'DEVELOPMENTAL ANTIGENS IN CANCER AND IMMUNE SUPPRESSION'

by

Ross Samuel Savvas, B.Sc.(HONS)

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Department of Animal Physiology
Waite Agricultural Research Institute
University of Adelaide

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This thesis is dedicated to Yahweh and Di

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SUMMARY

The malignant transformation, and the relevance of developmental antigens to the cancer process, is broadly reviewed. The two developmental antigens - foetal and placental - are then examined in experimental mouse and rat tumour systems.

An attempt to prepare a specific antiserum to foetal antigens is described. This type of antiserum, reported by Stonehill and Bendich (1970) as being able to detect murine onco-foetal antigens, could not be reproduced in these studies. Further examination revealed that the antiserum used by Stonehill was not detecting an onco-foetal antigen, but rather a circulating normal adult antigen.

A number of attempts were also made to demonstrate the appearance of placental antigens in rat tumours. Although a model was developed whereby at least 6 saline-soluble carcino-placental antigens could be detected, this system was found unsuitable for general laboratory use.

The reappearance of membrane-bound placental antigens in a chemically induced hepatoma was also examined. However, due to the inability to produce a specific anti-placental serum, these experiments were unsuccessful.

During the course of these studies, it was found that rabbits and mice were unable to raise antibodies to heterologous foetal extracts unless the immunogen was freeze-dried prior to inoculation. This unusual situation was unexpected, as lyophilization should not significantly alter the immunogenicity of the antigens. By

experimentally eliminating all other likely possibilities, it was proposed that the non-freeze dried foetal extract contained a repressor which specifically inhibited the production of antibodies to foetal antigens. Further, it was also postulated that the repressor may be a lyophilization-labile antigen-antibody complex.

The relevance of these repressor studies to foetal and tumour immunology is discussed.

DECLARATION

I declare that this thesis does not contain any material which has been submitted previously for any degree or diploma to any university; and to the best of my knowledge it does not contain any material previously published or written by another person, except where due reference is made in the text.

Ross Savvas

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PUBLICATIONS

Aspects of this study have been presented at scientific meetings in Australia. The abstracts appear in the following journals.

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Savvas, R.S., Bais, R. and Sabine, J.R. (1976). 'Carcino-placental antigens'. *Clin. Exp. Pharm. Phys.* *3* : 281.

ABBREVIATIONS

AFP	α-foeto protein
BSA	bovine serum albumin
CEA	carcino-embryonic antigen
DNA	deoxyribonucleic acid
FCA	Freund's complete adjuvant
FD	freeze dried
FIA	Freund's incomplete adjuvant
HCG	human chorionic gonadotrophin
IEP	immunoelectrophoresis
MeDaB	3-methyl-4-dimethyl amino azo benzene
NFD	non freeze dried
RNA	ribo nucleic acid

PREFACE

In this thesis, I have attempted to broadly review the current concepts of cancer research, with particular emphasis on tumour immunology. More specifically, I have concentrated on two emerging fields - carcinofoetal and carcinoplacental antigens.

Much of the current research is masked in a confusion of terminology. Further, there has been only a small attempt to answer the question, 'why do foetal and placental antigens re-appear in tumours?' - perhaps a fundamental question in the understanding of the cancer process. Part of the problem lies in the fact that the foetal antigens have not been characterized in terms of a specific non-immunological function. The experimental work presented in this thesis is to some degree also guilty of this. However, my approach from the start has been three-fold:

1. Demonstrate the presence of these onco-developmental antigens in experimental tumours.
2. Determine at which point of time following carcinogen application they appear.
3. Attempt to assign a non-immunological, biological function to these antigens.

Thus, while still keeping the third area of study in mind, it was first important to demonstrate the presence of these antigens in the tumours, and *then* to answer the question, 'What are they doing there?'.

In the final chapters of this thesis, I describe a new phenomenon - that of antibody synthesis repression by foetal antigens. I say new, not because the idea is new, but its concept and relationship to specific biological problems is now presented in the form of models as a basis for solving these problems.

CHAPTER I

LITERATURE REVIEW

SECTION I : GENERAL CHARACTERISTICS OF CANCER

1. The Disease

Cancer is a disease of multi-cellular organisms. It is characterized by uncontrolled cellular division, resulting in the production of tumours. Many such cancerous growths are able to spread to other tissues, often resulting in death of the host due to direct impairment of essential function, and/or wasting.

Although studies of cancer have been almost exclusively in mammalian systems, the disease has also been shown to occur in vertebrates (Halver, 1965), insects (Harshbarger and Taylor, 1968), and plants (El Khalifa and Lippincott, 1968). In this review, only mammalian cancer is considered, in order to integrate the many and diverse studies of oncology into a broad summary of cancer. These studies are presented at three organizational levels:

(a) tumour development

(b) genetic changes which initiate tumours and those which result from tumour development

(c) phenotypic products of these gene changes.

This thesis is principally concerned with a particular phenotypic characteristic of the tumour - the reappearance, in carcinomas, of properties normally associated with embryonic development.

2. Tumour Development - the clonal evolution of tumour cells

Before discussing any phenotypic changes which occur during the malignant transformation, it is necessary to describe the mechanism of tumour development from an initial cancerous cell. Such a discussion is warranted because many tumours share a number of phenotypic products, and yet still maintain their individuality. It is the path of tumour development which may determine the characteristics of a tumour.

Nowell (1976) has formulated a model which describes the clonal evolution of cancerous cells, and their subsequent development into a tumour. In this model, tumour initiation occurs by an induced change in a single, previously normal, cell. Neoplastic proliferation then proceeds either immediately or after a latent period. This small number of cells may escape immunological surveillance due to insufficient stimulation of the immune system (Bonmasser *et al.*, 1976), and hence the number of cancer cells grows. As the mass of cells increases some may ultimately be destroyed by the immune system. Others, however, undergo mutation due to genetic instability in the tumour cell population. Although many of these mutant cells are destroyed, either immunologically or by metabolic disadvantages, one mutant may arise which has a selective advantage and hence survives. This cell then becomes the precursor of a new predominant sub-population of cancer cells. This sequential selection process continues over a period of time, with the cells becoming increasingly abnormal both genetically and metabolically. However, it is important to note that this sequence

of events is not completely random. Consequently, different tumours may acquire certain similarities. The divergence or tumour-specific characteristics develop due to the effect of local conditions influencing the emergence of variant sub-lines. Ultimately, the fully developed malignancy as it appears clinically, has a genetic make-up uniquely different from the original non-neoplastic cell. Associated with this is an aberrant metabolic behaviour and "new" antigenic properties.

3. Genomic Alterations During Transformation

In the model presented above, a change in the normal cell was required in order to initiate the neoplastic process. Except in the case of viral transmission, the initiator of the transformation has yet to be isolated. However, it is generally considered that tumour initiation occurs due to some degree of escape from normal controls (e.g. local 'chalones', hormonal, intracellular). The cause of this "escape" may be due to an alteration in the intracellular DNA, e.g. a number of workers have shown that ionizing radiation, carcinogenic chemicals, and oncogenic viruses can react with the host cell genome in a variety of ways, in order to bring about the required alterations in gene function (Elkind and Whitmore, 1967; Temin, 1971; Miller and Miller, 1974). Phenotypic studies of fully developed tumours have resulted in the following classification of genome changes (reviewed by Anderson and Coggin, 1971):

(a) new DNA is added

(b) some DNA is lost

(c) nucleotide sequences are misread or not read

(d) DNA which is normally not read, is now coding for mRNA.

Although any one of these changes may have the potential to initiate carcinogenesis, they may however be a product of the 'selective evolution', i.e. not necessarily a cause of the loss of control, but rather a secondary effect caused by the clonal evolution of the initial cancer cells.

(a) New DNA (neo-genes)

Due to a "mutation" (induced by carcinogen, virus, radiation or by vertical transmission (Huebner and Todaro, 1967)), new DNA nucleotide sequences appear and an extragenomic molecule appears. Thus the observed phenotypic effect is an altered structural protein or a neo-antigen.

(b) Loss of DNA

Again due to a mutation, certain nucleotide sequences are lost, resulting in protein deletions. Consequently enzymes, antigens and other molecules characteristic of the normal cell are lost.

(c) Misreading of nucleotide sequences

Although not specifically involving a nucleotide change, this genetic abberation results in certain sequences being misread or not read at all. Such a defect may be caused by producer gene expression being altered.

(d) Re-expression of archeo-genes

Genes which are expressed only during the embryonic

development of the cell, are once again 're-activated'. These genes begin coding for phenotypic products generally only found associated with normal embryonic development.

These genome changes are reflected in the phenotypic alterations known to occur in experimental and human cancers. The phenotypic profile of tumour cells reveals that there is a loss of certain "normal" cell structures and functions, and a concommitant acquisition of 'new' cancer-characteristic properties. This is consistent with the genomic profile which essentially involves a loss or misreading of DNA, and a gain or reactivation of existing DNA. This loss and acquisition of properties has been demonstrated in studies on tumour antigens (Ingleton, 1971; Kuusela *et al.*, 1965; Collins and Black, 1973; Kyriazis and Wissler, 1972; Baldwin and Glaves, 1972), metabolism and enzymology (reviewed by Weinhouse, 1973) and cell receptors (Sharon and Lis, 1972; Fox *et al.*, 1971; Garrido *et al.*, 1974).

4. Summary

The ultimate phenotypic expression of a tumour may be influenced by the genetic "evolution" of cancerous cells during tumour development. Nowell's model predicts that tumours possess a number of tumour-specific characteristics which have arisen due to these evolutionary processes. The production of tumour specific antigens (Kyriazis and Wissler, 1972; Baldwin and Glaves, 1972) is an example of such specificity. However,

Nowell's model also envisages similarities between a number of tumours due to the non-randomness of the evolution. Such similarities include many common enzymes (Weinhouse, 1973), metabolism features such as high glycolytic rates (Warburg, 1923, 1956), and the acquisition of embryonic-associated properties (i.e. foetal and placental). It is this acquisition of embryonic-associated properties, demonstrated in many human and experimental tumours, which may be an important event in the sequential evolution, or even the initiation, of tumour development. Alternatively, the acquisition of these properties may be a consequence of the carcinogenic state (i.e. a secondary effect). These two alternatives are examined below, by considering foetal properties in cancer, the non-malignant and healthy states.

SECTION II : ACQUISITION OF EMBRYONIC-ASSOCIATED PROPERTIES

1. Introduction

The relationship between embryonic development and neoplasia has been a central theme of cancer research since 1901, when Ehrlich first formulated the concept of "misplaced cell rests" (see Alexander, 1972). Embryonic and cancerous cells share many properties. These include a number of biological processes, ranging from DNA synthesis enzymes, general synthesis of proteins, antigens, and of cell division.

2. Cell Surface Characteristics and Cell Division

The fundamental similarity between the cancerous and embryonic cell is their ability to divide rapidly. Differentiated adult cells do not generally divide, and are difficult to culture *in vitro*. Embryonic and cancerous cells, however, may be cultured with relative ease (Elkind and Whitmore, 1967).

The ability of cells to stop dividing, when a certain cell mass has been reached, is thought to be dependent on cell-cell surface membrane interactions (Burger, 1973). The ability of cells to bind the plant lectin, concanavalin A, is dependent on membrane receptors. Becker (1974) has found that malignant and foetal hepatocytes were able to bind this lectin, whereas non-dividing liver cells did not. This differential lectin binding suggests that there may be changes in cell surface receptors of tumour cells which are common to embryonic cell receptors.

3. DNA Production

Stafford and Jones (1972) and Piddington *et al.* (1976) have shown that thymidine kinase, an enzyme involved in DNA synthesis in the normal cell, differs to the kinase found in the cancer cell. They have reported the presence of a foetal-specific isozyme of thymidine kinase in human He La and KB tumour cells. This particular isozyme, which catalyses the production of the DNA precursor, thymidine triphosphate, is the predominant variant in the developing foetal cell, and is generally in very low quantities in the normal cell.

4. RNA

Although there has not been any evidence that the enzymes controlling mRNA synthesis in cancer cells differ to those of the normal cell, it has been shown that tumour cells produce a different type of tRNA.

Gonano *et al.* (1971) have studied the tRNA species in 5123c Morris hepatomas, and have found that these substances have different chromatographic characteristics than those of the adult liver. Further, they have also shown that the chromatographic pattern of the tumour phenylalanine accepting tRNA ($tRNA^{phe}$) is the same as the $tRNA^{phe}$ isolated from rat embryonic livers (Gonano *et al.*, 1973). Similarly, Yang (1971) has reported the presence of foetal tyrosine accepting tRNA in Reuber hepatoma. Again, this form of $tRNA^{tyr}$ was not detectable in the adult liver.

5. Enzyme Patterns

Foetal metabolism is poorly understood. However, it is clear that foetal tissues contain a large number of enzymes which, although performing the same function, vary slightly in amino acid composition and tissue specificity to the homologous adult enzyme. Many of these foetal isozymes have also been found in the tumour cell. Together with this reappearance of foetal enzyme forms, there is a simultaneous loss or decrease in the activity of the adult isozyme (Knox, 1972).

The summary of a few of these foetal enzymes (Table 1.1) suggests that there is a broad enzyme pattern established during carcinogenesis, i.e. there is a shift from adult to foetal isozyme synthesis. It is

not clear what special advantage these isozymes may provide to the malignant cell. Maugh (1974) has suggested that they may be better suited to the more rapid sustained growth of tumour and foetal tissues. However, it may be equally possible that no selective advantage is gained, and their appearance, as with other foetal properties, is a simple consequence of the carcinogenic state rather than a specific necessity.

6. Developmental Antigens

The antigenic structure of cancer cells also exhibits a reappearance of characteristics normally associated with embryonic development. Many workers have reported the appearance of antigens which are found in foetal cells, but not in the corresponding normal adult tissues (see Sections 2 and 3). However, it is important to note that the presence of these antigens in tumours is only one example of the reappearance of developmental antigens. The other types of developmental antigens are those which are found in the placenta and tumour, but not in foetal and normal adult cells (reviewed by Bagshawe, 1974).

7. Summary

From the data presented above, it can be seen that the acquisition of embryonic properties is associated with, perhaps a necessary association with, the malignant transformation. The mechanism and reason for the re-expression of the controlling genes, however, is not well understood. Potter (1969, 1970) has proposed

TABLE 1.1

ENZYME	SPECIFICITY			REFERENCE
	FOETAL	TUMOUR	ADULT	
Alanine amino transferase	M	M	S & M	Nakata *et al.* (1964)
Aspartate amino transferase	M	M	S & M	Nakata *et al.* (1964) Shied *et al.* (1965)
Hexokinase	G	G	H	Sato *et al.* (1969) Sugimura *et al.* (1966 a,b)
Pyruvate Kinase	Mu	Mu	L	Tanaka *et al.* (1967)
DNA polymerase	D	D	N	Ove *et al.* (1969) Ono & Umehara (1968)
Glutaminase	K	K	L	Knox *et al.* (1969) Horowitz & Knox (1968)
Arylesterase	A	A	-*	Failly-Crepin & Uriel (1973)

Enzymes which exhibit foetal isozymes during carcinogenesis.

M = membrane bound

S = soluble, cytoplasmic

G = Type IV isozyme

H = Type I, II or III isozyme

Mu = muscle tissue specific

L = liver tissue specific

K = kidney tissue specific

A = arylesterase

* absent from healthy adult tissues

that following carcinogen application, a normal population of cells is damaged. Many of these damaged cells are replaced by "new", rapidly-dividing cells. The cancer cells arise from the dividing cells which fail to respond to normal differentiation control mechanisms, possibly due to some block in one or more ontogenic pathways. Thus these cells remain essentially in the embryonic stage of the differentiation program. Consequently, cancer cells would be expected to exhibit a range of foetal properties, including biochemical functions, antigens, and cell division properties. The information described above confirms this expectation.

Potter's hypothesis is also consistent with the tumour, clonal evolution concepts of Nowell (1976). In this theory, the initial cancer cell would exhibit embryonic properties, and these would subsequently be passed on to future generations. During further evolution, some of these archeo-genes would be lost due to the "mutations" which occur. Those which remain active may produce molecules which give the tumour cell some selective advantage (Maugh, 1974; Weinhouse, 1973). The tumour which ultimately develops, would hence exhibit a number of embryonic properties, some unique to a group of tumours and others shared by all tumours.

The reappearance of developmental antigens in tumours is an example of such embryonic properties exhibited by a wide range of species. These antigens have been studied extensively by many workers. It will be seen in the following section that the reappearance of these antigens occurs in many tumours. This onco-foetal association may be an important factor in understanding the carcinogenic process.

SECTION III : ONCO-DEVELOPMENTAL ANTIGENS - FOETAL ORIGIN

1. Introduction - Tumour Cell Antigens

Three main groups of onco-associated antigens have been found in the fully developed tumour:

(i) tumour specific or transplantation-associated antigens (TAA)

(ii) foetal antigens

(iii) placental antigens.

The tumour-specific antigens differ from the other two types because each antigen is specific to the particular tumour from which it has been isolated. This type of antigen has been demonstrated in a variety of tumours, and is often found to be intimately associated with the cell membrane (Wissler, 1956; Baldwin *et al.*, 1971; Baldwin *et al.*, 1972; Prehn, 1967).

Foetal and placental antigens may be found in cancerous and embryonic tissue (foetus or placenta), but not in appreciable quantities in normal adult cells.

In this section, the association between foetal antigens and cancer cells is explored by examining the nomenclature and classification of these antigens, and the types of tumours which exhibit them.

2. Foetal Antigens - Classification and Nomenclature

The first significant attempt to classify foetal antigens was by Alexander (1972). His system, (Table 1.2), was based on three immunological properties of the onco-foetal molecule:

(i) immunogenicity in homologous and heterologous hosts

(ii) cytotoxicity induction in tumour-bearing hosts

(iii) cellular localization.

It can be seen from Table 1.2 that these properties are studied by employing two basic techniques. Specifically, these are:

(a) Using heterologous antiserum to detect foetal antigens

(i) in tumour-bearer's serum (e.g. α-foetoprotein (Abelev *et al.*, 1963); carcino-embryonic antigen (Gold and Freedman, 1965)) and tumour extracts (Stonehill and Bendich, 1970). In Alexander's system these are termed onco-foetal associated antigens;

(ii) on the cell surface membrane of tumour cells (Ishimoto *et al.*, 1974; Cauchi *et al.*, 1974).

(b) Examining animals immunized with either foetal extracts or cell suspensions for their ability to resist challenge against a tumour forming dose of cancer cells or virus, e.g. such onco-foetal associated transplantation antigens have been demonstrated by Coggin *et al.* (1970).

TABLE 1.2

Molecules in foetus and tumour	Immunogenicity		Cytotoxicity	Suggested Name	Example and Reference
	Heterologous	Homologous			
and some normal adult tissues	+	-	-	Onco-foetal associated antigens (OFA-assoc.)	CEA (Gold and Freedman, 1965) AFP (Abelev *et al.*, 1963) Stonehill and Bendich (1970)
absent from all normal tissues but induce tolerance because they occur in late foetal life	+	-	-	Onco-foetal specific heteroantigen (OFA-specific)	Ishimoto *et al.* (1974) Cauchi *et al.* (1974)
only occur in early foetal life, or in a cryptic form, and hence are recognized as foreign					
(a) cytoplasmic	+	+	-	Onco-foetal specific auto antigen (OFA-auto)	Chemically-induced hepatoma (Baldwin *et al.*, 1972)
(b) plasma membrane-bound	+	+	+	Onco-foetal trans-plantation-type antigen (OFA-transpl.)	Hamster, viral tumours (Coggin *et al.*, 1970)

The classification of onco-foetal antigens as proposed by Alexander (1972).

Although Alexander's nomenclature is specific about the immunological nature of the foetal molecule, it does not assign a specific function or role to the antigen under study. Further characterization of the antigen is desirable because

(a) much of the confusing terminology could be eliminated if each foetal antigen could be referred to by its functional name;

(b) a knowledge of its function may provide a clearer understanding of the biochemical and physiological processes which occur in the tumour.

Spasmodic attempts have been made to achieve this, e.g. Raftell *et al.* (1974) have detected and assigned a function to onco-foetal antigens in rat tumours. Ruoslahti *et al.* (1976) have demonstrated that the hepatoma-teratoma associated foetal antigen, α_1-foetoprotein, shares amino acid sequence homology with albumin. Thus it may be possible that alpha-foetoprotein is a "foetal" albumin. However, despite these attempts, and a current trend towards elucidating the functional role of foetal antigens, there still remain a number of misnomers in foetal antigen terminology, e.g. carcino-embryonic antigen (Gold and Freedman, 1965), is a general term which is used to describe a specific colon carcinoma-associated antigen.

3. The Reappearance of Foetal Antigens in Tumours

The reappearance of foetal antigens in tumours is not restricted to one particular animal species. From Table 1.3, it can be seen that a wide variety of tumour types in different species produce these antigens.

It is important to note that one certain tumour may produce two or three different foetal antigens, e.g. chemically-induced hepatomas produce α-foetoprotein, membrane-bound and cytoplasmic foetal antigens (Cauchi *et al.*, 1974). Conversely, a variety of different tumours may produce the same foetal antigens (Stonehill *et al.*, 1971), while others have been shown to produce only one (Banwo *et al.*, 1974; Mukherji and Hirshaut, 1973).

Thus it can be seen that the reappearance of foetal antigens is a characteristic of a large number of tumours. Perhaps the most studied onco-foetal antigens are α_1-foetoprotein and carcino-embryonic antigen. These antigens have been examined principally as a diagnostic and prognostic parameter in the clinical management of cancer (reviewed by Laurence and Neville, 1972; Maugh, 1974). These two antigens are briefly discussed in the next section in order to demonstrate that the reappearance of foetal antigens in the diseased state is not necessarily specific for cancer. This observation is of particular importance as its non-specificity for cancer suggests that their presence may simply be a function of the rapidly-dividing state rather than the malignant transformation.

TABLE 1.3

SPECIES	TUMOUR TYPE	CARCINOGEN	REFERENCE
Human	leukemia	unknown	Hughes *et al.* (1973)
	liver	unknown	Abelev *et al.* (1965)
	ovary	unknown	Abelev *et al.* (1968)
	pancreas	unknown	Banwo *et al.* (1974)
	skin	unknown	Fritze *et al.* (1976) Mukherji & Hirshaut (1973) Hollinshead *et al.* (1972)
	colon	unknown	Gold & Freedman (1965)
	brain	unknown	Wahlstrom (1973)
	stomach	unknown	Häkkinen (1972)
Hamsters	SV-40 tumour cell line	SV-40 virus	Coggin *et al.* (1970)
Guinea Pigs	liver	chemically	Borsos *et al.* (1973)
	sarcoma	chemically	Grant *et al.* (1974)
Mice	liver	chemically	Stonehill *et al.* (1971)
	liver	spontaneous	Sabine *et al.* (1975)
	teratoma	unknown	Artz *et al.* (1973)
	BO-IV (transplantable)	unknown	Rejthar (1974)
	mammary	spontaneous	Sabine & Stonehill (1972)
	SV-40 tumour cell line	SV-40 virus	Ting *et al.* (1973)
	neuroblastoma	unknown	Stonehill & Bendich (1970)
	leukemia	radiation	Stonehill *et al.* (1971)
Rats	sarcoma	chemically	Thompson & Alexander (1973)
	intestine	chemically	Martin *et al.* (1975)
	liver	chemically	Sell and Morris (1974) Baldwin *et al.* (1972)

Some human and experimental tumours in which foetal antigen re-expression has been demonstrated.

4. α_1-Foetoprotein (AFP)

(a) Definition

AFP has been defined as the first α-globulin to appear in mammalian sera during development, and the dominant serum protein during embryonic life (Annual Report of the International Agency for Research into Cancer, 1969). The foetal liver and yolk sac are the primary sites of synthesis during embryogenesis. After birth, small quantities of AFP are detected only by radio-immunoassay, and its synthesis is restricted to parenchymal liver cells (Abelev, 1971).

(b) AFP and carcinoma specificity

AFP is also synthesized by primary and metastatic hepatomas (Abelev *et al.*, 1963; Abelev, 1971). From Table 1.4, it can be seen that AFP has been detected in the sera of a variety of hepatoma-bearing hosts. However, it has also been found in a few sera of other non-hepatic carcinoma hosts (Table 1.4). From this table it is concluded that, other than hepatomas, only teratomas synthesize AFP to any significant degree.

(c) AFP and the non-malignant state

(i) *Normal serum values*

AFP has been detected in the serum of apparently healthy humans (Ruoslahti and Seppälä, 1971), mice (Pihko and Ruoslahti, 1973), and rats (Sell and Gord, 1973). The level of AFP in these cases can only be detected by the sensitive radio-immunoassay technique. However, the tumour-associated levels may

TABLE 1.4

SPECIES	TUMOUR TYPES	AFP INCIDENCE	REFERENCE
Human	1^{o} hepatoma	68%	Laurence & Neville (1972)
	1^{o} teratoma	45%	Laurence & Neville (1972)
	other non-hepatic	0.03%	Laurence & Neville (1972)
Rats	1^{o} hepatoma	n.a.	Kroes *et al.* (1972)
	ascites hepatoma	n.a.	Watabe *et al.* (1972)
	transplantable solid	n.a.	Sell and Morris (1974)
	non-hepatic	n.e.	---
Mice	1^{o} hepatoma	n.a.	
	transplantable hepatoma	n.a.	Abelev *et al.* (1963)
	ascites hepatoma	n.a.	Koda *et al.* (1973)
	non-hepatic	n.e.	---
Dogs	1^{o} hepatoma	n.a.	Shinomiya *et al.* (1973)
Monkeys	1^{o} hepatoma	n.a.	Hull *et al.* (1969)

Species in which the presence of AFP-bearing carcinomas have been demonstrated.

(It is important to note that the references cited, and the specific tumours studied, are only a representative sample of a large number of publications in which AFP-production has been demonstrated, using a number of carcinogens to induce these tumours.

n.a. = not applicable

n.e. = not examined

be measured by relatively insensitive methods such as counter-immunoelectrophoresis, and immunodiffusion (Abelev, 1971).

(ii) *The regenerating and experimentally injured liver*

Conflicting data has been collated concerning AFP synthesis in the regenerating liver. Perova (1969) and Stanislawski-Birrencwaig (1967) failed to demonstrate the appearance of AFP in partially hepatectomized animals. However, further investigation by Perova *et al.* (1971) using immuno-autoradiography, showed that AFP could be secreted by partially hepatectomized rats. These results have since been confirmed in mice by Pihko and Ruoslahti (1973).

Ruoslahti *et al.* (1973) have found a sharp increase in serum AFP levels in mice with carbon tetrachloride-induced liver injury. They have concluded that even in the non-malignant liver, regeneration depends on a relatively primitive (or embryonic) cell population which produces or passes through an AFP-producing stage during the differentiation phase of regeneration.

(iii) *Non-malignant diseases*

Elevated serum levels of AFP have been shown to be associated with non-cancerous hepatic diseases. Abelev (1971) and Silver *et al.* (1973) have shown such increases in serum hepatitis occurring during the recovery phase of the disease (Ruoslahti *et al.*, 1973). Elevated levels have also been associated with alcoholic hepatitis (Meshkinpour *et al.*, 1974), cirrhosis and

trauma (Ruoslahti *et al.*, 1973; Nishi and Hirai, 1973). Ruoslahti *et al.* (1974a) have shown that these AFP levels are generally lower than those associated with primary liver carcinoma. These authors have further suggested that in some cases of hepatic coma, caused by acute hepatitis, the high level may be due to the regeneration of damaged liver tissue.

These results further support Ruoslahti's concept that regenerating liver cells pass through an embryonic AFP-producing stage, i.e. in non-malignant diseases, AFP production is a function of the dividing state.

(d) Summary

There are two important conclusions that may be drawn from the AFP studies presented:

(i) AFP secretion is not specific for hepatomas;

(ii) AFP synthesis is also associated with other non-cancerous diseases.

These conclusions suggest that AFP synthesis may be a function of the rapidly-dividing state (i.e. either due to malignancy or regeneration), rather than a specific property of the malignant state.

This observation has a particular significance in determining whether foetal antigens are a necessary pre-requisite for cancer, or simply a secondary consequence.

5. Carcino-embryonic Antigen (CEA)

(a) Definition

CEA is defined as the perchloric acid-soluble glycoprotein antigen found in the human foetal gut between 2 and 6 months of gestation, but not in significant quantities in healthy adult gastro-intestinal tracts.

(b) CEA and human carcinoma

Using the double immunodiffusion technique, Gold and Freedman (1965) found CEA in 12 cases of colonic adeno-carcinoma. The CEA synthesized by the tumours is secreted into the serum of tumour-bearing hosts.

From Table 1.5 it can be seen that CEA secretion occurs in a majority of gastrointestinal carcinomas. Although initially thought to be a specific product of these tumours and their metastases, it may also be associated with other malignancies (Table 1.5). However, it has been proposed by Warner (1976) that colonic, breast and bronchial tumour CEA's may be different to the others. This is supported by evidence from Coligan *et al.* (1973), who showed that CEA was a heterogeneous mixture rather than a single well-defined molecule.

(c) CEA and the non-malignant state

With the advent of the sensitive radio-immunoassay, there has been a concentrated study of CEA levels in tissues and blood of patients with non-malignant diseases.

TABLE 1.5

SITE	DISORDER	NO. OF CEA +VE PLASMAS	%
Gastrointestinal tract	Carcinoma of:		
	Colon and Rectum	230/316	73
	Pancreas	48/52	92
	Liver	12/18	67
	Others	51/85	60
Breast	Malignant	82/159	52
	Benign	1/24	4
Respiratory tract	Carcinoma of:		
	Bronchus	65/90	72
	Upper respiratory tract	8/15	53
Healthy Individuals		1/335	0.3

Plasma CEA levels in gastrointestinal, bronchial and mammary disorders. A serum is classified as CEA +ve if it contains more than 10 ng/ml of antigen.

(Collated from Zamcheck *et al.*, 1972; LoGerfo *et al.*, 1972; Reynso *et al.*, 1972; Laurence *et al.*, 1972.)

(i) *Normal serum*

Like AFP, CEA has been detected in the sera of apparently healthy adults. However, these values are very low (< 10 ng/ml, Laurence *et al.*, 1972). Any appearance of CEA in the diseased state is classified as an elevation in serum quantities above an arbitrary normal range.

(ii) *CEA and non-malignant diseases*

CEA has also been found in the sera of patients with non-malignant diseases (Table 1.6). However, the highest incidence of plasma CEA elevation is amongst diseases of the gastrointestinal tract. The levels of CEA in these sera vary, and are often in the range characteristic of colonic carcinoma (Laurence *et al.*, 1972).

(d) Summary

From the data presented here, it can be seen that CEA is also produced in patients with a number of non-malignant gastrointestinal diseases, as well as those with gastrointestinal carcinoma. This lack of specificity for the malignant state suggests that the reappearance of CEA is due to the presence of rapidly-dividing cells.

TABLE 1.6

SITE	DISORDER	NO. OF CEA +VE PLASMAS	%
Gastrointestinal tract	Polyps of:		
	Colon and Rectum	6/67	9
	Inflammatory:		
	Ulcerative colitis, Crohn's disease	20/95	21
	Diverticulitis, peptic ulcer	7/33	21
	Cirrhosis	28/66	42
	Alcoholic pancreatitis	17/32	53
	Others	2/43	5
Breast	Reactive fibroadenosis	5/70	7
Respiratory tract	Inflammatory:		
	Pulmonary tuberculosis	8/21	38
	Chronic bronchitis & emphysema	16/63	25

Plasma CEA levels in patients with non-malignant diseases (Laurence and Neville, 1972).

SECTION IV : ONCO-DEVELOPMENTAL ANTIGENS - PLACENTAL ORIGIN

1. Introduction

The second type of developmental "antigen" produced by the cancer cell is of placental origin, i.e. synthesized by the placenta, and not foetal or healthy adult tissues. The methods used to study placental-tumour relationships differ from those used in onco-foetal antigen studies. Foetal molecules have been studied as a function of their antigenicity. Placental molecules have, however, been demonstrated in tumours by conventional biochemical assays, rather than antigenic behaviour. There has not been a specific rationale for the study of onco-placental antigens - most of these molecules were discovered because elevated serum levels were found in tumour-bearing patients.

The onco-placental molecules so far reported in the literature are:

(a) Regan isozyme

(b) Human chorionic gonadotrophin

(c) Placental lactogen

(d) Pyruvate kinase

(e) Histaminase

These substances, and their appearance in human carcinoma, are discussed below.

2. Onco-placental Molecules

(a) Regan isozyme (placental alkaline phosphatase)

Placental alkaline phosphatase is produced by trophoblastic cells and is secreted into the serum. It is not produced by foetal or non-trophoblastic adult cells. Its association with human cancer was first reported by Fishman *et al.* (1968), and has since been studied by a number of other workers (Doellgast, 1976; Suzuki *et al.*, 1976; Laurence and Neville, 1972). Regan isozyme production has since been demonstrated in a number of human tumours (Table 1.7). From Table 1.7, it can be seen that only a small percentage of tumour types produce this enzyme. Further, the incidence within one particular group of tumours is low compared to CEA (*c.f.* Table 1.5) incidence in colonic carcinoma, and AFP in hepatomas (*c.f.* Table 1.4).

(b) Human placental lactogen (HPL)

Placental cells produce human placental lactogen during the third trimester of pregnancy. It has also been detected in elevated levels in the sera of patients with trophoblastic tumours (Samaän *et al.*, 1966) and in 8% of patients with a variety of non-gonadal tumours (Weintraub and Rosen, 1971). Again the incidence of this particular antigen, as compared to foetal antigens, is very low.

TABLE 1.7

CARCINOMA	INCIDENCE	%
Bronchus	7/51	13.7
Mammary	6/49	12.24
Genito-urinary	10/55	18.18
Lymphoma	2/25	8.00
Melanoma	0/5	0
Gastrointestinal	10/81	12.35

Regan isozyme incidence amongst cancer patients showing an elevated serum alkaline phosphatase level (from Laurence and Neville, 1972).

(c) Human chorionic gonadotrophin (HCG)

HCG is a hormone which has also been found in the serum of patients with teratoma, seminoma, insuloma and gastro-intestinal carcinoma (Braunstein *et al.*, 1973). It is important to note, however, that the synthesis of this hormone by malignant cells may be a case of ectopic hormone synthesis, and not necessarily a true reappearance of a placental antigen, due to a general de-repression of archeogenes.

(d) Pyruvate kinase

Spellman and Fottrell (1973) have examined the isozymes of pyruvate kinase in placental, foetal, adult, and five tumour tissues. They found the placental-specific isozyme only in extracts of lung, stomach and jejunum carcinoma.

(e) Histaminase

Chei-Wei and Kirley (1976) have shown that ascites fluid obtained from human ovarian cancers contains a placental-specific isozyme of histaminase. Although elevations of histaminase were also associated with other tumours, only the ovarian carcinoma produced the placental isozyme of this enzyme.

3. Conclusions

The appearance of placental-specific molecules in human tumours supports the concept that placental antigen reappearance is associated with the malignant state. However, the low

incidence amongst certain tumours, and complete absence in many others, suggests that the reappearance of placental antigens may not be a major characteristic of carcinomas. Comparatively, the association of foetal antigens and cancer is very strong. The establishment of suitable animal models may provide the necessary methodology to determine whether the appearance of placental antigens is associated with cancer to the same degree as foetal antigens.

SECTION V : CONCLUSIONS

1. Introduction

In the preceding sections, three important problems in tumour immunology have been raised.

(a) The current nomenclature is unsuitable and often misleading when discussing embryonic-associated tumour antigens.

(b) There has not been a significant attempt to define the metabolic or physiological function of foetal antigens in tumours.

(c) Is the presence of these antigens a pre-requisite for a cell to undergo malignant transformation?

The first question has been examined by many workers, and many meetings have been convened to examine the problem (*c.f.* First Conference on Embryonic and Foetal Antigens in Cancer, Oak Ridge, Tennessee, U.S.A., 1971). This problem may be considerably reduced if a specific function could be assigned to the antigens.

The third problem is possibly of higher importance as it may provide a basis for an understanding of the many complex events which occur during malignant transformation. Essentially, it is important to determine whether the appearance of embryonic properties in general is necessary before transformation may occur, or whether these antigens appear later during the evolution of the first cancer cell clones.

The presence of AFP in patients with non-malignant diseases, suggests that the reappearance of this embryonic property in these cases is probably a function of rapidly-dividing cells (i.e. the antigen is produced by undifferentiated, embryonic cells which replace those destroyed by the disease agent). By extrapolation, AFP production in tumours must be produced by "embryonic-type" cells which replace adult cells destroyed by carcinogens. However, in this case, these replacement cells fail to respond to normal differentiation control mechanisms. This argument has been postulated by Potter (1969, 1970). Thus, this proposition would support the concept that the reappearance of embryonic properties is an integral characteristic of the first cancer cell(s) which ultimately undergo a selective evolution, and hence develop into a tumour.

However, there is some evidence to suggest that the embryonic properties may be acquired during the sequential evolution of the first cancer cells. Becker (1974) has shown that foetal and tumour cells exhibit differential lectin binding, whereas adult and dividing hepatocytes did not. Thus, this property is not simply a function of the undifferentiated, rapidly-dividing state. Rather it is a specific characteristic of embryonic and tumour cells. From these results it may be concluded that this particular onco-foetal characteristic does not necessarily need to be associated with the cells which replace those damaged by carcinogens. If this theory is valid, then this property, and possibly other onco-foetal characteristics, may have appeared during the sequential evolution of the first cancer cells.

The evidence in favour of either alternative, however, is insufficient to produce a definite conclusion. It would be useful to determine whether there are any cancer-characteristic properties which precede and possibly contribute to the appearance of embryonic characteristics in the developing tumour. Such studies may help establish a sequence of the events which occur during tumorigenesis.

SECTION VI : INTRODUCTION TO PROPOSED STUDY

Perhaps the fundamental question in experimental oncology is 'What is cancer?'. At the molecular level, cancer is often defined as a mass of cells that have lost a number of mechanisms which control normal cellular functions. However, it has yet to be established whether many of these lost control mechanisms are a primary event of carcinogenesis, or simply a secondary consequence of the malignant transformation. The appearance of developmental antigens in tumours may be an example of such a secondary event. The initial objective of the proposed study was to help resolve this question by determining the role of these antigens in experimental tumour systems, i.e. why do they appear? The following questions were asked in an attempt to characterize and to determine the role of onco-developmental antigens in the malignant transformation.

(a) At what point of time after the application of the carcinogenic stimulus do the developmental antigens first appear?

(b) Is their appearance correlated with the appearance or disappearance of other parameters known to be indicative of the carcinogenic state?

(c) Do these antigens have a biological function other than just being antigenic?

(d) What is the cellular localization of these antigens?

By following the sequence of events which lead up to the first appearance of developmental antigens, it was hoped to determine whether there was a correlation between this appearance and other events which are characteristic of the cancerous state.

The foetal antigens chosen for this study were first described by Stonehill and Bendich (1970). They were chosen because they could be detected by relatively simple means (immunodiffusion), and as their appearance was demonstrated in 72 different tumours of mice, this permitted their use in virtually any tumour system. The placental antigens of rat tumours were examined by raising antisera to both saline soluble and cell-membrane bound antigens.

Before these antigens could be studied, it was necessary to raise the appropriate antisera, and to detect developmental antigens in the tumour systems available in our laboratory. The anti-foetal serum was not able to detect onco-foetal molecules in the tumours examined - a direct contradiction of the work of Sabine and Stonehill (1972). It was concluded from these studies that Stonehill's original antiserum was probably detecting antigens of adult and not foetal origin. Although the antiserum raised against saline-soluble placental antigens could detect these antigens in rat tumours, the antigen extraction and detection methods were unsuitable for studying the problems proposed above. Attempts at raising an antiserum to membrane bound placental antigens were unsuccessful.

The inability to produce an antiserum and a system which could detect developmental antigens in tumours resulted in the proposed questions remaining unanswered. Consequently, the methodology of this study was critically re-examined, and modifications to these techniques theoretically evaluated.

As a consequence of the study on the onco-foetal antigens, an interesting and unusual observation was made. It was found that non-freeze-dried foetal extracts were unable to raise antibodies in rabbits, an effect easily reversed by lyophilization. It was subsequently proposed that the foetal extracts contained a lyophilization-labile immune repressor which specifically inhibited the production of antibody to foetal antigens. This proposition was examined further and confirmed. The relevance of these studies to tumour immunology was also discussed in the form of a model.

CHAPTER II

MATERIALS & METHODS

SECTION 1 : MATERIALS

1. Animals

(a) Rats

The rats used were of the Buffalo strain which had been bred by the University's Central Animal House. Tumours of this strain were obtained originally from Dr. Harold P. Morris of Harvard University, Washington D.C., and subsequently maintained in our Animal House by serial transplantation into animals of the same strain.

Other rats used were 3 month old male and female Hooded Wistars, also obtained from the University's Animal House. The rat (Wistar) bearing the 3-methyl-4-dimethylaminoazobenzene-induced hepatoma was a gift from Mr. K. Matthaei of the Australian National University.

(b) Mice

Swiss Albino and C3H.A^{vy} mice were used in this study. The latter were obtained originally from Dr. W.E. Heston of the National Cancer Institute, Bethesda, Maryland, and have since been bred and maintained in our Animal House on a cedar-shaving bed, as described by Sabine *et al.* (1973).

(c) Rabbits

All rabbit antisera were raised in New Zealand Whites, purchased from the University of Sydney Veterinary Pathology Animal House.

2. Reagents

Collagenase - Sigma Chemical Co., St. Louis, Mo., U.S.A.

Sephadex G-200 - Pharmacia Fine Chemicals, Uppsala, Sweden.

Freund's adjuvants - Difco Laboratories, Detroit, Michigan, U.S.A.

Bovine Serum Albumin - Sigma Chemical Co., St. Louis, Mo., U.S.A.

Agarose - BDH Chemicals, Poole, Dorset, England.

SECTION II : METHODS

1. Preparation of antigens

(a) Saline-soluble extracts

Soluble extracts were prepared by homogenizing the appropriate tissue with an equal volume of 0.85% saline, in an Ultra-turrax homogenizer at maximum speed, for 1.5 mins. The homogenate was then centrifuged at 1500 g for 15 mins, and the supernatant dialysed against several changes of physiological saline. In one study with murine foetal material (Chapter III), extracts were first freeze-dried and then reconstituted to 15 mg solid matter per ml, with distilled water (Stonehill and Bendich, 1970).

(b) Cell surface bound antigens

A crude suspension of plasma membranes was prepared from normal adult tissues (heart, lung, kidney, spleen, liver and intestine), by homogenizing each tissue with phosphate-buffered

saline in a teflon homogenizer. The homogenate was centrifuged at 1500 g for 30 mins and the pellet resuspended in PBS and washed three times. Following the final wash, the membrane pellet (and some nuclei) was centrifuged at 105,000 g for 60 mins.

Solubilization of the membrane-bound antigens was achieved by the addition of 1 ml of 0.5% sodium deoxycholate and 1% Triton X-100 to the membrane pellet and resuspending the pellet. Following a 5 min incubation at $37^{o}C$, the mixture was centrifuged at 105,000 g for 60 mins, and the supernatant dialysed against PBS for 24 hours. The presence of solubilized antigens in this extract was verified by double immunodiffusion.

Cellular membranes and solubilized antigens were similarly prepared from rat placenta, foetus and MeDaB-induced hepatoma.

(c) Whole placental cells

Single cell suspensions were prepared from rat placenta using the method detailed by Muller *et al.* (1972), with the following modifications:

(i) The dissociation medium outlined by Berry (1973) was found to give a better yield and viability of cells.

(ii) Centrifugation at 800 g for 60 seconds was performed following each dissociation. Each pellet was resuspended in 0.5 ml of physiological saline, and pooled.

Cell counts were carried out in a Neubauer haemocytometer, and viability established using the trypan blue exclusion test.

Prior to injection, the cells were killed with 10 mM sodium

arsenate, and then washed three times with 0.85% saline.

2. Antisera

Antisera were raised according to the schedules described in Appendices I-V. Blood was taken from the marginal ear vein of rabbits. Mice were given live *Mycobacterium phlei* during the final injections in order to develop an ascites fluid rich in antibodies (Sommerville, 1967). This fluid was tapped 7 days after the final injection, allowed to coagulate, and centrifuged to obtain a clear antibody solution.

3. Immunological Tests

(a) Immunodiffusion

This was performed on 1.5% agarose slides using the double diffusion pattern described by Ouchterlony (1968). Following the addition of antiserum and antigen (adjusted to 10 mg protein/ml) to the wells, the slides were incubated at room temperature for 72 hrs, washed, dried, and stained in 0.3% Ponceau S.

(b) Immunoelectrophoresis

1.0% agarose slides were used as the electrophoresing medium. 0.04 M sodium barbitone buffer, pH 8.2, was used as the electrode buffer, and 0.08 M sodium barbitone, pH 8.2, as the gel buffer. 2 µl of antigen (concentrated to 10 mg protein/ml) was electrophoresed for 2.25 hrs at 6 volts per cm of gel. Care was taken not to allow the current to exceed 20 mAmps at any stage during the run. On completion of the electrophoresis, antibody was added to the

central trough and the slides allowed to incubate for 72 hrs at room temperature, washed, dried, and stained in 0.3% Ponceau S.

CHAPTER III

ONCO-FOETAL ANTIGENS

SECTION I : RATIONALE OF THE PROPOSED STUDY

The biochemical and physiological events which occur in the cancerous and pre-cancerous cell have been reported by a number of workers in different fields. Time course studies before and during the pre-cancerous stage, and ultimately in the fully developed tumour, have met with limited success. Such a study would be useful because it would lead to some understanding of the time sequence of events which occur during the malignant transformation process.

The purpose of this study was to determine at what stage during the pre-cancerous stage did foetal antigens first appear. It has been mentioned previously, that these antigens may only be an effect rather than a cause of the cancerous state. To help in the clarification of this theory, known cancer associated parameters were examined by other workers to determine whether foetal antigens reappeared before or after these markers of the carcinogenic state (Sabine *et al.*, 1975). These parameters included:

(a) AFP (Dr. E. Ruoslahti, University of Helsinki);

(b) adenyl cyclase (Professor A.W. Murray, Flinders University of South Australia);

(c) DNA polymerase II (Dr. L.A. Burgoyne, Flinders University of South Australia);

(d) histology (Dr. R. Rac, Institute of Medical & Veterinary Science, Adelaide).

SECTION II : THE SYSTEM OF STUDY

1. The antigen

Perhaps one of the simplest systems of study of murine foetal antigens is that reported by Stonehill and Bendich (1970). They produced an antiserum which was capable of detecting the presence of foetal antigens in 72 different tumours, from a variety of mice strains. These onco-foetal associated antigens were chosen for this study because such an antiserum allows a comprehensive examination of any tumour system available.

2. The tumours

We have maintained in our laboratories, a strain of mice which have a 100% incidence of cancer when housed under the appropriate conditions (Sabine *et al.*, 1973). This 100% incidence may be altered almost to zero if the animals are born and bred on a different bedding. This presents an excellent test and control system for a unified study of the malignant transformation process.

A further impetus to the proposed study has been presented by Sabine and Stonehill (1972). They have shown that both mammary and liver cancers from these tumour-prone C3H.A^{vy} mice produce foetal antigens of the Stonehill type. They have also shown that these antigens could be detected in the livers of animals between 6 and 7.5 months of age, even though they were completely devoid of any obvious signs of tumours.

FIGURE 3.1

Immunodiffusion examination of unadsorbed serum from a rabbit immunized with murine foetal extracts.

F = foetal

a = composite adult

S = skin

T = mammary tumour

H = hepatoma

A = unadsorbed anti-foetal serum

FIGURE 3.2

Immunodiffusion examination of exhaustively adsorbed rabbit anti-foetal serum.

F = foetal

a = composite adult

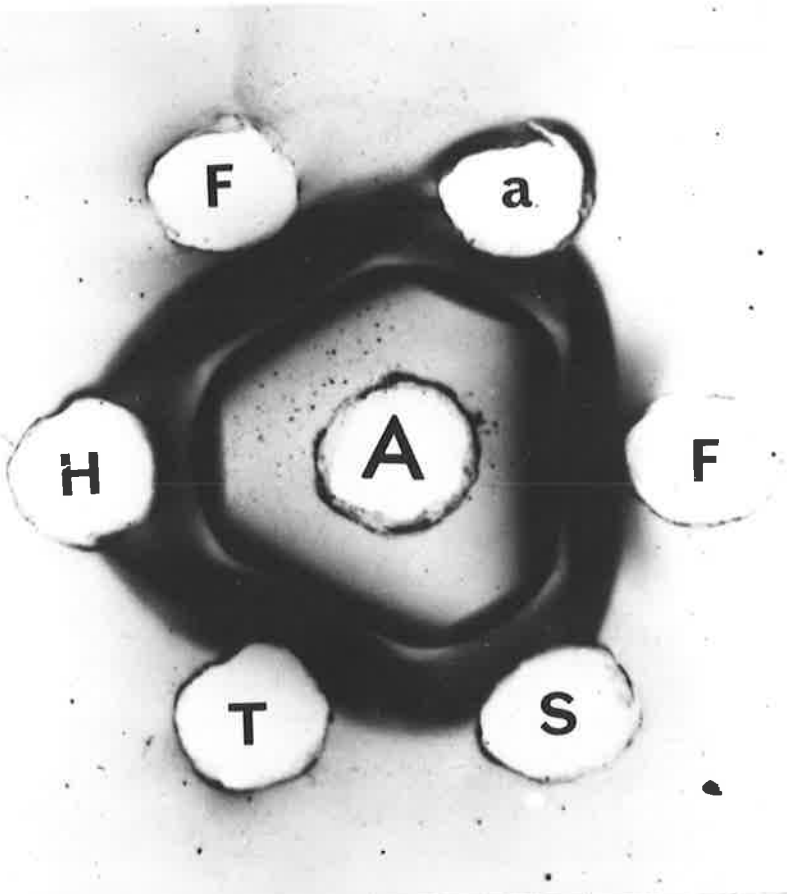
C = NaCl control

S = skin

T = C3H.A^{vy} mammary tumour

H = C3H.A^{vy} hepatoma

3.1



3.2

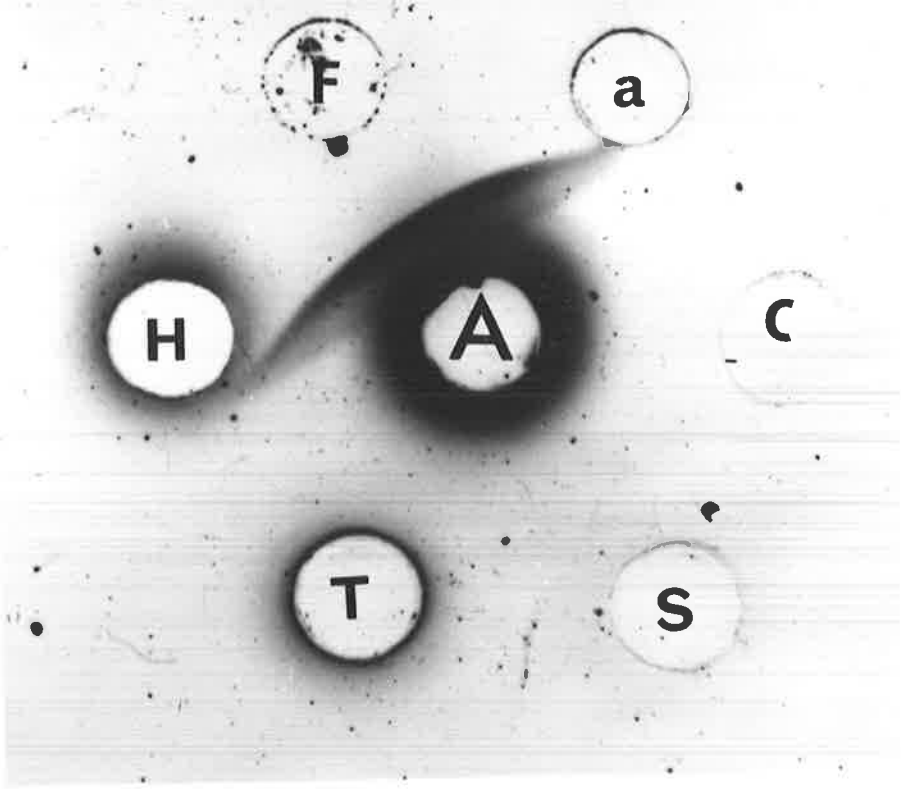


FIGURE 3.3

Immunodiffusion examination of rabbit anti-mouse foetal extract. This serum was given a primary adsorption with 3 mg of a composite mouse adult extract.

F = foetal

A = adult

C = NaCl control (band associated with this well is an artefact produced during the drying procedure)

T = C3H.A^{vy} mammary tumour

H = C3H.A^{vy} hepatoma

S = skin

A_1 = 1^o adsorbed anti-mouse foetal serum

FIGURE 3.4

Immunodiffusion examination of rabbit anti-mouse foetal extract. This serum has been adsorbed twice with 3 mg of a composite mouse adult extract.

f = foetal

c = NaCl control

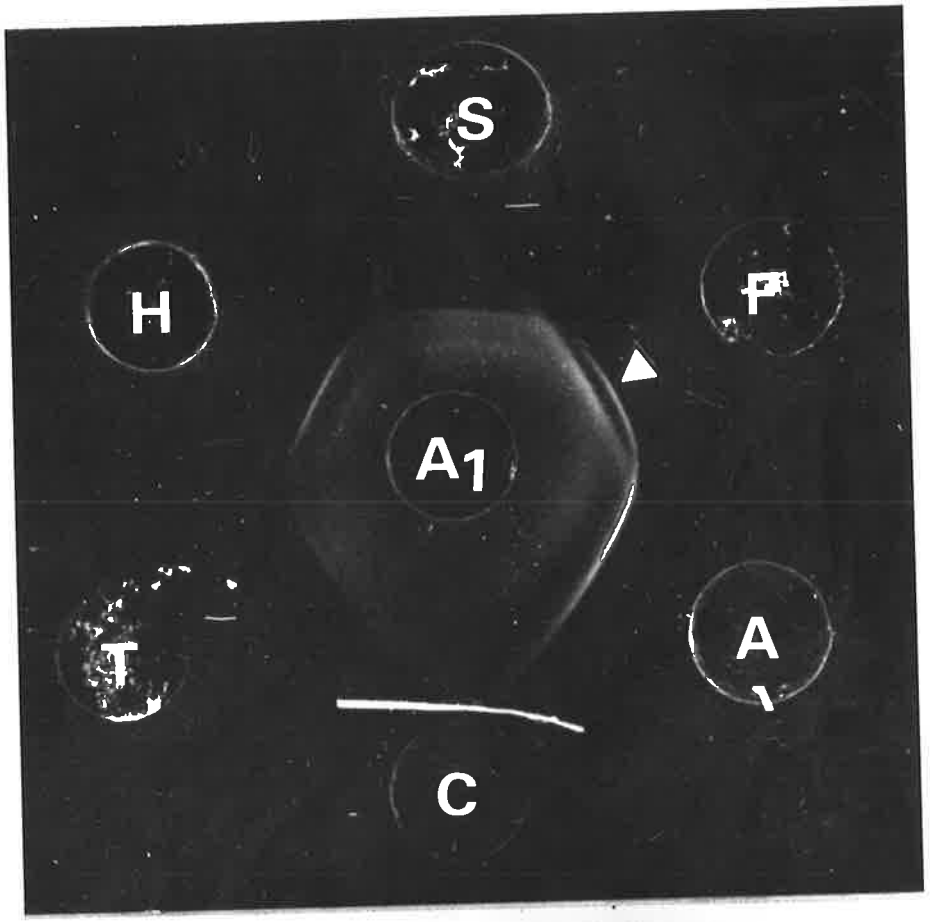
t = C3H.A^{vy} mammary tumour

a = composite adult

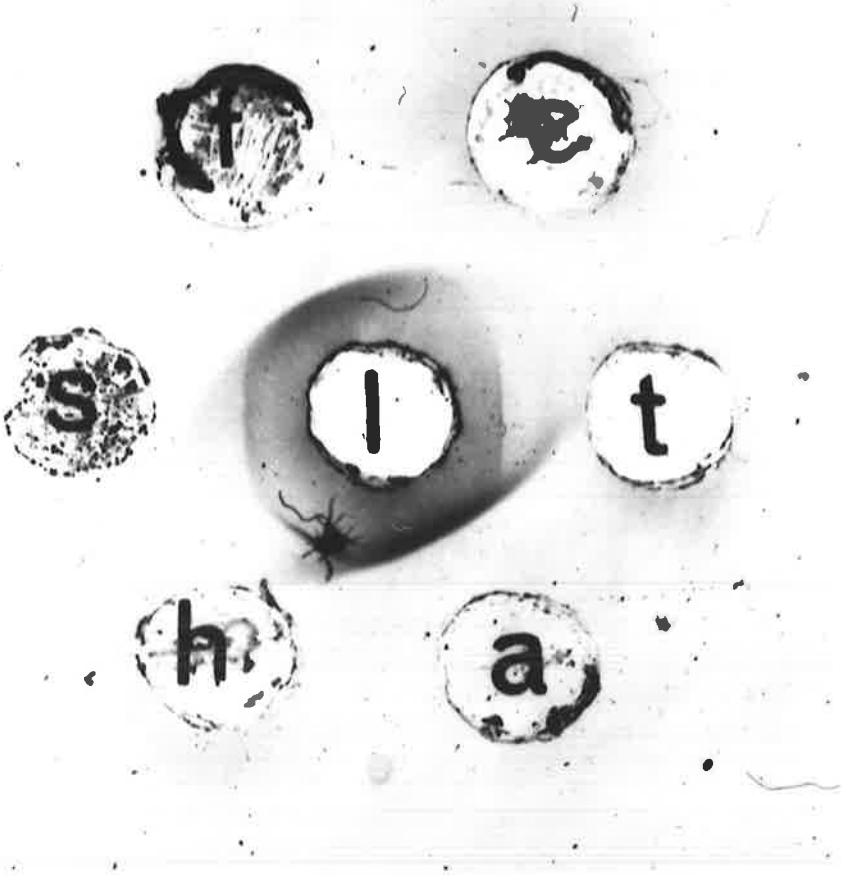
h = C3H.A^{vy} hepatoma

s = skin

1 = 2^o adsorbed anti-mouse foetal serum



3.4



This study was designed to determine the point of time during carcinogenesis at which foetal antigens first reappear. This was to be achieved by following the loss of foetal antigens from the neo-natal liver, and their subsequent reappearance in the livers of adult mice which had been maintained on the 100% tumour incidence environment.

SECTION III : CHARACTERIZATION OF THE FOETAL ANTISERUM

Since Sabine and Stonehill (1972) had shown that a number of foetal antigens reappeared in C3H.A^{vy} hepatomas, an antibody to a foetal extract was raised in our laboratory, in the same manner as Stonehill's. The unadsorbed antiserum was found to react strongly against foetal, skin, mammary and liver cancer, and a composite adult extract (Figure 3.1). Following exhaustive adsorption with the composite adult extract (i.e. 15 mg adsorbent per ml of antiserum), the residual serum only continued to produce double diffusion precipitation lines with the foetal extract (Figure 3.2). This result differed to that of Stonehill and Bendich (1970), who found that their adsorbed serum reacted with skin and tumour extracts as well.

To further examine this discrepancy, a sequential adsorption was performed by adding 3 mg of adsorbant to each ml of antiserum, removing the immune precipitate and testing the residual serum against the antigens used previously (Figure 3.3). This adsorption and retesting was repeated a second time (Figure 3.4).

Following the first adsorption, the antiserum still reacted against all the extracts. More importantly, the band indicated with an arrow on the precipitation pattern has a line of identity with a band found in all the other extracts. Since there is a line of identity with the adult extract, it may be concluded that it represents a precipitation of adult antigens which may also be found in the foetus. This type of precipitation band is similar to that found by Stonehill and his colleagues. In their system, this band represented the tumour-associated foetal antigens (i.e. they were not found in the adult extracts; Stonehill *et al.*, 1971). When a secondary adsorption was performed on the serum raised in our laboratory (Figure 3.4), the arrowed band slowly faded until it disappeared following exhaustive adsorption (Figure 3.2).

Thus, although a foetal-specific antiserum was obtained, it failed to react against either of our two tumour extracts - a direct contradiction of the work of Stonehill and his associates.

SECTION IV : CONCLUSIONS

1. The antibody

It is difficult to explain the discrepancy between the results described here and those obtained by Stonehill. There are a number of conclusions, however, which may be drawn from the adsorption studies, and from Stonehill's published material. These suggest that his antiserum was detecting normal adult antigens which circulate in healthy adult mice, and which are adsorbed by rapidly dividing cells such as skin, tumour, and intestine.

(a) Adsorption studies

Since there is a line of identity between a foetal precipitin band and the other immune precipitin bands in Figure 3.3, it may be concluded that the foetal, adult, skin, and tumour extracts all share a common antigen(s). Further, since this common band disappears from all the extracts after adsorption with adult adsorbant (Figure 3.2), the antigens which precipitate in this band must be normal adult antigens. According to Stonehill's system, antigens which precipitate in this cross band contain the tumour-associated foetal antigens. Assuming that the cross band found in our study detects the same antigen as that found by Stonehill and Bendich (1970), and there is no reason to assume otherwise since the same precipitation patterns were obtained, it is proposed that Stonehill's antiserum was only detecting adult antigens, and that these may have been of intestinal origin. This conclusion is drawn after consideration of the adult adsorbing mixtures used in both our laboratory and Stonehill's. Our composite mixture contained extracts of heart, lung, spleen, kidney, liver and intestine. Stonehill, however, did not add intestine to his adsorbant. Thus it may be that Stonehill's antiserum was detecting an antigen(s) which is shared by both tumours and adult intestine, i.e. a normal adult antigen(s), and one which is also expressed in tumours.

(b) Reactivity with skin extracts

Another possible explanation of this discrepancy lies in the ability of Stonehill's antiserum to react against skin tissue. Boone, commenting on Stonehill's antibody (Stonehill *et al.*, 1971),

suggested that perhaps Stonehill's antiserum was detecting collagen or connective tissue antigens, which, in addition to being found in skin extracts, may also be found in tumours. In reply, Stonehill admitted that the only difference he found between the tumour and skin antigens which were detected by his serum, was in molecular weight. The skin antigen had a molecular weight of 15,000, and the tumour antigen, 67,000. It is, however, quite possible that the larger molecule may consist of a number of sub-units of the smaller molecule. This would make the two antigens immunologically indistinguishable, and hence corroborate Boone's idea that the tumour-associated antigen was merely collagen, or a connective tissue-associated antigen.

(c) Circulating adult antigens

To explain the appearance of a skin antigen in tumour cells, it is proposed that the skin antigen circulates in the tumour bearing animal and is absorbed by the tumour cells. A similar analogy exists with the Lewis blood group antigens, which are absorbed by human red blood cells. Evidence to support this concept again comes from Stonehill's own published material. Stonehill *et al.* (1971) found that freshly excised tumour cells were antigen positive. After some time in tissue culture, however, they no longer produced any antigens capable of reacting with their anti-serum. When the cells were reinjected into non-tumour bearing mice, they again began to 'produce' the antigen(s). The concept of a circulating antigen would explain this phenomenon. In further corroboration of this idea, Stonehill *et al.* (1971), reported that on examination of healthy mouse sera, they detected an antigen which reacted against their anti-

serum. They dismissed this as being a different antigen than the tumour associated foetal antigen, because it had a molecular weight of 250,000 - 350,000. The presence of this high molecular weight antigen in the serum has not been explained by Stonehill. It is possible that the tumour, skin and serum antigens are the same, the difference in molecular weights being a reflection of the degree of sub-unit dissociation. In other words, the antigen detected by Stonehill is a normal adult antigen of high molecular weight. This antigen circulates in the host, and is adsorbed by rapidly dividing cells such as tumour, skin and intestine. Adsorption may be in the sub-unit form, or alternatively, these cells may absorb the high molecular weight antigen in the multiple aggregate form, and on extraction of the tissue the antigen breaks down into the sub-unit structure. (It is interesting to note that skin tissue requires a more vigorous homogenization than tumour, and hence a high molecular weight antigen would be subjected to a greater degree of breakdown of sub-unit structure. This is in keeping with the observed results that the skin antigen has a molecular weight of approximately 15,000, and the tumour antigen, 67,000.)

Nevertheless, whatever the explanation for the discrepancy between the results presented here and those of Stonehill, it is clear that his antiserum system could not be reproduced, and this could not be used in this study.

2. The time course study

The doubtful nature of Stonehill's antigen system raised a number of interesting points concerning the use of a heterologous antibody. The work of Stonehill and his colleagues has been extensively cited in the literature as the principal example of foetal antigen reappearance in tumours of the mouse. Their method, however, of injecting a mouse foetal extract into heterologous hosts, such as rabbits, results in the majority of the antibody response being directed to the high concentration of adult antigens in the extract. The foetuses used by both Stonehill, and in the study presented here, were of late gestation (16-19 days). A foetus of this age would be expected to have a low foetal-specific component and a relatively high concentration of adult antigens. Perhaps the most suitable method of overcoming this problem would be to immunize heterologous hosts with a purified foetal antigen(s). This has been done with AFP (Savvas, unpublished results) and CEA (Gold, 1965). For this proposed study, however, the only mouse antigen that has been purified sufficiently is AFP, but the levels of this were measured in Dr. Ruoslahti's laboratory (Sabine *et al.*, 1975).

Another possible substitute for Stonehill's system would be to use membrane-bound foetal antigens as a parameter of study. This would involve the use of early gestation foetal cells to raise an antiserum in homologous hosts. This would ensure that there would be a relatively high concentration of foetal antigens on the cell membrane, and by using homologous hosts, eliminate the production of antibodies to the

adult antigens. Further, since membrane-bound antigens would be used as the immunogen, this should facilitate a strong immunological reaction.

In view of the invalidity of the Stonehill antigens, and the unavailability of new cancerous and pre-cancerous tissue, it was decided to abandon this parameter in the time course study, and seek a new system for study. Carcino-placental antigen production in rat tumours was examined.

CHAPTER IV

ONCO-PLACENTAL ANTIGENS

SECTION I : INTRODUCTION

Examples of the appearance of carcino-placental molecules in human carcinoma have been described in Chapter I. The majority of research on these substances has been limited to the use of HCG and Regan isozyme in the clinical management of cancer. There has been little research into the use of these substances as biological, as opposed to clinical, markers of the malignant transformation. Perhaps the reason for this, is that these carcino-placental 'antigens' have so far been found only in human tumours, and hence ethical considerations have placed a limit on the experimental studies that may be done.

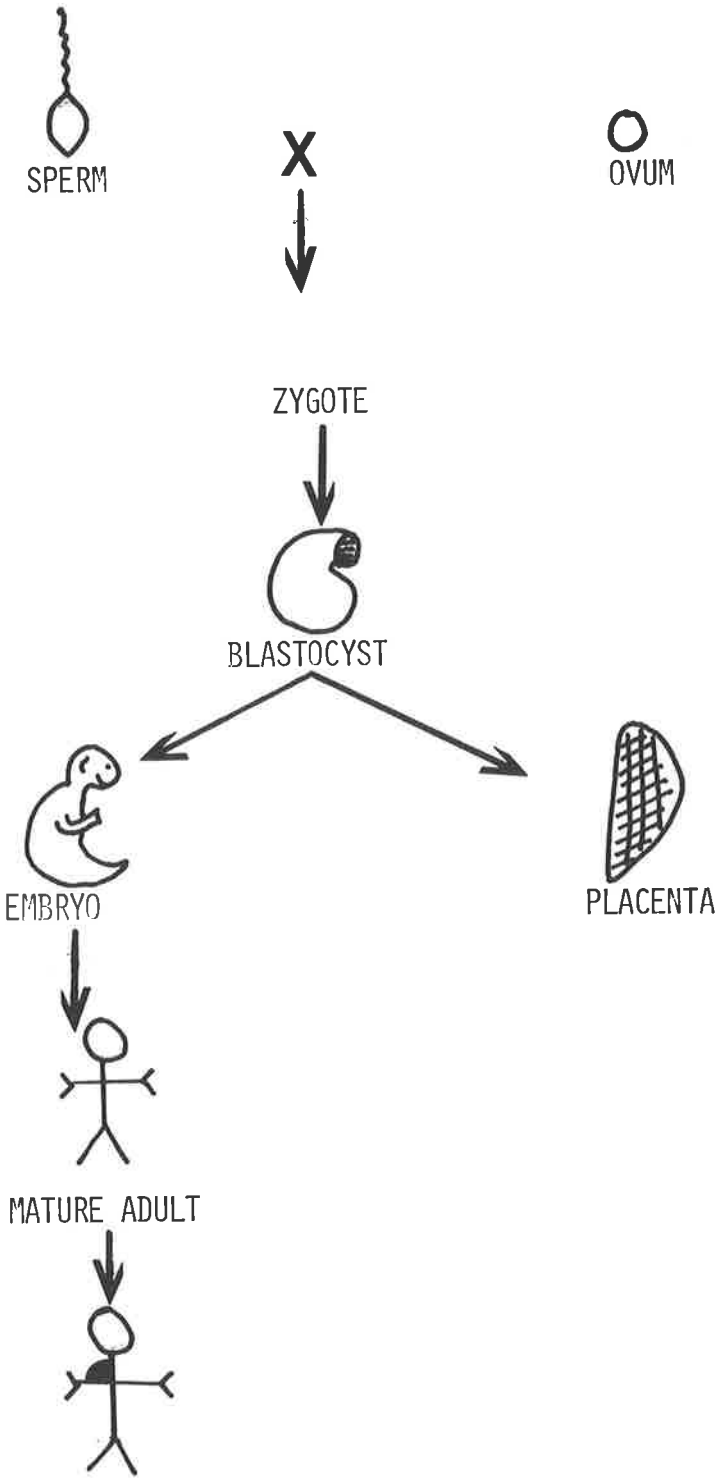
In this chapter, a hypothesis is presented to explain the appearance of these antigens, and attempts to demonstrate the presence of these antigens in experimental animals are described.

SECTION II : RATIONALE FOR THE REAPPEARANCE OF PLACENTAL ANTIGENS

An explanation for the appearance of foetal antigens in cancer cells is summarized diagrammatically in Figure 4.1. In terms of gene expression, it is proposed that during the malignant transformation certain genes which once appeared early in the developmental program of the animal, and have since been inactive, are once again re-expressed. This gives rise to the many foetal characteristics known to prevail in tumours. In arbitrary terms, the cell has undergone a "de-differentiation".

FIGURE 4.1

The developmental sequence of mammalians. The blastocyst goes on to develop into the embryo and placenta. At each stage of development, there are certain genes that are active. As development proceeds, some genes are "switched off" and new genes activated.



SINCE THE TUMOUR TISSUE REVERTS BACK TO AN EMBRYONIC STATE. PERHAPS THE DEDIFFERENTIATION GOES BACK FURTHER TO THE BLASTOCYST STAGE WHERE POTENTIAL PLACENTAL ANTIGENS MAY BE FOUND

This concept may also be used to explain the mechanism of appearance of placental antigens. It is hypothesized that the de-differentiation of the tumour cell goes back further, past the embryo/foetal stage, to the blastocyst. At this stage of development, there are four gene types which are active:

(a) blastocyst only

(b) placental only

(c) foetal only

(d) foetal and adult

Each of these gene types code for a specific type of phenotypic product. Consequently, the placental cells which develop from the blastocyst will synthesize all four phenotypic products. Thus the de-differentiation of the normal cell to the cancerous could result in the reappearance of phenotypic products of all the blastocyst gene types. It is difficult to look for blastocyst-specific products in tumours, since this tissue is so small in experimental animals that accurate immunological studies are impossible. However, since foetal and placental products do appear in human tumours, there is considerable support for this theory of de-differentiation.

In the proposed study, a direct immunological examination of rat tumours for the presence of placental antigens is described. The immunological approach was chosen rather than the biochemical, because the currently known onco-placental molecules (e.g. alkaline, phosphatase, pyruvate kinase), may not necessarily be the placental antigens re-expressed in the rat tumours. Further, since the onco-

placental antigens may be membrane bound, a study of cell-surface bound and cytoplasmic antigens was undertaken.

The experiments presented in this chapter have two distinct but related aims:

1. To determine whether soluble and membrane-bound placental antigens reappeared during carcinogenesis.

2. If so, to use these antigens as markers of the transition of normal liver cells to the cancerous state, and in particular to determine the earliest time of appearance following the application of chemical carcinogens.

Attempts to demonstrate the reappearance of these antigens in rat tumours were only partially successful. The presence of soluble placental antigens was demonstrated in three rat tumours. However, the complexity of this system prevented its use as a means of studying placental antigens in cancer.

Membrane-bound placental antigens were then examined to determine whether these antigens reappeared in rat tumours.

SECTION III : SALINE-SOLUBLE CYTOPLASMIC PLACENTAL ANTIGENS

1. Immunodiffusion studies

(a) Method

The methodology of this study was similar to that of the foetal antigen program, described in the previous chapter. Rat placentae (Buffalo strain) were homogenized in 0.85% saline, and the soluble extract prepared as outlined in the Materials and Methods. Antibodies to this extract were raised in rabbits. Since this antiserum would contain antibodies to foetal, adult and placental antigens, it was adsorbed first with a composite adult extract and then with a foetal extract in an attempt to render it placental-specific. It was then planned to use this antiserum to detect placental antigens in tumour extracts using double immunodiffusion plates.

(b) Results

From Figure 4.2 it can be seen that the unadsorbed antiserum reacted very strongly against the placental, foetal, adult and tumour (5123c Morris hepatoma) extracts. Following exhaustive adsorption with a composite adult extract the serum failed to react with the adult and tumour antigens, with only the placental and foetal extract exhibiting precipitin bands (Figure 4.3). Further, since the foetal and placental bands exhibited a reaction of Identity (Ouchterlony, 1968) it seems likely that the adsorbed antiserum was detecting only the foetal component of the placenta. Thus in this system, the antibody which

FIGURE 4.2

Immunodiffusion pattern of unadsorbed anti-placental serum tested against tumour, foetal, placental and normal adult extracts.

P = placental

F = foetal

H = heart/lung

K = kidney/spleen

L = liver/intestine

T = transplantable hepatoma

ap = anti-placental serum

FIGURE 4.3

Immunodiffusion pattern of anti-placental serum tested against placental, foetal, tumour and normal adult extracts.

P = placental

F = foetal

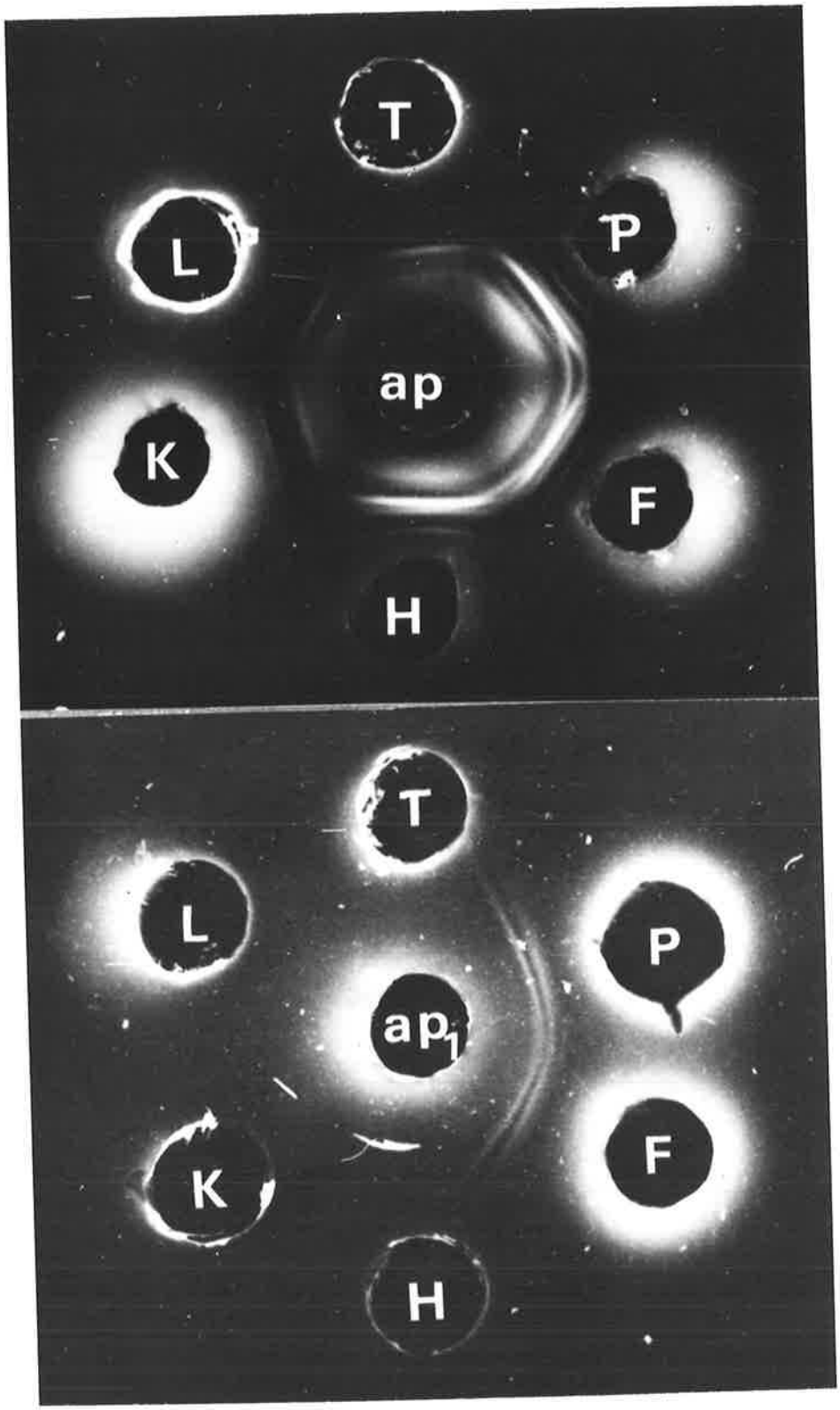
H = heart/lung

K = kidney/spleen

L = liver/intestine

T = Morris 5123c transplantable hepatoma

ap_1 = anti-placental serum adsorbed with adult tissue extracts


T
L
P
ap
K
F
H
T
L
P
ap1
K
F
H

was raised in rabbits seemed to be directed against the adult and foetal antigens, and not against the placental-specific molecules. A major consideration here, however, is the effect that adsorption has on the antiserum, and this is considered below.

2. Immunoelectrophoretic studies

(a) Rationale

In the previous study, there was a possibility that placental-specific antibodies may have been removed during adsorption of the antiserum with adult antigens. This might occur, for instance, if the particular antibody in the serum was directed against a placental enzyme which has an adult isozyme.* Should the antibody bind to the antigenic determinant on both isozymes, adsorption with an adult extract would remove the antiplacental activity from the serum. To examine this particular example of adsorption effects placental, tumour, foetal and adult extracts were to be individually immunoelectrophoresed. Antigens which were found in the placenta were visualized by adding unadsorbed antiplacental serum to the IEP trough. By comparing the precipitin bands of the four extracts, it was hoped to locate molecules which are common to the tumour and placenta, but not found in either the adult or foetus. This technique would eliminate any problems which may have been caused by adsorption. Discrimination of the adult and placental isozyme should occur because different forms of the same enzyme often have different electrophoretic mobilities.

*Such sharing of antigenic determinants has been demonstrated in the case of placental and adult (intestinal) alkaline phosphatase (Doellgast, 1976).

(b) Direct Immunoelectrophoresis

Firstly, only placental and foetal extracts were loaded directly on the gel and electrophoresed, in an attempt to determine which of the placental antigens detected by the antiserum were not present in the foetus. A similar method has been used extensively in the identification of abnormal serum proteins (Ouchterlony, 1968). Figure 4.4 demonstrates the unsuitability of the system as applied here, since there was ineffective separation of the extracts. This was probably due to steric hindrance, caused by the large number of molecules which constitute the extracts examined. This idea of ineffective separation is supported by the immunodiffusion patterns seen in Figure 4.2. The precipitation bands on that plate suggest that the unadsorbed antiserum should detect at least 6 distinct precipitation bands when the antibody is reacted against the placental extract. However, the immunoelectrophoresis pattern of Figure 4.4 shows the presence of only 4 bands. (Unpublished results from our laboratory have shown a similarly poor separation when whole placental, tumour and foetal extracts are electrophoresed on polyacrylamide gels.)

(c) Column Separation/Immunoelectrophoresis

(i) *Method*

In order to reduce the large number of molecules being applied to the gels, each extract was first fractionated on Sephadex G-200 columns, before electrophoresis. A protein profile of the column eluant was obtained (Figure 4.5), and then each peak was pooled and adjusted to a protein concentration of 10 mg/ml. The pools were examined for the presence of placental antigens by testing against

FIGURE 4.4

Direct immunoelectrophoresis of placental and foetal extracts.

p = placental

f = foetal

Central trough contains unadsorbed anti-placental serum.

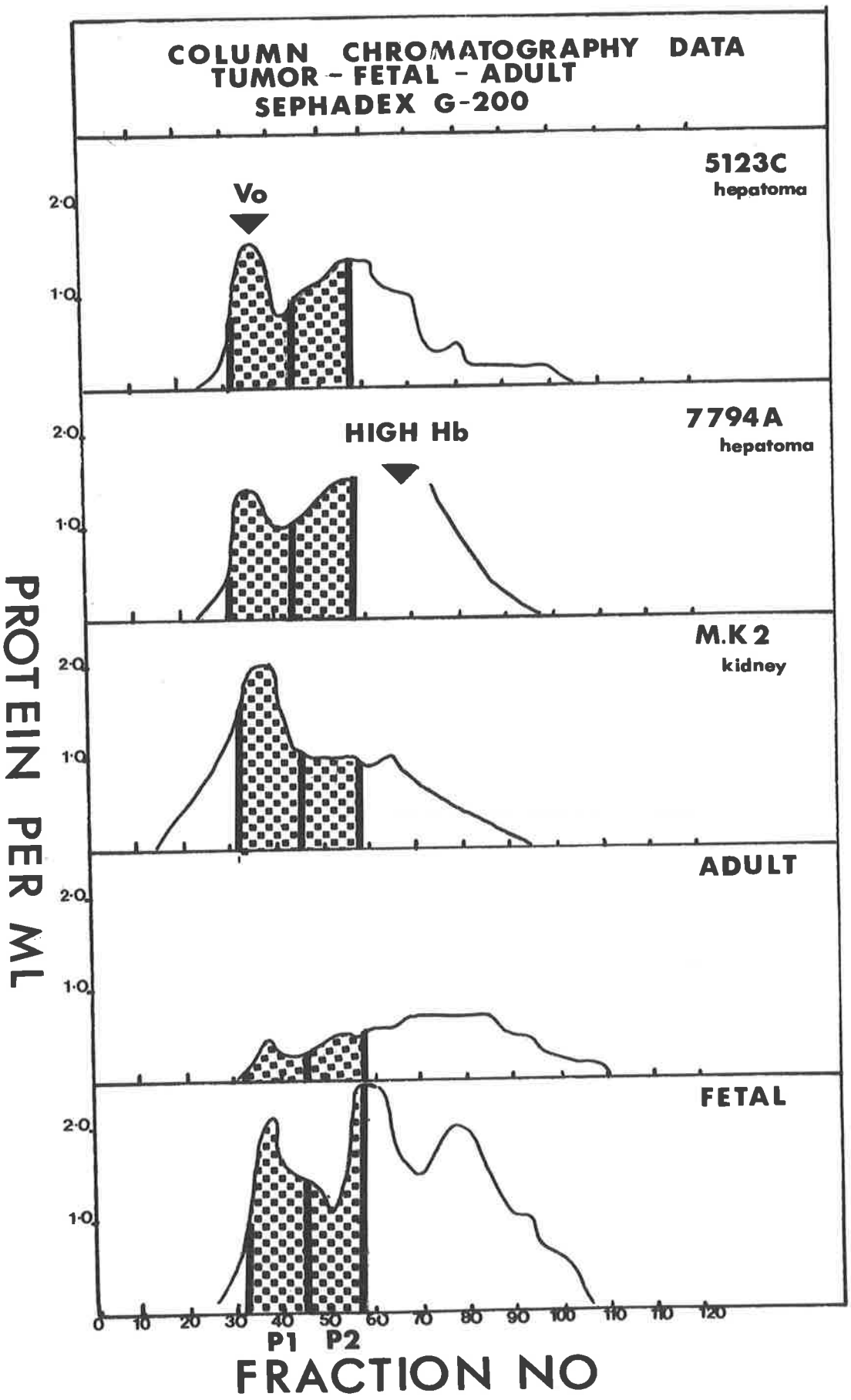
4.4

FIGURE 4.5

Gel filtration of Morris transplantable tumours, foetal and adult extracts on 40 cm x 2.5 cm Sephadex G-200 columns. Each extract was adjusted to 50 mg protein/ml, 3 mls applied to the column, and eluted with phosphate buffered saline. 2 ml fractions were collected and assayed for protein by the method of Lowry *et al.* (1951). Shaded areas represent those fractions which reacted positively against unadsorbed anti-placental serum.

V_o = void volume

P_1,P_2 = pooled fractions


COLUMN CHROMATOGRAPHY DATA
TUMOR - FETAL - ADULT
SEPHADEX G-200
5123C
hepatoma
Vo
2·0
1·0
7794A
hepatoma
HIGH Hb
M.K 2
kidney
ADULT
FETAL
0 10 20 30 40 50 60 70 80 90 100 110 110 120
P1 P2
PROTEIN PER ML
FRACTION NO

unadsorbed anti-placental serum, in Ouchterlony double-diffsuion plates. To demonstrate that some of the antigens found in the tumour were placental-specific, placental-positive peaks from each tumour were immunoelectrophoresed alongside the corresponding peaks from the foetal and adult extracts (i.e. Peak P_1 from each tumour, foetal and adult, then P_2 from the same extracts - Figure 4.5). By comparing the precipitin bands, placental-specific antigens were designated as those which were found in tumours, but not in the foetal nor adult extracts.

(ii) *Results and Discussion*

The precipitation bands which formed after electrophoresis are shown in Figures 4.6 (Pool 1) and 4.7 (Pool 2). The number of separate placental-specific antigens (i.e. specific by the above criteria) and their relative electrophoretic mobilities, are summarized in Tables 4.1 and 4.2. Direct comparisons of electrophoretic mobilities is valid because all the samples were run on the same slide.

By comparing the electrophoretic mobilities summarized in Table 4.1, it can be seen that the 5123c and 7794A tumours share an antigen with a common mobility and molecular weight range (Pool 1, CPA-1 and CPA-5). This antigen is absent, however, from the MK-2 tumour. Similarly, the 5123c and MK-2 tumours share another antigen (CPA-2 and CPA-4), which is absent from the 7794A. In addition to these antigens, the MK-2 tumour also produces an additional antigen, which is not found in either of the two hepatomas.

The electrophoretic patterns of the second pool of the tumour's column profile (Table 4.2) reveal that each tumour produces a specific

FIGURE 4.6

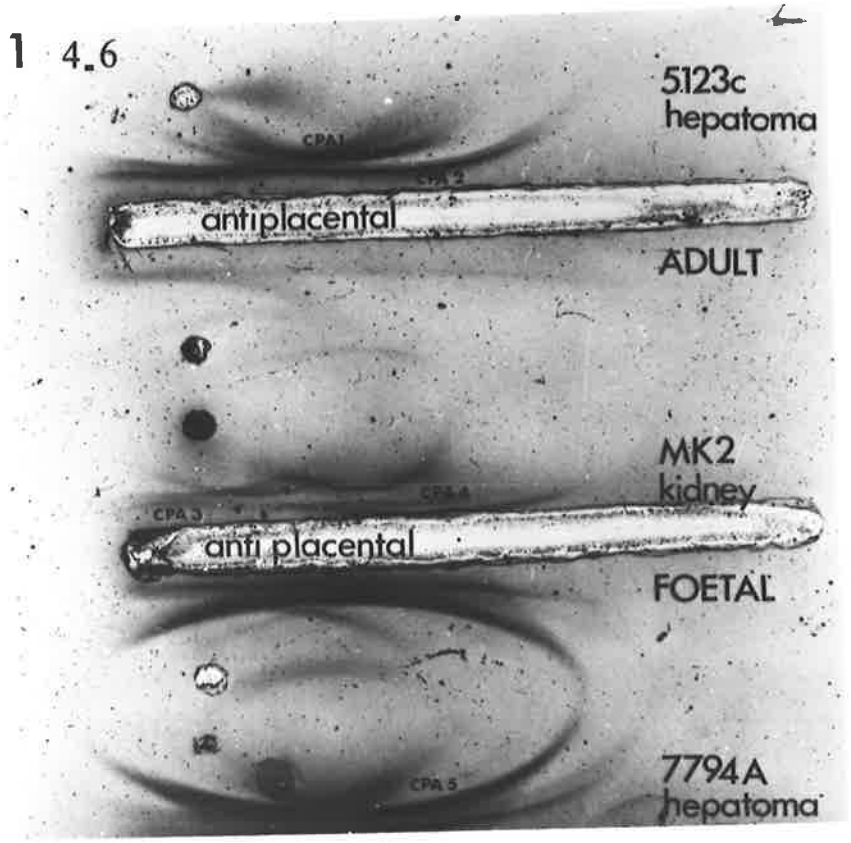
Immunoelectrophoresis of Morris transplantable tumour, foetal and adult extracts. Pool 1 from each of these extracts were electrophoresed alongside each other and placental antigens visualized immunologically by adding unadsorbed anti-placental serum to the central trough.

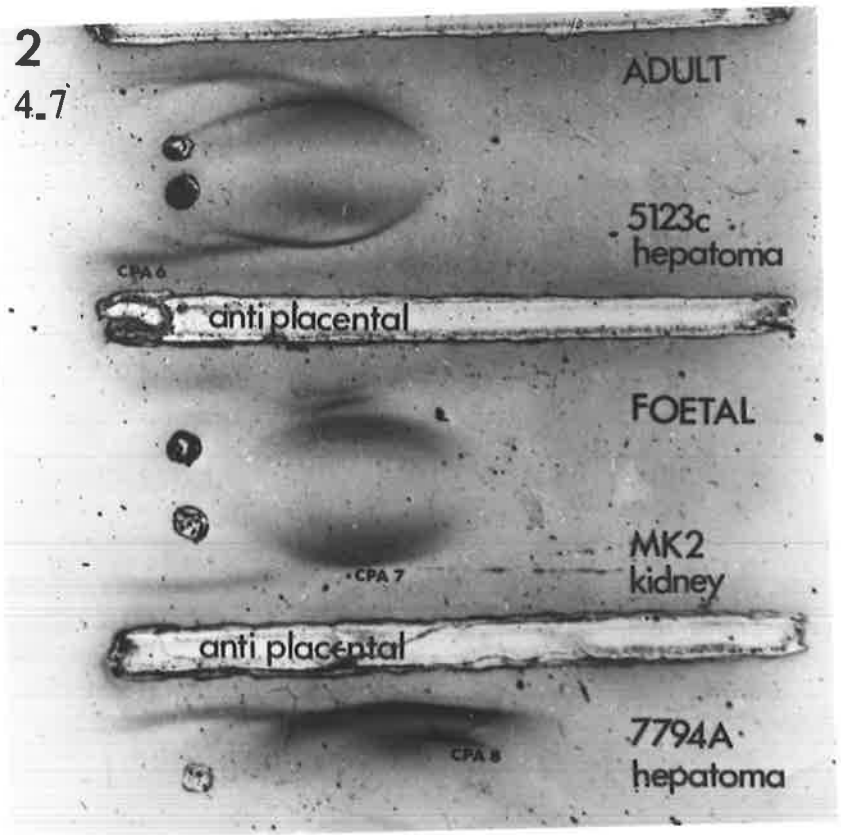
CPA = antigens found in tumours but not adult or foetal

FIGURE 4.7

Immunoelectrophoresis of Morris transplantable tumour, foetal and adult extracts. Pool 2 from each of these extracts were electrophoresed alongside each other and placental antigens visualized immunologically by adding unadsorbed anti-placental serum to the central trough.

CPA = antigens found in tumours but not adult and foetal


1
4.6
5123c
hepatoma
CPA1
CPA 2
anti placental
ADULT
MK2
kidney
CPA 4
CPA 3
anti placental
FOETAL
CPA 5
7794A
hepatoma


2
4.7
ADULT
5123c
hepatoma
CPA 6
anti placental
FOETAL
MK2
kidney
CPA 7
anti placental
CPA 8
7794A
hepatoma

placental antigen which is not shared with any of the other tumours examined here (CPA-6, CPA-7, CPA-8). Such tumour specificity for developmental antigens has been described previously for a number of foetal antigens (Kroes *et al.*, 1972; Häkkinen, 1974), and is considered a valid conclusion in these studies.

Thus, based on electrophoretic mobility comparisons, 6 distinct placental antigens have been shown to reappear in Morris transplantable tumours.

(iii) *Conclusions*

Although the methodology employed here is suitable for an initial demonstration of the presence of carcino-placental antigens, it is, however, unsuitable if these antigens are to be monitored in a large number of samples. This is because the method of column separation, followed by immunoelectrophoresis is a long and tedious procedure, requiring approximately 3 weeks to complete per sample. The technique also requires a large quantity of tissue (approximately 1.0 gm wet weight), which is generally unavailable in the cancerous and pre-cancerous stages. Therefore, a technique was sought which would enable:

(a) a small quantity of tissue to be utilized,

(b) simple extraction of tissue,

(c) detection of the antigens with relative ease.

Membrane-bound antigens conform with these criteria. Consequently, this type of antigen was chosen in a further attempt to demonstrate the reappearance of placental antigens in rat tumours.

TABLE 4.1

TUMOUR	ELECTROPHORETIC MOBILITY GRADIENT		
	SLOW	ANTIGEN NO.	FAST
5123c (Liver)		CPA-1	CPA-2
MK-2 (Kidney)	CPA-3		CPA-4
7794A (Liver)		CPA-5	

TABLE 4.2

TUMOUR	ELECTROPHORETIC MOBILITY GRADIENT		
	SLOW	ANTIGEN NO.	FAST
5123c (Liver)	CPA-6		
MK-2 (Kidney)		CPA-7	
7794A (Liver)			CPA-8

SECTION IV : MEMBRANE-BOUND PLACENTAL ANTIGENS

1. Introduction

Membrane-bound placental antigen reappearance in rat tumours was examined using a similar experimental approach to that previously described for saline-soluble antigens. In this study, an antiserum was raised to the membrane bound antigens, and attempts were made to render it placental-specific by adsorbing the antiserum with adult tissue membrane extracts.

Briefly, it was found that the two immunogens (i.e. whole placental cells and a crude preparation of their cell membranes), stimulated the production of an antiserum with activity directed principally against the adult antigen component of the placenta, and to a lesser degree, the foetal portion. In both cases, a placental-specific antibody was unobtainable. These attempts are described below.

2. Intact Placental Cells

(a) Preparation of antibody

Placentas from pregnant rats (Wistar) of gestation age 13-19 days, were minced into small pieces. Isolated cells were prepared as outlined in the Materials and Methods, and antibodies to the cells raised in rabbits. Antibodies in the rabbit serum were detected by testing against antigens which had been solubilized from tumour (MeDaB induced hepatoma), placental and adult membranes.

From Figure 4.8(a), it can be seen that the antiserum reacted strongly against all the antigens examined.

(b) Adsorption with adult antigens

Antibody against adult antigens was removed by incubating 1 ml of immune serum for 30 mins at room temperature, with 50 µl of packed, normal, adult red blood cells. This adsorption was repeated a further two times. On re-testing the adsorbed serum, all reactivity against the tumour, placental and adult antigens had disappeared (Figure 4.9).

(c) Conclusion

Since all immune reactivity was removed by adsorption with adult red blood cells, it was concluded that all the antibody raised against placental cells was directed against either antigens of the adult red blood cells, or adult isozymes of placental-specific enzymes. This result was similar to that obtained following adsorption of antiserum raised against saline-soluble extracts of rat placenta (Chapter IV, Section III).

3. Placental Plasma Membrane Fraction

The previous experiments were repeated with an antiserum raised against a crude preparation of placental cell-surface membranes, rather than whole cells.

(a) Heterologous antibody

(i) *Preparation of antibody*

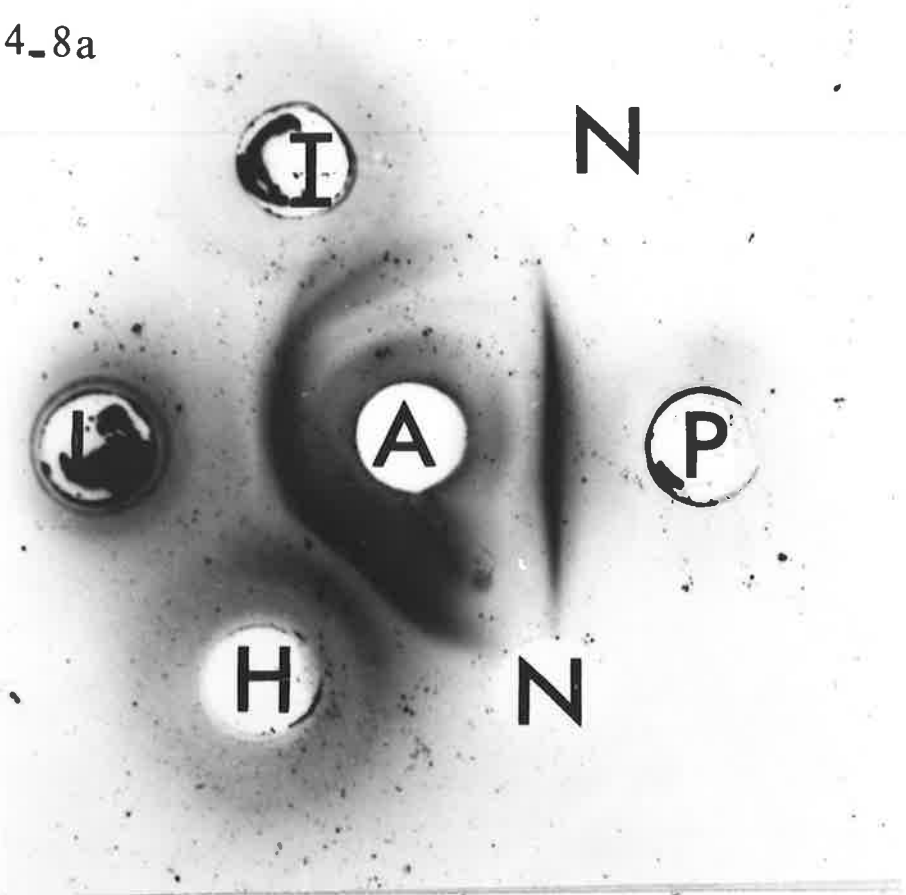
FIGURE 4.8

Immunodiffusion slide of antiserum from rabbits immunized with whole placental cells. The peripheral wells contain the test antigens, i.e. antigens solubilized off the appropriate tissue cell membranes, and the central wells contain the antiserum.

(a) P = placental
N = NaCl control
H = heart
N = NaCl control
L = lung
I = intestine
A = anti-whole placental cells

(b) T = tumour
C = NaCl control
S = spleen
A = Antiserum
N = NaCl control
K = kidney
Li = liver
A = unadsorbed anti-whole placental cells

4_8a



4_8b

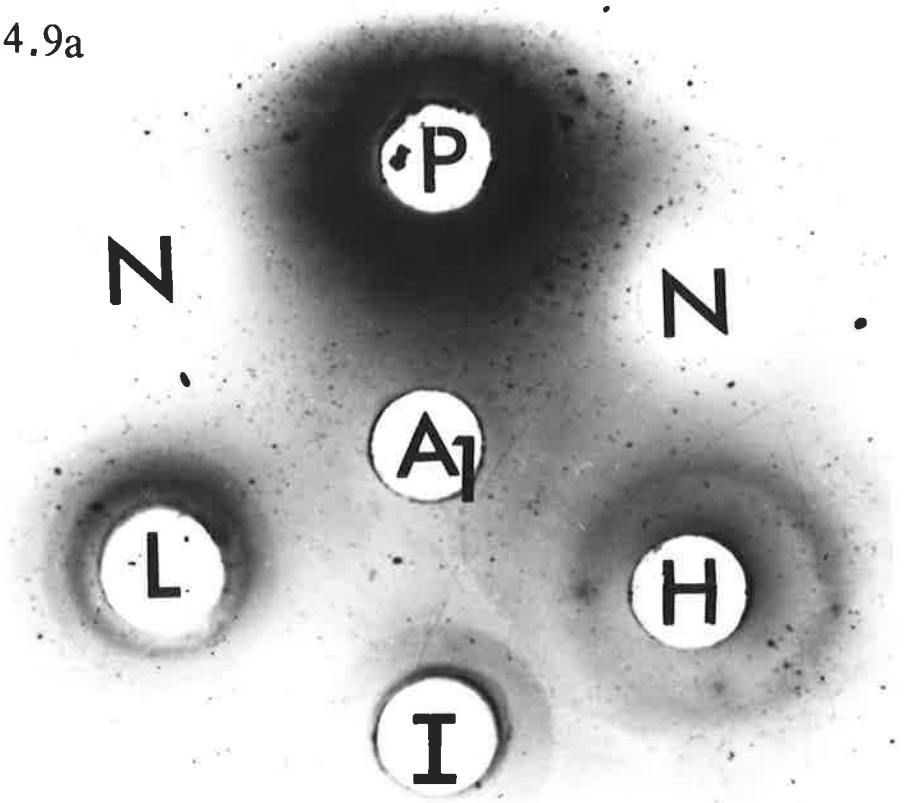
FIGURE 4.9

Immunodiffusion slide of antiserum from rabbits immunized with whole placental cells. The serum had been adsorbed three times with packed rat red blood cells before testing. The peripheral wells contain the test antigens, i.e. antigens solubilized off the appropriate tissue cell membranes, and the central wells contain the adsorbed antiserum.

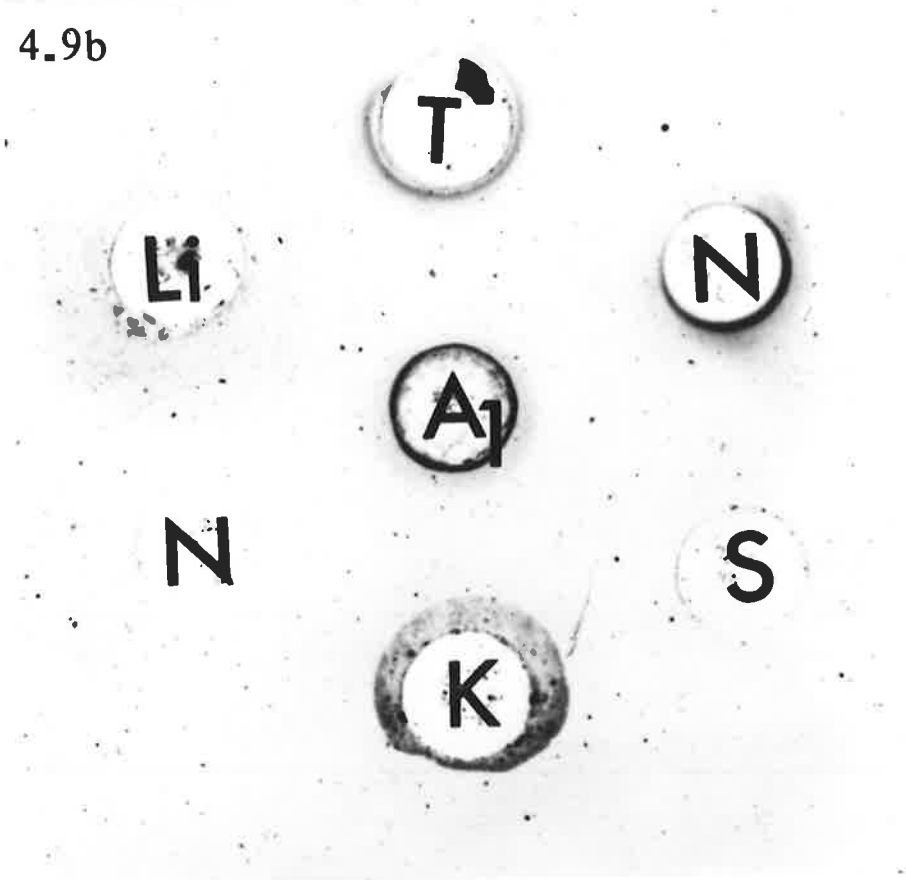
(a) P = placental
N = NaCl negative control
H = heart
A = adsorbed anti-whole placental cells (concentrated x10)
N = NaCl negative control
L = lung
I = intestine

(b) T = tumour
C = NaCl negative control
S = spleen
A = adsorbed anti-whole placental cells (concentrated x10)
N = NaCl negative control
K = kidney
Li = liver

4.9a



4.9b



Washed placental membranes were prepared from 13-19 day gestational pregnant Wistar rats. Rabbits were immunized with this membrane preparation, and the presence of antibodies again verified by testing the rabbit serum against membrane solubilized MeDaB hepatoma, placental and adult antigens. The antibody reacted strongly against all the antigens examined by double immunodiffusion (Figure 4.10).

(ii) *Adsorption with adult antigens*

1 ml of immune serum was adsorbed with 50 µl of packed normal adult red blood cells as described previously. The adsorption markedly reduced the intensity of precipitation of the antigens by the immune serum (Figure 4.11). However, a residual anti-adult activity still remained in the serum.

1 ml of this antiserum was then further adsorbed with a composite mixture of adult tissue membranes. On repeated adsorption, all reactivity with the adult and tumour disappeared from the antiserum (Figure 4.12). The resultant serum still reacted with the placental extract, although the degree of activity was considerably diminished. This adsorbed antiserum was tested to determine whether the remaining antibody was placental-specific, or simply directed against the foetal component of the placental cell membrane. This was done by testing the residual antiserum by immunodiffusion, against a solubilized membrane extract from Wistar rat foetus. This antiserum was able to precipitate antigens from the rat foetus (Figure 4.13). Further, since the precipitin bands of the placental and foetal antigens

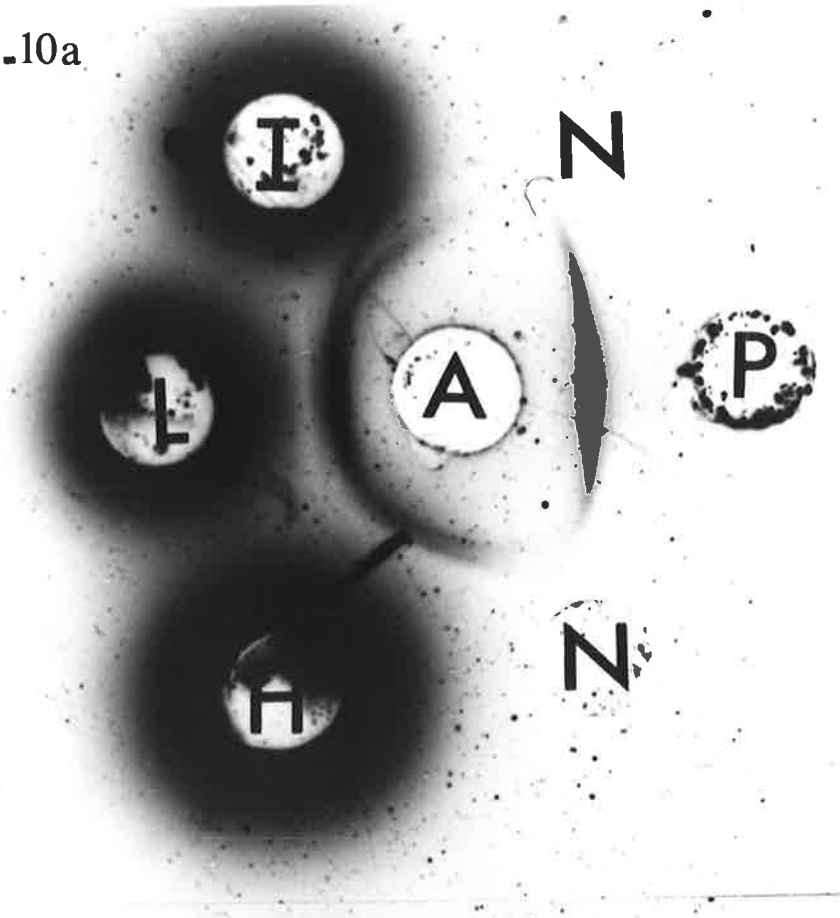
FIGURE 4.10

Immunodiffusion slide of antiserum from rabbits immunized with a cell membrane fraction prepared from rat placental cells. The peripheral wells contain the test antigens, i.e. antigens solubilized off the appropriate tissue cell membranes, and the central wells contain the adsorbed antiserum.

(a) P = placental
N = NaCl negative control
H = heart
N = NaCl negative control
L = lung
I = intestine
A = unadsorbed anti-placental cell membranes

(b) S = spleen
N = NaCl negative control
T = tumour
C = NaCl negative control
K = kidney
Li = liver
A = unadsorbed anti-placental cell membranes

4.10a



4.10b

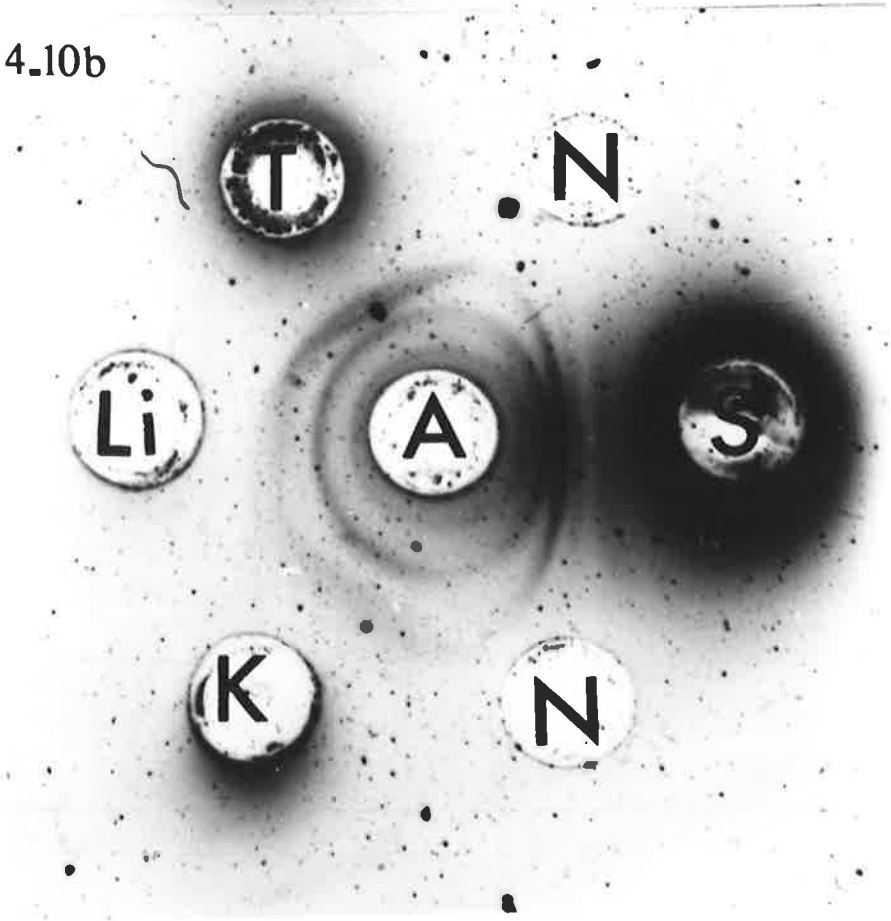


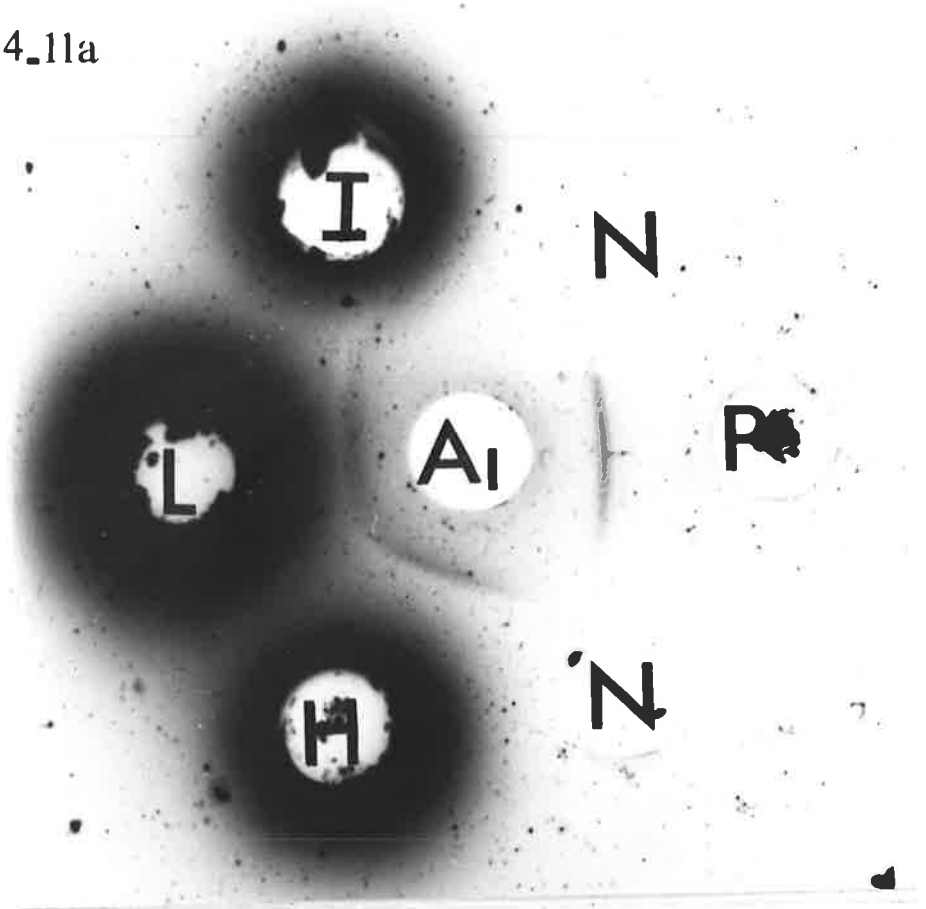
FIGURE 4.11

Immunodiffusion slide of antiserum from rabbits immunized with a cell membrane fraction prepared from rat placental cells. The serum had been adsorbed three times with packed rat red blood cells prior to testing. The peripheral wells contain the test antigens, i.e. antigens solubilized off the appropriate tissue cell membranes, and the central wells contain the adsorbed antiserum.

(a) P = placental
N = NaCl control
H = heart
N = NaCl control
L = lung
I = intestine
A = RBC adsorbed anti-placental membranes

(b) S = spleen
N = NaCl negative control
T = tumour
N = NaCl negative control
K = kidney
Li = liver
A = RBC adsorbed anti-placental membranes

4_11a



4_11b

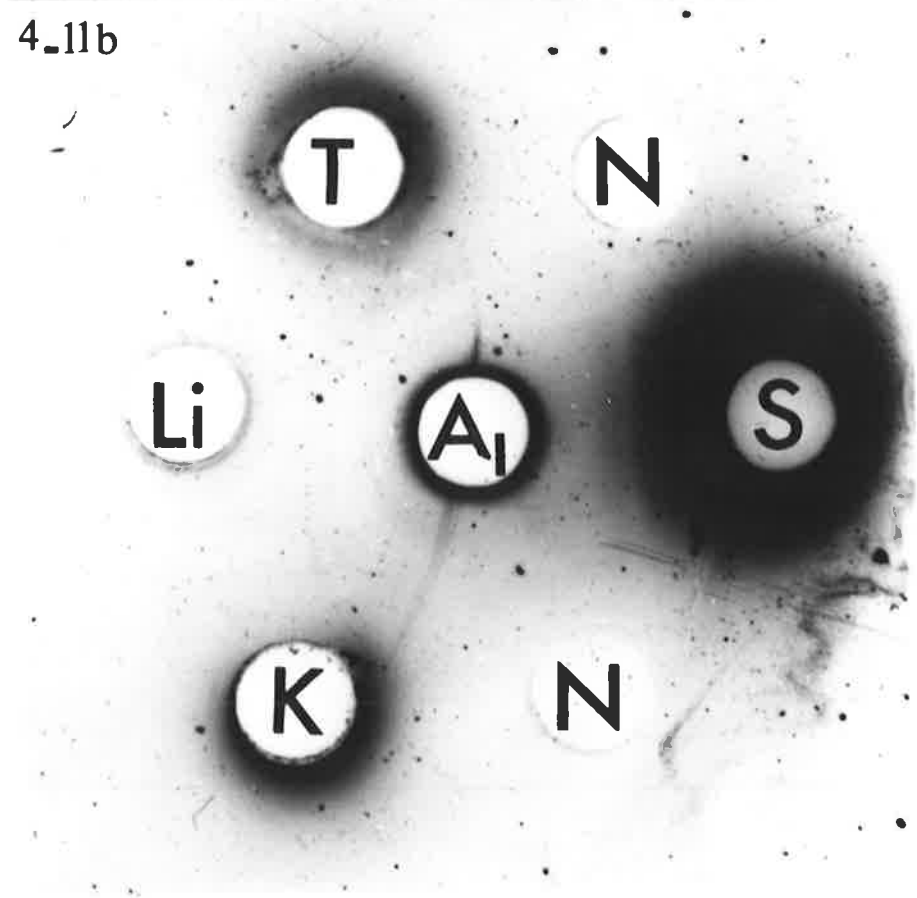


FIGURE 4.12

Immunodiffusion slide of antiserum from rabbits immunized with rat placental cell membrane fraction. The antiserum had been adsorbed three times, with packed rat red blood cells, and then three times with a composite mixture of membrane from normal adult tissues. The peripheral wells contain the solubilized test antigens, and the central wells the adsorbed antiserum.

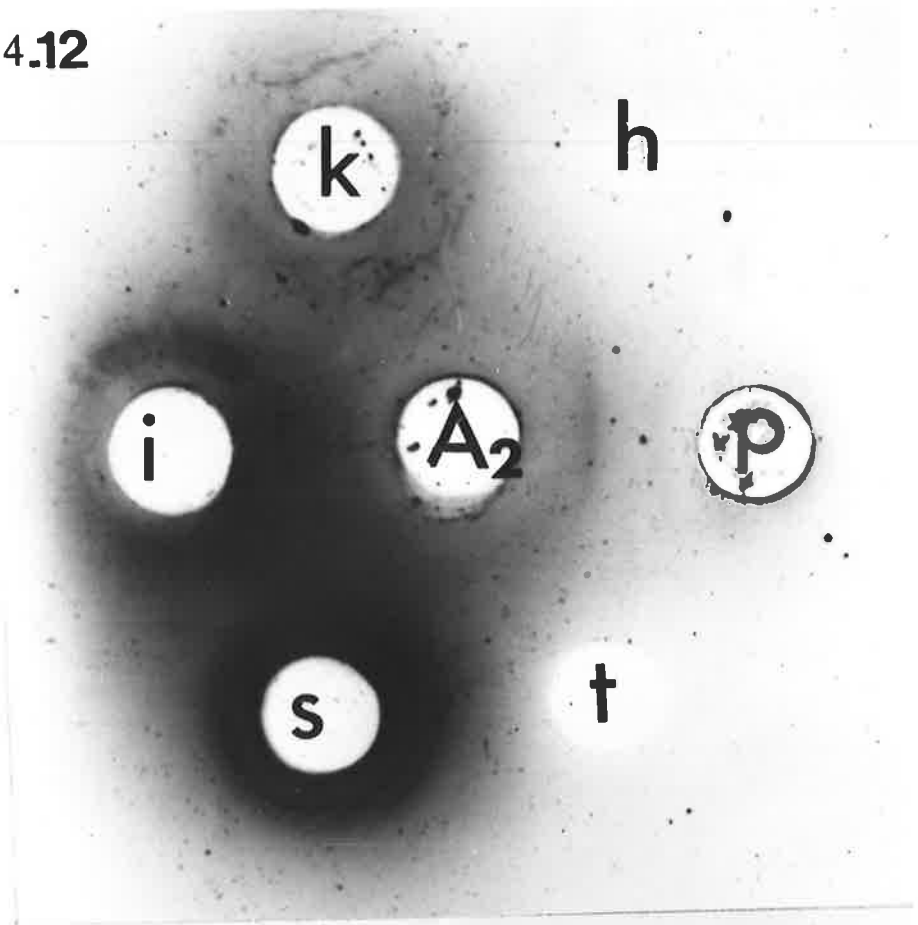
p	=	placenta	t	=	tumour
s	=	spleen	i	=	intestine
k	=	lung	h	=	heart

FIGURE 4.13

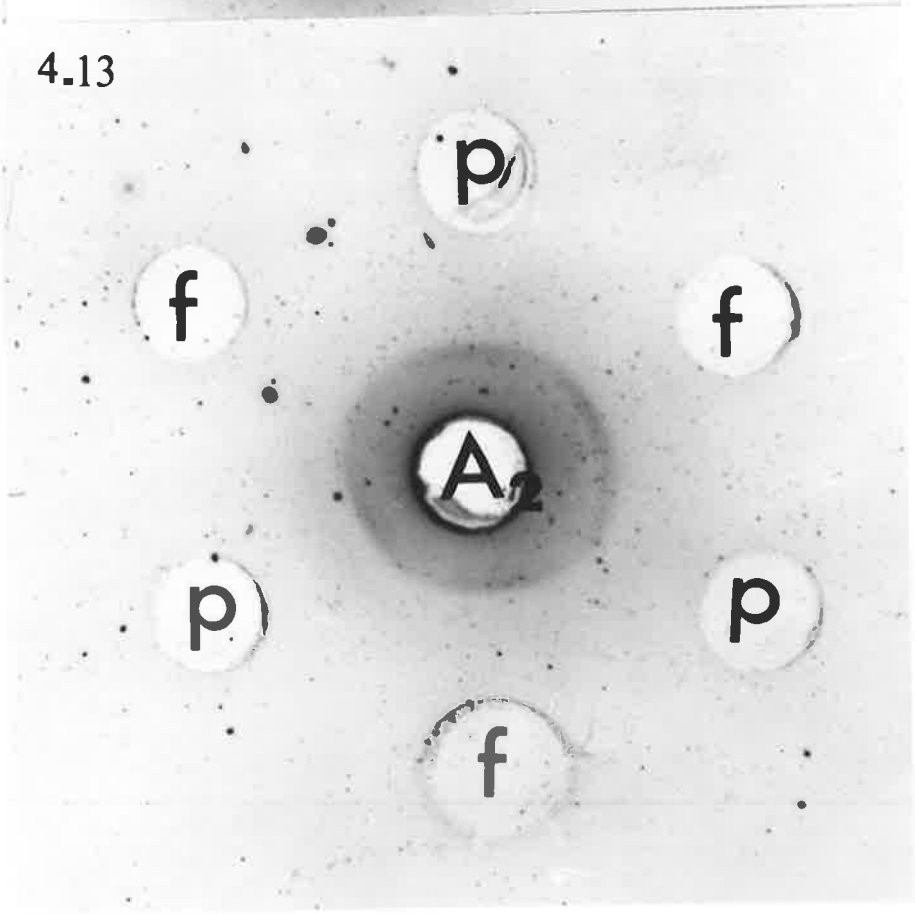
Immunodiffusion slide of antiserum from rabbits immunized with placental cell membrane fraction. The antiserum has been adsorbed with rat red blood cells and a composite adult membrane suspension.

P	=	placental
F	=	foetal

4.12



4.13



exhibited a reaction of identity, the antiserum activity was exclusively directed against the foetal component of the placenta.

(iii) *Conclusion*

The results obtained here indicate that the antiserum which was raised against the placental membrane fraction was directed against the foetal and adult (or adult isozyme of a placental enzyme) component only. This effect is again similar to that which occurred during the preparation of an antibody to saline-soluble extracts of the rat placenta. Consequently, this method of raising an antibody was also unsuitable. A method of eliminating the production of antibody to the large quantity of adult antigens was thus sought.

(b) Homologous antibody

(i) *Introduction*

The attempts to raise an antibody to placental-specific, membrane-bound antigens had been unsuccessful so far, since most of the immune response was directed against the high concentration of adult antigens on the cell surface membrane, and to a lesser degree against those of foetal origin. In an attempt to overcome this problem, rats were immunized with homologous placental membranes. Since the rats should not recognize the adult antigens as foreign, the antibody response to these antigens should be eliminated. This would allow the production of placental and foetal-specific antisera. The amount of antibody to these antigens should also be considerably higher because

the immunological response to the adult antigens (principally, the highly immunogenic transplantation antigens), which would normally "swamp" the host's immune system, would be eliminated.

(ii) *Results and discussion*

The attempt to immunize rats with homologous placental membranes was unsuccessful. The sera from the 12 immunized rats failed to react against the solubilized placental membrane extracts (Figure 4.14). This inability to raise an antibody to the placental antigens was probably due to either the small quantity of placental-specific antigens which constitute the placental cell membrane, or to the host's immunological tolerance to these antigens. In the first circumstance, this could possibly be overcome by continual immunization over periods of 6 months. Chang *et al.* (1976) have overcome this problem in such a manner, and have successfully raised, in rats, an antibody to two soluble rat placental enzymes. The second problem of immunological tolerance would be difficult to solve. This would require the formation of placental antigen haptens, to facilitate their recognition by the rat's immune system. A similar method has been used by Ruoslahti *et al.* (1974) to break homologous tolerance to AFP. With placental antigens, however, many technical difficulties prevail, since a pure solution of placental-specific antigens is not available. Any attempt to haptenate an impure mixture of placental, foetal and adult antigens, would probably result in the production of antibodies to the adult antigens as well, since adult antigens would be haplenated to such an extent,

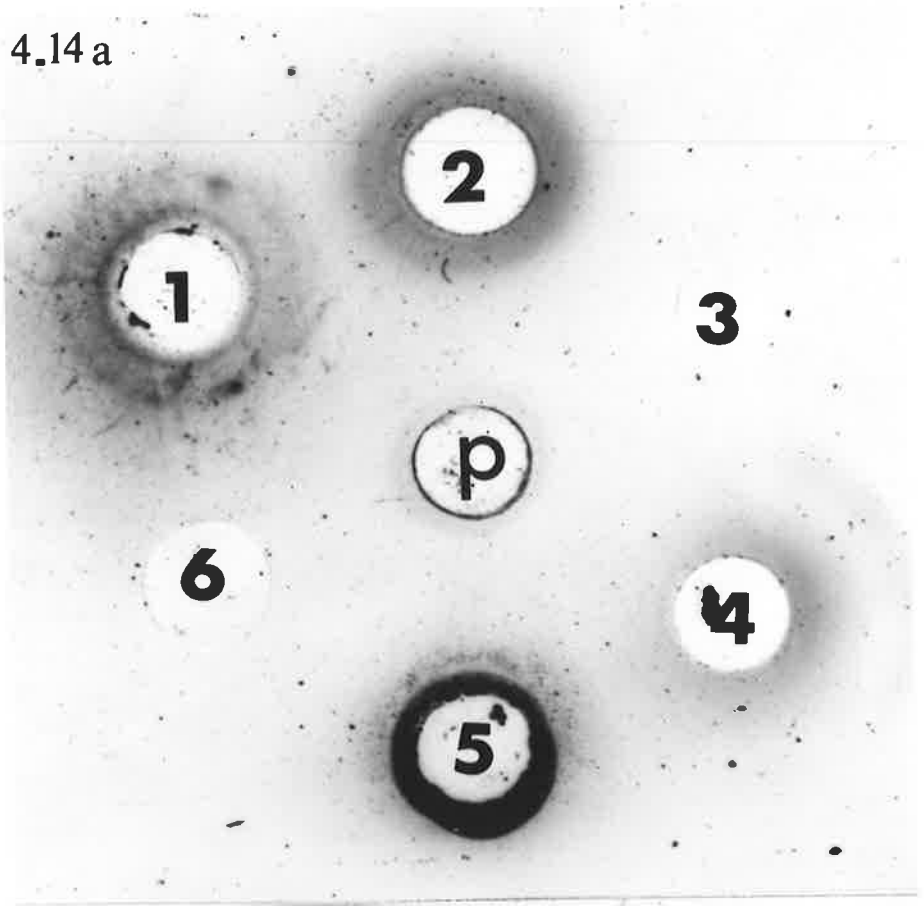
FIGURE 4.14

Immunodiffusion slides of sera taken from Wistar rats immunized with homologous rat placental membranes. The peripheral wells contain the antisera and the central wells contain an antigen extract prepared from rat placental cell membranes.

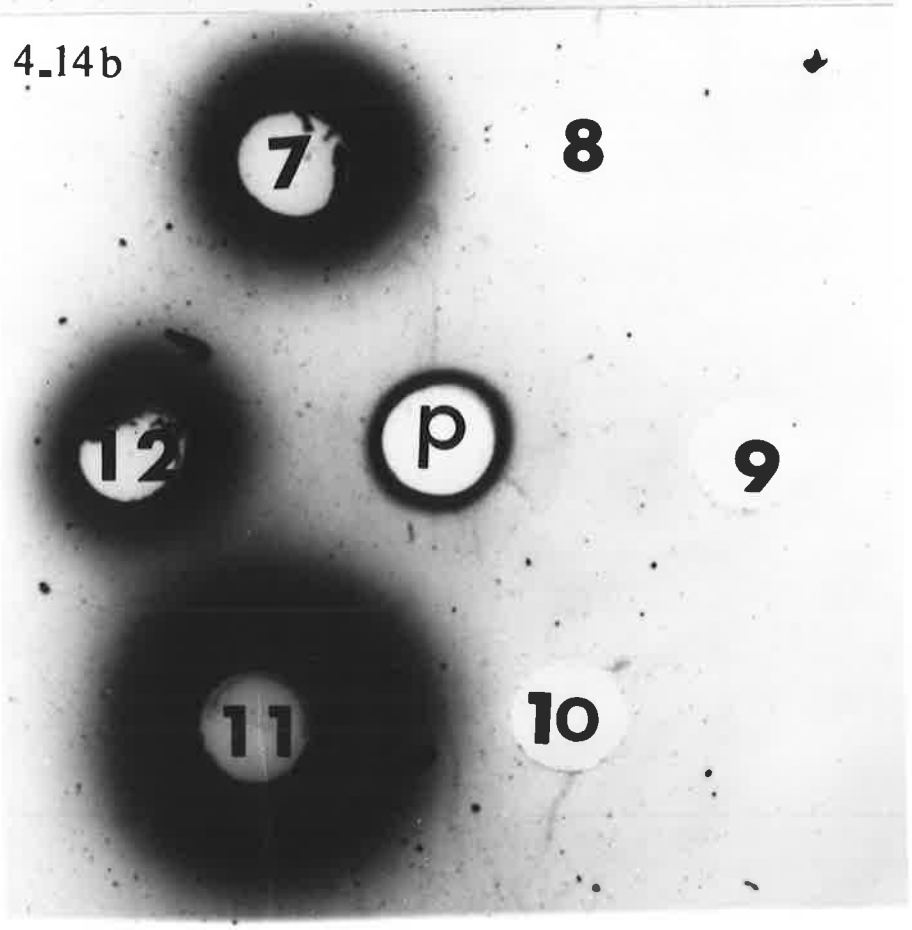
p = placental membrane antigens

1 ... 12 = rat sera numbers

4.14a



4.14b



that immunological tolerance to these would be broken, resulting in the unwanted production of antibodies to adult molecules.

SECTION V : SUMMARY

The aim of this study had been to

(a) demonstrate the appearance of placental antigens in experimental rat tumours;

(b) to use these antigens in a time course study as markers of the malignant state.

The first aim has been achieved. However, the second has not been attempted because of the inability to raise an anti-placental serum, which could be used to detect placental antigens in cancerous tissues. In the majority of attempts to obtain such a serum, two important observations were noted:

(i) adsorption of an anti-placental serum with adult tissues removed most of the antibody activity;

(ii) any residual activity after such adsorption was directed against the foetal component of the placenta.

The first observation was examined further and it was found that the unadsorbed antiserum to saline-soluble placental extracts could be used to detect placental antigens in tumour extracts. However, the particular antigens against which this activity was directed probably

had adult isomers, hence their detection with an absorbed antiserum was not possible because absorption with adult extracts removed the antibodies to these antigens. Although a similar situation was not shown in the membrane studies, this may have occurred here also.

Thus, although placental antigen reappearance in rat tumours was demonstrated by indirect means, it was not possible to raise an antibody to a placental-specific antigen (i.e. one without a similar adult form). There may be two reasons for the inability to do this:

(a) they do not exist;

(b) although synthesized by placental cells, they exist in small quantities and are unable to raise a sufficient immune response from the inocculated host.

The complete absence of such molecules would limit the use of placental antigens in experimental studies of the malignant transformation. Such studies would employ placental molecules which also have an adult form, e.g. the Regan isozyme, HCG, etc. of humans.

If the presence of placental-specific antigens is to be verified, however, the methods used in these studies require considerable modifications. The alterations to this methodology are reviewed further in Chapter VI.

CHAPTER V

FOETAL ANTIGENS AND IMMUNE REPRESSION

SECTION I : INTRODUCTION

Considerable difficulty was observed in raising antibody to a 1500 g extract of mouse foetal homogenates (see Chapter III). This difficulty was overcome by freeze-drying the foetal material, and reconstituting prior to immunization - an unusual situation because freeze-drying is not normally associated with any change in the immunogenicity of an antigen.

To explain this unusual phenomenon, the presence, in the foetal extract, of a lyophilization-labile immune repressor was proposed. Although this possibility was initially thought to be unlikely, the importance of this finding was such as to warrant further investigation. During this study:

(1) the immunogenicity of the foetal extracts was examined;

(2) the inability to raise antibodies to foetal extracts was also repeated in mice immunized with non-freeze-dried rat foetal extracts; and

(3) it was found that this repression of antibody synthesis was specific for foetal antigens only.

From the data presented, it has been proposed that the inability to raise antibodies to non-freeze-dried foetal extracts was due to the action of a lyophilization-labile repressor, possibly an antigen-antibody complex.

SECTION II : SUPPRESSION OF ANTIBODY SYNTHESIS TO FOETAL EXTRACTS

1. Rabbit Immunity

(a) Aim and Methods

During the attempts to raise an antibody to mouse foetal extracts, with which foetal antigens of murine tumours could be detected (Stonehill and Bendich, 1970), two groups of rabbits were immunized - one with non-freeze-dried (NFD) foetal extract, and the other with freeze-dried and reconstituted (FD) extract.

Immunizations were given to the animals via a number of injection routes, using three different concentrations of antigen (Tables 5.1 and 5.2). Antibody production was monitored by double immunodiffusion, using non-freeze-dried foetal extracts as the precipitating antigen.

(b) Results

The raising of antibodies to non-freeze-dried foetal extracts is summarized in Table 5.1. All attempts, using a variety of inoculation protocols, and antigen concentrations, were unsuccessful. In contrast to these results, antibody production was induced in the second group of animals which had been given freeze-dried extract (Table 5.2). In this case, the response varied but was present in all animals examined.

The difference in the responses of the two groups of animals is difficult to explain since both groups apparently received the same antigen, and in four cases, in comparable concentrations. It is unusual that freeze-drying of the antigens should alter the immunogenicity of the extract, since lyophilization is a standard biophysical technique which rarely alters the chemical integrity of

TABLE 5.1

Immune responses of rabbits inoculated with murine foetal extracts

RABBIT NO.	ANTIGEN CONC.[1]	SITE[2]	RESPONSE[3]
1	8	s.c./i.m.	-
2	3	i.v./s.c./i.m.	-
3	3	i.v./s.c./i.m.	-
4	10	f.p.	-
5	10	f.p.	-
6	3	i.m.	-
7	3	i.m.	-
8	8	s.c./i.m.	-
9	3	s.c./i.m.	-
10	3	s.c./i.m.	-

Rabbits were immunized with various concentrations of non-freeze-dried murine foetal extracts and their ability to raise antibodies to the extract examined. Protocols are given in Appendix IV.

[1]mg protein/ml per injection

[2]the route of administration of the antigen

s.c. = subcutaneous

i.v. = intravenous

i.m. = intramuscular

f.p. = footpad

Where multiple sites were used, the extract was divided into equal portions.

[3]The humoral immune response was measured by testing the rabbit serum for antibodies in immunodiffusion plates. The antigen used was non-freeze-dried mouse foetal extract, at a concentration of 10 mg protein per ml.

any molecule.

It was decided to examine this unusual situation further, and in particular to see if these results could be repeated using other animal species.

2. Murine Immunity

(a) Aim and Methods

To determine whether non-freeze-dried foetal extracts are able to raise antibodies in other heterologous species, mice were immunized with NFD and FD rat (Wistar) foetal extracts and antibody production examined by immunodiffusion. This system was chosen for three reasons:

(i) pregnant rats are available at regular intervals from the University Animal House;

(ii) the rat foetus is relatively large, and a considerable quantity of tissue can be obtained from a single pregnancy;

(iii) previously unpublished results from this laboratory showed that NFD rat foetal extracts were unable to induce antibody production in rabbits. (Immunization with freeze-dried rat foetal extracts had not, however, been attempted, and hence a comparison of these results with the murine foetal results was not considered valid.)

The rat foetal extracts were prepared as described in Chapter II and

TABLE 5.2

RABBIT NO.	ANTIGEN CONC.[1]	SITE[2]	RESPONSE[3]
1	8	i.v./s.c./i.m.	+
2	8	i.v./s.c./i.m.	++
3	3	i.v./s.c./i.m.	++
4	3	i.v./s.c./i.m.	++
5	3	i.m.	++++
6	3	i.m.	+++

[1]mg protein per ml (8 mg protein per ml per injection).
Concentration was calculated from a freeze-dried extract reconstituted to 15 mg solid matter per ml with distilled water.

2)
) as in Table 5.1
3)

inoculation of Swiss Albino mice performed according to the protocol described in Appendix V.

(b) Results

Two groups of animals were immunized with either freeze-dried or non-freeze-dried rat foetal extracts. The ascites fluid of each animal was examined for the presence of antibodies to rat foetal antigens by immunodiffusion, and the results and statistical analysis recorded in Table 5.3. The χ^2 analysis of this data showed a significant difference in the ability of the two groups to raise antibodies to foetal antigens, *viz.* the group given freeze-dried extract contained twice the number of animals which produce antibody to the foetal extract.

These results are similar to those obtained in the rabbit/murine foetal system. However, repression of antibody production in the rabbits occurs in all the animals given non-freeze-dried foetal extract, whereas repression is not complete in mice given rat foetus. The statistical analyses, however, do show that the difference between both groups of mice is significant.

There are two possible explanations for the incomplete repression of the murine immune system:

(a) The degree of repression may vary amongst different animal species.

(b) Incomplete repression may also occur in rabbits given NFD foetal extracts. However, because of the relatively

TABLE 5.3

	Freeze dried	Non-freeze-dried
No. animals positive	18	6
Total no. of animals per group	29	20
% no. raising anti-bodies	62.07	30

χ^2 Analysis : $p < 0.05$, therefore difference is significant.

Number and percentage of antibody positive animals immunized with either freeze-dried or non-freeze-dried rat foetal extract.

small number of rabbits, animals with positive antibody production may not have been isolated.

Whatever the explanation for this difference, it is clear that repression of antibody synthesis to rat foetal extracts in mice may only occur if the lyophilization procedure is omitted during the preparation of the extract.

3. Conclusions

From the results presented here, it is clear that NFD murine foetal extracts were unable to evoke a humoral immune response in rabbits. Similarly, a statistically significant number of mice were unable to raise antibodies to NFD rat foetal extracts. In both cases this negative antibody response was reversed by freeze-drying the foetal extract prior to inoculation.

Four possible explanations of this phenomenon are proposed here:

(i) Immunogenicity of the foetal extract

The extract used to raise the antibody was a 1500 g supernatant of whole foetus homogenate. Consequently, the extract contains many small cellular organelles which may not have been disrupted during homogenization. Following lyophilization, these organelles may have been broken, hence releasing new immunogenic material. It is proposed here that it is this material which causes the production of antibody in the host animals. Conversely, if lyophilization is omitted, the organelles remain intact and no immunogenic molecules are available to cause antibody production.

(ii) Immunocompetence of the animals

The raising of antibodies in the FD group of animals and not in those given NFD foetal extracts may have been due to the non-immuno-competence of the animals inoculated with NFD extracts. This is, however, very unlikely because production of rabbit and mouse antisera to other antigens studied in this laboratory has been successful (antigens include AFP, gamma globulins, placental extracts and membrane-bound antigens).

(iii) Antigen modifier

The third possible explanation of the observed phenomenon is that the foetus contains a modifier which renders the antigen non-immunogenic. Further, this modifier may be destroyed by freeze-drying, permitting the resumption of antibody synthesis.

(iv) Immunological repressor

The foetus produces, or contains, an immunological suppressor which is found in the 1500 g extract of the tissue homogenate. This repressor acts on the rabbit's humoral immune system, preventing the production of antibodies to the foetal antigens. The lyophilization of the extract destroys the repressor, and antibody synthesis now occurs.

In the following sections, these four concepts are examined further,to determine which may suitably explain this unusual phenomenon.

SECTION III : IMMUNOGENICITY OF MURINE FOETAL EXTRACT

In the previous section, it was proposed that freeze-drying may cause the disruption of cell organelles, hence releasing 'new' immunogenic material. Alternatively, it was also theorized that lyophilization may destroy a modifier, which somehow prevents antigens from being immunogenic.

These two possibilities were examined by characterizing the antigens, both quantitatively and electrophoretically. Murine foetal extracts were chosen for these experiments because these NFD preparations were unable to evoke antibody production in any immunized rabbits, whereas rat foetal extracts did produce some humoral response in inoculated mice.

1. Cell Organelle Antigens

To determine whether lyophilization significantly adds to the immunogen pool by disrupting cell organelles, fresh mouse foetuses were homogenized and centrifuged at 105,000 g for one hour to remove all undisrupted cell organelles. The resultant supernatant was dialysed and examined for the foetal antigens which are capable of evoking humoral immunity. The antibody used to detect these antigens was raised to FD foetal extracts. If the concept that immunogenic material is released only *after* freeze-drying is correct, then antigens should not be detected in the 105,000 g supernatant since their source (the cell organelles) has been removed. The

immunodiffusion patterns of Figure 5.1, however, show that the 105,000 g extract does contain antigens which are capable of evoking immunity. Further, these antigens exhibit a reaction of identity with both FD and NFD 1500 g extracts. It may be argued that although the antigens are present in the 105,000 g extract, their presence may be due to earlier disruption of the organelles, and lyophilization results in complete breakage, causing the quantity of immunogens to rise to a level high enough to evoke humoral immunity. This is, however, unlikely as the quantity of immunocompetent antigens in both the 105,000 g extract and the FD, 1500 g extract is comparable (Table 5.4).

Thus it would seem that the breakage of cell organelles would not significantly add any more antigens to the immunogen pool.

2. Antigen Modifier

The presence of an antigen modifier was examined by determining the number of antigens in the foetal extract, which could be detected by the anti-foetal serum.

If an antigen modifier is active in these extracts, then it is unlikely that one such molecule will alter the immunogenicity of a variety of antigens. From Figure 5.2, it can be seen that there are at least four, possibly five, antigens which by their reactivity against this antiserum are able to raise an antibody in rabbits. Further, these antigens have a diverse range of electrophoretic mobilities, some also being found in adult tissues as well.

IGURE 5.1

Immunodiffusion examination of 105,000 g extract of murine foetus for the presence of foetal antigen.

h = 105,000 g extract

d = 1500 g non-freeze-dried extract

f = 1500 g freeze-dried extract

a = adult tissue extracts (This extract was used as a positive control. Previous studies from this laboratory have shown that foetal extracts do contain some adult antigens.)

5_1

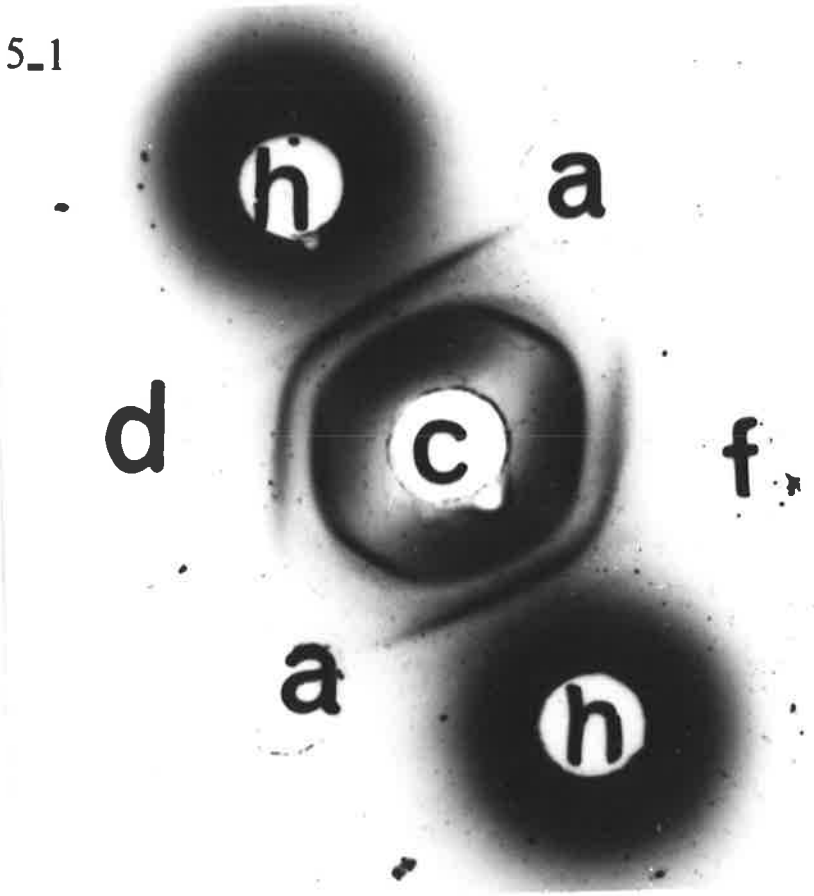


TABLE 5.4

Antibody Precipitation Response of:	ANTIGEN DILUTION* NEAT	2	4	8	16	32	64	128	256
105,000 g**	+++†	+++	+++	+++	++	++	++	+	-
1500 g (FD)	+++	+++	+++	+++	++	++	++	+	-

The ability of a constant concentration of anti-foetal serum to precipitate various concentrations of antigens from a 105,000 g and 1500 g extract of mouse foetal extract.

*The antigen dilution is recorded as a reciprocal dilution (e.g. 2 = ½).

**Each extract was initially adjusted to 10 mg of protein per ml.

† +++ → + indicates the degree of precipitation.

FIGURE 5.2.

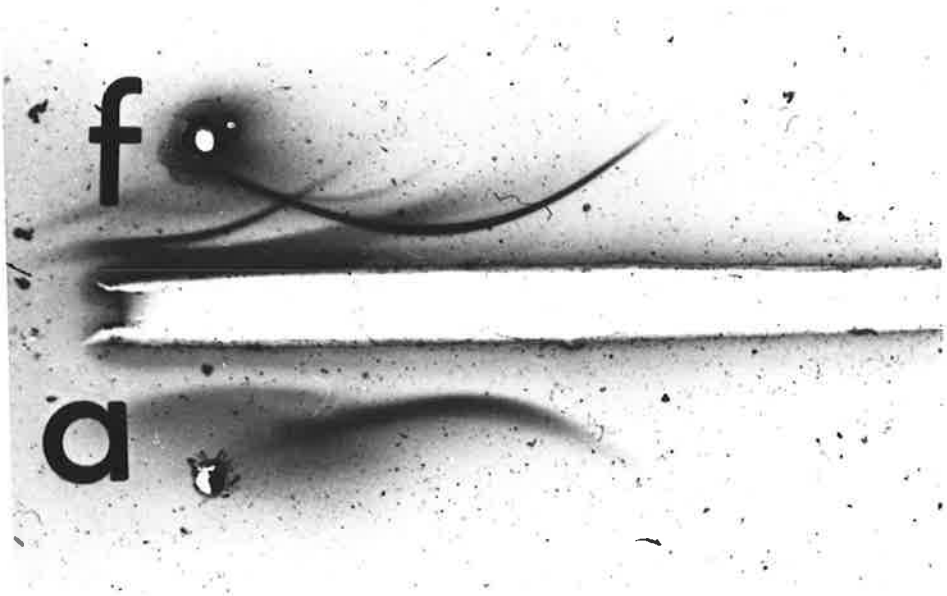
Immuno-electrophoresis of murine foetal, and adult extracts

The central trough contains anti-serum raised against F.D. foetal extracts.

f = N.F.D. foetal extract

a = adult extract

5.2



It is unlikely that a single modifier can alter the immunogenicity of such a diverse range of antigens. It is equally unlikely that the foetus needs to produce a large number of modifiers, and more importantly, each one being lyophilization-labile. The concept that freeze-drying makes the antigens more immunogenic would require a large number of antigens to be lyophilization-labile - an improbable situation as such antigens are rare.

3. Conclusions

From these results it was concluded that the inability of NFD foetal extracts to raise an antibody in rabbits was not due to the presence of an antigen modifier or to the lack of immunogenic material due to the undisrupted state of cell organelles. With the further improbability that the animals used in this study were non-immunocompetent, it was proposed that the foetal extract contained an immunological repressor, i.e. a substance which would inhibit the production of antibodies to the foetal extracts.

To accurately determine whether such a repressor was present, an isolation or partial purification of the mediating molecule(s) was required. Two such methods were considered:

(a) Column chromatography on Sephadex G-200 using an 0.85% saline solvent.

(b) Ammonium sulphate fractionation.

The first technique is probably the better of the two, because the inert Sephadex and saline would not be expected to alter the activity of the repressor. The second method, although considered "safe" by biochemists, may have a similar effect as freeze-drying - particularly since both of these techniques remove water. Thus the "denaturation" of the repressor(s) by the ammonium ions may permit the production of antibodies to non-freeze-dried foetal extracts - a negative result which would neither confirm nor reject the presence of a repressor.

Attempts at partially purifying murine foetal extracts by column chromatography were not successful. Consequently, the only remaining method for directly showing that the foetus produced an immune repressor was by using the foetal extract to impair antibody production to an antigen, not produced by the foetus.

Because such an experiment required a large number of animals, it was decided to inoculate mice with rat foetal extracts, rather than rabbits with murine foetus. The use of a different animal system was considered as valid because both rabbit and murine antibody production to NFD foetal extracts was repressed.

SECTION IV : IMMUNOLOGICAL REPRESSION

1. Introduction

The presence of a lyophilization-labile immunological repressor was examined by immunizing mice with bovine serum albumin (BSA), and non-freeze-dried foetal extracts. If an immune repressor was present in the foetal extracts, then there should have been a repression of antibody production to BSA.

2. Methodology

Three groups of mice were inoculated with either BSA and saline (group 1), BSA and NFD rat foetal extract (group 2), or BSA and FD foetal extract (group 3). Antibody production by the first group will confirm the use of BSA as a suitable antigen. The third group of animals serves as a control to determine whether injecting BSA and foetal extracts overloads the immune system, resulting in immune paralysis. If this should occur, there would be no antibody synthesis in the second group, causing confusion between inhibition by a repressor, and immune paralysis mediated repression.

The BSA was mixed with the foetal extract and given intra-peritoneally according to the dosage and inoculation routes described in Appendix V.

3. Results

The antibody production by animals in the three groups is presented in Table 5.5. All animals of the group given BSA and saline (group 1) were able to raise antibodies to this antigen. Thus the concentration of antigen given is sufficient to raise antibodies in mice. The animals given BSA and NFD foetal extract were also able to raise antibodies to the antigen. Conversely, the animals immunized with FD foetal extract did not develop antibodies to the BSA. Initially, it was thought that immune paralysis had occurred in the animals of the third group. However, this is unlikely because the group given BSA and NFD foetal extract had the same quantity of antigenic material injected as the group given BSA and FD foetal extract.

These results are a direct contradiction of the supposition that the NFD foetal extract contains an active immunological suppressor which should prevent the production of antibodies to BSA.

This apparent contradiction could be explained by proposing that the repressive process still occurred, but its inhibitive action was specific only for foetal antigens and did not significantly alter the antibody production to BSA. Under these conditions, it would be expected that group 2 animals would raise an antibody to BSA only and not foetal extracts. From Table 5.6 it can be seen that this situation did occur. Similarly, group 3 animals would be expected to raise antibodies to both BSA and foetal extracts, as repression has now been eliminated by freeze-drying. This, however, did not occur.

TABLE 5.5

	1 BSA + Saline (CONTROL)	2 BSA + NFD	3 BSA + FD
No. positive	12	9	0
Total No. animals	16	12	12
% positive	75	75	0

χ^2 Analysis : $p < 0.05$, therefore difference between groups 2 & 3 is significant.

Percentage number of animals able to raise antibodies to BSA, when given concurrent immunizations of saline, non-freeze-dried or freeze-dried foetal extracts.

TABLE 5.6

	Group 2 BSA + NFD Foetal	Group 3 BSA + FD Foetal
ANTI-BSA ACTIVITY		
No. positive	9	0
Total No. animals	12	12
% positive	75	0
ANTI-FOETAL ACTIVITY		
No. positive	2	11
Total No. animals	12	12
% Positive	17	92

STATISTICS:

(1) Anti-BSA activity : (see Table 5.5).

(2) Anti-foetal activity : χ^2 analysis - $p < 0.05$, therefore the two groups are significantly different.

Percentage of animals inoculated with BSA and foetal extracts, able to develop humoral immunity to these antigens.

The animals of group 3 only raised antibody to the foetal antigens and not BSA - an unusual result as both antigens should have raised an antibody in mice.

Although these results confirm that repression is still occurring in animals given NFD foetal extracts, it is unusual that antibodies to BSA did not develop in animals immunized with BSA and FD foetal extracts (group 3). A possible explanation is that an antigen in the FD foetal extract competed with the BSA for the production of antibodies. Antigenic competition between BSA and other antigens has been extensively reported in the literature (see Liacopoulos and Ben-Efraim, 1975). These authors have concluded that competition may occur between two antigens given simultaneously in the same Freund's adjuvant emulsion. In the experiments described in this chapter, the BSA and foetal extracts were mixed together and emulsified with Freund's adjuvant.

Although this hypothesis is tenable, it would be expected that the NFD foetal antigens would also compete with the BSA, hence eliminating the latter immune response. However, this did not occur in these experiments (Table 5.6). To explain why competition did not occur in this case, it is necessary to consider the mechanism of antigen competition. In the mouse experiments described here, it has been proposed that a specific repressor prevents the production of antibody to foetal antigens. In the next section of this chapter, it is further proposed that this repression occurs at the macrophage site. Gottlieb *et al.* (1972) have reported that antigen competition probably occurs at the macrophage site, during antigen handling and/or

processing. Consequently, when BSA is given together with the foetal antigens, the immune reaction to the latter antigens is suppressed, hence eliminating the competition with BSA. Thus BSA antibody synthesis now occurs. When FD foetal extract is given with the BSA, however, repression no longer exists, competition between both antigen types is initiated, and an immune response to the foetal antigens only occurs.

The concept of a competition between BSA and a foetal antigen is further supported by evidence from Ruoslahti *et al.* (1976) who have shown that human albumin, and the foetal antigen AFP, show considerable amino acid sequence homology. (AFP is present in large quantities in rat foetal homogenates.) Further, Harel *et al.* (1972) have shown that another globulin, bovine gamma globulin, is able to compete with BSA, resulting in elimination of the latter's antibody production.

Thus, from these reports, it is probable that antibody production against BSA when injected simultaneously with FD foetal extracts, is suppressed because of antigen competition. The results presented here suggest that repression of antibody synthesis is specific for antigens found in the foetal extract.

SECTION V : DISCUSSION

The inability of rabbits and mice to raise antibodies to heterologous foetal extracts has been demonstrated. After eliminating all other likely explanations of this phenomenon, it was proposed that the foetal extracts contained a substance which was able to

specifically repress the production of antibodies to the foetal antigens. Further, this repressor was lyophilization-labile.

Three important questions have arisen from this work:

(i) What is the nature of the repressor?

(ii) What type of immune inhibition does this molecule(s) exhibit?

(iii) How does freeze-drying destroy the repressor(s)?

It is difficult to answer these questions without further experimentation. However, there is a considerable amount of information in the literature from which a model of the nature and mechanism of the repression may be formulated, and ultimately examined experimentally.

(i) Nature of the repressor

To answer the first question, it is desirable to list two important criteria which the repressor(s) must fulfil:

1. Since the repression is specific for antigens in the foetal extracts, then the antigens themselves must somehow be mediators of the repression. It is unlikely that the antigens are immunosuppressive.* Rather, another molecule(s) directly interacts with the antigens and this blocks the humoral immune system from raising antibodies to these antigens.

2. Since there are a large number of antigens, each with a different electrophoretic mobility (see Chapter V, Section II), there must also be an equal number of repressors, each

*There have been a number of studies reported, which suggest that α_1-foetoprotein may be an immuno-regulator (Tomasi *et al.*, 1976; Sheppard *et al.*, 1976; Goeken and Thompson, 1976). However, these studies have been inconclusive, as yet.

being lyophilization-labile, and specific for one antigen. Consequently, the repressors should be within one class of chemical substance, i.e. sharing many characteristics, but still able to confer specificity on a particular antigen - in this case, foetal antigens.

Antibodies are molecules which conform to both of these criteria. Although a different antigen specificity exists, the chemical composition of the antibodies is essentially the same. It is difficult to envisage any other class of molecules which may conform to these established criteria. Assuming that antibody molecules may be the repressors, it is then possible to consider the mechanism of their action and their lability to freeze-drying.

(ii) Mechanism of action

It is unlikely that repression is caused by antibody masking every antigenic determinant on all molecules in the extracts. A more plausible explanation is that only some of the antigens are bound to antibodies, and these complexes specifically block the production of "new" antibodies. A similar situation has been reported by Mishell and Dutton (1967), who showed that when anti-sheep red blood cell was mixed with an immunogenic dose of sheep red blood cells, this conjugate was able to repress the appearance of antibody-forming cells to this antigen. Nossal and Ada (1971), reviewing this area of immunology, have concluded that the repression occurs early in the immune process, possibly at antigen-macrophage interaction.

The complexing antibody must be of either foetal or maternal origin. Although some specific globulin synthesis by the foetus has been reported (Solomon, 1971), it is improbable that this antibody forms the antigen-antibody complexes. Consequently, this antibody may be of maternal origin. A number of workers have reported the presence of maternal antibody against antigens of the developing foetus. Further, they have shown that antibody synthesis in neonatal animals may be repressed by the presence of large quantities of specific maternal antibody (Greengard and Bernstein, 1935; Cooke *et al.*, 1948; Barr *et al.*, 1953). The proposed mechanism of action of the repressor found in the foetal extracts described in this chapter is consistent with the results reported by other workers, i.e. the maternal antibody in the foetus forms antigen-antibody complexes, which may prevent the production of antibody to these antigens by heterologous hosts.

(iii) Lyophilization-lability of repression

The experiments described here have shown that the repression phenomenon is labile to freeze-drying. To explain this lability, in terms of an active antigen-antibody repressor, freeze-drying must either destroy the antibody portion of the immune complex or its bond with the antigen be broken. It is unlikely that lyophilization destroys the antibody itself, because freeze-drying is routinely used to preserve antisera from degradation. Thus the labile point must be the bond between the antigen and antibody.

There has not been any report in the literature which describes a dissociation of antibody-antigen complexes by freeze-drying. There have, however, been numerous reports of high salt concentrations causing the dissociation of these complexes into free antibody and antigen (Heidelberger *et al.*, 1936; Heidelberger and Kendall, 1936). It is possible that the foetal extracts used in these studies had a hypertonic salt content after freeze-drying and subsequent re-constitution. This would result in a breaking of the antigen-antibody bond, and the subsequent elimination of the repressive phenomenon. The validity of this observation is clearer if the equilibrium reaction for antigen-antibody complex formation is considered:

viz. Ag (antigen) + Ab (antibody) $\underset{\text{hypertonic salt}}{\overset{\text{isotonic salt}}{\rightleftharpoons}}$ Ag-Ab (complex)

During the preparation of murine foetal extracts, for rabbit inoculation, the freeze-dried inoculum was re-constituted to 15 mg of solid materials per ml of distilled water. This results in the salt concentration of the inoculum being elevated, hence shifting the equilibrium reaction to the left. Raffel (1961) has reported that only a 15% increase in salt content is sufficient to cause this shift. Consequently, it may be possible that a small elevation in salt concentration in the murine foetal extracts was able to destroy the repressive antibody-antigen complexes.

Foetal rat extracts, for inoculation into mice, were re-constituted to their pre-lyophilization volume. Thus they would not be expected to contain a significant quantity of dissociated immune complexes, because the concentration of the salt has not been altered. However, during the lyophilization procedure, there is a concentrating of the salt that is present, and hence dissociation may occur here. However, on reconstitution, reassociation would be expected over a period of time because the extract solution has been restored to isotonic strength (see equilibrium reaction). This situation is probably reflected experimentally in Table 5.3, where 37.5% of animals given FD foetal extracts unexpectedly did not raise antibodies. This may have been due to re-aggregation of antigen and antibody into immune complexes.

Thus it is proposed here that the immune repression demonstrated in these studies is not specifically reversed by lyophilization. Rather it is the elevation of salt concentrations brought about by lyophilization/reconstitution procedure that reverses the repression.

(iv) Summary

In this chapter it has been demonstrated that antibody synthesis against foetal extracts occurs when lyophilization is included in the preparative procedure. This phenomenon is thought to be due to the action of an antigen-specific humoral immune system repressor. A theoretical model, formulated to explain the mode of repression, has been presented. However, it is important to note that,

while this model is speculative, the literature covering the field strongly supports the concepts of the model. The experimental testing of the proposed model is discussed further in the next chapter.

CHAPTER VI

CONCLUDING REMARKS

SECTION I : DEVELOPMENTAL ANTIGEN REAPPEARANCE IN CANCER

In this thesis, the methodology for the preparation of antibody to both foetal and placental antigens has been the same, i.e. an antiserum was raised to each extract, and then an attempt was made to render it specific for either foetal or placental antigens by absorption with adult tissue powders. This technique was designed to detect a range of foetal and placental antigens, without specifying the tissue of origin of the antigen. However, one major problem prevented the effective use of these systems to detect developmental antigens in tumours - both foetal and placental extracts contained a high concentration of adult antigens. Thus the antisera to these extracts had a high proportion of antibody which was directed against the normal adult antigens, e.g. the antiserum to intact placental cells was exclusively reactive against adult red blood cells, and not placental antigens.

For this type of system to be used successfully, the immune response to the adult antigens should be eliminated or significantly reduced. This may be done in one of two ways:

(a) Immunization of homologous hosts instead of heterologous animals.

(b) Prior purification of the extracts, resulting in a relatively higher concentration of developmental antigens being available for stimulation of the immune system.

Both methods have been briefly discussed in the earlier chapters. The second concept of purification, however, requires some further discussion as immunization of homologous hosts may not always result in antibody production (see Chapter IV).

The purification of developmental tissue extracts may be achieved by either:

(a) fractionating the extract into smaller pools, or

(b) selecting a specific foetal organ or placental tissue.

The first method of purification may involve either Sephadex G-200 or ammonium sulphate fractionation. These two techniques may ensure that each fraction contains a higher proportion of certain foetal antigens than the unfractionated extract. Thus the immunizing host would receive a larger quantity of foetal antigens. This method may also be applied to placental antigens.

The second method involves the selection of one particular foetal organ and raising an antibody to either a soluble or membrane extract of this tissue. Again, this may eliminate excess antigens, and in particular, a large quantity of adult antigens. This type of approach has been used successfully in this laboratory for the preparation of anti-AFP,

viz, rather than used whole foetus, amniotic fluid was used as the vaccine for the preparation of the antiserum (Okita *et al.*, 1974).

A similar approach may also be adopted to raise antibody to both membrane and cytoplasmic extracts of the placenta. In this method, it is desirable to firstly perfuse the placenta with isotonic saline

in order to remove as much blood as possible. Following this, a particular area of the placenta is selected and an extract (either membranous or cytoplasmic) prepared for immunization. Similarly, other areas of tissue may be chosen. Thus there is an elimination of excess antigen, and an effective increase in the concentration of placental specific antigen entering the immunizing host.

In conclusion, although the methods proposed above may still be effective as a means of preparing specific antibody for detecting developmental antigens in tumours, it is not possible to produce a single antiserum with multiple foetal and placental antigen specificity.

SECTION II : IMMUNOLOGICAL REPRESSION

The aim of this study has been to determine whether the foetus produces a lyophilization-labile immune repressor or antigen modifier. It has been proposed in this thesis that the rat and mouse foetal extracts contain 'modified' antigens which are able to specifically inhibit the production of antibodies to these antigens in xenogenic hosts. Further, this modification process is an attachment of antibodies to the foetal antigens, and it is this antigen-antibody complex which represses antibody production to the foetal antigens.

However, it is important to note that this concept is largely speculative, and although supported by a number of experiments

reported in the literature, no direct experimental evidence has been presented in this thesis. Further, a direct contradiction of this theory is evident from the results of Nishi (1970), who has been able to raise an antibody to AFP by immunizing horses with AFP-anti-AFP complexes. This evidence emphasizes the need to establish the validity of the theory presented in Chapter V and to use this to possibly determine Nishi's discrepancy.

There are three important aspects of this theory which may be examined experimentally:

(a) Are there significant numbers of antigen-antibody complexes in the NFD foetal extracts?

(b) Can these complexes, if they exist, be dissociated during freeze-drying and further reconstitution with distilled water?

(c) Will the addition of antigen-antibody complexes to a FD foetal extract result in the inhibition of antibody production to the foetal antigens?

These experiments may assist in confirming or rejecting the proposed theory.

The relevance of the proposed model to tumour immunity may be considered in view of the many similarities between foetal and cancerous cells (Chapter I). Currie (1969) has shown that maternal T-lymphocyte attack against the developing foetus may be repressed by blocking factors which consist of antigen-antibody factors. Such

blocking factors have also been implicated as a defense mechanism which tumours possess to prevent destruction by the host's immune system (Sjogren et al 1971 Chalmers *et al.*, 1976).

Evidence in favour of B-lymphocyte (i.e. antibody production) suppression by antibody-foetal antigen complexes has been limited to a few studies from our laboratory. Six attempts have been made to raise antibody to NFD 1500 g extracts of hepatoma tissue. Each attempt was found to be unsuccessful. By repeating the experiments in the previous chapter with tumour rather than foetal extracts, the possible role of antibody-foetal antigen complexes in the regulation of B-lymphocyte immunity to the developing tumour may be determined.

The model of B-lymphocyte suppression presented in this thesis, requires further examination and verification. However, its importance in tumour, foetal and transplantation immunology, is such that the basic concepts of this model may provide an impetus for further investigation.

ADDENDA

CHAPTER IV

Sabine (personal communication), using the same methods described in this Chapter, has also been unable to produce a specific antiserum capable of detecting carcino-placental antigens in syngeneic rat tumours.

CHAPTER V

An important observation has been made by a number of colleagues, during discussions of the results presented in this Chapter. They have noted that if the repressor is specific for foetal antigens only, then the rabbits and mice should have raised antibodies to the adult component of the foetal extract. However, it is important to note that the adult component consists of both maternal and paternal antigens. Consequently, the foetal extract would contain paternal antigen-antibody complexes, because these antigens would be recognized as foreign by the maternal immune system. Hence antibody synthesis to these antigens in heterologous hosts would be inhibited.

The maternal antigens should stimulate antibody production in heterologous hosts, as there should be no antigen-antibdoy complexes present. However, the level of these antigens in the foetal extracts is probably too low to produce enough antibody which could be detected by the insensitive immunodiffusion system employed in these studies.

APPENDICES

APPENDIX I

IMMUNIZATION SCHEDULE FOR THE PRODUCTION OF RABBIT ANTI-SERUM TO MOUSE FOETAL EXTRACTS.

Day	Injection No.	Inoculum Dose			Site***
		Antigen (ml)*	Adjuvant Type**	: (ml)	
1	1	1.0	FCA	1.0	i.m.
8	2	1.0	FCA	1.0	i.m.
15	3	1.0	FCA	1.0	i.m.
29	4	1.0	FIA	1.0	i.m.

*Antigen concentration was 15 mg solid matter (after lyophilization).

**FCA : Freund's Complete Adjuvant

FIA : Freund's Incomplete Adjuvant

***s.c. : subcutaneous

i.m. : intramuscular

Animals were bled 10 days after the final inoculation.

APPENDIX II

IMMUNIZATION SCHEDULE FOR THE PRODUCTION OF RABBIT ANTISERUM TO SALINE SOLUBLE EXTRACTS OF RAT PLACENTA.

Day	Injection No.	Inoculum Dose			Site
		Antigen* (ml)	Adjuvant Type :	(ml)	
1	1	1.0	FCA	1.0	s.c.
8	2	1.0	FCA	1.0	s.c.
15	3	1.0	FCA	1.0	s.c.
29	4	1.0	FIA	1.0	i.m.

*antigen concentration was 8 mg protein per ml.

Animals were bled 10 days after the final inoculation.

APPENDIX III

IMMUNIZATION SCHEDULE FOR THE PRODUCTION OF ANTISERUM TO RAT PLACENTAL ANTIGENS.

1. HETEROLOGOUS ANTISERUM TO WHOLE PLACENTAL CELLS.

Day	Injection No.	Inoculum Dose* (ml)	Site**
1	1	2	n.p.,i.m.
6	2	2	i.v.
11	3	2	i.v.

*concentration of cells was 3.6×10^8 per ml.

**n.p. : nape of neck

i.m. : intramuscular

i.v. : intravenous

Animals were bled at 7 day intervals after the final inoculation.

2. HETEROLOGOUS ANTISERUM TO CRUDE PLACENTAL CELL MEMBRANE EXTRACT

Day	Injection No.	Inoculum Dose			Site
		Antigen (ml)*	Adjuvant Type	: (ml)	
1	1	1.0	FCA	1.0	i.m.
15	2	1.0	FCA	1.0	i.m.
29	3	1.0	FCA	1.0	i.m.
43	4	1.0	FIA	1.0	i.m.

*concentration of antigen was 10 mg protein per ml.

Animals were bled 14 days after the final inoculation.

3. HOMOLOGOUS ANTISERUM TO CRUDE PLACENTAL MEMBRANE EXTRACTS

Day	Injection No.	Inoculum Dose			Site**
		Antigen (ml)*	Adjuvant Type	: (ml)	
1	1	0.5	FCA	0.5	i.p.
15	2	0.5	FCA	0.5	i.p.
22	3	0.5	FCA	0.5	i.p.
43	4	0.5	FCA	0.5	i.p.
57	5	0.5	FIA	0.5	i.p.

*antigen concentration was 10 mg protein per ml.

**i.p. : intraperitoneal.

Animals were bled 14 days after the final inoculation.

APPENDIX IV

IMMUNIZATION SCHEDULES FOR THE PRODUCTION OF ANTIBODY TO NFD MURINE FOETAL EXTRACTS.

1. FOOTPAD PROTOCOL

Day	Injection No.	Inoculum Dose			Site
		Antigen (ml)*	Adjuvant Type :	(ml)	
1	1	0.5	FCA	0.5	f.p.
11	2	0.5	FIA	0.5	f.p.

*concentration of the antigen was 10 mg protein per ml.

Animals were bled 10 days after the final inoculation.

2. SUBCUTANEOUS-INTRAMUSCULAR PROTOCOL (i.e. s.c./i.m.)

Day	Injection No.	Inoculum Dose			Site
		Antigen (ml)*	Adjuvant Type :	(ml)	
1	1	1.0	FCA	1.0	s.c.
8	2	1.0	FCA	1.0	s.c.
15	3	1.0	FCA	1.0	s.c.
29	4	1.0	FIA	1.0	i.m.

*concentration of the antigen was either 8 or 3 mg protein per ml (see Table 5.1).

Animals were bled 10 days after the final inoculation.

3. INTRAMUSCULAR PROTOCOL

Day	Injection No.	Inoculum Dose			Site
		Antigen (ml)*	Adjuvant Type	: (ml)	
1	1	1.0	FCA	1.0	i.m.
15	2	1.0	FCA	1.0	i.m.
29	3	1.0	FIA	1.0	i.m.

*concentration of the antigen was 8 mg protein per ml.

Animals were bled 10 days after the final inoculation.

4. INTRAVENOUS-SUBCUTANEOUS-
INTRAMUSCULAR PROTOCOL

Day	Injection No.	Inoculum Dose			Site
		Antigen (ml)*	Adjuvant Type	: (ml)	
1	1	0.5	-		i.v.
1	2	0.5	FCA	0.5	s.c.
8	3	1.0	FCA	1.0	s.c.
15	4	1.0	FCA	1.0	s.c.
29	5	1.0	FIA	1.0	i.m.

*concentration of the antigen was 3 mg protein per ml.

Animals were bled 10 days after the final inoculation.

The same immunization schedules were employed when raising antibodies to FD extracts which use these protocols (Table 5.2).

APPENDIX V

IMMUNIZATION SCHEDULE FOR THE PRODUCTION OF MURINE ANTISERUM TO RAT FOETAL EXTRACTS, AND BOVINE SERUM ALBUMIN.

Day	Injection No.	Antigen Type	Antigen Dose (ml)	Adjuvant Type	Adjuvant Dose (ml)	Site
1	1	Saline	0.2	FCA	0.2	i.p.
15	2	Foetal*	0.2	FIA	0.2	i.p.
21	3	Foetal	0.2	FIA	0.2	i.p.
25	4	Foetal	0.2	FIA	0.2	i.p.
30	5	Foetal	0.2	FCAM**	0.2	i.p.
37	6	Foetal	0.2	FCAM	0.2	i.p.

*concentration of foetal inoculum was 2.7 mg protein per ml. Where BSA was given also, it was added to the foetal extract (or saline) prior to inoculation, to give a final concentration of 15 mg per ml.

**FCAM : Freund's Complete Adjuvant with 5 mg per cent live *Mycobacterium phlei* added (Sommerville, 1967).

Ascites fluid from these animals was tapped 8 days after the final inoculation.

REFERENCES

ABELEV, G.I. (1965). Antigenic structure of chemically induced hepatomas. Prog. Exp. Tumour Res. *7*: 104-157.

ABELEV, G.I. (1968). Production of embryonal serum α-globulin by hepatomas: Review of experimental and clinical data. Cancer Res. *28*: 1344-1350.

ABELEV, G.I. (1971). Alpha-fetoprotein in ontogenesis and its association with malignant tumours. Adv. Cancer Res. *14*: 295-358.

ABELEV, I., S.D. PEROVA, N.L. KHARMKOUA, Z.A. POSTNIKOVA and I.S. IRLIN (1963). Production of embryonal globulin by transplantable mouse hepatomas. Transplant *1*: 174-180.

ALEXANDER, P. (1972). Foetal antigens in cancer. Nature *235*: 137-140.

ANDERSON, N.G. and J.H. COGGIN (1971). Models of differentiation, retrogression and cancer. Proc. 1st Conf. Embryonal and Fetal Antigens in Cancer, pp.7-37. AAEC: Oak Ridge.

ARTZ, K., P. DUBOIS, D. BENNETT, M. CONDAMINE, C. BABINET and F. JACOB (1973). Surface antigens common to mouse clearage embryos and primitive teratocarcinoma cells in culture. Proc. Nat. Acad. Sci. *70*: 2988-2992.

BAGSHAWE, K.D. (1974). Tumour-associated antigens. Br. Med. Bull. *30*: 68-73.

BALDWIN, R.W. and D. GLAVES (1972). Deletion of liver cell-surface membrane components from amino-azo dye induced rat hepatomas. Int. J. Cancer *9*: 76-85.

BALDWIN, R.W., D. GLAVES, J.R. HARRIS and M.R. PRICE (1971). Tumour-specific antigens associated with amino-azo dye induced rat hepatomas. Transpl. Proc. *3*: 1189-1190.

BALDWIN, R.W., D. GLAVES and B.M. VOSE (1972). Embryonic antigen expression in chemically induced rat hepatomas and sarcomas. Int. J. Cancer *10*: 233-243.

BANWO, O., VERSEY, J. and J.R. HOBBS (1974). A new oncofoetal antigen for human pancreas. Lancet i.: 643-645.

BARR, M., A.T. GENNY and J.W. HOWIE (1953). Active immunization of ewes and their lambs. J. Pathol. Bacteriol. *65*: 155.

BECKER, F.F. (1974). Differential lectin agglutination of fetal, diving, post natal and malignant hepatocytes. Proc. Nat. Acad. Sci. *71*: 4037-4041.

BERRY, M. (1973). High yield preparation of morphologically intact isolated parenchymal cells from rat liver. Methods in Enzymology *32*: 625-632.

BONMASSAR, E., E. MENCONI, A. GOLDIN and G. CUDKOWICZ (1974). Escape of small numbers of allogeneic lymphoma cells from immune surveillance. J. Natl. Cancer Inst. *53*: 475-479.

BORSOS, T., A.K. RICHARDSON, S.H. OHANIAN and E.J. LEONARD (1973). Immunochemical detection of tumour-specific and embryonic antigens of di-ethyl nitrosamine induced guinea pig tumours. J. Natl. Cancer Inst. *51*: 1955-1960.

BRAUNSTEIN, G.D., J.L. VAITUKAITIS, P.P. CARBONE and G.T. ROSS (1973). Ectopic production of human chorionic gonadotrophin by neoplasms. Ann. Intern. Med. *78*: 39-45.

BURGER, M.M. (1973). Surface changes in transformed cells detected by lectins. Fed. Proc. *32*: 91-101.

CAUCHI, M.N., J.B.W. HALLEY, M.G. IRVING and J.F. WILLIAMS (1974). Hepatocellular localization of fetal antigens during induction of rat liver tumours by 31-methyl-4-dimethylamino-azo-benzene. Cancer Res. *8*: 1808-1812.

CHALMERS, P.J., N. MATTHEWS and R.C. NAIRN (1976). Characterization of serum factors modulating splenic cytotoxicity in a syngeneic rat tumour system. Br. J. Cancer *34*: 645-650.

CHANG, C.H., T. NISHIYAMA, D.A. ANGELLIS and C. CONNERY (1976). Iso-immunogenic placental proteins. Proc. Conf. Oncodevelopmental Gene Expression, p.26.

CHEI-WEI, L. and S.D. KIRLEY (1976). Properties of onco-placental histaminase. Proc. Conf. Oncodevelopmental Gene Expression, p.56.

COGGIN, J.H., K.R. AMBROSE and N.G. ANDERSON (1970). Fetal antigen capable of inducing transplantation immunity against SV-40 hamster tumour cells. J. Immun. *105*: 524-526.

COLIGAN, J.E., P.A. HENKART, C.W. TODD and W.D. TERRY (1973). Heterogeneity of the carcinoembryonic antigen. Immunochem. *10*: 591-599.

COLLINS, J.J. and P.H. BLACK (1973). Analysis of surface antigens on Simian virus 40-transformed cells: I. Unique antigenicity of Simian virus 40-transformed outbred hamster kidney cell lines. J. Natl. Cancer Inst. *51*: 95-114.

COOKE, J.V., J. HOLLOWACH, J.E. ATKINS and J.R. POWERS (1948). Antibody formation in early infancy against diphtheria and tetanus toxoid. J. Pediat. *33*: 141-146.

CURRIE, G.A. (1969). The foetus as an allograft: the role of maternal "unresponsiveness to paternally derived foetal antigens". In: Foetal Autonomy. (Eds.) G.E. Wolstenholme and M. O'Connor. Churchill : London. pp.32-52.

DOELLGAST, G.J. (1976). Homogeneous human placental alkaline phosphatase shares antigenic determinants with human intestinal alkaline phosphatase. Proc. Conf. Oncodevelopmental Gene Expression, p.41.

ELKHALIFA, M.D. and J.A. LIPPINCOTT (1968). Quantitative measurement of interactions between crown gall tumours and the pinto bean host. Am. J. Bot. *55*: 382-391.

ELKIND, M.M. and G.F. WHITMORE (1967). The radiology of cultured mammalian cells. Gordan and Breach : N.Y.

FAILLY-CREPIN, C. and J. URIEL (1973). A fetal carboxylic esterase of mouse origin common to normal embryonic brain and murine experimental teratocarcinoma. FEBS Letters *33*: 351-354.

FISHMAN, W.H., N.R. INGLISS, S. GREEN, C.LANTISS and N.K. GOSH (1968). Immunology and biochemistry of Regan enzyme of alkaline phosphatase in human cancer. Nature *219*: 697-699.

FOX, T.O., J.R. SHEPPARD and M.M. BURGER (1971). Cyclic membrane changes in animal cells. Transformed cells permanently display a surface architecture detected in normal cells only during mitosis. Proc. Nat. Acad. Sci. *68*: 244-247.

FRITZE, D., D.H. KERN, C.R. DROCEMULLER and Y.H. PILCH (1976). Production of antisera with specificity for malignant melanoma and human fetal skin. Cancer Res. *36*: 458-466.

GARRIDO, J., M. BURGLEN, D. SAMOLYK, R. WICKER and W. BERNHARD (1974). Ultrastructural comparison between the distribution of concanavalin A and wheat germ agglutin cell surface receptors of normal and transformed hamster and rat cell lines. Cancer Res. *34*: 230-243.

GOEKEN, N.E. and J.S. THOMPSON (1976). Immuno-suppressive properties of human alpha-fetoprotein and human cord plasma labumin. Proc. Conf. Oncodevelopmental Gene Expression, p.26.

GOLD, P. and S.O. FREEDMAN (1965). Demonstration of tumour-specific antigens in human colonic carcinomata by immunological tolerance and absorption techniques. J. Exp. Med. *121*: 439-462.

GONANO, F., V.P. CHIARUGI, G. PIRRO and M. MARINI (1971). Transfer ribonucleic acids in rat liver and Morris 5123 minimal deviation hepatoma. Biochemistry *10*: 900-908.

GONANO, F., G. PIRRO and S. SILVETTI (1973). Foetal liver $tRNA^{phe}$ in rat hepatoma. Nature New Biol. *242*: 236-237.

GOTTLIEB, A.A., R.H. SCHWARTZ and S.R. WALDMAN (1972). Competition between L- and D-synthetic copolymers at the level of the macrophage. J. Immun. *108*: 719-725.

GRANT, J.P., S. LADISCH and S.A. WELLS (1974). Immunologic similarities between fetal cell antigens and tumour cell antigens in guinea pigs. Cancer *33*: 376-383.

GREENGARD, J. and H. BERNSTEIN (1935). Passive immunity in infants and their response to diphtheria toxoid. J. Am. Med. Assoc. *105*: 341-342.

HALVER, J.E. (1965). Aflatoxins and rainbow trout hepatoma. In: "Mycotoxins and foodstuffs", Ed. by G.N. Wogan. Cambridge : MIT Press.

HÄKKINEN, I. (1972). Immunochemical relationship of the carcino-embryonic antigen and the fetal sulphoglycoprotein antigen. Immunochem. *9*: 1115-1119.

HAREL, S., P. LIACOPOULOS and S. BEN-EFRAIM (1972). Inhibition of immunological components by antigenic competition, and other immuno-depressing procedures. Immunol. *22*: 515-524.

HARSHBARGER, J.C. and R. TAYLOR (1968). Neoplasms in insects. Ann. Rev. Entom., *13*: 159-190.

HEIDELBERGER, M. and F.E. KENDALL (1936). Quantitative studies on antibody purification. I. The dissociation of precipitates formed by pneumococcus specific polysaccharides and homologous antibodies. J. Exp. Med. *64*: 161-172.

HEIDELBERGER, M., F.E. KENDALL and T. TEORELL (1936). Quantitative studies on the precipitin reaction. J. Exp. Med. *63*: 819-826.

HOLLINSHEAD, A., C. McWRIGHT, T. ALFORD, D. GLEW, P. GOLD and R.B. HERBERMAN (1972). Separation of skin reactive intestinal cancer antigen from the carcino-embryonic antigen of Gold. Science *177*: 887-889.

HOROWITZ, M.L. and W.E. KNOX (1968). A phosphate active glutaminase in rat liver different from that in kidney and other tissues. Enzym. Biol. Clin. *9*: 241-255.

HUEBNER, R.J. and G.J. TODARO (1969). Oncogenes of RNA tumour viruses as determinants of cancer. Proc. Nat. Acad. Sci. *64*: 1087-1094.

HUGHES, D.W.O'G., P.R. LAM-PO-TANG and B. WEBSTER (1973). Foetal haemoglobin levels in acute myeloid leukemia of childhood: Their value in predicting remission. Med. J. Aust. *2*: 1003-1004.

HULL, E.W., P. CARBONE, D. GITLIN, R.W. O'HARA and M.G. KELLEY (1969). α-Fetoprotein in monkeys with hepatoma. J. Natl. Cancer Inst. *42*: 1035-1044.

INGLETON, P.M. (1971). Acute effects of hepatocarcinogens on mouse liver microsomal antigens. Eur. J. Cancer *7*: 501-504.

ISHIMOTO, A., Y. SUZUKI, T. YOSHIDA and Y. ITO (1974). Further studies on mouse fetal antigen cross reacting with Rauscher leukemia. Cancer Res. *34*: 2338-2343.

KNOX, W.E. (1972). Enzyme patterns in foetal, adult and neoplastic tissues. pp.164-184. Karger : Basil.

KNOX, W.E., M.L. HOROWITZ and G.M. FRIEDELL (1969). The proportionality of glutaminase content to growth rate and morphology of rat neoplasms. Cancer Res. *29*: 669-680.

KODA, T., S. ISHIGAMI and S. TANABE (1973). Studies on liver cancer. II. Production of α-fetoprotein by experimental hepatoma, and its characterization. Biken. J. *16*: 129.

KROES, R.M., J.M. SONTAG, J.H. WEISBURGER, P. NEWBERNE and G.N. WOGAN (1972). α-Foetoprotein in rats with hepatomas induced by Aflatoxin B_1. Nature *240*: 240-241.

KUUSELA, P., E. RUOSLAHTI and A. VAHERI (1975). Polypeptides of a glycoprotein antigen (S.F.) present in serum and surface of normal but not of transformed chicken fibroblasts. Biochem. Biophys. Acta *379*: 295-303.

KYRIAZIS, A.P. and R.W. WISSLER (1972). Demonstration and purification of a specific antigen in Morris hepatoma 5123. Lab. Investigation *26*: 178-183.

LAURENCE, D.J. and A.M. NEVILLE (1972). Foetal antigens and their diagnosis and clinical management of human neoplasms. A review. Br. J. Cancer *26*: 335-355.

LAURENCE, D.J.R., V. STEVENS, R. BETTLELHEIM, D. DARCY, C. LESSE, C. TURBERVILLE, P. ALEXANDER, E.W. JOHNS and A.M. NEVILLE (1972). Role of plasma carcino-embryonic antigen in diagnosis of gastro-intestinal, mammary and bronchial carcinoma. Br. Med. J. *3*: 605-609.

LIACOPOULOS, P. and S. BEN-EFRAIM (1975). Antigenic competition. Prog. in Allergy *18*: 97-204.

LoGERFO, P., F. LoGERFO, F. HERLER, H.G. BARKER and H.J. HANSEN (1972). Tumour associated antigens in patients with carcinoma of the colon. Am. J. Surg. *123*: 127-131.

LOWRY, O.H., N.J. ROSEBROUGH, A.L. FARR and R.J. RANDALL (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193*: 265-275.

MARTIN, F., S. KNOBEL, M. MARTIN and M. BORDES (1975). A carcinofetal antigen located on the membrane of cells from rat intestinal carcinoma in culture. Cancer Res. *35*: 333-336.

MAUGH, T.H. (1974). Fetal antigens - A biochemical assay for cancer. Science *184*: 147-148.

MESHKINPOUR, H., H.T. WESPIC and W.R. SCHMALHORST (1974). Alpha-1-fetoprotein and alcoholic hepatitis. Am. J. Dig. Dis. *19*: 709-713.

MILLER, E.C. and J.A. MILLER (1974). The molecular biology of cancer. p.377. Academic Press, N.Y.

MISHELL and DUTTON (1967). Immunization of dissociated spleen cell cultures from normal mice. J. Exp. Med. *126*: 423-444.

MUKHERJI, B. and Y. HIRSHAUT (1973). Evidence for fetal antigen in sarcoma. Science *181*: 440-442.

MULLER, M., M. SCHREIBER, J. RARTENBECK and G. SCHREIBER (1972). Preparation of single-cell suspensions from normal liver, regenerating liver and Morris hepatomas 9121 and s123tc. Cancer Res. *32*: 2568-2576.

NAKATA, Y., T. SUESMATSU and Y. SAKAMOTO (1964). Transaminase activities in rapidly growing tissues. J. Biochem. Tokyo *55*: 199-201.

NISHI, S. (1970). Isolation and characterization of a human fetal α-globulin from the sera of fetuses and a hepatoma patient. Cancer Res. *30*: 2507-2513.

NISHI, S. and H. HIRAI (1970). Radio-immunoassay of α-fetoprotein in hepatoma, other diseases and pregnancy. Gann Mono. Cancer Res. *14*: 79-98.

NOSSAL, G.J.V. and G.L. ADA (1971). In antigens, lymphoid cells, and the immune response. Academic Press : N.Y.

NOWELL, P.C. (1976). The clonal evolution of tumour cell populations. Science *194*: 23-28.

OKITA, K., M. GRUENSTEIN, M. KLAIBER and E. FARBER (1974). Localization of α-fetoprotein by immunofluorescence in hyperplastic nodules during hepatocarcinogenesis induced by 2-acetylaminofluorene. Cancer Res. *34*: 2758-2763.

ONO, T. and Y. UMEHARA (1968). Heterogeneity of DNA polymerases in rat liver and hepatomas. Gann Mono. *6*: 97-107.

OUCHTERLONY, O. (1968). Handbook of immuno-diffusion. Ann Arbor. Humphrey Sciences : London.

OVE, P., J. LASZLO, M.D. JENKINS and H.P. MORRIS (1969). Increased DNA polymerase activity in a series of rat hepatomas. Cancer Res. *29*: 1557-1561.

PEROVA, S.D. (1969). Dissertation thesis. University of Moscow. (Cited from Perova *et al.*, 1971).

PEROVA, G.D., D.A. EL'GORT and G.I. ABELEV (1971). α-Foetoprotein in rat sera after partial hepatectomy. Byvll. Eksp. Biol. Med. *71*: 45-46.

PIDDINGTON, S.K., J.E. NEWELL and R.M. FOX (1976). Are foetal genes expressed in human lymphocytes. Clin. Exp. Pharm. Phys. *3*: 277.

PIHKO, H. and E. RUOSLAHTI (1973). High level of alpha-fetoprotein in sera of adult mice. Int. J. Cancer *12*: 354-360.

POTTER, V.R. (1969). Recent trends in cancer biochemistry: The importance of studies on fetal tissue. Can. Cancer Conf. *8*: 9.

POTTER, V.R. (1970). Environmentally induced metabolic oscillations as a challenge to tumour autonomy. In: Miami Winter Symposium *2*: 291. North-Holland Publ. Co. : Amsterdam.

PREHN, R.T. (1967). The significance of tumour-distinctive histocompatability antigens. In: "Cross-reacting antigens and neoantigens." Ed. by J.J. Trentin, Williams & Wilkins : Baltimore, p.105.

RAFFEL, S. (1961). Antigen-antibody reactions in immunity, p.145. Ed. S. Raffel. Appleton, Century, Crofts : N.Y.

RAFTELL, M., F. BLOMBERG and P. PERLMANN (1974). A comparison of enzyme-active membrane antigens from two different 4-dimethylamino-azo-benzene induced rat hepatomas with those of adult and foetal rat liver. Cancer Res. *34*: 2300-2306.

REJTHAR, A. (1974). Detection of tumour-associated transplantation antigens of BO-IV carcinoma on somatic cells of tumour-free syngeneic mice: IV. Search for this antigen on syngeneic embryonic tissues. Folia Biol. *20*: 252-257.

REYNSO, G., T.M. CHU, D. HOYYOK, E. COHEN, T. NEMOTO, J.J. WANG, J. CHUANG, P. GUINAN and G. MURPHY (1972). Carcinoembryonic antigen in patients with different cancers. J. Am. Med. Assoc. *220*: 361-365.

RUOSLAHTI, E. and M. SEPPALA (1971). Studies on carcino-fetal proteins. III. Development of a radio-immunoassay for α-fetoprotein. Demonstration of α-fetoprotein in serum of healthy human adults. Int. J. Cancer *8*: 374-383.

RUOSLAHTI, E., M. SEPPALA, T.A. RASANEN, P. VUOPIO and T. HELSKE (1973). Alpha-foetoprotein and hepatitis B antigen in acute hepatitis and primary cancer of the liver. Scand. J. Gastroent. *8*: 197-202.

RUOSLAHTI, E., H. PIHKO and M. SEPPALA (1974a). Alpha-fetoprotein: Immunochemical purification and chemical properties. Expression in normal state and in malignant and non-malignant liver disease. Transplant. Rev. *20*: 38-59.

RUOSLAHTI, E., H. PIHKO, M. BECKER and O. MAKELA (1974b). Rabbit alpha-fetoprotein: normal levels and breakage of tolerance with haptenated homologous alpha-fetoprotein. Eur. J. Immun. *5*: 7-10.

RUOSLAHTI, E., E. ENGVALL, H. JALANKO and H. PIHKO (1976). Alpha-fetoprotein: sequence homology with albumin and attempts towards use in tumour immunotherapy. Proc. 1st Conf. Oncodevelopmental Gene Expression, p.47.

SABINE, J.R. and E.H. STONEHILL (1972). Appearance of "tumour-specific" embryonal antigens in pre-tumourous tissue. Proc. Aust. Biochem. Soc. *5*: 44.

SABINE, J.R., B.J. HORTON and M.B. WICKS (1973). Spontaneous tumours in C3H.A^{vy} and C3H.A^{vy}fB mice: High incidence in the United States and low incidence in Australia. J. Natl. Cancer Inst. *50*: 1237-1242.

SABINE, J.R., R.S. SAVVAS, A.K. VERMA, A.W. MURRAY and E. RUOSLAHTI (1975). Time course of cellular changes during spontaneous hepatic carcinogenesis in mice. Clin. Exp. Pharm. Phys. *2*: 85.

SAMAAN, N., S.C.C. YEN, H. FRIESCEN and O.H. PEARSON (1966). Serum placental lactogen levels during pregnancy and trophoblastic disease. J. Clin. Endocrinol. Metab. *26*: 1303-1308.

SELL, S. and D. GORD (1973). Rat alpha-fetoprotein III. Refinement of radio-immuno-assay for detection of one ng rat α_1F. Immunochem. *10*: 439-442.

SELL, S. and H.P. MORRIS (1974). Relationship of rat α_1-fetoprotein to growth rate and chromosome composition of Morris hepatomas. Cancer Res. *34*: 1413-1417.

SHARON, N. and H. LIS (1972). Lectins: Cell agglutinating and sugar-specific proteins. Science *177*: 949-958.

SHEPPARD, H., M. POLER, P. TREFTS and S. SELL (1976). Effect of A.F.P. on immune phenomena in rats and mice. Proc. Conf. Oncodevelopmental Gene Expression, p.46.

SHIED, B., H.P. MORRIS and J.S. ROTH (1965). Distribution and activity of aspartate amino transferase in some rapidly proliferating tissues. J. Biol. Chem. *240*: 3016-3022.

SHINOMIYA, Y., H. KAZUYA, K. MATSUMURA, A. IMAGAWA, Y. ENOMOTO, Y. FUKAI and N. ITO (1973). α-Fetoprotein during hepatocarcinogenesis in dogs treated with chemical carcinogens. Gann Mono. *14*: 301-313.

SILVER, H.R., P. GOLD, S. FEDER, S.O. FREEMAN and J. SHUSTE (1973). Proc. Nat. Acad. Sci. *70*: 526-530.

SILVER, H.K., J. DENAULT, P. GOLD, W.G. THOMPSON, J. SHUSTER and S.O. FREEDMAN (1974). The detection of α_1-fetoprotein in patients with viral hepatitis. Cancer Res. *34*: 244.

SJOGREN, H.O., I. HELLSTROM, S.C. BANSAL and K. HELLSTROM (1971). Suggestive evidence that blocking antibodies of tumour-bearing individuals may be antigen-antibody complexes. Proc. Nat. Acad. Sci. *68*: 1372-1375.

SOLOMON, J.B. (1971). Maternal antibody. III. Protective effects and immunological hazard. In: "Foetal and Neonatal Immunology" North-Holland Publ. Co. : Amsterdam, p.115-138.

SOMMERVILLE, R.G. (1967). The production of fluorescent antibody reagents for virus diagnosis in the albino mice. I. Hyper-immune anti-species serum. Arch. fin. die Gesmate virusforschung. *20*: 445-452.

SPELLMAN, C.M. and P.F. FOTTRELL (1973). Similarities between pyruvate kinase from human placenta and tumours. FEBS Letters *37*: 281-284.

STAFFORD, M. and O.W. JONES (1972). The presence of fetal thymidine kinase in human tumours. Biochim. Biophys. Acta *277*: 439-442.

STANISLAWSKI-BIRRENCWAIG, B., E.J. URIEL and P. GRABAR (1967). Association of embryonic antigens with experimentally induced hepatic lesions in the rat. Cancer Res. *27*: 1990-1997.

STONEHILL, E.H. and A. BENDICH (1970). Retrogenetic expression: The re-appearance of embryonal antigens in cancer cells. Nature *228*: 370-371.

STONEHILL, E.H., E. BORENFREUND and A. BENDICH (1971). Anachronistic genetic expression recognized by the production of foetal antigens in cancer cells. Proc. Conf. on Embryonal and Foetal Antigens in Cancer *1*: 85-104. AAEC : Oak Ridge.

SUGIMURA, T., K. IKEDA, K. HIROTA, M. HOZUMI and H.P. MORRIS (1966a). Chemical enzymatic and cytochrome assays of microsomal fraction of hepatomas with different growth rates. Cancer Res. *26*: 1711-1716.

SUGIMURA, T., T. MATSUSHIMA, T. KAWACHI, Y. HIRATA and S. KAWABE (1966b). Molecular species of aldolases and hexokinases in experimental hepatomas. Gann Mono. *1*: 143-149.

SUZUKI, H., K. MIKI, S. IINO, H. NIWA and T. ODA (1976). Alkaline phosphatase isoenzymes in cancer tissues of the stomach. Proc. 1st Conf. on Oncodevelopmental Gene Expression, p.58.

TANAKA, T., Y. HARANO, F. SUE and H. MORIMURA (1967). Crystallization, characterization and metabolic regulation of two types of pyruvate kinase isolated from rat tissues. J. Biochem. Tokyo *62*: 71-91.

TEMIN, H.M. (1971). Mechanism of cell transformation by RNA tumour viruses. Ann. Rev. Microbiol. *25*: 609-648.

THOMPSON, D.M.P. and P. ALEXANDER (1973). A cross-reacting embryonic antigen in the membrane of rat sarcoma cells, which is immunogenic in the syngeneic host. Br. J. Cancer *27*: 35-47.

TING, C.C., J.R. ORTALDO and R.B. HERBERMAN (1973). Expression of foetal antigens and tumour-specific antigens in SV-40 transformed cells. I. Serological analysis of the antigenic specificities. Int. J. Cancer *12*: 511-518.

TOMASI, T.B., R. THOMPSON, R. KELLOR and R. DATTWYLER (1976). Immune regulation by alpha-fetoprotein. Proc. 1st. Conf. on Oncodevelopmental Gene Expression, p.45.

WAHLSTROM, T. (1973). Glia-specific antigens in cell cultures from rabbit brain, human foetal, adult brain and gliomas. Acta Path. Microbiol. Scand. (Micro et Immunol.) *81*: 768-774.

WARBURG, O. (1923). Versache en uberlebendem carcinogewebe. Biochem. *14*: 317-333.

WARBURG, O. (1956). On the origin of cells. Science *123*: 309-314.

WARNER, N.L. (1976). Experimental tumour immunology. Clin. Exp. Pharm. Phys. *3*: 293.

WATABE, H., H. HIRAI and H. SATOH (1972). α-Fetoprotein in rats transplanted with ascites hepatoma. Gann 63:I89

WEINHOUSE, S. (1973). Metabolism and isozyme alterations in experimental hepatomas. Fed. Proc. *32*: 2162-2167.

WEINTRAUB, B.D. and S.W. ROSEN (1971). Ectopic production of human chorionic sommatomammotropin by non-trophoblastic cancers. J. Clin. Endocrinol. Metab. *32*: 94-101.

YANG, W.K. (1971). Iso-accepting transfer RNAs in mammalian differentiated cells and tumour tissues. Cancer Res. *31*: 639-643.

ZAMCHECK, N., T.L. MOORE, P. DHAR and H. KUPCHIK (1972). Immunological diagnosis and prognosis of human digestive cancer: Carcinoembryonic antigen. N. Eng. J. Med. *286*: 83-86.