PRODUCTION AND EVALUATION OF A TWO-SITE IMMUNORADIOMETRIC ASSAY FOR ALPHA-1-FOETOPROTEIN USING MONOCLONAL ANTIBODIES

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Lateat scintulla forsan ?

LIST OF ABBREVIATIONS USED IN THE TEXT

^{o}C	degrees Celsius
AAWD	Anterior abdominal wall defect (gastroschisis, exomphalos or body stalk anomaly)
AF-AFP	Amniotic fluid alpha-foetoprotein
AFP	Alpha-1-foetoprotein
BD	Bad duplicate (difference between duplicate counts exceeds a preset limit)
cm	centimetre - m $x10^{-2}$
CV	Coefficient of Variation
CVS	Chorionic Villus Sampling
DS	Down's Syndrome
EPPS	N-2-hydroxyethyl-piperazine-N'-3-propane-sulphonic acid
EQAS	External Quality Assessment Scheme for AFP (RIA Section, Biochemistry Department, Royal Infirmary, Edinburgh)
MoM	Multiples of the Median
MS-AFP	Maternal serum alpha-foetoprotein
ng	nanogram - g $x10^{-9}$
NTD	Neural tube defect
NHS	National Health Service
SAPU	Scottish Antibody Production Unit (Law Hospital, Carluke, ML8 5ES, UK)
SD	Standard Deviation
ug	microgramme - g $x10^{-6}$
uL	microlitre - L $x10^{-6}$
um	micrometre - m $x10^{-6}$

SUMMARY

Alpha-1-foetoprotein (AFP) is a glycoprotein present at high concentration in foetal serum until birth, with residual low levels present throughout adult life. The most frequent cause of elevated AFP concentration in adults is pregnancy, during which foetal AFP passes across the placenta and the amnion into the maternal circulation. The concentration of maternal serum AFP (MS-AFP) is related to the gestational age, and to the clinical status of the foetus and placenta. Elevated MS-AFP is a marker of several clinical entities, of which the most important and frequent are neural tube defects. In view of this, since 1975, the entire mid-trimester pregnant population of the West of Scotland has been offered MS-AFP screening and at the start of this project the West of Scotland maternal-serum screening programme used a RadioImmunoAssay (RIA) technique with ^{125}-Iodine labelled AFP and sheep anti-human AFP antiserum. Separation was effected by 16-hour incubation in polyethylene glycol 6000. 30,000 samples per year were being assayed and the workload was increasing. Disadvantages of the RIA included the long incubation time, lack of robustness and reliability, and poor sensitivity and precision.

The potential superiority of a commercially available two-site ImmunoRadioMetric Assay (IRMA) with respect to sensitivity, precision and working range was confirmed during local evaluation but was invalidated for routine use in the screening programme by its cost. Accordingly, this project was initiated in order to manufacture an in-house IRMA which would be available to NHS laboratories.

Polyclonal antibodies were generated by immunizing mice intra-peritoneally with 10 microgrammes (ug) of AFP (in amniotic fluid) suspended in Freund's adjuvant. Two boosts were given at two weeks and four weeks, and a pre-fusion intravenous boost of 4 ug was administered 3 days before post-mortem removal of the spleen. Monoclonal antibody-secreting cell lines were produced by polyethylene glycol 1500 fusion of mouse spleen cells 1:1 with mouse myeloma X63 Ag8.653 cells. Non-hybrid myeloma cells were killed by inclusion of hypoxanthine-aminopterin-thymidine (HAT) in the medium and non-hybrid spleen cells were not viable in the culture

conditions. Preparatory work on the fusion technique had shown that the presence of mouse peritoneal macrophages increased the likelihood of finding viable hybrid colonies from 6 per 96 wells to >50 per 96 wells, and rapidly reduced the quantity of debris present. Thus cells from each fusion experiment were plated into 480 wells previously inoculated with 3,000 macrophages per well.

In order to investigate only high avidity/affinity antibodies, an RIA method was used to screen supernatant from all wells for antibody secretion. All supernatants demonstrating high binding were then assessed at an appropriate dilution for displacement. For this the fluid was incubated in the presence and absence of antigen. Supernatants showing considerable displacement were selected for further investigation; the cell lines were cloned by limiting dilution and large quantities of monoclonal antibody produced by injecting the clone into mouse peritoneum.

Of nine mice immunized, one was killed before it was realised that the myeloma cells were non-viable owing to high incubator temperature. Two mice were used for six fusion experiments, and over 90% of the 2880 wells plated showed viable, hybrid colonies. 1136 wells (39.4%) contained anti-AFP secreting colonies (arbitrarily defined as >10% binding of ^{125}I-labelled-AFP), many of which will have contained several clones. Supernatant from 291 wells (10.1%) bound greater than 30% of added label, and were subjected to displacement testing. 67 wells were selected for storage after some growth, of which 16 wells, exhibiting the best growth-rate, antibody avidity and secretion characteristics, were cloned by limiting dilution. Two episodes of infection, one by Pseudomonas (from a bought-in reagent) and one by an unidentified bacterium, prevented more than two clones being used to generate large quantities of monoclonal antibody in mouse ascitic fluid for solid-phase coupling and ^{125}I-labelling studies.

The two antibodies were of similar avidity and had similar specificity, which prevented the development of a double-monoclonal assay. Characterization of the antibodies showed that they were of the IgG1 isotype and had avidities of approximately 10^{10}.

IRMA assay conditions were optimized, starting from a straightforward, low-cost base. A large-scale evaluation of the completed technique was performed using both density gradient

sedimentation, and saline wash methods for separating the bound and free fractions.

Development of a practicable IRMA protocol showed that incubation time of 2.5 hours gave minimum drift,and that the addition of sheep serum and detergent to the incubate was necessary to minimise non-specific binding effects and maximise sensitivity. After optimization of the IRMA it was apparent that the parameters of the assay (based on precision profile data) were; sensitivity <1kU/L (compared to RIA - 15kU/L), inter-assay CV 4% (compared to RIA - 10%), and working range 5->500kU/L (compared to RIA - 30-400kU/L). No dose difference was observed between the separation methods investigated; density sedimentation was used for the West of Scotland maternal serum screening programme because of its suitability for large assays and the availability of suitable equipment. Linear regression analysis of 2291 samples assayed in parallel by both RIA and IRMA demonstrated a gradient of 1.29 (for local reasons, 1.25 was expected), intercept +3.1kU/L and correlation coefficient 0.956. The IRMA assay was consequently introduced into routine use.

Experience in routine use demonstrated that previously undetectable shifts of 2% between standard preparations were detectable. This improvement in precision has enabled a clinically significant reduction in the intervention level from the 97th to the 95th centile. Improved sensitivity has enabled the initiation of low-MS-AFP screening to give a risk factor for Down's syndrome in each pregnancy where maternal age and weight are supplied. Eight other laboratories are using the labelled antibody (AF5/A2) reagent, three in Scotland, four in England and one in Wales. Each laboratory has adjusted the protocol for their own circumstances.

The antibody has been purchased for use in a commercial alpha-1-foetoprotein, immunofluorimetricassay method which is widely marketed for both maternal serum screening and tumour marker studies.

CHAPTER 1 INTRODUCTION

1A CHEMISTRY OF ALPHA-1-FOETOPROTEIN

i) Discovery

The first unequivocal identification of alpha-1-foetoprotein was made by Bergstrand and Czar (1956) when they demonstrated a major protein component of early human foetal serum which was not present in adults and which migrated slightly more slowly than albumen on electrophoresis. Ultracentrifugation indicated a molecular weight of 65,000 daltons.

ii) Structure

Nishi (1970) used antigen-antibody precipitation to purify AFP from two source materials, cardiac serum from ten aborted human foetuses at 7-10 weeks gestation and serum from an histologically confirmed case of primary hepatocellular carcinoma. The two AFPs were characterized separately and were indistinguishable chemically; in nitrogen, sugar, sulphur and amino-acid constitution, and physicochemically by determination of Svedberg and diffusion coefficients, disc electrophoresis and double (Ouchterlony) immunodiffusion.

Investigation of the amino acid sequences, of albumen and AFP, obtained by cyanogen bromide and peptic digestion led Brown (1976) to recognize 3 domains, each with 3 subdomains, which he postulated as derived, by duplication and subsequent divergence, some 700 million years ago from a 190 amino acid ancestral polypeptide.

Sodium dodecyl sulphate gel studies showed that AFP has a single polypeptide chain; Ruoslahti and Hirai (1978) reported estimates of molecular weights ranged from 64600-70000, and carbohydrate content, consisting of N-acetylglucosamine, mannose, galactose and sialic acid residues, from 3% to 4.5%. In addition, aggregation, especially to a dimeric form, was noted as was the similarity of the amino acid composition to that of albumen (Ruoslahti & Terry 1976). They concluded that there was some heterogeneity but they did not accept that AFP had substantial variants. Thus although two N-terminal sequences had been found, one lacking a tripeptide, both were present in foetal and hepatoma serum

and in equal proportions.

The carbohydate content of bovine AFP was investigated by Krusius & Ruoslahti (1982) using protease digestion and hydrazinolysis. They found; N-acetyl neuraminic acid, N-acetylglucosamine, mannose, galactose and a small amount of fucose. There were three variants separable by filtration through concanavalin-A immobilized on sepharose. Human AFP has been reported to have only one glycan (Smith and Kelleher 1980) and Morinaga et al. (1983) report a 12 residue glycan bound to asparagine 232, which they found to be the only available attachment point on the polypeptide chain. Nonetheless, Toftager-Larsen et al. (1983) showed the existence of several variants in the carbohydrate moiety by banding patterns with plant lectins.

iii) Gene

Electron microscopy (Kioussis et al. 1981) of AFP and albumen mRNAs hybridized to genomic DNA displayed 15 exons and 14 introns in both proteins. Eiferman and colleagues (1981) investigated the exon junctions of murine AFP with cloned genomic DNA and showed that the protein sequence, and nucleotide sequences of exons 3 to 14 corresponded to a threefold repeat of four exons per domain. They suggested an evolutionary mechanism of triplication and divergence after duplication of the ancestral gene 300-500 million years ago to form AFP and albumen.

Beattie and Dugaiczyk (1982) identified human AFP mRNA extracted from foetal liver (17-20 weeks gestation) using a mouse cDNA probe. The protein sequence deduced (amino acids 405-532) showed good agreement with cyanogen bromide degradation and demonstrated 44% homology with the 3rd domain of albumen. They showed that the rate of evolution for AFP was greater than that for albumen.

Harper and Dugaiczyk (1983) used two cDNA clones prepared from mRNA and about 1600 and 384 bases long for in situ hybridization studies and showed that albumen and AFP are linked. They localized both genes to bands q11-22 on chromosome 4.

Morinaga et al. (1983) sequenced three cDNA clones and a genomic clone to produce the complete alpha-foetoprotein mRNA structure. The deduced amino acid sequence contains 590 residues

(mouse AFP has 586) and is shown in Figure 1. The calculated molecular weight is 66,300 or, with 4% carbohydrate, 69,030 daltons. The protein showed 39% overall homology with human albumen and the major structural difference was found to be lack of two disulphide bridges with, consequently, the formation of a "hinge" between domains 2 and 3 in AFP.

A genomic clone from fibroblasts which were not producing either AFP or albumen isolated by Urano et al (1984) and inserted in bacteriophage lambda (Charon 4A) showed that the genes were in tandem 14.5 kilobases apart and in the same orientation with albumen upstream. They also concluded that no gross rearrangement of the genes was necessary for expression of the proteins.

Olsson et al. (1977) reported the discovery of persistent high AFP in the mouse strain BALB/c/J and that it is controlled by one recessive Mendelian gene. The hereditary persistence of human hyperalpha-foetoproteinaemia (HP-AFP 10414; McKusick 1988) in a large kindred was reported by Ferguson-Smith et al. (1983). This was shown to be an autosomal dominant trait, and cosegregation of HP-AFP, albumen, AFP and GC (vitamin D binding alphaglobulin) was observed with restriction fragment length polymorphisms detected using a cDNA albumen probe (Ferguson-Smith et al. 1985).

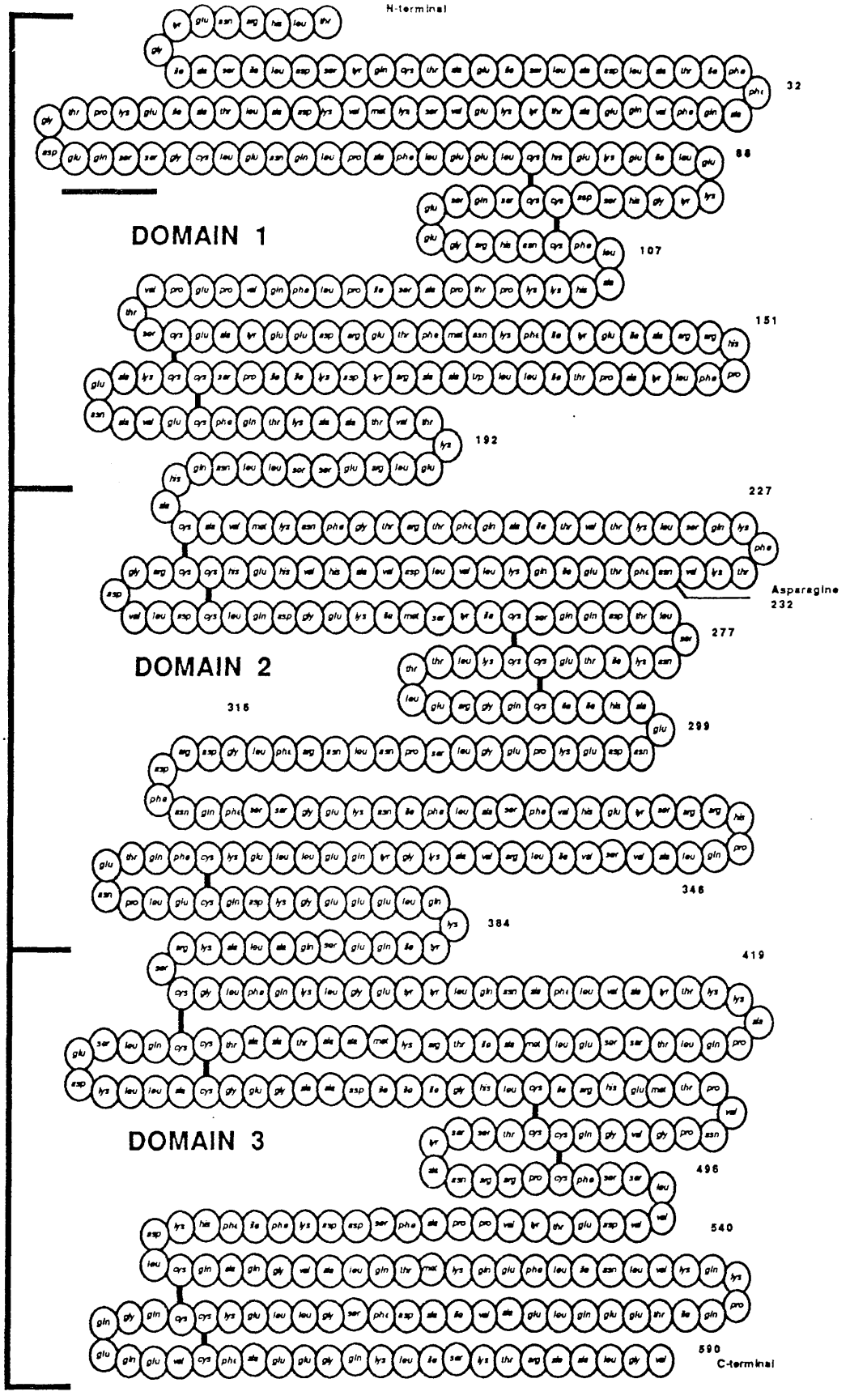


FIGURE 1 The 590 amino-acid sequence of Alpha-1-foetoprotein. The 4-amino-acid group absent from mouse AFP is underlined and the carbohydrate side-chain attachment point (at asparagine-232) is indicated.

1B PHYSIOLOGY OF ALPHA-1-FOETOPROTEIN

i) Function

No convincing evidence has been given for the existence of specific functions for AFP but since it is linked, and closely related, to albumen, and appears in its stead in the foetus, efforts have been made to show that it acts as a transport protein. Soloff et al. (1971) found that rat AFP binds oestriol, but this has not been confirmed in man. Belanger et al. (1981) reported dexamethasone suppression of AFP synthesis in newborn rat liver, and Sakai et al. (1985) found a 9 base-pair sequence in the 5-prime flanking region of the human gene which is similar to the glucocorticoid response sequence (Schmid 1982).

Immunosuppressive activity has been reported by Murgita and Tomasi (1975) and would be consistent with the presence of AFP during pregnancy.

ii) Site of Synthesis

The yolk sac is almost certainly the source of early AFP, but production later shifts to the liver. There is some evidence of AFP synthesis in the gastrointestinal tract (Gitlin 1975). This production pattern is reflected in the pathology.

Studies on the binding of AFP to jack bean (Canavalia ensiformis) lectin concanavalin-A (terminal mannosyl and glucosyl residue binding protein) revealed the existence of binding and non-binding fractions (Krusius & Ruoslahti 1982). Lentil (Lens culinaris) lectin was shown to possess not only non-reactive and reactive variants, but also a weakly reactive fraction (Toftager-Larsen et al. 1983). In early pregnancy a substantial proportion of foetal AFP is concanavalin-A non-reactive, whereas the proportion of concanavalin-A reactive AFP increases with gestation which suggests the liver is the source of this variant. As there is only one AFP gene, the lectin binding patterns show either post-translational processing differences or mRNA processing at the various production sites.

iii) Production pattern

Production of AFP in the foetus begins with the development of the yolk sac at around 4-6 weeks and the foetal serum concentration peaks at about 13 weeks gestation, when it may constitute up to 1/3 of total serum protein, although production may peak at 20-30 weeks (Gitlin 1975).

In normal adults the normal range of AFP has been reported to be 2-20ng/mL (Talermann et al. 1978). This is equivalent to about 1.6-16kU/L of AFP using the British Standard cord serum preparation BS 72/227 (Sizaret et al. 1975).

iv) Distribution in normal pregnancy

Gitlin and Boesman (1966) describe the biology of AFP and its production during pregnancy which they found low at 6.5 weeks gestation reaching a plateau from 22 to 32 weeks followed by steady fall. They reported the highest concentration of AFP in foetal serum to be 3g/L at 13 weeks and the post-natal half-life to be five days. They suggested the placenta as the site of synthesis. Production of albumen was also low at 6.5 weeks (concentration of 0.15g/L), rising in parallel with AFP concentration but delayed by 4-6weeks, to a plateau of 30 g/L (approximately normal adult level) at 26 weeks. They remarked on the higher concentration of albumen found in amniotic fluid compared to foetal urine and concluded that it must have a different source. In 1970, Gitlin and Perricelli detected uptake of radiolabelled amino acids by cultures of minced human yolk sac at 5.5 weeks gestation, but not in the solid, non-vascular 11.5 week material, and subsequently identified radioactive AFP as well as prealbumen, alpha-1-antitrypsin and transferrin. They determined that the production rate of these materials in the yolk sac at 5.5 weeks was at least a match for the liver synthesis at this period.

Foetal serum AFP may reach 3 million kU/L (3g/L) at 13 weeks, afterwards diminishing to a level of 30,000 to 100,000kU/L at 40 weeks (May et al. 1988). Neonatally, AFP appears to have a half life of about 5 days, which is consistent with production having ceased. Amniotic fluid AFP concentration is about 1% of the foetal serum level, the bulk normally being derived from foetal urine. It is assumed that ingestion and digestion of AFP by the foetus is the main route for consumption (Brock 1983).

Maternal serum AFP rises from normal adult levels at 4-6 weeks gestation to a peak of 200-400 kU/L at about 25 weeks, as the size of the placenta increases, after this the decreasing foetal serum AFP outweighs the size increase of the placenta and MS-AFP at birth may be 50-200kU/L (Clayton-Hopkins et al. 1982).

After allowing for the effects of increasing gestation, Los et al. (1985) showed a weak correlation between amniotic fluid albumen and maternal serum AFP in 81 paired samples. They showed mathematically that the transplacental route must be the major source of MS-AFP in normal pregnancies, and only in abnormal pregnancies can the greatly raised amniotic fluid AFP contribute substantially to the MS-AFP. This would explain the lack of correlation which they found between amniotic fluid AFP and maternal serum AFP.

1C PATHOLOGICAL IMPLICATIONS

i) Tumours

The prenatal production pattern is reflected in the types of neoplasms associated with elevated AFP concentration, these are generally primary hepatocellular or endodermal sinus tumours, including germ cell and teratoma types. Rarely, other AFP secreting tumours are found, for example, in the gastrointestinal tract.

Nishi (1970) used an insensitive, radial immunodiffusion technique to assay sera from 500 patients and showed one abnormal result: a case of carcinoma with liver metastases. By contrast, 23 of 31 patients with primary hepatocellular carcinoma had high concentrations of AFP.

Prospective studies using specially sensitized radioimmunoassays (RIA) showed that AFP secretion can be detected as much as two years before clinical manifestation. The biological half life of 5 days (Milford-Ward & Bates 1979) has been widely used as an indicator of completeness of resection of tumour material and serial sampling is a valuable prognostic tool in the post-operative period.

ii) Neural Tube Defect Diagnosis and Screening

It was postulated that the failure during embryogenesis of the neural tube to close would allow large amounts of AFP into the

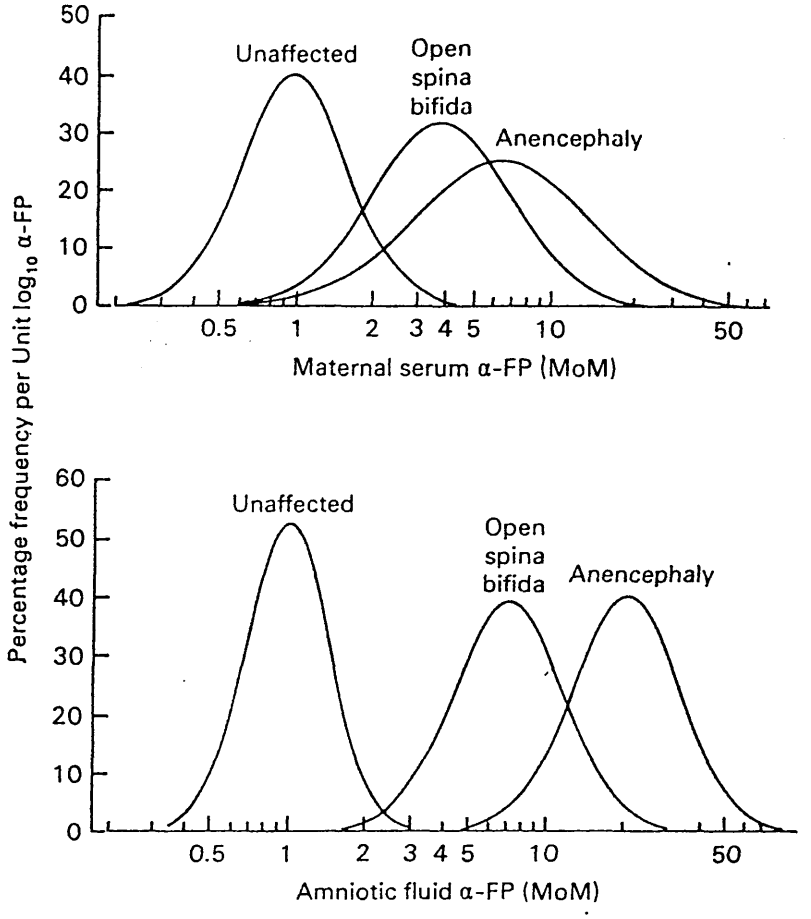
amniotic fluid. Accordingly Brock and Sutcliffe (1972) subjected amniotic fluids, which had been stored at -25°C for up to 3 years, from 37 abnormal pregnancies to quantitative "rocket" immunoelectrophoresis for AFP. All 31 anencephalic pregnancies collected between 25 and 35 weeks and one out of three open spina bifida pregnancies showed abnormal concentrations, but the 3 hydrocephalics were within normal limits. They concluded that the assay was unreliable for detection of foetal abnormality above 35 weeks. Little overlap between normal and abnormal ranges was noted, see figure 2. They also predicted that the elevated amniotic fluid alpha-1-foetoprotein (AF-AFP) would cause increased AFP passage through the amnion into the maternal circulation and thus raise MS-AFP. Amniotic fluids from pregnancies affected by various foetal and maternal abnormalities, particularly the effects of Rhesus isoimmunization, were studied by Brock & Sutcliffe (1973), who confirmed that anencephalus was the main cause of raised AF-AFP.

The RIA techniques (pioneered by Yalow & Berson 1960) in use for tumour marker studies were sensitive but slow, taking up to 4 days (Chayvialle & Ganguli 1973). Use of a specially developed RIA, designed to allow rapid turnround of large numbers of MS-AFP samples (Vince et al. 1975), in a prospective pilot study for evaluation of this theory led to full scale maternal serum screening programme in the West of Scotland.

The normal range for MS-AFP overlaps the range for anencephalus and substantially overlaps the range found in open spina bifida, as shown in figure 2. Serum assay cannot therefore be used as a diagnostic test. The overlap has important implications for the precision and robustness of assay methods used in the screening programme.

Maternal serum AFP screening programmes have been restricted to 16-20 weeks inclusive since the Report of the UK Collaborative Study (1977) because before this the MS-AFP is not reliably raised, and afterwards it is impractically late in gestation.

Crandall and colleagues (1983) showed that black women had higher (about 10%) MS-AFP levels than caucasian, hispanic or oriental women if all values were corrected for the effects of maternal weight.



Overlap is shown between unaffected, open spina bifida and anencephalic pregnancies for MSα-FP levels (top) and amα-FP levels (bottom). The choice of intervention point between 2 and 3 MoMs (multiples of the median) determines the proportion of false-positive and false-negative results. (The unit of $\log_{10}$ α-FP used is 0.2163).

From UK Collaborative Study, Fourth Report (1982)

FIGURE 2 Frequency distribution of Alpha-1-foetoprotein concentrations at 16 to 18 weeks gestation for maternal serum and amniotic fluid.

iii) Other Conditions in Pregnancy

Brock (1976) suggested mechanisms for the maternal serum and amniotic fluid AFP concentrations found in various conditions by assuming that foetal urine is the source of amniotic fluid and of AF-AFP, and that ingestion of the amniotic fluid and digestion of constituent proteins is the main route of AFP catabolism. Thus high AF-AFP is expected where foetal blood or serum enters the amniotic fluid. If the AF-AFP is high enough, MS-AFP will be raised because of increased AFP passage across the amnion.

This would account for the pattern seen in NTD and in some cases of anterior abdominal wall defects (AAWD) where the covering membrane is thin. A similar pattern will be evident in cases with abnormal or impaired kidney function; thus in congenital (Finnish) nephrosis (25630; McKusick 1988) AF-AFP is grossly elevated (Ryyanen et al. 1983). In this 2-year study of 10,724 women in Kuopio and North Karelia (Finland), 96% of pregnancies in the area were studied and the outcome determined for 10,584. 509 raised AFP samples were reduced to 267 by exclusion of multiple pregnancy, wrong dates and normal second sample. 225 proceeded to amniocentesis and of the 16 foetuses which gave rise to AF-AFP concentrations >10 standard deviations above the median (SDAM), 6 suffered from congenital nephrosis, 6 had serious abnormalities (including 45XO, 47XXY, 47XYY, neural tube defect and exomphalos) and 4 were normal. No missed cases were discovered and the incidence of Finnish congenital nephrosis was assessed as 1:2600. In this condition, large quantities of protein pass through the kidney as a result of microscopic glomerular abnormality. It is notable that the placenta is greatly enlarged in this condition. Infants invariably die before 1 year.

The elevated concentrations of AFP found in muscle and skin (Brock 1983) may be the reason for elevated AF-AFP found in foetus papyraceous (Lange et al. 1979).

Lack of foetal urine will cause oligohydramnios with (possibly) low AF-AFP and raised MS-AFP because of increased transplacental AFP movement. This is the case in Potters Syndrome (Freling et al. 1983) and has been seen in atresia of the urethra in a trisomy 18 foetus (Heller et al. 1980). In a similar foetus but showing marked thinning of the abdominal wall, absence of the urethra associated

with trisomy (Nevin et al. 1983) the AF-AFP was raised and the acetylcholinesterase (AChE) pattern was similar to a NTD. Fifty-eight trisomy 18 foetuses were compared by Lindenbaum et al. (1987) and they confirmed the tendency to low MS-AFP and raised AF-AFP concentrations.

Quantitation of foetomaternal transfusion, which causes raised MS-AFP, during medical procedures has proved useful, especially in amniocentesis and chorionic villus sampling (Mariona et al. 1986).

Clayton-Hopkins et al. (1982) investigated the use of MS-AFP in late (33-40 weeks) pregnancy as an indicator of onset of foetal distress, maternal hypertension and pre-eclampsia. They showed that maternal hypertension was associated with raised AFP concentration and that serial sampling may indicate the severity of foetal distress.

Non-NTD causes of raised MS-AFP include underestimated gestation, multiple pregnancy (Ghosh et al. 1982; Thom et al. 1984), anterior-abdominal-wall defect (Mann et al. 1984), intra-uterine death; threatened, missed or incomplete abortion, hydrocephalus, foetal neoplasm, placental haemangioma (Mann et al. 1983), amniotic band syndrome (Aitken et al. 1984a), foetal skin defects and HP-AFP. Studies have shown raised MS-AFP concentration to be associated with; placental dysfunction and/or infarction and increased size (Boyd & Keeling 1986), and low birth-weight (Brock et al. 1977, Brock et al. 1979, Brock, Barron and Raab 1980). Purdie et al. (1983) screened 7223 pregnancies and reported that 37 out of 141 high MS-AFP (over 2.5 MoM at 16-20 weeks) samples led to a birthweight less than the 10th centile and that there was a highly significant association between raised AFP and abruption, presumably because of foetomaternal tranfusion. Some factors may be excluded by careful dating using ultrasound as used by Purdie, and others by detailed (diagnostic) ultrasonography. A second sample is used, in the West of Scotland, to prevent possible errors of identification and to detect falling AFP which may suggest threatened abortion or transient foetal distress as the cause of the elevated AFP concentration. Perhaps the use of genealogy to exclude HP-AFP is appropriate.

The effect of foetal viraemia on maternal serum AFP has been investigated in parvovirus infection as a measure of aplastic crisis

(Carrington et al. 1987) following a suggestion by Anand et al. (1987).

The association of low concentrations of MS-AFP linked to foetal trisomy, which was reported by Merkatz et al. (1984), stimulated widespread interest in the estimation of MS-AFP and increased the need for improved sensitivity and working range.

iv) Other Conditions outwith Pregnancy

Ataxia telangiectasia (20890; McKusick 1988) has been associated with raised serum AFP (Waldmann & McIntire 1972) and used for diagnosis. In this condition the liver is immature. The position in several immunodeficiency syndromes was stated by Amman et al. (1986) who noticed raised serum AFP in the absence of liver disease in severe combined immunodeficiency (with and without adenosine deaminase deficiency), in combined T-cell and B-cell deficiency and in gastrointestinal disease.

Antenatal liver disease is implicated in the elevated cord serum AFP reported in Type I Tyrosinaemia (27670; McKusick 1988) reported by Hostetter et al. (1983).

1D CLINICAL APPLICATIONS

i) Diagnostic Assay

The local risk of having a second NTD pregnancy was assessed as 1/20, considerably higher than the population risk of 5.7/1000 (local figures for 1974). For patients at risk, amniocentesis and diagnostic AFP (in amniotic fluid using immunoelectrophoresis) assay have been available in the West of Scotland since 1973 and the first report of successful prenatal diagnosis using AF-AFP was given by Allan et al. in the same year.

Following Smith CJ et al. (1979) and Brock (1979), the use of concanavalin-A binding pattern was suggested as a means of eliminating uncertainties in the diagnosis of NTD. Unfortunately, the assay is relatively difficult to perform and has consequently not been widely used.

Smith AD et al. (1979) suggested the use of amniotic fluid acetylcholinesterase as an adjunct to the AFP diagnostic assay. In

order to diagnose foetal NTD, the use of a cut-off at 5 standard deviations above the normal median level of AF-AFP had been successful, but the advent of specific-inhibitable acetylcholinesterase (AChE) electrophoresis banding pattern promised reduced false positive and false negative results. The status of amniotic fluid tests at this time was reviewed by Brock (1983). Aitken et al. (1984b) reported their experience in estimating AFP and AChE in 3785 amniotic fluids. Of these, 541 were taken from frozen storage and quantitative AChE assay was unsuccessful in discriminating between normals and open neural tube defect. Qualitative polyacrylamide gel electrophoresis identified 251 out of 255 NTDs and 29 out of 31 false positive AFP results. These results led to the prospective analysis of 3244 fluids and the identification of all 170 open NTDs and 20 other foetal abnormalities. The false positive rate of 0.4% is better than the 1.8% of the AF-AFP test. The appearance of atypical low intensity second bands and second bands in fluids contaminated by maternal blood constituted 11 of the 13 false positive AChEs. They concluded that the use of AChE would improve the sensitivity and specificity compared to the use of AF-AFP alone. In a recent paper, Brock & Barron (1988) suggest the use of a monoclonal antibody, specific for the fast-moving AChE band, as the basis for a quantitative assay to replace the qualitative gel electrophoresis technique and thereby reduce operator dependence. Both methods are affected by contamination with old blood.

ii) Prenatal screening for neural tube defect

Prenatal screening for neural tube defects was first carried out in areas of high incidence (e.g. West of Scotland; Ferguson-Smith et al. 1978, North East of Scotland; Thom et al. 1984) and has since been successfully applied to low incidence areas (Ghosh et al. 1986) by using different intervention criteria. The intention of screening is to identify a group of mothers at increased risk of carrying a foetus affected by one of several abnormalities. The proportion of pregnancies followed up is based on data collected by each screening centre using a combination of practicability, assay precision, and incidence. The intervention level is usually between 2 and 3 multiples of the median AFP level (MoM) and 1 to 5 percent of pregnancies will require further action. MS-AFP screening is at

present performed at 16-20 (inclusive) completed weeks of gestation, after experience of missed anencephalic pregnancies at 15 weeks. Sufficient time must be allowed for repeat serum assay and diagnostic amniotic fluid assay in order to give the option of termination well before 24 weeks.

Figure 2 shows the distribution of the serum AFP concentrations, in multiples of the median (MoM), for maternal serum from "normal" pregnancies (including closed lesions) and those affected by open spina bifida and anencephaly (UK Collaborative Study 1982).

1E DIFFICULTIES WITH CURRENT RIA

The normal (highest would be 60,000 kU/L at 16 weeks) and abnormal levels of amniotic fluid AFP and the extreme elevation of serum AFP (over 1,000 kU/L and often over 100,000kU/L) seen in neonates and certain neoplasms were detectable by quantitative rocket immunoelectrophoresis. This technique is still widely used for the diagnostic amniotic fluid assay where the lowest detectable level of 2,000kU/L and minimum reportable level of 5,000kU/L is not a disadvantage.

The normal adult concentration of serum AFP was not amenable to assessment until the development of sensitive radioimmunoassays (Ruoslahti and Seppala 1971, Chayvialle and Ganguli 1973). The assay techniques used for tumour marker studies were unsuited to maternal serum screening because they were slow, technically demanding and unnecessarily sensitive. Vince et al. (1975) developed a single-stage RIA, using polyethylene glycol separation (a review of separation methods is given in Ratcliffe 1983), to allow a high-throughput assay. This was optimized to give best precision at 60-100 kU/L with a minimum detectable concentration of 25 kU/L. This is an unsatisfactory sensitivity for tumour marker assay and as a result a separate, specially sensitized assay was maintained for this purpose. The insensitivity and poor precision precluded the investigation of the significance of small changes of AFP concentration.

The RIA being used at the start of this project showed typical assay parameters, based on precision profile data, to be; sensitivity

15kU/L, inter-assay CV 10%, and working range 30-400kU/L. Although many improvements had been applied, the limitations of the basic technology were apparent in internal quality control data and these were supported by performance in the UK External Quality Assessment Scheme (EQAS: Seth et al. 1988).

Evident were: restricted working range, poor precision, lack of robustness (seen in operator dependency), number of repeat estimations necessary and periodic episodes of high or low internal quality control (which were usually confirmed by EQAS), and very variable weekly follow-up rate. This was nominally 3%, but internal quality control data showed extreme results for a week of 0.6% and 6.6% despite the large sample number (approximately 600). The apparent AFP value was markedly, and variably, raised when assaying plasma samples. Incubation time of 16 hours was necessary, limiting the turnround and through-put of samples and this was most important in preventing replacement of the immunoelectrophoresis for AF-AFP. Performance data; a dose-response curve and a precision profile (Ekins 1983) for the RIA method as applied to AFP assay are shown in Figure 3.

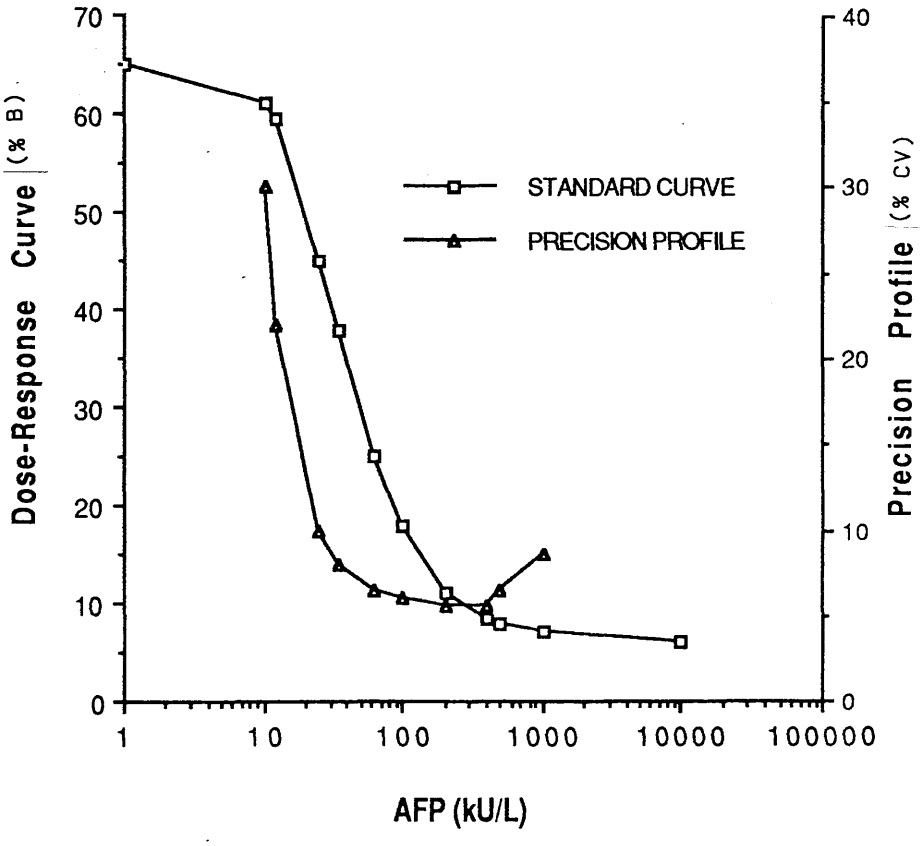


FIGURE 3 Dose-response curve and precision profile for the RadioImmunoAssay used in the West of Scotland MS-AFP Screening Programme until June 1985.

1F AIMS OF THE PRESENT STUDY

In view of the limitations of the RIA outlined above, the purpose of this project was three-fold:

A) to produce a two-site IRMA method for the estimation of AFP in biological fluids, using monoclonal antibody technology, and which would be available to all National Health Service laboratories,

B) to validate the new technique scientifically and in clinical practice, and

C) to introduce it into the West of Scotland maternal serum alpha-1-foetoprotein screening programme.

CHAPTER 2 MATERIALS AND METHODS

2A PREPARATION OF MONOCLONAL ANTIBODIES

The insulin radioimmunoassay described by Yalow & Berson (1960) was the first *in vitro* use of the extreme sensitivity available in biological systems to quantitate analytes which are present at very low concentration in the blood. Despite the sensitivity obtained it was clear that the production of antibodies in whole animals led to the generation of antisera containing components with a wide range of specificities and avidities. These reagents were very poorly characterized and were dependent on the batch of antigen, its purity and the immune capacity of the animals used.

Kohler & Milstein (1975) reported the consequences of fusion of pluripotent myeloma cells obtained from mice with fully differentiated cells obtained from mouse spleen. They generated immortalized cell lines derived from a single cell which continued to secrete a single antibody species of the desired specificity and in large quantities. This allowed the suggestions of Miles & Hales (1968) to be realized in the development of two-site immunoassays. Until the advent of monoclonal antibodies careful purification of the polyclonal antibodies was required for these techniques and the quantities of antibody required made widespread use impracticable.

i) Immunization of mice

A sample (250 uL) of amniotic fluid from a pregnancy affected by an anencephalic foetus and which contained a high concentration (200mg/L) of AFP was diluted 1+3 in Freund's Complete Adjuvant (Difco, East Molesey, UK). The mixture was emulsified by ultrasound at 4 ^{o}C. The emulsion was taken up into a 1mL syringe and approximately one-third (10ug) injected (26 gauge, 1/2 inch needle) while still cold into the peritoneum of each of three anaesthetised, mature, female BALB/c mice (University of Glasgow stock).

Three secondary immunization injections, into each animal, were performed in each animal at two week intervals by the same method but using Freund's Incomplete Adjuvant (ibid) which lacks killed mycobacteria. At each of these stages the tail vein was punctured and a drop of blood absorbed into filter paper for assessment of anti-AFP titre (see section 2A v)). The absorbed blood was tested

for the presence of anti-human chorionic gonadotrophin and anti-prolactin activity. On confirming high titres of anti-AFP activity in the immunized mice, another six were treated similarly but using a different, high-AFP concentration, amniotic fluid.

ii) Preparation of myeloma cells

Myeloma cell culture medium was prepared from RPMI-1640 (containing 20mM HEPES) to which was added 10% foetal bovine serum, 2mM L-glutamine, 100,000U/L penicillin, 100mg/L streptomycin and 2.5mg/L Amphotericin-B (all purchased from Flow Labs, Irvine, U.K.).

All stages of <u>in vitro</u> cell culture used macrophages obtained by washing out the peritonea of freshly killed BALB/C mice with RPMI-1640 and diluting the product to 30,000 cells per mL in myeloma medium with added 20% foetal bovine serum.

Living cells from mouse myeloma cell line X63 Ag8.653 were purchased from Flow Labs. These were multiplied until sufficient stocks were available for freezing down at 5 million cells per 0.5mL aliquot. The method used for storing cells in liquid nitrogen (vapour phase) was to transfer 5 million cells to a sterile universal container and then to centrifuge the tube at 1500g for 7 minutes, followed by resuspension of the cells in 0.5mL of ice-cold freezing mixture. This was prepared by dropwise addition of 10mL warm DMSO (dimethyl sulphoxide; Sigma, Poole, UK) to 100mL ice-cold, foetal, bovine serum.

To establish a colony of healthy, multiplying cells suitable for hybridization, one pellet was thawed at 37^{o}C and transferred aseptically to a sterile plastic container. The cells were washed by twice resuspending in 5mL myeloma medium at 37^{o}C, added slowly with gentle agitation, followed by centrifugation at 1500g for 7 min and aspiration of the supernatant. After further resuspension the concentration of cells was assessed in a haemocytometer and adjusted to 200,000 per mL by addition of further medium. The suspension was transferred to a 25cm^{2} cell culture flask (J. Bibby, Stone, Staffs., UK) in which the concentration of cells and their condition, assessed by viability counting using Trypan Blue, was monitored. The plates were sealed and incubated at 37^{o}C without carbon dioxide. Medium was added, and the incubate expanded into 75cm^{2} flasks when necessary in order to maintain cell density between 100,000 and 800,000 per mL.

iii) Hybrid generation

Three days prior to fusion, 20uL (containing 4ug of AFP) of amniotic fluid diluted with 80uL of sterile saline was injected into the tail vein of the selected mouse (Fazekas de St Groth & Scheidegger 1980).

On the fusion day, after sacrifice, all available blood was removed by cardiac stab and the spleen was dissected and transferred to 5mL RPMI-1640. Serum was stored for control purposes. The spleen was teased out thoroughly before transfer (discarding detritus) into a centrifuge tube. Cells were centrifuged at 1500g for 7 min, the supernatant was removed and 20mL RPMI-1640 was added. The cells were resuspended and counted in dilute solution (to lyse erythrocytes) before further washing and resuspension in 15 mL of RPMI-1640. This was divided equally into three universal containers.

A ratio of 1:1 for myeloma to spleen cells was sought. Sufficient myeloma cells (typically 50 million per fusion) which had been maintained in log-phase growth for at least seven days, were concentrated and divided into three universal containers. One aliquot of myeloma cells was mixed thoroughly with one aliquot of spleen cells, centrifuged (1500g, 7 min) and the supernatant removed completely. With continual swirling in a 37 ^{o}C water bath, 0.8mL 50% (10g plus 10ml, passed through 0.22um filter to ensure sterility) polyethylene glycol 1500 (molecular weight range 1430-1570) in RPMI-1640 was added (more slowly at first) dropwise over 60 sec. After 90 sec reaction time, the mix was rehydrated with 10 mL RPMI-1640 which was added, more slowly at first, over 5min. Swirling was then stopped and the fusion products washed and diluted to 50mL. A 100uL aliquot was introduced into each well of five 96-well tissue culture plates (Flow Labs., Irvine, UK) which had been already prepared by the addition of 100uL of macrophage preparation (3,000 cells).

iv) Chemical selection of hybrids

Mouse spleen lymphocytes do not multiply in the culture conditions used. The myeloma cell line grows rapidly and indefinitely in these conditions but it lacks the enzyme hypoxanthine guanine ribosyl transferase (HGPRT). The scheme devised by Littlefield (1964) relies on the inability of cells which lack this

enzyme to synthesize purine nucleotides when the main metabolic pathways are blocked by the presence of aminopterin (folic acid antagonist). $HGPRT^+$ cells would be able to use hypoxanthine or guanine via the "salvage" pathway for their nucleotide synthesis. The addition of hypoxanthine (final concentration of 13.611 mg/mL), aminopterin (0.176 mg/mL), and thymidine (3.876mg/mL), supplied combined as HAT medium x50 concentrate (Flow Laboratories, Irvine, UK), to the myeloma medium kills unfused myeloma cells, but hybrids containing the part of the spleen genome (X chromosome) which includes the gene for HGPRT will survive. Although HAT may be omitted from the medium used to feed the hybrids after 7 days, in this study it was included throughout, in order to eradicate unstable hybrids and revertants.

v) RIA for detection of anti-AFP activity

Antibody secreting hybridomas were detected by incubating 0.05mL aliquots of the well supernatant with 0.2mL ^{125}-Iodine labelled AFP (Department of Obstetrics and Gynaecology, Dundee) for 16 hours prior to addition of 0.2mL sheep anti-mouse gamma-globulin (Scottish Antibody Production Unit, Law Hospital, UK) linked (see sections 2B i) to vi)) to Sepharose CL-4B (Pharmacia, Milton Keynes, UK) and agitating for 60 min. Separation of reacted (bound) and unreacted (free) label was effected by three cycles of wash (3mL of 0.9% saline, 0.2% Tween 20 (Sigma, Poole, UK) in distilled water), centrifugation and aspiration.

Mouse anti-AFP activity was detected similarly using mouse serum or 6mm circles of filter paper (see section 2A i)). The serum and filter paper circles were similarly used to assay anti-hCG and anti-prolactin.

In order to select for cloning only high-avidity antibody secreting cell lines all supernatants with high binding were reassayed, at several dilutions, in the presence of 25 uL 100kU/L AFP standard.

vi) Cloning and choice of antibody

The growth of cells on the plates was monitored so that the wells could be tested for the presence of cross reactivity, anti-AFP activity and displacement with AFP, before attaining confluence. It

is important that cloning is only performed on cells in logarithmic-phase growth.

The cells from each supernatant containing high avidity antibody (high binding plus good displacement - section 2A v)) were transferred with fresh macrophages to 24-well plates, and allowed to become confluent. At this point they were cloned by limiting dilution. Cell concentration was assessed and adjusted to 800 cells per mL with medium (Row A). Seven, doubling, serial dilutions of this were made, so that the last dilution (Row H) contained, on average, less than 1 cell per 100uL. Twelve aliquots were taken from each dilution and placed in wells 1 to 12 of the appropriate row in a 96-well microtitre plate prepared with 3000 macrophages/well.

vii) Multiplication of clone

After two cloning stages, the continued production of anti-AFP was confirmed and the assumption made that all cells present were of monoclonal origin. The clone was grown up, using macrophages as feeder cells, via 24-well plates and 25 & 75cm^2 flasks until they were in sufficient quantity (in log-phase growth) to inject mice.

viii) Preparation of ascitic fluid

Each of five mice was injected intraperitoneally with pristane (2,6,10,14 tetramethylpentadecane, Sigma, Poole, UK.), to improve acceptance of the tumour cells, at least 14 and preferably 21 days before they were given a similar injection of $5x10^6$ hybridoma cells. The mice were inspected daily and the peritonea were drained of ascitic fluid as necessary.

2B DEVELOPMENT AND OPTIMIZATION OF AN IMMUNORADIOMETRIC ASSAY

i) Preparation of immunoglobulin fraction from polyclonal antibody

Following the method of Steinbuch and Audran (1969), 20 mL of sheep serum (high anti-AFP titre) were adjusted to pH 5.0 by addition of 0.1M acetic acid (0.572mL glacial acetic acid in 100mL water). 1.76mL of n-octanoic acid was dripped in slowly while stirring. After mixing by rotation for 30min. the tube was centrifuged for 20 min. at 1500g. The supernatant was removed and the pellet washed with 20mL 0.01M sodium bicarbonate, pH 8.0 (4.2g of sodium bicarbonate in 5L water) and recentrifuged. Washings and supernatant were pooled and dialysed for 24 hours against 0.1M sodium bicarbonate. The IgG was stored at -20 ^{o}C after concentration by ultrafiltration to about 50mg/mL.

ii) Preparation of immunoglobulin fraction from monoclonal antibody

The above method was used but using appropriate volumes of ascitic fluid, 2mL for testing, 20mL for larger-scale preparation.

iii) Protein assay

To assess the efficiency of solid-phase coupling, and to quantitate the production of monoclonal antibody in the ascitic fluids, the protein concentration was measured by the modified Lowry method of Schacterle and Pollack (1973).

Aqueous standards were prepared by doubling, serial dilution of a solution containing 50mg bovine serum albumen in 50mL of water. The working range of the assay was 0.063 to 1mg/mL; the protein solution to be analysed was prediluted between 1:50 and 1:200 for IgG fractions and solid phases, and between 1:10 and 1:25 for ascitic fluid.

0.2mL of solution was sampled and 0.2mL of alkaline copper reagent (10% sodium carbonate with 2% sodium hydroxide, 0.1% sodium potassium tartrate and 0.05% copper sulphate) added, the tube mixed and allowed to stand for 10 min. at room temperature. 1mL of working Folin-Ciocalteau reagent, stock solution diluted 1+25 with distilled water, was added and the tubes vortexed before incubation at 55 ^{o}C for 5 min.. After rapid cooling the absorbance was measured at 650 um

and unknowns calculated by interpolation from a plot of the standard points and multiplication by the predilution factor.

iv) Solid phase activation

Following the method of Chapman & Ratcliffe (1982), 500mL of Sepharose CL-4B (Pharmacia, Milton Keynes, UK) was allowed to stand overnight and was made up to twice the settled gel volume with isotonic saline (9g Sodium chloride in 1 litre of water). The solution was well mixed and 200mL transferred to a porosity 3 sintered glass funnel (>500mL capacity) for dehydration with 500mL of successively; distilled water, 30%, 50%, 70% and 100% acetone. Care was taken to prevent the Sepharose drying out, and it was stirred gently to resuspend it before each wash. The solid-phase slurry was transferred to a conical flask (calibrated to 200mL) using a small amount of acetone to prevent loss. Further acetone was added to bring the volume up to 200mL. 4.87g of fresh carbonyldiimidazole was added and the flask sealed with a ground glass stopper. It was stirred for at least 1 hour and then transferred back to the sinter funnel and rehydrated with, successively, 500mL of each of 100%, 70%, 30% acetone and distilled water.

v) Coupling

The activated, rehydrated Sepharose was immediately washed through with 500mL of coupling buffer (0.1M EPPS [N-2-hydroxyethyl -piperazine-N'-3-propane-sulphonic acid], 25.23 g in water) and washed into a 500mL calibrated conical flask. 100mg of prepared immunoglobulin fraction of the antiserum was added and the volume made up to 200mL. This was stirred overnight and washed slowly with 500mL of 0.5M Sodium bicarbonate (4.2g in 1 litre), 0.1M acetic acid (5.72mL in 1 litre water), water and 0.9% Sodium chloride. The reagent was stored at 50% settled gel in saline at 4 ^{o}C.

vi) Production batches of solid-phase

Using a purpose-built 2 litre sinter with an overhead stirrer, the above volumes (sections 2B iv) and v)) were tripled and the dehydration simplified to washing through 1 litre each of; water, 50% and 100% acetone. The 100% acetone was retained for the rehydration

procedure. Activation and coupling were effected in the sinter vessel.

vii) Test batches of Solid-phase monoclonal antibody.

The same protocol was followed as in iii) and iv) but the volumes reduced to one tenth of the above.

viii) Iodination of immunoglobulin fraction from monoclonal antibody

The quantities and constitution of the reagents for iodination (Karonen et al. 1975)were as follows. 10 uL of 0.5M phosphate buffer pH 7.4, 5uL carrier-free ^{125}-Iodine (Amersham, UK), 10uL lactoperoxidase linked to microcrystalline cellulose, and 10 uL hydrogen peroxide (10uL of 100vol hydrogen peroxide solution in 250mL distilled water), each separated by 10uL of air, were drawn up into a narrow polytetrafluoroethylene (PTFE) tube using a 100uL glass syringe. The contents were carefully expelled into 20uL of 0.05M phosphate buffer, pH 7.4, containing approximately 20ug of IgG. The reaction mix was vortexed briefly and incubated at room temperature for thirty minutes, mixing every 10 minutes. The reaction was stopped using 200uL column buffer (0.05M EPPS, pH 8.0). The products were passed through a Sepharose 6B column to remove unreacted iodine and to separate immunoreactive and non-immunoreactive fractions.

A similar method was used for comparison, but substituting 1ug of chloramine T in 10 uL phosphate buffer (pH 7.4) for the lactoperoxidase, omitting the hydrogen peroxide and incubating for thirty seconds. This method was also used for iodination of IgG stored in sodium azide solution.

ix) Assay optimization

The choice of buffer, and concentrations of label, solid phase, sheep serum, Tween-20 and sample were investigated, and the time course of the reaction determined, and the centrifugation and density sedimentation (Wright & Hunter 1983) separation methods compared.

x) Standards and quality controls

One ampoule of human cord serum BS72/227 (Sizaret et al. 1975) was reconstituted in water and stock standard prepared by diluting to 100mL in EPPS buffer pH 8.0 (see IRMA protocol section following).

1mL aliquots were stored at -70 ^{o}C.

Several possible matrices for the preparation of standards were evaluated by addition of known amounts of standard material.

Recovery was similarly assessed in 6 human sera.

xi) IRMA protocol for small batches

Buffer (0.1M EPPS, 0.2% Tween-20, 0.1% sodium azide) was prepared by dissolution of 25.23g of EPPS, 2mL Tween-20 and 1g sodium azide in 750mL of distilled water, adjusting the pH to 8.0 with solid sodium hydroxide and making up to 1000mL. "Mixed solid phase/label" reagent was one part of well-mixed polyclonal sheep-anti-human AFP covalently linked to Sepharose CL-4B (stored as 50% settled gel in isotonic saline) plus three parts buffer; with 4uL (2% final concentration) sheep serum and 5ng ^{125}-Iodinated monoclonal antibody (AF5/A2) per 200uL.

The optimised protocol for small batches (100 tubes) was as follows. Using a semi-automatic pipette (or manually), a 25 uL sample was diluted with 200uL buffer. 400uL "mixed solid phase/label" reagent was added by Hamilton PB600-10 or Boehringer 8000 repeating pipette and the tubes incubated for 2.5hours at room temperature while being shaken at 250rpm. Separation was effected by three passes of 3mL of 0.9% saline, brief centrifugation to 1500g and aspiration by multi-probe vacuum pump. The bound fraction was counted on a NE1600 gamma-counter (Nuclear Enterprises, Edinburgh, UK) and the data generated processed using the single-binding-site option in the World Health Organization "law of mass action" model-based data reduction programme (Edwards & Ekins 1983) or by manual interpretation of a graph of percent bound ($\log_{10}$) versus AFP concentration ($\log_{10}$).

xii) IRMA protocol for large batches

Large batches of up to 400 tubes were performed as follows. The Micromedic APS-2 was used to take up 200uL buffer plus 25uL sample. On completion of all sample dilution, 200ul of "mixed solid-phase/label" reagent was added rapidly to each tube using a Hamilton PB600-10 or Boehringer 8000 repeating pipette. Incubation was for 2.5 hours at room temperature (10-30 ^{o}C) on an orbital shaker (250 rpm). After separation of bound and free radioactivity using

two passes of; addition of 1mL 0.9% saline and 1.0% Tween-20 (2mL Cornwall syringe) followed by addition to the bottom of the tube of 3mL 10% sucrose and 1% Tween-20 (40-tube peristaltic pump) and aspiration of the supernatant (40-tube vacuum pump), the bound fraction was counted on a Rackgamma 1270 (LKB-Pharmacia, Milton Keynes, UK) and the data generated reduced using a 5 parameter log-logit transformation in the Edinburgh RIAPAC Programme (McKenzie & Thompson 1983: Raab 1983).

xiii) Statistical methods

Results from the two assay methods were compared using linear regression analysis and Student's 't'-statistic. Quality control values were assessed using standard deviation and coefficient of variation estimates.

CHAPTER 3 RESULTS

The results are divided into three Sections; A) Preparation of Monoclonal Antibodies, B) Development and Optimization of an Immunoradiometric Assay (IRMA), and C) Routine Use and Experience.

3A PREPARATION OF MONOCLONAL ANTIBODIES

i) Immunization and fusion

Following immunization of the mice as in section 2A i), binding of ^{125}-I AFP by blood samples from three mice at 0 weeks was 4% (equivalent to zero); at 4 weeks two mice showed 47% and one 20% and at 6 weeks all had reached 63%. All subsequent mice reached high, stable anti-AFP titres in 4-6 weeks.

Two mice were used for six fusion experiments. In fusions AA-AC 207 million spleen to 130 million myeloma cells (89% viable, ratio 1.59:1) were used and in fusions AD-AF 105 million spleen to 153 million myeloma (92% viable, 0.686:1).

Over 90% of the 2880 wells plated from all six fusions showed visible colonies at 4 days. A third set of three fusions was abandoned because the myeloma cells were 45% viable, an arbitrary minimum acceptable viability of 85% was applied.

ii) Testing

As shown in section 2A v), the testing scheme used, a) dilutions of serum from one of the high-anti-AFP-titre mice, and b) a commercial monoclonal antibody as positive controls. At 1 in 1000 dilution the monoclonal bound 22.0% of added label, and 8.0% with 500ng/ml AFP standard added (displacement of 64%). Comparable figures for the mouse serum were 1 in 16,000, 21.4% and 3.2% (displacement of 85%). The mouse serum did not bind ^{125}-Iodine labelled prolactin or human chorionic gonadotrophin.

A positive well was arbitrarily defined as >10% binding of added ^{125}I-AFP label. There were 384 positive wells in fusions AA-AC (Table 4), and 752 positive wells in fusions AD-AF (Table 5), a total of 1136. Thus 39.4% of wells contained anti-AFP secreting colonies, many of which would have contained several secreting and/or non-secreting clones. 256 wells (8.9% of wells, 22.5% of positives)

were subjected to displacement testing. Those with binding greater than 40% from fusions AA-AC were assayed at dilutions of 1:3 (>40%), 1:9 (>50%) and 1:27 (>60%). Those with binding greater than 30% from fusions AD-AF were assayed at dilutions of 1:3 (>30%), 1:9 (>40%), 1:27 (>50%) and 1:81 (>60%). The results are displayed in Tables 6 and 7.

The 19 "high binders" from fusions AA-AC were not diluted sufficiently and the cell lines were lost due to cell overgrowth before they could be retested at a higher dilution.

From fusions AA-AC, colonies from the 38 wells with greatest displacement were taken on but seven were lost before storage. Concurrently the best 8 were cloned. Colonies from the 48 wells with greatest displacement from fusions AD-AF were taken on into 2mL wells but 10 did not survive to be frozen down.

PERCENT BOUND	<10	10-20	20-30	30-40	40-50	50-60	>60
PLATE				Fusion A			
AA1	70	9	6	1	2	8	0
AA2	76	7	4	1	2	4	2
AA3	73	13	2	1	3	4	0
AA4	72	5	7	2	4	4	2
AA5	72	8	6	4	3	3	0
				Fusion B			
AB1	73	9	3	4	4	2	1
AB2	70	11	2	3	3	5	2
AB3	64	12	9	4	1	6	0
AB4	67	13	7	3	3	3	0
AB5	65	16	5	4	2	2	2
AB6	62	22	6	2	2	1	1
				Fusion C			
AC1	71	14	7	2	0	2	0
AC2	78	8	4	1	1	2	2
AC3	68	10	3	3	2	9	1
AC4	75	8	5	0	0	3	5
TOTAL	1056	165	76	35	32	58	18
AA-AC PERCENT	73.3	11.5	5.28	2.43	2.22	4.03	1.25
% OF +ves		43.0	19.8	9.11	8.33	15.10	4.69

Table 4 Binding of ^{125}-Iodinated AFP by Supernatant from 96-well plates at 11 days post-fusion

PERCENT BOUND	<10	10-20	20-30	30-40	40-50	50-60	>60
PLATE			Fusion D				
AD1	51	25	5	5	6	2	2
AD2	56	18	15	2	3	1	1
AD3	65	19	6	1	1	3	1
AD4	62	20	7	3	0	4	0
AD5	59	29	4	2	2	0	0
			Fusion E				
AE1	26	38	20	7	2	1	2
AE2	24	35	18	10	7	2	0
AE3	36	36	15	7	0	2	0
AE4	42	32	10	7	1	2	2
AE5	42	34	12	4	3	1	0
			Fusion F				
AF1	42	32	9	7	5	1	0
AF2	43	33	10	5	2	3	0
AF3	42	41	5	5	1	2	0
AF4	48	36	7	2	2	1	0
AF5	50	29	4	5	5	2	1
TOTAL	688	457	147	72	40	27	9
AD-AF PERCENT	47.8	31.7	10.2	5.00	2.78	1.87	0.62
% OF +ves		60.8	19.6	9.57	5.32	3.59	1.20
GRAND TOTAL	1744	622	223	107	72	85	27
AA-AF PERCENT	60.6	21.6	7.74	3.72	2.50	2.95	0.94
% OF +ves		54.8	19.6	9.42	6.34	7.48	2.38

Table 5 Binding of ^{125}-Iodinated AFP by Supernatant from 96-well plates at 7 days post-fusion.

PERCENT DISPLACEMENT						
	<10	10-40	40-60	60-80	>80	HIGH BINDER
PLATE			Fusion A			
AA1	0	5	0	1	0	4
AA2	0	3	2	0	0	3
AA3	1	2	1	1	2	0
AA4	0	8	0	1	1	0
AA5	0	1	2	3	0	0
			Fusion B			
AB1	2	2	2	0	0	1
AB2	0	2	2	1	1	4
AB3	0	1	2	1	1	2
AB4	0	4	0	1	0	1
AB5	3	1	0	0	2	0
AB6	0	3	0	0	0	1
			Fusion C			
AC1	0	1	0	0	0	1
AC2	1	1	1	1	1	0
AC3	1	6	2	2	0	1
AC4	0	3	2	2	0	1
TOTAL	8	43	16	14	8	19

Table 6 Displacement of ^{125}-Iodinated AFP by Supernatant from 96-well plates at 12 days post-fusion

PERCENT DISPLACEMENT					
	<10	10-40	40-60	60-80	>80
PLATE			Fusion D		
AD1	0	0	2	8	4
AD2	0	0	0	2	5
AD3	0	0	0	3	3
AD4	0	0	1	4	2
AD5	0	0	0	2	2
			Fusion E		
AE1	0	0	1	7	4
AE2	0	1	1	10	7
AE3	0	0	2	4	3
AE4	$ 6	0	2	2	3
AE5	$ 4	0	0	1	4
			Fusion F		
AF1	0	0	3	8	2
AF2	0	1	0	5	4
AF3	0	0	2	1	4
AF4	0	0	1	2	2
AF5	0	0	2	6	5
TOTAL	10	2	17	65	54

$ - Assay errors.

Table 7 Displacement of ^{125}-Iodinated AFP by Supernatant from 96-well plates at 8 days post-fusion.

iii) Cloning

From fusions AA-AC, 8 cell lines were cloned from the 96-well plates, of which 5 were no longer producing antibody when tested for displacement. The other three "clones" were positive and wells showing good growth were taken on into 2mL wells and 25 cm^2 flasks, from which they were frozen down. A severe contamination event prevented second cloning. From fusions AD-AF, 6 cell lines were cloned after growing up in 2 mL wells of which 2 were not producing antibody when tested for displacement. Cell lines from the four producing "clones" which were showing good growth, were taken on into 2mL wells and then 25 cm^2 flasks, from which they were frozen down. Two maintained their growth-rate, displacement and secretion parameters and were cloned a second time, by limiting dilution. Both survived the procedure and maintained their characteristics.

iv) Antibody Selection

The two second-clone cell lines (AF5/A2/G11/H10 and AF4/B11/C1/B2 known henceforth as AF5/A2 and AF4/B11) were used to generate ascitic fluid (up to 9 taps were taken from each mouse without distress) and subsequently for solid-phase coupling and ^{125}-Iodination studies.

Protein concentration and volumes of ascitic fluid obtained from the 10 mice are shown in Table 8. The protein concentrations of immunoglobulin fractions of individual taps are shown in Table 9.

The two cell lines had shown similar growth characteristics and by preparing ^{125}-Iodinated (sections 2B ii & viii)) and solid-phase linked (sections 2B ii) iv) v) & vii)) versions of each antibody from the immunoglobulin fractions of the ascitic fluids, their specificity was investigated. Table 10 shows the inhibition of binding of labelled AF5/A2 by solid-phase AF4/B11 and vice versa and with solid-phase SAPU polyclonal. This similar specificity prevented the development of a double-monoclonal assay. Figure 11 shows the higher potency of ascitic fluids taken from AF5/A2 mice and that the potency of the taps increases with the tap number.

	Cell Line							
	AF5/A2				AF4/B11			
Tap Number	Protein mg/mL	IgG mg/mL	Volume mL	Total Protein	Protein mg/mL	IgG mg/mL	Volume mL	Total Protein
1	55	7.1	11	605	35	3.5	6	210
2	48	8.0	9	432	42	4.6	9	378
3	63	7.1	5	315	30	4.8	3	90
4	43	8.4	4	172	35	5.9	5	175
5	89	12.3	7	623	39	6.5	3	117
6	64	25.3	7	448	49	7.8	9	441
7	89	26.0	7	623	40	9.6	3	120
8	56	10.4	4	224				
9	63	12.0	3	189				

Table 8 Protein concentration and volumes of ascites fluid obtained from the 12 mice.

Antibody	Mouse	Tap	IgG mg/mL
AF5/A2	Black	3	7
	Blank	3	15
		4	13
	Green	3	30
		4	30
		5	17
AF4/B11	Blue	4	11
	Red	3	7

Table 9 The protein concentrations of immunoglobulin fractions of selected individual taps.

	Solid-phase		
	AF4/B11	AF5/A2	polyclonal antibody
label AF4/B11	1.6%	1.5%	52.3%
label AF5/A2	1.2%	0.99%	63.0%

Table 10 The binding of monoclonal and polyclonal antibodies in the presence of 25uL 500kU/L AFP standard.

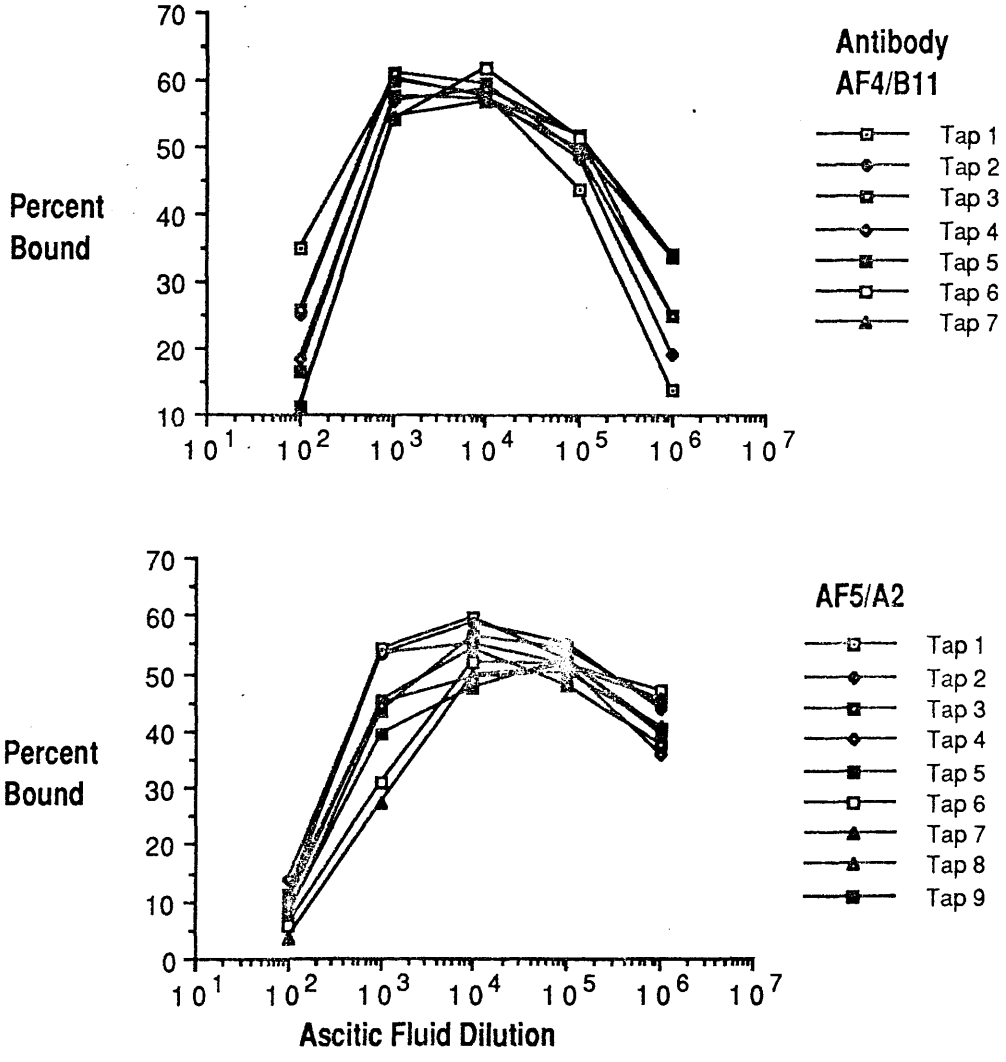


FIGURE 11 Binding of ^{125}-Iodinated alphafoetoprotein by dilutions of ascitic fluids.

v) Characterization of candidate antibodies

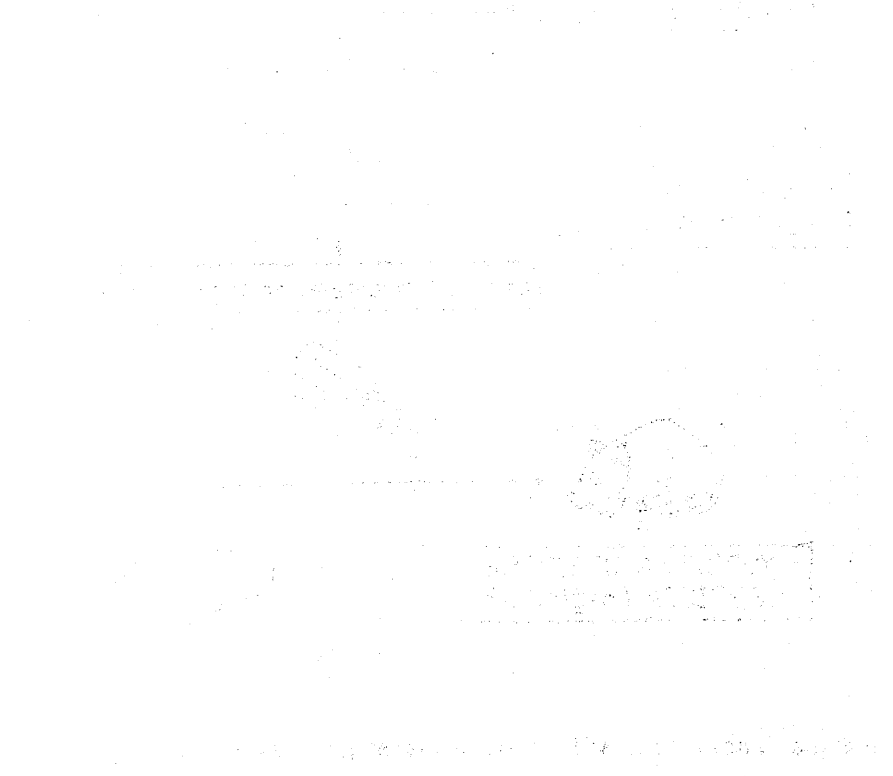
Both monoclonals, AF5/A2 and AF4/B11 were shown by radial immunodiffusion, using a kit supplied by Serotec (Bicester, UK), to form precipitin arcs with antiserum to the IgG1 isotype. No reaction was noted with antisera to immunoglobulins G2a, G2b, G3, A or M.

vi) Determination of avidity

Scatchard analysis of the second-clone product, AF5/A2, using an RIA similar to the testing scheme showed that the association constant was greater than 10^{10} L/mol. Figures 12 and 13 show data for the monoclonal AF5/A2 and for the sheep polyclonal antibodies.

vii) Flow charts

The overall results of immunisation and antibody production are summarised in Figures 14 and 15.



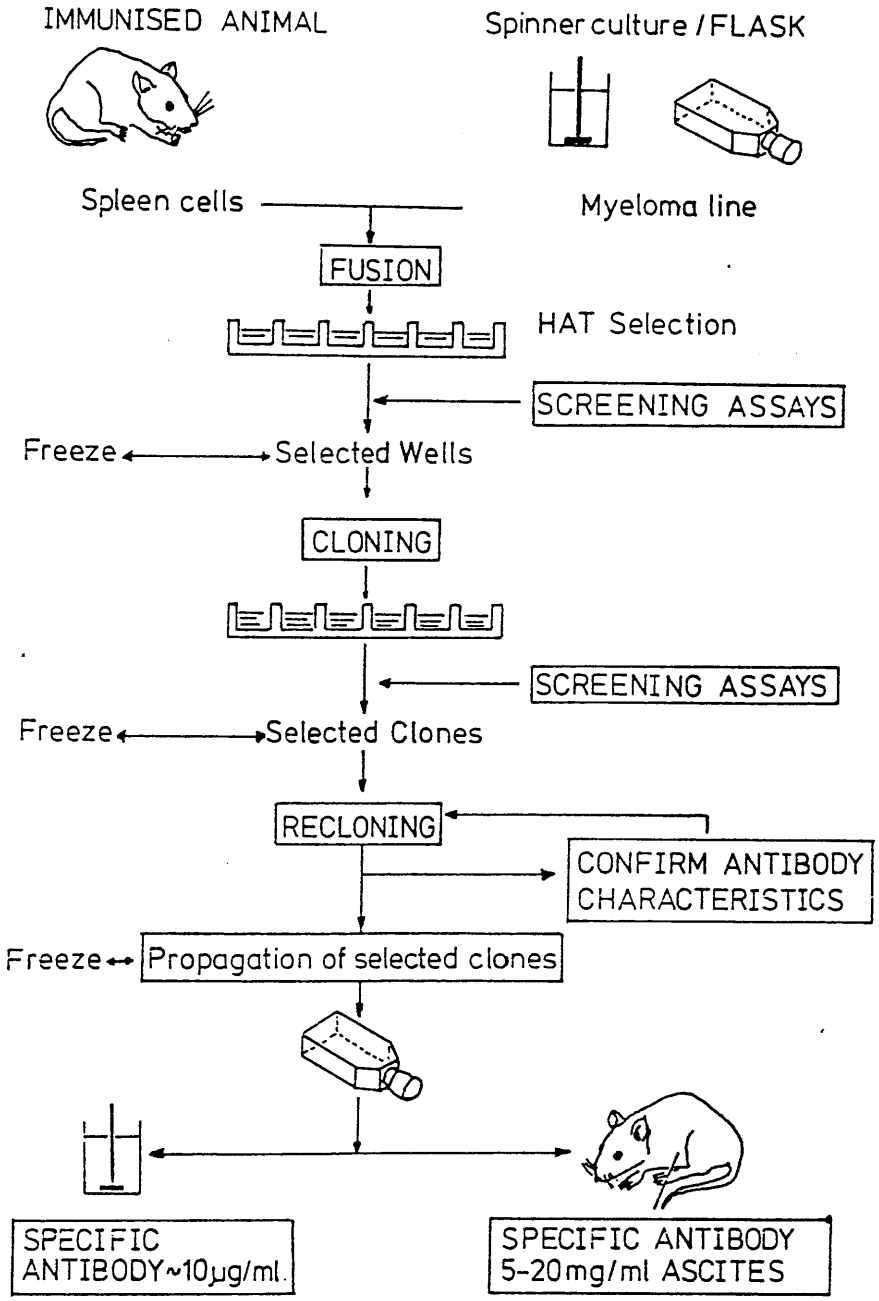


FIGURE 14 Diagrammatic representation of the monoclonal antibody generation system.

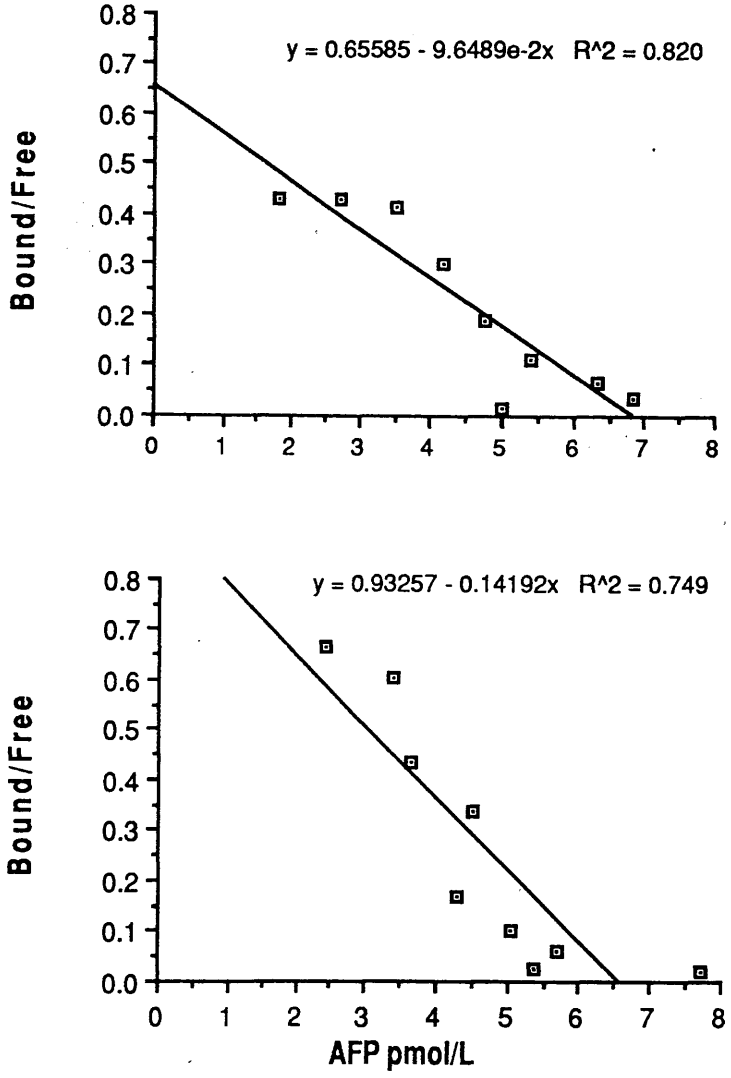


FIGURE 12 Two Scatchard plots for the monoclonal antibody AF5/A2, showing avidities of 9.6×10^{10} and 1.4×10^{11}.

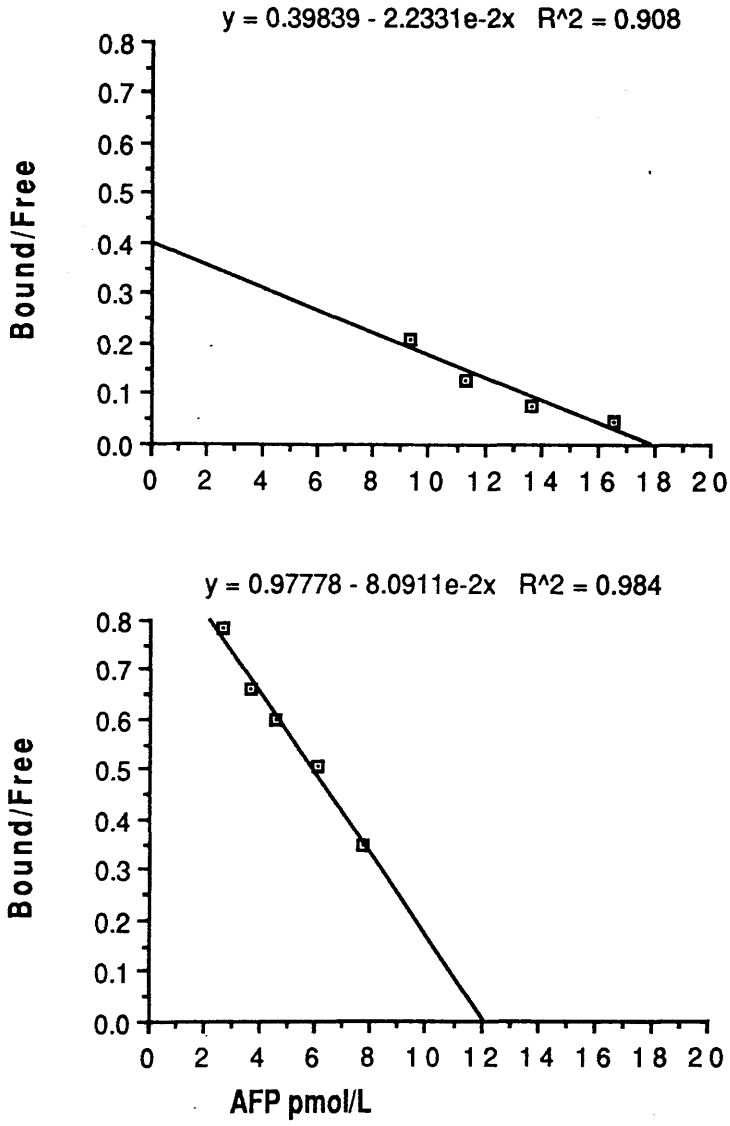


FIGURE 13 Scatchard plots for the low avidity and high avidity groups of the sheep polyclonal antibody, showing avidities of 2.2×10^{10} and 8.1×10^{10}.

FUSION

Total cells available Hybrid formation: 235,000,000

TESTING

Colonies (approx.)		14400
	in	2880 wells
Producers (>10%)		1136

TISSUE CULTURE

Stored	67
Cloned	16
Ascites,	2

VALIDATION OF ASSAY

^{125}I-Iodination	2
Solid-phase	2

ROUTINE USE 1

Figure 15 Flow Diagram of Monoclonal Antibody Production

viii) Observations

Two episodes of infection during the tissue culture stages did not prevent the generation of a large number of hybrid cell lines. From these, two were obtained which expressed avid anti-AFP, grew rapidly in the incubation conditions and maintained apparently stable constitutions and antibody secretion characteristics. It was clear from the binding characteristics that they would be suitable even for a radioimmunoassay method, and that they would be good candidates for their inclusion in a sensitive immunoradiometricassay.

3B DEVELOPMENT AND OPTIMIZATION OF AN IMMUNORADIOMETRIC ASSAY

i) Calibration curve

As shown in section 3A iv), the monoclonal antibodies have the same specificity but either is compatible with the sheep anti-human-AFP polyclonal antibody supplied by SAPU. Figure 16 shows typical dose-response curves for AF5/A2 monoclonal/SAPU polyclonal, SAPU monoclonal/SAPU polyclonal and commercial double-monoclonal assays under optimized conditions.

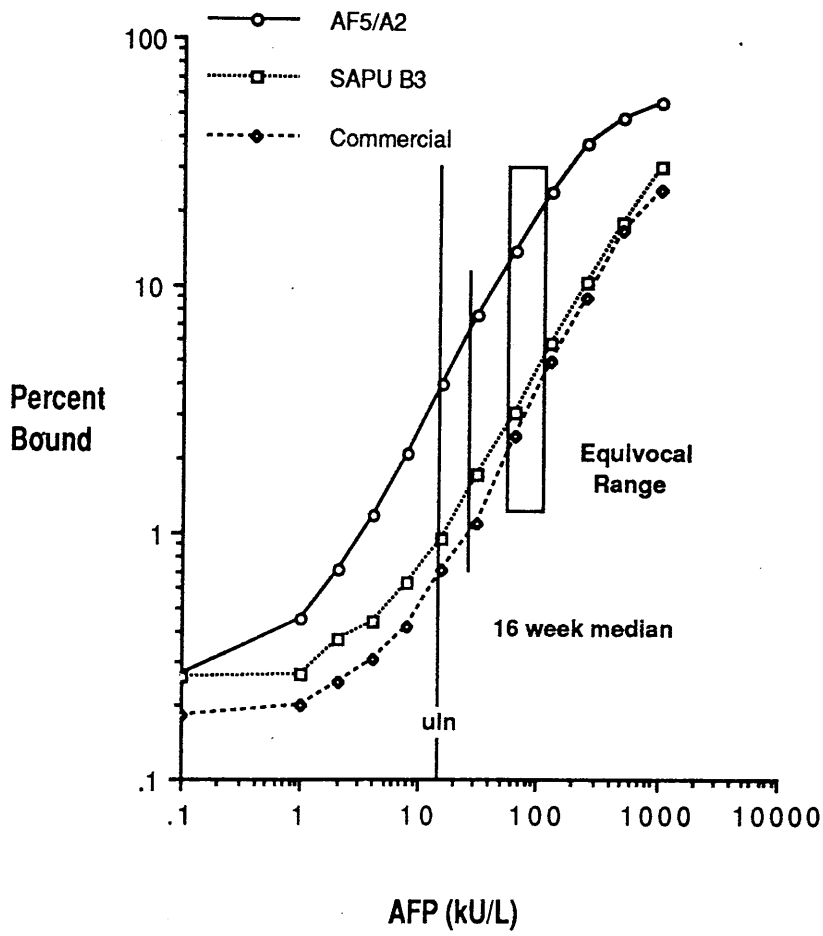


FIGURE 16 Dose-response curves for three monoclonal antibody based AFP-IRMAs.

uln = upper limit of "normal" males.

ii) Differences between candidate antibodies

The two antibodies chosen showed little evidence of difference in binding properties or in the growth and stability of the parent cell lines. AF5/A2 was selected because it gave slightly higher binding on iodination.

iii) Specificity

Using the present combination of monoclonal and polyclonal antibodies, there has been no evidence of interference from other molecular species. The lack of cross-reaction to prolactin and hCG was demonstrated at the antibody production phase (sections 2A i) & v)). Of the many high-titre AFP samples tested none has shown the non-linearity on dilution which would suggest that the antigen was not AFP.

iv) Solid phase

Little difference in the uptake of the immunoglobulin fractions was noted. Typical loading of the solid-phase with different antibodies were: AF4/B11 - 1.2mg/mL using 10mg of IgG per mL of added, settled gel, AF5/A2 - 1.3mg/mL (21mg/mL added), polyclonal 1.2mg/mL (6.8mg/mL added). A modest excess was used for routine preparation, 240mg per 200mL settled gel.

v) Differences between batches and stability

Personal observations on the small batches (3mL to 200mL settled gel) prepared during the development of the technique showed little variation in the shape of standard curve and plateau length. Quality Assessment parameters were set as (using saline separation and assuming freshly ^{125}-Iodinated AF5/A2) binding at 0kU/L $<0.4\%$, at 61kU/L 14% and plateau to 25,000kU/L. The larger routine batches showed greater variation, especially in plateau length. It was also quickly noticed that the standard curve gradually shifted to the right during the 3-4 months that each batch was in use. This problem was overcome by washing the solid-phase before use, or periodically.

vi) Iodination

Figure 17 shows a typical iodination profile for monoclonal antibody AF5/A2. The lactoperoxidase iodination performance was

consistent using the partially purified IgG aliquots. Each iodination is sufficient for about 10,000 tubes. The labelled protein supplied by SAPU using a large scale, chloramine-T iodination technique gave indistinguishable results.

Calculation of iodination parameters for the iodination shown in the figure.

P = Total Protein peak 1,309,700 counts
U = Immunoreactive Protein peak 1,076,800 counts
I = Unreacted Iodine peak 112,200 counts

Incorporation	= P/(P+I)	= 92%
Activity	= 1500*U/(P+I)	= 1136uCi
Useable Protein	= 57.6*(U/P)	= 47.4ug
Specific Activity	= 1136/47.4	= 23.9uCi/ug
Concentration	= (47.4/10)ug/mL	= 4740ng/ml

Table 18 and Figure 19 demonstrate the immunoreactivity of the individual fractions collected from a similar iodination of monoclonal antibody AF4/B11. Label preparations with specific activities greater than 25uCi/ug generated low-binding standard curves.

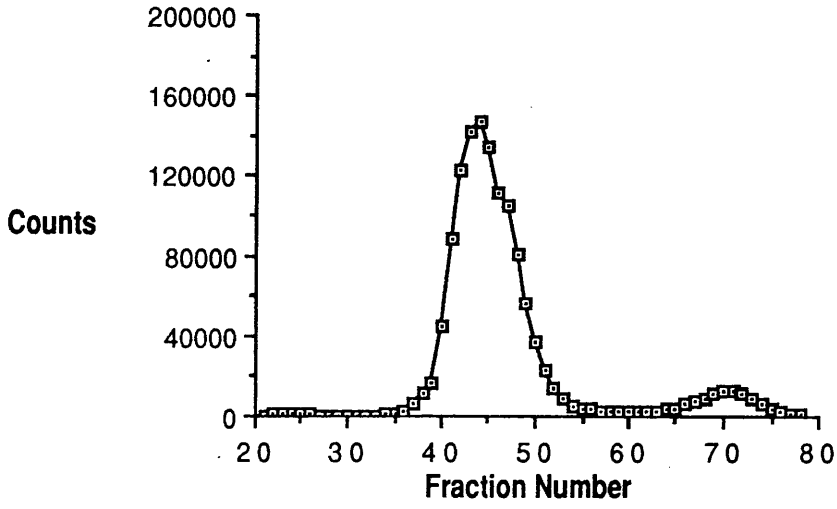


FIGURE 17 A typical iodination profile for monoclonal antibody AF4/B11.

Fraction number	33	34	35	36	37	38
Counts ($x10^3$)	97	175	215	210	165	99
% Bound						
1000kU/L	48.2	46.2	45.2	44.7	37.6	32.5
333	40.5	39.4	36.6	36.5	30.2	26.6
111	26.9	25.4	23.7	23.4	19.5	16.5
34	9.15	9.17	8.40	8.44	7.04	6.10
11	5.39	5.28	5.15	4.92	4.22	3.78
3.4	2.71	2.72	2.63	2.60	2.25	2.12
1.1	1.70	1.70	1.77	1.81	1.75	1.60
.34	1.36	1.45	1.44	1.54	1.36	1.34
0	1.12	1.25	1.32	1.34	1.29	1.28

Table 18 Immunoreactivity of individual column aliquots from a typical iodination (AF4/B11).

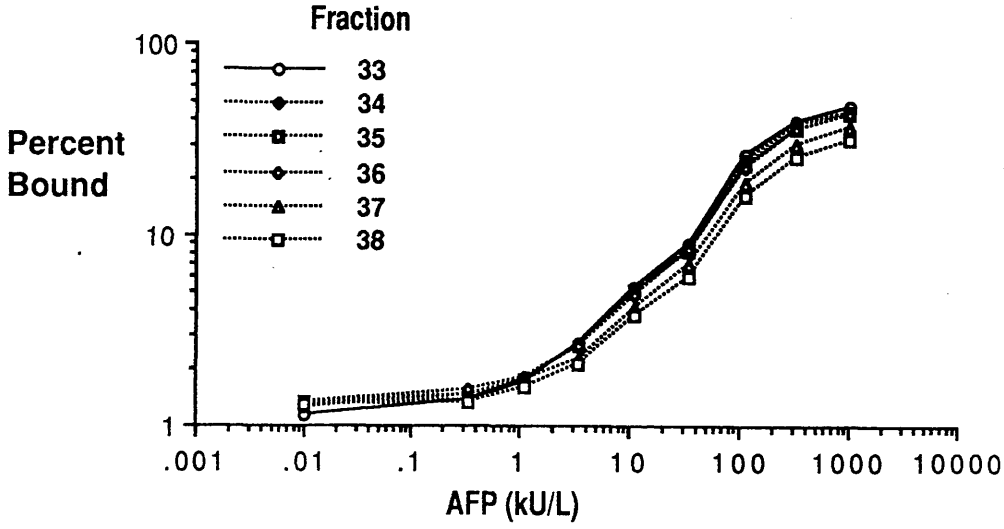


FIGURE 19 Immunoreactivity of individual fractions of a typical iodination of AF4/B11

vii) Differences between batches and stability

Personal observations on the batches prepared for the development of the technique showed little variation in specific activity using the solid-phase lactoperoxidase method, and dose response curves, for fresh label, were generally superimposable. The label was stable for at least 7 weeks losing about 20% of the original binding, but the counting time was by then too great for routine use.

The scaled-up routine label preparations (chloramine T iodination) show greater variation, especially in the loss of binding during the 4-week working life of the label. Nonetheless, the extensive quality control data rarely show any effect on the reported values.

Figure 20 shows the dose-response curves obtained using one tracer preparation at 10 and 40 days. Figure 21 compares the dose-response curves for two monoclonal antibodies using the same preparation of solid-phase reagent.

vii) Optimization

The working buffer was 0.25M EPPS with 2% sheep serum, 0.1% Tween-20 and 0.1% Sodium azide, unless stated otherwise, prepared by dissolving 25.23g EPPS in 750mL distilled water, adjusting the pH to 8.0 and adding 1mL Tween-20 before making up to 1 litre. Sheep serum was added at time of preparation of the label reagent. During optimization all results were obtained using the middle value of triplicates.

Sheep Serum Concentration

The effect on binding of addition of increasing quantities of sheep serum in the absence of Tween-20 is shown in Table 22. Maximum bindings were unaffected by standards matrix; minimum bindings were reduced with increasing sheep serum concentration and the use of serum standards. Although binding was lost at high levels of AFP in the density sedimentation separation system, this was more than compensated for by the reduction at low levels, with concomitant improvement in sensitivity.

Tween-20

The requirement for detergent in the assay was investigated for both separation methods. Table 23 shows a slight increase in maximum bindings with increasing Tween concentration. Diluent (assay buffer) standards gave higher binding.

Wash Protocol - Saline/Centrifugation

Two extensive washing experiments were performed to determine the number of washes required using 0.9% Saline with 0.2% Tween-20. Table 24 and Figure 25 demonstrate that although there was considerable advantage from 4, 5 or 6 washes, three gives the best compromise between speed and efficiency. This separation method was chosen for small batches because of the greater speed. Time taken for the separation stage depends on batch size.

Wash Protocol - Density Sedimentation

Figure 26 shows the results of one extensive experiment using 1mL wash (1% Tween-20 in water) and 3mL 10% sucrose in water (added to the bottom of the tube). Two passes showed good reproducibility

and this system was chosen for large routine assays; with increasing batch size the time disadvantage became less, and less operator intervention is required. Time taken for the separation stage is independent of batch size.

Time and Separation Method

For the two-stage assay 20uL sample and 200uL label were mixed thoroughly and subjected to various incubation times on an orbital shaker. Then 200uL of solid-phase was added and the tubes further incubated for 1 hour on the shaker. Separation was by either; three passes of 0.9% saline containing 0.2% Tween-20, brief centrifugation and vacuum pump aspiration of the supernatant; or two passes of 1mL 1% Tween-20 in water and 3mL 10% sucrose, 1% Tween-20 in water (the latter added to the bottom of each tube using a suitable probe) and vacuum pump aspiration of the supernatant.

The superiority of density sedimentation separation is marked. Sixty minute, separate-addition assay gave satisfactory binding for good working range, precision and sensitivity as shown in Tables 27 and 28.

Sample Volume

The effect of sample volume on the dose-response curve was investigated by 1 and 2 hour incubation of mixed reagent with 25uL or 50uL of 0, 100 and 5000kU/L AFP. The results are shown in Table 29.

Mixed Reagent Addition

Incubation of sample with label for 60 minutes, followed by a second 60 minute incubation with solid-phase (on an orbital shaker) was compared with a single-stage 60 minute incubation. Both separation methods were used and the points investigated were 5000, 100 and 0kU/L AFP. Table 30 shows that the reaction rate at 60 minutes incubation in the single-stage assay was still high, which suggested the probability of drift in a large assay. The preferred solution would be to have timed addition of the solid-phase/label and wash reagents. This was impracticable with the equipment available and the other options were, either to use the two-stage assay or to increase the incubation time. The assessment of a 2.5hour assay with mixed reagent addition showed that reagent could be added over as

long as 20 minutes without significant drift (Table 31). In practice, only 5 minutes is necessary for addition of mixed reagent to 400 tubes which is performed after completion of the sampling and dilution of specimens.

The Table shows that binding at 125kU/L AFP for 2.5 hour incubation with mixed reagent addition is equivalent to the 90 minute separate addition assay.

Standards Matrix

Buffer standards gave very variable recovery (data not shown).

All candidate sera, and serum derivatives, tested gave recoveries between 96 and 109% and the results are summarized in Table 32. Using bovine serum standards, recovery from four male human sera with 25, 50 and 100 kU/L of AFP standard added averaged 102%, 105% and 108%.

Sensitivity of the optimized assay to concentration of solid-phase and tracer

Figure 33 shows the effect of adding three times, and one third as much tracer and (separately) solid-phase. Complete standard curves were assayed and to investigate concentrations over 500kU/L, dilutions of a cord serum, approximately 50,000kU/L, were assayed. The quality control results were not affected by changes in concentration of either reagent. Neither incorrect solid-phase, nor incorrect tracer concentration has marked effect on the binding of the ascending curve. The descending curve is greatly affected by solid-phase concentration as expected.

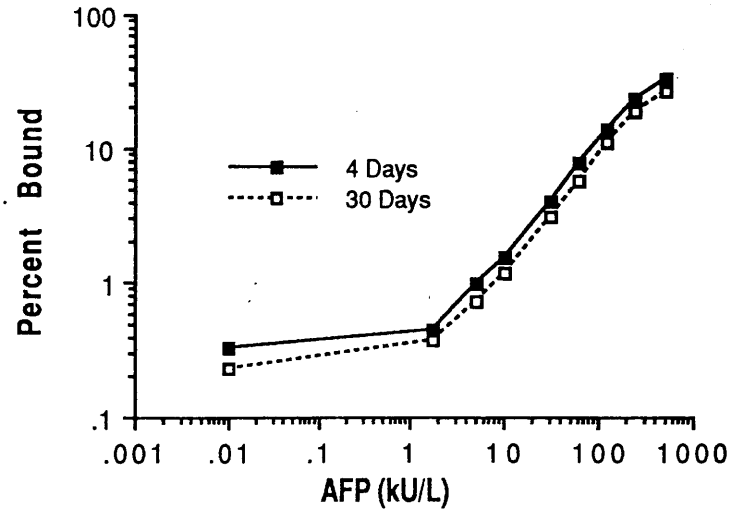


FIGURE 20 The effect of time on the binding of a lactoperoxidase iodination of AF5/A2.

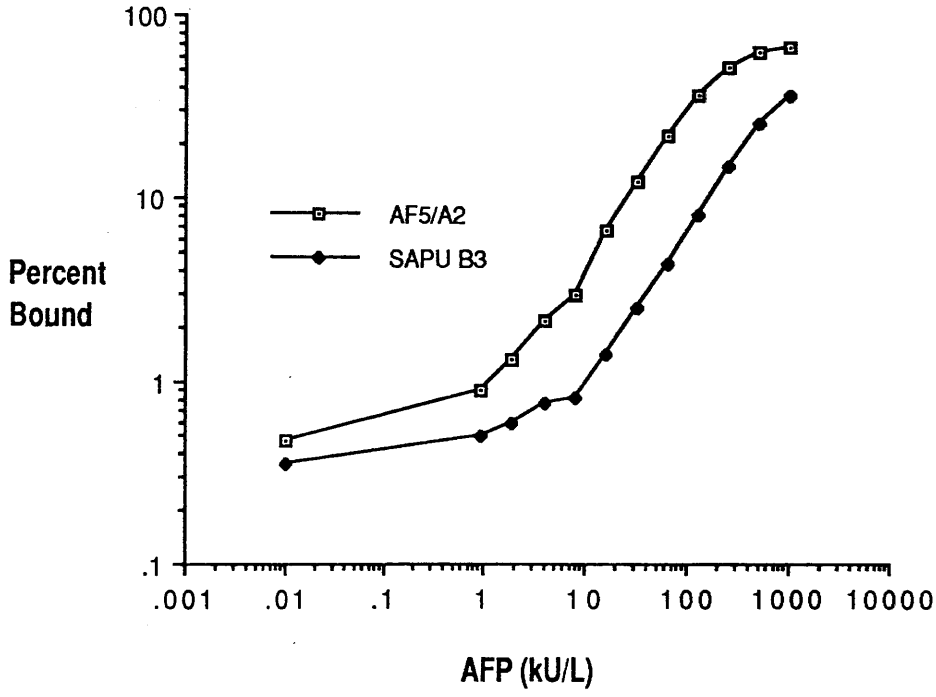


FIGURE 21 Dose-response curves for two optimized IRMAs using the same preparation of solid-phase reagent.

	Concentration of sheep serum % (v/v).				
	0.125	0.25	0.5	1.0	2.0
Diluent Standards					
3 saline washes					
0kU/L	2.0	1.3	0.87	0.73	0.72
100kU/L	32.5	32.3	32.9	33.8	32.2
2 density sedimentation washes					
0kU/L	0.47	0.37	0.21	0.17	0.15
100kU/L	24.4	24.4	24.4	23.9	24.4
Serum Standards					
3 saline washes					
0kU/L	0.55	0.66	0.39	0.34	0.44
100kU/L	29.5	28.0	30.4	30.7	29.0
2 density sedimentation washes					
0kU/L	0.13	0.13	0.085	0.075	0.076
100kU/L	22.3	20.3	22.2	22.3	22.2

Table 22 Binding characteristics of labelled monoclonal antibody in the presence of sheep serum, using two wash methods. No detergent added. Percentage of added tracer bound at two concentrations of AFP.

	Concentration of Tween-20 % (v/v)				
	0.1	0.2	0.4	0.8	1.6
Diluent Standards					
3 saline washes					
0kU/L	0.53	0.46	0.53	0.36	0.55
100kU/L	33.8	33.2	34.4	35.7	36.4
2 density sedimentation washes					
0kU/L	0.11	0.12	0.10	0.11	0.13
100kU/L	25.9	24.8	25.8	26.8	28.0
Serum Standards					
3 centrifugation washes					
0kU/L	0.37	0.34	0.37	0.25	0.34
100kU/L	30.6	30.9	31.8	33.1	33.4
2 density sedimentation washes					
0kU/L	0.073	0.060	0.073	0.067	0.060
100kU/L	22.3	20.3	22.2	22.3	22.2

Table 23 Binding characteristics of labelled monoclonal antibody in the presence of detergent, using two wash methods. No serum added. Percentage of added tracer bound at two concentrations of AFP.

	NUMBER OF WASHES							
Binding (%)	1	2	3	4	5	6	9	12
at 1000kU/L	67.7	62.1	61.0	59.9	58.3	56.6	53.7	52.7
125kU/L	32.4	24.2	22.3	21.9	21.0	20.1	17.7	17.1
15kU/L	11.5	5.1	4.3	4.0	3.8	3.7	2.8	2.7
1kU/L	12.0	2.0	1.0	0.85	0.84	0.81	0.67	0.71
0kU/L	12.2	2.1	0.94	0.68	0.65	0.67	0.57	0.60
Ratio 1000/0	5.5	29.6	64.9	88.1	89.7	84.5	94.2	87.8

Table 24 The effect of number of passes on efficiency of centrifugation separation method.

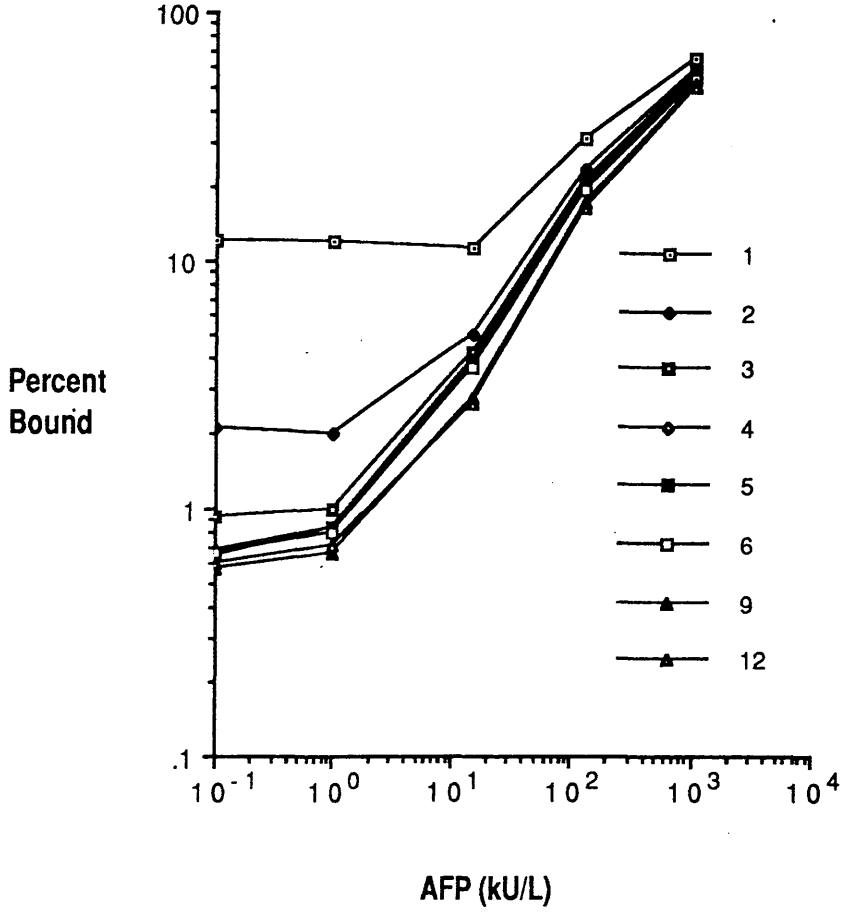


FIGURE 25 The effect of number of passes of centrifugation washes on the dose-response curve.

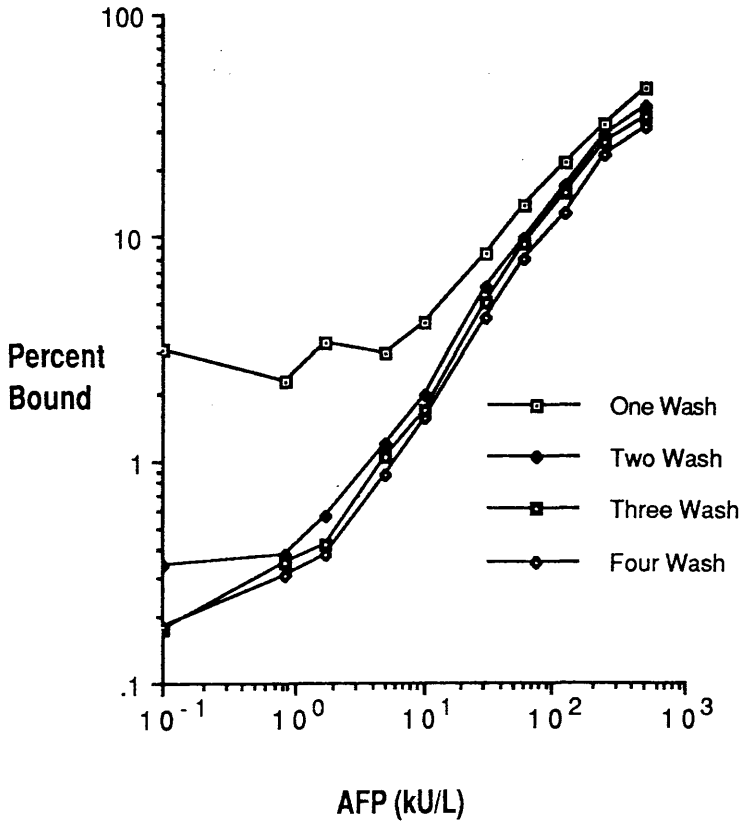


FIGURE 26 The effect of number of passes of density sedimentation washes on the dose-response curve.

		INCUBATION TIME (minutes)				
Binding (%)		20	40	60	90	120
at	5000kU/L	52.1	52.5	53.8	52.7	53.4
	100kU/L	16.6	21.3	25.0	28.2	31.3
	0kU/L	0.31	0.28	0.28	0.21	0.19
	Ratio 5000/0	168	188	192	251	281

Table 27 Two-stage assay: the rate of reaction followed by centrifugation separation (three passes). (Background Subtracted)

	Time (minutes)				
Binding (%)	20	40	60	90	120
5000kU/L	48.9	49.2	50.4	49.8	49.1
100kU/L	14.6	18.6	21.9	24.8	26.6
0kU/L	0.090	0.081	0.057	0.038	0.038
Ratio 5000/0	543	607	884	1310	1292

Table 28 Two-stage assay: the rate of reaction followed by density sedimentation (two passes) separation. (Background Subtracted)

AFP Concentration	0	100	5000 kU/L
60 minute incubation		% Binding	
25uL	0.41	16.2	46.0
50uL	0.35	16.2	47.0
120 minute incubation			
25uL	0.36	23.5	52.7
50uL	0.35	22.8	52.3

Table 29 Choice of sample volume for two possible incubation times.

AFP Concentration	0	100	5000 kU/L
Centrifugation		% Binding	
Single Stage	0.23	10.7	53.2
Two-Stage	0.28	25.0	53.8
Density Sedimentation			
Single-Stage	0.084	8.8	48.8
Two-Stage	0.057	21.9	50.4

Table 30 Choice of single- or two-stage reagent addition. 60 minute incubation.

	Percent Bound		
Time before shaking	20 min	10 min	0 min
500kU/L	36.6	35.5	35.2
125kU/L	26.1	24.4	23.8
4kU/L	1.9	2.0	1.9
0kU/L	0.48	0.48	0.43

Table 31 The effect of reagent addition for the single-stage assay taking up to 20 minutes before a 2.5hour incubation.

AFP (kU/L)	25	50	100	Mean	S.D.
Horse Serum (HS)	104	104	102	103.3	1.2
HS/EPPS Buffer	101	99	106	102.0	3.6
Newborn Calf Serum (NCS)	102	101	96	99.7	3.2
NCS/EPPS Buffer	109	107	107	107.7	1.2
Human Serum	106	101	98	101.7	4.0
Mean	104.4	102.6	101.8		
S.D.	3.2	3.0	4.8		

Table 32 Recovery of exogenous AFP at three concentrations from several possible "standards diluent" matrices

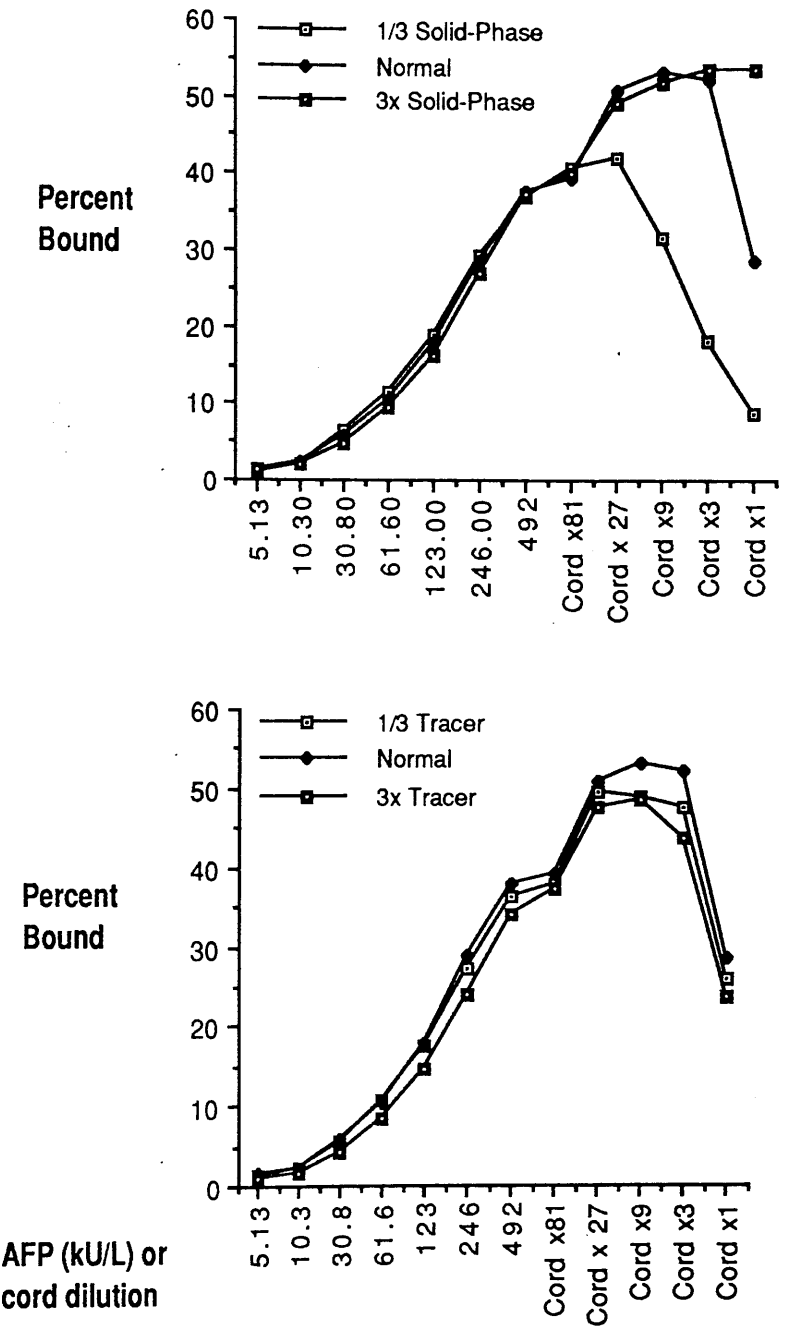


FIGURE 33 The consequences of varying the concentration of solid-phase or tracer reagents.

ix) Comparison with radioimmunoassay

Protocol for the IRMA was as given in 2B xi) and xii).

The routine RIA was used as the basis for comparison. Buffer used was 0.05M barbitone with 1% bovine gamma globulin, 0.1% sodium azide and 8.4% polyethylene glycol 6000 (PEG). 1ng of labelled AFP was used per tube. All samples were assayed in duplicate. 700uL of buffer/PEG/label and 40uL of sample were taken up and 100uL of 1:50,000 (in buffer) goat-anti-human AFP added by Micromedic APS-2. Reaction tubes were vortex-mixed immediately after processing and allowed to stand at room temperature (15-25^{o}C) for 16-64hr. All tubes were centrifuged at 2000rpm for 30min at +4^{o}C in a Damon-6000 refrigerated centrifuge. The supernatant was aspirated manually and the bound fraction was counted. Calculation was by 4-parameter log-logit method.

The results of linear regression analysis of 2291 samples assayed, in parallel by both RIA and IRMA, are summarized in Figure 34. Demonstrated are; gradient of 1.29 (for local reasons, 1.25 was expected), Y-intercept +3.1kU/L and correlation coefficient 0.956.

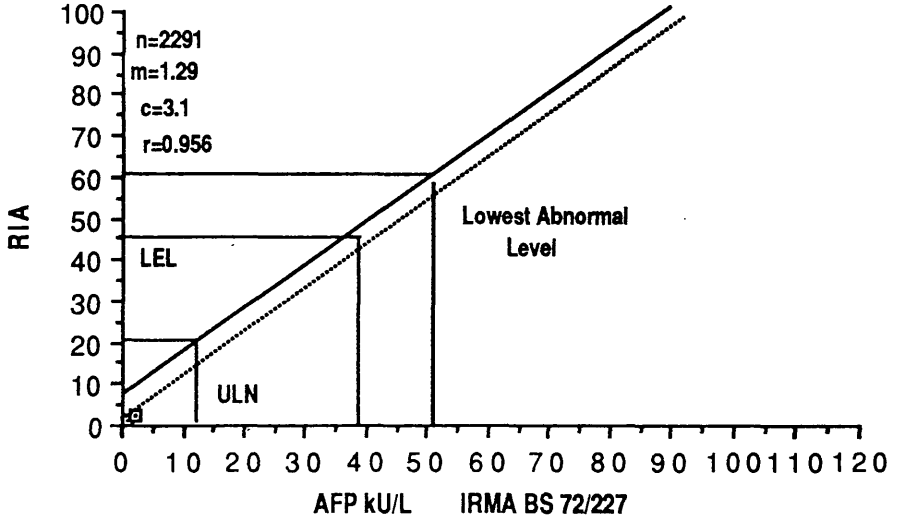


FIGURE 34 Regression analysis for the 2291 maternal serum samples assayed by RIA and IRMA. The regression line (solid) is plotted, with the line of equivalence (dotted). LEL= lowest equivocal level. ULN= upper limit of normal.

x) Comparison with commercial immunoradiometric assay

Each assay was run as per protocol. In-house standards, which had been prepared using bovine serum, were compared with kit standards prepared from human serum in one assay. 35 samples were assayed giving a correlation coefficient of 0.9978. The equation of the linear regression analysis was Y(Kit Results) = 1.28(IRMA Results) - 7.3kU/L. Although the gradient is 1.28, Student's "t" statistic of 1.70 for the difference between methods did not attain significance ($p>0.05$). This may be due to the small number of samples. The difference is, however, outside the usual variation inherent in data reduction and suggests significant differences between standard preparations.

To compare the assay methods, 5 assays were run in parallel containing a total of 217 samples (data not shown). The correlation coefficient was 0.9953, and the equation of the regression line, Y(Kit Results) = 1.07(IRMA Results) - 2.5kU/L. Despite the much smaller gradient, the larger number of points gave a "t" statistic of 3.28 which was highly significant ($0.005>p>0.0005$). This degree of bias is probably accounted for by the difference demonstrated between standards.

Figure 16 (After Page 41) shows dose-response curves for AF5/A2 monoclonal/SAPU sheep polyclonal, SAPU monoclonal/SAPU sheep polyclonal, and the commercial assay.

3C ROUTINE USE AND EXPERIENCE

i) Scottish Antibody Production Unit

On completion of assessment of the developed assay, the Scottish Antibody Production Unit (SAPU) based at Law Hospital, Carluke, Lanarkshire, undertook to maintain routine supplies of reagents to National Health Service (NHS) laboratories.

The solid-phase reagent is prepared at Carluke from the immunoglobulin fraction of SAPU sheep-anti-human AFP polyclonal antibody, in 5-litre batches, using the carbonyldiimadazole method described in sections 2B i), iv), v) and vi). Large scale preparation of the ^{125}-Iodinated monoclonal antibody is effected by the Radioimmunoassay Laboratory at the Department of Obstetrics and Gynaecology, Ninewells Hospital and Medical School, Dundee, using the Chloramine-T technique.

Quality assessment of reagents is performed at both sites and at the Institute of Biochemistry in Glasgow Royal Infirmary.

ii) External Quality Assessment Scheme (EQAS)

The percentage deviation from the all-laboratory trimmed mean (ALTM: Cameron 1978: Bacon et al. 1983) for each sample in 6 distributions is tabulated in Table 35 for the period during 1985 which included the changeover from RIA to IRMA. Performance for each sample is shown and the results for each month is given. To reduce bias from assessment in only one month, the figures are combined to give two-monthly estimates. The usual method of inspecting a six-month window is not suitable for this purpose, but is shown for the 24 month period of the changeover in Table 36; the display is of the six-month window where possible and the IRMA advantages are clear.

The variable bias of the RIA was replaced by a substantial positive bias in the IRMA. The variance, as shown by the ranking in Table 35 and the Figure 36, was markedly improved. In the comparison of the RIA and IRMA techniques the gradient of 1.29 was greater than the expected value of 1.25 which suggested a positive bias of 4% using routine pregnancy samples. The EQAS used pooled human sera and this may also add a systematic bias not detectable in the internal quality control data. Comparison of RIA and IRMA values for 50

stored EQAS samples had shown a positive bias to the latter of 11.9% (Stevenson et al. 1987). The IRMA shows a positive bias compared with RIA for all values, probably because of the lack of competition between the different specificities and avidities of antibodies present in polyclonal antisera.

There was no evidence of dose dependent recovery in either assay.

	Assay Method					
	<----------RIA---------->				<--------IRMA->	
Batch Number	118	119	120	121	122	123
Sample 1	- 3.2	- 3.7	- 5.5	- 5.1	+11.0	+ 7.3
2	+ 2.7	- 4.4	- 9.3	- 1.1	+13.0	+ 7.5
3	- 8.6	- 6.4	+13.0	- 5.7	+ 7.8	+ 4.7
4	+ 6.8	- 8.5	- 5.8	- 7.4	+ 5.7	+10.0
5	+ 5.3	- 0.60	+11.0	- 3.7	+ 5.8	+ 5.7
Monthly Bias	+ 0.60	- 4.7	+ 0.68	- 4.6	+ 8.7	+ 7.0
Monthly Variance	6.4	3.0	10.4	2.4	3.2	2.0
Two Month Bias		- 2.1		- 2.0		+ 7.9
Ranking		19/41		18/51		29/38
Two Month Variance		5.5		7.7		2.6
Ranking		34/84		61/84		1/84

Table 35 Results of analysis of six batches of the EQAS for alphafoetoprotein, distributed from March to August 1985.

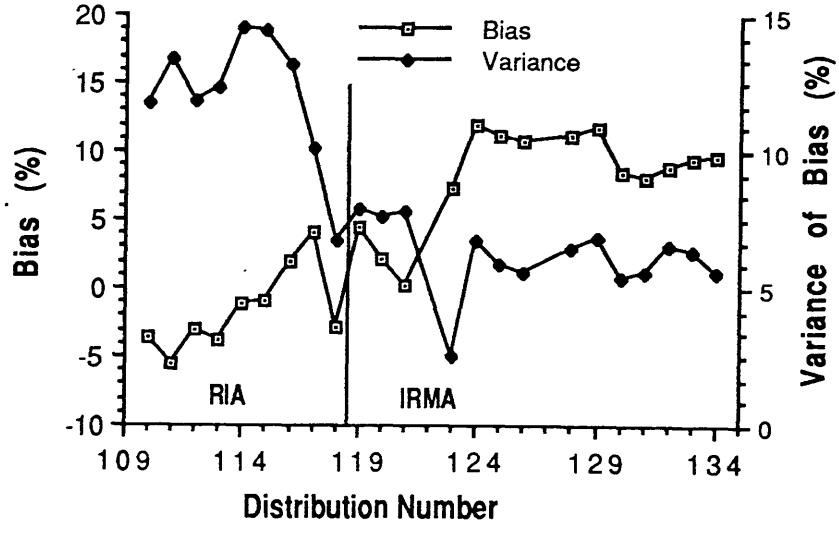


FIGURE 36 EQAS results for the 24 month period around the changeover from RIA to IRMA. Six-month windows for BIAS and variance of the bias are plotted where available.

iii) Internal Quality Control

To monitor the daily assay performance, 4 quality control sera, prepared from normal human serum pools with added cord serum or amniotic fluid, are run at the beginning and at the end of each assay. A similar sample is used for determination and estimation of internal assay drift and is assayed every fifteen specimens. Commercially available pooled serum tested for the absence of HIV and HBsAg are used (HD Supplies, Aylesbury, UK). They are targetted at points of clinical significance on the dose response curve (Figure 16 following page 41); 3, 25, 65, 110 and over 300 kU/L which represent; normal adult (3 kU/L), typical "Down's risk" pregnancy at 16 weeks gestation (25 kU/L), intervention levels at 16 and 20 weeks gestation (65 and 110 kU/L), and "very high" which is typical of anencephalus (over 300 kU/L). About fifteen of the 160-tube batches, each containing up to 57 serum samples, are processed each week.

Details of the workload are recorded for each month. A complete frequency distribution for each week of pregnancy from 16 to 20 completed weeks is generated every three months and this is used to validate the intervention levels and medians.

The medians at 16 and 17 weeks are noted for periods of 1 week, 1 month and three months. Typical numbers of samples are (at 16 weeks) 250, 1100 and 3300. Follow-up rates are collated similarly.

Several assay parameters are recorded, and in Table 38 it is clear that for month 3, the binding of the top standard was lower than usual. This was associated with a particular batch of label and caused the month to show the lowest median figures, although the follow-up rate and quality control data appear satisfactory.

Table 37 shows the present monthly workload, and Table 38 a summary of the latest presentation of workload, internal quality control and distribution of sample results submitted to the Endocrinology Section of the Institute of Biochemistry at Glasgow Royal Infirmary for external audit.

BATCHES Performed 61 Drift 3 Lack of Fit 1

(No samples reassayed)

SAMPLES	First Samples		Samples reassayed		
Less than 16 weeks	188	8.80%	Swapped Racks	14	0.5%
16-20 weeks	1942	90.9 %	Bad Duplicate	$270	8.6%
More than 20 weeks	6	0.28%			
Total	2136				
Amniotic Fluids	177		Distribution of results		
Repeat Blood Samples	134	6.27%	"Trisomy Risk"	174	8.1%
Other Blood Samples	803		90th-95th Centile	142	6.0%
Internal QC Samples	29		Over 95th Centile	142	6.0%
Total	1116		Median at 16 wks	33	n= 998
GRAND TOTAL	3145		Median at 17 wks	37	n= 631

INTERNAL QUALITY CONTROL

Control	Target	Mean	S.D.	C.V.	Number	BD#	Low	High	Range
Q1	2.9	2.39	0.53	22 #	46	6	6	1	2- 4
Q2	17.4	17.7	1.2	6.9	111	10	2	3	15- 19
Q3	77.9	77.7	3.0	3.9	108	14	2	11	72- 83
Q4	195	193	11.1	5.8	108	16	2	4	185-225
P	108	108	5.0	4.6	61	-	0	0	107-117

only integral values collated, thus not accurate

SENSITIVITY Minimum 0.43kU/L Maximum 3.8kU/L

$ Vacuum pump maladjusted

Target for "Trisomy Risk" is 6.7%. BD = bad duplicate

Table 37 One month of internal quality control; February 1989.

1988/1989	OCT	NOV	DEC	JAN	FEB	MAR
WORKLOAD						
Reported Batches	62	71	62	64	61	74
First Samples	2163	2668	2246	2396	2136	2573
Other Samples	1030	963	945	974	1009	1212
Rejected Estimations	162	261	199	131	270	161
DISTRIBUTION and MEDIANS						
90-95th centile (%)	5.3	5.3	4.5	5.8	5.7	4.4
>95th centile (%)	5.9	5.3	5.7	6.2	5.3	5.2
"Trisomy Risk" (%)	7.3	7.2	7.7	5.9	8.1	7.6
Median at 16 weeks	33	33	32	35	33	33
at 17 weeks	37	36	36	38	37	37
STANDARDS						
Mean % Bound	32.0	33.5	31.9	35.1	29.6	37.5
Minimum Zero (%)	0.118	0.179	0.129	0.137	0.147	0.128
QUALITY CONTROL						
Q1 n=100 (CV%)	21@	24	--	25	22	15
Q2 n=100 (CV%)	5.8	6.2	6.6	6.4	6.9	7.2
Q3 n=100 (CV%)	3.0	3.3	3.6	4.4	3.9	3.2
Q4 n=100 (CV%)	4.4	4.9	4.2	4.2	5.8	5.3
Q5 n=50 (mean6)	2.6	2.4	4.3	3.5	4.6	3.8

-- Assay separation problems, insensitive assays.

@ Integer values collected, thus high CVs.

Table 38 Six months of internal quality control: October 1988 to March 1989

iv) General Routine Experience

The labelled protein supplied by SAPU prepared using a chloramine-T iodination technique gives good results. In the long term this method seems to produce a more variable and more fragile reagent, as shown by monitoring the change in binding during the 4 weeks of its currency, than the lactoperoxidase technique which was used during the validation of the method.

In routine use, leakage of antibody from the 5-litre batches occurred during the 3-4 month shelf life of the reagent. This caused a slow but severe shift of the dose response curve to the right (Figure 39) and associated loss of sensitivity and precision. Accordingly, the assay protocol was amended to include routine replacement of the supernatant before use (or periodically).

Internal quality control data for the IRMA showed coefficient of variation of <2% over the range 5-500kU/L compared with RIA data of <5% over the range 50-500kU/L. This improvement in precision had considerable effect on the weekly follow up figures. The extreme range for RIA using 3% nominal follow-up was 0.6 to 4.6% and use of 5% nominal follow-up would have given even greater range. The markedly improved precision allowed a reduction in the intervention level from the 97th to the 95th centile, and the extreme range of follow-up over 26 weeks was 3.4% to 6.6%. Improved precision has enabled the initiation of Down's syndrome screening using low-MS-AFP to give a risk factor in mothers aged 25 years and over. Practical effects of the precision available are shown in Table 40 (taken from Stevenson et al. (1987)) and suggest its suitability for use in tumour marker monitoring.

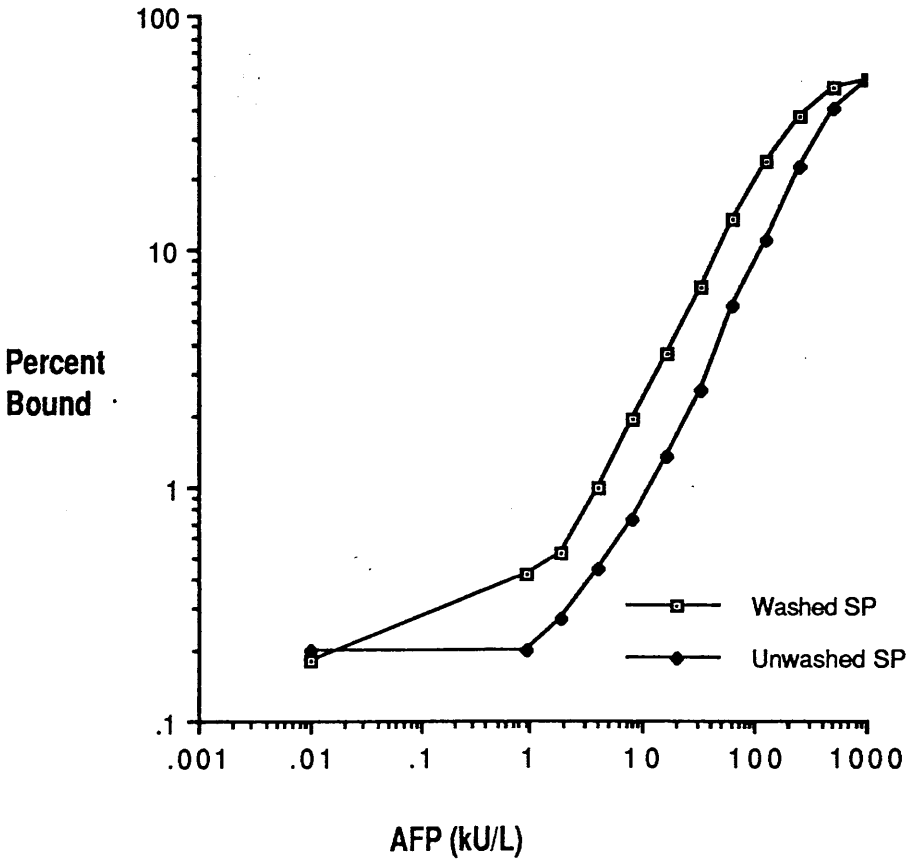


FIGURE 39 Dose-response curves, before and after washing (3 replacements of supernatant), following six-months storage of solid-phase.

Serum Sample from	Typical AFP result (kU/L)	95% confidence limit of result (kU/L) IRMA	RIA-tumour	RIA-maternal
Normal subject				
Treated tumour	5	4- 6	<range	<range
Chorionic villus sample (6-10 week pregnancy)	10	9-11	6-14	<range
Down's pregnancy	25	23-27	19-29	16-34
Normal pregnancy (median 16-17 weeks)	35	33-37	31-39	29-41
Abnormal pregnancy 16 weeks	65	62-68	59-72	59-72
Normal (but equivocal level) pregnancy 20 weeks	100	96-104	90-110	90-110

Table 40 The effect of improvement of precision on the confidence limits achieved by three AFP assay methods in various clinical situations.

v) Amniotic fluid assay

A limited assessment of the suitability of the IRMA for diagnostic AFP assay was performed using 38 consecutive routine, and 12 stored abnormal amniotic fluids. Each sample was processed in the routine, quantitative, "rocket" immunoelectrophoresis assay and then diluted 1:1000 in each of three diluents; EPPS IRMA assay buffer, bovine serum and distilled water.

Rocket assay - X mg/L, IRMA - Y MU/L

Buffer	r= .9977	Gradient = .4126	Intercept = . +2.098
Serum	r= .9974	.3867	+ .340
Water	r= .9985	.4060	+2.525

This batch of serum gives lower recovery than the other diluents and before routine use was started several other sera were investigated; particular batches of pooled sheep or human sera were shown to give recovery of >95% (data not shown) and good parallelism.

The rocket assay unitage is unstandardized against external calibrants, thus no comment can be made concerning the gradients, but is based on internal data collected over 6 years and seven thousand pregnancies. Correlation coefficients are very good. Preparation of 1:1000 dilutions is reliable using two-stage dilution, manually or using the Hamilton Microlab M (Bonaduz, Switzerland).

Routine IRMA-based diagnostic assay will only require the use of external calibrant and internal standard for ample quality control of the dilution and assay procedures.

vi) Observations

The two monoclonal antibodies generated in this study (AF5/A2 and AF4/B11) have shown their superiority over commercial and other antibodies and a satisfactory and convenient assay generated from one of them.

The MS-AFP screening programme cut-off for structural abnormalities of the foetus has been raised from 3% to 5% of all pregnancies. Table 41 shows the number and type of lesions detected by serum screening in this area during the years 1984-5 to 1987-8. Different total numbers of lesions reflect the varying incidence rate. The effect of the change to IRMA technology is quantitated by noting the pregnancies in which the old intervention limit would not have detected the abnormality. Overall detection rate for open spina

bifida increased by 16.5%. In addition, two anencephalic foetuses would have been missed if the RIA had still been used for screening.

The wide working range of the assay allows assessment of tumour marker follow-up and maternal serum samples in the same batch. It is necessary to dilute all amniotic fluids to 1:1000 and unknown tumour marker samples to 1:1000 in a carefully selected diluent. The assay is used for for monitoring small numbers of tumour marker patients, large numbers of maternal sera and about 1200 amniotic fluid samples per year. The method is also used for routine tumour marker studies at Glasgow Royal Maternity Hospital.

LESION					
YEAR	Anencephalus	Spina Bifida	Anterior Abdominal Wall Defect	Body Stalk Abnormality	Others
RIA					
1984-5	18	24	6	1	3
IRMA					
1985-6	26 (0)	33 (7)	4 (0)	2	0 (0)
1986-7	24 (2)	35 (4)	13 (2)	0	3 (0)
1987-8	17 (0)	31 (3)	6 (0)	0	4 (1)

Table 41 Effect of changing intervention limits from 97th to 95th centile. The figures in brackets are lesions which would have been missed using the RIA.

CHAPTER 4 DISCUSSION

The primary objective of this project was to develop a robust, low-cost, sensitive and specific IRMA for AFP assay in biological fluids, by using hybridoma technology to generate high avidity, monoclonal antibodies. The objective has been achieved, and the discussion is divided into six sections; A: Antibody generation methods, B: AFP assay techniques, C: Limitations of the developed assay, D: Possible improvements, E: Clinical impact on NTD screening in the West of Scotland, F: Autosomal trisomy and G: Concluding observations.

4A ANTIBODY GENERATION METHODS

The strategy (see Fazekas de St. Groth & Scheidegger 1980 and James et al. 1984) employed in the project was to immunise mice using amniotic fluid containing a high concentration of AFP with subsequent creation and selection of anti-AFP secreting, hybridoma cell lines. The very high success rate with this approach may represent a combination of factors.

Experience of generation of polyclonal antibodies at the Scottish Antibody Production Unit since the inception of maternal serum screening programme AFP assays in 1975 has shown that the molecule is highly immunogenic, and it is common to find high-affinity antibodies in immunized animals (sheep and goats, unpublished observations). The reasons for this are possibly connected with its presumed function as a transport protein for which it would require a variety of immunogenic binding sites which would act as epitopes. AFP also contains about 4% carbohydrate (Ruoslahti and Hirai 1978).

The fusion technique was based on several published methods, but possibly important local modifications included the use of a 1:1 ratio of myeloma to spleen cells and the ubiquitous use of 3000 cells/well (96-well plate) macrophage feeder cells. It proved very successful in generating very large numbers of hybrid cell lines (several per well) and large numbers of antibody-secreting, hybrid cell lines. Robustness of the cell lines was ensured by allowing

them to grow in competition with non-secreting lines and the lines chosen for extensive investigation proved stable through all procedures.

To ensure that only suitable antibodies were investigated, the initial screening procedure was biphasic. The first stage detected the presence of anti-AFP secreting cell lines, but could not distinguish between robust colonies secreting large amounts of low-affinity antibody and slower growing colonies secreting small amounts of high-affinity antibody. The second stage, displacement testing, enabled a rapid assessment of the potential affinity of the antibodies. This rigorous selection also reduced the need to clone large numbers of cell lines.

At each stage and periodically, especially after each cloning procedure and before freezing down, the cell lines were tested for displacement to confirm the maintenance of the desired characteristics.

The system for anti-AFP antibody generation was part of a small, highly motivated team working in parallel on a number of antigens; this conferred efficiency, speed and flexibility on the system.

4B AFP ASSAY TECHNIQUES

Following the paper written by Brock & Sutcliffe (1972) and the in-house development of a suitable RIA (Vince et al. 1975), there has been a maternal serum screening programme for AFP in the West of Scotland since 1975. Experience and cost-benefit analysis (Ferguson-Smith et al. 1978, Chamberlain 1978) led to widespread availability of the test; 23 laboratories were participating in the United Kingdom External Quality Assessment Scheme in 1976 (Seth et al. 1988), and that figure is now 103. This includes several laboratories whose sole use for AFP assay is as a tumour marker. Although the principle of IRMA had been expounded in 1968 by Miles and Hales, introduction of reliable methods had to await the development of high-affinity monoclonal antibodies available in large quantity. RIA accounted for all of the 23 assays in 1976. In early 1988, 78 laboratories still used RIA (30 using one of three types of

commercial kits) and only 13 used IRMA (10 using one of three commercial kits the other three use the described in-house method). For the rest, 11 use one of 6 non-isotopic commercial kits and one laboratory uses an in-house non-isotopic method.

The potentially desirable characteristics (Hunter 1982) of a monoclonal antibody based IRMA are shown graphically in Figure 42 and have been realised with the development of a precise and comprehensive assay. It is robust, convenient to use and undemanding of routine skills. The reagents are accessible and straightforward in preparation and have been made available to all NHS laboratories (without charge in Scotland). The high avidity antibody provides the sensitivity necessary to investigate the significance of small changes in concentration at low levels and to give a more responsive follow up for tumour marker studies. The follow-up rates and monthly median figures are stable and appropriate.

Lipaemia and haemolysis have not been shown to affect the apparent AFP level and the new assay is much less sensitive to matrix effects, allowing the use of plasma and serum samples in the assay. With dilution in a suitable matrix, the method is useful for amniotic fluid AFP estimation.

The monoclonal antibody AF5/A2 has been purchased by a commercial company to form one half of a double monoclonal, non-isotopic immunoassay.

The bias shown in the EQAS scheme has been investigated: EQAS recovery samples are always positively biased. The magnitude of the bias has varied from 5% to 12% despite the in-house use of BS72/227 standards. Internal quality controls are prepared from human pooled sera with added high-titre AFP for which accurate recovery data is not collated.

A limited assessment (section 3B ix)) comparing the newly developed assay with a commercial double-monoclonal, isotopic IRMA showed the in-house method to have better sensitivity (Figure 16 After Page 41), but in other respects was not distinguishable.

4C LIMITATIONS OF THE DEVELOPED ASSAY

The following advantages of IRMA were considered germane; smaller sample volume, greater working range, greater sensitivity, greater precision, robustness and shorter incubation time. It was also important that the final protocol was straightforward and required only cheap, accessible reagents and no investment in expensive, specialized equipment. The sole disadvantage to be expected was the presence of the "high-dose hook" effect. This is an inevitable consequence of the two-site technique; with normal or high levels of AFP in the sample, the label precipitated is proportional to the concentration of AFP. However, with a gross excess of analyte in the sample both reagent antibodies are separately bound to the antigen and the label precipitated is then inversely proportional to increasing AFP concentration. The length of the plateau can be quantitated easily and problems minimized by rigorous quality assessment of new batches of the solid-phase reagent.

The high-dose hook effect (shown in Figure 42), which causes decreasing bindings over about 25,000kU/L with these reagents, is not a problem in maternal serum screening programmes. In 250,000 maternal sera in the West of Scotland none has exceeded 16,000kU/L. To use this assay method in tumour marker studies, in which levels of 250,000kU/L are not uncommon, it is recommended that all unknown samples be assayed twice; neat and at a dilution of 1 in 1000 (Stevenson et al. 1987).

The need to use serum standards necessitates careful assessment of candidate matrices. It is also possible that biological fluids other than serum might require different diluents to maintain acceptable recovery and parallelism. Thus amniotic fluid IRMA assay, which requires 1 in 1000 predilution of all samples, needs separate assessment of diluent before introduction into routine use. Similar recovery and parallelism experiments should be performed when the batch of diluent is changed or, for e.g. tumour marker samples, where the AFP is from a different source and may possess different epitopes.

The mixed reagent addition assay compromises the ultimate sensitivity and requires longer incubation time, but reduces operator interventions and operator time considerably. The use of a

radioactive label as tracer on the monoclonal antibody limits the shelf life of this reagent to 60 days. The shelf life of the solid-phase reagent is at least 12 months, but it is necessary to wash the reagent before use to prevent apparent loss of sensitivity.

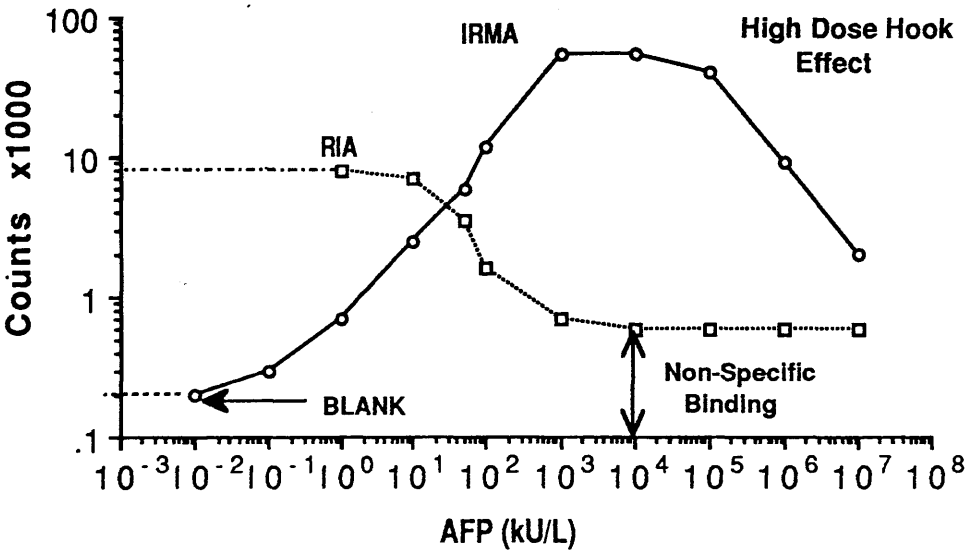


FIGURE 42 The symmetrical standard curve typical of IRMA techniques, showing comparable data for an RIA.

4D POSSIBLE IMPROVEMENTS

In the short-term a magnetic solid-phase, using Dynospheres (TM Dynal Ltd, Liverpool UK) with an affinity-purified polyclonal antibody, is being evaluated because of the potential reduction in incubation and separation times and increase in precision associated with this type of reagent.

The reliability of the IRMA assay during routine use (despite the use of a partially purified, polyclonal antiserum linked to the solid phase) is reassuring, but the assay could be considerably improved by using another monoclonal antibody to form a monoclonal-monoclonal sandwich. Although this would not be likely to have such a dramatic effect on AFP assay as the change from RIA to IRMA it would be expected to have some or all of the following effects; shorter incubation time, smaller sample volume, improved working range, more economical use of the solid phase and/or label and reduced operator dependence. Owing to the great specificity of the monoclonal antibody, it would require extensive comparison with the existing technique to ensure no loss of practical sensitivity, which is the detection of abnormal pregnancies.

The pattern of binding of AFP to plant agglutinins (lectins), which recognize carbohydrate side-chains, shows that AFP possesses some degree of heterogeneity, presumably depending on the source of AFP and the number and nature of the barriers through which it passes. The extreme specificity of antibodies should be able to make use of this heterogeneity to produce disease- or abnormality-specific assays and thus improve differential diagnosis.

The heterogeneity is a potential problem in IRMA where only one (or two) highly specific monoclonal antibody(ies) is (are) used; it may be that, for maternal serum screening, a mixture of 2 to 5 labelled monoclonal antibodies and 2 to 5 solid-phase antibodies would give better parallelism, even less dependence on matrix and better differential diagnosis.

The development of a highly sensitive immunoassay for AFP using conventional ^{125}-I technology, despite the development of time-resolved fluorescence (Suonpaa *et al*. 1985) and several other labelling techniques, was dictated by the in-house availability of equipment. The monoclonal antibody is suitable for the more modern

assay methods, although these have not yet demonstrated marked superiority over the IRMA technique now in routine use, as has been demonstrated by its commercial acceptability.

Although the detection and quantitation of radioactivity is straightforward, the theoretical sensitivity is severely limited when compared with systems in which the signal can be amplified by the label, e.g. particle-counting (Colet-Cassart et al. 1981), chemiluminescence (Weeks et al. 1983 and John et al. 1986), enzymes (ELISA; McDonald & Kelly 1978, Brock et al. 1982, Chan et al. 1986) and fluorescence (Suonpaa et al. 1985 and Chan et al. 1987). Although these reagents are less hazardous than radioactivity, this may not apply to their preparation and a greater dependence on highly-developed commercial kits may ensue. The techniques require sophisticated technology in the preparation of the labels and in the detection systems. The availability of high-avidity, monoclonal antibodies has stimulated investment in these methods, which are potentially superior in ease of starting up MS-AFP and Down's Syndrome screening programmes.

4E CLINICAL IMPACT ON NTD SCREENING IN THE WEST OF SCOTLAND

The impact of MS-AFP screening on open neural tube defects in England and Wales is described by Cuckle & Wald (1987) and the status in Scotland is given by Ferguson-Smith (1983). Table 43 shows the amniocentesis rate (per thousand patients screened) in the West of Scotland during the years 1976-1986 and the results of careful and complete ascertainment of the total incidence of neural tube defects. During this period the prevalence of NTD fluctuated between 2.4 and 5.4 per thousand, while birth incidence was reduced by termination of 61% of total NTDs. The effect of IRMA on the number of amniocenteses is marked, but has reduced (for NTD indication) since 1986 as a result of improved ultrasound techniques and equipment.

As shown in Table 42, the effect of the introduction of the IRMA and the consequent reduction of the intervention level from the 97th to the 95th centile, overall detection rate for open spina bifida increased by 16.5%. In addition, two anencephalic foetuses would have been missed if the RIA had still been used for screening.

The relationship between maternal weight and serum AFP and its effect on screening for neural tube defects was noted by Haddow et al. (1981) and quantified by Wald et al. (1981). It was suggested as a way of reducing screening programme false positives and false negatives, but was not introduced into the West of Scotland MS-AFP system until the onset of risk estimation for Down's Syndrome screening in July 1987. The precision of the IRMA at relatively low AFP concentrations gave considerable refinement to the risk estimate.

Table 43 Incidence of Neural Tube Defects in the West of Scotland during the period 1976-1986.

Year of Confinement	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986
Incidence per thousand births	4.5	4.4	5.1	5.2	4.2	4.7	4.2	3.2	3.0	2.4	3.0
Amniocentesis rate per thousand screened	6.4	6.2	6.2	12.7	9.8	9.7	10.9	9.3	11.0	8.3	16.5

4F AUTOSOMAL TRISOMY

i) Background

The first reported association of low MS-AFP with autosomal trisomy by Merkatz et al. in 1984 was the result of assiduous questioning of the medical staff by a patient. This led to a retrospective study of 278 pregnancies and considerable interest in the possible prenatal diagnosis of aneuploidies. Cuckle et al. (1984) found a significant reduction in AFP level at 14-20 weeks gestation (0.72 multiples of the median (MoMs) compared to 0.98 in unaffected pregnancies) and suggested that a screening test for diagnostic amniocentesis and karyotyping with the cut-off at 0.5 MoMs would identify 21% of Down's Syndrome (DS) foetuses for a 5% amniocentesis rate if gestational age confirmed with ultrasound (Wald et al. 1982). They predicted that routine amniocentesis of all mothers over 38 years combined with age dependent MoM cutoff would detect 40% of Down's Syndrome foetuses for a 6.8% amniocentesis rate.

A retrospective study by Seller (1984) found only one DS for 500 amniocenteses but a carefully controlled study by Murday and Slack (1985) gave encouraging results. Palomaki (1986), using only one serum result (Haddow et al. 1986), gave results of a large study (51,000 screening tests) in which 21% of estimated DS cases were detected while subjecting only 2.1% to amniocentesis.

Comment in the Lancet (Editorial 1985) stressed the importance of the reduced risk where the AFP level was relatively high (>2.5 MoM). Cuckle et al. (1985) found a mean MoM in amniotic fluid from 48 affected pregnancies of 0.64 compared to 1.02 in 4005 normal pregnancies. Hershey et al. (1986) and Palomaki and Haddow (1987) gave individual odds calculations for the screening programme.

Screening in the first trimester, using a sensitive IRMA, has been suggested by Brambati et al. (1986) and Barkai et al. (1987) and an extensive pilot study is in progress in the West of Scotland.

ii) Screening

Using the comprehensive risk estimates published for Down's Syndrome by Cuckle et al. (1987), the IRMA has been used, since July 1987, for antenatal screening for foetal autosomal trisomy in women over the age of 25 years in the West of Scotland. This requires that

the assay be accurate and precise from 8 (0.25 Mom at 16 weeks) to 100 (upper limit of normal at 20 weeks) kU/L. The algorithm uses ultrasonic confirmation of gestation, where available, with maternal age and a maternal weight correction (as Wald et al. 1981) to calculate the MoM. The action limit was set at a combined mid-trimester risk of Down's Syndrome of 1:250, in the light of the local incidence of pregnancies in patients aged over 35 years. The expected proportion of the population to be identified was 5.4% but only 3.6% were detected as a result of disparity between local data and published data. Results from the first year of screening are detailed in Table 44. Subsequently, local data for AFP concentrations in autosomal trisomy pregnancies (chromosomes 13, 18 and 21) using screened-population age-distributions (Crossley et al. 1988) were used from July 1988. The cut-off risk factor of 1:280 was equivalent to the mid-trimester population age-related risk of a 35 year old woman. The predicted follow-up rate was 6.6%; using age alone, and maternal age 35 years as cut-off 6.7% of pregnancies would be followed-up. Actual follow-up is close to that predicted.

The decision to offer further investigation (amniocentesis) to the patients was taken by the obstetrician. Prior to the start of Down's Syndrome screening by AFP assay, 30% of mothers over 35 years had amniocentesis and none below 35. Now over 60% of mothers aged over 35 years, and some 30% of mothers under 35 all of whom are given a risk factor of >1:280 are investigated. Age-related diagnostic assay was associated with amniocentesis rate of 3%, but 6.7% of mothers were eligible. AFP-related Down's screening would be associated with 6.6% amniocentesis rate if all mothers identified were to have amniocentesis.

Patients screened during period	29800 + 360 #	
Combined risk estimate >1:250	956 + 200 #	
Percentage combined risk >1:250	3.8%	
Maternal age less than 35 years	334	
Amniocentesis performed	93	(28%)
Autosomal Trisomy (21^3)	3	
Other Abnormalities (47XXY)	1	
Average risk for Autosomal Trisomy	1:110	
Maternal age over 35 years	547	
Amniocentesis performed	277	
Chorionic Villi sampled	50	
Total interventions	327	(60%)
Autosomal Trisomy (21^3)	4	
Other Abnormalities (45X/46XY mosaic)	1	
Average risk for Autosomal Trisomy	1:140	
TOTAL AUTOSOMAL TRISOMY	(11/1156) 1:105	
Calculated Average Risk for age >35 years	1:125	

665 patients were aged over 35 years but assigned a low-risk of Down's Syndrome. 220 of these had amniocentesis or CVS (33%). Two abnormalities (21^3) were found in the whole group; ratio 1:333

360 patients did not have serum AFP assay before amniocentesis, of whom 200 would have been identified as "at risk". This sub-group of 200 contained 4 of the 5 abnormalities (2 x 13^3 3 x 21^3) in the whole group.

Table 44 West of Scotland maternal serum AFP Screening Programme - results of Down's Syndrome Screening for combined AFP, maternal age and weight risks for July 1987 to June 1988.

4G CONCLUDING OBSERVATIONS

Neural tube defect screening using AFP assay has been fully proven and the value of AFP estimation has been greatly enhanced by the advent of Down's Syndrome screening. Quantitation of other analytes, especially hCG (Wald et al. 1988a) and unconjugated oestriol (Wald et al. 1988b), in conjunction with AFP is evidently of value in this area and the generation of a double-label IRMA, or mixed AFP-IRMA and hCG-RIA (because of the high concentration of hCG), method for simultaneous and cost-effective AFP and HCG assessment is being actively investigated. Microheterogeneity of AFP suggests that there are further opportunities for specific diagnosis.

An improved method for AFP determination has been thoroughly evaluated and introduced into the West of Scotland maternal serum alpha-1-foetoprotein screening programme. This IRMA technique is precise, robust and has a wide working range. It has allowed the maternal serum screening programme intervention point to be lowered to the 95th centile (2.0 MoM) and has enabled the development of Down's Syndrome screening. There is no longer a need to maintain separate assay protocols for tumour marker and screening assays.

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