Steciak, 1986) where it is presumably catabolized, since both membrane-bound (Nardacci et al., 1975) and cytosolic (Walter and Shlank, 1975) AVP-inactivating peptidases were reported in renal tissue. Impaired renal function can drastically alter glomerular filtration and tubular reabsorption (Pruszczynski et al., 1984) so that although significant correlations between plasma and urinary AVP concentrations were reported in normal subjects (Khokhar et al., 1975; Thomas and Lee, 1976; Thibonnier et al., 1981; Stern and LaRochelle, 1982), no correlation was found in infants (Smith et al., 1990) or children (Bald et al., 1988) with renal damage. AVP excretion does not therefore reliably reflect either the secretion rate or plasma concentration of AVP during renal failure. Even in normal individuals, however, AVP excretion fluctuates significantly

with changes in the total solute clearance rate, independently of changes in AVP secretion or plasma concentration (Robertson, 1977), so that urinary AVP values can be difficult to interpret.

For many years, the only way to assay AVP was to determine its biological activity in experimental animals. These laborious bioassays, which required intricate surgical skills, relied on the detection of increased blood pressure (Dekanski, 1952), increased urine conductivity (Yoshida et al., 1963) or decreased urine flow (Czaczkes et al., 1964) in response to injections of AVP, and had the disadvantage that few samples could be assayed per animal. The pressor assay (Dekanski, 1952), although reproducible and precise, was insensitive because physiological AVP concentrations do not increase blood pressure (Elijovich and Krakoff, 1985) therefore it could only be used to assay highly concentrated samples. The antidiuretic bioassays (Yoshida et al., 1963; Czaczkes et al., 1964), on the other hand, were reasonably sensitive but were susceptible to interference from other antidiuretic substances in the sample (Robertson, 1977).

More recently, rat kidney segments were used in extremely sensitive cytochemical bioassays which relied on the detection of AVP-stimulated increases in the Na^+/K^+ ATP-ase activity of the cells in the thick ascending limb of Henle's loop (Smith and McIntosh, 1984; Baylis et al., 1986). These assays could measure the AVP content of unextracted plasma samples, even those from water-loaded subjects whose low AVP concentrations were undetectable by other methods (Baylis et al., 1986). However, although sensitive, these cytochemical assays were still very laborious and suffered from high intra-assay and inter-assay coefficients of variation (Smith and McIntosh, 1984; Baylis et al., 1986).

Following the pioneering RIA work of Yalow and Berson (1959), many research groups set out to produce anti-AVP antisera and develop RIAs for AVP. Immunization was difficult because AVP is only weakly immunogenic, so many antisera had relatively low AVP binding affinities and produced insensitive assays, although a few high affinity antisera were reported (Oyama et al., 1971; Czernichow et al.,

1975; Fyhrquist et al., 1976; Rooke and Baylis, 1982). Under normal physiological conditions, plasma contains only 1-5 pg AVP/ml (Robertson, 1977), a concentration range below the detection limit of most RIAs therefore plasma samples usually had to be concentrated before assay. In contrast, urine contains higher concentrations of AVP (Miller, 1984; Claybaugh and Sato, 1985) therefore several RIAs were able to measure AVP in unextracted urine samples (Oyama et al., 1971; Freisenhausen et al., 1976; Lishajko et al., 1981; Smith and McIntosh, 1986; Bald and Rascher, 1990; Panzali et al., 1990).

Although a few RIAs were sufficiently sensitive to measure plasma AVP in unconcentrated samples (Robertson et al., 1973; Skowsky et al., 1974; Sadler et al., 1983; Ysewijn-Van Brussel and De Leenheer, 1985), macromolecular substances in human plasma interfered with RIA determinations (Robertson et al., 1970; Beardwell, 1971) by causing enzymatic degradation of ^{125}I-AVP (Robertson et al., 1973; Fyhrquist et al., 1976) so that falsely high AVP concentrations were estimated in unextracted plasma. This interference varied between antisera (Robertson, 1977), due to differences in binding specificity (Chard, 1973; Thomas and Lee, 1976), but all RIAs were affected to some extent (Robertson, 1977). Plasma extraction was therefore essential to remove the interfering substances, although ε-aminocaproic acid was reported to prevent tracer degradation and allow RIA determinations in unextracted plasma (Fyhrquist et al., 1976), which avoided the problem of the variable AVP recoveries that were found with some extraction methods (Uhlich et al., 1975; Shimamoto et al., 1976; Moulin et al., 1978; Glänzer et al., 1984). Serum was also reported to cause tracer damage (Robertson, 1977) yet RIA data from unextracted serum correlated well with bioassay data (Skowsky and Fisher, 1972a) and with the physiological status of the serum donor (Wagner et al., 1977), which suggested that serum interference was negligible in some RIAs.

Unextracted urine was not reported to cause tracer degradation, but it contained widely varying concentrations of NaCl and urea which could disrupt antigen-antibody binding (Miller and Moses, 1972; Chard, 1973) therefore urine was

often extracted to remove this source of RIA interference. However, some antisera were unaffected by high osmolalities (Oyama et al., 1971) so the AVP concentrations estimated in extracted and unextracted urine samples were similar (Seckl et al., 1986; Smith and McIntosh, 1986; Panzali et al., 1990). Several research groups also showed that urine contained immunoreactive, non-AVP, substances that only cross-reacted with certain antisera (Thomas and Lee, 1976; Bald and Rascher, 1990) but which co-extracted with AVP and were likely to be AVP metabolites (Thomas and Lee, 1976; Tausch et al., 1983; Claybaugh and Sato, 1985; Bald and Rascher, 1990). It was therefore important to ensure that the antisera selected for RIA were specific for the intact peptide.

AVP has been extracted from plasma and urine by many different methods, most of which relied on non-specific adsorption of the peptide onto solid supports such as Florisil (Beardwell, 1971; Uhlich et al., 1975; Thomas and Lee, 1976), Spherosil (Edwards et al., 1970; Khokhar et al., 1978), Vycor (Ratcliffe and Edwards, 1971; Ten Haaf et al., 1992), Fuller's earth (Johnston, 1972), Bentonite (Skowsky et al., 1974; Shimamoto et al., 1976), Isorex 50 (Thibonnier et al., 1981), Amberlite XAD-2 (Gerbes et al., 1992), Amberlite CG-50 (Miller and Moses, 1971; Merkelbach et al., 1975) and Rexyn H^+ (Fressinaud et al., 1974), although minor procedural differences between laboratories which used the same adsorbent produced a wide range of AVP recoveries. However, batch-to-batch variation was reported with Florisil (Uhlich et al., 1975), which co-extracted substances that caused RIA interference (Ratcliffe and Edwards, 1971), while urinary AVP recoveries from Spherosil decreased with increasing urine osmolality (Khokhar et al., 1978) despite a report that Spherosil was unaffected by changes in osmolality (Ratcliffe and Edwards, 1971). More recently, octadecasilyl-silica, in the form of C_{18} Sep-Pak columns, produced improved extractions with consistently high AVP recoveries (LaRochelle et al., 1980; Ysewijn-Van Brussel and De Leenheer, 1985) from both plasma and urine (Glänzer et al., 1984), and had the advantage that AVP and ANP were extracted simultaneously (Hartter and Woloszczuk, 1986; Bodola and Benedict, 1988), although octyl-silica (C_8)

columns produced even better results because they co-extracted fewer impurities and allowed almost 100% recovery of AVP (Van de Heijning et al., 1991; Ten Haaf et al., 1992). Other AVP extraction methods have included gel filtration on Sephadex G-25 (Robertson et al., 1970) or G-50 (Lishajko, 1983), protein precipitation by acetone (Robertson et al., 1973; Moulin et al., 1978; Camps et al., 1983), dioxane (Rougon-Rapuzzi et al., 1977) or ethanol (Camps et al., 1983), and specific binding by anti-AVP antibody-coated Sepharose (Morton and Riegger, 1978), although these methods were only suitable for plasma extraction and could not be used with urine.

The most efficient extraction methods isolated enough AVP from 1 ml plasma for measurement by sensitive RIAs (Robertson et al., 1973; Skowsky et al., 1974; Van de Heijning et al., 1991), although many extractions required 5-10 ml plasma (Edwards et al., 1970; Beardwell, 1971; Johnston, 1972; Baylis and Heath, 1977) particularly if they were combined with an insensitive assay. Prompt extraction was important because AVP was unstable in plasma during storage at -20°C (Robertson et al., 1973; Fyhrquist et al., 1976; Lishajko, 1983), although some workers disagreed (Beardwell et al., 1975; Thomas and Lee, 1976; Baylis and Heath, 1977) which may indicate that their antisera cross-reacted with degraded AVP (Beardwell et al., 1975). After extraction, the solvent was removed by evaporation (Beardwell, 1971; Camps et al., 1983) or lyophilization (Robertson et al., 1970; Rougon-Rapuzzi et al., 1977), which increased the total preparation time significantly but allowed concentration of the sample for RIA.

Very low antiserum concentrations were used in RIAs (Chard, 1973) therefore long incubation periods were necessary to ensure maximal binding and, thus, sensitivity. RIA sensitivity could, however, be increased by preincubation of the sample and antiserum before addition of the tracer (Skowsky et al., 1974; Beardwell et al., 1975). The total incubation period reported for AVP RIAs varied from 24 hours (Beardwell et al., 1975; Burd et al., 1984) to 7 days (Robertson et al., 1973), but most assays were incubated for 3-5 days (Beardwell, 1971; Husain et al., 1973; Skowsky et al., 1974; Baylis and Heath, 1977) before separation of bound and free tracer.

Many different separation methods have been reported. Separations which relied on the adsorption of unbound tracer by charcoal coated with BSA (Miller and Moses, 1972; Gerbes et al., 1992), dextran (Edwards et al., 1972; Johnston, 1972; Moulin et al., 1978), Ficoll 400 (Ten Haaf et al., 1992) or plasma (Thomas and Lee, 1976; Glänzer et al., 1984) could usually be carried out in less than 30 minutes, as could antibody precipitation by ammonium sulphate (Edwards et al., 1970; Khokhar et al., 1978), ethanol (Edwards et al., 1970), or PEG 8000 (Husain et al., 1973; Robertson et al., 1973). However, high non-specific binding decreased the sensitivity of assays which used these methods (Chard, 1973), while prolonged exposure to the separating agent caused dissociation of antibody-bound AVP (Moulin et al., 1978; Valiquette and Neubort, 1989). Precipitation by a second antibody avoided these problems but added 1-2 days to the total assay time (Beardwell, 1971; Uhlich et al., 1975; Baylis and Heath, 1977; Rooke and Baylis, 1982) although precipitation by PEG-assisted (Lishajko et al., 1981; Tausch et al., 1983) or pre-precipitated second antibody (Ysewijn-Van Brussel and De Leenheer, 1985) could be carried out within 1-2 hours. Separation by chromatoelectrophoresis (Oyama et al., 1971; Husain et al., 1973) was unpopular because it was less convenient than other methods, while equilibrium dialysis assays (Rougon-Rapuzzi et al., 1977) required specialized dialysis devices but avoided the need for a discrete separation step because incubation and separation occurred simultaneously. Separation could also be achieved simply by centrifugation if the RIA antiserum was coupled to a solid phase such as microcrystalline cellulose (Burd et al., 1984).

A competitive enzyme immunoassay for AVP was also reported (Uno et al., 1982). However, it was a liquid-phase assay with a second antibody separation step and therefore did not offer any advantage in terms of speed, sensitivity or convenience when compared with RIA methods.

1.2 ATRIAL NATRIURETIC PEPTIDE

The existence of a natriuretic hormone was postulated for many years (De Wardener, 1977). ANP was finally discovered when De Bold et al. (1981) showed that intravenous injections of atrial extract caused profound natriuresis and diuresis in experimental animals, while ventricular extracts had no effect. α-ANP is a 28-amino acid peptide (Flynn et al., 1983; Kangawa and Matsuo, 1984) that has a 17-amino acid disulphide-bonded loop which is essential for biological activity (Misono et al., 1984; De Bold, 1985). Human ANP is synthesized as a 151-amino acid prepropeptide (De Bold, 1985; Kangawa et al., 1985) that is processed to form γ-ANP, a 126-amino acid propeptide (Ballermann and Brenner, 1985; Kangawa et al., 1985) which is stored in secretory granules within the atrial myocytes (De Bold, 1985). α-ANP is cleaved from the C-terminal of γ-ANP simultaneously with secretion (Sei et al., 1992) so that both α-ANP and a 98-amino acid peptide are released into circulation (Michener et al., 1986; Itoh et al., 1988; Sundsfjord et al., 1988; Meleagros et al., 1989; Sei et al., 1992). The right atrium synthesizes more ANP than the left atrium (Gutkowska et al., 1984a, 1984b), while a very small amount is also synthesized in the ventricles (Cernacek et al., 1988) and in the brain (Morii et al., 1985; Samson, 1987; Ueda et al., 1987). However, brain ANP consists mainly of α-ANP(4-28) and α-ANP(5-28), which are N-terminally truncated forms of α-ANP (Ueda et al., 1987) therefore precursor processing in the brain differs from that in the heart. β-ANP, a 56-amino acid antiparallel dimer of α-ANP, was reported to exist in human atria (Kangawa et al., 1985) and in the plasma of neonates (Ito et al., 1990) and chronic renal failure patients (Itoh et al., 1988; Marumo et al., 1992) but was not found in normal adult plasma (Theiss et al., 1987; Marumo et al., 1992) so its significance was uncertain.

ANP controls the blood volume and so protects the heart from overload. There is a close relationship between atrial pressure and plasma ANP concentration because distensional stimulation of the atrial wall stretch receptors causes a dose-dependent increase in ANP secretion into the coronary sinus (Nicholls et al., 1987; Cernacek et

al., 1988). ANP is therefore secreted in response to extracellular volume expansion due to drinking (Larose et al., 1985), isotonic infusion (Lang et al., 1985; Burrell et al., 1990), sodium loading (Sagnella et al., 1987; Naomi et al., 1988), renal failure (Nicholls et al., 1987; Shenker et al., 1987) or congestive heart failure (Shenker et al., 1985; Cernacek et al., 1988), central hypervolemia due to postural change (Larose et al., 1985; Burrell et al., 1990) or immersion in water (Anderson and Bloom, 1986), and increased perfusion pressure due to atrial pacing (Gutkowska et al., 1985), paroxysmal tachycardia (Anderson and Bloom, 1986; Nicholls et al., 1987) or hypertension (Arendt et al., 1985; Gutkowska et al., 1987).

ANP binds reversibly to the B-ANP receptors that are located in the kidney (Napier et al., 1984; Cernacek et al., 1988), aorta (Napier et al., 1984; Schenk et al., 1985), adrenal cortex (Anderson and Bloom, 1986; Takayanagi et al., 1987) and central nervous system (Jacobs et al., 1987; Samson, 1987), thereby activating particulate guanylate cyclase which produces the cGMP that mediates the biological effects of the peptide (Schenk et al., 1985; Scarborough, 1989). The guanylate cyclase-free C-ANP receptors, on the other hand, rapidly clear ANP from circulation (Maack et al., 1987; Scarborough, 1989) by receptor-mediated endocytosis and transport to the lysosomes (Scarborough, 1989), so that it has a plasma half-life of only 2-5 minutes *in vivo* (Gutkowska et al., 1987; Nicholls et al., 1987; Scarborough, 1989). The C-ANP receptors also clear ANP metabolites since they can bind truncated peptides that are not recognized by the B-ANP receptors (Takayanagi et al., 1987; Scarborough, 1989), although only a small proportion of ANP undergoes extracellular degradation *in vivo* (Gutkowska et al., 1987; Condra et al., 1988; Scarborough, 1989).

ANP acts at three main sites within the kidney, producing haemodynamic and tubular effects which cause natriuresis and diuresis. It boosts hydraulic pressure in the glomeruli by dilating afferent arterioles and constricting efferent arterioles, whilst simultaneously increasing capillary permeability, therefore the glomerular filtration rate rises (Cernacek et al., 1988). In the collecting duct, sodium reabsorption and AVP-dependent water reabsorption are inhibited while sodium transport into the

urine is increased (Raine et al., 1989; Zeidel, 1989). ANP also decreases renin secretion from the juxtamedullary cells (Anderson and Bloom, 1986; Raine et al., 1989; Zeidel, 1989) therefore angiotensin II-stimulated water reabsorption in the proximal tubule and aldosterone-mediated sodium reabsorption in the distal tubule are indirectly inhibited (Raine et al., 1989).

ANP is a potent hypotensive agent which inhibits the arterial smooth muscle response to vasoconstrictors such as AVP and angiotensin II (Ballermann and Brenner, 1985; Anderson and Bloom, 1986). It also increases capillary permeability so that fluid shifts towards the extravascular space (Nicholls et al., 1987; Cernacek et al., 1988), rapidly decreasing the blood volume and thus the heart's workload.

In addition to inhibiting renin secretion, and thus indirectly decreasing angiotensin II and aldosterone secretion, ANP acts on the adrenal cortex to directly inhibit both the synthesis and secretion of aldosterone (Anderson and Bloom, 1986; Cernacek et al., 1988). ANP also decreases AVP secretion by action on the posterior pituitary (Samson, 1987; Allen et al., 1988) and on the hypothalamus (Samson, 1987; Cernacek et al., 1988), where it was shown to inhibit the firing of AVP-containing neurones (Standaert et al., 1987). The functional antagonism between ANP, AVP and the sodium-conserving renin-angiotensin-aldosterone system is therefore complemented by the inhibitory effect of ANP on the secretion of the other hormones, so that fluid and electrolyte balance are finely controlled.

Plasma ANP concentrations were reported to fluctuate diurnally (Donckier et al., 1986; Richards et al., 1987a; Winters et al., 1988) but the fluctuations were small (Richards et al., 1987a) and the timing of the peak concentration was in fact posture-related, occurring shortly after the subjects assumed a recumbent position (Bell et al., 1990), despite a claim to the contrary (Winters et al., 1988). However, ANP is secreted in a pulsatile manner, with secretory bursts every 9 minutes and longer-term oscillations of the plasma ANP concentration every 34 minutes (Haak et al., 1990), therefore true diurnal variation would be difficult to detect and the ANP concentration of a single plasma sample may not seem to correlate with the subject's

physiological status.

Although most ANP is cleared from circulation by receptor-mediated processes, a small proportion is filtered by the kidney (Scarborough, 1989) and excreted in the urine (Greenwald et al., 1986; Marumo et al., 1986). Urinary ANP concentrations have rarely been reported, but are roughly similar to plasma ANP concentrations (Marumo et al., 1986, 1990, 1992). However, no correlation was found between plasma and urinary ANP concentrations in normal adults (Marumo et al., 1990) and neonates (Ito et al., 1990), or in patients with renal damage (Marumo et al., 1987; Ando et al., 1988; Marumo et al., 1990), even though elevated plasma ANP concentrations were reflected by elevated urinary ANP (Ando et al., 1988; Ito et al., 1990). It is therefore doubtful whether urinary ANP measurements could offer any advantage over plasma ANP determinations in the assessment of an individual's fluid balance status.

The earliest studies monitored ANP activity using bioassays which relied on its natriuretic and diuretic properties in experimental animals (De Bold et al., 1981; Gutkowska et al., 1984a) or vasorelaxant effect on preconstricted smooth muscle strips from rabbit aorta (Misono et al., 1984; Napier et al., 1984) or chick rectum (Kangawa and Matsuo, 1984; Misono et al., 1984). These bioassays readily detected ANP in atrial extracts but were insufficiently sensitive to measure plasma ANP concentrations. However, ANP research proceeded so rapidly that the peptide had been sequenced (Flynn et al., 1983; Kangawa and Matsuo, 1984), synthesized and used to generate anti-ANP antisera (Gutkowska et al., 1984b) within 3 years of its discovery. Many research groups subsequently developed RIAs for ANP, encountering similar problems to those which occurred with RIAs for AVP.

Under normal physiological conditions, plasma contains 5-70 pg ANP/ml (Polesi et al., 1989; Watanabe et al., 1989), a concentration range at the limit of detection of many RIAs therefore samples were usually extracted to concentrate the peptide before assay. Some RIAs were apparently unhindered by unextracted plasma (Gutkowska et al., 1985; Marumo et al., 1986), although it was frequently reported to interfere with RIA determinations by causing enzymatic degradation of ^{125}I-ANP

(Yamaji et al., 1985; Hartter et al., 1986; Nishiuchi et al., 1986; Cernacek et al., 1988) so that the ANP concentration of unextracted plasma was overestimated by 50-80 pg/ml (Yandle et al., 1986; Gutkowska et al., 1987; Cernacek et al., 1988; Sarda et al., 1989). Some plasma interference seemed to be platelet-related since high-speed centrifugation decreased both the platelet content and measurable ANP concentration of the sample (Richards et al., 1987b), a phenomenon that was also observed in a RIA for AVP (Sadler et al., 1983). Platelets have high affinity receptors for both ANP (Schiffrin et al., 1986) and AVP (Thibonnier et al., 1984) and could therefore cause interference if the RIA antiserum recognized receptor-bound peptide or if sample processing released peptide from the receptors, although Tan et al. (1989) found no evidence of platelet interference in a RIA for ANP with either extracted or unextracted plasma. However, adequate centrifugation removes the risk that platelets might affect the RIA result.

An albumin-like ANP-binding protein, which was detected by RIAs for ANP, was recently discovered in plasma (Kato et al., 1988; Wilson et al., 1991b). Acidification released ANP from the binding protein (Wilson et al., 1991a), which explains the increased extractable ANP concentrations (Buckley et al., 1987) and increased extraction efficiencies (Richards et al., 1987b; Sarda et al., 1989) that were observed after plasma acidification, since extraction separates protein-bound ANP from free ANP (Wilson et al., 1991b). However, the value of acidifying plasma before extraction will remain uncertain until the physiological significance of the ANP-binding protein is fully understood, particularly as acidification also releases ANP from receptors (Jacobs et al., 1987) so that platelets could influence the final assay result. Nevertheless, plasma extraction was essential to remove all sources of RIA interference and allow accurate determination of ANP concentrations.

Non-specific adsorption onto C_{18} Sep-Pak columns has been the most popular ANP extraction method (Gutkowska et al., 1987; Cernacek et al., 1988), with the advantage that ANP and AVP were extracted simultaneously (Hartter and Woloszczuk, 1986; Bodola and Benedict, 1988), although minor procedural differences

between laboratories produced a wide range of reported ANP recoveries (Yandle et al., 1986; Richards et al., 1987b; Naomi et al., 1988; Sarda et al., 1989). Other ANP extraction methods have included non-specific adsorption onto Amberlite XAD-2 (Arendt et al., 1985), Vycor (Gutkowska et al., 1987) or Florisil (Burrell et al., 1990), protein precipitation by ethanol (Winters et al., 1988) or heat treatment (Iinuma et al., 1987), removal of protein by ultrafiltration (Cernacek et al., 1988), and immunoextraction by anti-ANP antibody coated onto Sepharose (Yamaji et al., 1985) or magnetic particles (Török and Penke, 1991).

Most extraction methods isolated sufficient ANP from 1-2 ml plasma for measurement by RIA (Yamaji et al., 1985; Gutkowska et al., 1987; Burrell et al., 1990) although some extractions required 5-15 ml plasma (Arendt et al., 1985; Hartter et al., 1986), while the methods described by Török and Penke (1991) and Iinuma et al. (1987) needed only 0.25 ml and 0.5 ml plasma, respectively, because they were combined with sensitive RIAs. Prompt extraction was important because ANP was unstable in plasma (Nishiuchi et al., 1986; Richards et al., 1987b; Artner-Dworzak et al., 1991) and serum (Hartter et al., 1986), even during storage at -80°C (Lijnen et al., 1988; Burrell et al., 1990; Nelesen et al., 1992), and ANP activity was also decreased by freeze-thawing (Bürgisser et al., 1985; Tan et al., 1990). After extraction, the solvent was usually removed by evaporation (Richards et al., 1987b; Burrell et al., 1990) or lyophilization (Yamaji et al., 1985; Hartter and Woloszczuk, 1986; Sarda et al., 1989), a step which increased the total preparation time significantly and which was unnecessary in some methods (Iinuma et al., 1987; Török and Penke, 1991). Many extractions were therefore time-consuming, although the heat treatment method could be carried out in less than 30 minutes (Iinuma et al., 1987). However, even after extraction and lyophilization, ANP was still unstable at -80°C (Nelesen et al., 1992) therefore prolonged storage before assay was undesirable.

Long incubation periods were reported for ANP RIAs, with most workers preincubating the sample and antiserum before addition of the tracer (Hartter et al., 1986; Gutkowska et al., 1987; Cernacek et al., 1988). The total incubation period

varied from 16 hours (Iinuma et al., 1987) to 6 days (Rasmussen et al., 1990), but most ANP RIAs were incubated for 2-3 days (Gutkowska et al., 1987; Cernacek et al., 1988; Sarda et al., 1989) before separation of bound and free tracer.

The most popular separation methods have been antibody precipitation by a second antibody (Yamaji et al., 1985; Iinuma et al., 1987; Cernacek et al., 1988; Sarda et al., 1989) or PEG-assisted second antibody (Gutkowska et al., 1985; Jüppner et al., 1986; Rosmalen et al., 1987), and adsorption of unbound tracer by dextran-coated charcoal (Gutkowska et al., 1984a; Lang et al., 1985; John et al., 1986; Sarda et al., 1989). Other separation methods reported for ANP RIAs have included antibody precipitation by PEG 8000 (Larose et al., 1985), or by second antibody coupled to cellulose (Rasmussen et al., 1990) or Sephacryl S-1000 (Prowse et al., 1989), and adsorption of unbound tracer by BSA-coated charcoal (Arendt et al., 1985).

A competitive enzyme immunoassay for ANP was reported (McLaughlin et al. 1987) but it was less sensitive than many RIAs and the total assay time was still 1-2 days. Its second antibody-coated microtitre plate format was more convenient for handling than liquid-phase assays, and avoided the need for a discrete separation step because incubation and separation occurred simultaneously, but it did not offer any other significant advantage over RIA methods.

A few research groups also developed radioreceptor assays for ANP, using receptor-containing membranes derived from rat kidney glomeruli (Ballermann, 1988; Gutkowska et al., 1988) or bovine adrenal cortex (Bürgisser et al., 1985; Sagnella et al., 1987; Ong et al., 1988; Capper et al., 1990), tissues which contain mainly B-ANP receptors. Binding to these high affinity receptors occurred rapidly therefore the incubation periods reported for radioreceptor assays varied from only 1 hour (Bürgisser et al., 1985; Gutkowska et al., 1988) to 22 hours (Ballermann, 1988). Bound and free ^{125}I-ANP were then separated either by filtration (Bürgisser et al., 1985; Ballermann, 1988; Gutkowska et al., 1988; Ong et al., 1988) or by centrifugation (Sagnella et al., 1987; Capper et al., 1990), so the total assay time was significantly shorter than that required for most RIAs yet radioreceptor assay sensitivity was roughly similar to RIA

sensitivity. However, B-ANP receptors recognize all biologically-active forms of ANP therefore radioreceptor assays cross-react with any peptide that has a 17-amino acid disulphide-bonded loop and an intact C-terminal (Gutkowska et al., 1988; Capper et al., 1990). In contrast, RIA specificity varies between antisera, some of which cross-react significantly with biologically-inactive ANP metabolites (Gutkowska et al., 1985; Ballermann, 1988; Ong et al., 1988). All competition assays for ANP are therefore likely to detect a range of ANP-related peptides in addition to α-ANP.

1.3 TWO-SITE IMMUNOMETRIC ASSAYS

In immunometric assays, labelled antibody binds non-competitively to the analyte (Miles and Hales, 1968) and high reagent concentrations drive the reaction to completion so that binding equilibrium is attained rapidly (Baker et al., 1985; Ekins, 1987). In contrast, competitive assays (Figure 1.1A) use low reagent concentrations to achieve maximal sensitivity therefore long incubation periods are essential to ensure that equilibrium is reached (Chard, 1987). Immunometric assays are therefore much faster than competitive immunoassays.

In two-site immunometric, or 'sandwich', assays (Figure 1.1B), a labelled antibody and a solid phase-bound antibody bind simultaneously to separate epitopes on the analyte. Two-site assays are therefore more specific than competitive assays because a positive response is obtained only when both antibodies are bound (Baker et al., 1985; Chard, 1987; Ekins, 1987; Gosling, 1990). Structurally-similar materials, which cross-react in competitive assays by displacing the labelled analyte, are less likely to participate in two-site binding (Baker et al., 1985) although cross-reaction can occur if binding to one antibody increases the affinity of the second antibody for the cross-reacting substance (Boscato et al., 1989). However, materials which bind to only one antibody will cause interference by decreasing the capacity for two-site binding so that the analyte concentration may be underestimated (Baker et al., 1985; Boscato et al., 1985). Anti-immunoglobulin antibodies, which are found in the plasma

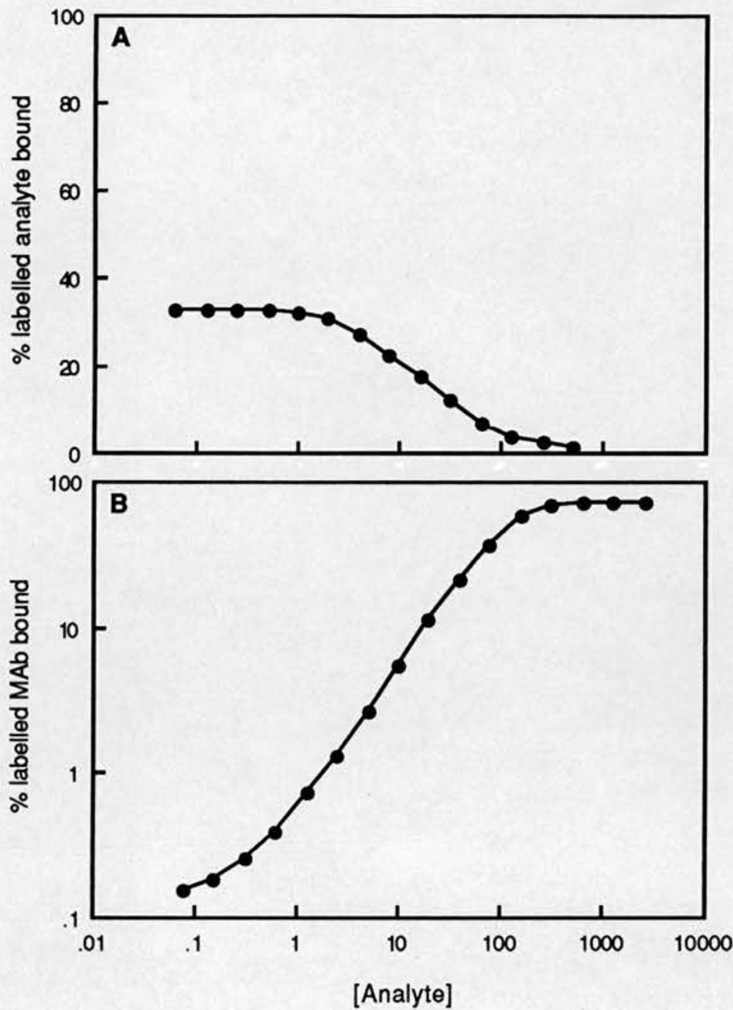

FIGURE 1.1

<u>Comparison of the standard curves obtained in competition assays and in two-site immunometric assays</u>

In competitive assays (**A**), unlabelled analyte competes with a fixed amount of labelled analyte for binding to a limited quantity of antibody or receptor molecules, in immunoassays or radioreceptor assays, respectively. As the unlabelled analyte concentration increases, a decreasing proportion of labelled analyte is recovered in the bound fraction. The example shown is a typical RIA standard curve obtained by the method described in Section 2.4.2.

In two-site immunometric assays (**B**), excess labelled antibody and a second, solid phase-bound, antibody bind to separate epitopes on the analyte therefore an increasing amount of labelled antibody becomes bound to the solid phase as the analyte concentration of the sample increases. The example shown is a typical direct IRMA standard curve obtained with two analyte-specific MAbs as described in Section 2.5.2.1.

of some individuals, can also interfere in two-site assays because they cross-link the reagent antibodies in the absence of analyte although this is prevented simply by the addition of normal serum from the species in which the reagent antibodies were raised (Hunter and Budd, 1981; Baker et al., 1985; Boscato and Stuart, 1988; Nahm and Hoffmann, 1990; Levinson, 1992).

Competitive immunoassay sensitivity is dependent on the affinity of the chosen antibody (Baker et al., 1985; Gosling, 1990). In contrast, the sensitivity of an immunometric assay is largely determined by the specific activity of the labelled antibody (Baker et al., 1985; Ekins, 1987) and is less dependent on its binding affinity (Gosling, 1990). Immunometric assays are therefore more sensitive than competitive assays that are based on the same antibody (Hunter and Budd, 1981; Baker et al., 1985; Chard, 1987; Ekins, 1987). Two-site immunometric assay sensitivity can be improved by decreasing the non-specific binding of labelled antibody, which increases the signal-to-noise ratio of the system (Baker et al., 1985; Ekins, 1987). Assay sensitivity will also increase if cooperativity between the antibodies produces circular antibody-analyte complexes in which the two antibodies are linked through both binding sites by two analyte molecules, with the effect that their affinity for the analyte is enhanced (Ehrlich et al., 1982; Ehrlich and Moyle, 1984).

Immunometric assays usually have a wider working range than competitive immunoassays (Hunter and Budd, 1981; Baker et al., 1985). This is advantageous for those analytes which have a broad physiological concentration range because samples are less likely to require dilution before assay. However, two-site assays are prone to a high-dose 'hook' effect where the binding of labelled antibody decreases with increasingly high analyte concentrations (Figure 6.23). This effect, which causes the underestimation of very high analyte concentrations, occurs because the antibodies are no longer in excess therefore free analyte competes with labelled antibody-bound analyte for binding to the solid phase-coupled antibody (Baker et al., 1985; Chard, 1987). This problem, if expected, can be overcome either by increasing the antibody concentration on the solid phase or by assaying the samples at more than one dilution

(Baker et al., 1985).

Analyte labelling can alter the specificity of a competitive immunoassay if the label obscures an epitope that is recognized by some of the antibodies in the antiserum (Miles and Hales, 1968; Chard, 1987). An immunometric assay avoids this problem, which is most severe with small analytes (Chard, 1987), because the antibody is labelled instead of the analyte. Labelled antibodies are relatively stable molecules (Chard, 1987; Ekins, 1987) which are unaffected by the plasma proteases that can degrade labelled analytes such as ^{125}I-AVP or ^{125}I-ANP, therefore immunometric assays can determine the analyte concentration of unextracted plasma without risking the overestimation that can occur in competitive assays as a result of tracer degradation. The sample analyte would still be prone to proteolytic attack in unextracted plasma but this would be less serious during the short immunometric assay incubation period than during prolonged competitive assay incubations and might be adequately decreased by protease inhibitors.

The main disadvantage of the two-site immunometric assay is that large quantities of purified antibody are required for reagent preparation. Polyclonal antisera contain a relatively small proportion of specific antibody and therefore have to be immunoaffinity-purified to produce the highly specific reagents that two-site assays demand (Hunter and Budd, 1981; Baker et al., 1985). This procedure requires large amounts of pure analyte which may be difficult to obtain, while the extreme conditions that are needed to elute high affinity antibodies from the immobilized analyte may cause denaturation (Siddle, 1985; Tijssen, 1985) therefore polyclonal antibodies have not been widely used in two-site immunometric assays.

1.4 MONOCLONAL ANTIBODIES

Immunization stimulates antigen-specific B cells to proliferate and mature into antibody-secreting plasma cells, each of which produces antibody specific for a single epitope. Most immunogens have a large number of different epitopes and therefore

stimulate many different B cells, producing a polyclonal antiserum that contains a range of antibodies with different specificities, affinities and isotypes (Roitt et al., 1985). Individual animals respond differently to the same immunogen, while subsequent antigen contact alters the spectrum of antibodies secreted by any one animal (Campbell, 1984; Tijssen, 1985). The properties of polyclonal antisera are therefore extremely variable, while the supply of a particular quality of antiserum is strictly limited to the volume obtained in one bleed.

The development of hybridoma technology (Köhler and Milstein, 1975, 1976) allowed the production of monoclonal antibodies, which are homogeneous with respect to specificity, affinity and isotype because each MAb is secreted by an isolated clone of cells. Plasma cells have a finite lifespan and will not grow in culture but can be fused with myelomas, which are plasma cell tumours, to produce hybridoma cell lines that secrete antibody and will grow indefinitely (Galfrè and Milstein, 1981; Campbell, 1984). However, it is important to select a myeloma which does not itself produce antibody, or the resulting hybridomas would secrete mixed-chain antibodies in addition to the desired specificity (Galfrè and Milstein, 1981) thus necessitating immunoaffinity purification. After cell fusion, a selection procedure ensures that the only cells to survive are the hybridomas, which are then separated to produce monoclonal cell lines that can be grown either *in vitro* or *in vivo* (Galfrè and Milstein, 1981).

MAbs usually have lower binding affinities than polyclonal antisera that are raised against the same antigen (Siddle, 1985; Ehrlich et al., 1982) therefore they make poor competitive immunoassay reagents. However, MAbs are ideal for immunometric assays because they are monospecific, available in unlimited quantities (Baker et al., 1985; Siddle, 1985), and can be purified by simple methods since they are the only immunoglobulins in spent culture supernatant and can comprise up to 90% of the antibody in ascitic fluid (Galfrè and Milstein, 1981). Monospecificity is advantageous in assays that have to distinguish substances with closely-related structures, such as AVP and LVP (Table 5.3), but is a disadvantage for analytes

which exhibit polymorphism since MAb-based assays may not recognize all forms of the analyte (Campbell, 1984; Siddle, 1985). Unfortunately, MAbs may also bind to epitopes on unrelated substances that could cause significant assay interference (Campbell, 1984), a problem which does not occur with polyclonal antisera, whose specificities are determined by the combined reactions of many different antibodies (Goding, 1986). MAb-based assays are therefore prone to unexpected cross-reactions which can only be eliminated by careful MAb selection, a problem that is counter-balanced by the reagent standardization that MAbs permit.

1.5 AIMS OF THE PROJECT

AVP and ANP act together to control the fluid balance of the body. The neonatal kidney responds to these hormones (Birk et al., 1989; Kojima et al., 1990) although renal function is not fully-developed at birth (Bell and Oh, 1979). The urine concentrating capacity is initially low (Aperia et al., 1983; Rees et al., 1984a; Wiriyathian et al., 1986) and gradually increases over the first few months of life (Svenningsen and Aronson, 1974; Aperia et al., 1983; Sulyok, 1988). However, healthy neonates have a good diluting capacity so they can excrete excess water efficiently (Aperia et al., 1983). In contrast, premature and sick infants often have fluid balance problems that can decrease their survival prospects (McIntosh and Smith, 1985). Clinical management of these individuals is difficult because their symptoms can be misinterpreted. For example, while hypernatraemia is usually a result of inadequate fluid intake, hyponatraemia might be caused by either sodium loss or water retention, and incorrect treatment would worsen the infant's condition (Bell and Oh, 1979; Rees et al., 1984b). Premature infants have a particularly low capacity to compensate for inappropriate fluid and electrolyte administration (Bell and Oh, 1979).

Major haemodynamic changes occur at birth, when the lungs replace the placenta as the site of gaseous exchange. In the normal infant, heart rate and cardiac output increase, pulmonary vascular resistance decreases, and the ductus arteriosus and

foramen ovale close so that the blood is directed through the lungs before delivery to the peripheral circulation (Birk et al., 1989). These changes increase the left atrial pressure, causing raised plasma ANP concentrations in the first few days of life (Tulassay et al., 1987; Ito et al., 1988; Kikuchi et al., 1988), and are probably responsible for the diuresis and natriuresis that cause postnatal contraction of the extracellular fluid (Birk et al., 1989).

In the premature infant, the ductus arteriosus may remain open, or may reopen, so that blood flows from the aorta into the pulmonary artery causing pulmonary hypertension, further left atrial stretch and high plasma ANP concentrations (Andersson et al., 1987) that correlate significantly with the severity of ductal blood flow (Pesonen et al., 1990). However, glomerular immaturity blunts the postnatal increase in glomerular filtration rate (Aperia et al., 1981) and so decreases the efficiency of ANP-induced water excretion. Over-hydration increases the risk that premature infants will develop patent ductus arteriosus, congestive heart failure (Bell et al., 1980), respiratory distress syndrome (Stegner et al., 1987) and bronchopulmonary dysplasia (Brown et al., 1978).

Infants with respiratory distress syndrome usually receive positive-pressure ventilation. The increased thoracic pressure causes decreased venous return to the heart and so stimulates the baroreceptors to promote AVP secretion (Rees et al., 1984a; Wiriyathian et al., 1986; Stegner et al., 1987) that correlates with the ventilation pressure (Stegner et al., 1987) and may cause water retention (Bell and Oh, 1979; Stegner et al., 1987). However, tubular function develops later than glomerular function so premature infants have a lower capacity than full-term infants to carry out AVP-induced water reabsorption (Aperia et al., 1981). Ventilation was also reported to compress the atria, decreasing stretch receptor stimulation and thus ANP secretion in volume-expanded individuals, which would tend to further inhibit water excretion although the ANP concentrations did not fall to normal levels (Leithner et al., 1987). Kojima et al. (1990) reported elevated plasma AVP and ANP concentrations in ventilated infants with bronchopulmonary dysplasia, and suggested

that ANP is raised in an attempt to compensate for water retention induced by the AVP that is secreted in response to pulmonary abnormalities.

Inappropriate AVP secretion is common in premature infants, occurring in response to non-osmotic stimuli such as birth asphyxia (Wiriyathian et al., 1986; Smith et al., 1990), respiratory distress, pneumothorax, intracranial haemorrhage (Rees et al., 1984a; Wiriyathian et al., 1986; Sulyok, 1988) or meningitis (Rees et al., 1980; Sulyok, 1988). The highest AVP concentrations occur in the most severely ill infants (Stegner et al., 1987; Smith et al., 1990), who are therefore least able to excrete a water load. Birth asphyxia causes particularly high AVP concentrations (Wiriyathian et al., 1986; Smith et al., 1990) but may be complicated by tubular damage that decreases the urine concentrating capacity (Svenningsen and Aronson, 1974; Aperia et al., 1983) or by glomerular damage which decreases urine output (Smith et al., 1990).

Relatively little is known about the concentrations of AVP and ANP in neonatal plasma, or about the way in which these hormones influence the fluid balance and cardiovascular systems of premature and sick infants, because most assays require larger samples than can be obtained from small infants. Urinary measurements have provided most of the published AVP data, although there is no correlation between urinary and plasma AVP concentrations in infants with renal damage (Smith et al., 1990). All reported AVP and ANP assays require long incubation periods, while sample extraction can increase the total assay time significantly. The current assays are therefore unsuitable for diagnostic purposes because the condition of sick and premature infants changes rapidly. The fastest AVP assay takes 26 hours to produce a result from 2 ml plasma (Burd et al., 1984), while the fastest ANP assay takes 19 hours and requires 0.5 ml plasma (Iinuma et al., 1987). Although these assays measure physiological peptide concentrations, the sample volumes are too large and the incubations too long to be clinically-useful in the context of neonatal care.

There is a need for rapid assays that can measure physiological concentrations of AVP and ANP in small volumes of unextracted plasma within a few hours of

sampling. These assays would facilitate research on the effects of AVP and ANP in sick and premature infants, and could ultimately allow more sophisticated management of fluid balance than is currently possible. Immunometric assays could provide the necessary speed while MAbs ensure fine specificity and continuity of reagent supplies. The aims of this project were therefore:

1. To generate MAbs specific for AVP and ANP.

2. To determine whether two-site immunometric assays for AVP and ANP could be developed with appropriate pairs of MAbs.

3. To determine whether these assays would have the sensitivity required to measure the concentrations of AVP and ANP in unextracted plasma.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 ANIMALS

Mice (BALB/c) and rats (Sprague-Dawley and Wistar strains) were supplied by Bantin and Kingman, Hull, and housed in the Medical Faculty Animal Area.

2.1.2 TISSUE CULTURE MEDIA

Foetal calf serum, 200 mM L-glutamine, HAT supplement, HT supplement, High Protein Hybridoma Medium, 50 mM 2-mercaptoethanol, penicillin-streptomycin, RPMI 1640 with 20 mM HEPES without L-glutamine, RPMI 1640 without HEPES, and 100 mM sodium pyruvate were obtained as sterile solutions from Gibco BRL, Paisley.

Acridine orange, ethidium bromide, polyethylene glycol 4000 and Oxoid Dulbecco 'A' PBS tablets were obtained from BDH Laboratory Supplies, Glasgow.

Dimethyl sulphoxide was supplied by Sigma Chemical Company, Poole.

2.1.3 IMMUNOCHEMICALS

A Mouse Monoclonal Antibody Isotyping Kit was supplied by Amersham International, Amersham.

Bovine (new-born calf) serum was obtained from Gibco BRL, Paisley.

Immunopure streptavidin-HRP conjugate was supplied by Pierce, Rockford, Illinois, USA.

Normal rabbit serum, donkey anti-rabbit IgG HRP conjugate and sheep anti-mouse γ-globulin antiserum were provided by the Scottish Antibody Production Unit, Carluke.

Rabbit (IgG fraction of antiserum) anti-mouse IgG (whole molecule) HRP conjugate, affinity-isolated goat anti-mouse 'polyvalent immunoglobulins' HRP

conjugate and affinity-isolated sheep anti-mouse IgG (whole molecule) HRP conjugate were obtained from Sigma Chemical Company, Poole.

Rabbit polyclonal antibodies (anti-AVP serum, anti-ANP serum, anti-PAG serum, TG1 antiserum, affinity-purified anti-AVP-thyroglobulin and affinity-purified anti-PAG) were provided by Dr. A. Smith, Department of Child Life and Health.

The murine anti-ANP MAbs ESA 4, ESA 5 and ESA 9 were a gift from Dr. C. Prowse, Edinburgh and South-East Scotland Blood Transfusion Service.

2.1.4 PEPTIDE HORMONES AND ANALOGUES

^{125}I-ANP (human) was supplied by Amersham International, Amersham.

AVP, dCDAVP, dDAVP, dNMeAVP, Hep, OT, PAG and tGLVP were obtained from Ferring AB, Malmo, Sweden.

ANP (human), AVP (acetate salt), AVT (acetate salt) and LVP (acetate salt) were supplied by Sigma Chemical Company, Poole.

2.1.5 OTHER REAGENTS

Na^{125}I was obtained from Amersham International, Amersham.

Biotinyl-ε-aminocaproic acid N-hydroxysuccinimide ester (Biotin-X-NHS) was obtained from Calbiochem Novabiochem (UK) Ltd., Nottingham.

Pre-packed PD-10 columns, Protein A-Sepharose CL-4B, Sephacryl S-300, Sephacryl S-500, Sephadex G-25 and Sephadex G-50 were supplied by Pharmacia, Milton Keynes.

N-hydroxysulphosuccinimide and Iodogen came from Pierce, Rockford, Illinois.

Non-fat dried milk was purchased from J. Sainsbury Ltd., London.

Bovine albumin (fraction V), L-cysteine hydrochloride (anhydrous), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride, Freund's adjuvant (complete and incomplete), glycerol, hydroxylamine (hydrochloride),

2-iminothiolane (hydrochloride), keyhole limpet haemocyanin, 2-mercaptoethanol, m-maleimidobenzoic acid N-hydroxysuccinimide ester, N,N-dimethylformamide, polyethylene glycol 8000, pristane (2,6,10,14-tetramethylpentadecane), Rivanol (6,9-diamino-2-ethoxyacridine lactate, monohydrate), sodium borohydride, sodium m-periodate, streptavidin, 3,3',5,5'-tetramethylbenzidine, thyroglobulin (bovine), Triton X-100 and Tween 20 (polyoxyethylenesorbitan monolaurate) were obtained from Sigma Chemical Company, Poole.

Alhydrogel (aluminium hydroxide gel, alum) came from Superfos Speciality Chemicals, Vedback, Denmark.

All other chemicals were of the highest grade available.

2.1.6 DISPOSABLE EQUIPMENT

Membrane-bottomed 96-well plates and a Single Vacuum Manifold were supplied by Amersham International, Amersham

Sterilin RT30 polystyrene test tubes (75 x 12 mm) were supplied by BDH Laboratory Supplies, Glasgow.

Immulon 1 and Immulon 2 flat-bottomed 16-well MicroELISA Duo-strips and Immulon 4 16-well MicroELISA Divida-strips were supplied by Dynatech Laboratories, Billingshurst.

Nunc Microwell Modules (Maxisorp, F-16) were obtained from Gibco BRL, Paisley.

2.1.7 BUFFERS

The main buffers used in this project were as follows :

BUFFER	COMPOSITION
A	0.25 M tri-sodium citrate/citric acid, pH 4.0
B	0.02 M sodium acetate/acetic acid, pH 5.0
C	0.1 M sodium acetate/acetic acid, pH 5.0
D	0.1 M sodium acetate/citric acid, pH 6.0, 5 mM EDTA, 0.02% Tween 20
E	0.05 M Na_2HPO_4/NaH_2PO_4, pH 7.0
F	0.05 M Na_2HPO_4/NaH_2PO_4, pH 7.0, 2% bovine serum
G	0.05 M Na_2HPO_4/NaH_2PO_4, pH 7.0, 2% bovine serum, 3% Tween 20
H	0.05 M Na_2HPO_4/NaH_2PO_4, pH 7.0, 1% Tween 20
I	0.5 M Na_2HPO_4/NaH_2PO_4, pH 7.4
J	0.05 M Na_2HPO_4/NaH_2PO_4, pH 7.5
K	0.05 M Na_2HPO_4/NaH_2PO_4, pH 7.5, 0.9% NaCl
L	0.05 M Na_2HPO_4/NaH_2PO_4, pH 7.5, 0.9% NaCl, 1% BSA, 0.1% Tween 20
M	0.05 M Na_2HPO_4/NaH_2PO_4, pH 7.5, 0.9% NaCl, 1% BSA, 0.5% Tween 20
N	0.05 M Na_2HPO_4/NaH_2PO_4, pH 7.5, 0.9% NaCl, 0.5% Tween 20
O	0.25 M Na_2HPO_4/NaH_2PO_4, pH 7.5
P	0.25 M Tris/HCl, pH 8.5, 2% bovine serum
Q	0.25 M Tris/HCl, pH 8.5, 2% bovine serum, 1% Tween 20
R	0.25 M Tris/HCl, pH 8.5, 2% bovine serum, 4% Tween 20
S	0.25 M Tris/HCl, pH 8.5, 1% Tween 20
T	0.05 M $NaHCO_3/NaOH$, pH 9.0
U	0.05 M $NaHCO_3/NaOH$, pH 9.0, 0.5% Tween 20
V	0.1 M $NaHCO_3/NaOH$, pH 9.0

2.2 MONOCLONAL ANTIBODY PRODUCTION

2.2.1 PREPARATION OF IMMUNOGENS

Small peptides, such as AVP and ANP, are poor immunogens so they are usually conjugated to large carrier proteins to increase their immunogenicity. Bovine thyroglobulin and KLH were the carrier proteins used in this project.

2.2.1.1 AVP-Thyroglobulin (EDC Method)

AVP was conjugated to bovine thyroglobulin essentially by the method of Skowsky and Fisher (1972b) :

0.86 mg AVP and 4.3 mg thyroglobulin were dissolved in 0.3 ml distilled water. 0.1 ml 4 mg/ml 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) was added, followed by 2 µl 10 M NaOH which neutralized the solution and prevented precipitation of thyroglobulin. The reaction mixture was vortexed and incubated at room temperature for 24 hours, then 0.4 ml 1 M hydroxylamine was added. After incubation for a further 6 hours, 0.1 ml aliquots were stored at -20°C.

2.2.1.2 AVP-Thyroglobulin (Enhanced EDC Method)

AVP was also conjugated to thyroglobulin in the presence of N-hydroxysulphosuccinimide, which has been reported to enhance the coupling yield of water-soluble carbodiimide-mediated reactions (Staros et al., 1986).

4.3 mg AVP and 21.5 mg thyroglobulin were dissolved in 1 ml distilled water. 0.5 ml 20 mM N-hydroxysulphosuccinimide and 5 µl 10 M NaOH were added, followed by 0.5 ml 3 mg/ml EDC. The mixture was incubated at room temperature for 24 hours, then 2 ml 1 M hydroxylamine was added. After incubation for a further 6 hours, 0.1 ml aliquots were stored at -20°C.

2.2.1.3 AVP/LVP-KLH

A mixture of AVP and LVP was coupled to KLH by a modification of the method of Lerner et al. (1981). m-Maleimidobenzoic acid N-hydroxysuccinimide ester (MBS), a heterobifunctional reagent, was used to link the primary amine groups of KLH to free sulphydryl groups that had been introduced into AVP and LVP.

Activation of KLH by MBS

10 mg KLH was dissolved in 625 µl 0.1 M sodium phosphate buffer, pH 8.0, 0.9% NaCl. 88 µl 2% MBS in DMF was added dropwise and the mixture was stirred at room temperature for 20 minutes. Unreacted MBS was removed by gel filtration on a Pharmacia PD-10 column equilibrated with degassed 0.1 M sodium phosphate buffer, pH 6.7, 0.9% NaCl, 5 mM EDTA (PBS-EDTA). The buffer contained EDTA to chelate oxidising metals which might interfere in the second stage of the reaction.

Thiolation of AVP and LVP

Neither AVP nor LVP contained free sulphydryl groups therefore these were introduced using Traut's reagent, 2-iminothiolane (Traut et al., 1973; Jue et al., 1978). All buffers were degassed to prevent oxidation of the sulphydryl groups.

A stock solution of 20 mg/ml 2-iminothiolane was prepared in Buffer C. AVP (0.86 mg) was dissolved in 194 µl PBS-EDTA and mixed with 6 µl 20 mg/ml 2-iminothiolane. Simultaneously, 4.3 mg LVP was dissolved in 938 µl PBS-EDTA and mixed with 62 µl 20 mg/ml 2-iminothiolane. Both reactions were incubated on ice for 30 minutes.

Conjugation of m-maleimidobenzoyl-KLH to thiolated AVP and LVP

Immediately after their preparation, 5 mg m-maleimidobenzoyl-KLH was mixed with the thiolated AVP and LVP. The pH was adjusted to 7.0-7.5, using 10 M NaOH, then the incubation mixture was stirred at room temperature for 3 hours. 0.1 ml aliquots were stored at -20°C.

Unconjugated vasopressin was not dialysed away because it was reported that antibodies could be raised against unconjugated AVP (Roth et al., 1966b; Wu and

Rockey, 1969) and LVP (Roth et al., 1966a; Bichet et al., 1986).

2.2.1.4 ANP-Thyroglobulin

ANP-thyroglobulin conjugate, a gift from Dr. C. Prowse, had been prepared by the following method :

Bovine thyroglobulin was dissolved in distilled water then dialysed overnight against 0.05 M sodium phosphate buffer, pH 7.2, 0.1 M NaCl, at 4°C. 4 ml 2.1 mg/ml thyroglobulin was mixed with 6 mg EDC, added dropwise to 1 ml 1 mg/ml ANP in 0.15 M NaCl, and stirred for 15 minutes. The mixture was incubated overnight at 4°C, then an additional 6 mg EDC was added whilst stirring. After incubation at room temperature for 2 hours, the mixture was dialysed overnight against 0.15 M NaCl at 4°C, then stored at -20°C in 0.25 ml aliquots.

2.2.2 IMMUNIZATION OF MICE

All immunogens were injected intraperitoneally.

Twelve-week-old female BALB/c mice were primed with immunogen emulsified in an equal volume of Freund's complete adjuvant so that a drop floated on water as a discrete creamy-white globule which did not disperse. Each mouse was given either 50 µg AVP or 5 µg ANP, conjugated to carrier protein, in a total volume of 0.1-0.2 ml. After 5 weeks, they were boosted with immunogen emulsified in Freund's incomplete adjuvant then, after a further 5 weeks, with immunogen in alum (aluminium hydroxide gel).

Tail-bleeding was carried out 14 days later. The mice were warmed in a ventilated box positioned over a 25 W light bulb, until their tail veins were well-dilated. Each mouse was restrained in a 50 ml polypropylene conical centrifuge tube, with its tail protruding through a 0.5 cm-wide slot, then one of the lateral tail veins was cut with a clean razor blade. Blood was collected into heparinized

haematocrit tubes by capillary action, then the cells were removed by centrifugation in a haematocrit centrifuge for 1 minute. The serum was assayed for anti-peptide antibody by RIA or ELISA (Section 2.2.4).

Further boosts of immunogen in alum were given, if necessary, at intervals of at least 5 weeks until antibody titres of more than 1/3000 were obtained. Three days before cell fusion, the mice received a final boost of immunogen in Dulbecco 'A' PBS.

2.2.3 CELL CULTURE

All cells were cultured under sterile conditions in a 5% CO_2/air atmosphere with 90% humidity, at 37°C. Tissue culture flask caps were kept loose to allow for gas exchange.

2.2.3.1 Preparation of Culture Media

Foetal calf serum (FCS) was heat-inactivated by incubation at 56°C for 30 minutes (Campbell, 1984). All medium supplements were stored at -20°C, except sodium pyruvate which was stored at 4°C.

500 ml RPMI 1640 with 20 mM HEPES without L-glutamine was supplemented with 5 ml 200 mM L-glutamine, 2.5 ml penicillin-streptomycin solution (containing 10,000 IU/ml penicillin and 10,000 µg/ml streptomycin), 5 ml 100 mM sodium pyruvate and either 25 ml, 50 ml or 75 ml heat-inactivated FCS, to produce 5%, 10% or 15% FCS medium, respectively.

HAT medium, which contained 100 µM hypoxanthine, 0.4 µM aminopterin and 16 µM thymidine, was prepared by the addition of 10 ml HAT supplement to 500 ml 15% FCS medium. HT medium contained 100 µM hypoxanthine and 16 µM thymidine, but no aminopterin, and was prepared by the addition of 10 ml HT supplement to 500 ml 15% FCS medium.

Serum-free medium was prepared by the addition of 5 ml 200 mM L-glutamine,

2.5 ml penicillin-streptomycin solution and 0.5 ml 50 mM 2-mercaptoethanol to 500 ml High Protein Hybridoma Medium. Sodium pyruvate was not required.

Confirmation of culture medium sterility

Freshly-prepared culture medium was stored at room temperature, in the dark, for 24 hours. A few drops were added to 4 ml sterile nutrient broth and incubated at 37°C for 7-10 days. Sterility was confirmed if the nutrient broth remained clear, whereas contamination caused turbidity. Severely contaminated media were discarded but slight contamination could be removed by passage through a bottle top filter (0.2 μm membrane).

Prepared culture media were stored at -20°C until sterility was confirmed. Thawed media were discarded after 4 weeks at 4°C because the L-glutamine was unstable (Goding, 1986). All media were warmed to 37°C in a water bath before use. Multiple cultures of any cell line were fed from different bottles of medium to decrease the risk of losing all the cells to microbial contamination.

2.2.3.2 Cell Counting

Viable cell numbers were estimated with a vital stain which consisted of 5 μg/ml acridine orange and 5 μg/ml ethidium bromide in Dulbecco 'A' PBS (Parks et al., 1979). The stain solution was stored in the dark at 4°C.

10 μl stain and 10 μl cell suspension were mixed and immediately transferred to a haemocytometer chamber (improved Neubauer type) for counting. When viewed under a fluorescence microscope, live cells were green and dead cells were orange.

The ruled area of the haemocytometer contained nine 1 mm^2 sections and the chamber was 0.1 mm deep therefore the volume of each section was 0.1 μl. The viable cell concentration was calculated from :

$$X = Y \times Z \times 10^4$$

where X = number of live cells/ml, Y = number of live cells/section and Z = dilution factor of the cells in stain (usually 2).

2.2.3.3 Preparation of Mixed-Thymocyte Culture-Conditioned Medium (MTM)

MTM was kindly prepared by Dr. L. Micklem, according to the method of Micklem et al. (1987) :

A Sprague-Dawley rat and a Wistar rat (5-6-week-old females) were killed by cranial impact. The fur was soaked with 70% ethanol then the skin was cut open with sterile scissors. A fresh set of sterile instruments was used to open the thoracic cavity and remove the thymus from each rat.

A cell suspension was prepared by homogenization of the thymi in 5 ml Dulbecco 'A' PBS. The cells were washed three times with 25 ml aliquots of Dulbecco 'A' PBS, collected by centrifugation (200 x g for 10 minutes), and finally resuspended in 15% FCS medium at 5×10^6 cells/ml. After incubation for 48 hours, in 75 cm^2 tissue culture flasks, the cells were removed by centrifugation (2000 x g for 10 minutes). The supernatant (MTM) was filtered through a 0.2 µm membrane then stored in 10 ml aliquots at -20°C.

2.2.3.4 Preparation of Myeloma Cells

The NS-0 myeloma cell line, which was derived from BALB/c mice and does not express immunoglobulin chains (Galfrè and Milstein, 1981), was the fusion partner used in this work.

Three days before cell fusion, a vial of NS-0 cells was thawed at 37°C and the contents immediately transferred into a 75 cm^2 tissue culture flask containing 30 ml 10% FCS medium. After incubation for 5-6 hours, 20 ml 5% FCS medium was added. As soon as the cells were confluent (usually by the next day), the culture was expanded by division. A sharp smack dislodged the cells from the plastic surface, half of the resulting cell suspension was transferred to a fresh 75 cm^2 flask, then 25 ml 5% FCS medium was added to each flask. This procedure was repeated daily to ensure that the cells were in exponential growth.

On the day of cell fusion, NS-0 cells were harvested by centrifugation (100 x g for 8 minutes) then washed twice by resuspension in sterile Dulbecco 'A' PBS followed by centrifugation. The final cell pellet was resuspended in 10 ml Dulbecco 'A' PBS then a 10 µl sample was removed for counting.

2.2.3.5 Preparation of Spleen Cells

Three days after receiving a final intraperitoneal boost of immunogen in PBS, the immunized mouse was killed by cervical dislocation. Its fur was soaked with 70% ethanol then the abdominal skin was cut open with sterile scissors. A fresh set of sterile instruments was used to cut open the left side of the peritoneal cavity and remove the spleen with as little connective tissue as possible.

A cell suspension was prepared by homogenizing the spleen in 5 ml sterile Dulbecco 'A' PBS. After allowing any undisrupted tissue to settle out, the cells were transferred into a sterile plastic universal container, washed three times with 25 ml aliquots of Dulbecco 'A' PBS and collected by centrifugation (200 x g for 8 minutes). The final cell pellet was resuspended in 10 ml Dulbecco 'A' PBS then a 10 µl sample was removed for counting.

2.2.3.6 Preparation of 50% PEG 4000

One day before cell fusion, 1 g PEG 4000 was autoclaved in a glass bijou bottle. While the PEG was still hot, 1 ml RPMI 1640 without HEPES was added and mixed thoroughly. The resulting solution was stored in the dark at room temperature. Before use, 10 µl sterile 2 M Tris was added to adjust the pH to 8.0-9.0, then the solution was warmed to 37°C.

2.2.3.7 Cell Fusion

This method was a modification of that of Galfrè and Milstein (1981) :

Spleen and NS-0 cells were mixed together (1.2×10^8 cells, at a spleen : NS-0 ratio of either 2:1 or 4:1) then centrifuged at 200 x g for 5 minutes. The supernatant was carefully decanted (complete removal was essential) then the cell pellet was loosened by gentle vibration. 1 ml 50% PEG 4000 was added and the cells were resuspended by gentle swirling. At this point cell agglutination could be seen. After centrifugation (80 x g for 5 minutes), 5 ml RPMI 1640 without HEPES was carefully added over 1-2 minutes without disturbing the cell pellet. Gentle swirling for 3-4 minutes gradually diluted the PEG solution and resuspended the cells, which were then collected by centrifugation (150 x g for 5 minutes). The supernatant was discarded then 5 ml HAT/MTM medium (from a mixture of 100 ml HAT medium and 20 ml MTM) was slowly added without disturbing the cell pellet. After 7 minutes, the cells were resuspended by gentle swirling, persistent clumps were dispersed by pipetting, and the remaining 115 ml HAT/MTM medium was added to give a final concentration of 1×10^6 cells/ml. The cell suspension was distributed amongst the central 60 wells (0.2 ml/well) of each of ten 96-well flat-bottomed tissue culture plates. The outermost wells were not used because they had a higher risk of contamination and were also prone to evaporation.

2.2.3.8 Cell Fusion with *In Vitro*-Boosted Spleen Cells

Spleen homogenates often contained more cells than were required for one fusion. The surplus cells were boosted *in vitro* by culture with antigen (Reading, 1982) because *in vitro* stimulation of immune spleen cells was reported to enhance the efficiency of antigen-specific hybridoma production (Erich et al., 1989).

Spleen cells were cultured in MTM/FCS medium (3 parts MTM : 7 parts 15% FCS medium) containing either AVP (2 μg/ml) or ANP (1 μg/ml), in a

25 cm^2 tissue culture flask (5 x 10^6 cells/ml), and harvested after 4 days. Any cells which remained attached to the plastic were collected after EDTA treatment. 5 ml 0.02% EDTA in Dulbecco 'A' PBS was added to the flask, then 30-60 seconds later the adherent cells could be resuspended by a sharp smack. The *in vitro*-boosted spleen cells were collected by centrifugation, washed, and fused with NS-0 cells as above.

2.2.3.9 Culture of Nascent Hybridomas

The plates of fused cells were incubated undisturbed for 5 days (by which time unfused NS-0 cells were dead), then examined for hybridoma growth with an inverted phase-contrast microscope. One drop of HAT medium was added to each well on day five and at intervals thereafter as necessary. Exchange feeding (where half of the culture medium was replaced with fresh HAT medium) was carried out on any wells which had been undisturbed for more than 21 days.

Antibody screening began 10-14 days after cell fusion when large hybridoma colonies occupied about 25% of the area of their culture wells. Supernatant samples were removed aseptically, replaced with fresh HAT medium, and assayed for anti-peptide antibody by RIA or ELISA (Section 2.2.4). Hybridoma cultures which produced positive assay results were immediately cloned and also expanded to produce cells for cryopreservation.

2.2.3.10 Dilution Cloning

Hybridoma cells were resuspended by repeated pipetting of the culture well contents. A 10 μl sample was diluted to 1 ml in HT medium supplemented with 10% MTM, while a second 10 μl sample was taken for cell counting. The cells in HT/MTM were further diluted to 25, 5 and 2.5 cells/ml, distributed into a 96-well tissue culture plate (0.2 ml/well, 30 wells/concentration), incubated undisturbed for 7 days, then examined for clonal growth. The number of clones in each well was

recorded before feeding (which often dispersed the loosely-adherent cell clusters). One drop of HT medium was added to each well. Wells containing single clones were screened for antibody production when they were sufficiently well-grown.

Three positive clones were selected for expansion and cryopreservation. The best clone was subcloned as described above, except that 15% FCS medium containing 10% MTM was used. Three subclones were selected for expansion and cryopreservation. The best subclone was used for bulk antibody production, either in culture or in ascites.

2.2.3.11 Expansion of Hybridoma Cultures

Hybridoma cultures from 96-well plates were resuspended by repeated pipetting then transferred into 0.5 ml medium containing 10% MTM, in a 24-well tissue culture plate. (Parent hybridomas were transferred into HT medium, clones and subclones into 15% FCS medium.) The cultures were fed 0.5 ml medium, without MTM, at 3-4 day intervals. As soon as a culture occupied more than half the area of its well, the cells were resuspended and transferred into 5 ml 15% FCS medium in a 25 cm^2 tissue culture flask, then fed as required with 10% FCS medium.

At least three flasks were set up for each cell line. The cells were either harvested and stored in liquid nitrogen, or used for bulk antibody production.

2.2.3.12 Cryopreservation of Cell Lines

Cell lines were stored at all stages of preparation (i.e. parent hybridoma, clones and subclones) to insure against loss resulting from contamination or overgrowth by non-secreting variants (Galfrè and Milstein, 1981).

Exponentially-growing cells were dislodged from the surface of the flask by a sharp smack, then harvested by centrifugation (100 x g for 8 minutes). The supernatant was decanted and the cell pellet loosened by gentle vibration. The cells were resuspended in ice-cold freezing medium (90% FCS, 10% DMSO), at about

4×10^6 cells/ml, then 0.5 ml aliquots were transferred into chilled freezing vials and immediately placed in the vapour phase above liquid nitrogen. After 24 hours, the vials were transferred into the liquid nitrogen for long-term storage (Reading, 1982).

NS-0 myeloma cells were stored in 1 ml aliquots.

2.2.3.13 Recovery of Hybridomas from Liquid Nitrogen

Frozen cells were thawed rapidly in a 37°C water bath then immediately transferred into 10 ml 15% FCS medium in a 25 cm^2 tissue culture flask. Some cell lines did not readily recover from freezing therefore, as a precaution, 2 ml of the cell suspension was transferred into a second flask containing 1 ml MTM. Once cell growth was established the flasks were fed with 10% FCS medium.

2.2.3.14 Bulk Antibody Production in Ascites

Ten-week-old male BALB/c mice were primed by intraperitoneal injection of 0.3 ml pristane (2,6,10,14-tetramethylpentadecane), 10 days and 3 days before injection of hybridoma cells.

Monoclonal cell lines were grown in 10% FCS medium, harvested by centrifugation (100 x g for 8 minutes), washed, and resuspended in Dulbecco 'A' PBS at 2.5×10^6 cells/ml. Each mouse received 5×10^5 cells intraperitoneally. Ascites tumours developed in 2-3 weeks. The ascitic fluid was tapped at 1-2 day intervals and collected into heparinized tubes. Cells were removed by centrifugation (1000 x g for 10 minutes) then sodium azide (final concentration 0.1%) was added to the ascitic fluid, which was stored at 4°C.

2.2.3.15 Bulk Antibody Production in Culture

Monoclonal cell lines were cultured in 10% FCS medium until exponential

growth was established, then transferred into 75 cm^2 (and subsequently 150 cm^2) tissue culture flasks. The culture volume was doubled every 1-2 days, using 5% FCS medium, then the cells were left to grow to exhaustion, which usually took 10-14 days after the final addition of medium. The cells were removed by centrifugation (2000 x g for 10 minutes) then sodium azide (final concentration 0.1%) was added to the supernatant, which was stored at 4°C.

2.2.4 SCREENING ASSAYS

All incubations were carried out at room temperature.

2.2.4.1 AVP-Capture ELISA

Rabbit TG1 antiserum (Smith and McIntosh, 1986) was diluted 1/10,000 in Buffer T then adsorbed onto MicroELISA Duo-strips (100 µl/well) by overnight incubation. Each well was rinsed three times with Buffer T, incubated for 30 minutes with 400 µl 1% BSA in Buffer T to block remaining protein-binding sites, rinsed once with Buffer T and dried by evaporation. The coated Duo-strips were incubated with 50 ng/ml AVP in Buffer L (100 µl/well) for 16 hours, then sealed and stored at -20°C, or used immediately, after rinsing with Buffer K.

The wells were incubated with 100 µl hybridoma culture supernatant, HAT medium (negative control) or immune mouse serum (1/1000 dilution in Buffer L, positive control) for 16 hours, then with 100 µl rabbit anti-mouse IgG (whole molecule) HRP conjugate (1/200 dilution in Buffer L) for 1 hour. Unbound HRP conjugate was removed by five washes with Buffer D before the addition of 100 µl freshly-prepared substrate (100 µg/ml 3,3',5,5'-tetramethylbenzidine, 0.002% H_2O_2 in Buffer B). Colour development was stopped after 30 minutes by the addition of 50 µl 2 M H_2SO_4. The absorbance at 450 nm was immediately determined in a Dynatech MR700 automatic microplate reader.

2.2.4.2 Antigen-Coated-Plate ELISA

Either 1 µg/ml AVP or 0.1 µg/ml ANP, in Buffer K, was adsorbed onto MicroELISA Duo-strips (100 µl/well) by overnight incubation. Unbound antigen was removed by three washes with Buffer K then the wells were dried, sealed and stored at -20°C until required.

Hybridoma culture supernatant, HAT medium (negative control) and immune mouse serum (positive control) were diluted 1/2, 1/2 and 1/2000, respectively, with Buffer L then duplicate 100 µl samples were incubated in the wells for 16 hours. Incubation with rabbit anti-mouse IgG (whole molecule) HRP conjugate, and the remainder of the assay, was carried out as described for the AVP-capture ELISA.

2.2.4.3 Radioimmunoassay with PEG Separation (PEG-RIA)

Duplicate 100 µl samples of hybridoma culture supernatant, HAT medium or immune mouse serum (diluted 1/1000 in Buffer L) were incubated with 50 µl ^{125}I-AVP (7 pg, 1700 µCi/µg, in Buffer L) in RT30 tubes for 16 hours, then antibody-bound and free tracer were separated by the method of Desbuquois and Aurbach (1971). 50 µl bovine serum and 500 µl 25% (w/v) PEG 8000, in distilled water, were added to the incubation mixture, vortexed and immediately centrifuged at 2000 x g for 30 minutes. The supernatant was decanted and the inverted tubes were drained on tissue paper for at least 30 minutes, then the radioactivity of the precipitate was determined in a gamma counter.

2.2.4.4 Radioimmunoassay with Second Antibody Separation (SAM-RIA)

100 µl hybridoma culture supernatant, HAT medium or immune mouse serum (diluted 1/1000 in Buffer L) was incubated with 50 µl ^{125}I-AVP (7 pg, 1700 µCi/µg, in Buffer L) for 16 hours, then 50 µl SAM-S-500 solid phase (25% (settled volume) slurry

in Buffer R) was added. The mixture was agitated on an orbital shaker for 1 hour then bound and free tracer were separated by the sucrose layering technique (Wright and Hunter, 1983). 1 ml Buffer S was added and the solid phase was allowed to settle for 5 minutes, then 2 ml 10% (w/v) sucrose in Buffer S was pumped beneath the mixture. The solid phase settled to the bottom of the tube then unbound tracer, and most of the sucrose, was aspirated. Sucrose layering was repeated once, then the radioactivity of the solid phase was determined in a gamma counter.

2.3 PREPARATION OF ASSAY REAGENTS

2.3.1 PURIFICATION OF MONOCLONAL ANTIBODIES

Before reagent preparation, MAbs were concentrated and partially purified by sodium sulphate precipitation from ascitic fluid or by PEG 8000 precipitation from culture supernatant. When required, highly purified MAb was isolated from culture supernatant by affinity chromatography on Protein A-Sepharose.

2.3.1.1 Sodium Sulphate Precipitation

The solubility of sodium sulphate is very temperature-dependent (Tijssen, 1985) therefore antibody precipitation had to be performed at 20°C. Sodium sulphate is insufficiently soluble to precipitate antibodies at lower temperatures.

Debris was removed from ascitic fluid by centrifugation (2000 x g for 20 minutes) then anhydrous sodium sulphate was gradually added, to a final concentration of 18% (w/v), whilst stirring. After standing for 30 minutes, the precipitate was collected by centrifugation (2000 x g for 15 minutes) then washed twice by resuspension in 10 ml aliquots of 18% (w/v) sodium sulphate followed by centrifugation. The final precipitate was redissolved in a small volume of Buffer V, dialysed against 5 litres Buffer V for 16 hours at 4°C, then stored at -20°C.

2.3.1.2 PEG 8000 Precipitation

Hybridoma culture supernatant was mixed with 30% (w/v) PEG 8000 in Buffer E, to give a final PEG concentration of 12.5% (for IgG antibodies) or 15% (for IgA), then incubated at 4°C for 4 days. The precipitate was collected by centrifugation (2000 x g for 30 minutes), redissolved in a small volume of Buffer V, then stored at -20°C after removing insoluble particles by centrifugation.

2.3.1.3 Protein A-Sepharose Affinity Chromatography

This protocol, based on the method of Ey et al. (1978), was used to purify IgG1 from spent culture supernatant. All buffers contained 0.1% sodium azide to prevent microbial growth.

3 g Protein A-Sepharose CL-4B was swollen in 0.01 M sodium phosphate buffer, pH 8.0, at room temperature for 1 hour. The gel was degassed, packed into an adjustable column (1.0 x 13 cm), then equilibrated with 0.1 M Tris/HCl buffer, pH 8.7, at a flow rate of 40 ml/hour.

Hybridoma culture supernatant was adjusted to pH 8.7 by dropwise addition of 10 M NaOH, then pumped through the Protein A-Sepharose column at 20 ml/hour. The column was washed with 0.1 M Tris/HCl buffer, pH 8.7, until the absorbance of the effluent at 280 nm decreased to zero, then IgG1 was eluted in 1.9 ml fractions by 0.1 M sodium citrate buffer, pH 5.0. Antibody-containing fractions were neutralized by the addition of 110 µl 2 M Tris, then pooled, concentrated to 0.5 ml using an Amicon Minicon-B15 Concentrator, and stored at -20°C.

2.3.2 PURIFICATION OF POLYCLONAL ANTIBODIES

Several rabbit polyclonal antibody preparations were used in this work. Some of these were obtained in an affinity-purified form. The others were received as

unprocessed antisera which, if haemolysed, were purified by PEG 8000 fractionation followed by Rivanol precipitation. Non-haemolysed antisera were purified by sodium sulphate precipitation, as described in Section 2.3.1.1.

2.3.2.1 PEG 8000 Fractionation of Antisera

Haemolysed antiserum was centrifuged at 2000 x g for 20 minutes to remove particulate material. 25 ml 45% (w/v) PEG 8000 in Buffer E was mixed with 200 ml antiserum then, after standing at room temperature for 2 hours, the precipitate (0-5% PEG cut) was collected by centrifugation (2000 x g for 20 minutes). Another 33 ml 45% (w/v) PEG 8000 was added to the supernatant. A gelatinous precipitate (5-10% PEG cut) formed immediately and was harvested by centrifugation, as above. Most of the haemoglobin remained in the supernatant.

The 0-5% and 5-10% PEG precipitates were redissolved in small volumes of 50 mM sodium acetate then dialysed against 5 litres 50 mM sodium acetate for 16 hours at 4°C, in preparation for Rivanol precipitation.

2.3.2.2 Rivanol Precipitation

The PEG 8000 fractions from haemolysed antisera were further purified by Rivanol precipitation, which removed contaminants such as albumin and IgM, leaving relatively pure IgG in solution (Franek, 1986).

3% (w/v) Rivanol (6,9-diamino-2-ethoxyacridine lactate), pH 8.2, was added dropwise to the antibody preparation (180 μl Rivanol/ml antibody) whilst stirring with a glass rod. The mixture was left at room temperature for 20 minutes, with occasional stirring, then the precipitate was removed by filtration through dampened Whatman No. 1 filter paper. The filtrate was freed of Rivanol by the addition of NaCl (to a final concentration of 5%) followed by centrifugation (2000 x g for 15 minutes) to remove the yellow precipitate. The IgG-containing supernatant was

concentrated by PEG 8000 precipitation. An equal volume of 30% (w/v) PEG 8000 in Buffer E was added then, after incubation at 4°C for 16 hours, the precipitate was collected by centrifugation, redissolved in 3-5 ml Buffer V, and stored at -20°C.

2.3.3 ESTIMATION OF ANTIBODY CONCENTRATION

The concentrations of purified antibody preparations were estimated from their absorbances at 280 nm, assuming an extinction coefficient of 14 for a 10 mg/ml solution in a 1 cm optical pathway (Ey et al., 1978).

2.3.4 LABELLING METHODS

2.3.4.1 Radioiodination of AVP

^{125}I-AVP was kindly prepared by Mrs. R. Stephen, using a protocol based on the Iodogen method of Salacinski et al. (1981) :

200 µg/ml Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril), in methylene chloride, was aliquoted into 1 ml microcentrifuge tubes (25 µl/tube), vortexed, then dried under a stream of nitrogen gas. Coated tubes were stored in a desiccator at 4°C.

5 µl 1 mg/ml AVP in 0.2% acetic acid, 5 µl Buffer I and 10 µl 100 mCi/ml Na^{125}I were added to an Iodogen-coated tube. The mixture was vortexed immediately, and at 2 minute intervals for 10 minutes, then loaded onto a Sephadex G-25 column (fine, 1 x 40 cm) equilibrated with 0.2% acetic acid, 0.2% BSA. The reaction tube was rinsed with 100 µl Buffer I, which was added to the column, then three peaks of radioactivity were eluted by 0.2% acetic acid, 0.2% BSA, in 1.75 ml fractions. Fractions from the second peak, which contained monoiodinated AVP, were stored in 50 µl aliquots at -20°C.

2.3.4.2 Radioiodination of Monoclonal Antibodies

MAbs were iodinated by a modification of the chloramine-T method (Hunter and Greenwood, 1962) using 10 μCi $Na^{125}I$/μg MAb :

1 g Sephadex G-50 (fine) was swollen in Buffer P at room temperature for 4 hours, then packed into a Quickfit column (1.1 x 10.5 cm). Solutions of 2.5 mg/ml chloramine-T in Buffer O, 0.625 mg/ml cysteine hydrochloride in Buffer J, and 5 mg/ml KI in Buffer J, were prepared immediately before iodination. MAbs were diluted to 4 mg/ml in Buffer O.

5 μl MAb, 5 μl Buffer O, 2 μl 100 mCi/ml $Na^{125}I$ and 5 μl chloramine-T were vortexed for 15 seconds in an RT30 tube, then 100 μl cysteine hydrochloride was added to stop the reaction. The mixture was diluted to 1 ml with KI then its radioactivity was determined in an SR3 Scaler-Ratemeter (Nuclear Enterprises Ltd).

The iodination mixture was carefully loaded onto the Sephadex G-50 column. The reaction tube was rinsed with two 1 ml aliquots of Buffer P, which were added to the column, then the residual radioactivity in the tube was determined. (After a successful iodination, about 10% of the total counts remained in the reaction tube because iodinated protein adsorbed to the plastic surface.) Two peaks of radioactivity were eluted from the column by Buffer P. 1 ml fractions were collected until the radioactivity of the eluate decreased to background levels. Up to three fractions from the first peak were pooled (rinsing the tubes with 0.5-1.0 ml Buffer P to maximize antibody recovery), then stored at 4°C until further purified.

The specific activities and yields of ^{125}I-MAb were calculated from :

$$\text{Specific Activity } (\mu Ci/\mu g) = \frac{A \times B}{C}$$

$$\text{where } A = \frac{D + E}{E + F}$$

$$\text{Yield } (\mu g) = \frac{G}{D + E} \times C$$

where A = fractional incorporation of ^{125}I, B = μCi ^{125}I in reaction mixture, C = μg protein in reaction mixture, D = total counts in MAb peak, E = residual counts in reaction tube, F = total counts eluted from Sephadex G-50 column, and G = counts in MAb fractions selected for pooling.

2.3.4.3 Purification of Iodinated Monoclonal Antibodies

Most unreacted ^{125}I was removed during the Sephadex G-50 gel filtration step whereas high molecular weight contaminants co-eluted with ^{125}I-MAb. These iodinated contaminants, which made an additional purification step necessary, were removed (along with any remaining free ^{125}I) by gel filtration on a Sephacryl S-300 column (1.8 x 57 cm) equilibrated with Buffer P containing 0.1% sodium azide.

Up to 2.5 ml ^{125}I-MAb was applied to the column and eluted with Buffer P containing 0.1% sodium azide, in 2 ml fractions. The radioactivity in a 10 μl sample from each fraction was determined in a gamma counter. The peak MAb-containing fractions were pooled, rinsing the tubes with 1 ml Buffer P to maximize antibody recovery, then stored at 4°C.

Purified ^{125}I-MAb was usable for 4-5 weeks.

2.3.4.4 Preparation of ^{125}I-Streptavidin

Radioiodination of streptavidin was carried out as in Section 2.3.4.2, except that 10 μg streptavidin was reacted with 500 μCi $Na^{125}I$. Iodinated streptavidin was purified by gel filtration on Sephacryl S-300 (Section 2.3.4.3) then stored at 4°C.

2.3.4.5 Biotinylation of Monoclonal and Polyclonal Antibodies

MAbs and polyclonal antibodies were biotinylated with biotinyl-ε-aminocaproic acid N-hydroxysuccinimide ester (Biotin-X-NHS) at a biotin : antibody

ratio of 20 : 1 as follows :

A Pharmacia PD-10 column was prepared by BSA-blocking its protein-binding sites. 4 ml 5% (w/v) BSA in Buffer V was applied to the column and eluted with 50 ml Buffer V.

25 μl freshly-prepared 2.42 mg/ml Biotin-X-NHS in DMF was added to 1 ml 1 mg/ml antibody in Buffer V, then incubated at room temperature for 30 minutes with continuous agitation on a roller-mixer. The reaction mixture was applied to the BSA-blocked PD-10 column and eluted in 1 ml fractions with Buffer V. Antibody-containing fractions were stored at 4°C.

2.3.5 PREPARATION OF ANTIBODY-COATED SOLID PHASES

Antibodies were immobilized either by covalent coupling to periodate-oxidized Sephacryl or by adsorption onto polystyrene MicroELISA strips and nitrocellulose membrane-bottomed 96-well plates. All incubations were carried out at room temperature.

2.3.5.1 Oxidation of Sephacryl

Periodate oxidation of Sephacryl S-300 and Sephacryl S-500 was achieved by the method of Wright and Hunter (1982) :

150 ml (settled volume) Sephacryl was thoroughly washed with 10 litres distilled water, in a sintered glass funnel (No. 2 porosity) attached to a Venturi pump. The gel was resuspended in 500 ml 5 mM sodium m-periodate in Buffer C, then agitated on an orbital shaker for 1 hour. 5 ml 10% (v/v) glycerol was added to use up excess periodate, then agitation continued for another hour. The oxidized Sephacryl was washed with 10 litres Buffer V, resuspended in Buffer V, then stored at 4°C as a 50% (settled volume) slurry.

Oxidized Sephacryl was stable for at least 6 months.

2.3.5.2 Covalent Coupling of Antibodies to Sephacryl S-300

Antibodies were coupled to oxidized Sephacryl S-300, with subsequent stabilization of the Schiff's base linkage by borohydride reduction, according to the method of Wright and Hunter (1982) :

3 ml (settled volume) oxidized Sephacryl S-300 was washed with 50 ml Buffer V in a sintered glass funnel, resuspended in 4 ml 1 mg/ml MAb or affinity-purified polyclonal antibody (or 8 mg/ml partially-purified polyclonal antibody) in Buffer V, then incubated for 16 hours with continuous agitation on a roller-mixer. Unreacted antibody was removed by filtration then the Sephacryl was washed with Buffer K, resuspended in 25 ml Buffer K, and allowed to settle for 30 minutes. The buffer was decanted, which removed fines, then the gel was resuspended in 20 ml Buffer K and reacted with 100 mg sodium borohydride for 30 minutes with occasional stirring. The reduced antibody-S-300 solid phase was washed four times with Buffer A, five times with Buffer Q, then resuspended in 22 ml Buffer R to give a 12.5% (settled volume) slurry, and stored at 4°C.

Assessment of antigen-binding ability

Some antibodies were damaged by covalent linkage to Sephacryl therefore all new antibody-S-300 solid phases were assayed for the ability to bind antigen. Duplicate 100 μl aliquots of antibody-S-300 solid phase (12.5% (settled volume) slurry in Buffer R) were incubated with 50 μl ^{125}I-Ag (7 pg ^{125}I-AVP, or 6.4 pg ^{125}I-ANP, in Buffer P) in RT30 tubes for 1 hour, with agitation on an orbital shaker. Bound and free ^{125}I-Ag were separated by sucrose layering (Section 2.2.4.4) then the radioactivity of the solid phase was determined.

Preparation for immunoradiometric assay

Antibody-S-300 solid phase was rinsed before use, to remove any free antibody which had leaked from the gel (Hornsey et al., 1986; Bessos et al., 1991). The buffer was decanted and replaced with an equal volume of Buffer G. The solid phase was resuspended and allowed to settle, then the buffer was exchanged once more.

2.3.5.3 Preparation of SAM-S-500 Solid Phase

Sheep anti-mouse γ-globulin (SAM) antiserum was coupled to oxidized Sephacryl S-500 without prior antibody purification.

15 ml (settled volume) oxidized Sephacryl S-500 was washed with Buffer V, resuspended in 20 ml 25% (v/v) SAM antiserum in Buffer V, then incubated, washed, de-fined and reduced (with 500 mg sodium borohydride and 100 ml Buffer K), as described in Section 2.3.5.2. After washing with Buffers A and Q, the reduced solid phase was resuspended in 50 ml Buffer R to give a 25% (settled volume) slurry, and stored at 4°C. The buffer was exchanged at weekly intervals to remove any free antibody which had leaked from the solid phase.

2.3.5.4 Adsorption of Antibodies onto MicroELISA Strips

Monoclonal and polyclonal antibodies were diluted to 6 μg/ml in Buffer T, then adsorbed onto MicroELISA strips (200 μl/well) by overnight incubation. Each well was rinsed three times with Buffer T, incubated for 30 minutes with 400 μl 1% BSA in Buffer T to block remaining protein-binding sites, then rinsed once with Buffer T and dried by evaporation. The coated strips were sealed and stored at -20°C.

2.3.5.5 Adsorption of Antibody onto Nitrocellulose Membrane-Bottomed Plates

Monoclonal antibody (5-30 μg/ml, in Buffer T) was incubated in nitrocellulose membrane-bottomed 96-well plates (100 μl/well) for 1 hour, then drawn through the membrane by a vacuum manifold attached to a Venturi pump. The wells were washed four times by drawing Buffer U through the membrane, then blotted on tissue paper and used immediately in an immunofiltration assay (Section 2.5.3).

2.4 CHARACTERIZATION OF MONOCLONAL ANTIBODIES

2.4.1 ISOTYPE DETERMINATION

MAbs in hybridoma culture supernatant (diluted 1/3 in 20 mM Tris/HCl buffer, pH 7.6, 137 mM NaCl, 0.1% Tween 20) were isotyped with an Amersham Mouse Monoclonal Antibody Isotyping Kit, using the protocol supplied.

2.4.2 ESTIMATION OF ANTIBODY AFFINITY

MAb affinity constants were estimated by Scatchard analysis of RIA data (Scatchard, 1949) :

Serial dilutions of hybridoma culture supernatant in Buffer L (100 µl/tube) were incubated with 50 µl ^{125}I-Ag (7 pg ^{125}I-AVP or 6.4 pg ^{125}I-ANP, in Buffer L) for 16 hours at room temperature. MAb-bound ^{125}I-Ag was separated from free ^{125}I-Ag by incubation with SAM-S-500 followed by sucrose layering (Section 2.2.4.4) for IgG isotypes, or by the PEG separation method (Section 2.2.4.3) for other isotypes. The radioactivity of the bound fraction was used to estimate the MAb dilution which produced half-maximal ^{125}I-Ag binding. This dilution was then used in an RIA standard curve.

Duplicate 100 µl aliquots of diluted MAb were incubated with 50 µl ^{125}I-Ag and 100 µl 0-1000 ng/ml unlabelled Ag in Buffer L for 16 hours at room temperature, then separation and counting of the antibody-bound fraction was carried out as above. Non-specific binding was determined by replacing MAb with 100 µl Buffer L. At each point on the standard curve,

$$B = T \times M \qquad \text{and} \qquad F = T - B$$

where B = MAb-bound antigen concentration (moles/litre of assay mixture), T = total antigen concentration (^{125}I-Ag + Ag, moles/litre), M = MAb-bound fraction of ^{125}I-Ag (minus non-specific binding) and F = free antigen concentration (moles/litre).

A plot of B/F against B produced a line with a gradient of −K, where K was

the antibody affinity constant (litres/mole).

2.4.3 ASSESSMENT OF ANTIBODY SPECIFICITY

Anti-AVP MAb specificities were determined by RIA (Section 2.4.2). The inhibition of ^{125}I-AVP binding caused by AVP was compared with that caused by various peptide analogues, then the cross-reactivity of MAb with each analogue was calculated from :

$$\text{Cross-reactivity (\%)} = \frac{[\text{AVP}]^{50}}{[\text{Analogue}]^{50}} \times 100$$

where $[\ \ ]^{50}$ = peptide concentration causing 50% inhibition of ^{125}I-AVP binding.

2.5 TWO-SITE IMMUNOMETRIC ASSAYS

All incubations were carried out at room temperature.

2.5.1 TWO-SITE ELISA

The assay protocols below were modified in some experiments, as described in Chapters 6 and 8.

2.5.1.1 Separate Incubation Protocol

Antibody-coated MicroELISA Duo-strip wells (Section 2.3.5.4) were incubated with duplicate 100 µl aliquots of antigen standard in Buffer L for 20 hours, then with 100 µl 2 µg/ml biotin-antibody in Buffer L containing 1% normal mouse serum for 24 hours. After incubation with 100 µl 2 µg/ml streptavidin-HRP conjugate in Buffer L for 15 minutes, the wells were washed five times with Buffer D, then incubated with 100 µl freshly-prepared substrate (100 µg/ml 3,3',5,5'-tetramethylbenzidine,

0.002% H_2O_2 in Buffer B). Colour development was stopped after 30 minutes by the addition of 50 µl 2 M H_2SO_4. The absorbance at 450 nm was immediately determined in a Dynatech MR700 automatic microplate reader.

Alternatively, MAb-coated MicroELISA wells were incubated with 100 µl antigen in Buffer L for 20 hours, then with 100 µl 6 µg/ml unlabelled rabbit polyclonal antibody in Buffer L for 24 hours, and finally with 100 µl donkey anti-rabbit IgG HRP conjugate (1/500 dilution in Buffer L) for 1 hour. Plate-washing, and the remainder of the assay, was carried out as above.

2.5.1.2 Simultaneous Incubation Protocol

Duplicate 100 µl aliquots of antigen standard in Buffer L and 50 µl 2 µg/ml biotin-antibody in Buffer L containing 4% normal mouse serum were incubated in antibody-coated MicroELISA wells for 16 hours, then 50 µl 2 µg/ml streptavidin-HRP conjugate in Buffer L was added. The assay mixture was swirled gently to ensure homogeneity, then incubated for another 15 minutes. Plate-washing, and the remainder of the assay, was carried out as described for the separate incubation protocol.

2.5.2 IMMUNORADIOMETRIC ASSAY (IRMA)

IRMA binding was detected directly using ^{125}I-MAb, or indirectly using ^{125}I-streptavidin as a universal label in combination with biotinylated antibodies. Assay protocols were modified in some experiments, as described in Chapters 6 and 8.

2.5.2.1 Direct IRMA

Duplicate 100 µl aliquots of antigen standard in Buffer E containing 5% BSA were incubated with 100 µl 50 ng/ml ^{125}I-MAb in Buffer F for 2 hours, then 100 µl antibody-S-300 solid phase (12.5% (settled volume) slurry in Buffer G) was added.

The mixture was agitated on an orbital shaker for 2 hours then bound ^{125}I-MAb was isolated by the sucrose layering technique (Wright and Hunter, 1983). 1 ml Buffer H was added and the solid phase was allowed to settle for 5 minutes, then 2 ml 10% (w/v) sucrose in Buffer H was pumped beneath the mixture. The solid phase settled to the bottom of the tube then free ^{125}I-MAb, and most of the sucrose, was aspirated. Sucrose layering was repeated once then the radioactivity of the solid phase was determined in a gamma counter.

2.5.2.2 Indirect IRMA

Duplicate 100 µl aliquots of antigen standard in Buffer E containing 5% BSA were incubated with 100 µl 50 ng/ml biotin-antibody in Buffer F for 2 hours, then 100 µl antibody-S-300 solid phase (12.5% (settled volume) slurry in Buffer G) was added. The mixture was agitated for 2 hours, then 100 µl 20 ng/ml ^{125}I-streptavidin in Buffer F was added. After agitation for a further 15 minutes, bound and free ^{125}I-streptavidin were separated by the sucrose layering technique, as described in Section 2.5.2.1.

2.5.3 IMMUNOFILTRATION ASSAY

A MAb-coated nitrocellulose membrane-bottomed 96-well plate (Section 2.3.5.5) was incubated with AVP standard in Buffer M (100 µl/well) for 30 minutes, then the AVP was drawn through the membrane under vacuum. The wells were washed three times by drawing Buffer N through the membrane, blotted on tissue paper, then incubated with 100 µl 50 ng/ml ^{125}I-antibody in Buffer M for 30 minutes. The wells were washed with Buffer N as before, then the membranes were excised and their radioactivity was measured in a gamma counter.

CHAPTER 3

HYBRIDOMA SCREENING ASSAYS

3.1 INTRODUCTION

Hybridoma screening assays need to be capable of rapidly processing large numbers of samples, and must be optimized before MAb production can begin. They are usually set up with serum from the immune spleen donor (Campbell, 1984; Goding, 1986) and, ideally, should detect only those antibodies which are specific for the antigen of interest. The assay format is determined by the intended use of the antibodies therefore ELISA and RIA screening are often used when MAbs are required for immunoassays. Unfortunately, several problems may be encountered.

The radiolabelled antigens used in RIA will have some altered epitopes which are no longer recognised by the appropriate antibodies. This may be acceptable for large protein antigens but is a serious problem with small peptides. For example, iodination of AVP increases its molecular weight by 11% and significantly increases the risk of obtaining a false negative screening assay result. An anti-AVP MAb which does not bind well to ^{125}I-AVP has been reported (Hou-Yu et al., 1982; Valiquette et al., 1986; Valiquette and Neubort, 1989).

ELISA methods avoid this problem. However, if conformational changes occur when antigens, such as large proteins, adsorb to the solid phase, ELISA may detect antibodies which cannot recognise the native antigen in solution (Mierendorf and Dimond, 1983; Vaidya et al., 1985; Schwab and Bosshard, 1992) and may fail to detect antibodies which are specific for the native antigen (Mierendorf and Dimond, 1983; Brennand et al., 1986; Overall et al., 1989). On the other hand, small peptides may not bind well to polystyrene ELISA plates (Briand et al., 1985; Presentini et al., 1989) so they are often conjugated to large carrier molecules to improve adsorption, but both the coupling agent and carrier must differ from those used in the immunogen if the assay is to be specific for anti-peptide antibodies and avoid cross-reaction with anti-carrier and anti-coupling agent specificities (Briand et al., 1985; Edwards et al., 1989; Peeters et al., 1989). Alternatively, if the antigen is big enough to bind two antibodies simultaneously, a capture antibody can be used to efficiently immobilize it

without loss of its native conformation (Smith and Wilson, 1986).

At the start of this project, an anti-ANP screening assay was locally available (Prowse et al., 1989) whereas an anti-AVP hybridoma screening assay had to be developed. Immune serum was the only source of mouse anti-AVP antibody so it was used for assay development. This caused several unexpected problems.

3.2 PRELIMINARY STUDIES

Iodination of the tyrosine residue of AVP masks an important epitope because the adjacent phenylalanine is the only amino acid which distinguishes the AVP ring structure from that of oxytocin (Table 5.3). Consequently, hybridoma screening by RIA is unlikely to detect antibodies specific for this important region of the AVP molecule. ELISA seemed to be a more appropriate screening assay format because it does not require labelled peptide.

An AVP-coated-plate ELISA, with BSA-blocking, was used in initial experiments with tail bleed sera from immunized mice. It seemed to work, because non-specific binding to BSA-blocked wells was lower than the specific binding to AVP-coated wells (Figure 3.1) although only one of four mice showed a significant antibody response. However, antibody binding did not reach equilibrium if serum dilutions were only incubated in the wells for 1 hour. Longer incubations produced higher absorbances (Figure 3.2), but the cell culture work was incompatible with same-day completion of assays requiring more than 1 hour for antibody binding. Maximal binding would ensure that screening assays were sensitive, so a standard 16 hour first incubation period was selected for routine use with all screening assay formats because longer or shorter incubations were impractical.

When the same tail bleed sera were assayed by SAM-RIA, in an attempt to confirm the validity of the ELISA data, no specific anti-AVP binding could be detected. This result was surprising because immune serum had been expected to contain some antibodies capable of binding ^{125}I-AVP. However, the SAM-S-500 solid

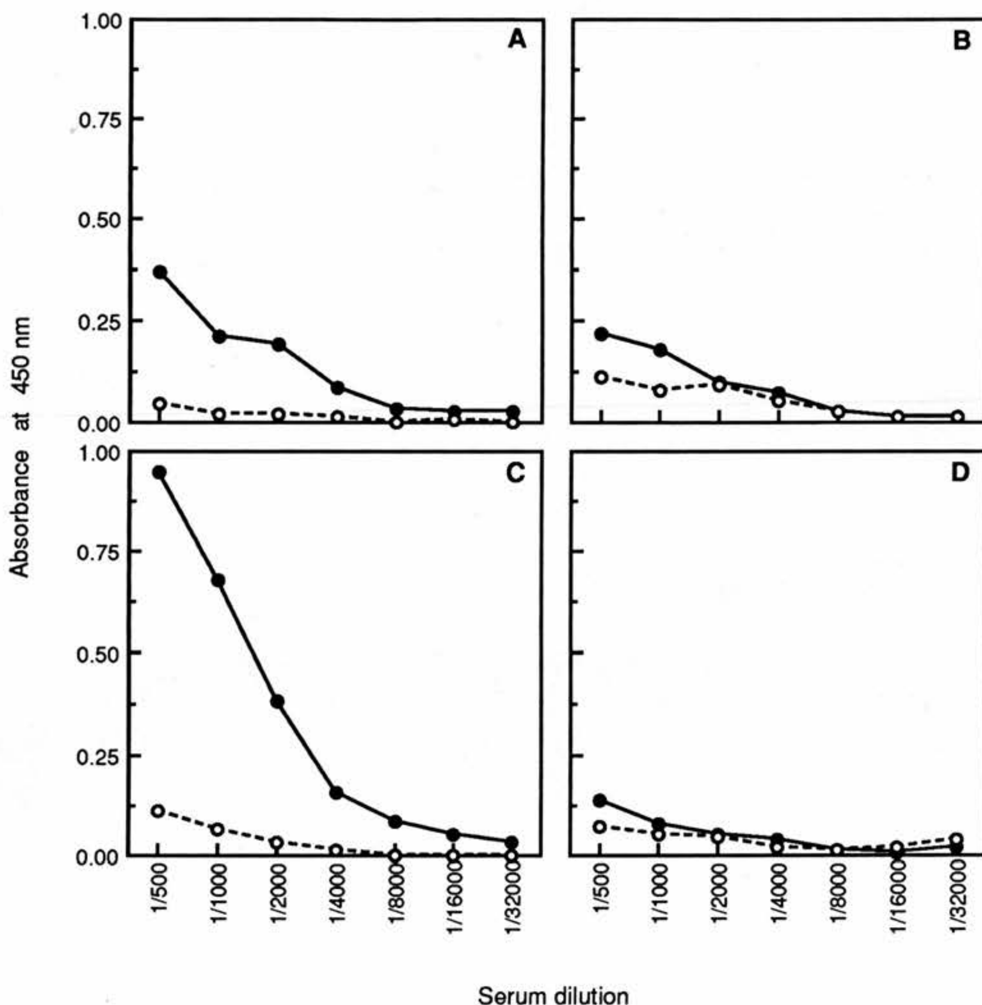

FIGURE 3.1

Response of immune mouse serum in an AVP-coated-plate ELISA

Serum dilutions from mouse nos. 3363 (**A**), 3364 (**B**), 3365 (**C**), and 3366 (**D**), immunized with AVP-thyroglobulin conjugate, were assayed in AVP-coated (●), and AVP-free (○), BSA-blocked Microwells.

Nunc Maxisorp Microwells were coated by incubation with 100 µl 10 ng/ml AVP in Buffer K for 16 hours, rinsed 3 times with Buffer K, incubated with 400 µl 1% BSA in Buffer K for 30 minutes, rinsed once with Buffer K, then dried by evaporation. Serum dilutions (100 µl, in Buffer L) were incubated in the wells for 1 hour, followed by 100 µl goat anti-mouse 'polyvalent immunoglobulins' HRP conjugate (1/100 dilution in Buffer L) for 30 minutes. The remainder of the assay was carried out as described in Section 2.2.4.1.

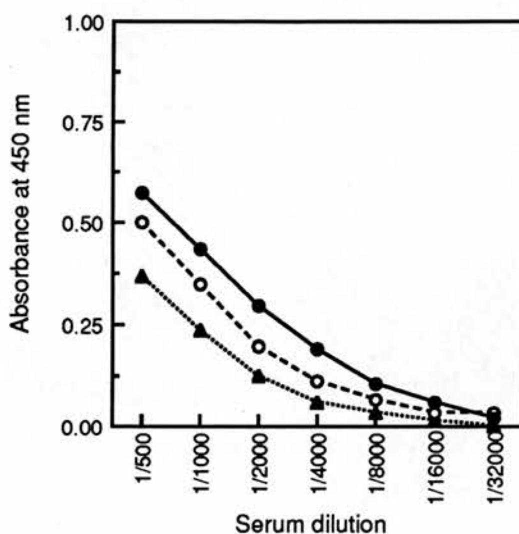

FIGURE 3.2

Effect of incubation time on AVP-coated-plate ELISA

Serum from mouse 3365, diluted in Buffer L, was incubated for 1 (▲), 2 (o), or 3 (●) hours in Microwells which had been coated with 10 ng/ml AVP and BSA-blocked. The assay was completed as described in Figure 3.1. Non-specific binding to BSA-blocked wells has been subtracted from the absorbances shown.

phase, which was used in the RIA, bound mainly to IgG isotypes whereas the goat anti-mouse 'polyvalent immunoglobulins' HRP conjugate, which had been used in the ELISA work, was equipotent for IgG, IgA and IgM. When the 'polyvalent' conjugate was compared with rabbit anti-mouse IgG (whole molecule) HRP conjugate, in an AVP-coated-plate ELISA, extremely low anti-IgG results were obtained (Figure 3.3). This suggested that either the immunization schedule had failed to generate a significant IgG response or, because animals immunized against vasopressin tend to develop the symptoms of diabetes insipidus (Morton and Waite, 1972; Skowsky and Fisher, 1972b), that the mouse anti-AVP IgG had bound *in vivo* to circulating endogenous AVP, leaving only low affinity IgM antibodies available to bind AVP in the screening assays. Both interpretations of the data raised doubts about the validity of the AVP-coated-plate ELISA and the SAM-RIA as hybridoma screening assays because no suitable positive control samples were available. It could not be proved that either assay would efficiently detect IgG antibodies specific for AVP.

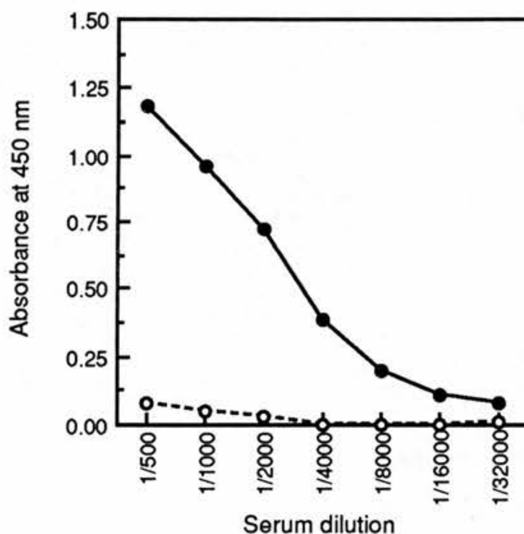

FIGURE 3.3

Effect of HRP conjugate specificity on AVP-coated-plate ELISA

Serum from mouse 3365, diluted in Buffer L, was incubated for 16 hours in Microwells which had been coated with 10 ng/ml AVP and BSA-blocked, then antibody binding was revealed with goat anti-mouse 'polyvalent immunoglobulins' HRP conjugate (1/100 dilution in Buffer L, ●) or rabbit anti-mouse IgG (whole molecule) HRP conjugate (1/200 dilution in Buffer L, o). Non-specific binding to BSA-blocked wells has been subtracted from the absorbances shown.

3.3 AVP-CAPTURE ELISA

An AVP-capture ELISA, with a polyclonal capture antibody of non-mouse origin, seemed an ideal alternative to the AVP-coated-plate ELISA and SAM-RIA screening formats because previous unpublished work had suggested that a two-site assay was feasible for AVP (N. McIntosh, personal communication). Theoretically, the immobilized capture antibody would bind pre-formed antigen-antibody complexes, whereas free antibodies would only bind after preincubation of the capture antibody with antigen (Smith and Wilson, 1986).

Rabbit TG1 antiserum (Smith and McIntosh, 1986) was the only source of non-mouse anti-AVP polyclonal antibody available when the AVP-capture ELISA was set up. Four plate-coating concentrations of TG1 antiserum were compared by assaying serum from an immunized mouse in coated wells which had been BSA-blocked and

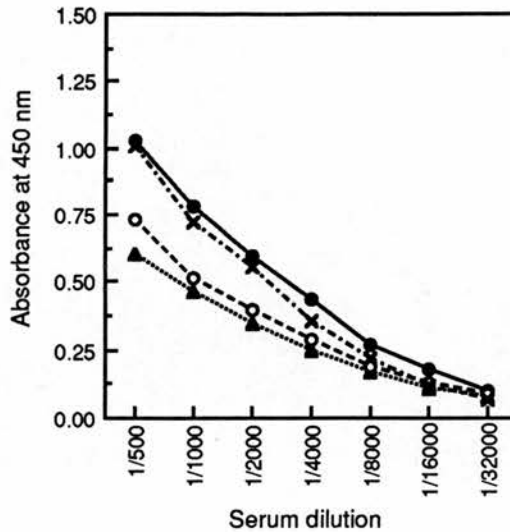

FIGURE 3.4

Influence of capture antibody coating concentration on the AVP-capture ELISA

TG1 antiserum, diluted 1/100 (▲), 1/1000 (○), 1/10,000 (●) or 1/100,000 (×) in Buffer T, was adsorbed onto Nunc Maxisorp Microwells (100 µl/well) by overnight incubation. The wells were rinsed and BSA-blocked, incubated with 100 µl 10 ng/ml AVP in Buffer L for 16 hours, rinsed with Buffer K, then used to assay serum dilutions from mouse 3365. Antibody binding was revealed with goat anti-mouse 'polyvalent immunoglobulins' HRP conjugate (1/100 dilution in Buffer L).

preincubated with AVP (Figure 3.4), using the 'polyvalent immunoglobulins' HRP conjugate to reveal antibody binding because the reason for the low anti-IgG response of immune serum in the AVP-coated-plate ELISA (Figure 3.3) had not yet been determined. A 1/10,000 dilution of TG1 antiserum produced the highest absorbances and so was selected for routine use in the AVP-capture ELISA.

3.3.1 ASSESSMENT OF THE AVP-CAPTURE ELISA

When immune mouse serum was analysed in the AVP-capture ELISA, the 'polyvalent immunoglobulins' HRP conjugate produced higher absorbances than the anti-IgG HRP conjugate (Figure 3.5) which suggested, in agreement with the AVP-coated-plate ELISA data (Figure 3.3), that the immunized mouse had more anti-AVP IgM than free anti-AVP IgG in its serum. No importance could be attached

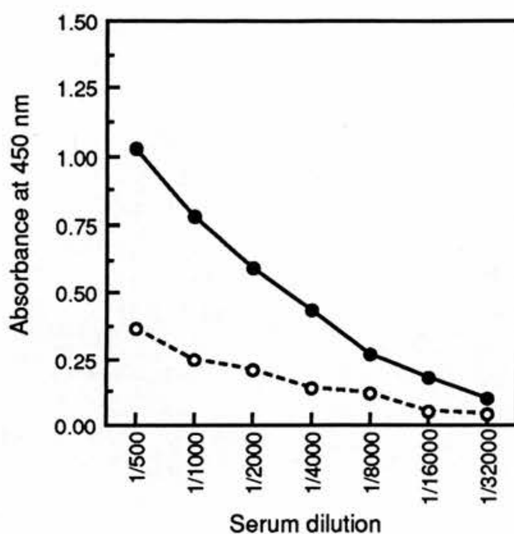

FIGURE 3.5

Effect of HRP conjugate specificity on AVP-capture ELISA

Serum dilutions from mouse 3365 were assayed in AVP-capture ELISA wells (1/10,000 dilution TG1 antiserum, BSA-blocked, 10 ng/ml AVP). Antibody binding was revealed with goat anti-mouse 'polyvalent immunoglobulins' HRP conjugate (●) or sheep anti-mouse IgG (whole molecule) HRP conjugate (○). The absorbances caused by non-specific binding of the HRP conjugates have been subtracted from the values shown.

to the fact that a higher IgG response was obtained in the AVP-capture ELISA (Figure 3.5) than in the AVP-coated-plate ELISA (Figure 3.3) because different serum samples had been used in the two assays.

Immune mouse serum antibodies bound to immobilized TG1 antiserum whether or not it had been preincubated with AVP (Figure 3.6), which gave the impression that the serum contained both AVP-free and AVP-bound antibodies. In the absence of added AVP, antibody binding was detected by both the 'polyvalent immunoglobulins' and the anti-IgG HRP conjugates (Figure 3.7), which was taken to mean that some of the AVP-bound antibody was IgG. This binding was much higher if BSA-blocking was omitted (Figure 3.7), but the antibodies did not adsorb to unoccupied sites on unblocked ELISA wells because the mouse serum had been diluted in Tween 20-containing buffer which virtually eliminated non-specific binding to polystyrene (Table 3.4), as reported by Mohammad and Esen (1989). Instead, it seemed that plate-blocking caused steric

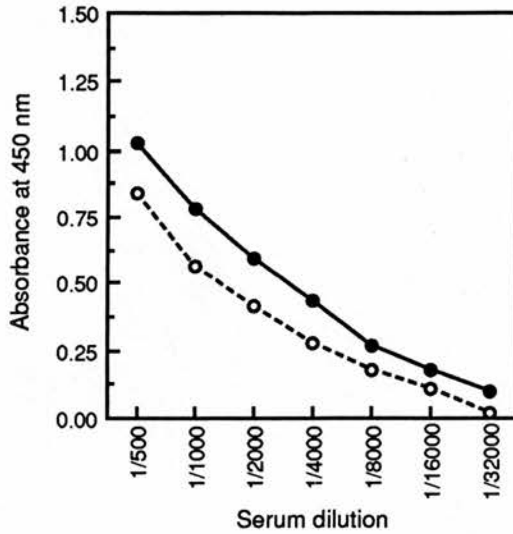

FIGURE 3.6

Effect of omitting AVP from the AVP-capture ELISA

Microwells coated with TG1 antiserum, and BSA-blocked, were incubated with 100 µl 10 ng/ml AVP in Buffer L (●), or 100 µl Buffer L (o), for 16 hours, rinsed with Buffer K, then used to assay dilutions of serum from mouse 3365. Antibody binding was revealed with goat anti-mouse 'polyvalent immunoglobulins' HRP conjugate.

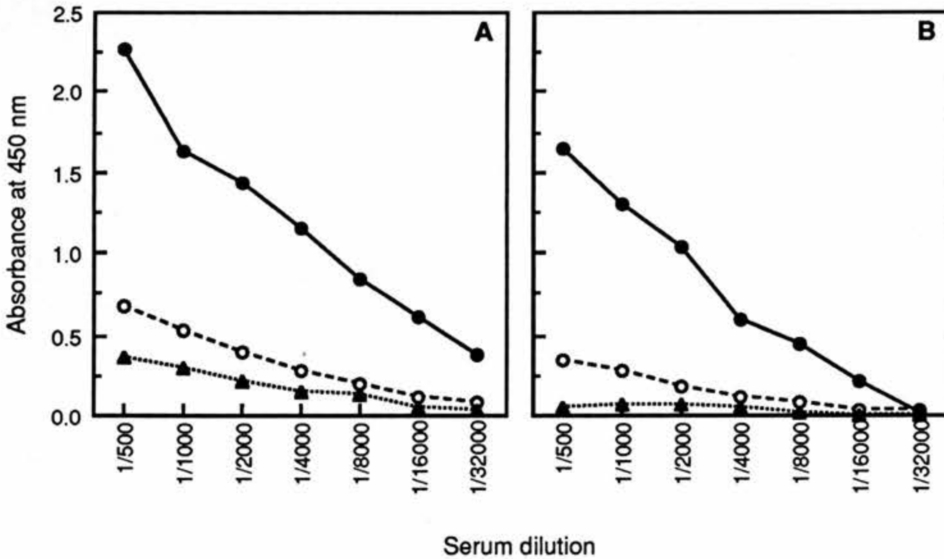

FIGURE 3.7

Binding of immune mouse serum to TG1 antiserum and BSA

Serum dilutions from mouse 3365 were assayed in Microwells coated with TG1 antiserum only (●), TG1 antiserum with BSA-blocking (o), or BSA-blocked wells (▲). Antibody binding was revealed with goat anti-mouse 'polyvalent immunoglobulins' HRP conjugate (**A**) or sheep anti-mouse IgG (whole molecule) HRP conjugate (**B**). The absorbances caused by non-specific binding of the HRP conjugates have been subtracted from the values shown.

hindrance of binding.

Immune mouse serum contained anti-BSA antibody which caused almost half of the binding response that was detected by the 'polyvalent immunoglobulins' HRP conjugate in TG1-coated, BSA-blocked, wells (Figure 3.7A). Cell fusion was therefore expected to generate hybridomas that secreted anti-BSA antibodies, which would bind to BSA-blocked AVP-capture ELISA wells causing false positive results. However, the anti-IgG HRP conjugate detected much less anti-BSA activity (Figure 3.7B), indicating that most anti-BSA antibodies were either IgM or IgA. Fewer anti-BSA specificities would be detected if an anti-IgG HRP conjugate was used in hybridoma screening.

Non-specific binding of the affinity-isolated anti-IgG HRP conjugate to AVP-capture ELISA wells caused inconveniently high background absorbances. Despite the presence of BSA and Tween 20 in the assay buffer, the conjugate bound to BSA, TG1 antiserum, normal rabbit serum, bovine serum (NBCS) and foetal calf serum, but not to uncoated wells (Table 3.1), which indicated that it bound to the coating materials and not to polystyrene. The 'polyvalent immunoglobulins' HRP conjugate did

TABLE 3.1

Non-specific binding of the affinity-isolated sheep anti-mouse IgG (whole molecule) HRP conjugate to coated ELISA wells

Various plate-coating solutions, in Buffer T, were adsorbed onto Immulon 1 MicroELISA wells by overnight incubation, then antigen-coated-plate ELISA was carried out with 100 µl HAT medium in place of sample. The absorbances shown are the means of duplicate determinations.

Coating solution	Absorbance at 450 nm
None	0.000
0.01% TG1	0.184
0.01% NRS	0.080
1% BSA	0.159
2% NBCS	0.217
2% FCS	0.042

not produce high background readings, but was unsuitable for hybridoma screening because it would detect IgM and IgA in addition to the desired IgG isotypes.

Preincubation of immobilized TG1 antiserum with AVP could not be optimized when the AVP-capture ELISA was set up, because immune serum was the only source of mouse anti-AVP antibody available and its binding did not seem to depend on the addition of AVP (Figures 3.6 and 3.7). Preincubation with 50 ng/ml AVP was selected for hybridoma screening because this concentration was thought certain to saturate the binding sites of immobilized TG1 antiserum and so maximize MAb detection.

Despite these problems, the AVP-capture ELISA was used for hybridoma screening because it produced higher immune mouse serum absorbances than an AVP-coated-plate ELISA that used the same amount of AVP (Figure 3.8), so it appeared to be a more sensitive assay. It also seemed reasonably specific for anti-AVP IgG because the binding response of immune mouse serum detected by the anti-IgG HRP conjugate was much higher than that of normal mouse serum (Figure 3.9).

3.3.2 HYBRIDOMA SCREENING BY AVP-CAPTURE ELISA

The first cell fusion (VP1, Table 4.6) generated hybridomas in 378 culture wells. Undiluted supernatants from 26 wells were considered antibody-positive because they produced AVP-capture ELISA absorbances which exceeded 0.3. However, when antibody dilution analysis was carried out to identify the highest affinity anti-AVP antibodies (Van Heyningen, 1986), dilution in assay buffer almost eliminated antibody binding in many culture supernatants, even when the dilution factor was as low as two (Table 3.2, Experiment 1). Dilution in cell culture medium did not have this drastic effect (Table 3.2, Experiment 2), which was seen particularly clearly with supernatant from hybridoma VP1/169 (Figure 3.10). Something in the assay buffer obviously prevented the hybridoma antibodies from binding to the TG1 antiserum-coated solid phase.

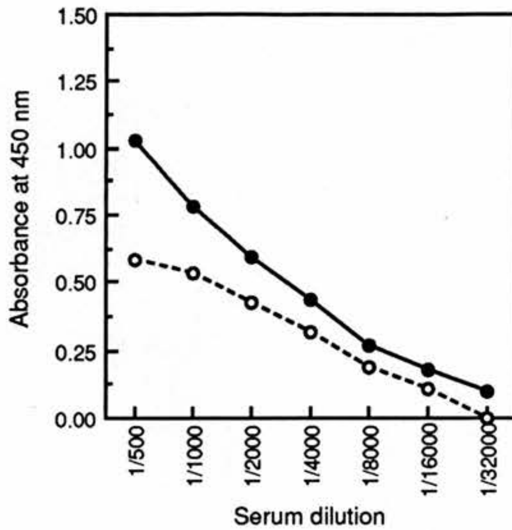

FIGURE 3.8

<u>Comparison of the binding responses of immune mouse serum in the AVP-capture ELISA and in the AVP-coated-plate ELISA</u>

Serum dilutions from mouse 3365 were simultaneously assayed in AVP-capture ELISA wells (1/10,000 dilution TG1 antiserum, BSA-blocked, 10 ng/ml AVP, ●) and in AVP-coated-plate ELISA wells (10 ng/ml AVP, BSA-blocked, ○). Antibody binding was revealed with goat anti-mouse 'polyvalent immunoglobulins' HRP conjugate.

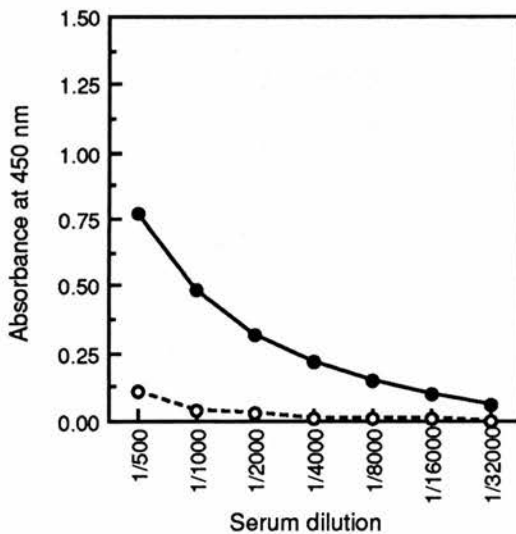

FIGURE 3.9

<u>Response of immune mouse serum and normal mouse serum in the AVP-capture ELISA</u>

Dilutions of immune serum from mouse 3365 (●), and normal mouse serum (○), were assayed by AVP-capture ELISA. Antibody binding was revealed with sheep anti-mouse IgG (whole molecule) HRP conjugate.

TABLE 3.2

Response of AVP-capture ELISA-positive culture supernatants to dilution

The AVP-capture ELISA response of undiluted hybridoma culture supernatant was compared with the response obtained after dilution with an equal volume of Buffer L (Experiment 1), or 15% FCS medium (Experiment 2). The absorbance caused by non-specific binding of the sheep anti-mouse IgG (whole molecule) HRP conjugate has been subtracted from the values shown.

Hybridoma	Absorbance at 450 nm			
	Experiment 1		Experiment 2	
	Undiluted	Diluted 1/2 in Buffer L	Undiluted	Diluted 1/2 in 15% FCS medium
VP1/10	0.525	0.441	0.544	0.568
VP1/89	1.005	0.126	1.422	1.193
VP1/121	1.522	0.049	1.869	1.788
VP1/140	>2.050	0.300	>2.050	>2.050
VP1/169	1.620	0.743	1.916	>2.050
VP1/271	1.991	0.040	>2.050	2.028
VP1/367	0.712	0.089	0.469	0.411

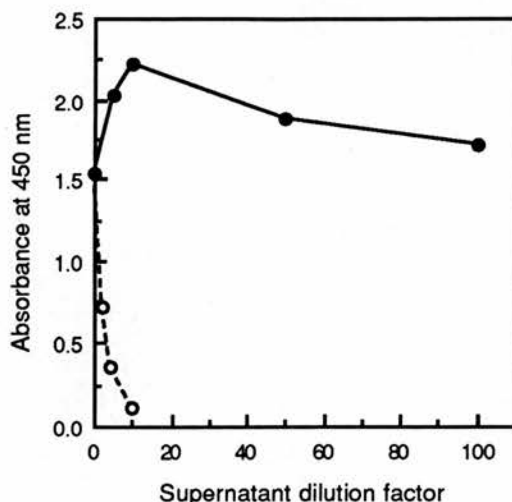

FIGURE 3.10

Effect of diluent composition on the AVP-capture ELISA response of culture supernatant from hybridoma VP1/169

Culture supernatant was diluted in 15% FCS medium (●), or Buffer L (○), then assayed by AVP-capture ELISA.

3.3.3 ANALYSIS OF AVP-CAPTURE ELISA-POSITIVE SUPERNATANTS

The immunized mice had produced an anti-BSA response (Figure 3.7) therefore some AVP-capture ELISA-positive hybridomas were expected to secrete anti-BSA antibodies. However, the BSA used in plate-blocking, and in the assay buffer, was only 98% pure and so was likely to contain other substances, such as thyroglobulin, to which the hybridoma antibodies might bind. Pure BSA was not available therefore BSA-blocked wells had to be avoided in specificity assays.

Antibody binding to thyroglobulin was assayed in wells blocked with non-fat dried milk, which was reported to be an effective plate-blocking agent (Vogt et al., 1987). All AVP-capture ELISA-positive supernatants, except those from hybridomas VP1/10 and VP1/367, bound equally to thyroglobulin-coated wells and 1% dried milk-blocked control wells (Table 3.3), giving the impression that 1% milk was insufficient to provide adequate plate-blocking. However, higher concentrations of dried milk could not eliminate binding (Figure 3.11), which suggested that most of the

TABLE 3.3

Binding response of AVP-capture ELISA-positive culture supernatants to thyroglobulin

Nunc Maxisorp Microwells were coated by incubation with 100 µl 10 µg/ml bovine thyroglobulin in Buffer T for 16 hours, rinsed three times with Buffer T, incubated for 1 hour with 400 µl 1% non-fat dried milk in Buffer T to block remaining protein-binding sites, rinsed once with Buffer T, then dried by evaporation.

Undiluted hybridoma culture supernatants were incubated in the coated wells and in dried milk-blocked control wells for 16 hours, then antibody binding was revealed with sheep anti-mouse IgG (whole molecule) HRP conjugate. Serum from mouse 3365, diluted 1/1000 in Buffer L, was included for comparison. The absorbances caused by non-specific binding of the HRP conjugate have been subtracted from the values shown.

Sample	Absorbance at 450 nm	
	10 µg/ml Thyroglobulin 1% Dried Milk	1% Dried Milk
VP1/10	0.008	0.006
VP1/89	0.830	0.935
VP1/121	0.662	0.525
VP1/140	>2.050	1.877
VP1/169	1.703	1.653
VP1/271	1.674	1.987
VP1/367	0.004	0.009
Immune serum	>2.050	1.778

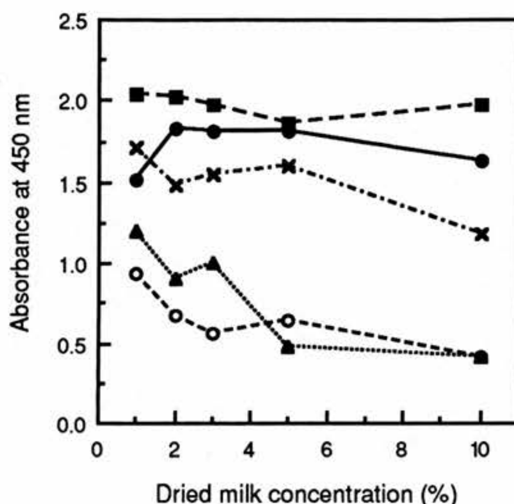

FIGURE 3.11

Binding of AVP-capture ELISA-positive hybridoma culture supernatants to non-fat dried milk-blocked Microwells

Nunc Maxisorp Microwells were incubated for 1 hour with 400 µl 1-10% non-fat dried milk in Buffer T, to block protein-binding sites, rinsed with Buffer T, then used to assay undiluted culture supernatants from hybridomas VP1/89 (o), VP1/140 (■), VP1/169 (×), and VP1/271 (▲). Immune serum from mouse 3365, diluted 1/1000 in Buffer L (●), was included for comparison. Antibody binding was revealed with sheep anti-mouse IgG (whole molecule) HRP conjugate.

antibodies bound specifically to a component of the milk preparation. Further evidence was provided by the fact that immune mouse serum bound strongly to milk-blocked wells (Tables 3.3 and 3.4, Figure 3.11), despite dilution in Buffer L which prevented non-specific binding to polystyrene (Table 3.4).

The effect of plate-blocking was checked by comparing antibody binding in dried milk-blocked, BSA-blocked and unblocked wells (Table 3.4). Immune mouse serum and AVP-capture ELISA-positive culture supernatants bound to BSA-blocked wells more strongly than to unblocked wells, which confirmed that there was specific binding to the BSA preparation. In contrast, most samples bound less strongly to non-fat dried milk-blocked wells than to unblocked wells, which suggested that milk had a reasonable plate-blocking effect even though specific binding also occurred. Non-specific binding to unblocked polystyrene was a measure of the antibody content of undiluted samples therefore the polyclonal supernatant from *in vitro*-boosted spleen

TABLE 3.4

Effect of blocking proteins on the adsorption of antibodies onto uncoated ELISA wells

Uncoated Nunc Microwells were blocked by incubation with 400 µl 3% dried milk, or 1% BSA, in Buffer T for 1 hour, rinsed once with Buffer T, dried by evaporation, and used to assay undiluted culture supernatants from AVP-capture ELISA-positive hybridomas, *in vitro*-boosted spleen cells and ESA 4, an anti-ANP MAb. Immune serum from mouse 3365, diluted 1/1000 in Buffer L, was included for comparison. The absorbance caused by non-specific binding of the sheep anti-mouse IgG (whole molecule) HRP conjugate to the plate-blocking proteins has been subtracted from the values shown.

Sample	Absorbance at 450 nm		
	3% Dried Milk	1% BSA	Unblocked
VP1/10	0.000	0.713	0.044
VP1/89	0.424	1.689	0.290
VP1/121	0.164	>2.050	1.645
VP1/140	0.033	0.971	0.020
VP1/169	0.907	>2.050	>2.050
VP1/271	0.263	1.376	0.126
ESA 4	0.028	0.000	0.658
Spleen supernatant	0.078	0.266	1.231
Immune serum	1.454	0.187	0.049

cells contained a lot of antibody, but relatively few anti-BSA and anti-dried milk specificities, because it bound more strongly to uncoated, unblocked, wells than to BSA-blocked or dried milk-blocked wells (Table 3.4).

Since the AVP-capture ELISA-positive culture supernatants bound to BSA and dried milk, they might also have bound to other commonly-used blocking agents therefore it was essential to carry out specificity assays in unblocked ELISA wells. However, the Nunc Maxisorp Microwells, which had been used in all previous work, had a high protein binding capacity so non-specific binding could have been a problem in unblocked wells. Antibody adsorption to uncoated, unblocked, Nunc Microwells was therefore compared with that to Immulon 1 and Immulon 2 MicroELISA wells (Table 3.5). Undiluted culture supernatants adsorbed more strongly to Nunc Microwells than to Immulon 2 MicroELISA wells, while the lowest adsorption was recorded in Immulon 1 MicroELISA wells whose low binding capacity suggested that coating proteins might occupy all possible protein-binding sites thus making plate-blocking unnecessary.

When specificity assays were carried out in unblocked Immulon 1 MicroELISA wells, most undiluted AVP-capture ELISA-positive hybridoma culture supernatants bound strongly to BSA, TG1 antiserum, normal rabbit serum and bovine serum, while their response to foetal calf serum was similar to, or lower than, the adsorption to uncoated wells which occurred during the 16 hour incubation period (Table 3.6). Most supernatants also seemed to bind to AVP-coated wells but this binding was probably non-specific because it was much lower than that to TG1 antiserum, normal rabbit serum, bovine serum or BSA, and no anti-AVP antibody could be detected by RIA. Hybridomas VP1/10 and VP1/367 appeared to secrete anti-BSA antibodies.

Foetal calf serum sometimes contains antibodies (Kniazeff and Rimer, 1967) but is usually considered to be antibody-free, whereas high antibody concentrations are present in non-foetal sera. BSA preparations are often contaminated by antibodies (Boscato and Stuart, 1988) while dried milk, to which many hybridoma supernatants bound (Figure 3.11), was also likely to contain bovine antibodies. Most undiluted

TABLE 3.5

Comparison of the adsorption properties of three types of polystyrene ELISA well

Undiluted culture supernatants from AVP-capture ELISA-positive hybridomas, *in vitro*-boosted spleen cells and ESA 4, an anti-ANP MAb, were incubated in uncoated, unblocked, ELISA wells for 16 hours, then antibody adsorption was revealed with sheep anti-mouse IgG (whole molecule) HRP conjugate. Immune serum from mouse 3365, diluted 1/1000 in Buffer L, was included for comparison.
ND = not determined.

Sample	Absorbance at 450 nm		
	Nunc Maxisorp	Immulon 1	Immulon 2
VP1/10	0.044	0.000	0.001
VP1/89	0.290	0.026	0.331
VP1/121	1.645	0.034	ND
VP1/140	0.020	0.002	0.087
VP1/169	>2.050	0.207	1.559
VP1/271	0.126	0.032	0.000
ESA 4	0.658	0.141	0.561
Spleen supernatant	1.231	0.365	0.732
Immune serum	0.049	0.000	0.000

TABLE 3.6

Specificity of AVP-capture ELISA-positive hybridoma culture supernatants

Undiluted culture supernatants were assayed in Immulon 1 MicroELISA wells coated with the materials indicated, without any additional plate-blocking agent. The absorbance caused by non-specific binding of the sheep anti-mouse IgG (whole molecule) HRP conjugate to each plate-coating material has been subtracted from the values shown. Immune serum from mouse 3363, diluted 1/1000 in 15% FCS medium, was included for comparison.

Sample	Absorbance at 450 nm						
	Uncoated	2% FCS	2% NBCS	1% BSA	0.01% TG1	0.01% NRS	10 ng/ml AVP
VP1/10	0.066	0.123	0.177	0.777	0.040	0.088	0.090
VP1/89	0.190	0.135	0.603	1.658	1.073	1.120	0.410
VP1/121	0.409	0.181	0.717	1.855	1.449	1.609	0.822
VP1/140	0.485	0.256	1.000	>2.050	1.606	>2.050	0.746
VP1/169	1.733	1.843	>2.050	>2.050	>2.050	>2.050	>2.050
VP1/271	0.290	0.378	1.484	>2.050	>2.050	>2.050	1.063
VP1/367	0.045	0.045	0.088	1.083	0.040	0.152	0.073
Immune serum	0.063	0.072	0.799	0.319	0.964	0.971	0.158

AVP-capture ELISA-positive culture supernatants bound strongly to all of the antibody-containing materials, but not to foetal calf serum (Table 3.6), therefore it was concluded that they contained anti-immunoglobulin antibodies which would have caused false positive results in the AVP-capture ELISA, even in the absence of a blocking protein, by binding directly to the antibody fraction of TG1 antiserum. Dilution in BSA-containing buffer decreased binding in these samples (Table 3.2) because the anti-immunoglobulin antibodies bound to antibody contaminants in the BSA and so were unavailable to bind to the AVP-capture ELISA wells.

3.3.4 FURTHER AVP-CAPTURE ELISA STUDIES

Immune mouse serum contained high concentrations of anti-immunoglobulin and anti-BSA antibodies. Even after dilution in Buffer L, it bound strongly to AVP-capture ELISA wells (Figure 3.12A) yet no anti-AVP IgG could be detected by SAM-RIA. Higher AVP-capture ELISA binding was recorded if the serum was diluted in culture medium (Figure 3.12A) but the difference was less dramatic than that observed with AVP-capture ELISA-positive hybridoma culture supernatants (Figure 3.10, Table 3.2).

The anti-immunoglobulin and anti-BSA antibodies had probably been raised in response to contaminants in the thyroglobulin that was used for immunogen preparation, because serum from a mouse immunized with PTH 53-84 conjugated to thyroglobulin (by the method in Section 2.2.1.4) also bound to AVP-capture ELISA wells (Figure 3.12B). PTH 53-84 is structurally unrelated to AVP so the serum contained no anti-AVP activity, and its AVP-capture ELISA binding was therefore attributed to anti-thyroglobulin or anti-contaminant antibodies. Lower AVP-capture ELISA absorbances were obtained when the PTH-thyroglobulin antiserum was diluted in Buffer L rather than culture medium (Figure 3.12B), showing that it contained antibodies which behaved similarly to those raised against AVP-thyroglobulin.

Although the AVP-thyroglobulin antiserum did not seem to contain anti-AVP IgG, its AVP-capture ELISA binding was higher than that of the PTH-thyroglobulin

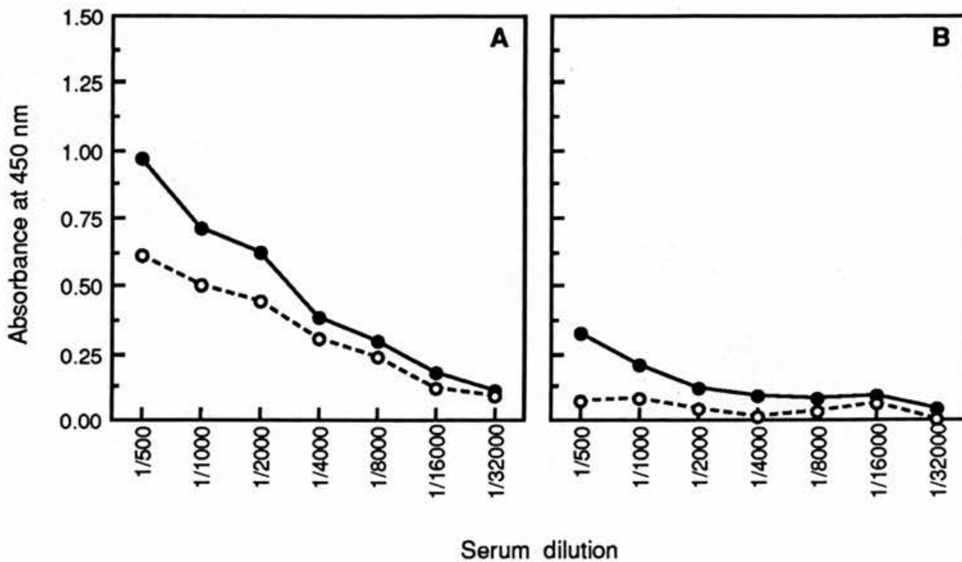

FIGURE 3.12

Effect of diluent composition on the AVP-capture ELISA response of immune mouse serum

Serum from mouse 3365, immunized with AVP-thyroglobulin conjugate (**A**), and from a mouse immunized with PTH 53-84 conjugated to thyroglobulin (**B**), was diluted in 15% FCS medium (●), or Buffer L (o), then assayed by AVP-capture ELISA. Antibody binding was revealed with sheep anti-mouse IgG (whole molecule) HRP conjugate.

antiserum (Figure 3.12). This may have been caused by a difference in the concentration of contaminants in the thyroglobulin preparations used for immunogen production or, alternatively, the larger size of the PTH 53-84 peptide may have influenced the immunogenicity of the conjugate so that more of the response was directed towards the peptide than the carrier. Antiserum against ANP-thyroglobulin was not available for comparison when these experiments were carried out.

Antibody concentrations in hybridoma culture supernatants are initially low therefore screening assays must be very sensitive to avoid false negative results, and supernatants are commonly assayed without dilution (Galfrè and Milstein, 1981; Robert et al., 1985; Vaidya et al., 1985; Smith and Wilson, 1986; Gustafsson, 1990). However, false positive results were a major problem when undiluted culture supernatants were assayed by AVP-capture ELISA. Dilution of supernatants with an equal volume of Buffer L did not always eliminate false positive binding (Table 3.2),

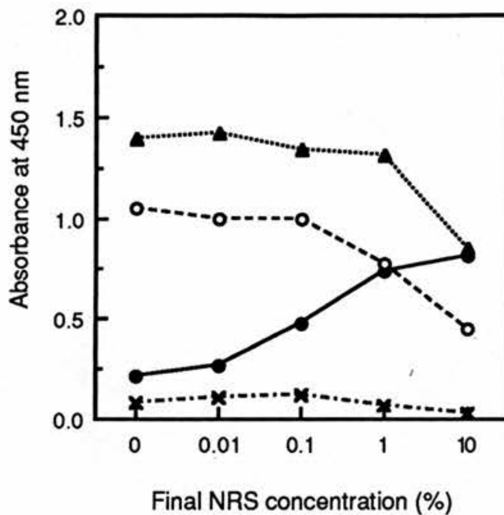

FIGURE 3.13

Effect of normal rabbit serum on the binding of AVP-capture ELISA-positive culture supernatants to TG1 antiserum

Normal rabbit serum dilutions (10 µl, in 15% FCS medium) were added to TG1 antiserum-coated, BSA-blocked, Microwells, which were immediately used to assay undiluted culture supernatants (90 µl/well) from hybridomas VP1/103 (●), VP1/169 (o), VP1/271 (▲) and VP1/367 (×). Antibody binding was revealed with sheep anti-mouse IgG (whole molecule) HRP conjugate.

and higher dilution factors would have increased the risk of false negative results, so the addition of non-immune serum to the supernatant samples seemed the best way to remove anti-immunoglobulin antibody interference.

When AVP-capture ELISA-positive culture supernatants were incubated with normal rabbit serum (NRS) in TG1 antiserum-coated BSA-blocked wells, binding tended to decrease as the NRS concentration increased (Figure 3.13) but a final concentration of 10% NRS could not eliminate all false positive binding, while the binding of supernatant from hybridoma VP1/103 actually increased with the NRS concentration. Similar results were obtained whether NRS was added to the sample or substituted for BSA in the assay buffer (Table 3.7), even though the buffer was only present during conjugate incubation. NRS could therefore displace anti-immunoglobulin antibodies which had already bound to the coated wells. Binding decreased further if NRS was present in both the sample and the buffer (Table 3.7) but this still did not

TABLE 3.7

Effect of normal rabbit serum on false positive AVP-capture ELISA results

Undiluted culture supernatants from AVP-capture ELISA-positive hybridomas, and *in vitro*-boosted spleen cells, were assayed in AVP-capture ELISA Microwells (90 µl/well) to which either 10 µl 15% FCS medium or 10 µl NRS had been added. Antibody binding was revealed with sheep anti-mouse IgG (whole molecule) HRP conjugate, diluted in either Buffer L (BSA buffer) or Buffer K containing 2% NRS and 0.1% Tween 20 (NRS buffer). Immune serum from mouse 3365, diluted 1/1000 in 15% FCS medium, was included for comparison. The absorbance caused by non-specific binding of the HRP conjugate has been subtracted from the values shown.

	Absorbance at 450 nm			
Sample	BSA Buffer		NRS Buffer	
	10 µl Medium	10 µl NRS	10 µl Medium	10 µl NRS
VP1/10	0.345	0.207	0.194	0.121
VP1/89	0.265	0.072	0.042	0.013
VP1/103	0.227	0.435	0.089	0.140
VP1/121	0.438	0.079	0.150	0.008
VP1/140	0.865	0.415	0.302	0.216
VP1/169	1.090	0.538	0.533	0.199
VP1/271	1.401	0.799	0.694	0.417
VP1/367	0.120	0.000	0.023	0.003
Spleen supernatant	0.321	0.179	0.137	0.077
Immune serum	1.284	0.944	0.472	0.303

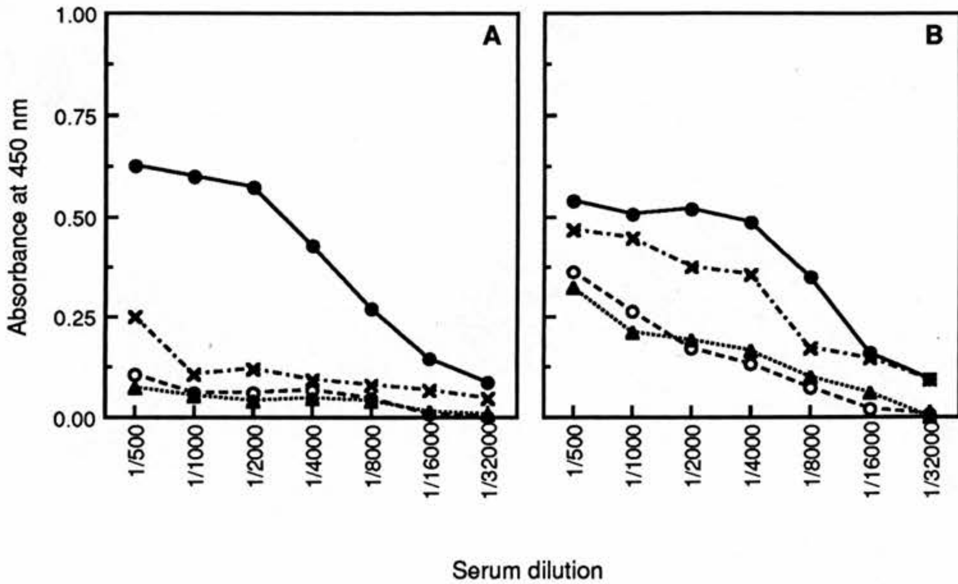

FIGURE 3.14

Binding specificity of anti-AVP antibody-containing mouse serum

Serum from mouse 3476, immunized with AVP-thyroglobulin conjugate, was diluted in Buffer K containing 2% bovine serum and 0.1% Tween 20, then assayed in Immulon 1 MicroELISA wells coated with 10 ng/ml AVP (×), 0.01% normal rabbit serum (▲), 0.01% TG1 antiserum (○), or 0.01% TG1 antiserum preincubated with 50 ng/ml AVP (AVP-capture ELISA, ●), without any additional plate-blocking agent. Antibody binding was revealed with sheep anti-mouse IgG (whole molecule) HRP conjugate (**A**) or goat anti-mouse 'polyvalent immunoglobulins' HRP conjugate (**B**).

The absorbances caused by non-specific binding of the HRP conjugates to each plate-coating material have been subtracted from the values shown.

eliminate all false positive results.

When tail bleed sera from other AVP-thyroglobulin-immunized mice became available, the AVP-capture ELISA was shown to be genuinely capable of detecting anti-AVP antibodies (Figure 3.14) although low titre sera gave ambiguous results. The anti-IgG HRP conjugate detected strong binding to AVP-capture ELISA wells and weak binding to AVP-coated wells (Figure 3.14A), whereas the 'polyvalent immunoglobulins' HRP conjugate recorded strong binding to both types of well (Figure 3.14B). This suggested that the AVP-capture ELISA bound all isotypes equally, while the AVP-coated wells bound anti-AVP IgM more efficiently than IgG.

Immune serum bound equally to TG1 antiserum and to normal rabbit serum (Figure 3.14), which suggested that direct binding to immobilized TG1 antiserum had

been caused by anti-immunoglobulin antibodies and not, as previously thought, by AVP-bound antibodies. The anti-IgG HRP conjugate detected less anti-immunoglobulin binding (Figure 3.14A) than did the 'polyvalent immunoglobulins' HRP conjugate (Figure 3.14B), confirming previous suggestions that many of these antibodies were either IgM or IgA isotypes (Figure 3.7).

Despite these observations, the problem of preventing false positive binding remained unresolved therefore the AVP-capture ELISA could not be used for further hybridoma screening.

3.4 RADIOIMMUNOASSAY

Initial experiments with SAM-RIA failed to detect anti-AVP antibody in immune mouse serum. There had been no other mouse anti-AVP antibody available so there was no positive control for the assay and it was impossible to establish whether the problem had been assay failure or absence of antibody.

In the SAM-RIA, antibody-bound and free tracer were separated by the SAM-S-500 solid phase which was specific for mouse antibodies and bound mainly to IgG isotypes. The PEG-RIA was not species-specific because it achieved separation by PEG 8000 precipitation of antibodies. Consequently, rabbit TG1 antiserum could be used as a positive control in the PEG-RIA but not in the SAM-RIA.

3.4.1 PEG SEPARATION METHOD (PEG-RIA)

When tail bleed sera from eight AVP-thyroglobulin-immunized mice were assayed by PEG-RIA, all of the sera bound ^{125}I-AVP although a lot of the anti-AVP antibody might have been IgM because PEG 8000 precipitated all isotypes. None of the AVP-capture ELISA-positive hybridoma culture supernatants (Section 3.3.2) could bind ^{125}I-AVP, backing up previous data which had suggested that these supernatants contained only anti-immunoglobulin and anti-BSA antibodies (Section 3.3.3).

The PEG-RIA was prone to high background readings (5-10% of added tracer) and poor reproducibility, which was caused by inefficient separation of unbound ^{125}I-AVP although longer tube-drainage times produced better results. The assay was also unlikely to detect antibodies specific for epitopes close to the tyrosine residue of AVP. However, despite these problems, the PEG-RIA was used to screen hybridoma supernatants from the next two groups of cell fusions (Table 4.6).

The PEG-RIA detected ^{125}I-AVP binding in an *in vitro*-boosted spleen cell culture supernatant even though unlabelled AVP was present. However, when 51 hybridoma culture supernatants were assayed, only one bound ^{125}I-AVP. The antibody in this supernatant was not detected by the AVP-capture ELISA or by an AVP-coated-plate ELISA (10 ng/ml AVP, unblocked wells) which detected anti-AVP antibody in immune mouse serum and in the *in vitro*-boosted spleen cell supernatant. Nevertheless, the PEG-RIA-positive hybridoma was cloned, and subcloned, to produce a monoclonal cell line which secreted ESVP 1, an anti-AVP MAb.

3.4.2 SECOND ANTIBODY SEPARATION METHOD (SAM-RIA)

Immune mouse serum, ESVP 1 supernatant and an *in vitro*-boosted spleen cell supernatant, which all bound ^{125}I-AVP in the PEG-RIA, were also SAM-RIA-positive. Although the supernatants produced similar results in both assays, higher immune serum titres were estimated by PEG-RIA than by SAM-RIA (Table 3.8), which suggested that the serum contained significant amounts of anti-AVP IgM.

Since the early attempts to assay immune serum by SAM-RIA had been unsuccessful, two of the original tail bleed sera were compared with serum samples taken from the same mice after additional boosting (Figure 3.15). Both mice were SAM-RIA-negative before additional boosting but positive afterwards, which suggested that the initial sera contained no anti-AVP IgG. Initial serum from mouse 3365 had produced strong positive AVP-coated-plate ELISA results with the 'polyvalent immunoglobulins' HRP conjugate (Figure 3.1), indicating that the serum

TABLE 3.8

Comparison of the immune serum anti-AVP antibody titres estimated by PEG-RIA and by SAM-RIA

Tail bleed sera from mice immunized with AVP-thyroglobulin conjugate were diluted in Buffer K containing 2% bovine serum and 1% Tween 20, then assayed by PEG-RIA and by SAM-RIA. Antibody titres were defined as the serum dilutions which produced half-maximal ^{125}I-AVP binding.

Mouse no.	Antibody titre	
	PEG-RIA	SAM-RIA
3474	1/2000	<1/100
3476	1/35,000	1/3200
3477	1/2700	1/160

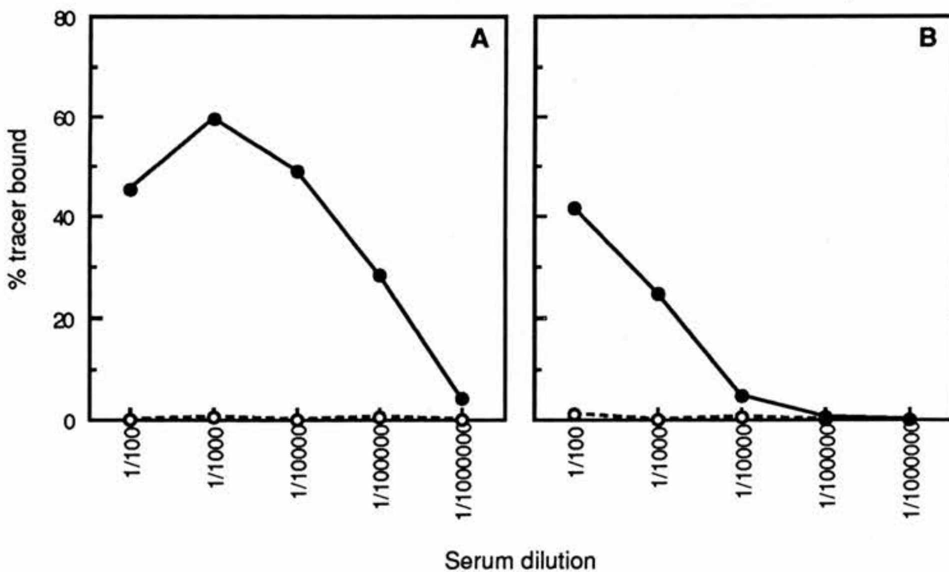

FIGURE 3.15

The effect of additional boosting on the SAM-RIA response of serum from immunized mice

Tail-bleeding was carried out after basic immunization (priming dose of AVP-thyroglobulin conjugate and two boosts, o) and after additional boosting (●). The serum was diluted in Buffer K containing 2% bovine serum, then assayed by SAM-RIA. Mouse 3365 (**A**) had received three additional boosts of immunogen in alum, while mouse 3366 (**B**) had received two additional boosts.

contained a lot of anti-AVP antibody, but this must all have been IgM.

The SAM-RIA was superior to the PEG-RIA because its background readings were much lower (0.5-1.0% of added tracer) and it was selective for the desired IgG isotypes. It was therefore used to screen the next group of cell fusions, using ESVP 1 MAb as a positive control. However, 288 hybridomas were SAM-RIA-negative, even though the spleen donor (mouse 3476, Table 4.2) was well-immunized and its *in vitro*-boosted spleen cell supernatant was SAM-RIA-positive. Some anti-AVP antibodies might have been missed because they could not bind ^{125}I-AVP therefore screening by AVP-coated-plate ELISA was investigated as an alternative to RIA.

3.5 AVP-COATED-PLATE ELISA

The original AVP-coated-plate ELISA was set up using rabbit TG1 antiserum as the sample, with donkey anti-rabbit IgG HRP conjugate to detect binding. When immune mouse serum became available, coating with 10 ng/ml AVP had still seemed satisfactory because serum from mouse 3365 produced a good dilution curve with the 'polyvalent immunoglobulins' HRP conjugate (Figure 3.1), although poor results were obtained with the anti-mouse IgG HRP conjugate (Figure 3.3) due to a lack of anti-AVP IgG (Figure 3.15). However, even when the AVP-capture ELISA identified immune serum which contained anti-AVP IgG, the AVP-coated-plate ELISA recorded very low serum IgG binding (Figure 3.14A) and could not detect ESVP 1, an anti-AVP IgG1 MAb, in undiluted culture supernatant.

Investigation of the AVP-coated-plate ELISA revealed that ESVP 1 binding increased dramatically as the AVP-coating concentration increased (Figure 3.16A). In contrast, there was a relatively small effect on TG1 antiserum (Figure 3.16B) which bound well at all AVP-coating concentrations. This showed that the high avidity TG1 antiserum had misleadingly suggested that a coating of 10 ng/ml AVP would detect anti-AVP IgG antibodies, when much higher AVP-coating concentrations were actually needed for efficient MAb detection.

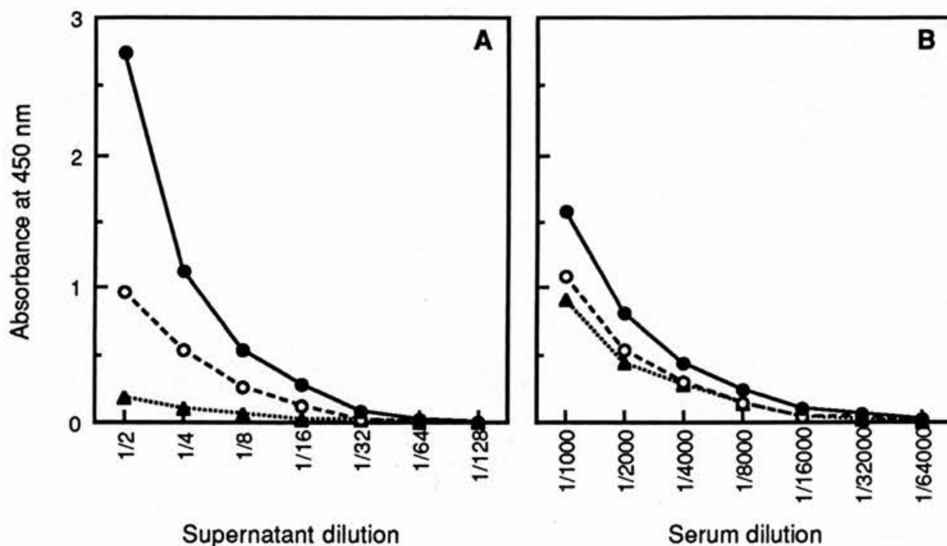

FIGURE 3.16

Effect of AVP-coating concentration on antibody binding in the AVP-coated-plate ELISA

ESVP 1 MAb culture supernatant (**A**), and rabbit TG1 antiserum (**B**), were diluted in Buffer L, then assayed in Nunc Microwells coated with 10 ng/ml (▲), 100 ng/ml (o), or 1 μg/ml AVP (●), without any additional plate-blocking agent. ESVP 1 binding was revealed with rabbit anti-mouse IgG (whole molecule) HRP conjugate (1/200 dilution in Buffer L), while TG1 antiserum binding was revealed with donkey anti-rabbit IgG HRP conjugate (1/500 dilution in Buffer L). The absorbances caused by non-specific binding of the HRP conjugates have been subtracted from the values shown.

An AVP-coated-plate ELISA with a coating of 1 μg/ml AVP, and using rabbit anti-mouse IgG (whole molecule) HRP conjugate, seemed appropriate for hybridoma screening because it recorded strong ESVP 1 MAb binding (Figure 3.16A). It also produced immune mouse serum titre estimates which were roughly similar to those produced by SAM-RIA (Table 3.9). A few sera had higher AVP-coated-plate ELISA titres than SAM-RIA titres (e.g. mouse 3477, Table 3.9) because they contained some antibodies that could bind immobilized AVP but not ^{125}I-AVP. This demonstrated that the ELISA could detect anti-AVP specificities that the SAM-RIA would miss.

Plate-blocking was omitted from the AVP-coated-plate ELISA because BSA caused non-specific binding of the anti-mouse IgG HRP conjugate (Table 3.1), allowed false positive binding of anti-BSA and anti-immunoglobulin antibodies (Table 3.6),

TABLE 3.9

Comparison of the immune serum antibody titres estimated by AVP-coated-plate ELISA and by SAM-RIA

Tail bleed sera from mice immunized with AVP-thyroglobulin conjugate were diluted in Buffer L then assayed by AVP-coated-plate ELISA (1 µg/ml AVP, unblocked Immulon 2 MicroELISA wells) and by SAM-RIA. ELISA binding was revealed with rabbit anti-mouse IgG (whole molecule) HRP conjugate. The antibody titre was defined as the serum dilution which produced an absorbance of 1.0 at 450 nm (AVP-coated-plate ELISA) or half-maximal ^{125}I-AVP binding (SAM-RIA).

Mouse no.	Antibody titre	
	ELISA	SAM-RIA
3470	1/4000	1/1800
3471	1/300	1/440
3472	1/2400	1/2400
3475	1/4200	1/3200
3477	1/3500	1/400

TABLE 3.10

Effect of BSA-blocking on antibody binding in the AVP-coated-plate ELISA

ESVP 2 MAb culture supernatant and immune serum from mouse 3475 (diluted 1/2 and 1/2000, respectively, in Buffer L) were assayed in BSA-blocked, or unblocked, Immulon 2 MicroELISA wells coated with 1 µg/ml AVP. The absorbance caused by non-specific binding of the rabbit anti-mouse IgG (whole molecule) HRP conjugate has been subtracted from the values shown. Colour development occurred rapidly therefore the absorbance at 630 nm was recorded before H_2SO_4 addition.

Sample	Absorbance at 630 nm	
	Blocked	Unblocked
ESVP 2	1.085	1.297
Immune serum	0.534	0.614

and decreased anti-AVP antibody binding by steric hindrance (Table 3.10). All hybridoma culture supernatants were therefore diluted with an equal volume of Buffer L, which introduced Tween 20 and so prevented non-specific binding to the unblocked wells during the 16 hour incubation period (Mohammad and Esen, 1989). The initial hybridoma cultures were so small that only 150 µl supernatant could be taken for screening but dilution increased the sample volume and so allowed duplicate assay wells to be set up, which improved the reliability of the result. As an additional benefit, sample dilution often increased the binding of anti-AVP antibodies (Table 3.11) so that false negative results were less likely to occur.

Fusion VP12, which generated hybridomas in 466 culture wells (Table 4.6), was screened by AVP-coated-plate ELISA, AVP-capture ELISA and SAM-RIA. One monoclonal cell line was established after it was detected by both the AVP-coated-plate ELISA and the SAM-RIA, but it could not be detected by the AVP-capture ELISA (ESVP 2 MAb, Table 3.11). Undiluted culture supernatants from 110 hybridomas were AVP-capture ELISA-positive but did not contain anti-AVP antibody because AVP-coated-plate ELISA binding was prevented by dilution with an equal volume of Buffer L (Table 3.11) and all were SAM-RIA-negative. Dilution also decreased the binding of these supernatants to thyroglobulin, BSA, NRS, TG1 antiserum and AVP-capture ELISA wells (Table 3.11). Immune mouse serum and *in vitro*-boosted spleen cell supernatants reacted strongly with thyroglobulin even after dilution in Buffer L because they contained anti-thyroglobulin antibodies, and in comparison their binding to BSA, NRS and TG1 antiserum was weak (Table 3.11).

The remaining cell fusions were screened by AVP-coated-plate ELISA, with RIA confirmation of positive results, and two further monoclonal cell lines were established. These secreted ESVP 3 and ESVP 4, which were anti-AVP IgM and IgA MAbs, respectively, therefore the anti-mouse IgG HRP conjugate was not specific for mouse IgG. In addition to binding mouse IgM and IgA, the conjugate showed some cross-reaction with rabbit and bovine sera, even if it was an affinity-isolated preparation (Table 3.1).

TABLE 3.11

Effect of sample dilution on ELISA binding responses

Culture supernatants from anti-AVP MAbs (ESVP 1 and ESVP 2), AVP-capture ELISA-positive hybridomas (VP12/33, VP12/58 and VP12/181), and *in vitro*-boosted spleen cells, were assayed in Immulon 2 MicroELISA wells coated with the materials indicated. Immune serum from mouse 3475, diluted 1/1000 in 10% FCS medium, was included for comparison. All samples were assayed before, and after (figures in parentheses), dilution with an equal volume of Buffer L. The absorbance caused by non-specific binding of the rabbit anti-mouse IgG (whole molecule) HRP conjugate to each plate-coating material has been subtracted from the values shown, which are the means of duplicate determinations.

Sample	Absorbance at 450 nm					
	Antigen-coated-plate ELISA					AVP-capture ELISA
	1 μg/ml AVP	1% BSA	10 μg/ml Thyroglobulin	0.01% NRS	0.01% TG1 1% BSA	0.01% TG1, 1% BSA 50 ng/ml AVP
ESVP 1	1.906 (>2.050)	0.090 (0.079)	0.020 (0.021)	0.022 (0.033)	0.100 (0.000)	0.040 (0.000)
ESVP 2	>2.050 (>2.050)	0.029 (0.021)	0.001 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)
VP12/33	0.137 (0.003)	0.148 (0.097)	0.760 (0.674)	0.341 (0.128)	0.214 (0.163)	0.054 (0.052)
VP12/58	0.070 (0.019)	0.156 (0.013)	0.037 (0.000)	0.115 (0.053)	0.953 (0.743)	0.509 (0.483)
VP12/181	0.332 (0.000)	0.225 (0.002)	0.845 (0.372)	0.681 (0.076)	0.455 (0.000)	0.359 (0.000)
Spleen supernatant	0.910 (1.447)	0.037 (0.068)	>2.050 (>2.050)	0.453 (0.296)	0.162 (0.163)	0.139 (0.164)
Immune serum	0.701 (1.328)	0.000 (0.000)	>2.050 (>2.050)	0.286 (0.143)	0.105 (0.083)	0.137 (0.153)

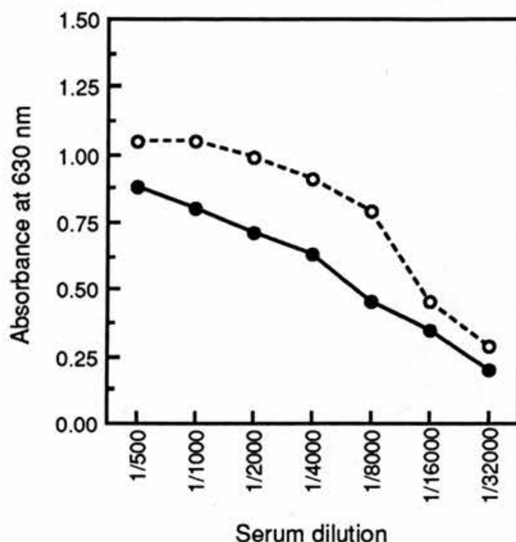

FIGURE 3.17

Effect of BSA-blocking on the ANP-coated-plate ELISA response of immune mouse serum

Serum from mouse 3478, immunized with ANP-thyroglobulin conjugate, was diluted in Buffer L then assayed in BSA-blocked (●), or unblocked (○), Immulon 2 MicroELISA wells coated with 100 ng/ml ANP. Antibody binding was revealed with rabbit anti-mouse IgG (whole molecule) HRP conjugate. Colour development occurred rapidly therefore the absorbance at 630 nm was recorded before H_2SO_4 addition.

3.6 ANP-COATED-PLATE ELISA

The original ANP-coated-plate ELISA method (Prowse et al., 1989), which used BSA-blocked wells coated with 100 ng/ml ANP, was likely to cross-react with the anti-BSA and anti-immunoglobulin antibodies that were expected to be generated in response to immunization with ANP-thyroglobulin conjugate. This cross-reaction was avoided by the omission of BSA-blocking, while non-specific binding to the unblocked wells was prevented by dilution of all samples in Buffer L. Higher anti-ANP antibody binding was recorded in the absence of BSA-blocking (Figure 3.17) because BSA caused steric hindrance.

Antibody binding increased with the ANP-coating concentration (Figure 3.18), but the effect on ESA 4 MAb detection (Figure 3.18A) was less dramatic than that on ESVP 1 MAb detection when the AVP-coating concentration increased (Figure 3.16A).

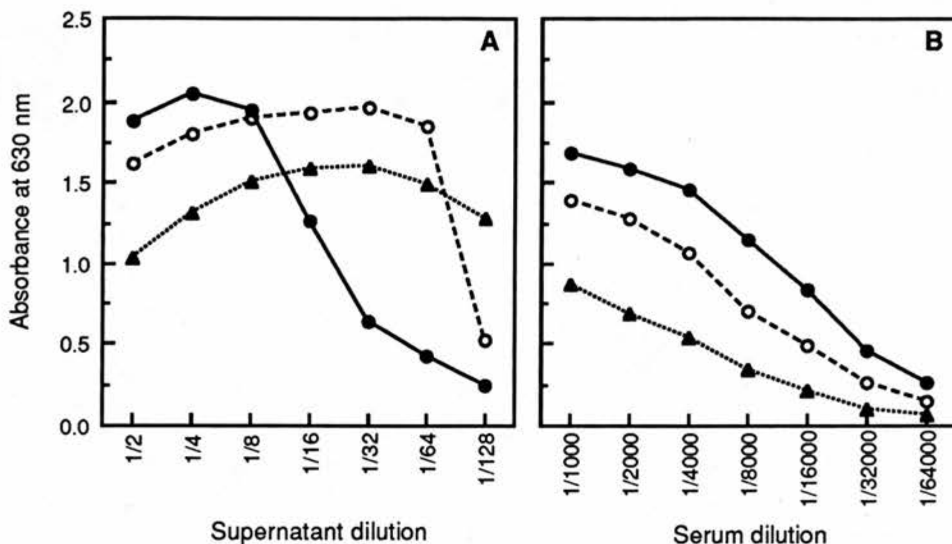

FIGURE 3.18

Effect of ANP-coating concentration on antibody binding in the ANP-coated-plate ELISA

ESA 4 MAb culture supernatant (**A**), and serum from mouse 3479, immunized with ANP-thyroglobulin conjugate (**B**), were diluted in Buffer L then assayed in Immulon 2 MicroELISA wells coated with 10 ng/ml (▲), 100 ng/ml (○), or 1 µg/ml ANP (●), without any additional plate-blocking agent. Antibody binding was revealed with rabbit anti-mouse IgG (whole molecule) HRP conjugate. Colour development occurred rapidly therefore the absorbance at 630 nm was recorded before H_2SO_4 addition.

This may have been due to the difference in binding affinities because ESA 4 had a much higher affinity constant (Table 7.1) than ESVP 1 (Table 5.1). ESA 4 MAb and immune mouse serum bound strongly to all of the ANP-coating concentrations which were tested, but 100 ng/ml ANP produced the best MAb detection because high absorbances were obtained over a wide range of ESA 4 dilutions (Figure 3.18).

An ANP-coated-plate ELISA with 100 ng/ml ANP-coated, unblocked, wells was selected for hybridoma screening. Culture supernatants were diluted with an equal volume of Buffer L, and antibody binding was revealed with rabbit anti-mouse IgG (whole molecule) HRP conjugate. This assay only detected anti-ANP antibodies, whose binding was often increased by dilution in Buffer L (Table 3.12).

Some hybridomas which were raised against ANP-thyroglobulin conjugate secreted antibodies that bound strongly to BSA-blocked, rabbit TG1 antiserum-coated

TABLE 3.12

Effect of sample dilution on ANP-coated-plate ELISA binding response

Culture supernatants from anti-ANP MAbs (ESNP 1 and ESNP 3), an anti-immunoglobulin hybridoma (NP6/27), and *in vitro*-boosted spleen cells derived from mouse 3478, were assayed in Immulon 1 MicroELISA wells coated with the materials indicated. Serum from mouse 3481, immunized with ANP-thyroglobulin conjugate, diluted 1/500 in 15% FCS medium, was included for comparison. All samples were assayed before, and after (figures in parentheses), dilution with an equal volume of Buffer L. The absorbance caused by non-specific binding of the rabbit anti-mouse IgG (whole molecule) HRP conjugate to each plate-coating material has been subtracted from the values shown, which are the means of duplicate determinations.

Sample	Absorbance at 450 nm			
	100 ng/ml ANP	1% BSA	10 μg/ml Thyroglobulin	0.01% NRS
ESNP 1	0.868 (2.020)	0.007 (0.000)	0.002 (0.007)	0.005 (0.003)
ESNP 3	1.266 (1.765)	0.001 (0.000)	0.004 (0.015)	0.000 (0.004)
NP6/27	0.000 (0.000)	0.066 (0.026)	0.187 (0.075)	0.063 (0.036)
Spleen supernatant	0.006 (0.016)	0.185 (0.061)	0.220 (0.342)	0.087 (0.042)
Immune serum	0.359 (0.766)	0.025 (0.001)	1.819 (>2.050)	0.020 (0.019)

wells but not to ANP, while an *in vitro*-boosted spleen cell supernatant which contained very little anti-ANP antibody could bind to thyroglobulin and to BSA (Table 3.12), indicating that anti-thyroglobulin, anti-BSA and anti-immunoglobulin antibodies had been produced in response to the immunogen. In contrast, immune mouse serum bound strongly to ANP and thyroglobulin yet appeared to contain negligible anti-BSA and anti-immunoglobulin activity (Table 3.12) even when diluted in culture medium rather than Buffer L, which suggested that BSA and immunoglobulins were probably very minor components of the ANP-thyroglobulin immunogen.

RIA was not used to screen for anti-ANP antibodies. ^{125}I-ANP could not be prepared locally because there were no HPLC facilities for the separation of unreacted ^{125}I, while purchased ^{125}I-ANP was expensive and had a short shelf-life. The ANP-coated-plate ELISA was therefore the most cost-effective assay.

3.7 DISCUSSION

A two-site antigen-capture ELISA, using rabbit polyclonal antibodies to immobilize the antigen (Smith and Wilson, 1986), had seemed ideal for hybridoma screening because MAbs suitable for two-site immunometric assay development were required. This type of screening assay was reported to be more sensitive than antigen-coated-plate ELISA (Al Moudallal et al., 1984; Brennand et al., 1986) and was the only ELISA format that could detect all of the antigen-specific MAbs which were tested by Brennand et al. (1986).

Initial tests had suggested that the AVP-capture ELISA detected anti-AVP antibodies, despite slight cross-reaction with anti-BSA antibodies (Figure 3.7) and non-specific binding of the anti-mouse IgG HRP conjugate to the solid-phase components (Table 3.1). Serum from an AVP-thyroglobulin-immunized mouse produced much higher AVP-capture ELISA absorbances than normal mouse serum (Figure 3.9) or serum from a mouse immunized with PTH 53-84-thyroglobulin conjugate (Figure 3.12). The anti-AVP-thyroglobulin serum bound to TG1 antiserum-coated wells whether or

not they were preincubated with AVP (Figures 3.6 and 3.7), but this was assumed to indicate the presence of AVP-bound mouse antibodies. However, the AVP-capture ELISA-positive culture supernatants failed to bind after dilution in Buffer L (Table 3.2), even though significant adsorption to uncoated wells (Tables 3.5 and 3.6) had suggested that they contained high concentrations of antibody (Miller et al., 1983). Investigation of this phenomenon revealed that undiluted AVP-capture ELISA-positive culture supernatants also bound strongly to rabbit TG1 antiserum, normal rabbit serum, bovine serum and BSA (Table 3.6), while their response to FCS was the same as to uncoated wells.

Many BSA preparations are contaminated by immunoglobulins (Boscato and Stuart, 1988), which were also present in all of the sera used in this work, except FCS, therefore most of the AVP-capture ELISA-positive supernatants probably contained anti-immunoglobulin antibodies. Since antibodies are raised against all immunogen components, it is likely that the bovine thyroglobulin used in immunogen production (and described by Sigma as 'electrophoretically heterogeneous') was contaminated by immunoglobulins. Anti-immunoglobulin antibodies have often been reported to cause interference in immunoassays and have been described as heterophilic antibodies (Boscato and Stuart, 1988; Nahm and Hoffmann, 1990; Levinson, 1992). The thyroglobulin preparation presumably also contained BSA because anti-BSA antibodies were detected in immune mouse serum (Figure 3.7) and in some hybridoma culture supernatants (e.g. hybridoma VP1/367, Table 3.6). When supernatants were diluted with an equal volume of Buffer L, the anti-BSA and anti-immunoglobulin antibodies reacted with the BSA and immunoglobulins in the buffer so that binding to AVP-capture ELISA wells was prevented, although high titre supernatants could still show some binding due to inadequate neutralization (Table 3.2). Dilution in cell culture medium did not have this effect (Table 3.2, Figure 3.10) because it contained no immunoglobulins and relatively little BSA.

Traditional ELISA methods include a blocking step during which an inert protein is adsorbed onto unoccupied sites on the solid phase to prevent non-specific

binding of the assay components (Campbell, 1984), although there are reports which suggest that blocking is unnecessary (Engvall and Perlmann, 1972; Mohammad and Esen, 1989; Gosling, 1990). The effectiveness of any particular blocking protein seems to depend on the assay system in which it is used (Kenna et al., 1985; Vogt et al., 1987; Pruslin et al., 1991) and there is a risk of interference from anti-blocking-protein antibodies (Schønheyder and Andersen, 1984; Kemeny and Chantler, 1988). BSA is a commonly-used blocking agent (Reading, 1982; Douillard and Hoffman, 1983; Gustafsson, 1990; Pruslin et al., 1991) although casein (Kenna et al., 1985; Vogt et al., 1987) and non-fat dried milk (Vogt et al., 1987; Mohammad and Esen, 1989) have been suggested as cheaper alternatives and serum has been used occasionally (Hou-Yu et al., 1982; Campbell, 1984). However, these materials are all likely to contain immunoglobulins and so will be bound by anti-immunoglobulin antibodies. Campbell (1984) suggested the use of antigen-free plates to confirm that antibodies are antigen-specific and do not bind to the blocking protein or the plastic surface, but this increases the work and expense involved in hybridoma screening and introduces the risk that antigen-specific antibodies might be overlooked if they are present in supernatants which also contain anti-blocking-protein antibodies.

Omission of BSA-blocking from the AVP-coated-plate and ANP-coated-plate ELISAs prevented the binding of anti-BSA and anti-immunoglobulin antibodies, decreased non-specific binding of the anti-mouse IgG HRP conjugate (Table 3.1), and increased specific antibody binding (Table 3.10, Figure 3.17) by removing the steric hindrance (Hobbs, 1989; Mohammad and Esen, 1989) that was caused by the large BSA molecules (67,000 Daltons) surrounding the small AVP (1,084 Daltons) and ANP (3,077 Daltons) peptides. The binding of anti-immunoglobulin antibody to TG1 antiserum-coated wells was also increased in the absence of BSA-blocking (Figure 3.7). Since the Fc portion of IgG is hydrophobic (Ishikawa, 1987) it is likely to be the part of the molecule which adsorbs to the solid phase. BSA-blocking will sterically hinder binding to this region therefore many of the anti-immunoglobulin specificities were probably directed against the Fc portion of the antibody molecule.

Dilution of hybridoma culture supernatants with an equal volume of Buffer L introduced Tween 20 which prevented non-specific binding to the unblocked ELISA wells (Mohammad and Esen, 1989). At the same time, specific antibody binding often increased (Tables 3.11 and 3.12), probably as a result of buffering the sample because spent culture supernatants tend to be acidic (Campbell, 1984) while most antibodies bind optimally at neutral pH values. However, sample dilution may also have decreased the steric hindrance which occurs when second antibody-enzyme conjugates bind to high concentrations of solid phase-bound antibody (Koertge and Butler, 1985), and there may have been a prozone effect because concentrated antibody solutions bind univalently to immobilized antigen and can then leach off the assay plate during subsequent incubation and washing steps (Nygren et al., 1985; Vos et al., 1987).

Caution is required when immune sera are used to develop hybridoma screening assays, because their binding properties differ from those of MAbs so that they may bind strongly under conditions in which MAbs are undetectable (Al Moudallal et al., 1984). Normal mouse serum may not be an appropriate negative control because it does not produce the false positive binding which can be caused by antibodies raised against contaminants in the immunogen. Serum from an animal immunized with an unrelated peptide conjugated to the same carrier protein is more likely to give an indication of possible problems, particularly if it is diluted in culture medium instead of assay buffer. In this work, serum from a PTH 53-84-thyroglobulin-immunized mouse bound more strongly to AVP-capture ELISA wells if it was diluted in 15% FCS medium rather than Buffer L (Figure 3.12B), which showed that it contained some anti-immunoglobulin or anti-BSA antibodies. However, the PTH 53-84-thyroglobulin (Figure 3.12B) and ANP-thyroglobulin (Table 3.12) conjugates did not produce such a strong anti-immunoglobulin response as the AVP-thyroglobulin conjugate (Figure 3.12A, Table 3.11), which may indicate that there were batch-to-batch variations in the contaminant concentrations present in the thyroglobulin which was used for immunogen production.

Immune serum was used to set up the AVP-capture ELISA and the

AVP-coated-plate ELISA but gave misleading results because two major problems were concealed. Firstly, the presence of anti-immunoglobulin and anti-BSA antibodies was masked when immune serum was diluted in Buffer L, and only became obvious when undiluted culture supernatants were assayed. This problem, which was also encountered when screening hybridomas raised against thyroglobulin-conjugated peptides derived from PTH (unpublished observation) and HIV (J. Boyd, personal communication), might have been prevented by using an alternative carrier protein in the immunogen. However, Skowsky and Fisher (1972b) showed that the choice of carrier is important in determining the immune response to small peptides. Bovine thyroglobulin was used because it was known to be a good carrier for AVP and had been successfully used in anti-AVP antibody production by many research groups (Baylis and Heath, 1977; Hou-Yu et al., 1982; Jones et al., 1985; Robert et al., 1985; Work et al., 1985; Smith and McIntosh, 1986).

Secondly, the anti-AVP MAbs were undetectable by AVP-capture ELISA (Table 3.11) and difficult to detect at low AVP-coating concentrations in the AVP-coated-plate ELISA (Figure 3.16A), even though the polyclonal anti-AVP antibodies of immune serum produced high absorbances under these conditions (Figure 3.16B). MAbs bind poorly at low epitope densities (Lew, 1984), especially when they have low binding affinities (Nimmo et al., 1984; Nygren et al., 1985; Peterman et al., 1985), whereas the high affinity antibodies in immune serum bind strongly and give the impression that an appropriate screening assay has been developed even though much higher antigen-coating concentrations may be needed for efficient MAb detection.

All of the anti-AVP MAbs, and many anti-ANP MAbs, reported by other workers were raised against AVP-thyroglobulin and ANP-thyroglobulin conjugates, respectively. The anti-AVP MAbs were detected by liquid-phase RIA with ^{125}I-AVP (Hou-Yu et al., 1982; Robert et al., 1985) or ^{3}H-AVP (Jurzak et al., 1990), by solid-phase RIA using ^{125}I-anti-mouse F(ab)$_2$ (Hou-Yu et al., 1982), by ELISA (Robert et al., 1985; Jurzak et al., 1990) and by an immunohistochemical method (Robert et

al., 1985). Most workers screened for anti-ANP MAbs by liquid-phase RIA with ^{125}I-ANP (John et al., 1986; Naomi et al., 1987; Mukoyama et al., 1988a; Rathinavelu and Isom, 1989; Prowse et al., 1989) although solid-phase RIA with ^{125}I-anti-mouse IgG (Glembotski et al., 1987; Milne et al., 1987) and ELISA (Prowse et al., 1989) screening methods have also been reported.

Liquid-phase RIA with ^{125}I-Ag is unlikely to produce false positive screening results but can give false negative results if antibodies are directed to parts of the antigen into which ^{125}I has been incorporated. This is a particular problem with AVP because of its small size, and one anti-AVP MAb which does not readily bind to ^{125}I-AVP has been reported (Hou-Yu et al., 1982; Valiquette et al., 1986). On the other hand, solid-phase screening methods, in which the binding of antibodies to unlabelled, immobilized, antigen is revealed by a second antibody, can easily give rise to false positive results especially when blocking proteins are present. Several workers used BSA-blocking (Robert et al., 1985; Prowse et al., 1989; Jurzak et al., 1990), serum-blocking (Hou-Yu et al., 1982), or an immobilized antigen-BSA conjugate (Glembotski et al., 1987; Milne et al., 1987; Jurzak et al., 1990) in their solid-phase anti-AVP and anti-ANP screening assays, so interference from anti-BSA and anti-immunoglobulin antibodies could be expected. However, false positive binding was not reported even though undiluted culture supernatants were assayed, although problems may have occurred because Robert et al. (1985) and Jurzak et al. (1990) suggested that supernatants should be screened with more than one type of assay. This is a wise precaution, particularly with small peptide antigens, because no screening assay is entirely problem-free. In this work, anti-AVP MAbs were screened by AVP-coated-plate ELISA and positive results were confirmed by RIA. Anti-ANP MAbs were only screened by ANP-coated-plate ELISA but anti-ANP activity was confirmed if the addition of liquid-phase ANP decreased antibody binding to immobilized ANP.

Early screening of hybridoma supernatants is important because cultures often contain more than one colony of cells and non-producers are liable to overgrow

antibody-secreting cells (Goding, 1986). Screening assays must be sensitive to avoid false negative results therefore supernatants are often assayed without prior dilution. This is acceptable in liquid-phase RIA screening because buffer is introduced with the labelled antigen and only specific binding is detected, but ELISA screening of undiluted supernatants is prone to many problems. Hybridoma culture supernatants, particularly those raised against thyroglobulin-conjugated peptides, should therefore be diluted in buffer which contains BSA and Tween 20 prior to ELISA screening. This simple step will prevent non-specific binding and so make plate-blocking unnecessary, maximize specific binding by buffering the sample, and neutralize most of the anti-immunoglobulin and anti-BSA antibodies which are produced in response to contaminants in the thyroglobulin preparation.

Hybridomas are usually cloned as soon as positive screening results are obtained (Goding, 1986), at which time there is often insufficient supernatant available for repeat assays or specificity testing. If cloning is delayed until these results are known, valuable cell lines may be lost because of overgrowth by non-secreting variants. Valiquette et al. (1986) considered that false positive results were more acceptable than false negatives, but a lot of time can be wasted cloning hybridomas which are unsuitable because they secrete antibodies that bind to the wrong antigen. The screening assay therefore needs to be specific for the antigen of interest. This is particularly important if a successful cell fusion produces so many positive hybridomas that they cannot all be handled. Hybridomas which produce the highest initial screening results are generally selected for further study, so if false positive results are higher than genuine positive binding, as happened with the AVP-capture ELISA, there is a danger that cultures containing antigen-specific antibodies will be rejected in favour of those which contain irrelevant antibodies. Consequently, it is important to select hybridoma screening assays which minimize the occurrence of false positive binding while avoiding false negative results.

CHAPTER 4

ANTIBODY PRODUCTION

4.1 INTRODUCTION

The supply of any polyclonal antiserum is ultimately limited by the size and lifespan of the immunized animal, while the properties of antisera vary between bleeds (Campbell, 1984; Tijssen, 1985). Antibody-secreting plasma cells will not grow in culture and can only survive for a few days, but after fusion with myeloma cells to produce hybridomas (Köhler and Milstein, 1975) they can be cultured indefinitely to yield unlimited quantities of monoclonal antibodies which, in contrast to polyclonal antisera, have uniform properties. Hybridomas are usually prepared from spleen cells but other tissues such as lymph nodes (Galfrè and Milstein, 1981) can also be used.

The immune system can synthesize antibodies against an almost infinite number of antigens before antigen contact occurs, each B cell secreting antibody which is specific for a single epitope. Antibody-secreting hybridomas can therefore be prepared from the cells of non-immunized animals (Campbell, 1984; Westerwoudt, 1985) although immunization produces better results because it induces clonal expansion of antigen-specific B cells and so increases the number available for fusion. The primary antibody response, which occurs after the first exposure to antigen, is dominated by relatively low affinity IgM antibodies. Subsequent antigen contact produces a much higher secondary antibody response which consists mainly of IgG isotypes, and the average antibody affinity is increased as a result of the selective expansion of high affinity antibody-secreting clones, a process known as affinity maturation (Roitt et al., 1985; Tijssen, 1985). Immunization therefore increases the probability that a cell fusion will produce hybridomas which secrete the high affinity antibodies that are necessary for the development of sensitive immunoassays.

4.2 IMMUNIZATION OF MICE

Although immunization with unconjugated AVP has been reported (Roth et al., 1966b; Wu and Rockey, 1969), small peptides tend to be poor immunogens so they are

usually conjugated to large carrier molecules to induce a better antibody response. However, antibodies raised against the carrier and the coupling agent (Briand et al., 1985; Edwards et al., 1989; Peeters et al., 1989) can cause problems with some screening assay formats. The AVP-capture ELISA, for example, produced false positive results (Section 3.3.3) because it detected antibodies that had been raised against components of the carrier preparation. Assays therefore need to be very carefully selected when immunization has been carried out with a peptide-carrier conjugate.

Bovine thyroglobulin, which is highly immunogenic (Skowsky and Fisher, 1972b), is the most commonly-reported carrier molecule for AVP and ANP, but BSA (Freisenhausen et al., 1976; Jüppner et al., 1986), KLH (Reilly and Root, 1986; Rosmalen et al., 1987), IgG (Wu and Rockey, 1969), IgA (Rougon-Rappuzi et al., 1977; Nishiuchi et al., 1986), polyvinylpyrillidone (Reilly and Root, 1986) and succinylated poly-L-lysine (Vallotton, 1971) have also been used. All of the reported anti-AVP MAbs (Hou-Yu et al., 1982; Jones et al., 1985; Robert et al., 1985; Work et al., 1985; Jurzak et al., 1990), and several anti-ANP MAbs (Milne et al., 1987; Mukoyama et al., 1988c; Prowse et al., 1989; Watanabe et al., 1989), were produced following immunization with thyroglobulin-conjugated peptides. ANP-KLH conjugate has also been used to produce some anti-ANP MAbs (John et al., 1986; Glembotski et al., 1987; Naomi et al., 1987).

Bovine thyroglobulin was the main carrier molecule used in this project but KLH was also tested as an alternative carrier for AVP.

4.2.1 AVP-THYROGLOBULIN

The first batch of AVP-thyroglobulin conjugate was prepared by the technical staff, and immunization begun, so that immune mice would be available at the start of this project. The conjugate was accidentally prepared with cyanamide (carbodiimide, mol. wt. 42.04) rather than EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride, mol. wt. 191.7) so the reaction conditions were incorrect,

which would have affected the composition of the final product. Mouse nos. 3363-3366 were primed and boosted twice with cyanamide-coupled conjugate then tail-bled for antibody titre estimation. Serum from mouse 3365 produced moderate AVP-coated-plate ELISA binding but the other sera contained very little anti-AVP antibody (Figure 3.1, Table 4.1) and all of them were SAM-RIA-negative (Table 4.1). Mouse 3365 was boosted with a larger dose of the cyanamide-coupled conjugate in week 12 of the immunization schedule but its serum antibody titre did not improve.

All subsequent work was carried out with EDC-coupled conjugate. The first batch was prepared without the neutralization step (Section 2.2.1.1) and, as a result, the acidic conditions caused immediate thyroglobulin precipitation which may have hindered conjugation to AVP. Boosting with this material, in week 16, had only a minor effect on the serum antibody titres estimated by AVP-coated-plate ELISA, and mouse 3366 was still SAM-RIA-negative (Table 4.1). Nevertheless, mouse 3363 was used in fusion VP1 (Table 4.6) which produced no anti-AVP MAbs but revealed that the AVP-capture ELISA (Section 3.3) was unsuitable for screening hybridomas raised against AVP-thyroglobulin conjugate.

After boosting with correctly-prepared conjugate (Section 2.2.1.1), in week 36, mouse nos. 3364-3366 became SAM-RIA-positive (Table 4.1). It was concluded that the anti-AVP response during the first four months of the immunization schedule had consisted entirely of IgM antibodies, which could be detected by the goat anti-mouse 'polyvalent immunoglobulins' HRP conjugate but not by the SAM-S-500 solid phase which bound mainly to IgG isotypes. The mice began to produce anti-AVP IgG after boosting with the correctly-prepared conjugate, but only mouse 3365 responded sufficiently well to be used in a cell fusion.

The next group of mice (nos. 3470-3477) was primed and boosted once with EDC-coupled conjugate prepared without the neutralization step, then boosted with correctly-prepared conjugate. All of the initial tail bleed sera were PEG-RIA-positive (Table 4.2). After a boost in week 18, the SAM-RIA titres of sera from mouse nos. 3474-3477 were less than 10% of their PEG-RIA titres (Table 4.2), which showed

TABLE 4.1

Immunization with AVP-thyroglobulin conjugate

Mouse nos. 3363-3366 were immunized by intraperitoneal injection of AVP-thyroglobulin conjugate at the intervals shown below. The indicated dose corresponds to the amount of AVP in the immunogen. Serum samples were assayed by AVP-coated-plate ELISA (10 ng/ml AVP, BSA-blocked wells, binding revealed by goat anti-mouse 'polyvalent immunoglobulins' HRP conjugate) or by SAM-RIA (results in parentheses). The antibody titre was defined as the serum dilution which produced an absorbance of 1.0 at 450 nm (AVP-coated-plate ELISA) or half-maximal ^{125}I-AVP binding (SAM-RIA). ND = not determined.

During the first 12 weeks of the immunization schedule a cyanamide-coupled conjugate was used. Subsequent boosting was carried out with a conjugate prepared by the EDC method (Section 2.2.1.1). A dash indicates an occasion when a mouse was not boosted.

Week	Dose	Serum antibody titre			
		3363	3364	3365	3366
0	40 μg	ND	ND	ND	ND
4	40 μg	ND	ND	ND	ND
8	40 μg	<1/500 (0)	<<1/500 (0)	~1/500 (0)	<<1/500 (0)
12	150 μg	–	–	~1/500 (0)	–
16	60 μg	<1/500	<1/500	~1/500	<1/500 (0)
22	50 μg	Cell fusion	–	–	–
36	50 μg		(<1/100)	(1/66,000)	(1/520)
40	50 μg		–	Cell fusion	–

TABLE 4.2

Immunization with AVP-thyroglobulin conjugate (EDC preparation)

Mouse nos. 3470-3477 were immunized by intraperitoneal injection of AVP-thyroglobulin conjugate prepared by the EDC method, the dose indicating the amount of AVP in the immunogen. Serum dilutions were assayed by PEG-RIA (asterisked results), by SAM-RIA, or by AVP-coated-plate ELISA (1 μg/ml AVP, unblocked wells, results in parentheses). ELISA binding was revealed with rabbit anti-mouse IgG (whole molecule) HRP conjugate. The antibody titre was defined as the serum dilution which produced an absorbance of 1.0 at 450 nm (AVP-coated-plate ELISA) or half-maximal ^{125}I-AVP binding (PEG-RIA and SAM-RIA). ND = not determined. A dash indicates an occasion when a mouse was not boosted.

Week	Dose	Serum antibody titre			
		3470	3471	3472	3473
0	10 μg	ND	ND	ND	ND
5	10 μg	ND	ND	ND	ND
10	10 μg	*1/600	*<1/100	*1/1000	*1/4000
14	10 μg	–	–	–	Cell fusion
24	50 μg	1/160	<1/100	1/100	
30	50 μg	ND	ND	ND	
32	50 μg	1/230	1/1000	1/240	
42	50 μg	1/1600 (1/4000)	1/440 (1/300)	1/2400 (1/2400)	
69	50 μg	Cell fusion	–	–	
		3474	3475	3476	3477
0	20 μg	ND	ND	ND	ND
5	20 μg	ND	ND	ND	ND
10	20 μg	*1/600	*1/1300	*1/9000	*1/500
18	50 μg	*1/2000 <1/100	*1/3500 ND	*1/35,000 1/3200	*1/2700 1/160
21	60 μg	–	–	1/32,000	–
24	50 μg	1/400	1/560	Cell fusion	1/130
30	50 μg	ND	ND		ND
32	50 μg	1/560	1/1000		1/125
42	50 μg	–	1/3200 (1/4200)		1/400 (1/3500)
54	40 μg	–	–		Cell fusion

that the anti-AVP IgG response was much smaller than the IgM response. The SAM-RIA titres gradually increased with additional boosting, but the later samples were not assayed by PEG-RIA so it is not known whether the ratio of anti-AVP IgG : IgM increased. Four of the mice were eventually used in cell fusions (Table 4.6).

One batch of AVP-thyroglobulin conjugate was prepared by the enhanced EDC method (Section 2.2.1.2). The inclusion of 5 mM N-hydroxysulphosuccinimide in the reaction mixture was reported to enhance the coupling of peptides to carrier molecules (Staros et al., 1986) so a stronger immune response was expected with this preparation. However, mouse nos. 3542-3549 produced such low anti-AVP IgG responses to the enhanced EDC-coupled conjugate, as determined by SAM-RIA (Table 4.3), that their serum antibody titres could not be estimated, whereas mouse nos. 3470-3477 had produced moderate anti-AVP IgG responses to the normal EDC-coupled conjugate (Table 4.2). This suggested that the enhanced EDC-coupled conjugate was actually less immunogenic than the normal EDC-coupled material, but there was no time available to carry out further study of this phenomenon.

4.2.2 AVP/LVP-KLH

Initial hybridoma screening results had shown that immunization with AVP-thyroglobulin conjugate generated anti-BSA and anti-immunoglobulin antibodies which caused false positive ELISA results if undiluted culture supernatants were assayed in wells coated with antibodies or BSA (Section 3.3.3). KLH was therefore investigated as an alternative carrier molecule because it was less likely to generate antibodies that would cross-react with mammalian proteins, so ELISA screening of undiluted culture supernatants might have been possible. This would have had the advantage of decreasing the workload, whilst minimizing the risk that antibody would be lost by adsorption onto equipment surfaces (Campbell, 1984), because the supernatant samples could have been transferred directly into the assay wells.

KLH was coupled to a mixture of AVP and LVP by m-maleimidobenzoic acid

TABLE 4.3

Immunization with AVP-thyroglobulin (enhanced EDC preparation)

Mouse nos. 3542-3549 were immunized by intraperitoneal injection of AVP-thyroglobulin conjugate prepared by the enhanced EDC method (Section 2.2.1.2). The dose shows the amount of AVP in the immunogen. Serum dilutions were assayed by SAM-RIA but low binding prevented antibody titre estimation. Non-specific binding has been subtracted from the values shown, which indicate the % ^{125}I-AVP bound by serum diluted 1/100 in Buffer L. ND = not determined.

Week	Dose	% ^{125}I-AVP bound							
		3542	3543	3544	3545	3546	3547	3548	3549
0	50 µg	ND	ND	ND	ND	ND	ND	ND	ND
5	48 µg	ND	ND	ND	ND	ND	ND	ND	ND
11	50 µg	6.6	0.3	1.5	6.4	0.0	0.6	0.7	5.1
36	50 µg	15.3	1.2	1.6	1.0	4.6	13.3	0.9	18.4

TABLE 4.4

Immunization with AVP/LVP-KLH conjugate

Mouse nos. 3538-3541 were given intraperitoneal injections of AVP/LVP-KLH conjugate (Section 2.2.1.3), where the dose indicates the total amount of AVP and LVP in the immunogen. Serum dilutions were assayed by SAM-RIA but low binding prevented antibody titre estimation. Non-specific binding has been subtracted from the values shown, which indicate the % ^{125}I-AVP bound by serum diluted 1/100 in Buffer L. ND = not determined.

Week	Dose	% ^{125}I-AVP bound			
		3538	3539	3540	3541
0	50 µg	ND	ND	ND	ND
5	40 µg	ND	ND	ND	ND
11	50 µg	0.5	0.4	0.5	0.7
36	50 µg	0.3	0.3	0.0	0.2

N-hydroxysuccinimide ester (MBS), a heterobifunctional reagent which links primary amine groups to free sulphydryl groups. AVP and LVP do not contain free sulphydryl groups so these were introduced by 2-iminothiolane (Traut et al., 1973; Jue et al., 1978) which acted on the N-terminal α-amino groups of AVP and LVP, and the lysine ε-amino group of LVP. The thiolated peptides were expected to react with MBS-modified KLH in such a way that both the AVP tail tripeptide and the LVP ring structure (which is identical to that of AVP) would be presented for antibody recognition on the surface of the KLH molecule. Although some LVP molecules would have coupled to KLH through their α-amino groups, so that antibodies would also have been raised against the LVP tail region, immunization with the AVP/LVP-KLH conjugate was expected to maximize the probability of raising antibodies against opposite ends of the AVP peptide and, therefore, of developing two-site AVP assays. However, when mouse nos. 3538-3541 were immunized with the AVP/LVP-KLH conjugate, no significant anti-AVP IgG responses were detected either by SAM-RIA (Table 4.4) or by AVP-coated-plate ELISA (data not shown) so none of these mice could be used in cell fusions.

4.2.3 ANP-THYROGLOBULIN

Mouse nos. 3478-3481 were immunized with a batch of EDC-coupled ANP-thyroglobulin conjugate which had been successfully used in previous immunizations (Prowse et al., 1989). After four injections of this immunogen, all of the mice produced reasonable anti-ANP IgG responses, as determined by ANP-coated-plate ELISA (Table 4.5), so all of them were used in cell fusion experiments (Table 4.8).

TABLE 4.5

Immunization with ANP-thyroglobulin conjugate

Mouse nos. 3478-3481 were immunized by intraperitoneal injection of ANP-thyroglobulin conjugate (Section 2.2.1.4) as indicated, each dose of immunogen containing 5 µg ANP. Serum dilutions were assayed by ANP-coated-plate ELISA (100 ng/ml ANP, unblocked wells). The antibody titre was defined as the serum dilution which produced an absorbance of 1.0 at 450 nm. ND = not determined.

A dash indicates an occasion when a mouse was not boosted.

Week	Dose	Serum antibody titre			
		3478	3479	3480	3481
0	5 µg	ND	ND	ND	ND
5	5 µg	ND	ND	ND	ND
10	5 µg	1/1000	1/2800	1/1000	1/700
40	5 µg	1/19,000	1/10,500	1/5300	1/5000
62	5 µg	–	Cell fusion	–	–
76	5 µg	Cell fusion		–	–
81	5 µg			Cell fusion	–
84	5 µg				Cell fusion

4.3 CELL FUSIONS

4.3.1 AVP FUSIONS

Fifteen cell fusions were carried out in an attempt to produce anti-AVP MAbs (Table 4.6). Ten of these fusions were done with freshly-prepared spleen cells from AVP-thyroglobulin-immunized mice, and the remainder were carried out after surplus spleen cells had been boosted *in vitro* (Section 4.3.3).

Fusion VP1 generated hybridomas in 378 culture wells (Table 4.6). Undiluted supernatants from 26 wells were AVP-capture ELISA-positive but investigation revealed that they did not contain anti-AVP antibodies (Section 3.3.3). Although the AVP-capture ELISA bound the anti-AVP antibody from high titre serum samples (Figure 3.14), it was unsuitable for hybridoma screening because it also bound anti-BSA and anti-immunoglobulin antibodies but did not bind anti-AVP MAbs (Table 3.11). No other screening assay was available at that time so if any anti-AVP antibody-secreting hybridomas were present they remained undetected.

Fusion VP1 was carried out by the standard method (Section 2.2.3.7) whereas fusion VP2, which used spleen cells from the same mouse, was done with NS-0 cells which had been adapted to grow in serum-free medium, and the fused cells were cultured in serum-free HAT/MTM medium. Fusion VP2 produced a few hybridomas but they were weak and did not survive. Their frailness was probably caused by the serum-free-adapted NS-0 cells, which were less robust than those grown in FCS medium, so no further serum-free fusions were attempted.

Fusions VP3 and VP4 were distributed into 25 cm^2 tissue culture flasks (5 ml/flask) so that large volumes of supernatant would be available for screening by PEG-RIA. (Replicate samples were essential because inefficient separation of unbound ^{125}I-AVP caused poor reproducibility in this assay.) The concentration of anti-AVP antibody in the supernatant was expected to be low therefore large samples (450 µl) were assayed to maximize sensitivity. All of the cultures were PEG-RIA-negative but

TABLE 4.6 AVP fusions

Suspensions of fused cells were diluted to 1×10^6 cells/ml and seeded into cultures of 0.2 ml, unless otherwise stated. The fusion efficiency was the number of cultures which contained hybridomas, expressed as a percentage of the total number of cultures seeded.

Mouse no.	Fusion no.	*In vitro* boost	Spleen : NS-0 ratio	Cultures seeded	Hybridoma cultures	Fusion efficiency (%)	Assay method	Ab-positive cultures	MAbs produced
3363	VP1	No	4:1	600	378	63	AVP-capture ELISA	26	0
	VP2	No	4:1	90	0	0	–	–	–
3473	VP3	No	1:1	4 x 5 ml	2	50	PEG-RIA	0	0
	VP4	No	3:2	5 x 5 ml	5	100	PEG-RIA	0	0
	VP5	Yes	1:2	2 x 7 ml	0	0	–	–	–
3365	VP6	No	4:1	48 x 1 ml	23	48	PEG-RIA	1	1
	VP7	No	4:1	48 x 1 ml	12	25	PEG-RIA	0	0
	VP8	Yes	3:2	48 x 0.5 ml	9	19	PEG-RIA	0	0
3476	VP9	No	5:1	300	126	42	SAM-RIA	0	0
	VP10	No	3:1	400	156	39	SAM-RIA	0	0
	VP11	Yes	5:6	180	6	3	SAM-RIA	0	0
							AVP-capture ELISA	110	
3477	VP12	No	2:1	585	466	80	AVP-coated-plate ELISA	1	1
							SAM-RIA	1	
	VP13	Yes	4:3	48 x 1 ml	34	71	AVP-coated-plate ELISA	0	0
3470	VP14	No	2:1	577	531	92	AVP-coated-plate ELISA	4	2
	VP15	Yes	2:1	180	0	0	–	–	–

this might have been caused by excessive antibody dilution if the cultures contained only a few anti-AVP antibody-secreting cells.

Fusions VP6 and VP7 were distributed into 1 ml cultures, which still allowed duplicate sampling for the PEG-RIA but decreased the dilution factor of any antibody in the supernatant. Fusion VP6 generated hybridomas in 23 cultures but only one was PEG-RIA-positive. This hybridoma was cloned, and subcloned, to produce a monoclonal cell line which secreted ESVP 1 MAb (Table 4.7). Fusion VP7 produced 12 hybridoma cultures but they were all PEG-RIA-negative.

The remaining cell fusions were distributed into 0.2 ml cultures.

Fusions VP9 and VP10 generated hybridomas in 282 culture wells. These were all SAM-RIA-negative, even though the spleen donor (mouse 3476, Table 4.2) had produced a high serum anti-AVP antibody titre in response to immunization, but some anti-AVP antibodies might have been missed because they could not bind ^{125}I-AVP.

Fusion VP12 generated hybridomas in 466 culture wells. One hybridoma secreted anti-AVP antibody but was difficult to clone because it was unstable. The first cloning plate became contaminated, and by the time a second cloning plate was set up, 10 days after first identifying the hybridoma, non-secreting cells were rapidly taking over the parent culture so the clones were all antibody-negative. Although cells were stored before the parent hybridoma stopped secreting antibody, most of them were non-secretors therefore hundreds of cloning wells had to be seeded to maximize the probability of isolating antibody-secreting cells. The stored cells produced clones in 2535 culture wells, 11 of which were SAM-RIA-positive but only one clone, from a well which contained multiple cell colonies, was sufficiently stable to produce antibody-positive subclones. The best subclone was cloned again to produce the final monoclonal cell line which secreted ESVP 2 MAb (Table 4.7).

Fusion VP14 generated hybridomas in 531 culture wells, four of which were anti-AVP antibody-positive. Two hybridomas were lost because they stopped secreting antibody before the parent cells could be stored, and all of their clones were antibody-negative. The other two hybridomas were cloned, and subcloned, to produce

TABLE 4.7

Monoclonal cell lines produced in AVP fusions

Hybridoma cell line	MAb secreted
VP6/7.1.4	ESVP 1
VP12/339.5.3.2	ESVP 2
VP14/247.6.1	ESVP 3
VP14/308.1.1	ESVP 4

monoclonal cell lines which secreted ESVP 3 MAb and ESVP 4 MAb (Table 4.7).

4.3.2 ANP FUSIONS

Three anti-ANP MAbs (ESA 4, ESA 5 and ESA 9) were available at the start of this project. In an attempt to produce additional anti-ANP MAbs, six cell fusions were carried out (Table 4.8), four of which were done with freshly-prepared spleen cells from ANP-thyroglobulin-immunized mice, while two fusions were done with *in vitro*-boosted spleen cells (Section 4.3.3).

Fusion NP1 had a disappointingly low fusion efficiency (Table 4.8). It produced only three hybridoma cultures, which were all ANP-coated-plate ELISA-negative.

In contrast, fusion NP2 generated hybridomas in 553 culture wells, four of which were ANP-coated-plate ELISA-positive. Two hybridomas soon stopped secreting antibody, while another was abandoned because it always produced low assay results and was found to secrete an IgM antibody. The remaining hybridoma was cloned, and subcloned, to produce a monoclonal cell line which secreted ESNP 1 MAb (Table 4.9).

Fusion NP4 produced hybridomas in 416 culture wells, three of which were ANP-coated-plate ELISA-positive. Two hybridomas were easy to clone, and subclone, to produce monoclonal cell lines which secreted ESNP 2 MAb and ESNP 4 MAb

TABLE 4.8

ANP fusions

Suspensions of fused cells were diluted to 1×10^6 cells/ml and seeded into 0.2 ml cultures. The fusion efficiency was the number of cultures which contained hybridomas, expressed as a percentage of the total number of cultures seeded. Culture supernatants were screened by ANP-coated-plate ELISA, and were considered antibody-positive if they produced absorbances which exceeded 0.3. Some hybridomas subsequently ceased to secrete antibody.

In vitro-boosted spleen cells were used in fusions NP3 and NP6.

Mouse no.	Fusion no.	Spleen : NS-0 ratio	Cultures seeded	Hybridoma cultures	Fusion efficiency, %	Ab-positive cultures	MAbs produced
3479	NP1	2:1	600	3	0.5	0	0
3478	NP2	2:1	607	553	91.1	4	1
	NP3	2:1	180	1	0.5	0	0
3480	NP4	2:1	600	416	69.3	3	3
3481	NP5	2:1	558	44	7.9	0	0
	NP6	1:1	280	31	11.1	0	0

TABLE 4.9

Monoclonal cell lines produced in ANP fusions

Hybridoma cell line	MAb secreted
NP2/63.1.1	ESNP 1
NP4/186.3.1	ESNP 2
NP4/213.1.1	ESNP 3
NP4/279.11.4	ESNP 4

(Table 4.9). The third hybridoma initially produced positive clones but they soon stopped secreting antibody, and 73 subclones were all antibody-negative. The parent hybridoma was still strongly positive so it was recloned but no further antibody-positive clones were obtained. However, the antibody-secreting cells released so much antibody that cultures which contained few cells could generate high ELISA absorbances. Non-secreting cells were therefore concealed until they had taken over a culture, because the ELISA readings decreased only when exchange feeding diluted the residual antibody. The parent hybridoma eventually stopped secreting antibody but cells had been stored before this happened. The stored cells produced clones in 1185 culture wells, six of which were ANP-coated-plate ELISA-positive although only two clones continued to secrete antibody. One of these was subcloned to produce the final monoclonal cell line which secreted ESNP 3 MAb (Table 4.9).

Fusion NP5 had a low fusion efficiency and produced only 44 hybridoma cultures, all of which were ANP-coated-plate ELISA-negative.

4.3.3 FUSIONS WITH *IN VITRO*-BOOSTED SPLEEN CELLS

Most spleen homogenates contained more cells than were required for a cell fusion. The surplus cells were boosted *in vitro* then used in additional cell fusions

because it was reported that memory B cells, generated by *in vivo* immunization, can be activated *in vitro* (De Boer et al., 1988) to produce an increase in both the fusion efficiency and the production of antigen-specific antibodies (Erich et al., 1989).

Many workers favour serum-free medium for *in vitro* immunization (Reading, 1982; Van Ness et al., 1984; Darfler, 1987; De Boer et al., 1989), but when spleen cells from mouse 3473 were *in vitro*-boosted in serum-free medium only 16% of the cells survived the 4 day culture period (Table 4.10). In contrast, there was a 46% survival rate when spleen cells from mouse 3365 were *in vitro*-boosted in 15% FCS medium. All other *in vitro* boosts were therefore done in 15% FCS medium, and cell survival rates of 34-60% were recorded (Table 4.10).

In vitro-boosting did not produce the expected increase in fusion efficiency. Instead, it seemed to decrease the ability of spleen cells to undergo successful cell fusion, because most fusions which were carried out with *in vitro*-boosted cells had lower efficiencies than those done with freshly-prepared cells from the same mouse (Tables 4.6 and 4.8). Fusion NP6 was the exception because it had a higher efficiency than fusion NP5 which was done with freshly-prepared spleen cells (Table 4.8), but this difference occurred because the cells from fusion NP5 had a low viability as a result of an unusually long exposure to PEG 4000.

Five cell fusions were carried out with *in vitro*-boosted spleen cells from AVP-thyroglobulin-immunized mice (Table 4.6). Fusions VP5 and VP15 did not produce any hybridomas at all. Fusions VP8, VP11 and VP13 generated hybridomas in 9, 6 and 34 culture wells, respectively, but no anti-AVP antibodies were detected.

Two cell fusions were carried out with *in vitro*-boosted spleen cells from ANP-thyroglobulin-immunized mice (Table 4.8). Fusion NP3 generated one hybridoma culture, while fusion NP6 generated 31 hybridoma cultures, but all of these were ANP-coated-plate ELISA-negative.

TABLE 4.10

Survival rate of *in vitro*-boosted spleen cells

Immune mouse spleen cells were boosted *in vitro* by culture in the presence of antigen (Section 2.2.3.8). Cells from mouse 3473 were cultured with EDC-coupled AVP-thyroglobulin conjugate in serum-free medium containing 30% serum-free MTM (Section 2.2.3.3, prepared using serum-free medium instead of 15% FCS medium), while those from mouse nos. 3365, 3470, 3476 and 3477 were cultured with unconjugated AVP in MTM/FCS medium. Spleen cells from mouse nos. 3478 and 3481 were cultured with unconjugated ANP in MTM/FCS medium.

Mouse no.	No. cells cultured	No. cells surviving	% cells surviving	Fusion no.
3473	15×10^6	2.4×10^6	16.0	VP5
3365	30×10^6	14×10^6	46.7	VP8
3476	50×10^6	17×10^6	34.0	VP11
3477	50×10^6	27×10^6	54.0	VP13
3470	55×10^6	23×10^6	41.8	VP15
3478	73×10^6	28×10^6	38.4	NP3
3481	50×10^6	30×10^6	60.0	NP6

4.4 BULK PRODUCTION OF MONOCLONAL ANTIBODIES

Large quantities of MAbs were required for the two-site immunometric assay work (Chapters 6 and 8). The monoclonal anti-AVP and anti-ANP cell lines were therefore either injected into pristane-primed mice for ascitic fluid production (Section 2.2.3.14) or grown in bulk culture (Section 2.2.3.15).

4.4.1 MONOCLONAL ANTIBODIES TO AVP

Ascitic fluid production was attempted with all four anti-AVP MAb-secreting cell lines (Table 4.7) but only three of them induced ascites tumours.

Hybridoma VP6/7.1.4 was injected into ten mice, all of which produced ascitic fluid containing ESVP 1 MAb (Table 4.11). Ascitic fluid tapping began 10-21 days after injection of the cells and was completed by day 25 (total volume 65.5 ml), no mouse surviving for more than 7 days after the start of tapping. Despite some fluctuation, the ESVP 1 MAb titres of the ascitic fluid tended to increase over the tapping period and titres ranging from 1/6200 to 1/125,000 were recorded (Table 4.11).

Five mice, which had been injected with hybridoma VP14/247.6.1, produced 34.5 ml ESVP 3 MAb-containing ascitic fluid (Table 4.12). The tapping period was similar to that for ESVP 1 MAb-containing fluid (Table 4.11) but the ESVP 3 MAb titres were lower, ranging from 1/2200 to 1/18,000 (Table 4.12).

In contrast, hybridoma VP14/308.1.1 was slow to produce ascites tumours. It was injected into five mice but only one produced ESVP 4 MAb-containing ascitic fluid, on day 39 (Table 4.13). When the other four mice were given a second injection of cells, three of them produced ascitic fluid although one mouse was not ready for tapping until 62 days after the second injection. A total of 37 ml ascitic fluid was eventually collected. The ESVP 4 MAb titres were lower than expected (maximum titre 1/540) and could not be estimated in ascitic fluid from two of the mice (Table 4.13), therefore hybridoma VP14/308.1.1 was also grown in bulk culture to

TABLE 4.11

ESVP 1 MAb production in ascitic fluid

Mouse nos. 3504-3513 were pristane-primed then given intraperitoneal injections of hybridoma VP6/7.1.4 (5×10^5 cells/mouse). Ascitic fluid was collected whenever abdominal distension was evident. Antibody titres were defined as the ascitic fluid dilutions which produced half-maximal ^{125}I-AVP binding in the SAM-RIA. A dash indicates an occasion when fluid was not collected.

Day	Ascitic fluid antibody titre									
	3504	3505	3506	3507	3508	3509	3510	3511	3512	3513
10	–	–	–	1/8000	–	–	–	1/25,000	–	–
11	–	–	–	–	–	–	–		–	–
12	–	–	–	1/6200	–	–	1/10,000		–	–
13	–	–	–	1/10,000	–	–	–		–	–
14	–	–	–	1/11,000	–	–	1/35,000		–	–
15	–	–	–	–	–	–	–		–	–
16	–	–	–	1/58,000	1/21,000	1/20,000	1/62,000		–	–
17	–	–	–		1/40,000	1/28,000			–	–
18	–	1/6600	–		1/18,000	–			–	–
19	–	–	–		–	1/11,000			–	–
20	–	–	1/14,000		1/100,000	1/23,000			1/16,000	1/6200
21	1/23,000	1/20,000	–		–	–			–	–
22	–	1/27,000	1/47,000		1/125,000	1/100,000			–	1/114,000
23	1/8000		1/11,000						1/70,000	–
24	–									1/18,000
25	1/45,000									

TABLE 4.12

ESVP 3 MAb production in ascitic fluid

Pristane-primed mice (nos. 3584-3588) were given intraperitoneal injections of hybridoma VP14/247.6.1 (5×10^5 cells/mouse). Ascitic fluid was collected whenever abdominal distension was evident. The antibody titre was defined as the ascitic fluid dilution which produced an absorbance of 1.0 at 450 nm (AVP-coated-plate ELISA, using sheep anti-mouse IgG (whole molecule) HRP conjugate to reveal binding). A dash indicates an occasion when fluid was not collected.

Day	Ascitic fluid antibody titre				
	3584	3585	3586	3587	3588
11	–	1/5000	–	–	–
12	–	–	–	–	–
13	–	1/7400	1/5000	1/2200	1/5400
14	–	1/9000	1/7000	1/4800	
15	–		1/12,000	–	
16	–			1/6200	
17	–			–	
18	–			1/13,000	
19	–				
20	1/3500				
21	1/5300				
22	–				
23	–				
24	1/9000				
25	–				
26	1/18,000				

TABLE 4.13

<u>ESVP 4 MAb production in ascitic fluid</u>

Pristane-primed mice (nos. 3589-3593) were given intraperitoneal injections of hybridoma VP14/308.1.1 (5×10^5 cells/mouse), but only mouse 3592 produced ascitic fluid in response. The other mice were given a further dose of pristane on day 39, followed by 1×10^6 hybridoma VP14/308.1.1 cells/mouse on day 44. Ascitic fluid was collected whenever abdominal distension was evident, and was assayed by PEG-RIA but low binding prevented antibody titre estimation in some samples. Non-specific binding has been subtracted from the values shown, which indicate the % ^{125}I-AVP bound by ascitic fluid diluted 1/100 in Buffer L. The antibody titres which could be estimated are shown in parentheses, and were defined as the ascitic fluid dilutions which produced half-maximal ^{125}I-AVP binding.

No ascitic fluid was produced by mouse 3591. Dashes indicate occasions when fluid was not collected.

Day	% ^{125}I-AVP bound			
	3589	3590	3592	3593
39	-	-	60.5 (1/400)	-
60	-	9.6		-
68	-			46.0 (1/180)
70	-			53.0 (1/300)
71	-			60.0 (1/400)
73	-			74.2 (1/540)
106	11.5			
110	11.0			
112	17.2			
113	20.2			
115	25.9			
117	31.1			

TABLE 4.14

Anti-AVP MAb production in culture

When monoclonal anti-AVP cell lines were grown to exhaustion in FCS medium, the antibody titres obtained were typically as shown below. Antibody titres were defined as the culture supernatant dilutions which produced half-maximal ^{125}I-AVP binding in the SAM-RIA (for ESVP 1 and ESVP 2) or the PEG-RIA (for ESVP 3 and ESVP 4).

MAb	Supernatant antibody titre
ESVP 1	1/320
ESVP 2	1/56
ESVP 3	1/6
ESVP 4	1/13

produce 500 ml ESVP 4 MAb-containing culture supernatant (Table 4.14).

Hybridoma VP12/339.5.3.2 would not grow in ascites. It was injected into five mice, which had not responded by day 46 so they were given an additional dose of pristane followed, 4 days later, by 1×10^6 hybridoma VP12/339.5.3.2 cells/mouse. No ascites tumours formed so the cells were grown in bulk culture instead, to produce 1 litre ESVP 2 MAb-containing culture supernatant (Table 4.14).

Hybridomas VP6/7.1.4 and VP14/247.6.1 were not grown in bulk culture, but the ESVP 1 and ESVP 3 MAb titres attainable in spent culture supernatant have been included in Table 4.14 for comparison.

4.4.2 MONOCLONAL ANTIBODIES TO ANP

The cause of the poor ascites response of hybridomas VP12/339.5.3.2 and VP14/308.1.1 (Section 4.4.1) was unknown so no attempt was made to grow the anti-ANP MAb-secreting cell lines (Table 4.9) in ascites. They were instead grown in bulk culture, each producing 500 ml anti-ANP MAb-containing supernatant (Table 4.15).

TABLE 4.15

Anti-ANP MAb production in culture

Bulk production of anti-ANP MAbs was achieved by culturing the monoclonal cell lines to exhaustion in FCS medium. The antibody titre was defined as the culture supernatant dilution which produced an absorbance of 1.0 at 450 nm in the ANP-coated-plate ELISA.

MAb	Supernatant antibody titre
ESNP 1	1/440
ESNP 2	1/840
ESNP 3	1/3000
ESNP 4	1/530

4.5 PURIFICATION OF MONOCLONAL ANTIBODIES

4.5.1 MONOCLONAL ANTIBODIES TO AVP

4.5.1.1 Sodium Sulphate Precipitation

ESVP 1 and ESVP 3 MAbs were partially purified and 5-fold concentrated from ascitic fluid by sodium sulphate precipitation (Section 2.3.1.1). 370 mg ESVP 1 and 137 mg ESVP 3 were obtained from 65.5 ml and 34.5 ml ascitic fluid, respectively. However, ascitic fluid also contains endogenous mouse antibodies which co-precipitate with MAbs (Galfrè and Milstein, 1981; Baines et al., 1990) so the ESVP 1 and ESVP 3 preparations would have been contaminated by these antibodies. Few other components were present, because iodinated ESVP 1 eluted from Sephacryl S-300 as one major peak of radioactivity corresponding to IgG (Figure 4.1A), while iodinated ESVP 3 eluted as two peaks (Figure 4.1B) which corresponded to a major peak of IgM (^{125}I-ESVP 3) and a smaller peak of endogenous mouse IgG.

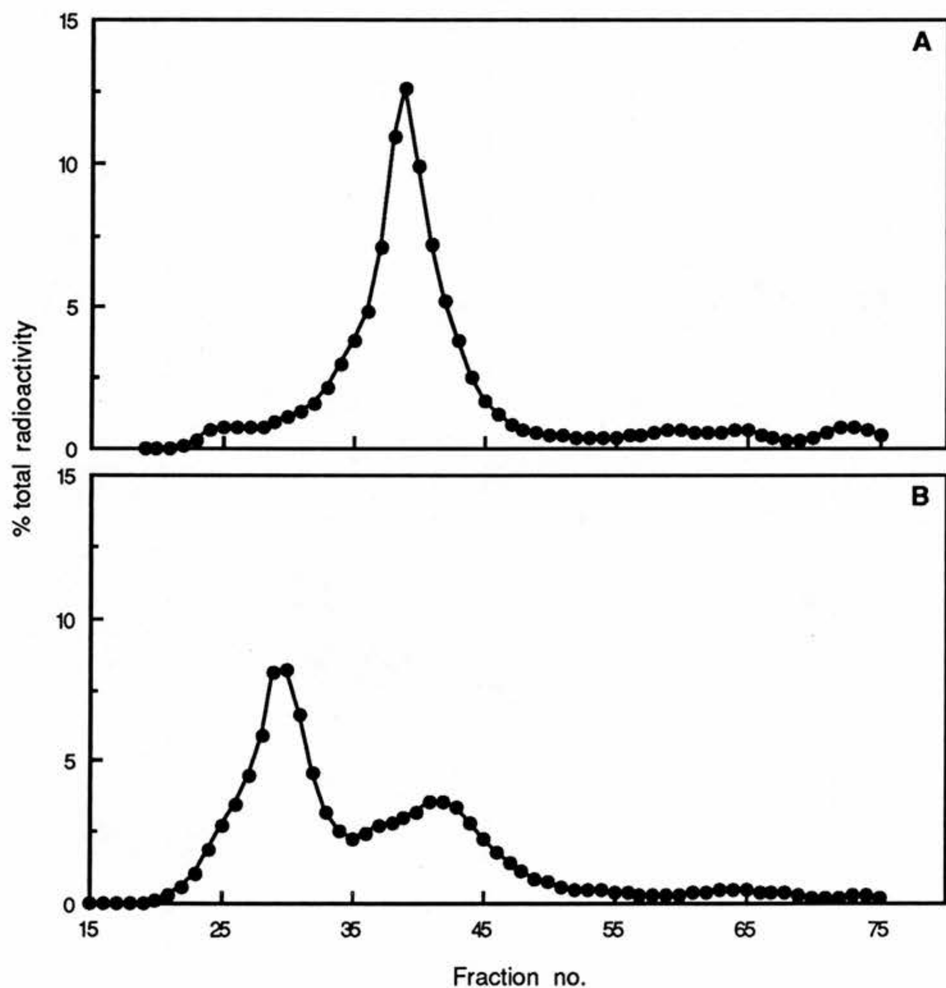

FIGURE 4.1

Elution of ^{125}I-ESVP 1 and ^{125}I-ESVP 3 from Sephacryl S-300

ESVP 1 (**A**) and ESVP 3 (**B**) were prepared from ascitic fluid by sodium sulphate precipitation, then radioiodinated (Section 2.3.4.2) and purified by gel filtration on a Sephacryl S-300 column (Section 2.3.4.3). The radioactivity of each fraction is expressed as a percentage of the total applied to the column.

4.5.1.2 PEG 8000 Precipitation

ESVP 2 and ESVP 4 MAbs were concentrated from spent culture supernatant by PEG 8000 precipitation (Section 2.3.1.2) because sodium sulphate was reportedly unsuitable for precipitating MAbs from culture supernatants (Baines et al., 1990).

In a preliminary study, the ESVP 2 MAb precipitated by 10% (w/v) PEG 8000 was collected after a 2 hour incubation period but the antibody titre of the supernatant indicated that a significant amount of ESVP 2 remained in solution (Table 4.16, Experiment 1). The bulk culture supernatant (450 ml) was therefore incubated with PEG 8000 for 16 hours before collection of the precipitate, but even this was insufficient to achieve maximal precipitation because a second ESVP 2 MAb-containing precipitate formed during storage of Supernatant I (Table 4.16, Experiment 2) and the antibody titre of Supernatant II suggested that it still contained about half of the original ESVP 2 activity. Precipitate II was purer than Precipitate I because it had a higher antibody titre despite a lower protein concentration (Table 4.16).

IgA precipitation requires higher PEG concentrations than are needed for IgG precipitation (Cripps et al., 1983) therefore 15% (w/v) PEG 8000 was selected for the preparation of ESVP 4 MAb from culture supernatant. In contrast to ESVP 2 MAb, a preliminary experiment showed that more than 90% of ESVP 4 activity was precipitated after a 2 hour incubation period (Table 4.17). For convenience, however, the ESVP 4 MAb-containing bulk culture supernatant (450 ml) was incubated with PEG 8000 for 4 days before collection of the precipitate (86.6 mg).

4.5.1.3 Protein A-Sepharose Affinity Chromatography

Highly purified ESVP 2 MAb was prepared from spent culture supernatant by affinity chromatography on Protein A-Sepharose (Section 2.3.1.3). ESVP 3 and ESVP 4 could not be purified in this way because mouse IgM and IgA antibodies do not bind

TABLE 4.16

PEG 8000 precipitation of ESVP 2 from culture supernatant

ESVP 2 MAb-containing culture supernatant was mixed with 30% (w/v) PEG 8000 in Buffer E, to give a final PEG concentration of 10% (w/v), then incubated at room temperature for 2 hours (Experiment 1) or at 4°C for 16 hours (Experiment 2) before collection of the precipitate (Section 2.3.1.2). Further precipitation occurred during storage, at 4°C, of Supernatant I from Experiment 2. Precipitate II formed within 24 hours but was not collected until day 4.

The antibody titre was defined as the sample dilution which produced an absorbance of 1.0 at 450 nm in the AVP-coated-plate ELISA. ND = not determined.

Sample	Experiment 1			Experiment 2		
	Volume (ml)	Antibody titre	Protein (mg/ml)	Volume (ml)	Antibody titre	Protein (mg/ml)
Starting material	35	1/135	ND	450	1/56	ND
Supernatant I	52.5	1/64	ND	675	1/35	ND
Precipitate I	3.5	1/500	2.2	8.0	1/420	6.6
Supernatant II	–	–	–	675	1/28	ND
Precipitate II	–	–	–	2.0	1/1800	4.2

TABLE 4.17

PEG 8000 precipitation of ESVP 4 from culture supernatant

ESVP 4 MAb-containing culture supernatant was mixed with an equal volume of 30% (w/v) PEG 8000 in Buffer E, then incubated at room temperature for 2 hours before collection of the precipitated protein.

The antibody titre was defined as the sample dilution which produced an absorbance of 1.0 at 450 nm in the AVP-coated-plate ELISA. ND = not determined.

Sample	Volume (ml)	Antibody titre	Protein (mg/ml)
Culture supernatant	44	1/10	ND
PEG supernatant	88	<1/2	ND
PEG precipitate	4.4	1/90	3.0

to protein A (Ey et al., 1978).

Mouse IgG1 was reported to have a weak affinity for Protein A-Sepharose (Underwood et al., 1983; Jiskoot et al., 1991), binding at high pH values and eluting in the pH range 6.0-7.0 (Ey et al., 1978). However, an initial trial showed that there was still some interaction between protein A and ESVP 2 at pH 6.0 because elution was retarded in 0.1 M sodium citrate buffer, pH 6.0, so that ESVP 2 emerged in a broad peak of fifteen fractions (data not shown). In contrast, pH 5.0 buffer eluted ESVP 2 in a sharp peak of only three fractions (Figure 4.2). Elution at pH 5.0 was therefore selected for bulk purification of ESVP 2 because it produced the most concentrated antibody solution.

The Protein A-Sepharose column (bed volume 10 ml) was expected to bind about 50 mg IgG (Ey et al., 1978) but it failed to bind all of the ESVP 2 from 390 ml culture supernatant, even though only 4.07 mg protein could be eluted at pH 5.0. The column initially extracted about 99% of ESVP 2 from the culture supernatant but became less efficient as loading progressed, so that the final drops of supernatant to pass through the column had almost the same antibody titre as the starting material. There was

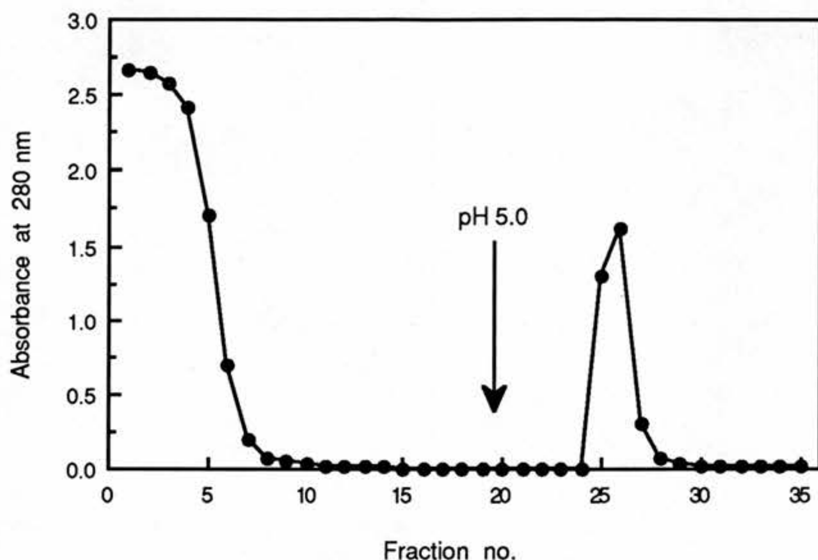

FIGURE 4.2

Elution of ESVP 2 MAb from Protein A-Sepharose

ESVP 2 MAb-containing culture supernatant (390 ml, pH 8.7) was loaded onto a Protein A-Sepharose column as described in Section 2.3.1.3. Fraction collection began when all of the supernatant had been applied. Bound antibody was eluted by 0.1 M sodium citrate buffer, pH 5.0.

also continual SAM-RIA-detectable leakage of ESVP 2 activity during the washing step, which suggested that ESVP 2 did not bind tightly to protein A at pH 8.7 despite a report that IgG1 was fully retained at pH 8.0 (Ey et al., 1978). However, Underwood et al. (1983) showed that MAbs of the same isotype could have widely differing affinities for protein A and that the concentration of IgG bound by a Protein A-Sepharose column was dependent on the binding affinity. ESVP 2 may therefore have had a particularly low affinity for protein A.

Iodination of ESVP 2, followed by gel filtration (Figure 4.3), revealed that protein A-purified MAb was much purer than PEG-precipitated MAb since the latter contained a large quantity of high molecular weight material which was unable to bind to the SAM-S-500 solid phase (Figure 4.3A). This result was confirmed when 1 ml PEG-precipitated ESVP 2 (6.6 mg protein) was protein A-purified and only 0.5 mg protein was retained on the column, which implied that more than 90% of the

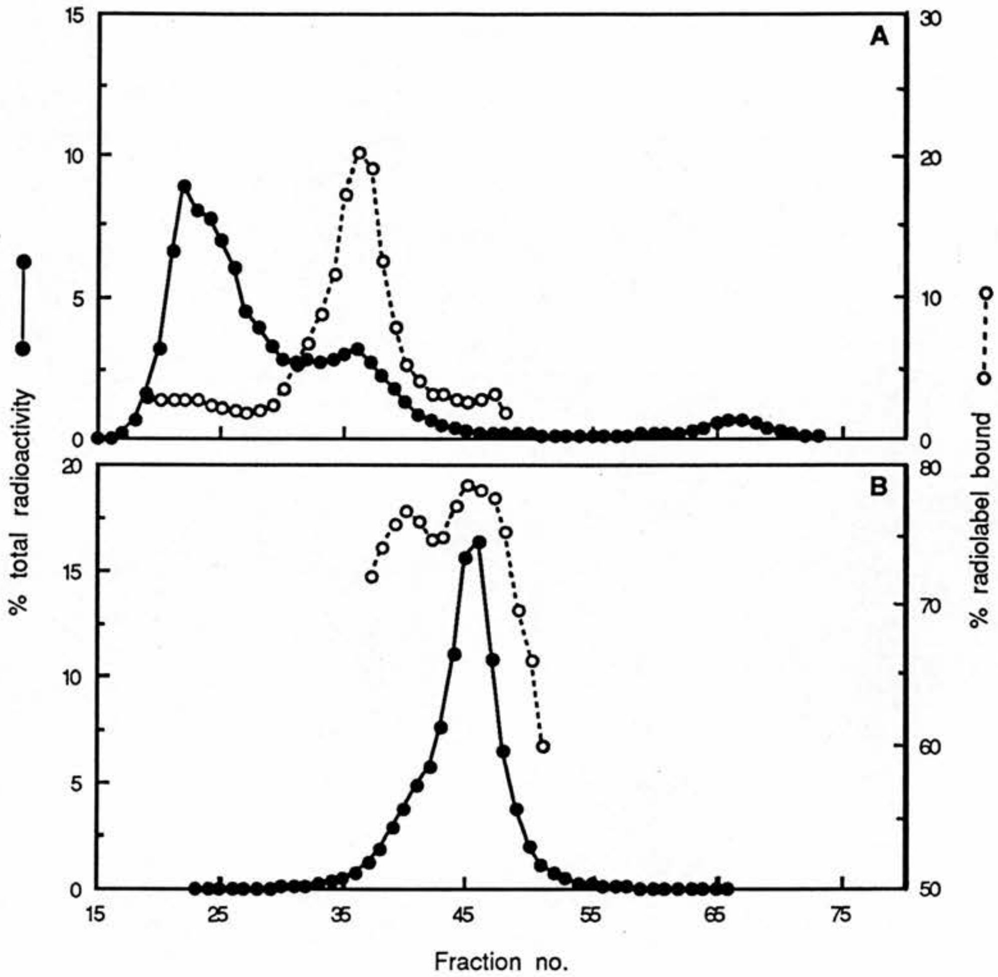

FIGURE 4.3

Elution of ^{125}I-ESVP 2 from Sephacryl S-300

ESVP 2 MAb was prepared from spent culture supernatant by PEG 8000 precipitation (**A**; Precipitate I (Experiment 2), Table 4.16) or Protein A-Sepharose affinity chromatography (**B**), then radioiodinated (Section 2.3.4.2) and purified by gel filtration on a Sephacryl S-300 column (Section 2.3.4.3). Iodinated material (●) was eluted in 2.3 ml (**A**) or 1.95 ml (**B**) fractions. The radioactivity of each fraction is expressed as a percentage of the total applied to the column.

The eluate was assayed for ^{125}I-IgG (○) by agitating a mixture of 50 µl column fraction and 50 µl SAM-S-500 solid phase (25% (settled volume) slurry in Buffer R) on an orbital shaker for 1 hour at room temperature. Bound ^{125}I-IgG was separated from unbound ^{125}I-proteins by the sucrose layering technique (Section 2.2.4.4).

PEG-precipitated preparation consisted of non-IgG substances. In contrast, there were no high molecular weight materials in the protein A-purified ESVP 2 preparation, although it seemed to contain two IgG components which were partially separated by gel filtration on Sephacryl S-300 (Figure 4.3B).

4.5.2 MONOCLONAL ANTIBODIES TO ANP

ESNP 1, ESNP 2, ESNP 3 and ESNP 4 MAbs were each concentrated from 500 ml spent culture supernatant by precipitation with 12.5% (w/v) PEG 8000 (Section 2.3.1.2), yielding 101.9, 69.4, 108.5 and 104.0 mg protein, respectively. A preliminary study had shown that significant amounts of MAb remained in solution after protein precipitation with 10% (w/v) PEG 8000 (Table 4.18, Experiment 1), as previously seen with ESVP 2 MAb (Table 4.16). Increasing the PEG 8000 concentration to 12.5% (w/v) decreased the residual antibody titre of the supernatant (Table 4.18, Experiment 2) but still did not precipitate all of the MAb. Centrifugation at 40,000 x g, rather than 2000 x g, made no difference to the antibody titre of the supernatant (data not shown) so the failure to collect all of the MAb was not due to inadequate centrifugation.

Samples of ascitic fluid containing ESA 4, ESA 5 and ESA 9 (2.5 ml each), which were a gift from Dr. C. Prowse, were purified by sodium sulphate precipitation (Section 2.3.1.1), yielding 33.0, 23.1 and 15.3 mg protein, respectively.

4.6 PURIFICATION OF POLYCLONAL ANTIBODIES

4.6.1 ANTI-AVP SERUM

The anti-AVP serum was a pool of rabbit antisera which had been raised against both unconjugated AVP and AVP-BSA. Insoluble material was removed by centrifugation at 2000 x g then the antibody was partially purified by sodium sulphate precipitation (Section 2.3.1.1), yielding 6.956 g protein from 476 ml antiserum.

TABLE 4.18

Effect of PEG 8000 concentration on anti-ANP MAb precipitation

Anti-ANP MAbs were precipitated from culture supernatant by PEG 8000 in Buffer E, at a final PEG concentration of 10% (w/v) (Experiment 1) or 12.5% (w/v) (Experiment 2), and collected after incubation at room temperature for 30 minutes (Experiment 1) or at 4°C for 4 days (Experiment 2). The supernatants were assayed by ANP-coated-plate ELISA before, and after, PEG 8000 precipitation. The antibody titre was defined as the sample dilution which produced an absorbance of 1.0 at 450 nm.

MAb	Antibody titre			
	Experiment 1		Experiment 2	
	Starting material	PEG supernatant	Starting material	PEG supernatant
ESNP 1	1/150	1/45	1/440	1/40
ESNP 2	1/470	1/250	1/840	1/70
ESNP 3	–	–	1/3000	1/400
ESNP 4	1/1100	1/240	1/530	1/24

4.6.2 ANTI-PAG SERUM

The haemolysed rabbit anti-PAG serum, which had been raised against a PAG-thyroglobulin conjugate, was purified by PEG 8000 precipitation, a method which was reportedly more suitable for haemolysed sera than was salt precipitation (Tijssen, 1985). Cripps et al. (1983) showed that half of the IgM in human serum was precipitated by 4% (w/v) PEG 8000 while most of the IgG was precipitated by 4-8% (w/v) PEG 8000, therefore the anti-PAG serum was fractionated by 0-4%, 4-8% and 8-13% (w/v) PEG 8000. When the fractions were assayed (Table 4.19), most of the antibody activity was found in the 4-8% PEG cut while almost none remained in the 13% PEG supernatant, which contained most of the haemoglobin.

Iodination of ESVP 2 MAb had shown that PEG precipitates were very impure (Figure 4.3) therefore the PEG cuts were further purified by Rivanol precipitation (Section 2.3.2.2), which removes most serum proteins leaving relatively pure IgG in solution (Franek, 1986). The PEG cuts were not dialysed before Rivanol precipitation because PEG fractionation was expected to have decreased the concentration of chloride ions, in which Rivanol is insoluble. However, the Rivanol formed a yellow precipitate from the PEG cuts, rather than the expected sticky brown paste, and the 4-8% and 8-13% PEG cuts were still reddish-brown after filtration. This was thought to indicate that some of the Rivanol had been precipitated by residual NaCl in the PEG cuts, particularly as a second volume of Rivanol, added to the 4-8% and 8-13% PEG cuts, precipitated an orange-brown paste and produced a clear yellow filtrate.

The 0-4% PEG cut was expected to consist mostly of IgM (Cripps et al., 1983), which is precipitated by Rivanol (Franek, 1986), so the large decrease in antibody titre produced by Rivanol precipitation (Table 4.19) had been anticipated. However, Rivanol precipitation also produced an unexpectedly large decrease in the antibody titres of the 4-8% and 8-13% PEG cuts (Table 4.19), which should have consisted mainly of IgG (Cripps et al., 1983). Rivanol was not expected to precipitate IgG (Franek, 1986) but the extra Rivanol which was added to the 4-8% and 8-13% PEG

TABLE 4.19

Preparation of IgG from anti-PAG serum

Haemolysed anti-PAG serum (400 ml) was purified by PEG 8000 fractionation followed by Rivanol precipitation (Section 4.6.2). Antibody titres were defined as the sample dilutions which produced half-maximal ^{125}I-AVP binding in the PEG-RIA. spnt = supernatant.

PEG cut	After PEG fractionation			After Rivanol precipitation			
	Volume (ml)	Antibody titre	% Antibody activity	Volume (ml)	Antibody titre	% Antibody activity	Protein (mg)
0-4%	25	1/26,000	16.1	24	1/170	53.0	48
4-8%	45	1/70,000	78.2	47	1/65	39.7	62
8-13%	25	1/9000	5.6	28	1/20	7.3	38
13% spnt	600	<1/4	<0.1	–	–	–	–

TABLE 4.20

Preparation of IgG from anti-ANP serum

Antibody was collected from 200 ml haemolysed anti-ANP serum by PEG 8000 fractionation (Section 2.3.2.1). Each PEG cut was dialysed against 50 mM sodium acetate then purified by Rivanol precipitation (Section 2.3.2.2). The antibody titre was defined as the sample dilution which produced an absorbance of 1.0 at 450 nm in the ANP-coated-plate ELISA, using donkey anti-rabbit IgG HRP conjugate to reveal binding. spnt = supernatant.

PEG cut	After PEG fractionation			After Rivanol precipitation			
	Volume (ml)	Antibody titre	% Antibody activity	Volume (ml)	Antibody titre	% Antibody activity	Protein (mg)
0-5%	10	1/14,500	22.0	12.5	1/2000	9.3	24
5-10%	22	1/21,000	70.0	25.5	1/8000	75.9	257
10-15%	26	1/2000	7.9	33	1/1200	14.7	359
15% spnt	300	<1/2	<0.1	–	–	–	–

cuts must have significantly affected the solubility of the IgG because more antibody activity remained in the 0-4% PEG cut than in either the 4-8% or 8-13% PEG cuts (Table 4.19). The 0-4% PEG cut was therefore selected for use in the two-site immunometric assay work (Section 6.3.2).

4.6.3 ANTI-ANP SERUM

The haemolysed rabbit anti-ANP serum was purified by PEG 8000 fractionation, using 0-5%, 5-10% and 10-15% (w/v) PEG cuts (Section 2.3.2.1). When the PEG cuts were assayed by ANP-coated-plate ELISA, using donkey anti-rabbit IgG HRP conjugate to detect binding, most of the antibody activity was found in the 5-10% PEG cut (Table 4.20) which would have consisted mainly of IgG (Cripps et al., 1983). The 10-15% PEG cut contained relatively little antibody activity (Table 4.20) and was very impure because most of the haemoglobin was precipitated from the antiserum by 15% (w/v) PEG 8000.

The anti-ANP serum PEG cuts were dialysed against 50 mM sodium acetate then further purified by Rivanol precipitation (Section 2.3.2.2). A yellow-brown precipitate was removed from the PEG cuts, rather than the expected sticky brown paste, and almost none of the haemoglobin was precipitated from the 10-15% PEG cut which was still dark red after filtration.

The 0-5% PEG cut contained most of the IgM, and some IgG (Cripps et al., 1983), so the large decrease in antibody titre produced by Rivanol (Table 4.20) was due to IgM precipitation. This also showed that the anti-rabbit IgG HRP conjugate was not specific for rabbit IgG, but cross-reacted with IgM. The 5-10% PEG cut may also have contained some IgM because more antibody was lost during Rivanol precipitation than expected, although this fraction still contained the largest portion of antibody activity (Table 4.20) and was therefore selected for use in two-site immunometric assay work (Section 8.3). Relatively little antibody was lost from the 10-15% PEG cut, which would have contained mainly IgG (Cripps et al., 1983).

4.7 DISCUSSION

Anti-AVP and anti-ANP MAb-secreting hybridomas were generated by the fusion of NS-0 myeloma cells with spleen cells from immunized BALB/c mice. However, immunization against AVP was difficult because AVP is present in almost all mammals, including mice, and it was only weakly immunogenic even after conjugation to a carrier molecule. Immunization against ANP was easier because human ANP differs from mouse ANP in one amino acid residue, so moderate anti-ANP antibody titres were obtained after four doses of ANP-thyroglobulin conjugate (Table 4.5).

The cyanamide-coupled AVP-thyroglobulin conjugate was a particularly poor immunogen which produced a weak anti-AVP response that consisted entirely of IgM antibodies (Table 4.1). A peptide : thyroglobulin : cyanamide molar ratio of 118 : 1 : 1400 had been used to prepare this conjugate, although Skowsky and Fisher (1972b) reported that optimal conjugation occurred with a peptide : thyroglobulin : EDC ratio of 118 : 1 : 200. Both the quantity and the type of carbodiimide used in the conjugation procedure had therefore been incorrect, which would have influenced the composition of the final product. The correctly-prepared EDC-coupled AVP-thyroglobulin conjugate was more immunogenic than the cyanamide-coupled material and so produced anti-AVP IgG, although good responses were obtained only after many injections (Tables 4.1 and 4.2).

The enhanced EDC conjugation method was expected to produce a better immunogen by increasing the incorporation of AVP onto thyroglobulin (Staros et al., 1986) but the enhanced EDC-coupled AVP-thyroglobulin conjugate actually generated a weaker anti-AVP IgG response (Table 4.3) than the normal EDC-coupled material (Table 4.2). Peeters et al. (1989) reported that conjugate immunogenicity decreased with increasing peptide incorporation, and suggested that peptide antigenic determinants are masked by close-packing on the carrier, so the low immunogenicity of the enhanced EDC-coupled conjugate might have resulted from epitope masking.

However, the compositions of the AVP-thyroglobulin conjugates were not analysed so it is not known whether there was a correlation between AVP incorporation and immunogenicity.

Carbodiimides catalyse the formation of amide bonds between carboxyl groups and amino groups (Skowsky and Fisher, 1972b; Staros et al., 1986). Proteins contain many such groups therefore carbodiimide conjugation generates many different products (Peeters et al., 1989), including self-coupled proteins (Liu et al., 1979). In contrast, heterobifunctional reagents, such as MBS, can be used in a step-wise manner to produce specific cross-linking of peptides and proteins with minimal self-coupling (Liu et al., 1979). The N-hydroxysuccinimidyl group of MBS reacts with the primary amino groups of a sulphydryl-free protein in the first step, then the MBS maleimido group reacts with a sulphydryl-containing peptide to produce a conjugate in which the peptide and protein are linked by a spacer arm. In this project, KLH was reacted with MBS by the method of Lerner et al. (1981) then mixed with thiolated AVP and LVP to produce the final conjugate. The composition of the product was not analysed so it is not known whether the thiolation and conjugation procedures were successful, but the failure of the AVP/LVP-KLH preparation to produce a significant anti-AVP IgG response (Table 4.4) suggests that very little peptide had conjugated to KLH. The anti-AVP IgM response to this material was not determined.

KLH is often used as a carrier molecule because it is strongly immunogenic (Goding, 1986). An EDC-coupled AVP-KLH conjugate was reported to generate anti-AVP IgG in BALB/c mice, although eight injections produced a maximum serum antibody titre of only 1/2500 (Reilly and Root, 1986). However, the EDC-coupled AVP-thyroglobulin conjugate could generate anti-AVP IgG titres of more than 1/30,000 (Tables 4.1 and 4.2) which suggested that thyroglobulin was superior to KLH as a carrier for AVP. The absence of an anti-AVP IgG response to the AVP/LVP-KLH preparation may therefore have been only partly due to poor conjugation and partly due to the KLH carrier because Skowsky and Fisher (1972b) showed that the choice of carrier is important in determining the immune response to small peptides.

Thyroglobulin was better than BSA, ovalbumin or amino acid polymers as a carrier for LVP and PTH (Skowsky and Fisher, 1972b), and it was better than either BSA or KLH as a carrier for ANP (Prowse et al., 1989).

Despite conjugation to thyroglobulin, however, the antibody responses to AVP and ANP were still relatively low. Recently, Fiori and Rappelli (1991) showed that intravenous injection of PPD tuberculin, a polyclonal B cell activator (Sultzer and Nilsson, 1972), stimulated mouse spleen cells so that the specific antibody response to soluble antigens increased dramatically and subsequent cell fusions generated a higher frequency of antigen-specific hybridomas. PPD tuberculin treatment of mice, prior to immunization, might therefore be of value in future attempts to generate anti-AVP and anti-ANP MAbs.

Alternatively, *in vitro* immunization can be used to raise antibodies against highly conserved and weakly immunogenic antigens, including 'self'-antigens (Reading, 1982; Van Ness et al., 1984; Borrebaeck and Möller, 1986). Successful *in vitro* immunizations have been achieved under a wide range of culture conditions. Many workers used serum-free medium because it contained fewer competing antigens than FCS medium (Ossendorp et al., 1986; Darfler, 1987; De Boer et al., 1989), in which only the most immunogenic antigens produced specific antibody responses (Van Ness et al., 1984), and because some batches of serum were reported to inhibit *in vitro* immunizations (Reading, 1982). However, serum proteins are often considered essential for B cell division so some workers added FCS to their *in vitro* immunization cultures after a serum-free induction period (Van Ness et al., 1984; Erich et al., 1989), while others used culture medium containing either FCS (Takahashi et al., 1987; Rathinavelu and Isom, 1989) or rabbit serum (Borrebaeck and Möller, 1986) throughout the procedure. The minimum antigen concentration required to produce an antibody response varied between antigens (Van Ness et al., 1984; Borrebaeck and Möller, 1986; Ossendorp et al., 1986; Takahashi et al., 1987), with the highest concentrations being necessary for the most highly conserved antigens (Van Ness et al., 1984), while a range of stimulation periods (3-8 days) have all been reported as optimal (Borrebaeck

and Möller, 1986; Takahashi et al., 1987; De Boer et al., 1989; Erich et al., 1989). However, a comparison of reported *in vitro* immunizations is difficult because most workers used different cell concentrations and different culture medium supplements, which would have affected the final result, and the optimum conditions for *in vitro* immunization may well prove to be different for each antigen.

In vitro immunization tends to produce only a primary immune response so the hybridomas generated in subsequent cell fusions usually secrete IgM antibodies (Van Ness et al., 1984; Darfler, 1987; De Boer et al., 1989). A few IgG-secreting hybridomas occasionally arise (Ossendorp et al., 1986; Rathinavelu and Isom, 1989) although optimized stimulation conditions can produce them at higher frequencies (Takahashi et al., 1987). Alternatively, memory B cells which have been generated by *in vivo* immunization can be activated *in vitro* to produce a secondary immune response (Reading, 1982; De Boer et al., 1988), increasing both the fusion efficiency and the antigen-specific IgG-secreting hybridoma production of subsequent cell fusions (De Boer et al., 1988; Erich et al., 1989). *In vitro*-boosting was therefore carried out whenever surplus immune spleen cells were available.

Comparatively few spleen cells survived *in vitro*-boosting in serum-free medium (Table 4.10) even though B cell survival was reported to be higher in serum-free medium than in serum-containing medium (Iscove and Melchers, 1978). However, no optimization work had been carried out so the antigen concentrations chosen for *in vitro*-boosting may have been too low. Cell survival was reportedly higher in antigen-stimulated cultures than in control cultures (Ossendorp et al., 1986), and increased as the antigen concentration increased (Van Ness et al., 1984), so the higher survival rate in FCS medium (Table 4.10) may have been caused by the serum proteins acting as additional antigens.

The fusion efficiencies of the *in vitro*-boosted spleen cells were usually much lower than those of freshly-prepared cells (Tables 4.6 and 4.8), which was surprising because higher fusion efficiencies had been expected (Erich et al., 1989). However, hybridomas tend to be generated from immature, actively-dividing B cells (Reading,

1982) since fully differentiated cells do not fuse efficiently (Tijssen, 1985). The mice were boosted 3 days before splenectomy, then the spleen cells were *in vitro*-boosted for 4 days so B cell maturation may have progressed too far. The final *in vivo* boost could not be omitted because most fusions used freshly-prepared cells and only the surplus cells were boosted *in vitro*, but a shorter *in vitro*-boosting period might have produced better results. However, the memory B cells may not have responded to *in vitro*-boosting because most *in vitro*-boosts were done with unconjugated peptides (Table 4.10) whereas the donor mice had been immunized with thyroglobulin conjugates (Section 4.2). Small peptides produce optimal secondary responses only if they are conjugated to the carrier molecule that was used in an earlier antigen challenge (the 'carrier effect'; Roitt et al., 1985). The *in vitro* antibody responses to AVP and ANP may also have been inhibited by FCS proteins in the culture medium (Van Ness et al., 1984), although Rathinavelu and Isom (1989) were able to generate an anti-ANP IgG-secreting hybridoma from spleen cells which had been *in vitro*-immunized in FCS medium.

AVP and ANP are such weak antigens that immunization produced few antigen-specific B cells therefore subsequent cell fusions could not generate many antigen-specific hybridomas (Erich et al., 1989), although high fusion efficiencies increase the probability of hybridoma generation from rare B cells. All of the fusions that produced antigen-specific MAbs were done with freshly-prepared spleen cells and, with the exception of fusion VP6 (Table 4.6), had efficiencies of more than 69% and generated more than 400 hybridomas, less than 1% of which were antigen-specific (Tables 4.6 and 4.8). Fusion VP6 was done with spleen cells from mouse 3365, which had responded exceptionally well to immunization (Table 4.1). Many AVP-specific B cells would have formed so an anti-AVP MAb-secreting hybridoma was generated despite the low efficiency of fusion VP6 (Table 4.6), and culture supernatant from the *in vitro*-boosted surplus cells was strongly PEG-RIA-positive even though unlabelled AVP was present. Surplus spleen cells from other mice failed to secrete significant amounts of antigen-specific antibody into the culture medium.

The AVP and ANP cell fusions (Tables 4.6 and 4.8) generated 2796 hybridoma-containing cultures, thirteen of which were originally antigen-specific although four were lost at an early stage. Hybridomas VP12/339 and NP4/213 would also have been lost if stored cells had not been available for re-cloning. Some antigen-specific hybridomas are lost because they are overgrown by unrelated cells. Hybridoma NP4/213 (Section 4.3.2, Table 4.9) came from a culture well which contained two hybridoma colonies so the antibody-secreting cells were probably overgrown by the second hybridoma, although they were eventually isolated from frozen cell stocks. In contrast, hybridoma VP12/339 (Section 4.3.1, Table 4.7) came from a culture well which contained only a single hybridoma colony. The non-secreting cells which rapidly overgrew the culture were therefore derived from hybridoma VP12/339, and a stable MAb-secreting clone was difficult to obtain.

Hybridomas have two sets of chromosomes and are genetically unstable, tending to lose chromosomes until a more stable genotype is achieved (Galfrè and Milstein, 1981; Goding, 1986). Loss of antibody chain expression occurs frequently (Köhler and Milstein, 1976). Although the genes which code for the heavy and light antibody chains are located on different chromosomes, there is preferential loss of the heavy chain because free heavy chains seem to be toxic for the cells (Köhler, 1980). Hybridomas which stop secreting antibody have therefore lost the chromosome which controls heavy chain synthesis, but may continue to produce light chains.

Both the spleen cells and the NS-0 myeloma cells (Galfrè and Milstein, 1981) were derived from BALB/c mice so the anti-AVP and anti-ANP hybridomas could theoretically be grown as ascites tumours in BALB/c mice (Köhler and Milstein, 1976). Hybridomas VP6/7.1.4 and VP14/247.6.1 produced high titre ascitic fluid containing ESVP 1 and ESVP 3 MAbs, respectively (Tables 4.11 and 4.12). Fluid tapping began 10-21 days after injection of the cells and the antibody titres increased over the tapping period as reported by Brodeur et al. (1984). In contrast, hybridoma VP14/308.1.1 produced ascitic fluid only after a long delay (Table 4.13) and its low antibody titre suggested that the ascites tumours contained few ESVP 4 MAb-secreting

cells, although the average fluid volume collected per mouse was similar to that obtained from hybridomas VP6/7.1.4 and VP14/247.6.1 (Section 4.4.1). However, myelomas are induced in 40% of BALB/c mice within one year of receiving three intraperitoneal pristane injections (Goding, 1986). The slow ascitic fluid response to hybridoma VP14/308.1.1 may therefore have been largely due to pristane-induced myeloma formation, particularly in the case of mouse 3589 (Table 4.13) which produced exceptionally low titre ascitic fluid and did not do so until 62 days after a second dose of hybridoma cells.

Hybridoma VP12/339.5.3.2, however, would not grow in ascites at all. Some hybridomas lose the ability to grow as tumours after long periods of *in vitro* culture (Hoogenraad and Wraight, 1986; Mattes et al., 1991), presumably because they have lost a myeloma-derived gene which confers tumorigenicity since Mattes et al. (1991) showed that backcrossing these cell lines with the parent myeloma restored their ability to produce ascites. The parent hybridoma, VP12/339, had been difficult to clone (Section 4.3.1) because it was unstable and tended to stop secreting antibody, which seemed to be a result of chromosome loss. It is therefore quite likely that stabilization of the final ESVP 2 MAb-secreting cell line was achieved by the loss of a chromosome which carried the gene for tumorigenicity.

Ascitic fluid usually contains 2-20 mg MAb/ml (Goding, 1986). It also contains high concentrations of non-MAb proteins which, if not removed, would compete with the MAb during labelling reactions and solid phase preparation (Neoh et al., 1986). Spent culture supernatant, on the other hand, has lower concentrations of non-MAb proteins but typically contains only 5-50 µg MAb/ml (Goding, 1986) and so needs to be concentrated to allow for efficient MAb labelling. MAbs were therefore partially purified from ascitic fluid by sodium sulphate precipitation (Tijssen, 1985), and concentrated from hybridoma culture supernatant by PEG 8000 precipitation (Baines et al., 1990) or Protein A-Sepharose affinity chromatography (Ey et al., 1978).

Baines et al. (1990) recommended a final concentration of 10% (w/v) PEG 8000 for IgG MAb precipitation from hybridoma culture supernatant because Neoh et al.

(1986) had reported that maximal precipitation occurred within the range 5-10% (w/v) PEG 8000, at a concentration that was characteristic for each MAb, although this information was obtained with ascitic fluid. However, 10% (w/v) PEG 8000 produced inefficient precipitation of ESVP 2 (Table 4.16), ESNP 1, ESNP 2 and ESNP 4 MAbs (Table 4.18, Experiment 1) because it left about half of the MAb activity in the PEG supernatant. IgG precipitation was improved by increasing the PEG 8000 concentration to 12.5% (Table 4.18, Experiment 2) but the concentration of FCS-derived contaminants also rose because increasing amounts of non-antibody protein precipitated from serum and ascitic fluid as the PEG 8000 concentration was raised above 7% (Cripps et al., 1983; Neoh et al., 1986). Recently, Brooks et al. (1992) reported that 15% (w/v) PEG 8000 was optimal for IgG MAb precipitation from culture supernatant, so the exact composition of the MAb-containing sample may influence PEG 8000 precipitation even though it was supposedly insensitive to the solution conditions (Ingham, 1984). However, high PEG concentrations precipitate large amounts of contaminating protein and so produce very impure MAb preparations. Towards the end of this project, 8% (w/v) PEG 8000 was shown to precipitate a lot of protein but relatively little ESVP 2 activity from ESVP 2 MAb-containing culture supernatant, while more than half of the total ESVP 2 activity was present in an 8-12.5% PEG cut which contained less protein (Table 4.21). PEG fractionation was therefore a better way to concentrate MAbs from spent culture supernatant because a large portion of the non-MAb protein was removed from the final product.

PEG precipitation was reported to be a rapid procedure, requiring only a short equilibration period of up to 1 hour before centrifugation (Ingham, 1984; Neoh et al., 1986; Baines et al., 1990). However, PEG precipitation of IgG MAbs from culture supernatant was slow and continued for more than 16 hours, because further ESVP 2 MAb-containing precipitates formed during storage of the PEG supernatants that remained after collection of ESVP 2 MAb Precipitate I (Table 4.16, Experiment 2) and 8-12.5% PEG cut I (Table 4.21). In contrast, IgA precipitation by 15% (w/v) PEG 8000 occurred rapidly so that more than 90% of ESVP 4 MAb activity was precipitated

TABLE 4.21

Preparation of ESVP 2 MAb by PEG 8000 fractionation of culture supernatant

ESVP 2 MAb-containing culture supernatant was mixed with 40% (w/v) PEG 8000 in Buffer E, to give a PEG concentration of 8% (w/v), then incubated at 4°C for 24 hours before collection of the precipitate (0-8% PEG cut) as described in Section 2.3.1.2. Additional 40 % (w/v) PEG 8000 in Buffer E was added to the 8% supernatant, to give a final PEG concentration of 12.5% (w/v), then the mixture was incubated at 4°C for 16 hours before the precipitate (8-12.5% PEG cut I) was collected. Further precipitation occurred during storage of 12.5% supernatant I at 4°C, although the 8-12.5% PEG cut II was not collected until day 3. Even after this, an additional ESVP 2 MAb-containing precipitate formed, and was harvested, from 12.5% supernatant II (data not shown).

The antibody titre was defined as the sample dilution which produced an absorbance of 1.0 at 450 nm in the AVP-coated-plate ELISA. ND = not determined.

Sample	Volume (ml)	Antibody titre	Protein (mg/ml)
Starting material	450	1/56	ND
8% supernatant	565	1/44	ND
0-8% PEG cut	5.5	1/100	12.1
12.5% supernatant I	650	1/15	ND
8-12.5% PEG cut I	5.5	1/2900	8.4
12.5% supernatant II	650	1/10	ND
8-12.5% PEG cut II	3.5	1/475	1.9

within 2 hours (Table 4.17), although this material was very impure because 30% of total serum proteins were reported to precipitate in 14% (w/v) PEG 8000 (Cripps et al., 1983).

Protein A-Sepharose affinity chromatography was an inefficient purification method for ESVP 2 MAb (Section 4.5.1.3) because mouse IgG1 antibodies do not bind strongly to protein A (Underwood et al., 1983; Jiskoot et al., 1991) and the binding capacity of the Protein A-Sepharose column for ESVP 2 was low, although the eluted MAb was relatively pure (Figure 4.3B). ESVP 2 was produced by bulk culture in FCS medium, and although FCS is usually considered to be antibody-free (Goding, 1986) it often contains a low concentration of bovine IgG (Kniazeff and Rimer, 1967; Underwood et al., 1983; Jiskoot et al., 1991) which was reported to co-purify with mouse IgG1 on Protein A-Sepharose (Underwood et al., 1983; Jiskoot et al., 1991). Bovine IgG may therefore have been a contaminant of protein A-purified ESVP 2, which seemed to contain two ^{125}I-IgG components after radioiodination and gel filtration on Sephacryl S-300 (Figure 4.3B). The SAM-S-500 solid phase, which detected the ^{125}I-IgG (Figure 4.3), was supposed to be specific for mouse antibodies but was shown to cross-react with a sheep antiserum (unpublished observation) and might therefore also cross-react with bovine IgG.

MAbs prepared from ascitic fluid by sodium sulphate precipitation were contaminated by endogenous mouse antibodies (Galfrè and Milstein, 1981; Baines et al., 1990) but relatively few other components (Figure 4.1). In contrast, MAbs prepared from spent culture supernatant by PEG 8000 precipitation were very impure and contained large quantities of high molecular weight materials (Figure 4.3A) which were not present in protein A-purified ESVP 2 (Figure 4.3B) and which interfered with reagent preparation for the two-site immunometric assay work (Chapters 6 and 8). The specific activities attainable for ^{125}I-MAbs were decreased by impurities in the MAb preparations (Table 4.22) because different proteins have different iodination efficiencies (Goding, 1986). Monoiodinated IgG has a specific activity of 10 μCi/μg (Hunter et al., 1983) but protein A-purified ESVP 2 was the only material that could

TABLE 4.22

Effect of MAb purification method on the specific activity of ^{125}I-MAb

MAbs were partially purified from ascitic fluid by sodium sulphate precipitation, and concentrated from hybridoma culture supernatant by PEG 8000 precipitation or Protein A-Sepharose affinity chromatography, then radioiodinated. The specific activities of the ^{125}I-MAbs were calculated as described in Section 2.3.4.2. The Sephacryl S-300 column profiles of ^{125}I-ESVP 1, ^{125}I-ESVP 3 and the ^{125}I-ESVP 2 preparations are shown in Figures 4.1 and 4.3.

MAb	Specific activity (μCi/μg)		
	Sodium sulphate precipitation	PEG 8000 precipitation	Protein A-Sepharose affinity chromatography
ESVP 1	6.7	-	-
ESVP 2	-	2.1	9.5
ESVP 3	7.1	-	-
ESVP 4	-	3.0	-
ESNP 1	-	4.7	-
ESNP 2	-	3.2	-
ESNP 3	-	2.8	-
ESNP 4	-	2.6	-
ESA 4	6.5	-	-
ESA 5	5.8	-	-
ESA 9	3.5	-	-

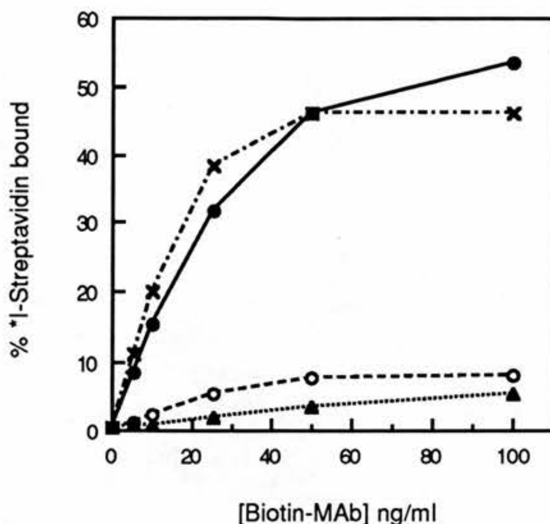

FIGURE 4.4

Effect of MAb purification method on the binding of ^{125}I-streptavidin to biotin-MAbs

ESVP 1 (●) and ESVP 3 (×) MAbs were partially purified from ascitic fluid by sodium sulphate precipitation, while ESVP 2 (o) and ESVP 4 (▲) were concentrated from hybridoma culture supernatants by PEG 8000 precipitation, then the MAbs were biotinylated as described in Section 2.3.4.5.

The biotin-MAbs were assayed for ^{125}I-streptavidin binding by incubation of 100 µl biotin-MAb in Buffer L with 100 µl 20 ng/ml ^{125}I-streptavidin in Buffer R for 15 minutes at room temperature, then 100 µl SAM-S-500 solid phase (25% (settled volume) slurry in Buffer R) was added. The mixture was agitated on an orbital shaker for 1 hour, then bound and free ^{125}I-streptavidin were separated by the sucrose layering technique (Section 2.2.4.4).

be iodinated to a specific activity which approached this figure (Table 4.22). Low specific activities were obtained with the very impure PEG 8000-precipitated MAbs, while sodium sulphate preparations iodinated to an intermediate specific activity (Table 4.22). The impurities decreased the efficiency of MAb biotinylation such that less ^{125}I-streptavidin bound to biotin-MAbs prepared from PEG 8000 precipitates than to those prepared from sodium sulphate precipitates (Figure 4.4), and lower two-site ELISA binding occurred with biotin-ESVP 2 prepared from a PEG 8000 precipitate than with that prepared from protein A-purified material (Figure 6.13). The impurities also interfered with solid phase preparation by binding to sites which MAbs would otherwise have occupied, although this effect was less noticeable because of the high binding capacity of the solid phases (Chapter 6). These problems could have been

avoided by further purification of the MAbs but there was no time available to investigate suitable methods.

Purification of the rabbit polyclonal antibodies (Section 4.6) also presented difficulties because the antisera contained irrelevant antibodies in addition to the anti-AVP and anti-ANP specificities, while two of the antisera were badly haemolysed. The anti-AVP serum (Section 4.6.1) was purified by sodium sulphate precipitation only, so the product consisted of a mixture of IgM and IgG antibodies. The haemolysed anti-PAG and anti-ANP sera (Sections 4.6.2 and 4.6.3), on the other hand, were purified firstly by PEG 8000 fractionation then by Rivanol precipitation, so the final preparations consisted mainly of IgG antibodies (Franek, 1986).

PEG 8000 precipitation acted more efficiently on antisera (Tables 4.19 and 4.20) than it did on hybridoma culture supernatants (Tables 4.16, 4.18 and 4.21). As reported by Cripps et al. (1983), more than 90% of polyclonal antibody activity was rapidly precipitated from serum by 8% (w/v) PEG 8000 (Table 4.19), a concentration which had little effect on ESVP 2 MAb (Table 4.21), while IgG MAb precipitation from culture supernatant by 10% or 12.5% (w/v) PEG 8000 was slow (Tables 4.16 and 4.21, respectively) and left up to half of the MAb in the PEG supernatant (Tables 4.16, 4.18 and 4.21). A PEG 8000 concentration of 10% (w/v) did not improve polyclonal antibody precipitation (Table 4.20) but merely increased the precipitation of impurities (Cripps et al., 1983).

Rivanol precipitation of the anti-PAG serum PEG cuts did not proceed as expected (Sections 4.6.2) so a second volume of Rivanol was added to the 4-8% and 8-13% PEG cuts. This increased the final Rivanol concentration from 0.46% (w/v) to 0.79% (w/v) and, surprisingly, precipitated most of the antibody activity (Table 4.19). Rivanol precipitates negatively-charged proteins (Franek, 1986) so at neutral pH values it should, for example, precipitate serum albumin but not IgG (which have isoelectric pH values of 4.9 and 6.6, respectively; Lehninger, 1975). However, the anti-PAG serum PEG cuts had neither been dialysed nor had their pH values checked so the pH was probably higher than expected. The extra volume of Rivanol (pH 8.2)

would have raised the pH further so that IgG, and the traces of haemoglobin (isoelectric pH 6.8) that were precipitated by 13% (w/v) PEG 8000, became negatively charged and were therefore precipitated by the Rivanol. In contrast, the anti-ANP serum PEG cuts were dialysed before Rivanol precipitation and only one volume of Rivanol was added, so the pH was correct and most of the IgG activity therefore remained in solution (Table 4.20). The nature of the Rivanol precipitate was still not as expected, however, but this was due to the low serum albumin content of the PEG cuts (Cripps et al., 1983). Albumin is a major component of the material precipitated from whole serum or ascitic fluid by Rivanol (Franek, 1986) and is the main contributor to the sticky precipitate which had been expected.

The polyclonal antibody preparations could have been further purified by affinity chromatography on immobilized antigen (Goding, 1986) which would have removed the irrelevant antibodies. However, this was not deemed worthwhile because the polyclonal antibodies were mainly used in feasibility studies (Chapters 6 and 8) and the aim of the project was to produce two-site immunometric assays which used only monoclonal antibodies.

CHAPTER 5

CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO VASOPRESSIN

5.1 INTRODUCTION

Anti-AVP MAbs were difficult to produce because AVP is only weakly immunogenic, even after conjugation to a carrier molecule. The AVP fusions (Section 4.3.1) generated 1748 hybridoma-containing cultures but only six of these were anti-AVP antibody-positive. Four hybridomas were eventually cloned, and subcloned, to produce monoclonal cell lines which secreted anti-AVP MAbs (Table 4.7).

Antibody purification was not necessary for the MAb characterization work, which was done with samples of spent culture supernatant.

5.2 ANTIBODY ISOTYPES AND AFFINITIES

The isotypes of the anti-AVP MAbs, determined using an Amersham Mouse Monoclonal Antibody Isotyping Kit, are shown in Table 5.1. All of the MAbs had κ light chains.

Affinity constants were determined by Scatchard analysis (Scatchard, 1949) of solution-phase RIA binding (Section 2.4.2). The mean values shown in Table 5.1 were calculated from the results of seven (ESVP 1), five (ESVP 2) and two (ESVP 3 and ESVP 4) experiments.

TABLE 5.1

Isotypes and affinities of the anti-AVP MAbs

MAb	Isotype	Affinity (l/mole)
ESVP 1	IgG1	2.8×10^8
ESVP 2	IgG1	2.2×10^8
ESVP 3	IgM	1.1×10^7
ESVP 4	IgA	1.9×10^7

5.3 ANTIBODY SPECIFICITIES

ESVP 1 MAb bound to the AVP ring structure. It cross-reacted strongly with LVP, Hep, dDAVP and tGLVP (Table 5.2), which have similar ring structures to AVP but different tail regions (Table 5.3). The ESVP 1 epitope was unlikely to include the N-terminal cysteine residue of AVP because deamination (in dDAVP) had little effect on binding, while the addition of three glycine residues (in tGLVP) produced a 4.7-fold increase in cross-reactivity, relative to LVP (Table 5.2), which was presumably caused by conformational changes that allowed greater access to the ring structure. In comparison, cross-reactivity with dNMeAVP was low, even though it has an identical ring structure to dDAVP (Table 5.3), therefore the N-methylarginine residue in its tail region must have caused steric hindrance of ESVP 1 binding. The ring structures of OT, AVT and dCDAVP differ from that of AVP, so ESVP 1 bound poorly to these peptides and did not bind at all to the isolated tail tripeptide (PAG).

ESVP 2, ESVP 3 and ESVP 4 MAbs all bound to the AVP tail region. They cross-reacted strongly with AVT, which has the same tail tripeptide as AVP, and bound poorly to analogues which have different tail structures (Tables 5.2 and 5.3). However, the ring structure is important in maintaining the correct conformation of the tail tripeptide therefore cross-reactivity with isolated PAG was low.

5.4 DISCUSSION

The result of isotyping the anti-AVP MAbs (Table 5.1) was surprising. The MAbs were all expected to be IgG antibodies because they were all bound strongly by the anti-mouse IgG (whole molecule) HRP conjugate in the AVP-coated-plate ELISA, and were also bound by the SAM-S-500 solid phase which had only ever detected IgG MAbs in previous (unpublished) hybridoma screening work. However, while the AVP-coated-plate ELISA and the SAM-RIA produced similar results with ESVP 1 and

TABLE 5.2

Cross-reactivity of the anti-AVP MAbs with some AVP analogues

Cross-reactivity was calculated from RIA data, as described in Section 2.4.3. The structures of the AVP analogues are shown in Table 5.3.

Peptide	Cross-reactivity (%)			
	ESVP 1	ESVP 2	ESVP 3	ESVP 4
AVP	100.0	100.0	100.0	100.0
LVP	66.6	<0.1	1.9	0.4
OT	6.7	<0.1	<0.1	<0.1
AVT	10.4	59.0	65.8	80.0
Hep	37.6	<0.1	<0.1	<0.1
PAG	<0.1	2.3	0.4	0.3
dDAVP	70.8	8.7	6.8	4.5
dCDAVP	0.6	6.2	1.6	1.5
dNMeAVP	2.3	13.9	15.6	6.9
tGLVP	317.4	0.2	4.7	0.6

TABLE 5.3

Amino acid sequences of AVP and AVP analogues

The sites at which the AVP analogues differ from AVP are shown in bold type. d = desamino.

Peptide	Sequence
AVP	NH_2-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2 (S–S bridge between Cys residues)
LVP	NH_2-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-**Lys**-Gly-NH_2 (S–S bridge between Cys residues)
OT	NH_2-Cys-Tyr-**Ile**-Gln-Asn-Cys-Pro-**Leu**-Gly-NH_2 (S–S bridge between Cys residues)
AVT	NH_2-Cys-Tyr-**Ile**-Gln-Asn-Cys-Pro-Arg-Gly-NH_2 (S–S bridge between Cys residues)
Hep	NH_2-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-**OH** (S–S bridge between Cys residues)
PAG	**NH_2**-Pro-Arg-Gly-NH_2
dDAVP	**d**-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-**D-Arg**-Gly-NH_2 (S–S bridge between Cys residues)
dCDAVP	**d**-Cys-Tyr-Phe-Gln-Asn-**Cys**-Pro-**D-Arg**-Gly-NH_2 (S–**CH_2** bridge between Cys residues)
dNMeAVP	**d**-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-**N-MeArg**-Gly-NH_2 (S–S bridge between Cys residues)
tGLVP	NH_2-**Gly-Gly-Gly**-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-**Lys**-Gly-NH_2 (S–S bridge between Cys residues)

ESVP 2, the binding obtained with ESVP 3 and ESVP 4 was always much lower in the SAM-RIA than in the AVP-coated-plate ELISA (data not shown). This was originally thought to indicate that ESVP 3 and ESVP 4 bound near to the tyrosine residue of the AVP ring structure, because an anti-AVP MAb which did not bind well to ^{125}I-AVP had been reported (Hou-Yu et al., 1982; Valiquette et al., 1986), but high PEG-RIA binding later showed that these MAbs bound strongly to ^{125}I-AVP. The low SAM-RIA binding of ESVP 3 and ESVP 4 was therefore the result of a weak cross-reaction of the SAM-S-500 solid phase with IgM and IgA isotypes. The anti-mouse IgG HRP conjugate, on the other hand, bound strongly to IgM and IgA MAbs and so was clearly not specific for mouse IgG, particularly as it had also shown some cross-reaction with rabbit and bovine sera (Table 3.1).

The affinity constants of ESVP 1 and ESVP 2 were disappointingly low, even though they were an order of magnitude higher than those of ESVP 3 and ESVP 4 (Table 5.1), because MAbs with much higher affinities were needed if sensitive two-site immunometric assays, which could measure physiological AVP concentrations, were to be developed. However, the cell fusions had been unlikely to produce high affinity anti-AVP MAbs because the weak immunogenicity of AVP made immunization difficult, so that most of the mice produced low titre antisera (Sections 4.2.1 and 4.2.2) which were unlikely to contain high affinity anti-AVP antibodies. Cell fusions produce MAbs with a range of binding affinities that reflect the affinity distribution of the antibodies generated by the immunized animal, so affinities which are rare *in vivo* will also be rare amongst the MAbs that are subsequently produced (Goding, 1986).

AVP consists of a hexapeptide ring and a C-terminal tripeptide tail (Table 5.3), which folds back over the ring structure (Jurzak et al., 1990) and seems to be stabilized by hydrogen bonding between the side chains of the asparagine residue in the ring and the arginine residue in the tail (Langs et al., 1986). The aromatic side chains of the tyrosine and phenylalanine residues interact with each other and are directed away from the AVP ring structure. In contrast, the side chains of the tyrosine

residues in OT and AVT cannot form this type of interaction with isoleucine, so they lie over the ring structures of these peptides which therefore have an entirely different conformation from the AVP ring structure (Langs et al., 1986).

ESVP 1 was specific for the AVP ring so it did not bind well to OT or AVT, and bound even less well to dCDAVP (Table 5.2) which has a distorted ring structure due to the absence of a sulphur atom from residue 6 (Table 5.3). The ESVP 1 epitope probably contained the phenylalanine, glutamine and asparagine residues of AVP, and possibly also the cysteine residue at position 6, but did not include tyrosine because ESVP 1 bound strongly to ^{125}I-AVP. The slightly decreased cross-reactivity of LVP and dDAVP (Table 5.2), which have similar ring structures to AVP (Table 5.3), was probably caused by steric hindrance from their altered tail tripeptides which would have interacted differently with the side chain of asparagine. However, this steric hindrance was small compared with that caused by the N-methylarginine residue in the tail of dNMeAVP (Table 5.2), so the N-methyl group must be orientated towards the hexapeptide ring, blocking the access of ESVP 1 to its epitope. The hexapeptide ring of Hep has the same sequence as that of AVP, but residues 8 and 9 are absent from the Hep tail (Table 5.3). The asparagine side chain of the Hep ring is therefore likely to assume the conformation which occurs in pressinoic acid, where it lies over the ring structure and hydrogen bonds to the nitrogen atom of the tyrosine residue (Langs et al., 1986). The decreased cross-reactivity of Hep, relative to AVP (Table 5.2), was therefore likely to be caused by the different conformation of the Hep ring. Pressinoic acid, which consists of the hexapeptide ring of AVP without the tail tripeptide, was not available for comparison so the influence of the proline residue on ESVP 1 binding could not be determined. There were also no analogues available in which glutamine was replaced by another residue, but its position between the important phenylalanine and asparagine residues make it a probable component of the ESVP 1 epitope.

ESVP 2, ESVP 3 and ESVP 4 were all specific for the AVP tail region but had different patterns of cross-reactivity (Table 5.2) and so bound to overlapping, but not

identical, epitopes. The arginine residue was an important component of these epitopes because the anti-tail MAbs cross-reacted strongly with AVT but did not bind either to the truncated tail of Hep or to OT (Table 5.2), in which a leucine residue replaces arginine (Table 5.3). Cross-reactivity with LVP and tGLVP was also very low, while the D-arginine residues of dDAVP and dCDAVP produced large decreases in cross-reactivity, relative to the L-arginine residue of AVP (Table 5.2). Surprisingly, dNMeAVP cross-reacted more strongly with the anti-tail MAbs than with the ring-specific ESVP 1 MAb (Table 5.2), so the N-methylarginine side chain of the dNMeAVP tail is probably located between the tail and the ring structure, where the N-methyl group would cause decreased cross-reactivity, relative to AVP, by altering the tail conformation rather than by directly blocking the epitopes of the anti-tail MAbs. The conformation of the tail tripeptide was stabilized by the ring structure (Jurzak et al., 1990), which was important for MAb binding, so the cross-reactivity of the anti-tail MAbs with isolated PAG was low (Table 5.2). Unfortunately, there were no analogues available with an alteration at position 9, so the importance of the glycine residue to the anti-tail MAb epitopes could not be determined.

The ESVP 2 epitope was probably closer to the C-terminal of AVP than the epitopes of ESVP 3 and ESVP 4. ESVP 2 cross-reacted less well with AVT than did the other anti-tail MAbs (Table 5.2), possibly because the different conformation of the AVT ring, relative to AVP, altered the orientation of the glycine residue. It also bound to isolated PAG more readily than the other MAbs, while the altered residue at position 6 of dCDAVP produced only a small decrease in cross-reactivity relative to dDAVP, which has the same tail tripeptide (Table 5.3). The cysteine residue at position 6 was therefore unlikely to be part of the ESVP 2 epitope.

The ESVP 3 and ESVP 4 epitopes seemed to be closer to the ring structure because ESVP 3 and ESVP 4 cross-reacted more strongly with AVT, and less strongly with PAG, than ESVP 2, while the distorted ring structure of dCDAVP produced a larger decrease in cross-reactivity with these MAbs than with ESVP 2. The ESVP 3 and ESVP 4 epitopes may therefore have included the cysteine residue at position 6.

However, these epitopes were not identical, because the cross-reactivity of ESVP 3 with dNMeAVP was stronger than that of ESVP 4 (Table 5.2) so the MAbs probably bound to different sides of the AVP tail.

Despite the low affinities of the anti-AVP MAbs (Table 5.1), ESVP 1 and ESVP 2 were interesting because they were IgG1 antibodies which bound to different parts of the AVP molecule and so could potentially be used in two-site immunometric assays. ESVP 4 was also interesting because IgA MAbs are rare (Komisar et al., 1982; Campbell, 1984) and only one other anti-AVP IgA MAb has been reported (Jurzak et al., 1990), but ESVP 3 was unlikely to be useful because IgM antibodies are not usually suitable for immunometric assays.

CHAPTER 6

TWO-SITE IMMUNOMETRIC ASSAYS FOR VASOPRESSIN

6.1 INTRODUCTION

At the start of this project, there were no published reports of two-site immunometric assays for AVP. However, unpublished work had suggested that two-site assays were feasible (N. McIntosh, personal communication) even though AVP consists of only nine amino acids.

Four anti-AVP MAbs (Chapter 5) were generated in this project, and four polyclonal antisera were available in sufficient quantity for immunometric assay studies. ESVP 1 MAb was generated several months before the other anti-AVP MAbs therefore a large portion of the two-site assay work was carried out using ESVP 1 in combination with the polyclonal antibodies. However, the aim of the project was to develop an assay with a pair of MAbs and, therefore, to avoid the problems caused by the variable quality and limited supplies of polyclonal antisera.

6.2 TWO-SITE ELISA

Preliminary experiments produced poor results but suggested that ESVP 1 MAb and polyclonal anti-AVP antibodies could bind simultaneously to AVP in a two-site ELISA (data not shown). Two-site binding to AVP seemed to occur with liquid-phase anti-AVP serum in ESVP 1 MAb-coated ELISA wells, but there was no evidence of two-site binding when liquid-phase ESVP 1 was tested in ELISA wells coated with unpurified anti-AVP serum. Non-specific binding of the liquid-phase antibody caused inconveniently high background absorbances that increased as the concentration of anti-AVP serum increased, irrespective of whether it was in the liquid phase or coated onto the solid phase. Non-specific binding of the donkey anti-rabbit IgG HRP conjugate, or the rabbit anti-mouse IgG (whole molecule) HRP conjugate, made a much smaller contribution to the background absorbances.

Further studies were conducted after partial purification of the anti-AVP serum (Section 4.6.1), which removed most of the non-antibody material and allowed

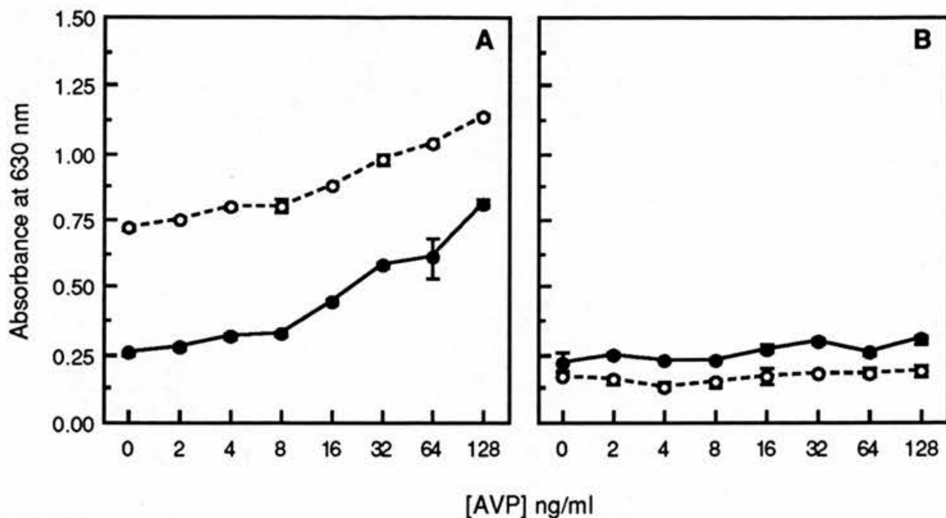

FIGURE 6.1

Effect of antibody orientation on a two-site ELISA with ESVP 1 MAb and anti-AVP serum

Serial dilutions of AVP in Buffer L were incubated in ESVP 1 MAb-coated (**A**), or partially-purified anti-AVP serum-coated (**B**), BSA-blocked, Immulon 2 MicroELISA wells for 22 hours. The wells were washed once with Buffer L, then incubated with either biotinylated (2 μg/ml, ●) or unlabelled (6 μg/ml, ○) partially-purified anti-AVP serum (**A**), or ESVP 1 (**B**), in Buffer L for 19 hours. The remainder of the assay was carried out as described in Section 2.5.1.1, using 2 μg/ml streptavidin-HRP conjugate (●), 1/500 dilution donkey anti-rabbit IgG HRP conjugate (**A**, ○) or 1/200 dilution rabbit anti-mouse IgG (whole molecule) HRP conjugate (**B**, ○) to reveal binding. Colour development occurred rapidly therefore the absorbance at 630 nm was recorded before H_2SO_4 addition. The symbols show the mean of duplicate estimates, and the vertical bars show the difference between those estimates.

biotinylation (Section 2.3.4.5) to be carried out. Initial results with partially-purified anti-AVP serum confirmed the previous observation that detectable binding occurred only when anti-AVP serum was in the liquid phase (Figure 6.1). Antigen-specific antibodies usually comprise less than 5% of the immunoglobulin fraction of an antiserum (Baker et al., 1985) therefore the failure to detect two-site binding in anti-AVP serum-coated ELISA wells was probably caused by the adsorption of high concentrations of irrelevant antibodies, and very little AVP-specific antibody, onto the polystyrene surface. The epitope density of the AVP that subsequently bound to immobilized anti-AVP antibody was therefore too low to efficiently bind ESVP 1 MAb (Figure 3.16A; Lew, 1984), particularly as some anti-AVP antibodies would have recognized the same epitope as ESVP 1 and, thus, have been unable to participate in

two-site binding with the MAb.

Non-specific binding of unlabelled anti-AVP serum antibodies to ESVP 1 MAb-coated, BSA-blocked, ELISA wells was much higher than that of biotinylated anti-AVP serum (Figure 6.1A). The anti-AVP serum was a pool of antisera that had been raised against both unconjugated AVP and AVP-BSA, so it contained both anti-AVP and anti-BSA antibodies. Unlabelled anti-AVP serum therefore bound directly to BSA-blocked ELISA wells despite the presence of BSA in the assay buffer. Biotinylated anti-AVP serum contained few anti-BSA antibodies because unreacted biotin was removed by gel filtration on a BSA-blocked PD-10 column which absorbed most of the anti-BSA activity, therefore similar background binding occurred in both BSA-blocked (Figure 6.1) and unblocked (Figure 6.2) ESVP 1 MAb-coated ELISA wells. Biotinylated anti-AVP serum also produced a slightly steeper dose-response curve than the unlabelled material (Figure 6.1A) and was therefore selected for further two-site ELISA studies.

Increasing the ESVP 1 MAb-coating concentration from 6 μg/ml to 12, 18 or 24 μg/ml had no effect on either specific or non-specific binding to unblocked wells (data not shown), which suggested that a coating of 6 μg/ml ESVP 1 filled most of the protein-binding sites and that reagent adsorption onto polystyrene was unlikely to cause the remaining non-specific binding. However, AVP adsorbed onto uncoated, BSA-blocked, wells causing a dose-dependent increase in biotinylated anti-AVP serum binding (Figure 6.2). This was lower than the binding recorded in ESVP 1 MAb-coated unblocked wells (Figure 6.2), which was presumed to be genuine two-site binding, but implied that considerable assay interference could result from non-specific binding of liquid-phase reagents to solid phase-bound ELISA components.

Higher background absorbances occurred in ESVP 1 MAb-coated, unblocked, ELISA wells than in uncoated, BSA-blocked, wells (Figure 6.2), which suggested that the biotinylated anti-AVP serum contained anti-mouse IgG specificities that bound directly to ESVP 1 MAb. This was confirmed when the absorbance recorded in AVP-free ESVP 1 MAb-coated ELISA wells was shown to increase as the concentration

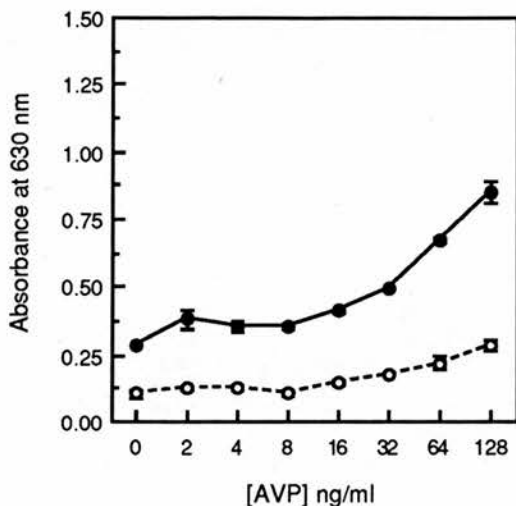

FIGURE 6.2

Comparison of the binding of AVP and biotinylated anti-AVP serum to ESVP 1 MAb and to BSA

Serial dilutions of AVP in Buffer L were incubated in ESVP 1 MAb-coated, unblocked, MicroELISA wells (●), or in uncoated, BSA-blocked, wells (○), for 20 hours. The wells were washed once with Buffer L, then incubated with biotinylated partially-purified anti-AVP serum (2 µg/ml in Buffer L) for 19 hours. Biotin-antibody binding was revealed with streptavidin-HRP conjugate. Colour development occurred rapidly therefore the absorbance at 630 nm was recorded before H_2SO_4 addition. Vertical bars show the difference between duplicate estimations.

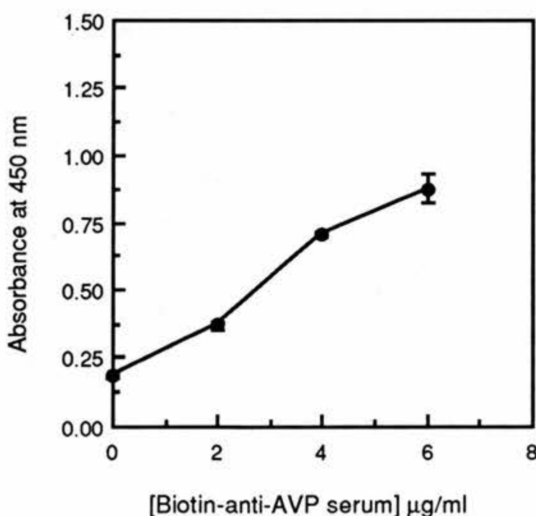

FIGURE 6.3

Binding of biotinylated anti-AVP serum to ESVP 1 MAb

Duplicate 100 µl aliquots of biotinylated partially-purified anti-AVP serum in Buffer L were incubated in ESVP 1 MAb-coated, BSA-blocked, MicroELISA wells for 18 hours, then biotin-antibody binding was revealed with streptavidin-HRP conjugate (2 µg/ml in Buffer L).

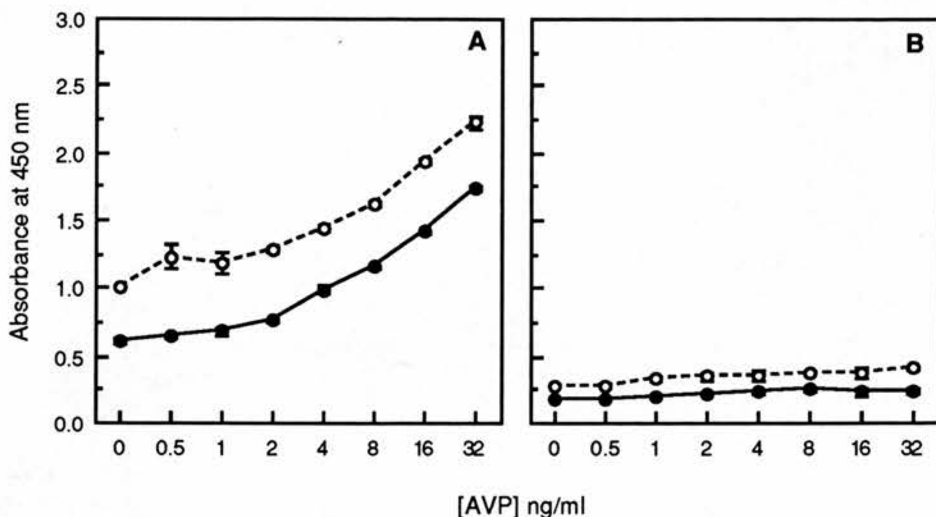

FIGURE 6.4

Effect of incubation protocol on a two-site ELISA with biotinylated anti-AVP serum and ESVP 1 MAb

Serial dilutions of AVP in Buffer L were assayed in ESVP 1 MAb-coated, BSA-blocked, MicroELISA wells, using the separate (Section 2.5.1.1, **A**) or simultaneous (Section 2.5.1.2, **B**) incubation protocols with biotinylated partially purified anti-AVP serum in the presence (●) or absence (○) of normal mouse serum. Biotin-antibody binding was revealed with streptavidin-HRP conjugate. Vertical bars show the difference between duplicate estimations.

of biotinylated anti-AVP serum increased (Figure 6.3), and to decrease when normal mouse serum was added to the assay buffer (Figure 6.4). A final concentration of 1% (v/v) normal mouse serum was therefore routinely added to all liquid-phase antibodies in subsequent two-site AVP ELISA work.

The incubation protocol had a significant effect on the magnitude of the binding recorded with biotinylated anti-AVP serum in ESVP 1 MAb-coated ELISA wells (Figure 6.4). Reasonable dose-dependent binding was obtained with the separate incubation protocol (Figure 6.4A), in which each reagent was added to the wells after the unbound fraction of the previous reagent had been removed and maximal specific binding occurred because there was no competition between reagents. In contrast, specific binding was barely noticeable with the simultaneous incubation protocol (Figure 6.4B), in which AVP and biotinylated anti-AVP serum were preincubated in the coated wells then streptavidin-HRP conjugate was added so that all of the assay

components were in the well simultaneously. Partially-purified anti-AVP serum contained irrelevant antibodies that could not take part in two-site binding to AVP but were biotinylated and therefore competed with anti-AVP antibodies for binding of the streptavidin-HRP conjugate, while anti-AVP antibodies competed with ESVP 1 MAb for AVP binding if their epitope specificities overlapped with that of ESVP 1. Consequently, minimal specific, and non-specific, binding was recorded (Figure 6.4B) because the streptavidin-HRP conjugate was more likely to react with unbound biotinylated antibodies than with solid phase-bound assay components.

Although the simultaneous incubation protocol was unsuitable for partially purified polyclonal antibodies, it could significantly decrease the total assay time if competition between reagents was eliminated. Two affinity-purified antisera were therefore compared with partially-purified anti-AVP serum to determine whether the removal of irrelevant antibodies would improve two-site ELISA binding. Initial results obtained with unlabelled polyclonal antibodies in ESVP 1 MAb-coated, BSA-blocked wells, using the separate incubation protocol, showed that affinity-purified anti-PAG produced a steeper dose-response curve and lower non-specific binding than either affinity-purified anti-AVP-thyroglobulin or the partially-purified anti-AVP serum (Figure 6.5). The high non-specific binding of unlabelled anti-AVP serum was largely due to anti-BSA antibodies, despite the presence of BSA in the assay buffer. Affinity-purified anti-AVP-thyroglobulin was not expected to contain anti-BSA antibodies, but the cause of its high non-specific binding (Figure 6.5) was not investigated because affinity-purified anti-PAG produced superior results.

The non-specific binding of biotinylated affinity-purified anti-PAG was unexpectedly high compared with that of biotinylated anti-AVP serum (Figure 6.6), which may indicate that the harsh conditions needed to elute antibodies from an immunoaffinity matrix had caused structural damage that increased the probability of non-specific adsorption onto coated ELISA wells. Nevertheless, the signal-to-noise ratio was higher than that obtained with biotinylated anti-AVP serum (Figure 6.6) because affinity-purified anti-PAG contained a higher concentration of anti-AVP

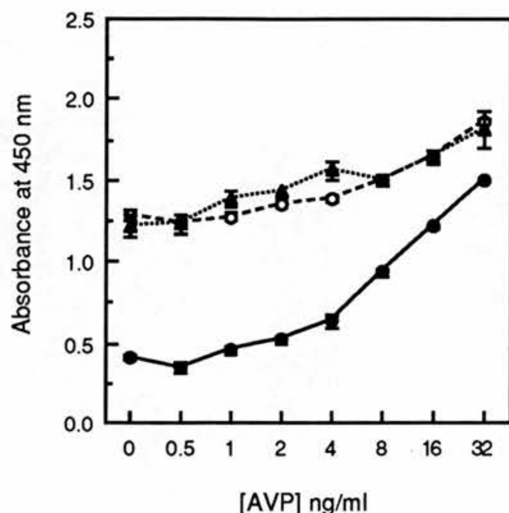

FIGURE 6.5

Comparison of three polyclonal antibody preparations in a two-site ELISA

ESVP 1 MAb-coated, BSA-blocked, Immulon 2 MicroELISA wells were incubated with serial dilutions of AVP in Buffer L, then with unlabelled affinity-purified anti-PAG (●), affinity-purified anti-AVP-thyroglobulin (▲) or partially-purified anti-AVP serum (o) in Buffer L containing 1% normal mouse serum, as described in Section 2.5.1.1. Antibody binding was revealed with donkey anti-rabbit IgG HRP conjugate. Vertical bars show the difference between the absorbances recorded in duplicate wells.

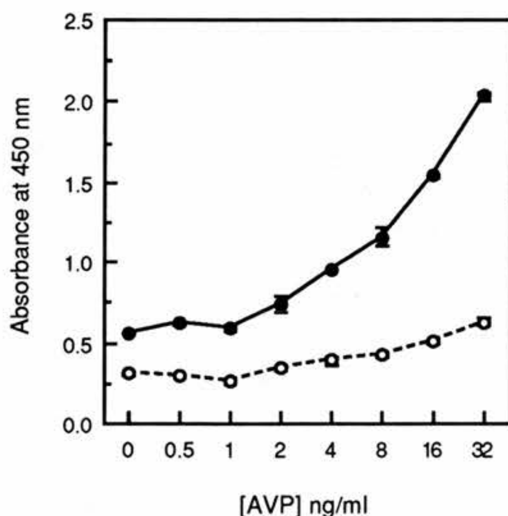

FIGURE 6.6

Comparison of two biotinylated polyclonal antibody preparations in a two-site ELISA

ESVP 1 MAb-coated, BSA-blocked, MicroELISA wells were incubated with serial dilutions of AVP in Buffer L, then with biotinylated affinity-purified anti-PAG (●) or partially-purified anti-AVP serum (o) in Buffer L containing 1% normal mouse serum (Section 2.5.1.1). Biotin-antibody binding was revealed with streptavidin-HRP conjugate. Vertical bars show the difference between duplicate estimations, although most bars are smaller than the symbols used in this Figure.

antibodies than the partially-purified anti-AVP serum and was less likely to compete with the ring-specific ESVP 1 MAb (Section 5.3) for AVP binding because it consisted mainly of anti-tail antibodies. However, no two-site binding was detected when the simultaneous incubation protocol was used with biotinylated affinity-purified anti-PAG and ESVP 1 MAb-coated, unblocked, wells (data not shown). This suggested that the anti-PAG epitopes were so close to the ESVP 1 epitope that steric hindrance between the antibodies severely limited two-site binding to AVP, therefore unbound biotinylated anti-PAG antibodies successfully competed for streptavidin-HRP conjugate binding. Affinity purification had therefore failed to eliminate competition between reagents so the separate incubation protocol, which maximized specific binding, was used in all subsequent ELISA work with polyclonal antibody preparations.

Sephadex G-25 gel filtration of radioiodinated AVP separates ^{125}I-AVP from unreacted ^{125}I and AVP (Roth et al., 1966a), eluting three peaks of radioactivity (Figure 6.7) which correspond to unreacted ^{125}I, monoiodinated and diiodinated AVP, respectively (Sadler et al., 1986). When the elution profile was analysed in a two-site ELISA with biotinylated affinity-purified anti-PAG and ESVP 1 MAb-coated, BSA-blocked wells (Figure 6.7), 28% of detectable AVP was shown to elute just before unreacted ^{125}I, presumably as BSA-bound peptide (Sadler et al., 1986), 34% eluted in two overlapping peaks of cold AVP and only 38% coincided with monoiodinated AVP. A similar result was obtained when the elution profile of a second radioiodinated preparation was analysed (data not shown). Diiodinated AVP was not recognized by the two-site ELISA. Tyrosine was not a component of the epitopes bound by either ESVP 1 (Section 5.4) or affinity-purified anti-PAG, but the addition of two ^{125}I atoms increased the molecular weight of AVP by 23% and so caused significant steric hindrance. The ability of the individual antibody preparations to bind diiodinated AVP was not tested but it was reportedly bound by tail-specific antisera (Sadler et al., 1986) therefore its failure to react in the two-site ELISA was probably caused by distortion of the ESVP 1 epitope.

Radioactivity in the ELISA plate could not be accurately measured during the

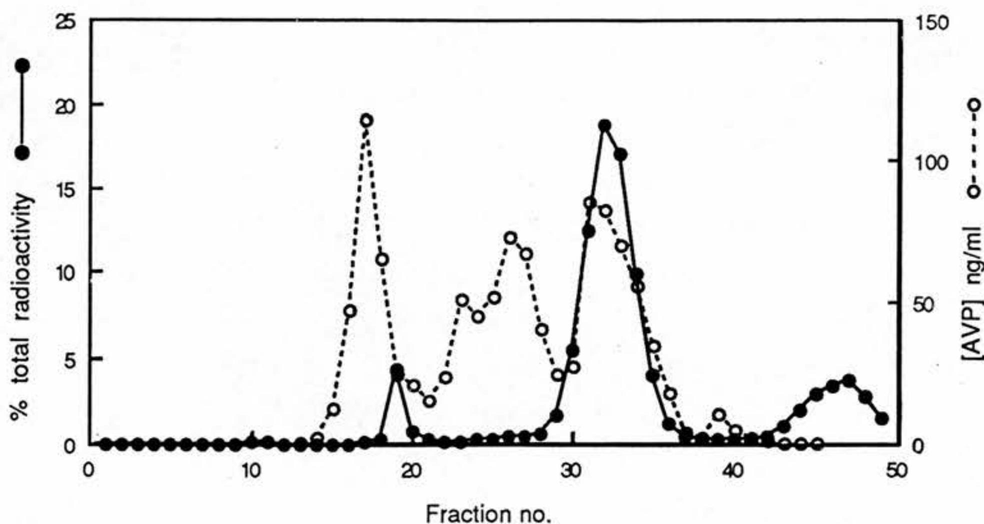

FIGURE 6.7

Two-site ELISA analysis of ^{125}I-AVP elution from Sephadex G-25

Radioiodinated AVP was purified on a Sephadex G-25 column as described in Section 2.3.4.1. A sample from each fraction was diluted in 3 volumes Buffer L, then assayed in a two-site ELISA using ESVP 1 MAb-coated, BSA-blocked, MicroELISA wells, biotinylated affinity-purified anti-PAG and the separate incubation protocol (Section 2.5.1.1). Standard AVP was prepared in a mixture of 3 volumes Buffer L and 1 volume 0.2% acetic acid, 0.2% BSA.

two-site assay but was roughly estimated with a Geiger counter after each step of the separate incubation protocol. Although the monoiodinated AVP peak comprised 72% of the radioactivity eluted from the column (Figure 6.7), only 25% of the original radioactivity remained in the ELISA wells after sample incubation and plate-washing with Buffer L to remove loosely-adherent material, which suggested that the ESVP 1 MAb-coated wells had a low AVP-binding capacity. Incubation with biotinylated affinity-purified anti-PAG removed another 20% of the radioactivity because some of the anti-PAG antibodies displaced ^{125}I-AVP that had already bound to ESVP 1 MAb, but no further radioactivity was lost after incubation with the streptavidin-HRP conjugate. Less than 5% of the original radioactivity remained in the ELISA plate at the end of the assay period, adding to the evidence which suggested that steric hindrance caused competition between the antibodies that severely limited two-site binding to AVP.

The radioactivity of column fraction 33 (Figure 6.7), which was selected for use as a RIA tracer, was 115 μCi/ml (assuming 2.2×10^6 disintegrations/min/μCi and an 80% counting efficiency) while the two-site ELISA data suggested that it contained 70 ng AVP/ml. The specific activity of the ^{125}I-AVP in fraction 33 was therefore 1643 μCi/μg, which is close to the theoretical maximum for monoiodinated AVP of 1861 μCi/μg (Czernichow et al., 1975) and to the previously reported values of 1710 ± 155 Ci/mmol (Baylis and Heath, 1977) and 1756 ± 98 Ci/g (Sadler et al., 1986).

ESVP 3 was the next anti-AVP MAb to become available for immunometric assay work. It bound the AVP tail tripeptide (Section 5.3) and so could theoretically substitute for affinity-purified anti-PAG in a two-site ELISA with ESVP 1 MAb. However, no detectable two-site binding occurred between ESVP 1 and ESVP 3, irrespective of whether ESVP 3 was in the liquid phase (Figure 6.8A) or coated onto the solid phase (Figure 6.8B). This was probably due to the IgM isotype of ESVP 3, which would have caused much greater steric hindrance than an IgG antibody, although the high non-specific binding of the biotinylated MAbs possibly obscured a specific binding response that might have been revealed under different solution conditions (Wadsley and Watt, 1987).

Unexpectedly, dose-dependent binding of biotinylated affinity-purified anti-PAG occurred in both ESVP 1 MAb-coated (Figure 6.8A) and ESVP 3 MAb-coated (Figure 6.8B), BSA-blocked, ELISA wells. ESVP 3 and affinity-purified anti-PAG had similar epitope specificities so they were unlikely to bind to AVP simultaneously, which suggested that the observed binding response was caused by non-specific adsorption of AVP onto the solid phase. This was confirmed when further investigation (Figure 6.9) revealed that similar dose-dependent binding occurred whether the ELISA wells were coated with ESVP 1, ESVP 3 or ESA 5, an anti-ANP MAb that did not bind dDAVP (Prowse et al., 1989) and was unlikely to bind AVP although its cross-reactivity with AVP was not tested. The same dose-dependent binding also occurred in uncoated BSA-blocked wells (Figure 6.9A) but not in uncoated unblocked wells (Figure 6.9B), yet omission of BSA-blocking failed to decrease AVP

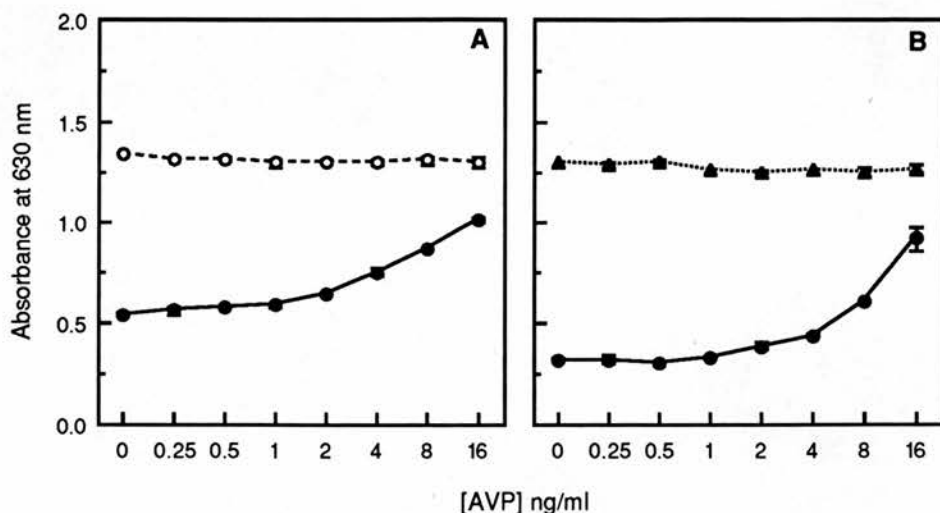

FIGURE 6.8

Comparison of ESVP 1 and ESVP 3 MAbs in a two-site ELISA

BSA-blocked Immulon 2 MicroELISA wells, coated with ESVP 1 (**A**) or ESVP 3 (**B**), were incubated with duplicate 100 µl aliquots of AVP in Buffer L for 26 hours, then with biotinylated affinity-purified anti-PAG (●), biotin-ESVP 3 (o) or biotin-ESVP 1 (▲) for 17 hours (separate incubation protocol). Biotin-antibody binding was revealed with streptavidin-HRP conjugate. Colour development occurred rapidly therefore the absorbance at 630 nm was recorded before H_2SO_4 addition.

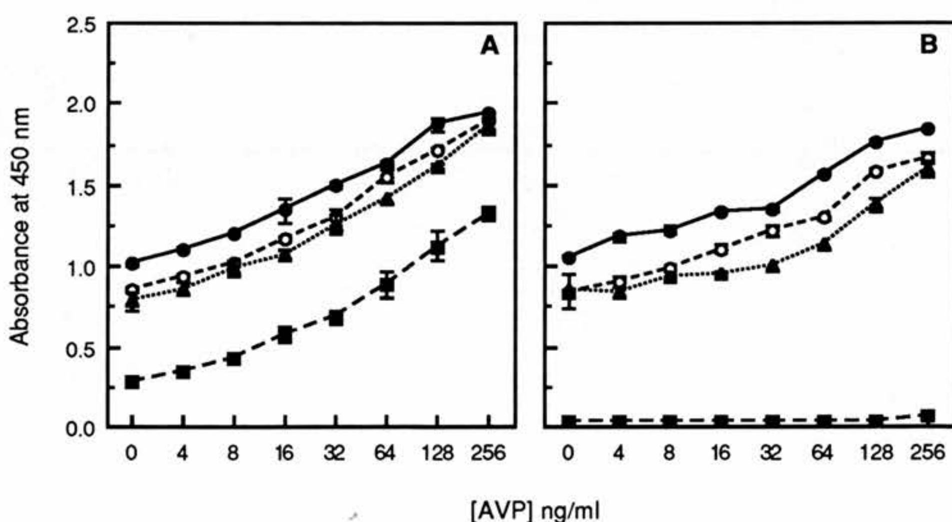

FIGURE 6.9

Comparison of the binding of AVP and biotinylated anti-PAG to MAbs and to BSA

BSA-blocked (**A**) or unblocked (**B**) MicroELISA wells, coated with ESVP 1 (●), ESVP 3 (o), ESA 5 (▲) or nothing (■), were incubated with serial dilutions of AVP in Buffer L for 23 hours, then with biotinylated affinity-purified anti-PAG for 18 hours. Biotin-antibody binding was revealed with streptavidin-HRP conjugate. Vertical bars show the difference between the absorbances recorded in duplicate wells.

adsorption onto MAb-coated ELISA wells (Figure 6.9B). This implied that AVP adsorbed equally onto MAbs and onto BSA, although AVP adsorption onto BSA had previously been lower than the two-site binding response (Figure 6.2).

The signal-to-noise ratio in these experiments (Figures 6.8 and 6.9) was much lower than in previous work that had suggested that two-site binding to AVP occurred with biotinylated affinity-purified anti-PAG in ESVP 1 MAb-coated ELISA wells (Figure 6.6). However, several months had elapsed between the two sets of experiments, during which time the affinity-purified anti-PAG preparation was stored at -20°C. Some antibodies are labile to freeze-thawing (Goding, 1986) therefore antibody deterioration had probably occurred, particularly as affinity purification and subsequent lyophilization were likely to have caused structural damage that increased the lability of some specificities. Affinity-purified anti-PAG contained antibodies against a range of overlapping epitopes on the AVP tail tripeptide, some of which were unable to form a two-site assay with ESVP 1 MAb. Antibodies whose epitopes were too close to the ESVP 1 epitope to allow two-site binding would nevertheless recognize non-specifically adsorbed AVP if it was orientated in such a way that their epitopes were accessible. If the ESVP 1 MAb-compatible anti-PAG specificities were the most labile, antibody deterioration would inhibit two-site binding yet the more robust antibodies would still detect non-specifically adsorbed AVP. Unfortunately, this phenomenon could not be investigated because no other batches of affinity-purified anti-PAG were available.

ESVP 2 and ESVP 4 MAbs, which also bound to the AVP tail tripeptide (Section 5.3), produced no evidence of two-site ELISA binding with ESVP 1 in initial studies (data not shown). However, these MAbs were very impure because they were prepared from spent culture supernatant by PEG 8000 precipitation (Section 4.5.1.2) and so contained high concentrations of non-MAb protein (Figure 4.3A) that could not take part in two-site binding to AVP but which decreased the potential signal-to-noise ratio of the assay system (Figure 4.4). ESVP 4 MAb was not used in any further ELISA work because it also failed to produce evidence of two-site binding in the direct IRMA

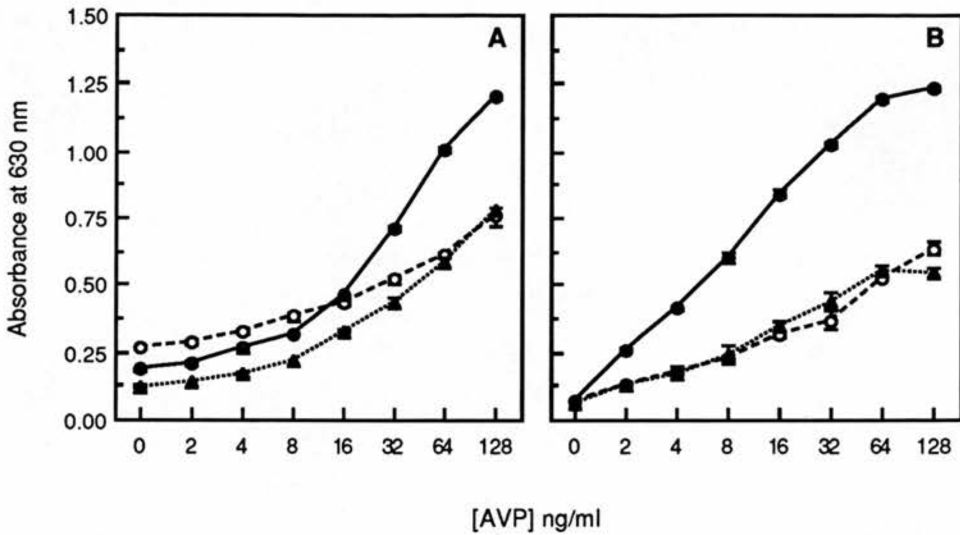

FIGURE 6.10

Effect of MAb orientation and post-coating treatment on two-site ELISA binding with ESVP 1 and ESVP 2 MAbs

BSA-blocked (▲) or unblocked (● and o) Nunc Maxisorp Microwells, coated with protein A-purified ESVP 2 (**A**) or ESVP 1 (**B**) and rinsed with Buffer T (▲ and o) or with Tween buffer (●, 0.01 M Tris/HCl, pH 7.8, 0.9% NaCl, 0.01% Tween 20), were incubated on a plate-shaker with serial dilutions of AVP in Buffer L for 2 hours, then with biotin-ESVP 1 (**A**) or biotinylated protein A-purified ESVP 2 (**B**) for 2 hours. The remainder of the assay was carried out as described in Section 2.5.1.1. Colour development occurred rapidly therefore the absorbance at 630 nm was recorded before H_2SO_4 addition. The symbols show the mean of duplicate estimates, while the vertical bars show the difference between those estimates.

format (Section 6.3.1).

When protein A-purified ESVP 2 became available, ESVP 1 and ESVP 2 MAbs were shown to bind simultaneously to AVP in a two-site ELISA (Figure 6.10) although the magnitude of the binding response was unexpectedly dependent on the post-coating treatment of the MAb-coated wells. Most of the two-site ELISA work in this project was done with antibody-coated wells that were Buffer T-washed then BSA-blocked, as described in Section 2.3.5.4. BSA-blocking decreased the non-specific binding of biotin-ESVP 1 to ESVP 2 MAb-coated Buffer T-washed wells (Figure 6.10A) and so increased the signal-to-noise ratio, but it had no effect on either the specific or non-specific binding of biotin-ESVP 2 to ESVP 1 MAb-coated Buffer T-washed wells (Figure 6.10B). In contrast, a post-coating Tween buffer wash dramatically increased

the dose-dependent binding response in unblocked wells compared with that obtained in Buffer T-washed wells, regardless of the MAb orientation (Figure 6.10), therefore assay sensitivity was increased. (The sensitivity, or detection limit, of an assay was defined as the analyte concentration that produced a binding response equal to the sum of the mean zero analyte response plus three standard deviations of that response.) Interestingly, the augmentative effect of Tween buffer on dose-dependent binding in MAb-coated wells was not unique to ESVP 1 and ESVP 2 because it also occurred in a microtitre plate-based immunometric assay for proinsulin (unpublished observation). However, the binding response failed to increase if MAb-coated wells were BSA-blocked before the Tween buffer wash, which suggested that Tween buffer altered the conformation of the adsorbed MAb in such a way that subsequently-bound antigen was more readily accessible to the liquid-phase MAb and that BSA-blocking caused steric hindrance which inhibited the conformational change.

Irrespective of the post-coating treatment, a more sensitive assay was obtained with biotinylated protein A-purified ESVP 2 in ESVP 1 MAb-coated wells (Figure 6.10B) than with biotin-ESVP 1 in ESVP 2 MAb-coated wells (Figure 6.10A). Further investigation showed that the dose-dependent binding response resulted from specific interaction of AVP with the binding sites of ESVP 1 and ESVP 2 MAbs, and not from non-specific adsorption of AVP onto the coated ELISA wells. No significant dose-dependent binding of biotin-ESVP 2 occurred in unblocked wells coated with ESNP 2, an anti-ANP IgG1 MAb (Chapter 7), or in uncoated BSA-blocked wells, while the adsorption of AVP onto uncoated unblocked wells was negligible compared with the specific binding response in ESVP 1 MAb-coated wells (Figure 6.11). Short incubation periods were used in these experiments because a plate-shaker became available, whereas long incubations had been used in the earlier work with polyclonal antibody preparations (Figures 6.1 to 6.9) to ensure that binding equilibrium was reached in the unshaken wells. The previously-observed non-specific adsorption of AVP onto wells coated with BSA (Figures 6.2 and 6.9A) or with MAbs (Figure 6.9B) was likely to have been a consequence of these long incubation periods.

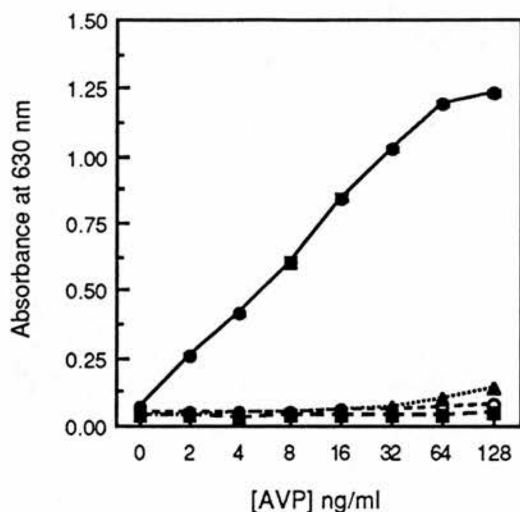

FIGURE 6.11

Effect of plate-coating protein on ELISA binding of biotin-ESVP 2

Unblocked Microwells, coated with ESVP 1 (●), ESNP 2 (o) or nothing (▲), were rinsed with Tween buffer (Figure 6.10) then incubated on a plate-shaker with serial dilutions of AVP in Buffer L for 2 hours, followed by biotinylated protein A-purified ESVP 2 MAb for 2 hours. Biotin-ESVP 2 binding was revealed with 1 µg/ml streptavidin-HRP conjugate. Uncoated, BSA-blocked, wells (■) were included for comparison. Colour development occurred rapidly therefore the absorbance at 630 nm was recorded before H_2SO_4 addition. Vertical bars showing the difference between duplicate estimations are smaller than the symbols used in this Figure.

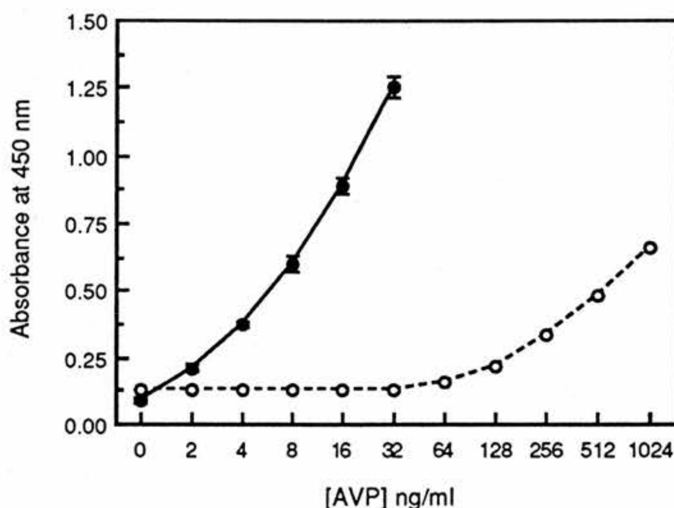

FIGURE 6.12

Comparison of the binding of AVP and biotin-ESVP 2 to ESVP 1 MAb and to ESVP 2 MAb

Unblocked Microwells, coated with ESVP 1 (●) or protein A-purified ESVP 2 (o) then rinsed with Tween buffer (Figure 6.10), were incubated on a plate-shaker with duplicate 100 µl aliquots of AVP in Buffer L for 2 hours, then with biotinylated protein A-purified ESVP 2 for 2 hours (separate incubation protocol). Biotin-ESVP 2 binding was revealed with 0.1 µg/ml streptavidin-HRP conjugate.

Unexpectedly, dose-dependent binding of biotin-ESVP 2 was recorded in ESVP 2 MAb-coated wells (Figure 6.12), although extremely high non-specific binding of biotin-ESVP 1 to ESVP 1 MAb-coated wells obscured any dose-dependent response that might have occurred when ESVP 1 was on both sides of the 'sandwich' (data not shown). AVP is too small to bind two molecules of the same MAb simultaneously but it binds to BSA (Sadler et al., 1986) therefore the 'pseudo-two-site' binding of biotin-ESVP 2 to ESVP 2 MAb-coated wells probably resulted from MAb cross-linkage by an AVP-BSA complex in which several AVP molecules were attached to each BSA molecule. Nevertheless, the 'pseudo-two-site' binding response was less than 2% of the genuine two-site response recorded with biotin-ESVP 2 in ESVP 1 MAb-coated wells (Figure 6.12), which suggested that only a small proportion of the AVP was complexed to BSA.

The incubation protocol had a significant effect on the magnitude of the dose-dependent binding recorded with biotin-ESVP 2 in ESVP 1 MAb-coated ELISA wells (Figure 6.13). However, unlike partially-purified anti-AVP serum and affinity-purified anti-PAG, which produced no significant evidence of two-site binding with ESVP 1 MAb in the simultaneous incubation protocol because they contained antibodies whose epitope specificities overlapped with that of ESVP 1, ESVP 2 MAb recognized a completely different epitope from ESVP 1 (Section 5.4) so there was no competition between the MAbs for AVP binding. Reasonable dose-response curves were therefore obtained with biotinylated protein A-purified ESVP 2 in all incubation protocols (Figure 6.13A). The partial simultaneous incubation protocol, in which AVP and biotin-ESVP 2 were incubated together in the coated wells then unbound material was removed before addition of the streptavidin-HRP conjugate, produced the most sensitive assay (with a detection limit of 200 pg AVP/ml; Figure 6.13A) because it maximized the recorded signal by preventing competition between bound and unbound biotin-ESVP 2 for binding of the streptavidin-HRP conjugate. The much shallower dose-response curve and decreased sensitivity (500 pg AVP/ml) recorded with biotinylated protein A-purified ESVP 2 in the simultaneous incubation protocol showed

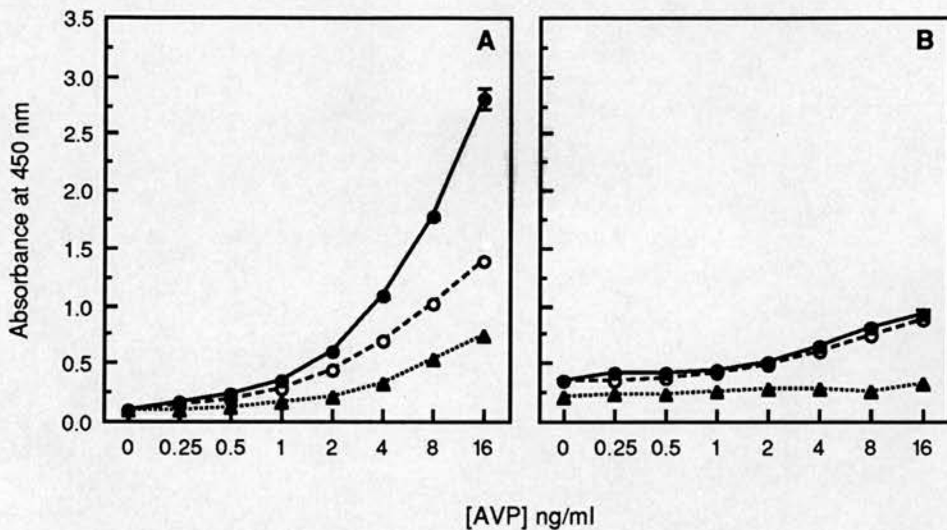

FIGURE 6.13

Effect of incubation protocol and ESVP 2 MAb purity on two-site ELISA binding with biotin-ESVP 2 and ESVP 1 MAb

Serial dilutions of AVP in Buffer L were assayed in ESVP 1 MAb-coated, unblocked, Tween buffer-washed Microwells on a plate-shaker, using the separate (○), partial simultaneous (●) or simultaneous (▲) incubation protocols with biotinylated protein A-purified ESVP 2 (**A**) or biotinylated PEG 8000-precipitated ESVP 2 (**B**; Precipitate I (Experiment 2), Table 4.16).

In the separate incubation protocol, the coated wells were incubated with 100 µl AVP for 2 hours, then with 100 µl 2 µg/ml biotin-ESVP 2 for 2 hours, followed by 100 µl 0.1 µg/ml streptavidin-HRP conjugate for 15 minutes, before plate-washing and incubation with 100 µl substrate. In the partial simultaneous incubation protocol, 100 µl AVP and 100 µl 2 µg/ml biotin-ESVP 2 were incubated together for 2 hours, then the wells were incubated with 200 µl 0.1 µg/ml streptavidin-HRP conjugate before plate-washing and incubation with 200 µl substrate. In the simultaneous incubation protocol, 100 µl AVP and 50 µl 4 µg/ml biotin-ESVP 2 in Buffer L containing 2% normal mouse serum were incubated together for 2 hours, before the addition of 50 µl 0.4 µg/ml streptavidin-HRP conjugate. After 15 minutes, the wells were washed and incubated with 200 µl substrate.

The symbols show the mean of duplicate estimates, and the vertical bars show the difference between those estimates. Bars which are not shown are smaller than the symbols used in this Figure.

the desensitizing effect of competition for binding of the conjugate (Figure 6.13A) because the simultaneous and partial simultaneous protocols differed only in their conjugate incubation conditions. The dose-response curve produced by the separate incubation protocol was intermediate between those of the simultaneous and partial simultaneous protocols (Figure 6.13A), and had a sensitivity of 250 pg AVP/ml.

The purity of the ESVP 2 MAb preparation had a major influence on the signal-to-noise ratio and thus the sensitivity of two-site ELISA binding in ESVP 1 MAb-coated wells (Figure 6.13). PEG 8000-precipitated ESVP 2 contained a lot of non-MAb protein (Figure 4.3A) that decreased the effective MAb concentration of biotinylated PEG 8000-precipitated ESVP 2 so that it produced lower dose-dependent binding in the partial simultaneous and separate incubation protocols (Figure 6.13B) than biotinylated protein A-purified ESVP 2 (Figure 6.13A), while the impurities increased non-specific binding and limited the assay sensitivity to 1 ng AVP/ml. No dose-dependent response was observed with the simultaneous incubation protocol (Figure 6.13B) because the streptavidin-HRP conjugate was more likely to bind to biotinylated impurities than to solid phase-bound biotin-ESVP 2.

Interestingly, initial studies with biotinylated PEG 8000-precipitated ESVP 2 had failed to detect two-site binding in ESVP 1 MAb-coated wells with the separate incubation protocol, whereas the above experiment clearly showed dose-dependent binding with both the separate and partial simultaneous incubation protocols (Figure 6.13B). However, the initial work was done in Buffer T-washed wells while the more recent studies used Tween buffer-washed wells, which were shown to produce higher dose-dependent binding responses (Figure 6.10). Further investigation confirmed that biotinylated PEG 8000-precipitated ESVP 2 produced no detectable dose-dependent binding in ESVP 1 MAb-coated Buffer T-washed wells (data not shown), and so emphasized the importance of the post-coating treatment in determining the sensitivity of two-site ELISA binding.

The concentration of streptavidin-HRP conjugate used in these experiments was much lower than in earlier work because the conjugate was in short supply. The lower

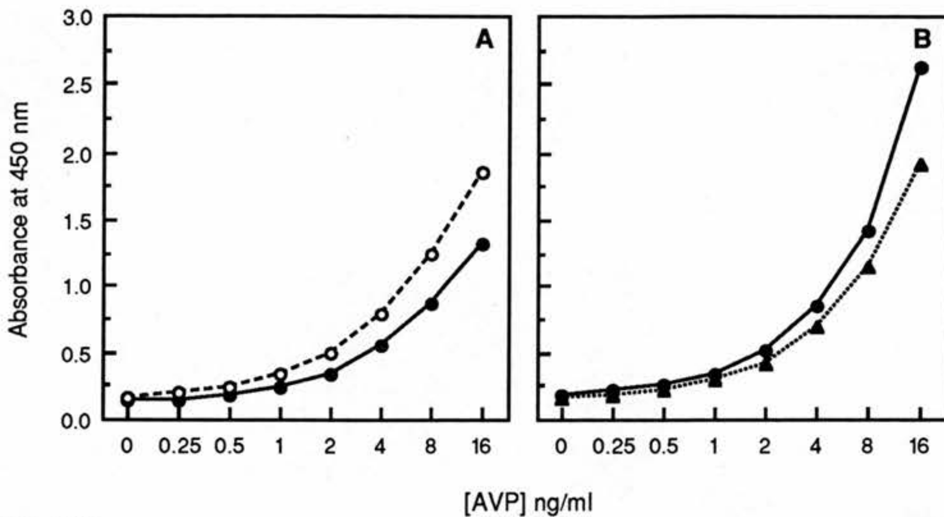

FIGURE 6.14

Effect of streptavidin-HRP concentration on two-site ELISA binding with biotin-ESVP 2 and ESVP 1 MAb

Serial dilutions of AVP in Buffer L were assayed in ESVP 1 MAb-coated, unblocked, Tween buffer-washed Microwells on a plate-shaker, using the separate (**A**) or partial simultaneous (**B**) incubation protocols as described in Figure 6.13. Binding of biotinylated protein A-purified ESVP 2 was revealed with either 100 µl (**A**) or 200 µl (**B**) of 0.05 µg/ml (▲), 0.1 µg/ml (●) or 0.2 µg/ml (○) streptavidin-HRP conjugate. The difference between duplicate estimations was smaller than the symbols used in this Figure.

concentration caused an 8-fold molar excess of biotin-ESVP 2 over streptavidin-HRP conjugate and was therefore responsible for the competition that occurred between bound and unbound biotin-ESVP 2 for binding of the conjugate in the simultaneous incubation protocol (Figure 6.13). However, reasonable dose-response curves were still obtained with the separate and partial simultaneous incubation protocols because the total amount of two-site binding was relatively low. All incubation protocols used the same mass of sample and biotin-ESVP 2, but the separate incubation protocol had an assay volume of only 100 µl and used half as much streptavidin-HRP conjugate as the other protocols, which had 200 µl assay volumes (Figure 6.13). Lower dose-dependent binding of biotinylated protein A-purified ESVP 2 was recorded with the separate incubation protocol than with the partial simultaneous protocol (Figure 6.13A) but this was only partly caused by the difference in conjugate mass (Figure 6.14). If the conjugate concentration was adjusted so that both protocols used the same conjugate

mass, the partial simultaneous protocol still produced higher dose-dependent binding (Figure 6.14) because its larger assay volume allowed a larger area of the ESVP 1 MAb-coated ELISA well to take part in two-site binding with AVP and biotinylated protein A-purified ESVP 2, which suggested that ESVP 1 MAb availability limited the binding response. In contrast, biotinylated PEG 8000-precipitated ESVP 2 produced similar dose-response curves with both the separate and partial simultaneous incubation protocols (Figure 6.13B) because the low ESVP 2 content of the impure preparation limited the amount of two-site binding that could occur.

The amount of ESVP 1 MAb that adsorbed onto the surface of the ELISA wells was not determined but Sorensen and Brodbeck (1986) reported that 350-375 ng rabbit IgG adsorbed onto Nunc Microwells from 100 µl of a 6 µg/ml solution. If ESVP 1 adsorbed in a similar manner, the coated wells would have contained an excess of ESVP 1 MAb over AVP, irrespective of the assay volume, therefore the separate and partial simultaneous incubation protocols should have produced identical dose-response curves with any given mass of conjugate. The higher binding that occurred with biotinylated protein A-purified ESVP 2 in the partial simultaneous incubation protocol (Figure 6.14) was therefore evidence of steric hindrance between ESVP 1 and ESVP 2 MAbs, particularly as the percentage increase in binding at high AVP concentrations was exactly matched by the percentage increase in surface area available for binding when compared with the separate incubation protocol.

The low conjugate concentration also limited the recorded binding response but this was less important than the effect of steric hindrance between the MAbs because doubling the conjugate mass did not double the recorded signal (Figure 6.14). However, the large size of the streptavidin-HRP conjugate was likely to cause steric hindrance that limited the amount of conjugate binding to biotin-ESVP 2 (Koertge and Butler, 1985). Nevertheless, these experiments showed that AVP could bind to two MAbs simultaneously in a two-site ELISA, although the binding response was ultimately limited by steric hindrance and only increased if the area of the MAb-coated surface increased.

6.3 IMMUNORADIOMETRIC ASSAY (IRMA)

IRMA solid phases were prepared by covalently coupling MAbs and polyclonal antibodies onto periodate-oxidized Sephacryl S-300 (Section 2.3.5.2). Some antibodies might have coupled in such a way that their antigen-binding capacity was destroyed therefore the antibody-S-300 solid phases were assayed for the ability to bind ^{125}I-AVP before they were used in IRMA work. All of the antibody preparations, except ESVP 3 and ESVP 4, produced solid phases that bound significant amounts of ^{125}I-AVP (Table 6.1). Knutson et al. (1991) reported that IgM MAbs were irreversibly inactivated by immobilization, which may explain the failure of ESVP 3-S-300 to bind ^{125}I-AVP. ESVP 4 was an IgA MAb, an isotype that is so rare amongst MAbs that little is known about its properties. The failure of ESVP 4-S-300 to bind ^{125}I-AVP might have been caused either by coupling the MAb too close to its binding site or by immobilization-induced denaturation.

Oxidized Sephacryl S-300 had a high protein-coupling capacity therefore impurities in the antibody preparations had little effect on the ability of the antibody-S-300 solid phases to bind ^{125}I-AVP. ESVP 2-S-300 was prepared from impure PEG 8000-precipitated ESVP 2 (Precipitate II (Experiment 2), Table 4.16) yet it bound almost as much ^{125}I-AVP as the protein A-purified ESVP 2-S-300 (Table 6.1) that was prepared from material that contained no detectable non-IgG substances (Figure 4.3B). Similarly, solid phases prepared from partially-purified antisera bound only slightly less ^{125}I-AVP than solid phases prepared from affinity-purified antisera (Table 6.1). Impurities that coupled to the solid phases were therefore unlikely to cause the drastic inhibition of two-site binding that was observed in the ELISA format (Section 6.2).

6.3.1 DIRECT IRMA

Initial IRMA studies with ^{125}I-ESVP 1 (Figure 4.1A, Table 4.22) and three

TABLE 6.1

Binding of ^{125}I-AVP to antibody-S-300 solid phases

Solutions of 1 mg/ml anti-AVP MAb or affinity-purified antibody, 8 mg/ml partially-purified anti-AVP serum (Section 4.6.1) or 4 mg/ml partially-purified anti-PAG serum (Section 4.6.2) were coupled to oxidized Sephacryl S-300 then assayed for antigen-binding ability as described in Section 2.3.5.2.
Pro A-p = protein A-purified; Aff-p = affinity-purified; Par-p = partially-purified.

Solid phase	% ^{125}I-AVP bound
ESVP 1-S-300	86.4
ESVP 2-S-300	54.0
Pro A-p ESVP 2-S-300	56.6
ESVP 3-S-300	3.2
ESVP 4-S-300	2.1
Aff-p anti-PAG-S-300	68.1
Aff-p anti-AVP-thyroglobulin-S-300	80.1
Par-p anti-AVP serum-S-300	54.1
Par-p anti-PAG serum-S-300	62.5

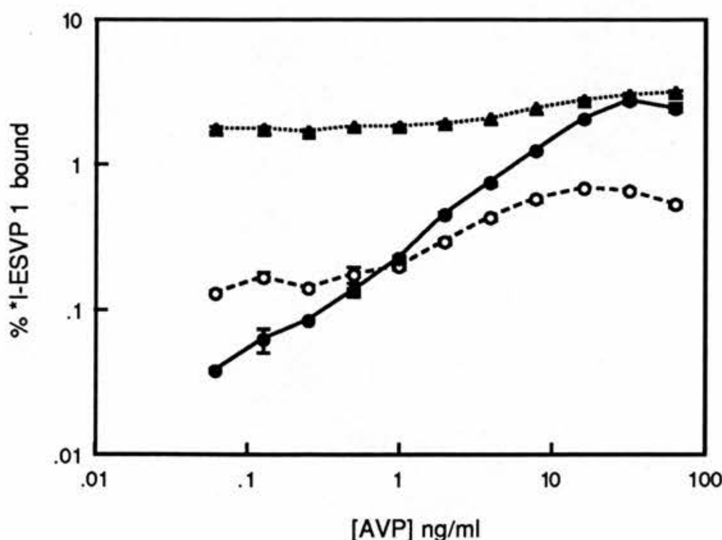

FIGURE 6.15

Comparison of three polyclonal antibody-S-300 solid phases in a direct IRMA

Serial dilutions of AVP, in Buffer E containing 5% BSA, were assayed by direct IRMA as described in Section 2.5.2.1, using ^{125}I-ESVP 1 with affinity-purified anti-PAG-S-300 (●), affinity-purified anti-AVP-thyroglobulin-S-300 (o) or partially purified anti-AVP serum-S-300 (▲) solid phases. Non-specific binding of 0.04% (●), 0.12% (o) and 1.72% (▲) of added ^{125}I-ESVP 1 was observed with these solid phases.

The symbols show the mean of duplicate estimates, while the vertical bars show the difference between those estimates. Bars which are not shown are smaller than the symbols used in this Figure.

polyclonal antibody-S-300 solid phases (Figure 6.15) confirmed the ELISA evidence (Figure 6.5) that two-site binding to AVP occurred with these antibody combinations. The partially-purified anti-AVP serum-S-300 solid phase produced an insensitive assay because high non-specific binding of ^{125}I-ESVP 1 (Figure 6.15), that was probably caused by anti-mouse IgG antibodies (Section 6.2), resulted in a low signal-to-noise ratio. However, 1% (v/v) normal mouse serum failed to decrease this non-specific binding (data not shown) therefore the partially-purified anti-AVP serum-S-300 solid phase was not used in any further direct IRMA work. Lower non-specific binding occurred with the affinity-purified anti-AVP-thyroglobulin-S-300 solid phase but low dose-dependent binding of ^{125}I-ESVP 1 produced a poor assay (Figure 6.15). This solid phase bound well to ^{125}I-AVP (Table 6.1) therefore the low two-site binding indicated that the affinity-purified anti-AVP-thyroglobulin preparation contained a

high proportion of antibodies that recognized epitopes which were too close to the ESVP 1 epitope to allow simultaneous binding. In contrast, the affinity-purified anti-PAG-S-300 solid phase, which bound less ^{125}I-AVP (Table 6.1), produced a better dose-response curve (Figure 6.15) because it contained a larger proportion of ESVP 1 MAb-compatible antibodies, while exceptionally low non-specific binding and good precision resulted in an assay with a sensitivity of 130 pg AVP/ml. However, the maximum binding response was only 3% of added ^{125}I-ESVP 1 which was further evidence to suggest that steric hindrance between the antibodies limited two-site binding to AVP.

Polyclonal antibodies tend to have higher average binding affinities than MAbs (Campbell, 1984; Goding, 1986) so affinity-purified anti-PAG probably had a significantly higher affinity than ESVP 1 MAb (Table 5.1). IRMA sensitivity is maximized when the labelled antibody has the highest possible affinity constant (Baker et al., 1985) therefore iodinated affinity-purified anti-PAG seemed likely to produce a more sensitive assay than ^{125}I-ESVP 1. However, the specific activity of the label is also important in determining IRMA sensitivity (Baker et al., 1985; Ekins, 1987). Iodination is most efficient in concentrated solutions (Chard, 1987) but, unfortunately, the affinity-purified anti-PAG preparation was fairly dilute therefore it failed to iodinate to a high specific activity and could not reliably reveal two-site binding with ESVP 1-S-300 solid phase (data not shown). The effect of using labelled affinity-purified anti-PAG could therefore only be studied in the indirect IRMA format (Section 6.3.2).

Most experiments were done with 100 µl AVP samples although an assay intended for neonatal studies would ideally use no more than 50 µl plasma per test. However, as expected, decreasing the sample volume to 50 µl decreased the sensitivity of the IRMA with ^{125}I-ESVP 1 and affinity-purified anti-PAG-S-300 solid phase (Figure 6.16). Since this IRMA was insufficiently sensitive to measure normal physiological AVP concentrations, no attempt was made to use a plasma matrix for the standard AVP solutions which were instead prepared in 5% BSA. However, AVP

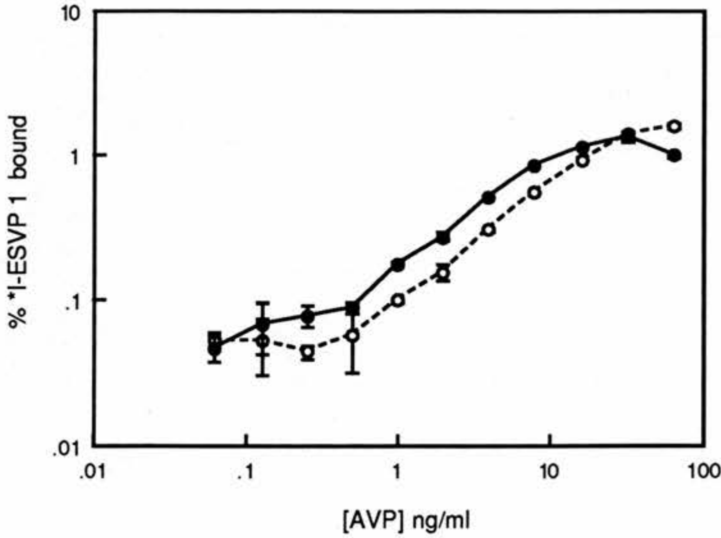

FIGURE 6.16

Effect of sample volume on direct IRMA binding

Duplicate 50 µl (o) or 100 µl (●) aliquots of AVP, in Buffer E containing 5% BSA, were assayed by direct IRMA, using ^{125}I-ESVP 1 with affinity-purified anti-PAG-S-300 solid phase. The non-specific binding corresponded to 0.03% of added ^{125}I-ESVP 1, irrespective of sample volume. The symbols show the mean of the duplicate estimates, while the vertical bars show the difference between those estimates.

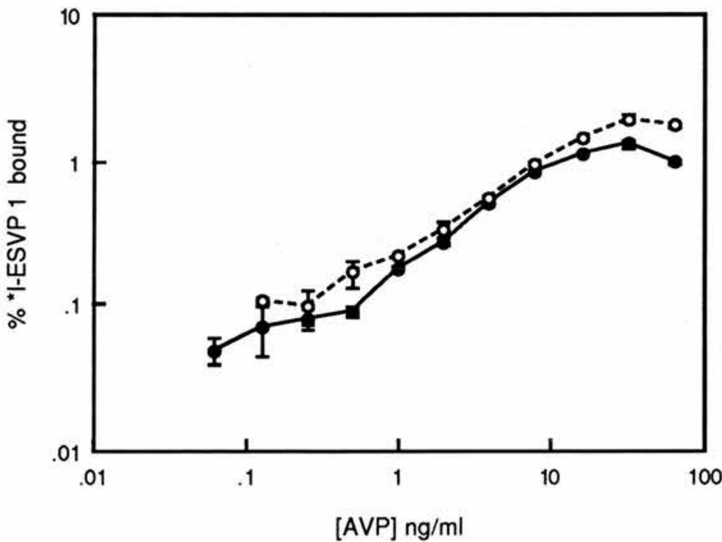

FIGURE 6.17

Effect of sample matrix composition on direct IRMA binding

Serial dilutions of AVP, in Buffer E containing 5% BSA (●) or in Buffer F (o), were assayed by direct IRMA, using ^{125}I-ESVP 1 with affinity-purified anti-PAG-S-300 solid phase. Non-specific binding of 0.03% (●) or 0.09% (o) of added ^{125}I-ESVP 1 was recorded. Vertical bars show the difference between duplicate estimations.

adsorbed non-specifically to BSA (Figures 6.2, 6.7 and 6.9), which might have caused significant assay interference, so the 5% BSA matrix was compared with assay buffer which contained 2% bovine serum (Buffer F). Lower specific and non-specific binding was recorded in 5% BSA than in Buffer F (Figure 6.17) but assay sensitivity was almost unchanged therefore the BSA matrix did not seem to adversely affect the IRMA. Decreased binding was also reported in other IRMAs when the protein concentration of the matrix was increased (Hunter et al., 1983; Wright et al., 1984).

ESVP 3 and ESVP 4 MAbs were unsuitable for IRMA work because they could not be coupled to the solid phase (Table 6.1), and they produced no evidence of two-site binding when ^{125}I-ESVP 3 and ^{125}I-ESVP 4 were combined with ESVP 1-S-300 solid phase (data not shown). In contrast, ESVP 1 and ESVP 2 MAbs combined to produce a dose-dependent response in the IRMA format, although ^{125}I-ESVP 2 binding was poor and better results were obtained with ^{125}I-ESVP 1 and ESVP 2-S-300 solid phase (Figure 6.18). Protein A-purified ESVP 2 iodinated to a higher specific activity than PEG 8000-precipitated ESVP 2 (Table 4.22) but this did not improve the binding response therefore ^{125}I-ESVP 2 was not used in any further work.

The dose-dependent binding of ^{125}I-ESVP 1 to ESVP 2-S-300 solid phase was caused by specific interaction of AVP with the binding sites of the MAbs, and not by non-specific adsorption of AVP onto the solid phase, since no dose-dependent response occurred between ^{125}I-ESVP 1 and MAb-free solid phase (data not shown) or between ^{125}I-ESVP 1 and ESNP 2-S-300 solid phase (Figure 6.19). However, dose-dependent binding was observed between ^{125}I-ESVP 1 and ESVP 1-S-300 solid phase (Figure 6.19). This 'pseudo-two-site' binding was only 10% of the genuine two-site response that was recorded with ^{125}I-ESVP 1 and ESVP 2-S-300 solid phase, but it was larger than the 'pseudo-two-site' response that had been observed between biotin-ESVP 2 and ESVP 2 MAb-coated ELISA wells (Figure 6.12) even though the same AVP preparation was used in both experiments. This observation provided further evidence of the assay interference that resulted from MAb cross-linkage by AVP-BSA complexes, and showed that the degree of interference was dependent on the assay format.

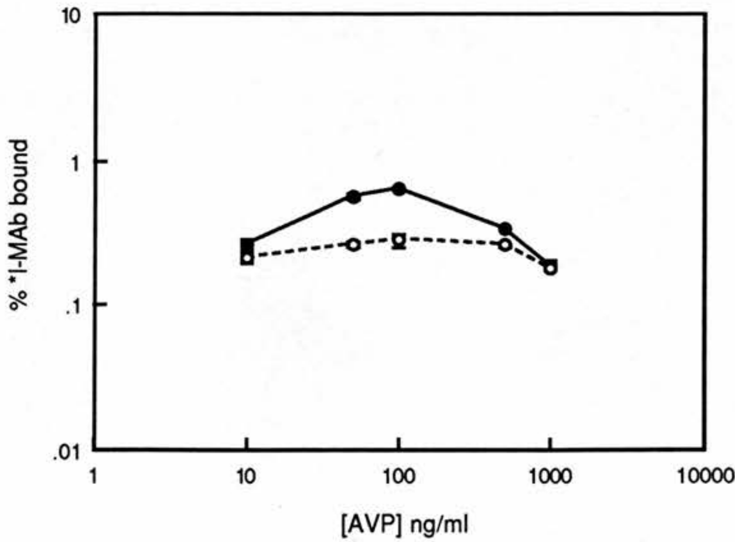

FIGURE 6.18

Effect of MAb orientation on a direct IRMA with ESVP 1 and ESVP 2 MAbs

Dilutions of AVP in Buffer E containing 5% BSA were assayed by direct IRMA, using ^{125}I-ESVP 1 with ESVP 2-S-300 solid phase (●), or ^{125}I-ESVP 2 with ESVP 1-S-300 solid phase (o). Non-specific binding of 0.05% (●) or 0.18% (o) of added ^{125}I-MAb was recorded. Vertical bars showing the difference between duplicate tubes are smaller than the symbols used in this Figure.

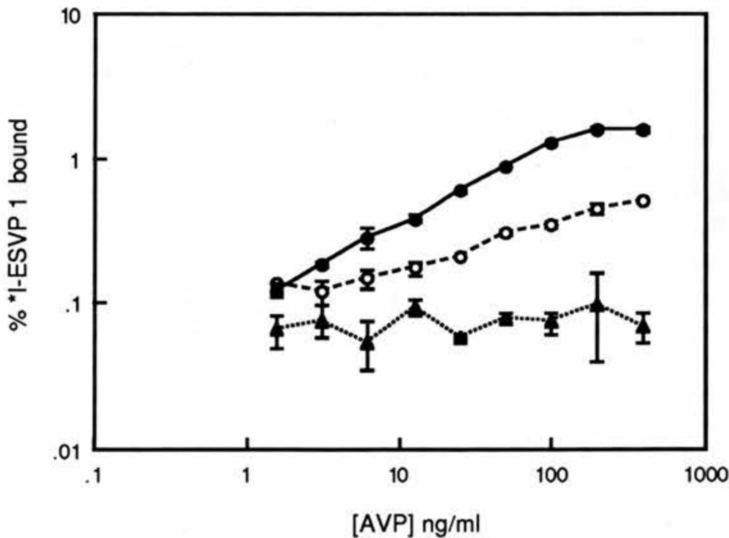

FIGURE 6.19

Comparison of three MAb-S-300 solid phases in a direct IRMA with ^{125}I-ESVP 1

Serial dilutions of AVP, in Buffer J containing 5% BSA, were assayed by direct IRMA, using ^{125}I-ESVP 1 with ESVP 2-S-300 (●), ESVP 1-S-300 (o) or ESNP 2-S-300 (▲) solid phases. Non-specific binding of 0.04% (●), 0.14% (o) and 0.05% (▲) of added ^{125}I-ESVP 1 was recorded with these solid phases. The symbols show the mean of duplicate estimates, while the vertical bars show the difference between those estimates.

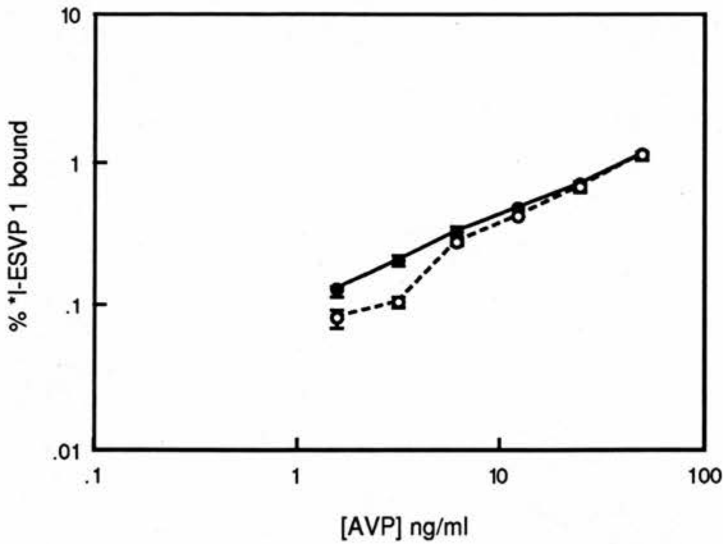

FIGURE 6.20

Effect of incubation protocol on a direct IRMA with ESVP 1 and ESVP 2 MAbs

Serial dilutions of AVP, in Buffer E containing 5% BSA, were preincubated with ^{125}I-ESVP 1 for 2 hours then ESVP 2-S-300 solid phase was added and incubation continued for a further 2 hours (●), or they were incubated with ^{125}I-ESVP 1 and ESVP 2-S-300 solid phase simultaneously for a total of 2 hours (○), before separation of bound and free ^{125}I-ESVP 1 by the sucrose layering technique. Non-specific binding of 0.05% of added ^{125}I-ESVP 1 was recorded with both incubation protocols. Vertical bars show the difference between duplicate estimations.

Most of the IRMA work was done with a total incubation period of 4 hours, where the sample was preincubated with ^{125}I-MAb for 2 hours before addition of the antibody-S-300 solid phase, since this type of incubation protocol was reported to produce the best IRMA sensitivity (Hunter et al., 1983). When the incubation period was decreased to 2 hours by omission of the preincubation step, the sensitivity of the IRMA with ^{125}I-ESVP 1 and ESVP 2-S-300 solid phase decreased from 1 ng AVP/ml to 4 ng AVP/ml, although the maximum binding response was unchanged (Figure 6.20).

A concentration of 50 ng ^{125}I-ESVP 1/ml was used in most IRMA work, producing roughly similar binding with both the affinity-purified anti-PAG-S-300 and the ESVP 2-S-300 solid phases. IRMA sensitivity can be increased by decreasing the concentration of added ^{125}I-MAb so that a greater proportion of the ^{125}I-MAb becomes bound (Hunter et al., 1983). When the ^{125}I-ESVP 1 concentration was decreased, the

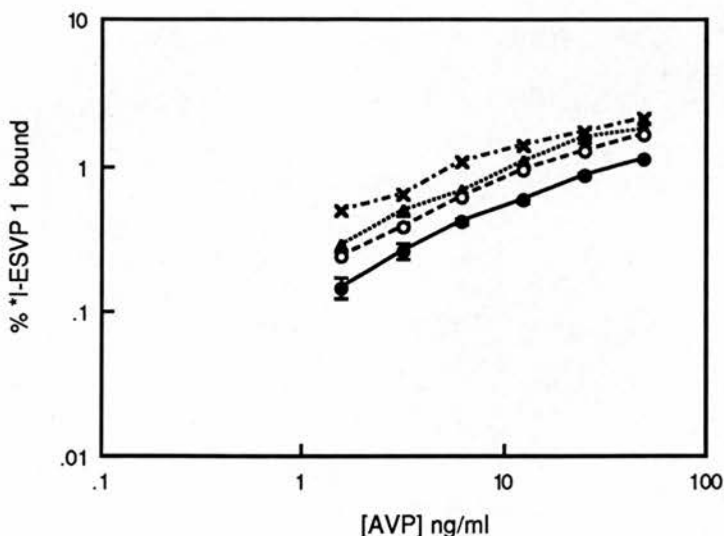

FIGURE 6.21

Effect of ^{125}I-ESVP 1 concentration on direct IRMA binding

Serial dilutions of AVP in Buffer E containing 5% BSA were assayed by direct IRMA, using 5 (×), 10 (▲), 25 (○) or 50 (●) ng/ml ^{125}I-ESVP 1 in Buffer F with protein A-purified ESVP 2-S-300 solid phase in Buffer R. Non-specific binding of 0.14% (×), 0.04% (▲), 0.01% (○) or 0.01% (●) of added ^{125}I-ESVP 1 was recorded. The difference between duplicate estimations with 50 ng/ml ^{125}I-ESVP 1 is indicated by vertical bars. For clarity, equivalent data for the other ^{125}I-ESVP 1 concentrations has been omitted from this Figure.

percentage binding to the ESVP 2-S-300 solid phase increased as expected (Figure 6.21) but the total amount of bound radioactivity was so low that increased counting errors decreased the precision of the assay (Klee and Post, 1989) and the sensitivity was not improved. There was therefore no advantage to be gained from decreasing the ^{125}I-ESVP 1 concentration used in the IRMA.

Further studies with ^{125}I-ESVP 1 and ESVP 2-S-300 showed that variation of the solid phase volume had little effect on IRMA binding. Similar results were obtained with 100 µl and 200 µl ESVP 2-S-300 solid phase at AVP concentrations up to 25 ng/ml (Figure 6.22), although 200 µl solid phase produced better ^{125}I-ESVP 1 binding at higher AVP concentrations. Impurities in the ESVP 2 preparation had no effect on IRMA sensitivity because solid phases that were prepared from protein A-purified ESVP 2 and PEG 8000-precipitated ESVP 2 produced similar IRMA binding at AVP concentrations up to 25 ng/ml (Figure 6.23). However, protein A-purified ESVP 2-S-300

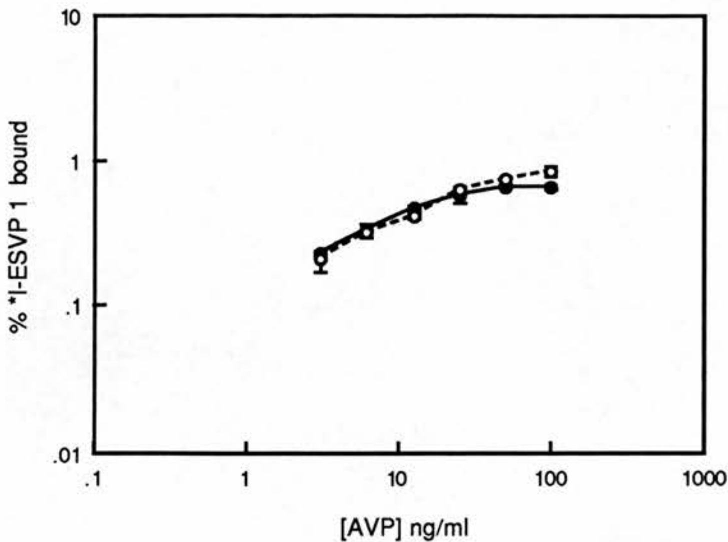

FIGURE 6.22

Effect of ESVP 2-S-300 solid phase volume on direct IRMA binding

Serial dilutions of AVP, in Buffer E containing 5% BSA, were assayed by direct IRMA, using ^{125}I-ESVP 1 with either 100 µl (●) or 200 µl (○) ESVP 2-S-300 solid phase. Non-specific binding was 0.05% (●) or 0.1% (○) of added ^{125}I-ESVP 1. Vertical bars show the difference between duplicate estimations, although most of the bars are smaller than the symbols used in this Figure.

solid phase had a higher AVP-binding capacity, presumably because fewer impurities coupled to sites that ESVP 2 MAb would otherwise have occupied, therefore at high AVP concentrations it allowed higher IRMA binding than the PEG 8000-precipitated ESVP 2-S-300 solid phase (Figure 6.23).

In the IRMA format, the concentration of ^{125}I-MAb is usually much lower than that of the solid phase-coupled antibody therefore 'plateau' binding occurs at high analyte concentrations when all of the ^{125}I-MAb becomes linked to the solid phase (Figure 1.1B). If the analyte concentration increases further, it eventually exceeds the binding capacity of the solid phase and causes the high-dose 'hook' effect when free analyte competes with ^{125}I-MAb-bound analyte for binding to the solid phase antibody (Figure 6.23). In this project, 4 ml 1 mg/ml protein A-purified ESVP 2 coupled to 3 ml (settled volume) oxidized Sephacryl S-300 with an efficiency of 25%, producing sufficient solid phase for 240 tests, therefore each aliquot of protein A-purified ESVP 2-S-300 solid phase contained about 4 µg ESVP 2. The AVP IRMA used only

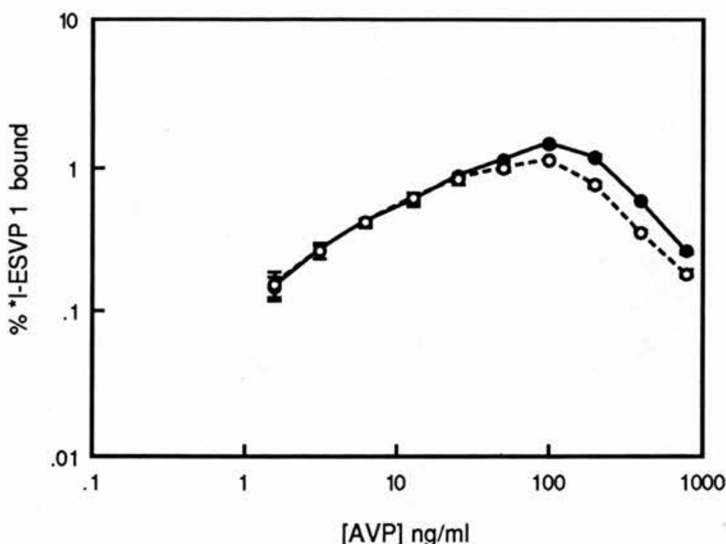

FIGURE 6.23

Effect of ESVP 2 MAb purity on the direct IRMA binding of ESVP 2-S-300 solid phase

ESVP 2 MAb was prepared from spent culture supernatant by PEG 8000 precipitation (Precipitate II (Experiment 2), Table 4.16, ○) or Protein A-Sepharose affinity chromatography (●), then solutions of 1 mg/ml ESVP 2 were coupled to oxidized Sephacryl S-300 (Section 2.3.5.2).

Serial dilutions of AVP, in Buffer E containing 5% BSA, were assayed by direct IRMA, using ^{125}I-ESVP 1 with the ESVP 2-S-300 solid phases. The non-specific binding was 0.01% of added ^{125}I-ESVP 1 for both solid phases. Vertical bars show the difference between duplicate estimations.

5 ng ^{125}I-ESVP 1 per tube therefore the ESVP 2-S-300 solid phase had an AVP-binding capacity that was 800-fold larger than that of ^{125}I-ESVP 1 and 'plateau' binding was expected to occur at high AVP concentrations. However, ^{125}I-ESVP 1 binding continued to increase until the binding capacity of the solid phase was reached at 100 ng AVP/ml and no 'plateau' binding was observed (Figure 6.23). This was further evidence of steric hindrance between the antibodies because 100 ng AVP/ml corresponded to a 300-fold molar excess of AVP over ^{125}I-ESVP 1. In the absence of steric hindrance, and assuming that both ESVP 1 binding sites were accessible to AVP, maximal binding of ^{125}I-ESVP 1 to the ESVP 2-S-300 solid phase would have been expected to occur at a concentration closer to 0.6 ng AVP/ml.

Most experiments were done at pH 7.0. However, investigation of the effect of pH on the IRMA with ^{125}I-ESVP 1 and ESVP 2-S-300 solid phase showed that optimal

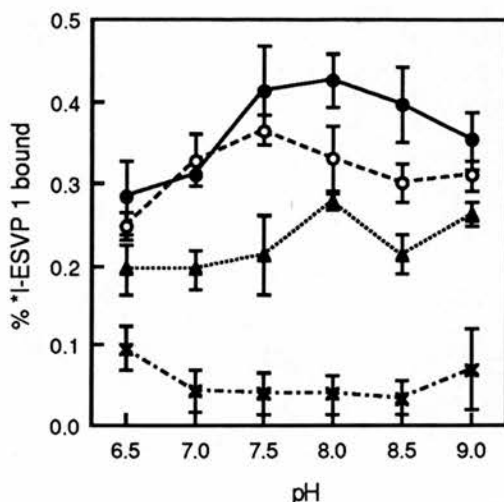

FIGURE 6.24

Effect of pH on a direct IRMA with ^{125}I-ESVP 1 and ESVP 2-S-300 solid phase

Quadruplicate 50 µl aliquots of AVP (0 (×), 6.25 (▲), 25 (●) and 100 (○) ng/ml in 0.01M sodium phosphate buffer, pH 7.0, containing 5% BSA) were incubated with 50 µl assay buffer and 100 µl 50 ng/ml ^{125}I-ESVP 1 in assay buffer for 2 hours, then 100 µl ESVP 2-S-300 (12.5% (settled volume) slurry in assay buffer containing 3% Tween 20) was added. The mixture was agitated on an orbital shaker for 2 hours, then bound and unbound ^{125}I-ESVP 1 were separated by sucrose layering, at the appropriate pH. The symbols show the mean of the quadruplicate estimations and the vertical bars show the standard deviation of those estimations.

The assay buffers were Universal buffer solutions (Britton and Robinson type; Dawson et al., 1969) in the pH range 6.5-9.0, containing 2% bovine serum.

binding seemed to occur in the range pH 7.5-8.0 (Figure 6.24). Non-specific binding was unaffected by pH changes in the range pH 7.0-8.5 therefore the sensitivity of the IRMA could be increased by raising the pH of the assay buffer, although the gain in sensitivity would be relatively small. Unfortunately, there was no time available to carry out further studies of this phenomenon.

6.3.2 INDIRECT IRMA

The affinity-purified anti-PAG preparation did not iodinate well therefore it could not be used as the liquid-phase antibody in a direct IRMA. Biotinylated affinity-purified anti-PAG had, however, produced reasonable two-site ELISA binding (Figure 6.6) therefore it was studied in the indirect IRMA, where ^{125}I-streptavidin

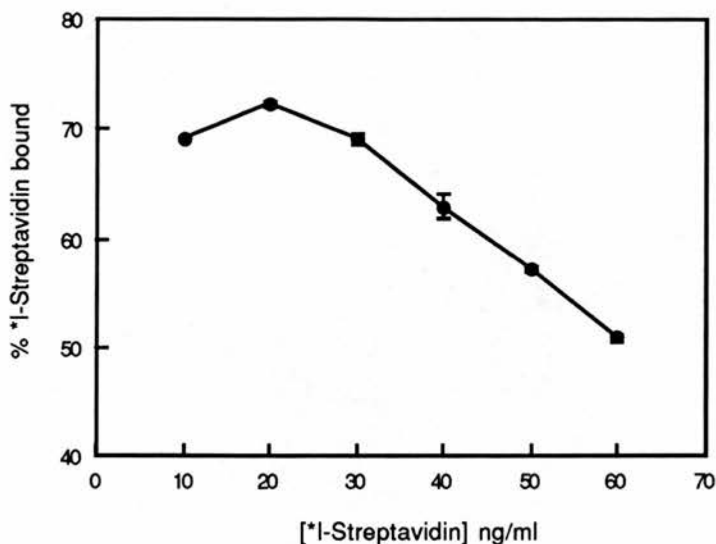

FIGURE 6.25

Optimization of ^{125}I-streptavidin binding to biotin-ESVP 1

Duplicate 100 µl aliquots of ^{125}I-streptavidin in Buffer F were incubated with 100 µl 50 ng/ml biotin-ESVP 1 in Buffer F for 15 minutes, then 100 µl SAM-S-500 solid phase (25% (settled volume) slurry in Buffer G) was added. The mixture was agitated on an orbital shaker for 1 hour, then bound ^{125}I-streptavidin was isolated by the sucrose layering technique, as described in Section 2.5.2.1. Vertical bars show the difference between the duplicate estimations.

was used as a universal label to reveal the binding of biotin-antibody. The indirect IRMA had the advantage that it was not prone to the high non-specific binding that frequently occurred in the two-site ELISA format (Section 6.2).

A preliminary study with 50 ng/ml biotin-ESVP 1 showed that optimal signal generation occurred with 20 ng/ml ^{125}I-streptavidin (Figure 6.25), which corresponded to a 1:1 molar ratio of streptavidin to MAb. It had to be assumed that the same molar ratio would also provide optimal detection of biotinylated affinity-purified anti-PAG because there was no solid phase available that would allow the study of ^{125}I-streptavidin binding to biotinylated rabbit antibodies.

The AVP-binding affinity of the affinity-purified anti-PAG preparation was unknown but was expected to be higher than that of ESVP 1 MAb. Maximal IRMA sensitivity is obtained when the labelled antibody has the highest binding affinity (Baker et al., 1985) therefore higher indirect IRMA binding was expected with

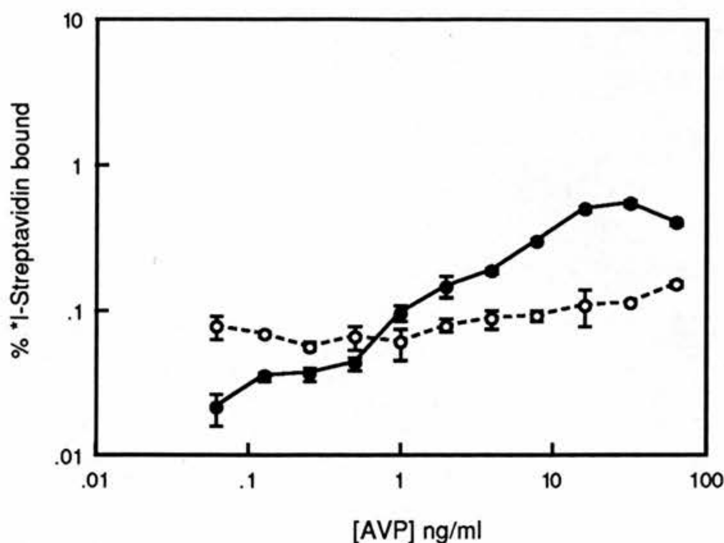

FIGURE 6.26

Effect of antibody orientation on an indirect IRMA

Dilutions of AVP, in Buffer E containing 5% BSA, were assayed by indirect IRMA (Section 2.5.2.2), using biotin-ESVP 1 with affinity-purified anti-PAG-S-300 solid phase (●) or biotinylated affinity-purified anti-PAG with ESVP 1-S-300 solid phase (o). Non-specific binding of 0.01% (●) and 0.06% (o) of added ^{125}I-streptavidin was recorded. The symbols show the mean of duplicate estimates, while the vertical bars show the difference between those estimates.

biotinylated affinity-purified anti-PAG and ESVP 1-S-300 solid phase than with biotin-ESVP 1 and affinity-purified anti-PAG-S-300 solid phase. However, two-site binding was barely detectable with biotinylated affinity-purified anti-PAG, while biotin-ESVP 1 produced a reasonable dose-response curve (Figure 6.26). This surprising result provided additional evidence to support the theory that some of the anti-tail antibodies in the affinity-purified anti-PAG preparation bound to epitopes that were so close to that of the ring-specific ESVP 1 MAb that they could not combine with ESVP 1 to form a two-site AVP assay. AVP bound to all biotin-antibody specificities during the 2 hour preincubation period but the antibody-S-300 solid phase could only cross-link with biotin-antibodies that recognized compatible epitopes. Although steric hindrance prevented a high binding response, all of the biotin-ESVP 1 MAb-bound AVP was available for binding to the multispecific affinity-purified anti-PAG-S-300 solid phase. In contrast, the monospecific ESVP 1-S-300 solid phase could only bind a

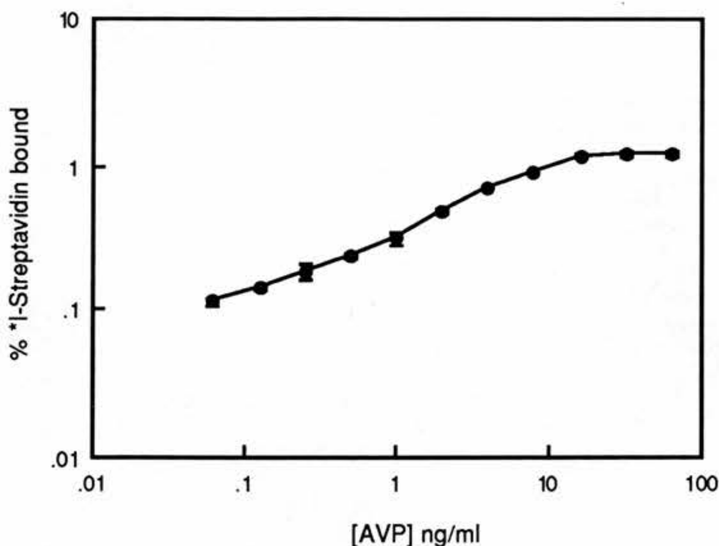

FIGURE 6.27

Indirect AVP IRMA using two polyclonal antibody preparations

Serial dilutions of AVP, in Buffer E containing 5% BSA, were assayed by indirect IRMA, using biotinylated affinity-purified anti-PAG with partially-purified anti-AVP serum-S-300 solid phase. The non-specific binding was 0.11% of added ^{125}I-streptavidin. Vertical bars showing the difference between duplicate estimations are smaller than the symbols used in this Figure.

small fraction of the biotinylated affinity-purified anti-PAG-bound AVP therefore extremely low dose-dependent binding was recorded (Figure 6.26).

Interestingly, biotinylated affinity-purified anti-PAG produced a much better dose-response curve with partially-purified anti-AVP serum-S-300 solid phase (Figure 6.27), which confirmed that the low binding obtained with ESVP 1-S-300 solid phase (Figure 6.26) was due to steric hindrance and not to sub-optimal signal generation since the same ^{125}I-streptavidin concentration was used in both experiments. These results also showed that the partially-purified anti-AVP serum contained antibodies to both the ring and tail structures of AVP since it could form a two-site assay with ESVP 1 MAb (Figures 6.2 and 6.15) and with affinity-purified anti-PAG (Figure 6.27) provided that AVP bound to the first antibody before the partially purified anti-AVP serum was added (Figures 6.1 and 6.4).

Although 'plateau' binding was not observed with any other antibody combination, it occurred in the indirect IRMA with biotinylated affinity-purified

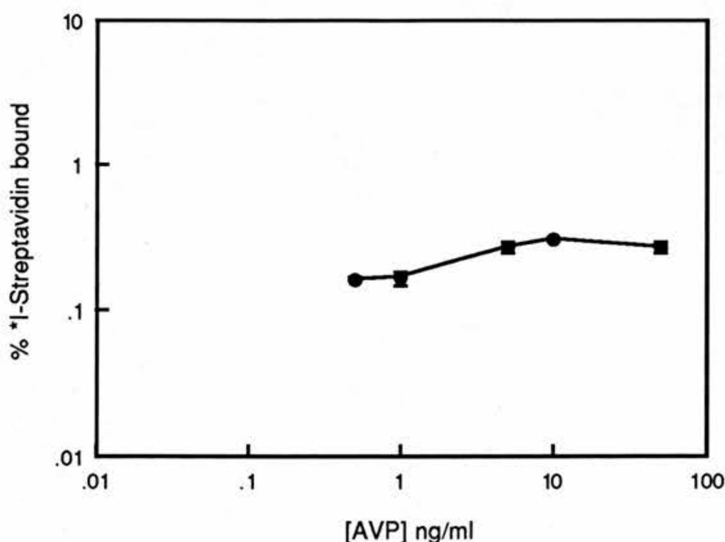

FIGURE 6.28

Indirect IRMA with biotin-ESVP 1 and anti-PAG serum-S-300 solid phase

Dilutions of AVP, in Buffer E containing 5% BSA, were assayed by indirect IRMA, using biotin-ESVP 1 with partially-purified anti-PAG serum-S-300 solid phase (prepared from the 0-4% PEG cut of anti-PAG serum, after further purification by Rivanol precipitation; Section 4.6.2). Non-specific binding was 0.15% of added ^{125}I-streptavidin. Vertical bars show the difference between duplicate estimations.

anti-PAG and partially-purified anti-AVP serum-S-300 solid phase (Figure 6.27). This suggested that the anti-AVP serum antibodies that bound to AVP simultaneously with affinity-purified anti-PAG probably recognized epitopes at the opposite side of the AVP molecule from the anti-PAG epitopes, so that there was less steric hindrance between these antibodies than between ESVP 1 MAb and the anti-tail antibody preparations (Figures 6.23 and 6.26). Nevertheless, the total binding was only 1.2% of added ^{125}I-streptavidin, which suggested that few anti-AVP serum antibodies were compatible with affinity-purified anti-PAG in a two-site assay. Further purification might have increased the binding response obtained with the anti-AVP serum preparation, but there was no time available to investigate suitable methods because the aim of the project was to develop a MAb-based assay.

Antibody-S-300 solid phase preparation required large amounts of antibody (Section 2.3.5.2), so few experiments could be done with the affinity-purified anti-PAG-S-300 solid phase because very little affinity-purified antibody was

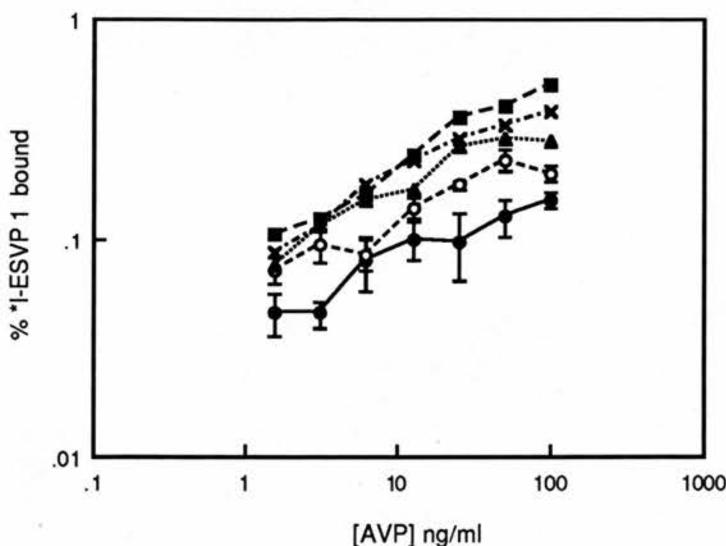

FIGURE 6.29

Immunofiltration assay with ^{125}I-ESVP 1 and PEG-precipitated ESVP 2 MAb

PEG 8000-precipitated ESVP 2 MAb (Precipitate II (Experiment 2), Table 4.16) was coated onto nitrocellulose membrane-bottomed wells (Section 2.3.5.5) at 5 (●), 10 (○), 15 (▲), 20 (×) or 30 (■) µg/ml. Duplicate 100 µl aliquots of AVP in Buffer M were assayed by immunofiltration through the coated membranes, using ^{125}I-ESVP 1 MAb to detect binding (Section 2.5.3). Non-specific binding was 0.05% (●), 0.06% (○), 0.07% (▲), 0.07% (×) or 0.08% (■) of added ^{125}I-ESVP 1.

Vertical bars show the difference between the duplicate estimations in wells coated with 5 (●) or 10 (○) µg ESVP 2/ml. For clarity, the equivalent data for the other coating concentrations has been omitted from this Figure.

available. The partially-purified anti-PAG serum preparation (Section 4.6.2) could not substitute for affinity-purified anti-PAG because biotin-ESVP 1 produced poor indirect IRMA binding with partially-purified anti-PAG serum-S-300 solid phase (Figure 6.28) compared with that produced by affinity-purified anti-PAG-S-300 solid phase (Figure 6.26). Consequently, no further indirect IRMA studies of ESVP 1 MAb in combination with anti-PAG antibodies could be carried out.

6.4 IMMUNOFILTRATION ASSAY

Immunoassay reaction rates at solid-liquid interfaces tend to be limited by the rate of diffusion of the reactants (Stenberg and Nygren, 1988) although high reagent concentrations minimize this effect and drive the reaction to completion (Ekins, 1987).

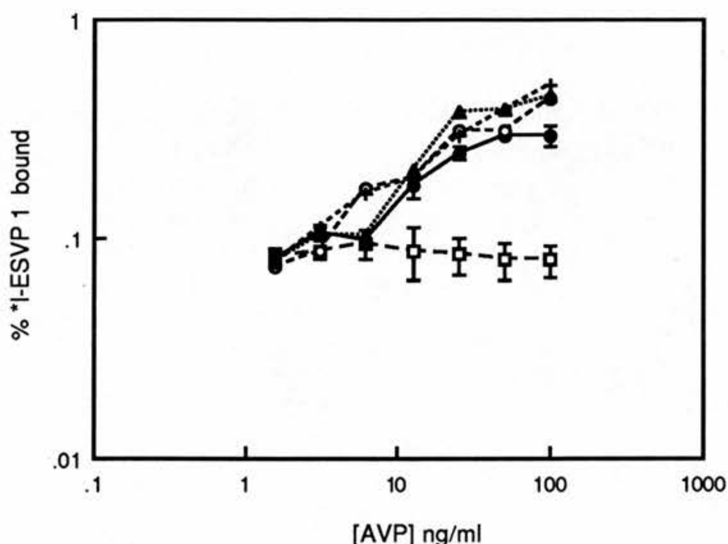

FIGURE 6.30

Immunofiltration assay with ^{125}I-ESVP 1 and protein A-purified ESVP 2

Protein A-purified ESVP 2 MAb was coated onto nitrocellulose membrane-bottomed wells (Section 2.3.5.5) at 5 (●), 10 (○), 15 (▲) or 25 (+) μg/ml, then immediately used to assay serial dilutions of AVP in Buffer M by immunofiltration (Section 2.5.3). Uncoated wells (□), washed four times with Buffer U, were included for comparison. The non-specific binding was 0.09% (□), 0.08% (●), 0.07% (○), 0.07% (▲) and 0.06% (+) of added ^{125}I-ESVP 1.

Vertical bars show the difference between duplicate estimations in uncoated wells (□) and in those coated with 5 μg ESVP 2/ml (●). For clarity, equivalent data for the other coating concentrations has been omitted from this Figure.

Immunofiltration assays bring the reactants into close proximity as they pass through a coated membrane therefore maximal reaction rates are achieved and incubation periods can be very short (Valkirs and Barton, 1985; IJsselmuiden et al., 1989). Immunofiltration therefore had the potential to produce a rapid AVP assay.

An initial study showed that two-site binding in an immunofiltration assay with ^{125}I-ESVP 1 and ESVP 2 MAb-coated nitrocellulose membranes increased dramatically as the coating concentration of PEG 8000-precipitated ESVP 2 increased from 5 μg/ml to 30 μg/ml (Figure 6.29), although the total binding of ^{125}I-ESVP 1 was lower than that observed in a direct IRMA (Figure 6.23). In contrast, there was only a small increase in binding as the coating concentration of protein A-purified ESVP 2 increased from 5 μg/ml to 10 μg/ml and there was no significant improvement at higher coating concentrations (Figure 6.30), which compared well with the previous

observation that 6 μg/ml ESVP 1 filled most of the protein-binding sites in polystyrene wells (Section 6.2). However, the maximum amount of dose-dependent ^{125}I-ESVP 1 binding that occurred was the same with both ESVP 2 preparations therefore the impurities in PEG 8000-precipitated ESVP 2 were unlikely to adsorb onto the membrane but instead interfered with coating by decreasing the effective concentration of ESVP 2 MAb in the coating solution. The efficiency of membrane coating was not determined but the low ^{125}I-ESVP 1 binding suggested that the ESVP 2 MAb-coating capacity of the nitrocellulose membrane was lower than that of oxidized Sephacryl. No dose-dependent binding occurred on uncoated membranes (Figure 6.30) therefore AVP did not adsorb onto nitrocellulose under the conditions of the assay.

Although the total assay time was only 1 hour, low binding and poor precision limited the sensitivity of the immunofiltration assay to 10 ng AVP/ml therefore it was 10-fold less sensitive than a direct IRMA with ^{125}I-ESVP 1 and ESVP 2-S-300 solid phase (Figure 6.19). Variation of the assay parameters might have improved the binding of ^{125}I-ESVP 1 but the sensitivity of the immunofiltration assay was ultimately limited both by steric hindrance between the MAbs and by the low coating capacity of the nitrocellulose membrane therefore it did not offer any significant advantage over the direct IRMA.

6.5 DISCUSSION

Two-site immunometric assays for AVP could be carried out in the ELISA, IRMA and immunofiltration assay formats, despite the small size of the AVP molecule, although steric hindrance between the antibodies was a major problem which severely limited the total amount of binding that occurred. The epitopes that were recognized by the anti-AVP MAbs seemed to consist of 3-4 amino acids (Section 5.4). Epitopes are usually reported to contain 4-6 amino acids (Sela, 1969; Barbet et al., 1981; Baker et al., 1985; Siddle, 1985) therefore many workers consider that peptides of 15-20 amino acids are the smallest that will allow two-site binding

(Siddle, 1985; Gosling, 1990), although a minimum peptide size of 10-12 amino acids has also been suggested (Baker et al., 1985). AVP consists of only nine amino acids and is therefore likely to be one of the smallest molecules that can be measured in a two-site assay, despite claims that AVP could not bind simultaneously to two antibody molecules (Hashida et al., 1991b) and that the entire AVP molecule would occupy an antibody binding site as a single epitope (Wu and Rockey, 1969). An antibody binding site is widely considered to be a pocket or cleft (Roitt et al., 1985) into which the appropriate epitope will fit, although recent studies have shown that binding sites can be relatively flat or even protrude from the antibody molecule (Wilson et al., 1991). Antibodies that bind simultaneously to AVP may therefore have particularly shallow binding sites that enable them to bind in close proximity.

Non-specific binding was a serious problem in the two-site ELISA format with most of the available antibody combinations. Polyclonal antibody preparations that were contaminated by anti-BSA (Figure 6.1A) or anti-mouse IgG (Figures 6.3 and 6.4) specificities bound to MAb-coated BSA-blocked ELISA wells, causing high background absorbances despite the presence of BSA and normal mouse serum in the assay buffer. Affinity-purified antisera were free from these contaminants but probably contained some antibodies that had been structurally-damaged during the purification procedure. Non-specific binding of this material was recorded in coated ELISA wells (Figures 6.5 and 6.9) but not in uncoated wells (Figure 6.9B), which showed that the binding was caused by protein-protein interactions and not by adsorption onto the polystyrene surface under the conditions of the assay. Non-specific binding of biotin-MAbs (Figures 6.8, 6.10A and 6.13B) was reportedly a pH-related phenomenon since biotinylation shifted the isoelectric pH of MAbs towards more acidic values, causing decreased solubilities and therefore higher non-specific binding at pH values of 7.0 or less, so that the highest signal-to-noise ratios were obtained at pH 8.0-9.0 (Wadsley and Watt, 1987). All of the two-site AVP ELISAs were done at pH 7.5, and the effect of pH was not investigated, but non-specific binding might have been lower at pH 8.0.

Impurities in the biotin-MAb preparations contributed to non-specific binding

and significantly decreased the signal-to-noise ratio because they decreased the effective concentration of MAb in solution and, thus, the dose-dependent binding response. Protein A-purified ESVP 2 contained no detectable non-antibody substances (Figure 4.3B) and, when biotinylated, produced steep dose-response curves and negligible non-specific binding in a two-site ELISA with ESVP 1 MAb-coated wells (Figures 6.10B and 6.13A). In contrast, the very impure PEG 8000-precipitated ESVP 2 (Figure 4.3A) biotinylated to produce a reagent that gave higher non-specific binding and extremely poor dose-response curves in ESVP 1 MAb-coated wells (Figure 6.13B). Relatively high background absorbances also occurred with biotin-ESVP 1, although it produced reasonable dose-response curves in ESVP 2 MAb-coated wells (Figure 6.10A). The ESVP 1 MAb preparation contained little non-antibody material (Figure 4.1A) but it was prepared from ascitic fluid by sodium sulphate precipitation (Section 4.5.1.1) and would therefore have contained a low concentration of endogenous mouse IgG (Galfrè and Milstein, 1981; Baines et al., 1990). The solubilities of some of the mouse IgG contaminants may have been decreased by biotinylation (Wadsley and Watt, 1987), thus causing the observed non-specific binding. Further purification of ESVP 1 MAb might have decreased this non-specific binding and increased the signal-to-noise ratio obtained with biotin-ESVP 1 in ESVP 2 MAb-coated wells, although it seems unlikely that any improvement would be sufficient to produce a more sensitive ELISA with this MAb orientation than with biotinylated protein A-purified ESVP 2 in ESVP 1 MAb-coated wells (Figure 6.10).

Long incubation periods were used in most of the two-site ELISA studies, both for convenience and to ensure that maximal binding occurred because static incubations are subject to diffusion-limited reaction rates (Stenberg and Nygren, 1988) and no plate-shaking facilities were available. However, these long incubations contributed to non-specific binding and to intra-assay variation (Kemeny and Chantler, 1988). AVP adsorbed onto BSA (Figures 6.2 and 6.9A) and onto MAbs (Figures 6.8 and 6.9) during long incubation periods but not during the shorter incubations that were used towards the end of this project once a plate-shaker became available (Figure 6.11),

while greater variation between duplicate ELISA estimations was recorded after long incubations (Figures 6.5 and 6.9A) than after shorter incubation periods (Figures 6.10 and 6.12). A 15 minute incubation period was always used with the streptavidin-HRP conjugate because streptavidin binds rapidly to biotin-antibody and incubations of more than 20 minutes were reported to decrease the signal-to-noise ratio (Kendall et al., 1983). Nevertheless, the streptavidin-HRP conjugate adsorbed to coated ELISA wells in the absence of biotin-antibody (Figure 6.3) and so increased the background absorbances, although this effect was insignificant in later work that used very low conjugate concentrations (Figures 6.12, 6.13 and 6.14).

Antigen-antibody interactions at solid-liquid interfaces are considered to be practically irreversible (Stenberg and Nygren, 1988), particularly as only 1% of antigen was reported to desorb from antibody-coated ELISA wells in 3 days (Kemeny and Challacombe, 1988). However, when a two-site AVP ELISA was used to analyse the elution of ^{125}I-AVP from Sephadex G-25 (Figure 6.7), biotinylated affinity-purified anti-PAG displaced a significant amount of ^{125}I-AVP from the ESVP 1 MAb-coated wells. This suggested that some anti-PAG antibodies recognized epitopes that were so close to the ESVP 1 epitope that steric hindrance between the antibodies prevented two-site binding. Nevertheless, these anti-PAG epitopes were exposed on the surface of ESVP 1 MAb-bound AVP and so were accessible to high affinity anti-PAG antibodies that bound more strongly to ^{125}I-AVP than did ESVP 1, which eventually released the ^{125}I-AVP molecule because electrostatic repulsion between the antibodies exceeded its binding affinity for ^{125}I-AVP. However, some ^{125}I-AVP remained bound to the ESVP 1 MAb-coated ELISA wells, allowing analysis of the Sephadex G-25 elution profile (Figure 6.7), because some anti-PAG antibodies recognized epitopes that were sufficiently far from the ESVP 1 epitope to allow two-site binding to ^{125}I-AVP.

The polyclonal antibody preparations contained a variety of anti-AVP specificities, many of which recognized epitopes that overlapped with that of ESVP 1 MAb and so were unable bind to AVP simultaneously with ESVP 1. Consequently, the incubation protocol played an important role in determining the magnitude of the

dose-dependent binding response with these preparations because competition for AVP binding was only prevented if AVP was allowed to bind to the ESVP 1 MAb-coated ELISA wells before addition of the polyclonal antibodies (Figure 6.4). However, assay sensitivities were poor because partially-purified antisera contained irrelevant specificities that decreased the effective concentration of anti-AVP antibodies while, even with the affinity-purified antisera, a proportion of anti-AVP specificities were unable to take part in two-site binding with ESVP 1 MAb. Many specificities would also have been incompatible with ESVP 2 MAb in a two-site assay, although ESVP 2 was not tested for two-site binding in combination with the polyclonal preparations.

In contrast, the epitopes recognized by ESVP 1 and ESVP 2 MAbs did not overlap therefore two-site binding was recorded with biotinylated protein A-purified ESVP 2 and ESVP 1 MAb-coated wells in all incubation protocols (Figure 6.13A). However, steric hindrance between the MAbs limited the amount of two-site binding that occurred therefore the dose-dependent response only increased if the surface area available for binding increased (Figure 6.14), even though ESVP 1 MAb was in excess, while the signal-to-noise ratio was improved if competition between bound and unbound biotin-ESVP 2 for binding of the streptavidin-HRP conjugate was eliminated (Figure 6.13A). The partial simultaneous incubation protocol therefore produced the most sensitive two-site AVP ELISA, with a detection limit of 200 pg AVP/ml (Figure 6.13A), because its large assay volume maximized the area available for binding and it was free from competition for the streptavidin-HRP conjugate.

Immobilization of MAbs by direct adsorption onto polystyrene was reported to significantly decrease their antigen-capture capacities due to conformational changes that affected their binding sites (Suter and Butler, 1986), whereas biotin-MAbs that were immobilized via a streptavidin 'bridge' onto biotinylated carrier-protein-coated ELISA wells had antigen-capture capacities that were as much as 400-fold greater than those of directly-adsorbed MAbs (Suter and Butler, 1986; Suter et al., 1989). The effect of this immobilization method on the two-site AVP ELISAs was not tested, although it might have increased the dose-dependent binding response and therefore

the sensitivity of the assays. However, the results obtained in this project showed that two-site ELISA binding was also affected by the post-coating treatment of the MAb-coated wells since higher dose-dependent responses were recorded if post-coating washes were done with Tween buffer instead of Buffer T (Figure 6.10). This unexpected observation seemed to result from a change in the orientation of the MAbs on the polystyrene surface because it was prevented if coated wells were BSA-blocked before the Tween buffer wash. Proteins adsorb onto polystyrene through hydrophobic bonds (Cantarero et al., 1980). Although the Fc region is the most hydrophobic portion of IgG (Ishikawa, 1987), parts of the Fab region will also exhibit varying degrees of hydrophobicity therefore antibodies are likely to adsorb randomly onto polystyrene in many different orientations. Non-ionic detergents, such as Tween 20, prevent hydrophobic interactions (Tijssen, 1985) therefore the post-coating Tween buffer wash probably disrupted the weaker hydrophobic bonds so that IgG molecules which had originally adsorbed onto the polystyrene surface through several different parts of their structures were eventually bound only by the most hydrophobic areas of the Fc region. The Fab regions would therefore become oriented towards the solvent, allowing more efficient analyte binding (Tijssen, 1985). In the case of very small analytes, such as AVP, reorientation of the solid phase-bound MAb would also minimize the effects of steric hindrance between the antibodies because the liquid-phase MAb would have easier access to the immobilized analyte.

The dose-dependent binding response of biotinylated protein A-purified ESVP 2 in ESVP 1 MAb-coated ELISA wells was doubled by a post-coating Tween buffer wash (Figure 6.10B), although there was a much smaller increase in the binding response of biotin-ESVP 1 in ESVP 2 MAb-coated wells (Figure 6.10A). Since the two-site binding response of a proinsulin assay also increased when MAb-coated wells were given a post-coating Tween buffer wash, it seems likely that Tween 20 will have a similar effect on many assays which use MAb-coated polystyrene solid phases. There have been many studies on the adsorption of antibodies onto various surfaces (Pesce et al., 1977; Cantarero et al., 1980; Herrmann, 1981; Soos et al., 1984) but they have tended

to concentrate on the absolute quantity of antibody adsorbed and not on its functional activity. There is no consensus on post-coating amongst other workers so the effect on two-site binding may not be widely appreciated, particularly as general ELISA texts (e.g. Tijssen, 1985; Kemeny and Chantler, 1988; Kemeny and Challacombe, 1988) rarely mention post-coating procedures. Reported methods have included plate-blocking with dried milk (Wadsley and Watt, 1987), BSA (Soos et al., 1984; Aoyagi et al., 1991), or BSA followed by Tween buffer (Phillips et al., 1994), and Tween buffer washing of unblocked wells (Butler et al., 1986; Sorensen and Brodbeck, 1986). Although traditional ELISA methods include a plate-blocking step, blocking proteins are often unnecessary, particularly if Tween 20 is included in all buffers (Mohammad and Esen, 1989; Gosling, 1990), and they can hinder two-site binding responses by preventing Tween-induced reorientation of solid phase-bound antibodies. The sensitivity of many microtitre plate-based immunometric assays could probably be improved simply by omitting the blocking protein and including Tween 20 in all buffers.

The most sensitive two-site AVP assay that was produced in this project had a detection limit of 130 pg AVP/ml and used ^{125}I-ESVP 1 with affinity-purified anti-PAG-S-300 solid phase in the direct IRMA format (Figure 6.15). Very low non-specific binding was recorded but the total amount of dose-dependent ^{125}I-ESVP 1 binding was severely limited by steric hindrance. Good precision was obtained with freshly-prepared ^{125}I-ESVP 1, but the sensitivity of the assay decreased significantly as the radioactivity of ^{125}I-ESVP 1 decayed during storage. The total amount of radioactivity that was bound in the IRMA became so small that significant imprecision was caused by counting errors (Klee and Post, 1989). The two-site ELISA did not have this problem because the biotin-MAbs and streptavidin-HRP conjugate could be stored for long periods without significant losses of activity.

The ESVP 2-S-300 solid phase (Figure 6.19) produced a less sensitive direct IRMA than the affinity-purified anti-PAG-S-300 solid phase (Figure 6.15) because the AVP binding affinity of ESVP 2 MAb was lower than the affinity of the polyclonal preparation. However, unlike the two-site ELISA with ESVP 1 and ESVP 2 MAbs, the

direct IRMA was unaffected by the volume of solid phase, except at very high AVP concentrations (Figure 6.22), because the large surface area of Sephacryl S-300 minimized the effect of steric hindrance between adjacent solid phase-bound MAbs on the observed dose-dependent binding response. The immunofiltration format produced the least sensitive assay because the low coating capacity of the nitrocellulose membrane limited the binding response to such an extent that counting errors significantly decreased the precision of estimations.

Impurities in the antibody preparations caused fewer difficulties in the direct IRMA format than were observed in the two-site ELISAs. Non-antibody contaminants decreased the efficiency of MAb iodination (Table 4.22) but were removed when the ^{125}I-MAbs were purified by gel filtration (Figures 4.1 and 4.3) and therefore did not cause any interference with the IRMA binding response. The high protein-coupling capacity of oxidized Sephacryl S-300 produced antibody-S-300 solid phases which bound reasonable amounts of ^{125}I-AVP, irrespective of the degree of purity of the antibody preparations (Table 6.1). However, solid phases that were coupled to polyclonal preparations which contained irrelevant antibodies, or specificities which recognized epitopes that overlapped with that of ESVP 1 MAb, produced poorer binding responses (Figure 6.15) because they contained extremely low concentrations of ESVP 1 MAb-compatible antibodies.

In contrast, impurities in the antibody preparations decreased indirect IRMA sensitivity because they decreased the binding of biotin-antibody to ^{125}I-streptavidin (Figure 4.4). Two-site binding was therefore detected more efficiently with ^{125}I-ESVP 1 (Figure 6.16) than with biotin-ESVP 1 (Figure 6.26). Interestingly, however, the indirect IRMA format could produce an assay which had polyclonal antibody preparations on both sides of the 'sandwich' (Figure 6.27), a combination that was impossible in the direct IRMA format because affinity-purified anti-PAG was too dilute to iodinate efficiently while irrelevant specificities in the partially-purified anti-AVP serum, which would have iodinated, could not participate in binding to AVP and would therefore significantly decrease the signal-to-noise ratio.

A clinically-useful assay, in the context of assisting neonatal care, would measure physiological AVP concentrations in small volumes of unextracted plasma. It would therefore need to have a sensitivity of about 1 pg AVP/ml and, ideally, should produce a result within a few hours of sampling. It should be specific for AVP and so should not cross-react with closely-related peptides, such as OT and AVT, or with any other substances in the sample. Good precision and accuracy are also necessary to ensure that the results are reliable, where precision describes the variation of replicate estimations around the mean value obtained for any concentration of analyte while accuracy implies that the estimated concentration is as close as possible to the true value (Ekins, 1983).

The assays that were developed in this project were insufficiently sensitive to measure AVP in unextracted plasma because the weak immunogenicity of AVP resulted in the production of relatively low affinity MAbs, while the small size of the peptide caused steric hindrance which severely limited the total amount of two-site binding that occurred, even with high affinity polyclonal antibody preparations. Nevertheless, ESVP 1 and ESVP 2 MAbs could be used to produce rapid assays in the two-site ELISA, IRMA and immunofiltration assay formats, with total incubation periods of 1-5 hours. However, although the most sensitive AVP assay was produced in the direct IRMA format (Figure 6.15), ^{125}I-ESVP 1 and ESVP 2-S-300 solid phase produced a relatively insensitive direct IRMA because the low radioactivity of the bound fraction decreased the assay precision (Klee and Post, 1989). Better sensitivity was obtained when these MAbs were used in the two-site ELISA format with the partial simultaneous incubation protocol (Figure 6.13A) which produced extremely low non-specific binding and good precision. Immunofiltration produced a rapid assay with these MAbs, but the bound radioactivity was lower than that observed in the direct IRMA because the nitrocellulose membrane had a lower protein-binding capacity than oxidized Sephacryl S-300 therefore the assay was very insensitive.

No other two-site immunometric assays for AVP have been reported, although an alternative type of noncompetitive AVP assay was recently developed by Hashida

et al. (1991b). This hetero-two-site complex transfer enzyme immunoassay had a detection limit of 1.1 fg AVP/tube, which made it the most sensitive AVP assay so far reported. It was 450-fold more sensitive than a competitive enzyme immunoassay which used the same antiserum (Uno et al., 1982) but it was extremely complicated with 11 separate steps during which AVP was biotinylated then transferred between various different solid phases and the total assay time was 4 days. A simpler version (Hashida et al., 1991a) detected 11 fg AVP/tube but still had a total assay time of 50 hours so both of these assays were too slow to be clinically-useful.

Despite steric hindrance, two-site assays for AVP were possible with ESVP 1 and ESVP 2 MAbs, with ESVP 1 in combination with any of four different polyclonal antibody preparations, and with a pair of polyclonal antibodies. The sensitivity of these assays varied with the format but none of them had a detection limit below 130 pg AVP/ml therefore none could be used to measure normal physiological AVP concentrations (1-5 pg AVP/ml plasma; Robertson, 1977). However, the very high AVP concentrations that can occur in infants with birth asphyxia (up to 550 pg AVP/ml plasma; Smith et al., 1990) were just within range of the direct IRMA which used ^{125}I-ESVP 1 and affinity-purified anti-PAG-S-300 solid phase (Figure 6.15), and also the two-site ELISA which used biotinylated protein A-purified ESVP 2 with ESVP 1 MAb-coated wells (Figure 6.13A). Enzyme amplification systems (Stanley et al., 1985) using luminescent or fluorescent labels (Ekins, 1987; Ishikawa, 1987), which are detected more sensitively than colorimetric substrates (Ishikawa, 1987), could increase the sensitivity of two-site AVP assays provided that non-specific binding did not increase, but the sensitivities of the existing assays would need to be increased about 200-fold before they would be clinically-useful. Alternatively, better assays might be produced with a pair of high affinity MAbs specific for epitopes at opposite sides of the AVP molecule so that steric hindrance is minimized. ESVP 1 and ESVP 2 MAbs, although able to bind simultaneously to AVP, had relatively low AVP-binding affinities (Table 5.1). The highest reported anti-AVP MAb affinity was 1×10^9 l/mole (Jones et al., 1985), but if anti-AVP MAbs with affinities of 10^{10} l/mole could be

generated it may be possible to produce rapid two-site assays that can measure physiological AVP concentrations. Unfortunately, the weak immunogenicity of AVP will make this difficult to achieve.

CHAPTER 7

CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO ATRIAL NATRIURETIC PEPTIDE

7.1 INTRODUCTION

Three anti-ANP MAbs (ESA 4, ESA 5 and ESA 9; Prowse et al., 1989) were available at the start of this project. Additional anti-ANP MAbs were produced by the ANP fusions (Section 4.3.2), which generated 1048 hybridoma-containing cultures although only seven were ANP-coated-plate ELISA-positive. Four hybridomas were eventually cloned, and subcloned, to produce monoclonal cell lines which secreted anti-ANP MAbs (Table 4.9).

MAb characterization work was done with unpurified samples of spent culture supernatant or ascitic fluid.

7.2 ISOTYPES

The four anti-ANP MAbs which were generated in this project were isotyped with an Amersham Mouse Monoclonal Antibody Isotyping Kit (Table 7.1). All of these MAbs had κ light chains. The isotypes shown for ESA 4, ESA 5 and ESA 9 (Table 7.1) are quoted from Prowse et al. (1989), and were not determined in this work. Their light chain type was not specified but may be κ because more than 95% of mouse light chains are κ chains (Goding, 1986).

7.3 ANTIBODY AFFINITIES

The affinity constants of the anti-ANP MAbs were determined by Scatchard analysis (Scatchard, 1949) of solution-phase RIA binding, as described in Section 2.4.2. The mean values shown in Table 7.1 were calculated from the results of three (all MAbs except ESNP 1) or four (ESNP 1) experiments.

The anti-ANP MAb titres which had been determined by RIA, in preparation for the affinity constant estimations, were in most cases significantly different from the titres estimated by ANP-coated-plate ELISA (Table 7.2). ESNP 2, ESNP 3 and

TABLE 7.1

Isotypes and affinities of the anti-ANP MAbs

MAb	Isotype	Affinity (l/mole)
ESNP 1	IgG2a	2.4×10^9
ESNP 2	IgG1	1.2×10^9
ESNP 3	IgG1	1.6×10^9
ESNP 4	IgG1	8.3×10^7
ESA 4	IgG2a	6.5×10^{10}
ESA 5	IgG2b	4.1×10^{10}
ESA 9	IgG1	2.9×10^{10}

TABLE 7.2

Comparison of the anti-ANP MAb titres estimated by ANP-coated-plate ELISA and by RIA

Serial dilutions of anti-ANP MAb-containing culture supernatant, in Buffer L, were assayed by ANP-coated-plate ELISA (100 ng/ml ANP, unblocked wells) and by RIA (6.4 pg ^{125}I-ANP, 624 μCi/μg, in Buffer L, SAM-S-500 solid phase, as described in Section 2.4.2). The antibody titre was defined as the culture supernatant dilution which produced an absorbance of 1.0 at 450 nm (ANP-coated-plate ELISA) or half-maximal ^{125}I-ANP binding (RIA).

MAb	Antibody titre	
	ELISA	RIA
ESNP 1	1/440	1/350
ESNP 2	1/840	1/40
ESNP 3	1/3000	1/20
ESNP 4	1/530	1/10
ESA 4	1/40	1/16,000

ESNP 4 had much lower RIA titres than ELISA titres, whereas the RIA titre of ESA 4 was 400-fold higher than its ELISA titre. In contrast, the titres estimated for ESNP 1 were roughly similar in both assays. Unlike ESVP 3 and ESVP 4, which produced higher binding in the AVP-coated-plate ELISA than in the SAM-RIA because their isotypes were more readily bound by the anti-mouse IgG HRP conjugate than by the SAM-S-500 solid phase (Section 5.4), the titre differences observed with the anti-ANP MAbs did not seem to be isotype-related because the MAbs were all IgG antibodies (Table 7.1). The differences were also unlikely to be related to the affinity constants of the MAbs, but were probably indicative of different binding specificities. Unfortunately, no ANP analogues were available so the specificities of the anti-ANP MAbs could not be determined.

7.4 DISCUSSION

Human ANP is more immunogenic than AVP, in mice, because human ANP differs from murine ANP in one amino acid residue whereas human and murine AVP molecules are identical. Immunization with ANP-thyroglobulin conjugate therefore produced higher average anti-peptide antibody titres (Table 4.5) than were obtained with the AVP-thyroglobulin conjugates (Tables 4.1, 4.2 and 4.3), and presumably generated antibodies with higher average binding affinities because the affinity constants of ESNP 1, ESNP 2 and ESNP 3 (Table 7.1) were an order of magnitude higher than those of the anti-AVP MAbs (Table 5.1). The binding affinity of ESNP 4, however, was disappointingly low (Table 7.1), and none of the MAbs which were produced in this project had affinities that were as high as those of ESA 4, ESA 5 and ESA 9 (Table 7.1).

The binding specificities of the anti-ANP MAbs were not determined in this work, but differences in the MAb titres estimated by ANP-coated-plate ELISA and by RIA (Table 7.2) suggested that the MAbs recognized at least three different epitopes. Prowse et al. (1989) showed that ESA 4 bound to an epitope in the central loop of

ANP, between residues 7 and 12, an area which is part of the most hydrophobic region of the ANP molecule. Since adsorption to polystyrene occurs through hydrophobic bonds (Cantarero et al., 1980), the ESA 4 epitope is likely to be in contact with the surface of ANP-coated ELISA wells and therefore relatively inaccessible to ESA 4, so that the ANP-coated-plate ELISA produced low ESA 4 titre estimates (Table 7.2). This epitope would be further obscured by close-packing of the ANP molecules, which accounts for the observation that ELISA estimates of ESA 4 titres decreased as the ANP-coating concentration increased (Figure 3.18A). In contrast, ESA 4 bound readily to ^{125}I-ANP in solution therefore the liquid-phase RIA produced a much higher ESA 4 titre estimate (Table 7.2).

ESNP 2, ESNP 3 and ESNP 4, on the other hand, did not bind well to ^{125}I-ANP so the RIA estimated low titres for these MAbs (Table 7.2). The ^{125}I atom of ^{125}I-ANP is substituted into the C-terminal tyrosine residue therefore the ESNP 2, ESNP 3 and ESNP 4 epitopes are probably close to the C-terminal of ANP. If the central loop of ANP is the main area which adsorbs to polystyrene, the C-terminal would be readily accessible for MAb binding in the ANP-coated-plate ELISA, which accounts for the much higher titre estimates produced for ESNP 2, ESNP 3 and ESNP 4 in the ANP-coated-plate ELISA than in the RIA (Table 7.2).

ESNP 1 bound to an epitope which was equally accessible in the RIA and in the ANP-coated-plate ELISA, because roughly similar titres were estimated by both assays (Table 7.2). The ESNP 1 epitope is therefore unlikely to be in either the hydrophobic portion of the central loop or the extreme C-terminal of ANP, but may be located in the N-terminal portion of the molecule.

CHAPTER 8

TWO-SITE IMMUNOMETRIC ASSAYS FOR ATRIAL NATRIURETIC PEPTIDE

8.1 INTRODUCTION

Shortly before this project began, one research group reported a two-site ANP ELISA which used a solid phase-bound MAb and a liquid-phase affinity-purified polyclonal antibody (Hashida et al., 1988a, 1988b; Mukoyama et al., 1988c). This assay was very sensitive because it detected 0.6 pg ANP/ml unextracted plasma, with a sample volume of only 50 µl. However, the total assay time was more than 49 hours so this ELISA was too slow to be clinically-useful in the context of neonatal care.

Four anti-ANP MAbs (Chapter 7) were generated in this project. Three other anti-ANP MAbs (ESA 4, ESA 5 and ESA 9; Prowse et al., 1989) and a polyclonal antiserum were also available for immunometric assay studies.

8.2 TWO-SITE ELISA

Prowse et al. (1989) showed that ESA 4 and ESA 5 MAbs bound to epitopes which differed from that of ESA 9 MAb, although they could not bind to ANP simultaneously with ESA 9 to form a two-site assay. No ANP analogues were available during the course of this project therefore the epitope specificities of ESNP 1, ESNP 2, ESNP 3 and ESNP 4 MAbs could not be determined. However, differences in the MAb titres estimated by ANP-coated-plate ELISA and by RIA (Table 7.2) suggested that ESNP 1 bound to an epitope which differed from those recognized by ESNP 2, ESNP 3 and ESNP 4 MAbs, or from that bound by ESA 4 (Section 7.4). All MAb combinations were therefore studied in the two-site ELISA format to determine whether any of them could form a two-site ANP assay. Partially-purified anti-ANP serum (Section 4.6.3) was not available for comparison when this work was carried out.

Initial experiments with the separate incubation protocol produced poor results but suggested that two-site binding to ANP occurred with most MAb combinations (data not shown), although non-specific binding of assay reagents to the MAb-coated,

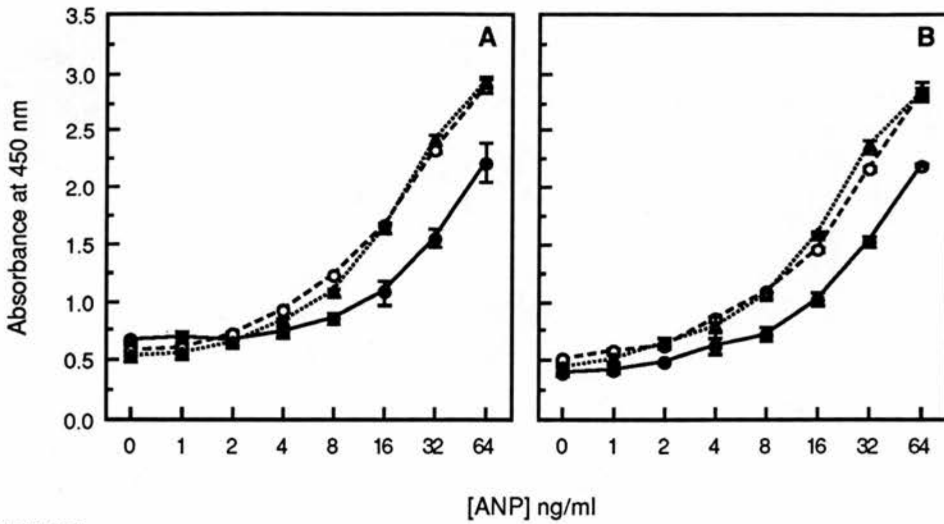

FIGURE 8.1

Binding of ANP and biotin-MAbs to MAb-coated ELISA wells

Unblocked Nunc Maxisorp Microwells, coated with ESNP 4 (●), ESA 4 (○) or ESA 5 (▲), were incubated with serial dilutions of ANP in Buffer L, then with biotin-ESA 4 (**A**) or biotin-ESA 5 (**B**) in Buffer L containing 1% normal mouse serum (Section 2.5.1.1). Biotin-MAb binding was revealed with streptavidin-HRP conjugate. The symbols show the mean of duplicate estimates, while the vertical bars show the difference between those estimates.

unblocked, ELISA wells caused high background absorbances. However, further studies showed that the binding response was determined by the biotin-MAb, irrespective of of the solid phase composition. Although biotin-ESA 4 (Figure 8.1A) and biotin-ESA 5 (Figure 8.1B) bound to ESNP 4 MAb-coated ELISA wells in a dose-dependent manner, much higher binding occurred if the wells were coated with either ESA 4 or ESA 5 (Figure 8.1). ANP consists of only 28 amino acids, with no repeated sequences, and is therefore unlikely to have epitopes that would allow two molecules of the same MAb to bind simultaneously, which suggests that the observed binding response was entirely caused by non-specific adsorption of ANP onto the coated ELISA wells. This was confirmed when further investigation revealed that dose-dependent binding of biotin-ESA 5 to ESA 5 MAb-coated, unblocked, wells was an artefact of the assay method because it occurred only with the separate incubation protocol (Figure 8.2). In the simultaneous incubation protocol, ANP was more likely to bind to liquid-phase ESA 5 than to adsorb non-specifically onto the solid phase. The streptavidin-HRP

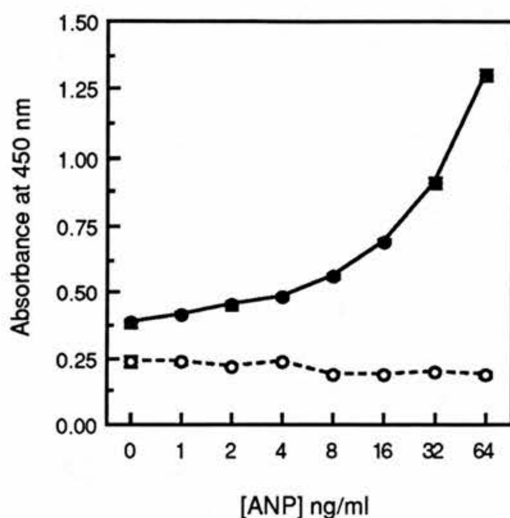

FIGURE 8.2

Effect of incubation protocol on the binding of ANP and biotin-ESA 5 to ESA 5 MAb-coated ELISA wells

Serial dilutions of ANP in Buffer L were assayed in ESA 5 MAb-coated, unblocked Immulon 2 MicroELISA wells, using the separate (Section 2.5.1.1, ●) or simultaneous (Section 2.5.1.2, o) incubation protocols with biotin-ESA 5. Binding was revealed with streptavidin-HRP conjugate. Vertical bars showing the difference between duplicate estimations are smaller than the symbols used in this Figure.

conjugate was also more likely to react with unbound biotin-ESA 5 than to adsorb onto the solid phase-bound assay components therefore the simultaneous incubation protocol produced lower background absorbances than the separate incubation protocol (Figure 8.2). Interestingly, non-specific binding in the simultaneous incubation protocol seemed to decrease slightly with increasing ANP concentrations (Figure 8.2), which suggested that ANP occupancy of the ESA 5 MAb binding site caused minor structural changes (Wilson et al., 1991) that decreased the ability of biotin-ESA 5 to adsorb onto the solid phase.

Although lower background absorbances were recorded in BSA-blocked wells (Figure 8.3A) than in unblocked wells (Figure 8.3B), with the separate incubation protocol, BSA-blocking did not prevent ANP adsorption onto ESA 5 MAb-coated wells (Figure 8.3A) because ANP also adsorbed onto BSA (Figure 8.3A). Unblocked wells coated with ESVP 2, an anti-AVP MAb (Chapter 5), also adsorbed ANP (Figure 8.3B) although to a lesser extent than either ESA 5 MAb or BSA (Figure 8.3A). The long

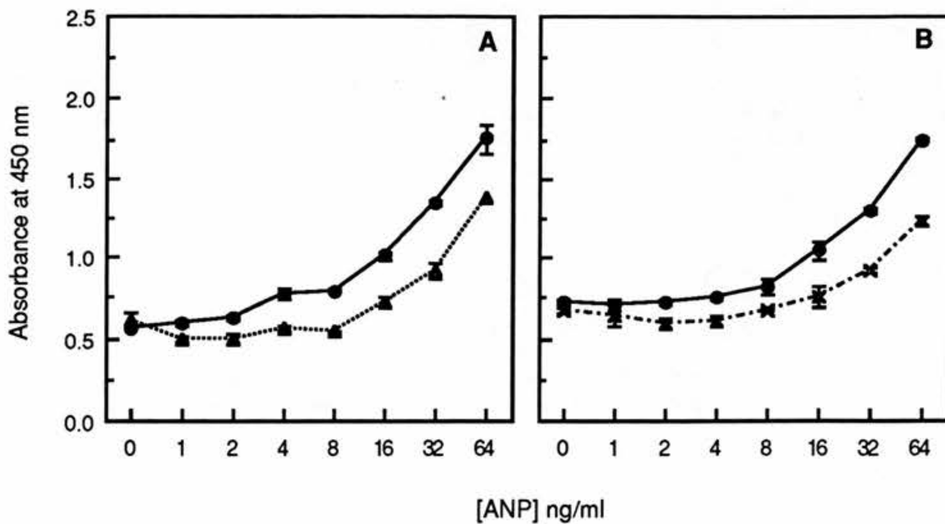

FIGURE 8.3

Comparison of the non-specific binding of ANP and biotin-ESA 5 to MAbs and to BSA

BSA-blocked (**A**) or unblocked (**B**) MicroELISA wells, coated with ESA 5 (●), ESVP 2 (×) or nothing (▲), were incubated with serial dilutions of ANP in Buffer L, then with biotin-ESA 5 in Buffer L containing 1% normal mouse serum (Section 2.5.1.1). Binding was revealed with streptavidin-HRP conjugate. Vertical bars show the difference between the absorbances recorded in duplicate wells.

incubations that were used in most of the ELISA work probably contributed to the non-specific binding (Kemeny and Chantler, 1988), but significant non-specific binding also occurred when the incubation periods of ANP and biotin-ESA 5 were decreased from 20 hours and 24 hours to 3 hours each (data not shown). Most of the two-site ELISA work was done in Immulon 2 MicroELISA wells. Different types of polystyrene have significantly different protein-binding capacities (Kemeny and Challacombe, 1988) but there was no advantage to be gained from using other ELISA wells because non-specific adsorption of ANP also occurred in Immulon 1 (Figure 8.4A), Immulon 4 and Nunc maxisorp (Figure 8.4B) wells, although the degree of adsorption varied between the different types of wells. Triton X-100 was reported to prevent ANP adsorption onto polystyrene (Lindberg and Andersson, 1991) but substitution of Triton X-100 for Tween 20 in the assay buffer failed to prevent ANP adsorption onto ESA 5 MAb-coated wells, although it decreased background absorbances significantly (Figure 8.5).

Non-specific adsorption of ANP onto the solid phase during the separate

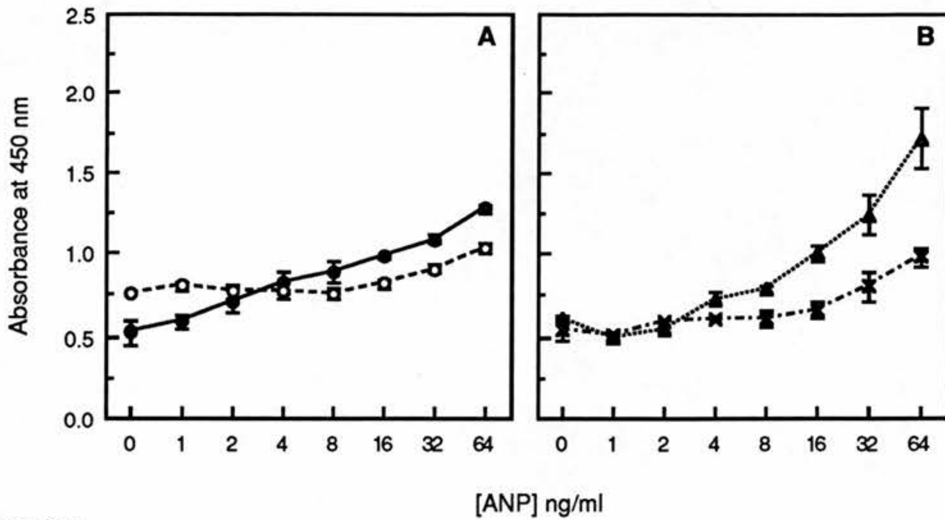

FIGURE 8.4

<u>Non-specific binding of ANP and biotin-ESA 5 to four types of polystyrene ELISA well coated with ESA 5 MAb</u>

ESA 5 MAb-coated, unblocked Immulon 1 (●), Immulon 2 (○), Immulon 4 (▲) or Nunc Maxisorp (×) ELISA wells were incubated with serial dilutions of ANP in Buffer L, then with biotin-ESA 5 in Buffer L containing 1% normal mouse serum (separate incubation protocol). Binding was revealed with streptavidin-HRP conjugate. Vertical bars show the difference between the absorbances recorded in duplicate wells.

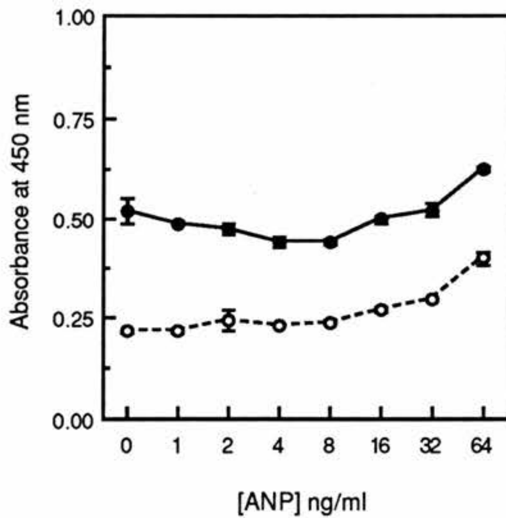

FIGURE 8.5

<u>Effect of Triton X-100 on non-specific binding of ANP and biotin-ESA 5 to ESA 5 MAb</u>

Unblocked ESA 5 MAb-coated MicroELISA wells were incubated with duplicate 100 µl aliquots of ANP for 19 hours, then with biotin-ESA 5 for 22 hours. Biotin-ESA 5 binding was revealed with streptavidin-HRP conjugate. ANP, biotin-ESA 5 and streptavidin-HRP conjugate were diluted either in Buffer L (●) or in Buffer K containing 1% BSA and 0.1% Triton X-100 (○).

incubation protocol was a major problem that could not be prevented and made ELISA results difficult to interpret. The simultaneous incubation protocol was free of this problem (Figure 8.2) but was not used for two-site ANP ELISA work because studies with the AVP ELISAs had shown that small amounts of two-site binding were difficult to detect since unbound biotin-antibody (Figures 6.4 and 6.13A) and biotinylated impurities (Figure 6.13B) competed with the solid phase-bound assay components for binding of the streptavidin-HRP conjugate. Further purification of the very impure PEG 8000-precipitated anti-ANP MAbs would have improved the detectability of two-site binding (Figure 6.13), as would the use of a post-coating Tween buffer wash in unblocked MAb-coated ELISA wells (Figure 6.10), but unfortunately there was no time available to carry out appropriate investigations. No further two-site ELISA work was done because neither incubation protcol could reliably detect specific ANP binding to the available MAb preparations.

8.3 IMMUNORADIOMETRIC ASSAY (IRMA)

All of the anti-ANP antibody preparations were coupled to periodate-oxidized Sephacryl S-300, then assayed for the ability to bind ^{125}I-ANP (Section 2.3.5.2) before use in the IRMA work. Most of the solid phases bound significant amounts of ^{125}I-ANP, although lower binding occurred with the partially-purified anti-ANP serum-S-300 solid phase than with the MAb-S-300 solid phases (Table 8.1). However, a solid phase produced from unpurified anti-ANP serum, which was included for comparison, failed to bind ^{125}I-ANP (Table 8.1). This result was surprising because the SAM-S-500 solid phase was also prepared from unpurified antiserum (Section 2.3.5.3) and yet bound strongly to mouse antibodies. The anti-ANP serum was therefore likely to have contained relatively little anti-ANP activity but, unfortunately, no other polyclonal anti-ANP antisera were available for immunometric assay work.

TABLE 8.1

Binding of ^{125}I-ANP to antibody-S-300 solid phases

Solutions of 1 mg/ml anti-ANP MAb, 8 mg/ml partially-purified anti-ANP serum (Section 4.6.3) or 25% (v/v) unpurified anti-ANP serum were coupled to oxidized Sephacryl S-300 then assayed for antigen-binding ability as described in Section 2.3.5.2. Par-p = partially-purified; Un-p = unpurified.

Solid phase	% ^{125}I-ANP bound
ESNP 1-S-300	71.9
ESNP 2-S-300	77.5
ESNP 3-S-300	81.2
ESNP 4-S-300	71.0
ESA 4-S-300	84.4
ESA 5-S-300	81.4
ESA 9-S-300	81.3
Par-p anti-ANP serum-S-300	49.3
Un-p anti-ANP serum-S-300	0.4

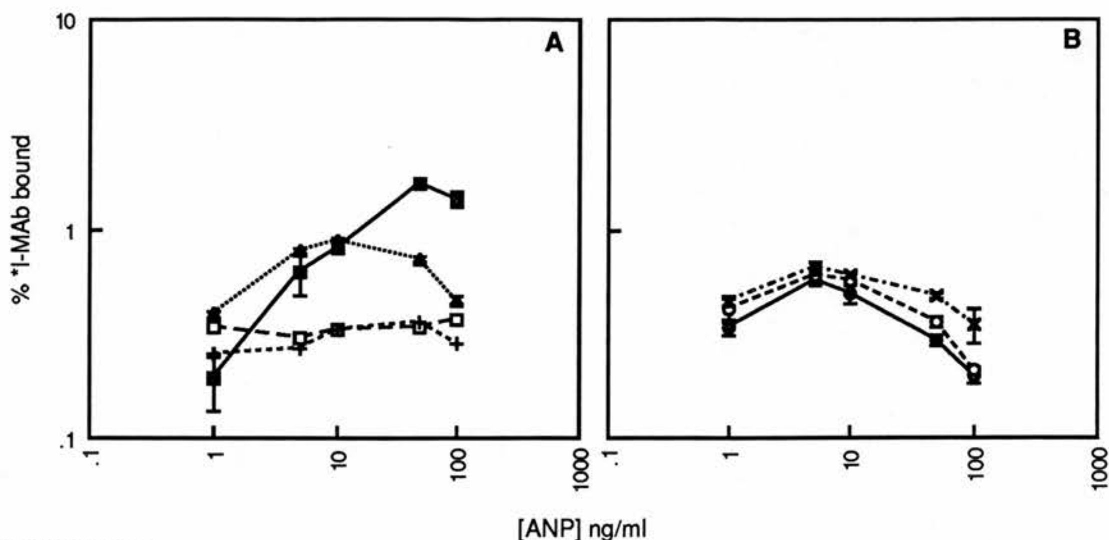

FIGURE 8.6

Direct ANP IRMA with ^{125}I-MAbs and anti-ANP serum-S-300 solid phase

Dilutions of ANP, in Buffer E containing 5% BSA, were assayed by direct IRMA (Section 2.5.2.1) using ^{125}I-ESNP 1 (▲), ^{125}I-ESNP 2 (□), ^{125}I-ESNP 3 (+), ^{125}I-ESNP 4 (■), ^{125}I-ESA 4 (●), ^{125}I-ESA 5 (o) or ^{125}I-ESA 9 (×) with partially-purified anti-ANP serum-S-300 solid phase (prepared from a 5-10% PEG cut of anti-ANP serum after further purification by Rivanol precipitation; Section 4.6.3). Non-specific binding was 0.17% (▲), 0.30% (□), 0.12% (+), 0.09% (■), 0.11% (●), 0.13% (o) or 0.24% (×) of added ^{125}I-MAb. The symbols show the mean of duplicate estimations, while vertical bars show the difference between those estimations. For clarity, bars are not shown for ^{125}I-ESNP 2 (□) and ^{125}I-ESNP 3 (+).

8.3.1 DIRECT IRMA

Initial IRMA studies with all possible ^{125}I-MAb and MAb-S-300 solid phase combinations failed to produce any evidence of two-site binding to ANP (data not shown). This result was disappointing because the anti-ANP MAbs had seemed likely to recognize three different epitopes (Section 7.4), although confirmation was not possible because there were no ANP analogues available for epitope specificity studies. However, the MAb epitopes were probably so close together that steric hindrance prevented detectable two-site binding.

Further studies showed that most of the ^{125}I-MAbs could take part in two-site binding to ANP in combination with the partially-purified anti-ANP serum-S-300 solid phase, although the maximum binding responses were very low (Figure 8.6). No

significant dose-dependent binding occurred with either ^{125}I-ESNP 2 or ^{125}I-ESNP 3 (Figure 8.6A) which suggested that iodination disrupted the binding sites of these MAbs. ESNP 4 had the lowest ANP-binding affinity (Table 7.1) and produced the least sensitive assay yet, unexpectedly, the maximum binding response of ^{125}I-ESNP 4 was higher than that of any other MAb and it produced a high-dose 'hook' effect at a higher ANP concentration than the other MAbs (Figure 8.6A).

The analyte concentration required to saturate the binding sites of an antibody-S-300 solid phase is a fixed characteristic that should not be affected by the liquid-phase assay reagents. Nevertheless, the ANP concentration required to produce a high-dose 'hook' effect was inversely related to the binding affinity of the ^{125}I-MAb, as was the maximum binding response (Figure 8.6). This surprising result suggested that the partially-purified anti-ANP serum preparation consisted mainly of low affinity antibodies, many of which would have recognized epitopes that were too close to the MAb epitopes to allow simultaneous binding. High affinity ^{125}I-MAbs, such as ESA 4 (Table 7.1, Figure 8.6B), bound strongly to ANP and were likely to remain in the liquid phase because steric hindrance severely limited simultaneous binding of the low affinity partially-purified anti-ANP serum antibodies. Higher two-site binding occurred with low affinity MAbs, such as ESNP 4, which were less able to compete for exclusive binding of the ANP molecule because their affinities were more closely matched by those of the polyclonal antibodies. Antibody binding sites have widely varying structures (Wilson et al., 1991) therefore the low affinity MAbs possibly had shallower binding sites that minimized steric hindrance and so allowed more two-site binding.

Additional work showed that ^{125}I-ESA 4 produced a more sensitive direct IRMA than ^{125}I-ESA 5 (Figure 8.7) but the total amount of bound radioactivity was so low that counting errors decreased the precision of the assay (Klee and Post, 1989) and limited the sensitivity to 1 ng ANP/ml. However, the high-dose 'hook' effect occurred at 5 ng ANP/ml therefore the range of this assay was too narrow to be of practical use.

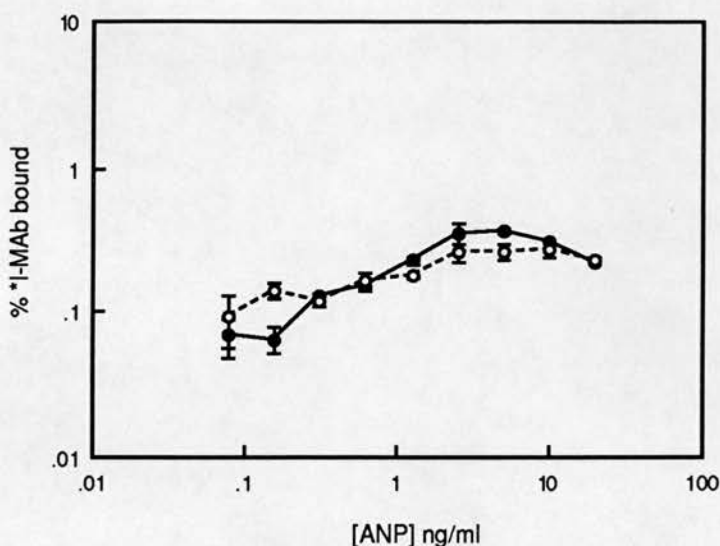

FIGURE 8.7

Comparison of ^{125}I-ESA 4 and ^{125}I-ESA 5 in a direct IRMA

Serial dilutions of ANP in Buffer E containing 5% BSA were assayed by direct IRMA, using ^{125}I-ESA 4 (●) or ^{125}I-ESA 5 (o) with partially-purified anti-ANP serum-S-300 solid phase. Non-specific binding was 0.05% (●) or 0.09% (o) of added ^{125}I-MAb. Vertical bars show the difference between the binding in duplicate tubes.

8.3.2 INDIRECT IRMA

Iodination seemed to disrupt the binding sites of ESNP 2 and ESNP 3 because they produced no significant direct IRMA binding with the partially-purified anti-ANP serum-S-300 solid phase (Figure 8.6A). In contrast, biotin-ESNP 2 and biotin-ESNP 3 bound readily to liquid-phase ANP (data not shown) therefore the anti-ANP MAbs were studied in the indirect IRMA format with partially-purified anti-ANP serum-S-300 solid phase. However, the only detectable two-site binding occurred with biotin-ESA 4 and the total amount of bound radioactivity was very low (Figure 8.8). Further investigation revealed that impurities in the biotin-MAb preparations decreased the potential signal-to-noise ratio of the assay since PEG 8000-precipitated MAbs bound much less ^{125}I-streptavidin than MAbs that were prepared from ascitic fluid by sodium sulphate precipitation (Figure 8.9), a phenomenon that was also observed with the anti-AVP MAbs (Figure 4.4). Two-site

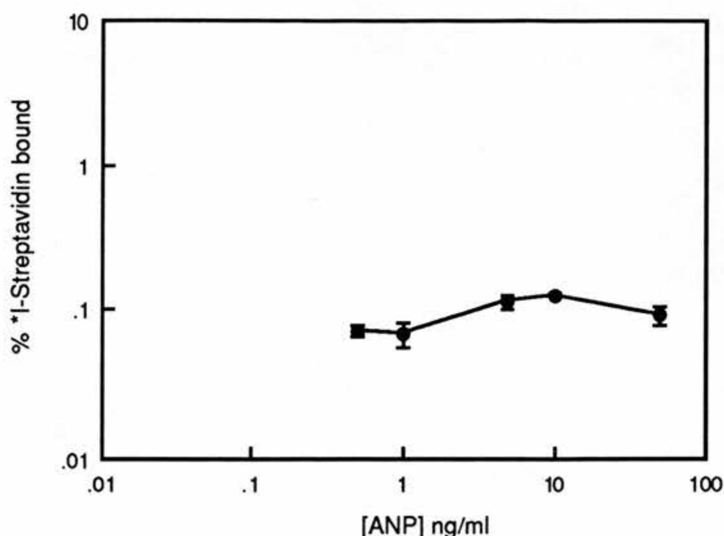

FIGURE 8.8

Indirect IRMA with biotin-ESA 4 and anti-ANP serum-S-300 solid phase

Dilutions of ANP, in Buffer E containing 5% BSA, were assayed by indirect IRMA as described in Section 2.5.2.2, using biotin-ESA 4 with partially-purified anti-ANP serum-S-300 solid phase. Non-specific binding was 0.05% of added ^{125}I-streptavidin. Vertical bars show the difference between duplicate estimations.

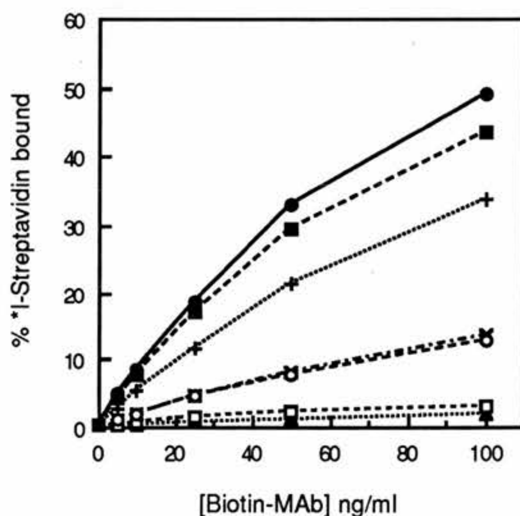

FIGURE 8.9

Binding of ^{125}I-streptavidin to biotinylated anti-ANP MAbs

ESA 4 (●), ESA 5 (■) and ESA 9 (+) MAbs were partially purified from ascitic fluid by sodium sulphate precipitation, while ESNP 1 (▲), ESNP 2 (□), ESNP 3 (×) and ESNP 4 (o) MAbs were concentrated from spent culture supernatants by PEG 8000 precipitation. The MAbs were biotinylated (Section 2.3.4.5), then assayed for ^{125}I-streptavidin binding as described in Figure 4.4.

binding was detected more efficiently in the direct IRMA format therefore no further indirect IRMA work was carried out.

8.4 DISCUSSION

Two-site binding to ANP was observed only when liquid-phase MAbs were combined with partially-purified anti-ANP serum-S-300 solid phase in the IRMA format (Section 8.3). However, the anti-ANP serum antibodies seemed to have low binding affinities so two-site IRMA binding was limited both by steric hindrance and by the poor quality of the solid phase, although non-specific binding was very low.

In contrast, non-specific binding was a major problem in the two-site ELISA format (Section 8.2). Many workers report that plate-blocking decreases non-specific ELISA binding (Campbell, 1984; Vogt et al., 1987; Pruslin et al., 1991). Detergents such as Tween 20 (Campbell, 1984; Mohammad and Esen, 1989) or Triton X-100 (Campbell, 1984) also inhibit adsorption onto the solid phase, although Kenna et al. (1985) reported that non-reactive proteins, such as casein or BSA, were more effective buffer additives, while ANP adsorption onto polystyrene was reportedly prevented by buffers containing 0.1% BSA (Artner-Dworzak et al., 1991), 0.1% human serum albumin or 0.1% Triton X-100 (Lindberg and Andersson, 1991). However, ANP adsorbed onto MAbs (Figures 8.1 and 8.3B) and onto BSA (Figure 8.3A) during the separate incubation protocol, despite the presence of 1% BSA and 0.1% Tween 20 in the assay buffer (Figure 8.2), and high background absorbances in the absence of ANP were caused by adsorption of the assay reagents onto the coated ELISA wells. Triton X-100 also failed to prevent ANP adsorption, although it decreased non-specific binding of the assay reagents (Figure 8.5). ANP adsorption onto uncoated polystyrene was not tested, but it seemed more likely that ANP adsorbed onto the protein coating than onto the polystyrene surface, as observed with AVP (Figure 6.9B), therefore small differences between ELISA well types (Figure 8.4) were probably caused by differences in the concentration of surface-adsorbed MAb. The amount of ESA 5 that adsorbed onto

the wells was not determined, but there were only slight differences between the adsorption of ANP onto ESA 5 MAb-coated Nunc Maxisorp, Immulon 1 or Immulon 2 wells. In contrast, much higher ANP adsorption was recorded in Immulon 4 wells (Figure 8.4B) which have a very high protein-binding capacity and so would have adsorbed significantly more ESA 5 MAb.

Interestingly, vitronectin and clusterin were recently reported to adsorb onto polystyrene in the presence of detergents, and clusterin adsorption was actually increased by Tween 20 (Høgåsen et al., 1993). These adhesive proteins adsorbed more readily onto Nunc Maxisorp ELISA wells than onto Immulon 2 wells, and this adsorption was not prevented by plate-blocking with BSA, gelatin or FCS, although it decreased if the buffer pH was decreased to 6.0 (Høgåsen et al., 1993). The effect of pH on ANP adsorption was not tested.

The two-site ANP ELISAs that were reported by Hashida et al. (1988a) used an affinity-purified C-terminal-specific polyclonal Fab'-HRP conjugate in combination with solid phase-bound MAbs specific for either the ANP N-terminus (Mukoyama et al., 1988a) or the N-terminal half of the disulphide-bonded loop (Mukoyama et al., 1988b). The N-terminal-specific MAb had the highest binding affinity (Mukoyama et al., 1988a) but did not produce the most sensitive assay (Hashida et al., 1988a), which suggests that there was more steric hindrance between this MAb and the polyclonal Fab'-HRP conjugate than between the loop-specific MAb and the conjugate. The hydrophobic Fc portion of IgG causes most non-specific binding (Ishikawa, 1987), therefore use of the liquid-phase Fab' fragment ensured extremely low background absorbances so that two-site binding could be sensitively detected by fluorimetry (Hashida et al., 1988a, 1988b). However, preparation of the affinity-purified Fab'-HRP conjugate was very laborious and liable to variation between batches because supplies of polyclonal antisera are limited. The use of Fab' fragments might have decreased the non-specific binding problems that occurred with the two-site ELISA format in this project but, unfortunately, there was no time available to investigate this possibility.

While this project was in progress, three other research groups also reported two-site immunometric assays for ANP (Lewis et al., 1989; Watanabe et al., 1989; Tattersall et al., 1990). None of these assays used a pair of MAbs, but all were sufficiently sensitive to measure physiological ANP concentrations in unextracted plasma. Watanabe et al. (1989) described a two-site ELISA which required a 50 µl sample and used wells coated with the $F(ab')_2$ fragment of a MAb against the C-terminal half of the disulphide-bonded loop, in combination with an affinity-purified C-terminal-specific polyclonal Fab'-HRP conjugate, but which had a total assay time of 44 hours. An IRMA which used an ^{125}I-MAb specific for the disulphide-bonded loop, and polyclonal antibody-coated microtitre wells, was reported by Lewis et al. (1989). This assay required a 150 µl sample and had an incubation period of 16 hours, although the maximum recorded binding was only 6.6% of added ^{125}I-MAb. However, Tattersall et al. (1990) obtained a maximum of 19% binding in a liquid-phase IRMA which used iodinated affinity-purified C-terminal-specific rabbit antibodies with a mouse polyclonal anti-ANP antiserum and achieved separation of bound and unbound radioactivity by second antibody precipitation. This IRMA required 400 µl samples and had a total assay time of 23 hours.

A noncompetitive hetero-two-site enzyme immunoassay, which used a MAb specific for the disulphide-bonded loop, was also reported for ANP (Hashida et al., 1991c). This assay was less complicated than the hetero-two-site assay reported for AVP (Hashida et al., 1991a, 1991b) but still had a total incubation time of 50 hours. However, it was 4-fold more sensitive than a colorimetric two-site ELISA that used the same MAb (Watanabe et al., 1989) because very low non-specific binding allowed the use of a fluorometric detection system.

All of the reported two-site ANP assays were very sensitive, but they required long incubation periods and so were too slow to be clinically-useful in the context of neonatal care. The sample volumes required by the IRMAs (Lewis et al., 1989; Tattersall et al., 1990) were also too large to be obtained on a routine basis from small infants. Nevertheless, these reports showed that antibodies could be raised

against four different regions of the ANP molecule, and that C-terminal-specific antibodies could combine with antibodies against the N-terminus (Hashida et al., 1988a), or against either half of the disulphide-bonded loop (Hashida et al., 1988a; Watanabe et al., 1989), to produce two-site ANP assays.

The reaction kinetics of MAb-based assays are simpler than those of assays which use polyclonal antisera and so reach equilibrium more rapidly (Baker et al., 1985). Two-site assays which use a pair of MAbs therefore require the shortest incubation periods. Unfortunately, the MAbs that were studied in this project were unable to bind simultaneously to ANP, and two-site binding was observed only when MAbs were combined with a polyclonal solid phase. Nevertheless, it should be possible to develop rapid two-site immunometric assays for ANP if pairs of MAbs with appropriate specificities can be produced, although steric hindrance is likely to limit the binding of all antibody combinations.

CHAPTER 9

CONCLUSION

Despite problems caused by the weak immunogenicities of AVP and ANP, four anti-AVP MAbs and four anti-ANP MAbs were generated in this project. Most of these MAbs were produced in bulk culture and then concentrated by PEG 8000 precipitation, a method which was reportedly suitable for isolating MAbs from spent culture supernatants (Baines et al., 1990; Brooks et al., 1992). However, all of the PEG 8000-precipitated MAbs were contaminated by large quantities of high molecular weight materials (Figure 4.3A) that decreased the specific activities of ^{125}I-MAbs (Table 4.21) and biotin-MAbs (Figures 4.4 and 8.9) and increased non-specific binding (Figure 6.13) so that the signal-to-noise ratios of assays which used these preparations were decreased. Impurities in the MAb preparations had a much smaller effect on the ability of MAb-S-300 solid phases to bind ^{125}I-Ag (Tables 6.1 and 8.1), although they decreased the efficiency of MAb adsorption onto nitrocellulose (Section 6.4) and presumably also onto polystyrene.

Nevertheless, two-site binding to AVP was observed with ESVP 1 and ESVP 2 MAbs, which bound to the AVP ring structure and the tail tripeptide, respectively. These MAbs had relatively low binding affinities and so produced insensitive assays, although AVP consists of only nine amino acids therefore steric hindrance was a major problem that severely limited the amount of two-site binding that occurred. Two-site binding was also recorded between ESVP 1 MAb and four different polyclonal antibody preparations, and between a pair of polyclonal preparations, but none of these combinations produced an assay that was sufficiently sensitive to measure physiological AVP concentrations.

The binding specificities of the anti-ANP MAbs were unknown but there was no evidence to suggest that any pair of MAbs could bind simultaneously to ANP. Two-site binding was observed only when the MAbs were combined with a poor-quality polyclonal anti-ANP preparation in the IRMA format.

Non-specific binding was a serious problem during the prolonged incubations that were used in most of the two-site ELISA work. Adsorption of the biotin-antibody preparations and the streptavidin-HRP conjugate onto coated ELISA wells caused high

background absorbances, while dose-dependent adsorption of AVP and ANP occurred during the separate incubation protocol but not during the simultaneous incubation protocol. However, the simultaneous incubation protocol was unsuitable for two-site ELISA work because competition between the assay reagents inhibited the detection of small amounts of specific binding and therefore decreased assay sensitivity. Non-specific binding of biotin-antibody preparations might have been decreased by the use of Fab' fragments instead of intact IgG molecules (Ishikawa, 1987), or by higher buffer pH values (Wadsley and Watt, 1987), but analyte adsorption onto the coated wells would still have occurred. Shorter incubation periods, which were possible when a plate-shaker became available, decreased the non-specific adsorption of AVP onto MAb-coated ELISA wells while an improved plate-coating procedure dramatically increased the two-site binding response and so increased assay sensitivity.

Low non-specific binding occurred in the IRMA and immunofiltration assay formats but specific ^{125}I-MAb binding was also low therefore counting errors decreased the precision of these assays. Nevertheless, these formats showed that rapid two-site assays for AVP and ANP could be carried out with relatively small sample volumes.

The materials that were available during this project could not be used to develop sensitive two-site assays capable of measuring the concentrations of AVP and ANP in unextracted plasma, but the results showed that such assays could probably be developed with appropriate pairs of MAbs. Steric hindrance is likely to be a problem with all antibody combinations but sensitive assays might be produced in alternative formats that use high specific activity labels (Ekins, 1987) to increase the efficiency of signal detection, provided that non-specific binding remains low.

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PUBLISHED PAPERS

Part of the work described in this thesis has been published in the following papers :

1. Smith, S. C., Smith, A., James, K. and McIntosh, N. (1990) A two-site immunometric assay for arginine vasopressin. Biochem. Soc. Trans. 18 : 1273-1274.

2. Smith, S. C., McIntosh, N. and James, K. (1993) Pitfalls in the use of ELISA to screen for monoclonal antibodies raised against small peptides. J. Immunol. Methods 158 : 151-160.

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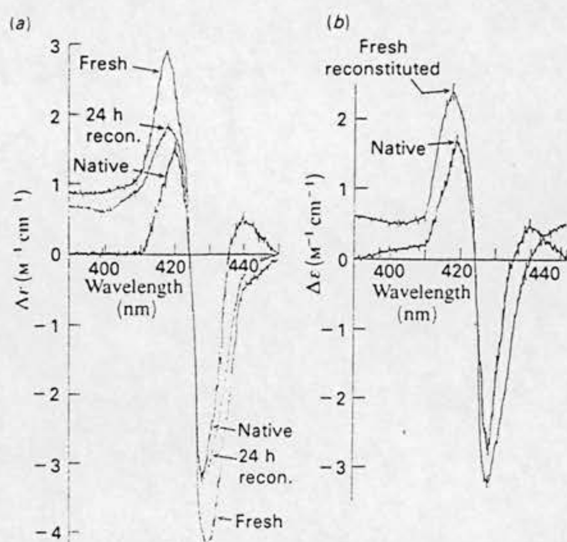

Fig. 1. *Circular dichroism spectra obtained of ferrocytochrome b_5; (a) at pH 7.4, (b) at pH 5.5*

the c.d. spectra. The concentrations of cytochrome b_5 solutions used for c.d. analysis were adjusted by appropriate dilutions in order that all the samples had identical absorances in the Soret region.

Although identical in their absorption spectra (both ferri and ferro protein) the native and the treated protein were significantly different in their c.d. spectra. Fig. 1 shows these spectra for protein which is returned either to pH 7.4 (Fig. 1*a* or to pH 5.5 (Fig. 1*b*). The c.d. of the native protein is closely similar to that reported by Strittmatter & Huntley [8].

Immediately following 'pH-jump' the positive peak is seen to have increased in amplitude and to have shifted from 420 nm to 418 nm. Similarly the negative peak increases in amplitude and moves from 428 nm to 430 nm. With the elapse of tine (2–3 days) the spectrum of the 'pH-jump' sample reverted to a spectrum very close to that of the native protein. An indication of the time course is given in Fig. 1(*a*), where spectrum of the protein 24 h after exposure to low pH is shown. It may be noted that all spectra pass through the $\Delta\varepsilon = 0$ point located at 424 nm.

By analogy with the results of Aojula *et al.* [6], who showed similar c.d. changes to be a reflection of haem disorder in myoglobin, we interpret the results shown in Fig. 1, to be an indication that the 'pH-jump' procedure induces haem orientational disorder in cytochrome b_5. The relaxation of the c.d. spectrum back to that of the native protein indicates that the 'minor' form becomes populated (at this time to an unknown extent) on 'pH-jump' and shows that slow reorientation of this form occurs returning the protein to the equilibrium (90% major, 10% minor) seen by proton n.m.r. [1].

The kinetics of the reorientation in ferricytochrome b_5 at pH 7.4 appears to be significantly more rapid than those observed for sperm whale myoglobin [6] and approaches the rate ($t_{1/2} = 16$ h) found for sperm whale myoglobin at pH 6.5 by Bellelli *et al.* [7], who also used the c.d. method. The kinetics of reorientation have been discussed by these latter authors in terms of the rate constant for dissociation of the haem from the protein. This implies that the dissociation constant of haem for ferricytochrome b_5 is greater than the corresponding constant for sperm whale myoglobin under the same conditions. This is a somewhat unexpected result in that the protein provides two ligands to the iron, thus helping to stabilize the haem-protein complex in cytochrome b_5. This enhanced reorientation rate must reflect a more general flexibility of the haem pocket in cytochrome b_5.

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A two-site immunometric assay for arginine vasopressin

SHIRLEY C. SMITH,* ALBERTO SMITH,† KEITH JAMES‡ and NEIL McINTOSH*

**Department of Child Life and Health, University of Edinburgh, Edinburgh EH9 1UW, U.K., ‡Department of Surgery, University of Edinburgh, Edinburgh EH8 9AG, U.K. and †Department of Child Health, St George's Hospital Medical School, University of London, London SW17 0QT, U.K.*

Arginine vasopressin (AVP), the hormone produced by the magnocellular cells of the hypothalamus and released by the posterior pituitary gland in response to osmolar and volume stimuli, is traditionally associated with the control of water balance in the human. More recently AVP has been identified in the adrenal gland, testis, ovary and thymus gland where its function is less clear. AVP is a nonapeptide composed of a 6-residue ring structure (Cys-Tyr-Phe-Gln-Asn-Cys) and a 3-residue *C*-terminal tail region (Pro-Arg-Gly-amide). Measurement of AVP in plasma or urine is normally achieved by radioimmunoassay although an enzyme immunoassay has been recently reported [1]. Physiological levels of plasma AVP are extremely low (1–5 pmol/l) so extraction of the samples is required both to achieve concentration and to remove substances which interfere with the assay by causing tracer degradation or increasing non-specific binding [2]. In addition, all assays so far reported require a prolonged incubation time of usually 3–5 days to achieve maximum sensitivity. Although this may be acceptable for research programmes and even for clinical diagnosis, it is of little use for everyday clinical management.

Two-site immunometric assays have increased both the sensitivity and the speed of performance [3] of many clinically important assays. They depend on the binding of two different antibodies to separate epitopes on the analyte. Using a mouse monoclonal antibody, ESVP1, which recognizes the ring structure of AVP, together with an affinity

Abbreviation used: AVP, arginine vasopressin.

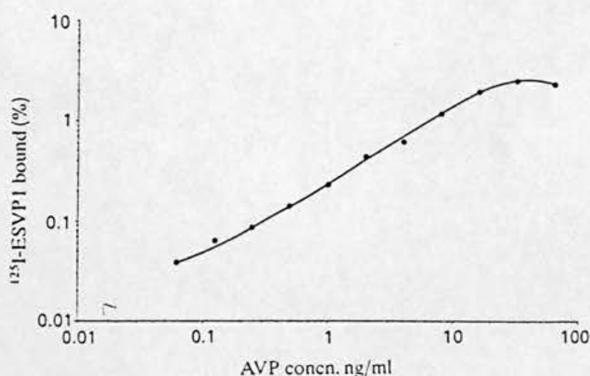

Fig. 1. *Standard curve for immunoradiometric assay of AVP*

100 μl (5 ng. 7.6 μCi/μg) ^{125}I-ESVP1, prepared by the chloramine T method [6] was incubated with 100 μl AVP sample in assay buffer [0.05 M-sodium phosphate buffer, pH 7.0, containing 2% (v/v) bovine serum albumin] for 2 h at room temperature. A sample (100 μl) of a 12.5% (settled volume) slurry of Sephacryl S300, coupled to affinity purified rabbit anti-AVP tail region at 1 mg/ml [7], in assay buffer containing 3% (v/v) TWEEN 20 was added, then the assay tubes were agitated for 2 h at room temperature. Separation of bound radioactivity was achieved by the sucrose-layering technique [8, 9].

purified rabbit polyclonal antiserum specific for the tripeptide tail region of the molecule, we have developed a two-site immunoradiometric assay for AVP (Fig. 1). This assay has a sensitivity of 200 pmol/l and is therefore considerably less sensitive than existing radioimmunoassays but may be carried out in a few hours rather than days. The monoclonal antibody ESVP1, which has an affinity constant of 4.5×10^8 l/mol, appears to be the factor limiting the sensitivity of the assay. The maximum binding achieved was only 2.6% of added ^{125}I-ESVP1 which is very low compared with the binding that we have achieved in other immunoradiometric assays [4]. Selection of a monoclonal antibody with a much higher affinity constant should increase the maximum binding and also the sensitivity of the assay. A useful diagnostic assay would have a sensitivity of 0.5–1.0 pmol/l and be able to measure AVP in unextracted plasma or urine samples.

We have shown that it is possible to develop a two-site immunoradiometric assay for a peptide containing only nine amino acids. Since an epitope is thought to consist of at least 4–6 amino acids [5], AVP may be one of the smallest molecules that can be measured in a two-site assay.

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Lactate utilization by neonatal rat liver *in vitro*

ANGELES ALMEIDA, JUAN P. BOLAÑOS and JOSÉ M. MEDINA

Departamento de Bioquímica y Biología Molecular, Facultad de Farmacia, Universidad de Salamanca, Post Box 449, E-37080 Salamanca, Spain

Lactate accumulates in blood during late gestation and the first minutes of extrauterine life to reach concentrations higher than 9 mM [1–3]. However, it is noteworthy that most of the lactate accumulated is utilized within the first 2 h of extrauterine life, i.e. before the onset of suckling takes place [2–4]. During this presuckling period, liver glycogenolysis is not induced [1] or its rate is very low [2, 5]; glycaemia is very low [1, 2, 6] and other relevant substrates are negligible [1, 2]. Consequently, under these circumstances lactate plays an important role as a source of energy and carbon skeletons for neonatal tissues. It has been reported that lactate is probably the main metabolic fuel for the brain during the presuckling period because its rate of utilization under these circumstances is much higher than other well-known brain fuels such as glucose and ketone bodies [7, 8]. In addition, other tissues such as the lung [9] and the heart (E. Fernández & J. M. Medina, unpublished work) may also contribute to the high rate of lactate utilization observed during the first 2 h after delivery [10]. The present work was thus designed to study the possible role played by the liver in the postnatal utilization of lactate. It should be mentioned that the gluconeogenic capacity of the liver within the presuckling period is negligible [6, 10]. Accordingly, we studied the uptake of lactate into CO_2 and lipids in early neonatal rat (1 h) liver incubated *in vitro*. The effect of hypoxia and maternal starvation on the rate of lactate utilization was also investigated.

Well-fed or 48 h-starved pregnant rats at 21.5 days of gestation were sacrificed by cervical dislocation and the fetuses were delivered by rapid hysterectomy. The newborns were carefully wiped and the umbilical cords tied and cut in a cabin at 37°C. They were then weighed and sacrificed by decapitation. Their livers were immediately removed and used for the experiments. Partially sliced whole livers (PSWL) were incubated at 37°C in 3 ml of phosphate physiological saline [11], containing 0.25 μCi of L-[U-^{14}C]-lactate and the desired concentration of the unlabelled substrate. The gas phase was O_2 except for the experiments on hypoxia in which O_2 was substituted by N_2. Incubations were stopped after 1 h with 0.2 ml of 20% (v/v) $HClO_4$, although shaking was continued for a further 20 min. The radioactive CO_2 trapped by 20% (v/v) KOH was measured by liquid-scintillation counting. Blanks without PSWL were carried out in parallel to measure volatile radioactivity, which was subtracted from other values. After incubation, PSWL were

Abbreviation used: PSWL, partially sliced whole livers.

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Pitfalls in the use of ELISA to screen for monoclonal antibodies raised against small peptides

S.C. Smith [a], N. McIntosh [a] and K. James [b]

[a] *Department of Child Life and Health, University of Edinburgh, Edinburgh EH9 1UW, Scotland, UK* and [b] *Department of Surgery (WGH), University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, Scotland, UK*

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Small peptides are often conjugated to large carrier proteins such as thyroglobulin to increase their immunogenicity. Antibodies are raised against peptide, thyroglobulin and contaminants, such as immunoglobulins or BSA in the thyroglobulin preparation, but anti-contaminant antibodies will not be revealed if immune serum is diluted in buffer containing immunoglobulin-contaminated BSA. This may lead to the development of an inappropriate hybridoma-screening assay which detects more anti-contaminant than anti-peptide antibodies if ELISA plates are coated with a capture antibody or blocked with BSA. Dilution of culture supernatants in buffer containing BSA and Tween 20 minimises the risk of false positive results and makes plate-blocking unnecessary.

The high affinity peptide-specific antibodies in immune serum, which are more readily detected than lower affinity monoclonal antibodies, may result in an inadequately sensitive hybridoma-screening ELISA.

Key words: Monoclonal antibody, anti-peptide; ELISA; Supernatant screening; Anti-immunoglobulin antibody; False positive results

Introduction

Hybridoma-screening assays must be capable of rapidly processing large numbers of samples. The assays have to be optimised before monoclonal antibody production can begin, so they are frequently set up using serum from the immune spleen donor (Campbell, 1984; Goding, 1986). Enzyme-linked immunosorbent assays (ELISA) and radioimmunoassays (RIA) which detect specific binding to the antigen have proved useful in screening for monoclonal antibodies which are intended for use in immunoassays. However, problems arise with both types of assay.

The radiolabelled antigens used in RIA may have altered epitopes which are no longer recognised by the appropriate antibodies. This is a particular problem with small peptides such as arginine vasopressin (AVP) which contains only nine amino acid residues. Iodination of AVP increases its molecular weight by 11% and significantly increases the risk of obtaining a false negative screening assay result. An anti-AVP mono-

Correspondence to: N. McIntosh, Department of Child Life and Health, 17 Hatton Place, Edinburgh EH9 1UW, Scotland, UK. Tel.: 031-667 2617; Fax: 031-668 2605.

Abbreviations: AVP, arginine vasopressin; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; FCS, foetal calf serum; i.p., intraperitoneally; IRS, immune rabbit serum; NBCS, newborn calf serum; NRS, normal rabbit serum; RIA, radioimmunoassay.

clonal which does not bind well to ^{125}I-AVP has been reported (Hou-Yu et al., 1982; Valiquette et al., 1986; Valiquette and Neubort, 1989).

ELISA methods seem superior because they avoid this problem, but may detect antibodies which cannot recognise the native antigen in solution if conformational changes occur when the antigen adsorbs to the solid phase (Mierendorf and Dimond, 1983; Vaidya et al., 1985). However, if the antigen is large enough to bind two antibodies simultaneously, a capture antibody can be used to immobilise it without loss of its native conformation (Smith and Wilson, 1986).

Successful monoclonal antibody production requires that the spleen donor be adequately immunised. Small peptides such as AVP are poor immunogens and therefore they are usually conjugated to a large protein such as thyroglobulin (Skowsky and Fisher, 1972) in order to induce a better antibody response. Unfortunately this procedure causes difficulties when hybridoma culture supernatants are screened by ELISA. We report the problems encountered during screening for anti-AVP monoclonals raised against an AVP-thyroglobulin conjugate and describe a simple way to avoid the problem of false positive ELISA results.

Materials and methods

Immunisation

12-week-old female BALB/c mice were immunised i.p. with 300 μg AVP-thyroglobulin conjugate containing 50 μg AVP (prepared by a modification (Staros et al., 1986) of the method of Skowsky and Fisher (1972) using bovine thyroglobulin (T1001, Sigma Chemical Company, Poole, UK)) emulsified in an equal volume of Freund's complete adjuvant. After 6 weeks, the mice were boosted i.p. with 300 μg immunogen emulsified in Freund's incomplete adjuvant and, after a further 6 weeks, with 300 μg immunogen in alum. 3 days prior to cell fusion a final i.p. boost of 300 μg immunogen in PBS (0.05 M phosphate, 0.9% NaCl, pH 7.5) was given. Animal care was in accordance with institutional guidelines.

Cell fusion

Hybridomas were generated by polyethylene glycol 4000-induced fusion of immune spleen cells with NS-0 murine myeloma cells (Galfrè and Milstein, 1981) at a spleen cell-to-myeloma cell ratio of 2:1. The fused cells were seeded into 96-well tissue culture plates at 2×10^5 cells/well, in 0.2 ml HAT medium (RPMI 1640, (Gibco, Paisley, Scotland, cat. no. 041-02402) containing 15% heat-inactivated foetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin, 100 μM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine), supplemented with rat mixed-thymocyte culture-conditioned medium (Micklem et al., 1987). Culture supernatants were assayed for the presence of antibody as soon as large colonies of cells were observed.

Screening assays

All incubations were carried out at room temperature, using 100 μl of each reagent unless otherwise stated.

Antigen-capture ELISA

Polyclonal rabbit anti-AVP serum (0.01% (v/v) TG1 (Smith and McIntosh, 1986) in 0.05 M sodium bicarbonate buffer, pH 9.0) was adsorbed onto 16-well flat-bottomed Immulon 1 microtiter duo-strips (M17916A, Dynatech Laboratories, Billingshurst, Sussex) by overnight incubation. Each well was washed three times with bicarbonate buffer and incubated with 400 μl 1% BSA (A7030, Sigma Chemical Company), 0.1% lactose, in bicarbonate buffer, for 30 min, to block remaining protein-binding sites. After washing, 50 ng/ml AVP in PBS, 1% BSA, 0.1% Tween 20 (PBS-BSA-T) was added and incubated for 16 h. The assay strips were stored at -20°C, or used immediately, after rinsing with PBS.

Hybridoma culture supernatant was incubated in the wells for 16 h. After removing the supernatant, rabbit anti-mouse IgG (whole molecule) horseradish peroxidase conjugate (A2028, Sigma Chemical Company, 1/200 dilution in PBS-BSA-T) was added and incubated for 1 h. Unbound peroxidase conjugate was removed by rinsing five times with wash buffer (0.1 M acetate-citrate buffer, pH 6.0, 5 mM EDTA, 0.02% Tween 20)

prior to the addition of freshly prepared substrate (100 μg/ml 3,3′,5,5′-tetramethylbenzidine, 0.002% H_2O_2 in 20 mM acetate buffer, pH 5.0). Colour development was stopped after 30 min by adding 50 μl 2 M H_2SO_4 and the absorbance measured at 450 nm on a Dynatech MR700 automatic microplate reader. Negative control wells contained HAT medium in place of culture supernatant.

Antigen-coated-plate ELISA

AVP (1 μg/ml) or other test material in PBS was adsorbed onto Immulon 1 microtiter duostrips by overnight incubation. After three washes with PBS, hybridoma culture supernatant was incubated in the wells for 16 h. Incubation with rabbit anti-mouse IgG peroxidase conjugate, and the remainder of the assay, was performed as above.

Radioimmunoassay

Hybridoma culture supernatant was incubated with 50 μl ^{125}I-AVP (7 pg, 1700 μCi/μg) in PBS, 2% NBCS, 1% Tween 20, for 16 h before separation of bound from free tracer by the polyethylene glycol (PEG) precipitation method (Desbuquois and Aurbach, 1971). 50 μl NBCS was added to the incubation mixture, followed by 500 μl PEG 6000 (25% (w/v) in distilled water). The assay tubes were vortexed and then immediately centrifuged at $2000 \times g$ for 30 min. The supernatant was decanted and the inverted tubes allowed to drain on tissue paper before counting the radioactivity of the precipitate.

Results

Antigen-capture ELISA

The antigen-capture ELISA appeared to be specific for anti-AVP antibodies because immune mouse serum produced higher absorbances than non-immune serum (Fig. 1), and its response to IRS-bound AVP was greater than to IRS alone (Fig. 2). RIA confirmed that immune mouse serum contained anti-AVP antibodies. However, high background absorbances were caused by non-specific binding of the second antibody-peroxidase conjugate to wells coated with IRS, NRS, NBCS, FCS or BSA (Table I), despite the pres-

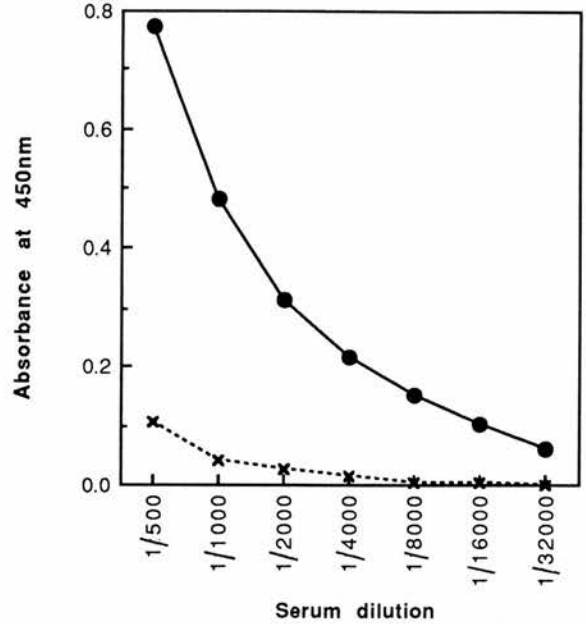

Fig. 1. Binding response of immune mouse serum (•) and normal mouse serum (×) in the antigen-capture ELISA. Serum dilutions were prepared in PBS-BSA-T.

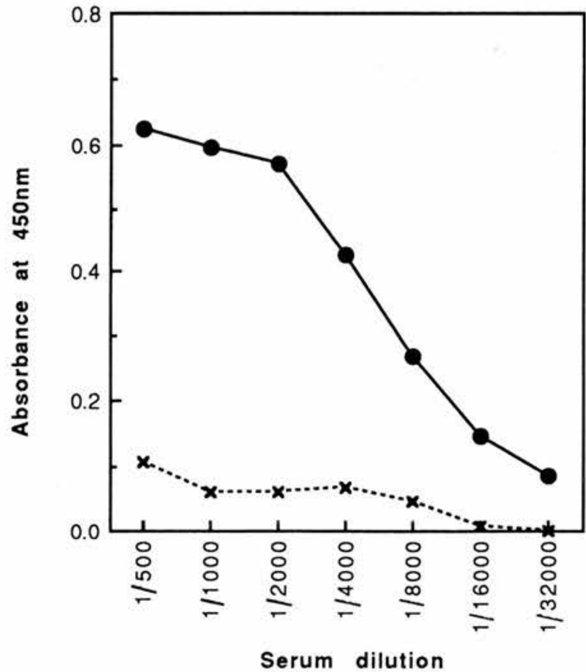

Fig. 2. Binding response of immune mouse serum to IRS-bound AVP (antigen-capture ELISA, •) and to IRS alone (×). Serum dilutions were prepared in PBS-BSA-T.

TABLE I

NON-SPECIFIC BINDING OF ANTI-MOUSE IgG PEROXIDASE CONJUGATE TO MICROTITER WELLS

Various protein-containing solutions were adsorbed onto Immulon 1 microtiter wells. The antigen-coated-plate ELISA was performed as described in materials and methods, except that 100 μl HAT medium replaced hybridoma culture supernatant. The absorbances shown are the means of duplicate determinations.

Coating solution	Absorbance
None	0.000
0.01%IRS	0.183
0.01% NRS	0.082
1% BSA	0.057
2% NBCS	0.183
2% FCS	0.020

ence of BSA and Tween 20 in the assay buffer (PBS-BSA-T). It did not bind to uncoated wells.

One cell fusion generated hybridomas in 378 culture wells. Undiluted supernatants from 26 hybridomas were considered antibody-positive because their absorbances in the antigen-capture ELISA exceeded 0.3. These supernatants were subjected to antibody dilution analysis (Van Heyningen, 1986) to identify high affinity anti-AVP antibodies. However, dilution in assay buffer virtually eliminated antibody binding in most samples, even with a dilution factor as low as 2 (Table IIA). Dilution in culture medium did not have this drastic effect (Table IIB, Fig. 3). In contrast, although dilution of immune mouse serum in assay buffer caused lower readings than dilution in culture medium, the recorded absorbances were still substantial (Fig. 4).

Antigen-coated-plate ELISA

The specificities of antigen-capture ELISA-positive culture supernatants were investigated by antigen-coated-plate ELISA (Table III). Undiluted supernatants bound strongly to wells coated with 1% BSA and all, except that from hybridoma 6, also bound to coatings of 0.01% IRS, 0.01% NRS, and 2% NBCS. Readings from wells coated with 2% FCS were similar to those from uncoated wells, which were caused by sample adsorption during the 16 h incubation period. All supernatants appeared to bind AVP at a low level (compared with their response to IRS and BSA) but this was probably non-specific. Many supernatants bound strongly to wells coated with

TABLE II

RESPONSE OF HYBRIDOMA CULTURE SUPERNATANTS IN THE ANTIGEN-CAPTURE ELISA

Culture supernatants were assayed undiluted, and after dilution with an equal volume of assay buffer (PBS-BSA-T) (A), or HAT medium (B). The absorbance due to non-specific binding of the second antibody-peroxidase conjugate has been subtracted from the values shown.

Hybridoma	Absorbance	
	Undiluted	Diluted (1/2)
A 1	0.976	0.097
2	1.493	0.020
3	> 2.000	0.271
4	1.591	0.714
5	1.962	0.011
6	0.683	0.060
B 1	1.368	1.139
2	1.815	1.734
3	> 2.000	> 2.000
4	1.862	> 2.000
5	> 2.000	1.974
6	0.415	0.357

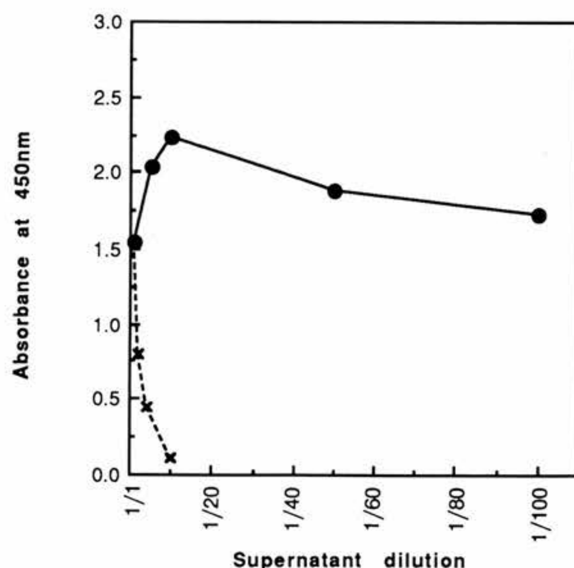

Fig. 3. Effect of diluting culture supernatant from hybridoma 4 on its response in the antigen-capture ELISA. Supernatant was diluted in culture medium (•) or PBS-BSA-T (×).

TABLE III

BINDING OF HYBRIDOMA CULTURE SUPERNATANTS IN THE ANTIGEN-COATED-PLATE ELISA

Undiluted culture supernatants were assayed in microtiter wells coated with a range of test materials. The absorbance due to non-specific binding of the second antibody-peroxidase conjugate to each plate-coating material has been subtracted from the values shown.

Hybrid	Plate-coating material							
	None	10 ng/ ml AVP	2% FCS	2% NBCS	1% BSA	0.01% IRS	0.01% NRS	1% milk
1	0.190	0.410	0.135	0.603	1.658	1.073	1.120	0.924
2	0.409	0.822	0.181	0.717	1.855	1.449	1.609	0.553
3	0.485	0.746	0.256	1.000	> 2.000	1.606	> 2.000	> 2.000
4	1.733	> 2.000	1.843	> 2.000	> 2.000	> 2.000	> 2.000	1.693
5	0.290	1.063	0.378	1.484	> 2.000	> 2.000	> 2.000	1.184
6	0.045	0.073	0.045	0.088	1.084	0.040	0.102	0.019

1% non-fat dried milk (Table III). None bound to ^{125}I-AVP as determined by RIA.

Dilution of supernatants in assay buffer prevented non-specific binding to AVP-coated wells and decreased binding to IRS, NRS, BSA and thyroglobulin (Table IV). Immune mouse serum reacted strongly with thyroglobulin, even after dilution in assay buffer, because it contained anti-thyroglobulin antibodies in addition to anti-AVP. In comparison its response to IRS, BSA and NRS was weak (Table IV).

A second cell fusion, which generated 461 hybridomas, was screened by antigen-capture ELISA, AVP-coated-plate ELISA and RIA. Undiluted supernatants from 110 hybridomas were positive in the antigen-capture ELISA but did not contain specific anti-AVP antibody because all binding was prevented by dilution with an equal volume of assay buffer. One monoclonal cell line was established after its detection by AVP-coated-plate ELISA and RIA. It could not be detected by antigen-capture ELISA (hybridoma 7, Table IV).

The anti-AVP antibodies in immune mouse serum were detected by the antigen-capture ELISA (Fig. 2), although low titre sera gave poor results (data not shown). In contrast, the monoclonal antibodies which we produced were not detected even though they could participate in two-site binding (Smith et al., 1990). They also responded poorly to low AVP concentrations in the AVP-coated-plate ELISA. Higher coating

TABLE IV

EFFECT OF SAMPLE DILUTION ON ELISA BINDING RESPONSE

Culture supernatants were assayed undiluted and after dilution (1/2, figures in parentheses) with assay buffer (PBS-BSA-T). Hybridomas 7 and 8 produced specific anti-AVP monoclonals. Immune mouse serum (1/1000 in culture medium, and after further dilution (1/2) with assay buffer) was included for comparison. The absorbances shown are the means of duplicate determinations.

Sample	Antigen-coated-plate ELISA					Antigen-capture ELISA
	1 μg/ml AVP	1% BSA	10 μg/ml bovine thyroglobulin	0.01% NRS	0.01% IRS + 1% BSA	0.01% IRS + 1% BSA + 50 ng/ml AVP
7	> 2.000 (> 2.000)	0.028 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.004)
8	1.266 (1.346)	0.070 (0.000)	0.020 (0.000)	0.022 (0.020)	0.089 (0.028)	0.040 (0.000)
9	0.054 (0.007)	0.013 (0.000)	0.037 (0.000)	0.115 (0.049)	0.953 (0.764)	0.509 (0.483)
10	0.300 (0.000)	0.223 (0.000)	0.845 (0.371)	0.681 (0.077)	0.455 (0.000)	0.359 (0.000)
Immune mouse serum	0.700 (1.324)	0.000 (0.000)	> 2.000 (> 2.000)	0.279 (0.140)	0.118 (0.074)	0.159 (0.208)

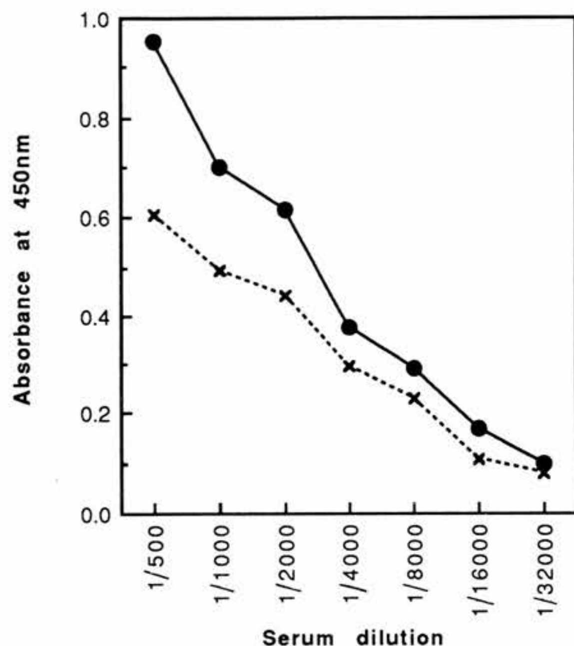

Fig. 4. Effect of diluent composition on the binding response of immune mouse serum in the antigen-capture ELISA. Serum was diluted in culture medium (•) or PBS-BSA-T (×).

concentrations improved their detectability but had relatively little effect on the binding response of immune serum (Fig. 5).

Plate-blocking was omitted from the AVP-coated-plate ELISA because BSA increased non-specific binding of the second antibody-peroxidase conjugate (Table I) and decreased specific binding of anti-AVP antibodies (Table V). Dilution of culture supernatants in buffer containing Tween 20 prevented non-specific binding to the plate and thus rendered plate blocking unnecessary.

Discussion

Arginine vasopressin and oxytocin are closely related nonapeptides, differing in only two amino acid residues. Iodination of the tyrosine residue of AVP masks an important epitope because the adjacent phenylalanine is the only amino acid which distinguishes the AVP ring structure from that of oxytocin. Hybridoma screening by RIA is unlikely to detect antibodies specific for this region of AVP. We therefore chose to use ELISA screening which avoids this problem because it does not require labelled peptide.

A two-site antigen-capture ELISA (Smith and Wilson, 1986), using rabbit antiserum to immobilise AVP, seemed ideal for supernatant screening because we required monoclonal antibodies suitable for two-site immunometric assay development (Smith et al., 1990). This type of screening assay was reported to be more sensitive than those involving direct adsorption of antigen onto plastic (Al Moudallal et al., 1984; Brennand et al., 1986) and was the only system capable of detecting all antigen-specific monoclonals (Brennand et al., 1986). Initial tests with immune mouse serum suggested that the antigen-capture ELISA was specific for anti-AVP antibodies (Figs. 1 and 2) despite non-specific binding of the second antibody-peroxidase conjugate to the solid-phase components. However, the antibodies detected in undiluted culture supernatants failed to bind after dilution in assay buffer (Table IIA), even though significant adsorption to uncoated wells (Table III) had suggested that the supernatants contained high antibody concentrations (Miller et al., 1983). Investigation of this phenomenon revealed that undiluted positive culture supernatants bound strongly to IRS, NRS, BSA and NBCS (Table III), whereas the response to FCS was the same as to uncoated wells.

TABLE V

EFFECT OF BSA-BLOCKING ON ANTI-AVP ANTIBODY BINDING IN THE AVP-COATED-PLATE ELISA

Immune mouse serum and a monoclonal antibody-containing culture supernatant diluted in PBS-BSA-T (1/2000 and 1/2 respectively) were assayed in AVP-coated wells, as described in materials and methods, or after blocking the coated plates with BSA (1% in PBS). The absorbance due to non-specific binding of the second antibody-peroxidase conjugate has been subtracted from the values shown, which are the means of duplicate determinations.

Sample	Absorbance	
	Blocked	Unblocked
Monoclonal culture supernatant	1.041	1.296
Immune mouse serum	1.297	1.659

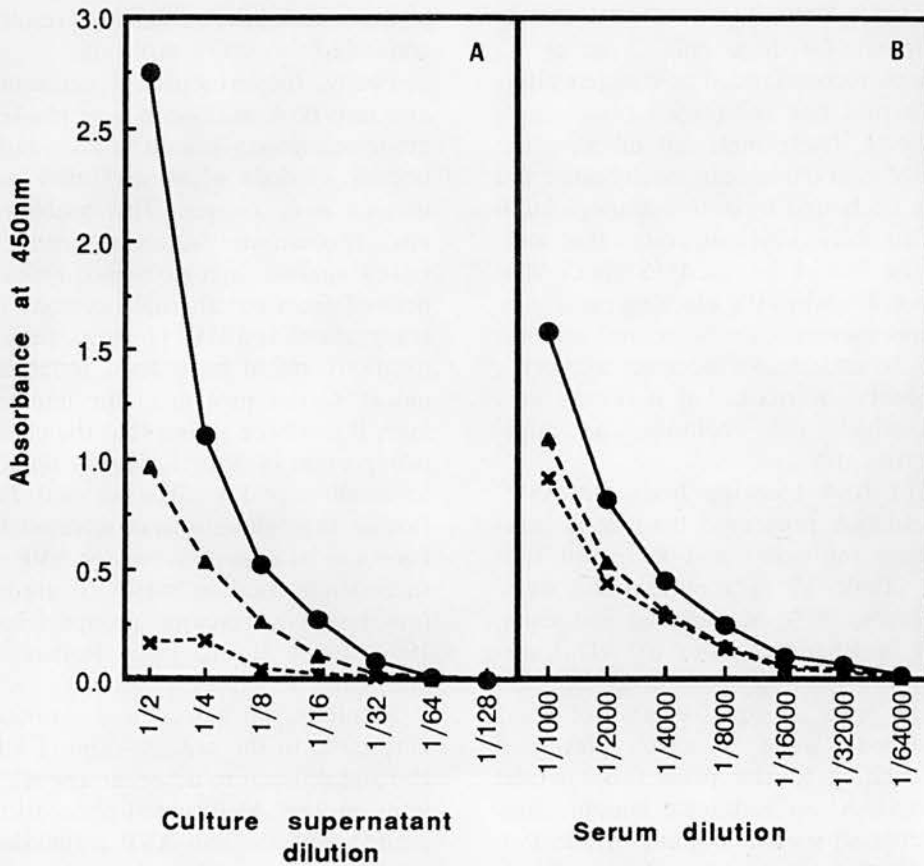

Fig. 5. Binding response of a monoclonal antibody-containing culture supernatant (A), and immune mouse serum (B), in the antigen-coated-plate ELISA, with AVP coated at 10 ng/ml (×), 100 ng/ml (▲) and 1 μg/ml (●).

Many BSA preparations are contaminated by immunoglobulins (Boscato and Stuart, 1988) which were also present in all the sera used in this work, except FCS. Since antibodies are raised against all immunogen components, it seems likely that the bovine thyroglobulin (Sigma, T1001, described as 'electrophoretically heterogeneous') used in immunogen production in this study, was contaminated by immunoglobulins, and that anti-immunoglobulin antibodies were contained in the antigen-capture ELISA-positive supernatants. Anti-immunoglobulin antibodies have often been reported to cause interference in immunoassays and have been described in the literature as heterophilic antibodies (Boscato and Stuart, 1988; Nahm and Hoffmann, 1990). The thyroglobulin preparation also contained BSA because a specific anti-BSA antibody was detected (hybridoma 6, Table III). When culture supernatants were diluted 1/2 in assay buffer, anti-BSA and anti-immunoglobulin antibodies reacted with the BSA and immunoglobulin contaminants in the buffer and so binding in the antigen-capture ELISA was prevented (although a few high titre supernatants might still show some binding due to inadequate neutralisation, Fig. 3). Dilution in culture medium did not have this effect because it contained no immunoglobulins and relatively little BSA.

Traditional ELISA methods include a blocking step whereby an inert protein adsorbs onto unoccupied sites on the solid phase to prevent non-specific binding of reagents (Campbell, 1984), but there is then a risk of interference by anti-blocking-protein antibodies (Schonheyder and Andersen, 1984; Kemeny and Chantler, 1988). BSA is commonly used as a blocking agent (Gustafsson,

1990; Pruslin, 1991) although casein (Kenna et al., 1985) and non-fat dried milk (Vogt et al., 1987) have been recommended as cheaper alternatives and serum has been used occasionally (Campbell, 1984). These materials all have the disadvantage of containing immunoglobulins and will therefore be bound by anti-immunoglobulin antibodies. Campbell (1984) suggested that antigen-free plates should be used to check that antibodies do not bind to the blocking protein or plastic, but this increases the work and expense involved and is undesirable because a specific antibody might be overlooked if it occurs in a supernatant which also contains an anti-blocking-protein antibody.

Omission of BSA blocking from the AVP-coated-plate ELISA prevented binding of anti-blocking-protein antibodies and increased specific binding (Table V) by removing the steric hindrance (Hobbs, 1989; Mohammad and Esen, 1989) caused by BSA molecules (67 kDa) surrounding the AVP peptide (1.084 kDa). Dilution of supernatants with an equal volume of assay buffer introduced Tween 20 which prevented non-specific binding to the plate (Mohammad and Esen, 1989). As an additional benefit, dilution often increased specific binding (Table IV), probably by a buffering effect because spent culture supernatants tend to be acidic (Campbell, 1984) while most antibodies bind optimally at neutral pH values. There may also have been a prozone effect because concentrated antibody solutions tend to bind univalently and may leach off the assay plate during subsequent incubation and washing steps (Nygren et al., 1985; Vos et al., 1987).

Caution is required when immune serum is used to develop a hybridoma-screening assay because the binding properties of antisera differ significantly from those of monoclonal antibodies (Al Moudallal et al., 1984) and normal mouse serum may not be an appropriate negative control. Serum from an animal immunised with an unrelated peptide conjugated to the carrier protein is more likely to give an indication of possible problems, particularly if it is diluted in culture medium instead of assay buffer. The antigen-capture ELISA and the AVP-coated-plate ELISA described above were set up using immune mouse serum which gave misleading results because it concealed two major problems.

Firstly, the presence of anti-immunoglobulin and anti-BSA antibodies was masked when immune serum was diluted in assay buffer and only became obvious when undiluted culture supernatants were assayed. This problem, which was also encountered when screening hybridomas raised against thyroglobulin-conjugated peptides derived from parathyroid hormone (unpublished observation) and HIV (J. Boyd, personal communication), might have been reduced by using a mouse carrier protein in the immunogen. However, it has been shown that the choice of carrier is important in determining the immune response to small peptides (Skowsky and Fisher, 1972). Bovine thyroglobulin was selected because it is known to be a good carrier for AVP and has been successfully used in anti-AVP antibody production by many research groups (Hou-Yu et al., 1982; Jones et al., 1985; Robert et al., 1985; Smith and McIntosh, 1986).

Secondly, our monoclonal antibodies were undetectable in the antigen-capture ELISA (Table IV) and difficult to detect at low AVP concentrations in the AVP-coated-plate ELISA (Fig. 5) even though the anti-AVP antibodies in immune serum produced high absorbances under these conditions. Monoclonal antibodies do not bind well at low epitope densities (Lew, 1984) particularly if they have low binding affinities (Nygren et al., 1985) whereas the high affinity antibodies in immune serum will bind strongly, leading to the belief that an appropriate screening assay has been developed when much higher antigen-coating concentrations may be needed for efficient monoclonal detection.

Early screening of hybridoma supernatants is vital because cultures often contain more than one colony of cells and non-producers are liable to overgrow antibody-secreting cells (Goding, 1986). Antibody concentrations are initially low, therefore screening assays must be very sensitive to avoid false negative results and supernatants are commonly tested without prior dilution (Galfrè and Milstein, 1981; Robert et al., 1985; Vaidya et al., 1985; Smith and Wilson, 1986; Gustafsson, 1990). While this is acceptable in liquid-phase RIA screening (because buffer is

introduced with the labelled antigen and only specific antibodies are detected), ELISA screening of undiluted supernatants is prone to many problems. These may not be anticipated by laboratories whose previous experience of hybridoma-screening has been restricted to RIA.

We recommend that all hybridoma supernatants, particularly those raised against thyroglobulin-conjugated peptides, are diluted in buffer containing BSA and Tween 20 prior to ELISA screening. This will prevent non-specific binding and so make plate blocking unnecessary, maximise specific binding by buffering the sample, and neutralise most anti-immunoglobulin and anti-BSA antibodies.

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