DEVELOPMENT OF A MONOCLONAL ANTIBODY-BASED IMMUNORADIOMETRIC ASSAY FOR THE MEASUREMENT OF THE FREE ALPHA-SUBUNIT OF HUMAN CHORIONIC GONADOTROPHIN

by

RAAZIA BE HANEEF

Submitted in partial fulfilment of requirements for the degree of Master of Medical Science in the Department of Chemical Pathology, University of Natal

1990

Various advantages result even from the publication of opinions; for though we are very liable to error in forming them, yet their promulgation, by exciting investigation, and pointing out the deficiencies of our information, cannot be otherwise than useful in the promotion of science.

John Abernethy [1764 - 1831]

In this research the statistical analyses and recommendations arising from these analyses have been performed in consultation with the Institute for Biostatistics of the Medical Research Council.

CONTENTS

PREFACE

The experimental work described in this thesis was carried out in the Department of Chemical Pathology, University of Natal Medical School, Durban.

These studies represent original work by the author and have not been submitted to any other university. Where use was made of the work of others, it has been duly acknowledged in the text.

The following is a publication from this work:
Norman RJ, Haneef R, Buck RH, Joubert SM. Measurement of the free alpha-subunit of human glycoprotein hormones by a monoclonal antibody-based immunoradiometric assay, and further exploration of antigenic sites on the choriogonadotrophin molecule. *Clin Chem* 1987; **33:** 1147-1151.

ACKNOWLEDGEMENTS

I wish to thank all those who have given me valuable assistance during the preparation of this thesis. I am grateful to Professor SM Joubert, former Head of the Department of Chemical Pathology, University of Natal Medical School, for giving me the opportunity to carry out this work, and for his advice and interest. I thank Professor GMB Berger, present Head of the Department of Chemical Pathology, for allowing me to complete this thesis. I wish to extend my thanks to Dr RJ Norman, now of Queen Elizabeth Hospital, Adelaide, Australia for initiating this project. My sincere thanks to Dr Rosemary Pegoraro for her valuable guidance and supervision during the writing of this thesis. I am also grateful to Dr RH Buck for his advice on the scientific and clinical aspects of this work.

I acknowledge the technical advice of Mr Walter Deppe. I also thank Mrs Moyra Kemp for her technical assistance and for proof reading this thesis.

I greatly appreciate the co-operation of the Department of Obstetrics and Gynaecology, University of Natal, in particular Professor RW Green-Thompson. I am grateful to Mrs Marichen Conradie of the Natal Institute of Immunology, Pinetown, for her help and advice on cell-fusion techniques. I acknowledge the assistance of the staff of the Medical Illustration Unit for preparation of the many figures. I am extremely grateful to the South African Medical Research Council and University of Natal for financial support.

I greatly appreciate the assistance and typing skills of Belinda Barrett.

Finally, I thank my husband and family for their continued support and encouragement throughout this work.

ABBREVIATIONS

α	-	alpha
Ab	-	antibody
Ag	-	antigen
ß	-	beta
B	-	bound radioactivity
ßhCG	-	free ß-subunit or intact hCG
BMD	-	benign molar disease
BSA	-	bovine serum albumin
CO_2	-	carbon dioxide
cpm	-	counts per minute
CV	-	coefficient of variation
°C	-	degree Celsius
DMAC	-	p-dimethylaminocinnamaldehyde
DMF	-	dimethyl formamide
DMSO	-	dimethyl sulphoxide
ELISA	-	enzyme-linked immunosorbent assay
F	-	free radioactivity
FSH	-	follicle stimulating hormone
g	-	gram
g	-	force equivalent to that of gravity
GTD	-	gestational trophoblastic disease
h	-	hour
HAT	-	hypoxanthine, aminopterin, thymidine
hCG	-	human chorionic gonadotrophin
HGPRT	-	hypoxanthine guanine phosphoribosyl transferase
HRPO	-	horse radish peroxidase
HT	-	hypoxanthine and thymidine
HUCS	-	human umbilical cord serum
Ig	-	immunoglobulin

i.p.	-	intraperitoneal
IRMA	-	immunoradiometric assay
IRP	-	International Reference Preparation
IS	-	International Standard
IU	-	international units
i.v.	-	intra-venous
k	-	kappa
K_a	-	association constant
K_d	-	dissociation constant
k_1	-	association rate constant
k_{-1}	-	dissociation rate constant
kg	-	kilogram
λ	-	lambda
l	-	litre
LH	-	luteinising hormone
MBq	-	mega becquerel
mg	-	milligram
min	-	minute
ml	-	millilitre
mmol	-	millimole
mol	-	mole
mw	-	molecular weight
N	-	normal (concentration)
ng	-	nanogram
nm	-	nanometre
NSB	-	non-specific binding
OD	-	optical density
OPD	-	1.2 phenylenediamine
%	-	percentage
PBS	-	phosphate buffered saline
PEG	-	polyethylene glycol
pH	-	negative logarithm of hydrogen ion concentration
PPLO	-	pleuropneumonia-like organisms
PMD	-	persistent molar disease

RIA	-	radioimmunoassay
s	-	second
SD	-	standard deviation
S.E.M.	-	standard error of mean
SPE	-	Serum Protein Electrophoresis
T	-	total binding
TSH	-	thyroid stimulating hormone
TST	-	Tris-saline-Tween
μg	-	microgram
μl	-	microlitre
v/v	-	volume per volume

INTRODUCTION

Research is fundamentally a state of mind involving continual reexamination of the doctrines and axioms upon which current thought and action are based. It is, therefore, critical of existing practices.

Theobald Smith [1859 - 1934]

Almost a century has elapsed since the antigen-antibody interaction was first recognised as the basis of an immune response (Ehrlich, 1897). However, it was only in the 1930s, with the development of improved technologies that this concept was better understood, and led to the discovery of the amazing diversity and specificity of antibody molecules (Landsteiner, 1933). Theoretically, it is possible to make antibodies to a variety of biological substances and other chemicals, and therefore they are ideally suited as specific recognition elements to be used for analytical, cytological, functional, therapeutic and biochemical purposes.

The development of the radioimmunoassay (RIA) thirty five years ago, revolutionised research in many areas of clinical and scientific investigation. This technique evolved rapidly from the discovery made by Berson *et al.* in 1956 that antibodies to insulin could be detected in patients treated with this hormone, by measuring the binding of radiolabelled insulin to these antibodies.

Although in the past RIAs have been the most important assay system employing antibody and labelled tracer, the limitation was that reliance had to be placed on the chance development of a good polyclonal antibody. These shortcomings stimulated the search for monospecific antibodies of reproducible quality and sufficient quantity.

The development and introduction of monoclonal antibody technology brought about a revolution in immune serology (Kohler and Milstein, 1975). Establishment of immortal cell lines which contained the genetic elements of antibody-producing cells

was achieved by fusion between a myeloma cell line and spleen cells from an immunised donor. The resulting hybrids had the essential properties of both parents, namely, permanent growth and a high capacity for the synthesis and secretion of immunoglobulins, normally characteristics of plasmacytomas, together with the genetic elements defining a specific antibody.

Gestational trophoblastic disease (GTD) is a neoplastic condition of the trophoblast and occurs as molar pregnancy in a benign or invasive form, or as choriocarcinoma in a malignant form. Effective therapy has been developed for the treatment of both choriocarcinoma and molar pregnancy, but the key to successful management of these patients lies in their prompt diagnosis and careful monitoring of response to treatment (Green-Thompson, 1986). Fortuitously, these tumours elaborate the human chorionic gonadotrophin hormone (hCG) and its free alpha (α) and beta (ß) subunits and hence a ready marker for the tumour exists.

Human chorionic gonadotrophin is one of a group of glycoprotein hormones, which includes luteinising hormone (LH), follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH). These hormones are composed of two dissimilar subunits designated α and ß, which are bound non-covalently in the intact molecule. The ß-subunit of each glycoprotein hormone is unique and is responsible for the respective biological and immunological properties of the glycoproteins. In contrast, all four hormones possess an identical α-subunit which is coded for by a single gene (Fiddes and Goodman, 1979).

The measurement of hCG and its free ß-subunit, as so-called ßhCG, for the diagnosis and monitoring of therapy in patients with GTD is now routinely practised throughout the world (Vaitukaitis *et al.*, 1972). However it has been demonstrated by Bagshawe (1975) that when serum ßhCG can no longer be measured by current RIA methods, up to 10^4 tumour cells may remain undetected. In addition, there have been isolated reports of two patients with choriocarcinoma in whom ßhCG was undetectable in the serum but who appeared to be secreting only the α-subunit (Dawood *et al.*, 1977). Furthermore, it has been suggested that measurement of free α-subunit rather than intact hCG or the free ß-subunit is a more effective means of detecting persistent trophoblastic disease as well as tumour recurrence following

treatment (Quigley *et al.*, 1980a and b).

Radioimmunoassays which measure the free α-subunit of hCG have been developed, but in general lack the specificity and sensitivity required (Gaspard *et al.*, 1980; Kohorn *et al.*, 1981). These assays employ polyclonal antisera which also detect epitopes common to the pituitary gonadotrophins. Thus there is a need to produce monoclonal antibodies which recognise regions of the free α-subunit which are hidden in the intact gonadotrophins. Such antibodies would provide the required specificity for use in RIAs but are limited in their use by their inherent lack of high affinity for the antigen.

Fortunately, this drawback may be overcome by using monoclonal antibodies as labelled reagents in an alternative assay system, the immunoradiometric assay (IRMA), described by Miles and Hales (1968). The IRMA, particularly the two-site sandwich version of the assay, has been shown to provide greater sensitivity in addition to allowing enhanced specificity. This is a consequence of the use of two antibodies in excess to detect the analyte, each directed at a different epitope on the target molecule. The first antibody, referred to as the capture antibody, is usually linked to a solid-phase to facilitate easy separation and is added in excess relative to the target hormone to enhance antibody-antigen interaction, thereby allowing increased sensitivity in the measurement of analyte. The second antibody, referred to as the detection antibody, is labelled with a radioactive isotope or an enzyme to detect antigen already bound to the capture antibody.

The application of monoclonal antibodies specific for the free α-subunit to a highly sensitive IRMA format is an obvious need. Hence this study was undertaken firstly, to raise and characterise monoclonal antibodies to the free α-subunit, secondly to develop an IRMA using these antibodies and finally to establish whether measurement of free α-subunit has any clinical advantage.

CHAPTER 1

PRODUCTION OF MONOCLONAL ANTIBODIES TO THE ALPHA-SUBUNIT OF HUMAN CHORIONIC GONADOTROPHIN

The role of reason in research is not so much in exploring the frontiers of knowledge as in developing the findings of the explorers.

Beveridge WIB [1908 -]

1.1 INTRODUCTION

Monoclonal antibodies derive their name from the fact that they are produced by a clone of cells which is descended from a single hybrid cell known as a hybridoma. All the cells in the clone produce antibody molecules of identical amino acid sequence which bind to a single epitope on the antigen. Thus monoclonal antibodies have an extremely high degree of specificity and their use in immunoassays, especially in two-site immunometric assays, allows reliable quantitation of biological analytes (Miles and Hales, 1968). The purpose of this study was to raise monoclonal antibodies to the free α-subunit of human chorionic gonadotrophin (hCG) for use in a two-site immunoradiometric assay.

The method chosen for the raising of monoclonal antibodies was essentially that described by Kohler and Milstein (1975). This method is based on the fusion of myeloma cells, which are capable of permanent growth in culture, with actively dividing splenic B lymphocytes triggered by antigenic stimulation of the donor animal. The success of this technique relies on the selection of only the hybrid cells. While spleen cells are unable to grow in culture, unfused myeloma cells soon overgrow the small percentage of newly generated hybridomas unless they can be selectively excluded. To this end, the myeloma cell is first made genetically defective in its

nucleotide salvage biosynthetic pathway by selecting for mutants that are resistant to either 8-azaguanine, 6-thioguanine or 5-bromo-2-deoxyuridine. Such cell lines are unable to grow in Littlefield's 'HAT' medium, containing hypoxanthine, aminopterin and thymidine (Littlefield, 1964). As aminopterin blocks *de novo* biosynthesis of purines and pyrimidines, a cell's survival in HAT is dependent upon its ability to utilise exogenous hypoxanthine and thymidine through these salvage biosynthetic pathways. Kohler and Milstein used a myeloma cell line selected for resistance to 8-azaguanine which also has a deficiency in the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT), making it unable to grow in HAT medium. Fusion of these cells with normal spleen cells which possess this enzyme, however, produces hybridomas resistant to HAT selection.

The major steps involved in the raising of monoclonal antibodies to the free α-subunit are the immunisation of a suitable spleen donor with the free α-subunit using an appropriate immunisation protocol; the maintenance in culture of a myeloma cell line deficient in an enzyme of the nucleotide salvage pathway; the fusion of immunised spleen cells with myeloma cells; the selection of hybridomas using HAT medium; the screening for the antibody in supernatant fluids; the maintenance of selected hybridomas; the production of ascitic fluid and finally the isolation of the antibody. These steps are described in detail in this chapter.

1.2 IMMUNIZATION OF MICE

When a foreign substance or antigen is injected into an animal, the appropriate B lymphocyte populations are stimulated to produce antibodies which recognise many different sites on the antigenic molecule. It is possible to generate antibodies against substances that are not normally antigenic, eg. hormones, by using an adjuvant which renders them antigenic (Freund, 1956). In addition, it is known that repeating injections of the same antigen in a highly aggregated form favours a strong antibody response (Goding, 1986).

1.2.1 Choice of Lymphocyte Donor

The mouse is preferred as the lymphocyte donor because of the availability of suitable mouse myeloma cell lines. All currently available mouse myeloma cell lines used for hybridoma production are of Balb/c origin and this strain of mouse was therefore chosen for initial immunization in this study. Moreover, the choice of Balb/c mice for immunisation allows the resulting hybridomas to be grown as tumours in the same mouse strain.

1.2.2 Materials and Methods

The immunization protocol used is based on that described by Siddle and Soos (1981).

Ten Balb/c mice (obtained from the Medical Research Council, Research Institute for Diseases in a Tropical Environment, Durban) aged between 6 and 12 weeks were used. Primary immunization was carried out subcutaneously or intraperitoneally with free α-subunit of hCG (CR123 reference preparation; section 3.3) in doses ranging from 5 - 10 μg in Freund's complete adjuvant. After a rest period of 2 to 3 months, a booster dose was given intraperitoneally in Freund's incomplete adjuvant.

The production of antibodies against the free α-subunit was monitored by examining serum antibody titres in blood taken from the tail vein four days after the first booster dose was given. To obtain blood the mice were prewarmed to 40°C and held in a fixed position in a cone. The tail vein was swabbed with alcohol and a nick made into the artery with a clean scalpel blade. Blood was collected in a microfuge tube and allowed to clot; after centifugation the supernatant serum was collected using a pasteur pipette.

The serum was screened for antibodies to the free α-subunit in an enzyme-linked immunosorbent assay (ELISA) system described in section 1.7.

Three to four weeks after sufficiently high titres were obtained a divided

intravenous/intraperitoneal dose ranging between 50 and 150 μg of free α-subunit (CR123 reference preparation) in saline was injected into a mouse showing a high titre of the desired antibody. This final boost was given to allow most of the circulating antibodies to be cleared from the blood stream of the mouse, resulting in a stronger immune response.

1.2.3 Results and Comment

In Table 1.1 the antibody titres obtained four days after the first booster dose was given, are represented. Good titres were achieved in all 10 mice immunised, indicating the use of an adequate antigen dose and a suitable immunisation protocol.

Immunisation protocols for the generation of immune lymphocytes for hybrid formation are varied, but they are comparable in procedure. The timing between injections and the amount of antigen used must be determined for each antigen. A critical feature of all immunisation protocols is the timing of the final antigen boost relative to the time of cell fusion. This boost should be delivered 3 - 4 days prior to removal of the spleen for hybridisation with the myeloma cell line. In a series of early hybridisation experiments, Kohler found that the yield of specific, antibody-producing hybridomas increased up to 3 days after the booster injection and subsequently decreased (Milstein, 1986).

For many antigens, special measures must be taken to increase the yield of hybridomas secreting monoclonal antibody of the desired specificity and/or affinity. Stahli *et al.* (1980), for example, found it necessary to hyperimmunise mice with the hormone hCG by repeated injections with the glycoprotein in Freund's complete adjuvant. Final immunisation was without adjuvant and required large amounts of antigen injected i.v. in 200 μg aliquots on each of three successive days, beginning on day 4 prior to fusion. Similar immunisation regimens have succeeded in producing hybridomas to the human leucocyte interferons (Staehelin *et al.*, 1981). In the current study final immunisation required a large divided intravenous/intraperitoneal dose of free α-subunit ranging between 50 - 150 μg which was injected 3 - 4 days prior to fusion.

Table 1.1 Mean optical densities obtained after sera of immunised mice were tested in the α-subunit ELISA.

	OD at varying serum dilutions			
Mouse Number	1/100	1/1000	1/10000	1/100000
1	>2	1.6	1.0	0.8
2	>2	1.7	0.8	0.5
3	>2	>2	1.8	0.8
4	1.6	1.7	1.6	0.5
5	1.8	1.7	1.1	0.4
6	1.8	1.6	1.1	0.4
7	1.9	1.7	1.2	0.5
8	>2	1.8	1.1	0.6
9	1.9	1.6	1.0	0.6
10	>2	>2	1.5	0.6

Results represent means of duplicate samples of mice sera.

The purity of the antigen is another important factor in determining the final yield of antigen-specific hybridomas. Immunisation with crude preparations results in the stimulation of many B-cell clones producing antibodies to epitopes other than those of interest. Thus, a relatively pure free α-subunit preparation was used for immunisation to yield a higher percentage of hybridomas producing antibody of the desired specificity.

1.3 MYELOMA CELL LINE

A myeloma cell line which lacks HGPRT activity is required for fusion with immunised spleen cells to allow for the selection of hybridomas. In species which are dependent on exogenous purines or purine nucleosides for the formation of purine nucleotides, the salvage of preformed hypoxanthine or guanine can be initiated by HGPRT activity. Clearly, in a cell line which lacks HGPRT activity, the cells cannot grow or survive in a medium in which the sole source of exogenous purine is hypoxanthine and guanine; nor can HGPRT activity be blocked by 8-azaguanine, which is a guanine derivative and substrate analogue.

These cells therefore only grow in media which are enriched with purine nucleotides. The enzyme deficiency can be exploited to favour cells which have fused with cells not lacking in the enzyme by the simple expedient of manipulating the composition of the growth medium.

The cell line chosen for fusion should be of the same species as the immunised mice so that the resulting hybrids can be grown in the same strain for large scale antibody production. Furthermore, for a higher yield of spleen cell derived immunoglobulins, the myeloma cell line should preferably not secrete any immunoglobulin molecule. The most commonly used cell lines for hybridoma production are descendents of MOPC-21 which have been selected for HAT sensitivity and loss of endogenous immunoglobulin heavy chains (eg. P3-NS1-Ag 4-1; abbreviated NS) or loss of both heavy chains (eg. X63-Ag 8.653, abbreviated, NS-1). Of these, the NS-1 cell line is the most commonly used.

1.3.1 Choice of Myeloma Cell Line

The NS-1 cell line was chosen for fusion with spleen cells in this study because it was readily available and most importantly, is of the same species as the immunised animal. In addition, NS-1 cells do not express the heavy chain of the immunoglobulin but only synthesise the *k* light chains which are degraded intracellularly and not secreted. Fusion of NS-1 cells with spleen cells results in the production of active antibody-secreting hybrids. The immunoglobulins secreted by the hybrid cell consist of only spleen cell heavy chains, but the *k* light chains are derived from both spleen and NS-1 cells.

1.3.2 Method of Culture

1.3.2.1 Preparation of Culture Medium

Medium for the culture of NS-1 cells was made up of the following components:

372 ml RPMI 1640 with $NaHCO_3$ (liquid) [Flow Laboratories, Scotland, UK]

50 ml horse serum (heat inactivated) [M.A.Bioproducts, Walkersville, MD, USA]

50 ml fetal calf serum (heat inactivated) [Flow]

10 ml hepes buffer (1 mol/*l*) [Sigma Chemical Company, St Louis, USA]

10 ml anti-PPLO Agent [Gibco, UK]

5 ml L-glutamine (200 mmol/*l*) [Flow]

2 ml penicillin/streptomycin (5000 IU/ml / 5000 μg/ml) [Flow]

1 ml fungizone (250 μg/ml) [Flow]

1.3.2.2 Preparation of 8-Azaguanine Medium

A vial of lyophilized concentrate of 8-azaguanine (Sigma) was reconstituted in 10 ml of sterile distilled water. One ml of this solution was dissolved in 50 ml of culture medium, prior to use.

1.3.2.3 Maintenance of Myeloma Cells

NS-1 cells (gift from M.Conradie, Natal Institute of Immunology, Pinetown) were grown in RPMI 1640 culture medium at a concentration of 1 - 8 x 10^5 cells/ml. Every two months NS-1 cells were passaged through 8-azaguanine medium to ensure that a HGPRT deficient cell line was maintained.

A week before fusion cells were stabilized at a logarithmic phase of growth (2 - 10 x 10^5 cells/ml). To ensure this, cells were counted in a haemocytometer and the culture was appropriately diluted with fresh medium every second day. This growth phase is crucial because rapidly dividing cells are a requirement for successful hybridisation. Furthermore, the viability of cells during the exponential growth phase is generally greater than 95%. Cells were grown to a sufficient culture volume to yield 10^7 cells for each spleen used.

On the day of fusion, cells were pooled in a 50 ml conical centrifuge tube (Nunc, Kamstrup-DK-4000, Ruskilde, Denmark), harvested by centrifugation at 400 *g* for 5 min; cells were then washed 3 times with 10 ml serum-free culture medium and recovered by centrifugation each time. The final pellet was resuspended in 10 ml serum-free culture medium; the viability was determined by the trypan blue exclusion test and the concentration adjusted to 10^6 cells/ml.

1.4 SPLEEN CELLS

Only one percent of spleen cells actively secrete immunoglobulins while up to ten percent of hybrid cell lines secrete antibody (Goding, 1986). This may be explained by preferential fusion of myeloma cells with activated B cells. On the other hand it may be that myeloma cells have the ability to activate non-secreting B cells to rapid secretion (Eshhar *et al.*, 1979). It is known that the spleen cells which fuse with myeloma cells are larger than average and have recently undergone antigenic stimulation and blast formation (Andersson and Melchers, 1978). This consideration stresses the need to ensure adequate antigenic stimulation shortly before fusion

attempts.

1.4.1 Removal of Spleen

On the day of fusion a mouse immunised 3 - 4 days previously was sacrificed by cervical dislocation. The animal was swabbed with 70% alcohol, the superficial skin pinched up over the left side of the abdomen and a small cut made over the spleen to expose the peritoneum. The spleen was removed under aseptic conditions and transferred to a petri-dish containing serum-free culture medium.

1.4.2 Preparation of Spleen Cells

A spleen cell suspension was prepared by teasing out the cells with a pair of bent sterile needles. Clumps were broken up to suspend cells. The debris was allowed to settle under gravity and the cell suspension was transferred to a sterile centrifuge tube and centrifuged at 400 *g* for 5 min; the supernatant was aspirated, the pellet resuspended in 5 ml 0.85% NH_4Cl and left on ice for 10 min to allow red cell lysis.

Spleen cells were washed 3 times with 10 ml serum-free culture medium and the cells recovered between each wash by centrifugation. The final pellet of spleen cells was resuspended in 10 ml serum-free culture medium. The number of viable cells per ml was determined by the trypan blue exclusion test and the cell number adjusted to 10^7 cells/ml.

1.5 FUSION PROTOCOL

Successful hybridisation requires fusion of the membranes of both cells. The Sendai virus was used earlier as a fusing agent but most hybridomas are currently produced using polyethylene glycol (PEG) (Pontecorvo, 1976; Galfr'e *et al.*, 1977). The mechanism of fusion is still poorly understood and it has been suggested that both

the PEG itself and an unidentified contaminant in the PEG are required for fusion (Wojciezsyn *et al.*, 1983).

Fusion results in the formation of multinucleated cells called heterokaryons (Ringertz and Savage, 1976). The nuclei of heterokaryons fuse at the subsequent cell division, and thereafter daughter cells possess an approximately equal share of genetic material.

The fusion method chosen in this study is a modification of that described by Oi and Herzenberg (1980).

1.5.1 Materials and Methods

1.5.1.1 Preparation of PEG solution

PEG 1500 (3.5 g, Sigma) was autoclaved (120°C,1.2 kg/cm^2) and 500 μl of dimethyl sulphoxide (DMSO) (Merck, Darmstadt, W.Germany) and 6.5 ml sterile serum-free culture medium were added whilst the PEG was still liquid. The pH of the solution was adjusted to 8.0; 1 ml portions of this solution were stored at 4°C.

1.5.1.2 Fusion

Spleen cells (1 x 10^8) and NS-1 cells (1 x 10^7) were pooled in a conical 50 ml plastic centrifuge tube. The cell suspension was centrifuged and washed once in 10 ml serum-free medium. The medium was aspirated after centrifugation and the cells resuspended in the remaining medium by gently tapping the side of the centrifuge tube. At room temperature, 1 ml of the PEG solution was added slowly, over 1 min with constant stirring. This was followed by a series of dilutions as follows:
1 ml serum-free medium was added slowly whilst shaking for 1 min,
2 ml serum-free medium was added slowly whilst shaking for 2 min,
4 ml serum-free medium was added slowly whilst shaking for 2 min,
8 ml serum-free medium was added slowly whilst shaking for 3 min.
The cells were then pelleted gently (200 *g* for 10 min).

1.5.2 Comment

Several technical factors were found to be of importance in increasing the yield of hybrids and the following points summarise the critical aspects of the various procedures. Of major importance for successful fusion was the use of actively dividing myeloma cells, maintained in mid-log growth for a week before fusion. The concentration of PEG used in the fusion was also important; it is known that concentrations less than 30% result in very few hybrids being formed while concentrations greater than 50% are toxic. A recommended concentration of PEG (35%) was chosen to ensure that a slight overexposure of PEG to cells did not result in damage to cells (Gefter *et al.*, 1977). Dimethyl sulphoxide was added to the PEG to increase the pliability of the cell wall, allowing cells to fuse easily (Norwood *et al.*, 1976). The pH of the solution was adjusted to 8.0 to increase hybrid frequency as shown by Sharon *et al.* (1980).

1.6 SELECTION OF HYBRIDOMAS

The fusion procedure results in the formation of various cell combinations. In addition to myeloma-spleen cell fusions, spleen-spleen and myeloma-myeloma fusions also occur. Thus, a selection procedure is necessary to produce a long-term hybrid cell line. The most commonly used selection procedure is that described by Littlefield (1964).

1.6.1 Principle

This procedure depends on the fact that when the main synthetic pathway for purines and pyrimidines is blocked by the folic acid antagonist, aminopterin, there is an alternative 'salvage' pathway by which the cells are able to synthesise these nucleotides utilising an exogenous source of hypoxanthine and thymidine (Fig. 1.1).

Unfused myeloma cells and cells arising from myeloma-myeloma fusions, lack the

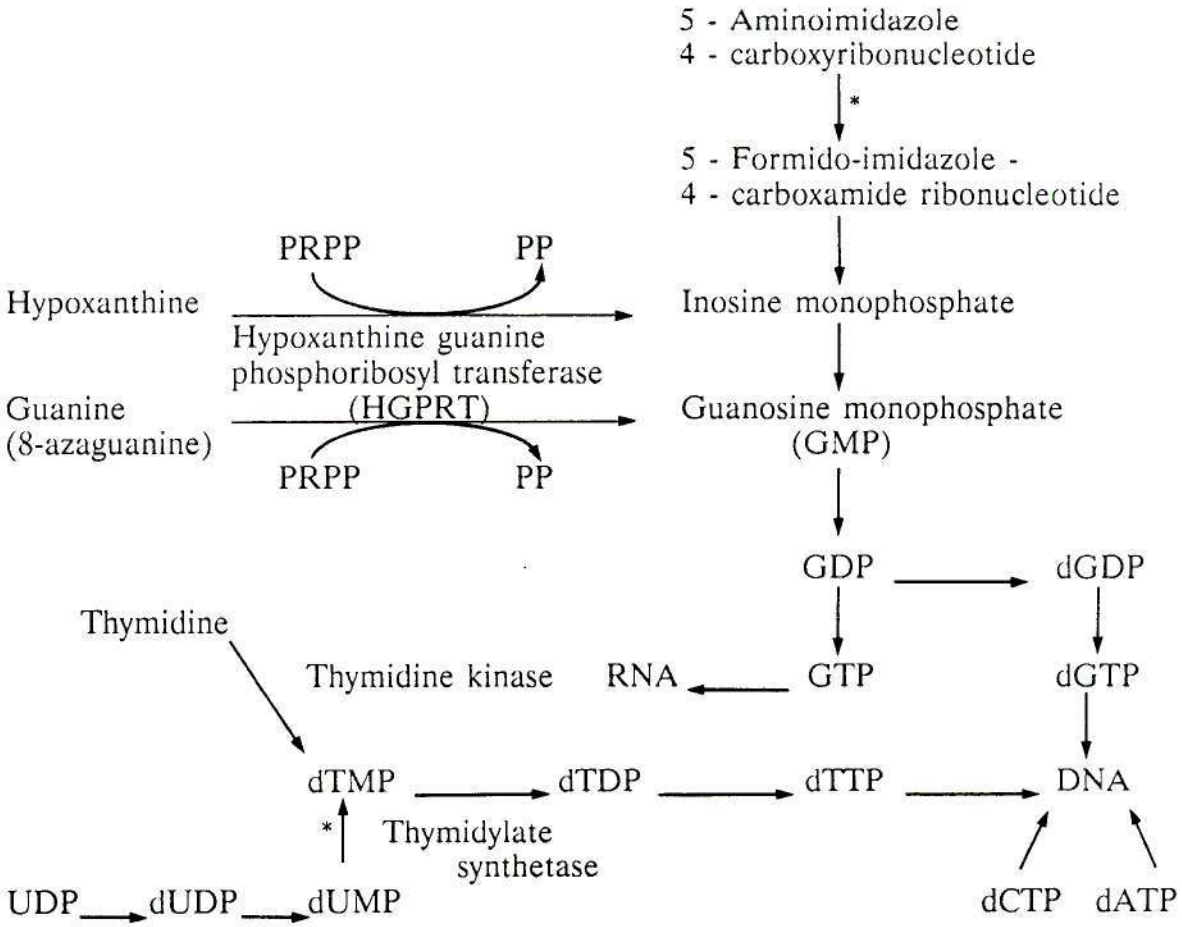


Figure 1.1 Metabolic pathways relevant to hybrid selection in medium containing hypoxanthine, aminopterin and thymidine (HAT). When the main synthetic pathways are blocked with the folic acid analogue aminopterin (*), the cell must depend on the 'salvage' enzymes HGPRT and thymidine kinase. (after Goding, 1986)

enzyme HGPRT and are therefore incapable of growing in HAT medium, because both the main and salvage pathways are blocked. There is no positive selection against the growth of spleen cells and spleen-spleen cell fusions in this scheme. Passive selection takes place because normal spleen cells have a limited growth potential in culture. However, a HGPRT negative myeloma cell can be made to grow in HAT medium if it is provided with the missing enzyme by fusion with an HGPRT positive spleen cell.

1.6.2 Materials and Methods

1.6.2.1 Preparation of HAT and HT medium

A vial of lyophilised HAT or HT (Sigma) was reconstituted in 10 ml of sterile distilled water. Aliquots of this stock solution were stored at 4°C. One ml of this solution was added to 50 ml of culture medium, prior to use.

1.6.2.2 Selection of Hybrids

After fusion, the hybridised cells were resuspended in 50 ml of HAT selective medium which contained 5% human umbilical cord serum (HUCS). It has been shown that HUCS is a potent growth promoter of hybridoma cells at low plating densities (Westerwoudt *et al.*, 1983). The cell suspension was distributed into 5 x 96-well plates in 100 μl portions. Plates were incubated at 37°C in an adequately humidified and aerated (5% CO_2-in-air) incubator (Flow) for 7 days.

After this period, a further 100 μl of HAT medium containing HUCS was added to each well. When the medium became acidic (approximately 14 days after fusion) as indicated by a change in the colour and when the growth of hybridomas was seen under the microscope, 100 μl supernatant was removed for screening from each well showing growth (see section 1.7). The wells were replenished with HT medium. Selection in HAT was terminated after two weeks when it was assumed that all the parental myeloma cells were dead and that any growing cells were myeloma-spleen hybrids.

Once the results of the screening became available, clones secreting the desired antibody were transferred in HT medium to 24-well plates. The clones were grown in HT medium to dilute out the aminopterin and to allow the hybrids to regain their enzyme activity. The selected clones were then cultured in large numbers for freezing and cloning. These procedures took place once a healthy, debris-free hybridoma culture was obtained.

1.6.3 Results and Comment

Between 10 and 60% of all wells plated had growth of hybrids. This range represents the results of seven fusions.

The initial cloning efficiency was low (10%) but improved after each performance possibly due to a more careful control of the NS-1 crucial exponential growth phase. It was also necessary to perform several fusions before hybridomas secreting the desired antibodies were obtained.

Only the screening results of the successful fusion are discussed. Two supernatants, 71C3 and 75C8, were positive for α-subunit antibody in the ELISA, with optical density (OD) readings of 1.55 and 1.90, respectively. These positive clones were screened on several occasions to ensure that they were not lost by overgrowth of a negative clone in the same well. In addition, all negative clones were tested two to three times to make sure that positive clones in low concentration were not missed.

1.7 SCREENING PROCEDURE

The choice of an appropriate screening assay is of major importance to hybridoma production. In principle, any method capable of detecting an antibody of the desired specificity may be used but a method that is accurate, reproducible and rapid is desirable; quick decisions about which culture wells to save or discard are essential to contain the volume of cultures. Quantitative results are mostly unnecessary in

hybridoma screening since the cells can be cloned if volume is required. The most appropriate assay for the screening of these hybridomas is an enzyme-linked immunosorbent assay (ELISA).

1.7.1 Principle

ELISAs utilize an antigen bound to a solid phase which in turn binds to a specific antibody. A second antibody conjugated to an enzyme such as horseradish peroxidase (HRPO) then binds to the antigen. After washing away unbound material, the bound enzyme is revealed by the addition of a substrate which undergoes a colour change proportional to the amount of enzyme present.

1.7.2 Materials and Methods

1.7.2.1 Preparation of Buffers

Carbonate buffer - 0.015 mol/*l* Na_2CO_3, 0.036 mol/*l* $NaHCO_3$, 0.02% NaN_3; pH 9.6

Phosphate buffered saline (PBS) with BSA - 0.15 mol/*l* NaCl, 0.04 mol/*l* Na_2HPO_4, 0.01 mol/*l* $NaH_2PO_4.H_2O$, 0.4% BSA, 0.02% NaN_3; pH 7.4

Tris-saline-Tween (TST) buffer - 0.05 mol/*l* Tris, 0.1 mol/*l* NaCl, 0.05% Tween-20; pH 8.0

1.7.2.2 Screening Procedure of Supernatant Fluid

1.7.2.2.1 Coating of Microwell Plates

Free α-subunit antigen (100 μl at a concentration of 5 μg/ml; CR123 reference preparation) in carbonate buffer was dispensed into each well of a 96-well ELISA plate (Nunc, high binding capacity) and incubated in a humidified box overnight at room temperature. The unbound antigen solution was aspirated the following day and

the wells were washed once with PBS containing 0.4% BSA. To block any unbound sites, wells were incubated at 37°C for 2 - 3 hours with 250 µl of PBS containing 0.4% BSA. Wells were then washed once with TST buffer and finally with distilled water. After washing, plates were dried by tapping on absorbent paper and stored at 4°C in a dry atmosphere.

1.7.2.2.2 Screening for Antibody in Supernatant Fluid

Supernatant fluid was screened for antibodies by adding 50 µl from each culture well to an ELISA plate well. Culture medium was used as negative control; as positive control a 1/100 dilution of ascitic fluid (designated 34E2) from Balb/c mice with peritoneal implants of free α-subunit producing hybridomas (Norman *et al.*, 1985) was used. ELISA plates were incubated at 45°C for 30 min in a humidified box. After incubation, wells were washed 3 times with TST buffer.

Fifty microlitres of conjugate (sheep anti-mouse IgG HRPO, courtesy Dr. J. Conradie, Natal Institute of Immunology, Pinetown) at an appropriate dilution (see section 1.7.3) were dispensed into each well and incubated at 45°C for 30 min in a humidified box. The wells were then washed 3 times with TST buffer to remove excess second antibody.

1.2 phenylenediamine dihydrochloride (7.2 mg) [OPD-2HCl] was dissolved in 6 ml substrate solution (Natal Blood Transfusion Services, Pinetown) a short while before use and 50 µl were dispensed into each well. Plates were left covered for about 20 min for colour development. One hundred microlitres of 1.5 N HCl (Merck) were then added to each well to stop the reaction and the absorbance was read at 498 nm on an ELISA plate reader (Biodata Instruments Inc., USA).

1.7.2.3 Optimization of ELISA

The following steps were undertaken to optimise assay conditions:

i) ELISA plates (Nunc) were coated with 100 µl free α-subunit (CR123 reference preparation) ranging from 1 - 10 µg/ml.

ii) Fifty microlitres of conjugate (sheep anti-mouse IgG HRPO) at dilutions ranging from 1/50 - 1/150 or PBS as blank were added.

iii) Wells were washed, between incubation steps, either once, twice or three times using an Eppendorf multipipette.

1.7.3 Results and Comment

Table 1.2 shows that at a conjugation dilution of 1/50 little difference in the OD readings of the positive control sample in wells coated with varying concentrations of antigen was seen. The concentration of coating antigen chosen for use in the ELISA was 5 μg/ml. The optimal conjugate dilution, however, was found to vary (results not shown) from batch to batch of conjugate preparation necessitating assessment of each batch before use.

The number of times the wells were washed made a considerable difference to the background reading especially when a low conjugate dilution was used. Three washes after each incubation step were optimal in reducing the background reading.

The coefficients of variation between inter- and intra-assay of samples were found to be $<10\%$ at the optimal conditions described above.

In practice, the assay was found to be simple, rapid, specific and reproducible, allowing for large numbers of supernatants to be screened accurately.

1.8 MAINTENANCE OF HYBRIDOMAS

After identifying the cultures with positive antibody production, the cells were transferred to wells in 24-well culture plates; 1 ml medium was added to each well prior to incubation. This was the first step in expanding the cell lines for cloning and freezing. Cells were frozen and stored in liquid nitrogen as soon as a sufficient number of cells was obtained as an insurance against contamination and genetic drift. When required, cells were thawed.

Table 1.2 Mean optical densities obtained after the positive control sample was tested in an ELISA under varying assay conditions.

OPTICAL DENSITY at 498 nm					
Conjugate Dilution	Free α-Subunit Concentration (μg/ml) 10	5	2.5	1	
1/50	0.85	0.97	0.95	1.05	
1/75	0.80	0.69	0.68	0.59	WASH
1/100	0.54	0.26	0.25	0.24	x 1
1/150	0	0	0	0	
1/50	1.65	1.65	1.70	1.83	
1/75	0.91	0.83	0.78	0.66	WASH
1/100	0.33	0.30	0.27	0.16	x 2
1/150	0	0	0	0	
1/50	1.87	1.88	1.80	1.89	
1/75	0.92	0.86	0.82	0.70	WASH
1/100	0.45	0.35	0.29	0.25	x 3
1/150	0	0	0	0	

Results represent means of duplicate samples.

Hybridomas secreting the desired antibody were cloned to reduce the risk of overgrowth by non-producer cells and to ensure homogeneity and monospecificity of the antibody. Cloning by limiting dilution is the most commonly used procedure.

1.8.1 Principle of Cloning Procedure

If cells are grown in small numbers, the fraction of wells with growth should follow the Poisson distribution, $f(0) = e^{-\lambda}$ (Lefkovits and Waldermann, 1979) where f (0) is the fraction of wells with no growth and lambda is the average number of clones per well. If lambda = 1 , then $f(0) = e^{-1} = 0.37$ (natural antilog).

In other words, to obtain a reasonable probability that wells with growth contain single clones, at least 37% of wells should have no growth (this analysis assumes a cloning efficiency of 100% and that there is no clumping of cells).

1.8.2 Materials and Methods

1.8.2.1 Freezing

Cells were prepared for freezing during the log phase of growth. They were pooled, centrifuged (400 *g* for 5 min) and then resuspended in the appropriate culture medium at a concentration of approximately 2 x 10^6 cells/ml. The cell suspension was placed on ice to cool and after cooling an equal volume of cold culture medium containing 20% DMSO (a cryoprotectant) was added dropwise to the cold cell suspension whilst swirling. One millilitre portions of the final cell suspension were dispensed into cryotubes and then placed in a polystyrene box, covered with dry ice and left for 24 hours. The following day the cryotubes were transferred to a liquid nitrogen cryostat (Union Carbide, USA).

1.8.2.2 Thawing

Vials were removed from liquid nitrogen and thawed quickly at 37°C until only a

little ice remained. The thawed cells were added to 9 ml of appropriate culture medium and centrifuged (400 *g* for 5 min). The supernatant was aspirated and the cells resuspended in culture medium to give a cell concentration of 1 - 2 x 10^5 cells/ml. The cells were then incubated at 37°C and the medium changed after 24 hours to ensure that all the DMSO was removed.

1.8.2.3 Cloning

Cells were resuspended, counted and an appropriate plating concentration selected (duplicate plates of each culture at 40 cells/ml and 10 cells/ml were used). Each cell suspension was initially diluted (1/100) in HT medium and further diluted in HT medium supplemented with HUCS. Portions (100 μl) of each cell suspension were distributed into a 96-well plate. When growth was observed in the wells (approximately 7 days after plating), each well was replenished with 100 μl of medium. A sample of supernatant was removed for screening once good growth was visible (approximately 14 days after plating) and wells were replenished with fresh medium. Plates with a percentage growth of less than 60% were used to ensure monoclonality (see section 1.8.1). Clones were then selected (based on clone size and appearance) and transferred to a larger dish for re-cloning and freezing. This procedure was repeated until stable clones were established.

1.8.2.4 Large Scale Cultures

Once the monoclone was established, it was grown in HT medium for a further 2 - 3 passages. The cells were then gradually transferred to progressively larger cultures vessels (6-well plates, followed by 5 ml petri-dishes), taking care to maintain exponential growth. When large cell numbers were achieved, some cells of each clone were frozen. The remaining cells were grown in medium containing half the concentration of HT, then quarter the concentration and finally in normal culture medium; culture supernatants were removed and screened for antibody activity at frequent intervals.

1.8.3 Results and Comment

Hybridomas 71C3 (71) and 75C8 (75) often grew slowly and were intolerant of low cell densities. The volume of each cell culture was therefore expanded gradually, at a rate that was determined empirically. In addition, each hybrid cell line showed a different growth rate. When sufficient cell numbers were obtained cell stocks were frozen in liquid nitrogen and at the same time, some cells were subjected to a cloning procedure.

After the first cloning, two clones (baby clones) were selected from each parent cell line, viz., 71C3C7, 71C3E3, 75C8C4 and 75C8E5. Each baby clone was grown in large numbers and stocks of each were frozen away before recloning. After recloning each clone selected (71C3C7G9, 71C3C7H2, 71C3E3C7, 75C8C4C2, 75C8C4G9, 75C8E5G3) was assumed to be homogeneous and referred to as a monoclone. Each monoclone was grown to large numbers and stocks of each were frozen in liquid nitrogen. Of these monoclones, two (71C3C7G9 and 75C8E5G3) were chosen for peritoneal implants in mice.

Further recloning of the established antibody-producing cell lines became necessary when these cells had been grown for long periods. Somatic mutation or chromosome loss may have occurred because it was observed that some cells of each line lost the ability to produce antibody.

The Poisson distribution shows that if cells are plated at a concentration of 5 cells/ml and growth is observed, the clone must have originated from a single cell (Lefkovits and Waldermann, 1979). Since the cloning efficiency and visibility is seldom 100%, cells were plated at a higher concentration. Cloning efficiency was improved by the presence of 5% HUCS in the culture medium. Recloning was carried out to allow cultures to eliminate any variant cells, especially spontaneous non-secreting variants. To ensure that the hybridomas were stable and single-cell cloned, the cloning procedure was repeated until every well tested was positive.

1.9 PRODUCTION OF ASCITIC FLUID

Large amounts of antibody can be obtained when cells are grown as tumours in appropriate histocompatible mice. The serum or ascitic fluid can contain approximately 1000 times the antibody concentration found in culture (Goding, 1986). In most instances, intraperitoneal injection (i.p.) of 10^6 - 10^7 hybridoma cells into histocompatible mice will result in tumour formation after 2 weeks. The injection of the peritoneal irritant pristane (2, 6, 10, 14-tetramethylpentadecane) (Sigma) a few days prior to injection of the cells, increases the success rate of tumour development and the probability of ascitic fluid formation (Goding, 1986).

1.9.1 Materials and Methods

1.9.1.1 Preparation of Hybridoma Cells

The cells chosen for ascites production were pooled and centrifuged (400 *g* for 5 min). The supernatant was removed and the cells washed once in saline. The cells were then resuspended in a known volume of saline and the cell number adjusted to 2 - 20 x 10^6 cells/ml.

1.9.1.2 Peritoneal Implantation of the Hybridomas

Mice were primed with 0.5 ml pristane i.p. 3 - 10 days prior to injection of cells. Between 1 - 10 x 10^6 cells suspended in 0.5 ml saline were injected i.p. into each mouse. After 14 - 28 days, ascitic fluid was tapped and collected in a clean tube. Cell debris was pelleted by centrifugation and the ascitic fluid collected and stored at -20°C.

1.9.2 Results

An average of between 2 ml and 5 ml ascitic fluid was collected (14 days after inoculation) over a period of one week from each mouse injected with monoclone

71C3C7G9 (71) and 75C8E5G3 (75), respectively.

1.10 ISOLATION OF ANTIBODY

Although ascitic fluid contains a high titre of monoclonal antibody, it also contains a variety of proteins, some of which may be immunoglobulin molecules of unknown specificity. Isolation of the desired immunoglobulin is therefore essential.

Knowledge of the antibody class is useful in determining the method of purification.

1.10.1 Choice of Purification Method

Affinity chromatography on Protein A-Sepharose was the method chosen for the purification of ascitic fluid because it was both readily available and suitable.

1.10.2 Principle

Staphylococcal protein A is a major cell component of most strains of *Staphylococcus aureus*. This protein has the property of binding the Fc-region of IgG with high affinity and specificity (Goding, 1978). Protein A, covalently attached to Sepharose beads serves therefore as a useful matrix for the binding of immunoglobulins. After attachment of the IgG to Protein A, the immunoglobulin is eluted under mild acid conditions.

1.10.3 Materials and Methods

1.10.3.1 Immunoglobulin Subtyping

Immunoglobulins were subtyped by using an anti-mouse monoclonal isotyping kit

(Serotec, Oxford, England) which distinguishes between the IgG, IgM and IgA classes and the different subclasses of IgG, namely IgG1, IgG2a, IgG2b and IgG3.

1.10.3.2 Protein Determinations

The protein concentrations in ascitic fluid were determined by the method of Lowry *et al.* (1951). The electrophoretic composition of the preparations was determined using a Paragon Serum Protein Electrophoresis (SPE) Kit (Beckman). The protein pattern was visually interpreted by densitometry.

1.10.3.3 Purification of Ascitic Fluid

The electrophoretic pattern of the ascitic fluid before purification was first determined. Thereafter the immunoglobulins were precipitated by saturating the fluid with ammonium sulphate (about 400 g/*l*). The precipitate was collected by centrifugation, washed once with 10 ml 40% ammonium sulphate solution and then dissolved in Tris-HCl buffer (1 mol/*l*, pH 9.0). To remove the ammonium sulphate the solution was dialysed against PBS (0.15 mol/*l*, pH 7.2), using Spectrapor membrane tubing (Spectrum Medical Industries Inc., LA, USA) with a molecular weight cut off of 12000 to 14000 daltons.

Immunoglobulin G was recovered from the crude preparation by affinity chromatography on Protein A-Sepharose (Pharmacia Laboratories, Uppsala, Sweden) as described below. A glass column (25 x 1 cm) was packed with Protein A-Sepharose gel and washed thoroughly with 50 ml 0.1 mol/*l* phosphate buffer, pH 8.0. A sample of the crude preparation containing approximately 15 mg of protein was applied to the column and left for a few hours to allow binding of IgG to Protein A. The column was then washed with 30 ml phosphate buffer, pH 8.0 and fractions were collected. Fractions containing high concentrations of protein were pooled (eluate 1). The eluting buffer (30 ml, 0.1 mol/*l* citrate buffer, pH 5.5) was then applied and fractions were collected. Fractions containing high concentrations of protein were pooled (eluate 2). Glycine-HCl (20 ml, 0.2 mol/*l*, pH 4.0) was then added to the column to elute any immunoglobulins that were still bound and fractions were collected. Each of these fractions was immediately neutralized with 1 mol/*l* Tris-

HCl, pH 9.0.

Each pooled fraction was thoroughly dialysed as described previously and the electrophoretic protein pattern of each eluate was determined.

1.10.4 Results and Comment

Both monoclonal antibodies 71 and 75 were found to be of the IgG1 subtype.

Only the purification results of ascitic fluid 75 are presented; purification results of 71 were very similar. The electrophoretic separation of ascitic fluid 75, before purification revealed the presence of a single gammaglobulin peak and various other proteins (Fig. 1.2).

After purification by Protein A affinity chromatography, elution with phosphate buffer yielded some albumin and globulins but no gammaglobulin (Fig. 1.3). The pooled citrate buffer eluate however contained essentially gammaglobulin (Fig. 1.4).

Although there have been reports on the inefficiency of Protein A affinity chromatography in the purification of mouse IgG1 monoclonal antibodies (Goding, 1980), good results were obtained when the above protocol was followed. The use of Protein A-Sepharose in the purification of IgG1 subclass is also favourably reported by Ey *et al.* (1978). The results in this study are clearly not in doubt and hence this technique was adopted for isolation.

1.11 SUMMARY

Using the mouse hybridoma technique two clones secreting monoclonal antibodies to the free α-subunit of hCG were successfully cultured. These hybridoma clones were identified after screening all culture supernatants in an optimised α-subunit ELISA. Each hybrid was single-cell cloned several times until homogeneous stable

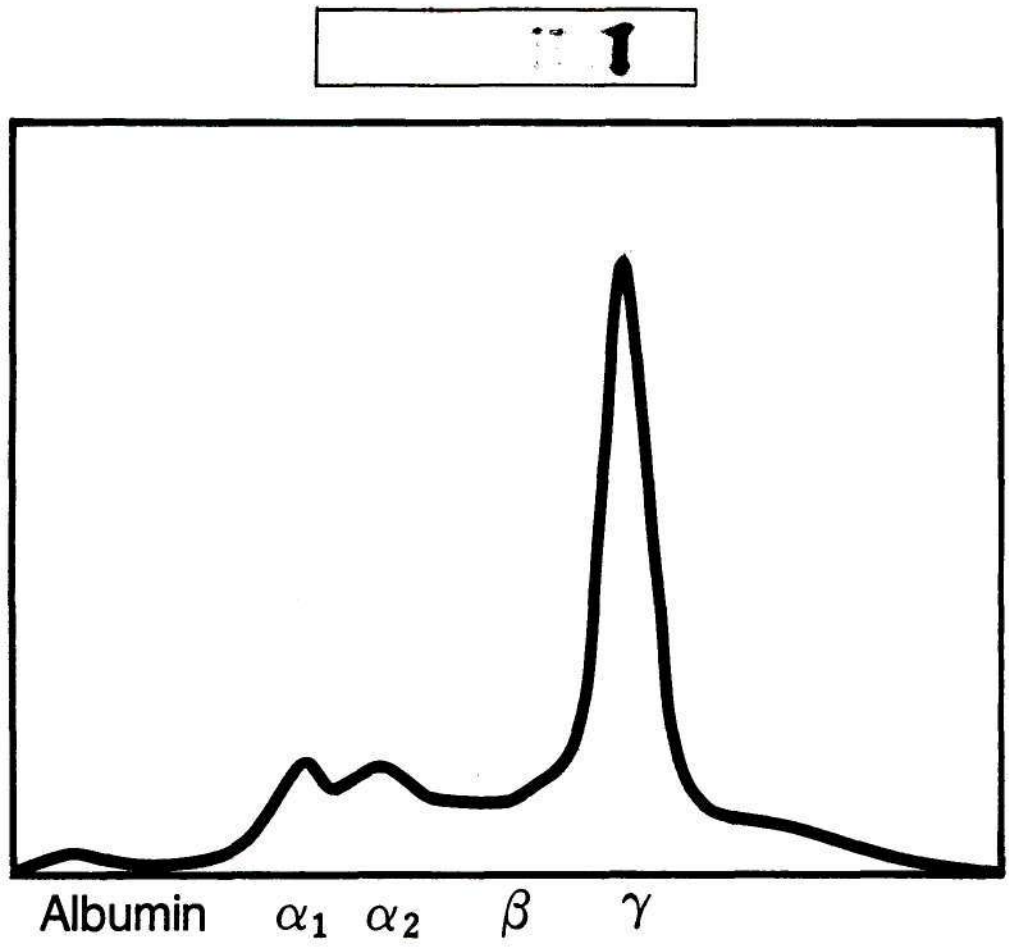


Figure 1.2 Electrophoretic pattern of ascitic fluid before purification, showing albumin, $\alpha_1\alpha_2$, ß and gammaglobulins.

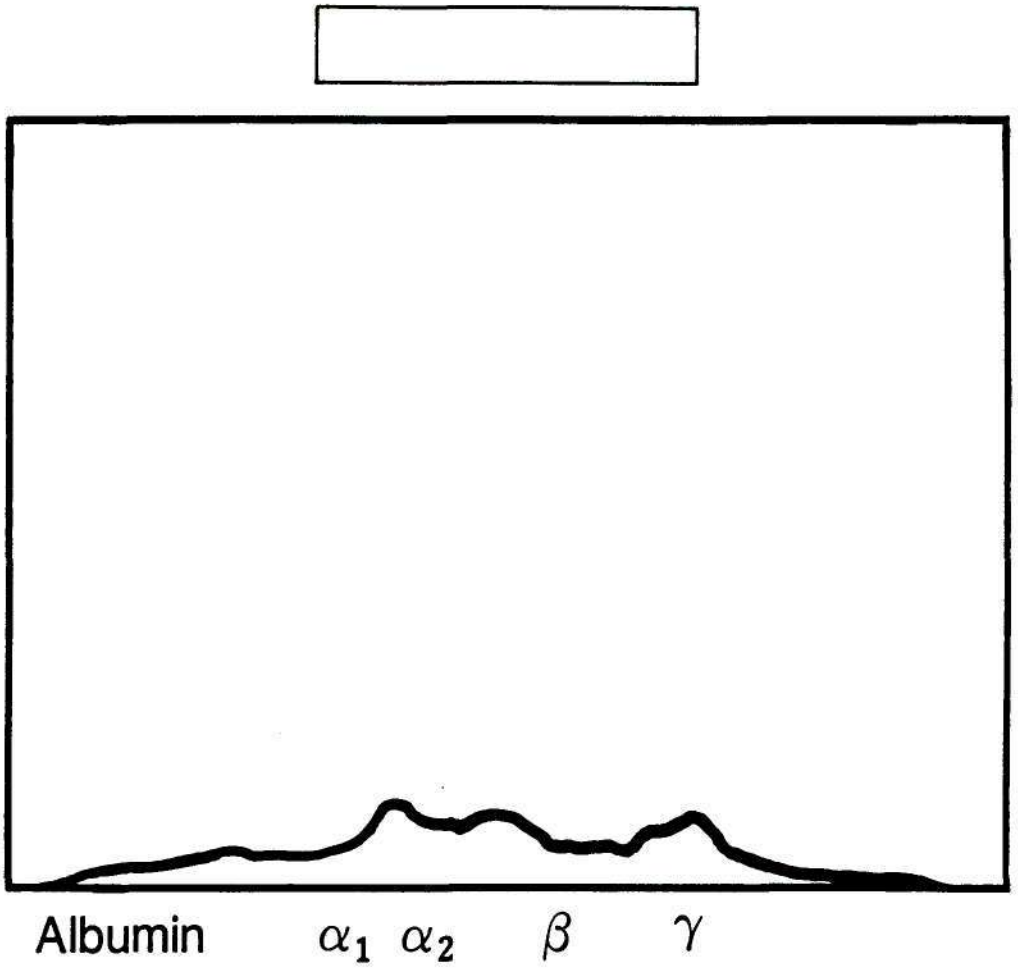


Figure 1.3 Electrophoretic protein pattern of eluate 1 obtained after washing with 0.1 mol/*l* phosphate buffer, pH 8.0.

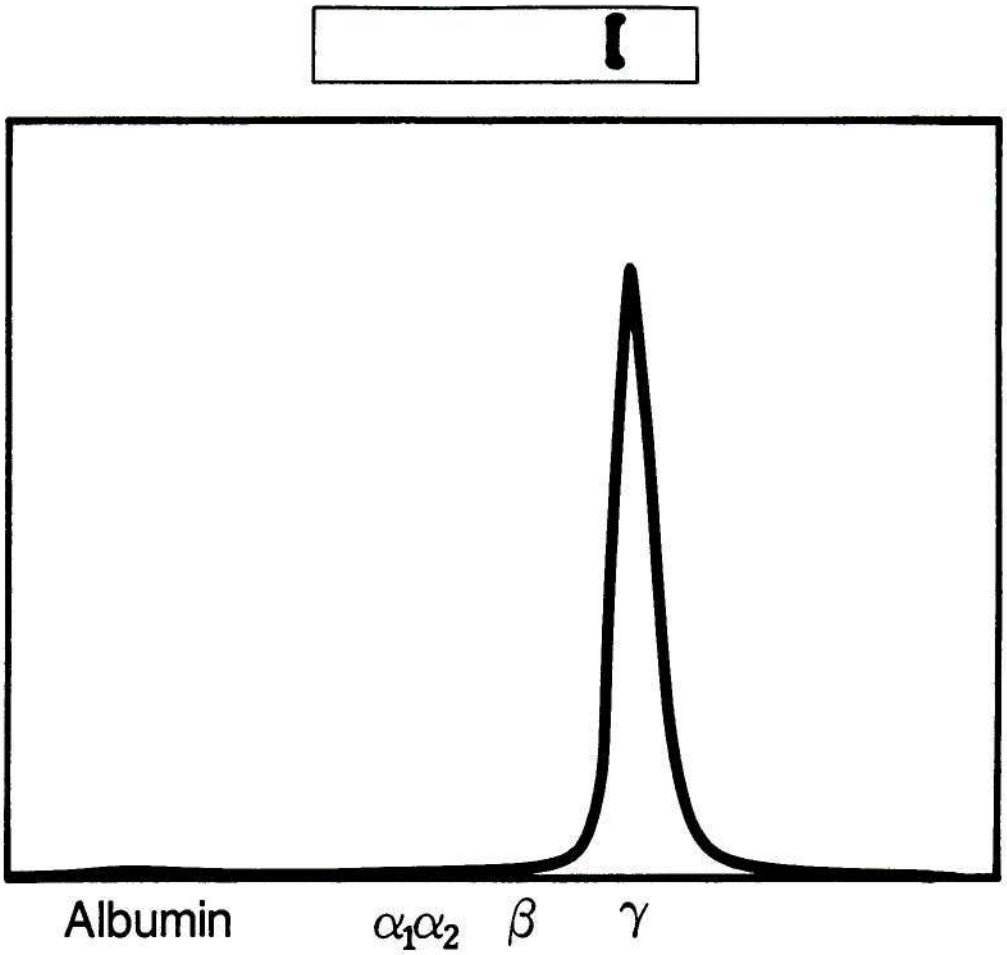


Figure 1.4 Electrophoretic protein pattern of eluate2 after elution with 0.1 mol/*l* citrate buffer, pH 5.5, showing a purified preparation of gammaglobulin.

cell lines were established. The monoclones were expanded and used for large-scale production of antibody in ascitic fluid. On subtyping, both antibodies were found to be of the IgG1 subclass. Immunoglobulin G was recovered from ascitic fluid by affinity chromatography on Protein-A Sepharose.

CHAPTER 2

CHARACTERISATION OF THE MONOCLONAL ANTIBODIES TO THE FREE ALPHA-SUBUNIT OF HCG

The man of science has learned to believe in justification, not by faith, but by verification.

Thomas Huxley [1825 - 1895]

2.1 PURPOSE OF THE STUDY

Monoclonal antibodies to the free α-subunit of hCG were raised and purified as described in the preceding chapter. The next step was to assess the quality of these antibodies to determine whether they meet the requirements of a specific and sensitive assay system.

The purpose of this study therefore was to examine the two monoclonal antibodies (71 and 75) that were reactive with the free α-subunit of hCG and characterise them with respect to their specificity, affinity, ability to recognise distinct epitopes on the free α-subunit molecule and display cooperativity in binding. The characterisation of another monoclonal antibody, designated antibody 42 (described by Norman *et al.*, 1985) which is also reactive to the free α-subunit of hCG, is included in this study for purposes of comparison.

2.2 SPECIFICITY OF THE MONOCLONAL ANTIBODIES

The specificity of the monoclonal antibodies for the free α-subunit was determined

by assessing cross-reaction with intact hCG and related glycoprotein hormones. Since an epitope present on the free α-subunit may also be exposed on the intact molecule, the specificity of the monoclonal antibody is defined by its ability to recognise an epitope on the α-subunit that is hidden in the intact dimeric form.

2.2.1 Choice of Method

The cross-reaction of the three monoclonal antibodies with the related glycoprotein hormones was assessed by a method which uses limiting concentrations of antibody.

2.2.2 Materials and Methods

Cross-reaction was expressed in percentage form as the ratio of intact glycoprotein hormone to free α-subunit which caused 50% inhibition of binding of a given quantity of radiolabelled free α-subunit to antibody when the antibody concentration was the limiting factor.

The dilution of antibody which bound 50% of the measured amount of labelled free α-subunit was determined as a first step. This was done by diluting the antibody preparation serially (dilutions ranged from 1/20 to 1/5120 depending on the antibody) in phosphate buffer (0.067 mol/*l*, pH 7.4). To 100 μl of each dilution, 100 μl (approximately 4 x 10^4 cpm) [^{125}I]-free α-subunit (iodination procedure described in section 3.4) were added. The tube contents were thoroughly mixed and left overnight at 4 °C.The following day, labelled free α-subunit bound to antibody was precipitated by the addition of 200 μl 1% gammaglobulin (Sigma) and 1.0 ml 20% PEG (Merck). After vortex mixing, the precipitate was collected by centrifugation (1000 *g* x 10 min), the supernatant discarded and the tubes drained. The bound radioactivity in the precipitate was measured in a Berthold multihead gamma spectrometer. The percentage radioactivity bound was plotted against the antibody dilution and the dilution of antibody which bound 50% of the radiolabelled free α-subunit was read off the graph. This limiting antibody dilution was then used in the measurement of cross-reactions with the related glycoprotein hormones.

Antibody (100 μl) at the appropriate dilution and 100 μl [^{125}I]-free α-subunit (approximately 4 x 10^4 cpm) were incubated with 100 μl volumes of increasing concentrations (ranging from 0.05 to 100 ng/ml) of unlabelled free α-subunit of hCG (CR123 preparation; section 3.3), hCG, LH, FSH and TSH (2nd IRP, a gift of National Pituitary Programme, National Institute of Health, USA) standards in phosphate buffer (0.067 mol/*l*, pH 7.4) at 37 °C for 4 hours. The bound complex was precipitated by the addition of 200 μl 1% gammaglobulin and 1.0 ml 20% PEG and mixed well before centrifugation (1000 *g* for 10 min). The supernatant was decanted and the bound radioactivity in the pellet counted in a Berthold gamma spectrometer.

The ratio (mass/mass) of glycoprotein hormone to free α-subunit expressed as a percentage which yielded a 50% inhibition of binding of the labelled free α-subunit to antibody was then calculated.

2.2.3 Results and Comment

The antibody dilution curves for antibodies 71 and 75 are shown in Figs. 2.1 and 2.2, respectively. The dilutions of antibodies 71 and 75 which bound 50% of labelled α-subunit were read as about 1/130 and 1/1000, respectively, and these dilutions were used in the cross-reactivity studies.

Crossreactivities with the glycoprotein hormones as calculated for the three antibodies are presented in Table 2.1; results are expressed as a percentage mass/mass ratio.

Both antibodies 75 and 71 showed little cross-reaction to the intact glycoprotein hormones and are therefore more likely to recognise epitopes on the free α-subunit which are hidden by the folding of the ß-subunit in the intact molecule. Antibody 71 showed a high cross-reaction with FSH initially, but after purification of the FSH preparation on Sephadex G100 (described in detail in section 3.6), the cross-reactivity decreased by 40 - 50%.

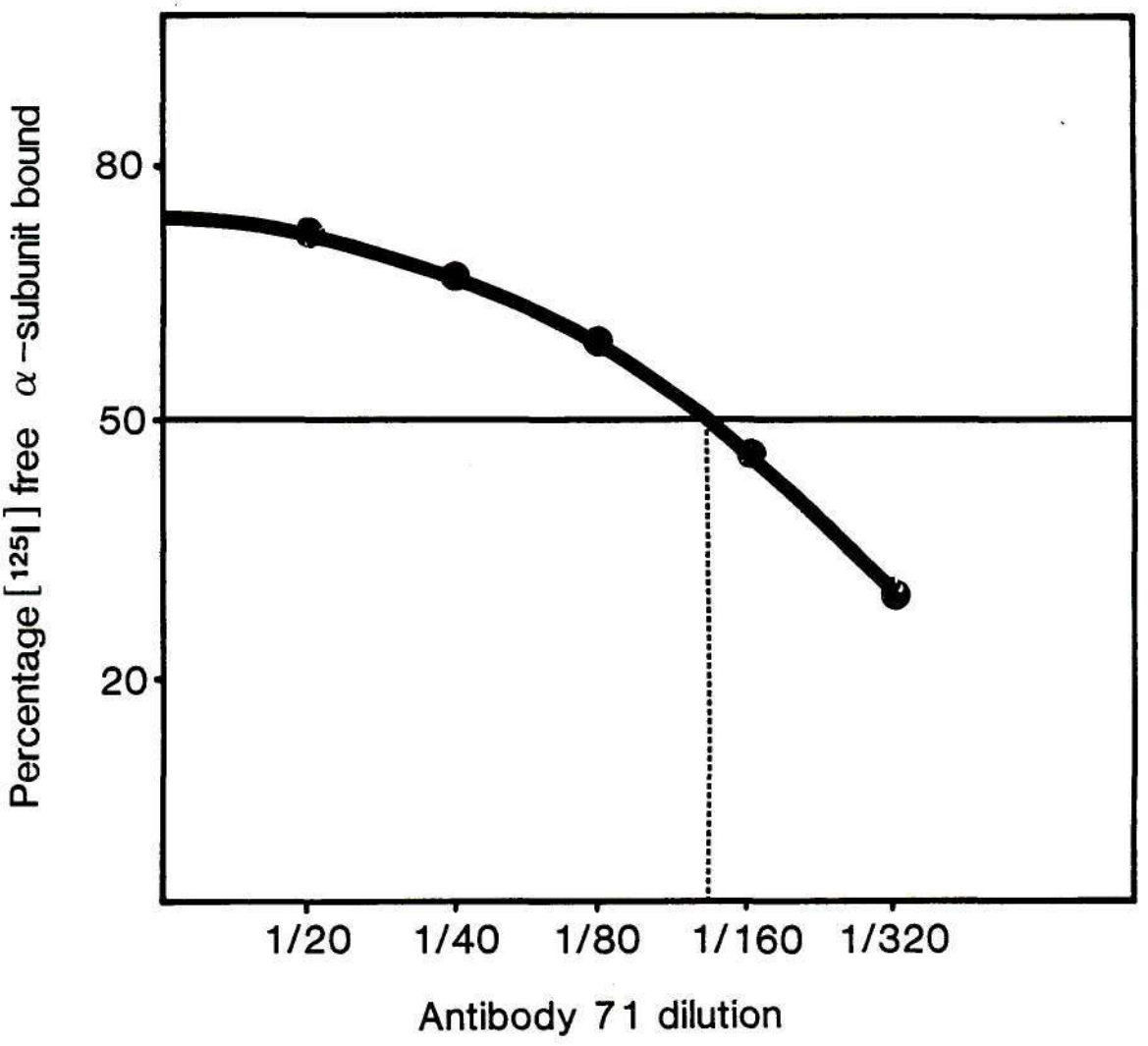


Figure 2.1 Antibody dilution curve of antibody 71 showing the dilution which bound 50% of the free α-subunit.

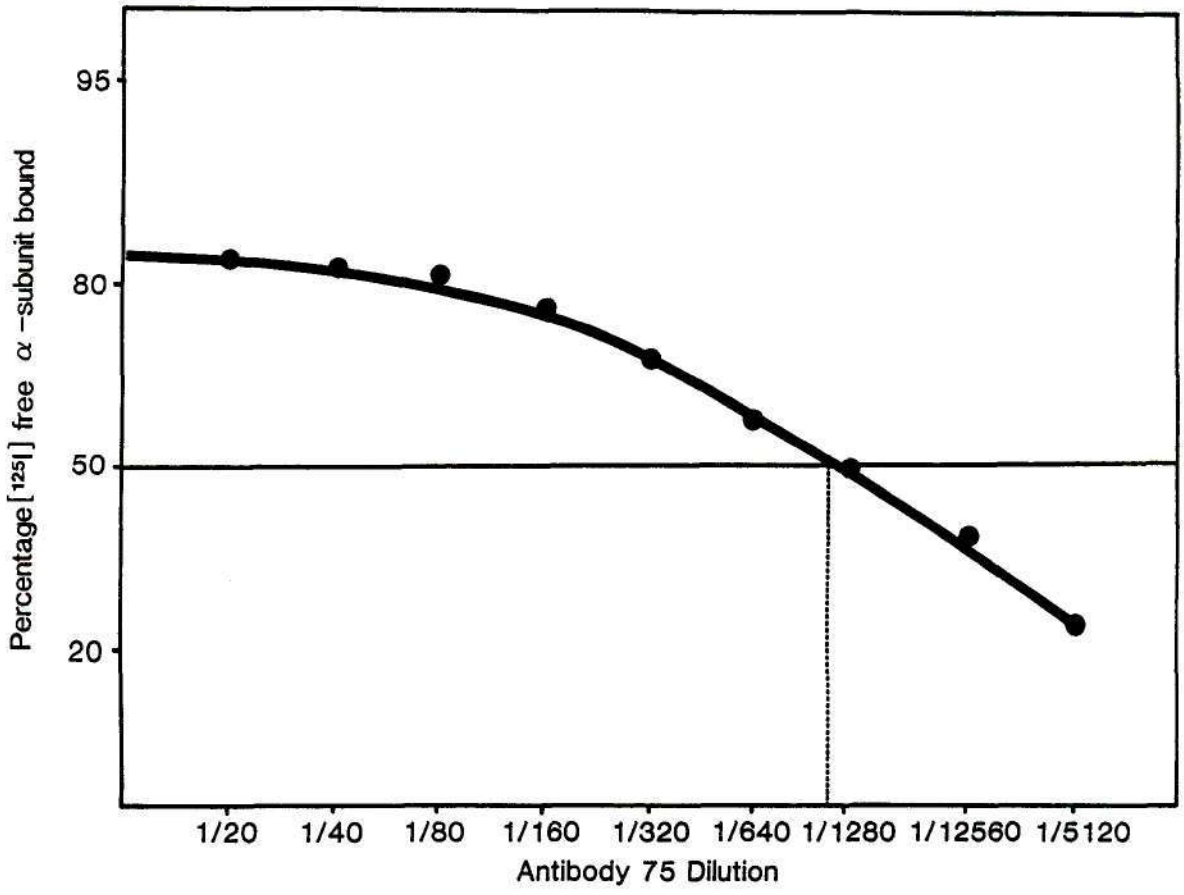


Figure 2.2 Antibody dilution curve of antibody 75 showing the dilution which bound 50% of the free α-subunit.

Table 2.1 Percentage cross-reactivity of the anti-α antibodies with the intact glycoprotein hormones.

	α-subunit	hCG	FSH	LH	TSH
antibody 75	100%	3%	5%	8%	15%
antibody 71	100%	5%	25%	14%	4%
antibody 42	100%	330%	300%	100%	300%

Cross-reactivity of the intact glycoprotein hormones is expressed as a percentage relative to binding of the free α-subunit (arbitrarily represented as 100%).

Antibody 42 on the other hand showed more binding to the intact glycoprotein hormones than to the free α-subunit. It would appear that this antibody recognises an epitope that is preferentially exposed when the α-subunit is combined with the ß-subunit. This non-specificity for the free α-subunit renders it of little use in an α-subunit immunoassay.

2.3 BINDING AFFINITIES OF THE MONOCLONAL ANTIBODIES

The strength or affinity of binding of an antibody to an antigen can be assessed in several ways. The method used in this study was that of Scatchard (1949).

2.3.1 Principle

Using Scatchard analysis the affinity of an antibody for its antigen is determined by plotting the ratio of bound to free antigen against the concentration of bound antigen. A straight line indicates that only one binding site or epitope is recognised while the slope reflects the affinity of binding; the ratio of the y and x intercepts (y/x) estimates the association constant, K_a, and the reciprocal x/y the dissociation constant, K_d. The relationship between the dissociation constant, or alternatively the association constant, and the affinity of the binding may be explained as follows:

In terms of the law of mass action, the rate of a reaction in dilute solutions is proportional to the concentration of the reactants and is represented by the equation:

$$[\text{antigen}] + [\text{antibody}] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [\text{antigen-antibody complex}]$$

where k_1 and k_{-1} represent the association and dissociation rate constants, respectively.

The forward reaction is a second order reaction in which the rate constant k_1 is

proportional to the product of the concentrations of the reactants and has the dimensions $\frac{1}{\text{conc x time}}$, usually $l\text{mol}^{-1}\text{s}^{-1}$. The reverse reaction is a first order reaction in which the rate constant k_{-1} is proportional to the concentration of only one reactant and has the dimensions of the reciprocal of time, usually s^{-1}.

At equilibrium the rate of the forward reaction is equal to the rate of the reverse reaction, that is,

k_1 [Ag] [Ab] = k_{-1} [AbAg]

The equilibrium constant of dissociation can therefore be determined from the above equation:

$$\frac{[Ab]\,[Ag]}{[AbAg]} = \frac{k_{-1}}{k_1} \quad \frac{s^{-1}}{l\text{mol}^{-1}\text{s}^{-1}}$$

$$= K_d \text{ mol}/l$$

Since K_d is the reciprocal of K_a, it follows that the smaller the value of the K_d the greater will be the K_a or binding affinity.

2.3.2 Materials and Method

The concentration of each antibody used was that which bound 50% of the radiolabelled free α-subunit (section 2.2.2). Portions (100 μl) of antibody were incubated with 100 μl [^{125}I]-free α-subunit (about 0.38 ng, 40 000 cpm) and 100 μl amounts of increasing concentrations of unlabelled free α-subunit, ranging from 0 to 2000 ng/ml. The non-specific binding (NSB) was estimated by incubating 200 μl phosphate buffer (0.067 mol/*l*, pH 7.4) and 100 μl [^{125}I]-free α-subunit. The incubation procedure and precipitation of bound radioactivity using gammaglobulin and PEG were as described in section 2.2.2. The concentration of bound labelled free α-subunit (B) was determined by multiplying the proportion of labelled α-subunit bound by the concentration (mol/*l*) of the total α-subunit (labelled and unlabelled) available for binding. The unbound labelled free α-subunit (F) was calculated by subtracting the amount bound in cpm (specific and non-specific) from the total counts (T). The ratio of B/F was plotted against concentration of B for

each antibody using linear regression analysis.

2.3.3 Results and Comment

Scatchard plots of both antibodies 71 and 75 were straight lines indicating the presence of a single class of binding site or epitope. The dissociation constants of these two antibodies were 0.92 x 10^{-8} and 4.08 x 10^{-8} mol/*l*, respectively. The binding data for antibodies 71 and 75 are shown in Figs. 2.3 and 2.4, respectively. The dissociation constant of antibody 42, calculated by Norman *et al.* (1985) was 1.0 x 10^{-7} mol/*l*.

All three antibodies showed relatively low affinities for the α-subunit which is not unexpected for monoclonal antibodies (Soos and Siddle, 1982). The binding affinities of antibodies 71 and 75, obtained in this study, differ from the data published by Norman *et al.* (1987) due to an error in the calculation of the dissociation constants in the previous study. In the current study antibody 71 showed an approximately 4 and 10 fold higher affinity for the free α-subunit than antibodies 75 and 42, respectively.

The comparatively low affinities of the monoclonal antibodies do not limit their value in immunoassays. Antibody affinity is not critical for the sensitivity of an immunoradiometric assay as the antibody is added in excess to maximise reaction with antigen (Miles and Hales, 1968). In this context therefore, the monoclonal antibodies 71 and 75, if produced in large quantities could be of great value.

2.4 EPITOPE ANALYSIS

A detailed analysis of the antigen binding sites for the different antibodies was carried out to determine whether the three antibodies recognise separate and distinct epitopes.

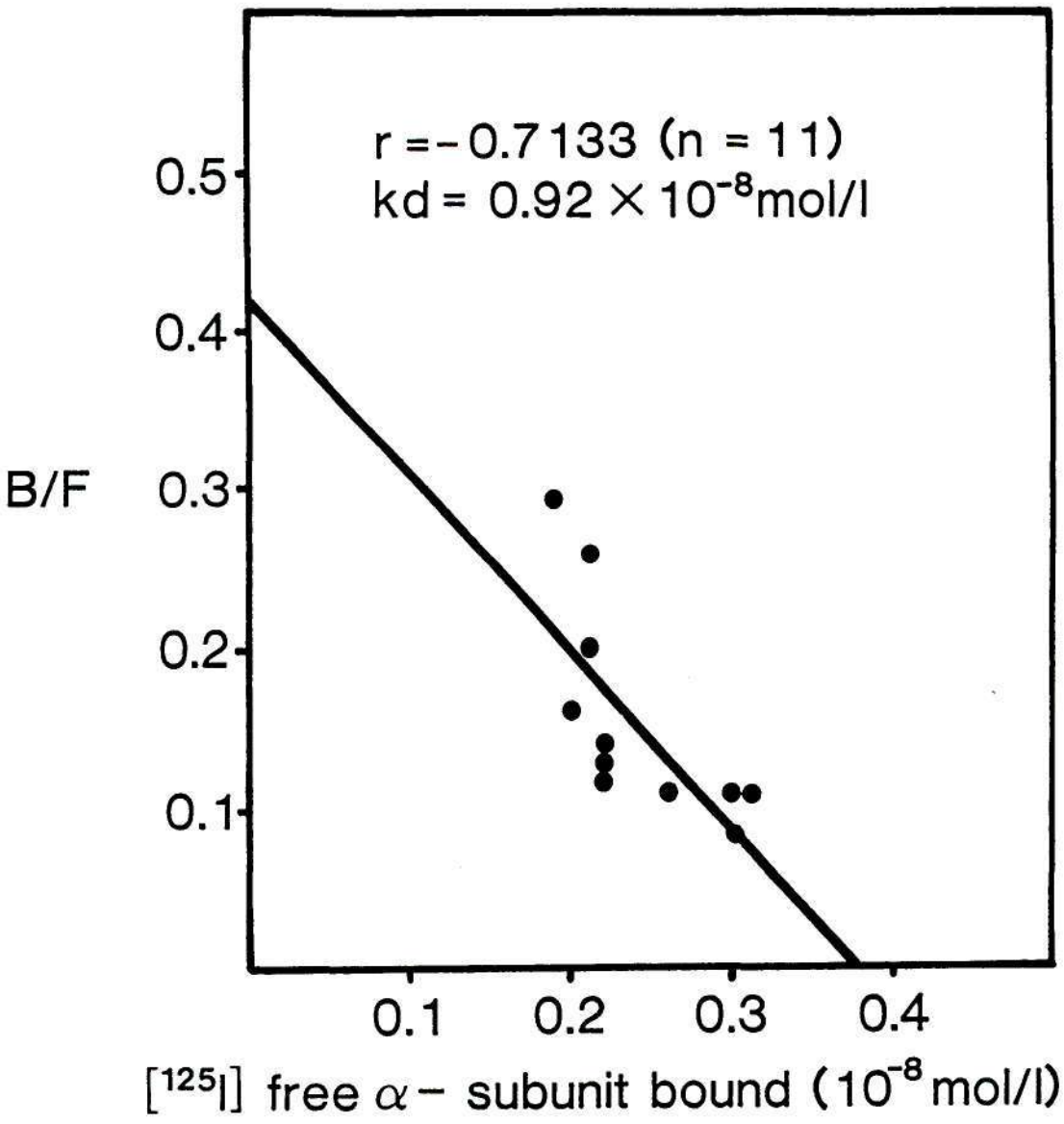


Figure 2.3 Scatchard plot of antibody 71. The concentration of antibody 71 which bound 50% of [^{125}I] α-subunit was incubated with labelled α-subunit and increasing concentrations of unlabelled α-subunit. Bound label (B) was measured and the unbound or free [^{125}I] α-subunit (F) was calculated by subtracting B from the total counts (T). The dissociation constant (K_d) was determined by calculating x/y intercepts.

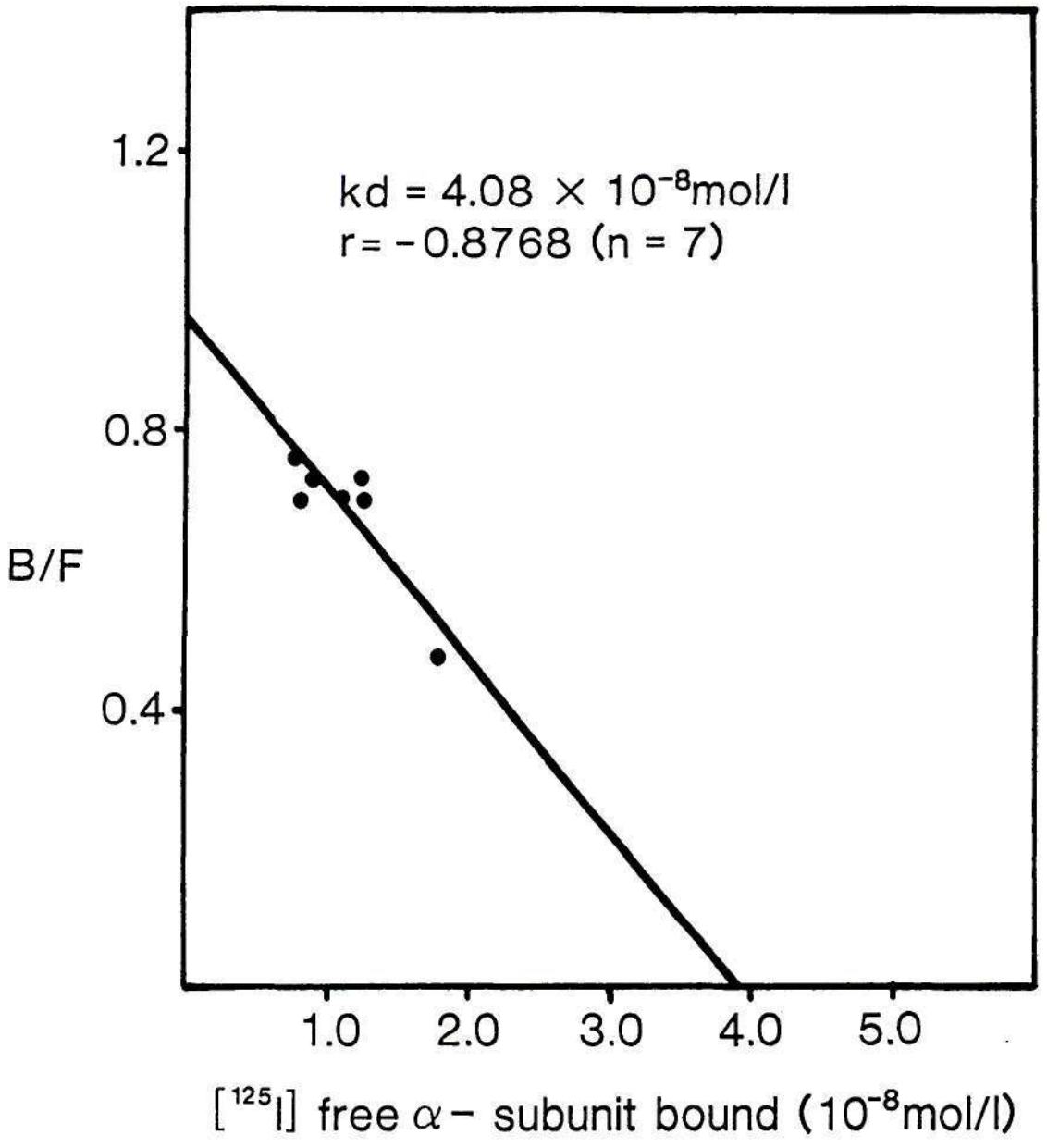


Figure 2.4 Scatchard plot of antibody 75. The concentration of monoclonal antibody 75 which bound 50% of [^{125}I] free α-subunit was incubated with labelled α-subunit and increasing concentrations of unlabelled α-subunit. Bound label (B) was measured and the unbound or free [^{125}I] α-subunit (F) was calculated by subtracting B from the total counts (T). The dissociation constant (K_d) was determined by calculating x/y intercepts.

2.4.1 Principle

The ability of pairs of antibodies to bind simultaneously to an antigen is assessed when one antibody, immobilised on a solid phase (ELISA plate or cellulose) binds the antigen and having done so, exposes an epitope on the antigen which binds to another radiolabelled antibody. The amount of label bound is a measure of two-site binding and hence antibody recognition of two distinctly different antigenic determinants.

2.4.2 Materials and Methods

All possible combinations of labelled and solid phase antibodies were assessed for two-site binding by the following two methods.

2.4.2.1 ELISA Plate as Solid Phase

One antibody was coated on to microtitre wells as described in the ELISA method (section 1.7); the final amount of antibody bound, determined empirically, was 5 μg/well. The other antibody was labelled with ^{125}I (Amersham) using the Chloramine-T method as described in section 3.4.

One hundred microlitres of free α-subunit (0.5 mg/l in 0.067 mol/l phosphate buffer, pH 7.4) or phosphate buffer as NSB were added to the wells and incubated at 37 °C for 7 hours. The wells were then washed 3 times with Tris-saline-Tween (TST) buffer, pH 8.0 (section 1.7). Labelled antibody (about 100 000 cpm) was added to each well and the plates incubated overnight at 4 °C.The wells were washed 3 times with TST buffer, pH 8.0 to remove unbound radioactivity. Each well was then cut out individually, placed in polystyrene tubes and the amount of radioactivity bound to the wells measured in a Berthold multihead gamma spectrometer. This procedure was carried out with antibodies 71, 75 and 42 in the 9 possible combinations.

2.4.2.2 Cellulose as Solid Phase

Cellulose was activated using 1.1-carbonyldiimidazole which derivatises cellulose and hence enables coupling to antibody (Chapman *et al.*, 1983).

Five grams of microparticulate cellulose (Sigmacell type 20, Sigma, London, UK) were activated by adding 1.1-carbonyldiimidazole (Sigma) (0.61 g/25 ml 0.15 mol/*l* acetone) and the mixture vigorously stirred at room temperature for 1 hour. The activated imidazolyl carbamate cellulose was recovered by filtration over a glass microfibre filter (Whatman GF/A, retention efficiency 2 μm, Whatman Labsales LTD., UK), washed with three 100 ml portions of acetone, dried in air and stored in a tightly sealed container at -20°C until required for protein coupling.

Two hundred milligrams of activated cellulose and 4.5 mg of antibody in 3.0 ml borate buffer (0.1 mol/*l* $Na_2B_4O_7.10H_2O$, pH 9.0) were vortex shaken to a slurry and then placed on a rotating shaker at room temperature for 16 hours. After the reaction, the protein remaining in solution was recovered by centrifugation (1200 *g* for 20 min) and the concentration in the supernatant measured by the method of Lowry *et al.* (1951). The percentage of protein coupled to cellulose was calculated by difference. The pellet was washed by centrifugation with 10 ml portions of several buffers in the sequence set out below:
bicarbonate buffer (0.5 mol/*l* NH_4HCO_3, pH 8.0), rotated for 20 min; repeated;
acetate buffer (0.1 mol/*l* CH_3COONH_4, pH 4.0), rotated for 20 min;
acetate buffer (pH 4.0) rotated 16 - 20 hours;
phosphate buffer (0.067 mol/*l*, pH 7.4, containing 1% BSA, 0.1% Tween-20 and 0.1% sodium azide) rotated for 20 min; repeated. The cellulose-bound antibody was stored at 4°C until required.

One hundred microlitres of a suspension of cellulose-bound antibody in about 16 volumes of phosphate buffer and 100 μl free α-subunit (5 μg/ml) in phosphate buffer were mixed and rotated overnight at room temperature. The NSB was measured by substituting 100 μl buffer for free α-subunit in one set of tubes. The incubate was centrifuged and the resulting pellet washed twice with 1.0 ml phosphate buffer (0.067 mol/*l*, pH 7.4 containing 0.1% BSA, 0.1% Tween-20 and

0.1% sodium azide). This was followed by a further incubation with 100 μl [^{125}I]-labelled antibody to the α-subunit (approximately 100 000 cpm, section 3.4) for 8 hours. After washing, the incubate was centrifuged at 3000 *g* for 10 min and the supernatant decanted and the tubes drained. Bound radioactivity in the cellulose pellet was counted in a Berthold gamma spectrometer.

2.4.3 Statistics

Each experiment was performed in quadruplicate and the significance of the differences in cpm, between the sample and NSB was tested by applying the unpaired t-test.

2.4.4 Results and Comment

The binding data of the three antibodies to the free α-subunit in a two-site assay in which the stationary medium was varied are presented in Table 2.2.

There are a number of relevant observations arising from these results. Firstly, enhanced binding occurred with combinations of different antibodies, particularly antibodies 71 and 75, irrespective of which solid-phase was used. No further binding took place when the same antibody was used in combination. It can therefore be concluded that each antibody is directed at a separate epitope on the free α-subunit molecule.

Secondly, binding of the free α-subunit to antibody 42, a relatively non-specific antibody (section 2.2.3) was generally very low. It would appear that antibody 42 binds at an epitope which may be near the epitopes of antibodies 71 and 75 and thus sterically interferes with binding. Alternatively, tertiary structural changes in the free α-subunit after binding to antibody 42 may obscure the epitopes for antibodies 71 and 75. In either event, antibody 42 is manifestly unsuitable for use in a two-site assay with either antibodies 71 and 75.

Table 2.2 Bound radioactivity measured in cpm after simultaneous incubation of pairs of anti-α antibodies with free α-subunit.

Solid-phase-bound antibody	Labelled Antibody		
	42	71	75
ELISA plate			
42	18	928*	726*
71	47	383	24459*
75	714*	27158*	24
Cellulose			
42	0	2980*	5941*
71	706*	0	37490*
75	1047*	45736*	0

Results shown indicate the difference in cpm between 500 ng and 0 ng of free α-subunit (NSB); * indicates a significant difference ($p < 0.05$).

By contrast antibodies 71 and 75 function exceptionally well in a two-site assay. It seems immaterial whether they are used as liquid or solid-phase antibodies, nor does the nature of the solid-phase seem to have any effect on the binding efficiency.

2.5 ASSESSMENT OF COOPERATIVE BINDING

The possibility of the phenomenon of cooperative binding occurring with the antibodies raised in this study was examined by comparing the binding following simultaneous incubation of free α-subunit with antibodies 71 and 75 or antibody 75 and a non-specific antibody in a radioimmunoassay (RIA).

2.5.1 Principle

The ability of two antibodies which bind two distinct epitopes on an antigen to form a circular complex resulting in enhanced affinity and sensitivity of measurement, is referred to as cooperative binding (Moyle *et al.*, 1983b).

2.5.2 Materials and Method

Antibody 75 coupled to cellulose (section 2.4.1.2) was diluted 100 fold in assay buffer (phosphate 0.067 mol/*l*, 0.1% BSA, 0.01% Tween-20; pH 7.4). One hundred microlitres of diluted antibody were incubated for 4 hours at 37°C with 100 μl of [^{125}I]-labelled free α-subunit (approximately 30 000 cpm) or buffer as NSB. Various dilutions (1/6 to 1/768) of antibody 71 in 100 μl portions or the mouse monoclonal antibody 2/6 (which recognises the ß-subunit of hCG but not α-subunit) as control were then added and further incubated for 4 hours at 37°C. The incubates were centrifuged and the pellet washed once with assay buffer. After centrifugation the radioactivity in the pellets was counted.

2.5.3 Results and Comments

Results shown in Table 2.3 indicate that the addition of a specific second monoclonal antibody, in this case antibody 71, led to a higher binding of the free α-subunit by the solid-phase-bound antibody 75 when compared to the addition of the 'non-specific' monoclonal antibody 2/6 as control.

It would appear therefore that antibodies 75 and 71 display cooperativity in binding to the antigenic molecule, that is, addition of 71 to an incubate of radiolabelled α-subunit bound to 75 induced further binding of the α-subunit (up to 250% at certain dilutions of antibody). This enhancement in binding may be explained by the phenomenon of circular complex formation described by Moyle *et al.* (1983b). The binding of the second specific antibody to the antigen is thought to induce circular complexes which render the epitopes present on the antigen molecule more accessible to further binding with the solid-phase antibody. Using the proposals of Moyle *et al.* (1983a) a possible structure for such a circular complex is illustrated in Fig 2.5.

2.6 SUMMARY

Two monoclonal antibodies 71 and 75 were characterised with respect to specificity, affinity for the α-subunit, ability to recognise different epitopes and display co-operativity in binding. A comparison was made with the published data on a third monoclonal antibody (antibody 42). Antibodies 71 and 75 showed a high degree of specificity for the free α-subunit, implying that they recognise epitopes hidden by the folding of the ß-subunit in the intact dimeric protein. Antibody 42 appeared to recognise an epitope that was preferentially exposed on the intact molecule of hCG, TSH, FSH and LH since the cross-reaction of this antibody with the intact hormone was greater than that with the isolated α-subunit.

Both antibodies 71 and 75 showed comparatively low affinities for the α-subunit molecule, with dissociation constants of 0.92×10^{-8} and 4.08×10^{-8} mol/*l*, respectively.

Table 2.3 Bound radioactivity measured in cpm after the incubation of a second antibody with antibody 75 and radiolabelled free α-subunit in a RIA.

Dilution of antibody	Second Antibody	
	71	2/6
1:6	8000 (208)*	4904 (127)
1:12	8855 (230)	4072 (106)
1:24	9897 (257)	3721 (97)
1:48	8925 (232)	3980 (104)
1:96	9102 (237)	4214 (110)
1:192	9759 (254)	3022 (79)
1:350	9454 (246)	3245 (85)
1:768	9703 (253)	5501 (143)

* Results are shown as radioactive counts after precipitation of the pellet (results in parentheses indicate non-specific binding).

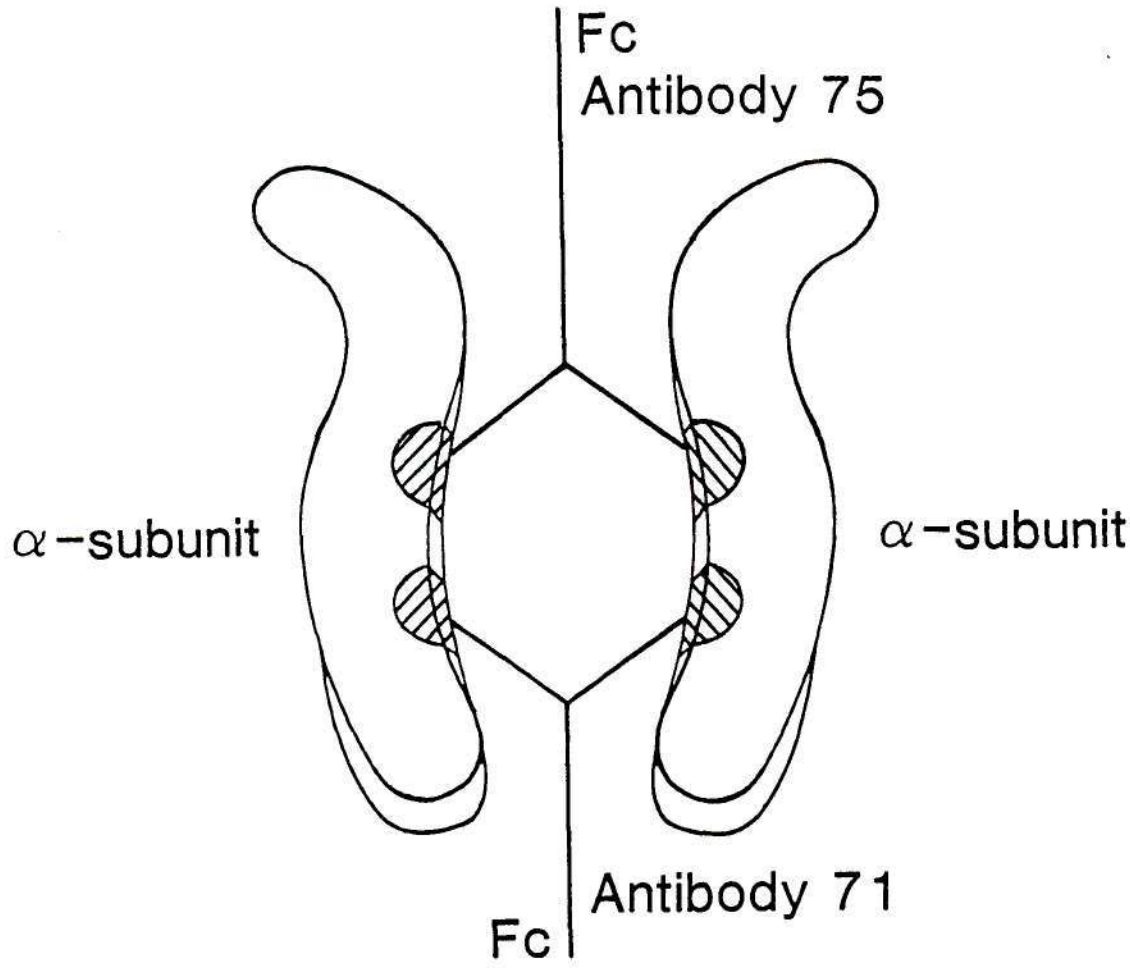


Figure 2.5 Circular complex formation of antibodies 71 and 75 with two molecules of the free α-subunit. This diagram represents a possible circular complex that is proposed to be responsible for the binding enhancement observed in some mixtures of monoclonal antibodies.

Pairwise combinations of the three antibodies were shown to bind simultaneously to the α-subunit, indicating that they recognise three distinctly different epitopes on the molecule. The two antibodies 71 and 75 displayed cooperativity in binding as demonstrated by an enhancement in binding to the free α-subunit when incubated simultaneously.

CHAPTER 3

THE DEVELOPMENT OF AN IMMUNORADIOMETRIC ASSAY FOR THE MEASUREMENT OF FREE ALPHA-SUBUNIT OF HCG

Good methods can teach us to develop and use to better purpose the faculties with which nature has endowed us, while poor methods may prevent us from turning them to good account. Thus the genius of inventiveness, so precious in the sciences, may be diminished or even smothered by a poor method, while a good method may increase and develop it.

Claude Bernard [1813 - 1878]

3.1 INTRODUCTION

The use of monoclonal antibodies in the immunoradiometric assay (IRMA) format (Norman *et al.*, 1985), has led to greatly increased sensitivity and specificity in a variety of hormone assays. This chapter describes the development of an IRMA for the measurement of free α-subunit of hCG using monoclonal antibodies raised and characterised as described in chapters 1 and 2.

3.1.1 Principle

In the commonly used two-site IRMA, the antigen becomes 'sandwiched' between two antibodies that are directed against distinct and sterically distant epitopes. One antibody, called the 'capture antibody', is usually bound to a solid phase matrix (plastic, cellulose, etc.) and its concentration exceeds that of the antigen. This antibody binds to the antigen in amounts proportional to the concentration of the

antigen in the sample to be analysed. The bound antigen is subsequently detected by a second antibody, designated the 'detection antibody', which carries a label such as a radioactive isotope or an enzyme.

3.1.2 Choice of Method

An ideal assay procedure is one which is simple, robust, specific and highly sensitive with a wide working range. The classical two-site IRMA is a two step procedure in which the first incubation step involves extraction of the antigen with a solid-phase coupled antibody and the second allows binding of radiolabelled antibody with the coupled antigen. The use of monoclonal antibodies in the IRMA format offers the advantage of increased specificity for the antigen. In addition, use of a solid-phase medium for separation, and a capture antibody in excess, provide for optimal binding of analyte and thorough washing at the separation stage. Both these factors are advantageous in providing high sensitivity, at least 3 orders of magnitude higher than in RIAs, and a wide assay working range.

Odell *et al.*(1986) described a unique assay design that is both simple and of greater sensitivity than the common IRMA. This assay procedure uses polystyrene beads and the avidin-biotin reaction as a solid-phase separation system. The capture antibody labelled with biotin is initially in the liquid phase and is thus a modification of the conventional two-site assay. The design of the IRMA is shown in Fig 3.1 which schematically represents a polystyrene bead coated with BSA, biotinylated BSA and avidin after incubation with biotinylated capture antibody, free α-subunit and radiolabelled detection antibody.

Biotin has a high affinity (K_d = 10^{-15} mol/l) for avidin and the reaction between the two molecules results in the formation of a very stable avidin-biotin complex. Avidin has four binding sites; when used in a two-site IRMA it acts as a bridge between two biotinylated reagents (eg. biotinylated BSA and biotinylated first or capture antibody) linking both antibodies and analyte to solid-phase. The two remaining sites on the avidin provide additional amplification and allow for further binding of capture antibody to solid-phase.

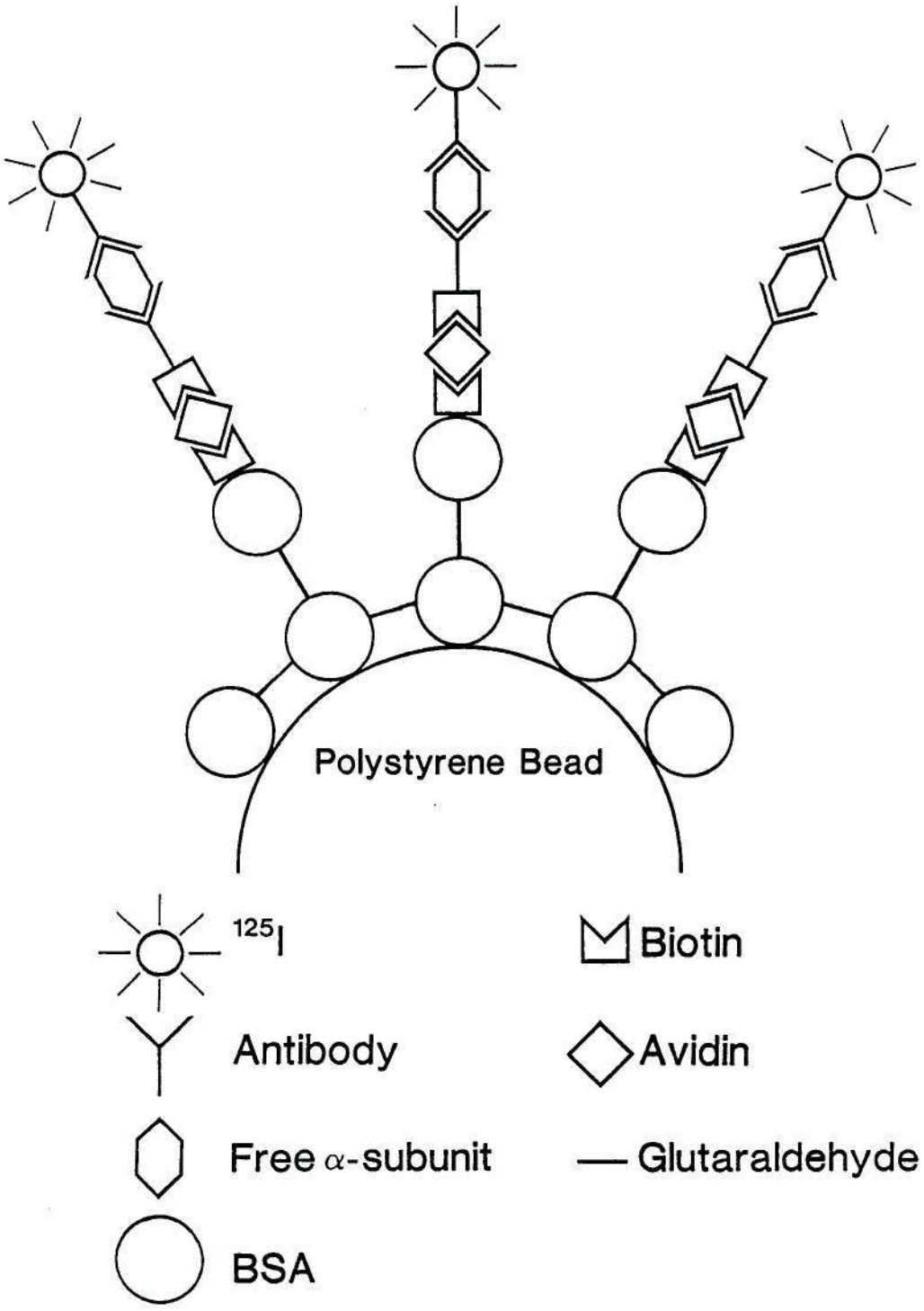


Figure 3.1 Schematic representation of a polystyrene bead coated with BSA, biotinylated BSA and avidin after incubation with biotinylated antibody, free α-subunit and radiolabelled antibody.

The ease with which BSA can bind to polystyrene, the affinity and specificity with which biotin binds to avidin and the ease with which biotin binds to any protein has been utilised to provide a solid-phase support that is both simple and reliable. In addition thorough washing of the solid-phase after the incubation period is a simple matter so that the precision and sensitivity of the assay can be fully exploited. Centrifugation is not required and the polystyrene bead therefore provides a simple method of separation.

The various procedures involved in the establishment of the IRMA are described in the following sections.

3.2 PREPARATION OF SOLID PHASE COMPONENTS

Careful selection of the solid phase and optimisation of the method of separation is essential in the overall design of an IRMA. The procedures involved in the preparation of the solid phase components and optimisation of the separation system are discussed in this section.

3.2.1 Coating of Polystyrene Beads

The technique used for coating of the polystyrene beads with biotin-avidin is essentially that described by Odell *et al.* (1986).

Two thousand polystyrene beads (8 mm in diameter, Precision Plastic Ball Co., Illinois, USA) were washed several times with distilled water (dH_2O), before being added to 600 ml of phosphate buffered saline (PBS) containing 0.1% BSA and shaken gently for two hours. The beads were rinsed once with dH_2O and allowed to stand in 600 ml of a 1% glutaraldehyde solution (BDH, U.K.) for 24 h to allow linkage between the BSA molecules. They were then washed 10 times with dH_2O and incubated for 6 - 8 h in 600 ml of PBS containing 20 mg of biotinylated BSA (the biotinylation procedure is described in section 3.2.2.2.2). After washing, the

beads were incubated with 600 ml PBS containing 1% BSA and 20 mg of streptavidin (Amersham) or avidin (Boehringer Mannheim, W. Germany). The beads were rinsed with dH_2O and finally incubated with PBS containing 0.1% BSA and 0.25% sucrose for 20 min. The coated beads (hereafter referred to as beads coated with avidin) were then spread on absorbent paper and allowed to dry thoroughly. The dried beads were stored in plastic containers in liquid nitrogen.

3.2.1.1 Comment

Streptavidin, a cell wall protein from *Streptomyces avidinii*, has been suggested in the literature as an alternative to avidin (Haeuptle *et al.*, 1983; Mayer and Walker, 1987). It has similar binding properties to avidin and is believed to have an added advantage in minimising background binding. There was however no change in the NSB of the assay when streptavidin was substituted for avidin in the coating procedure (results not shown) and avidin was therefore used for further coating of beads in this study.

The reproducibility of the coating of the polystyrene bead was carefully assessed for each new batch of solid-phase. When a new batch of coated beads was required, it was prepared as described and compared in the IRMA, using standards, to the previous lot. This was essential to the reproducibility of the assay.

3.2.2 Preparation of Biotinylated Antibody and BSA

The co-enzyme biotin is a relatively polar molecule and is coupled with high affinity to proteins under very mild conditions with little disruption to their structure. Before it can be coupled to protein, however, it must be derivatised to allow formation of stable amide bonds under very mild conditions. The most convenient method of coupling uses the N-hydroxysuccinimide ester of biotin (Bayer and Wilcheck, 1974; Bayer *et al.*, 1979). Both antibody 75 and BSA were labelled with biotin using the Enzotin reagent which is a commercially available N-hydroxysuccinimide ester of biotin. The degree of biotinylation of the antibody preparation was assessed using a method recommended by the manufacturer of the Enzotin reagent.

3.2.2.1 Principle

The mechanism of coupling of biotin to protein involves the activated ester of biotin and the nucleophilic unprotonated e-amino groups of the lysine residues on the protein, resulting in the formation of an amide bond and release of N-hydroxysuccinimide (as shown in Fig. 3.2). Succinimide esters are very prone to hydrolysis, and during coupling there is a competing hydrolytic reaction. The extent to which coupling and hydrolysis compete depends on the protein concentration. At high concentrations (1 - 12 mg/ml), the coupling reaction is quite efficient while at low concentrations (< 1 mg/ml), the competing hydrolysis reaction predominates.

The method used to determine the degree of biotinylation takes advantage of the fact that biotin reacts under anhydrous conditions with p-dimethylaminocinnamaldehyde in sulphuric acid-ethanol to produce a reddish-orange colour which is maximally absorbed at 533 nm. The ureido carbonyl function is a prerequisite for colour response and the stereospecificity of the colour reaction is for the sulphur end of biotin (McCormick and Roth, 1970).

3.2.2.2 Materials and Methods

3.2.2.2.1 Buffers and Reagents

i. Phosphate Buffered Saline (PBS)

130 mmol/*l* NaCl

7.0 mmol/*l* Na_2HPO_4

2.6 mmol/*l* $NaH_2PO_4.H_2O$

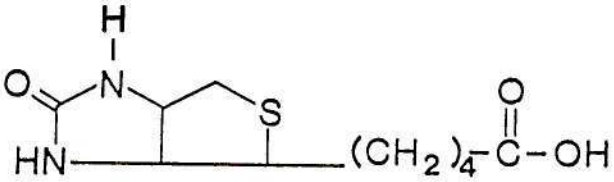
pH 7.4.

ii. p-dimethylaminocinnamaldehyde (DMAC) Solution

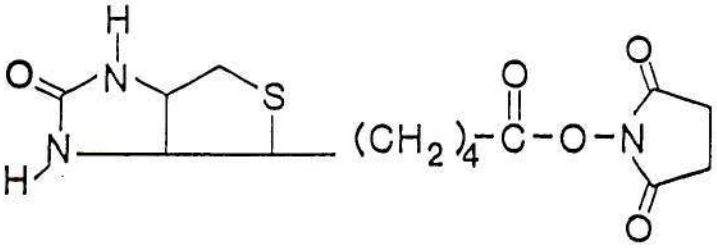
DMAC (200 μg) [Sigma] was dissolved in 100 ml of 2% (v/v) concentrated H_2SO_4 in ethanol.

3.2.2.2.2 Biotinylation Procedure

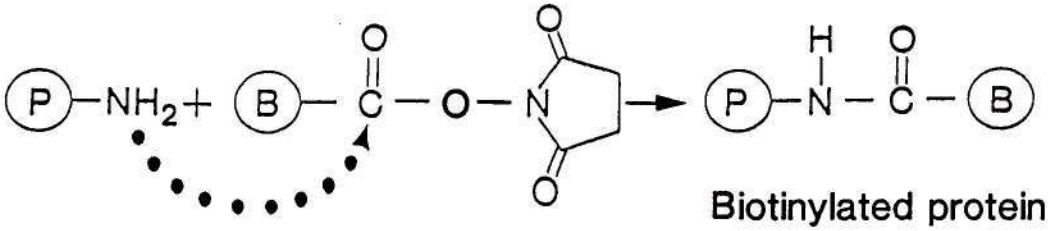
The protein solution (antibody 75 or BSA) was dialysed (using Spectrapor dialysis tubing with a molecular weight cut off of 12 000 - 14 000 daltons) overnight against



Biotin

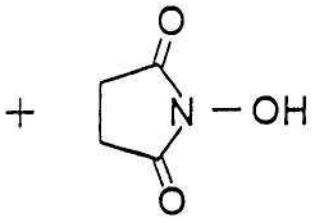


Biotin-NHS



Biotinylated protein

Reaction mechanism



NHS

NHS - N-hydroxysuccinimide ester
P - protein
B - biotin

Figure 3.2 Structure of biotin and its succinimide ester (biotin-NHS), and the mechanism of coupling to proteins.

1800 ml of 0.1 mol/*l* $NaHCO_3$ and the protein concentration adjusted to between 2 and 5 mg/ml with 0.1 mol/*l* $NaHCO_3$. The Enzotin reagent (Biochem Incorporation, New York, USA) was dissolved in dimethyl sulphoxide (DMSO) at the same concentration (mg/ml) as the protein. Enzotin (120 μl / ml protein solution) was added, mixed immediately, and incubated for 4 h at room temperature. After incubation, the solutions were dialysed (as described previously) against 2 *l* PBS overnight at 4 °C.

3.2.2.2.3 Determination of Degree of Biotinylation

Portions containing between 0.2 and 0.5 mg of biotinylated protein were added to an equal volume of concentrated HCl in a glass ampoule which was then sealed. The contents were heated in boiling water for 2 h and thereafter lyophilised. Another ampoule containing an equal amount of unbiotinylated protein or BSA was prepared as a blank.

Standards were prepared by dissolving the Enzotin reagent to a concentration of 1 mg/ml in dimethylformamide (DMF). Portions (ranging between 1 and 100 μl) of this solution were added in duplicate to the glass ampoules and lyophilised. To each ampoule 200 μl unbiotinylated protein or BSA (0.2 - 0.5 mg) were added. Concentrated HCl (200 μl) was then added and ampoules sealed. The contents were heated in boiling water for 2 h and then lyophilised.

The samples, standards and blank were redissolved when required in 800 μl of 2% concentrated H_2SO_4 in ethanol (v/v). One hundred microlitres of the chromogen (DMAC solution) were added to each ampoule followed by incubation for 1 h at room temperature.

The absorbances at 533 nm were read against unbiotinylated protein or BSA using a Beckman DU5 Spectrophotometer. A standard curve of absorbance against the amount of Enzotin was then plotted and used to determine the degree of biotinylation of the BSA and antibody.

3.2.2.2.4 Assessment of Binding of Each Biotinylated Antibody Preparation

Antibody 75 was labelled with varying amounts of Enzotin reagent (120, 240 and

360 μl) as described in section 3.2.2.2.2. Each preparation of biotinylated antibody 75 (50 μl) in concentrations ranging from 2 to 80 μg/ml phosphate buffer (0.067 mol/l with 0.1% BSA, pH 7.4) was incubated with 150 μl (about 1 x 10^5 cpm) ^{125}I labelled free α-subunit (see section 3.4) and a polystyrene bead coated with avidin (as described in section 3.2.1) on a rotating mixer for 2 h at room temperature. The bead was washed twice with phosphate buffer (0.067 mol/l, 0.2% Tween-20, pH 7.4) and the bound radioactivity counted in a Berthold multihead gamma spectrometer. The preparation of biotinylated antibody exhibiting maximal binding with free α-subunit was determined.

3.2.2.3 Results and Comment

3.2.2.3.1 Degree of Biotinylation

A standard curve relating the optical density readings at 533 nm to the amount of Enzotin (biotin ester) in micrograms is shown in Fig. 3.3.

After determining the amount of biotin which bound to antibody 75 from this curve, the ratio of unbiotinylated protein to biotin was calculated as shown in the following example:

molecular weight of antibody	= 150 000 daltons
molecular weight of biotin ester	= 454 daltons
Initial concentration of antibody	= 2.5 mg/ml
Concentration of biotin ester	= 2.5 mg/ml
Volume of biotin ester solution added	= 120 μl
Final concentration of protein mixture	= 2.5/1.12 = 2.2 mg/ml

250 μl (0.55 mg) of biotinylated antibody solution was used in the assessment.

Therefore moles of biotinylated antibody	= 550/150 000
	= 0.004 μmol

The absorbance of the chromogen labelled biotinylated protein was read at 533 nm.

Amount of labelled biotin ester read from graph = 29 μg

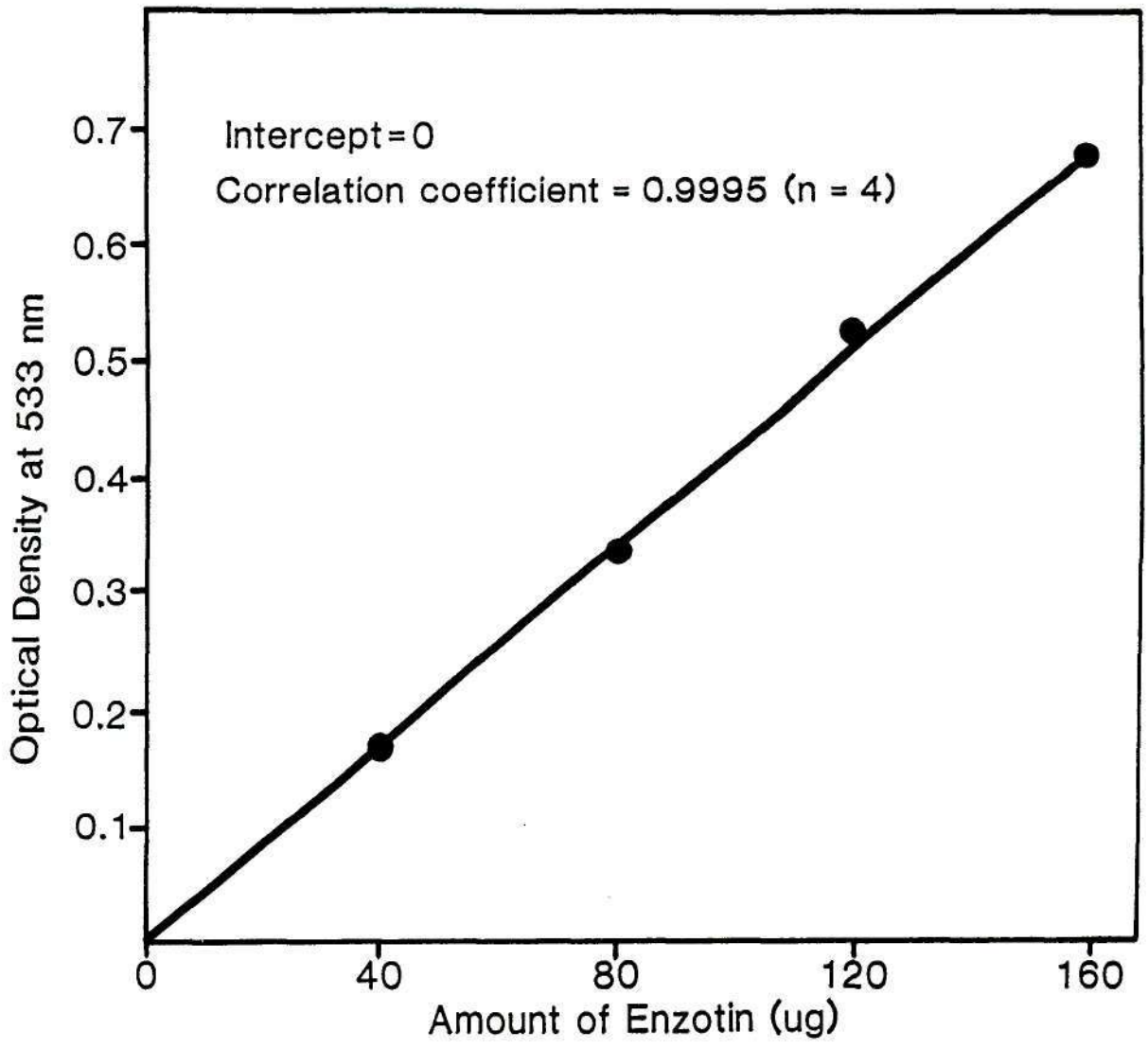


Figure 3.3 Standard curve for the estimation of the degree of protein biotinylation.

Therefore moles of biotin ester (Enzotin) = 29/454 = 0.064 μmol

Molar ratio of protein to biotin after labelling = 1:16

Similarly, the molar ratios of protein to biotin after the addition of increasing amounts of biotin ester in volumes of 240 and 360 μl were calculated as 1:21 and 1:29 respectively.

The possibility of conjugating several biotin molecules to each antibody has an obvious benefit in increasing the sensitivity of detection. However, overbiotinylation may lead to masking of the antigen-binding site, and hence inactivation of the antibody.

3.2.2.3.2 Assessment of Binding of Each Biotinylated Antibody Preparation

The three samples of antibody 75 labelled with different amounts of biotin (120, 240 and 360 μl) showed varying degrees of binding to the [^{125}I]-labelled free α-subunit (Fig. 3.4). The diluted preparation of antibody 75 labelled with 120 μl biotin per ml antibody showed highest overall binding with the radiolabelled α-subunit. The 10 μg/ml concentration of this preparation exhibited maximal binding and this concentration was therefore chosen for further use.

When determining the degree of biotinylation, that is the substitution of amino groups in the antibody molecule by biotin or the number of moles of biotin bound to each mole of antibody 75, it was found to be increased with increasing amounts of biotin ester (section 3.2.2.3.1). However, in the assessment of each preparation, it was found that the antibody binding capacity was decreased with more extensive substitution of amino groups by biotin; 120 μl biotin per ml antibody showed maximal binding with the range of standards used. This volume of biotin ester preparation per ml of protein is generally recommended for labelling of proteins (Goding, 1986).

In determining the optimal biotinylated antibody concentration, it was found that high concentrations of antibody inhibited binding, demonstrating the high dose 'hook' effect, a phenomenon very common to IRMAs (Hunter *et al.*, 1983). To overcome this problem, the labelling procedure was optimised to preserve antigen binding

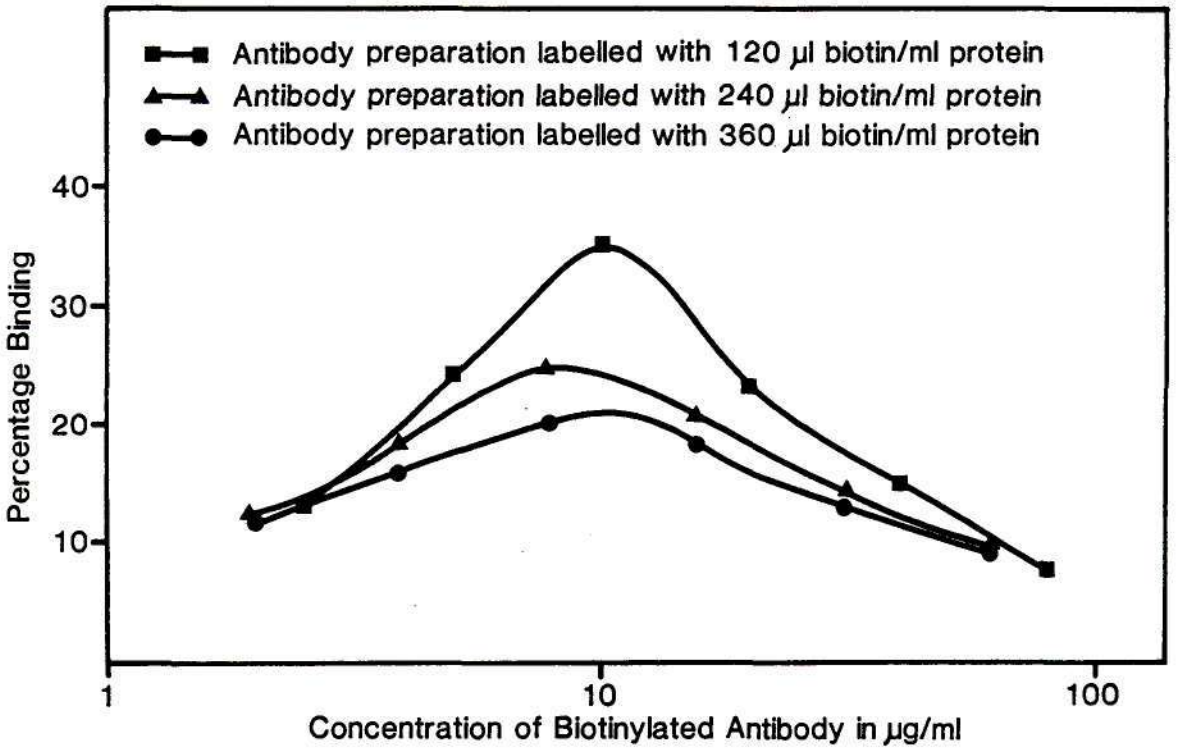


Figure 3.4 Antibody concentration curves for the various preparations of biotinylated antibody 75.

capacity while maintaining maximal sensitivity.

3.3 ALPHA-SUBUNIT STANDARDS

Standards were prepared from lyophilised free α-subunit (1st International Reference Preparation [IRP] 75/569, a gift from the World International Laboratory for Biological Standards, Holly Hill, UK) by dissolving 70 μg in 1.0 ml of phosphate buffer (0.067 mol/*l*, pH 7.4). Portions (40 μl) of this solution (master standard) were then stored at -20°C until use, at which time 32.5 μl were diluted with 7 ml undiluted horse serum to give a stock solution of 323 ng/ml. The stock solution was appropriately diluted in horse serum to prepare a set of standards as shown in the Table 3.1. The standards were divided into portions of 300 μl and stored at -20°C until use.

A reference preparation was required for the measurement of the free α-subunit of hCG because this component is not a single, chemically-defined substance. This material was prepared from the CR123 preparation of intact hCG by hydrogen bond cleavage with urea and separation by ion exchange chromatography (Morgan *et al.*, 1973). Although it possesses no intrinsic bioactivity, the α-subunit has been assigned a unit value per unit mass (1 mIU = 1 ng). However, for the purpose of the present study it was elected to express concentrations in terms of mass per unit volume. Where molar concentrations have been used, these values were calculated using an assigned molecular weight of 15 000 daltons for free α-subunit (Bellisario *et al.*, 1973).

Standards were initially prepared in phosphate buffered saline but subsequently standards were prepared in undiluted horse serum to improve the sensitivity of the assay (section 3.6.1.1).

The standards were found to be stable at -20°C for at least 6 months. When a new batch of working standards was required, this was prepared from the master standard as described above and compared to the preceding batch. The intra-and inter-assay

Table 3.1 Dilution protocol for the preparation of free α-subunit standards.

Stock Solution (ul)	Horse Serum (ml)	Total Volume of Standard (ml)	Final Concentration of Standard (ng/ml)
3200	12.80	16	64.60
1600	14.40	16	32.30
640	15.36	16	12.90
320	15.68	16	6.46
160	15.84	16	3.23
80	15.92	16	1.62
40	15.96	16	0.81
20	15.98	16	0.40
10	15.99	16	0.20
5	15.99	16	0.10
2.5	15.99	16	0.05

coefficients of variation between two batches of standards were required to be less than 10% over the range of standards used in the assay.

3.4 PREPARATION OF RADIOLABELLED ANTIBODY

The detection antibody 71 was radioiodinated by the Chloramine T method (Greenwood *et al.*, 1963).

3.4.1 Principle

Under mild alkaline conditions free ^{125}I released from [^{125}I]-NaI by an oxidising agent (Chloramine T), forms a covalent bond with tyrosine residues in the protein molecule. The oxidation reaction is terminated by the addition of a reducing agent (sodium metabisulphite) or alternatively, excess tyrosine, and quenched with buffer containing high concentrations of protein. The iodinated protein is isolated by gel filtration.

3.4.2 Materials and Methods

3.4.2.1 Buffers

i. 0.067 mol/*l* Phosphate Buffer, pH 7.4
Solution A = 0.067 mol/*l* KH_2PO_4
Solution B = 0.067 mol/*l* $Na_2HPO_4.2H_2O$

The pH of solution B was measured while adding solution A until a pH of 7.4 was obtained. Sodium azide was added to the buffer at a final concentration of 0.1%. Hereafter referred to as phosphate buffer 1.

ii. 0.33 mol/*l* Phosphate Buffer, pH 7.4
Solution A = 0.33 mol/*l* KH_2PO_4
Solution B = 0.33 mol/*l* $Na_2HPO_4.2H_2O$

The pH of solution B was measured while adding solution A until a pH of 7.4 was

obtained. Sodium azide was added to the buffer at a final concentration of 0.1%.

3.4.2.2 Reagents

i. Chloramine T Solution

Chloramine T (100 mg; Merck) was dissolved in 40 ml of phosphate buffer 1, pH 7.4, to give a concentration of 2.5 μg/ml.

ii. Sodium Metabisulphite Solution

One hundred milligrams of sodium metabisulphite (Merck) were dissolved in 16 ml of phosphate buffer 1, pH 7.4 to give a concentration of 6.25 μg/ml.

3.4.2.3 Preparation of Sephadex G100 Column

Deaerated Sephadex G100 (Pharmacia) in phosphate buffer 1 was poured into a 25 x 1 cm glass column. The column was packed and two volumes of buffer were washed through the column before use.

3.4.2.4 Iodination Procedure

Antibody 71 (40 μg), 5μl of phosphate buffer (0.33 mol/*l*, pH 7.4), 18.5 MBq in 5 μl of [^{125}I] sodium iodide solution (specific activity approximately 548 MBq/μg, Amersham) and 5 μl (2.5 μg/ml) of Chloramine T solution were added to a microfuge vial and mixed gently for 20 seconds. Five microlitres (6.25 μg/ml) of sodium metabisulphite solution were then added to the iodination vial and mixed for 1 minute. The reaction was quenched by the addition of 75 μl of phosphate buffer 1 containing 1% BSA.

The final solution was applied to the Sephadex G100 column and eluted with phosphate buffer 1 at a flow rate of 5 ml/h. Fractions of 500 μl or 10 drops/tube were collected and the radioactivity counted in a multihead gamma spectrometer (Berthold). The radioactive fractions (antibody peak) eluting after the void volume were pooled, suitably diluted with phosphate buffer 1 and assayed for antibody activity.

3.4.2.5 Assessment of Labelled Antibody

Fifty microlitres of labelled antibody 71 (approximately 200 000 cpm) were incubated with 50 μl (0.5 μg) of biotinylated antibody 75, 100 μl of the various α-subunit standards or undiluted horse serum (as blank) and one avidin-coated bead on a rotating mixer for 2 h at room temperature. After incubation the bead was washed twice with 2 ml of phosphate buffer 1 containing 0.2% Tween-20 and the bound radioactivity counted as above. This assay is described in greater detail in sections 3.5 and 3.6.

3.4.2.6 Calculation of Specific Activity of Radiolabelled Antibody

The percentage of ^{125}I incorporated by the antibody was determined as follows:

$$\% \text{ Incorporation} = \frac{\text{Bound } ^{125}\text{I}}{(\text{Bound} + \text{Free})\ ^{125}\text{I}} \times 100$$

$$= \frac{\text{cpm in } ^{125}\text{I antibody peak} \times 100}{\text{cpm in } ^{125}\text{I antibody peak} + \text{cpm in free } ^{125}\text{I peak}}$$

The specific activity per unit mass of radiolabelled antibody was then calculated as follows:

$$\text{specific activity} = \frac{\% \text{ incorporation} \times x\text{MBq} \times y}{\text{mass } (\mu\text{g})}$$

where x is the amount of radioactivity added and y is the decay factor.

3.4.3 Results and Comment

Because Chloramine T is a harsh oxidising agent, care was taken to ensure precise timing of the iodination reaction. Incorporation generally exceeded 90% when relatively high concentrations of protein were used (>1.0 mg/ml). Purification of the radiolabelled preparation revealed two radioactive peaks, with the larger antibody peak eluting first followed by a smaller free ^{125}I peak, as shown in Fig. 3.5.

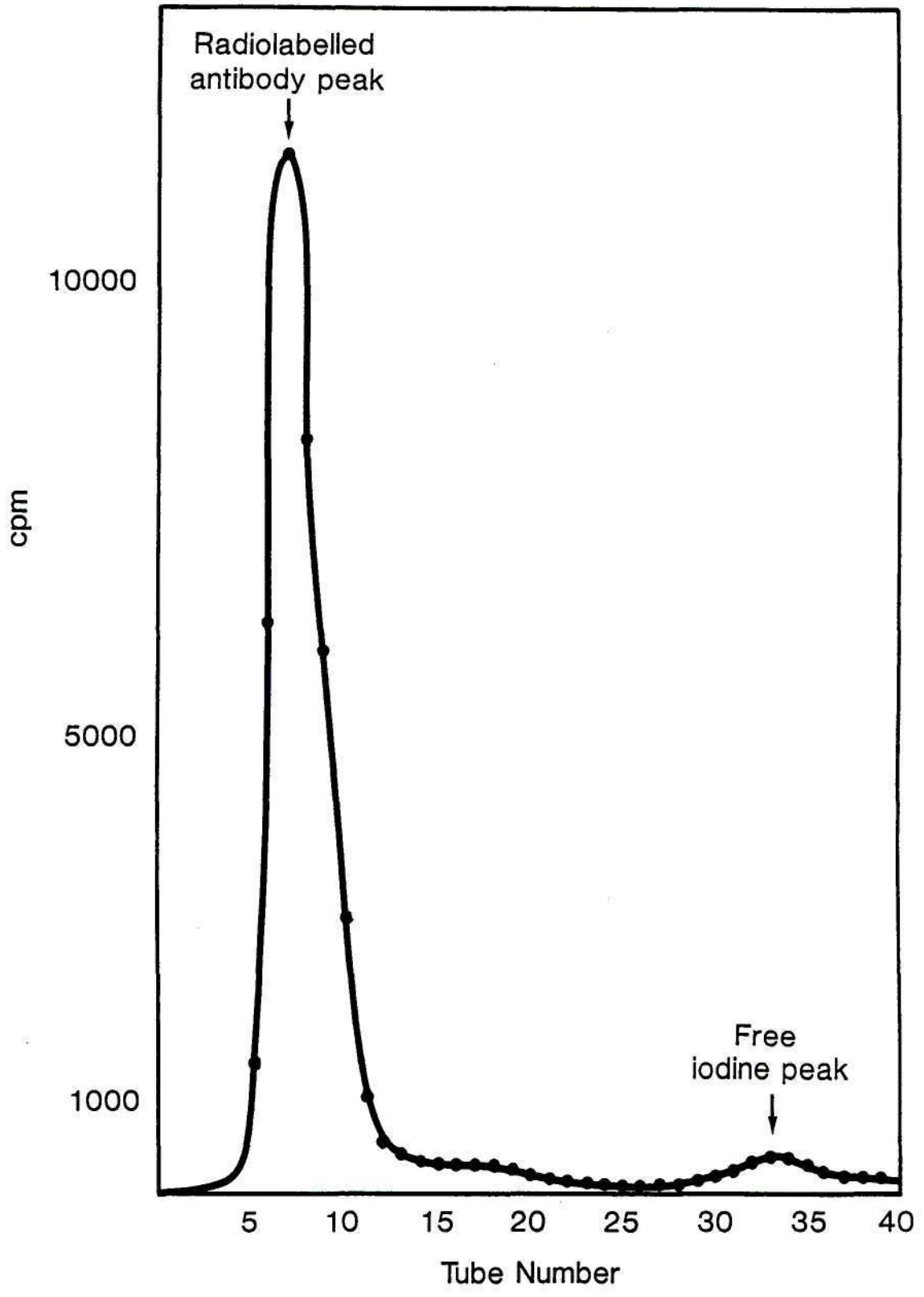


Figure 3.5 Radioactive peaks obtained after purification of radiolabelled antibody preparation by gel filtration on Sephadex G100.

An equimolar ratio of ^{125}I and antibody was chosen for use in the iodination reactions to achieve an average substitution of approximately one iodine atom per molecule of protein, as illustrated in the following example:

moles of antibody (40 μg, mw 150 000 daltons) = 0.27 nmol

moles of ^{125}I (18.5 MBq at specific activity of 548 MBq/μg)

$$= \frac{18.5 \text{ MBq}}{548 \text{ MBq}/\mu\text{g} \times 125} = 0.27 \text{ nmol}$$

The equimolar ratio was adopted because antibody preparations of low specific activity have a high proportion of unlabelled antibody which decreases assay sensitivity while in preparations of high specific activity, increased sensitivity is achieved at the cost of increased radiation damage.

The specific activity of the radiolabelled antibody was generally calculated to be about 420 MBq / μg of antibody. The radiolabelled antibody demonstrated good immunoactivity, the highest standard showing an average percentage binding of 60% for up to 4 weeks when stored in phosphate buffer 1 with 0.1% BSA at 4 °C.

3.5 IRMA PROCEDURE

Using the various reagents which have been prepared as described, the IRMA for the free α-subunit was empirically designed as follows: 50 μg biotinylated antibody 75 in 50 μl phosphate buffer 1, radiolabelled antibody 71 in the same buffer (50 μl; approximately 200 000 cpm) and 100 μl of free α-subunit standard diluted in PBS (pH 7.4) were incubated in a test tube containing an avidin-coated bead at room temperature on a rotating shaker for 2 h. PBS (100 μl) was assayed as an assessment of non-specific antibody (NSB). After incubation, the solution was aspirated and the bead washed twice with 2 ml phosphate buffer 1 containing 0.2% Tween-20 (Merck). The radioactivity on each bead was counted for 1 min in a gamma spectrometer.

3.6 EVALUATION OF THE IRMA

Having developed an IRMA for the measurement of free α-subunit, the next step was to examine systematically the various stages of the assay procedure for the purpose of optimising the assay conditions for sensitivity, specificity, precision and accuracy.

When evaluating the merits of the assay procedure it is imperative to define certain concepts. The sensitivity of an assay is defined as that concentration of analyte which gives a level of bound radioactivity equivalent to the mean NSB plus two standard deviations above the mean in any individual assay system. The specificity of a monoclonal antibody-based IRMA for an antigen is defined by the specificities of the antibodies for epitopes which are unique to that particular antigen and is usually determined by assessing cross-reaction with related molecules. The precision of an assay refers to the reproducibility of the results, usually assessed by determining the standard deviation of replicate measurements of the same sample within an assay (intra-assay precision) or between assays (inter-assay precision). Good sensitivity is therefore dependent on both a low NSB and precise measurement of the NSB.

In the following section, various aspects of the assay procedure which are important determinants of the assay sensitivity, specificity, precision and accuracy are examined and described in detail.

3.6.1 Materials and Methods

3.6.1.1 Assay Optimisation

3.6.1.1.1 Effect of Protein on Binding and Sensitivity

The effect of various protein solutions as assay diluents on NSB was examined with a view to optimising assay sensitivity. PBS (pH 7.4) as well as PBS containing 0.1% BSA, serum from a patient with panhypopituitarism, serum from a pregnant patient and horse serum were assayed in the IRMA. In addition, free α-subunit standards ranging in concentrations from 0.05 - 0.8 ng/ml were prepared in PBS with 0.1%

BSA, horse serum and serum from a patient with panhypopituitarism respectively, and assayed in the IRMA. The effect of these protein solutions on binding and sensitivity was assessed by means of dose response curves and by applying the z-test for each solution.

3.6.1.1.2 Effect of Sequential Incubation on Binding and Sensitivity

To examine the effect of sequential incubation of antibodies, free α-subunit standards (100 μl) diluted in horse serum and ranging in concentrations from 0 -32 ng/ml were incubated for 1 hour with avidin coated beads (1 bead/tube) and 100 μl biotinylated antibody 75 (10 μg/ml). After incubation, each bead was washed once with phosphate buffer 1 containing 0.2% Tween-20, the second radiolabelled antibody (200 μl, approximately 200 000 cpm) added and the tubes incubated for a further 1 h. The beads were then washed once with phosphate buffer 1 with 0.2% Tween-20 and the bound radioactivity counted in a gamma spectrometer.

Concurrently, an assay with simultaneous incubation of antibodies using horse serum as assay diluent was also carried out.

The specific binding at different concentrations of standard were compared by dose response curves and the sensitivity of each assay determined by applying the z-test.

3.6.1.1.3 Effect of Incubation Time on Assay

The assay was carried out as described in section 3.5 but varying incubation times of 1, 2, 4, 24 or 72 h and horse serum as assay diluent were used. The effect on assay binding at various standard concentrations and the sensitivity of the assay with increasing incubation time was determined.

3.6.1.1.4 Effect of Sample Volume on Assay Sensitivity

The effect of increasing incubation volumes on sensitivity was studied by assaying α-subunit standards diluted in horse serum, in concentrations ranging from 0.0125 - 0.2 ng/ml in volumes of 100-1000 μl, as described in section 3.5. The sensitivities of the assays using different sample volumes were compared by applying the z-test.

3.6.1.1.5 Effect of Washing on Binding, Precision and Sensitivity

The assay was carried out as described in section 3.5 except that the beads were washed once, twice or three times with phosphate buffer 1 containing 0.2% Tween-20 and horse serum was used as an assay diluent instead of PBS. The effect of washing on the NSB and the precision in the measurement of standards in the assay were then assessed.

3.6.1.2 Characteristics of the IRMA

After establishing the optimal assay conditions for the measurement of the free α-subunit, the IRMA procedure described in section 3.5 was suitably modified and then characterised with respect to its dose response curve, sensitivity, specificity and working range.

3.6.1.2.1 Dose Response Curve

A dose response curve was constructed by plotting the specific bound radioactivity measured in cpm against the free α-subunit concentration.

3.6.1.2.2 Sensitivity and Precision

Using the optimal assay procedure, the sensitivity of the assay was assessed by determining the lowest free α-subunit standard concentration which gave a level of bound radioactivity that was equivalent to or greater than the mean NSB + 2 SD above the mean. Intra-assay variation was assessed in a precision dose profile by quadruplicate assay of standards at concentrations ranging from 0 - 32 ng/ml, and inter-assay variation by the assay of the same standards in four separate assays. Precision was expressed as the percentage coefficient of variation (%CV) of intra- and inter-assay results.

3.6.1.2.3 Working Range

To determine the optimal concentration range of antigen over which the assay can be used, a serum sample with high concentrations of free α-subunit (32 ng/ml) was serially diluted to about 0.03 ng/ml in horse serum and assayed in the optimised IRMA. Expected-to-measured free α-subunit concentrations were compared and the optimal working range for serum determined.

3.6.1.2.4 Assay Specificity

To determine assay specificity for free α-subunit, standards of free α-subunit of hCG (1st IRP), free ß-subunit of hCG (1st IRP), intact hCG, LH, FSH and TSH (2nd IRP) were assayed in the optimised IRMA. The degree of cross-reaction was determined by calculating the ratio (mass/mass) of cross-reacting material to free α-subunit which yielded equivalent binding to labelled antibody. Cross-reactivity was expressed as a mass ratio rather than a molar ratio for purposes of comparison with the literature.

Contamination of the FSH standard by the free α-subunit has been reported by Thotakura and Bahl (1985). It was therefore necessary to purify the standard to determine its true cross-reaction in the free α-subunit IRMA. One ml of FSH standard (5 x 10^4 IU/l), dextran blue, [^{125}I]-FSH (100 µl, Amersham FSH kit) and [^{125}I]-free α-subunit (100 µl) were premixed and applied to a column of Sephadex G100 (Pharmacia; 450 x 15 mm) equilibrated with phosphate buffer 1. The column was eluted with the same buffer at a downward flow rate of 8 ml/h and fractions of 1.1 ml collected. All fractions were assayed for FSH by a commercial RIA (Amersham) and for free α-subunit by the IRMA. The percentage of free α-subunit contamination contributing to FSH cross-reaction (mass/mass) was determined by calculating the areas under the respective curves as measured in the free α-subunit IRMA. True cross-reaction was determined by comparing free α-subunit immunoreactivity with the FSH concentration in the fractions eluting with [^{125}I]-FSH.

3.6.2 Statistics

The z-test was applied to compare statistically the effect of related parameters on the sensitivity of the IRMA (Rimm *et al.*, 1980). Using this method, the actual measurements can be converted to a standard scale. The method assumes that if the total area under the normal distribution curve is 1.00 (100%), then it is possible to use the curve in a probabilistic manner. The standard normal distribution, denoted by z, has a mean equal to 0 and standard deviation equal to 1. To calculate an area under the normal distribution curve, the actual measurements are converted to a

corresponding point z on the z scale, using the following formula:

$$z = x2 - x1 / SD,$$

with x2 referring to the lowest standard mean, x1 referring to the NSB mean and SD to the standard deviation of the NSB duplicates. This formula expresses the mean value of the standard as being a specified number of standard deviation units from the mean NSB value. A value of $z > 2$ (which implies $p \leq 0.05$) indicates significant sensitivity and the highest z value indicates the best sensitivity.

The precision of replicate sample measurements was expressed as the percentage coefficient of variation (%CV) which was calculated by dividing the standard deviation (SD) by the mean value and then multiplying by 100.

The non-parametric Signed Rank test was applied to determine the significance of the differences in binding between pairs of the same samples, tested under varying assay conditions. When multiple comparisons were made (>2), the significance level was adjusted by dividing the p value by the number of variables assessed (Bonferroni adaptation).

3.6.3 Results and Comments

3.6.3.1 Optimisation of the IRMA

3.6.3.1.1 Effect of Protein on Binding and Sensitivity

Table 3.2 shows the NSB (cpm mean and SD) and calculated assay detection limit (mean NSB + 2SD) for each assay diluent tested. Horse serum demonstrated the lowest NSB and assay detection limit (NSB + 2SD).

The sensitivity of the IRMA when various protein solutions were used to dilute to the lowest standard (0.05 ng/ml) is shown in Table 3.3. Horse serum was found to give the highest z value and hence the best sensitivity. Fig. 3.6 shows the dose response curves when these protein solutions were used to dilute the free α-subunit standard. The percentage specific binding of standards diluted in horse serum was not significantly higher over the range of standards used, compared to those diluted

Table 3.2 Mean cpm, SD and mean + 2SD of non-specific binding for each assay diluent.

Assay diluent	NSB (cpm)		
	mean (n=2)	SD	mean + 2SD
PBS	6341	385	7111
PBS with 0.1% BSA	5148	115	5378
pregnancy serum	1859	37	1933
horse serum	392	142	676*
panhypopituitary serum	723	73	869

* indicates the assay diluent giving the lowest assay detection limit (mean + 2SD).

Table 3.3 Binding observed following dilution of the lowest free α-subunit standard with various protein solutions.

	cpm			
Protein solution	mean NSB (n=2)	SD	mean NSB + 2SD	0.05 std (ng/ml)
PBS with 0.1% BSA	6998	32	7062	6835[1]
horse serum	300	26	352	497[2]*
panhypopituitary serum	839	85	1009	919[3]

(1) z = -5.1

(2) z = 7.6

(3) z = 0.9

* indicates the protein solution giving the best sensitivity.

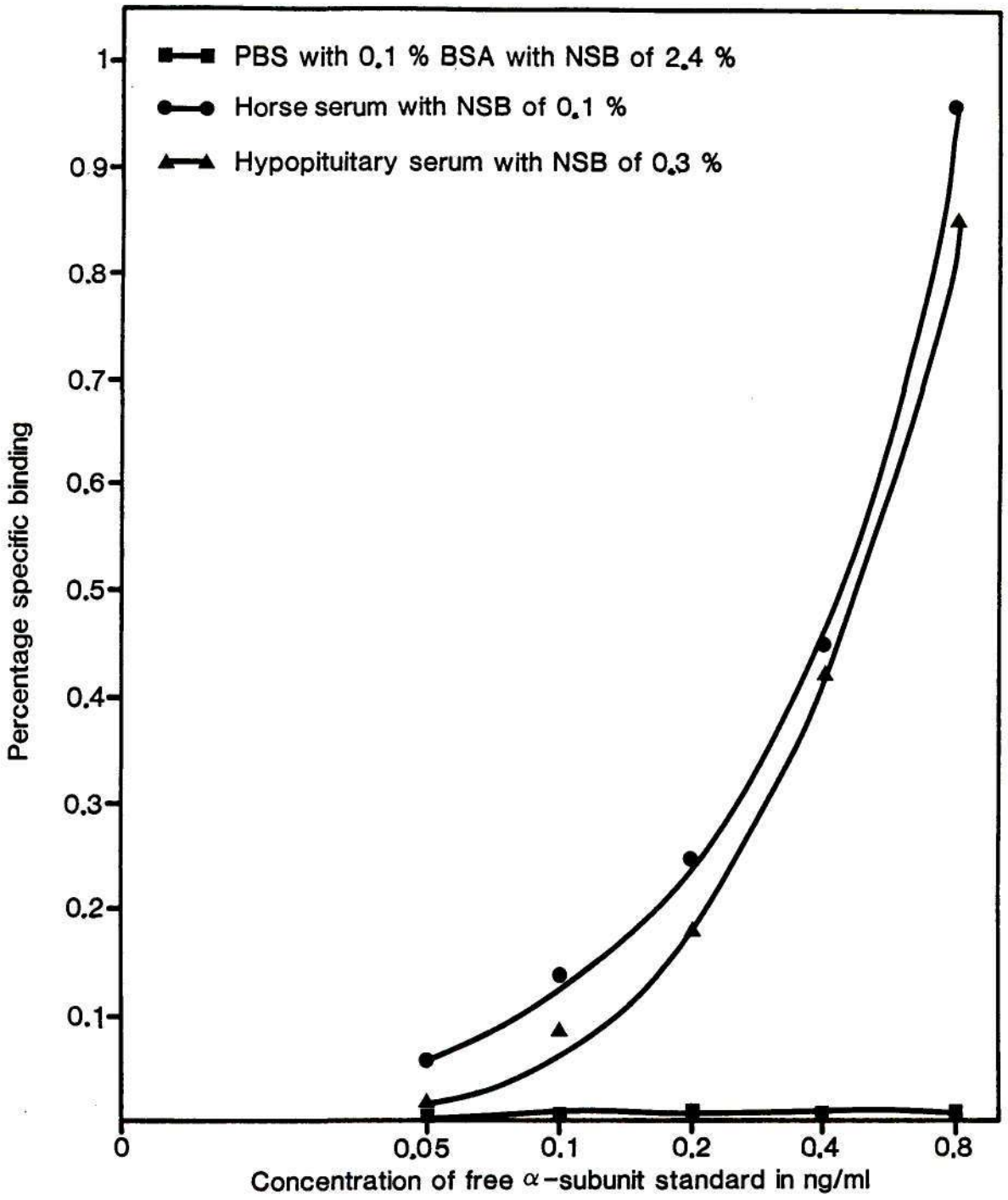


Figure 3.6 Dose response curves for α-subunit in the IRMA following dilutions with various protein solutions.

in serum from a patient with panhypopituitarism. Horse serum gave the best sensitivity and was therefore chosen for use as an assay diluent in the preparation of standard and control samples.

The choice of an appropriate diluent for the assay standards is important for optimal assay performance because it affects the NSB and therefore the sensitivity of assay. Solutions containing protein are commonly used, although the ideal matrix for a standard is the same as the sample to be tested. Although serum from patients with panhypopituitarism is most suitable as matrix when measuring free α-subunit the limited availability necessitated the search for a reliable substitute. Horse serum was found in this study to serve as an appropriate matrix. The use of horse serum as supporting matrix for assay standards is well documented by other workers (Bolton and Hunter, 1986; Odell and Griffin, 1987).

3.6.3.1.2 Effect of Sequential Incubation on Binding and Sensitivity

A comparison of the effects of simultaneous and sequential incubation of antibodies on sensitivity of the assay is shown in Table 3.4. The assay sensitivity was enhanced when both antibodies and analyte were incubated simultaneously. The percentage binding at free α-subunit concentrations of 0.05 - 6.4 ng/ml was significantly higher ($p=0.03$) as illustrated by the dose response curves (Fig.3.7).

The enhanced binding observed following simultaneous incubation may be attributable to the fact that these antibodies display co-operative binding when co-incubated, a phenomenon described in the characterisation studies (section 2.5).

3.6.3.1.3 Effect of Incubation Time on Assay

Fig. 3.8 shows the time course of binding in the IRMA for each concentration of free α-subunit studied when the incubation was performed at room temperature. Although binding at all concentrations of free α-subunit increased gradually with time, there was no meaningful increase in specific binding due to a simultaneous increase in NSB. As a consequence, the calculated detection limits and sensitivity of the assay did not increase with increasing incubation time (Table 3.5). Although an incubation time of 2 h showed a lower z-value, there was no effective change in the sensitivity of the assay. A 2 h incubation was found to be the most practical and

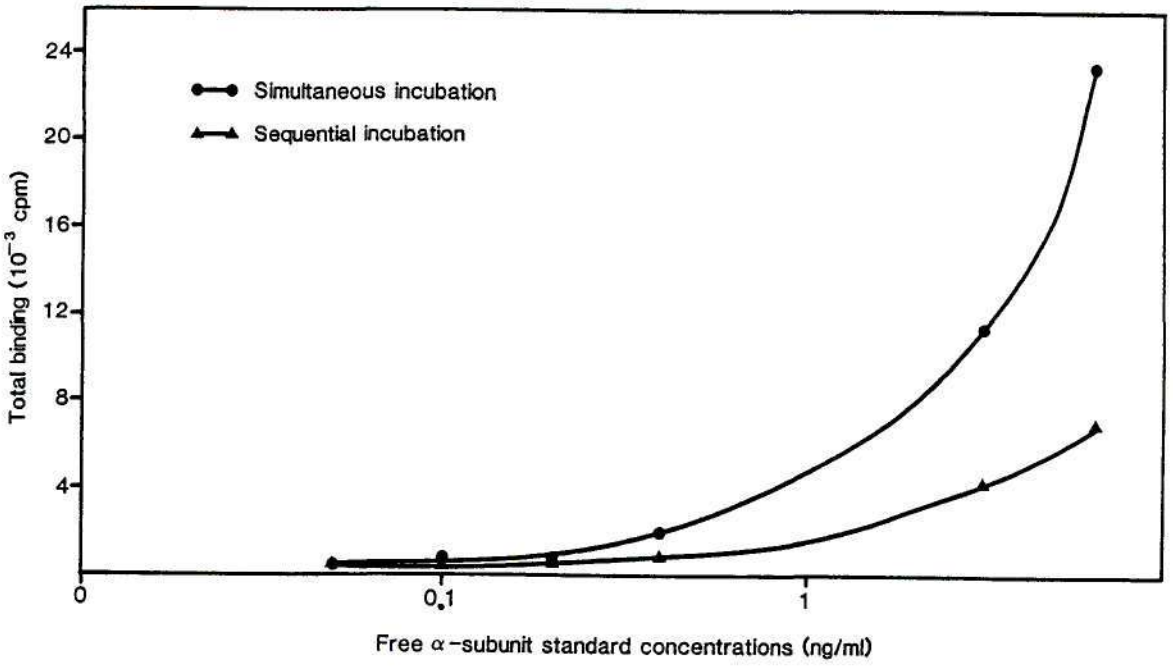
Table 3.4 Comparison of binding between simultaneous and sequential incubations in the IRMA.

	cpm			
Assay design	mean NSB (n=2)	SD	mean NSB + 2SD	0.05 std (ng/ml)
simultaneous incubation	328	4.2	336	559[(1)]*
sequential incubation	315	33	381	342[(2)]

(1) z = 55

(2) z = 0.82

* indicates the assay design giving the better sensitivity.



(1) vs (2) p = 0.03

Figure 3.7 Dose response curves of the IRMA after simultaneous and sequential incubations of the capture and radiolabelled antibodies.

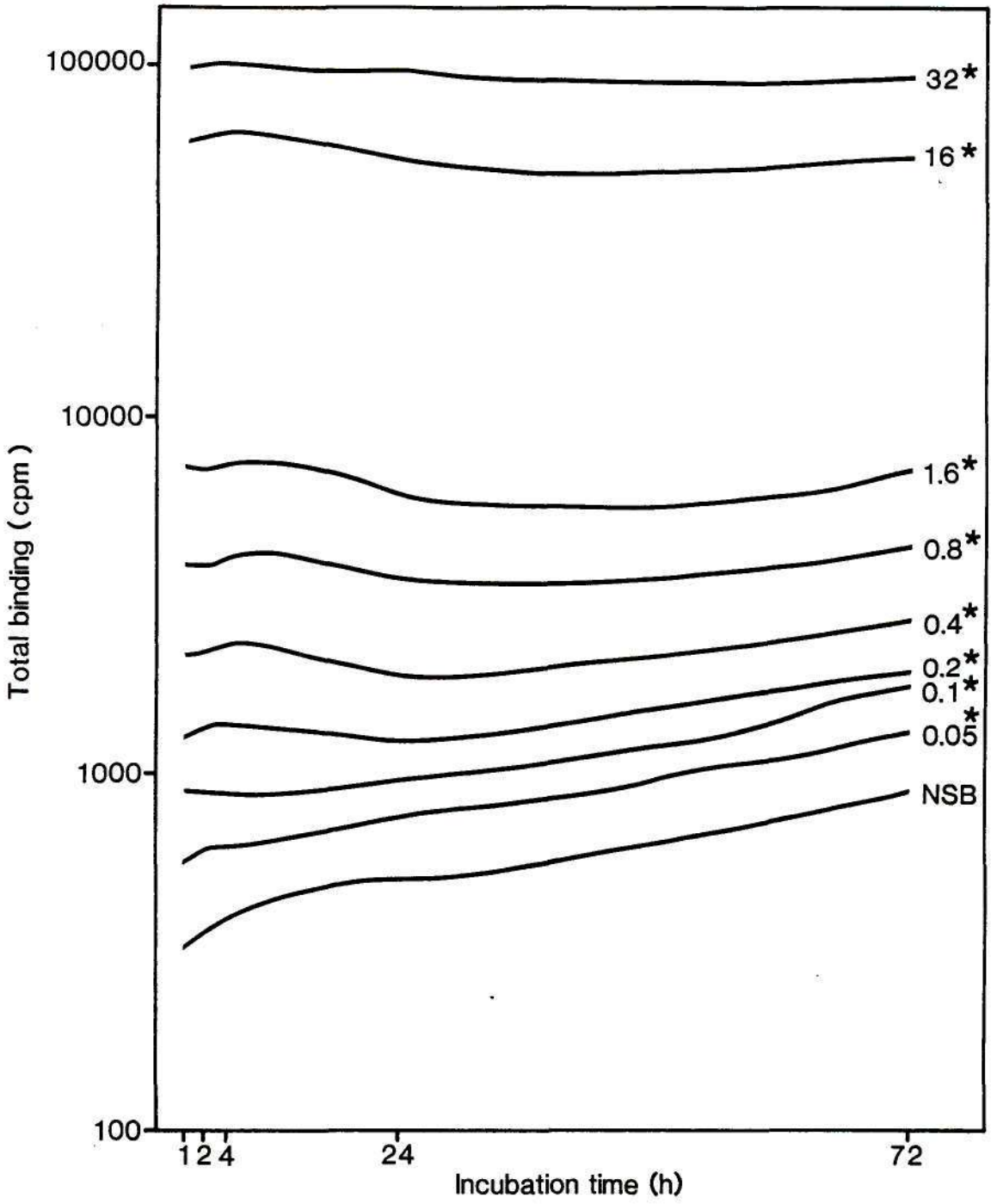


Figure 3.8 Time course of binding for IRMA when increasing concentrations of the α-subunit standard were incubated with biotinylated and radiolabelled antibodies at room temperature (* concentration of α-subunit standards in ng/ml).

Table 3.5 Comparison of binding at different incubation times in the IRMA.

Incubation time (h)	cpm mean NSB (n=2)	SD	mean NSB + 2SD	0.05 std (ng/ml)
1	323	3.5	330	558[(1)]*
2	374	31.8	438	622[(2)]
4	409	6.4	421	617[(3)]
24	500	13.4	527	762[(4)]
72	935	33.2	1001	1323[(5)]

(1) z = 67.1
(2) z = 7.8
(3) z = 32.7
(4) z = 19.5
(5) z = 11.7

* indicates the incubation time giving the best sensitivity.

was therefore selected for routine use.

The amount of specifically bound radioactivity in an IRMA is determined by both the rate of reaction of the antigen with the capture antibody and the rate of dissociation of the labelled antibody-antigen complex. The reaction with capture antibody is generally fast owing to its presence in large excess. The possibility that during the reaction the labelled antibody complex may dissociate from the capture antibody is minimal since most antibody-antigen systems are known to have negligible dissociation over a 4 h period at room temperature (Hales and Woodhead, 1980). The short incubation time necessary for the IRMA described here is therefore most likely due to a combination of the use of biotinylated capture antibody in excess and the stability of the biotinylated antibody-antigen-radiolabelled antibody complex.

3.6.3.1.4 Effect of Sample Volume on Assay Sensitivity

Table 3.6 shows both specific and non-specific binding when increasing sample volumes of free α-subunit standard (ranging from 0.025 - 0.2 ng/ml) were assayed. Neither assay binding nor sensitivity changed with increasing sample volume.

Although an increase in sample volume would be expected to result in increased binding, no change in binding was observed. A possible explanation for this may be that an increase in sample volume changes the capture antibody concentration required for optimal binding thereby resulting in no effective change in binding (see section 3.3.2.3.2).

3.6.3.1.5 Effect of Washing on Binding, Precision and Sensitivity

The lowest concentration of free α-subunit detected after each successive wash of the bead is shown in Table 3.7. Assay sensitivity and NSB improved with two washes of the bead compared to one wash but no significant increase in specific binding, at any free α-subunit concentration was shown.

There was however an improvement in the precision of measurement of quadruplicate samples at free α-subunit standard concentrations $\geq$0.2 ng/ml but no improvement in the precision of quadruplicates at low standard concentrations with further washing (Table 3.8). The coefficients of variation of quadruplicates were

Table 3.6 Comparison of binding with increasing incubation volumes of free α-subunit standards.

	cpm						
volume of sample (ml)	NSB		specific binding				
	mean	mean + 2SD	free α-subunit concentrations (ng/ml)				
			0.0125	0.025	0.05	0.1	0.2
0.1	137	243	123	222	270[(1)]*	414	774
0.2	72	190	141	178	272[(2)]*	474	944
0.5	149	305	95	102	203	415[(3)]*	866
1.0	64	146	56	72	163[(4)]*	217	485

(1) z = 2.5
(2) z = 3.4
(3) z = 3.4
(4) z = 2.4

* indicates the lowest standard concentration of free α-subunit in each incubation volume giving a sensitivity score or z value >2.

Table 3.7 Comparison of binding after each successive wash.

	cpm					
	NSB		specific binding			
number of washes	mean	mean + 2SD	free α-subunit concentrations (ng/ml) 0.025	0.05	0.1	0.2
1	1175	1638	1185	1441	1571	2000[1]*
2	93	130	147[2]*	249	379	670
3	64	107	109[3]*	169	270	488

(1) z = 3.6

(2) z = 2.9

(3) z = 2.1

* indicates the lowest concentration of α-subunit standard after each successive wash giving a sensitivity score of z >2

Table 3.8 Percentage coefficient of variation in measuring free α-subunit in quadruplicates at various standard concentrations after three successive washes.

free α-subunit standard (ng/ml)	precision (mean %CV of cpm)		
	wash x 1	wash x 2	wash x 3
NSB	19.72	19.79	33.46
0.025	19.63	8.79	17.08
0.05	17.97	15.87	16.95
0.1	10.44	14.48	17.52
0.2	12.37	7.77	8.15
0.4	4.32	2.22	8.99
0.8	5.58	1.13	4.85
1.6	2.59	1.34	2.05
3.2	0.90	1.04	1.21
6.4	3.38	1.98	1.25
12.8	1.96	1.85	1.97
32.0	0.38	0.33	0.21

>10% at standard concentrations <0.2 ng/ml even after 3 washes, demonstrating the lack of precision when measuring free α-subunit concentrations <0.2 ng/ml. The minimal advantages offered by a third wash did not justify the additional procedure.

The solid-phase was washed twice to minimise NSB to undetectable levels, thereby enhancing sensitivity of the assay. The lack of precision in measuring free α-subunit at low standard concentrations may be due to the fact that the amount of radioactivity detected at these concentrations is low and thus small differences in the cpm of quadruplicate samples can result in a high %CV.

3.6.3.2 Characteristics of the IRMA

3.6.3.2.1 Dose Response Curve

A dose response curve relating bound radioactivity to free α-subunit concentration is shown in Fig. 3.9. Binding of the [^{125}I]-labelled antibody increased proportionally with increasing concentrations of free α-subunit standard over a wide working range (0.05 to 32 ng/ml) with a limit of detection as low as 0.05 ng/ml of free α-subunit.

The assay shows no evidence of the 'high dose hook' phenomenon, where a paradoxical fall in binding at very high antigen doses often occurs. This may be due to the fact that the capture antibody (biotinylated antibody 75) was used at an optimal concentration and very high levels of α-subunit were not measured.

3.6.3.2.2 Sensitivity and Precision

As shown previously (3.6.3.1), the assay sensitivity for free α-subunit under optimal assay conditions was as low as 0.05 ng/ml. A precision dose profile demonstrating intra-assay variation is shown in Fig 3.10. The intra-assay coefficient of variation under optimal assay conditions was <10% at free α-subunit concentrations ranging between 0.2 - 32 ng/ml. The inter-assay coefficient of variation over the concentration range of 0.4 to 32 ng/ml was < 10% (results not shown).

The sensitivity of the IRMA for the free α-subunit may be a result of the use of excess capture antibody which ensures optimal binding of antigen. Since the two antibodies used were of similar affinity (section 2.3), this sandwich assay technique

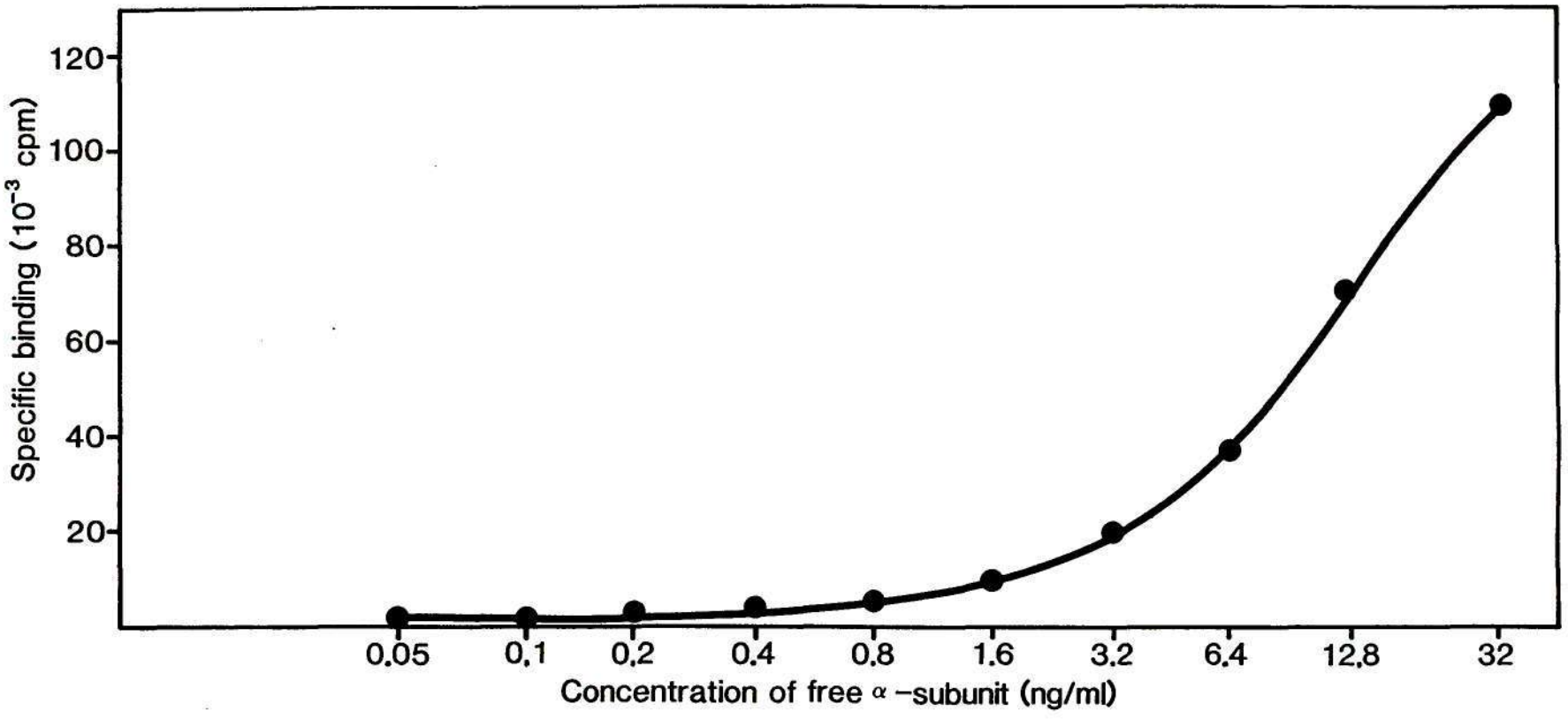


Figure 3.9 Dose response curve of free α-subunit in the two-site IRMA.

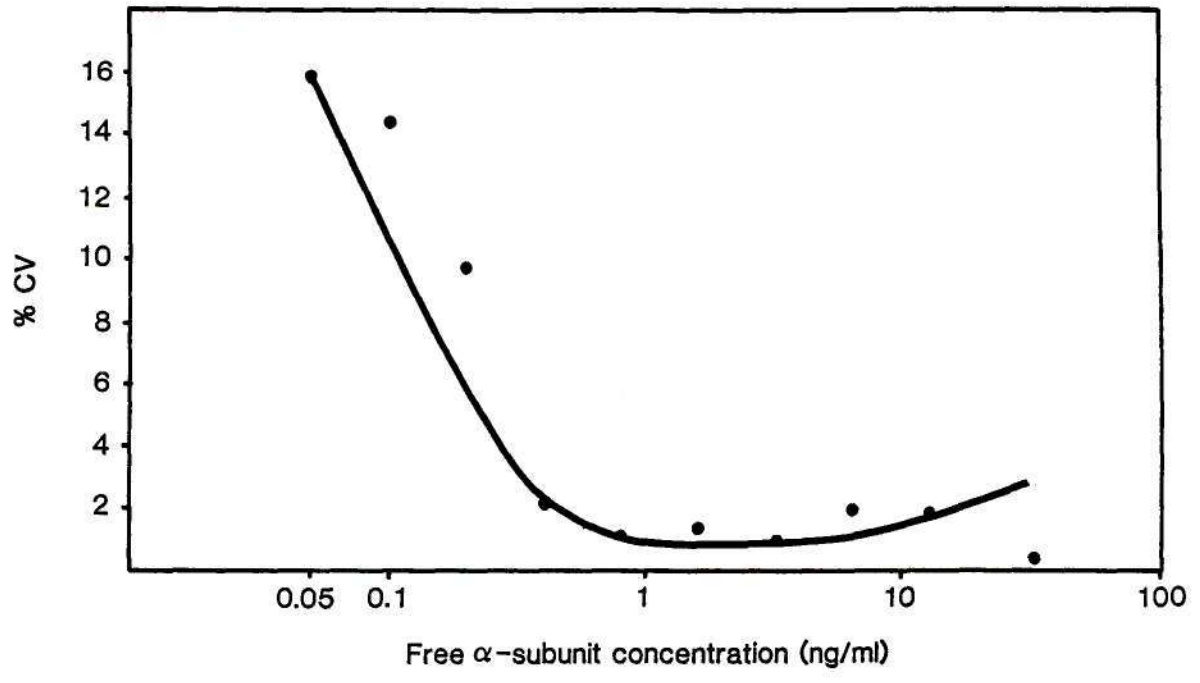


Figure 3.10 Precision dose profile of IRMA after assay of α-subunit standards.

should have a greater sensitivity, with almost all of the antigen reacting with both antibodies.

3.6.3.2.3 Working Range

A graph of expected versus measured serum free α-subunit concentrations in the range 0.03 - 32 ng/ml obtained by dilution is shown in Fig. 3.11. The levels of free α-subunit after dilution of the serum with horse serum were in good agreement with the expected values (slope 1.016 with a correlation coefficient of 0.9989). The optimal working range for the measurement of free α-subunit in serum was similar to that of the standards in the assay and extended over almost three orders of magnitude. Identification of the working range is important to ensure assay reliability, especially when choosing the appropriate dilution for samples with high free α-subunit concentrations.

3.6.3.2.4 Assay Specificity

Fig. 3.12 illustrates the percentage cross-reactivity of the glycoprotein hormone standard preparations in the IRMA.

Chromatography of the FSH standard (Fig. 3.13) demonstrated the presence of significant contamination of the preparation with the free α-subunit which was responsible for about 40 - 50% of the immunoactivity. Some cross-reaction of intact FSH in the free α-subunit IRMA was also observed. The percentage cross-reactions after chromatography of the FSH standard preparation are shown in Table 3.9.

The IRMA described for the free α-subunit shows minimal cross-reaction with the intact glycoprotein hormones or the free ß-subunit of hCG. This degree of specificity is attributed to the use of two monoclonal antibodies which recognise distinct sites on the antigen in a two-site binding format. In this way it is possible to trap and quantify a specific molecule. This is supported by the fact that when studied individually, the two selected monoclonal antibodies showed some cross-reaction with the intact hormones (section 2.2) but when used in this format, the cross-reaction was minimal.

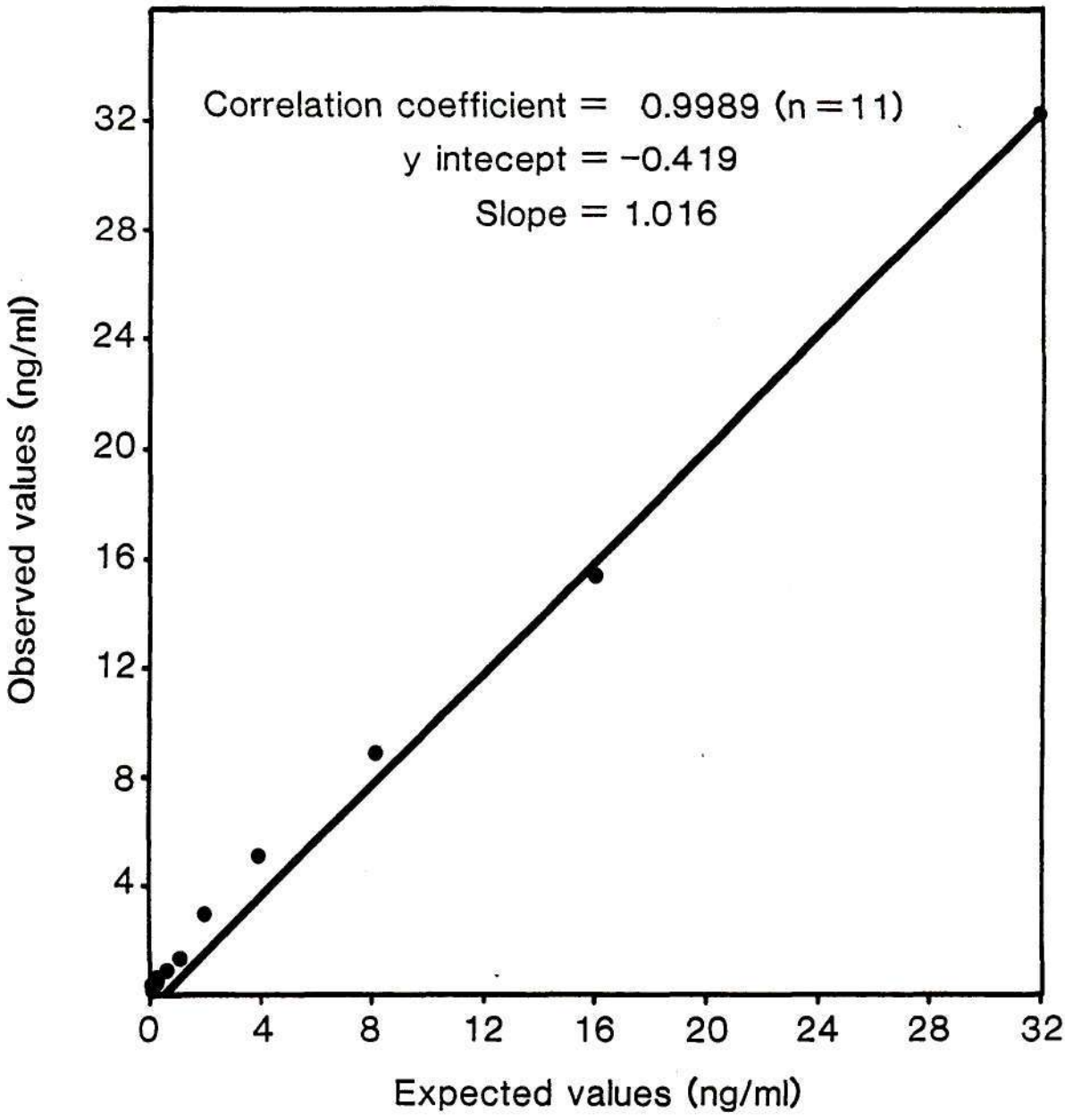


Figure 3.11 Graph of expected values against observed values of α-subunit when a serum sample was diluted with horse serum.

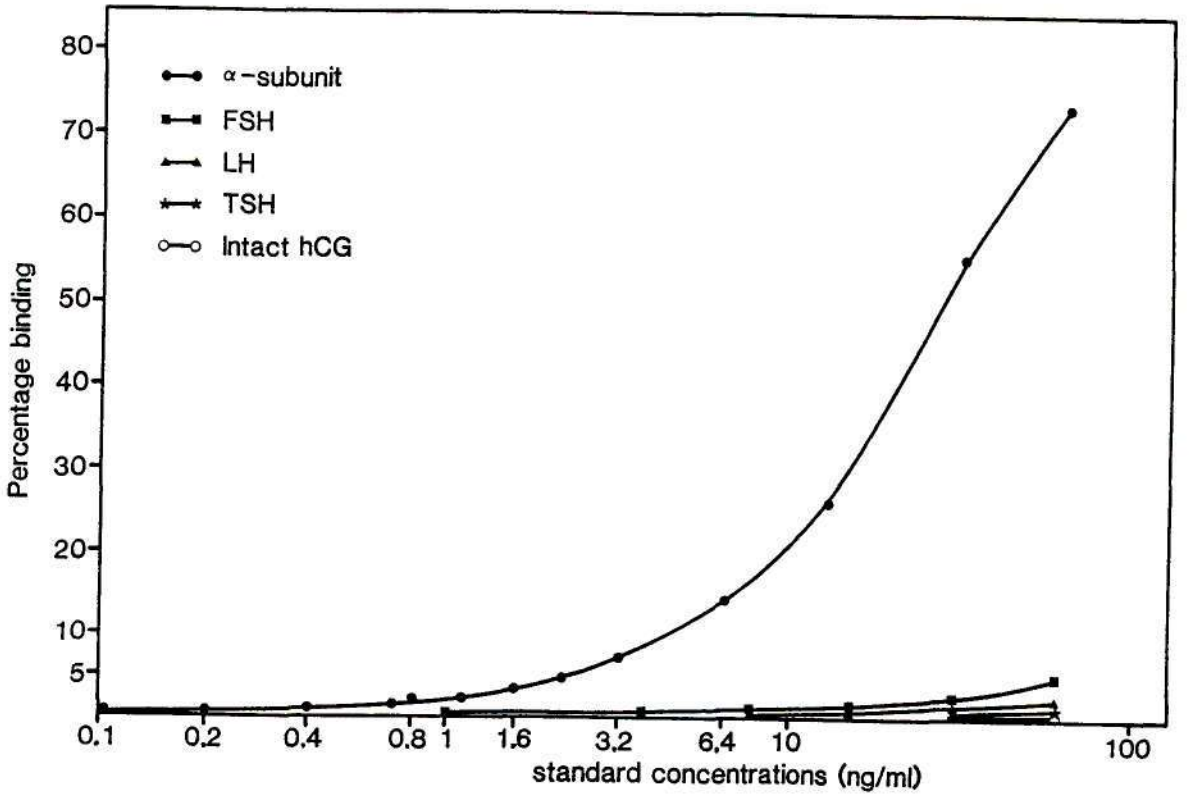


Figure 3.12 Dose response curves for IRMA showing cross-reaction of the related glycoprotein hormones (hCG 0.15%, LH 1.8%, FSH 4.5%, TSH 0.5%).

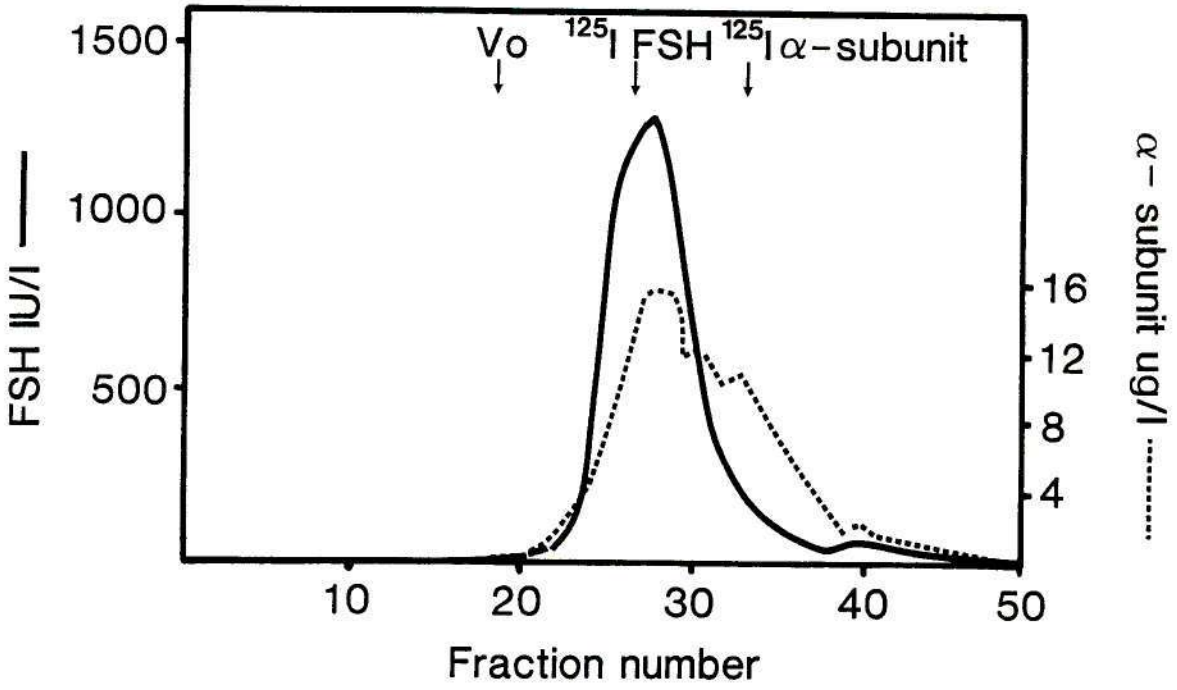


Figure 3.13 Sephadex G100 chromatography of FSH standard (2nd IRP 78/549). Standard (1.0 ml) was subjected to gel filtration on a column of Sephadex G100 equilibrated with phosphate buffer (0.067 mol/*l*, pH 7.4) and calibrated with [^{125}I] free α-subunit and [^{125}I]FSH. Dextran blue was used to determine the void volume (V_o). The column was diluted with the same buffer at a downward flow rate of 8 ml/h and fractions of 1.1 ml collected. All fractions were assayed for FSH by RIA and for free α-subunit by the IRMA. The solid line represents FSH immunoactivity and the broken line free α-subunit immunoactivity in the fractions collected. Chromatography of the FSH standard revealed the presence of free α-subunit contamination in the preparation and some cross-reactivity of intact FSH in the free α-subunit IRMA.

Table 3.9 Percentage cross-reaction of free ß-subunit of hCG and related glycoprotein hormones (LH, FSH, TSH and hCG) in the IRMA after purification of FSH standard.

glycoprotein	% cross-reaction
free ß-subunit of hCG	0
intact hCG	0.15
intact LH	1.80
intact FSH	2.50
intact TSH	0.50

3.7 SUMMARY

Using two monoclonal antibodies, one biotinylated as the capture antibody (75) and the other radiolabelled as the detection antibody (71), and polystyrene beads coated with biotin and avidin as a solid-phase separation system, an IRMA for the measurement of free α-subunit of hCG was developed.

Optimisation of the IRMA demonstrated that the use of horse serum as an assay diluent, the simultaneous incubation of assay reagents and two washes of the bead improved assay performance. On the other hand, longer incubation times and increased sample volumes did not have any advantage.

The assay was found to be specific for the free α-subunit and sensitive to levels of 0.05 ng/ml. The inter- and intra-assay CVs of the IRMA were $< 10\%$ at α-subunit concentrations ranging between 0.4 and 32 ng/ml. The optimal working range of the assay was similar to the standard range and extended over almost three orders of magnitude.

CHAPTER FOUR

MEASUREMENT OF THE FREE ALPHA-SUBUNIT OF HCG IN GESTATIONAL TROPHOBLASTIC DISEASE

Medical theory is truly useful, only when its practical results are beneficial. To judge correctly of these results, we must have recourse to the phenomena presented to clinical observers. It is by inductive reasoning thus experimentally obtained, that we can be prepared to detect the fallacy of each protean medical theory, whose shadowy being might long elude any other species of investigation.

Alexander M'Call [early 19th Century]

4.1 INTRODUCTION

As mentioned in the introduction to this thesis, several workers have suggested that serial measurement of the serum free α-subunit of hCG may be useful in the diagnosis and treatment of patients with gestational trophoblastic disease (GTD) [Dawood *et al.*, 1977; Gaspard *et al.*, 1977; Quigley *et al.*,1980a and b]. Consequently, this study was undertaken, to determine the levels of free α-subunit, using the IRMA described previously (chapter 3) in patients with GTD both at presentation and during the course of treatment.

In addition to establishing the diagnosis of GTD, there are several crucial clinical decisions with regard to the treatment of these patients. Because there are both benign and malignant forms of the disease, it is essential that this distinction be made as early as possible to allow for optimal therapy. It is also important to monitor closely the response to treatment because the objective of therapy is to remove all tumour cells as quickly as possible. Thus in the studies which follow, the clinical

utility of free α-subunit measurement is assessed with these objectives in mind. Since ßhCG is widely accepted as the tumour marker for the diagnosis and management of patients with GTD, the levels of free α-subunit are also compared to those of ßhCG, as measured by the ßRIA (Vaitukaitis *et al.*, 1972).

4.2 PATIENTS AND METHODS

4.2.1 Patients

Sixty one patients (fifty six Blacks and five Indians) ranging in age from 16 - 50 years, with histologically proven GTD were included in the study. The selection was based on the patient having a high serum ßhCG level (> 10 000 IU/l) at presentation and an adequate serial follow-up. Thirty four patients had molar disease and twenty seven choriocarcinoma. Of the patients with molar disease, twenty two were found to have benign molar disease (BMD) characterised by a rapid decline in ßhCG levels following treatment, and twelve had persistent molar disease (PMD, metastasizing or invasive moles) characterised by a more gradual decline or a rise in serum ßhCG levels following treatment as assessed at 6 - 8 weeks post-evacuation. Of the patients with choriocarcinoma, one had recurrence of the disease.

All patients with molar disease were treated initially by evacuation of the uterus. Patients subsequently classified as having BMD received no further therapy while those with PMD and those with choriocarcinoma were treated with cytotoxic chemotherapy. These patients were divided into three groups for the purpose of optimising treatment. The low risk group, consisting of patients with PMD, received several courses of standard doses of vincristine and methotrexate. The medium risk group comprised patients with choriocarcinoma whose disease either followed a term pregnancy, was diagnosed four months following an interrupted pregnancy or who at presentation had a ßhCG level of >40 000 IU/*l*. These patients were treated with several courses of chemotherapy, which included cis-platinum in addition to methotrexate and vincristine. The high risk group comprised patients in whom the above treatment had failed or who had brain or liver metastases. Chemotherapy

given to these patients included atoposide VP 16 in addition to the other agents. Treatment regimes were individualized, but in general each patient received a course of cytotoxic therapy at 3 weekly intervals while circulating serum ßhCG persisted. Therapeutic success was assumed when serum ßhCG was no longer detectable.

4.2.2 Blood Sampling

Blood was taken from each patient before therapy was initiated, at weekly intervals during therapy and weekly for several weeks after its cessation. After collection, blood was separated and the serum stored at -20° C until assay. The blood samples analysed were those submitted to the laboratory for routine ßhCG estimation.

4.2.3 Assays

Serum ßhCG was measured using the Amerlex ßRIA kit (Amersham); this assay utilises hCG standards calibrated against the 2nd International Standard (2IS). Thereafter, the remaining serum was stored at -20°C until assay in the free α-subunit IRMA described in the previous chapter. Samples from individual patients were assayed singly and in the same batch.

4.2.4 Analysis of Data

For purposes of analysis, patients were divided into 3 subgroups: BMD, PMD and choriocarcinoma. Where appropriate, patients were divided more broadly on the basis of benign or malignant (PMD and choriocarcinoma) disease. In addition to assessing the serum concentrations of free α-subunit and ßhCG in the various subgroups before and during therapy, the relationship between these two components was also investigated by calculating the percentage free α-subunit to ßhCG molar ratio before and during therapy. For this purpose the molecular weights of free α-subunit and hCG were taken as 15 000 and 38 000 daltons (Bellisario *et al.*, 1973, Hussa, 1981), respectively; the ßhCG value measured was assumed to represent

essentially intact hCG. Since the IU (2IS) = 2.07 IU (1st IRP), and the biological activity of the 1st IRP is 9.29 IU/μg or 9.29 mIU/ng,

$$1\ \text{IU}/l\ (\text{2IS}) = \frac{2.07\ \text{mIU/ml (1st IRP)}}{9.29\ \text{mIU/ng (1st IRP)}} = 0.22\ \text{ng/ml}$$

The %α/ßhCG molar ratio was calculated as follows:

$$\frac{[\text{free } \alpha\text{-subunit}]}{[\text{ßhCG}]} \quad \frac{\text{ng/ml}}{\text{IU}/l}$$

$$\frac{[\text{free } \alpha\text{-subunit}]}{[\text{ßhCG}] \times 0.22} \quad \frac{\text{ng/ml}}{\text{ng/ml}}$$

converting to %molar ratio

$$\frac{[\text{free } \alpha\text{-subunit}] / 15\,000}{[\text{ßhCG}] / 38\,000} \times 100 \quad \frac{\text{nmol/ml}}{\text{nmol/ml}}$$

$$= \frac{[\text{free } \alpha\text{-subunit}]}{[\text{ßhCG}]} \times 1152$$

4.2.4.1 Before Treatment

The pretreatment serum concentrations of free α-subunit and ßhCG in each of the 3 subgroups and the benign and malignant groups were compared directly as well as by calculation of the percentage free α-subunit to ßhCG (%α/ßhCG) molar ratios.

4.2.4.2 Response to Therapy

4.2.4.2.1 Free α-subunit and ßhCG Concentrations

The concentrations of free α-subunit and ßhCG and the %α/ßhCG molar ratios in patients with BMD and PMD were compared at each week following evacuation of the mole. The regression of free α-subunit and ßhCG after evacuation in BMD and PMD and during chemotherapy in choriocarcinoma, was also assessed by expressing these concentrations as a percentage of the free α-subunit and ßhCG level at

presentation.

4.2.4.2.2 Disappearance Time

The average time in weeks after treatment for the free α-subunit and ßhCG levels to reach normal levels of $\leq$0.6 ng/ml (Norman *et al.*, 1987) and <4 IU/*l* (Vaitukaitis *et al.*, 1972), respectively, was determined for each subgroup of patients and comparisons made between each group. Differences in the disappearance time between free α-subunit and ßhCG in each of the subgroups were also determined.

4.2.4.2.3 Free α-subunit Concentrations at Undetectable ßhCG Levels

The free α-subunit concentrations in patients who were successfully treated (ßhCG <4 IU/*l*, as defined in section 4.2.1) were assessed in each GTD patient.

4.2.4.3 Case Reports

Two patients who appeared to have unusual patterns of secretion of free α-subunit were studied in detail. The first of these patients (Patient A) had PMD, while the second (Patient B) had recurrence of choriocarcinoma.

4.2.5 Statistical Analysis

All values were expressed as mean ± S.E.M. The statistical analyses of unpaired data were based on non-parametric procedures when the data studied were not normally distributed. The statistical significance of differences between the means of the two groups was tested by the Mann-Whitney procedure; probability values were taken from two-tailed tables and only values of $p < 0.05$ were considered significant. When multiple (>2) comparisons were made, the Kruskall-Wallis analysis of variance on ranks was used to determine whether there were any significant differences between the groups studied. If a significant difference was found, pairwise comparisons were then made using the Mann-Whitney test with Bonferroni adjustments. The significance level in this case was adjusted by dividing the p value of 0.05 by the number of comparisons made.

The paired t-test was applied when data were normally distributed and used to determine the significance of the differences between pairs of data when samples were tested for two variables.

4.3 RESULTS

4.3.1 Before Treatment

The reference range for serum free α-subunit in pre-menopausal females is 0.05 - 0.6 ng/ml (Norman *et al.*, 1987). At presentation, all women with GTD had concentrations of free α-subunit above the reference range (mean 18.6 ± 1.2 S.E.M.; range 1.5 - 37.7 ng/ml). Table 4.1 shows the serum concentrations of free α-subunit and ßhCG, and the %α/ßhCG molar ratios, before treatment in each of the 3 subgroups and in patients with malignant disease. The free α-subunit levels in patients with BMD were similar to those in both PMD and choriocarcinoma when compared as individual groups or together as malignant disease. However, the free α-subunit levels in patients with PMD were found to be significantly higher ($p = 0.012$) than in patients with choriocarcinoma. There were no significant differences in ßhCG levels between the different subgroups, or between the benign and malignant groups. Similarly, no significant differences were found in the %α/ßhCG molar ratios.

4.3.2 Response to Therapy

4.3.2.1 Free α-subunit and ßhCG Concentrations

The serum levels of free α-subunit in almost all patients with GTD decreased progressively but erratically during treatment and roughly parallelled serum

Table 4.1 Free α-subunit and βhCG levels and percentage free α-subunit to βhCG molar ratios in patients with GTD before treatment.

Patients	free α-subunit (ng/ml)		βhCG (IU/*l*)		%α/βhCG molar ratio	
	mean ± S.E.M.	range	mean ± S.E.M.	range	mean ± S.E.M.	range
BMD (n=18)	19.9 ± 2.3	1.5 - 36.0	294489 ± 106546	18600 - 2040000	0.21 ± 0.03	0.001 - 0.42
PMD (n=12)	22.2 ± 2.6[(1)]	2.1 - 37.7	519063 ± 117223	23250 - 1289000	0.09 ± 0.02	0.03 - 0.21
choriocarcinoma (n=22)	15.6 ± 1.3[(2)]	1.9 - 28.9	302607 ± 58866	11100 - 1010000	0.21 ± 0.05	0.01 - 1.2
malignant disease (n=34)	17.9 ± 1.4	1.9 - 37.7	379003 ± 58032	11100 - 1289000	0.10 ± 0.04	0.01 - 1.2
all patients (n=52)	18.6 ± 1.2	1.5 - 37.7	349748 ± 52596	11100 - 2040000	0.02 ± 0.03	0.001 - 1.2

molecular weights of free α-subunit and βhCG taken as 15 000 and 38 000 daltons, respectively.

(1) vs (2) p = 0.012*

* Mann-Whitney test with Bonferroni adjustment (significance level p <0.0167)

concentrations of ßhCG.

The typical decreases in serum ßhCG and free α-subunit concentrations following treatment in patients with BMD, PMD and choriocarcinoma are illustrated in Figs. 4.1, 4.2 and 4.3, respectively. In patients with BMD both parameters decreased rapidly following evacuation until undetectable levels were achieved. A similar rapid decrease was demonstrated in patients with PMD in the first two weeks following evacuation, but thereafter the decrease was more gradual. The mean serum concentrations of free α-subunit and ßhCG were therefore not different in these groups of patients at the first week post-evacuation ($p > 0.05$), but thereafter (until the eighth week following therapy) the levels of both free α-subunit and ßhCG were significantly higher in patients with PMD ($p < 0.05$) as shown in Table 4.2. In patients with choriocarcinoma, both free α-subunit and ßhCG concentrations decreased gradually but erratically during cytotoxic therapy.

In patients with BMD and PMD, the %α/ßhCG molar ratio demonstrated a progressive rise following evacuation. However, the increase in the molar ratio was considerably greater in patients with BMD (> 100 fold) compared to patients with PMD (< 10 fold); hence the ratio was significantly higher in BMD from 2 - 7 weeks post-evacuation (Table 4.2). In contrast, the %α/ßhCG molar ratio fluctuated throughout the course of treatment in patients with choriocarcinoma.

Figures 4.4, 4.5 and 4.6 illustrate the patterns of change in serum free α-subunit and ßhCG, when the levels at each week post-evacuation in BMD and PMD, and each week of chemotherapy in choriocarcinoma, respectively, were expressed as a percentage of the values at presentation. In all 3 subgroups, ßhCG cleared far more rapidly than free α-subunit. The percentage decrease in free α-subunit and ßhCG levels relative to the level at presentation was similar in BMD and PMD in the first week following treatment but differed significantly ($p < 0.05$) thereafter and until 8 weeks post-evacuation (results not shown).

4.3.2.2 Disappearance Time

The time taken for the disappearance of both free α-subunit and ßhCG was

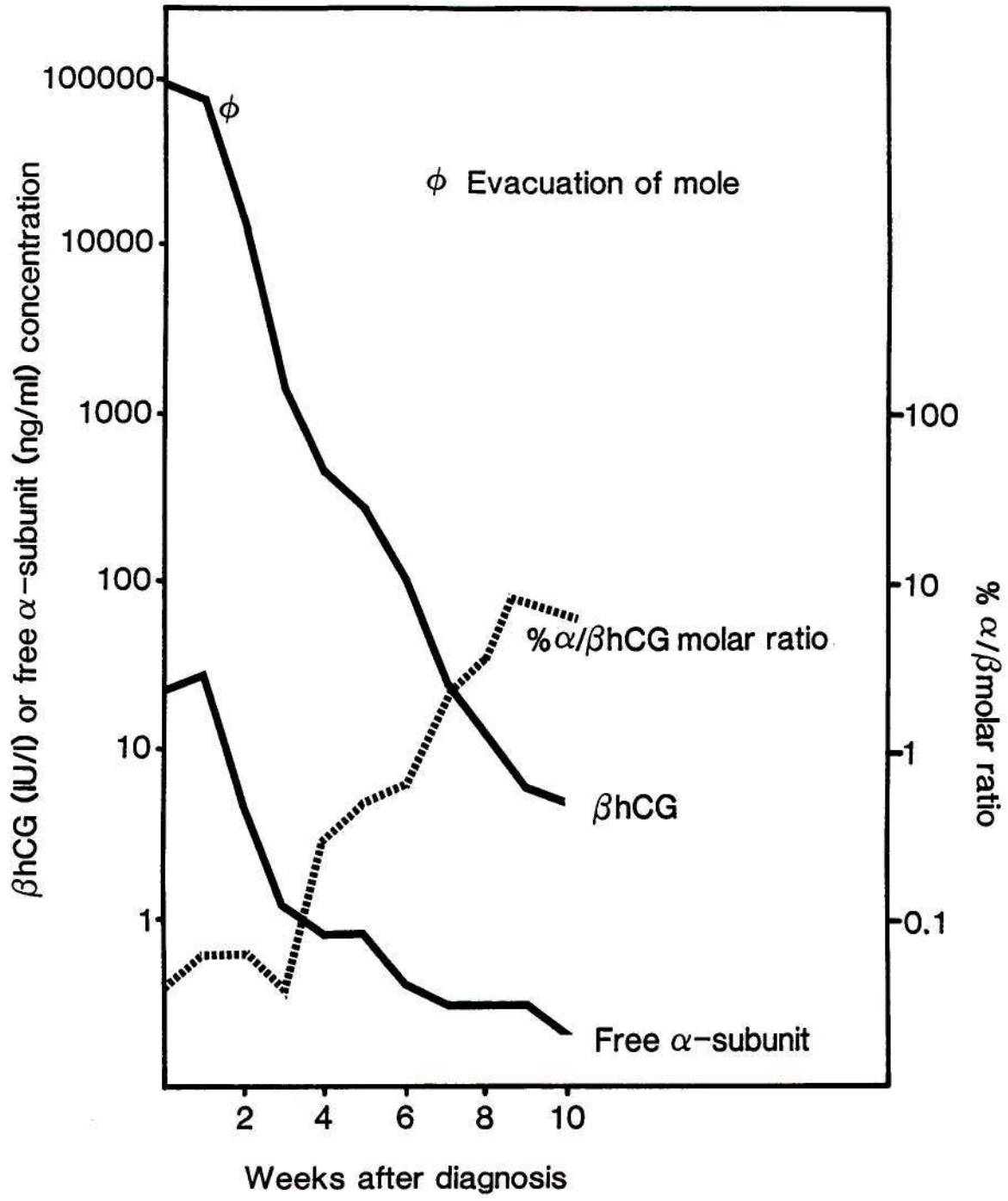


Figure 4.1 Serum free α-subunit and ßhCG concentrations, and the %α/ßhCG molar ratio in a typical patient with BMD treated by evacuation of the mole.

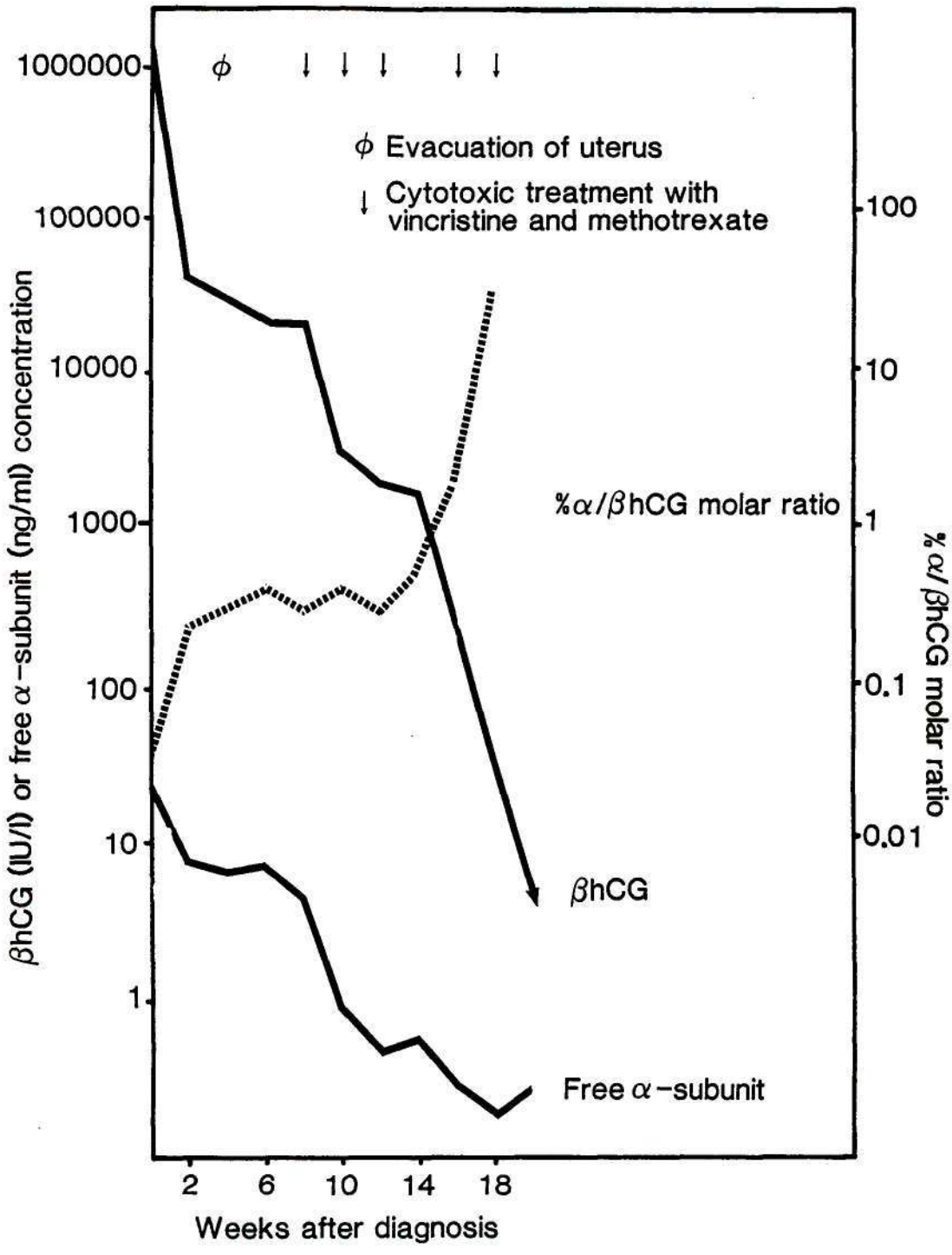


Figure 4.2 Serum free α-subunit and ßhCG concentrations, and the %α/ßhCG molar ratio in a typical patient with PMD treated by evacuation of mole and cytotoxics.

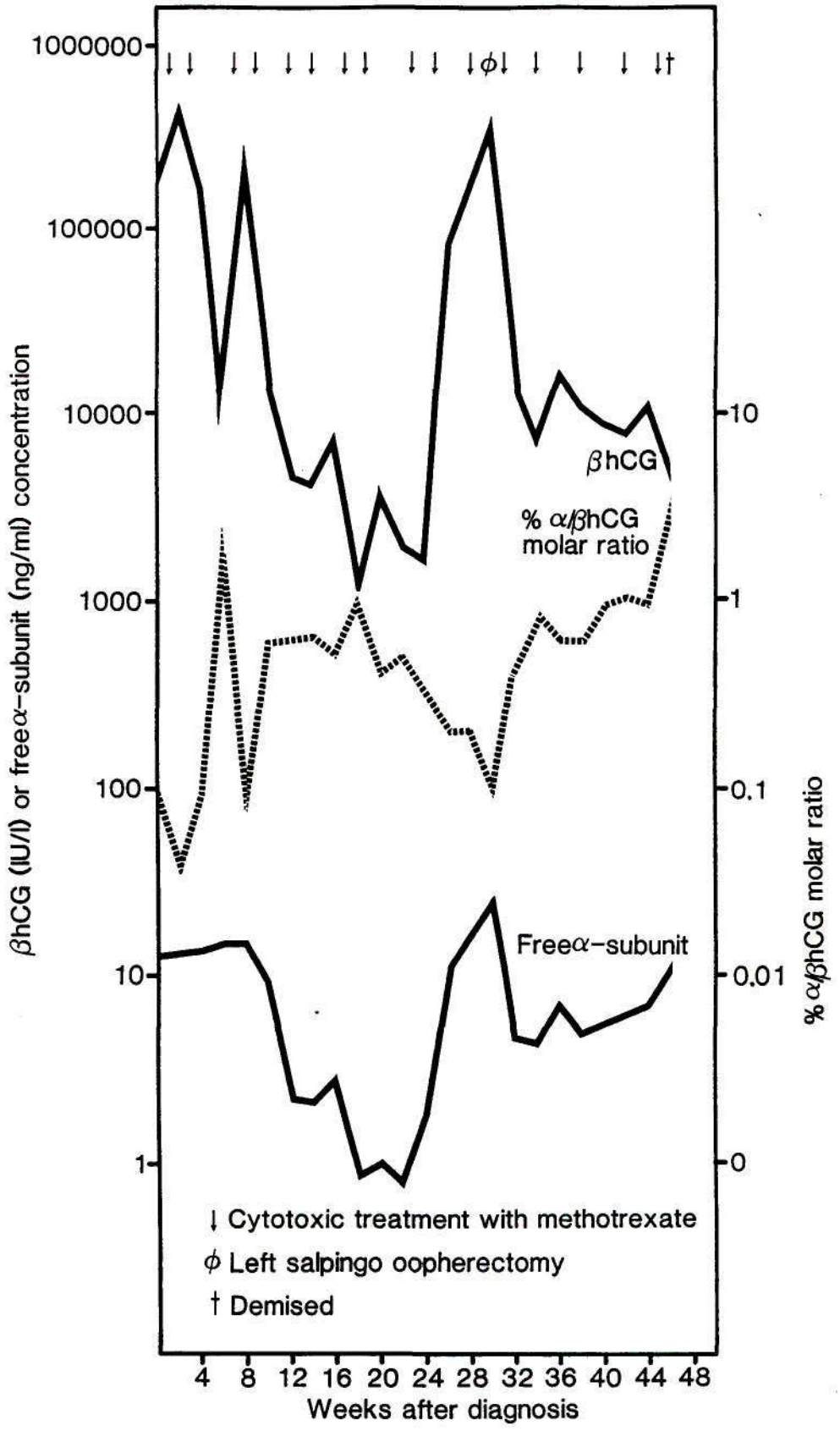


Figure 4.3 Serum free α-subunit and βhCG concentrations, and the %α/βhCG molar ratio in a patient with choriocarcinoma treated with cytotoxics.

Table 4.2 Mean free α-subunit and ßhCG concentrations in benign and persistent moles each week post-evacuation.

weeks	free α-subunit concentration (ng/ml)			ßhCG concentration (IU/l)			%α/ßhCG molar ratio		
	BMD mean ± S.E.M	PMD mean ± S.E.M	p value	BMD mean ± S.E.M	PMD mean ± S.E.M	p value	BMD mean±S.E.M	PMD mean±S.E.M	p value
1	5.2 ± 1.2	7.1 ± 1.3	>0.05	18949 ± 8900	23822 ± 7824	>0.05	0.09 ± 0.22	0.66 ± 0.12	>0.05
2	1.8 ± 0.40	4.2 ± 0.74	<0.05	1696 ± 440	9780 ± 3252	<0.002	2.0 ± 0.49	0.92 ± 0.13	<0.02
3	1.4 ± 0.35	4.9 ± 1.1	<0.05	788 ± 312	10991 ± 4649	<0.02	4.7 ± 1.3	1.0 ± 0.15	<0.002
4	0.89 ± 0.22	4.5 ± 0.85	<0.02	307 ± 177	11191 ± 3084	<0.02	1.3 ± 2.9	1.4 ± 0.65	<0.002
5	0.82 ± 0.21	3.1 ± 0.59	<0.05	541 ± 403	8341 ± 3472	<0.02	16 ± 5.1	1.2 ± 0.33	0.002
6	0.89 ± 0.21	3.2 ± 0.75	<0.05	412 ± 283	7521 ± 3769	0.02	45 ± 29	1.5 ± 0.37	<0.02
7	0.37 ± 0.13	3.3 ± 0.76	<0.05	19 ± 5.0	6069 ± 3617	0.002	38 ± 16	2.7 ± 1.0	<0.02
8	0.28 ± 0.09	2.5 ± 0.70	0.006	52 ± 39	7124 ± 5726	0.003	25 ± 8.4	3.7 ± 1.6	0.082

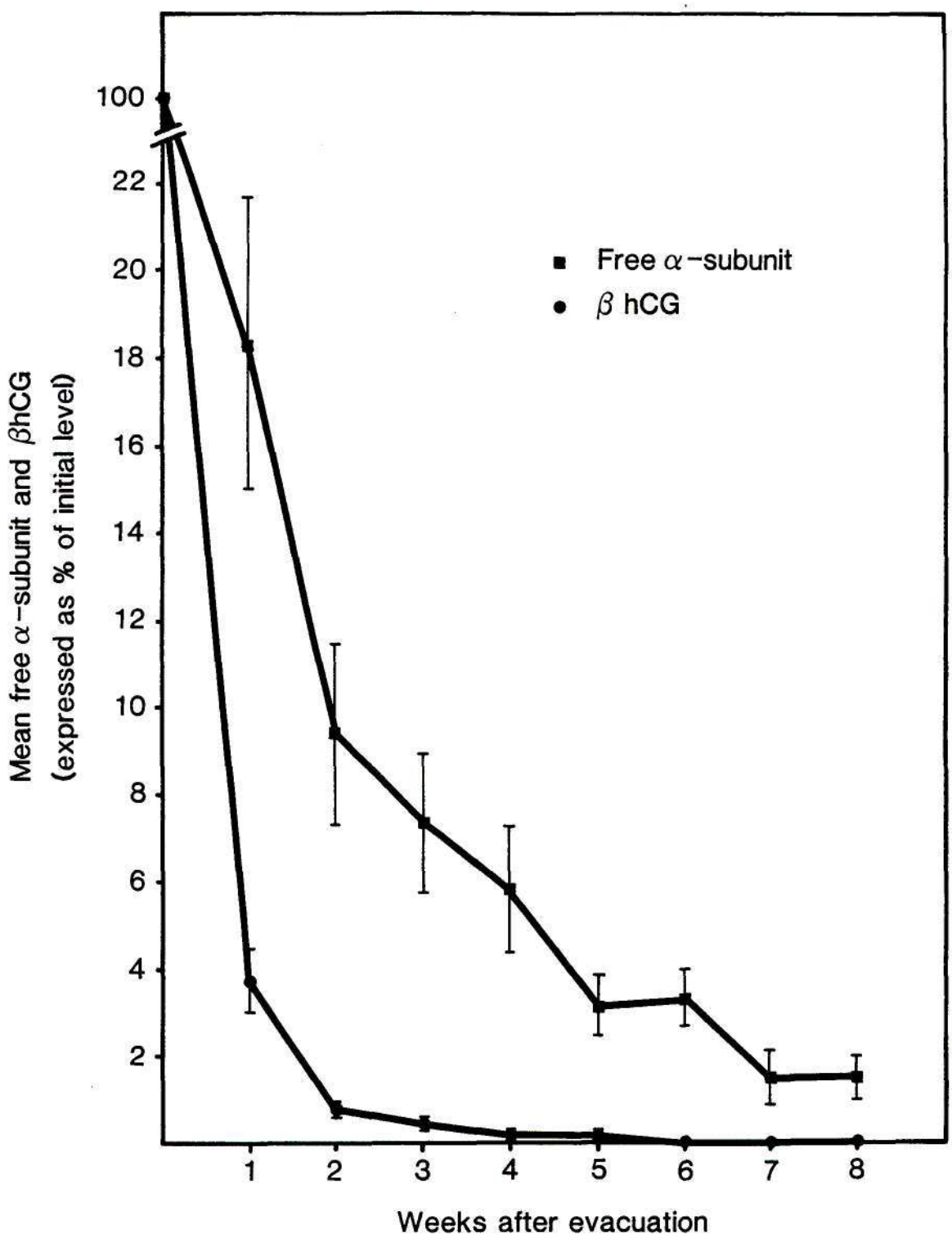


Figure 4.4 Post-evacuation disappearance of serum free α-subunit and ßhCG in patients with BMD.

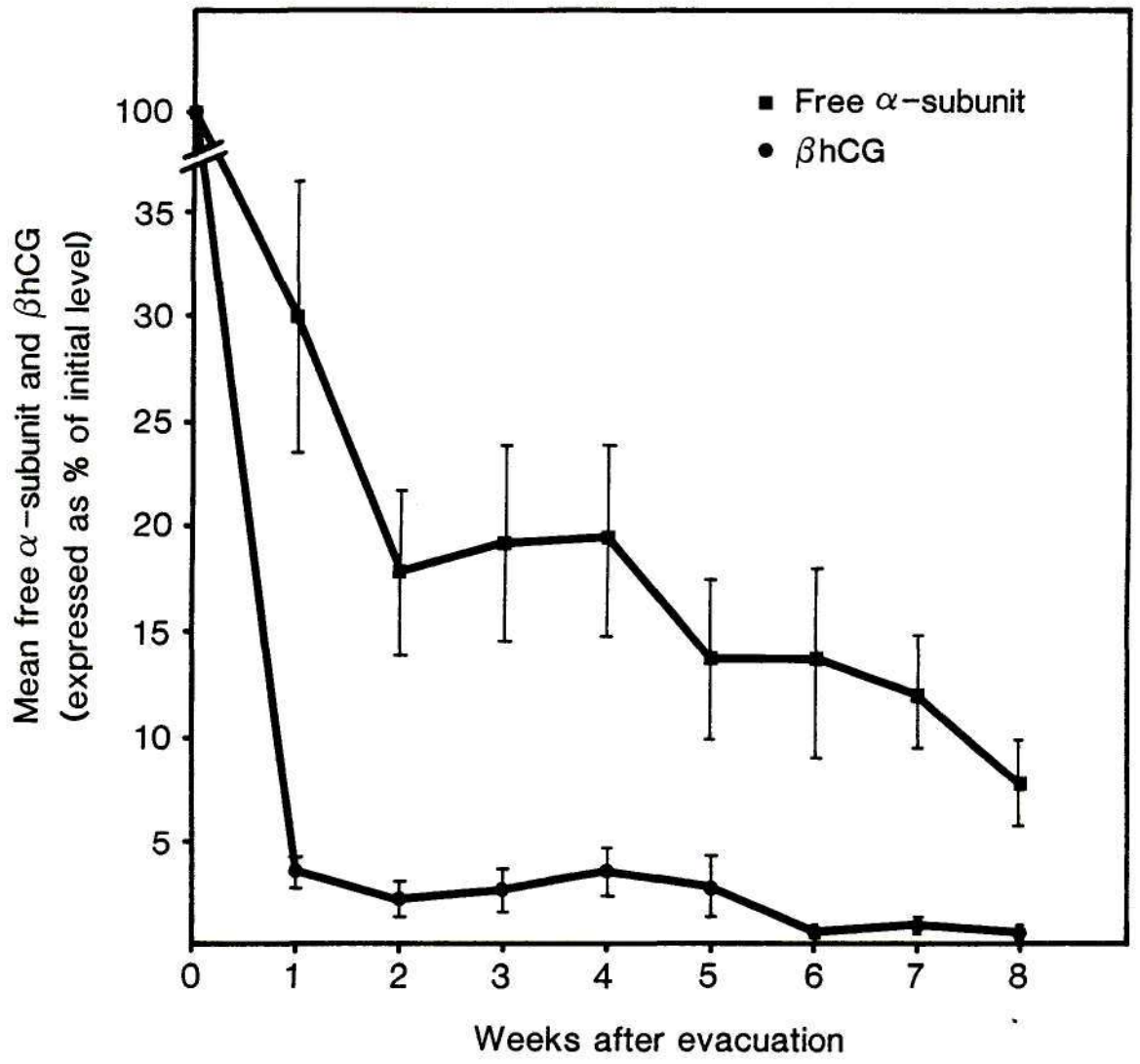


Figure 4.5 Post-evacuation disappearance of serum free α-subunit and ßhCG in patients with PMD.

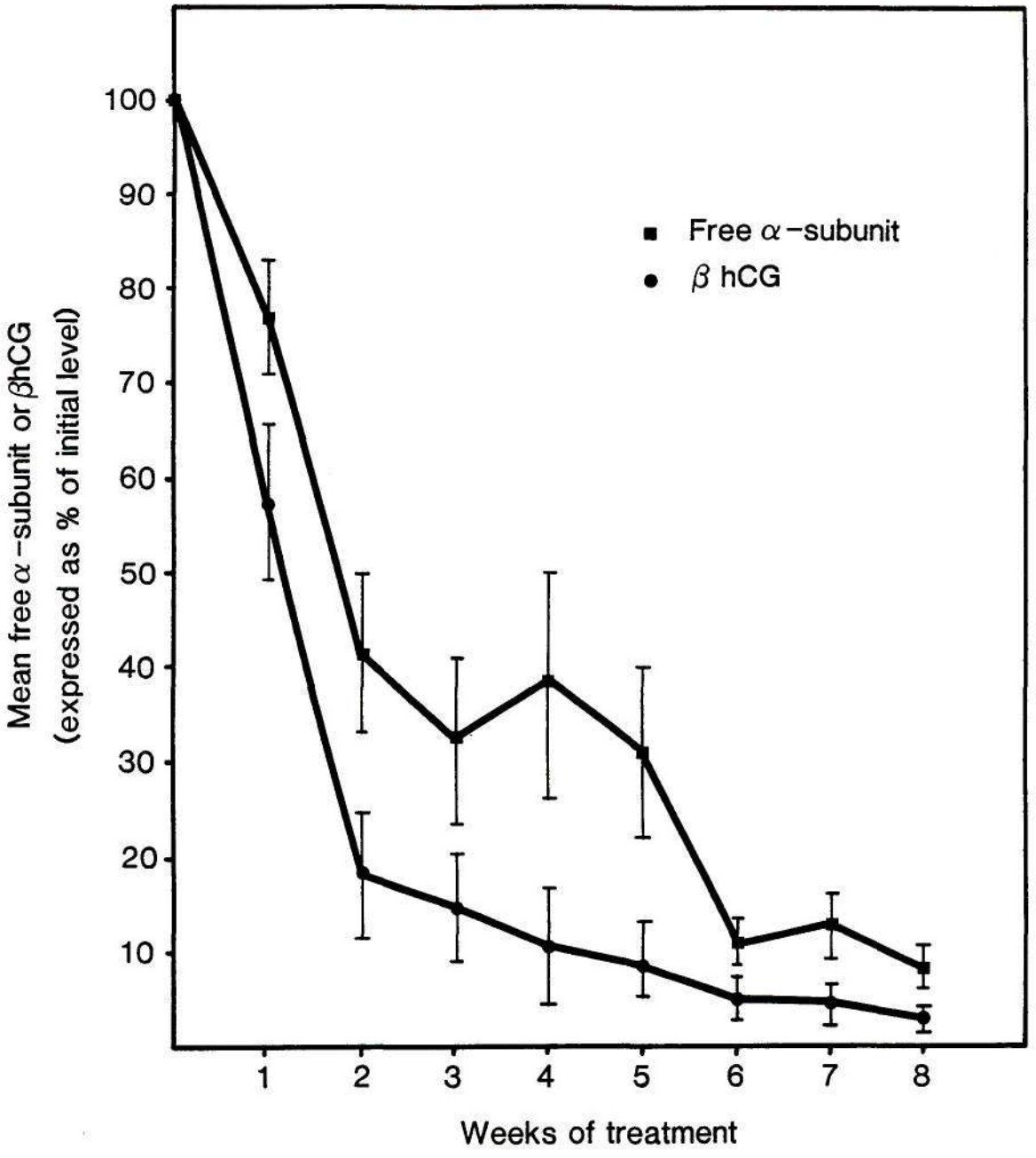


Figure 4.6 Disappearance of free α-subunit and ßhCG during chemotherapy in patients with choriocarcinoma.

significantly shorter in BMD than in either PMD ($p=0.006$) or malignant disease (PMD and choriocarcinoma; $p < 0.02$), but not significantly different to choriocarcinoma alone as shown in Table 4.3. In addition, the disappearance of free α-subunit was significantly earlier than that of ßhCG in each subgroup tested.

4.3.3 Free α-subunit Concentrations at Undetectable ßhCG Levels

The free α-subunit levels in patients with GTD in whom undetectable ßhCG levels had been achieved following treatment were all within the normal reference range for pre-menopausal females except one patient with PMD who had unusual levels of free α-subunit, as described in detail in section 4.3.4.

4.3.4 Case Reports

Fig. 4.7 demonstrates the changes in ßhCG and free α-subunit levels and the %α/ßhCG molar ratios in a patient with PMD (Patient A) during the course of the disease. The free α-subunit levels remained unchanged during treatment although ßhCG concentrations decreased to undetectable levels. The %α/ßhCG molar ratios therefore increased rapidly and were very high when ßhCG concentration was 7 IU/*l*. A rise in the ßhCG level (6 IU/*l*) after achieving an undetectable level, was noted in a serum sample taken 4 weeks later (not shown in illustration).

Fig. 4.8 illustrates the changes in free α-subunit and ßhCG levels, and the %α/ßhCG molar ratios in a patient with recurrent choriocarcinoma (Patient B). Two weeks after detection of recurrence (by a rising ßhCG level), the free α-subunit levels were increased above normal levels. This was reflected by a %α/ßhCG molar ratio of 45, which was higher (mean + 2 SD) than the mean pretreatment ratio in all GTD patients studied. The %α/ßhCG ratio fluctuated initially and then decreased gradually, but erratically during the course of the disease.

Table 4.3 Disappearance time of free α-subunit and ßhCG (in weeks) in GTD patients after treatment.

Patients	Weeks			
	free α-subunit		ßhCG	
	mean ± S.E.M.	range	mean ± S.E.M.	range
benign moles (n=8)	5.9 ± 0.93[1]	2.8 - 10.0	12.2 ± 1.4[5]	8.0 - 17.9
persistent moles (n=6)	14.1 ± 2.2[2]	5.6 - 21.0	21.1 ± 2.0[6]	14.9 - 27.7
choriocarcinoma (n=4)	10.4 ± 1.6[3]	7.1 - 14.6	15.9 ± 3.0[7]	10.7 - 23.7
malignant disease (n=10)	12.6 ± 1.5[4]	5.6 - 21.0	19.0 ± 1.8[8]	10.7 - 27.7

(1) vs (2) p = 0.006*
(1) vs (4) p < 0.02
(5) vs (6) p = 0.006*
(5) vs (8) p < 0.02
(1) vs (5) p = 0.007**
(2) vs (6) p = 0.015**
(3) vs (7) p = 0.033**

* Mann-Whitney test with Bonferroni adjustments (significance level p <0.0167)

**Paired t-test (significance level p <0.05)

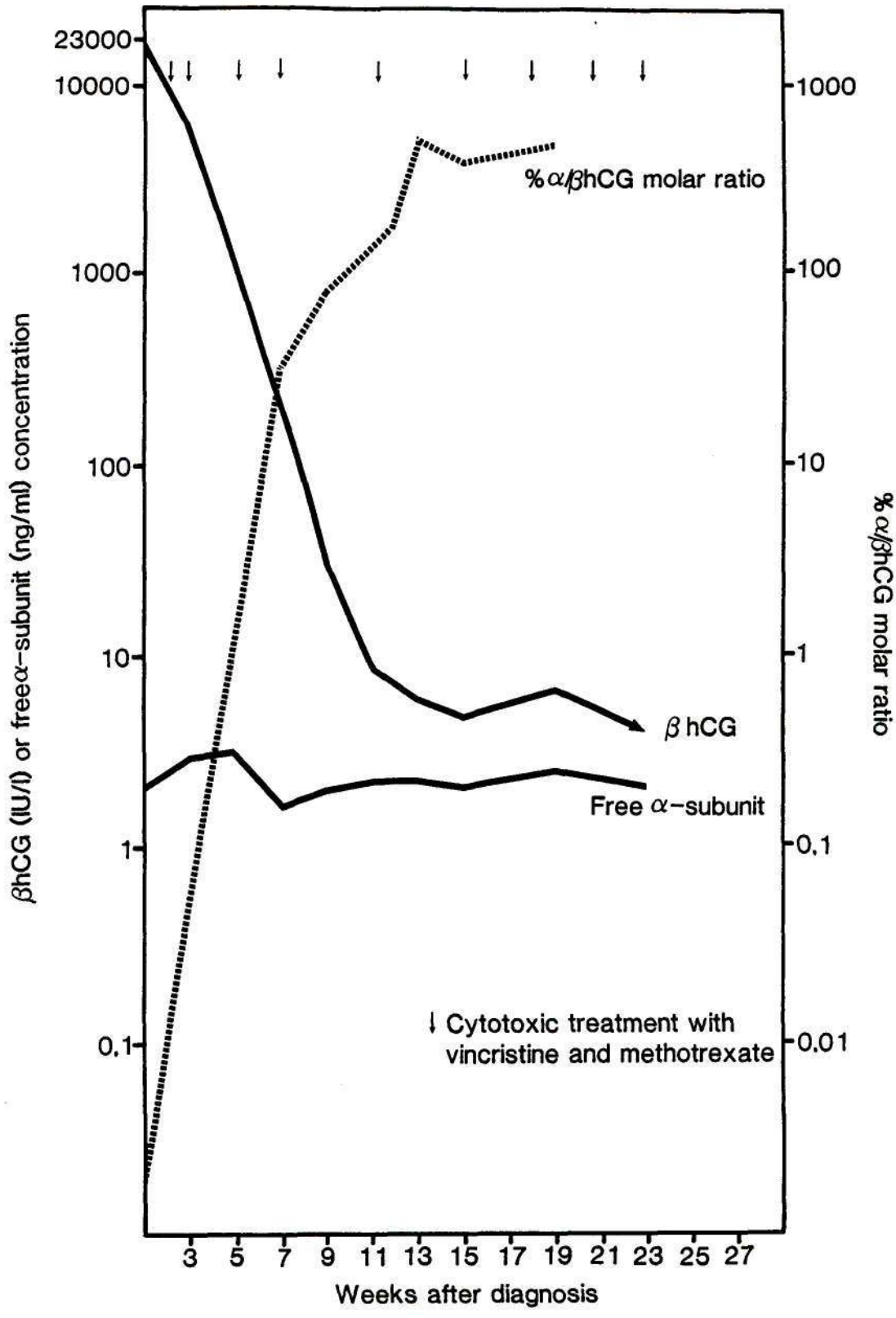


Figure 4.7 Serum free α-subunit and ßhCG concentrations, and %α/ßhCG molar ratios in a patient with PMD (Patient A).

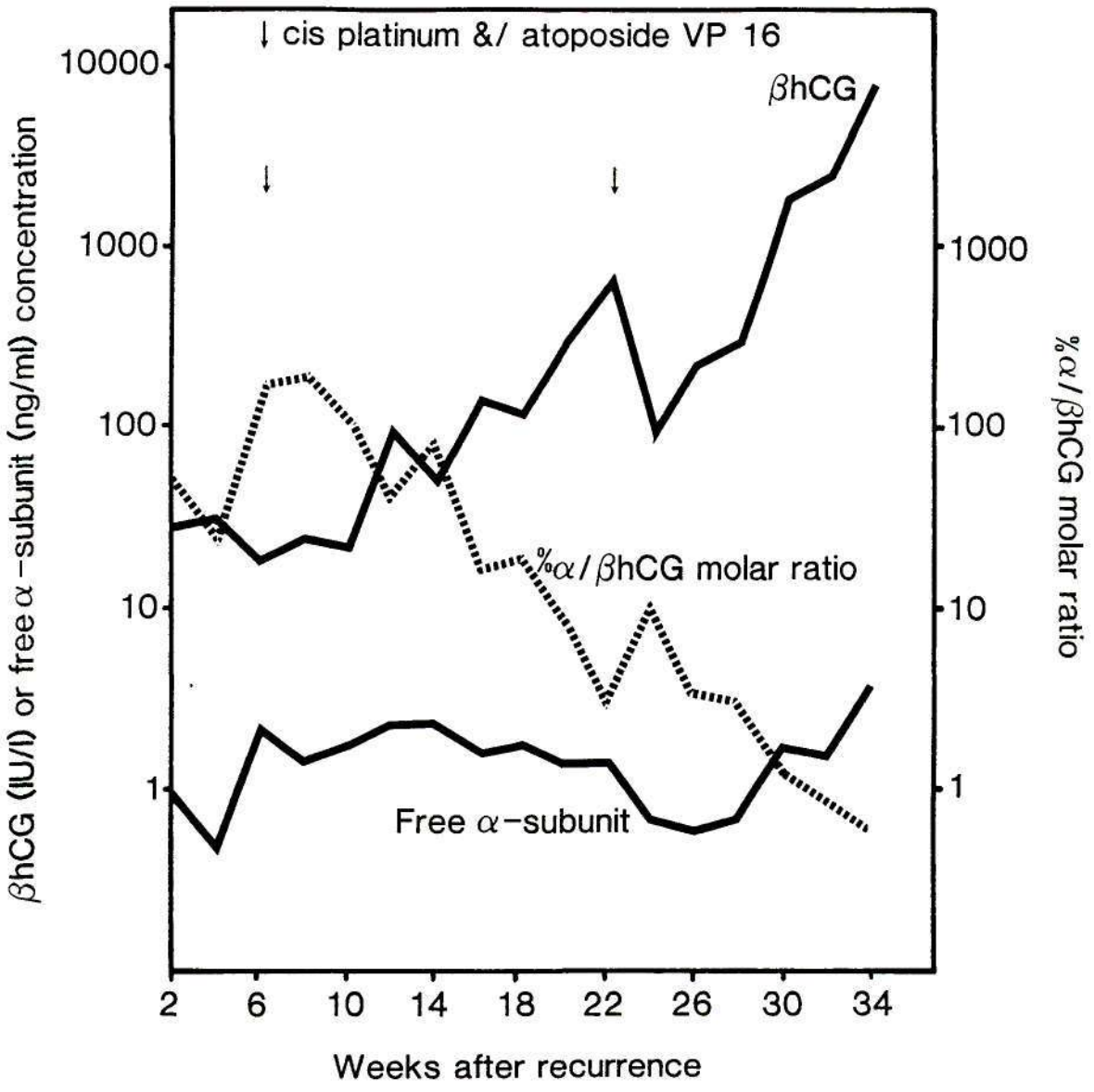


Figure 4.8 Serum free α-subunit and ßhCG concentrations, and %α/ßhCG molar ratios in a patient with recurrence of choriocarcinoma (Patient B).

4.4 DISCUSSION

There has been a great deal of controversy in the literature regarding the usefulness of routine free α-subunit estimations in the management of GTD. Although many workers have found free α-subunit measurement to be useful in the diagnosis and treatment of this disease, especially in high-risk patients (Schreiber *et al.*, 1976; Dawood *et al.*, 1977; Gaspard *et al.*, 1977; Nishimura *et al.*, 1979; Quigley *et al.*, 1980 a and b), others have found little use for free α-subunit estimations in these patients (Bagshawe 1980; 1984; Kohorn *et al.*, 1981). In these studies, however, polyclonal antibodies were used to measure free α-subunit and therefore could not be expected to have the sensitivity or specificity of the assay used in the present study. Hence, the true value of free α-subunit estimation in the management of GTD cannot be assessed from these earlier data. In the current study, a highly sensitive IRMA using monoclonal antibodies specific for the free α-subunit has been developed which has allowed the measurement of low levels of free α-subunit in the presence of high concentrations of intact hCG as found in trophoblastic disease. This specific IRMA was used to re-examine and evaluate the clinical usefulness of free α-subunit measurements in the diagnosis and the monitoring of tumour response to therapy in patients with GTD.

At presentation, the levels of free α-subunit in all patients in this study were considerably higher than the reference range for normal premenopausal females as determined by Norman *et al.* (1987) using a similar assay. However there would not appear to be any advantage in the measurement of free α-subunit for the initial diagnosis of GTD because the relative increase in this component is not as marked as that of βhCG. Furthermore, measurement of free α-subunit was of no value in differentiating between patients with benign and malignant disease at presentation, supporting an observation made by Hay (1988) who used a monoclonal-based IRMA to measure free α-subunit.

During the follow-up of patients with molar disease, however, both free α-subunit and βhCG concentrations were found to be significantly increased within 2 weeks of evacuation in PMD compared to BMD. In addition, the %α/βhCG molar ratios were significantly higher in patients with BMD than in those with PMD. Since

trophoblastic tumours display heterogeneity in tumour size, the free α-subunit and ßhCG concentrations were also expressed as a percentage of the levels at presentation. Similar differences between benign and malignant disease were observed and confirmed the above finding. Currently patients with molar disease are assessed at 6-8 weeks post-evacuation and grouped according to ßhCG concentrations. Results from this study show that differences in subgroups of patients were already apparent at 2 weeks, potentially allowing for an earlier distinction between BMD and PMD. Furthermore, the molar ratios may also be useful to differentiate the two subgroups.

Although ßhCG was present in much higher concentrations, its rate of disappearance was found to be faster than that of the free α-subunit in all groups of patients. This may be explained by either increased production of free α-subunit in relation to hCG by tumour tissue or by the relatively faster clearance of hCG. In BMD where it can be assumed that essentially all tumour tissue has been removed at surgery, it would appear that the observed rates of disappearance are more likely due to the enhanced clearance of hCG relative to the free α-subunit. The relative clearance rates of both free α-subunit and hCG in GTD in this study differ considerably from published data on half-life times ($t_{1/2}$) of hCG and the free α-subunit (Wehman and Nisula, 1979; 1981). These workers administered highly purified preparations of intact hCG and free α-subunit to normal subjects and found that the plasma $t_{1/2}$ of free α-subunit of hCG was significantly faster than that of intact hCG (1.27 vs 35.6 h). A possible explanation for the observed difference in clearance rates in GTD may be due to the presence of alternative forms of hCG and free α-subunit which have been described in some patients (Amr *et al.*, 1983; 1984). Heterogeneity in the carbohydrate content is believed to significantly influence the survival time in blood (Amr *et al.*, 1984). Desialylated forms of hCG which are rapidly cleared from the circulation have been found in the urine of patients with choriocarcinoma but are not present in normal pregnancy (Amr *et al.*, 1985). In addition, the secretion of large forms of free α-subunit has been observed in cultured human malignant trophoblasts (Hussa, 1977). These forms have a prolonged plasma half-life relative to the α-subunit found in normal pregnancy.

In patients with GTD, successful therapy is assumed when the concentration of ßhCG

reaches normal or undetectable levels. In all 3 groups of patients normal levels of free α-subunit were achieved earlier than ßhCG despite its slower clearance. Although this most likely relates to the lower initial concentrations of free α-subunit compared to ßhCG, free α-subunit estimation may therefore also be useful for the early identification of cure or remission in patients with GTD.

In the follow-up of two patients with malignant disease in detail, an unusual pattern of secretion of α-subunit and ßhCG was noted. The preferential secretion of α-subunit has also been demonstrated by Dawood *et al.* (1977) in two patients with choriocarcinoma whose α-subunit levels increased while hCG declined or were absent. Since the intact hCG levels were low or undetectable, the α-subunit measured was more likely to be free α-subunit and thus the persistence of the disease might have been missed if only the intact hCG levels were measured. It may therefore be necessary to measure both free α-subunit and ßhCG in the management of patients with GTD.

The preliminary data from this study indicate that the measurement of free α-subunit together with that of ßhCG may have an additional usefulness in the earlier identification of patients with malignant forms of GTD. In patients with molar disease, the use of prophylactic chemotherapy at the time of evacuation has met with little enthusiasm, since 80 - 85% of patients with BMD undergo spontaneous remission after evacuation (Khazaeli *et al.*, 1986). Differentiation between benign and persistent disease, as early as possible, is important to allow earlier chemotherapeutic intervention and possible prevention of high risk disease (cerebral and liver metastases). In addition, because of its earlier disappearance, measurement of free α-subunit may also be useful in the monitoring of tumour response to therapy. Since tumours may secrete free α-subunit preferentially, it is important to measure both the free α-subunit and ßhCG. Although the results obtained here are promising, this study was of a retrospective nature and included only a modest number of patients. A prospective study, with a larger number of patients and good follow-up would allow a thorough examination of free α-subunit levels in GTD and may lead to confirmation of these preliminary results.

4.5 SUMMARY

Increased levels of free α-subunit were found in the sera of all patients with GTD. The pre-treatment free α-subunit levels were significantly higher in PMD than in choriocarcinoma while no significant differences in the ßhCG and %α/ßhCG molar ratio within the subgroups were noted.

After evacuation and during chemotherapy the free α-subunit levels generally decreased in parallel with ßhCG levels. The free α-subunit and ßhCG concentrations in patients with molar disease, were found to be significantly higher from 2 - 8 weeks post-evacuation in PMD when compared with BMD. The %α/ßhCG molar ratios at each week following evacuation were significantly higher between 2 and 7 weeks post-evacuation in BMD. The disappearance of free α-subunit and ßhCG, expressed as a percentage of the initial levels, showed similar differences. However, the rate of disappearance of ßhCG was faster than that of free α-subunit. The disappearance time of both free α-subunit and ßhCG, in weeks, was significantly shorter in BMD than in PMD or malignant disease while the free α-subunit disappeared before ßhCG in each subgroup.

After treatment, detectable free α-subunit levels were found in patients who were cured or in remission; these concentrations were within the reference range for pre-menopausal females. In one patient with recurrence of disease, the %α/ßhCG molar ratio increased initially due to a paradoxical secretion of free α-subunit and ßhCG. Another patient presenting with PMD was found to have an unchanged serum free α-subunit level throughout the course of the disease.

SUMMARY AND CONCLUSION

The great art consists in devising decisive experiments, leaving no place to the imagination of the observer. Imagination is needed to give wings to thought at the beginning of experimental investigations on any given subject. When, however the time has come to conclude, and to interpret the facts derived from observations, imagination must submit to the factual results of the experiments.

Louis Pasteur [1822 - 1895]

The mouse hybridoma technique was successfully used to culture two clones secreting monoclonal antibodies to the free α-subunit of hCG. On subtyping, the antibodies designated 71 and 75 were found to be of the IgG1 subclass. Both antibodies 71 and 75 were very specific for the free α-subunit but showed comparatively low affinities for the molecule, with dissociation constants of 0.92 x 10^{-8} and 4.08 x 10^{-8} mol/*l*, respectively. Each antibody was shown to recognise two distinct epitopes on the free α-subunit molecule. In addition, the two antibodies 71 and 75 displayed cooperativity in binding to the free α-subunit when incubated simultaneously.

Using these two antibodies, one biotinylated as the capture antibody (75) and the other radiolabelled as the detection antibody (71), and polystyrene beads coated with avidin as a solid-phase separation system, an IRMA for the measurement of the free α-subunit of hCG was developed. The assay was found to be simple to perform, specific for the free α-subunit, sensitive to levels as low as 0.05 ng/ml and precise at α-subunit concentrations ranging between 0.2 - 32 ng/ml.

The assay was used to assess the usefulness of free α-subunit measurements in the diagnosis and management of patients with GTD. The levels of free α-subunit in these patients were found to be increased but were of no additional value to ßhCG in either the initial diagnosis or in differentiating between benign and malignant disease. After evacuation and during chemotherapy, the free α-subunit levels

decreased and generally followed the same pattern of change as seen in ßhCG. The disappearance time of free α-subunit was shorter than that of ßhCG. This important finding may allow an earlier identification of patients with persistent disease and may also play a role in the earlier detection of tumour response to chemotherapy. On the other hand, the rate of disappearance of ßhCG was found to be faster than that of the free α-subunit in all groups of patients. This unexpected observation may be explained by the possible presence of larger forms of free α-subunit or lower molecular weight desialylated forms of hCG in GTD. Because of the retrospective nature of this study and modest patient numbers the value of free α-subunit estimation in the management of GTD is still uncertain at this stage. A prospective study, with a larger patient population may confirm these preliminary results.

BIBLIOGRAPHY

Read not to contradict and confute, nor to believe and take for granted --- but to weigh and consider.

Francis Bacon [1561-1626]

Amr S, Wehman RE, Birken S, Canfield RE, Nisula BC. Characterisation of a carboxyterminal peptide fragment of the human choriogonadotropin ß-subunit excreted in the urine of a woman with choriocarcinoma. *J Clin Invest* 1983; **71:** 829.

Amr S, Rosa C, Wehman RE, Birken S, Nisula BC. Unusual molecular forms of hCG in gestational trophoblastic neoplasia. *Ann Endocrinol (Paris)* 1984; **45:** 321.

Amr S, Rosa C, Birken S, Canfield RE, Nisula BC. Carboxyterminal peptide fragments of the ß-subunit are urinary products of the metabolism of desialylated human choriogonadotropin in humans. *J Clin Invest* 1985; **76:** 350.

Andersson J and Melchers F. The antibody repertoire of hybrid cell lines obtained by fusion of X63-Ag8 myeloma cells with mitogen-activated B-cell blasts. *Current Topics Microbiol Immunol* 1978; **81:** 130.

Bagshawe KD. Trophoblastic tumours and teratomas. In: Bagshawe KD, ed. *Medical Oncology: Medical Aspects of Malignant Disease.* London: Blackwell Scientific Publications, 1975; 453.

Bagshawe KD. Marker proteins as indicators of tumour response to therapy. *Br J Cancer* 1980; **41 (4):** 186.

Bagshawe KD. Clinical applications of hCG. In: Patillo RA, Hussa RO, eds. *Human*

Trophoblastic Neoplasms. New York: Plenum Press, 1984; 313.

Bayer EA and Wilcheck M. Insolubilised biotin for the purification of avidin. *Meth Enzymol* 1974; **34:** 265.

Bayer EA, Shutelsky E, Wilchek M. The avidin-biotin complex in affinity cytochemistry. *Meth Enzymol* 1979; **62:** 308.

Bellisario R, Carlson PB, Bahl OP. Human chorionic gonadotropin: linear amino sequence of the α-subunit. *J Biol Chem* 1973; **248:** 6796.

Berson SA, Yalow RS, Bauman A, Rothschild MA, Newerly K. Insulin I^{131} metabolism in human subjects: demonstration of insulin-binding globulin in the circulation of insulin treated subjects. *J Clin Invest* 1956; **35:** 170.

Bolton AE and Hunter WM. Radioimmunoassay and related methods. In: Weir DM, ed. *Handbook of Experimental Immunology*. Oxford: Blackwell Scientific Publications, 1986; **1:** 26.1.

Chapman RS, Sutherland RM, Ratcliffe JG. Application of 1,1-carbonyldiimidazole as a rapid practical method for the production of solid phase immunoassay reagents. In: Hunter WM, Corrie JET, eds. *Immunoassays for clinical chemistry.* London: Churchill Livingstone, 1983; 178.

Dawood MY, Saxena BB, Landesman R. Human chorionic gonadotrophin and its subunits in hydatidiform mole and choriocarcinoma. *Obstet Gynaecol* 1977; **50:** 172.

Ehrlich P. Die wertbemessung des diphterieheilseums. *Klin Jahrb* 1897; **60:** 299. (English translation in *Collected Papers* 1956; **2:** 107. London: Pergamon)

Eshhar Z, Blatt C, Bergman Y, Haimovitch J. Induction of secretion of IgM from cells of the B cell line 38C-13 by somatic cell hybridisation. *J Immunol* 1979; **122:** 2430.

Ey PL, Prowse SJ, Jenkin CR. Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using protein A Sepharose. *Immunochemistry* 1978; **15:** 429.

Fiddes JC and Goodman HM. The cDNA for the α-subunit of human chorionic gonadotrophin suggests evolution of a gene by readthrough into the 3-untranslated region. *Nature* 1980; **286:** 684.

Freund J. The mode of action of immunologic adjuvants. *Adv Tuberc Res* 1956; **7:** 130.

Galfr'e G, Howe SC, Milstein C, Butcher GW, Howard JC. Antibodies to major histocompatibility antigens produced by hybrid cell lines. *Nature* 1977; **266:** 550.

Gaspard UJ, Reuter AM, Deville JC, Gaevert-Vrindts Y, Franchimont P. Typical pattern of hCG alpha and hCG beta serum concentrations in molar pregnancies. *Acta Endocrinol* 1977; **85 (212):** 33.

Gaspard UJ, Reuter AM, Deville JC, Gaevert-Vrindts Y, Bagshawe KD, Franchimont P. Serum concentration of human chorionic gonadotrophin and its alpha and beta subunits in trophoblastic tumours. *J Endocrinol* 1980; **13:** 319.

Gefter ML, Marguiles, DH, Scharff MD. A simple method for polyethylene glycol-promoted hybridization of mouse myeloma cells. *Somat Cell Genet* 1977; **3:** 231.

Goding JW. Use of Stapylococcal protein A as an immunological reagent. *J Immunol Methods* 1978; **20:** 241.

Goding JW. Antibody production of hybridomas. *J Immunol Methods* 1980; **39:** 285.

Goding JW. In: Goding JW, ed. *Monoclonal antibodies: principles and practice.* London: Academic Press, 1986.

Green-Thompson RW. Gestational trophoblastic disease. *CME* 1986; **4:** 13.

Greenwood FC, Hunter WM, Glover JS. The preparation of ^{131}I-labelled human growth hormone of high specific activity. *Biochemical Journal* 1963; **89:** 114.

Haeuptle M-T, Aubert ML, Djiane J, Kraehenbuhl J-P. Binding sites for lactogenic and somatogenic hormones from rabbit mammary gland and liver. *J Biol Chem* 1983; **258:** 305.

Hales CN and Woodhead JS. Labeled antibodies and their use in the immunoradiometric assay. *Meth Enzymol* 1980; **70:** 334.

Hay DL. Histological origins of discordant chorionic gonadotrophin secretion in malignancy. *J Clin Endocrinol Metab* 1988; **66 (3):** 557.

Herbert, WJ. Mineral-oil adjuvants and the immunisation of laboratory animals. In: Weir DM, ed. *Handbook of Experimental Immunology.* Oxford: Blackwell Scientific Publications, 1973; **3:** A2.1.

Hunter WM, Bennie JG, Budd PS, Heyningen V, James K, Micklem RL, Scott A. Immunoradiometric assays using monoclonal antibodies. In: Hunter WM and Corrie JET eds. *Immunoassays for Clinical Chemistry.* London: Churchill Livingstone, 1983; **12.2:** 531.

Hussa RO. Immunologic and physical characterization of human chorionic gonadotroin and its subunits in cultures of human malignant trophoblast. *J Clin Endocrinol Metab* 1977; **44(6):** 1154.

Hussa RO. Human chorionic gonadotrophin, a clinical marker: review of its biosynthesis. *Ligand Review* 1981; **3 (2).**

Khazaeli MB, Hedayat MM, Hatch KD, To ACW, Soong S-j, Shingleton HM, Boots LR, Lo Buglio AF. Radioimmunoassay of free ß-subunit of human chorionic gonadotrophin as a prognostic test for persistent trophoblastic disease in molar pregnancy. *Am J Obstet Gynaecol* 1986; **155 (2):** 320.

Kohler G and Milstein C. Continuous cultures of fused cells secreting antibody of pre-defined specificity. *Nature* 1975; **256:** 495.

Kohorn EI, Burton V, Cortes C, Cortes JM. Alpha subunit in gestational trophoblastic disease. *Placenta* 1981; (**3**)**:** 231.
Landsteiner K. *Die Spezifizitat der Serologishen Reaktionen.* Berlin: Springer, 1933. (English translation in: *The Specificity of Serological Reactions.* 2nd ed. New York: Dover, 1962.)

Lefkovits I and Waldmann H. *Limiting Dilution Analysis of Cells in the Immune System.* Cambridge: Cambridge University Press, 1979.

Littlefield, JW. Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinants. *Science* 1964; **145:** 709.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; **193:** 265.

Mayer RJ and Walker JH. Enzymes and proteins. In: Mayer RJ and Walker JH, eds. *Immunochemical Methods in Cell Biology*. London: Academic Press, 1987.

McCormick DB and Roth JA. Colorimetric determination of biotin and analogs. *Meth Enzymol* 1970; **18A:** 383.

Miles LEM and Hales CN. Labelled antibodies and immunological assay systems. *Nature* 1968; **219:** 186.

Milstein C. Overview: monoclonal antibodies. In: Weir DM, ed. *Handbook of Experimental Immunology.* Oxford: Blackwell Scientific Publications, 1986; **4:** 107.1.

Morgan EJ, Canfield RE, Vaitukaitis JL, Ross GT. Preparation and characterisation of the subunits of hCG. In: Berson SA and Yallow RS eds. *Methods of Investigative and Diagnostic Endocrinology, Part iii, Non-pituitary Hormones*. Amsterdam: North Holland Publishing Company, 1973; 733.

Moyle WR, Anderson DM, Erlich PH. A circular antibody-antigen complex is responsible for increased affinity shown by mixtures of monoclonal antibodies to human chorionic gonadotropin. *J Immunol* 1983a; **131:** 1900.

Moyle WR, Liu C, Corson RL, Erlich PH. Quantitative explanation for increased affinity shown by mixtures of monoclonal antibodies: Importance of a circular complex. *Mol Immunol* 1983b; **20:** 439.

Nishimura N, Ashikata Y, Tojo S. The clinical evaluation of the simultaneous measurements of human chorionic gonadotrophin and its alpha subunit in sera of patients with trophoblastic disease. *Endocrinol Jpn* 1979; **26:** 575.

Norman RJ, Haneef R, Buck RH, Joubert SM. Measurement of the free alpha subunit of human glycoprotein hormones by a monoclonal antibody-based immunoradiometric assay, and further exploration of antigenic sites on the choriogonadotropin molecule. *Clin Chem* 1987; **33:** 1147.

Norman RJ, Polton T, Gard T, Chard T. Monoclonal antibodies to human chorionic gonadotrophin: implications for antigenic mapping, immunoradiometric assays and clinical application. *J Clin Endocrinol Metab* 1985; **61:** 1031.

Norwood TH, Ziegler CJ, Martin GM. Dimethyl sulphoxide enhances polyethylene glycol-mediated cell fusion. *Somatic Cell Genet* 1976; **2:** 263.

Odell WD, Griffin J, Zahradnik R. Two-monoclonal-antibody sandwich-type assay for thyrotropin, with use of an avidin-biotin separation technique. *Clin Chem* 1986; **32(10):** 1873.

Odell WD and Griffin J. Pulsatile secretion of human chorionic gonadotropin in normal adults. *New Engl J Med* 1987; **317(27):** 1688.

Oi VT and Herzenberg LA. Immunoglobulin-producing hybrid cell lines. In: Mishell BB and Shiigi SM, eds. *Selected Methods in Cellular Immunology*. San Francisco: Freeman Press, 1980; 351.

Patillo RA and Hussa RO. Gestational trophoblastic disease and human chorionic gonadotrophin measurement. *J Reprod Med* 1987; **32 (9):** 629.

Pontecorvo G. Production of indefinitely multiplying mammalian somatic cell hybrids by polyethylene glycol treatment. *Somatic Cell Genet* 1976; **1:** 397.

Quigley MM, Tyrey L, Hammond CB. Alpha subunit in sera of choriocarcinoma patients in remission. *J Clin Endocrinol Metab* 1980a; **50:** 98.

Quigley MM, Tyrey L, Hammond CB. Utility of assay of alpha subunit of human chorionic gonadotrophin in the management of gestational trophoblastic malignancies. *Am J Obstet Gynecol* 1980b; **138:** 545.

Rimm AA, Hartz AJ, Kalbfeisch JH, Anderson AJ, Hoffmann RG. Z and t test-comparing two or more groups. *Basic Biostatistics in Medicine and Epidemiology*. New York: Appleton-Century Crofts. 1980; **13:** 207.

Ringertz NR and Savage RE. *Cell Hybrids*. New York: Academic Press, 1976.

Scatchard G. The attraction of proteins for small molecules and ions. *Ann NY Acad Sci* 1949; **51:** 660.

Schreiber JR, Rebar RW, Chen H, Hodgen GD, Ross GT. Limitations of the specific radioimmunoassay for human chorionic gonadotrophin in the management of trophoblastic neoplasms. *Am J Obstet Gynaecol* 1976; **125:** 705.

Sharon J, Morrison SL, Kabat, EA. Formation of hybridoma clones in soft agarose: effect of pH and medium. *Somat Cell Genet* 1980; **6:** 435.

Siddle K and Soos M. Monoclonal antibodies for human pituitary glycoprotein hormones and rabbit immunoglobulin. In: Albertini A and Ekins RP, eds. *Monoclonal antibodies and developments in immunoassay*. Amsterdam: Elsevier, 1981; 53.

Soos M and Siddle K. Characterisation of monoclonal antibodies directed against human thyroid stimulating hormone. *J Immunol Methods* 1982; **51:** 57.

Staehelin T, Durrer B, Schmidt J, Takac B, Stocker J, Miggiano V, Stahli C, Levy WP, Hershberg R, Pestka S. Production of hybridomas secreting monoclonal antibodies to the human leukocyte interferons. *Proc Natn Acad Sci USA* 1981; **78:** 1848.

Stahli C, Staehelin T, Miggiano V, Schmidt J, Haring P. High frequencies of antigen-specific hybridomas: dependence on immunisation parameters and production by spleen cell analysis. *J Immunol Methods* 1980; **32:** 297.

Thotakura NR and Bahl OP. Highly specific and sensitive hybridoma antibodies against the alpha-subunit of glycoprotein hormones. *Endocrinology* 1985; **117:** 1300.

Vaitukaitis J, Braunstein G, Ross GT. A radioimmunoassay which specifically measures human chorionic gonadotrophin in the presence of human luteinising hormone. *Am J Obstet Gynecol* 1972; **113:** 751.

Wehman RE and Nisula BC. Metabolic and renal clearance rates of purified human chorionic gonadotropin. *J Clin Invest* 1981; **68:** 184.

Wehman RE and Nisula BC. Metabolic clearance rates of the subunits of human chorionic gonadotropin in man. *J Clin Endocrinol Metab* 1979; **48:** 753.

Westerwoudt RJ, Blom J, Naipal AM, Rood JJ. Improved fusion technique 1: Human umbilical cord serum, a new and potent growth promoter compared with other B cell and hybridoma activators. *J Immunol Methods* 1983; **62:** 59.

Wojciezsyn JW, Schlegel RA, Limley-Sapanski K, Jacobson KA. Studies on the mechanism of polyethylene glycol-mediated cell fusion using fluorescent membrane and cytoplasmic probes. *J Cell Biol* 1983; **96:** 151.