Isolation and Characterisation of Tumour Associated Antigens and Their Significance in Host Immune Responses

Thesis submitted to the
University of Calicut under the **Faculty of Medicine**
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
IMMUNOLOGY

by

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Department of Biochemistry
Medical College
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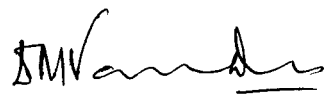
Certificate

This is to certify that the research work presented in this thesis entitled **"Isolation and Characterisation of Tumour Associated Antigens And Their Significance in Host-Immune Responses "** is based on the original work done by **Mr. Elyas K K**, under my guidance and supervision at the Department of Biochemistry, Medical College, Thrissur, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, and that no part of this work has been previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.



Dr. D M Vasudevan, MD, FRCPath
(Supervising Guide)
Principal
Amrita Institute of Medical Sciences
and Research Centre
Cochin- 682026

Cochin-26
Date: 1-06-2005

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Declaration

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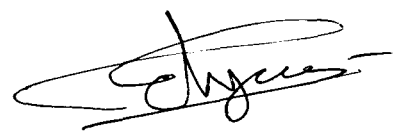

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LIST OF ABBREVIATIONS

A_{450}	–	Absorbance at 450 nm
A_{495}	–	Absorbance at 495 nm
ADC	–	Anti-coagulant Dextrose Citrate
ADCC	–	Antibody Dependent Cell Cytotoxicity
AFP	–	Alpha Fetoprotein
AIDS	–	Acquired Immuno Deficiency Syndrome
APC	–	Antigen-Presenting Cells
APS	–	Ammonium persulphate
BARC	–	Bhaba Atomic Research Centre
C1q	–	Complement 1q
CA	–	Cancer antigen/Carbohydrate antigen
CD	–	Cluster Designation Marker
CEA	–	Carcino Embryonic Antigen
CIC	–	Circulating Immune Complexes
CMI	–	Cell Mediated Immunity
CMV	–	Cytomegalovirus
CTL	–	Cytotoxic T-lymphocytes
dATP	–	deoxy Adenosine triphosphate
dCTP	–	deoxy Cytosine triphosphate
dGTP	–	deoxy Guanosine triphosphate
DNA	–	Deoxy ribonucleic acid
dNTP	–	Deoxy nucleotide phosphate
dTTP	–	Deoxy thymidine triphosphate
EBV	–	Epstein Barr Virus
EDTA	–	Ethylene Diamine Tetra Acetic acid
ELISA	–	Enzyme Linked Immuno Sorbent Assay
g	–	gram
h	–	hour
HARFC	–	High affinity rosette forming cells
HBV	–	Hepatitis B-virus
hCFHrp	–	Human complement factor H related protein

HIV	–	Human Immunodeficiency Virus
HLA	–	Human leukocyte antigen
HPV	–	Human papilloma virus
HSV	–	Herpes simplex virus
HTLV	–	Human T-Cell Leukemia Virus
IARC	–	International Agency for research on cancer
ICs	–	Immune complexes
IFN	–	Interferon
IgA	–	Immunoglobin A
IgD	–	Immunoglobin D
IgE	–	Immunoglobin E
IgG	–	Immunoglobin G
IgM	–	Immunoglobin M
IL	–	Interleukin
kDa	–	Kilo Dalton
l	–	Litre
LAK-cells	–	Lymphokine activated killer cells
LB	–	Luria Broth
LNL	–	Lymph node lymphocyte
LPS	–	Lipopolysaccharide
M	–	Molar
mg	–	Milligram
MHC	–	Major histocompatibility complex
ml	–	Millilitre
mM	–	Millimolar
MW	–	Molecular weight
NK Cells	–	Natural killer cells
nm	–	Nanometer
OD	–	Optical density
OPD	–	Orthophenaline diamine
PAGE	–	Polyacrylamide gel electrophoresis
PBL	–	Peripheral blood lymphocytes
PBS	–	Phosphate buffered saline
PEG	–	Poly ethylene glycol

PHA	–	Phyto heamagglutinin A
PSA	–	Prostate specific antigen
rIFN	–	Recombinant interferon
RNA	–	Ribonucleic acid
rpm	–	Revolutions per minute
SCC	–	Squamous Cell carcinoma
SDS	–	Sodium Dodecyl Sulphate
sRBC	–	Sheep red blood cells
SSC Buffer	–	Sodium Chloride Sodium Citrate Buffer
TA	–	Tumour Antigen
TAAs	–	Tumour associated antigens
TAE Buffer	–	Tris Acetic acid EDTA Buffer
TAM	–	Tumour infiltrating macrophage
TATAs	–	Tumour associated transplantation antigens
Tc cells	–	T cytotoxic cells
TEMED	–	N, N, N', N' Tetramethyl ethylene diamine
T_G cells	–	IgG Fc receptor bearing T cells
TIL	–	Tumour infiltrating lymphocytes
Tm cells	–	IgM Fc receptor bearing T cells
TMB	–	3, 3', 5, 5'-tetramethyl-benzidine
TNF	–	Tumour necrosis factor
TRFC	–	Total rosette forming cells
Tris	–	Tris (hydroxymethyl) aminomethane
TSAs	–	Tumour specific antigens
TSTA	–	Tumour specific transplantation antigen
V	–	Volt
WHO	–	World Health Organisation

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INTRODUCTION

Chapter I
INTRODUCTION

The tumours are thought to express unique tumour antigens that stimulates the host's immune system, initiates a response that will eventually kill and eliminate the tumour cells (Hole and Stern 1988). The reason the tumour is not rejected initially in the host was thought to be an insufficiency of the immune response, possibly caused by the tumour itself.

Immunologists distinguish between two types of tumour antigens: tumour- specific (TSAs) and tumour-associated (TAAs). TSAs are true tumour antigens, because they only occur on neoplastic cells and not on any normal cells at any stage of the individual's development and are non-self antigens (Chen *et. al.* 1991). TAAs occur on normal and neoplastic cells, but on the latter, under conditions in which the individual does not become tolerant of them and can therefore respond to the tumour (Lotnicker 1991). Since the method of detection of antigens is often done by transplantation, these antigens, TSAs and TAAs are called tumour specific transplantation antigens (TSTAs) and tumour associated transplantation antigens (TATAs), respectively. If a tumour expresses tumour antigens, it could theoretically induce both humoral and cellular immunity and one should therefore be able to demonstrate the antigen by both antibodies and sensitized T-lymphocytes. The tumour antigens can also be used by cytotoxic T (Tc) lymphocytes to kill neoplastic target cells (Van der *et. al.* 1991).

Based on the origin of tumour, three different types of TSAs have been identified. In the first case, each chemically induced tumour produces TSAs that are antigenically unique, so that the immunity induced by one tumour will not protect the individual from the growth of another tumour developed by the same chemical, although it would prevent the growth of the immunising tumour (Hellstrom *et. al.* 1979; Barth *et. al.* 1991). Carcinogenesis is likely to be accompanied by the induction of mutations in a variety of cellular genes. So the mutation may lead to alterations in cellular proteins, and the altered peptides of the mutated cellular proteins can appear on the cell surface bound to major histocompatibility complex (MHC) Class I glycoproteins and be recognized by T-cells as foreign (Yewdell and Bennink 1992). In the second category, in contrast to chemically induced tumours, virally induced tumours usually display TSAs shared by all tumours caused by the same virus (Hellstrom *et. al.* 1979; Murray *et. al.* 1992). These TSAs can be detected both by antibodies and sensitized T-lymphocytes. In the third category of spontaneous occurring tumours, it is difficult to demonstrate TSAs and the observed spontaneous tumours are the result of immuno-selection favouring TSTA–negative neoplastic cells.

Tumour-associated antigens (TAAs) are more common and widely used tumour marker. TAAs are found on tumour cells and on normal cells during foetal life, after birth in selected organs, or in many cells, but at much lower concentration than on tumour cells. Immune responses to TAAs may be suppressed because they are considered as self-antigens. TAAs are found on

the cell surface; including integrins, mucins, cadherins, growth factor receptors, membrane bound antigens and glycoproteins; which are known to play an important role in carcinogenesis (Pannall 1992; Sell 1993).

The pursuit of the ideal tumour marker has generated many tests for use in the diagnosis and management of cancer, several of which are now widely available. No test meets all of those requirements. Specifically, no marker has been established as a practical cancer-screening tool, either in a general healthy population or in most high-risk populations (Landman *et. al.* 1998; Nicolini *et. al.* 2003). The reason for this is the relative lack of sensitivity and specificity of the available tests, given the low prevalence of cancers in most populations. In general, even tests that are highly sensitive and specific may have low predictive values.

Nearly all markers can be elevated in benign disorders and most markers are not elevated in the early stages of malignancy (Gion 1999; Warnakulasuriya 2000). Extreme marker elevation often indicates a poor prognosis and in some malignancies can indicate the need for more aggressive treatment. Tumour markers have their greatest value when used to monitor therapy in patients with widespread cancer. Nearly all markers show some correlation with the clinical course of disease, with marker elevation in any stage declining to normal after a curative intervention (Granberg 2000).

Increased marker levels can accompany recurrent disease, but markers can detect an occult recurrence in only a few diseases, thereby facilitating a second attempt at cure. Although, it seems unlikely that an ideal tumour marker will be identified for every malignancy, several workable markers are already available. Increasing our knowledge about the capabilities and limitations of existing markers, will enable us to use them judiciously in the treatment of cancer, while improved understanding can lead to improvements in cancer therapy, prediction of prognosis and vaccine development (Lindblom and Liljegren 2000; Charan *et. al.* 2000). Tumour antigen(s) escape from immuno-surveillance represents the hurdle to be overcome in formulating truly effective cancer immunotherapy (Pawelec 2004).

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REVIEW OF LITERATURE

Chapter II
REVIEW OF LITERATURE

Tumour development occurs when genetic changes take place in the cells, with a progressive state of uncontrolled growth and division (Cairns 1981). Such neoplastic changes can occur in any cell of any tissue at any age. A benign tumour is a relatively slow growing mass of differentiated cells, that is restricted in anatomical location and is not life threatening. Malignant tumours are aggressive, life threatening and easily metastatic. Generally, malignant tumours penetrate, compress and ultimately destroy the surrounding normal tissues and/or its cellular product may prevent the normal functioning of the body. Malignant tumours of mesenchymal origin are termed sarcomas and those of epithelial origin are carcinomas.

Research has lead to the general acceptance of the view that cancer is the end result of acquired or inherited mutations in the structure of certain genes, contained within the DNA of the cell or its clonal descendants, which compose the cells of the tumour. The transformation of a normal cell to a tumour is a multi-step process (Knudson 1971). Development begins with initiation of the tumour spontaneously or by carcinogenic physiochemical or viral agents. In the next stage, during tumour promotion the preferential proliferation of the transformed cells takes place. Finally, during the tumour progression;

proliferation, invasion and metastasis production leads to formation of malignant tumour.

Many oncogenes are involved in the tumour development. They were first discovered in tumour viruses and are called viral oncogenes. Normal cells contain cellular oncogenes or the so-called proto-oncogenes and are cellular homologs of viral oncogenes (Brugge 1977). In addition, normal cells also contain another class of genes that are implicated in tumour development, called anti-oncogenes or tumour suppressor genes (Fearon and Vogelstein 1990). The normal expressions of these genes prevent the development of tumours. A proto-oncogene, the normal cellular form of oncogene, can be converted to an activated oncogenic form by variety of submicroscopic events including point mutations, insertions and inversions which increase the activity of the gene, where as tumour suppressor gene contribute to oncogenicity through their loss of function or gene inactivation. Thus, malignant transformation represents a multi-step process with mutations occurring in proto-oncogene and tumour suppressor genes, which leads to the aberrant expression of other cellular proteins. Many tumours take several years to develop from the time of the initiation of molecular mutation in the parental cells. In part, this may be due to the time required for a cell to accumulate sufficient growth transforming mutations for it to become free of the constraints of normal growth control (Vogelstein and Kinzler 1993; Ukraintseva and Yashina 2004).

2.1. Immunology of Tumour

Experiments had shown that, tumour cell lines induced *in vivo* by strong chemical carcinogens when transplanted to syngenic host demonstrated a clear immunogenicity (Woglom 1929). In these cases, the primary tumours may survive because tumours evolved were in a position to become poorly immunogenic in the host. In some cases, the tumour antigens that are apparently recognised by the component of the immune system have been identified, but these antigens may not be sufficient, per se to stimulate an effective anti-tumour immune response. The tumour development may be due to either internal changes occurring in the cellular gene or due to an external agent that brings about the internal changes leading to the uncontrolled proliferation. The significance of the immune system is that once appropriately activated against tumour specific determinants, it has very high level of antigen specificity and has inbuilt response amplification such that very small activation signals can produce long lasting body wide protection. Circumstantial evidences indicate that the immune system plays an important role in eliminating human tumours also. Many of the malignant diseases occur either in the early years of age or late years of age when there is ill developed or declined stages of immune response in the body (Gross 1965).

The role of immune response in controlling and eliminating tumour cells gave rise to the immune surveillance hypothesis of cancer (Burnet 1971). Such a protective immune response had been reported earlier in animal models. So the tumour cell can be immunogenic, poorly immunogenic or more

active to overcome the immune response (Hewitt *et. al.* 1976). Marked infiltrations of mononuclear immune cells found in improved prognostic condition of tumour rejection, indicates the involvement of cell in tumour rejection process (Wolf *et. al.* 1986). An increased incidence of cancer has been reported in immuno-compromised patients like transplant recipients and AIDS patients. The prevalence of Kaposis sarcoma and lymphoma are examples of this phenomenon in AIDS patients (Goedert *et. al.* 1998; Paparizos *et. al.* 2000 Krishnan *et. al.* 2003). These indicate that Immune system plays an important role in the prevention of human tumour development.

2.2. Mechanism of Tumour Recognition and Clearance

The mechanisms that operate in the recognition and elimination of tumours have been worked out. Humoral as well as cellular immunological responses are important in eliminating the neoplastic transformation in the body. The endogenously synthesized antigen is processed intracellularly within the proteosome of antigen presenting cells into short peptides typically about nine amino acid in length. These are selected into the molecular groove formed at the surface of the class I major-histocompatibility complex (MHC). The MHC peptide complex is then transported to the cell surface for antigen presentation to T-cells especially CTL, stimulating the expression of effector function and killing tumour cells (Bjorkman *et. al.* 1987). Many of the cytokines secreted by immune cells can mediate toxicity of tissue, either directly or via the recruitment of other inflammatory processes.

IL-2 has been demonstrated to synergize with anti-tumour antibodies in Murine models of melanoma and lymphoma (Eisenthal *et. al.* 1987; Berinstein and Levy 1987). In addition, a wide variety of chemotactic and vascular permeability factors that are involved in inflammatory responses also can indirectly mediate tumour destruction and may play a role in tumour immune phenomena. Cytokines and Interferons that modulate the lysis of tumour cells by immune effectors, has been known for sometime. Interferon and tumour necrosis factor (TNF) have been demonstrated to markedly increase MHC class I and class II antigens and many tumour-associated antigens (Houghton *et. al.* 1984; Pfizenmaier *et. al.* 1987).

TNF and lymphotoxin are two cytokines capable of direct destruction of tumour cell (Pennica and Goeddel 1987; Hamblin 1988). The key role played by CD-8, cytotoxic T- lymphocytes (CTL), were identified as an important effector population in the elimination of tumour (Greenberg 1991; Duraiswamy *et. al.* 2003). The CTL recognizes the processed antigens by T-cell receptors. For the completion of the action of immune response CD-4 T-Helper cells are required. This help in the form of cytokines can also activate CTL. The activated T-helper cells help B-cells to produce antibodies. Humoral response from B-lymphocytes with highly specific immunoglobulin mediated recognition of tumour antigens may also be important in generating antibody dependent cellular cytotoxicity (ADCC) or antibody dependent complement mediated lysis of tumour cells (Lloyed 1991). The best demonstration that specific T-cell mediated reactivity to tumour antigens exists, comes from the recovery of MHC-restricted

CD-8 T- cells, which have infiltrated the tumour. These T-cells called tumour-infiltrating lymphocytes (TIL) can be grown *in vitro* and subsequently lyse autologous tumour cells from the same histological type as long as they are MHC matched (Kawakami *et. al.* 1992). The extra cellular antigens found external to the cells are taken up by the professional antigen presenting cells (APC), such as dendritic cells or macrophages. These APCs must take up extracellular antigens; process it proteolytically via a separate endosomal antigen processing pathway and present antigenic peptides complexed with an MHC Class II molecule (Neefjes and Momburg 1993).

Natural Killer (NK) cells are also another population of activated leucocytes that have been shown to be front line effector cells in immuno-surveillance against tumours. These are non-B, non T-cell population of lymphocytes, which can lyse tumour cells nonspecifically and in an MHC unrestricted way (Klein and Mantovani 1993). Such cells migrate to the tumour site *in-vivo* and can be maintained *in-vitro* in high concentrations of IL-2. They are technically called lymphokine-activated killer (LAK) cells and are in trial for tumour therapy.

Tumour associated macrophages (TAM) are a group of nonspecific response against tumour. The high level of MHC II expression also allows them to present tumour antigen to CD-4 T-helper cells at the site of tumour development. This is important to promote the secretion of helper cytokines by antigen specific CD-4 cells and complete the antigen specific

activation of CTL. TAM also shows tumour cytotoxicity when activated with interferon (IFN) secreted from activated CD-4 T-cells. The TAM can kill tumours by necrosis factor, IL-1, free radical, protease and nitric oxide (Huang *et. al.* 1994). This T-cell activation may be induced by tumour-associated antigens. Many such antigens have been identified and shown to be capable of provoking T-lymphocyte responses (Boon *et. al.*1994; Duraiswamy *et. al.* 2003). Antigen-presenting cells (APCs) like dendritic cells, macrophages and B -cells form an integral part of the immune system. These cells serve very different immune functions; dendritic cells and macrophages are potent initiators of the immune response and in particular are responsible for inducing primary antigen-specific immune reaction. This implies that these cells are fundamental to intact immunity and antigen specificity in general (Peters *et. al.* 1996). Dendritic cells and macrophages are potent stimulators of T-cell activation and are capable of eliciting anti-tumour immune responses (Schwab *et. al.* 1999; Brode and Macary 2004).

2.3. Incidence of Cancer

Cancer is a disease showing dramatically differing frequencies in different parts of the world and is extremely frequent in human pathological conditions. In women, the predominant sites are the breast and the genitourinary organs, in particular the ovary and the uterine cervix. In contrary, the hospital based registers in India reveal that oral cancer accounts about forty percent of all malignancies in males (Desai 1983). In England and Wales, the most frequent sites for tumours in men are the respiratory and digestive organs; in particular

lung, stomach and large intestine (Quinn *et. al.* 2000). Even in developed countries, the incidence is high and it has been reported that cancer is the second leading cause of death among Americans (Jemal *et. al.* 2003). The distribution of tumour among various organs of the body is not uniform.

2.4. Incidence of Oral Cancer

Incidence of oral cancer varies greatly throughout the world. The incidence rate of oral cancers is also available from epidemiological survey in different potential places of India. In Manipur, the annual incidence rate was 21.4 (Wahi 1968), in textile mill workers of Ahmedabad, the crude incidence rate among individuals aged over 35 years was 25 (Malaowalla *et. al.* 1976). In a house-to-house ten year follow up study, the annual age adjusted incidence rate in Ernakulam and Srikakulam were 16 and 21 respectively per one lakh individuals (Gupta 1980). The annual age adjusted incidence rates of oral cancer per one lakh in several countries are reported (Waterhouse *et. al.* 1976; Padmanabhan and Vasudevan 1982; Moore 2000; Ries 2001). In America, the incidence rate varies from 4.4 in Colombia to 13.4 in Canada (Slavkin 1996). In Australia and New Zealand incidence rates of 2.6 and 7.5 respectively have been reported. In western countries, such as the United States, England and Wales, oral cancer accounts for 2-5% of cancer (Parkin *et. al.* 1988). In India, it is the commonest malignant neoplasm, accounting for 20-30% of all cancers (Nair *et. al.* 1990).

Each year, over 30,000 new cases of oral and pharyngeal cancer are diagnosed and over 8,000 deaths occur due to oral cancer and the survival rate of these cancer patients is about 50% (Silverman 1988; Vokes *et. al.* 1993). These numbers are low compared with 40% prevalence in Sri-Lanka and 50% in India (Desai 1983; Gupta *et. al.* 1992). Oral cancer is more common in men than women. The incidence of oral cancer in patients registered in the Regional Cancer Centre, Thiruvananthapuram was 27% when compared to 14% cervical cancer and 7% breast cancer (Padmakumary *et. al.* 1999).

2.5. Etiology of Oral Cancer

The etiology can be best understood using epidemiological data utilizing incidence patterns. Trauma and dental irritation from sharp teeth and dentures may act as co-carcinogens and there is adequate evidence suggesting that metastatic spread of malignant tumours can be affected by trauma (Gorsky and Silverman 1984). The single greatest risk factor is tobacco (Elwood *et. al.* 1984). While, cases of oral cancer are seen in patients who do not use tobacco, these constitute a very small percentage of oral cancers. All forms of tobacco use have been implicated as causative agents including cigarette, cigar and pipe tobacco as well as chewing tobacco (IARC 1985). Smokeless tobacco products of various types are accepted causes of oral cavity cancers in humans, where as tobacco smoking causes cancers of the lung, oral cavity, larynx, oesophagus, pancreas, kidney and bladder (IARC 1986).

Tobacco products are complex mixtures of chemical constituents, many of which have carcinogenic properties. Cigarette smoke contains at least 3,500 identified chemical constituents, of which more than forty percent are accepted carcinogens (IARC 1986; Hoffmann and Djordjevic 1997). Poly aromatic hydrocarbons like benzopyrene, aromatic amines like 2-naphthylamine and 4-aminophenyl, arsenic and chromium are reported to be having sufficient evidence for carcinogenicity by International Agency for Research on Cancer. It is also reported that the different tobacco specific nitrosamines are powerful carcinogens in laboratory animals independent of route of administration. Prevention of high-risk behaviours, which include cigarette, cigar or pipe smoking, use of smokeless tobacco and excessive use of alcohol are critical in preventing oral cancers (Blot *et. al.* 1988; Walsh and Epstein 2000).

Oral cavity and pharyngeal cancers occur on anatomic sites that lend themselves to early diagnosis and treatment (Raubenheimer and De Villiers 1989). Detection of oral cancer through periodic medical and dental examinations can significantly reduce the risk of these life-threatening cancers. Unfortunately, most patients do not seek consultation until advanced cancer is present with symptoms of persistent pain (Guggenheimer *et. al.* 1989). A strong synergetic effect between cigarette smoking and alcohol consumption and occurrence of upper digestive tract cancer, most notably oral cavity and oesophagus is seen (Elwood *et. al.* 1984; De Vita *et. al.*1993). In India and Sri Lanka, where chewing tobaccos is used with betel nuts and reverse smoking is in practice, there is a high incidence of oral cancer (Ghose and Pradia 1995). UV exposure is a

primary cause of lip cancer, while pipe smoking is also another factor (Osterlind 1993; Hindle *et. al.* 2000). Viruses are nowadays increasingly suspected as carcinogenic agents (Jacqueline *et. al.* 2001; Mork *et. al.* 2001).

2.6. Features of Oral Squamous Cell Carcinoma

It is universally accepted that squamous cell carcinoma can develop from pre-malignant lesions, however this concept may not be applicable in developed countries such as UK or US with low prevalence of oral cancer, since there are no reports available with clinically described pre-malignant lesions (Pindborg and Mehta 1968). There are two major clinically visible pre-malignant lesions namely, leukoplakia as white plaques and erythroplakia as red plaques (WHO 1978; Rajendran *et. al.* 1986). It is unlikely that oral squamous cell cancer arise from normal surface epithelium. Squamous cell carcinoma consists of irregular nests, columns and strands of malignant epithelial cells infiltrating sub-epithelially. Tumour cells may resemble any or all the layers of stratified squamous epithelium. Epithelial squamous cell carcinomas are characterised by strands, sheets or islands of epithelial cells invading the underline connective tissue and accompanied by a proliferating fibrous stroma of non-neoplastic origin.

The invading neoplastic epithelium resembles the original stratified squamous epithelium to various degrees. So, histologically squamous cell carcinomas are graded into well, moderately and poorly differentiated tumours. Two main features on which the grading is based are proliferation and

differentiation. Rapid abnormal proliferation is characterised by hyperchromatism, mitotic activity and cellular and nuclear pleomorphism. Differentiation of the tissue is marked by the presence of epithelial bridges and the production of keratin. An advanced degree of differentiation that is closeness of the structural characteristics of the tumour to the parent tissue is regarded as a feature of favourable prognosis, while lack of differentiation is considered to be a poor prognostic character (Evans *et. al.* 1982; Roland *et. al.* 1992). The surface epithelial cells undergo gradual changes from clinically undetectable pre-malignant lesion to clinically identifiable malignant lesions (Pindborg *et. al.* 1984). Over all, histological grading remains subjective and has little meaning for comparative studies or for prognosis of the individual patients (Burkhardt 1985). Pre-malignant states are often reversible and are readily curable. Screening alone can identify symptoms of pre-malignant conditions; however, most often these remain unnoticed. The patients report only after the disease advances to an irreversible malignant lesion (Guggenheimer *et. al.* 1989).

2.7. Cellular Immunological Phenomena in Oral Cancer

Direct and indirect evidences indicate that immunological derailment occurs during oral cancer development. Alterations of the surface characteristics of lymphocytes by anti-lymphocyte serum treatment (Martin and Miller 1967) or Neuraminidase treatment (Woodruff and Genser 1969) have been demonstrated. The impairment of cell-mediated immunity measured *in vitro* has been reported in patients with squamous cell carcinoma of the head and neck region (Scully 1982; Balaram and Meenattoor 1996). These studies involved

measurement of percentage and absolute numbers of lymphocyte population and/or their mitogen and alloantigen response. In some other studies, it was found that IFN failed to enhance NK cell activity *in vitro* in some cases and it was concluded that these patients were not suitable candidates for IFN therapy (Gore *et. al.* 1983). They also observed decreased NK cell activity with an increase in the tumour size. Increased involvement of regional lymph nodes however was accompanied by an increase in NK cell activity (Jamkar *et. al.* 1983).

A significant increase in the number and proportion of T_G-cells (IgG Fc receptor bearing T-cells) and a significant reduction in the T_M cells (IgM Fc receptor bearing T -cells) in the cancer patients have been reported (Balaram and Vasudevan 1983). The increase in T_G subset was obvious in the early stages of the disease, whereas the reduction in T_M sub-set was evident only in the advanced stages. Several other workers have reported decreased percentage and total T-lymphocyte counts in oral cancer patients. Difference in homing pattern of lymphocyte from oral cancer and that of normal healthy individual was observed, after administration of Neuraminidase to experimental animals (Vijayakumar and Vasudevan 1985). It was observed that the major deviations in immune parameters in oral cancer appears to be increased leucocytes and lymphocyte counts, increased B-cells and T-cell subpopulations, but have observed no change in percentage of T-lymphocyte or T-lymphocyte subpopulations in comparison to the normal healthy controls (Saranath *et. al.* 1985). Individual comparisons between the immune parameters pre and post operatively indicate that the major abnormalities in regulatory cells and T-cell

function still persisted in post surgery and they did not correlate with the load of tumour in the patients.

Total rosette forming cell (TRFC) and high affinity rosette forming cells (HARFC) were enumerated. HARFC were found to be decreased significantly while TRFC remained unaltered in the cancer patients (Vijayakumar and Vasudevan 1985). In a later study, it was reported that depression of HARFC could occur in patients with oral submucousfibrosis also (Rajendran *et. al.* 1986). Phyto-haemagglutinin (PHA) induced lymphocyte blastogenesis showed a significant impairment only when the tumour was well established and disseminated beyond its local confines (Das *et. al.* 1986). Reduced total lymphocyte counts in oral cancer patients are also reported (Pillai *et. al.* 1987). This study also revealed a significant increase in the proportion and count of B-lymphocyte in pre-malignant lesions. Reduction in the absolute number and proportion of T-cell was observed both in the malignant and pre-malignant lesions. The number of circulating T_G cells was considerably increased and that of T_M cells was decreased in both groups of patients. A decrease in the absolute count of peripheral lymphocytes and normal B-cell count is also reported in oral cancer patients (Sasidharan *et. al.* 1989).

Studies have been conducted to find out the role of IL-2 in T-cell responses, ability of activated macrophages to produce IL-1 and to exhibit tumouricidal activity in oral cancer. Although, the mitogen response was augmented by IL-2, the augmented levels of peripheral blood lymphocyte (PBL)

and tumour infiltrating lymphocyte (TIL) responses from oral cancer patients were still below those of healthy donors. IL-2 production in the PBL, TIL and lymph node lymphocytes (LNL) from oral cancer patients was normal, but was reduced in the PBL from leukoplakia patients (Murali *et. al.* 1989). It was observed that the production of IL-1 was in response to lipopolysaccharide (LPS) stimulation of macrophages from metastatic lymph nodes (Mukhopadhyaya *et. al.* 1989). The same group had studied the NK and K-cell cytotoxicity and the modulation of NK cell activity by recombinant interferon alpha (rIFNα). NK cells and antibody dependent cellular cytotoxicity functions were intact in PBL, but were severely depressed in LNL and TIL. Moreover, the NK activity of the LNL and TIL was not responsive to rIFNα.

2.8. Humoral Immune Response in Oral Cancer

Several studies have revealed the impairment of humoral immunity in oral cancer patients. A significant rise in the serum IgM and IgA levels in oral cancer patients was reported (Khanna *et. al.* 1982). These observations indicate the presence of oral cancer-associated antigens in patients (Scully *et. al.* 1982), which would induce the cellular and the humoral responses. The rise was more pronounced in advanced clinical stages. Elevated levels of IgG and IgA in the saline extracts of oral cancer and oral submucous fibrosis tissues were observed, but the elevation being more pronounced in oral cancer (Vivekanandan and Vasudevan 1982). An elevated level of IgA, IgD and IgE in cancer patients was also reported. IgA and IgD levels returned to normal levels in patients with clinical cure, where as IgE remained slightly elevated (Vijayakumar

et. al. 1986). Elevated levels of IgA and IgE in oral sub mucous fibrosis and IgG, IgD, IgA and IgE in oral cancer patients were reported (Rajendran *et. al.* 1986, Scully 1986). Tissue immunoglobulin levels have been elevated in oral cancer; IgG and IgA were found in the saline extracts of oral cancer tissues, but not in normal oral mucosal tissues (Ravindran *et. al.* 1986).

2.9. Viruses in cancer

The existence of living cells is a basic requirement for the multiplication of viruses, so also the immortalisation and transformation of the normal cell to cancerous cell is a basic strategy of the tumour viruses for its better survival. Numerous viruses have been identified that can induce cancer in vertebrates, including human. The evidence for the viral causation of cancer in humans long remained strong but only circumstantial; it is now clear, however that Burkitt's lymphoma, nasopharyngeal cancer and T-cell leukemia have a strong association with viruses.

Oncogenic viruses are classified according to the type of nucleic acid they contain. A common feature of oncogenic viruses is that they induce malignant transformation of target cells, which upon transplantation into suitable animal host; exhibits true autonomous growth, local invasion and metastasis to distant sites. Another common feature is physical integration of virus-specific genetic material in the DNA of the host cells. In the case of DNA tumour viruses, the viral genes are integrated directly, whereas with ribonucleic acid (RNA) viruses, the RNA is first transcribed into DNA, which is then integrated. The

integration of DNA as provirus can inactivate the normal genes making the cells neoplastic. The complexities of tumour viruses are such that progress to establish cause and effect has been quite difficult.

Herpes-type viruses have been found to be closely associated with two cancers in humans: Burkitt's lymphoma, a malignant tumour of lymphatic tissue and nasopharyngeal carcinoma, a squamous-cell carcinoma of the posterior part of the nasal cavity (Epstein 1979). Biopsies from both tumours grown in culture gave rise to cell lines in which a DNA virus of the herpes class was identified. Patients with either type of cancer have high levels of antibody directed against EB virus, indicating that they have encountered the virus and responded to it. Human blood lymphocytes can be transformed in culture by EB virus and there is experimental evidence that EB virus is also oncogenic for monkeys. In addition, EB virus is considered to be the causal agent for infectious mononucleosis, a distressing but self-limited viral infection. Yet, either Burkitt's lymphoma or nasopharyngeal carcinoma does not follow infectious mononucleosis. It is not known, why EB virus causes a self-limited disease in one instance and a variety of cancers in others. Evidence suggests that genetic factors may be important for development of the malignant tumour.

A second herpes virus that is becoming increasingly suspect as a human oncogen is the herpes simplex virus type 2 (HSV-2), a close relative of the virus that causes the common fever blister (Seth 1980). Other viruses that may be involved and are known to be transmitted by sexual contact are

papilloma virus and cytomegalovirus (CMV); both are capable of causing cell transformation in culture (Reeves *et. al.* 1989a; Reeves *et. al.* 1989b; Baichwal *et. al.* 1989). The experimental application of radioactive probes of antibodies to specific viral proteins and to components of the viral genome have localized either the integrated genome or the viral antigens of HSV-2, CMV and papillomavirus in the cells of cervical cancer in women. Although this cannot be taken as unequivocal evidence to show that the viruses are indeed the causal agents. The implication of virus as a cause of human cancer has been largely circumstantial. Furthermore, human T-cell leukemia virus (HTLV), an RNA virus, which bears no apparent relation to any of the known animal oncogenic RNA viruses, appears to be present in patients with T-cell leukemia and can be isolated from their tumour tissue with reasonable reproducibility (Greene *et. al.* 1989). Infection of normal blood T-lymphocytes in culture by HTLV leads to their uncontrolled growth. These tumours have been shown possibly to have an infectious basis.

Hepatitis virus infection with hepatitis B virus (HBV) is endemic in populations that also have a high incidence of liver cancer (Kekule *et. al.* 1990 Sarin *et. al.* 2001). This has led some researchers to conclude that the virus is the cause of liver cancer. As noted earlier, however, cancer is a multifactorial disease and liver cell cancer is an excellent example. HBV infection invariably leads to the augmented growth of liver cells, which renders them exquisitely sensitive to carcinogens. Since populations plagued by a high incidence of liver cancer are also chronically exposed to numerous toxic substances, some of

which are carcinogenic for the liver, it is difficult to dissect the interactions of virus and carcinogen with sufficient precision to either indict or exonerate HBV as a liver oncogen. Thus, HBV must be considered only as a possible carcinogen. HBV may well be considered a co-carcinogen, however; it may so increase the sensitivity of liver cells to carcinogens that it decreases the latent period during which the cells undergo malignant transformation and increases the number of tumours that ultimately develop.

Progress in tumour genetics and molecular biology may lead to a clearer understanding of how cells undergo malignant transformation. As stated earlier, RNA tumour viruses invariably integrate their DNA into the host's DNA (Kekule *et. al.* 1993). Cells of numerous vertebrate species, including humans, have such integrated DNA in their genes. The conservation of such genes throughout many diverse species suggests that they probably serve an important role in cells, perhaps in growth regulation. The fact that such genes are almost ubiquitous in their distribution and apparently not simply expressed in normal cells, led to the enunciation of the oncogene hypothesis, which suggests that silent (unexpressed) oncogenes or genes capable of inducing cancer can, upon proper stimulation become expressed and can cause a previously normal cell to become malignant.

Oncogenes have been found in chromosome 8 in Burkitt's lymphoma cells and in a variety of other human tumours. Introduction of such oncogenes in the DNA of normal cells from human tumour cells in culture causes

the normal cells to become transformed and behave like cancer cells. Activation of an oncogene in a human tumour has been shown to be accompanied by a minor change in its chemical structure. This suggests that the silent oncogenes may be activated by chemicals, radiation and viruses, all of which are known to alter DNA and to cause cancer. The oncogene may be the common denominator through which such diverse agents act. In recent years, there has been increasing evidence of the possibility of a casual relationship between viruses and various forms of cancer in humans. Report shows that the oncogene expression in virus-positive Burkitt's lymphoma tumour cells is dependent on the presence of EBV (Bell and Rickinson 2003).

2.10. Viral Immune evasion mechanisms

During the millions of years of coexistence with their hosts, viruses have learned how to manipulate host immune control mechanisms and evade the host immune system for their survival. Therefore, viruses have limited pathogenicity in an immuno-competent natural host. In turn, it is remarkable that, in the host-virus interaction process, individual virus families have targeted many immunological principles for its survival. In the case of RNA viruses, because of the small size of the genome, there is little room to allow immune defence to be encoded by individual genes. RNA viruses like HIV, owing to the low fidelity of RNA polymerase in their multiplication, confers the advantage of being able to use mutation to escape immune control through antigenic shift, so the immune response generated against the virus is not able to recognise the progeny virus at another time. Virus genomes maintained in host cells as prophage with limited

gene expression also helps the virus to evade the host immune response because of the inactive stage in the cell. In many DNA viruses, their genome size allows a larger number of genes to be devoted to control host defense. In certain cases, more than 50% of the genome may be devoted to tackle the immune recognition. There are two classes of viral immuno regulatory proteins: those encoded by genes with the sequence homology to the host cellular gene and those encoded by genes without sequence homology.

Interferons are known for their ability to protect cells from viral infection and viruses are known for their anti-interferon strategies. Viruses block interferon-induced transcriptional responses, signal transducers and activators of transcription signal transduction pathways, and also inhibit the activation of interferon effector pathways; that induce an antiviral state in the cell and limit virus replication. This is mainly achieved by inhibiting double stranded RNA-dependent protein kinase activation, the phosphorylation of eukaryotic translation initiation factor, the nuclease system that might degrade viral RNA and arrest translation in the host cell. Poxviruses encode soluble version of receptor for IFN-α, β and γ, which blocks the immune functions of interferon (Smith *et. al.* 1998). Several viruses produce blocking substances, which inhibit the synthesis or activity of other substances like interleukin-12 or interleukin-8 that is required for the production of interferons (Alcami *et. al.* 1998; Tortorella *et. al.* 2000). It has been recently observed that certain large DNA viruses like herpes virus produce substances that mimic cytokines, these virokines and the receptors also helps in the immune aversion (Lalani *et. al.* 2000). Soluble cytokine receptors might

neutralise cytokine activity and cytokine homologs might redirect the immune response for the benefit of the virus. Alternatively, viruses that infect immune cells might use homologs to induce signalling pathways in the infected cells that promote virus replication.

Apoptosis or programmed cell death can be triggered by a variety of inducers; including ligands, irradiation, cell-cycle inhibitors or infectious agents such as viruses. However, apoptosis might also facilitate virus dissemination. The cellular proteins implicated in the control of apoptosis are targeted by viral anti-apoptotic mechanisms. Viruses inhibit activation of caspases; encode homologs of the anti-apoptotic protein Bcl-2, which block apoptotic signals (Everett *et. al.* 2000). Viruses like herpes virus and Epstein-Barr virus (EBV) downregulate the action of antigen presentation either by destabilising the class 1 MHC molecule or by preventing the peptide degradation during antigen processing (Tortorella *et. al.* 2000). Virus encodes homologs of complement regulatory proteins that are secreted and block complement activation and neutralisation of the virus particle. The cowpox virus produces inflammation modulating protein which blocks immuno-pathological tissue damage at the site of infection by inhibiting production of the macrophage chemo-attractant factors C3a and C5a (Kotwal 2000). Viruses protect the membranes of infected cell and the lipid envelopes of virus particle from complement lysis by encoding homologs of inhibitors of the membrane attack complex.

Viruses such as HIV, human cytomegalovirus and vaccinia virus uses homolog to CD59, which normally protect cells from complement lysis and incorporating them into the viral envelope (Tortorella *et. al.* 2000; Kotwal 2000). The identification of novel immune-evasion strategies and the analysis of their functions in the viral infection should lead to a better understanding of the immune system, the interaction of the viruses with their hosts and their vital role in tumourogenesis. This will help to treat virus-induced pathology, to design safer and more immunogenic virus vectors as vaccines or gene delivery system and to identify new strategies for immune modulation. Circumstantial evidence has accumulated, suggesting that viruses that can adapt immune evasion mechanisms cause varieties of human cancers.

2.11. Role of HSV in Cancer

Herpesviridae family comprises about fifty different members and affects more than thirty animal species. HSV-1 and HSV-2 belongs to human herpes viruses that are classified in the alpha herpes virus subfamily of herpes viruses. Its capacity to establish latent infections with periodic recurrences, such as *Herpes labialis* was described as early as 1960s (Rawls *et. al.* 1968). Herpes viruses are symmetrical structures, consisting of a central core, which constitutes the viral DNA genome, the nucleocapsid and an envelope. The core is made up of a double stranded DNA, with a molecular weight of 8-150 x 10^6 Daltons and with very high cytosine-guanine content. Cancer of the mouth as well as cervix is predominantly squamous cell tumours.

Patients with pre-malignant lesions were shown to be associated with cell-mediated immunity against HSV (Lehner *et. al.* 1973). Investigations in recent years suggest that viruses may contribute to the etiology of some epithelial neoplasia. There is evidence for the association of herpes viruses with human uterine cervix carcinoma (Seth 1980, Galloway and Tension 1990). Oral cavity, as in the case of uterine cervix, is a major site of recurrent herpatic infections. Oral cancer has been shown to be associated with chewing tobacco. The role of viruses as one of the etiological agents or as a co-carcinogen, acting synergistically with chemical carcinogens, remains to be resolved. Earlier studies conducted using indirect haemagglutination and indirect haemagglutination-inhibition assays, have revealed elevated seroprevalence and titer of anti-HSV-I antibodies in oral cancer patients (Kumari *et. al.* 1982).

The classic presentation of primary HSV-2 infection is herpes genitalis, an infection characterised by the appearance of extensive bilaterally distributed lesions in the genital area (Corey *et. al.* 1983). The prevalence of HSV-1 infection increases gradually from childhood, reaching 70% to 80% in later adult years, whereas HSV-2 infection is typically acquired as a sexually transmitted disease, so that its incidence begins to increase in adolescence (Coleman *et. al.* 1983). Initial infection with HSV results in the establishment of viral latency, with the potential for subsequent viral reactivation. The initial muco-cutaneous infection caused by HSV-1 or HSV-2 is followed by latent infection of neuronal cells in dorsal root ganglia (Straus *et. al.* 1985). The classical presentation of primary HSV-1 infection is herpes gingivostomatitis, an infection

of the oral mucosa resulting in extensive painful vesicular lesions associated with marked submandibular lymphadenopathy. Other clinical manifestations of HSV-1 infection are conjunctivitis, keratitis and herpetic whitlow (Whitley 1988).

Infact, primary infection is often entirely asymptomatic. Because of the high prevalence of past HSV infections in the general population, many patients who develop malignancy, an immunodeficiency such as AIDS, or other disease that require immunosuppressive therapy may experience HSV-1 or HSV-2 infections. These infections, which may be primary or may arise from re-activation of a past infection, can be severe (Saral 1988). Assessing HSV immune status to document whether an individual has had past infection with HSV can be done by many serologic methods. Most methods are generally quite sensitive for detecting HSV IgG antibodies in individuals with past HSV infection regardless of whether the patient has had any recent signs of HSV disease. Serologic testing is accomplished most efficiently by using ELISA or latex agglutination procedure (Gleaves and Meyers 1988). The prevalence of HSV-2 infection ranges from 15% to more than 50% in adults, depending on a variety of demographic variables (Johnson *et. al.* 1989). Many individuals with primary HSV-1 or HSV-2 infection do not manifest characteristic clinical disease. Oral cancer has been shown to be associated with chewing tobacco, but other factors, including viruses as etiological agents, are to be carefully evaluated. Previous reports showing association of herpes virus with oral cancer are scanty. However, it has been reported that HSV-2 infection may act in conjunction with

HPV infection to increase the risk of invasive cervical carcinoma (Smith *et. al.* 2002).

2.12. Role of Human Papilloma Viruses in Cancer

The HPVs that cause the common warts, which grow on hands and feet, are different from those that cause certain types of cancer (Zur Hausen 1977). It has been implicated that the specific human papillomavirus types are involved in a wide range of epithelial malignancies. HPVs have been implicated in the pathogenesis of benign, premalignant and malignant lesions of human anogenital tract. DNA of specific HPV types is detectable in about 70% of cervical carcinoma, the most prevalent being HPV-16 and HPV-18 (Durst *et. al.* 1983). Infection with specific HPV types has been considered as the major etiological factor in cervical carcinoma. *In vitro* studies have clearly demonstrated the oncogenic potential of some HPV types. Thirty different HPV types can be passed from one person to another through sexual contact. Some types of HPVs may cause warts to appear on or around the genitals or anus. Genital warts, technically known as condylomata acuminatum, are most commonly associated with two HPV types, numbers 6 and 11 (Gissmann *et. al.* 1982; Gissmann *et. al.* 1983). HPVs are now recognized as the major cause of cervical cancer. Studies also suggest that HPVs may play a role in cancers of the anus, vulva, vagina and penis and some cancers of the oropharynx. Abnormal cervical cells can be detected when a Pap test is done during a gynecologic examination (Eddy 1990). HPVs may also cause flat, abnormal growths in the genital area and on the lower

part of the uterus that extends into the vagina. HPV infections often do not cause any symptoms.

Now HPVs are a group of more than 100 types of viruses (De Villiers 1994). They are called papillomaviruses because certain types may cause warts or papillomas, which are benign tumours (Jablonska and Majewski 1994). Some types of HPVs are referred to as "low-risk" viruses because they rarely develop into cancer; these include HPV-6 and HPV-11 and while the viruses that can lead to the development of cancer are referred to as "high-risk" viruses (Beaudenon *et. al.* 1986; De Villiers 1994). Both high-risk and low-risk types of HPVs can cause the growth of abnormal cells, but generally only the high-risk types of HPVs may lead to cancer. Sexually transmitted, high-risk HPVs have been linked with cancer in both men and women; they include HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, and 69. These high-risk types of HPVs cause growths that are usually flat and nearly invisible, as compared with the warts caused by HPV-6 and HPV-11. The majority of HPV infections goes away on their own and do not cause any abnormal growths. Of the women who do develop abnormal cell changes with high-risk types of HPV, only a small percentage will develop cervical cancer if the abnormal cells are not removed. Studies suggest that development of cervical cancer in woman depends on a variety of factors acting together with high-risk HPVs. The factors that may increase the risk of cancer in women with HPV infection include smoking, having many children and HIV infection (Winkelstein 1990; Chang *et. al.* 1994).

Laboratory research has indicated that HPVs produce proteins known as E6 and E7 (Dowhanick *et. al.* 1995). These proteins interfere with the cell functions that normally prevent excessive growth. For example, HPV E6 interferes with the human protein p53. p53 is present in all people and acts to keep tumours from growing. This research is being used to develop ways to interrupt the process by which HPV infection can lead to growth of abnormal cells and eventually, cancer. Vaccines for certain papillomaviruses, such as HPV-16 and HPV-18, are being studied in clinical trials for cervical cancer (Paavonen *et. al.* 2000).

2.13. Tumour Markers

An ideal tumour marker would be expected to fulfil certain criteria. To have clinical importance to these markers, the marker must have a therapeutic response to alteration in the state of neoplastic tissue, so that a change in the production of the marker follows tumour ablation or recrudescence would result in an appropriate change in the level of marker. Furthermore, elevated marker levels would be seen in patients with small amount of tumour tissue, so that residual primary or recurrent tumour could be detected at times appreciably earlier than would be possible by conventional diagnostic procedures. Thus, these markers can be used to determine the response to therapy and as an indicator of relapse during the follow-up period (Coomb's and Neville 1978). Both sensitivity and specificity of the marker are important in determining the value of a marker for a particular use. Virtually no tumour marker

fulfils the entire criterion cited above. The detection of such molecular marker to serve as an indicator for a tumour depends upon the constant production of that molecule by the tumour, but not at all, or not above the background level, by the non-tumour cells. However, the production of such molecule may vary not only between similar tumours from the same organ, but also from the same tumour. The production of such marker molecule may also be increased in normal tissue in response to certain physiologic stimuli, thus further confounding the utility of the molecule as a specific tumour marker (Houghton *et. al.* 1982). Since there is cellular heterogeneity within the same tumour represented by the difference in the rate of growth and the stage of differentiation; the expression of biochemical features could potentially be considered as marker for malignancy (Farber *et. al.* 1983).

A variety of tumour markers have been used in the serological diagnosis of cancer, some of which are expressed at the cell surface and are potentially exploitable for the therapy (Bates and Longo 1987). Recent data suggest that molecular markers may also be useful as markers of prognosis for these premalignant oral lesions (Sudbo *et. al.* 2001; Poh *et. al.* 2001). Although easily detected and often cured in its early stages, most oral cancers are moderately advanced at the time of diagnosis. Unfortunately, this pattern has not changed over time.

2.14. Tumour Associated Antigens

Extensive data have been accumulated in the last three decades revealing the existence of the tumour associated antigens on various types of human cancers, including hepatoma (Tatarinov 1964), osteogenic sarcoma (Byers *et. al.* 1975), melanoma (Hollinshed 1975), lung cancer (Bell and Seetharam 1976), experimentally induced animal tumour systems (Prince and Baldwin 1977), breast cancer (Nordquist *et. al.* 1978), leukemia (Greaves 1979), uterine cervix cancer (Heines *et. al.* 1981) and oral cancer (Abdul Kader *et. al.* 1981). The constant expression of an identifiable antigen is a useful diagnostic aid.

A breast cancer antigen, as a saline extract with a molecular weight 30kDa was isolated (Gentile and Flikinger 1972). This antigen gave positive reaction with 15 breast cancer patients, but not with any control tissues. Similarly, the presence of a tumour-associated antigen in the 3M KCl extract of tumour tissue with a reasonably high degree of specificity for breast carcinoma was also demonstrated (McCoy *et. al.* 1974). The existence of tumour-associated antigen in human oral cancer was demonstrated (Abdul Kader *et. al.* 1981).

Tumour associated antigen include many substances that are not systematically organized. Alpha-fetoprotein (AFP), a serum glycoprotein (MW 72,000 Da, single polypeptide) is a normal foetal serum protein synthesized by the liver, yolk sac and gastrointestinal tract that shares sequence homology with

albumin. In the foetus, AFP reaches its peak at about 15 weeks gestation and it decreases after birth, reaching adult values normally in 2 years; while in adults, AFP increases in pregnancy, liver regeneration, hepatitis, alcoholic liver disease, liver cirrhosis and biliary tract obstruction (Gitlin and Boesman 1966 and Lack *et. al.* 1982). Prolonged elevation on maternal AFP can occur in anencephaly, intrauterine foetal distress and congenital nephrosis. Primary liver cancer cells produce AFP. Elevation is more common in areas where hepatocellular carcinoma is endemic and may be useful in screening procedures, although absence of AFP has been shown in some hepatomas. Magnitude of increase and serial changes are important for management and assessment of recurrence. Elevation of AFP in cancer is much higher than that in non-malignant diseases.

A tumour-associated antigen (TA-4) was purified from human uterine cervical mucosa and the serum levels of the antigen were found to correlate with the stage of cervical squamous carcinoma (Kato *et. al.* 1983). In a follow-up study, elevated post-therapy serum levels were found in patients with short survival. An immunoassay for a subfraction of the TA-4 antigen, called squamous cell carcinoma associated antigen (SCC-Antigen) has been developed (Kato and Torigoe 1985).

Prostate Specific Antigen (PSA) glycoprotein and a serine protease (MW 34,000 Da, single polypeptide) are produced exclusively by the epithelial cells of acini and ducts of the prostate gland. It is a normal constituent of seminal fluid

and is tissue specific, but not cancer specific. Its elevation in serum can be detected in prostate cancer, prostatic hypertrophy and prostatitis. It is useful for diagnosis, staging and monitoring of prostate cancer (Andriole 1992).

Carcino embryonic antigen (CEA) is the best used tumour marker for cancers of the gastrointestinal tract (Moertel *et. al.* 1993). CEA is a cell-surface glycoprotein (MW 2, 00,000) of variable carbohydrate content. It is found in foetal intestine, liver and pancreas and is mainly produced in gut epithelium and is metabolised in the liver. In adults, CEA elevations have been detected in plasma and tissues cells in tumours of gastro intestinal tract, pancreas, liver, breast, lung, ovary etc. However, CEA production is not a consistent event. Increase in plasma CEA requires a disruption in the epithelial basement membrane which can also be caused by non-tumour conditions including inflammatory bowel disease, pancreatitis, gastritis, reduced liver metabolism, alcoholic liver disease, biliary obstruction hepatitis and liver cirrhosis. CEA should be regarded as a non-specific tumour marker. CEA is of particular use in colorectal cancer for indication of recurrence, monitoring treatment and prognostication.

Tumour specific antigen CA-125 is the most thoroughly studied serum marker for ovarian cancer. The raised serum CA-125 concentration is a powerful index of risk of ovarian cancer (Jacobs *et. al.* 1993). Carbohydrate antigen 19-9 (CA 19-9) has been used as a serum tumour marker for adenocarcinoma of the upper gastrointestinal tract, particularly primary

adenocarcinoma of the pancreas. This tumour marker has also been used to differentiate benign from malignant diseases of the pancreas (Forsmark *et. al.* 1994).

Studies identified bladder tumour associated antigen as human complement factor H-related protein (hCFHrp). hCFHrp is similar in composition, structure and function to human complement factor H (hCFH). In contrast, hCFHrp was produced by several human bladder cancer cell lines, but not by normal epithelial cell lines. hCFHrp was also not detected in the urine of individuals with other diseases (Pirtskalaishvili *et. al.* 1999).

There are currently many cancer vaccine immunotherapies being developed using different TAAs as targets (Anon 1999). A promising new candidate for immunotherapy is the h5T4 that was defined by a monoclonal antibody (mAb5T4) raised against human trophoblast glycoproteins. 5T4 is expressed by a wide spectrum of cancers, but is not detected on most normal adult tissue. In colorectal, gastric and ovarian carcinomas, tumour expression is associated with poorer clinical outcome. The human oncofoetal antigen 5T4 (h5T4) is a trans-membrane glycoprotein over expressed by a wide spectrum of cancers, including colorectal, ovarian and gastric, but with a limited normal tissue expression. Such properties make 5T4 an excellent putative target for cancer immunotherapy (Mulryan *et. al.* 2002).

Melanoma-associated antigens are at the center of many immunotherapeutic trials in melanoma. Little is known about the impact of antigen expression on the natural course of disease. For primary melanoma, melanoma associated antigens, gp100, MelanA/MART-1, MAGE-3, tyrosinase and HLA class I molecules has been used for immunotherapeutic trials (Hofbauer *et. al.* 2004).

Owing to the complex composition of cell membranes, isolation of tumour specific antigens from solid tumours, surgical specimens or cultured tumour cells has been difficult. Immune complexes seem to be an alternate source for the isolation and characterization of the antigen. Since circulating immune complexes (CIC) contain antigen as one of their components, a number of investigations have used CIC to isolate the potential antigens, which could be helpful in the diagnosis and management of diseases.

2.15. Immune Complexes in Cancers

The essential constituents of all immune complexes (ICs) are antigens and antibodies. Specific antibodies interact to antigen giving rise to antigen-antibody complexes. In any given immune complex, the number of antigen and antibody molecules may vary, depending on the characteristics of the reactants and the features of the antigen-antibody union. The biological properties of the immune complexes are related to the nature of the molecules forming the complexes as well as the number of the reactants in each complex. Immune complexes arise from various sources of antigen i.e. administered

antigens, those released from microorganisms, originating from endogenous tissues, derived from the diet and released from tumours. The valance of antigen molecule profoundly alters the lattice of the formed immune complexes. Only multivalent antigens form immune complexes with high degree of lattice and undergo immune precipitation. The molar ratio of antigen to antibody influences the degree of lattice formation, as illustrated by the classic precipitin curve. The association constant between the antigen and antibody influences the lattice formation of immune complexes. With all other variables constant, low affinity antibodies usually form smaller immune complexes than high affinity antibodies. The body fluids of cancer patients frequently contain immune complexes. To this extent, they differ little from the sera of patients with non-malignant, inflammatory or degenerative conditions of the same tissue or organ. Theoretically, circulating immune complexes detectable in the sera of cancer patients and patients with other pathological disorders contain several disparate antigens, and in the case of cancer patients some of these may be tumour associated antigens.

Two types of antigen-antibody complex formation can be envisaged in the patients with malignant diseases. In the first type, antitumour antibodies interact with cell-surface associated tumour antigens. Conversely, antigen excess immune complexes may facilitate binding of antigen to receptor bearing lymphocytes by an antibody-mediated cross-linking of antigen with resulting specific blockade of these cells (Feldmann and Diener 1972) and affect the traffic patterns of the lymphocytes (Stutman 1973). Otherwise, binding to lymphocytes Fc receptors may initiate complement fixation and lysis of the

effector cells or mediate indirect effect by preferential activation of suppressor T-cells (Gershan *et. al.* 1974); block activated T-cells that mediate delayed type hypersensitivity (Mackaness *et. al.* 1974) and inhibit the proliferation response of effector cells to tumour antigen (Hattler and Soehnlen 1975).

Immune complexes formed on the tumour cell surfaces may undergo endocytosis or be released into the cell environment (Robert *et. al.* 1976). In the second type, immune complexes are formed when the tumour–associated antigens shed from tumour cells circulate in body fluids and interact with antitumour antibodies (Prince and Baldwin 1977). The exact role of CIC in cancer patients has not been fully understood. The free antigen and/or immune complexes induce suppressor cells and other suppressor factors (Perry *et. al.* 1978) and anti-receptor antibodies (Huber and Lucas 1978). These effects would allow the immunogenic tumour to overcome host immunologic control. The mode, by which tumour antigen-antibody immune complexes block cellular defence mechanism in cancer, as well as the nature of the effector cell, has not yet been fully understood. It has been proposed that in the condition of antibody excess, immune complexes may mask, modulate or cause shedding of tumour-associated antigens, thus blocking the recognition by effector lymphocytes (Thung and Herberman 1975).

A variety of methods have been used for the detection and quantification of circulating immune complexes in the sera of patients with malignant and other diseases. The major problem in the assessment of immune

complex is that the type of assay employed may greatly influence the results obtained and in turn lead to discrepancies depending upon the kind of disease in which it is present. The methods vary widely in terms of disease sensitivity, specificity and clinical applicability. Broadly, they fall into three groups. First, those that utilizes the physical properties such as size or cryoprecipitability. Second, those that utilize biological properties of the IC, such as the ability to bind to specific receptors on culture cell, which includes method like Raji cell assay. The last one can look indirectly for secondary effects of ICs, such as the complement consumption of complement component. The C1q binding, the Raji cell assays and PEG precipitation methods are the frequently used methods in cancer studies (Baldwin *et. al.* 1979).

Studies have shown elevated levels of CIC in many types of human malignancies, such as lymphomas (Mukojima *et. al.* 1973), leukemia (Carpentier *et. al.* 1977), breast cancer (Hottken *et. al.* 1977), melanoma, gastrointestinal cancers (Rossen *et. al.* 1977), osteogenic sarcoma (Theofilopoulous *et. al.* 1977), animal tumour (Hoffkin *et. al.* 1978a; Hoffkin *et. al.* 1978b), ovarian cancer (Paulton *et. al.* 1978), neuroblastoma (Brandies *et. al.* 1978) and uterine cervix cancer (Vijayakumar *et. al.* 1986).

The formation of the immune complex in human cancers were studied using monoclonal antibodies and found that the complexes are cleared by liver within a half-life of eleven minutes in the case of ovarian cancer (Davies *et. al.* 1990). CIC is also important in prognosis of different cancers. A decrease

in CMI and subsequent increase in CIC indicate unfavorable prognostic in cancer patients and also precedes clinical manifestation of increased tumor mass in vivo (Aziz *et. al.* 1993). In an animal model, it has been demonstrated that the immune complexes are cleared from the body with the help of macrophages in clearing the viral pathogen (Reinagel and Taylor 2000).

2.16. CIC in Oral Cancer Patients

A progressive increase in CIC values in patients with cancer of oral cavity, uterine cervix and breast with clinical stages of the disease was reported and these solid tumour CIC levels could be used for monitoring prognosis (Vijayakumar *et. al.* 1986). Similar observations on cervical and oral cancer patients were also reported (Raghunath *et. al.* 1987). It has been reported that the CIC levels in the chewing controls were significantly raised when compared to normal controls; and the CIC levels in the patients with premalignant lesions were elevated almost to the same levels as in the oral cancer patients (Balaram *et. al.* 1987). In contrary, an elevated level of CIC was observed in the sera of the patients with oral submucous fibrosis; an oral pre-malignant lesion, however, the values were lower than those of oral cancer (Remani *et. al.* 1988). It was suggested that the level of CIC could be helpful in predicting the malignant transformation of oral submucous fibrosis. The methods currently used for measuring of CIC levels in cancer patients are non-specific and not capable of distinguishing tumour antigen containing CIC from the others. Therefore, these assays do not provide any direct evidence that the CIC detected are tumour related.

CIC also plays some regulatory role in certain antigens (Balaram *et. al.* 1994). Sera from patients with carcinoma of the buccal mucosa were analyzed for its regulatory effect on CD2 antigen expression using anti CD2 monoclonal antibodies and sheep erythrocyte rosetting assay. The sera from majority of the patients showed an inhibitory effect while sera from rest of the patients showed an enhancing effect on the CD2 antigen expression and the concentration of CIC was higher in the enhancer sera.

Biological properties of ICs include activation of complement system, interaction with cell receptors and the deposition in tissues. Other influences include alteration of the functions of lymphocytes, polyclonal B-cell and T-cell activation in autoimmune disorders. The complement system significantly influences the nature of the immune complexes by solubilising it. Investigations are undertaken to isolate and characterise the antigen moieties from the CIC. This may help in developing specific assays, which will be very useful, particularly for diagnosis. Furthermore, the analysis of tumour related immune complexes would help in the isolation of tumour marker substances, which induce a humoral and cellular response in the patients. Absolute proof of specific cellular recognition of tumour cell antigen will require the biochemical isolation of such antigens and the successful adoptive transfer of cells that can mediate anti tumour responses in humans.

The review of literature reveals that the immune response play an important role in cancer and these responses can be exploited for the diagnosis and prognosis of the cancer. The present study is to investigate the immunologic and virologic contributions to the prevalent cancer as well as the identification and analysis of molecular markers involved in this cancer. During cancer the derailment of immunological response may be because of the immune response itself which may act as a blocking factor, as in the case of circulating immune response. So the identification and modification of the tumour antigens or markers is a requirement for improving the immune response in a favorable way so that it can help to decrease the tumour burden. So identification of tumour antigen or marker has got an intense role in managing tumours.

2.17. Aims and Objectives of the present study

1. To compare the incidence of different cancers based on the hospital data, and selection of an appropriate cancer for the study of tumour associated antigens.
2. To find out the association of tumour virus with the cancer, by detecting specific antigen or nucleic acid of the virus from patient's serum and blood, respectively and to determine prevalence of these viruses with the specific cancer.
3. Detection and quantification of circulating immune complex in cancer patients as a source of tumour associated antigen.
4. To find out various classes of antibodies present in the immune complexes and to study its importance in immune reaction.
5. To Isolate, purify and characterise the tumour associated antigen from circulating immune complexes.
6. Production of polyclonal antibody against the isolated tumour specific antigen(s) and to determine the immunoreactivity of the antigen-antibody by Enzyme Linked Immunosorbent Assay and Protein blotting.

Isolation and Characterisation of Tumour Associated Antigens and Their Significance in Host Immune Responses

Thesis submitted to the
University of Calicut under the **Faculty of Medicine**
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
IMMUNOLOGY

by

ELYAS K. K.
Department of Biochemistry
Medical College
Thrissur
2005

MATERIALS AND METHODS

Chapter III
MATERIALS AND METHODS

3.1. Samples for the study

Patient's data for the epidemiological studies were collected from histo-pathological laboratory reports, during 1995-1998 periods from Amala Cancer Research Centre, Thrissur, a referral centre for cancer treatment and research in Kerala. The patient's data with the clinical symptoms and whose tissue samples were histo-pathologically examined were used for the study. The tissue samples were stained at Histo-pathology department of the centre using standard methods (David and James 1999). Tissue samples of all organs and lesions were preserved in neutral formalin; fixed tissues were embedded in paraffin, 5-7 µm thick sections were made and stained with hematoxylin and eosin stains. Special stains were used when needed.

Blood samples for the studies were collected from oral cancer patients and normal controls by venipunture into clean centrifuge tubes and were allowed to clot. The serum was separated by centrifugation, aliquoted and stored at -20^0C until use. Repeated freezing and thawing was avoided as this could cause aggregation of immunoglobulins leading to erroneous results. These samples were used for the isolation, characterisation of CIC and antigens from the serum.

Biopsies and surgery specimen, obtained from histo-pathologically proved oral squamous cell carcinoma cases were used for HPV-DNA hybridization

studies. Age matched control DNA samples were obtained from oral mucosa, from fresh autopsy specimens and minor oral surgical procedures. DNA samples were stored at -20^{0}C until use.

3.2. Detection of HSV-1 and HSV-2 infections in oral cancer patients by ELISA

HSV-1 and HSV-2 infections were determined by the assessment of specific antibodies in serum of patients and controls using commercially available ELISA kits (Human GmbH, Germany) (Bidwell 1977).

3.2.1. Reagents and Chemicals for the ELISA (Human GmbH, Germany)

1. **Dilution buffer** (pH 6.5 ± 0.2)
   - Phosphate buffer - 10 mmol/l
   - Sodium chloride - 8 g/l
   - Albumin - 10 g/l

2. **Substrate buffer** (pH 3.9 ± 0.2)
   - Potassium citrate - 0.2 mol/l
   - Hydrogen peroxide - 6 mmol/l

3. **Washing Solution** (pH 7.2 ± 0.2)
   - Tris Buffer - 10 mmol/l
   - Sodium chloride - 8 g/l

4. **TMB solution**
   3,3', 5,5'-tetra methyl-benzidine- 20 mmol/l

5. **Stopping solution**
   - Sulphuric acid - 1.5 mol/l

6. **Anti-IgG conjugate**
   a) Anti-Human IgG (Rabbit), Peroxidase-conjugated
   b) Anti-Human IgM (Rabbit), Peroxidase-conjugated

7. **Microtiter well strips coated with cell culture derived antigen**
   a) HSV-1 antigen for detection of IgG
   b) HSV-2 antigen for detection of IgG
   c) HSV-1 and HSV-2 antigen for detection of IgM

8. **Control serums**
   a) HSV, IgG/IgM-negative-controls (Human)
   b) HSV, IgG/IgM-positive-controls (Human)

3.2.2. Procedure for the ELISA

The presence of anti HSV-1 and anti HSV-2 IgG antibodies and anti HSV IgM antibodies were determined in patients serum samples. Mircrotitre strip wells as a solid phase were coated with cell culture derived HSV antigens. If corresponding specific antibodies were present in a patient sample or control, they were bound to the specific antigen on the solid phase. After a washing step to remove unbound material, anti immunoglobulin peroxidase conjugate was added, this binds specifically to the specific class of antibodies. The enzyme-linked complexes were detected by incubation with chromogenic solution and the subsequent development of the blue colour, which changed to yellow by stopping the enzymatic reaction with sulphuric acid. The colour developed is directly proportional to the antibodies present in the serum.

Hundred micro-liter of 1:100 diluted serum samples from oral cancer patients and normal healthy individuals were added to the microtitre wells and incubated for 30 minutes at room temperature. The wells were washed 3 times with washing solution to remove the unbound serum materials. The remaining fluid was removed by tapping the wells on a tissue paper. Added 100 µl of rabbit anti-immunoglobulin (anti-IgG or anti-IgM as the case may be) conjugated with

peroxidase enzyme and incubated for 30 minutes at room temperature. Wells were washed four times as described earlier. Added 100 µl TMB solution and incubated at room temperature for 15 min. in the dark and stopped the reaction by using 100 µl of sulphuric acid as the stopping reagent after the development of colour. The absorbance at 450nm (A_{450}) was measured in an ELISA Reader (Merck, Germany).

Negative and positive controls from the kit were also included during the experiment. Substrate blank was also incorporated in the experiment. All the tests were done in duplicates. Calculations were made as follows in accordance to the manufacturer's instruction.

COV = MNC + 0.1 MPC for IgG ELISA

COV = MNC + 0.2 MPC for IgM ELISA

COV: Cut Off Value

MNC: mean negative control absorbance

MPC: Mean Positive control absorbance

Results were interpreted as follows:-

A_{450} patient $\geq$ COV + 15% anti-HSV IgG/IgM antibody positive.

A_{450} patient < COV + 15% anti-HSV IgG/IgM antibody negative.

Positive patient's specimens were expressed in "Human Units"("HU/ml.")

$$\text{"HU/ml".} = \frac{\text{Absorbance of test sample}}{\text{Mean Absorbance of positive control}} \times 100$$

3.3. Detection of HPV-16 and HPV-18 DNA from oral cancer tissue samples

For the determination of the HPV association with oral cancer, hybridisation studies were conducted with the DNA isolated from oral cancer tissue samples.

3.3.1. Maintenance and storage of HPV DNA probes

Viral HPV probes were maintained as recombinant plasmid in pBR 322. These recombinant plasmids were used to transform the host *E. coli* by calcium chloride mediated chemical transformation (Sambrook *et. al.* 1989).

3.3.1.1. Reagents and chemicals for Transformation of *E. coli*

1. **Probe DNA**
   a) HPV-16 DNA Cloned at *Bam*H1 site of pBR 322.
   b) HPV-18 DNA Cloned at *Eco*R1 site of pBR 322.

2. **Host cell**
   E. coli. (HB 101)

3. **Luria Broth Medium (LB)**
   NaCl - 5.0 g (1%)
   Yeast extract - 2.5 g (0.5%)
   Bactotryptone - 5.0 g (1%)
   Autoclaved and stored at 4^0 C.

4. **$MgCl_2$** - 100mM

5. **$CaCl_2$** - 85 mM

6. **Antibiotics**

Antibiotic	Stock solution	Working Con.
Tetracycline	12mg/ml (in 70% Ethanol)	12µg/m
Ampicillin	4mg/ml (Water)	50µg/ml

3.3.1.2. Procedure for transformation of pBR 322-HPV recombinant probe plasmids in *E. coli*

HPV-16 and HPV-18 DNA cloned in the plasmid pBR 322 were used for the study. Cloned recombinant HPV-16 and HPV-18 DNA were used to transform and maintain in *E. coli* (HB 101). These clones were used for the preparation of the viral probes. The cloned HPV-16 DNA was at the *Bam*H1 site of pBR 322. HPV-18 was cloned at *Eco*R1 site of pBR322.

The following method was used to transform *E.coli* cells (Sambrook *et. al.* 1989). Single colony of *E. coli* (HB 101) was inoculated into 1ml of LB medium and incubated at 37^0C overnight with shaking at 250 rpm. One ml of the culture was inoculated into 50ml of LB medium and grown for 2-3 hours (until the cell density reached 0.6 OD_{600}). The culture was chilled on ice for 2 minutes and centrifuged at 3000 rpm for 5 minutes at 4^0C. The pelleted cells were resuspended in 9 ml of sterile 100mM $MgCl_2$ and subsequently centrifuged at 3000 rpm for 5 minutes at 4^0C. The pelleted cells were resuspended in 9 ml of ice-cold 100 mM $CaCl_2$ and incubated on ice for 40 minutes. Subsequently, the cells were pelleted and resuspended in 3.5 ml of a sterile solution containing 85mM $CaCl_2$ and 15% glycerol. The cells were dispensed into 100µl aliquots in pre-chilled microfuge tubes and stored at -70^0C. These cells were used for transformation.

About 50-100 ng HPV inserted pBR322 DNA was used to transform 100 µl aliquots of competent *E. coli* cells. DNA sample was added to the cells and incubated on ice for 40 minutes. Then the cells were incubated at 42^0C for 90 seconds and subsequently on ice for 5 minutes. One ml of antibiotic free LB

medium was added to the tube and cells were allowed to grow at 37^0C for one hour. The cells were plated on LB agar plates containing suitable antibiotics. Successful transformants were further propagated and maintained as continous culture or glycerol stock.

3.3.2. Isolation of Recombinant pBR322-HPV Plasmids DNA from *E. coli*

In order to get HPV-16 and HPV-18 for probing genomic DNA, HPV containing tranfomants were grown in appropriate antibiotic media and recombinant plasmids were isolated by alkali lysis method (Birnboim and Doly 1979).

3.3.2.1. Reagents and chemicals for Plasmid isolation

1. **Solution I (10ml)**

	Volume	Final concentration
Glucose (1M)	0.5ml	50mM
Tris-Cl (I M, pH 8.0)	0.25ml	25mM
EDTA (0.5 M, pH 8.0)	0.2ml	10mM

Distilled water added to make a final volume of 10ml

2. **Solution II (10ml)**

	Volume	Final concentration
NaOH (10N)	0.2ml	0.2N
SDS (10%)	1ml	1%

Distilled water added to make a final volume of 10ml

3. **Solution III (Sodium Acetate solution) (3M, pH 5.2)**
   Sodium acetate - 40.81g
   Distilled water - 80ml
   pH adjusted to 5.2 and distilled water added to make a final volume of 100ml

4. **Saturated Phenol**
   Distilled phenol - 100ml
   β-Hydroxyquinoline - 80mg (0.8mg/ml)
   β-Merocaptoethanol- 0.2%
   Tris-Cl (I M, pH 7.5)- 100ml

Mixed equal amounts of phenol with Tris-Cl (0.1M). It was allowed to settle down and aquous phase discarded. The extraction was repeated several times with equal volumes of 0.1 M Tris-Cl until the pH of the phenolic phase became 7. The equilibrated mixture was stored under an equal volume of 0.01 M Tris-Cl (pH 7.5) at 4^0C in dark glass bottles.

5. **Tris (1M, pH 8.0) stock solution**
   Tris base - 12.1 g
   Distilled water - 80 ml
   pH adjusted to 8.0 with concentrated HCl and final volume adjusted to 100ml with distilled water.

6. **EDTA (0.5 M, pH 8.0) stock solution**
   EDTA -18.6 g
   Distilled water - 80 ml
   pH adjusted to 8.0 and distilled water added to make a final volume of 100ml

7. **Tris-EDTA (TE) Working solution**

	Volume	Final concentration
Tris-Cl (I M, pH 8.0)	1.0ml	10mM
EDTA (0.5 M, pH 8.0)	0.2ml	1mM

   Distilled water added to make a final volume of 100ml. Autoclaved and stored at 4^0C.

8. **RNAse A (1ml) Solution**
   RNAse A - 10mg
   Tris-Cl (I M, pH 7.5) - 10µl (10mM)
   NaCl - 3µl (15mM)
   Distilled water - 973µl
   Heated to 100^0C for 15 minutes. Allowed to cool slowly at room temperature, stored at -20^0C in aliquots

3.3.2.2. Procedure for Plasmid Isolation

Single cell colony of the transformant was transferred into 5ml medium containing antibiotics and incubated at 37^0C with vigorous shaking (300 rpm) on a rotary shaker for 14 hrs. 1.5 ml of culture was taken in a microfuge tube and centrifuged at 10,000 rpm at 4^0C for 10min. The supernatant was discarded and the pellets were re-suspended in 100 µl of ice cold Solution I,

vortexed to mix the contents well and kept on ice for 5 min. 200 µl of freshly prepared Solution II was added, mixed gently by inverting the tubes several times and incubated on ice for 10 min. 150 µl of chilled Solution III was added and gently mixed by inverting the tubes several times and kept on ice for 15 min. Centrifuged at 12,000 rpm for 10 min at 4^0C and the supernatant was transferred to a fresh tube. 450 µl phenol-chloroform mixture (1:1) was added, mixed by inverting the tubes several times and kept on ice for 15 min and centrifuged at 12,000 rpm for 10 min at 4^0C. The supernatant was transferred to a fresh tube and added 2 volumes of chilled 100% alcohol, mixed by inverting the tubes several times and kept on ice for 30 min. Centrifuged at 12,000 rpm for 15 min at 4^0C. The supernatant was removed carefully and 1 ml of 70% ethanol was added to the pellet and mixed gently by inverting the tube several times. Centrifuged at 10,000 rpm at 4^0C for 15 min removed the supernatant carefully and the pellet (plasmid DNA) was dried at 37^0C for 1 hour. RNA was removed by treating the plasmid preparation in TE (pH 8) containing DNAase free pancreatic RNAase (20 µg/ml). Purity was checked by agarose gel electrophoresis. The prepared plasmids were kept at -20^0C until further use.

3.3.3. Excision of HPV probe DNA from recombinant vector

HPV-16 and 18 DNA cloned in PBR 322, were used for the preparation of probes for the study. The vectors containing the HPV inserts were digested with restriction enzymes, *Bam*H1 and *Eco*R1 for HPV-16 and HPV-18 respectively (Sambrook *et. al.* 1989).

3.3.3.1. Reagents and chemicals for restriction digestion

Restriction Enzyme	10X Buffer System
1. *Bam* H1	Tris 100 mM NaCl 1 M $MgCl_2$ 100 mM DTT 10 mM pH: 8
2. *Eco* R1	Tris HCl 500 mM NaCl 1 M $MgCl_2$ 100 mM 2-mercaptoethanol 50 mM

3.3.3.2. Procedure for restriction digestion

For restriction digestion, in a sterile microfuge tube 3 μl of HPV-plasmid DNA, 5 μl 10x buffer and 5 μl restriction enzyme (10 units/μl) were added and final volume made up to 50μl with distilled water and incubated at 37^0C for 3hr. After the digestion, electrophoresis was performed on agarose gel as in section 3.3.4. The HPV-DNA bands were cut out and the DNA was eluted from the gel by electroelution as in section 3.3.5. Eluted DNA was suspended in TE buffer (pH 8) and stored at -20^0C until use. These HPV inserts were labelled and used for DNA hybridization studies. Probes were prepared separately for HPV-16 and HPV-18 from the recombinant pBR 322 plasmids.

3.3.4. Agarose gel electrophoresis

This is a method widely used for the separation of DNA molecule based on their size and molecular weight (Slater 1989).

3.3.4.1. Reagents and chemicals for agarose gel electrophoresis of DNA

1. **Gel Running buffer TAE** (50 X)
   Tris-base - 24.20 g
   Glacial acetic acid - 5.71 ml
   0.5 M EDTA (pH 8) - 10.00 ml
   50X TAE was diluted to appropriate concentration prior to use with sterile distilled water.

2. **Agarose Gel**
   1% agarose in TAE buffer

3. **Ethidium bromide**
   Prepared as 10mg/ml stock solution in deionised water and stored in a dark bottle.

4. **DNA loading buffer** (6X)
   Glycerol - 30%
   Bromophenol blue - 0.25% (dissolved in sterile deionised water)

3.3.4.2. Procedure for agarose gel electrophoresis

Agarose gel electrophoresis was carried out in a horizontal matrix of agarose with TAE buffer. The DNA samples were diluted with 1/6 volume of 6X loading buffer and deionised water. Samples were loaded on agarose gel containing 0.5µg/ml ethidium bromide. Electrophoresis was performed at 5 V/cm until the run was complete in a horizontal electrophoresis apparatus (Hoefer USA). After completion of electrophoresis, the gel was visualized on a UV transilluminator (Hoefer USA).

3.3.5. Electoelution of DNA probes from agarose gel

The region of the gel containing the desired HPV DNA fragments was sliced out from the gel using a microscopic cover glass. DNA was eluted by electro elution method (Sambrook *et. al.* 1989).

3.3.5.1. Reagents and chemicals for electroelution

1) **TAE (Tris Acetic acid EDTA) ***

2) **Saturated Phenol ***

*Reagents were prepared as described in section 3.3.4.1

3.3.5.2. Procedure for the elution of DNA fragments from agarose gel

The gel slice containing the band of interest was kept in a dialysis bag, filled with 0.5X TAE buffer and the necks of the bag were sealed. The bag was immersed in an electrophoresis tank containing 0.5X TAE buffer and electric current of 50V was passed for 30 minutes. Polarity of the current was reversed for 30 seconds to release the DNA from the wall of the dialysis bag. The buffer was collected from the bag, extracted with phenol-chloroform (1:1), precipitated with absolute ethanol (100%) and the pellet was washed with 70% ethanol. The pellet was dried, resuspended in TE buffer and stored at -20^{0}C.

3.3.6. Genomic DNA extraction

DNA samples from biopsy and surgery specimens from histopathologically confirmed oral squamous carcinoma cases and from normal oral mucosa sample obtained from fresh autopsy specimens were used in DNA

hybridization studies. Tissue DNA was extracted by the standard phenol: chloroform: isoamyl alcohol method (Sambrook *et. al.* 1989).

3.3.6.1. Reagents and chemicals for genomic DNA extraction

1. **Tris EDTA buffer** *
2. **Saturated phenol** *
3. **RNAse A** *

*Reagents were prepared as described in section 3.3.2.1

4. **Sodium acetate** (3M, pH 5.2)

   Sodium acetate - 40.81 g

   Distilled water - 80 ml

pH adjusted to 5.2 and distilled water added to make a final volume of 100ml .

3.3.6.2. Procedure for genomic DNA extraction from tissue samples

The tissue samples were homogenized in Tris-EDTA buffer (1mg tissue/10ml buffer), 0.5% final concentration of SDS and proteinase-K 100 µg/ml were added to the homogenate and incubated for 3 hour to overnight at 37^0C. The digest was then extracted with water-saturated phenol. The aqueous layer was collected and re-extracted with phenol-chloroform–isoamyl alcohol (25:24:1). DNA was precipitated out with 1/10 volume of chilled 3M sodium acetate and 2.5 volumes of absolute ethanol.

The DNA pellet was washed with 70% ethanol, freeze-dried and resuspended in Tris-EDTA buffer. RNase was added to a final concentration of 100µg/ml and incubated at 37^0C for one hour. The RNA free DNA was purified by phenol/chloroform extractions, precipitated with ethanol, washed with 70% ethanol,

dried and suspended in TE buffer. Extreme care was taken to avoid cross contamination between samples.

3.3.7. Restriction digestion of genomic DNA

The following method was used for the complete digestion of the genomic DNA (Sambrook *et. al.* 1989)

3.3.7.1. Reagents and chemicals used for digestion of genomic DNA

Restriction Enzyme	10X Buffer System
Pst 1	Tris -100 mM Nacl - 1 M $MgCl_2$ -100 mM DTT - 10 mM pH: 8

3.3.7.2. Procedure for Restriction digestion of genomic DNA

In a sterile microfuge tube 8 µl of autoclaved distilled water, 10 µl of chromosomal DNA (approximately 0.2µg DNA) and 2 µl of 10X buffer were added and mixed well. I µl of *Pst*1 restriction enzyme was added, mixed thoroughly and incubated at 37^0C overnight. This DNA digest was used for Southern blotting. Digestion of genomic DNA was confirmed by agarose gel electrophoresis (section 3.3.4.) and observation of characteristic pattern (Sambrook *et. al.* 1989).

3.3.8. Capillary blotting of genomic DNA

The DNA was transferred from an agarose gel on to Hybond-N membrane by capillary action as described originally by Southern (Southern 1975). Restriction enzyme digested genomic DNA was electrophoresed in agarose gel

using sample buffer containing 0.25% (w/v) xylene-cyanol and 0.25% (w/v) bromophenol blue. After electrophoresis, placed the agarose gel in 0.25 M HCl until the dyes changed colour, and incubated for an additional 10 minutes. Rinsed gel in distilled water and incubated in denaturation buffer for 30 minutes at room temperature with shaking. Rinsed gel in distilled water and placed in neutralization buffer for 15 minutes at room temperature with shaking and repeated rinsing with neutralisation buffer for three times. The blotting was performed as described by Southern (Southern 1975).

3.3.8.1. Reagents and materials for capillary blotting

1. **Gel loading Dye (6X) for blotting**

   Bromophenol blue - 0.02g(0.25%)
   Xylene cyanol - 0.02g(0.25%)
   Ficoll (MW.400) - 1.5g (15%)
   Distilled water added to make a final volume of 10ml, aliquoted and stored at 4^0C
   Elecrtophoresis reagents were prepared as described in agarose gel electrophoresis section

2. **SSC (20X)**

   NaCl - 17.5g
   Sodium citrate - 8.8g
   Distilled water - 80ml
   pH adjusted to 7 and distilled water added to make a final vol. of 100ml

3. **Filter paper 3mm** (Whatman, UK)

4. **Hybond–N membrane** (Amersham, UK)

3.3.8.2. Procedure for capillary blotting

Glass tray filled with blotting buffer (20X SSC) was used for capillary blotting. Made a platform with a glass plate and covered it with a wick made from three sheets of Whatman 3mm filter paper which was saturated with

the blotting buffer from the tray. Placed the gel on the wick and avoided trapping air bubbles beneath it with cling film to prevent the blotting buffer being absorbed directly into the paper towels above. A sheet of Hybond-N membrane cut to the exact size of the gel was placed on the top of the gel. Trapping bubbles beneath the membrane was avoided. Air bubbles that appeared were squeezed out using a glass rod. Placed three sheets of 3mm paper, cut to size and wetted with blotting buffer on the top of the Hybond–N membrane. Placed a stack of absorbent paper towels on top of 3mm paper (approximately 5cm high). Placed a glass plate on top of the paper towels and kept a weight on top. Allowed transfer to proceed over night. After blotting, carefully dismantled the apparatus, marked sides of the membrane with pencil to allow later identification of the tracks. Washed the membrane briefly and carefully in 2X SSC to remove any adhering agarose. Baked the membrane in an oven at 80^0C for 2 hours under vacuum to fix.

3.3.9. HPV DNA probes labelling

Labelling of HPV-16 and HPV-18 DNA were done using Megaprime labelling system (Amersham, UK) (Feinberg and Vogelstein 1983).

3.3.9.1. Reagents and solutions for DNA labelling

(Megaprime labelling system, Amersham, UK.)

1. **Primer solution**
   Random nonamer primers in an aqueous solution

2. **Reaction buffer**
   A concentrated reaction buffer containing Tris HCl, pH 7.5, $MgCl_2$ and 2-mercaptoethanol

3. **Non radioactive nucleotide in buffer solutions**
   Nucleotides in concentrated buffer solutions containing Tris HCl, pH 8.0, 0.5mM EDTA.
   1. dATP
   2. dGTP
   3. dTTP

4. **Radioactive nucleotide**
   ^{32}P labelled dCTP (BARC, India)

5. **Enzyme solution**
   1U/µl DNA polymerase I "Klenow" fragment (cloned) in 50mM Potassium Phosphate pH 6.5, 10mM 2-mercaptoethanol and 50% Glycerol

6. **TE buffer** (Section 3.3.2.1)

3.3.9.2. Procedure for DNA labelling

Diluted the DNA to a concentration of approximately 5 µg/ml in 10 mM TE buffer. Added 25 ng (5 µl) of template DNA into a clean micro centrifuge tube and to it added 5 µl of primers. Denatured by heating to 95-100^{0}C for 5 minutes in a boiling water bath. Centrifuged briefly in a micro centrifuge to bring the contents to the bottom of the tube. Kept the tube at room temperature, added the 4 µl non-radioactive nucleotides and 5 µl 10X reaction buffer, 4 µl radiolabelled nucleotide, 2 µl DNA polymerase Klenow fragment enzyme and nuclease free water to make the final volume to 50 µl. Capped the tube and spun for a few seconds in a microcentrifuge to bring the contents to the bottom of the tube. Incubated at 37^{0}C for 10 minutes. Stopped the reaction by the addition of 5 µl of 0.2 M EDTA. For use in hybridization, denatured the labelled DNA by heating to 95-100^{0}C for 5 minutes and then chilled on ice.

3.3.10. Hybridization of Genomic DNA with labelled HPV Probes

The probe DNA was hybridized with the membrane transferred patient DNA to detect the presence of HPV (Meinkoth and Wahl 1984)

3.3.10.1. Reagents and chemicals for hybridization

1. **Denhardts solution (50X)**

Ficoll	– 5g
Polyvinyl pyrrolidone	– 5g
BSA (Pantex fraction V)	– 5g
Distilled water	– 500ml

Filter sterilized. Stored at –20^{0}C in aliquots.

2. **Prehybridization solution**

Formamide	– 50ml
SSC 5X	– 20ml (Section 3.3.8.1)
Denhardt's solution	– 0.02 ml
Herring sperm DNA	– 5mg
Trisodium phosphate 0.5M	– 10 ml
SDS	– 1 mg

Added distilled water and made upto 100 ml

3. **Hybridization solution**

Formamide	– 50ml
SSC 5X	– 20 ml (Section 3.3.8.1)
Denhardt's solution	– 0.02 ml
Herring sperm DNA	– 5mg
Trisodium phosphate 0.5M	– 10 ml
SDS	– 1 mg
Dextran sulphate	– 10%

Added distilled water and made upto 100 ml

3.3.10.2. Procedure for Probe Hybridization

The blots were prehybridized in prehybridization solution for two hour. Then prehybridized in a shaking water bath at 65^{0}C for 1 hour. For hybridization, denatured labelled HPV probe by heating to 100^{0}C for 5 minutes. Hybridization was carried out for 16-20 hour under stringent condition at 65^{0}C in

the hybridization solution. After hybridization, the membranes were subjected to stringent washes and then autoradiographed.

3.4. Isolation and quantification of circulating immune complexes (CIC) from oral cancer patients

Circulating immune complexes (CIC) are important source of cancer antigen. The antibody part of the CIC is a specific response against the CIC antigen and can be considered as a tumour associated antigen. Polyethylene glycol (PEG) precipitation is the simplest and the most widely used method for the isolation of CIC from other serum components. The isolated complexes are quantified by exploring the biological and physico-chemical properties of it. In the present study, the PEG precipitate was quantified by the ability of CIC to fix serum complement (Harkiss and Brown 1979). CIC fixes the limited amount of externally added complement in the reaction system, in proportion to its amount. The excess complement in the reaction system was quantified using sheep red blood cells (sRBC) and anti-sRBC antibody raised in rabbit as an indicator system. Excess complement acts on the indicator system; lyse the RBC and release haemoglobin. The amount of haemoglobin released depends on the amount of CIC.

3.4.1. Antibody to sheep red blood cells raised in rabbits.

In order to produce the above mentioned indicator system, anti-sheep RBC antibodies were raised in rabbits (Catty and Ray 1988).

3.4.1.1. Reagents and materials for production of anti-sRBC serum

1. **Anticoagulant Dextrose Citrate (ADC) 100 ml**
   Citric acid (anhydrous) – 0.8 g
   Sodium citrate (dihydrate) – 2.5 g
   Dextrose (anhydrous) – 12.0 g
   Sterilised by autoclaving

2. **sRBC** were collected from slaughterhouse, in sterile ACD solution.

3. **Alserver's solution 100 ml**
   Dextrose – 2.05 g
   NaCl – 0.42 g
   Trisodium citrate – 0.30 g
   Citric acid – 0.05 g
   Sterlised by filtration.

4. **Phosphate Buffered Saline** (5X)
   NaCl – 32.43 g
   KH_2PO_4 – 6.10 g
   Na_2HPO_4 – 24.72 g
   Distilled water – 1 litre
   PH – 7.2
   Diluted 1:5 in water prior to use

3.4.1.2. Procedure for production of anti-sRBC antibodies

Sheep red blood cells (sRBC) were harvested under sterile condition into anti-coagulant dextrose citrate (ADC) solution. Aliquotes of whole blood (20 ml) were centrifuged at 1500 rpm for 20 min in sterile centrifuge tubes and the plasma and buffy coat were removed. The cells were then washed four times in 20 ml Alserver's solution and resuspended in same volume. If necessary, they were stored for weeks at 4^0C in this condition. For immunization, the cells are washed twice in PBS and was made up to 10% (v/v) suspension in PBS that may contain approximately 10^9 cells/ml. For the good antibody response againt the sRBC, 10 intravenous injections of 1 ml cells/kg body weight was given in to the

lateral ear veins for the first four days and then on alternate days. Third day, after the final immunization, the rabbits were bleeded for harvesting serum. Blood was collected from the lateral ear veins and allowed to clot for 1h at 37^0C. The clots were freed from the walls of the container and left at 4^0C overnight for the clot to retract. The serum was spun at 1500 rpm to remove red cells and stored at -20^0C without any preservatives. Anti-sRBC antibodies were checked by haem-agglutination test.

3.4.2. Standardisation of number of sheep red blood cells (sRBC) and its sensitization

The number of erythrocytes used in complement consumption assay was standardised in order to reduce the day-to-day variation in the release of haemoglobin.

3.4.2.1 Reagents and chemicals for standardisation of number of sRBC

1) Borate buffer

Boric acid	– 6.18gm
Disodium tetraborate	– 4.38gm
NaN_3	– 1.00gm
Distilled water	– 1 litre
PH 8.4	

2) Phosphate buffered saline (Section 3.4.1.1.)

3) Sodium carbonate solution (1%)
1 g Na_2CO_3 in 100 ml water

3.4.2.2. Procedure for Standardisation of number of sRBC

For Standardisation of number of sRBC 10 ml of 50% sheep blood in ACD solution was centrifuged at 3000 rpm for 5min, removed the supernatant

and buffy coat. Washed the Sheep RBC (sRBC) three times with PBS solution, centrifuged at 3000 rpm for five minutes and 1ml of packed cells were suspended in 19 ml of PBS solution. After thorough mixing, 200µl of cell suspension was mixed with 2.2 ml of 1% Na_2CO_3 solution. After haemolysis, the cell debris was removed by centrifugation at 3000 rpm for 5 minutes and absorbance of the supernatant was measured at 545 nm in 1cm cuvette.

Following equation was used for the adjustment of the optical density (OD) of the supernatant to a final value of 1.50.

Vt=Vi (OD/1.50)

Vi = initial volume of PBS solution (20ml)

Vt = final volume of PBS in which the sRBC is to be suspended in order to get an OD of 1.5.

The suspended sRBC was sensitised by incubating with rabbit antiRBC antibody at a two fold dilution of its tire, in a shaking water bath at 37^0C for 30 minutes.

3.4.3. PEG precipitation and compliment consumption assay

3.4.3.1. Reagents and chemicals for the assay

1) Borate buffer (Section 3.4.3.1)

2) PEG-borate solution - 2.5%
2.1 g PEG in 100 ml of borate buffer

3) PEG-borate solution - 12.5%
125 g PEG in 1 liter of borate buffer

3.4.3.2. Procedure for PEG precipitation and compliment consumption assay

This assay was done as reported by Harkiss and Brown with modifications (Harkiss and Brown 1979). It is based on the isolation of CIC by PEG

precipitation and quantitation of the precipitate by their ability to activate the complement cascade. To 300µl of serum, 50µl of borate buffer (pH 8.4) and 50µl of 0.2M EDTA were added and mixed gently. An aliquot of 100µl of 12.5% PEG was added and the tubes were vortexed and then kept at 4^0C for 90 minutes. They were centrifuged at 1700 x g for 10 minutes at 4^0C; the supernatants discarded and the pellets were washed with 1 ml of 2.5% PEG at 1700Xg for 15 minutes at 4^0C. Once again, the supernatant was discarded and the pellet was dissolved in 30µl of warm borate buffer by vortexing. To this is added 15µl of pooled normal human serum (fresh) as a source of complement and incubated at 37^0C for 30 minutes and then kept on ice. Each sample was then made up to 750µl with warm borate buffer and then 250µl of warm 0.2% sensitized sheep RBC was added to each tube and further incubated at 37^0C for 15 minutes. The optical density of each sample was then noted at 545nm. A 100% haemolysis control, containing 30µl complement fixing diluent plus 15µl of pooled normal human serum and 0% haemolysis control, containing 45µl complement fixing diluent and no normal human serum was also included in the study. The results were expressed as the percentage of complement consumption (Raghunath *et. al.* 1987)

3.5. Determination of Immunoglobulin Classes in Circulating Immune Complex

A quantitative comparison of the CIC immunoglobulin classes present in various age groups of oral cancer patients and normal healthy individuals were studied.

3.5.1. Quantification of Immunoglobulin classes by ELISA

A specific, sensitive and reproducible solid-phase enzyme immunoassay was developed to perform the detection of total and CIC immunoglobulin classes in serum samples.

3.5.1.1. Reagents and chemicals for Quantification of Immunoglobulin classes by ELISA

1. **Phosphate buffered saline - 0.1M** (Section 3.4.1.1)

2. **Coating buffer (Carbonate/bicarbonate buffer)**
   Prepared (I) 0.2 M solution of anhydrous Na_2CO_3 (21.2g in 1000ml) and (II) 0.2 M solution of Na_2HCO_3 (16.8 g in 1000ml), used 16 ml of solution I and 34 ml of solution II and diluted to a total of 200 ml will yield the approximate pH of 9.6.

3. **Washing buffer**
   PBS with 0.05% Tween20

4. **Substrate buffer**
   Citric acid - 7.3 g
   $Na_2HPO_4.2H_2O$ - 11.8 g
   Dissolved in 1000 ml distilled water and adjust the pH to 5.0 with acid at the same molarity.

5. **Bovine Serum Albumin**- 1% solution in PBS

6. **Chromogen:** O-Phenylenediamine dihydrochloride 4mg in 10ml substrate buffer containing 2µl H_2O_2 solution.

7. **Antibody diluting buffer**
   PBS pH 7.4 with 1% BSA and 0.05% Tween20.

8. **Antibody conjugate** (Sigma USA)
   Antihuman IgG-peroxidase
   Antihuman IgM-peroxidase
   Antihuman IgA-peroxidase

9. **Standard Immunoglobulin** (Sigma USA)
   human IgG
   human IgM
   human IgA

3.5.1.2. Procedure for Quantification of Immunoglobulin classes by ELISA

Microplate wells were added separately with 100 µl of class specific anti-human Immunoglobulin antibodies (anti-IgG/ anti-IgM/ anti-IgA antibodies), 10µg/ml, diluted in coating buffer and incubated for 18-20h at 4°C. After washing three times with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T), the wells were incubated in PBS containing 2% BSA at 4^0C for 18-20 h for blocking unwanted sites on the plate. Washed again three times with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T). 100µl serum CIC in antibody diluting buffer were added and left for incubation for 1h at 37^0C. After washing three times with PBS-T wells were incubated with peroxidase conjugated class specific anti-human immunoglobulin antibodies diluted in PBS-T containing 2% BSA at 37^0C for 1h.

The plates were again washed three times with PBS-T and then 100µl of the orthophenaline diamine (OPD) substrate solution was added and left for 15-30 min at 37^0C till the colour developed. Adding 100µl of 2M H_2SO_4 stopped the colour reaction and the absorbance values were read on an ELISA reader at 495 nm (A_{495}). Each sample was tested in duplicate and their average Optical density was used for data analysis. The ELISA experiments were carried out on the same ELISA plates under identical conditions and appropriate blanks were also included in the test. Serially diluted immunoglobulins in PBS-T BSA were used as a standard.

3.6 Purification and Characterisation of Oral Cancer Antigen

3.6.1. Affinity purification of CIC using Protein-A column

Protein-A, synthesized from *Staphylococcus aureus* is a group specific ligand that binds to the Fc region of immunoglobulin from many species (Lindmark *et. al.* 1983, Hermanson *et. al.* 1992). Protein-A agarose column (Genei, Bangalore, India) with 5mg/ml binding capacity was used for the present study.

3.6.1.1. Reagents and Chemicals for Protein A Column

1. **Tris Buffer**
   0.1M Tris – 0.10 M
   NaCl – 0.15 M
   pH – 7.5

2. **Glycine HCl Buffer (elution buffer)**
   Glycine – 0.1 M

3. **Neutralising solution**
   KCl – 1 M

3.6.1.2. Procedure for purification of CIC

The column was equilibrated with Tris buffer. The immune complexes with a protein concentration of 5mg/ml were applied on the column. The column was then washed with Tris buffer until the absorbance at 280nm was less than 0.02. The bound IgG-Ligand complex was eluted with dissociating buffer and the eluted immune complex was neutralized immediately with 1M KCl. The absorbance of the eluted fractions was measured at 280 nm and the protein peak was calculated. Then, the sample with the highest peak was dialyzed against phosphate buffered saline and was stored at 4^0C.

3.6.2. Sephadex G-200 gel filtration of CIC

Sephadex G-200 (Pharmacia) filtration was performed according to the method of Andrews (Andrews 1965).

3.6.2.1. Reagents and materials required for the G-200 gel filtration.

1. **Sephadex – G 200** (Pharmacia)
2. **Phosphate buffered saline** (Section 3.10.1.1.)

3.6.2.2. Procedure for Sephadex G-200 gel filtration

The gel material was swelled in distilled water at 90^0C in a water bath, for 5 hrs. After cooling, the gel was packed into a 90 x 1.6 cm glass column to get a bed height of 70cm. The column was equilibrated for a flow rate of 12 ml/hr with 9M urea containing phosphate buffered saline and calibrated using standard molecular weight markers. A graph relating the molecular weight and the elution volumes were plotted (Andrews 1965)

Three ml of Protien-A purified CIC (1 mg/ml) was carefully applied on to the column and the fractions were collected in 5 ml volumes. The fractions were read at 280nm in a UV spectrophotometer to monitor protein content. The protein containing fractions were pooled, dialysed, concentrated and stored at -20^0C.

3.6.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)

Separation of various component proteins from CIC and determination of its molecular weight was done by SDS-PAGE both in presence and absence of β-mercaptoethanol, following modified Lammelli method (Lammelli 1970). Gel electrophoresis in presence of the detergent sodium dodecyl sulphate (SDS-PAGE) is widely used to determine the molecular weight of polypeptides. It is unmatched in its ability to resolve proteins and polypeptide in a complex protein mixture. The detergent SDS breaks hydrogen bonds and causes a considerable degradation of tertiary structure, but does not affect disulphide bonds. Addition of β-mercaptoethanol reduces disulphide bonds, completing the denaturation process.

3.6.3.1 Reagents and chemicals for SDS-PAGE

Stock solutions for SDS PAGE

1. **Sample treatment buffer**
   Tris – 1.21 g/l
   EDTA – 0.29 g/l
   SDS – 10.00 g/l
   Bromophenol blue (0.01%) – 10µl

2. **Stacking gel buffer**
   Tris – 1.5 M
   SDS – 4g/l
   pH was adjusted to 6.8

3. **Separating gel buffer**
   Tris – 1.5 M
   SDS – 4g/l
   pH adjusted to 8.8

4. **Acrylamide solution**
   Acrylamide – 300g/l
   Bis acrylamide – 8g/l
   Filtered and stored in amber coloured bottle.

5. **Electrophoresis buffer**
   Tris – 6.0 g/l
   Glycine – 28.8 g/l
   SDS – 1.0 g/l
   pH adjusted to 8.3.

Working solution for SDS PAGE

6. **Preparation of separating gel 10% (35ml)**
   Distilled water – 14.5 ml
   Separating gel buffer – 8.75 ml
   TEMED – 35 µl
   Acrylamide stock – 11.4ml
   APS (10%) – 350 µl

7. **Preparation of separating gel 15% (35ml)**
   Distilled water – 8 .8 ml
   Separating gel buffer – 8.75 ml
   TEMED – 35 µl
   Acrylamide stock – 17.1 ml
   APS (10%) – 350 µl

8. **Preparation of stacking gel 7% (10ml)**
   Distilled water – 5.3 ml
   Stacking gel buffer – 2.5ml
   TEMED – 10 µl
   Acrylamide stock – 2.1ml
   APS (10%) – 100 µl

9. **Gel fixative solution**
   Acetic acid – 50ml
   Methanol – 100ml
   Mixed and made upto 500ml

10. **Coomassie blue stain solution**
    Solution A – 0.2% Coomassie blue in 95% ethanol
    Solution B – 20% acetic acid in water

3.6.3.2. Procedure for SDS PAGE

A discontinuous buffer system with two gel components was used; where the 'stacking gel' concentrates and the 'separating gel' separates the protein sample. A vertical slab gel electrophoretic apparatus was used (Broviga, USA). Marker proteins (proteins of known molecular weight) were also run parallel. The vertical slab gel units were assembled in the casting mode using 1.5 mm thick spacers. Separating gel solution was taken leaving out APS and TEMED. Vaccum was applied for few minutes in a vaccum desiccator. TEMED and APS were added to the solution after de-aeration and mixed with swirling. Then, the solution was pipetted into the sandwich to a level of about 4.0 cm from the top. The gel was overlaid immediately with 1-2 ml butanol. When the gel was polymerized, butanol was poured off and stacking gel was casted over the separating gel layer. De-aeration of the stacking gel solution was also done as described above and mixed with APS and TEMED. The portion above the separating gel was filled with the stacking gel solution and the comb was inserted into it and allowed to polymerize for one to two hours at room temperature.

Standard marker proteins were used to compare the molecular weight of CIC components. The protein markers used were mixed at a final concentration of 2mg/ml, with sample treatment buffer. CIC was mixed in 1:1 proportion with sample treatment buffer containing bromophenol blue. The combs were carefully removed from the gels without disturbing the well dividers. The gel was placed in a vertical position in the electrophoresis apparatus and the upper and lower buffer chambers were filled with electrophoresis buffer up to the marked

level. Electrodes were connected to power pack and pre-electrophoresed the gel at 200-300 V for about 30 minutes. The power cables were disconnected after pre-electrophoresis and then the sample and protein marker mixture were applied in the wells in a volume of about 10-40 µl. Power supply was set to a constant voltage of 800 watts. When the dye reached to the bottom of the gel, indicating the completion of electrophoresis run, the power supply was turned off. The gel was removed from the apparatus and kept immersed in fixative for about three hours. The gel was put into the stain solution for 4-8 hours with gentle shaking and then transferred to destaining solution.

3.6.3.3. SDS-PAGE under reducing condition

The electrophoresis was repeated incorporating 5% β-mercapto-ethanol in protein sample treatment buffer. Pre heating of the CIC sample with the buffer for 10 minutes, in boiling water bath breaks the disulphide linkages in the protein molecule. Molecular weight of the polypeptides separated by the SDS-PAGE was calculated by comparing their mobility with that of marker proteins.

3.7. Detection of oral cancer antibody from patients' sera by ELISA

The detection and quantification of the oral cancer antibody from patient's sera were determined by the sandwich ELISA technique using purified oral cancer antigen (Engvall and Perlamann 1971).

3.7.1. Reagents for ELISA

1. **Phosphate buffered saline - 0.1M (PBS)**
   NaCl - 8 g
   KCl - 0.2 g
   KH2PO4 - 0.2 g
   Na2HPO4.2H2O - 1.48 g
   Distilled water - 1 L
   PH - 7.4

2. **Antigen coating buffer (Carbonate/bicarbonate buffer)**
   (I) Na_2CO_3 – 0.2 M
   (II) Na_2HCO_3 – 0.2 M
   16 ml of solution (I) and 34 ml of solution (II) diluted to a total of 200 ml will yield the approximate pH of 9.6.

3. **Washing buffer (PBST)**
   PBS – 0.1M (pH 7.4)
   Tween20 – 0.05%

4. **Diluting buffer PBST (Incubation buffer)**
   PBS – 0.1M (pH 7.4)
   Tween20 – 0.05%
   Bovine Serum Albumin – 1%

5. **Substrate buffer (100 ml)**
   Citric acid – 7.3 g
   $Na_2HPO_4.2H_2O$ –11.8 g
   pH – 5.0

6. **Chromogenic substrate**
   O-Phenyldiamine (OPD)– 4mg
   Substrate buffer – 10ml
   H_2O_2 – 2µl

7. **Antibody conjugate**
   Antihuman antibody conjugated to HRPO (Sigma USA)

3.7.2. Procedure for detection of oral cancer Antibody from patient's sera by ELISA

Hundred micro-liter purified antigen (1mg/ml) in coating buffer was added to a microtitre plate and incubated overnight at 4^0C. The wells were washed three times with PBS-Tween20 and blocked with 250µl BSA (1%) in antigen

coating buffer and incubated overnight at 4^0C. Washed three times, with PBS-Tween20. Oral cancer patient's serum or normal healthy control serum was diluted ten times in antibody diluting buffer and added 100µl into the microtitre wells. Incubated microtitre plates for 1h at 37^0C and again washed 3 times. 100 µl of secondary antibody conjugate in diluting buffer was added to the wells. Incubated for 1h at 37^0C and washed 3 times with PBS-Tween20 and added 100µl substrate solution (OPD/H_2O_2) for 15 min until a blue colour was developed. The reaction was stopped by adding 100µl of 2N H_2SO_4 and read the absorbance at 450nm in an ELISA reader. Appropriate antigen and antibody controls were incorporated in the test.

3.8. Production of Polyclonal antibody against the Oral Cancer antigen in rabbit.

3.8.1. Preparation of pure antigen from PAGE

Electrophoretically purified antigens were cut out and eluted from the reducing SDS PAGE and used for the immunization of rabbits.

3.8.1.1. Reagents and chemicals for antigen elution

1) **PAGE reservoir buffer** (Section 3.6.3.1)
2) **PBS** (Section 3.4.1.1)

3.8.1.2. Procedure for antigen elution from the PAGE

For pure antigen preparation, gel elution of oral cancer antigen was performed. Protein-A purified CIC fraction was electrophoresed and a portion of

the gel was cut and stained in Coomassie blue. This was matched with the remaining unstained portion of the gel for oral cancer antigen and cut out from the unstained gel. The pieces were chopped and transferred to dialysis tube. Three ml of reservoir buffer for Native PAGE was added and dialysis tube was closed with closure clips without any air bubbles. The protein was allowed to elute for overnight in a horizontal electrophoretic tank filled with native reservoir buffer at 80 V. After elution, the current was reversed for 30 seconds to allow the detachment of protein bound to dialysis membrane. The elutant was dialysed in phosphate buffer saline (PBS, pH 7, 0.1 M) and lyophilised. The preparation was used as purified oral cancer antigen.

3.8.2. Immunization of rabbit with oral cancer antigen

Purified antigens from PAGE were used for generating polyclonal antibody in rabbits (Stills 1994).

3.8.2.1. Reagents and Chemicals for immunization

1) **Freund's complete adjuvant** (GENEI Bangalore)
2) **Freund's incomplete adjuvant** (GENEI Bangalore)

3.8.2.2. Procedure for immunization of rabbit

Antibodies were raised against electrophoretically pure oral cancer antigen in New Zealand white rabbits by Intramuscular injection of 100µg of protein emulsified with an equal volume of Freund's complete adjuvant. Three booster injections were given at an interval of 15 days with 200 µg of protein emulsified

with an equal volume of Freund's incomplete adjuvant. After the 3rd booster injection, blood was collected from the lateral ear vein of the rabbit and allowed to clot at room temperature. Serum was separated by centrifugation at 1700 x g for 15 minutes, aliquoted and stored.

3.8.3. Purification of IgG by DEAE-Cellulose Chromatography

Partial purification of antibody was done by Diethylaminoethyl-cellulose (DEAE-cellulose) ion-exchange chromatographic method (Johnstone and Thrope 1996). IgG was purified from serum by ion-exchange chromatography. IgG has a higher or more basic isoelectric point than most serum proteins. Therefore, at pH below the isoelectric point, the immunoglobulins do not bind to an anion exchanger. This principle was used for partial purification of antibody from majority of other serum proteins that binds to the column matrix

3.8.3.1. Reagents and Chemicals for the ion exchange chromatography

1) **DEAE-Cellulose** (Pharmacia, Uppsala, Sweden)
2) **Sodium Phosphate buffer**

3.8.3.2. Procedure for the ion exchange chromatography

. The anion-exchange reactive group, DEAE covalently linked to cellulose was used for this purpose. Initially, globulin fraction was separated from the immune serum. Double dilution of the serum was made with phosphate buffer and added ammonium sulphate to get 0-30% saturation. The precipitate was centrifuged at 10,000 rpm for 10min, dissolved in minimum amount of phosphate

buffer, pH 6.3 and dialysed exhaustively against the same buffer for 24 hrs with 4 changes. Dialysed sample was applied to DEAE-Cellulose column (30 x 1 cm) which was pre equilibrated with the same buffer. After whole sample enters the column, it was washed with 2 column volumes of the same buffer and immediately fractions were collected till the absorbance at 280 nm falls to the baseline. The fraction was pooled, concentrated and stored in refrigerator; and used as purified polyclonal anti-oral cancer antibody.

3.8.4. Gel double diffusion (Ouchterlony method)

The production of polyclonal antibody was checked for its immunoreactivity by immunodiffusion of the serum samples from immunised rabbits (Ouchterlony 1958).

3.8.4.1. Reagents and Chemicals for gel double diffusion

Agarose 1% in PBS

3.8.4.2. Procedure for double diffusion

The method relies on passive diffusion of antibody and antigen solutions within the gel. Antigen and antibody solutions are placed in opposing wells cut into a horizontal agarose gel of approximately 1.5 mm depth. Diffusion occurs radially from the wells and precipitation line developed within the gel between opposing wells. Gels were prepared by adding 1 g of agarose powder to 100 ml of hot PBS in a conical flask and stirred on a hot plate magnetic stirrer. Poured enough molten agarose onto a clean and grease free glass slide with the help of pipette without disturbing the eddies, so as to get a uniform layer of gel on

the surface. Allowed the gels to set, and made wells using a gel punch. The plugs were removed by suction or gentle lifting. Filled the wells with antigen and antibodies using micropipettes. Placed the slide in a petridish on a dampened filter paper. Incubated the slides at 4^0C overnight to get the precipitation reaction. The slides were washed many times in PBS and dried in an incubator at 37^0C by applying water-dampened filter paper onto the gel surface. If necessary, stained the slides in Commassie blue stain and observed bands.

3.8.5. Antibody coupling to Horseradish peroxidase

The enzyme peroxidase was conjugated to the DEAE-cellulose purified IgG antibodies by periodate method (Nakane and Kawaoi 1974).

3.8.5.1. Reagents and Chemicals for enzyme coupling

1) HRPO Horse Radish Peroxidase (GENEI Bangalore)

2) Sodium periodate 0.1 M in 10 mM sodium phosphate

3) Sodium acetate buffer 1 mM (pH 4)
Acetic acid –1 mM
Sodium acetate – 1 mM
82 ml acetic acid and 18 ml sodium acetate to make 100 ml

4) Carbonate buffer
(I) Na_2CO_3 – 0.2 M
(II) Na_2HCO_3 – 0.2 M
16 ml of solution (I) and 34 ml of solution (II) diluted to a total of 200 ml will yield the approximate pH of 9.6.

3.8.5.2. Procedure for enzyme coupling

The polyclonal antibody raised in rabbits were conjugated to horseradish peroxidase by periodate coupling method. 5mg of Horseradish

peroxidase (HRPO) was dissolved in 1.2ml distilled water. 30 µl of freshly prepared 0.1 M sodium periodate in 10mM sodium phosphate (pH 7.0) was added to the above solution and incubated at room temperature for 20 min. The mixture was dialysed against 1 mM sodium acetate buffer (pH 4.0) at 4^0C with several changes of buffer. 5 mg antibody in 20mM carbonate buffer (pH 9.6) was added to the mixture and incubated at room temperature for 2h. The Schiff's bases thus formed had been reduced by adding 100 µl of 0.4% sodium borohydrate in water and incubated at 4^0C for 2h. It was dialysed against PBS and used directly with appropriate dilutions.

3.9. Detection of Oral cancer antibody in patients' serum by Immuno Blotting Technique

Identification of proteins separated by gel electrophoresis is compounded by the small pore size of the gel, which limits penetration by macro-molecular probes. Overcoming this problem can be achieved by blotting the proteins on to a nitrocellulose membrane (Towbin *et. al.* 1979; Johnstone and Thorpe 1996)

3.9.1. Reagents and Chemicals for Immuno Blotting

1. **Blotting buffer**
   Tris - 14.4 gm
   Glycine - 3.0 gm
   Methanol - 200 ml
   Water to make 1 liter

2. **Blocking solution** (in PBS pH 7.2)
   Tween 20 - 0.3%
   BSA - 1%

3. **Antibody dilution buffer**
   Tween 20 - 0.05% in PBS

4. **Washing buffer**
   Tween 20 - 0.05% in PBS

5. **Localising Chromogen** (GENEI Bangalore)
   TMB (tetramethyl benzidine) with H_2O_2

3.9.2. Procedure for Immunoblotting

The transfer apparatus was assembled and the tank was filled with transfer buffer. Two pieces of filter paper was cut to the size of the cassette clamp, soaked in transfer buffer and placed one on the cathodal side of the cassette on top of a wetted sponge pad. The specific oral cancer antigen was electrophoretically separated on a Polyacrylamide slab gel in presence of SDS.

Then the gel was placed on the filter paper covering the cathodal side of the cassette. The gel was kept wet all times with transfer buffer, soaked the nitrocellulose sheet (cut to the same size as the gel) in transfer buffer, and placed it on the gel. Trapping of air bubbles throughout the process was avoided. Filter paper was placed over the nitrocellulose and expelled all air bubbles between the nitrocellulose and gel. Finally, a wetted sponge pad was placed on top of the filter paper and clamped securely in the cassette in a tight fit manner. Then cassette was placed in the tank and the lid was fitted. Electrophoresis was done overnight at 0.5A. The nitrocellulose was processed.

The nitrocellulose paper was incubated for three hours in blocking solution. Then, washed thoroughly with washing solution and flooded with the

primary antibody solution followed by incubation at 37^0C for three hours. Washed with PBS Tween 20 solution, flooded the paper with Enzyme conjugate system and incubated for 45minutes. The substrate solution was added and incubated till the blue colour developed on the paper. The excess colour formation was stopped by washing the membrane with distilled water. The protein bands were observed as bluish bands on the nitrocellulose paper.

Isolation and Characterisation of Tumour Associated Antigens and Their Significance in Host Immune Responses

Thesis submitted to the
University of Calicut under the **Faculty of Medicine**
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
IMMUNOLOGY

by

ELYAS K. K.
Department of Biochemistry
Medical College
Thrissur
2005

RESULTS AND DISCUSSION

Chapter IV

RESULTS AND DISCUSSION

4.1. Epidemiology of cancer

Cancer incidence and mortality show striking geographic differences, varying significantly from country to country and within countries. The difference in incidence might be caused by a number of factors. The rapidly changing environmental and social conditions may affect the prevalence and pattern. Differences of incidence and mortality occur between the sexes, sites, various ethnic groups and various occupations (Parkin 1993). Incidence pattern is important for the understanding of epidemiology and etiology of cancers. Epidemiological analysis of such data continues to provide valuable clues and insights into the myriad factors that appear to be involved in the causation of cancer. These epidemiological data has got significance in planning, implementation and evaluation of the cancer control programmes and research.

The relevant data given below is based on the cancers reported at the Department of Pathology, Amala Cancer Research Centre, Trichur, Kerala for the years 1995 to 1998. A total number of 4023 cases were reported during this period. The biopsy and surgical specimens were diagnosed and analysed by experienced pathologist after staining with hematoxylin and eosin. In selected cases special stains were employed (David 1999).

4.1.1. Frequency of cancers at different sites.

Table 1 shows the order of frequencies affecting different systems and organ, when both sexes were taken into consideration. The data shows that the frequency is very high in the case of oral cancer. A relative frequency of 17.6 percent occurs as oral cancer. Taking into consideration the extent of area that we take under the oral region, the relative frequency is very high.

Table 1: Relative frequency of different cancers

Cancers at different sites	Percentage incidence
Oral	**17.6**
Reproductive system	13.6
Breast	11.8
Respiratory tract	9.0
Stomach	8.0
Oesophagus	7.4
Lymphatic system	5.1
Colon	4.1
Osteogenic	4.0
Skin	3.1
Thyroid	1.2
Salivary gland	1.2
Others	13.9

The data shows a slight increase over the relative frequency reported by hospital cancer registry, Regional Cancer Center Thiruvananthapuram (Padmakumary 1999). The difference can also be due to some extraneous factors that may play with this type of data collection, like

choice of the hospital by the patients, availability of the trained or experienced physicians at the centre and their accessibility etc.

4.1.2. Age distribution of Patient in Different cancer

All patients with oropharyngeal carcinoma were aged between 14 and 88. The average age of patient with different solid tumors are given in Table-2. The mean age of oral cancer patients is 48 years (SD±13), this shows that the oral cancer occur in this region at an early age than the cancers of other sites. All other cancers occur at late fifties or early sixties of age. Among the other cancer, the breast cancer only occurs at an early stage than oral cancer i.e. 42 (±13) which is a predominant cancer in females. This indicates that there are some additional factors responsible for the early incidence of oral cancers.

Table 2: Age distribution in different cancers

Cancers at different sites	**Age ± SD (in years)**
Breast	42.0 ± 13.0
Oral	**48.0 ±13.0**
Lymphatic system	50.0 ± 16.0
Skin	52.0 ± 11.0
Salivary gland	53.0 ± 22.0
Thyroid	55.0 ± 12.0
Colon	57.5 ± 14.5
Stomach	59.0 ± 12.5
Osteogenic	60.0 ± 12.0
Oesophagus	60.0 ± 13.0
Respiratory tract	61.0 ± 9.5
Reproductive system	63.0 ± 13.0

4.1.3. Male and Female ratio Of Oral Cancer patients

In male and female incidence percentage, male shows higher prevalence of oral cancer than female. In this study the ratio of male : female incidence was 1.6 : 1.0. This difference can be attributable to physiological, hormonal or habitual changes in each group.

4.1.4. Male and Female Patient Age distribution of Oral Cancer

The average age of male patients is 45 $\pm$12.1 while that of females is 54 $\pm$ 12.4. These data indicated that oral cancer incidence was more in males and that too it occured at an early age. Apart from genetic factors, this early incidence in male can be attributable to the tobacco and other behavioural aspects of males.

4.1.5. Locational distribution of Oral cancer

The oral cavity extends from the skin-vermilion junctions of the anterior lips to the junction of the hard and soft palates above and to the line of circumvallate papillae below. It is divided into the different areas, lip, anterior two-third of tongue, buccal mucosa, floor of mouth, lower gingiva, retromolar trigone, upper gingiva and hard palate. The distribution of cancer to different site was no way uniform (Table 3). The tongue was found to be the largest site in the oral region in the study locality (37%). Buccal mucosa and cheek were other major sites of cancer together constitute about thirty nine percent of oral cancer, while for tongue the incidence was as high as 37% of oral cancer. Many other studies support the finding that oral cancers appear

most often on the lower lip and floor of the mouth, apart on tongue. (Kroll and Hoffman 1976; Mashberg and Meyers 1976)

Table 3: Location of cancer at oral region

Oral region	Percentage
Tongue	37.0
Cheek	27.0
Buccal mucosa	12.0
Floor of mouth	9.0
Palate	7.0
Lip	6.0
Gingivobuccal sulcus	2.0

4.1.6. Histological types of oral cancer

Verrucous carcinoma is a form of epidermoid carcinoma of the oral cavity (Kayembe and Kalengayi 1999). The typical lesion is generally slow growing, chiefly exophytic and only superficially invasive with low metastatic potential. Here the epithelium is well differentiated and shows little mitotic activity, pleomorphism or hyperchromatism. Characteristically, cleftlike spaces lined by a thick layer of parakeratin extend from the surface deeply into the lesion. Significant chronic inflammatory cell infiltration in the underline may be present. Adenocytic or adenoid cystic carcinoma is a form of adenoid carcinoma of secretory glands of oral cavity. They are deeply staining uniform cells resembling basal cells arranged in a duct like pattern.

Epidermoid carcinoma better known as squamous cell carcinoma is the most common malignant neoplasm of the oral cavity

(Kayembe and Kalengayi 1999). These cells are generally large and show a distinct cell membrane, although intracellular bridges or tonofibrils often cannot be demonstrated. The nuclei of the neoplastic cells are large and may demonstrate a good deal of variability in the intensity of the staining reaction. In the present study, squamous cell carcinoma contributed 84%, verrucous carcinoma 6% and the rest included both adenocytic and papillomatosis lesions.

4.1.7. Histological differentiation of squamous cell carcinoma

Histologically oral squamous cell carcinomas are generally classified into three or four grades of differentiation (Fig.1), the various systems of classifications being mostly similar (Waldron 1970). The main criterion is usually the extent of keratinization. The classification being well-differentiated squamous cell carcinoma having more than 75% keratinisation, moderately differentiated squamous cell carcinoma 25-75% and poorly differentiated squamous cell carcinoma less than 25% keratinization. Other criteria believed to be relevant include number of mitosis, presence of vascular invasion, size of nucleus, degree of inflammatory infiltrate and type of invading border. In a well differentiated lesion mitotic figures may be found and appear as a sheet and nests of cells with obvious origin from squamous epithelium. One of the most prominent features of the well-differentiated epidermoid carcinoma is the presence of individual keratinization and the formation of numerous epithelial or keratin, pearls of varying size.

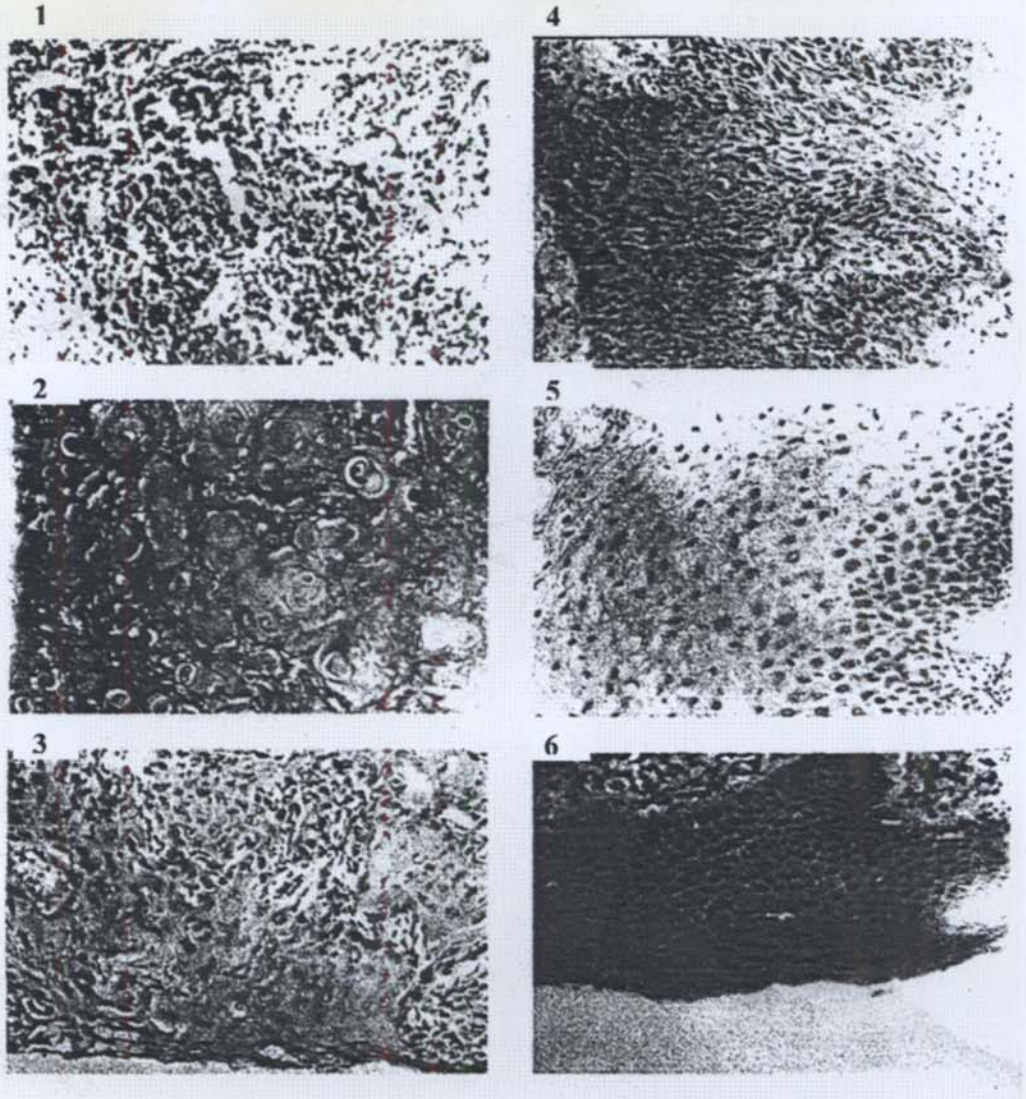


Fig 1. Photograph of histological types of Oral cancer

1. Poorly differentiated carcinoma
2. Moderately differentiated Carcinoma
3. Well differentiated Carcinoma
4. Moderate Dysplasia
5. Mild Dysplasia
6. Normal tissue

In the present data (Table-4) 47% were moderately differentiated and 31% well differentiated Oral Cancers, rest were non-differentiated or poorly differentiated oral cancers. The experience and the subjectivity of the pathologist is a major factor in grouping those cancers.

Table 4: Histological differentiation in Squamous Cell Carcinoma

Level of Differentiation	Percentage
Moderately differentiated	47.0
Well differentiated	31.0
Un-differentiated	14.0
Poorly differentiated	8.0

The result shows that in the cohort studied, oral cancer was the most widely occurring cancer. This cancer incidence was more in males at an early stage of their life. Among different histological types squamous cell carcinoma of oral cavity was more prevalent. Further studies were done using these cancers as a tumour model.

It should be remembered that apparent increase or decrease in cancer incidence over time may reflect changes in diagnostic methods or case reporting and under-reporting for cancers that may not be diagnosed in the laboratory, rather than true changes in cancer incidence. Also such facts must be considered in comparing the incidence pattern in different studies.

4.2. Viruses in Oral Cancer

Since the carcinogenic potential of viruses was first recognized, it has been hypothesised that numerous viruses are linked to the development of various cancers in human (Rawls *et. al.* 1977; Evans and Muller 1990; Reeves *et. al.* 1989a). Viruses are believed to be involved in the development of about fifteen percentage of human cancers (Zur Hausen 1991).

4.2.1. Herpes Simplex Viruses (HSV) and oral cancer

Herpes simplex virus, which includes HSV-1, a virus known to infect the oral mucosa and to result in canker sores and HSV-2, a sexually transmitted virus that has a predilection for infecting male and female genitalia, although it has also been shown to infect the oral mucosa (Corey and Spear 1986a). An important feature of HSV is its ability to become latent and to persist in the host for many years after infection (Corey and Spear 1986b). HSV-2 infection can be either symptomatic or asymptomatic. The frequency of infections that result in symptoms is unknown. However, serologic studies that have measured the prevalence of HSV-2 antibodies in different populations indicate that a sizeable proportion of infections with this virus are likely to be asymptomatic. Given the fact that these asymptomatic infections do not come to the attention of the medical community, it has been difficult to determine the overall prevalence of this virus. In this study, we have examined HSV antibody levels among the oral cancer patients and age matched controls to ascertain the prevalence of HSV infection in this segment of the population.

Results of the study are presented in Table 5, 6 and 7. One hundred and twenty six (84%) of the 150 patients and 37 (43.5 %) of the 85 normal subjects were positive for anti-HSV-1 IgG antibodies. Concentration of the antibody in patients ranged from 26-232.23 human units (mean,113.9 hu), and in normal subjects, from 30 -117.95 hu (mean, 75.9 hu).

Anti HSV-2 IgG antibody was detected in 135 (90%) patients and 18 (21.3%) normal subjects. Concentration of the antibody was 18.92-329 hu (mean 101.9 hu) in patients and 68.18-108.04 (mean, 82.8 hu) in control subjects. One hundred and five of the 150 patients (70%) harboured both anti HSV-1 and anti-HSV-2 IgG antibodies. Only 6 (7%) of the 85 normal subjects showed both antibodies.

Table 5. Seroprevalence of Anti –HSV antibodies in normal subjects and oral cancer patients

Subjects	Total No	Anti-HSV-1 IgG Antibody		Anti-HSV-2 IgG Antibody		Anti-HSV IgM Antibody	
		-ve	+ve	-ve	+ve	-ve	+ve
Normal control	85.0	48.0	37.0 43.5 %	67.0	18.0 21.2 %	78.0	7.0 8.0 %
Oral cancer patients	150.0	24.0	126.0 84.0%	15.0	135.0 90.0 %	30.0	120.0 80%

Table 6. Concentration of Anti –HSV antibodies (in human units) in oral cancer patients and control subjects

Subjects	Anti-HSV-I IgG Ab		Anti-HSV-2 IgG Ab		Anti-HSV IgM Ab	
	Range	Mean	Range	Mean	Range	Mean
Control	30.4 -117.95	75.9	68.18-108.04	82.8	19.9 - 61.69	22.7
Patients	26-232.93	113.9	18.9-329	101.9	15.42-442.79	51.7

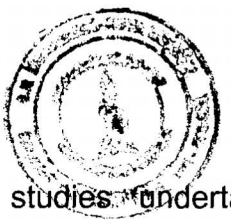
One hundred and twenty (80%) patients and 7 (8.2 %) normal subjects were positive for anti HSV IgM antibodies. Concentration of the IgM antibody in patients was 15.4 - 442.79 hu (mean, 51.7 hu) and in normal subjects, 19.9 to 61.69 hu (mean 22.7 hu). All the Anti-HSV IgM positive subjects were also positive for IgG antibodies to HSV-1 and /or HSV-2.

Table 7. Number of Positive cases of Anti-HSV antibodies (Ab) in normal subjects and in oral cancer patients

Subject	Total No.	HSV-1 & HSV-2 IgG Ab	HSV IgM & HSV-1 IgG Ab	HSV IgM & HSV-2 IgG Ab	HSV IgM& HSV-1 IgG & HSV-2 IgG Ab
Normal	85	6	4	2	0
Patient	150	105	56	64	42

Results of the present study strongly suggest an association between oral cancer and HSV. Here, we report on the elevated seroprevalence and concentration of anti-HSV-2 IgG antibodies in squamous epithelial carcinoma of oral cavity. An active HSV infection, as revealed by the presence of both anti-HSV IgG and IgM, has been observed in 89% of the

HSV positive patients as against 12.5% of normal subjects. The question whether this active state is due to primary infection or reactivation remains to be answered.



A few studies undertaken so far in India and abroad (Silverman *et. al.* 1976; Smith *et. al.* 1976; Shillitoe *et. al.* 1982; Kumari *et. al.* 1982; Shillitoe *et. al.* 1983; Vasudevan *et. al.* 1991b) have revealed elevated seroprevalence and titre of anti-HSV-1 IgG antibodies in oral cancer patients. Limited evidence suggests that HSV-2 might be involved in the etiology of other genital cancers (Kaufman *et. al.* 1981). Experimental *in-vitro* studies have revealed the transforming potential of HSV (Duff and Rapp 1971; Rapp 1981). HSV has been found more effective than some chemical carcinogens in amplifying SV40 DNA sequence in SV40-transformed hamster embryo cells associating via HSV-encoded DNA polymerase (Schlehofer *et. al.* 1983; Matz *et. al.* 1984; Matz *et. al.* 1985). The presence of RNA complementary to HSV-DNA in biopsy specimen from oral carcinoma but not from autologous normal mucosa has also been reported (Eglin *et. al.* 1983). The ability of HSV to serve as a cocarcinogen with tobacco (Hirsch *et. al.* 1984; Park *et. al.* 1986) or other chemicals (Larson *et. al.* 1989; Park *et. al.* 1991) has also been suggested.

Several studies indicated that women exposed to HSV-2 were at two to four-fold elevated risks of developing cervical cancer (Kaufman and Adam 1986). Laboratory evidence supported this hypothesis; *in-vitro* and *in-vivo* studies demonstrated the transforming ability of HSV-2 as

well as its potential carcinogenic effects (Wentz *et. al.* 1983). However, more recent evidence suggests that, while HSV-2 might be involved in the development of cervical cancer, it is not likely to play as important a role as was once believed. Evidence now points to human papilloma viruses (HPV) as the viruses that are likely to play a central role in the development of this disease (Reeves *et. al.* 1989b; Koutsky *et. al.* 1988). In animal studies it was found that immunization against HSV prevents the cocarcinogenic activity of HSV with dimethyl benzanthracene (Park *et. al.* 1990). Studies have revealed that HSV acts synergistically with tobacco-specific nitrosamine in cell transformations (Park *et. al.* 1991). DNA hybridisation studies have also furnished evidence for an association between oral cancer and HSV-1 by noting HSV-1 DNA segments in the genome of oral cancer tissues (Vasudevan *et. al.* 1991a). Any large scale investigation has not been undertaken on a possible association between HSV-2 and oral cancer, though a positive association of this virus has been reported in the squamous epithelial carcinoma of human uterine cervix.

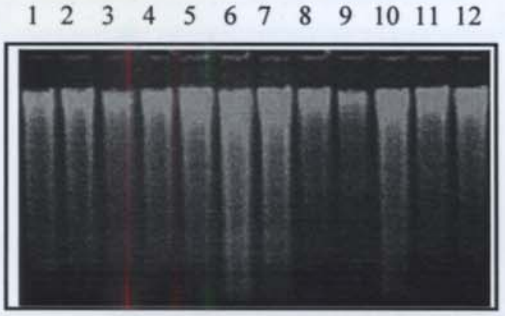
4.2.2. Human Papilloma Viruses (HPV) and oral cancer

In order to detect human papillomavirus infection in squamous cell carcinoma, DNA hybridisation studies were conducted. It has been already well established that HPV-16 and HPV-18 are two of the high-risk viruses that cause cervical cancer. It infects squamous epithelial cells of uterine cervix. Due to sexual behaviour of the individuals and the similarity in histology, the viruses may also possibly infect oral squamous cell epithelia.

For the present study, tissue DNA were isolated and hybridised with HPV-16 and HPV-18 DNA separately. These viruses are DNA viruses. These DNA viruses genome were maintained as recombinant plasmid in pBR322 in our laboratory. The whole genome of the viruses were cut out from recombinant plasmids and used to probe the presence of HPV infection in patient tissue DNA samples. Patient's tissue DNA samples were isolated using standard phenol-chloroform extraction method as described in materials and methods. Characteristic appearance of isolated tissue DNAs are shown in fig. 2. The *Pst*-1 digested DNA shows more smeared pattern compared to the undigested genomic DNA in 1% agarose gel. *Pst*-1 enzyme recognized CTGCAG site and restrict the DNA at the same region. On complete digestion of HPV-16, the enzyme gives five discrete characteristic DNA fragments of varying lengths (2.8, 1.9, 1.6, 1.0 and 0.5 kb). Whereas, the HPV-18 DNA gives a single characteristic 8.0 kb DNA fragment. The *Pst*-1 digested human genomic DNA was separated on agarose gel, blotted and hybridised with whole HSV DNA. The whole virus labelled with radioactive ^{32}P dCTP binds to the characteristic fragments of genomic DNA digest (Fig. 3)

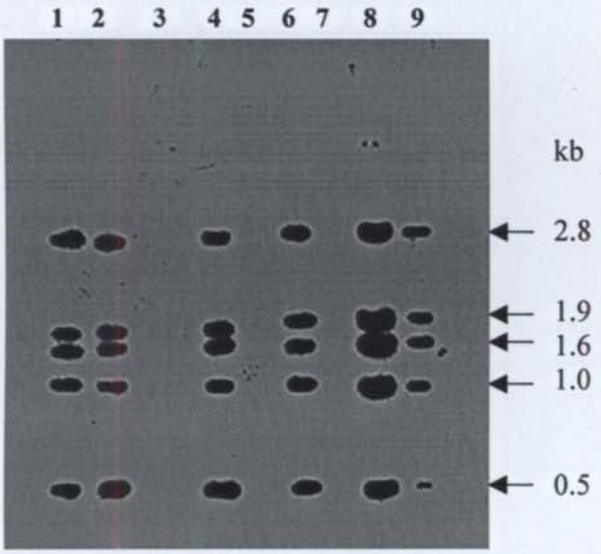
The Southern blot analysis employed for 34 patient DNA samples revealed HPV-16 DNA in 18 samples, which comes to 53% of total patient samples. None of the patient's sample showed HPV-18 DNA when hybridised with HPV-18 DNA probe. Fifteen age-matched samples from normal oral mucosa were found to contain no viral DNA on hybridisation with HPV DNAs. Majority of the positive samples were found to contain a rather low level of the viral genome. Five samples contained very high copy number of HPV-16 DNA.

Fig. 2 : Agarose gel electrophoresis of *Pst* 1 digested oral cancer patients tissue DNA



1-10- Patient DNA digested with Pst 1 restriction enzyme
11-12- Undigested Human DNA

Fig. 3 : Autoradiogram of Southern blot hybridisation analysis of HPV-16 DNA sequences in squamous cell carcinoma of oral cavity.



Lane 1-8 – Patient's Genomic DNA digested with *Pst* 1, electrophoresed in 1% agarose gel and hybridised with ^{53}P labelled HPV-16 DNA probe.
Lane 9 – Authentic *Pst* 1 cleaved pattern of HPV-16 DNA

In vitro laboratory studies have been able to isolate those fragments of the HPV-DNA that are likely to be responsible for its ability to transform cells in humans and HPV-DNA fragments have been detected in tumor samples obtained from cervical cancer patients. Given the high prevalence of HPV infection relative to the incidence of cervical cancer, it is believed that like HSV, HPV alone is not capable of inducing cervical cancer and that exposure to other factors, called cofactors, must be important determinants of which HPV infected women will develop the disease. Numerous exposures are currently under investigation as possible cofactors. These include host factors such as immunological status, including infection with HIV, as well as environmental exposures such as smoking, oral contraceptive use, diet, parity and other sexually transmitted diseases. HSV-2 and HPV are also believed to be associated together with the development of other cancers. However, a little is known about the link between these viruses and cancers. HPV has been linked to numerous cancers, including other genital tumors, as well as skin, oral, laryngeal and anal cancers, but causation is less firmly established. It has been suggested that HSV may act synergistically with HPV in carcinogenesis (Zur Hausen 1982).

Initial indication of an association between oral carcinoma and HPV originated from the demonstration of papilloma virus structural antigen in histological sections of the cancer tissues (Syrjanen *et. al.* 1983). Using DNA hybridisation techniques and polymerase chain reaction, few subsequent studies demonstrated the presence of HPV-DNA. It is reported that HPV-16 and HPV-2 DNA is present in tongue carcinoma (De Villiers *et. al.* 1985).

HPV comprises a family of viruses that includes more than 100 viral types. These viruses are known to be the causative agents of warts at various sites. About 20 types have been shown to infect the genital area (Koutsky *et. al.* 1988). In addition, various HPV types are known to infect the skin, oral cavity, larynx, and anus. As is the case of HSV-2, a large proportion of HPV infections are asymptomatic and may therefore go undetected. This has made it difficult to determine the prevalence of this virus in the general population; most estimates range from 10 to 40 percent, depending on the population studied, the method used to detect the virus and the viral types detected (Koutsky *et. al.* 1988).

The association between HPV and cervical cancer was first suggested by Harold Zur Hausen in 1974 (Zur Hausen 1989). Since that time, numerous epidemiologic and laboratory studies have supported an association between HPV and cervical cancer (Reeves *et. al.* 1989b; Koutsky *et. al.* 1988). It has been demonstrated that women who are infected with HPV are at higher risk of developing cervical intra-epithelial neoplastic as well as cervical cancer. (Koutsky *et. al.* 1988; Reeves *et. al.* 1989a). Two *in situ* DNA hybridisation studies abroad have noted HPV-16 and HPV-18 DNA in oral squamous cell carcinomas (Milde and Loning 1986; Syrjanen *et. al.* 1988). On the basis of epidemiological evidence, a possible interaction of HSV-2 with human papillomavirus 16/18 in the development of invasive cervical carcinoma has been noted (Hildesheim *et. al.* 1991). It has been shown that keratinocytes immortalized by HPV-16 DNA are tumorigenic in nude mice after transformation with HSV-DNA (Iwasaka *et. al.* 1988; Di Paolo

et. al. 1990). Many more reports from abroad are there on the presence of HPV genes in the genome of oral carcinoma tissues (Ostrow *et. al.* 1987; Syrjanen *et. al.* 1988). Possibly, at least in some cases HSV may act synergistically with HPV in oral cancer.

Studies have detected HPV-16 DNA in 76% of oral carcinomas in a group of Taiwanese patients (Chang *et. al.* 1989). HPV-18 and HPV-4 were detected in oral cell carcinoma and also a variant of HPV-16 in 46% of the oral cancer biopsies and in 5/12 normal oral mucosa samples were demonstrated (Maitland *et. al.* 1987; Yeudall and Campo 1991). Later HPV-16 DNA sequences were reported in oral cancer biopsies and in the cell lines derived from them (Maitland *et. al.* 1989).

In the present study, we could demonstrate papilloma virus -16 in a considerable number of oral squamous carcinoma tissue samples but not in normal oral mucosa. The result of our study was in general agreement with the previous studies that have suggested a more than accidental association of HPVs with oral squamous carcinoma. Recent studies (Howley 1991; Yeudall 1992) have shown that the cell transformation potential of specific HPV types in presence of cofactors. It is reported that E6 and E7 proteins of high risk HPVs can interact with human retinoblastoma gene product (pRb) and the tumour suppressor gene product p53, respectively in cell transformation. The high frequency occurrence of high risk HPVs in oral cancer and their known cell transformation potential suggest a possible aetiological role for HPV in oral neoplasia. Epidemiological studies have noted

betal quid chewing and other forms of abuse of tobacco and consumption of alcohol to be the major etiological factors in oral carcinoma. It may be the HPVs, in synergism with chemical carcinogens and /or other cofactors, play some role in carcinogenesis. Further studies are required to assess the roles of HSV and HPV in oral carcinogenesis (Gimenez-Conti and Slaga 1993).

The identification of viral association and the interaction with the host will lead to the better understanding of viral strategies and later the genes responsible in causing cancer. This will help to treat virus-induced pathology in a better way and design safer vaccine to treat these cancers.

4.3. Association of CIC with oral cancer

4.3.1. Quantification of CIC by complement conception test

The subject of antibody association with tumours is a controversial one; some author found no tumour cell associated immunoglobulin, while others have reported a relatively high incidence of anti tumour immunoglobulin (Kopf *et. al.* 1966; Nairn *et. al.* 1971; Gutterman *et. al.* 1973; Izsak *et. al.* 1974). It has been established that certain human neoplasm produce tumour associated antigens that may be expressed on the cell surface. These antigens shed to the circulation, combine with the antibodies and circulate in the serum as circulating immune complexes (Sjogren *et. al.* 1972). It has also been suggested that CIC has a role in immunoregulation (Hellstrom *et. al.* 1977) Antigen–antibody complexes are known to be involved in the pathogenesis of a variety of diseases. Circulating immune complexes are common features in neoplasia and a strong

correlation has been reported between their levels and the progress of cancer (Carpentier *et. al.* 1977; Gupta *et. al.* 1979).

The antigenic part of the CIC, depending on the origin and aetiology can be considered either as tumour associated or tumour specific antigen. It has been reported that tumour development may be due to the blocking effect of the CIC, which prevents the recognition of the transformed cells in the patients. The shed antigen from cancer cells combines with antibody to form CIC. Antigen non-specific methods used for the detection of CIC in serum are numerous. Each method depends on one or other limited characteristics of the complexes and the correlation between the results obtained by the different assays is often very poor (Lambert *et. al.* 1978; Fust *et. al.* 1980) PEG-turbidity assay has been used to demonstrate highly significant levels of CIC in different types of cancers in comparison to the normal (Rayner *et. al.* 1981; Chhajilani *et. al.* 1983). Precipitable immune complexes have been shown in 70% Hodgkin's disease cases as compared to the normal (Euler *et. al.* 1983).

In the present study, circulating immune complexes level in sera of oral cancer patients were compared with normal by antigen non-specific method viz., PEG-complement consumption assay. The concentration of CIC in sera of patients and healthy control of different age group is given in table 8. It was demonstrated that there was a significant increase in total CIC concentration in patients of age above 20 years in comparison to that of healthy controls ($P<0.001$). This increase in concentration of CIC was

approximately one and half times more in patients in comparison to controls. The incidences of positive results in the two groups were compared using the 95th percentile (mean + 2 SD) of the CIC levels in normal subjects as cut-off limit (Raghunath *et. al.* 1987); as many as 65 % were positive in this assay.

Table 8. Concentrations of Immune complex estimated by complement conception assay for oral cancer patients and normal healthy controls of different age group.

Age group Years	% Complement consumption	
	Oral Cancer Conc. ± SD (N)	Healthy controls Conc. ± SD (N)
0 –20	15.62 ± 3.8 (8)	15.25 ± 2.1 (50)
20 – 40	30.90 ± 4.0 (28)	18.06 ± 3.6 (50)
40 – 60	63.39 ± 4.8 (50)	25.61 ± 2.8 (50)
>60	60.25 ± 5.3 (50)	20.38 ± 2.3 (50)

N =Number of individuals studied.

Various investigators have suggested the use of CIC as a possible biological marker in cancer diagnosis (Baseler *et. al.* 1984; Vijayakumar *et. al.* 1986; Raghunath *et. al.* 1987; Remani *et. al.* 1988). Increased concentration of CIC have been detected in various malignancies (Carpentier *et. al.* 1977; Rossen *et. al.* 1977; Brown *et. al.* 1978; Raghunath *et. al.* 1987). A positive correlation was observed between CIC with CEA in patients with gastrointestinal cancer (Guidi *et. al.* 1991). They reported that CIC assay could be used in addition to other established markers for monitoring the clinical state of patients. An increased level of CIC was

associated with an increase in concentration of AFP in patients with hepatitis-B surface antigen positive hepatocellular carcinoma patients (Tsi Jung *et. al.* 1991).

Numerous techniques have been developed for the detection and quantification of CIC (Theofilopoulos and Dixon 1979; Ruddy and Moxley 1994). Among all, the crystallization of protein-protein complexes in which polyethylene glycol is used as precipitants; the most popular one employs PEG with MW between 3000 and 6000. The PEG concentration in protein-protein complex crystallization ranges from 5-30%, similar to that of normal soluble protein. In general, optimum concentration of PEG required to crystallise individual protein depends mostly on solubility and concentration of a given protein and can vary drastically from protein to protein. In many cases, the solubility of protein complex is less than that of its components, thus requiring a lower concentration of PEG for crystallization (Radaev and Sun 2002). Advantage of the complement conception test is that, this test mimics the events that actually take place *in vivo*. The activation of the complement occurs with the help of antigen-antibody complex, which can produce the tissue damage. So the *in vitro* complement conception test is an indication about how much damage may be produced in the body. Not all immunoglobulin can fix antibody in equal efficiency. The complex that containing antibody classes which cannot fix complement are not estimated in complement conception test.

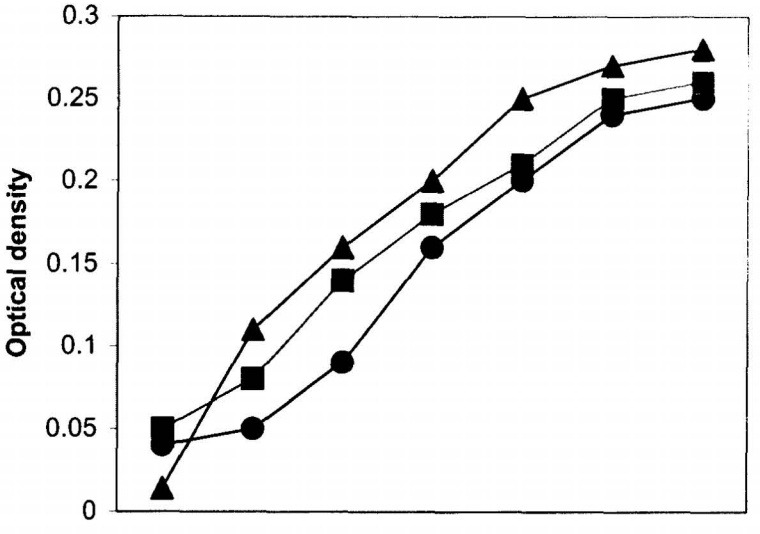
4.3.2. Various classes of Immunoglobulin present in the Circulating Immune complex

Because of the biological and clinical importance of non-random expression of immunoglobulin, the analysis of immunoglobulin classes in CIC of human oral cancer patients has became the interest of the present investigation. It is well established that the serum levels of immunoglobulin concentration changes during disease. The change can be either increase or decrease during the disease, depending on the type of disease, age and sex of the patients and the involvement of their immune system. Serum concentrations of different classes of Immunoglobulin show a change during disease. Monitoring the immunoglobulin class status is very useful in diagnosis, prognosis and disease management.

In the present study, the levels of immunoglobulin classes present in circulating immune complexes were measured by sandwich ELISA technique. The standard graph and patient data with normal healthy controls for the ELISA are given in Fig. 4 and Table 9 respectively. A marked increase in the concentration of immunoglobulin was seen in the case of oral cancer patients. This increase was more prominent and statistically significant in 40 - 60 age group ($P<0.001$) for all classes of immunoglobulin studied, i.e. IgG, IgM and IgA, when compared to age matched normal healthy controls. In the age group above 60 years, there was a significant increase for both IgG and IgA classes, when compared to control subjects. In addition, the IgA concentration in age group 20 to 40 also showed a significant difference. These indicated that there was more involvement of IgA in the immune

reactions of oral cancer patients in almost all age groups. Between the age group a significant ($P<0.001$) increase of IgA was observed when the concentrations of 40-60 and above 60 age groups were compared with 0-20 age groups.

Fig: 4 Standard curves of IgG/IgM/IgA ELISA



IgG ies1 IgM es2 IgA s3

It has been reported that increased concentrations of IgG and IgA can interfere with the action of tumour development by blocking the action of IgM in recognising the targets (Fugmann and Sigel 1967). IgM molecules are efficient fixers of complement that can help in clearing tumours. Same group has also reported that certain tumorigenic viruses like adenoviruses preferentially induce the production of certain classes of antibodies.

Table 9. IgG, IgM and IgA concentration in CIC of oral cancer patients and normal controls in various age groups

Age Group (years)	CIC IgG		CIC IgM		CIC IgA	
	Normal mg/dl±SD	Patient mg/dl±SD	Normal mg/dl±SD	Patient mg/dl±SD	Normal mg/dl±SD	Patient mg/dl±SD
0-20 N_n= 50 N_p = 8	**632.38** ± 141.66	**625.42** ± 134.53	**127.47** ±23.09	**120.49** ±50.79	**150.55** ±21.63	**150.83** ±45.00
20-40 N_n= 50 N_p = 28	**629.03** ± 120.22	**644.53** ±144.5	**107.14** ±29.48	**120.00** ±30.35	**210.93** ±50.93	**256.62** * ±50.55
40-60 N_n= 50 N_p = 50	**642.76** ±107.5	**755.58*** ±150.38	**122.29** ±39.87	**156.23*** ±39.88	**236.53** ±47.52	**447.68*** ±38.53
>60 N_n= 50 N_p = 50	**640.24** ±140.03	**730.40*** ±134.53	**128.05** ±29.50	**135.03** ±28.00	**227.83** ±45.52	**453.63*** ±65.00

* Indicates P value (<0.001) statistically significant for patients in comparison to normal.
N_P= Number of patients studied, N_n = Number of normal healthy individuals.

In view of the biological difference among the IgG subclasses, it is not surprising that these subclasses are selectively expressed. First, the antibody response to different types of antigens seems to favour certain classes or subclasses. For example, many antigens preferentially elicit IgG responses restricted to IgG3 in mice (Perlmutter *et. al.* 1978; Slack *et. al.* 1980) and IgG2 in humans (Riesen *et. al.* 1976; Yount *et. al.* 1968; Barrett and Ayoub 1986). In contrast, IgG1 dominates the IgG response to many protein antigens in both species (Shakib and Stanworth 1980; Stevans *et. al.* 1983; Skavril and Schilt 1984). Such preferential production of subclass can lead to the total increased production of various classes of antibodies. Secondly, acquisition of adult levels of IgG2 in humans is delayed during ontogeny (Schur *et. al.* 1979). It is speculated that the delayed maturation of the ability to produce an immune response to antigens is linked to this late maturation of various classes of antibodies. Interestingly, the response to

antigens is also delayed during ontogeny in mice (Mosier *et. al.* 1977). Thirdly, expression of certain IgG subclasses is selectively suppressed in certain immunodeficiencies. Patients with Ataxia-telangiectasia have selective deficiencies of IgA and of the IgG2 and IgG4 subclasses (Oxelius *et. al.* 1982) CBA/N mice have an X chromosome linked immunodeficiency that results in selective deficiency of the IgG3 subclass (Scher 1982). Lastly, a subclass specific lymphokine (BSF-1) has been identified in the mouse which preferentially facilitates IgG1 and decrease IgG3 expression following mitogen stimulation (Vitteta *et. al.* 1985). These observations strongly suggest a mechanism that selectively regulates expression of the immunoglobulin classes.

To explain selective immunoglobulin class and subclass expression, two contrasting models of B-cell development have been proposed. One model proposes that all B-cells develop in a single lineage and can express all IgG subclasses. In this model, selective expression of IgG subclasses is solely the result of exogenous factor, such as T-cells, inducing successive expression of the IgG subclasses according to their order in the genome (Teale and Klinman 1984; Gearhart *et. al.* 1980; Mongini *et. al.* 1982). The alternative model proposes that multiple B-cell lineages exist which are restricted in IgG subclass expression and which develop at different times in ontogeny (Abney *et. al.* 1978). Because the two models are of fundamental importance in understanding B-cell development and regulation, they have been intensely studied in experimental animals. So far, neither of the above models could fully explain all of the observations.

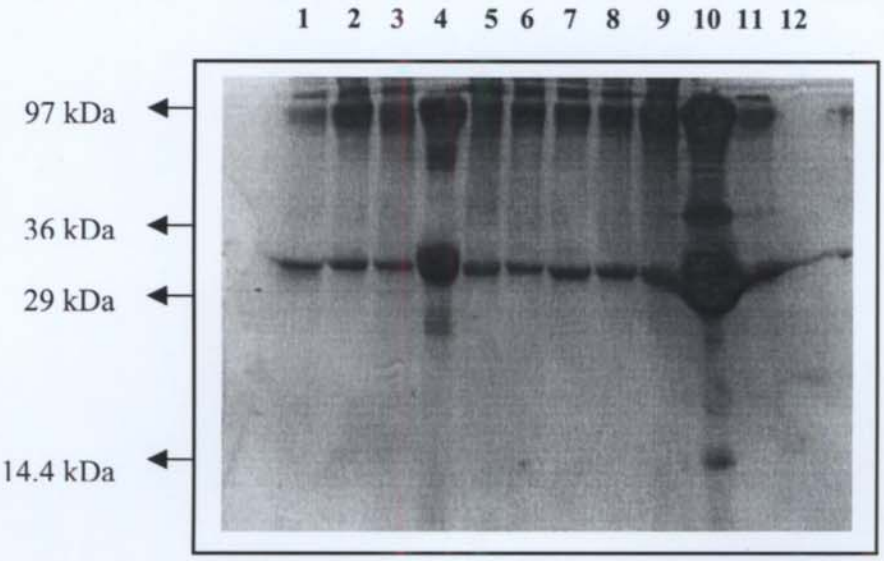
4.3.3. Purification and characterisation of Oral cancer antigens from CIC

If tumour bears surface antigen that differ from those of normal cells, they should evoke detectable immune response that could induce regression or retard metastasis. Detection and quantification of anti-tumour antigens and antibodies may be of value in diagnosis, clinical staging and monitoring the development of metastasis. Antibodies may be sought free in plasma, CIC or attached to the transformed cells (Pillai *et. al.* 1982). Isolation of tumour specific antigens and immunoglobulin and a study of their function might prove a good correlation of the prognosis and clinical staging. In view of the nature of the problem and its diagnostic value, the present investigation was carried out to look for the possible presence of tumour associated antigens in CIC of oral cancer patients.

4.3.3.1. SDS PAGE analysis of CIC

The PEG precipitation method serves as a simple and rapid method for the isolation of the immune complexes in large number of samples. Initial characterisation of CIC was done on SDS-PAGE under reducing and non-reducing conditions. Under non-reducing condition, the whole immunoglobulin dissociated from the CIC and a discreet band of antigen got separated at approximately 30 kDa regions on the SDS-PAGE (Fig. 5)

Fig. 5. Non-reducing SDS-PAGE of Circulating Immune Complex from Oral cancer patients

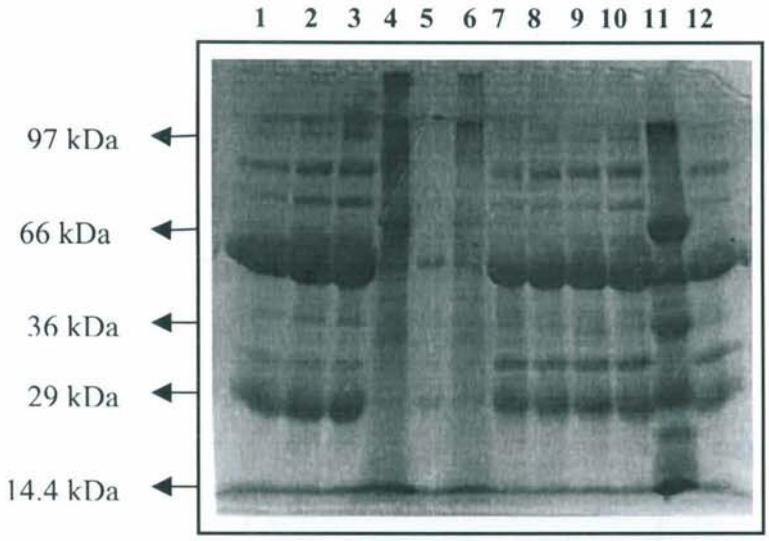


Lane 1-9 & 11-12 CIC from Oral cancer Patients.
Lane 10 – Marker Protein

Further more, it was found that in SDS under reducing condition three prominent bands were observed (Fig 6). Two of them corresponded to light and heavy chains of immunoglobulin molecule and all lots of other proteins that were getting precipitated. One prominent band that was seen near to the light chain region corresponds to the low molecular weight antigens of non-reducing SDS PAGE.

It seems that a whole spectrum of immune complex with different properties circulate in the blood of patients with various types of cancer. These complexes may differ from each other in size, in composition or in the nature of the antigen and antibody components. It is found that an antigen of approximately 30 kDa is a distinct antigen in oral cancer.

Fig. 6 Reducing SDS-PAGE of Circulating Immune complex (CIC) from Oral cancer patients



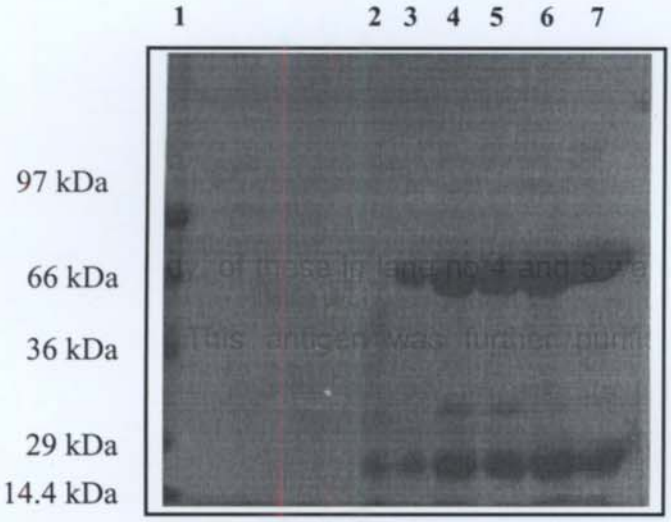
Lane 1-10 & 12 - CIC from Oral cancer Patients.
Lane 11 – Marker Protein

4.3.3.2. Protein A purification of CIC

The isolation of CIC by PEG is a crude and non-specific method, were many other serum proteins also may co-precipitate along with the immune complex. So in order to remove the other non CIC serum components it is essential to purify the PEG precipitate. In this study, further purification of the CIC was performed on an affinity chromatography using protein–A Sepharose as an affinity material. Protein A binds to many classes of human antibodies through Fc region (Lindmark 1983). So the CIC can also bind to the protein A Sepharose column. The bound fractions were eluted with glycine HCl buffer, neutralised with KCl and dialysed. Different fractions from the column were shown in Fig. 7. Lane numbers 2 to 7 contains light and

heavy chain of antibody, of these in lane no 4 and 5 we get authentic 30 kDa oral cancer antigen. This antigen was further purified by electro-elution method.

Fig. 7. SDS - PAGE (reducing condition) of Circulating Immune complex (CIC) purified by protein A Sepharose column



Lane 1 - Marker protein
Lane 2-7 - Various fractions eluted from protein A Sepharose column

4.3.3.3. Gel filtration analysis of CIC

Further, the isolation and separation of the antigen was done on Sephadex G-200 column, which was equilibrated with 9M urea. The calibration of the column and the elution profile of the columns are shown in fig. 8 and fig. 9 respectively. The protein, that came out as a last peak from the column corresponds to the characteristic 30 kDa antigen of oral cancer. Further, this antigen was used for production of polyclonal antibody. The authenticity of the antigen form PAGE and Sephadex-G200 column was compared by immunodiffusion technique.

Fig. 8 Calibration of Sephadex G-200 Gel Filtration column by known molecular weight protien

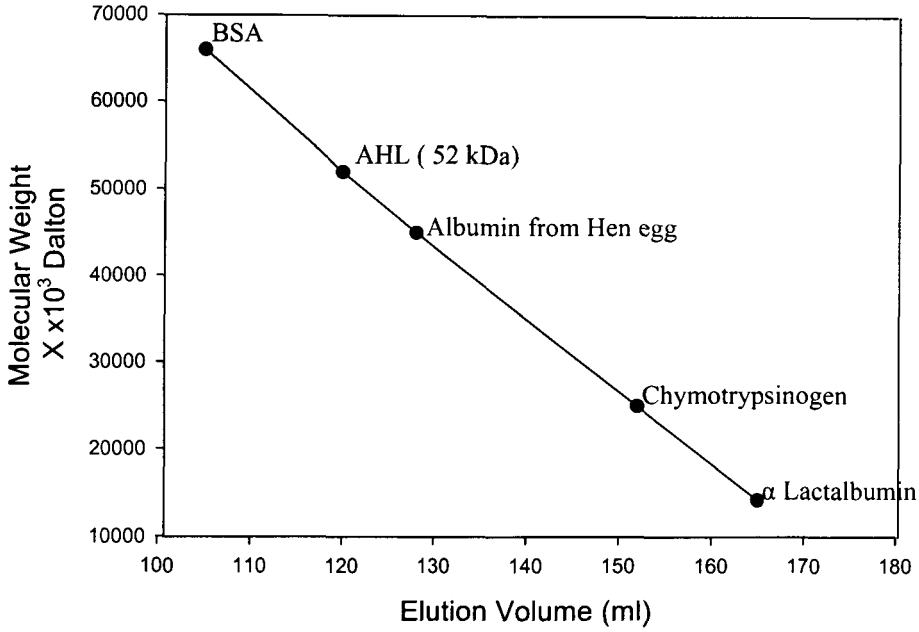
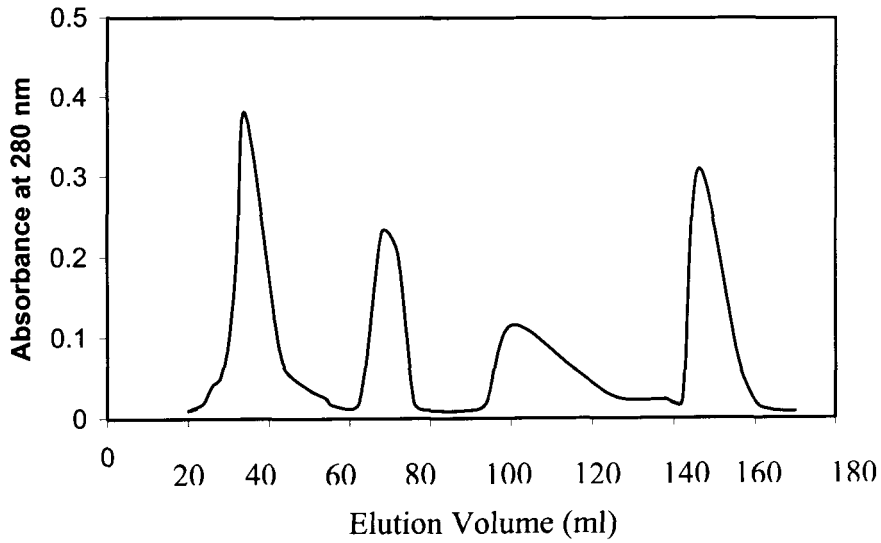


Fig. 9 Sephadex G-200 Gel filtration of crude oral cancer CIC



A remarkable work in immune complex purification and analysis has been done for isolating and classifying immune complexes on the basis of their size and composition with a view of identifying TAA and the

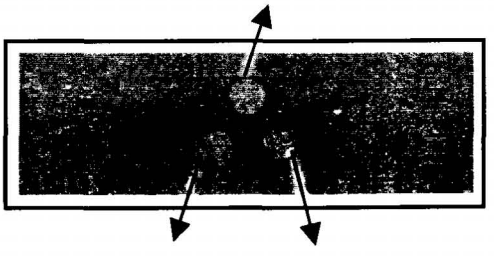
nature of CIC in which TAA are found (Phillips *et. al.* 1989). Various methods are tried for the isolation of antigen from circulating immune complexes (Eric and Lee 1995). Immune complexes in malaria infected mice and protein blot analysis were performed to characterise the isolated protein (Kusuhara *et. al.* 2000). In this method, antigens were isolated from immune complexes by eluting the antigen that is attached to the antibody through C1q bound micro well plates. Here, the antigens were eluted using 0.2 ml glycine buffer, containing 0.5 M NaCl. Such antigen was used to raise polyclonal antibody against the *Plasmodium berghei* and monitor infection by indirect immunofluorescence and immunoblot techniques.

4.3.4. Immunoreactivity of the oral cancer antigen

4.3.4.1. Immunodiffusion analysis

Polyclonal antibody raised against the antigen showed a banding pattern on immunodiffusion plates when it reacted with oral cancer antigen (Fig. 10).

Fig. 10. Immunodiffusion of oral cancer antigen with polyclonal antibody.



Oral cancer antigen from SDS PAGE

Oral cancer antigen from gel filtration chromatography

4.3.4.2. ELISA for the Detection of Tumor associated antibodies in cancer patients.

ELISA was performed using oral cancer antigens eluted from Sephadex G-200 column with patient serum. The results are shown in Table 10. It is found that a characteristic reactivity is seen in the case of oral cancer patient serum.

Table 10. Serum immunoglobulin concentration of anti-oral cancer antibodies in patient serum.

Age group Years	Male Oral cancer Patient (N)	Male Healthy normals (N)
0-20	- (16)	- (50)
20-40	0.87 ± 0.18 (30)	0.41 ± 0.15 (50)
40-60	**0.93 ± 0.18 (50)**	**0.39 ± 0.15 (50)**
>60	0.67 ± 0.14 (50)	0.41 ± 0.13 (50)

Age group Years	Female Oral cancer Patient (N)	Female Healthy normals (N)
0-20	- (8)	- (50)
20-40	0.38 ± 0.18 (10)	0.31 ± 0.15 (50)
40-60	**0.73 ± 0.18 (50)**	**0.33 ± 0.15 (50)**
>60	0.39 ± 0.17 (50)	0.36 ± 0.16 (50)

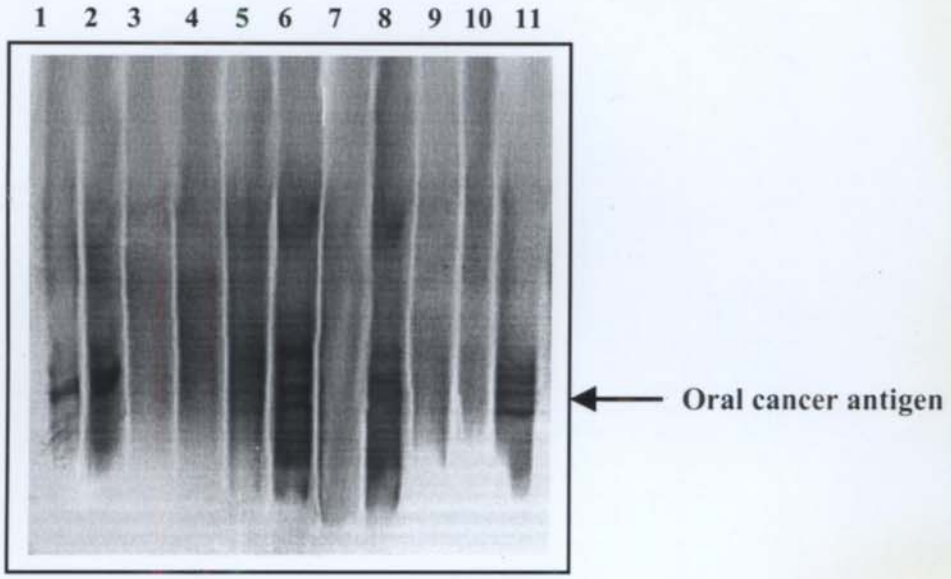
N- Number of samples

A statistically significant increase was observed in the case of 40 - 60 age group of patients in comparison to their age matched controls. The same group showed a significant increase with the other age groups of patients.

4.3.4.3. Protein blot analysis of oral cancer antigen from CIC

Protein blot analysis of the CIC isolated from patients showed the characteristic-banding pattern in the oral cancer antigen region (Fig. 11).

Fig. 11. Protein blot analysis of circulating immune complex from squamous cell carcinoma of oral cancer patients



Lane 1-Characteristic 30 kDa oral cancer antigen
Lane 2-11– Oral cancer patients CIC isolated by PEG precipitation, separated and blotted from non-reducing SDS PAGE probed with polyclonal anti-oral cancer antigen antibody raised in rabbit.

These antigen bands in patients correspond to the authentic tumour associated antigen, which was isolated and purified from patient CIC by previously discussed methods. There are many reports in which the protein blot analysis is used for the characterisation of tumour antigens (Dominique *et. al.* 2001, Jager *et. al.* 2002 and Koga *et. al.* 2004). Similar approach for the isolation, purification and characterization can be applied to any disease where there is a prevalence of CIC in the sera.

SUMMARY

Epidemiological analysis of cancer incidence showed that oral cancer is a predominant type of cancer in male population in the cohort studied. It was observed that there is an association of both HSV and HPV virus with oral cancer. The prevalence was more than 80% and 53% for HSV and HPV respectively. Among the various age groups studied for the presence of circulating immune complex (CIC), a statistically significant increase is observed in the case of above 40 age groups. In the analysis of various classes of antibodies associated in CIC, it was found that IgG and IgA shows a marked statistically significant increase in concentration when compared to healthy normal in most of the age groups studied. IgG concentrations in CIC was found to have significant difference in 40-60 age group in comparison to normal healthy individuals. An antigen, with an approximate molecular weight of 30 kDa was detected in immune complex of oral cancer patients. Further, it was purified on a Protein-A column and the antigen was immunised to rabbits to produce antibodies. These specific antibodies produced in rabbits were labelled with peroxidase enzyme and was used in protein blot analysis of patient sera and found that a corresponding molecule got lighted up at 30 kDa. region. Analysis for the presence of associated antibodies in patient serum samples revealed that there was a significant increase in concentration, especially in 40-60 age groups. Thus, this antigen can be further exploited for the prognostic, diagnostic and therapeutic use in our population.

Isolation and Characterisation of Tumour Associated Antigens and Their Significance in Host Immune Responses

Thesis submitted to the
University of Calicut under the **Faculty of Medicine**
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Doctor of Philosophy
In
IMMUNOLOGY

by

ELYAS K. K.
Department of Biochemistry
Medical College
Thrissur
2005

REFERENCES

Chapter V

REFERENCES

- Abdul Kader M., Ravindran A., Sudha L., Vijayakumar T. and Vasudevan DM. (1981) *Ind. J. Med. Res.* 74: 428.
- Abney ER., Cooper MD., Kearney JF., Lawton AR. and Parkhouse RM. (1978) *J. Immunol.* 120: 2041.
- Alcami A., Symons JA., Khanna A. and Smith GL. (1998) *Sem. Virol.* 8: 419.
- Andrews P. (1965) *Biochem. J.* 91:595-606.
- Andriole GL. (1992) *J. Clin. Onco.* 10: 1205.
- Anon. (1999) *J. Natl. Cancer Inst.* 91: 309.
- Aziz M., Akhtar S. and Malik A. (1993) *Cancer Detection and Prevention* 17(1).
- Baichwal VR., Hammerschmidt W. and Sugden B. (1989) *Curr. Top. Microbiol. Immunol.* 144: 233.
- Balaram P and Meenattoor G. (1996) *Singapore Dent. J.* 21(1):36.
- Balaram P. and Vasudevan DM. (1983) *Cancer.* 52: 1837.
- Balaram P., Meenattoor G., Nalinakumari KR., Hareendran NK., Padmanabhan TK. and Nair MK. (1994) *Eur. J. Cancer B. Oral Oncol.* 30B (1): 43-6.
- Balaram P., Pillai MR. and Abraham T. (1987) *J. Oral Pathol.* 16 (8): 389-91.
- Baldwin RW., Hollken K. and Robbins RA. (1979) *Recent Res. Cancer.* 67: 85.
- Barrett DJ. and Ayoub EM. (1986) *Clin. Exp. Immunol.* 63: 127.
- Barth RJ., Mule JJ. and Asher AL. (1991) *J. Immunol. Methods.* 140: 269
- Baseler MW., Marim PE. and Veltri FW. (1984) *Fed.Proc.* 43:1621.
- Bates SE. and Longo DL. (1987) *Semin. Oncol.*14: 102.
- Beaudenon S., Kremsdorf D., Croissant O., Jablonska S., Wain-Hobson S. and Orth G. (1986) *Nature.* 321: 246.
- Bell A. and Rickinson AB. (2003) *Trends Microbiol.* 11(11): 495.
- Bell CD. Jr. and Seetharam SA. (1976) *Int. J. Cancer.* 18: 605.
- Berinstein N. and Levy R. (1987) *J. Immunol.* 139: 971.
- Bidwell DE., Bartlett A. and Voller A. (1977) *J. Infect. Dis.* 136; 274.
- Birnboim HC. and Doly J. (1979) *Nucleic Acids Res.* 7(6): 1513.

- Bjorkman P., Saper MA., Samraoui B., Bennet WS., Strominger JL. and Wiley DC. (1987) *Nature* 329: 512.
- Blot WJ., McLaughlin JK. and Winn DM. (1988) Cancer Research 48:3282.
- Boon T., Cerottini JC., Van den Eynde B., Van der Bruggen P. and Van Pel A. (1994) *Ann. Rev. Immunol.* 12: 337.
- Brandies WE., Helson L., Wang Y. and Good RA. (1978) *J. Clin.Invest.* 62: 1201.
- Brode S. and Macary PA. (2004) *Immunology.* 112(3): 345.
- Brown AC., Hall CL., Kong JC., Carey K., Weitzman SA. and Aisenberg AC. (1978) *Amer. J. Med.* 64: 289.
- Brugge JS. and Erikson RL. (1977) *Nature.* 269: 346.
- Burkhardt A. (1985) *J. Oral. Pathol.* 14: 751.
- Burnet FM. (1971) *Transplantation Rev.* 7: 3.
- Byers VS., Levin AS., Johnson JO. and Hackett AJ. (1975) *Cancer Res.* 35: 2520.
- Cairns J. (1981) *Nature.* 289: 353.
- Carpentier NA., Lange GT., Fiere DM., Fournie GJ., Lambert PH. and Miescher PA. (1977) *J. Clin. Invest.* 60: 874.
- Catty D. and Ray Kundalia C. (1988) In: Antibodies – A Practical Approch Vol I, IRL Press, New York.
- Chang KW., Chang CS., Lai KS., Chou MJ. and Choo KB. (1989) *J. Med. Virol.* 28: 57.
- Chang Y., Cesarman E., Pessin MS., Lee F., Culpepper J., Knowles D. and Moore PS. (1994) *Science.* 226: 1865.
- Charan PS., Nachhattar S., Gurmeet K., Jagraj S. and Gagandeep S. (2000) *J. Ind. Acad. Clini. Med.* 1: 2.
- Chen I., Thomas EK., Hu SL. and Hellstorm KE. (1991) *Proc. Nat. Acad. Sci. USA.* 89: 110.
- Chhajilani V., Zinjde S., Adwani S. and Gothoskar B. (1983) *Leukemia Res.* 7:565.
- Coleman RM., Pereira L., Bailey PD., Dondero D., Wickliffe C. and Nahmias AJ. (1983) *J. Clin. Microbiol.* 18: 287.

- Coombs RC., Neville AM. and Stoll BA. (Ed.) (1978) Significance of tumour index substances in management. In Secondary spread in breast cancer, William Heinemann Medical Books, Chicago.
- Corey L. and Spear PG. (1986a) *N. Eng. J. Med.* 314(11):686.
- Corey L. and Spear PG. (1986b) *N. Eng. J. Med.* 314(12):749.
- Corey L., Adams HG., Brown ZA. and Holmes KK. (1983) *Ann. Intern. Medicine.* 98: 958.
- Das SN., Khanna NN. and Khanna S. (1986) *Cancer. Invest.* 4(3): 207.
- David GL. and James CEU. (Ed.) (1999) In Recent advances in histopathology, Churchill Livingstone.
- Davies KA., Hird V., Stewart S., Sivolapenko GB., Jose P., Epenetos AA. and Walport MJ. (1990) *J. Immunol.* 144(12): 4613.
- De Villiers EM. (1994) *Curr. Top. Microbiol. Immunol.* 86: 1
- De Villiers EM., Weidaner Otto H. and Zur Hausen H. (1985) *Int. J. Cancer.* 36: 575.
- De Vita VT., Hellman S. and Rosenberg SA. (1993) Cancer: Principles and Practises of Oncology 4th Ed. J.B. Lippincott Co., Philadelphia.
- Desai PB. (1983) *Ind. J. med. Res.* 78: 8.
- Di Paolo JA., Woodworth CD., Popescu NC., Koval DL., Lopez JV. and Doniger J. (1990) *Virology.* 177: 777.
- Dominique BG., Laetitia C., Cecile V. and Bignon YV. (2001) Oncology reports 8: 1363-1366
- Dowhanick JJ., McBride AA. and Howley PM. (1995) *J. Virology.* 69: 7791.
- Duff R. and Rapp F. (1971) *Nature new Biol.* 233: 48.
- Duraiswamy J., Burrows JM., Bharadwaj M., Burrows SR., Cooper L., Pimtanothai N. and Khanna R. (2003) *J. Virol.* 77: 7401.
- Durst M., Gissman L., Idenburg H. and Zur Hausen H. (1983) *Proc. Natl. Acad. Sci. USA.* 80: 3812.
- Eddy DM. (1990) *Ann. Int. Med.* 113: 214.
- Eglin RP., Scully C., Lehner T., Ward-booth P. and Mc Gregor IA. (1983) *Lancet* ii: 766.

- Eisenthal A., Lafreniere R., Lefor AT. and Rosenberg SA, (1987) *Cancer Res.* 47(11): 2771.
- Elwood JM., Pearson JCG., Skippen DH. and Jackson SM. (1984) *Int. J. Cancer* 34:603.
- Engvall JR. and Perlamann P. (1971) *Immunochemistry* 8: 871.
- Epstein MA. and Achong B. (Eds.) (1979) In The Epstein-Barr Virus, Springer Verlag, Berlin.
- Eric JT. and Lee CM. (1995) *J. Nat. Med. Assoc.* 87: 693.
- Euler HH., Beress R., Moldenhauer I. and Loffler H. (1983) *Int. J. Cancer.* 32: 437.
- Evans AS. and Mueller NE.(1990) *Ann Epidemiol.* 1(1):71.
- Evans SJ., Langdon JD., Rapidis AD. and Johnson NW. (1982) *Cancer.* 49: 773.
- Everett H., Barry M., Lee SF., Sun X., Graham K., Stone J., Bleackley RC. and Mc Fadden G. (2000) *J. Exp. Med.* 191: 1487.
- Farber H., Weatherall DJ., Ledingham JGG. and Worrell DA. (Eds.) (1983) In Neoplasia, The Oxford text book of medicine, United Kingdom, Oxford University press.
- Fearon ER. and Vogelstein B. (1990) *Cell.* 61: 759.
- Feinberg AP. and Vogelstein B. (1983) *Anal. Biochem.* 132: 6.
- Feldmann M. and Diener E. (1972) *J. Immunol.* 108: 93.
- Forsmark CE., Lambiase L. and Vogel SB. (1994) *Pancreas.* 9: 731.
- Fugmann RA. and Sigel MM. (1967) *J. of Virology* (4): 678.
- Fust G., Kavai M., Szegdi GY., Meretsy K., Falus A., Lenkey A. and Miez M. (1980) *J. Immunol. Meth.* 38: 281.
- Galloway DA. and Tension SA. (1990) *Mol. Biol. Med.* 7: 59.
- Gearhart PJ., Hurwitz JL. and Cebra JJ. (1980) *Proc. Natl. Acad. Sci. USA.* 77 : 5424.
- Gentile JM. and Flickinger JT. (1972) *Gynecol. Obstet.* 135: 61.
- Gershan RK., Mokyr MB. and Mitchell MS. (1974) *Nature.* 250: 594.
- Ghose UR. and Pradia BB. (1995) *Ind. J. Cancer.* 32: 95.
- Gimenez-Conti IB. and Slaga TJ. (1993) *J. Cell Biochem.* 17: 83.
- Gion M., Mione R., Leon AE. and Dittadi R. (1999) *Clin. Chem.* 45: 630.
- Gissmann L., Diehl V., Schultz-Coulon HJ., zur Hausen H. (1982) J. Virol. 44: 393.

- Gissmann L., Wolnik L., Ikenberg H., Koldovski U., Schnurch H C and Zur Hausen H. (1983) *Proc. Natl. Acad. Sci. USA* 80: 560.
- Gitlin D. and Boesman. (1966) *J. Clin. Invest.* 45: 1826
- Gleaves CA. and Meyers JD. (1988) *J. Clin. Microbiol.* 26: 1402.
- Goedert JJ., Cote TR., Virgo P., Scoppa SM., Kingma DW. and Gail MH. (1998) *Lancet.* 351: 1833.
- Gore MM., Tamkar AV., Kedarnath N., Banerjee AC., Metha MJ. and Gosh SN. (1983) *Ind. J. Cancer.* 20: 54.
- Gorsky M. and Silverman S. Jr. (1984) *J. Prosthet. Dent.* 52: 164.
- Granberg D., Wilander E., Oberg K. and Skogseid B. (2000) *J. Clin. Endocrinol. Metab.* 85(9): 3425.
- Greaves MR; Herberman RB. and Mc Entire KR. (Eds.) (1979) In Immuno diagnosis of cancer part I , Mercel Dekker, New York.
- Greenberg PD. (1991) *Adv. Immunol.* 49: 281.
- Greene WC., Bohnlein E. and Ballard DW. (1989) *Immunol. Today.* 10: 272.
- Gross L. (1965) *Cancer.* 18: 201.
- Guggenheimer J., Verbin RS., Johnson JT., Harkowitz CA. and Myers EN. (1989) *Cancer.* 64: 932.
- Guidi LC., Bartoloni R., Baroni C., Barone and Gambassi G. (1991) *Eos-Riv. Immunol. Immunofarmacol.* 11(2): 67.
- Gupta PC. (1980) *Commu. Denti. Oral. Epidem.* 8: 287.
- Gupta PC., Mehta FS., Pindborg JJ., Bhonsle RB., Murti PR., Daftary DK. and Aghi MB. (1992) *J. Oral. Pathol. Med* . 21(10): 433.
- Gupta R., Golub SH. and Morton DL. (1979) *Cancer Immunol. Immunother.* 6: 63.
- Gutterman JU., Rossen RD., Butter WT., Mc Credle KB., Bodey GP., Freireich FJ. and Hersh FM. (1973) *New. Engl. J. Med.* 288: 169.
- Hamblin AS; Male D. and Rickwood D. (Eds.) (1988) In lymphokines. IRL Press Ltd. Oxford.
- Harkiss GD. and Brown.GL. (1979) *Clin. Exp. Immunol.* 36: 117.
- Hattler BG. and Soehnlen B. (1975) *Science.* 114: 1374.
- Heines HG., McCoy JP., Hotheinz DE., Ng A B P., Nordquist SRB. and Laif RC. (1981) *J. Natl. Cancer. Inst.* 66: 465.

- Hellstrom I., Hellstrom KE., Zeidman L., Bernstein ID. and Brown JP. (1979) *Int. J. Cancer.* 23:555.
- Hellstrom KE., Hellstrom I. and Nepom JT. (1977) *Biochem. Biophys. Acta.* 473: 148.
- Hermanson GT., Mallia AK. and Smith PK. (1992) *In* Immobilised affinity Ligand Techniques. Academic San Diego, CA.
- Hewitt HB., Blake ER. and Walder AS. (1976) *Brit . J. Cancer.* 33: 241.
- Hildesheim A., Mann V., Brinton LA., Szklo M., Reeves WC. and Rawls WE. (1991) *Int. J. Cancer.* 49: 335.
- Hindle I., Downer MC. and Speight PM. (2000) *Commun. Dent. Health.* 17(3): 152.
- Hirsch JM., Johanson SL. and Vahine A. (1984) *J. Oral. Pathol.* 13: 52.
- Hofbauer GF., Burkhart A., Schuler G., Dummer R., Burg G. and Nestle FO. (2004) *J. Immunother.* 27(1): 73.
- Hoffkin K., Price MR., Mclanghlin PJ., Moore VE. and Baldwin RW. (1978a) *Int.J.Cancer.*21:496.
- Hoffkin K., Price MR., McLaughlin PJ., Moore VE. and Baldwin RW. (1978b) *Int.J.Cancer.*22: 576.
- Hoffmann D. and Djordjevic MV. (1997) *Advances Dent. Research* 11 :322.
- Hole N. and Stern PL. (1988) *Br. J. Cancer. 57*:239.
- Hollinshed AC. (1975) *Cancer.* 36:1282.
- Hottken K., Meredith ID., Robins RA., Baldwin RW., Davies CJ. and Blamey RM. (1977) *Brit. Med. J.* 2: 218.
- Houghton AN., Eisinger M., Albino AP., Cairncross JG. and Old LJ. (1982) *J. Exp.Med.* 156: 1755
- Houghton AN., Thomson TM., Gross D., Oettgen HF. and Old LJ. (1984) *J. Exp. Med.* 160: 255
- Howley PM. (1991) *Cancer Res.* (Suppl.) 51:5019.
- Huang AYC., Golumbek P., Ahmadzadeh M., Jaffee E., Pardoll D. and Levitsky H. (1994) *Science.* 264: 961
- Huber SA. and Lucas ZJ. (1978) *J. Immunol.* 121: 2485.
- IARC (International Agency for Research on Cancer) (1985) *Tobacco habits other than smoking; betel-quid and areca nut chewing; and some related nitrosamimes,*

ICAR monographs on the evaluation of the carcinogenic risk to humans, no 7. IARC, Lyon.

- IARC (International Agency for Research on Cancer) (1986) *Tobacco smoking* ICAR monographs on the evaluation of the carcinogenic risk of chemicals to humans, no 38. IARC, Lyon.
- Iwasaka T., Yokoyama M., Hayashi. and Sugimori H. (1988) *Am. J. Obstet. Gynecol.* 159: 1251.
- Izsak FC., Brenner HJ., Landes E., Ran M. and Witz I. (1974) *Israel J. Med. Sci.* 10: 642.
- Jablonska S. and Majewski S. (1994) *Curr. Top. Microbiol. Immunol.* 186: 157
- Jacobs I., Davies AP., Bridges J., Stabile I., Fay T. and Lower A. (1993) *BMJ* 306: 1030.
- Jacqueline RS., Janet RD., Dawn FE., Margaret MM., Rhoda A., Denise A., Galloway and Stephen MS. (2001*) Cancer Res.* 61, 8453.
- Jäger D., Elisabeth S., Julia K., Kristina H., Akin A., Michael A., Yao-Tseng Chen, Sacha G., Lloyd Old, Alexander Knuth and Elke Jäger (2002) *Cancer Immunity*, Vol. 2, p. 10
- Jamkar AV., Gore MM., Kedarnath N., Banerjee AC., Metha MJ. and Gosh SN. (1983) *Ind. J. Cancer.* 20: 49.
- Jemal A., Murray T., Samuels A., Ghafoor A., Ward E. and Thun MJ. (2003) *A cancer. J. for clinicians.* 53(1): 5.
- Johnson RE., Nehmias AJ., Magder LS., Lee FK., Brooks CA. and Snowden CB. (1989) *New Eng. J. Med.* 321: 7.
- Johnstone A. and Thorpe R. (1996) In *Immunochemistry in Practice*, 3rd ed.Blackwell Scientific, Oxford ,UK.
- Kato H. and Torigoe T. (1985) *Cancer.* 56: 302.
- Kato H., Morioka H. and Aramaki S., (1983) *Am. J. Obst. Gynecol.* 145: 350.
- Kaufman RH. and Adam E. (1986) *Clin. Obstet. Gynecol.* 29(3): 678.
- Kaufman RH., Dreesman GR., Burek J., Korhonen MO., Matson DO., Melnick JL., Powell KL., Purifoy DJ., Courtney RJ. and Adam E. (1981) *N. Eng. J. Med.* 305: 483.

- Kawakami Y., Zakut R., Topalian SL. Stotter H. and Rosenberg SA. (1992) *J. Immunol.* 148: 638
- Kayembe MKA. and Kalengayi MMR.(1999) *Odonto. Stomat. Trop.* 88: 29.
- Kekule AS., Lauer U., Meyer M., Caselman WH., Hofschneider PH. and Koshy R. (1990) *Nature.* 343: 457.
- Khanna MM., Das SM. and Khanna S. (1982) *J. Surg .Oncol.* 20: 46.
- Klein E. and Mantovani A. (1993) *Curr. Opin. Immunol.* 5: 741.
- Knudson AG. (1971) *Proc. Natl. Acad. Sci. USA.* 68: 820.
- Koga M., Komatsu N., Kawamoto N., Shichijo S., Itoh K and Yamada A. (2004) *Oncol. Rep.* 11:785-9.
- Kopf AW., Silberg I. and Cooper NS. (1966) *J. Invest. Drem.* 47: 83.
- Kotwal GJ. (2000) *Immunol. Today.* 21: 242.
- Koutsky LA., Galloway DA. and Holmes KK. (1988) *Epidemiol. Rev.* 10:122.
- Krishnan A., Zaia J. and Forman SJ. (2003) *Bone Marrow Transplant.* 32(8) :741.
- Kroll SO. and Hoffman S. (1976) *J. Am. Dent. Assoc.* 92: 571.
- Kumari TV., Shanmughan I . and Vasudevan DM. (1982) *Ind. J. Med. Res.* 75: 590.
- Kusuhara Y., Maeno Y., Nagase K., Sakai K., Nakazawa S., Kanbara H., Taniguchi K. and Nakabayashi T. (2000) *Int. J. Parasitology* 30: 609.
- Lack EE., Neve C. and Vawter GF. (1982) *Am. J. Surg. Pathol.* 6: 693.
- Lalani AS., Barrett JW. and McFadden I. (2000) *Immunol. Today.* 21: 100.s
- Lambert PH., Dixon FJ., Zubler RH., Agnello V., Cambiaso C., Casali P., Clarke J., Cowdery J., McDuffie F., May F., McLennan IC., Masson P., Müller-Eberhard H., Pentinnen K., Smith M., Tappeiner G., Theofilopoulos A. and Verrost PA. (1978) *J. Clin. Lab. Immunol.* 1:1.
- Lammelli UK. (1970) *Nature.* 227: 680.
- Landman J., Chang Y., Kavaler E., Droller MJ. and Liu BC. (1998) *Urology.* 52(3): 398.
- Larson PA., Johanson SL., Vahine A. and Hirsch JM. (1989) *J. Oral Pathol. Med.* 187.
- Lehner T., Wilton JMA., Shillitoe EJ. and Ivanji L. (1973) *Brit. J. Cancer.* 27: 351.
- Lindblom A. and Liljegren A. (2000) *BMJ.* 320: 424

- Lindmark R., Thoren-Tolling K. and Sjoquist J. (1983) *J. Immunol. Meth.* 62: 1.
- Lloyed KO. (1991) *Sem. Cancer Biol.* 2: 421.
- Lotnicker M., Pavesi F., Scarabelli M. (1991) *Int. J. Biolog Markers* 6:115
- Mackaness GB., Lagrange RH., Miller TE. and Ishibashi T. (1974) *J. Exp. Med.* 139: 543.
- Maitland NJ., Bromide T., Cox MF., Crane IJ., Prime SS. and Scully C. (1989) *Br. J. Cancer* 59:698.
- Maitland NJ., Cox MF., Lynas C., Prime SS., Meanwell CA. and Scully C. (1987) *Br. J. Cancer.* 59: 698.
- Malaowalla AM., Silverman S. Jr., Mani NJ., Bilimoria KF. and Smith LW. (1976). *Cancer.* 37: 1882.
- Martin WJ. and Miller JFAP. (1967) *Lancet ii.* 1285.
- Mashberg A. and Meyers H. (1976) *Cancer.* 37: 2149.
- Matz B., Schlehofer JR. and Zur Hausen H. (1984) *Virology.* 134: 328.
- Matz B., Schlehofer JR., Zur Hausen H., Huber B. and Fanning I. (1985) *Int. J. Cancer.* 35: 521.
- McCoy JL., Jerome LF.,Dean JH.,Cannon GB.,Alford TC.,Doering T. and Herberman RB. (1974) *J. Natl. Cancer. Inst.* 53: 11.
- Meinkoth J. and Wahl G.(1984) *Anal. Biochem.* 138: 267.
- Milde K. and Loning T. (1986) *J. Oral Pathol.* 15: 292.
- Moertel CG., Fleming TR., Macdonald JS., Haller DG., Laurie JA. and Tangen C. (1993) *JAMA.* 270: 943.
- Mongini PKA., Stein KE . and Metcalf ES. (1982) *J. Exp. Med.* 153: 884.
- Moore SR., Johnson NW., Pierce AM. and Wilson DF. (2000) *Oral Diseases* 6: 65.
- Mork J., Lie AK., Glattre E., Hallmans G., Jellum E., Koskela P., Moller B., Pukkala E., Schiller JT., Youngman L., Lehtinen M. and Dillner J. (2001) *N. Engl. J. Med.* 344 (15): 1125.
- Mosier De., Mond JJ. and Goldings EA. (1977) *J. Immunol.* 119: 1874.
- Mukhopadhyaya R., Tatake R J., Krishnan N., Rao R S., Fakih A R., Waik S L. and Gangal S G. (1989) In *National State of the Art Report on Cancer Immunology., Indian Association for Cancer Research, India .*
- Mukojima T., Gunven P. and Klein G. (1973) *J. Natl. Cancer. Inst.*51: 1319

- Mulryan K., Ryan MG., Myers KA., Shaw D., Wang W., Kingsman SM., Stern PL. and Carroll MW. (2002) In *Molecular Cancer Therapeutics* 1: 1129
- Murali PS., Somasundaram R., Rao RS., Fakih AR. and Gangal SG. (1989) *J. Oral Path. Med.* 18: 327.
- Murray RJ., Kurilla MG., Brooks JM., Thomas WA., Rowe M., Kieff E. and Rickinson A.B. (1992) *J. Exp. Med.* 176:157.
- Nair UJ., Friesen M., Richard I., MacClennan R., Thomas S. and Bartsch H. (1990) *Carcinogenesis* 11: 2145.
- Nairn RC., Nind APP., Guli EPG., Davies D., Rolland JM., McGiven AR. and Hughes E SR. (1971) *Brit. Med. J.* 4: 706.
- Nakane PK. and Kawaoi A. (1974) *J. Histochem. Cytochem.* 22: 1084
- Neefjes JJ. and Momburg S.H. (1993) *Curr. Opin. Immunol.* 5: 27.
- Nicolini A., Carpi A., Ferrari P. and Pieri L. (2003) *Tumor. Biol.* 24:275
- Nordquist RE., Anglin JH. and Lerner MP. (1978) *Brit. J. Cancer* .37: 776.
- Osterlind A. (1993) *Pharmacol. Toxicol.* 72 (1): 67.
- Ostrow R., Manias DA., Fong WJ., Zachow KR. and Faras AJ. (1987) *Cancer.* 59: 429.
- Ouchterlony O. Kallos P. and Waksman BH. (Eds) (1958) In *Progress in Allergy.* Karger, Basel.
- Oxelius VA., Berkel AL. and Hamson LA. (1982) *N. Engl. J. Med.* 206:575.
- Paavonen J., Halttunen M., Hansson B., Nieminen P., Rostila T. and Lehtinen M. (2000) *J. Clin. Virol.* 19: 25.
- Padmakumary G. and Varghese C. (Eds.) (1999) *Hospital cancer registry*, Regional cancer centre, Trivandrum. India.
- Padmanabhan TK. and Vasudevan DM. (1982) *Ind. J. Cancer.* 19: 189.
- Pannall P. (1992) *Clin. Biochem . Revs.* 13: 142.
- Paparizos VA., Kyriakis KP., Polydorou-Pfandl D., Hadjivassilou M. and Stavrianeas NG. (2000) *J. Acquir. Immune. Defic. Syndr.* 25:283-284.
- Park K., Cherrick H., Min BM. and Park NH. (1990) *J. Oral Pathol. Med.* 70: 186.
- Park NH., Dokko H., Li SL. and Cherrick HM. (1991) *J. Oral Maxillofae. Surg.* 49: 276.

- Park NH., Sapp JP. and Herbosa EG. (1986) *Oral. Surg. Oral. Med. Oral Pathol.* 62: 164.
- Parkin DM., Pisani P. and Ferlay J. (1993) *Int. J. Cancer.* 54: 594
- Parkin DM., Laara E. and Muir CS. (1988) *Int. J. Cancer.* 41: 184.
- Paulton TA., Crowther ME., Hay FC. and Niniham LJ. (1978) *The Lancet.* 2: 72.
- Pawelec G. (2004) *Cancer Immunol. Immunother* 53: 262.
- Pennica D., Goeddel DB., Well DR. and Goddel DV. (Eds.) (1987) In *Lymphokines* Academic press,New York. 13: 163.
- Perlmutter RM., Hansburg D., Briles DE., Nicolotti RA. and Davie JM. (1978) *J. Immunol.* 121: 566.
- Perry LL., Benacerrat B. and Greene MI. (1978) *J. Immunol.* 121: 2144.
- Peters JI., Giesler R., Thiele B. and Steinbach F. (1996) *Imm. Today.* 17: 273
- Pfizenmaier K., Scheurich P. and Schluter C. (1987) *J. Immunol.* 137: 975.
- Phillips TM., Queen WD. and Lewis MG; Reisfeld RA. and Ferrone S (Eds.) (1989) In *Melanoma, antigens and antibodies.* Plenum Press. NY
- Pillai R., Balaram P., Abraham T. and Nair MK. (1987) *Neoplasma.* 34: 469.
- Pillai S., Sasidharan VK., Balaram P., Vivekanandan S. and Vasudevan DM. (1982) *Ind. J. Cancer.* 19: 44.
- Pindborg JJ. and Mehta FS. (1968) *Br. J .Cancer.* 28: 646.
- Pindborg JJ., Murthy PR., Bhonsle RB., Gupta PC., Daftary DK. and Mehta FS. (1984) *Scand. J. Dent. Res.* 92: 224.
- Pirtskalaishvili G., Konety BR. and Getzenberg RH. (1999) *Postgrad. Med.* 106(6): 85
- Poh CF., Zhang L., Lam WL., Zhang X., An D., Chau C., Priddy R., Epstein J., and Rosin MP. (2001) *Lab Invest.* 81(4): 629.
- Prince MR. and Baldwin RW; Poste G and Nicolson GL (Eds.) (1977) In : Dynamic aspects of tumour surface organization, Cell Surface Review ,North Holland Amsterdam.3: 324.
- Quinn MJ., Babb PJ., Kirby EA. and Brock A. (2000) *Health statistics Quaterly.* 7: 71.
- Radaev S. and Sun PD. (2002) *J. Appl. Crystallogr.* 35: 674.

- Raghunath PN., Joseph CD., Sudheeran PC. and Vasudevan DM. (1987) *J. Exp. Clin. Cancer. Res.* 6: 173.
- Rajendran R., Sugathan CK., Remani P., Ankathil R. and Vijayakumar T. (1986) *Cancer.* 58(12): 2628.
- Rapp F., Essex M., Todaro G. and Zur Hausen H. (Eds.) (1981) In Viruses in naturally occurring cancers, Cold Spring Harbor Conference on cell proliferation. 7: 63.
- Raubenheimer EJ. and De Villiers PIA. (1989) *J. Dent. Ass. South Africa* 11:14.
- Ravindran A., Remani P., Vasudevan DM. and Vijayakumar T. (1986) *Cancer. J.* 1: 135.
- Rawls WE., Bacchetti S. and Graham FL. (1977) *Curr. Top. Microbiol. Immunol.* 77:71.
- Rawls WE., Tompkins WA., Figueroa ME. and Melnick JL. (1968) *Science* 161: 1255.
- Rayner AA., Steele G., Rodrick ML., Harte PJ., Munroe AE., Zamcheck N. and Wilson RE. (1981) *The American J. Surgery.* 141: 460.
- Reeves WC., Brinton LA., Garcia M., Brenes MM., Herrero R., Gaitan E., Tenorio F., de Britton RC. and Rawls WE. (1989a) *N. Eng. J. Med.* 320: 1437.
- Reeves WC., Rawls WE. and Brinton. (1989b) *Rev. Infect. Dis.* 11(3): 426.
- Reinagel ML. and Taylor RP. (2000) *J. Immunol.* 164(4): 1977.
- Remani P., Ankathil R., Vijayan KK., Beevi VMH., Rajendran R. and Vijayakumar T. (1988) *Cancer Lett.* 40: 185.
- Ries LA., Eisner MP. and Kosary CL. (Eds) (2001). In SEER Cancer Statistics Review 1973-1998. Bethesda, Md: National Cancer Institute.
- Riesen WF., Skavril F. and Brawn DG. (1976) *Biochem. Biophys. Res. Commun.* 66: 678.
- Robert M., Vincent C.,Arnauld P. and Ravillard JP. (1976) *Ann. Immunol.* 127: 145.
- Roland NJ., Caslin AW., Nash J. and Stell PM. (1992) *Head Neck.*14(3): 224.
- Rossen R D., Reisberg MA., Hersh EM. and Gutterman JU. (1977) *J. Natl. Cancer. Inst.* 58: 1205.
- Ruddy S. and Moxley G. (1994) *Diagn. Lab. Immunol.* 14: 387.

- Sambrook J., Fritsch EF. and Maniatis T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York.
- Saral R. (1988) *Amer. J. Med.* 85(2A): 57.
- Saranath D., Mukhopadhyaya R., Rao RS., Fakih AR., Naik SL. and Gangal SG. (1985) *Cancer.* 56: 1062.
- Sarin SK., Thakur V., Guptan RC., Saigal S., Malhotra V., Thyagarajan SP. and Das BC. (2001) *J. Gastroenterology and Hepatology* 16 (6): 666.
- Sasidharan VK., Kumari TV., Vijayakumar T. and Vasudevan DM. (1989) *Ind. J. Radiol. Image.* 39: 67.
- Scher JA., Titus SO., Sharrow JJ., Mond and Finkelman FD. (1982) *Ann. NY. Acad. Sci.* 399: 204-220.
- Schlehofer JR., Gissmann L., Matz B. and Zur Hausen H. (1983) *Int. J. Cancer.* 32: 99.
- Schur PH., Rosen F. and Norman ME. (1979) *Pediatr. Res.* 13:183.
- Schwab T., Schned AR. and Heaney JA. (1999) *J. Urol.* 162: 567.
- Scully C. (1982) *Oral Surg. Oral Med. Oral Path.* 53: 157.
- Scully C. (1986) *J. Max. Fac. Surg.* 26: 76.
- Sell. (1993) *Crit. Rev .Oncogenesis* .4: 419.
- Seth P. (1980) *ICMR Bull.* 10:98.
- Shakib F., Stanworth DR. (1980) Part 1 *La Ricera Clin. Lab* 10: 463.
- Shillitoe EJ., Greenspan D., Greenspan JS. and Silverman S. (1983) *Cancer.* 51: 65.
- Shillitoe EJ., Greenspan D., Greenspan JS., Hansen LS. and Silverman S. (1982) *Cancer.* 49: 2315.
- Silverman NA., Alexander JC., Hollinshead AC. and Chretien PB. (1976) *Cancer* .37: 135.
- Silverman S. Jr. (1988) *Cancer.* 62: 1796.
- Sjogren HO., Hellstorm I., Bansal SE., Warner GA. and Hellstorm K. (1972) *Int. J. Cancer.* 9: 274.
- Skavril F. and Schilt U. (1984) *Clin. Exp. Immunol.* 55: 671.
- Slack J., Der Balcian G., Nhim MH. and Davie JM.(1980) *J. Exp. Med.* 151:853.
- Slater GW., Turmel C., Lalande M. and Noolandi J. (1989) *Biopolymers* 28: 1793.

- Slavkin HC. (1996) *J. Am. Dent. Assoc.*, 127: 517.
- Smith GL., Symons JA. and Alcami A.(1998) *Sem. Virol.* 8: 409.
- Smith HG., Chretien PB., Henson DE., Silverman NA. and Alexander JC. (1976) *Am. J. Surg.* 132:541.
- Smith JS., Herrero R., Bosetti C., Munoz N., Bosch FX., Eluf-Neto J., Castellsague X., Meijer CJ., Van den Brule AJ., Franceschi S. and Ashley R. (2002) *J. Natl. Cancer Inst.* 94(21):1604.
- Southern EM. (1975) *J. Mol. Biol.* 98: 503.
- Stevans RD., Dick D., Keid B. and Heiner D. (1983) *J. Clin. Immunol.* 3: 65.
- Stills HF. (1994) Manning PJ., Ringler DH. and Newcomer CE. (Eds). In *the Biology of the Laboratory Rabbit.* Accademic press, San Diego.
- Straus SE., Rooney JF., Sever JL., Seidlin M., Nusinoff-Lehrman S. and Cremer K. (1985) *Ann. Int. Medicine.* 103: 404.
- Stutman O. (1973) *Transplant. Proc.* 5: 969.
- Sudbo J., Kildal W., Risberg B., Koppang HS., Danielsen HE and Reith A. (2001) *N Engl J Med.* 344(17):1270.
- Syrjanen K., Syrjanen S., Lamberg M., Pyrhonen S. and Nuutinen J. (1983) *Int. J. Oral. Surg.* 12: 418.
- Syrjanen S., Syrjanen KJ. and Happonen RP. (1988) *J. Oral. Pathol. Med.* 17: 273.
- Tatarinov YS. (1964) *Vopr. Med. Khim.* 10: 90.
- Teale JM and Klinman NR. (1984) *J. Immunol.* 133: 1811.
- Theofilopoulos AN. and Dixon FJ. (1979) *Adv. Immunol.* 28: 89.
- Theofilopoulos AN., Andrews BS., Urist MM., Morton DL. and Dixon FJ. (1977) *J. Immunol.* 119: 657.
- Thung CC. and Herberman RB. (1975) *Nature.* 257: 801.
- Tortorella D., Gewurz BE., Furman MH., Schust DJ. and Ploegh HL. (2000) *Annu. Rev. Immunol.* 18: 861.
- Towbin H., Staehelin T.and Gordon J. (1979) *Proc. Natl. Acad. Sci. USA.* 76:4350
- Tsi Jung FA., Juei- Hsiung TSA., When YC. and Tony CT. (1991) *Cancer Invest.* 9(2): 144.
- Ukraintseva SV. and Yashina AI. (2004) *Ann. NY. Acad. Sci.* 1019: 200.

- Van der BP., Traversari C. and Chomez P. (1991) *Science.* 254: 1643.
- Vasudevan DM., Reghunath PN., Shanavas KR., Vijayakumar T. and Antony A. (1991a) *J. Exp. Clin. Cancer Res.* 10: 291.
- Vasudevan DM., Shanavas KR., Kala V., Vijayakumar T. and Kumari TV; Varma AK(Ed) (1991b) In *Oral Oncology*, Mac Millan Press, India. 113.
- Vijayakumar T. and Vasudevan DM. (1985) Cancer *Lett.* 27: 339.
- Vijayakumar T., Ankathil R., Ramani P., Sasidharan VK., Vijayan KK. and Vasudevan DM. (1986) *Cancer Immunol. Immunother.* 22: 76.
- Vitteta ES., Ohara J., Myers CD., Layton JE., Kramer PH. and Paul WE.(1985) *J. Exp. Med.* 162: 1726.
- Vivekanandan S. and Vasudevan DM. (1982) *Ind. J. cancer.* 19: 44
- Vogelstein B. and Kinzler KW. (1993) *Trends in Genetics* 9(4): 138.
- Vokes EE., Weichselbaum RR., Lippman SM. and Hong WK. (1993) *N. Engl. J. Med.* 328:184.
- Wahi PN. (1968) *Bull. World Health Organ.* 38: 495.
- Waldron CA., Gorlin RJ. and Goldman HM. (Eds) (1970) In *Oral epithelial tumors*, Thoma's oral pathology, St. Louis, C.V. Mosby Co, 6.
- Walsh PM. and Epstein JB. (2000) *J. Canadian Dent. Ass.* 66 :22.
- Warnakulasuriya S. (2000) *J. Pathol.* 190: 407,
- Waterhouse J., Muir C., Correa P. and Powell J. (Eds.) (1976) *Cancer incidence in five continents*. International Agency for Research on Cancer. Lyon. IV
- Wentz WB., Heggie AD., Anthony DD. and Reagan JW. (1983) *Science.* 222(4628): 1128.
- Whitley RJ. (1988) *Amer. J. Med.* 85 (2A): 61.
- WHO (1978) *Oral Surg. Oral Med. Oral Path.*. 46: 517.
- Winkelstein W Jr. (1990) *Am. J. Epidemiol* .131: 945.
- Woglom WH. (1929) *Cancer Res.* 4: 129.
- Wolf GT., Hudson JL. and Peterso KA. (1986) *Head Neck surg.* 113: 1200.
- Woodruff JJ. and Genser BM. (1969) *J. Exp. Med.* 129: 551.
- Yeudall WA. (1992) *Eur. J. Cancer.* 28 B: 61.
- Yeudall WA. and Campo MS. (1991) *J. Gen. Virol.* 72: 173.
- Yewdell JW. and Bennink JR. (1992) *Adv. Immunol* 52: 1.

- Yount WJ., Dorner MM., Kunkel HG. and Kabat EA. (1968) *J. Exp. Med.* 127: 633.
- Zur Hausen H. (1977) *Curr. Top. Microbiol. Immunol.* 78: 1
- Zur Hausen H. (1982) *Lancet.* ii: 1370.
- Zur Hausen H.(1991) *Science* 254(5035): 1167.
- Zur Hausen H. and Klein G. (Eds.) (1989) In *Advances in Viral Oncology*, New York, Raven.p 8.