CIRCULATING TUMOUR ASSOCIATED ANTIGENS AS AID TO EARLY DIAGNOSIS OF CARCINOMA OF CERVIX

BY

BABATUNDE ADELUSI

M.B.,B.S.; Ph.D.; Cert. Immunol.; M.R.C.O.G.;
F.I.C.S.; F.W.A.C.S.; F.M.C.O.G.

A THESIS IN THE DEPARTMENT OF
OBSTETRICS AND GYNAECOLOGY

Submitted to the Faculty of Clinical Sciences & Dentistry
COLLEGE OF MEDICINE
in partial fulfilment of the requirements
for the Degree of

DOCTOR OF MEDICINE
of the
UNIVERSITY OF IBADAN

OCTOBER 1982.

To the memory of my late uncle

JOSEPH ONI OLADAYO OJO

whose inspiration and encouragement

led me into the field of Medicine.

And

To My Precious Love

IYABODE OLUREMI ADELUSI

A wife in a million.

ABSTRACT

Earlier studies by this author in 1976 provided evidence of an association between an infective, sexually transmitted agent, the Herpes Simplex Virus Type-2 (HSV-2) and human carcinoma of the cervix, irrespective of geographic location or ethnic origin of the individuals. Similarly, HSV-2 related antigens were demonstrated by the indirect immuno-fluorescent technique in desquamated cells of carcinoma of the cervix tissues from tumour bearing patients.

To highlight the potentials of the detection of tumour antigens in tumour-bearing patients as aid to early diagnosis of the cancer, isolation and purification of the Tumour Associated Antigens (TAA) of human cancer of the cervix were attempted by immunological and physicochemical procedures in this study. Two antigen preparations, one the Soluble Antigens (SA), and the other, the Membrane Bound Antigens (MBA) were prepared from a pool of cancer of the cervix (CaCx) tissues. The soluble antigens were extracted by homogenization in Earle's Balanced Salt Solution (EBSS) containing antibiotics, and the membrane-bound antigens were solublised by 3-molar potassium chloride (3 Molar KCl).

For isolation and purification of TAA, procedures for the purification of proteins were explored. These include physico-chemical stepwise ammonium sulphate fractionation, sephadex ion exchange chromatography, sephacryl S-200 gel filtration, and affinity chromatography to eliminate the normal tissue components of the antigen fractions. Hyperimmune rabbit sera were then prepared against the partially purified TAA.

Immunodiffusion studies, employing hyperimmune rabbit sera prepared against the partially purified TAA were used to demonstrate tumour associated antigens in the various cancer antigen preparations, and Circulating Tumour Associated Antigens (C-TAA) in the sera of tumour-bearing patients. The TAA in the cancer tissues showed lines of identity with the C-TAA in patients sera. Techniques involving adsorption by immuno-precipitation-in-gel with Normal Cervix (NCx) tissue antigen preparations, and pooled Normal Human Sera (NHuS) were utilized for the removal of antibodies to NCx and NHuS in the rabbit sera, after which there were no reactions with NCx indicating the specificity of TAA for CaCx.

A comparison of the soluble and 3 Molar KCl extracts of the cancer antigens by immuno-precipitation reaction, using adsorbed hyperimmune rabbit sera prepared against the partially purified CaCx TAA, demonstrated three TAA (TAA-1, close to the central antisera well, TAA-2, intermediate and TAA-3, close to and curving towards the peripheral antigen well) in the soluble antigen preparation. Only one TAA (probably identical to the TAA-3 of the soluble antigen) was demonstrated in the 3 Molar KCl extract.

Using the rabbit antisera against the partially purified CaCx TAA, adsorbed with NCx and NHuS, results of coded sera showed that immunodiffusion reaction was able to detect circulating TAA in 75.0 per cent of patients with cancer as compared with 5.6% in women with benign gynaecological diseases, 1.4% in pregnant women and 0.0% in healthy control women. The result indicates that the test has great potential for immunodiagnosis of cancer of cervix. Although the

sensitivity of the test method was low, the specificity was high, and could provide a means of early diagnosis of neoplastic changes in the cervix.

The detection of Circulating-TAA as tumour markers in sera of patients, may some day become routine, and thus make earlier diagnosis of cancer possible. Indications are that immuno-diagnostic procedures can be designed in ways that are reproducible, simple and reliable. Such serological tests would extend our present ability for detection and monitoring of malignancies.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Dr. B. Olusiji Osunkoya, Professor of Immunology and Director, Postgraduate Institute of Medical Research & Training, College of Medicine, University of Ibadan, who introduced to me the subject of Cancer Immunology, and who has continued to encourage me to pursue the study further. I am also very grateful to Dr. A.J. Nahmias (Andy), Professor of Paediatrics and Chief, Infectious Diseases and Immunology, Emory University School of Medicine, Atlanta, Georgia, and Dr. A.N. Ibrahim, Professor of Biology, Georgia State University, Atlanta, in whose laboratories some of the techniques employed in this study were acquired.

The International Research Fellowship of the Fogarty International Centre, National Institutes of Health, Bethesda, Maryland, U.S.A., which enabled me to advance my research techniques in two of the best laboratories in North America, is gratefully acknowledged. I thank the University of Ibadan which granted me sabbatical leave for the 1979/80 session to take up the International Research Fellowship, and later gave a Senate Research Grant to continue the studies at Ibadan. Appreciation goes to all my co-workers in Andy's laboratory for their cooperation, and for the healthy atmosphere they created (thanks to Andy's inspiration) which encouraged me to complete some vital parts of the study on schedule.

My appreciation also goes to my Consultant colleagues in the Department of Obstetrics and Gynaecology, University of Ibadan, for allowing the study of their patients, and particularly to Dr. O.A. Ojo

and Dr. O.A. Ladipo, both Professors of Obstetrics and Gynaecology in the University of Ibadan, who looked after my patients while I was away in the United States. I would like especially to thank the Resident Staff of the Department of Obstetrics and Gynaecology, University College Hospital, Ibadan, particularly Drs. Tony Marinho, Dosu Ojengbede and Soji Oluitan, for their help in the collection of materials for the study even when I was far away from the site of action in Ibadan.

My thanks go to the staff of the Biochemical Communications Department, University of Ibadan for preparation of the illustrations, Dr. D.A. Olatunbosun, Professor of Chemical Pathology, Dr. A. Adeloye, Professor of Neuro-Surgery, and Dr. A.O. Osoba, Professor of Medical Microbiology, all of the University of Ibadan, for their continued interest and encouragement throughout the period of the study. I am particularly grateful to Dr. E. Oluwole Akande, Professor of Obstetrics and Gynaecology and Provost of the College of Medicine University of Ibadan, Nigeria, who offered useful suggestions, and also encouraged and helped me in more ways than I can enumerate, to see that the study was successfuly completed. I would also like to acknowledge the secretarial assistance of Mr. Tolase Lawoyin and Mrs. Moji Toye of the Department of Obstetrics and Gynaecology, University College Hospital, Ibadan, Nigeria.

Finally, I would like to place on record, once again, the immeasurable support, both moral and material, of my devoted wife, Oluremi and loving children, Adeyinka, Aderonke, Adedotun and Adetokunbo

during the period of the study. In particular, they all gave one year of their precious lives to keep me company in the United States at some stage of the study, while making the best of the visit themselves.

It is pertinent to state that the materials used in this study in terms of the study population as well as tissues and sera specimens were collected personally, and with some help from three Resident Doctors from the Department of Obstetrics and Gynaecology, University College Hospital, Ibadan. The detailed laboratory work described in the study was carried out personally in the Laboratories of the Department of Paediatrics, Infectious Diseases and Immunology Division, Emory University School of Medicine, Atlanta, Georgia, and the Department of Biology, Georgia State University, Atlanta, Georgia. Laboratory assistance in the form of preparation of Buffer Solutions, immunization of rabbits, schematic representation and photography of immunodiffusion tests, as well as histopathology was provided by others who had been duly acknowledged.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

The natural history of carcinoma of the uterine cervix has been extensively investigated, and the gradation from the normal cell through intraepithelial neoplastic changes (dysplasia and carcinoma in-situ) to invasive carcinoma of the cervix has been well documented. This progression is suggested by the peak age distribution of dysplasias, which preceeds by several years that for carcinoma in-situ, which in turn also preceeds by many years, that of invasive carcinoma (Nahmias et al, 1980). Similar progression has also been observed in mouse models in which chemical carcinogens have been applied to the cervix (Christoperson, 1969).

It is believed that this cancerous change is initiated by the introduction of an oncogenic agent by the penis into the milieu of the cervix (Wynder et al, 1954; Terris and Oalman, 1960; Rotkin, 1967). The intraepithelial carcinoma of the cervix is thought to originate from the transitional cells that lie at the squamo-columnar junction through this "unknown-stimulus" (Fluhmann, 1960; Johnson et al, 1964). These proliferate in an abnormal fashion to form neoplastic sub-cylinderic cells which gradually develop into carcinoma in-situ, which in turn invade the stroma, and ultimately develop into clinically invasive carcinoma of the cervix (Johnson et al, 1960; Koss et al, 1963; Johnson, 1969; Richart and Baron, 1969; Hulka and Kupper, 1971).

Such a progression of disease does not appear to be an inevitable process (Ashley, 1966; Nahmias et al, 1980) and indeed, it is accepted by many investigators that dysplasias can regress in varying frequencies, and that not all cases of carcinoma in-situ progress to invasive cancer (Coppleson and Reid, 1967; Gusberg and Frick, 1970; Johnson, 1969). Nevertheless, ample evidence had accumulated to indicate that when invasive cancer occurs, it is practically always preceeded by a pre-invasive phase in its development, and that clinical progression of dysplasia and carcinoma in-situ to invasive cancer of the cervix exists. Various clinical (Fox, 1967; Hall and Walton, 1968; Richart and Baron, 1969), epidemiologic (Johnson et al, 1964; Baron and Richart, 1971; Hulka and Kupper, 1971) and laboratory studies (Richart, 1967) have now provided substantial evidence to support the view that cervical dysplasia, carcinoma in-situ and invasive carcinoma of the cervix represent a single spectrum of neoplastic change.

It is known that the normal cell contains a definite complement of antigens (Hollinshead et al, 1972; Frenkel et al, 1972), and the metamorphosis which occurs in the cervix tissue as it progresses from the normal state, through a dysplastic state and carcinoma in-situ, to invasive carcinoma (Fox, 1967; Richart, 1967; Hall and Walton, 1968; Johnson et al, 1968; Richart and Baron, 1969; Baron and Richart, 1971; Hulka and Kupper, 1971) may be accompanied by the acquisition of new antigenic substances. Indeed, in cells of animal tumours induced by DNA oncogenic viruses, transplantation antigens specific for the inducing virus have been demonstrated (Hellstrom and Hellstron, 1969),

and Herpes Simplex Virus (HSV) antigens have been detected by immuno-fluorescent techniques in cells exfoliated from cervical lesions (Royston and Aurelian, 1970; Adelusi et al, 1976).

Experimental evidence has accumulated, supporting the occurrence of structural changes on cellular membranes or within the endoplasm of individual cells during neoplastic transformation (Gross, 1943; Foley, 1953; Prehn and Main, 1957; Klein et al, 1960). The expression of neoantigens which are not expressed by their normal cell counterparts, at least in the adult host, results in an antigenic distinctness, and an elucidation of an immune response.

Various reports of tumour-specific or tumour-associated antigens have now accummulated. Abelev (1963, 1968), described the Alpha-Feto-Protein (AFP) associated with hepatomas; Gold and Freeman (1965) and Thompson et al (1969) demonstrated a Carcino-Embryonic Antigen (CEA) of the human digestive system. Hellstron et al, (1968, 1970) demonstrated antigens present in human neuroblastoma; Levi et al, (1969) and Levi (1971) described antigenecity of ovarian cysadenocarcinoma carried in tissue culture; McNeil et al, (1969) described cross-reactivity between the benign ovarian mucin and extracts of colon cancer, and Order et al, (1971) described evidence of tumour associated antigens of Hodgkins disease.

Other workers have provided evidence for the presence of tumour antigens in carcinoma of the cervix (Aurelian et al, 1971; Frenkel et al, 1972; Aurelian et al, 1973; Feorino and Palmer, 1973; Gall et al, 1973;

Chang et al, 1974). Gall et al, (1973) were able to demonstrate tumour antigens in all women with carcinoma of cervix but not in those with normal cervix, while the others were able to demonstrate tumour antigens induced or related to HSV-2.

Carcinoma of the cervix uteri is one of the most common cancers in women throughout the world. Indeed, it is by far the commonest female malignancy encountered in Nigeria, excluding the lymphoreticular tumours (Edington and Hendrickse, 1970). Because of its frequency and accessibility to methods of early detection, carcinoma of the cervix is among the most thoroughly studied of all human malignant diseases, and its natural history has been extensively investigated.

In the developed world, detection of cancer of the cervix has been accomplished without removal of tissue, mostly by cytologic examination of exfoliated cells, and in some instances, by direct magnified observations made with the colposcope. As a result, the mortality associated with the disease has decreased significantly in those places where these early detection methods are available.

However, in spite of its common occurrence in Nigeria, carcinoma of the cervix still constitutes one of the least studied diseases, and the mortality associated with it has shown little or no decline because of the absence of these early detection methods. Rather, the intraepithelial and early invasive stages of the disease are very infrequently detected by the traditional methods of history taking, speculum examination, palpation and probing, and even that, only when the patients decide to report in hospital.

The value of early detection and therefore, treatment of carcinoma of the cervix has never been in doubt. It is generally agreed that carcinoma of cervix is unique in that it is perhaps the only cancer of the female genital tract which can be detected before it becomes invasive. The recent development of routine cytologic screening of populations with completely normal appearing cervix has led to the diagnosis of many cases of very early cancer (in-situ) before the symptomatology of overt disease became apparent (Thomson et al, 1972).

The most comprehensive investigation of a patient with cellular atypia has included cold conisation as the ultimate procedure necessary to establish a definitive histologic diagnosis, especially in cases of in-situ carcinoma. In Ibadan, the preference has always been multiple punch biopsies of the squamo-columnar junction if the tumour is clinically obvious, (which happens to be most often), or cone biopsy where the cervix is suspect on clinical examination. For various reasons, cytologic screening methods have been on a limited scope (Adelusi, 1976).

Even then, the value of conisation has been limited by the demonstrated residual carcinoma, in-situ carcinoma or dysplasia in post-conisation hysterectomy specimens, suggesting that the total extent of the disease in 10 to 30 per cent of cases was not demonstrated or eliminated by this procedure (Silbar and Woodruff, 1966; Singleton and Rutledge, 1968). Furthermore, conisation demands hospitalisation and general anaesthesia, and is occasionally complicated by post-operative bleeding and late cervical dysfunction. Colposcopy on the other hand, though valuable, especially when complemented by other methods

(Navratil et al, 1958; Thomson et al, 1972) is available only in limited centres.

If procedures less traumatic than conisation and less expensive than colposcopy, can determine accurately, the presence and extent of the neoplastic process, the more operative procedure of conisation with its attendant hazards, could be greatly reduced, if not eliminated completely (Christopherson et al, 1967; Sabatelle et al, 1969; Channen and Hollycock, 1971). There is the need therefore, for a search for other diagnostic techniques for carcinoma of the cervix.

As a consequence of the demonstration of tumour associated antigens in a variety of human neoplasms by serological techniques, the prospect of employing these antigens as diagnostic tests has been investigated, extensively in recent years. Each of these tumour antigens types--viral antigens, transplantation antigens, membrane bound and cytoplasmic antigens, carcino-embryonic antigens--and the antibodies directed against them, have been detected on a sufficient variety of animal and human cancers to warrant consideration for their use in immunodiagnostic procedures (Balwin and Price, 1976; 1977; Ristow and McKahan, 1977).

A model system has been established to investigate the feasibility and reliability of Tumour Associated Antigens (TAA) detection as a diagnostic procedure. Ray (1974); Megaw (1974) and Ibrahim et al, (1975) using Herpes Simplex Virus Type-2 (HSV-2) associated hamster tumour (Nahmias et al, 1970), demonstrated the presence of tumour specific antigens The technique of immunodiffusion and immuno-electrophoresis, with or without immuno-adsorption in gel (Ibrahim, 1969) were used to establish

the possible application of TAA in immunodiagnosis of cancer. Supplemental research demonstrated cross-reactivity between the antigens of HSV-2 associated hamster tumours (OT-I and OT-II) and human carcinoma of the cervix (Ibrahim et al, 1976).

The rewards for isolation and purification of tumour antigens and tumour specific antibodies could be considerable. Purified material may facilitate the determination of the exact relationship between the antigens of virally induced tumours and the cause of malignancy. The prospects of a reliable diagnositc test may improve with the availability of purified reagents (Ibrahim et al, 1979). Purification may improve the detection of free antigens or antigen-antibody complexes in the circulation of tumour-bearing patients. This information may reveal the extent of the disease, the existence of metastasis and provide a prognostic indicator.

The eventual establishment of HSV-2, or any other virus, as the causative agent of a human neoplasm will first require a systematic attempt to isolate and separate the various tumour associated antigens of the specific human malignancy. The present research is directed towards the purification of tumour associated antigens of carcinoma of cervix tissues for immunisation of rabbits and the exploration of the possibility of using the anti-sera to detect TAA in the circulation of women with carcinoma of cervix.

CHAPTER 2

REVIEW OF THE LITERATURE

The current concept of the genesis and natural history of carcinoma of the cervix accepts that neoplastic transformation occurs over a period of time. It is believed that cancer development is initiated with the transformation of a single cell from normal, through dysplastic cell and carcinoma in-situ, to invasive carcinoma (Fox, 1967; Johnson et al, 1968; Richart and Barron, 1969; Hulka and Kupper, 1971; Barron and Richart, 1971), although controversies still exist about this (Richart, 1967; Johnson, 1969). Through genetic alterations, the abnormal cell gives rise to a multiplicity of similarly altered cells (Levi, 1971).

Such genetic transformation would induce the synthesis of new protein species - antigens - not expressed by their normal counterparts, and these new antigens would in turn be expected to provoke immune response by their host. The progression and proliferation of the malignancy may be due to impairment of immunologic responses of the host (Levi, 1971). Thus, the early fundamental events of malignancy appear to be an alteration of the initial cell by acquisition of new antigens, followed by immunologic failure of the organism to the newly acquired antigens. The alteration of the first cancerous cell subsequently involves escape from regulatory mechanisms, and changes in the relation of the malignant cell with its adjacent cells, leading to proliferation and invasion (Levi, 1971; Nahmias et al, 1980).

Several reports of experimental evidences have now accummulated to support this theory of the occurrence of structural changes on the cellular membranes or within the endoplasm of the individual cells during neoplastic transformation. The expression of neo-antigens which are not expressed by their normal counterparts, at least in the adult host, results in an antigenic distinctness (Gross, 1943; Foley, 1953; Klein et al, 1960; Hellstrom and Hellstrom, 1969; Levi et al, 1969; Order et al, 1971; Aurelian et al, 1971; Frenkel et al, 1972).

2.1: TUMOUR SPECIFIC ANTIGENS IN ANIMAL TUMOURS

The Study of tumour immunology, and more particularly of tumour associated antigens, has always relied heavily on transplantation experiments and efforts to immunize animals with tumour tissues or related antigen preparations. This was probably first successfully demonstrated between 1875 and 1876 by a Russian veterinarian, M.A. Novinsky, who successfully transplanted a malignant tumour from one animal to another (Shimkin, 1955; Gross, 1970). Although the transplants did not take in all the animals inoculated, the work suggested that the tumours themselves might be antigenic (Hellstrom and Hellstrom, 1967).

There was little attention paid to these and other reports on the transplantation antigens however, until the beginning of the twentieth century when large scale studies conducted in the United States (Loeb, 1902) and other centres (Bashford and Russel, 1910) confirmed the earlier reports. It should be noted however, that histocompatibility genes and the antigens determined by them had not, as yet, been reported.

Thus, the investigators failed to distinguish between normal tissue transplantation and tumour transplantation responses.

By the early 1940's, various investigators had established that susceptibility to tumour transplants was genetically controlled (Little et al, 1941). These reports, coupled with earlier findings of Loeb and Wright, (1927); Loeb and King, (1929); and Buttner, (1935) who worked with normal tissue transplants, produced the concept of histocompatibility antigens which are controlled by specific histocompatibility genes (Gorer, 1961).

The credit for the production of the first clear-cut evidence of the existence of a Tumour-Specific Transplantation Antigen (TSTA) goes to Gross (1943). Using an immune sarcoma, designated S-37, several in-bred syngeneic mice were inoculated, most of which developed tumours. After spontaneous regression and recovery, attempt to re-transplant the same tumour in these animals was unsuccessful in many instances, while control animals (mice of the same strain receiving their first inoculation of the tumour) all developed tumours. It was concluded that the immunity in the test animals was directed specifically against the tumour used for inoculation.

However, it was Prehn and Main (1977), who clearly demonstrated that a TSTA indeed did exist, when they induced tumours with methylcholanthrene, and the tumours produced specific immunity in the animals into which these were transplanted. It was also found that different methylcholanthrene induced sarcomas had individually distinct tumour antigens which, according to transplantation experiments, did not cross-react.

It was not until the study of Klein et al, (1960) that heterogeneity of animals could be completely ruled out in demonstrating a tumour specific transplantation antigen, when sarcomas were induced with methylcholanthrene. After several treatment with irradiated tumour cells from the sarcomas induced, the original host animal was challenged with the same tumour which had been growing in the isologous animal. Immunity was clearly demonstrated, and there was no cross-immunity for any of the different methylcholanthrene induced tumours produced. Serum from an immune animal had no effect on sarcoma cells in vitro, but neutralisation could be achieved with the lymph node cells of the immune animal. Furthermore, those untreated animals had no neutralising effect, and neither did immune lymph node cells which had been killed. Thus, it was believed that these immune reactions could be attributed to the antigenic distinctness of the expression of neo-antigens in the cancer tissue, but which were not expressed by their normal counterparts, at least in the adult animal.

Lack of immunologic cross-reactivity consistently reported for tumours induced by the same agent, even when different tumours had been induced by the agent in the same animal, was attributed to their being chemically induced (Klein et al, 1960; Old and Boyse, 1964; Baldwin and Barker, 1967; Baldwin et al, 1971; Leonard, 1975; Lo Gerfo, 1976).

A major break through was made in tumour specific transplantation antigen study when Sjogren et al, (1961), working with polyoma virus-induced tumours in in-bred mice, demonstrated that adult animals immunized with the polyoma virus were resistant to transplantation with

polyoma tumour cells. They attributed this finding to the presence, in the polyoma tumour cells, of specific antigens determined by the virus. A parallel study by Habel (1962) not only confirmed this, but also speculated that the resistance produced was specific.

Other studies (Sjogren, 1961) showed immunization with tumour cells induced resitance to the polyoma virus-induced tumours, and that all tumours produced by the polyoma virus in the same strain of animals showed antigenic cross-reactivity. Similarly, Huebner et al, (1963) demonstrated cross-reactivity for tumours induced by adenoviruses, and between tumours induced by different but related adenoviruses, even in virus-free tumours. It was thus concluded that tumours induced by one virus contain the same characteristic cellular antigen, even across species virus (Old and Boyse, 1964).

Further work on the tumour specific transplantation antigens was demonstrated by Hellstrom (1965) when he showed that antibodies to polyoma virus-induced tumour cells had a strongly limiting effect on the growth of the tumour cells in vitro, whereas, antiviral antibody did not. It was suggested that the virally-induced tumour specific transplantation antigen was not necessarily viral material itself, thus confirming the earlier studies of Habel (1962) which suggested that the detectable presence of the virus itself was not necessary for immunity. Rather, it was postulated that the virally transformed cells may lose certain normal antigens and gain new ones, with the information for the new antigens now residing in the genome of the transformed cells.

The mechanism of virus-induced cell transformation, and the nature of the common antigens produced were not clear until Rowe (1967) demonstrated a definite distinction between the types of tumour antigens possessed by DNA virus-induced and RNA virus-induced tumours. Since all antigens discussed were not of the transplantation type, it was therefore, suggested that the term "Tumour Associated Antigens (TAA)" should be applied to all tumour-related antigens.

2.2:TUMOUR ASSOCIATED ANTIGENS IN HUMAN CANCER:

Up till now, most of the evidence for viral etiology of cancer has been confined to animals. However, various experimental reports of cross-reacting or tumour-specific antigens of human cancer have started accumulating. Klein et al, (1966) demonstrated tumour specific immune reactions in Burkitt lymphoma patients by the membrane immunofluorescence reactions. Hellstrom et al, (1968, 1970)demonstrated antigens in human neuroblastoma, while Levi et al, (1969), described antigenicity of ovarian serous cystadenoma.

Other studies of tumour antigens include the description of cross-reactivity between the benign ovarian mucin and extracts of colon cancer (McNeil et al, 1969), the finding of antigens in malignant melanoma (Morton et al, 1970; Jehn et al, 1970; Carrell and Theilkase, 1973) and the report of immune response in urinary bladder tumours (Bubenik et al, 1970). Similarly, Order et al (1971) described evidence of tumour associated antigens of Hodgkins disease, and Levi (1971), demonstrated the presence of tumour antigens in carcinoma of cervix by the precipitin-in-gel method.

Apart from the non cross-reacting, individual and tumour specific antigens of chemically-induced tumours, and the cross-reacting virus-specific antigens of the virally induced tumours, another class of tumour associated antigens were delineated. Designated the "Embryonic antigens or Embryo-associated antigens", these antigens have been associated with certain cancerous conditions in humans.

Perhaps the first of these to be directly associated with cancerous condition was the Alpha Fetb Protein (AFP), a glycoprotein found in the fetal serum of all species of mammals, including man, and synthesized in the fetal liver, but which disappears soon after birth. Abelev (1963), demonstrated the presence of the antigen, associated with a primary transplantable mouse hepatoma which had been induced with ortho-aminoazo toluene in C_3H mice, and with sera and tissues of recipient animals, but not in the tissues or sera of normal adult animals. It was postulated that the presence of the antigens might indicate "simplification" of the tumour cell antigens and possible de-repression of genes that are active in embryonic development, but normally turned off in adult life.

Tatarinov (1964) found the antigen in association with human hepatomas, and Abelev (1971), found it associated with malignant teratomas. Current statistics (Buitin, 1972) indicated that 40 to 80 per cent of hepatomas and 25 per cent of malignant teratomas in adults, can be detected by the presence of AFP. In children, these figures could rise to 90 per cent and 75 per cent respectively. However, there is the disadvantage that the detection of AFP as a diagnostic tool could be

limited by the presence of the protein in regenerative hepatitis and certain other non-cancerous disorders. Nevertheless, the detection of AFP could have diagnostic value if a technique with limited sensitivity is employed.

Gold and Freedman (1965) and Thompson et al (1969) demonstrated another embryonic antigen associated with cancer. This glycoprotein, the carcino-embryonic antigen (CEA), normally produced by the fetal liver from two to six months in utero, and found only in trace amounts in normal adults, was found in association with human colonic cancer. It is now known to be associated with cancer of the entire length of the alimentary tract, pancreas, breast and bronchus (Gold, 1971; Reyensoro et al, 1972).

Since the application of CEA elicits antibody formation (Gold, 1967), a simple technique for demonstration of CEA has become available, and use of this as a diagnostic tool has been greatly enhanced by the development of radioimmunoassay technique for the detection of CEA in sera of patients (Thompson et al, 1969). However, elevated levels of CEA can be found frequently in conjuction with non-malignant lesions such as benign polyps, (including hemorroidal polyposis) alcoholic cirrhosis of the liver, gastric ulcers, pulmonary emphysema and ulcerative colitis (Temp et al, 1974). Low levels of CEA have also been reported in normal tissues (Rogers, 1976), and in sera from 10% of normal individuals (Terry et al, 1974).

A variety of other embryonic antigens have been described (Cinader, 1972; Buitin, 1972) although most of these have not been shown to have any clear diagnostic value with regards to the particular tumour.

Embryonic antigens have been found in association with certain animal tumours. For example, Brawn (1970), demonstrated embryonic antigens associated with methylcholanthrene-induced murine sarcomas, and Baldwin et al, (1971), described an embryonic antigen associated with aminoazo dye-induced rat hepatomas. The presence of cross-reacting embryonic antigens in the chemically-induced tumours provided evidence that chemically-induced Tumour Associated Antigens may not necessarily be entirely specific for each tumour.

The demonstration of immunological reactions of pregnant hamster serum with the surface of cells transformed by Simian virus-40 (Duff and Rapp, 1970), and the demonstration of fetal antigen capable of inducing immunity against the virus-induced hamster tumour cells (Coggin et al, 1970) indicated that embryonic antigens are probably universally associated with neoplastic tissues although they may be different from tumour specific antigens that are specific for a particular tumour, or tumours induced by particular virus (Ting et al, 1972).

2.3: HSV-2 ASSOCIATED ANTIGENS IN CANCER OF THE CERVIX:

In 1964, studies of an infant with neonatal Herpes Simplex Virus (HSV) infection, and his mother's cervical HSV infection, led to further investigations by the Emory University group which provided the first suspicion of an association between the virus and cervical neoplasia. This evidence was based on the observation that women cytologically detected with genital herpes had a greater frequency of cervical neoplasia than women in the general population (Naib et al, 1966). The later discovery (Nahmias and Dowdle, 1968) that about 95 per cent

of cases of genital herpes simplex virus infection are caused by a different viral type, Herpes Simplex Virus Type-2 (HSV-2), as distinct from that isolated from most non-genital herpetic infections (HSV-1), provided the means for comparing the frequency of HSV-2 antibodies in women with, and without, cervical neoplasia (Nahmias et al, 1970b).

Demographic surveys on cancer of the cervix have established patterns of high risk that strongly suggest that the disease may be a venereal disease (Beral, 1974; Nahmias et al, 1980). Numerous evidences have now accumulated to show that HSV-2 is a venereally transmitted disease (Hutfield, 1968; Rawls et al, 1971; Josey et al, 1972; Duenas et al 1972; Adelusi et al, 1976b; Kessler, 1977; Doll, 1977). It has thus been postulated that this virus might be an initiating or promoting carcinogenic agent transmitted to the female from the male during sexual intercourse.

Various multicentric approaches, from serologic and epidemiologic studies (Rawls, et al 1969; Aurelian et al, 1970; Nahmias et al, 1970b; Royston and Aurelian, 1970; Nahmias et al, 1972; Rotkin, 1973; Adelusi et al, 1975; Nahmias et al, 1976; Adelusi et al, 1981) to molecular studies (Feorino and Palmer, 1973; Anzai et al, 1975; Aurelian et al, 1976; Camacho and Spear, 1978; Heise et al, 1979; Shortland et al, 1979), have been employed to substanciate this association. However, despite all the studies, no definitive cause and effect association has been established.

The oncogenic potential of HSV-2 has been demonstrated by the report that intraperitoneal and intrathoracic inoculation of newborn hamsters with the virus has been shown to be associated with the

development of undifferentiated sarcomas at the site of inoculation (Nahmias et al, 1970). Such tumour bearing hamsters have been shown to possess HSV-2 antibodies in their sera and HSV-2 antigens can be demonstrated in varying number of tumour cells (Rapp and Duff, 1973; Kimura et al, 1975). Direct genital infection with the virus has also been shown to produce lesions identical to atypia and carcinoma in-situ (Nahmias et al, 1970c; Munoz, 1973).

Further convincing evidence of the oncogenic potential of HSV-2 has been provided by the transformation of hamster cells in vitro by the virus (Duff and Rapp, 1971, 1971b). Similarly, the virus has been shown to transform rat (McNab, 1974) and mouse (Kimura et al, 1975) cell cultures, and the cell lines derived from these transformations, when inoculated into other animals, have been shown to produce tumours consistently (Duff et al, 1974; Skinner, 1974; Copple and McDougall, 1976). Such interaction in-vitro between HSV-2 and most, if not all cells, results in lysis. Experimental transformation was achieved by eliminating viral infectivity, such as by ultraviolet inactivation (Duff and Rapp, 1971), or by using a subgenomic sequence of viral DNA (Camacho and Spear, 1978). Of possible relevance also was the in vitro demonstration of defective DNA of some of the virus strains (Roizman and Furlong, 1974).

Various attempts to detect HSV-2 antigens in cervical cells or biopsy specimens have been performed by immunofluorescence, immuno-peroxidate and radioimmunoassay techniques. For example, the indirect immunofluorescence technique was applied to exfoliated cells (Royston

and Aurelian, 1970; Aurelian, 1973; Adelusi et al, 1976), and the anticomplimentary immunofluorescence technique was applied to biopsy materials (Chiang et al, 1974; Nahmias et al, 1975). On the other hand, exfoliated cervical cells from patients without carcinoma of the cervix did not show evidence of viral antigens, except in those women diagnosed clinically as having herpetic cervicitis. However, problems of non-specific positive fluorescence have been known to mask such studies (Nahmias et al, 1980).

HSV-2 is a DNA virus, and therefore, unlikely to be demonstrated in transformed cells. However, significant portions of the viral genome and readily identifiable virally induced RNA, have been found in cancer cells by molecular biological studies, using hybridization techniques (Frenkel et al, 1972; Collard et al, 1973; Minson et al, 1976; Copple and McDougall, 1976). Unfortunately, the detection of viral DNA or RNA in cancer cells by molecular hybridization techniques was limited by the sensitivity of the assays, since if only a small fraction of the viral genome was present in the cancer tissue, the assay would not be sensitive enough to allow its detection. Furthermore, the cancer tissue being tested need to be examined for the proportion of cancer cells and normal cells, since it was crucial for the assay that cancer cells should comprise the large majority of cells in the tissues. In case of in-situ hybridization techniques performed on tissue sections (McDougall et al, 1980), problems of non-specific positive reactions were also limiting factors.

HSV-2 antigens, expressed in tumour cells have been suggested to be capable of stimulating specific immune responses as the transformed cells carrying them multiply. These antibodies might be less readily stimulated by productive infection, even with multiple recurrences (Nahmias et al, 1980). Thus women with carcinoma of cervix might then be found to possess antibodies to these antigens in high frequency than that occuring in women with primary or recurrent infections. This theory was used to explain the findings of higher frequency of antibodies to "early" or "non-viron" antigens reported by some investigators (Sabin and Tarro, 1973; Hollinshead et al, 1973; Aurelian et al, 1974). It should be stressed that these results require confirmation, since the initial positive observations made could not be reproduced by some workers (Sabin, 1974).

Increasing evidence for the presence of herpes simplex virus associated antigens in carcinoma of the uterine cervix has now accummulated (Gall and Haines; 1974; Ibrahim et al, 1975; Notter and Docherty, 1976; Notter et al, 1978). Among these antigens, the membrane associated HSV-TAA (Hollinshead et al, 1972, 1973, 1976) has been demonstrated in cancer of the cervix and in human cultured cells infected with herpes virus, but not in control tissues or un-infected control cultures (Hollinshead et al, 1972, 1973).

Antibodies to HSV-TAA have been found at much high frequencies in sera of patients with squamous carcinomas of the cervix than in control sera or sera from patients with other types of malignancies (Notter and Docherty, 1976; Hollinshead et al, 1976). The recent identification, separation and characterisation of the two polypeptide chains which

comprise HSV-TAA, permitted the production of highly specific hyperimmune antisera to these antigens (Hollinshead et al, 1976; Hollinshead and Stewart, 1979), and such antisera have been used to ascertain whether HSV-TAA could be demonstrated in cell lines of human carcinomas of the cervix (Auersperg et al, 1980).

Thus, the weight of circumstantial evidence supporting the role of herpes simplex virus type-2, in human carcinoma of cervix has increased, based on these various studies. However evidence supporting a similar role for many other DNA viruses as etiological agents of human cancer is lacking (Rangan et al, 1968).

2.4: COMMON ANTIGENS OF HSV-2, OT-1 AND CARCINOMA OF THE CERVIX:

Following the various reports on the association of genital herpes simplex virus infection with cancer of the cervix (Nahmias et al, 1980), and the demonstration of tumour associated antigens (TAA) in several animal and human cancers by a variety of in-vitro and in-vivo techniques (Hellstrom and Hellstrom, 1969; Cinader, 1972; Notter and Docherty, 1976; Notter et al, 1978), attempts were made to show TAA in sarcomas produced after inoculation of newborn hamsters with herpes simplex virus Type-2 (Nahmias et al, 1970), by the immunoadsorption-in-gel method (Ibrahim et al, 1975). Various physico-chemical methods were applied for the preparation and purification of the antigens.

It was of interest therefore, to ascertain whether HSV-2 related antigens could be detected in the sarcomas produced after HSV-inoculation into hamsters. Ibrahim et al, (1976) demonstrated cross-reactivity between anti HSV-2 sera and the HSV-2 associated hamster tumours (OT-I

and OT-II). Anti HSV-2 serum also reacted by passive haemaglutination with the purified TAA of OT-II, whereas, anti HSV-1 serum did not react with any of the hamster tumours. Adsorption of the anti HSV-2 serum with HSV-2 removed homologous reactivity as well as heterologous reactivity with the HSV-2 associated hamster tumours. This indicates the presence of common antigens between HSV-2, OT-I and OT-II.

Using the same technique (Levi, 1971; Ibrahim et al, 1976) antisera prepared against partially purified cancer of cervix preparations, adsorbed with pooled normal human plasma and normal cervix preparations, reacted reproducibly with cancer of the cervix tissue antigen preparations, thus indicating the presence of TAA in the antigen preparations from several cancer tissues. Rabbit antisera to normal cervix, similarly adsorbed, did not react with the test antigens or test sera. These findings suggested that antisera prepared against cancer of the cervix contains antibodies to TAA, and that these antibodies are not present in anti normal cervix sera.

Subsequent research has demonstrated cross-reactivity between the antigens of HSV-2, HSV-2 associated hamster sarcomas and human cancer of the cervix (Ibrahim et al, 1976; Adelusi, 1982). Hyperimmune rabbit antiserum to HSV-2 reacted with HSV-2, OT-I and OT-II (HSV-2 associated hamster sarcomas) and cancer of cervix; and a precipitin line of partial identity was noted between HSV-2, OT-I and OT-II and CaCx. The converse was also observed, where antiserum against CaCx. reacted with HSV-2, but not with HSV-I.

Adsorption of the anti HSV-2 serum with HSV-2 eliminated the reaction with CaCx., suggesting that the common antigen may be specific

to HSV-2. Similarly, antiserum to CaCx. reacted with HSV-2 associated hamster tumours, and adsorption of the serum with CaCx. antigens removed both homologous and hererologous reactivity. However, adsorption of the anti CaCx. serum with OT-I or OT-II did not eliminate reactivity with CaCx. Anti OT-I or anti OT-II sera, adsorbed with CaCx did not eliminate reactivity with the hamster tumour preparations either. The results of the studies suggested therefore, that both common and specific antigens may be present in the human and hamster tumours, and are probably related to HSV-2. TAA(Hollinshead et al, 1972; Nahmias et al, 1975; Ibrahim et al, 1976).

The demonstration of circulating-tumour associated antigen (C-TAA) in the sera of tumour bearing hamsters (Ibrahim et al, 1975) was equally significant, and was achieved by reacting antisera against purified OT-II tumour asscciated antigen with tumour bearing hamster serum (TBHS), using the precipitin-in-gel technique also. This showed a pattern of identity with TAA of OT-I and OT-II. Similarly, there was a pattern of identity when unadsorbed antiserum against TBHS was reacted against the purified TAA of OT-II, whereas the same antiserum, adsorbed with normal hamster serum (NHS) still reacted with TBHS as well as the crude OT-II tumour antigen preparation, but not with NHS.

The results of these various studies thus demonstrates further the possible role of HSV-2 in human cancer of the cervix. However, a definitive causal relationship would still require confirmatory studies. The isolation, purification and characterisation of tumour associated antigens in carcinoma of cervix, might be one way to demonstrate this relationship.

2.5:TUMOUR ASSOCIATED ANTIGENS IN CANCER OF THE CERVIX:

In order to explore host responses to its malignancy and simultaneously attain a deeper understanding of the cancerous process itself, especially with regards to the acquisition of new antigenic substances during the process of transformation, the antigenicity of gynaecologic tumours was studied, to search for and isolate tumour specific antigens (Levi, 1971). One of the aims of this study, incidentally, was to isolate tissue antigens not present in normal tissues, following which tumour specific antibodies would be produced.

The results of the study suggested the existence of "Specific" tumour antigens in squamous cell carcinoma of the cervix. Evidence was shown that the antigens were specific, that is, qualitatively distinct, rather than quantitative increases in normally present tissue components. Immunologic comparison of equivalent fractions eluted from Sephadex gels showed antigenic non-identity of the fractions derived from normal tissues with those from tumour tissues.

The possible application of any specific differences which might exist, in particular, the potential use of the tumour specific antigens in the diagnosis and therapy of the malignancy was also explored. Thus, it was determined that once the tumour specific antigenic fractions, and antibodies against them were available, Levi (1971) proceeded to investigate the sera of patients suspected of having the particular malignancies for the presence of circulating antigens in the hope that the findings would correlate with the post-operative pathological diagnosis.

A model was established to investigate the feasibility and reliability of TAA detection as a diagnostic method. Ray (1974) and Megaw (1974) demonstrated the presence of TAA in HSV-2 associated hamster sarcomas, using the technique of immunodiffusion and immunoelectrophoresis, with and without immunoadsorption-in-gel method. This has now been confirmed to be a simple method for demonstrating TAA in carcinoma of cervix (Ibrahim et al, 1979). It was established that unadsorbed rabbit anti CaCx. sera reacted with CaCx. and normal cervix antigens. However, after in-gel adsorption of the antisera with normal tissue extract and normal serum, precipitin reactions appeared monospecifically with the tumour antigens. Furthermore, the anti-normal tissue serum, when adsorbed with normal tissue, did not react with any test antigen, and anti CaCx. sera, adsorbed with the homologous tumour antigens, did not react with the cancer preparation.

Attempts were made to partially purify the TAA prepared from CaCx. (Ibrahim et al, 1979) by ammonium sulphate precipitation and DEAE exchange chromatographic methods. The two fractions obtained following DEAE-cellulose had TAA activity, but were only partially purified, as the unadsorbed anti CaCx. sera prepared from these still reacted with CaCx. and normal cervix. However, the antisera prepared were more readily adsorbed with normal human plasma and normal cervix than the antisera prepared against the crude antigen extracts. In addition, it was found that the volume of the innocula as well as the number of injections needed to prepare the antisera were less than those used with the crude antigen preparations.

Further attempts have been made to purify the CaCx. TAA (Adelusi, 1981). In addition to the ammonium sulphate precipitation and DEAE Ion Exchange Chromatography, for isolation of purified TAA used by Ibrahim et al (1979), other methods of purification of proteins were explored, including Sephacryl gel filtration.. Even though these methods eliminated a lot of impurities from the tumour antigens, the TAA were only partially purified, as these still reacted with antisera to normal cervix and normal human serum. After immunisation of rabbits, the unadsorbed anti CaCx. sera still reacted with CaCx. and NCx. As in previous experiments, the antisera prepared against the partially purified TAA of the CaCx. were more readily adsorbed with normal human serum and normal cervix. Furthermore, the number of precipitin lines with CaCx. was still inconsistent, varying from one to three, depending on the various antisera and antigens.

The demonstration of TAA in the circulation of tumour bearing patients added yet another dimension to the study of tumour immunology. Circulating tumour associated antigens (C-TAA) have been demonstrated in the sera of individuals with human cancers (Hellstrom and Hellstrom, 1972; Cinader, 1972), although many of these antigens were probably also expressed in fetal tissues (Gold, 1971; Cinader, 1972) and pregnancy has been shown to lead to the development of pregnancy-associated antigens that may cross-react with the cancer antigens (Rosen et al, 1975). However, the demonstration of C-TAA in the sera of tumour bearing hamsters (Ibrahim et al, 1975) has encouraged the exploration of the possibility of detecting TAA in the circulation of women with neoplasia of the cervix (Ibrahim et al, 1979; Adelusi et al, 1982).

Adsorbed rabbit antisera to CaCx. was noted to detect C-TAA in the sera from patients with cancer of the cervix, but not from normal control sera. That the TAA detected in the circulation of the cancer patients is related to the TAA of CaCx. was suggested by the pattern of identity with one of the precipitin lines in the cancer tissue, and the adsorption-in-gel technique has proven to be specific and of value in the detection of C-TAA in human cancers.

2.6:POTENTIAL OF C-TAA DETECTION IN IMMUNODIAGNOSIS OF CANCER

Consequent on the detection of elevated levels of the various tumour associated antigens in a variety of human malignancies (Levi et al, 1969; McNeil et al, 1969; Order et al, 1971; Levi, 1971; Cinader, 1972; Ibrahim et al, 1979), the prospect of employing these antigens as diagnostic and probable prognostic tool has now been accepted, and have been investigated extensively in recent years (Baldwin and Price, 1976; Ristrow and McKhann, 1977; Ibrahim et al, 1979; Adelusi, 1981; Adelusi et al, 1982).

To date, the most promising procedure for the diagnosis of cancer appeared to be the detection of circulating TAA in the sera of tumour bearing patients (Ibrahim et al, 1979; Adelusi, 1981) when it was found that with uncoded sera, all but one of 59 (Ibrahim et al, 1979) and all 30 (Adelusi, 1981) sera samples from normal individuals yielded negative reactions for TAA. On the other hand, a high percentage of the serum samples from cancer patients showed positive reactions.

The detection of C-TAA in significant percentage of the sera from women with invasive carcinoma of the cervix at various stages of development, and from women with in-situ cancer and cervical dysplasia,

might provide a means for the detection of pre-invasive neoplasia of the cervix. It has been suggested that such serologic test might differentiate between women with cervical dysplasia likely to develop into carcinoma of cervix and those who would not (Nahmias et al, 1980). Such a study could also demonstrate a progressive decline in the detection of C-TAA in post-operative sera from patients with carcinoma of cervix, thus suggesting that potential prognostic value of the procedure exists.

The findings that fewer injections were required for the preparation of antisera against the partially purified TAA of CACx. than against the crude antigen preparation, and the observation that the rabbit antisera against the partially purified CaCx. TAA were more readily adsorbed than those against the crude antigen preparation (Ibrahim et al, 1979; Adelusi, 1981), would suggest that use of further purification of TAA as immunogen might lead to the production of more specific antisera against CaCx. TAA. This might thus lead to detection of C-TAA more specifically. Even though some of these circulating antigens so far have proven to be embryonic in nature (Cinader, 1972; Lausch and Rapp, 1974), other circulating antigens in these patients have been shown to be tumour specific (Hollinshead et al, 1972; Nahmias et al, 1975; Ibrahim et al, 1976).

2.7:OBJECTIVES OF STUDY:

To date, there is no definitive information as to the number and types of TAA in cancer of the cervix, although progress is continuing, and TAA may eventually be standard laboratory tools for diagnosis and and immunotherapy of cancer. There is no doubt that the rewards of

isolation and purification of tumour associated antigens and tumour specific antibodies would be considerable.

The purified TAA would facilitate the determination of the exact relationship between the antigens of virally-induced tumours. and the cause of cancer. Furthermore, the prospects for a reliable diagnostic test may improve with the availability of purified antigens for production of monospecific antibodies against CaCx. for the detection of free antigens in the circulation of cancer patients. This information may relate to the extent of disease, the existence of metastasis or may provide a prognostic indicator.

The results obtained so far are promising for the development of a procedure for early diagnosis and monitoring prognosis of patients with carcinoma of cervix. It is the aim of this study to explore the possibility of further purification of the cancer of cervix antigens by other physico-chemical methods. Thus, the preparation of monospecific antibodies against the CaCx. antigens would enhance the detection of circulating TAA in the sera of cancer patients, and hence, aid in the early diagnosis of the disease.

CHAPTER 3

MATERIALS AND METHODS

3.1: STUDY MATERIALS

The study population and specimens consisted of the following:

a) Study Population:

i) The cancer bearing patients were 36 parous women who have clinically and histopathologically confirmed invasive carcinoma of the cervix. These are patients attending the gynaecological clinic of the University College Hospital, Ibadan, Nigeria. They are all Nigerians, of various ethnic origins. Their ages ranged between 25 and 66 years, with a mean of 40 years. The clinical stages of their disease varied from I to IV (FIGO Classification) (Table 3.1). The pathological grading of their malignancies ranged from I to III.

ii) There were 18 parous women with benign gynaecological problems and with no clinical or cytological evidence of carcinoma of cervix. Their ages ranged between 25 and 55 years, with a mean of 35 years, and were mostly from the same areas and socioeconomic classes as the cancer patients (Table 3.2). Their complaints were for benign gynaecologic diseases such as erosion of the cervix, cervicitis etc. and were selected mainly from the routine gynaecologic clinics.

TABLE 3.1

Age (Years)	I	II(a)	II(b)	III	IV	Total
≤ 25	1	1	0	0	1	3
26 - 35	2	4	1	2	0	9
36 - 45	1	1	6	4	0	12
46 - 55	0	1	3	2	1	7
56 - 65	0	0	2	2	0	4
66+	0	0	0	0	1	1
Total	4	7	12	10	3	36

$X^2 = 29.73$ on 20 df $0.10 > P > 0.05$

Age incidence and clinical stage of disease in patients with Carcinoma of the Cervix.

TABLE 3.2

Socio-Economic Class	I	II(a)	II(b)	III	IV	Total
Upper	0	0	0	0	0	0
Upper Middle	0	1	0	0	0	1
Lower Middle	3	1	5	4	1	14
Lower	1	5	7	6	2	21
Total	4	7	12	10	3	36

Analysis of Socio-economic classes of patients with carcinoma of cervix in relation to the clinical stage of the disease.

$X^2 = 7.63$ on 8 df $0.50 > P > 0.30$

iii) There were 72 pregnant women, whose ages ranged from 18 to 42 years, with a mean of 28 years. These were mainly women with normal pregnancies selected from the antenatal clinic of the University College Hospital, Ibadan, and the parity of the women ranged from 1 to 5. (Table 3.3).

iv) The healthy control women consisted of 36 women from the Family Planning Clinic of the University College Hospital, Ibadan, Nigeria. These had no gynaecologic problems. Their ages ranged from 21 to 40 years, with a mean of 36 years, and their parity ranged from 0 to 4.

b) Study Specimens:

i) Cancer Tissue Biopsies - Invasive carcinoma of cervix tissue biopsies were obtained from cancer patients in the study population at the time of surgery. Under sterile conditions in the operating theatre, patients were anaesthesized, cleaned and draped. The vagina was swabbed clean and biopsy specimens were taken from the cervix by punch forceps or as wedge resections. These were kept in sterile containers and stored at - 20° centigrade until processed.

ii) Normal tissue biopsies - Normal cervix tissue biopsies were obtained from about 10 post hysterectomy specimens of patients with uterine fibroids or ruptured uterus. The specimens were collected under sterile conditions and treated as in the cancer tissues above.

TABLE 3.3

Age (Years)	0	1	2	3	4	5	Total
< 20	2	1	0	0	0	0	3
21 - 25	2	6	8	2	0	0	18
26 - 30	1	6	12	1	2	0	22
31 - 35	1	2	7	4	2	1	17
36 - 40	0	2	4	1	2	1	10
41+	0	1	0	1	0	0	2
	6	20	31	9	4	2	72

Age incidence and parity of pregnant women in the study.

$X^2 = 35.06$ on 25 df $0.10 > P > 0.05$

iii) Serum Specimens - Serum samples were obtained from all the four groups in the study population. Under sterile conditions 10.0mls. of blood were obtained from each patient by vene-puncture. Each specimen of blood was allowed to clot at room temperature and serum was obtained from clotted blood by centrifugation. All sera were stored in 1.0ml aliquots, with two drops of 1:10,000 methiolate added to each vial to serve as preservative, and the specimens were kept at -20°C until tested.

3.2: TISSUE ANTIGEN PREPARATION

The following procedure was used for the preparation of antigens from normal and cancer tissues.

a) Tumour Tissue Antigens

i) The cancer tissue biopsies from all the tumour-bearing patients in the study population were pooled together and placed in a sterile petridish. Necrotic portions and fatty tissues were carefully removed as far as possible and discarded. The remaining tissues were weighed for purposes of determining the weight/volume (w/v) ratio tissue suspension in Earle's Balanced Salt Solution (EBSS) for homogenisation.

ii) The tissues were then minced thoroughly in about 5 to 10 mls. of EBSS containing 100 units of Penicillin/ml. and 100mg. of Streptomycin/ml (or such a value of EBSS that will permit thorough mincing of the tissues) using sterile scalpel and pair of scissors. The minced tissues

were transferred into 40.0ml. centrifuge tubes and washed three times with EBSS by centrifugation at 500 x g. for 30 minutes.

iii) A 20 per cent (w/v) tissue suspension in EBSS was prepared and the suspension was homogenized in the cold (with the glass tube held in an ice bath) by intermittent runs of one minute, for several cycles, using the Bunkman Polytron Homogenizer.

iv) The homogenate was centrifuged at 20,000 x g. for 30 minutes at 4°C, to yield Supernate-1 and Pellet-1. Supernate-1 was stored in the freezer.

v) Pellet-1 was re-suspended in EBSS to one-tenth of the original volume in a flask, and the cells ruptured by rapid freezing and thawing three times in an "acetone-dry ice" bath. The base of the flask containing the material was completely emmersed in the "acetone-dry ice" bath.

vi) The resultant material was centrifuged at 20,000 x g. for 30 minutes at 4°C, to yield Supernate-2 and Pellet-2.

vii) Supernate-1 and Supernate-2 were pooled together and concentrated 10-fold by dialysis against polyethylene glycol 20,000 (Cabowax flakes). The retentate was dialysed against Phosphate Buffered Saline (PBS) pH 7.6 for 48 hours at 4°C. This was then designated as the Soluble Antigen (SA) preparation.

viii) Pellet-2 was re-suspended in 3-Molar Potassium Chloride (KCl) in the proportion of 5ml. KCl per gram (of the initial weight) of the tissue. A few Crystals of Deoxyribonuclease (DNA-ase) were sprinkled to this, to prevent aggregation (Meltzer et al, 1971), and the suspension retained at $4^{o}C$ for 48 hours, with periodic mixing.

ix) The consequent material was centrifuged at 20,000 x g. for 30 minutes at $4^{o}C$, to yield Supernate-3 and Pellet-3. Pellet-3 was discarded.

x) Supernate-3 was dialysed for 24 hours against 200 volumes of de-ionized water at $4^{o}C$, with several changes of the dialysate. The resultant preparation was concentrated 4-fold as above, and re-dialysed again. This was designated as the Membrane Bound Antigen (MBA) preparation.

b) Normal Tissue Antigens:

i) The normal cervix specimens from all the individual post-operative biopsies in the study were pooled together and placed in a sterile petridish. These were weighed as above.

ii) The tissues were then minced thoroughly as for the cancer tissues, and the various steps for the preparation of the Soluble Antigens (SA) and the Membrane Bound Antigens (MBA) were followed as above.

iii) However, both the SA and the MBA for the normal cervical tissues were pooled together after the preparations for purposes of immunization and the various tests.

3.3: ANTISERA PREPARATION

Antisera were prepared, using two rabbits for each of the following immunogens:

a) Crude SA of the cancer tissues

b) Crude MBA of the cancer tissues

c) Partially purified SA of the cancer tissues

d) Partially purified MBA of the cancer tissues

e) Crude normal cervix (pooled SA and MBA antigen preparation) and

f) Pooled normal human serum.

a) Immunisation Procedure

i) Twelve (12) adult rabbits (3 to 5 month-old New Zealand White rabbits, each weighing 10 to 12.5 kilograms) were bled prior to the initiation of the process of immunization.

ii) The immunizing inoculum consisted of 1.0ml of the crude antigen preparation, or 0.5ml of the partially purified antigens, emulsified in an equal volume of complete Freund's Adjuvant.

iii) Each rabbit received 3 to 4 intramuscular injections of the immunogen at 10-day intervals.

iv) To maintain adequate levels of antibodies in each case, one booster dose of the immunogens were injected, without adjuvant, at 15 to 20-day intervals.

b) Collection of Antisera

i) Serum samples were obtained from each rabbit by simple incision opening of the ear marginal vein before each booster dose.

ii) When each rabbit has developed adequate precipitin reaction by the Immunodiffusion Adsorption (IDA) technique, large blood

samples (about 20 to 40mls.) were obtained.

iii) The serum was separated by centrifugation of clotted blood as previously described for human serum specimens, and were stored in 1.0 ml. aliquots at -20°C until used.

3.4: PARTIAL PURIFICATION OF TUMOUR ANTIGENS

For isolation of partially purified antigens, the crude antigen preparations (SA and MBA) were subjected to the following steps:

a) Protein concentration determination (Ibrahim et al, 1979)

5.0 mls. of filtered 1:5 dilution of Bio-Rad protein assay reagent (Bio-Rad Labs., Richmond, California), was added to 0.1ml. (100 ul) of the antigen preparation (or dilutions of this, if the protein concentration was too high to be read off in the spectrometer), and 0.1 ml. (100ul.) of PBS (as control). After thorough mixing, these were left at room temperature for 5 to 6 minutes, and the protein adsorbence at 595nm (wavelength) read off from the spectrometer, using the control to standardise the equipment. The protein concentration was then determined from the linear standard graph (Fig. 3.1).

Example - If the protein absorbence was 0.73, and the dilution of protein was 1:4. Then the protein

concentration at 0.73 = 120.0 ug/0.1 ml

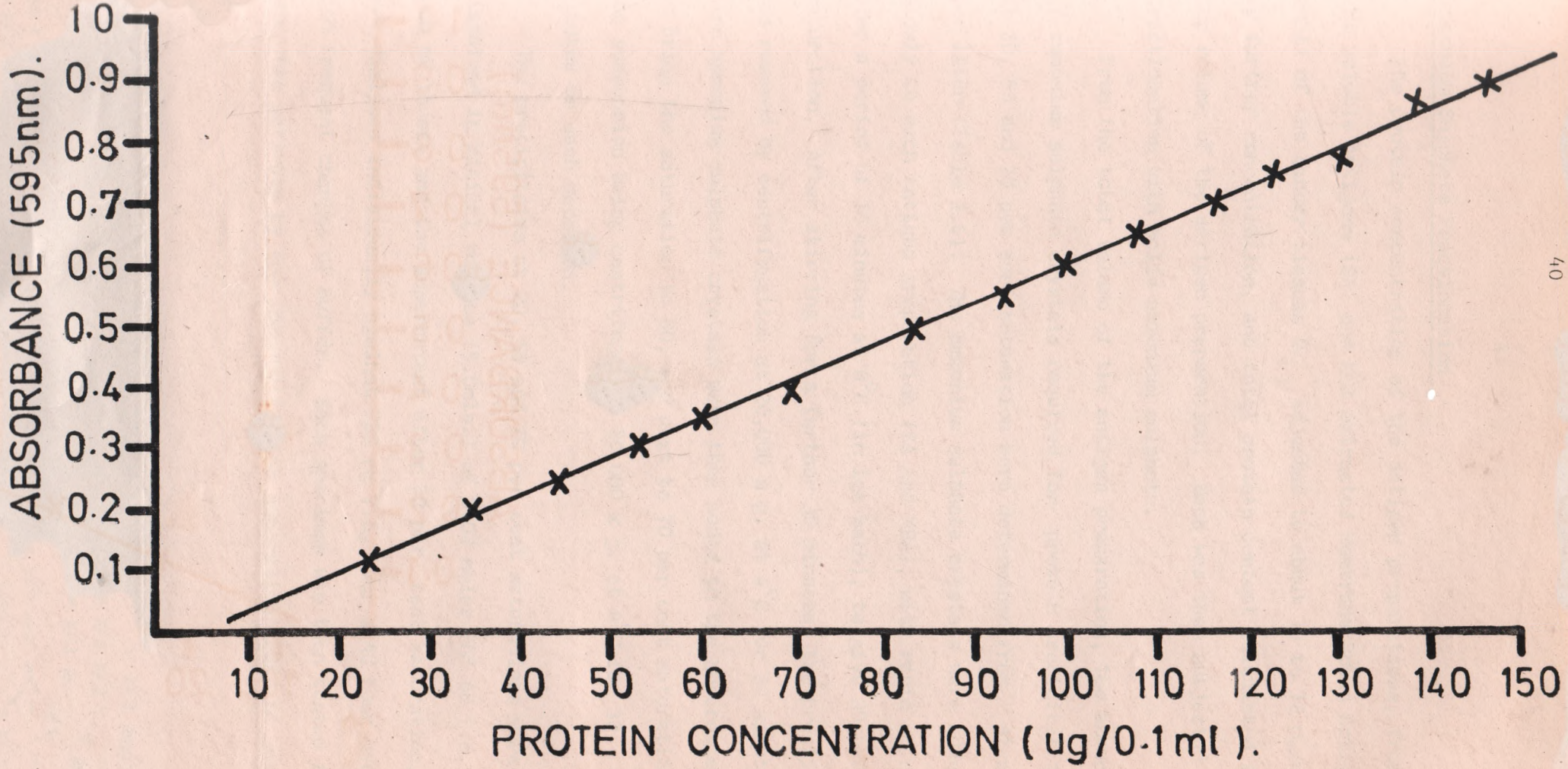
1:4 dilution = 120.0 X 4 ug/0.1ml.

= 480 ug/0.1 ml

Concentration = 4800ug/ml.

∴ Protein concentration = 4.8mg/ml.

BIO - RAD PROTEIN ASSAY



Graph for Protein Concentration Determination by Bio Rad Protein Assay Method

(Ibrahim et al, 1979)

b) Ammonium Sulphate Fractionation

The protein concentration of the antigen preparations, from the Soluble Antigens (SA) and KCl extracted Membrane Bound Antigens (MBA) of the cancer tissues were adjusted to about 10 to 12 mg/ml by further concentration, and total protein content calculated from the volume of the antigen preparation. Each was then subjected to fractionation with solid ammonium sulphate.

From the total volume of the antigen preparation, the amounts of ammonium sulphate crystals required for protein precipitation at 20, 50 and 70 per cent saturation were determined from standards per liter (Table 3.4). The ammonium sulphate crystals were added slowly to each antigen preparation (SA and MBA), with rapid stirring over a period of 10 minutes at 0°C (in ice bath), to give 20 per cent saturation. After stirring for a further 30 minutes, the precipitate was removed by centrifugation at 20,000 x g. at 4°C for 30 minutes. More ammonium sulphate crystals were then added to the supernatant to bring the saturation to 50, and next to 70 per cent saturation, the suspension being centrifuged at 20,000 x g. at 4°C for 30 minutes on each occasion.

The precipitates at 20, 50 and 70 per cent saturations were dissolved in minimal volumes (5.0mls.) of 0.02 molar PBS pH 7.6. Each of these, and the supernatant after 70 per cent saturation, were dialysed individually against PBS pH 7.6, over a 48 hour period, with frequent changes of buffer. Each specimen was then tested for CaCx. TAA activity by the immunodiffusion adsorption (IDA) in gel

Per Cent Saturation--Final Concentration of Ammonium Sulfate

Per Cent Saturation
Initial Concentration of Ammonium Sulfate

	10	15	20	25	30	33	35	40	45	50	55	60	65	70	75	80	85	90	95	100
0	56	84	114	114	176	196	209	243	277	313	315	390	430	472	516	561	610	662	713	767
10	-	28	57	86	118	137	150	183	216	251	288	326	365	406	449	494	540	592	640	694
15	-	-	28	57	88	107	120	153	185	220	256	294	333	373	415	459	506	556	505	657
20	-	-	-	29	59	78	91	123	155	189	225	262	300	340	382	424	471	520	569	619
25	-	-	-	-	30	49	61	93	125	158	193	230	267	307	348	390	436	485	533	583
30	-	-	-	-	-	19	30	62	94	127	162	198	235	273	314	356	401	449	496	546
33	-	-	-	-	-	-	12	43	74	107	142	177	214	252	292	333	378	426	472	522
35	-	-	-	-	-	-	-	31	63	94	129	164	200	238	278	319	364	411	457	506
40	-	-	-	-	-	-	-	-	31	63	97	132	168	205	245	285	328	375	420	469
45	-	-	-	-	-	-	-	-	-	32	65	99	134	171	210	250	293	339	383	431
50	-	-	-	-	-	-	-	-	-	-	33	66	101	137	176	214	256	302	345	392
55	-	-	-	-	-	-	-	-	-	-	-	33	67	103	141	179	220	264	307	353
60	-	-	-	-	-	-	-	-	-	-	-	-	34	69	105	143	183	227	269	314
65	-	-	-	-	-	-	-	-	-	-	-	-	-	34	70	107	147	190	232	276
70	-	-	-	-	-	-	-	-	-	-	-	-	-	-	35	72	111	153	194	237
75	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	36	74	115	155	198
80	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	38	77	117	157
85	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	39	77	118
90	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	38	77
95	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	39

Percent saturation of ammonium sulphate for Precipitation of Proteins in the CaCx antigen preparations at 25°C.

technique. The protein concentration of each fraction was next determined, and those reactive fractions (with TAA activity) were subjected to the next stage of purification.

c) DEAE Sephadex A-25 Ion Exchange Chromatography

The TAA-active fractions (20-50% and 50-70% ammonium sulphate precipitate) from the Soluble Antigens and KCl extracted Membrane Bound Antigens (Table 3.5) were pumped separately into a column (50 X 1.6 cm.) of Diethyl amino-ethyl (DEAE) Sephadex A-25 (Pharmacia Fine Chemicals, AB, Uppsala, Sweden) Ion Exchange Chromatography column equilibrated with 0.02 Molar Tris HCl, (pH 8.0) buffer. The flow rate of the buffer was calibrated to 30.0ml per hour.

i) Preparation and Packing of Column - The amount of Ion Exchanger (gel) required was stirred into an excess of starting buffer (Tris HCl, 0.02 Molar, pH 8.0) and swollen over a 48 hour period, at room temperature. Vigorous strirring was avoided in order not to damage the gel beads. The buffer was removed and replaced with fresh buffer several times during the swelling period (using a Buckner funnel), to ensure equilibration of the gel with the buffer.

The column was set up vertically, with the bed support covered by about 1 centimeter of eluent to ensure that no air remains in, or under the bed support, and that the column outlet is closed. The swollen gel was mixed thoroughly with the buffer to facilitate easy pouring, and avoid trapping air bubbles. This was poured gently down the column (down the side of

TABLE 3.5

Ca. Cx Antigen Prep.	Amm. Sulphate Precipitation			
	0 - 20%	20 - 50%	50 - 70%	>70%
Soluble Antigen	-	++	+	-
Membrane Bound Antigen	-	+	+	-

Demonstration of TAA activity in the ammonium sulphate fractions of the CaCx antigen preparations.

the chromatographic tube to avoid bubble formation), and allowed to stabilise. Excess elument was removed and more gel was poured in repeatedly until the required height of the column was reached. The column was then set up in cold chamber ($4^{o}C$) and equilibrated with the starting buffer over a period of 12 hours. This also stabilized the bed.

ii) Separation of Antigens - The column was connected to a fractions collector (LKB 2112 Redirac) designed to deliver constant volumes through the Spectrometer (Wavelength 280 um) into collection tubes. The elument (Tris HCl, 0.02 Molar, pH 8.0) was also connected to the column from above. The antigen fraction was gently layered on top of the gel, using a capillary tubing. The absorbence/Fluorescence analyser (Recorder), having been warmed up for about 2 hours prior to the fraction collection to adjust to optimum working conditions, was adjusted to a lamp current of 340 milliamps. The sensitivity and baseline of the recorder were adjusted as appropriate and the chart speed was set at 3.0 cm. per hour.

iii) Sodium Chloride Continuous Gradient Elusion - Elusion was performed, using a linear gradient of 0.02 to 0.1 Molar Tris HCl pH 8.0 containing 1.0 Molar sodium chloride. For this procedure, an equal amount of 0.1 Molar, Tris HCl, pH 8.0 was connected to 0.02 Molar Tris HCl, pH 8.0, using the same gradient apparatus for the solutions. This was connected to the column with the 0.02 Molar Tris HCl, pH 8.0 buffer beaker next to the column and a total of 200 ml. of the gradient was run through. Separation of antigen fraction collected in tubes within the recorded peaks was pooled, concentrated

against carbowax, dialysed against PBS, pH 7.6 and tested for:

a) TAA activity, using adsorbed anti-CaCx. serum by the IDA technique,

b) purity from normal tissue antigenic components, using unadsorbed anti-NCx and anti-NHuS sera

c) the protein concentrations and

d) titer of TAA activity.

d) Sephacryl S-200 Gel Filtration

Each of the active fractions from the DEAE Sephadex A-25 Ion Exchange Chromatography were next pumped separately into a column (100 X 1.6 cm) of Sephacryl S-200 (Pharmacia Fine Chemicals, AB, Uppsala, Sweden) gel filtration column, equilibrated with 0.02 Molar Tris HCl, pH 8.0 buffer. The flow rate was calibrated to 8.0 ml. per hour.

i) Preparation and Packing of Column - Sephacryl S-200 was hydrated with 0.02 Molar Tris HCl, pH 8.0 over a period of 3 days, avoiding excessive stirring to prevent rupture of the gel beads. The gel was washed three times with more buffer, and the final gel slurry (1 part buffer to 2 parts settled gel), was de-aerated before packing.

The column tube was mounted vertically and the dead space under the net and in the tube filled with eluent. The slurry was poured gently into the column, fitted with a flow adaptor at the bottom, and a gel and eluent reservoir which had been previously filled to approximately 15 per cent of its height with buffer, at the top. The gel was allowed to settle for

about 20 minutes prior to the opening of the column outlet. Hydrostatic pressure, measured from the liquid level in the gel reservoir to the tip of the outlet tubing, was maintained at 15 cm. Washing of the gel continued until a constant height (approximately 100 cm.) was attained, at which time an upper flow adaptor was intalled. The column was futher equilibrated by passage of two bed volumes of the Tris HCl buffer.

ii) Separation of Antigens - The TAA-active preparations as previously fractionated by DEAE Sephadex A-25 Ion Exchange Chromatography, was each applied to the bottom of the gel bed by capillary tubing. The column was connected to the fraction collector (LKB 2112 Redirac) and the eluent, with the various adjustments of lamp current and sensitivity. The antigen was eluted by upward flow of 8.0 ml per hour. Protein concentration in the effluent was monitored and recorded by an ultraviolet analyser.

The discharged material was collected in fractions by use of the fraction collector, and the individual fractions collected in tubes within the recorded peaks were pooled and concentrated 4-fold and dialysed against PBS pH 7.6. Each fraction was then tested for TAA activity, purity, protein concentration and titer of TAA activity as before.

e) Affinity Chromatography

Aminohexyl (AH) Sepharose 4B (Sigma Chemical Company, St. Louis, Mo, USA) activated with carbodiimide HCl, at acid pH, was used to immobilise both anti-NCx and anti-NHuS in a convenient one-step coupling method.

The required amount of freeze-dried powder of AH-Sepharose 4B (1 gm. of powder for every 200 mg. of protein to be coupled), was swollen in 0.5 Molar NaCl solution on a glass filter, and washed for 15 minutes with the same solution. Approximately 200 ml. solution was added in several aliquots for each gram of dry powder, and 100 gm. of the dried material gave a final gel volume of approximately 4.0 ml. Next, the required amount of 1-Ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) hydrochloride (Sigma Chemical Company, St. Louis, Mo, USA) was dissolved in de-ionized water (10 mg. of EDC per ml. of the gel) and mixed with the gel.

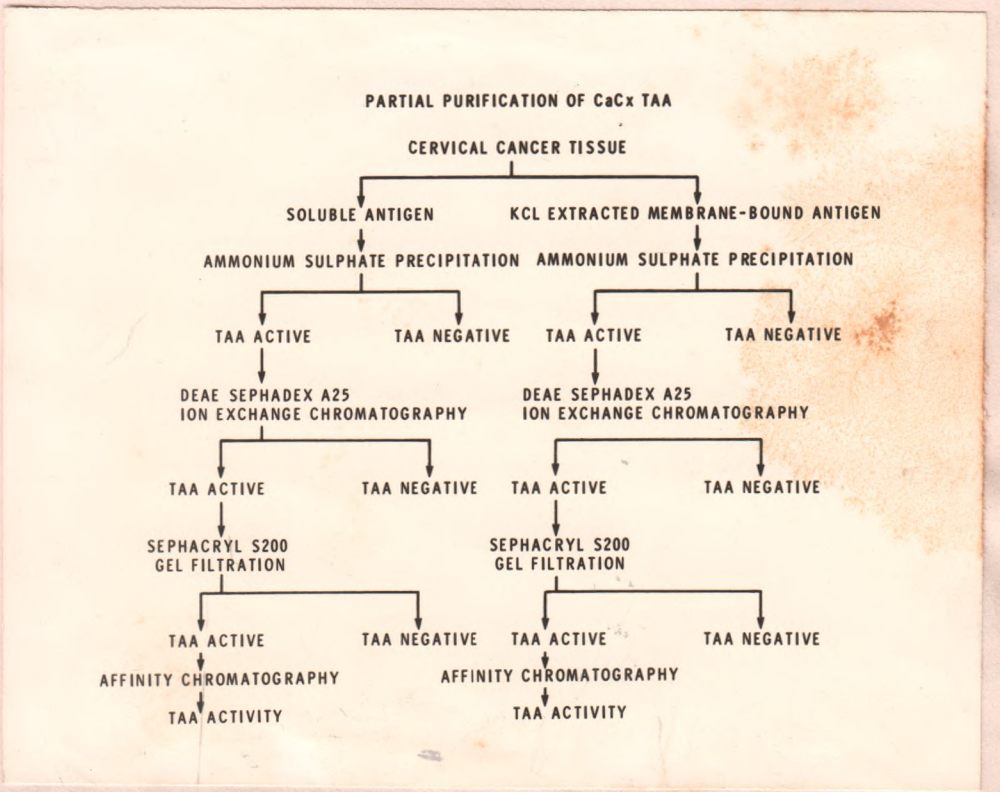
The sera to be coupled (rabbit anti-NCx serum and rabbit anti-NHuS serum, which had been partially purified by ammonium sulphate fractionation at 50 per cent saturation, and their protein concentrations determined) were mixed each with half the gel slurry (100 mg. per gm. of gel). The pH of the mixtures were adjusted to 5.0 by adding dilute 0.2 Molar HCl, and the mixtures were gently rotated in sealed containers overnight at room temperature.

The mixtures were poured on to glass filters and the eluents from each collected. The protein concentration of each was determined to ensure that coupling has occurred. After coupling was complete, the two halves of the gel slurry (coupled anti-NCx and anti-NHuS immunoglobulins) were mixed and washed with several aliquots of the coupling solution (de-ionized water) to remove uncoupled proteins. The gel slurry was reconstituted in Borate buffer pH 8.6 to be used in the affinity chromatography.

The antigen preparation obtained from Sephacryl S-200 gel filtration experiment, was mixed with the gel slurry and allowed to react for 24 hours at $4^{o}C$. A maximum initial concentration of 1.0 mg. of protein per ml. of Sepharose-protein conjugate was used. On completion of affinity chromatography, the gel was washed on glass filter to elute the antigen completely. This was dialysed against de-ionized water for 24 hours to get rid of all salt (Borate buffer salt), and concentrated against Borax wax. Each of the chromatographed antigen fractions was tested for TAA activity, purity, protein concentration and titer of TAA activity as usual.

In summary, by the various purification steps (Fig. 3.2), attempts were made to separate the TAA components in the cancer tissue antigen preparation by various methods of purification of proteins. The protein components that contain no TAA activity were thus disposed of, while those that contain the TAA were further purified.

FIG. 3.2



Summary of Step-wise Purification Procedures of the CaCx. Antigens.

3.5: SEROLOGIC TESTS

a) Immunodiffusion

The Ouchterlony microimmuno-precipitation technique of double diffusion in agar, described by Ibrahim and Hammon (1968), was followed. Pre-cleaned slides (35 x 75mm) were coated with 0.2 per cent suspension of noble agar (Difco Laboratories) in Borate buffer, pH 8.6 and allowed to dry at room temperature. A 3.5 ml. sample of 1.0 per cent melted agar in Borate buffer, or a 1.0 per cent agarose suspension in Barbital buffer, pH 8.6 was prepared onto each slide. Once the gel had hardened, the slides were kept in humidified chambers and stored at 4°C. A stainless steel template was used to cut a pattern of central well (7 mm. in diameter), and six peripheral wells (5 mm each) in the gel, the distance between the central well and each peripheral well, and between adjacent peripheral wells being 5 mm. In general, homologous and heterologous antigen preparations were introduced into the peripheral wells, and test antiserum into the central well. All slides were incubated in humidified chambers at 37°C, and the results read and recorded daily for 4 days.

b) Adsorption-in-Gel Immunoprecipitation Technique:

The immunoadsorption-in-gel technique described by Ibrahim (1969) was used. The procedure is designed to eliminate cross-activity between antiserum prepared against tumour antigen extracts and normal tissue and normal serum antigens (Tumour contaminants).

Adsorption of anti-tumour antisera was achieved by the introduction of the adsorbing antigen (NCx and NHuS in sequence) into the central well, followed by incubation at 37°C for 120 minutes. The introduction of NCx into the central well and incubation was followed by aspiration of the residual NCx before replacement with NHuS and incubation. After the aspiration of all residual adsorbing antigen, the antiserum to be adsorbed (Anti-CaCx serum) was introduced into the central well, and the peripheral wells were filled with the homologous antigen preparation and appropriate controls with which to determine the adequacy of the adsorption process. The test slides were incubated in humidified chambers at 37 °C for 24 to 96 hours, and the results read and recorded daily.

c) Immuno-electrophoresis Technique:

A modification of the single dimension immunoelectrophoresis (IE) method detailed by Ibrahim and Hammon (1968b) was used. Pre-cleaned glass slides (50 x 75 mm) were coated and layered with 0.1 ml. of agar as described for immunodiffusion. A template was employed to cut two central troughs (2 x 50 mm), and three wells (5 mm. diameter) at a distance of 5 mm. between each trough and well (one well between the two troughs and one well outside each trough), about midpoint of the troughs.

Each outside well was filled with a fraction of the test antigen preparation and the central well with the control antigen preparation. The slides were placed in an

electrophoretic tank (Gelman), and electrode bridges were made by six layers of moist Whatman's filter paper number 1. The antigens were electrophoresed at room temperature for 120 minutes at 5 volts per centimeter of gel (as demonstrated by appropriate dye). Constant power was supplied by a power source (Gelman Model 38201).

After electrophoresis, the slides were transferred to humidified chambers and the gel from the troughs removed. The troughs were filled with the test antiserum, and the slides were incubated at 37°C for 24 to 96 hours and the results read and recorded daily.

d) Adsorption-in-Gel Immunoelectrophoresis Technique:

The single dimension immunoelectrophoresis (IE) method was carried out as above. After immunoelectrophoresis, the slides were transferred to humidified chambers and the gel from the troughs removed. Adsorption of the anti-tumour antisera was carried out in the troughs with NCx and NHuS, as detailed above (Adsorption-in-Gel immunoprecipitation). After the aspiration of the remnants of the adsorbing antigens, the test antiserum to be adsorbed was introduced into the troughs and the slides were incubated in humidified chamber at 37°C for 24 to 96 hours, and the results were read and recorded daily.

e) Reading and Recording of Results:

To visualise the precipitin bands in their gel background, the slide was positioned at an oblique angle to a flourescent desk lamp, and a 4X Magnifying lens was used as an aid in

viewing the precipitin bands in the slides. A daily record of the bands was kept on prepared sheets with patterns made after the template.

To photograph the precipitin bands in their gel background, a viewing box with dark field illumination was used. Light was directed through the gel at an oblique angle from the inside of the box. A 35mm reflex camera with an extremely high resolution panchromatic film (Kodak High Contrast Copy M.136-36) was used for making permanent records.

3.6: CLINICO-PATHOLOGIC STUDIES

a) Clinical Studies:

Clinical data were collected from each patient with regard to age, and socio-economic status based on the level of education, occupation and family income. Details of the coital practice were assessed with regards to the marital status and the number of marriages, the age at first coitus, number of sex partners and frequency of coitus per week. The number of pregnancies, including abortions, as well as the number of living children were ascertained (Appendices III and IV)

All the patients were examined clinically. During pelvic examination, cervical and posterior vaginal smears were made on clean microscopic slides (25 x 75 mm). These were fixed in acetone at $4^{\circ}C$ and kept until processed for cytologic examination by the Papanicolau method. Microscopic examination was performed as previously described (Adelusi, 1976). During the clinical examination, the stage of the malignancy of the

cervix (FIGO Classification) was assessed, and biopsy materials (punch, cone or wedge) were taken for histopathologic examination.

b) Histopathologic Studies:

The microscopic diagnosis of the malignancy was based on the two chief characteristics of abnormalities in the constituent cells, and the abnormal architecture of the tissue cells. The histologic type of cell was determined on whether it was epidermoid or mucin secreting, well-differentiated cylinderic cells. Based on the cellular architecture, Grades I, II or III were recognised (Adelusi, 1976).

i) Grade I (Keratinising Carcinoma) - This was characterised by a predominance of large abnormal epithelial cells with a high degree of pleomorphism, bizzare, elongate and caudate forms, with moderately large, hyperchromatic, coarsely granualr, chromatin nuclei. Isolated nuclei and syncytia were relatively rare and epithelial pearls and isolated cell keratinisation were present. A low mitotic index was commonly observed. This histologic structure was that of a well differentiated squamous cell carcinoma of the cervix.

ii) Grade II (Large cell non-Keratinising Carcinoma) - This was characterised by many abnormal basophilic epithelial cells, varying in size. A high nuclear-cytoplasmic ratio was characteristic. The nuclei were opaque with coarse chromatin pattern and frequent micro nucleioli. Isolated nuclei and syncytia were more common than in the other two grades. There was a differentiated squamous cell

carcinoma with rare isolated cell keratinisation, and no epithelial pearls. There was a distinct variation in cell size, and moderately high mitotic index. The histologic structure in this was that of poorly differentiated squamous cell carcinoma of the cervix.

iii) Grade III (Small Cell Carcinoma) - This was characterised by predominance of uniformly small basophilic cells with a high nuclear-cytoplasmic ratio. The nuclei were opaque or coarsely granular. Macro nucleoli and syncytia were not as common as in Grade II. The histological architecture was remarkable for the uniformity of cell and nuclear size. Epithelial pearls and isolated keratinized cells were not observed, and a high mitotic index was common. The histologic structure was that of undifferentiated cell carcinoma of the cervix

Finally the results of the detection of circulating TAA (C-TAA) in the sera of tumour-bearing patients were correlated with the various clinico-pathologic findings, particularly the clinical stage and grade of the disease, and the findings were analysed statistically.

CHAPTER 4

RESULTS.

Two rabbits were each hyperimmunized with:

a) Crude Soluble Antigen (SA) of the carcinoma of cervix (CaCx.) tissues preparation;

b) Crude Membrane Bound Antigen (MBA) of the CaCx tissue preparations;

c) Crude Mixture of the SA and MBA of the cancer tissue preparations;

d) The partially purified SA preparation of the cancer tissue;

e) Normal Cervix (NCx) tissue antigen preparation; and

f) Normal Human Serum (NHuS) pool. There was not enough of the partially purified MBA for any immunisation procedures.

Subsequent to the third injection, the rabbits gave immunoprecipitation reactions with the homologous antigens and were given booster doses of the appropriate immunogen. The reactions intensified with these subsequent immunisations, and stabilised by the fifth or sixth inoculation. The most strongly reactive antiserum of each antigen was utilised for further test procedures.

4.1: DEMONSTRATION OF TAA IN CANCER TISSUE PREPARATIONS:

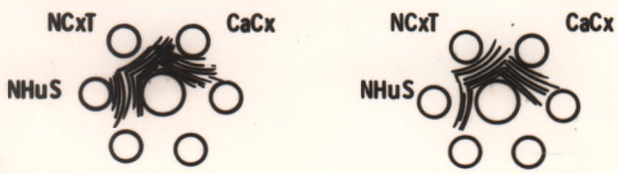
In a pilot study to demonstrate TAA in CaCx. tissues, immunodiffusion (ID) tests of the rabbit antiserum against the crude mixture of the SA and MBA of the cancer antigen preparations demonstrated 8 to 10 immunoprecipitin lines with the same cancer antigen preparation, 4 to 6 precipitin lines with normal cervix (NCx) tissue antigen

preparation and 6 to 8 precipitin lines with normal human serum (NHuS) pool (Fig. 4.1a). On the other hand, ID tests of the rabbit antiserum against NCx showed 4 to 6 precipitin lines with the cancer antigen preparation, 5 to 6 precipitin lines with NCx tissue antigen preparation, and 3 to 4 precipitin lines with NHuS pool (Fig. 4.1b). Similarly, ID tests of the rabbit antiserum against NHuS showed 6 to 8 precipitin lines with the cancer antigen preparation, 4 to 6 precipitin lines with NCx tissue antigen preparation and 10 to 12 precipitin lines with the NHuS pool (Fig. 4.1c).

Some variations in the number of precipitin lines were noted with different antisera tested, and with different tests. However, the consistency of the number of precipitin lines, on the average, was satisfactory. When the pilot study was repeated with the antiserum prepared against the crude SA and the crude MBA preparations respectively, similar results to that of the mixture of the antigens (SA and MBA) were obtained, although the number of precipitin lines were less.

In the definitive study, in-gel adsorption of the crude cancer antisera with NCx preparation and NHuS pool for 120 minutes consecutively (IDA technique) demonstrated precipitin lines only with the CaCx. antigen preparation, but not with NCx and NHuS. Three immunoprecipitin lines, designated TAA-1, TAA-2 and TAA-3, were consistently observed with the Soluble Antigen preparation. Only one precipitin line, probably identical to TAA-3 of the Soluble Antigen preparation, was demonstrated in the Membrane Bound Antigen preparation (Fig. 4.2). This was close to and curving towards the antigen well in the template.

FIG. 4.1

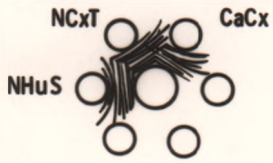


Rabbit anti CaCx

a

Rabbit anti NCx

b

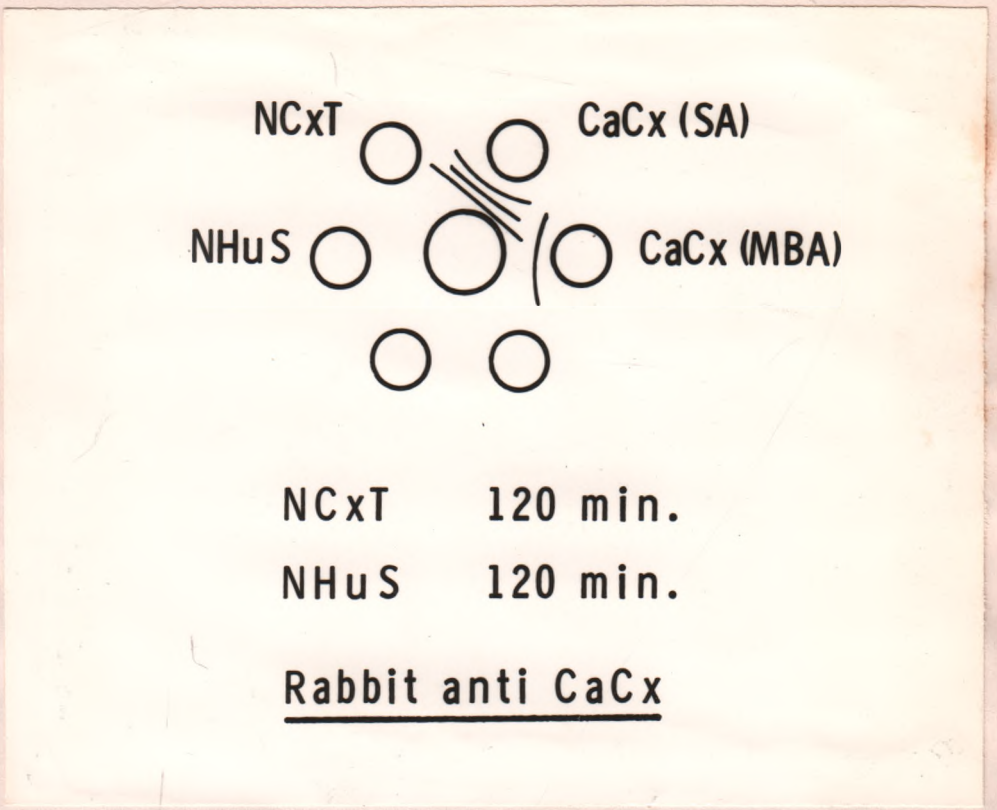


Rabbit anti NHuS

c

Schematic Representation of Immunodiffusion Reactions of Rabbit anti-CaCx, anti-NCx and anti-NHuS sera against Cervical Antigen Preparations.

FIG. 4.2



Schematic Representation of Immunodiffusion Reactions Between Adsorbed Rabbit anti-CaCx Sera with CaCx Soluble and Membrane Bound Antigens.

When the cancer antigen preparation was titrated to show at what dilution the various TAA would cease to be demonstrated with the crude cancer antisera, it was found that the concentration of the TAA varied one from the other. TAA-1 was demonstrated at 1:16 dilution whereas, TAA-2 was demonstrated at 1:8 dilution and TAA-3 was present only at 1:2 dilution. The titers of the three TAA in the cancer antigen preparation were recorded therefore as 16, 8 and 2 respectively.

None of the pre-immune serum samples reacted with either the homologous or heterologous antigen preparations. Similarly, the rabbit antisera against the NCx antigen preparation and the NHuS, when adsorbed with both the NCx tissue preparation and NHuS consecutively as above, failed to react with the homologous as well as the heretologous antigen preparations.

4.2:PARTIALLY PURIFIED TAA ACTIVITY:

Immunodiffusion (ID) and immunodiffusion adsorption (IDA)-in-gel test, were carried out on the various partially purified specimens of the cancer antigens:

a) Fractionation of TAA by Ammonium Sulphate Precipitation:

The TAA of CaCx. were constantly precipitated at salt saturations between 20 and 70 per cent. The majority of the TAA activity appeared to be present in the 20 to 50 per cent ammonium sulphate saturation fraction however (Table 3.5).

Immunodiffusion adsorption in-gel of rabbit anti-CaCx. (with NCx and NHuS, each for 120 minutes), showed 3 TAA (TAA-1, -2, and -3) with

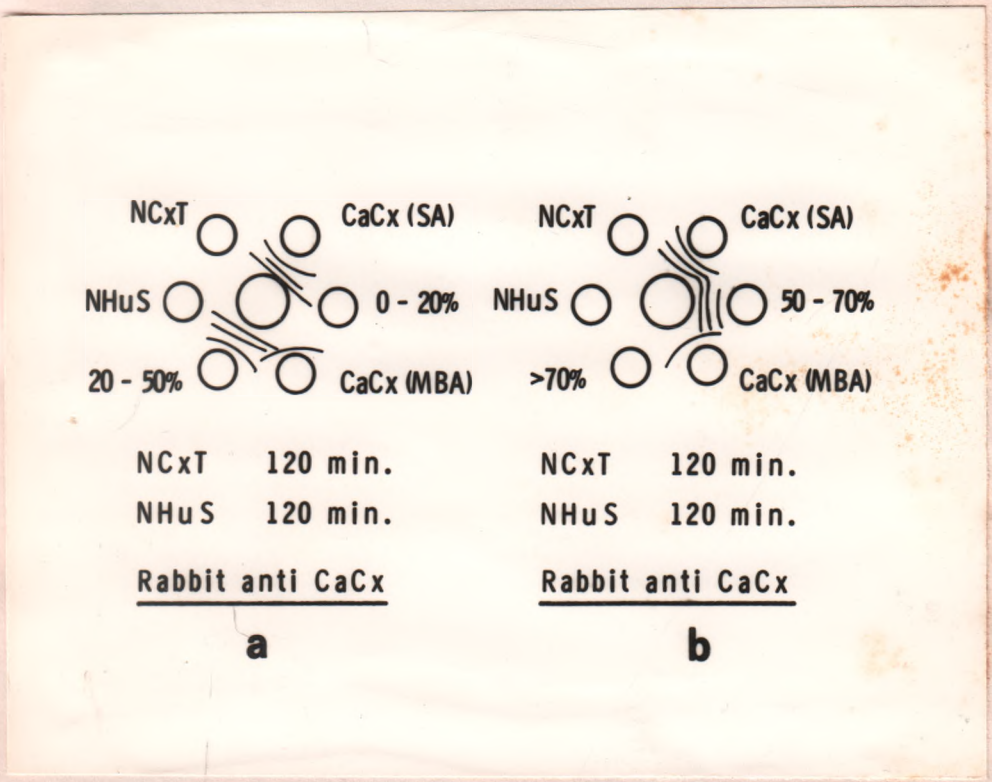
the 20-50% ammonium sulphate fraction, and also three TAA (TAA-1, -2, and -3) with the 50-70% fraction from the Soluble Antigen (SA) preparation. Both the 0-20% and the over 70% ammonium sulphate saturation fractions showed no reactions (Fig. 4.3).

Similarly, only the 20 to 70 per cent of the ammonium sulphate precipitated fraction of the Membrane Bound Antigen (MBA) preparation showed TAA activity (Fig. 4.4), and this was identical to TAA-3 of the SA. The resolution of the individual tumour-specific precipitin bands was generally enhanced without a corresponding loss in the actual number of precipitin reactions. While the number and intensity of reactive normal components of the tumour extract was visibly diminished by fractionation when reacted against the unadsorbed rabbit antisera to CaCx, the reduction was only minimal.

The protein concentration of the various ammonium sulphate precipitated fractions as well as the titers of the reactive TAA are shown in Table 4.1. From the starting protein content of 375 mg. of the CaCx SA preparation, TAA activity was demonstrated in 265 mg. contained in the 20 to 70 per cent ammonium sulphate precipitated fraction, and the titers of the various TAA activity were 16, 8 and 2 for TAA-1, TAA-2 and TAA-3 respectively.

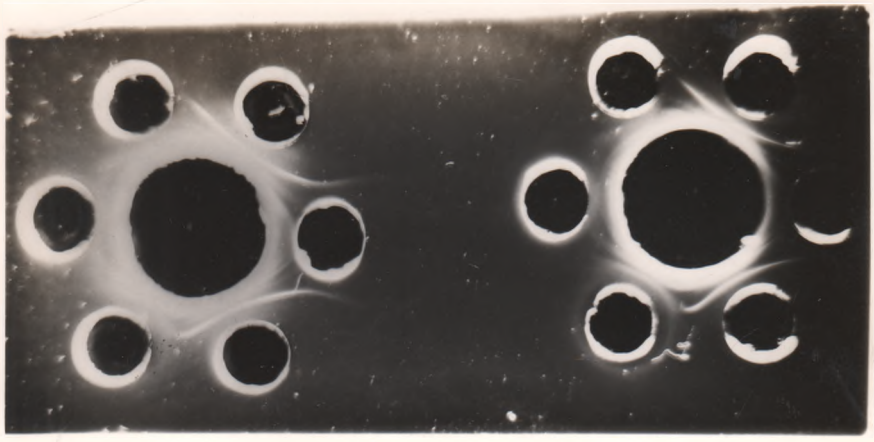
On the other hand, only the 80 mg. of protein contained in the 20 to 70 per cent salt fraction from the MBA preparation demonstrated TAA activity, and the titer of TAA activity, probably TAA-3, was found to be 8. The TAA active fraction (20-70%) were pooled for each antigen preparation for the next step of purification.

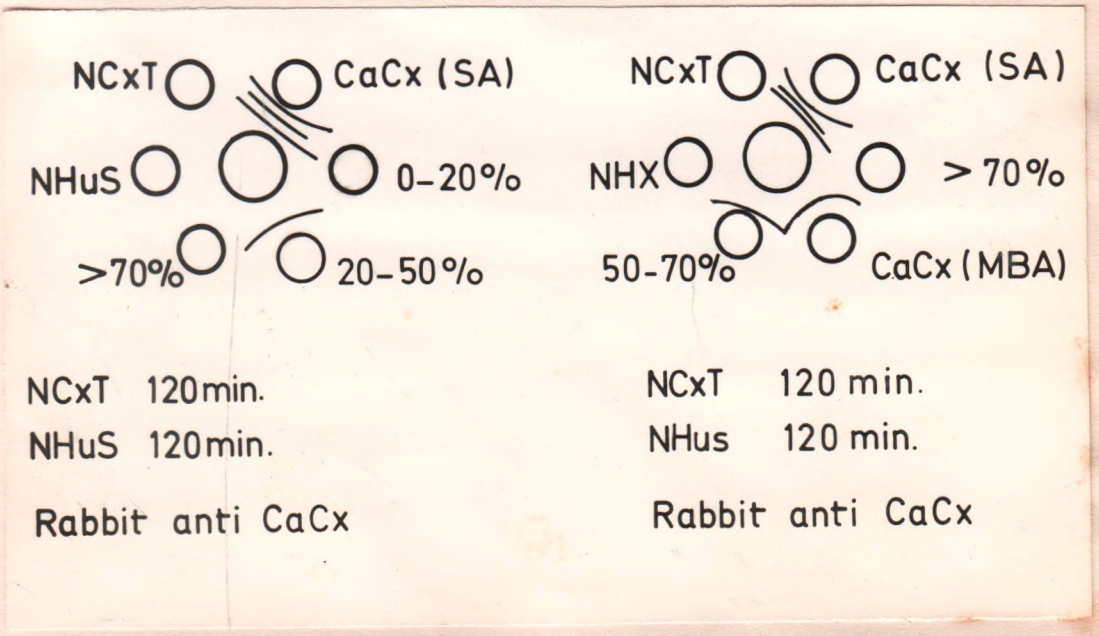
FIG. 4.3



Schematic Representation of Immunodiffusion Adsorption-in-Gel Reactions between anti-CaCx Sera and Ammonium Sulphate Fractions of CaCx Soluble Antigens.

FIG. 4.4





Schematic Representation of Immunodiffusion Adsorption-in-Gel Reactions of Rabbit anti-CaCx Sera with Ammonium Sulphate Fractions of CaCx Membrane Bound Antigen.

TABLE 4.1

ANTIGEN	Volume ml	Protein mg/ml	Total Protein mg	I.D. Titer	Comments
1. CaCx (SA) 375 mg					
0 - 20%	3.5	15.0	52.5	-	No TAA activity
20 - 50%	4.0	38.0	152.0	16,8,2	Active TAA-1,-2 & -3
50 - 70%	3.5	32.5	113.8	16,8,2	Active TAA-2,-2 & -3
> 70%	2.5	10.0	25.0	-	No TAA activity
2. CaCx (MBA) 196 mg					
0 - 20%	5.0	4.5	22.5	-	No TAA activity
20 - 50%	5.0	7.0	35.0	8	Active TAA (?TAA-3)
50 - 70%	10.0	4.5	45.0	8	Active TAA (?TAA-3)
> 70%	6.0	5.0	30.0	-	No TAA activity

Protein concentrations and titer of TAA activity in the ammonium sulphate fractions of CaCx antigens.

b) DEAE Ion Exchange Chromatography -

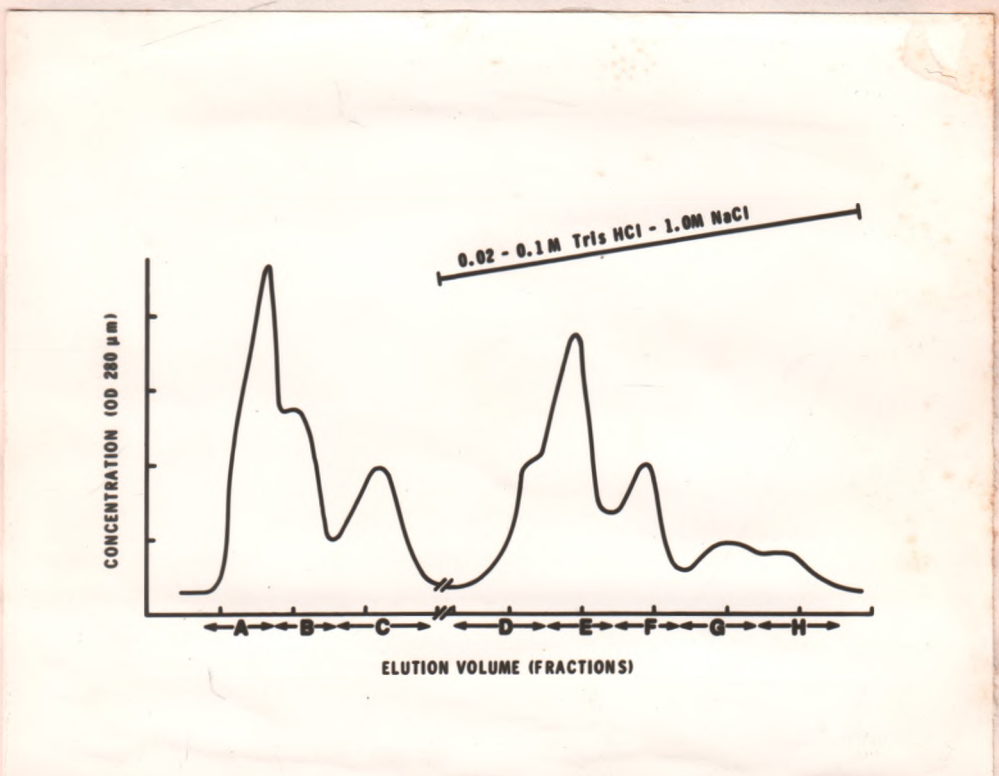
When the 20-70% ammonium sulphate fraction of the Soluble Antigen preparation was passed through the DEAE A-25 Ion Exchange Chromatographic column, the effluent record contained eight individual peaks (A to H), three before and five after the Sodium Chloride elution (Fig. 4.5).

Immunodiffusion adsorption in-gel of the individual fractions pooled from the peaks indicated the presence of immunologically reactive proteins within four of the eight peaks, two before and two after the Sodium Chloride elution. Fractions A and B, designated the "Unbound Fractions" of the SA preparation, demonstrated precipitin lines, both identical to TAA-2, and TAA-3, while fractions D and E, designated the "Bound Fractions" showed precipitin lines identical to TAA-1, (Fig. 4.6).

On the other hand, the 20-70% ammonium sulphate fraction of the Membrane Bound Antigen preparation when passed through DEAE A-25 Ion Exchange Chromatography, produced five individual peaks (A to E), two before and three after Sodium Chloride elution (Fig. 4.7).

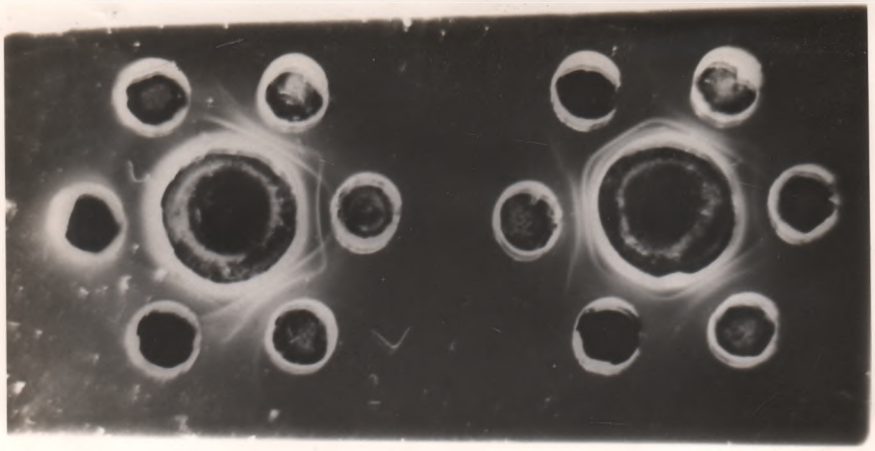
Immunodiffusion adsorption in-gel of the individual fractions pooled from the peaks indicated the presence of immunologically reactive proteins within two of the five peaks, both before the Sodium Chloride elution. Fractions A and B designated "Unbound Fractions", demonstrated precipitin lines both identical to TAA-3 (Fig. 4.8).

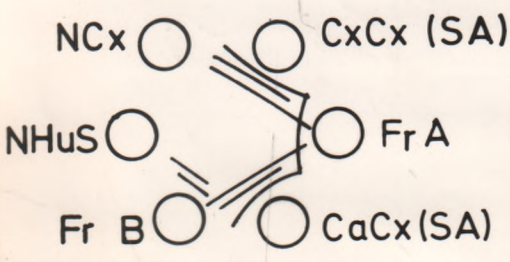
FIG. 4.5



Profile of the DEAE Ion Exchange Chromatography of the 20-70% Ammonium Sulphate Fraction of the CaCx Soluble Antigen Preparation.

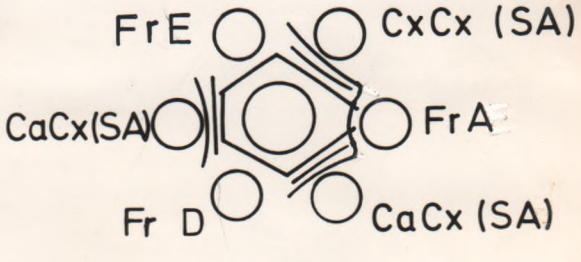
FIG. 4.6





NCxT 120 min.
NHuS 120 min.

Rabbir anti CaCx

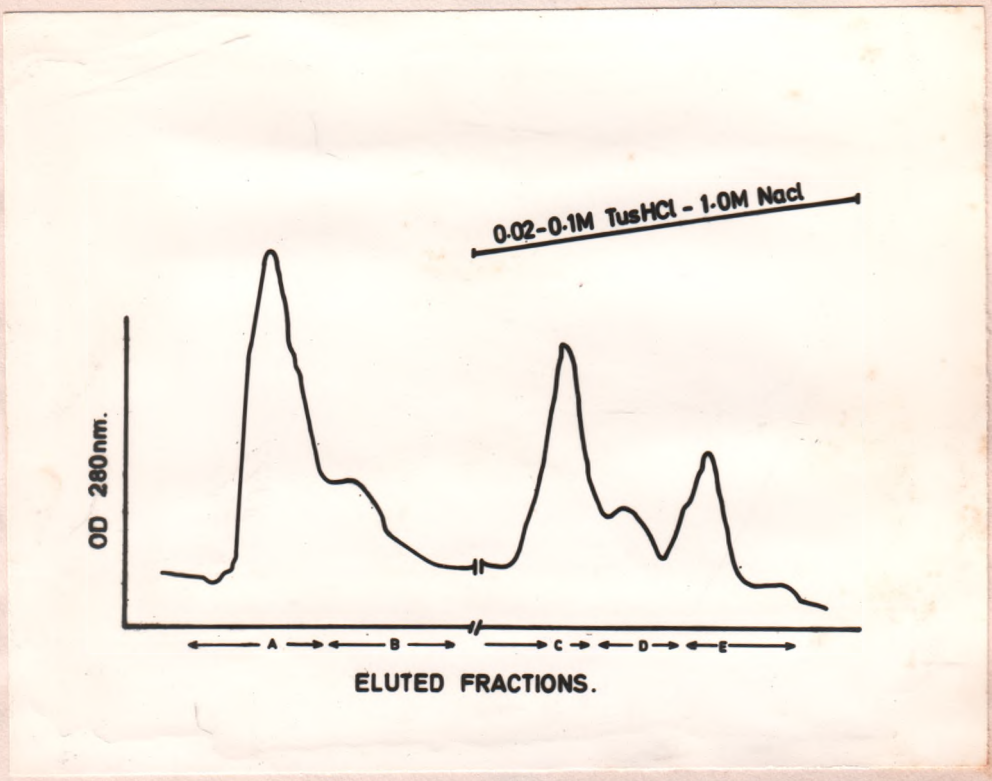


NCxT 120 min.
NHuS 120 min.

Rabbit anti CaCx

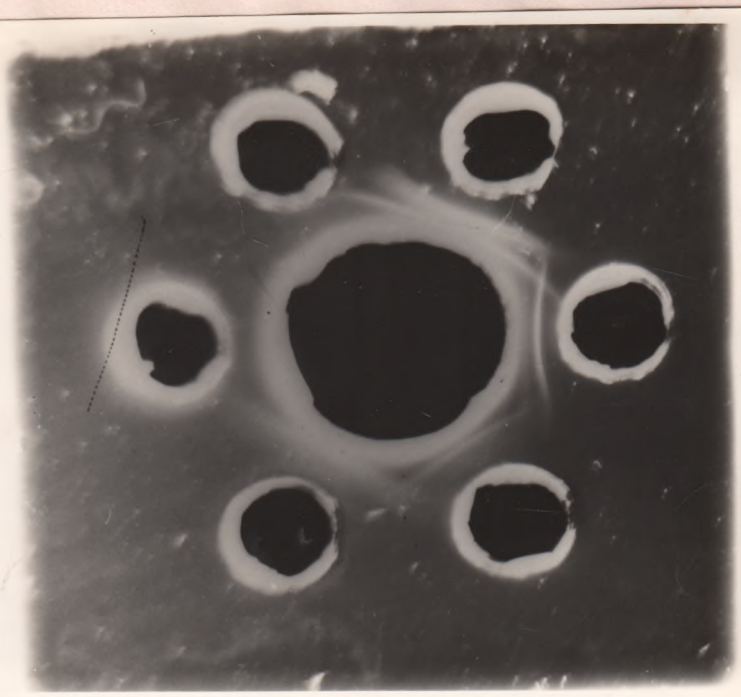
Immunodiffusion Adsorption-in-gel Reaction Between Rabbit anti-CaCx Sera and the DEAE Ion Exchange Chromatographic Fractions of the CaCx Soluble Antigen Preparation.

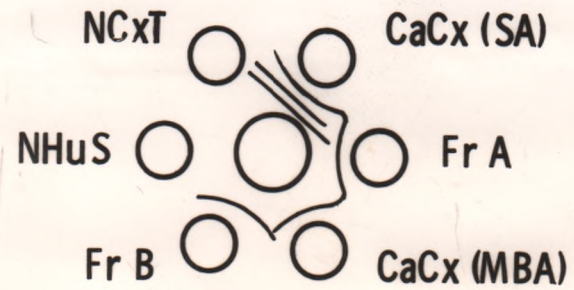
FIG. 4.7



Profile of the DEAE Ion Exchange Chromatography of the 20-70% Ammonium Sulphate Fraction of the CaCx Membrane Bound Antigen Preparation.

FIG. 4.8





NCxT 120 min.

NHuS 120 min.

Rabbit anti CaCx

Immunodiffusion Adsorption-in-gel Reaction Between Rabbit anti CaCx Sera and the DEAE Ion Exchange Chromatographic Fractions of the CaCx Membrane Bound Antigen Preparation.

The protein concentrations and the total protein content of the various fractions obtained from the DEAE A-25 Ion Exchange Chromatography of both the soluble and membrane-bound antigens preparations are shown in Table 4.2. Of the 90 mg. of total protein recorded from the Soluble Antigen preparation, TAA activity was fround in the 24.6 mg. of fractions A and B (Unbound) and the 30.4 mg. of the fractions D and E (Bound). On the other hand, of the 25.0 mg. recorded from the Membrane-Bound Antigen, TAA activity was demonstrated in the 12.2 mg. of the fractions A and B (Unbound). The titers of the various fractions were as shown in Table 4.2.

The "bound" and "unbound" fractions from the Soluble Antigen preparations of the CaCx. Antigen, were pooled separately to increase the amount of material for the next stage of purification. Similarly, the "unbound" fractions from the Membrane-bound Antigen preparation were pooled. When the purity of the various fractions were tested for by reacting these with unadsorbed rabbit antisera against NCx and NHuS, each of the purified antigens showed immunoprecipitin reactions (Fig. 4.9). Although the impure state of the antigens preparations were thus demonstrated, the number of the precipitin lines against anti-NCx and anti-NHuS sera were much less than with the crude antigen preparation.

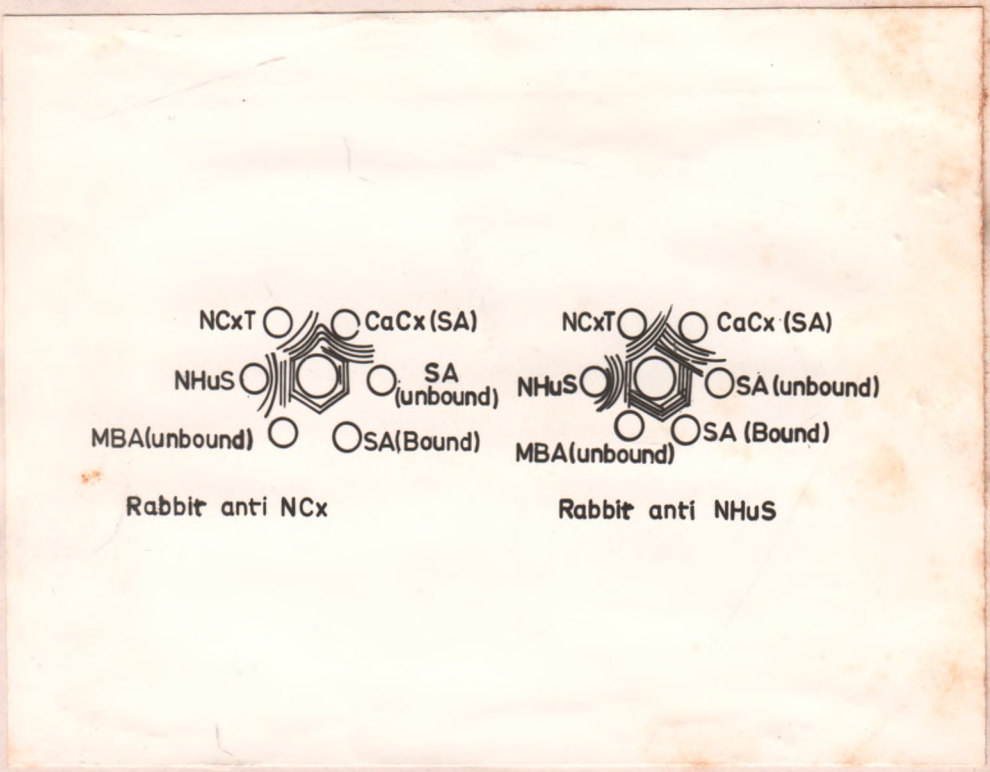
Occasionally, there were some inconsistencies in the precipitin lines in the IDA tests of the partially purified SA and MBA preparations with the numbers varying from one to two, showing that TAA-3 (occasionally, TAA-2) has been lost in the process of DEAE A-25 Ion Exchange Chromatography of the SA preparation. The three pooled fractions,

TABLE 4.2

ANTIGEN	Volume ml	Protein mg/ml	Total Protein mg	I.D. Titer	COMMENTS
CaCx (SA)					
Fraction A	2.5	4.3	10.8	4, 2	Active TAA 2, 3
B	3.0	4.6	13.8	4,2	Active TAA 2, 3
C	2.0	3.5	7.0	-	No TAA activity
D	2.5	6.8	17 0	4,2	Active TAA 1,2
E	2.0	6.7	13.4	4	Active TAA 1
F	2.0	5.7	11.4	-	No TAA Activity
G	1.5	4.0	6.0	-	No TAA Activity
H	2.5	3.8	9.5	-	No TAA Activity
CaCx (MBA)					
Fraction A	2.0	3.0	6.0	2	Active TAA 3
B	2.0	3.1	6.2	2	Active TAA 3
C	2.0	1.5	3.0	-	No TAA Activity
D	2.0	2.2	4.4	-	No TAA Activity
E	1.5	3.6	5.4	-	No TAA Activity

Protein concentrations and titer of TAA activity in the DEAE Ion Exchange Chromatographic Fractions of CaCx antigens.

FIG. 4.9



Schematic Representation of Immunodiffusion Reaction of Rabbit anti-NCx and anti-NHuS sera against the DEAE Ion Exchange Chromatographic Fractions of the CACx TAA, to test for level purification.

the "unbound" and "bound" fractions of the Soluble Antigen and the "unbound" fraction from the Membrane Bound Antigen preparation, were kept for further purification.

c) Sephacryl S-200 gel filtration -

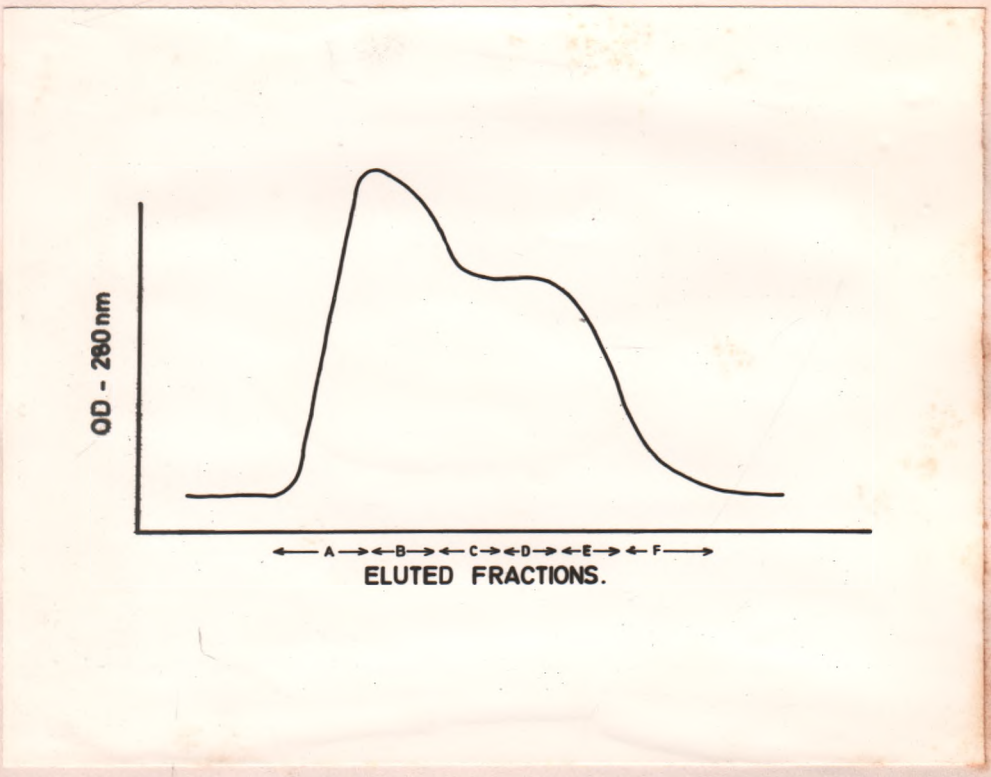
Passage of the "unbound" fractions of the Soluble Antigen preparation through Sephacryl S-200 gel column resulted in the elution of the individual proteins throughout a single peak with a broad base (Fig. 4.10). All the TAA-active fractions were found to be located within the contiguous fractions B, C, D and E, pooled from the various tube fractions. Immunodiffusion adsorption in-gel of rabbit anti-CaCx sera demonstrated TAA activity in fractions B and C, identical to TAA-2 and TAA-3, and in fractions D and E, identical to TAA-2 (Fig. 4.11).

From the "bound" fraction of the Soluble Antigen preparation, five fractions were obtained from the single peak with a broad base (identical to Fig. 4.10). Immunodiffusion adsorption in-gel of the tumour antisera showed TAA activity, identical with TAA-1 in only fraction A (Fig. 4.12).

The fraction from the Membrane Bound Antigen preparation, when passed through the gel filtration, gave rise to five fractions from the single peak with a broad base (identical to Fig. 4.10). Immunodiffusion adsorption in-gel of the tumour antisera showed showed TAA activity in fractions A and B, identical to TAA-3 (Fig. 4.13

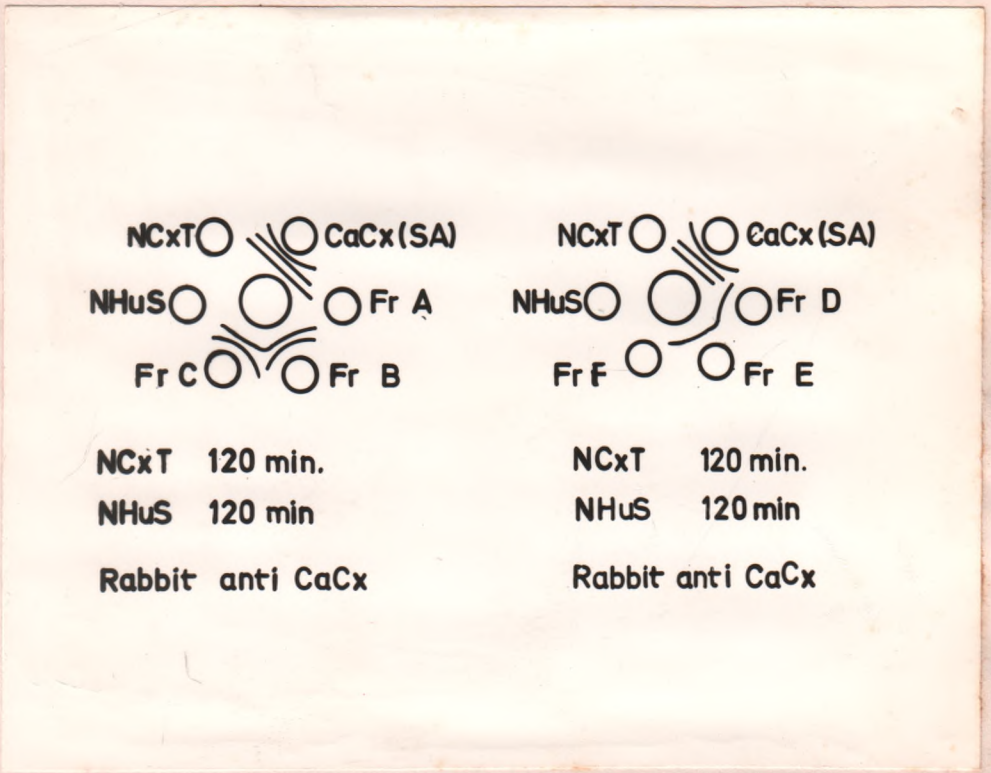
The protein concentrations, the total protein content and the titers of the TAA activity of the various fractions are shown in Table 4.3. Fractions B and C of the "unbound" fraction of the Soluble

FIG. 4.10



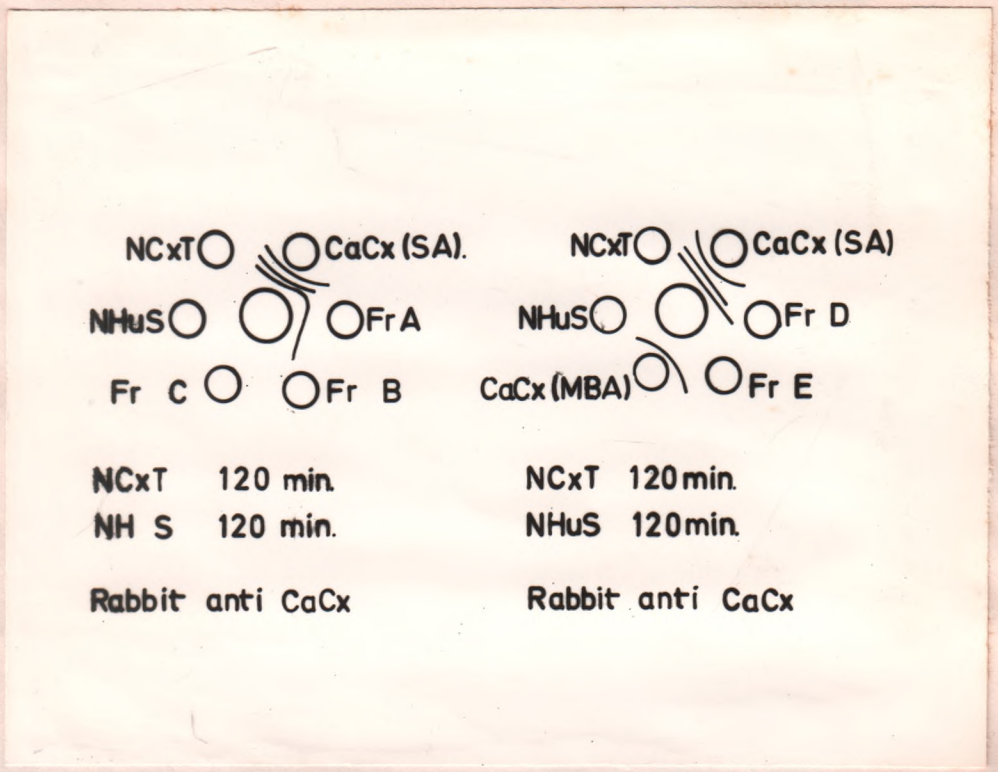
Profile of the Sephacryl S-200 gel Filtration of the Partially Purified CaCx TAA Fractions.

FIG. 4.11



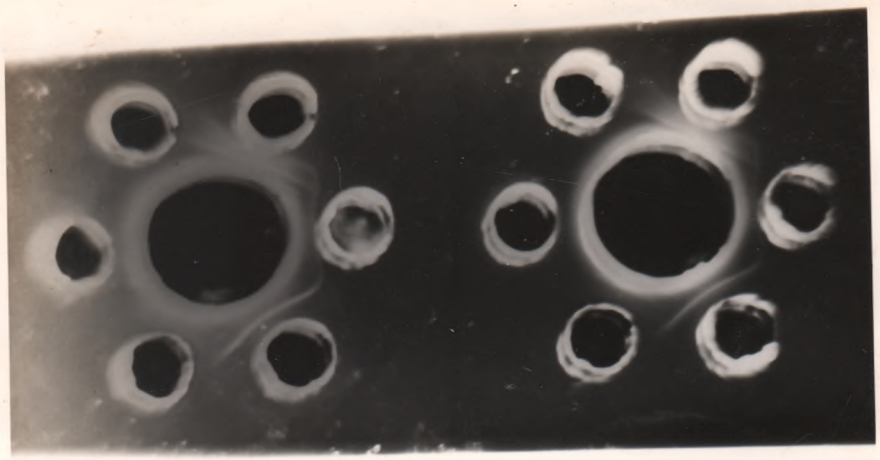
Schematic Representation of Immunodiffusion Adsorption-in-gel of Rabbit anti CaCx Sera with the Gel-purified DEAE "unbound" Fractions of the CaCx Soluble Antigen Preparation.

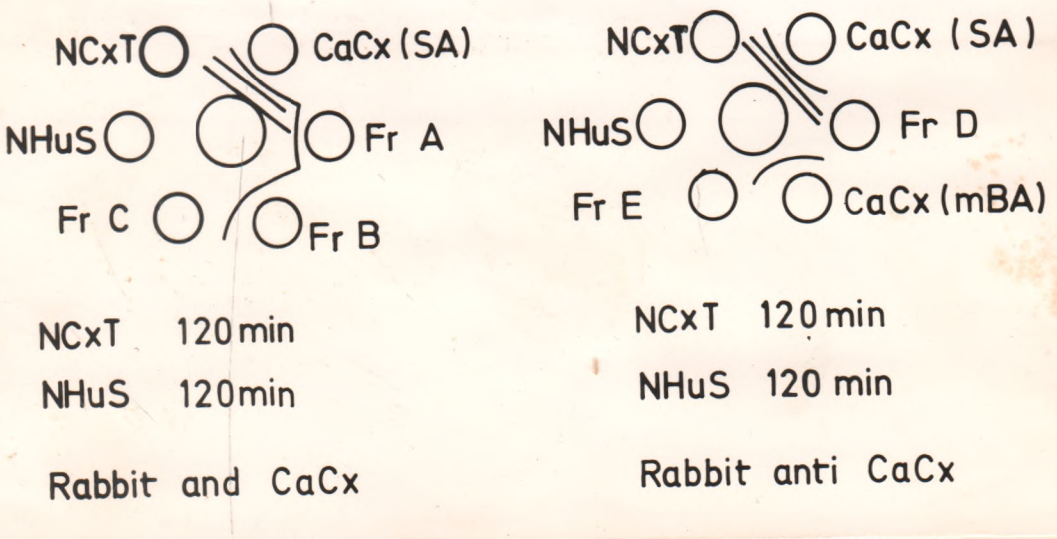
FIG. 4.12



Schematic Representation of Immunodiffusion Adsorption-in-gel of Rabbit anti CaCx Sera withe the gel-purified DEAE "bound" Fractions of the CaCx Soluble Antigen Preparation.

FIG. 413





Schematic Representation of Immunodiffusion Adsorption-in-gel between Rabbit anti CaCx Sera and the gel-purified DEAE "unbound" Fractions of the CaCx Membrane Bound Antigen Preparation.

TABLE 4.3

ANTIGEN		Volume ml	Protein mg/ml	Total Protein mg	I.D. Titer	COMMENTS
CaCx (SA) Unbound						
Fraction	A	2.0	0.5	1.0	-	No TAA Activity
	B	2.0	1.6	3.2	2,1	Active TAA 2,3
	C	1.5	1.3	2.0	2,1	Active TAA 2,3
	D	2.0	1.6	3.2	2	Active TAA 2
	E	1.5	1.4	2.1	2	Active TAA 2
	F	1.5	0.8	1.2	-	No TAA Activity
CACx (SA) Bound						
Fraction	A	2.0	1.3	2.6	2	Active TAA 1
	B	2.0	1.0	2.0	-	No TAA Activity
	C	1.5	0.7	1.5	-	No TAA activity
	D	2.0	0.5	1.0	-	No TAA ACtivity
	E	1.5	0.5	0.8	-	No TAA ACtivity
CACx (MDA) Unbound						
Fraction	A	2.0	1.2	2.4	2	Active TAA 3
	B	2.0	1.0	2.0	2	Active TAA 3
	C	1.5	0.8	1.2	-	No TAA Activity
	D	2.0	1.0	2.0	-	No TAA Activity
	E	2.0	0.8	1.6	-	No TAA Activity

Protein concentrations and titer of TAA activity of CaCx fractions after purification with Sephacryl S-200 gel filtration.

Antigen, showing TAA activity identical to TAA-2, and TAA-3, had 5.2 mg. of protein, while fractions D and E, which had TAA activity identical to TAA-2, contained 5.3 mg. On the other hand, the total protein content of the "bound" fraction of the Soluble Antigen preparation with TAA activity identical to TAA-1, was 2.6 mg. And the fractions A and B of the "unbound" Membrane Bound Antigen preparation with TAA activity identical to TAA-3, contained 4.4 mg. of proteins. The TAA titer varied from 1 to 2 only.

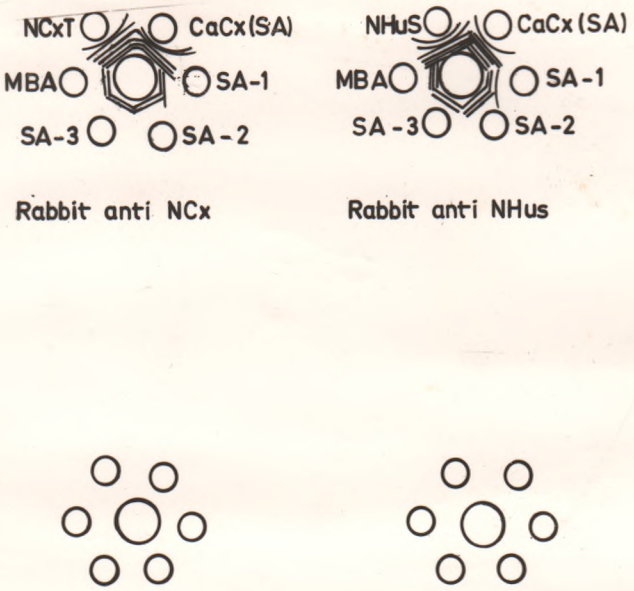
Fractions B and C of the "unbound" fraction from the Soluble Antigen Preparation were pooled and labelled "Soluble Antigen-1". Similarly, fractions D and E of the same fraction were pooled and designated "Soluble Antigen-2", and the active fraction A of the "bound" fraction was designated "Soluble Antigen-3". The active fractions A and B from the Membrane Bound Antigen was still designated "Membrane Bound Antigen".

Test of purity of the various fractions was evaluated by reacting these with unadsorbed antisera prepared against NCx and NHuS by the immunoprecipitin in-gel reaction (Fig. 4.14). Each of the fractions still reacted with anti-NCx and anti-NHuS sera. However, precipitin lines were much fewer, limited to only two or three lines, and the intensity was faint.

d) Affinity Chromatography -

All the four fractions, three from the Soluble and one from the Membrane Bound Antigen preparations, showed TAA activity after affinity chromatography. Immunodiffusion adsorption in-gel reaction of anti-CaCx. sera against the fractions showed that Soluble Antigen-1,

FIG. 4.14



Immunodiffusion Reactions of Rabbit anti NCx and anti NHuS Sera with the various gel-purified fractions of CaCx TAA to test for the level of purification.

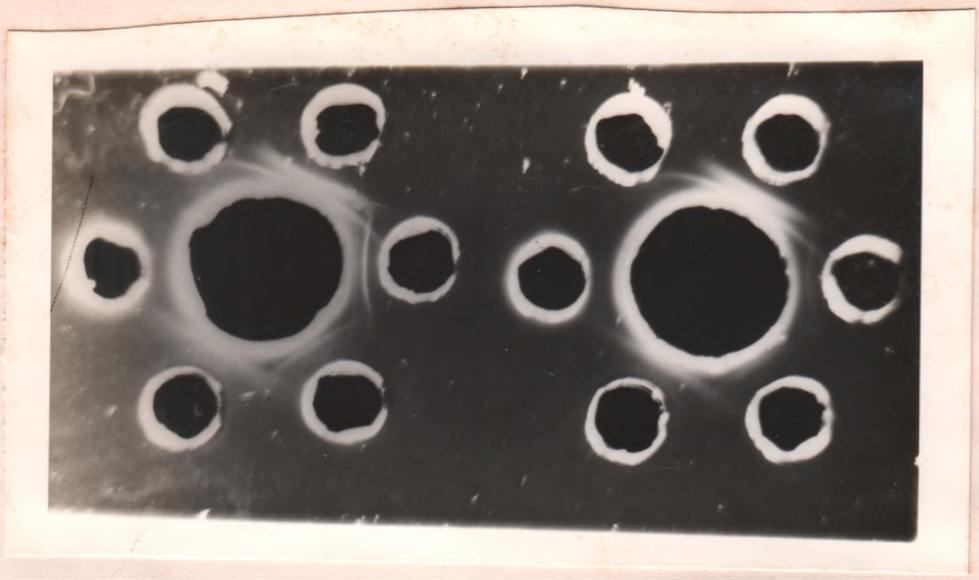
demonstrated one or two precipitin lines identical to TAA-2 and TAA-3, while Soluble Antigen-2 demonstrated only one line identical to TAA-2 and Soluble Antigen-3 showed one precipitin line identical to TAA-1. On the other hand, the Membrane Bound Antigen Fraction demonstrated only one precipitin line identical to TAA-3 (Fig. 4.15).

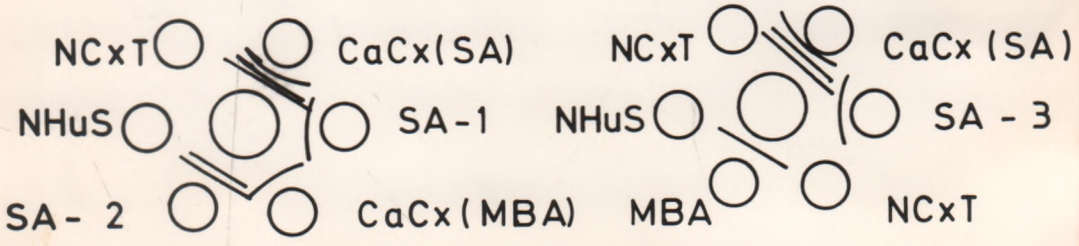
The protein concentration and the titers of the TAA activity in the various fractions are shown in Table 4.4. The protein concentration in all the fractions were rather low, and the titers of TAA activity were mostly in the undiluted fractions. However, the TAA in each fraction was still identical to those before affinity chromatography, although, there was a tendency for TAA-3 in the Soluble Antigen-1 to disappear.

When the fractions were reacted with the unadsorbed anti-NCx and anti-NHuS sera by the immunodiffusion tests, each fraction still produced precipitin lines (Fig. 4.16), demonstrating the presence of some normal components in the fractions. However, in each case, the number of precipitin lines was limited to only one or two with either antiserum, as compared with the precipitin lines when the crude antigen preparation was reacted with these unadsorbed antisera. Furthermore, the precipitin lines were very faint.

In summary, therefore, (Fig. 4.17), the "unbound" fractions passed through the Sephacryl S-200 gel filtration of the Soluble Antigen preparation produced two samples: "Soluble Antigen-1, which appeared to be TAA-2, but contaminated by TAA-3 and Soluble Antigen-2 which appeared to be TAA-2; while the "bound" fractions of the same process yielded only one sample, the Soluble Antigen-3, which appeared to be consistent with TAA-
The "unbound" fractions of the Membrane Bound Antigen yielded only one sample which appeared to be consistent with TAA-3.

FIG. 4.15





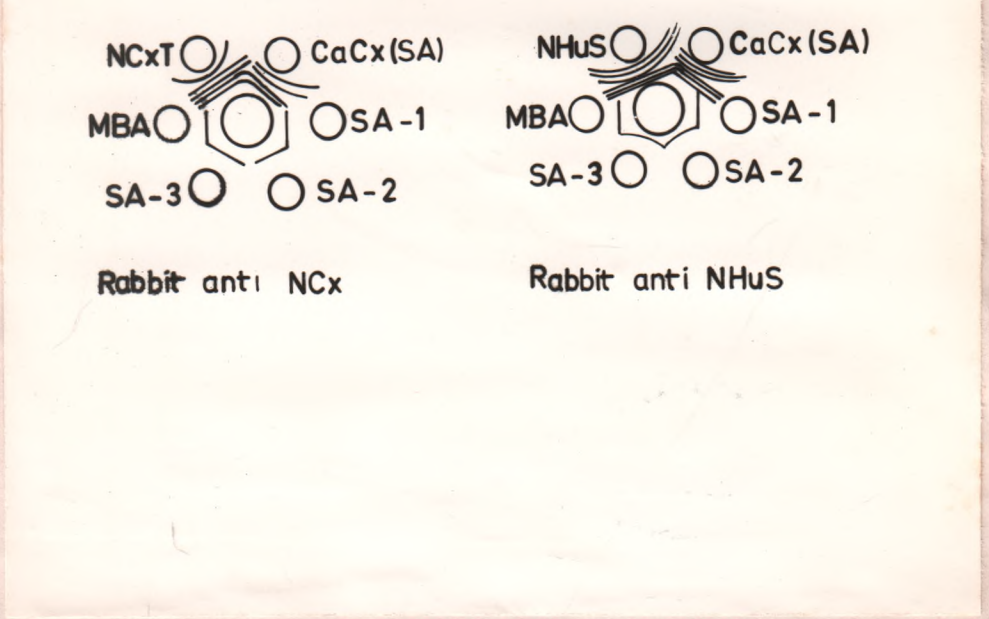
Schematic Representation of Immunodiffusion Adsorption-in-gel reaction between rabbit anti CaCx Sera and the CaCx TAA fractions obtained after affinity Chromatography.

TABLE 4.4

ANTIGEN	Volume ml	Protein mg/ml	Total Protein mg	I.D. Titer	COMMENTS
CaCx (SA)					
Soluble Antigen 1 (Unbound)	2.0	0.5	1.0	1	Active TAA 2 (?3)
Soluble Antigen 2 (Unbound)	2.0	0.8	1.6	2	Active TAA 2
Soluble Antigen 3 (Bound)	1.5	0.6	0.9	2	Active TAA 1
CaCx (MBA)					
Membrane Bound Antigen (Unbound)	1.0	0.5	0.5	1	Active TAA 3

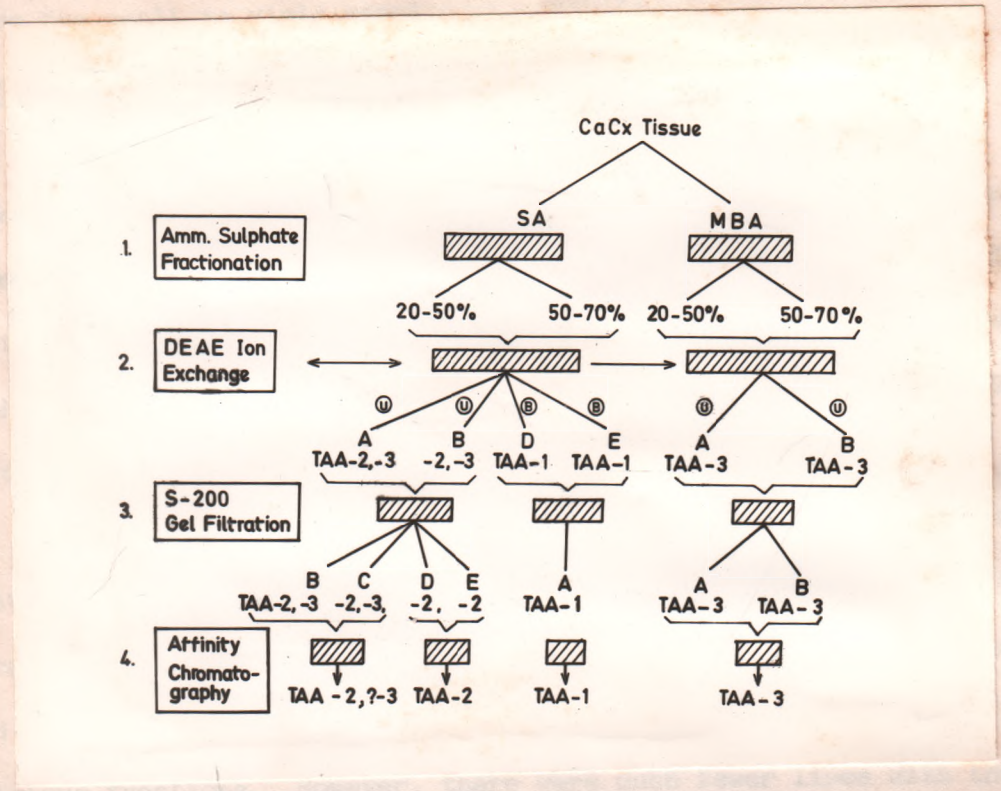
Protein concentrations and titer of TAA activity of CaCx TAA fractions after affinity Chromatography.

FIG. 4.16



Immunodiffusion Reactions Between RAbbit anti NCx and anti-NHuS Sera and the CaCx TAA Fractions obtained after affinity Chromatography to test for the level of purification.

FIG. 4.17



Analysis of the Results of Stepwise Purification of CaCx Tissues Antigen Preparations.

Further purification could not be continued because the samples were too small to yield meaningful results.

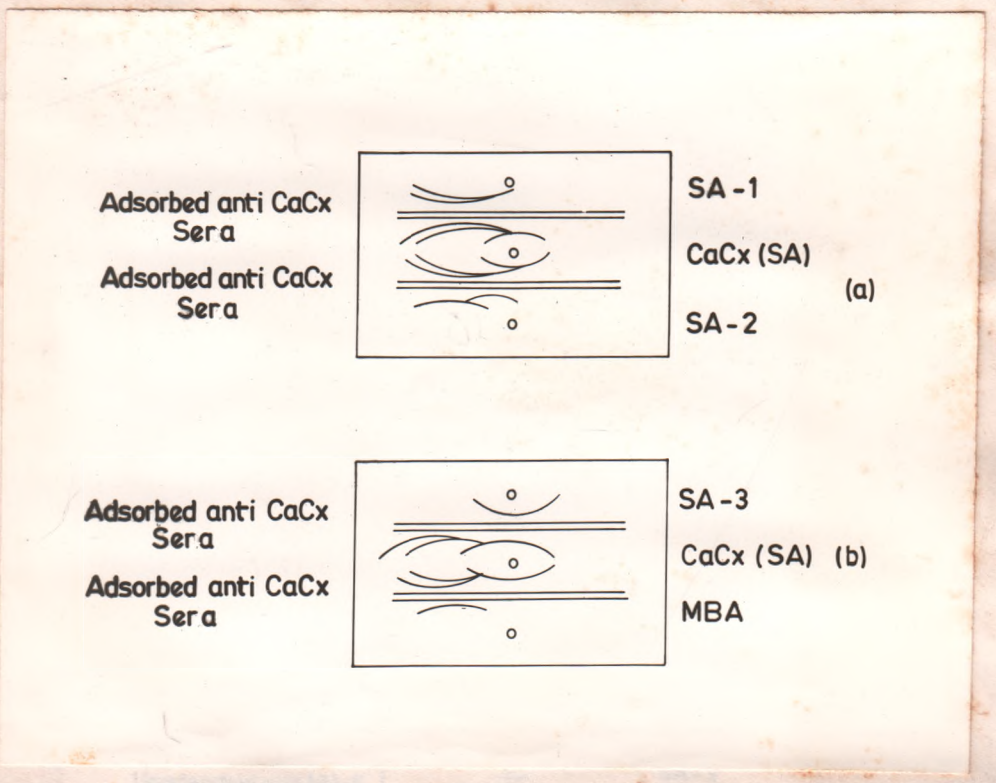
Single Dimension Immunoelectrophoresis:

Immunodiffusion adsorption of rabbit anti-CaCx. sera with the electrophoresed fractions obtained from affinity chromatography demonstrated one or two precipitin lines each (Fig. 4.18). The Soluble Antigen fractions 1 and 2 showed two precipitin lines each, while the others showed one line each, although, there were more than one line occasionally.

When the electrophoresed fractions were reacted with unadsorbed anti-NCx and anti-NHuS sera, there were two or three lines with each fraction (Fig. 4.19). With the resolution of the various proteins in the fractions, it was easy to demonstrate the impure state of the various fractions. However, there were much fewer lines with these fractions than with the crude cancer antigen preparation.

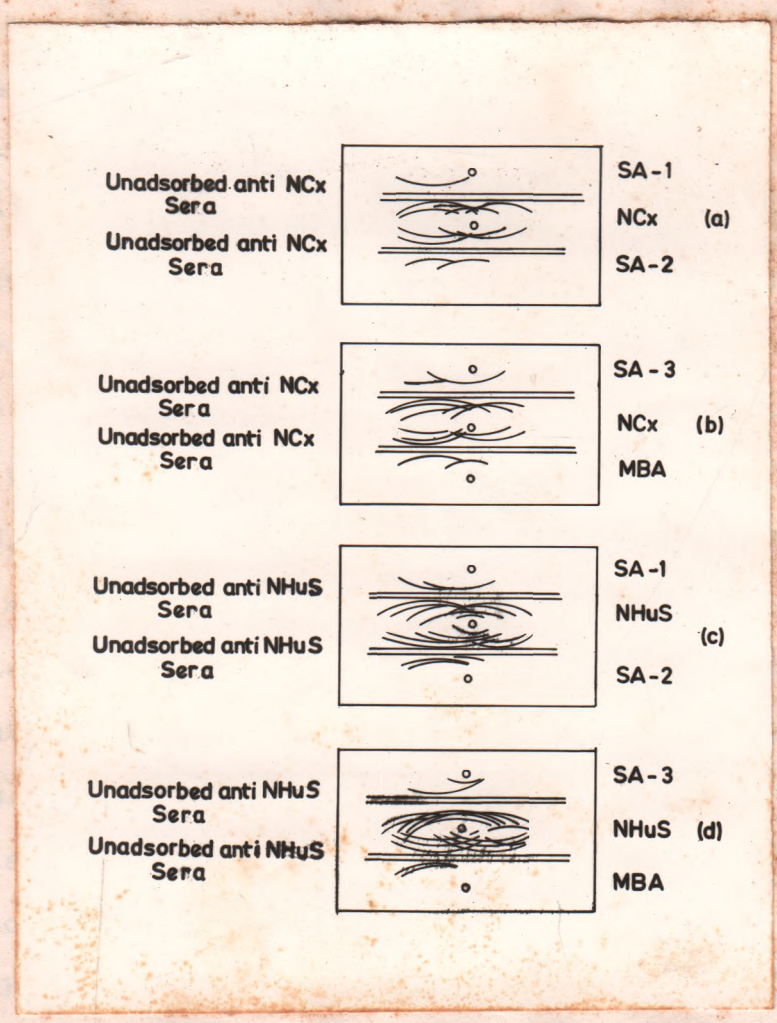
A ssample of the pooled TAA-active fractions of the partially purified cancer antigens which was used to immunise rabbit, yielded antisera which reacted with both the CaCx and NCx antigen preparations. However, the volume of the inocular (0.5 ml) as well as the number of injections required for producing adequate immunoprecipitin reactions, were much less than those used with the crude antigen preparations. Also, the antisera prepared against these were more readily adsorbed in-gel with pooled normal human sera and normal cervix antigen preparations.

FIG. 4.18



Schematic Representation of Immunoelectrophoresis Reaction of Adsorbed Rabbit anti-CaCx Sera and the CaCx TAA after affinity Chromatography.

FIG. 4.19



Schematic Representation of Immunoelectrophoresis Reaction of Rabbit anti-NCx and anti-NHuS Sera and the CaCx TAA after affinity chromatography to test for level of purification.

4.3: DEMONSTRATION OF C-TAA IN SERA CANCER PATIENTS:

The results obtained when adsorbed rabbit anti-CaCx. sera prepared against the partially purified TAA were reacted by immunodiffusion with coded sera from cancer patients and healthy controls are presented in Table 4.5. Circulating-TAA was detected in 27 of 36 (75%) sera from cancer patients. On the other hand, only 1 of 18 (5.6%) of the sera from patients with benign gynaecological diseases and 1 of 72 (1.4%) of sera from pregnant women, showed any precipitin reactions, when treated in the same manner. None of the sera from 36 healthy controls showed any precipitin reactions.

In all the sera from cancer patients where Circulating-TAA were demonstrated, the precipitin lines were identical to those shown by the CaCx tissue antigen preparations used as controls in the template. This would tend to show that the Circulating-TAA being demonstrated in the sera of cancer patients are the same as those present in the cancer antigen preparations.

Analysis of the precipitin results in the cancer patients in relation to the clinical stage of the disease is shown in Table 4.6. Overall, 3 (75%) of the patients in Stage I, 6 (86%) in Stage IIa and 9 (75%) in Stage IIb showed positive reactions. Furthermore, 7 (70%) of the patients in Stage III and 2 (67%) in Stage IV showed positive reactions. However, there was no statistically significant association between the clinical stage of the disease and the detection of Circulating-TAA in the cancer sera.

The majority (35) of the cancer tissue were of the squamous variety type. When the histopathologic grading of the specimens

TABLE 4.5

Serum Specimens	No. of Serum Specimens	No. of Positive Specimens	% of Positive Reactions
Cancer Patients	36	27	75.0
Patients with Cynecol. Diseases	18	1	5.6
Pregnant Women	72	1	1.4
Healthy Controls	36	0	0.0

$X^2 = 102.94$ on 3 df $P < 0.001$

Immunodiffusion reaction between adsorbed rabbit anti CaCx Sera and Sera from Cancer Patients and Controls.

TABLE 4.6

CaCx: Clinical Stage	No. of Serum Samples	No. of Positive Reactions	% of Positive Reactions
I=	4	3	75.0
II(a)	7	6	85.7
II(b)	12	9	75.0
III	10	7	70.0
IV	3	2	66.7
	36	27	75.0

$X^2 = 6.73$ on 4 df $0.20 > P > 0.10$

Immunodiffusion reactions between adsorbed rabbit anti CaCx Sera and Sera from Cancer Patients, in relation to the clinical stage of disease.

were analysed, 4 of the tissues were placed as Grade I, 5 as Grade II while the remainder, 27 were classified as Grade III. No association could be found between the detection of Circulating-TAA in the sera of patients and the pathologic grade of the disease.

CHAPTER 5

DISCUSSION

The concept that the normal cell contains a definite complement of antigens, the histocompatibility antigens (Hollinshead et al, 1972; Frenkel et al, 1972), and that the metamorphosis which occurs during neoplastic transformation induces the synthesis of new antigens not normally expressed by their normal counterparts (Hellstrom and Hellstrom, 1969; Levi et al, 1969; Frenkel et al, 1972), is now universally accepted. The results of the study of Levi (1971) on the antigenicity of squamous cell carcinoma of the cervix uteri suggested the existence of such tumour specific antigens, and the later studies of Ibrahim et al, (1979); Adelusi, (1981); Adelusi et al, (1982) have confirmed this finding.

DETECTION OF TAA IN CANCER OF CERVIX TISSUE PREPARATIONS:

The results of the current study has gone further to provide evidence that tumour specific antigens of carcinoma of cervix tissues are qualitatively distinct from,rather than mere quantitative increases in, normally present antigens of normal cervix tissue components. Immunological comparison of fractions obtained from the cancer tissue antigen preparations showed antigenic distinctness and non-identity to fractions derived from normal tissues.

Hyperimmune rabbit anti anti-CaCx serum, when unadsorbed, reacted with CaCx, NCx antigens, as well as NHuS pool. However, the same antiserum when adsorbed in gel with NCx and NHuS, demonstrated precipitin reactions only with CaCx. On the other hand, hyperimmune rabbit serum prepared against

NCx, when similarly adsorbed with NCx and NHuS, failed to react with both NCx and CaCx antigen preparations. This would tend to confirm that antisera prepared against CaCx contain antibodies specific for CaCx tumour associated antigens, and that such antibodies are not present in anti-NCx sera. According to Rawlins (1977), the possibility that the reactions observed in these studies could be attributed to HLA is unlikely since the extracted antigen preparations from a pool of normal cervix from healthy controls were used in adsorbing and testing the anti-CaCx sera.

There are now, convincing biochemical and immunochemical evidence for the existence of one or more CaCx TAA in the soluble extracts of the cancer tissues. Indeed, the present work has confirmed the earlier findings of Levi (1971) on the antigenicity of squamous cell carcinoma of the cervix, and Adelusi (1981) on the number of TAA (TAA-1, -2 and -3) in the cancer tissues. The tumour associated antigens are thought to be non-structural antigens present in all DNA virus-induced tumours, and possibly in some RNA virus-induced tumours as well (Tucker, 1979). They are found within the nucleus and cytoplasm of the tumour cells, and the cell membranes. Their chemical composition is not known (Deinhardt, 1974).

The antiserum utilized throughout these investigations was prepared against a pool of all the three supernate fluids obtained as part of the tumour antigen extraction process. This practice was established subsequent to the observation that in addtion to one or two common tumour-specific antigens, each of the supernates contain TAA which are distinct from those demonstrated in the other preparations.

This phenomenon may be attributable to the nature of the cellular components released by the different antigen extraction procedures. The Soluble Antigen (SA) is the product of simple homogenisation processes which release the intracellular antigen components. These cytoplasmic or nuclear antigens may represent enzymes or other proteins ectopically expressed by the cancerous cells (Tucker, 1979). The KCl-extracted Membrane Bound Antigen (MBA) on the other hand, is the solubilised cell membrane antigen. This has been suggested to represent the released tumour rejection antigens (Reisfeld et al, 1971), membrane antigens capable of eliciting an immunological attack on malignant cells.

Several other investigators have employed the 3-Molar KCl to extract Soluble Antigens from tumours. In studies with malignant melanoma, Roth et al, (1975) found that the 3-Molar KCl extracts exhibited a 20-fold increase in antigenicity over that of the crude antigen preparations. Stroehmann et al (1977) suggested that the KCl extraction is superior to papain digestion because biological activity of the proteins is retained. Meltzer et al (1971) showed that 3-Molar KCl solubilised antigens retained their specificity and remained stable at $4^{o}C$ for several months. Watson et al (1975) raised antisera in rabbits to fractions obtained from KCl extraction of a variety of histological types of lung tumours with which they were able to detect 2 TAA in Lung Cancer Tissues, and Boddie et al, (1975) demonstrated the presence of TAA in KCl-solubulised extracts of lung tumours, using human leukocyte migration inhibition assays in agarose gels, and concluded that antigens released by KCl extraction were more specific than those obtained by sonication of the tissues.

In the present study, the TAA obtained from the Membrane Bound Antigen fraction (designated TAA-3) was found to be distinct from the other two antigens from the Soluble Antigen fraction, in contradistinction to the earlier study of (Tucker 1979) who found no difference between the Soluble and the KCl extracts of CaCx. antigens. Furthermore, purification of the KCl extracts was more readily achieved. Even though it has been suggested that the KCl extracted TAA can be a source of false positive reactions if this is not eliminated from the test system (Leonard et al, 1972), this was not the case in this study.

It is hoped that the Soluble Antigen and the KCl extracted Membrane Bound Antigen would eventually be purified individually in large enough quantities for immunisation of rabbit to produce antibodies. Further, it is hoped that the individual antisera will be tested to determine which anti-TAA is capable of detecting Circulating-TAA in the sera of tumour bearing patients.

PURIFICATION OF TAA IN CANCER TISSUES:

Investigations in area of research on TAA have employed myriad of techniques in attempt to purify TAA in human cancers. Various methods of purification employed in this study, including Ammonium Sulphate precipitation, Sephadex Ion Exchange Chromatography, Sephacryl S-200 gel filtration, and Affinity Chromatography, resulted in partially purified invididual TAA.

Fractional precipitation of the antigens by ammonium sulphate was applied routinely as a preparatory step for further purification. Several pertinent facts were established by the repeated application of this procedure. Most notable is the consistent extraction of TAA in the 20-70% salt saturation

level. This is different from the results of Ibrahim et al, (1979) where the TAA were consistently extracted in the 20-50% saturation. The reasons may be varied.

Investigations with various cancerous tissues suggest that the point of saturation at which a particular TAA is precipitated by ammonium sulphate may be unique to, or characteristic of, the type of malignancy or its associated antigens. The majority of the tumour-specific antigens of lung cancer for example, precipitate consistently within the 60-70 per cent saturation range whereas those of carcinoma of cervix were known to precipitate within the 20-50 per cent saturation (Tucker, 1979).

Since the protein precipitation by ammonium sulphate is based on the charge on the proteins, the present technique has been able to precipitate TAA with higher affinity for the H_3O^+, that is, greater charge, by precipitating these at the higher saturation (50-70%). It is possible, however, that the immunoprecipitation test, regarded as the least sensitive of the serological techniques, may not have detected the TAA present in the other fractions in the study of Ibrahim et al (1979).

Attempted purification of TAA by column chromatography techniques achieved a degree of success not attained before by the more simplistic approaches employed by earlier studies (Tucker, 1979; Ibrahim et al, 1979). Individual TAA, designated TAA-1, TAA-2 and TAA-3, were isolated. Although these were still not completely purified, it was possible to eliminate the non-reactive proteins to a great extent. However, the production of purified material to this degree by these procedures also resulted in significant loss in the total number and concentration of detectable tumour-associated reactions. For example, the TAA-3 of the Soluble Antigen preparation was

invariably lost in the process, suggesting that this additional TAA remained in the unused fractions, although the loss of detection may be attributable to the poor sensitivity of the immunodiffusion test procedure.

Sephadex Ion Exchanges are derived from neutral Sephadex by introduction of functional groups attached by other linkages to the glucose units of the dextran chains. Proteins and other biological substances are not denatured by the hydrophilic polysaccaride net work, and hence the tumour specific reactivity is not adversely affected. However, separation of proteins vary with variations in the pH, ionic strength, temperature, column bed height and diameter as well as the flow rate. Hence, it is essential that care should be taken of these factors in the purification processes in order to achieve adequate separation of the various TAA.

While Sephadex A-25 Ion Exchanges separate the proteins according to their electrical charges, Sephacryl S-200 gel filtration on the other hand, separates them according to their molecular weight. It would be easy to determine the molecular weight of the TAA on this basis. Even though this was not caliberated in this study, the TAA were found to be in the group of low molecular weight proteins.

Affinity Chromatography, on the other hand, offered a unique method for the elimination of normal tissue and serum reactions without any significant loss in the number of discernible tumour specific reactions. Elimination of normal tissue and serum antigens was readily achieved by a single process once the optimum conditions of ligand preparations had been established. The technique ofers the possibility of achieving separation of proteins which are difficult or even impossible to separate when less specific techniques are used, because it exploits the unique specificity of

biological interactions to isolate proteins and glycoproteins.

In principle, the technique can be used to isolate either of the components of a reversibly reacting system. Thus by coupling of one component, the antibodies to NCx and NHuS, to an isoluble matrix, AH Sepharose-4^{B}, (Pharmacia Fine Chemicals) while still retaining its specific binding activity, it was possible to eliminate, substantially, the NCx and NHuS components of the CaCx TAA from the partially purified fractions.

Affinity Chromatography is a type of adsorption chromatography in which the bed material has biological affinity for the substance to be isolated. The specific adsorptive properties of the bed material are obtained by covalently coupling an appropriate binding ligand to an insoluble matrix. The binding ligands in this case, anti-NCx and anti-NHuS, are then able to adsorb from solution, the substances to be isolated, the NCx and NHuS components of the CaCx. antigen preparation.

Separations are rapid, since in most cases, only small bed volumes are required. The high specificities of the separations derive from the natural specificities of the antigen-antibody system. Affinity Chromatography provides opportunities for the isolation of substances according to their biological function, and thus differs radically from conventional chromatographic techniques in which separation depends on gross physical and chemical differences between substances.

However, as in previous studies (Tucker, 1979; Ibrahim et al, 1979; Adelusi, 1981), removal of reactivity against anti-NCx and anti-NHuS sera was incomplete. The hyperimmune rabbit antisera prepared against a pool of some of the purified fractions, when unadsorbed, still reacted, however slight, with normal tissues as well as normal human serum. Similarly,

hyperimmune rabbit antisera prepared against NCx and NHuS also reacted with the purified fractions, signifying the incompleteness of purification.

It was noticed however, that the antisera obtained from immunizing rabbits with the partially purified TAA were more readily adsorbed with NCx and NHuS than those from the crude antigen preparations. Also, the doses of immunogen as well as the number of immunisations of rabbits with the partially purified TAA were less than those with the crude antigens.

Greater effort at isolating individual and pure TAA, by such other methods of purification of proteins, such as Isoelectric foucssing, may be essential, for the production of anti-TAA which is specific and capable of detecting Circulating-TAA in the sera of tumour-bearing patients. On the other hand, there is the need to look for a more easily purifiable tissue antigen specimens, such as a continuous cancer cell line which will contain less normal tissues and normal human sera. This is worthy of further study following the use of antibodies to the continous cancer cell line (C_4II) to detect Circulating-TAA in the sera of patients with cancer of the cervix (Adelusi et al, 1982).

DETECTION OF CIRCULATING-TAA IN SERA OF CANCER PATIENTS:

The concept of transformed cell acquiring new antigenic substances not normally present in the normal tissues and which are shed into circulation (Levi, 1971), can be explored in the immunodiagnosis of malignancy. The demonstration of Circulating-TAA in the sera of tumour-bearing patients (Ibrahim et al, 1979; Adelusi, 1981; Adelusi et al, 1982) has added yet another dimension to the study of tumour immunology.

Using a modification of the technique of Ibrahim (1969) and Ibrahim et al, (1979), the results obtained in this study, when adsorbed anti-CaCx

sera prepared against the partially purified CACx TAA, were reacted with sera from cancer patients, patients with benign gynaecologic diseases, pregnant women and healthy controls, indicated that Circulating-TAA can be demonstrated in the sera of cancer patients, but not in healthy controls.

That the TAA detected in the cancer patient sera are related to the TAA of CaCx. is suggested by the pattern of identity with one of the CaCx. precipitin reactions. This finding is consistent with the findings of Ibrahim et al, (1969) and Adelusi, (1981). However, Circulating-TAA was apparently detected in the sera of non-cancerous patients, some of whom were pregnant women. This amay be indicative of an embryonic antigen, especially if all the affected tissues are of similar embryonic origin.

If this is the case, then the false positive controls could represent sera from women who were or had been pregnant, analogous to Gold's observation in 1971. On the other hand, it is also conceivable that the control group included women with cancer undetected by the routine Pap Smear as presently practised. Sera from pregnant women were included in as much as it has been shown that pregnancy may lead to the development of pregnancy associated antigens that cross-react with cancer antigens (Rosen et al, 1975).

In the present study, Circulating-TAA was detected in a significantly high percentage of sera from women with invasive carcinoma of the cervix at various stages of development. However, there was no significant relationship between the detection of Circulating-TAA and the clinical stage or pathological grade of the disease. Tucker (1979) and Ibrahim et al, (1979) on the other hand, were able to find differences in the detection of Circulating-TAA between women with invasive carcinoma and

and those with carcinoma in situ, as well as those with post operative follow-ups. A possible reason for this discrepancy is the fact that there was no case of in-situ carcinoma in the present study. Furthermore, since all the cases in this study are invasive carcinoma, it is possible that once the invasive carcinoma has been established, there may be no qualitative distinctness in the detection of Circulating-TAA in the sera of the tumour bearing patients.

With the development of the "Pap Smear" test for cancer of the cervix screening in 1943, epidemiologists predicted that the incidence of invasive carcinoma of the cervix in the United States would be close to zero by the early 1970's. Inspite of the routine widespread use of this smear test however, cancer of the cervix is the second most common malignant disease of women in the United States today (Goldberg and Gravell, 1976). In Nigeria, one the other hand, despite the fact that the disease is the commonest malignant disease of women (Edington and Hendrickse, 1970), the Pap Smear test is yet to be established, much less, have any effect in reducing the incidence of the disease.

On the other hand, cytology has many problems, First it requires that all women from adolescence onwards have repeat cytological screening at frequent intervals. One of the major probelms is that of obtaining "Pap Smears in those women who are at highest risk of developing carcinoma of the cervix. Since such women, mostly in the low socio-economic classes, are the least likely to seek preventive services, this approach requires a great deal of public education and community effort, apart from the problem of the enormous cost of routine screening programmes which include cost of educating qualified

personnel for this. Indeed, it is now thought that certain cancers of the cervix may have accelerated growth rates (Silverberg, 1977), and cytologic screening may not detect premalignant changes in these cases.

It is hoped that the detection of Circulating-TAA as tumour markers in sera of patients, may some day become routine, and thus make earlier diagnosis of cancer possible. The results of the present study indicate very high specificity, and potential immunodiagnostic application of these tests not only in the early diagnosis of cervical neoplasia, but also in the follow-up of post-surgical cancer patients. Indications are that immuno-diagnostic procedures can be designed in ways that are reproducible, simple and reliable. Such serological tests would extend our present ability for detection and monitoring of malignancies.

RELATIONSHIP OF CACX TAA TO ONCOFETAL ANTIGENS:

The discovery of Circulating-TAA in sera of cancer of cervix patients as well as those of other cancer patients has raised the specter of embryonic origin for TAA, especially if all affected tissues are of similar embryonic origin. Indeed, it has been suggested that the majority of human tumour antigens described to date are fetal antigens (Tucker, 1979).

Since the first association of fetal antigen with cancer was made by Abelev (1963), when he found an Alpha-Feto-Protein (AFP), a glycoprotein normally found in sera of new born mice, associated with a transplantable mouse hepatoma induced by ortho-aminoazololuene, various other fetal antigens have been described in animals and man (Tatarinov, 1964; Gold and Freedman, 1965; Thompson et al, 1969; Brawn, 1970; Balwin et al, 1971; Fuks et al, 1974; Leung et al, 1977; Von Kleist et al, 1977). Costanza and Nathanson (1974)

defined fetal antigens as "Biologic material identified through immunologic means that is found in both malignant tissue and in embryonic, fetal and plancetal tissue, but which cannot be detected in adult non-malignant tissues in amounts greater than 10.0ug/ml.".

It is believed that in malignant tissues, fetal antigens are located mainly in cell membrane (Lausch and Rapp, 1974; Tucker, 1979), although a few types are found only in the cell cytoplasm (Tucker, 1979). Both types may enter body fluids and be incorporated into normal cells, and are usually detected by serum assay. Because in experimentally induced hepatomas, some fetal antigens, especially AFP, have been detected before the appearance of cancer cells, it has led to speculation that perhaps, some carcinofetal alterations may preceed malignant transformation (Hirai, 1975). Sell and Skelly (1976) have also suggested that since non-carcinogenic compounds do not produce AFP elevations, it may be supposed that the induction of AFP production by chemical carcinogens is related to the carcinogenic process.

Recently, Goldenberg et al, (1978) reported the presence of a beta-globulin associated with cervical and colonic tumours which was also present in normal adult and fetal kidney and liver tissues. This antigen was shown to be identical to beta oncofetal antigen (BOFA), first described by Frische and Mach (1975). Antiserum to BOFA and antiserum to CaCx. antigen were shown to demonstrate a line of identity when reacted against carcinoma of cervix tissue antigens using the ID technique. However, the antigen did not cross-react with CEA, AFP or Colon-associated tumour antigens.

Since in the study of Ibrahim et al, (1979) it was shown that no cross-reactivity was observed between the CaCx antigens and any of the fetal antigens tested, including CEA, AFP, HCG or B-microglobulin, no further cross-reactivity

tests were performed in this study. Even though the study of Goldenberg at et al (1978) demonstrated quantitative increase of the Beta-globulin in carcinoma of certix tissues, because it is also present in large amounts in normal adult liver and kidney tissues, it no longer qualifies as an Oncofetal Antigen. Other workers (Ting et al, 1972) have indicated that although Fetal Antigens may be expressed in tumour cells, they are different from Tumour-Specific Antigens that are specific for a particular tumour, or for tumours induced by a particular virus. However, further work is still required, therefore, to determine the specificity and relationship of CaCx. TAA to the Fetal Antigens.

The finding that many tumours have antigens common to fetal tissues but not to normal adult tissue, has led to speculation that perhaps oncogenic viruses are able to cause re-expression of fetal antigens in transformed cells (Goggin et al, 1970; 1971) and that virally induced tumours possess common antigens (Sjogren, 1961; Habel, 1962; Leonard, 1975; Lo Gerfo, 1976). Individually distinct viral TAA have been demonstrated in mammary virus-induced tumours of mice (Heppner et al, 1976) and in polyoma induced tumours (Witz et al, 1976).

ASSOCIATION BETWEEN HSV-2 AND CARCINOMA OF THE CERVIX:

Another angle of looking at the development of a system for determining the existence of TAA in CaCx. is the causal relationship of HSV-2 in the genesis of Carcinoma of Cervix. The excessive cross-reactivity among cancer antigens, even across spieces lines (hamster and human) tend to indicate a viral etiology for some human cancers, particularly the cervix. The cross-reactivity of antigens of carcinoma of cervix with anti HSV-2 serum, but not with HSV-1 serum, tends to implicate HSV-2 as the viral etiological agent in CaCx. (Adelusi, 1982).

Many oncogenic DNA and RNA viruses are highly immunogenic and produce both hormonal and cell-mediated immune responses (Herberman, 1977). Indeed a number of studies have confirmed the presence of HSV-2 related antigens in exfoliated cancer of cervix cells (Royston and Aurelian, 1970; Nelson, 1974; Pascal et al, 1976; Adelusi et al, 1976; Bell et al, 1978). Significant proteins of HSV-2 viral genome and readily identifiable virally induced RNA have been found in cancer cells by molecular biological studies, using hybridization techniques (Duff and Rapp, 1971; Munyon et al, 1971; Frenkel et al, 1972; Minson et al, 1976; Couple and McDongall, 1976).

The association between HSV-2 and carcinoma of the cervix was strengthened by the cross-reactivity between the antigens of, and antisera prepared against the virus, HSV-2 associated hamster tumour, the tumours developing following inoculation of HSV-2, implicating the virus as a causative agent (Nahmias et al, 1970), and human cancer of the cervix, (Ibrahim et al, 1975). The observed cross-reactivity suggests that HSV-2 may be etiologically involved in human neoplasm, especially, carcinoma of cervix. The inter-relationship between the virus and cervical neoplasm is further strengthened by the identity between the precipitations of the TAA of CaCx. and HSV-2 (Adelusi, 1982).

Thus the weight of circumstantial evidence supporting the role of Herpes Simplex Virus Type-2 (HSV-2) in human carcinoma of the cervix has increased, based on these various studies. On the contrary, evidence supporting similar role for many other DNA viruses as etiological agents of human cancer is lacking. It is possible that the use of further purified cancer of the cervix antigens may be precipitated more specifically by antisera prepared against HSV-2 antigens. This would further assist in establishing a possible causative association between the virus and carcinoma of the cervix.

Elucidation of both the initial carcinogenic and subsequent immune reactions in host-tumour interaction has been partially impeded by the lack of detailed immunochemical characterization of the tumour specific antigenic determinants. The production and availability of purified water-soluble tumour antigens of CaCx. would be a significant advance in this direction, and may ultimately lead to a molecular basis for immunotherapy of cancer.

SPECIFICITY AND SENSITIVITY OF IMMUNODIAGNOSIS OF CANCER

Immunodiffusion, with adsorption-in-gel, was the standard procedure adopted for the demonstration of TAA in the cancer tissues, the test of purity of the CaCx. antigen, and the demonstration of Circulating-TAA in the sera of tumour-bearing patients in this work. This technique proved to be simple, reliable, reproducible and inexpensive, when applied to these investigations. It was easy to adsorb the anti-CaCx. serum with NCx and NHuS, thus reducing the number of anti-normal reactions in the resultant serum. Sequential adsorption, as employed in the study was able to minimize any deleterious effects of excessive adsorption.

However, immunodiffusion is regarded as one of the least sensitive serological tests, inspite of its very high degree of specificity. The possibility exists therefore, that some TAA could be found in low concentrations in normal and fetal tissues by more sensitive immunological reactions, such concentrations which may be too low to be detected by immunodiffusion. Bell (1976), investigatiang human lung cancer, for example, found that the non-reactive normal lung and fetal lung antigen preparations gave precipitin reactions when their protein content was adjusted to five times that of the reactive lung cancer preparation.

On the other hand, TAA may be present in tumour antigen suspensions, both crude and purified, but remain undetected because of super-imposition of two precipitates, antigen excess or non-precipitating reactions. The effect of adsorption-in-gel by homologous antiserum on the elucidation of TAA in cancer tissues or in sera of cancer patients has not been ascertained. The possibility of mechanical inhibition or adsorption of minimally cross-reacting anti-TAA which identifies patient identity between a TAA and a normal antigen, may adversely affect the total response in these tests. It is possible that these factors may account at least in part, for the occasional fluctuations in the number of tumour specific responses recorded from the individual purification procedures. Unequivocal demonstration of purity will necessarily require the application of more sensitive techniques.

Improvement in the sensitivity of this immuno-diagnostic technique would require the purification of the antisera being produced at present against the partially purified CaCx TAA. However, the detection of the Circulating-TAA in all cancer sera would require the use of a more sensitive assay system, such as the Enzyme Linked Immuno-Sorbent Assay (ELISA). Using ELISA, sensitivities, in all probability, would be increased.

Of course, a new approach to the whole problem of the specificity of the technique of immunodiagnosis of carcinoma of cervix may be in the new development of monospecific antibodies. This involves immunization of mice, and hybridization of the mouse spleen cells with myeloma cells, to produce monoclonal cells of the hybrid. This in turn produces monospecific antibodies against the antigens, and can be applied to detection of Circulating-TAA in the sera of tumour-bearing patients. Even though it is

in its early stages with regard to CaCx. TAA, it promises to be a probable solution to the problem of specificity of the immunodiagnostic test, while ELISA promises to be the solution to that of the sensitivity of the system.

ELIMINATION OF FALSE POSITIVE REACTIONS

Most of the false positive reactions in the sera of non-cancerous individuals could be attributable to:

a) C- reactive protein (CRP)

b) Rheumatoid factor (RA)

c) Histocompatibility Antigens (HLA)

d) Bacterial contamination of reagents, and

e) Allotypes of human immunoglobulins (Tucker, 1979).

Most cancer patient sera as well as sera from many individuals with non-malignant conditions contain CRP. Since CRP is a foreign protein in human sera, occasionally, it elicits an immune response, resulting in the production of circulating antibodies to CRP.

A number of rabbit antisera used in these studies contain CxRP, an animal protein analogous to human CRP. The appearance of this protein in rabbits was probably the result of a traumatic response to antigen injection, since Sherman (1969) has shown that subcutaneous injection of incomplete Freund's adjutant may trigger off production of CxRP. It is important therefore, to determine the presence of CxRP in the rabbit antiserum because it may react with human anti-CRP in sera. Fortunately, anti-CRP in the test sera may be easily removed by adsorption with human CRP. In an earlier study (Ibrahim et al, 1979), this possibility was found to account for some of the false positive results. However, adsorption of the test sera in this study removed some of these false positive results.

Some positive reactions with normal human sera, had been attributed to the failure of adsorption procedures to eliminate antibodies to histo-compatibility antigens and allotypes of human immunoglobulins (Tucker, 1979). This hypothesis was supported by the fact that after adsorption of anti-CaCx. sera with a single normal serum that was known to give a false positive reaction, reactivity with that serum was no longer observed, but reactions with other sera with similar reactions were not eliminated. With the adsorption of anti-sera with both pooled normal cervical antigens and pooled normal human sera consecutively over a longer period (120 minutes each) for this study, it was possible to eliminate these false positive results to a greater extent.

Because rheumatoid factor is an anti-immunoglobulin, any screening study for Circulating-TAA in the general population using currently available reagents, would require the testing of all positive sera for the presence of this factor. This is especially significant because, it is estimated that only 25% of those whose sera are RA positive actually have any clinical evidence of rheumatoid disease (Ball and Lawrence, 1961). Furthermore, previously negative test sera was noted to become positive upon becoming contaminated with bacteria (Hirata et al, 1973) because bacterial lipopolysaccharides of gram negative bacilli cross-react with human transplantation antigens. However, in the study of Adelusi (1981), neither the presence of rheumatoid factor in the sera, nor bacterial contamination of the sera could account for any false positive results.

From the results obtained so far, this study appears promising for the development of a potential procedure which could be employed, not only for

the diagnosis of Carcinoma of the Cervix, but also for monitoring the prognosis of patients after surgery for example. Indications are that such immuno-diagnostic procedures can be designed in a way that is simple, reproducible and reliable, and would extend our present ability for detection of malignancies. A large scale sero-epidemiologic study, using antiserum to carcinoma of cervix tissue antigens, especially fairly purified TAA, would be required to confirm the study. Furthermore, some of the positive cases, especially of the non-malignant cases (Severe dysplasia and carcinoma in-situ) would be of great value for follow-up study.

CHAPTER 6

SUMMARY AND CONCLUSIONS

Immunodiffusion, with adsorption-in-gel, was the technique employed in this work to demonstrate the presence of Tumour Associated Antigens (TAA) in the two types of antigens (the Soluble Antigen and the Membrane Bound Antigens), prepared from a pool of Carcinoma of Cervix tissues, and Circulating-Tumour Associated Antigens (C-TAA) in the sera of tumour-bearing patients. Hyperimmune rabbit sera prepared against partially purified CaCx TAA, adsorbed with normal cervical tissue (NCx), and pooled normal human sera (NHuS), produced precipitin reactions with CaCx. antigens, and with sera of most patients with carcinoma of cervix, using this technique.

The absence of reactivity of hyperimmune rabbit sera prepared against NCx and NHuS, adsorbed with the homologous antigen preparations, when reacted with NCx or the tumour antigen preparations, indicate the tumour-relatedness of the TAA in the tumour extract preparation. Evidence has been provided in the present study which suggests that carcinoma of the cervix uteri possess soluble antigens specific for the tumours, and antibodies raised against these antigens were shown to detect up to 75.0% of cancer bearing patients.

To improve the specificity of the test method, preparation of specific anti-TAA antibodies would require the extraction and isolation of individual and pure TAA, so that the presence of minute amounts of the antigens, in nanogram amounts, might be revealed earlier in the circulation

in cancer bearing patients. To improve the specificity of the antiserum used, various methods of protein purification, including Ammonium Sulphate fractionation, DEAE Ion Exchange Chromatography, Sephadex gel filtration and Affinity Chromatography were employed in this study. These enhanced the isolation of individual, even if only partially pure, CaCx. TAA. The ultimate goal is to pursue the isolation of individual and highly purified TAA to be used as immunogens, to produce specific anti-TAA antibodies capable of detecting Circulating-TAA in the sera of tumour bearing patients. Thus, the progress of patients with pre-cancerous lesions would be followed up both clinically and immunologically.

The possible approaches to control of cancer of the cervix include:

a) Eradication of the causal agents;

b) Prevention of transmission of the disease, whether or not the causal agents are known; and

c) Early detection and treatment of the disease, preferably in its pre-invasive form.

Of these three approaches, the most desirable in the long run is the first. Unfortunately, as at now, the causal agent is yet unknown. Indeed it is unknown at the present stage whether cell transformation involves the known HLA being susceptible to infective agents causing the cell transformation or the development of completely new antigens. And this is worthy of study.

As regards the second alternative, on the basis of epidemiological evidence that the disease may be "sexually transmitted", it is likely that sexual abscention could prevent the disease, but this will be very unethical and unacceptable. The third approach, which is the one currently employed

by way of cytology in most places, is associated with a great number of problems.

The development of an immunodiagnostic screening test which is cheap, reliable and reproducible will not only overcome most of the problems enumerated in the study, but is most likely to benefit the group of women most likely to be affected, the low socio-economic group of women. Immunodiffusion adsorption-in-gel was found to be simple, reliable, reproducible and inexpensive even,if it is one of the least sensitive serological tests. There is need for improving and increasing the specificity and sensitivity of the TAA, and hence, the antiserum to the TAA to be used in the tests.

Finally, based on the findings reported in this study, a sero-diagnostic screening test for Circulating-TAA in the sera of carcinoma of cervix patients is feasible. The immunodiffusion test for the detection of Circulating-TAA has a good potential as a diagnostic tool for cancer. Indications are that immunologic procedures can be designed in such ways that are simple, reliable and reproducible. Such serologic tests would extend our present ability for detection and monitoring of malignancies. This would be of particular interest also in such other malignancies as ovarian cancer for which there is no reliable diagnostic test at present.

BIBLIOGRAPHY

Abelev, G.I. (1963): Study of antigenic structures of tumours. Acta Union Int. Cancer 19; 80-82

Abelev, G.I. (1968): Production of embryonal serum alphaglobulin by Hepatomas - Review of experimental and clinical data. Cancer Res. 28; 1344-1350

Abelev, G.I. (1971); Alpha-feto-protein in ontogenesis and its association with malignant tumours. Adv. Cancer Res. 14; 295-298

Adelusi, B., Osunkoya, B.O. and Fabiyi, A. (1975): Antibodies to Herpes type-2 virus in carcinoma of the cervix uteri in Ibadan. Am. J. Obstet. Gynaec. 123; 758-761

Adelusi, B., Osunkoya, B.O. and Fabiyi, A. (1976): Herpes Type-2 Virus Antigens in Human Cervical Carcinoma. Obstet. Gynecol. 47; 544-548

Adelusi, B. (1976) Herpes Type-2 virus and carcinoma of the cervix uteri in Nigerians: Immunovirological Studies. Ph. D. Thesis, University of Ibadan.

Adelusi, B., Ayeni, O., Fabiyi, A. and Osunkoya, B.O. (1976b): Serological evidence for the venereal mode of transmission of Herpes virus Type-2 infection in Ibadan, Nigeria. Nig. Med. J. 6; 386-391

Adelusi, B., Naib, Z.M., Muther, J. and Nahmias, A.J. (1981): Epidemiological studies relating Genital Herpes Simplex Virus (HSV-2) to cervical neoplasia - An Update: In the Human Herpes Viruses--An interdisplinary perspective. (A. Nahmias, W., Dowdle and R. Schinazi; Eds.). Elseview-North Holland. New York.

Adelusi, B. (1981): Tumour associated antigens in cervical cancer tissues and in the sera of Nigerian women with cervical cancer.
Afri. J. Med. Sci. 11: 123-127

Adelusi, B. (1982): Common antigens of Herpes Simplex Virus Type-2 (HSV-2) and Carcinoma of the Cervix.
W. Afric. J. Med. (In Press)

Adelusi, B., Nahmias, A.J., Ibrahim, A.N. and Ausperg, N. (1982): Detection of circulating tumour associated antigens in the semen of women with cervical neoplasia: Use of rabbit antiserum against a continuous cancer cell line (C-4II).
Brit. J. Obst. Gynaec. 89: 238-241

Anzai, T., Dreasman, G.R., Courtney, R.V., Adam, E., Rawls, W.E. and Binyesh-Melnick, M. (1975): Antibody to Herpes Simplex Virus Type-2 Induced Non-sturctural proteins in women with cervical acancer and in control groups.
J. Natl. Cancer Inst. 54; 1051-1059.

Ashley, D.J. (1966): The biologic status of carcinoma in-situ of the uterine cervix.
J. Obstet. Gynecol. Brit. Comm. 73; 372-381.

Auersperg, N., Hollinshead, A.C., Lee, O.B. and Wong, K.S. (1980): Detection of Herpes Simplex Virus-Tumour Associated Antigens in human cell lines after long term cultivation.
Clin. Immunol Immunopathol. (In Press).

Aurelian, L., Royston, I. and Davis, H.J. (1970): Antibody to genital Herpes Simplex Virus: Association with cervical Atypia and Carcinoma in-situ.

J. Natl. Cancer Inst. 45; 455-464

Aurelian, L., Strandberg, J.D. Melendez, L.V., and Johnson, L.V. (1971): Herpes Virus Type-2 isolated from cervical tumour cells grown in tissue culture.

Science 174; 704-707

Aurelian, L. (1973): Virus and antigens of Herpes Virus Type-2 in Cervical Carcinoma.

Cancer Res. 33; 1539-1547

Aurelian, L., Davis, J.J. and Julian, C.G. (1973): Herpes Virus Type-2 induced Tumour-specific antigen in cervical cancer.

Am. J. Epidem. 98; 1-9

Aurelian, L., Sternsdberg, J.D. and Marcus, R.L. (1974): Neutralisation Immunofluorescence and Complement fixation tests in identification of antibody to Herpes virus Type-2 induced Tumour-specific antigen in sera from squamous cervical carcinoma. Immunology of Cancer.

Prog. in Exp. Tumour Res. 19; 165-181.

Aurelian, L., Smith, F. and Garnish, J.D. (1976): IgM antibody to a Tumour-associated antigen (AG-4) induced by Herpes Simplex Virus Type 2 - Its use in location of the antigen in infected cells.

J. Natl. Cancer. Inst. 56; 471-477

Baldwin, R.W. and Barker, D.R. (1967): Tumour-Specific antigenicity of Aminoazo dry induced rat hepatomas.

Int. J. Cancer 2; 355-361

Baldwin, R.W., Glaves, D., Harris, J.R. and Price, M.R. (1971): Tumour-Specific antigens associated with aminoazo dye induced rat hepatomas.

Transplant. Proc. 111; 1189-1190

Baldwin, R.W. and Price, M.R. (1976): Nature and expression of tumour antigens associated with experimental animals and human tumours

Ann. Clin. Biochem. 13; 488-494

Baldwin, R.W. and Price , M.R. (1977): Tumour rejection antigens associated with carcinogenic "induced tumours".

Ric. Clin. Lab. 7; 102-111

Ball, J. and Lawrence, J.S. (1961): Epidemiology of the Sheep cell agglutination test.

Ann. Rheum. Dis. 20; 235-239

Barron, B.A. and Richart, R.M. (1971): An Epidemiologic study of cervical neoplastic disease.

Cancer 27; 978-986

Bashford, E.F. and Russel, B.R.G. (1910): Further evidence of the homogenecity of the resistance to the implantation of malignant new growths.

Royal Soc.London Proc. 82; 283-306.

Bell, C.E. (1976): A normal adult and fetal lung antigen present at different quantitative levels in different histologic types of human lung cancer.

Cancer 37; 706-713

Bell, R.B., Smith, C.S. and Aurelian, L. (1978): Humoral and Cellular Immunity to Herpesvirus Type-2 (HSV-2) Antigens in exfoliated cervical tumour cells.

Proc. Ann. Ass. Cancer Res. 19; 193-196

Beral, V. (1974): Cancer of the Cervix - A sexually transmitted infection?

Lancet 1; 1037-1040

Boddie, A.W., Holmes, CE.C., Roth, J.A. and Morton, D.L. (1975): Inhibition of human leukocyte migration in agarose by KCl extracts of carcinoma of the lung.

Int. J. Cancer 15; 823-829

Brawn, R.J. (1970): Possible Association of Embryonal Antigens with severe primary 3-Methyl-Cholanthrene-induced Murine Sarcomas.

Int. J. Cancer 6; 245-249

Bubenik, J., Perlmann, P., Helmstein, K. and Moberger, G. (1970): Immune response to urinary bladder tumours in man.

Int. J. Cancer 5; 39-46

Buitin, P. (1972): Antigens in human cancers.

Ann. Biol. Clin. 30; 95-97

Camacho, A. and Spear, P.G. (1978): Transformation of Hamster Embryo fibroblasts by a specific fragment of the Herpes Simplex Virus genome.

Cell 15; 993-1002

Carrell, S. and Theilkaes, L. (1973): Evidence for a Tumour-associated antigen in human malignant melanoma.
Nature 242; 609-610

Chiang, G.C.N., Del Buono, I., Tunca, C. and Nahmias, A.J. (1974): Herpes Simplex Virus Type-2 antigens detected in human cervical smears by anti-complementary Immunofluorescence.
Unpublished Data.

Channen, W. and Hollycock, V.E. (1971): Colposcopy and Electro-coagulation Diathemy for cervical Dysplasia and Carcinoma in-situ.
Obstet. Gynecol. 37; 623-628

Christopherson, W.M., Gray, L.A. and Parker, J.E. (1967): Role of punch Biopsy in subclinical lesions of the uterine cervix.
Obstet. Gynecol. 30; 806-811

Christopherson, W.M. (1969): Concepts of the genesis and development in early cervical neoplasia.
Obstet. Gynecol. Survey 24; 842-850

Cinader, B. (1972): The future of tumour immunology.
Med. Clin. N. America 56; 801-836

Coggin, J.H., Ambrose, K.R. and Anderson, N.C. (1970): Fetal antigen capable of inducing transplantation immunity against SV-40 hamster tumour cells.
J. Immunol. 105; 524-525

Coggin, J.H., Ambrose, K.R., Bellomy, B.B. and Anderson, N.G. (1971): Tumour immunity in hamsters immunized with fetal tissues.
J. Immunol. 107; 526-533

Collard, W., Thornton, H. and Green, M. (1973): Cells transformed by HSV-2 Transcribe Virus specific RNA sequences shared by Herpes Virus Types 1 and 2.

Nature New Biol. 243; 264-266

Coppleson, M. and Reid, D.L. (1967): Pre-Clinical Carcinoma of the Cervix Uteri.

Oxford Pergamen Press, England.

Copple, C.D. and McDougall, J.K. (1976): Clonal derivatives of a Herpes Type-2 Transformed hamster cell line (333-8-9). Cytogenetic analysis, tumourigenicity and virus sequence detection.

Int. J. Cancer 17; 501-510

Costanza, M.E. and Nathanson, L. (1974): Carcinofetal antigens. In: Progress in Clinical Immunology Vol. II (Ed. R.S. Schwartz).

Grune and Stratton, New York.

Deinhardt, F. (1974): Antigens associated with DNA Virus - induced tumours. In: Progress in Immunology Vol. III (Eds. L. Bient and J. Holbonow).

North-Holland Publishing Co. Amsterdam. New York

Doll, R. (1977): Introduction to origins of human cancer (Eds. H.H. Hiatt, J.D., Watson, and J.A. Winsten).

Cold Spring Harbor Conferences on Cell Proliferation Vol.4

Deunas, A., Adam, E., Melnick, J.L. and Rawls, W.E. (1972): Herpes Virus Type-2 in a prostitute population.

Am. J. Epidem. 95; 483-439

Duff, R. and Rapp, F. (1970): Reaction of serum from pregnant hamsters with surface of cells transformed by simian virus -40 (SV-40).

J. Immunol 105; 521-523

Duff, R. and Rapp, LF. (1971): Oncogenic transformation of hamster cells after exposure to Herpes Simplex Virus Type-2.

Nature New Biol. 233; 48-50

Duff, R. and Rapp, F. (1971b): Properties of hamster embryo fibroblasts transformed in-situ after exposure to UV-irradiated Herpes Simplex Virus Type-2.

J. Virol 8; 469-477.

Duff, R., Kreider, J.W., Levy, B.M., Katz, M. and Rapp, F. (1974): Comparative pathology of cells transformed by Herpes Simplex Virus Type-1 or Type-2.

J. Natl. Cancer Inst. 53; 1159-1164

Edington, G.M. and Hendrickse, M. (1970): Cancer in Ibadan. In: Cancer Incidence in Five Continents Vol. II (R. Muir and J. Waterhouse Eds.).

IUCC. Spinger-Verlag. New York

Feorino, P.M. and Palmer, E.L. (1973): Incidence of antibody to envelope antigen of Herpes Simplex Virus Type-2 among patients with cervical carcinoma.

J. Infect. Dis. 127; 732-735

Fluhmann, C.F. (1960): Carcinoma in-situ and the transitional zone of the cervix uteri.

Obstet Gynecol. 16; 424-437

Foley, E.J. (1953): Antigenic Properties of Mehtylcholanthrene - Induced Tumour in mice of the strain of origin.
Cancer Res. 13; 835-837.

Fox, C.H. (1967): Biologic behaviour of dysplasia and carcinoma in-situ.
Am. J. Obstet. Cynecol. 99; 960-974

Frenkel, N., Roizman, B., Cassai, E. and Nahmias, A. (1972): A DNA Fragment of Herpes Simplex-2 and its transcription in human cervical cancer tissue.
Proc. Natl. Acad. Sci. (USA) 69; 3784-3789

Fritshe, R. and Mach, J.P. (1975): Identification of a new Oncofetal Antigen associated with several types of human carcinomas.
Nature 258; 734-737

Fuks, A., Banjo, C., Shuster, J., Freedman, S.O. and Gold, P. (1974): Carcinoembryonic Antigen (CEA): Molecular biology and Clinical significance.
Biochemica et Biophysica Acta 417; 123-152

Gall, S.A., Walling, J. and Pearl, J. (91973): Demonstration of Tumour-associated agents in human gyraecologic malignancies.
Am. J. Obstet. Gynecol. 45; 387-393

Gall, J.A. and Haines, H. (1974): Cervical Carcinoma Antigens and the relationship to HSV-2.
Gynecol. Oncology 2; 451-459

Gold, P, and Freedman, S.O. (1965): Demonstration of Tumour Specific Antigens in human colonic carcinoma by immunolgical tolerance and adsorption techniques.
J. Exptl. Med. 121; 439-452.

Gold, P. (1967): Circulating Antibodies against Carcinoembryonic antigens of the human digestive system.
Cancer 20; 1663 - 1667

Gold, P. (1971): Embryonic origin of human tumour specific antigens.
Prog. Exptl. Tumour Res. 14; 43-58

Goldberge, R.J. and Gravell, M. (1976): A search for Herpes Simplex Virus Type-2 markers in Cervical Carcinoma.
Cancer Res. 36; 759-799

Goldenberg, D.M., Garner, T.F., Pant, K.D. and Van Nagell, J.R. (1978): Identification of beta-oncofetal antigen in cervical squamous cancer and its demonstration in neoplastic and normal tissues.
Cancer Res. 38; 1246-1249

Gorer, P.A. (1961): The antigenic structure of tumours.
Adv. Immunol 1; 345-393

Gross, L. (1943): Intradermal immunisation of C_3H mice against sarcoma that originated in animal of the same line.
Cancer Res. 3; 326-333

Gross, L. (1970); Oncogenic Viruses. 2nd Edition
Pergman Press, New York

Gusberg, S.B. and Frick, H.C. (1970); Corscaden's Gynaecologic Cancer. (4th Edition).

Habel, K. (1961); Resistance of Polyoma Virus immune animals to transplanted polyoma tumours.
Prof. Soc. Exptl. Biol. Med. 106; 722-725

Habel, K. (1962): Antigenic properties of cells transformed by polyoma virus. Cold Spring Harbor Symposium. Quant. Biol. 27; 433-439

Hall, J.E. and Walton, L. (1968): Dysplasia of the Cervix: A prospective study of 206 cases. Am. J. Obstet. Gynecol. 100; 662-671

Heise, E.R., Kucera, L.S., Raben, M. and Homesley, H. (1979): Serological response patterns to Herpes Virus Type-2 early and late antigens in cervical carcinoma patients. Cancer Res. 39; 4022-4026

Hellstrom, I. (1965); Distinction between the effects of anti-viral and anti-cellular polyoma antibodies on polyoma tumour cells. Nature 206; 652-653.

Hellstron, K.E. and Hellstrom, I. (1967): Immunologic deferences against Cancer. In: Immunobiology. (Ed. R.A. Good). Sinauer Associates Inc. Stamford, Connecticut.

Hellstrom, I., Hellstrom, K.E., Pierce, G.E. and Bill, A.H. (1968): Demonstration of cell bound and humoral immunity against Neuroblastoma cells. Proc. Natl. Acad. Sci. (USA) 60; 1231-1238

Hellstron, K.E. and Hellstrom, I. (1969): In: Advances in Cancer Research. (Eds. J.P. Greenstein and A. Haddow) Vol. 12 Academic Press, New York

Hellstrom, I., Hellstrom, K.E., Bill, A.H., Pierce, G.E. and Yang, J.P.S. (1970): Studies on Cellular Immunity to human Neuroblastoma Cells. Int. J. Cancer 6; 172-188

Hellstrom, I. and Hellstrom, K.E. (1972): New concepts of cancer of the colon and rectum. Cellular immunity to human colonic carcinomas. Dis. Colon Rectum 15; 100-105

Heppner, G.H., Kopp, J.S. and Medina, D. (1976): Microcytotoxicity assay of immune responses to non-mammary tumour virus-induced pre-neoplastic, and neoplastic mammary lesions in BALB/C mice. Cancer Res. 36; 753-758

Herberman, R.B. (1977); Immunogenicity of tumour antigens. Biochemica et Biophysica Acta 473; 93-119

Hirai, H. (1975); Opening remarks of Conference on Carcinofetal proteins. N.Y. Acad. Sci. Ann. 259; 5

Hirata, A.A., McIntire, F.C., Terasaki, P.I. and Mittal, K.K. (1973); Cross-reactions between human transplantation antigens and bacterial polysaccharides. Transplantation 15; 441-445

Hollinshead, A., Lee, O.B., McKelway, W., Melnuck, J.L. and Rawls, W.E. (1972): Reactivity between Herpes Virus Type-2 related Soluble Cervical Tumour Cell Membrane Antigens and matched cancer and control sera. Proc. Soc. Exptl. Biol. Med. 145; 688-693

Hollinshead, A.C., Lee, O., Chietien, P.B., Tarplay, J.L., Rawls, W. and Adam, E. (1973): Antibodies to Herpes Virus "Non-virion" Antigens in Squamous Carcinomas. Science 182; 713-715

Hollinshead, A.C., Chietren, P.B., Lee, O.B., Tarpley, J.L., Kerney, J.E., Silverman, N.A. and Alexander, V.C. (1976): In vivo and in-vitro measurements of the relationship of human squamous carcinomas to Herpes Simplex Virus Tumour Associated Antigens.
Cancer Res. 36; 821-828

Hollinshead, A.C. and Stewart, T.H.M. (1979); In: Proc. 1976 III International Symposium on "Detection and Prevention of Cancer" Vol. IV Part 2.
Marcel Dekker

Huebner, R.J., Rowe, W.P., Turner, H.C. and Lane, W.T. (1963): Specific Adenovirus complement fixing antigens in Virus-free Hamster and Rat Tumours.
Proc. Nat. Acad. Sci. (USA) 50; 379-389

Hulka, B.S. and Kupper, L.L. (1971): An Epidemiologic Test of the Spectrum of disease concept in Cervical Neoplasia.
J. Natl. Cancer Inst. 47; 1215-1222

Hutfield, D.C. (1968); Herpes Genitalis.
Brit. J. Vener. Dis; 44; 241-250

Ibrahim, A.N. and Hammon, W.M. (1968); Application of Immunodiffusion Methods to the Antigenic Analysis of Dengue Viruses.
I: Precipitation in-gel double diffusion in two dimensions.
J. Immunol. 100; 86-92

Ibrahim, A.N. and Hammon, W.M. (1968b): Application of Immunodiffusion Methods to Antigenic Analysis of Dengue Viruses.
II: Immunoelectrophoresis
J. Immunol. 100; 93-98

Ibrahim, A.N. (1969): Elimination of most tissue reactions in Immunodiffusion and Immunoelectrophoretic studies of arboviruses Proc Soc. Exptl. Biol. Med. 130; 785-787.

Ibrahim, A.N., Ray, M. and Nahmias, A.J. (1975): Tumour Antigens in Hamsters with sarcomas associated with Herpes Virus Type-2. Proc. Soc. Exptl. Biol. Med. 148; 1025-1028

Ibrahim, A.N., Ray, M., Megaw, J., Brown, R. and Nahmias, A.J. (1976): Common Antigens of Herpes Simplex Virus Type-2 Associated with Hamster Tumours and Human Cervical Cancer. Proc. Soc. Exptl. Biol Med. 152; 343-347.

Ibrahim, A.N., Robinson, R.A., Marr, L., Abdelal, A.T.H., and Nahmias, A.J. (1979): Tumour associated antigens in Cervical Cancer tissues and in sera from patients with cervical cancer or with head and neck cancer. J. Natl. Cancer. Inst. 63; 319-323

Jehn, U.W., Nathenom, L., Schwartz, R.S. and Skinnerm, A. (1970): In vitro lymphocyte stimulation by soluble antigens from malignant melanoma. New Eng. J. Med. 283; 329-333.

Johnson, L.D., Hertig, A.G., Hinman, C.H. and Easterday, C.L. (1960): Pre-invasive cervical lesions in Obstetric patients - method of diagnosis, cause and clinical management. Obstet. Gynec. 16; 133-145.

Johnson, L.D., Easterday, C.L., Gore, H. and Hertig, A.G. (1964): The Histogenesis of Carcinoma in-situ of the Uterine Cervix. A preliminary report of the origin of carcinoma in-situ in

subcylinderical cell anaplasia.

Cancer. 17; 213-229

Johnson, L.D., Nickerson, R.J., Easterday, C.L., Stewart, R.S. and Hertig, A.G. (1968): Epidemiologic evidence for the Sprectrum of change from dysplasia through carcinoma in-situ to invasive carcinoma.

Cancer 22; 901-914

Johnson, L.D. (1969): The Histopathological approach to early cervical Neoplasia.

Obstet. Gynecol. Survey 24; 735-767

Josey, W.E., Nahmias, A.J. and Naib, Z.M. (1972): The Epidemiology of Type-2 (Genital) Herpes Simplex Infection.

Obstet. Gynec. Survey 27; 296-302.

Kessler, I.I. (1977): Veneral Factors in Human Cervical Cancer.

Cancer 39; 1912-1919

Kimura, S., Flannery, V.L., Levy, B. and Schaffer, P.A. (1975): Oncogenic Transformation of primary Hamster cells by Herpes Simplex Virus Type-2 (HSV-2) and HSV-2 temperature sensitive mutant.

Int. J. Cancer 15; 786-798

Klein, G., Sjogren, H.O., Klein, E. and Hellstrom, K.E. (1960): Demonstration of resistance against Methylcholanthrene-induced sarcomas in the primary authochthonous host.

Cancer Res. 20; 1561-1572

Klein, G., Clifford, P., Klein, E. and Stjeinsward, J. (1966): Search for Tumour-specific Immune Reactions in Burkitt Lymphoma patients the Membrane Immuno-Fluorescence reaction.
Proc. Nat. Acad. Sci. (USA) 55; 1682-1685

Koss, L.G., Stewart, F.W., Foote, F.W., Jordan, M.J., Barder, G.M. and Day, E. (1963): Some histological aspects of behaviour of epidemoid carcinoma in-situ and related lesions of the uterine cervix - Long Term Prospective Study.
Cancer 16; 1160-1211

Lausch, R.N. and Rapp, F. (1974): Tumour-specific antigens and re-expression of fetal antigens in mammalian cells.
Prog. Exptl. Tumour Res. 19; 45-58.

Leonard, E.J. E.J., Meltzer, S.M., Barsos, T. and Rapp, H.J. (1972); Properties of Tumour-specific antigens solubilised by hypertonic potassium chloride.
Natl. Cancer Inst. Monograph 35; 129-134.

Leonard, E.J. (1975): Cell-surface tumour associated antigens of chemically induced tumours. In: Cellular Membranes and Tumour Cells behaviour. 28th Annual Symposium on Fundamental Cancer Research.
Williams and Wilkins Co. Baltimore Med. pp.385-397

Leung, J.P., Plow, E.F., Eghdat, Y., Marchesi, V.T. and Edgington, T.S. (1977): Delineation of three classes of CEA Antigenic determinants. Identification of Membrane Associated CEA as an independent species of CEA.
J. Immunol. 119; 271-282

Levi, M., Keller, S. and Mandl, I. (1969): Antigenicity of a papillary serous cysadenocarcinoma tissue homogenate and its fractions. Am. J. Obstet. Gynecol. 105; 856-861.

Levi, M. (1971): Antigenicity of ovarian and cervical malignancies with a view towards possible immunodiagnosis. Am. J. Obstet. Gynecol. 109; 689-698

Little, C.C., (1941); Review of progress in study of genetics of spontaneous tumour incidence. J. Natl. Cancer Inst. 1; 727-736.

Loeb, L. (1902): Further investigations in transplantation of Tumours. J.N. Med. Res. 3; 44-73

Loeb, L. and Wright, S. (1927): Transplantation and individuality differentials in inbred families of guinea pigs. Am. J. Path. 3: 251-283

Loeb, L. and King, H.D. (1929): Individuality differentials, in stains of in-bred rats. Arch. Path. 12; 203-221

Lo Gerfo, P. (91976): Tumour Antigens associated with lung cancer. In: Lung Cancer (Eds. L. Israel and A.P. Chahinian). Academic Press, New York. pp. 51-93.

McDongall, J.K., Galloway, D.A. and Fenoglio, C.M. (1980): Cervical Carcinoma-detection of Herpes Simplex Virus RNA in cells undergoing neoplastic change. Int. J. Cancer 25: 1-8

McNab, J.C.M. (1974): Transformation of rat embryo cells to temperature sensitive mutants of Herpes Simplex Virus.
J. Gen. Virol 24: 143-153

McNeil, C., Ladle, J.N., Helmick, W.M., Trentleman, E. and Wentz, M.W.(1969) An Antiserum to ovarian mucinous cyst fluid with colon cancer specificity.
Cancer Res. 29: 1535-1540

Megaw, J.M. (1974): Immunological studies on Herpesvirus-associated Hamster tumours and possible relationship with human cervical cancer.
M.S. Thesis: Georgia State University

Meltzer, M.S., Leonard, E.J., Rapp, H.J. and Borsos, T. (1971): Tumour Specific Antigen solubilised by hypertonic KCl.
J. Natl. Cancer. Inst. 47: 703-709

Minson, A.C., Thouless, M.E., Eglin, R.P. and Darby, G. (1976): The Detection of virus DNA sequences in a herpes type-2 transformed hamster cell line (333-8-9).
Int. J. Cancer 17: 493-500

Morton, D.C., Elber, F.R., Malmgren, R.A. and Wood, W.C. (1970): Immunological factors which influence response to immunotherapy and malignant melanoma.
Surgery 68: 158-162

Munoz, N. (1973): Effects of Herpes virus Type-2 and Hormonal imbalance on the Uterine Cervix of the Mouse.
Cancer Res. 33: 1504-1508

Munyon, W., Krasielburd, E., Davis, D. and Mann, J. (1971): Transfer of Thymidine Kinase to Thymidine Kinaseless L Cells by infection with ultraviolet irradiated Herpes Simplex Virus.
J. Virol. 7: 813-820

Nahmias, A. and Dowdle, W. (1968): Antigenic and biologic differences in herpesvirus hominis.
Prog. Med. Virol. 10; 110-159

Nahmias, A.J., Naib, Z.M., Jose, W.E., Murphy, F.A. and Luce, C.F. (1970): Sarcomas after inoculation of newborn hamsters with Herpes Virus hominis Type-2 strains.
Proc. Soc. Exptl. Biol. Med. 134: 1065-1069

Nahmias, A.J., Josey, W.E., Naib, Z.M., Luce, C.F. and Guest, B.A. (1970b): Antibodies to Herpesvirus Hominis Types 1 and 2 in humans. II: Women with Cervical Cancer.
Am. J. Epidem. 91: 547-552.

Nahmias, A.J., Naib, Z.M., and Josey, W.E. (1970c): Herpes Virus Hominis Type-2 Infection. Association with cervical cancer and perinatal disease.
Perspect. Virol. 7: 73-88

Nahmias, A.J., Naib, Z.M. and Josey, W.E. (1972): Genital Herpes and Cervical Cancer - Can a causal relation be proven ?
In: Biggs, P.M., deThe, G. and Payre, L.N. (Eds.). Oncogenesis and Herpes Viruses.
IARC Scientific Publications. Lyon, France.

Nahmias, A., Del Buono, I. and Ibrahim, I. (1975): Antigenic relation between Herpes Simplex Virus, Human Cervical Cancer and HSV-

associated hamster tumours. In Herpesviruses and oncogenesis Vol.2 (Eds. G. deThe, W. Henle and F. Rapp). IARC Scientific Publications. Lyon, France

Nahmias, A.J., Josey, W.E. and Oleske, J.M. (1980): Epidemiology of cervical cancer. In: Viral Infections of Humans. Plenum. Publishing Corp. New York.

Naib, Z.M., Nahmias, A.J. and Josey, W.E. (1966): Cytology and Histopathology of cervical Herpes Simplex Viirus. Cancer. 19: 1026-1031

Navratil, E., Brughard, E. Bajardi, F. and Nash, W. (1958): Simultaneous Colposcopy and Cytology used in screening for carcinoma of the cervix. Am. J. Obstet. Gynecol. 75: 1292-1297.

Nelson, D.S. (1974): Antigens of carcinoma of the cervix uteri. A study by means of immunofluorescence. Clin. Expt. Immunol. 16: 53-62

Notter, M.F.D. and Docherty, J.J. (1976): Comparative diagnostic aspects of Herpes Simplex Virus Tumour associated Antigens. J. Natl. Cancer Inst. 57: 483-488

Notter, M.F.D., Docherty, J.J., Mortel, R. and Hollinshead, A.C. (1978): Detection of Herpes Simplex Virus Tumour-associated Antigen in Uterine cervical cancer tissue: Five case studies. Gynecol Oncol. 6: 574-581

Old, L.V. and Boyse, E.A. (1964): Immunology of experimental tumours. Ann. Rev. Med. 15: 167-186

Order, S.E., Porter, M. and Hellman, S. (1971): Hodgkins disease - Evidence for a tumour-associated antigen.
New Eng. J. Med. 285: 471-474.

Pasca, A.S., Kummerlander, B., Pejtsik, B., Krommer, K. and Pali, K. (1976): Herpes Simplex Virus-specific Antigens in exfoliated cervical cells from women with and without cervical anaplasia.
Cancer Res. 36: 2130-2132

Prehn, R.T., and Main, J.M. (1957): Immunity to Methyl-Cholanthrene-induced Sarcomas.
J. Natl. Cancer Inst. 18: 769-778

Rangan, S.R.S., Mukherjee, A.L. and Bang, F.B. (1968): Search for adenovirus etiology of human oral and pharyngeal tumours.
Int. J. Cancer 3: 819-828

Rawlins, D.R. (1977): Demonstration of tumour associated antigens in human lung cancer tissues and in sera of tumour bearing patients
M.S. Thesis. Georgia State Unitersity.

Rawls, W.E., Tompkins, W.A.F. and Melnick, J.L. (1969): The Association of Herpesvirus Type-2 and Carcinoma of the Uterine Cervix.
Am. J. Epidem. 89: 547-554

Rawls, W.E., Gardner, H.L., Flanders, R.W., Lowry, S.P., Kaufman, R.H. and Melnick, J.L. (1971): Genital Herpes in two social groups.
Am. J. Obstet. Gynec. 110: 682-689

Rapp, F. and Duff, R. (1973): Transformation of hamster embryo fibroplasts by Herpes Simplex Viruses Types 1 and 2
Cancer Res. 33: 1527-1534

Ray, M. (1974): Herpes Simplex Virus Type-2 associated tumour specific antigens in Hamsters.

M.S. Thesis: Georgia State University.

Reisfeld, R.A., Pelligrino, M.A. and Kahan, B.D. (1971): Salt extraction of soluble HLA antigens.

Science 172: 1134-1136

Reyensoro, G., Chu, T.M., Holyoke, D., Cohen, E., Nemoto, T., Wang, J.J. Chuang, J., Guinan, P. and Murphy, G.P. (1972): Carcino-embryo Antigen in patients with different cancers.

J.A.M.A. 220: 361-365

Richart, R.M. (1967): Natural history of cervical intraepithelial Neoplasia.

Clin. Obstet. Gynecol. 10: 748-784

Richart, R.M. and Barron, B.A. (1969): Follow-up study of patients with Cervical Dysplasia.

Am. J. Obstet. Gynecol. 105: 386-393

Ristow, S. and McKahn, C.F. (1977): Tumour associated antigens.

In: Mechanisms of Tumour Immunology (Eds. I. Green, S. Cohen and R.T. McCluskey).

John Wiley and Sons. New York.

Rogers, G.T. (1976): Heterogenecity of Carcinoembryonic antigen. Implications on its role as a tumour marker substance.

Biochemica et Biophysica Acta 458: 355-373

Roizman, B. and Furlong, D. (1974): The replication of Herpesviruses.

In: Comprehensive Virology Vol. 3 (H. Franekel-Comat and R.R. Wagner, Eds.).

Plenum Publishers. New York

Rotkin, I.D. (1967): Adolescent coitus and cervical cancer: Associations of related events with increased risk.

Cancer Res.: 27: 603-617

Rotkin, I.D. (1973): A Comparative review of key epidemiologic studies in cervical cancer related to current searches for transmissible agents.

Cancer Res. 33: 1353-1367

Roth, J.A., Holmes, E.C., Boddie, A.W. and Morton, D.L. (1975): Lymphocyte responses of lung cancer patients to TAA measured by leucine incorporation.

J. Thorac. Cadiovasc. Surg. 68: 613-618

Rosen, S.W., Weintraub, B.D., Vaitukaitis, J.L., Sussman, H.H., Hershman, J.M. and Muggia, F.M. (1975): Placental proteins and their sub-units as tumour markers.

Ann. Intern. Med. 82: 71-83

Rowe, W.P. (1967): Virus specific antigens in viral tumours. In: Cross reacting antigens and neoantigens (Ed. J.J. Trentin).

NAS-NRC. Washington D.C.

Royston, I. and Aurelian, L. (1970): Immunofluorescent detection of Herpes Virus antigens in exfoliated cells from human cervical carcinoma.

Proc. Natl. Acad. Sci. (USA) 67: 204-221

Sabatelle, R., Sedlis, A., Sall, S. and Tchertkoff, V. (1969): Cervical Biopsy versus Conisation.

Cancer 23: 663-666

Sabin, A.B. and Tarro, G. (1973): Herpes Simplex and Herpes Genitalis viruses in etiology of some human cancers.
Proc. Natl. Acad. Sci. (USA) 70: 3225-3229

Sabin, A.B. (1974): Herpes Simplex Genitalis Virus "Non-virion" antigens and their implication in certain human cancers: Unconfirmed.
Proc. Natl. Acad. Sci. (USA) 71: 3248-3253

Sell, S. and Skelly, H. (1976): Tissue sites of Alpha-fetoprotein Synthesis by the rat during pregnancy and hepatoma growth.
J. Natl. Cancer Inst. 56: 645-648

Sherman, M. (1969): Immune mechanisms in health and disease. In: A review of research grants supported by the National Heart and Lung Institute.
US. Department of H.E.W. Bethesda, Md.

Shimkin, M.B. (1955): A note on the history of transplantation of tumours.
Cancer 8: 653-655

Shortland, N.R., Monsansy, K., Fees, R.C. and Potter, C.W. (1979): Tumorigenicity of herpesvirus hominis type-2 transformed cells (Line 333-8-9) in adult hamsters.
J. Pathol. 129: 169-178

Silbar, E.L. and Woodruff, J.D. (1966): Evaluation of biopsy, cone and Hysterectomy in intra-epithelial carcinoma of the cervix.
Obstet. Gynecol. 27: 89-97

Silverberg, E. (1977): Cancer Statistics.
CA- A Cancer J. for Clinicians 27: 26-31

Singleton, W.P. and Rutledge, F. (1968): To cone or not to cone - The Cervix.
Obstet. Gynecol. 31: 430-436

Sjogren, H.O. (1961): Further studies on the induced resistance against Iso-transplantation of polyoma tumours.
Virol. 15: 214-218

Sjogren, H.O., Hellstrom, I. and Klein, G. (1961): Transplantation of polyoma virus-induced tumours in mice.
Cancer Res. 21: 329-337

Skinner, G.R.B. (1976): Transformation of primary hamster embryo fibroblasts by Type-2 Herpes Simplex Virus - Evidence of a "Hit and run" mechanism.
Brit. J. Cancer 22: 798-801

Stroehmann, I., Schmidt, R., and Dalkmann, B. (1977): Rat transplantation antigens. V: A variation of KCl-method releasing low molecular weight antigen.
Immunol 32: 227-231

Tatarinov, Y.S. (1964): Detection of embryo-specific globulin in the blood sera of patient with primary liver tumour.
Vopr. Med. Khim 10: 90-91

Terris, M. and Oalman, M.C. (1960): Carcinoma of the Cervix.
J.A.M.A. 174: 1847-1851

Terry, N.D., Hewkart, P.A., Coligan, J.E. and Todd, C.W. (1974): Carcino-embryonic antigen: Characterisation and clinical application.
Transplant Rev. 20: 100-125

Thompson, D.M.P., Krupey, V., Freedman, S.O. and Gold, P. (1969): The Radioimmunoassay of circulating carcinoembryonic antigen of the human digestive system.

Proc. Natl. Acad. Sci. (USA) 64: 161-167

Thomson, B.H., Woodruff, J.D., Davis, H.J., Julian, C.A. and Silva, F.G. (1972): Cytopathology, histopathology and colposcopy in the management of cervical neoplasia.

Am. J. Obstet. Gynecol. 114: 329-338.

Ting, C., Lavrin, D.H., Shiu, G. and Herberman, R.B. (1972): Expression of Fetal Antigens in Tumour Cells.

Proc. Natl. Acad. Sci. (USA) 69: 1664-1668

Tucker, R.A. (1979): Tumour antigens in Cervical Cancer Tissues.

M.S. Thesis, Georgia State University

Von Kleist, S., Charennel, G., and Burtin, P. (1972): Indentification of an antigen from normal human tissue that cross-reacts with the CEA.

Proc. Natl. Acad. Sci. (USA) 69: 2492-2494

Watson, R.D., Smith, A.G. and Levy, J.G. (1975): The detection by immunodiffusion of tumour associated antigenic components in extracts of human bronchogenic carcinoma.

Brit. J. Cancer 32: 300-309

Witz, I.P., Lee, N., and Klein, G. (2976): Serological detectable specific and cross-reactive antigens on the membrane of a polyoma virus induced murine tumour.

Int. J. Cancer 18: 243-249

Wynder, E.L., Cornfield, J., Schroff, T.D. and Docaiswani, K.R. (1954): Study of environmental factors in carcinoma of the cervix. Am. J. Obstet. Gynecol. 68: 1016-1052.

APPENDIX I

ABREVIATIONS

AFP	-	Alpha Fetoprotein
BOFA	-	Beta Oncofetal Antigen
CaCx	-	Carcinoma of Cervix
CCS	-	Carcinoma of Cervix Serum
CEA	-	Carcinoembryonic Antigen
CRP	-	C-Reactive Protein
C-TAA	-	Circulating Tumour Associated Antigen
CxRP	-	Rabbit protein analogous to Human CRP
DEAE	-	Diethylaminoethyl-cellulose
DNA	-	Deoxyribonucliec Acid
DN-ase	-	Deoxyribonuclease
EBSS	-	Earle's Balanced Salt Solution
EDC	-	1-Ethyl-3-(3-Dimethyl-aminoproyl) Carbodimidine Hydrochloride
FCA	-	Freund's Complete Adjutant
HLA	-	Human Leukocyte Antigen
HSV-2	-	Herpes Simplex Virus Type-2
ID	-	Immunodiffusion
IDA	-	Immunodiffusion Adsorption
IEP	-	Immunoelectrophoresis
KCl	-	Potassium Chloride
MBA	-	KCl Solubilised Membrane Bound Antigen

NaCl	-	Sodium Chloride
NHS	-	Normal Hamster Serum
NHuS	-	Normal Human Serum
OT-1, OT-II	-	HSV-2 Associated Hamster Tumours
PBS	-	Phosphate Buffered Saline
PSNB	-	Penicillin-Streptomycin-Neomycin-Bacitracin
RA	-	Rheumatoid arthritis factor
RNA	-	Ribonucleic Acid
SA	-	Soluble Antigen
TAA	-	Tumour Associated Antigen
TBHS	-	Tumour Bearing Hamster Serum
Tris-HCl	-	Tris (Hydroxymethyl) aminomethane-Hydrochloride
TSTA	-	Tumour Specific Transplantation Antigen

APPENDIX II

Earl's Balanced Salt Solution (EBSS)

50.0 ml	Earle's basic salt solution
5.0 ml	Antibiotic solution (PSNB)
0.5 ml	Mycostatin suspension
455.5 ml	Sterile deionized water
500.0 ml	Total Volume

Final pH is adjusted to 7.6 with 8.8% $NaHCO_3$. EBSS with 3X antibiotics requires 15.0 ml PSNB and 434.5 ml. sterile dH_2O in the above formula.

Antibiotic Solution (PSNB)

In 50 ml EBSS (1X), dissolve the following:-

5×10^6 units	Penicillin G.
5.0 gm.	Streptomycin sulphate
5.0 gm.	Neomycin sulphate
5×10^6 units	Bacitracin

Solution is diluted to 200 ml. with additional EBSS, and filter sterilized (Millipore 10.22 um). Concentrations in the final PSNB solution are as follows:-

Penicillin G	25,000 u/ml
Streptomycin	25,000 mg/ml
Neomycin	25,000 mg/ml
Bacitracin	250 u/ml.

Mycostatin Suspension

Mycostatin (Squibb, Nystatin) 500,000 units is suspended in 16.6 ml of EBSS (1X) to give a final concentration of approximately 30,000 u/ml. Suspension is prepared aseptically since mycostatin can neither be autoclaved nor filtered.

0.01 Molar Phosphate Buffered Saline (PBS) pH 7.6

0.45 gm	-;	NaN_2PO_4
1.80 gm	-	$Na_2H\ PO_4$
8.50 gm	-	NaCl
0.20 gm	-	NaN_3

Deionized water is added to bring the final volume to 1 liter. Solution is terilized by autoclaving.

0.12 Molar Tris HCl Buffer pH 8.0

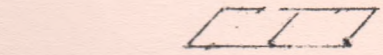
2.42 gm	-	Tris (Tham)

Deionized water is added to bring the volume to 1 liter. pH is adjusted to 8.0 with HCl.

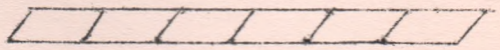
APPENDIX III

PATIENTS' DATA

1. Name (Initials Only)

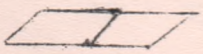


2. Hospital No.



3. Personal History:

i) Age (In years)



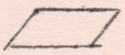
1) 20
2) 21 - 30
3) 31 - 40
4) 41 - 50
5) 51 - 60
6) 60

ii) Socio-economic Status

1) Upper Class
2) Upper Middle
3) Lower Middle
4) Lower Class

4. Gynaecologic History:

i) Marital Status -



1) Single
2) Married
3) Separated
4) Divorced
5) Widowed

ii) No. of marriages (if married) -

1) Don't know
2) None
3) 1
4) 2
5) 3
6) 4+

11

iii) Age at first coitus:

1) Don't know
2) Under 11
3) 11 - 15
4) 16 - 20
5) 21 - 25
6) 26+

12 13

iv) No. of sex partners:

1) Don't know
2) 1
3) 2
4) 3
5) 4
6) 5+

14

v) Frequency of intercourse:

1) Don't know
2) 1
3) 2
4) 3
5) 4
6) 5+

15

vi) Consorts Circumscission:

16

1) Don't know
2) Yes
3) No

vii) No. of Pregnancies:

17

1) 0
2) 1
3) 2
4) 3
5) 4
6) 5
7) 6
8) 7
9) 8+

5. Examination Findings:

18

i) Clinical Presentation:

1) Normal cervix
2) Cervical Erosion
3) Cervicitis
4) Ulcerative growth
5) Fungating growth

ii) Clinicopathologic stage ;

19

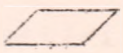
1) No malignancy
2) Stage I
3) Stage II(a)
4) Stage II(b)
5) Stage III
6) Stage IV

iii) Histologic type:

20

1) Well diff. Sq. Cell Ca.
2) Poorly diff. Sq. Cell Ca.
3) Undifferentiated Ca.
4) Adenocarcinoma Cx.
5) Adenosquamous Ca.
6) Others.

iv) Pathological Grade:



21

1) Grade 1
2) Grade 2
3) Grade 3
4) Others.

APPENDIX IV

BRIEF CLINICAL INFORMATION ON STUDY POPULATION

1. Geographic Places of Origin

All the women included in the study population are Nigerians. 110 (68.5%) of these were from the Yoruba speaking area of the country while the rest came from other parts, including the Ibo speaking (10.1%), Edo speaking (7.6%), Hausa speaking (5.8%), Itsekiri speaking (2.8%) and other areas (5.2%).

2. Age Incidence

For the cancer bearing women, the ages ranged between 25 and 66 years, with a peak age incidence of 36-45 years, (Table 3.1) and a mean of 40.2 years. Similarly, the youngest woman with benign gynaecological problem, but no cytological evidence of carcinoma of the cervix, was 24 years, and the oldest was 55 years. The peak age incidence was 36-45 years with a mean of 35.6 years.

On the other hand, the ages of the pregnant control women ranged from 18 to 42 years, with a peak age incidence of 26-30 years (Table 3.3) and a mean of 28.4 years. The healthy control women were aged 21 to 40 years, with a peak age incidence of 26-35 years and a mean of 36.1 years.

3. Socio-economic Status

Analysis of the cancer bearing women according to their socio-economic classes, based on the computation of their level of education, employment opportunities

and total family income, showed that the majority of these were from the lower socio-economic classes (Table 3.2). Even though a few of the women with benign gynaecological problems, pregnant women, and healthy controls were classified as upper socio-economic classes, there was not much difference in the distribution of these women on socio-economic basis, as compared with the cancer bearing women.

4. Marrital Status

Of the cancer bearing women, 30 (83.3%) were married, while 2 were divorced and 4 were widowed as at the time of the study. On the other hand, all the 18 women with benign gynaecological problems were married, while 25 of the healthy controls were married, 7 separated and 4 were single women. The marrital status of the pregnant women were not separately ascertained as at the time of the study.

5. Coital Practice

Analysis of age at first coitus among the cancer bearing women showed that 7 (19.5%) of these were known to have commenced heterosexual coital activities in early life (11-15 years), as compared with the healthy control women where 4 (11.2%) were known to have commenced heterosexual activities at the same age period. It was found that the cancer bearing women had intercourse more frequently than the healthy controls.

Whereas 14 (39.2%) of the cancer bearing women had intercourse three or more times a week only 6 (16.8%) of the healthy controls had intercourse three or more times a week. It was difficult to ascertain the frequency of intercourse or the age of onset of coitus among the pregnant women, hence this was not analysed. Similarly, in view of the inconsistent answers obtained with regards to the numbers of coital partners among most of the subjects this also was discountenanced.

6. Obstetric History

Many of the women under study were multiparous. However, while 18 (50.0%) of the cancer bearing women had six or more pregnancies, only 5 (27.8%) of the women with benign gynaecological problems, 18 (25.0%) of the pregnant women and 7 (33.4%) of the healthy controls have had six or more pregnancies.

7. Clinical Symptoms in Cancer Bearing Women

Abnormal vaginal bleeding was the commonest complaint (56.0%). This varied from postcoital and inter-menstrual bleeding to postmenopausal bleeding. Others include foul smelling vaginal discharge (35.4%), weight loss (33.6%), Pelvic pain (28.0%), and urinary symptoms such as dysuria, frequency and Hematuria (22.4%). Gastro-intestinal symptoms, Insomnia and peripheral edema were also complained of by a few of the women.

8. Clinico-pathologic Stage of Disease in Cancer Bearing Women

When the clinico-pathological stage of carcinoma of cervix was analysed, it was found that only 4 (11.2%) of the patients presented in Stage 1, while 7 (19.6%) presented in Stage II(a), 12 (33.6%) presented in Stage II(b) and 10 (28.0%) presented in Stage III. The remaining 3 (8.4%) women presented in Stage IV (Table 3.2). Even though there was not much difference between this distribution and an earlier study (Adelusi, 1976), there was never-the-less, a slight improvement in the number of patients reporting early for investigations, and a slight reduction in the very late stages (stage IV) of the disease.