mononuclear phagocyte function in head and neck cancer a.j.m.balm

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ABBREVIATIONS

AFP	-	alpha-feto protein
ALS	-	antilymphocyte serum
BCG	-	Bacillus Calmette-Guérin
CEA	-	carcinoembryonic antigen
CMV	-	cytomegalovirus
ConA	-	concanavelin A
DNCB	-	2,4 dinitro-1-chlorobenzene
EA	-	early antigen;erythrocyte amboceptor (sensitized erythrocytes)
EAC	-	erythrocyte amboceptor complement
EBV	-	Epstein-Barr virus
HEP	-	human encephalotogenic protein
HLA	-	human leucocyte antigen
HSV	-	herpes simplex virus
IgA (G, M)	-	immunoglobulin A (G, M)
Ia	-	I region-associated
IDC	-	interdigitating cell
ip	-	intraperitoneal
Ir	-	immune response
LMI	-	leucocyte migration inhibition
MAF	-	macrophage activating factor
MEM	-	macrophage electrophoretic mobility
MHC	-	major histocompatibility complex
MIF	-	migration inhibitory factor
MLC	-	mixed lymphocyte culture
MNC	-	mononuclear cell suspension
MPS	-	mononuclear phagocyte system
NBT	-	nitroblue tetrazolium
NSE	-	non-specific esterase
PHA	-	phytohaemagglutinin A
PWM	-	pokeweed mitogen
RES	-	reticuloendothelial system
sc	-	subcutaneous
SD	-	standard deviation
SEM	-	standard error of the mean
TAA	-	tumour associated antigen
TSTA	-	tumour specific transplantation antigen
VCA	-	viral capsid antigen

CONTENTS

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to Gea, Thomas, Rachel

PREFACE

For many years the major concern in tumour immunology has been the antitumour activity of antibodies and lymphocytes. More recently mononuclear phagocytes have found to be of importance in initiating and regulating the immune response and in making an important contribution to host's defence against tumourgrowth. This notion makes a study on the role of the mononuclear phagocytes in patients with head and neck cancer worthwhile.

CHAPTER I.

INTRODUCTION

The main purpose of this investigation was to study the relation of the presence of a tumour in the head and neck region and the function of the mononuclear phagocyte system (MPS) in these patients.

In the next three chapters an overview is given on the literature concerning this subject, especially focusing on immunological aspects of cancer in general, mononuclear phagocytes and cancer and immunological aspects of head and neck cancer in particular.

In Chapter V experimental questions are put forward for this study.

Chapter VI presents the results of some functional tests on blood monocytes in head and neck cancer patients e.g. the nitroblue tetrazolium (NBT) dye reduction capacity, chemotactic responsiveness, maturation capacity and ultrastructure. This chapter also describes age-related differences in monocyte function of healthy controls.

In Chapter VII data are reported on a correlation between monocyte chemotactic responsiveness and the number of macrophages infiltrating the tumour area.

The found results led to further tests on the influence of low molecular weight factors derived from head and neck carcinomas on macrophage migration (Chapter VIII).

CHAPTER II

IMMUNOLOGICAL ASPECTS OF CANCER

1. Some historical notes on tumour immunology.

Perhaps the earliest description of immunological resistance to tumour growth is that of Clowes and Baeslack (1905). They described animals in which spontaneous regression of transplanted tumours was a common phenomenon. They demonstrated further that when challenged with tissues from the same line of tumour, those animals whose tumours had regressed were resistant to challenge. This experimental work was biased because of the use of non-inbred strains of animals. Probably only allograft rejection of transplanted tumours had been observed.

Ten years earlier a serotherapy for cancer was described by Hericourt and Richet. They treated 50 patients with anti-tumour sera raised in dogs and in donkeys. Although poorly documented they claimed substantial beneficial effects from this treatment but needless to say no evidence of objective tumour regression was described. In the late 1890's Coley noted a spontaneous regression of a sarcoma of the tonsil following streptococcal infection. He then developed a bacterial vaccine which was a mixture of heat killed *Streptococcus pyogenes* and *Serratia marcescens*. Patients who received at least 7 injections of this vaccine following surgery appeared to have an improved survival compared to patients untreated after surgery.

In 1926 Murphy first described the participation of lymphocytes in the immunological reactions of the host to an implanted tumour. In that period this was a radical idea because only serum-mediated mechanisms were supposed to be involved in immunological reactions.

Only when syngeneic tumours are used can tumour antigenicity be studied and it was the introduction of inbred mouse strains which allowed Gross in 1943 to produce evidence for specific antigenicity of experimental tumours. He induced sarcomas by methylcholanthrene and transplanted them in syngeneic mice with

intradermal inoculation. About 20% of the intradermal tumours grew for a while and then regressed. Gross was able to show that mice in which the tumour had regressed were resistant to subsequent challenge with the same tumour line, indicating a specific antitumour reaction.

In 1953 Foley confirmed the work of Gross by challenging mice whose tumours had been excised. Later Prehn and Main (1957) showed that transplantation resistance induced in the strain of origin was a tumour specific phenomenon since the survival of intra-skin grafts was unimpaired in tumour immune hosts, thereby eliminating the involvement of allo-antigenic responses.

The foregoing remarks on the history of tumour immunology demonstrate that interest in the immune pathogenesis of tumours dates from the beginning of this century. The principles of tumour immunology await further clarification but continue to gain acceptance as new insights emerge in parallel with the growth of basic immunology.

2. The concept of immunological surveillance.

In 1959, Thomas, in a discussion on the nature of delayed hypersensitivity and homograft immunity spoke of the "universal requirement of multi-cellular organisms to preserve uniformity of cell type" and suggested that the "phenomena of homograft reaction will turn out to represent a primary mechanism for natural defence against neoplasia". This point of view was subsequently supported by Burnet (1967) and referred to under the name "immunological surveillance". This concept which suggests that host's immunesystem monitors cell replication with the elimination of abnormal cells, soon became widely accepted.

There are clinical data available which tend to confirm the existance of immunological surveillance mechanisms:

A. Immune deficiency and cancer.

It is well known that immune deficient patients have an increased cancer incidence and in the case of kidney grafted

patients taking immunosuppressive drugs the frequency as a high as 1% (Möller and Möller, 1978). Penn (1975) reported an overall corrected incidence of 5.6% compared with 0,058% for a normal aged-matched population. Similarly patients with natural immune deficiencies as congenital thymic aplasia and the Wiscott-Aldrich syndrome also show an increased incidence of tumour growth. The increased incidence of tumours at the extremes of human life (Castro, 1978), the depressed rejection of Moloney sarcomas in aged mice (Stutman, 1976) and the well known immunologic deficiencies in senescence as reported by Price and Makinodan (1972) also support the immunological surveillance hypothesis.

It is striking that the histological types of tumours arising in transplanted patients are markedly different from the normal population. Immunedeficient patients also exhibit a very restricted range of neoplasms: approximately 60% of the tumours are of lymphoid origin. In contrast there is no increase in a common human tumour like mammary carcinoma (Möller and Möller, 1978). This restricted range of tumour type weakens the concept of immunological surveillance, which implies that multiple tumours will develop in different organs. In addition tumour recurrences in patients treated with immunosuppressive drugs are nearly always regrowths of the original tumour and no new tumours arise. Based on this notion (Möller and Möller, 1976) suggest an involvement of a genetic mechanism in the induction of tumours. The observation of Rygaard and Povlsen (1976) that nude mice do not develop spontaneous tumours does not support the surveillance theory either. However, it may be that the protected environment used for these mice, because of their susceptibility to infections, may prevent viral infections leading to tumour growth.

The greatly increased incidence of Kaposi's sarcoma among recipients of renal allografts (Myers et al, 1974; Stribling et al, 1978; Harwood et al, 1979; Penn, 1979) and others receiving immunosuppressive therapy (Gange and Jones, 1978; Klepp et al, 1978; Hoshaw and Schwartz, 1980) re-emphasizes the relation between deficiencies in the immune system and

the development of cancer.

B. Spontaneous regressions.

There are convincing reports of spontaneous regression of established human tumours. Everson and Cole,(1966) reported regressions of neuroblastoma, hypernephroma, choriocarcinoma and melanoma. These findings may support the idea of defensive reactions to human tumours, although they need to be immunological determined.

C. Post-mortem prevalence.

Another possible evidence for a defensive reaction of the host against neoplasia may be the clinical pathological evidence that cancer cells may persist in the body for many years without the development of an overt malignancy and that many more malignant cells escape into the blood than ever give rise to progressive metastases. The reasons for this resistance are obscure and could well be other than pure immunological. It remains of considerable interest that there are at least three types of malignant tumours which are much more frequently diagnosed histologically in random pathological material, than they are found clinically in comparable populations. The three examples are: neuroblastoma of the adrenal in children (Beckwith and Perrin, 1963), thyroid carcinoma (Mortensen et al, 1955) and carcinoma of the prostate (Ashley, 1965).

The concept of immunological surveillance in its original form remains a theoretical explanation for the observation that tumours develop in man, since immunedeficient patients exhibit a restricted range of tumours (p.7), and several diseases with well known, though restricted immunesuppression like leprosy and sarcoidosis do not show an increased incidence of tumours (Stutman, 1975; Melief and Schwartz, 1975). In addition naturally T-cell immunedeficient mice (nude mice) or those made deficient by administration of antilymphocyte serum (ALS) do not show an increased incidence of tumours (p.7), although they are more susceptible to infections and viral oncogenesis. Such observations

whilst arguing against a surveillance mechanism mediated by T-lymphocytes (footnote 1, p.51) do not exclude immunological control of tumour growth executed by a non-T-lymphocyte population, perhaps macrophages.

These data from literature do not directly support a concept of immunological surveillance, they may, however, indicate the importance of immunological defence during the actual growth of malignant cells.

3. The concept of stimulation of the immune system in cancer.

Methods which stimulate immunity have been associated with a reduction in tumour growth. The scientific rationale for immune stimulation is based on the assumption that cancers may develop and proliferate as a result of a failure in the equilibrium between the proliferation of neoplastic cells and the ability of body's immunological mechanisms to control this process (Turk, 1978). In the case of tumours induced by viruses and chemical agents it is logical to attempt to restore the state of equilibrium by eliminating the cause of the development of mutant neoplastic cells. However, other cancers develop as spontaneous mutations and may be allowed to proliferate owing to a defect in the body's immunological mechanisms. Under these conditions it is logical to try to potentiate the body's immunological mechanisms in an attempt to restore the state of equilibium.

In the late 1950's Old et al demonstrated that bacillus Calmette-Guérin (BCG) was an active anti-tumour agent in mice by increasing the latent period of sarcoma induction by methylcholanthrene. In the 1960's BCG emerged as a prototype for the development of active non-specific immunotherapy. Morton and co-workers (1970) demonstrated that intralesional viable BCG organisms could lead to regression of metastatic intradermal deposits of malignant melanoma. Similarly Cohen et al (1976) reported regression of 119 out of 173 BCG injected nodules in 4 patients with melanoma and that in 25% of patients a complete regression was achieved. Rejection of

uninjected nodules occurred only in 15-20% of patients. Subsequently Mavligit and co-workers (1976, 1977) have reported significant prolongation of disease-free-interval and survival in 112 patients with Dukes C carcinoma of the colon and rectum who received adjuvant immunotherapy with BCG by scarification of chemo-immunotherapy with oral 5-fluoro-uracil and BCG postoperatively.

In addition to mycobacteria clinical investigations of the effect of corynebacterium organisms have been performed with success. In particular corynebacterium parvum has shown potent antitumour effects in a variety of animal systems and intravenous application of this organism appeared to have much greater activity against artificially induced pulmonary metastases in a murine fibrosarcoma model compared to subcutaneous applied corynebacterium parvum (Milas et al, 1974). In man, however, intravenous injection of corynebacterium parvum can be dangerous. Von Blomberg et al (1980) described harmful side effects of intravenous corynebacterium parvum in patients with inoperable lung carcinoma and in addition she found that survival times shortened significantly. On the other hand Mignot et al (1980) described beneficial effects of a single neighbourhood injection of 2 mg corynebacterium parvum in patients who received radical surgery for carcinoma of the uterine cervix. In their follow-up a significantly low relapse rate of 5% was established in the corynebacterium parvum treated group compared to a relapse rate of 29% in the controls. Zighelboim et al (1979) could not improve patient's immunereactivity with weekly administration of corynebacterium parvum in combination with chemotherapy in patients with head and neck malignancies. In addition Cheng et al (1982) could not achieve an improvement in the frequence of survival when corynebacterium parvum was used as adjuvant immunotherapy in head and neck cancer patients who received radiation therapy. They administered corynebacterium parvum into regional lymphnodes as well as by the intravenous route.

In view of the preceding brief summary on non-specific immune stimulation in cancer patients we may state that the immune system has a role to play in host defence against neoplasia, though it is not clear what exact role is played by it. There is now some evidence available which shows that improved host defence to cancer induced by non-specific immune stimulation is mediated by increased macrophage function. *(see also Chapter III, p.35).*

4. Evidence for tumour associated antigens (TAA).

One of the major aspects of cancer immunology lies in the actual detection of characteristic tumour antigens in tumour tissues and in body fluids of cancer patients. Only two tumour antigens, the carcino-embryonic antigen (CEA) and alpha-foetoprotein (AFP) have been well characterized and their clinical role extensively explored. These molecules are examples of antigenic reversion, in as much as they are normally present in human fetal and embryonic life but their synthesis is almost entirely repressed in differentiated cells. Synthesis of these molecules can, however, be reinitiated in the process of malignant transformation. Although sensitive and reproducible the radioimmunoassays used to detect these antigens suffer from a lack of specificity, since a variety of benign disorders are also associated with elevated levels of CEA (Egan et al, 1977). Only in colo-rectal cancer a fall in CEA values to a normal range indicate a radical surgical removal (Shuster et al, 1978). In addition the determination of the CEA level is of importance in postoperative follow-up because increases may be a sign of recurrence.

The highest incidence and levels of circulating AFP are seen in patients suffering from primary hepatoma, 90% positive, and in patients with embryonal carcinomas of the ovary and testis, 75% positive. Seldom are elevations of AFP observed with other tumour types (Ruoslahti et al, 1974). AFP appears also to be important in prenatal diagnosis, because elevated amniotic fluid AFP levels appear to correlate extremely well

with the presence of open neural tube defects (Milunsky and Alpert, 1976).

The existance of TAA's is also demonstrated by the presence of lymphocyte sensitization to these tumour associated antigens. If lymphocytes show killing or growth inhibition of tumour target cells in vitro it is arguable, though difficult to prove, that the same cell might exert a similar function in vivo. The initial technique to demonstrate sensitized lymphocytes to malignant cells was a colony inhibition technique designed by Hellström and Sjögren (1965) in a mouse sarcoma model. In this technique tissue cultured colonies of replicating tumour cells and non-tumour cells were exposed to lymphocytes from tumour bearers and suitable control sources and the number of colonies surviving this confrontation was assessed by visual counting from three uptil five days afterwards. In the hands of its originators this proved to be a very useful and reproducable technique and selective tumour cell colony inhibiting activity was demonstrated with the lymphocytes of patients with neuroblastoma and other malignancies including melanomas, sarcomas and cancer of colon, testis, endometrium, ovary and female breast (Hellström et al, 1971). The technology of this test is extremely demanding and few laboratories have been able to establish it in routine use.

TAA's might also be induced by viruses. In man the best evidence for such virus induced antigens is in Burkitt's lymphoma of African children. Two decades have passed since Denis Burkitt, a British surgeon working in Uganda, originally described the tumour that now bears his name. Classical descriptions of Burkitt's lymphoma define a distinct syndrome of large extranodal tumours affecting the bones of the jaws and abdominal viscera - mainly the kidneys, ovaries and retroperitoneal structures (Wright, 1970). It is a neoplasm of B-lymphocytes characterized by the presence of immunoglobulin and other B-cell markers on the cell surface (Mann, 1976). In 1964 Epstein et al desbribed a herpes-like virus in electron micrographs of a Burkitt's lymphoma cell-line.

African patients with Burkitt's lymphoma were found to have significantly elevated antibody titers to a variety of Epstein-Barr virus (EBV) determined antigens (Henle et al, 1969). A sero-epidemiological study of de-Thé et al (1978) revealed markedly higher EBV antibody titers in children in whom Burkitt's lymphoma later developed, ruling out an acute EBV infection as a proximate cause of Burkitt's lymphoma. This is consistent with the idea that EBV is an innocent "passenger", coincidently residing in a B-cell that undergoes transformation by other causes unrelated to EBV (Ziegler et al, 1977). In this respect probably chronic malaria acts as an etiologic cofactor since O'Connor (1970) reported a geographical incidence of Burkitt's lymphoma and hyperendemic or holo-endemic malaria.

EBV is an ubiquitous virus and infects the majority of all adult human populations in all countries (Niederman et al, 1970). In 1978 EBV was shown by Niederman et al to be the cause of infectious mononucleosis. Despite the unclear pathogenesis of the infectious mononucleosis syndrome, infectious EBV can be regularly recovered from throat washings of patients with the disease. (Gerber et al, 1972; Miller et al, 1973).

Remarks about the possible role of EBV in human cancer would not be complete without considering the relation between EBV and nasopharyngeal carcinoma (see also Chapter IV), which is mostly found in the Chinese population. These tumours also express EBV determined antigens (Klein et al, 1974) and antibodies against it are associated with these tumours (Sako et al, 1975).

Although all three diseases, infectious mononucleosis, Burkitt' lymphoma and nasopharyngeal carcinoma are supposed to be associated with EBV infections, the exact role in the pathogenesis is far from clear. One could hypothesize that the same EBV is a primary infective agent and that the manifestations of the infection depend upon local modifying factors and/or genetic factors. In this respect EBV infection in a healthy European may lead to infectious mononucleosis, whereas an African child with a chronic malaria develops a Burkitt's lymphoma and a Chinese person nasopharyngeal carcinoma.

In Kaposi's sarcoma a viral origin has also been suggested. Giraldo et al (1972) first suggested an association between Kaposi's sarcoma and cytomegalovirus (CMV) infection. The evidence for a viral etiology also includes the high prevalence of CMV antibodies among patients with Kaposi's sarcoma (Giraldo et al, 1975) and the incorporation of a CMV genome into Kaposi's tumour cells (Giraldo et al, 1980).

There is also an increasing body of evidence that uterine cervix carcinoma (Adam et al, 1974; Dreesman et al, 1980) and squamous cell carcinoma of the vulva (Kaufman et al, 1981) is associated with herpes simplex virus (HSV). HSV is a major cause of sexually transmitted disease at present and there is a parallel in the incidence of HSV infection and carcinoma of the female genital tract. The role of the virus in this transformation warrants further investigation.

In conclusion it can l ted that evidence is accumulating for the presence of TAA's in human malignancy (see also Chapter IV, p.45). If tumours bear these antigens and the patient's immune system responds to them , it is highly probable that there is a continuing interaction between tumour cells and their antigens on the one hand and the immune system on the other. This interaction may form the subject of investigations by determining corresponding functional activities of lymphocytes and macrophages.

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CHAPTER III

MONONUCLEAR PHAGOCYTES AND CANCER

1. Cells of the mononuclear phagocyte system (MPS) and their function in general.

The MPS is of great importance in host defence. Mononuclear phagocytes are derived from the bone marrow. They leave the bone marrow as monocytes, which circulate in the blood. Upon diapedesis from the vessels these cells are able to transform into specialized tissue macrophages, Kuppfer cells in the liver, alveolar macrophages in the lung and histiocytes in the connective tissue (Fig.1) (van Furth, 1980). Each of these mononuclear phagocytes has its own functional and biochemical characteristics.

FUNCTIONS

A. Phagocytosis

Microbial phagocytosis and killing are basic functions of phagocytes and defects in these function lead to increased incidence of infections and has been described in chronic granulomatous disease (David et al, 1968) myelomonocytic leucaemia (Cline, 1973) and after corticosteroid administration (Rinehart, 1975).

Phagocytosis is the best known of all the functions of the mononuclear phagocyte. During an inflammatory response the following events in the MPS will take place. Firstly the number of circulating monocytes increases, due to recruitment from the bone marrow. Secondly the cells start to migrate to the site of inflammation by penetrating through the endothelial cells of the blood vessels, a process that is called diapedesis. Upon arrival phagocytes move towards the foreign agent, or damaged tissues, a process known as chemotaxis. Well known chemotactic factors are products of stimulated phagocytes and lymphocytes themselves, complement components (Ward et al, 1969) some

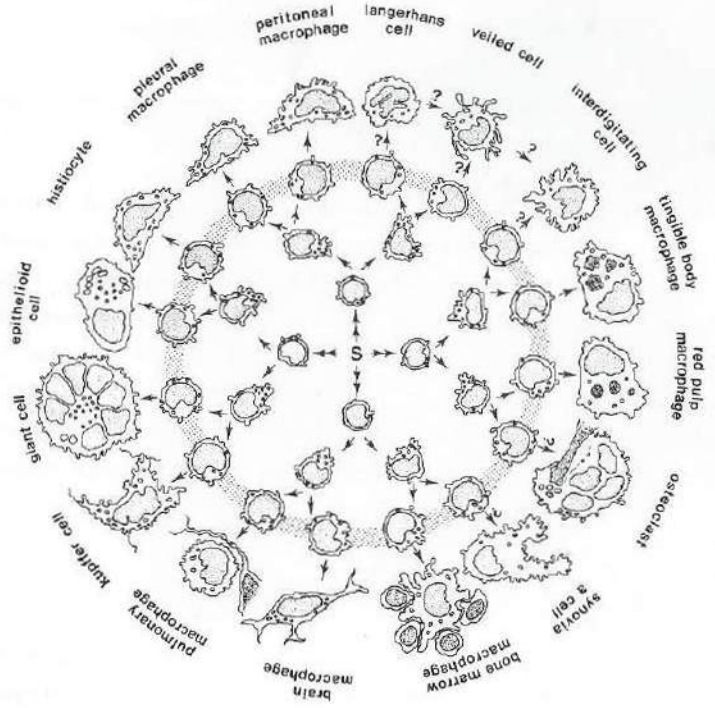


Fig. 1. Schematic representation of the mononuclear phagocyte system (MPS). By cell division of monoblasts (inner ring), promonocytes (second ring) are formed, which form monocytes (shaded ring) by division. Monocytes differentiate into macrophages (outer ring) with their different names and/or functions in different tissues. S = hemapoietic stem cell. ? = evidence to include these cells in the MPS is not yet conclusive. (Design and drawing by J.W.M. van der Meer, reproduced with his permission from "Biological Properties of bone marrow mononuclear phagocytes in long-term cultures, Ph.D. thesis, University Leiden, the Netherlands, 1982).

clotting factors (Weiss et al, 1973), but also bacterial products. Once at the inflammatory site micro-organisms, cell debris etc. can be phagocytosed. Although these materials are easily ingested by mononuclear phagocytes, the most efficient uptake is under the influence of serum factors called opsonins. Opsonisation is the process by which particles, for instance micro-organisms are covered by specific antibodies of the IgG or IgM class, with or without complement involvement; or by complement alone. The most important complement component involved is C3b (Stossel, 1976). Opsonized particles are recognized by specific receptors on the surface of phagocytic cells: Fc receptors (Messner and Jelinek, 1970) and C3b receptors for the C3b fragment of the complement system (Bianco, 1977). Nonspecific receptors exist mediating phagocytosis of non-opsonized particles (van Furth and Leyh, 1981) (Fig.2).

Attachment of an opsonized particle to a phagocytic cell is accompanied by the formation of pseudopodia, which protrude round and enclose the particle forming a membranous vacuole: the phagocytic vacuole or phagosome. Lysosomes fuse with these vacuoles allowing a discharge of a whole variety of hydrolytic enzymes such as acid phosphatase, naphtol AS acetate esterase, glucuronidase and others (Carr, 1978). Phagocytosis induces an increased respiratory activity: "the respiratory burst", which is the higher oxygen consumption, an increase in pentose shunt activity and the production of superoxide(O_2^-) and hydrogenperoxide (H_2O_2) (Reiss and Roos, 1978), which are essential for bacterial killing (Fig.3).

The importance of these products for the killing of certain types of micro-organisms is indicated by the inability of monocytes of patients with chronic granulomatous disease to kill several micro-organisms, such as candida albicans (Lehrer, 1970; 1975).

PHAGOCYTOSIS OF MONONUCLEAR PHAGOCYTE

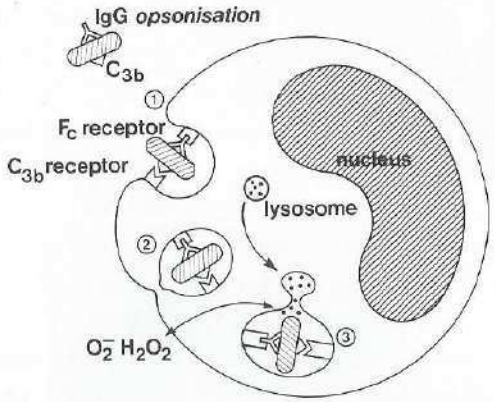
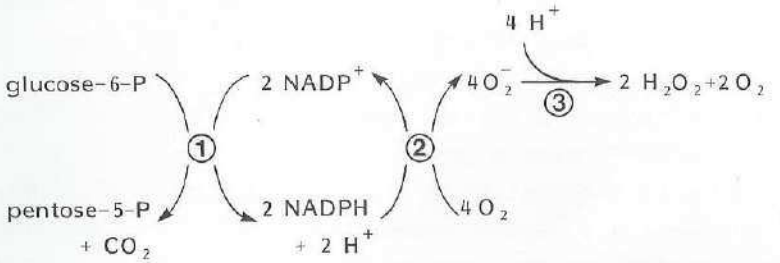


Fig. 2. Schematic representation of the phagocytic process.
1. The mononuclear phagocyte surrounds an opsonized bacterium mostly following attachment via Fc and/or C3b receptors.
2. Phagosomes are formed.
3. A lysosome fuses with the phagosome forming a phago-lysosome. Enzymes (amongst which peroxydases and hydrolases) are released leading to bacterial death and digestion in co-operation with antimicrobial oxygen products (O_2^- and H_2O_2).

B. Antigen presentation.

The cells of the MPS represent the non-specific element in immune responsiveness in as much as they pick up any antigen and present it to lymphocytes in a manner that allows a suitable antigen specific response, possibly by altering concentration, presenting multivalent forms (Mosier, 1976) or combining it with structures of human leucocyte antigen (HLA)-D(r) nature, coded for by the major histocompatibility complex (MHC) (Unanue, 1980). The lymphocytes on the other hand

PENTOSE SHUNT AND O_2^- + H_2O_2 FORMATION



① glucose-6-phosphate dehydrogenase + 6-phosphogluconate dehydrogenase

② NADPH oxidase

③ spontaneous reaction

Fig. 3. Schematic representation of the pentose shunt and the generation of antimicrobial oxygen products. The reducing equivalents are generated in the pentose shunt and reduce NADP+ into NADPH, which subsequently reduces oxygen into O_2^- using an NADPH-oxidase. In the phagosome O_2^- reacts spontaneously to H_2O_2, which is essential for bacterial killing.

are the cells responsible for exquisite antigen specificity and for the secondary or anamnestic responses - the basis of prophylactic immunizations. Due to this mechanism in vivo impairment of macrophage function has also an impact on specific immunological competence: in 1974 Twomey et al described a case of an adult man with mucocutaneous candidiasis, cutaneous anergy and impaired in vitro lymphoproliferative responsiveness to soluble antigens; his lymphocytes could be stimulated to incorporate ^{3}H-thymidine by Monilia antigen when cultured with monocytes from a control donor, but not in the presence of his own monocytes. Conversely monocytes from the patient were

unable to mediate a prolifetarive response of purified lymphocytes from a sensitized control donor. Nelson (1974) has subsequently presented other patients with chronic mucocutaneous candidiasis with a similar impairment of macrophage function.

It is of interest that macrophage and lymphocyte functions are highly interdependent and that these functions are integrated by the action of a set op genes of the MHC-locus. These genes termed immune-response genes (Ir-genes) are known to control lymphocyte recognition by expressing special determinants (Ia antigens = I region associated antigen), probably HLA-D(r) structures, on the surface of the cell membrane (Cullen et al, 1974). Probably only those mononuclear phagocytes which bear Ia molecules are capable of co-operating with lymphocytes. Lymphocytes will establish tenacious contact with these cells (Lipcombs et al, 1977) and this intimate contact is the first essential step in an immune reaction. A special family of cells probably belonging to the MPS namely the epidermal Langerhans cells, the lymph-borne veiled cells and the lymphnode interdigitating cells (IDC's) strongly express these Ia antigens (Rowden, 1977; Klareskog et al, 1977) and are able to form active cellular contact with lymphocytes in their vicinity (Drexhage et al, 1980)[1].

C. Secretory functions.

One of the other biological activities of cells belonging to the MPS that has been recognized in the last few years is their secretory capacity. Macrophages may synthesize and secrete a variety of biologically active products into their extracellular environment. These macrophage products are involved in inflammatory processes: as for instance lysozymes

1) Langerhans cells, veiled cells and interdigitating cells (IDC's) are cells, probably belonging to the MPS. They are involved in the antigen presentation to T-cells. These cells have now been described to be present in skin (Langerhans cells), lymph (veiled cells), lymph nodes (IDC) and Peyer's patches of the gut.

for bacteriolysis, several neutral proteinases for degrading vessel walls and artificial surfaces (collagenease and elastase) and colony stimulating factors which influence the growth and function of bone marrow stem cells and lymphocytes (Nathan et al, 1980).

D. Cytotoxicity

Macrophages may also exert cytotoxic activities against tumour cells. This functional aspect will be discussed in more detail on page 38.

TESTS OF MACROPHAGE FUNCTION

A. Chemotaxis.

The determination of the chemotactic activity of mononuclear phagocytes is an assay system used to get informed on migratory capacities of mononuclear phagocytes in clinical situations. The assays are performed using a millipore filter system in which cells are exposed to a concentration gradient of a chemotactic factor (Fig.4).

B. Nitroblue tetrazolium (NBT)-dye reduction.

The activity of the pentose shunt is also used as a parameter for the functional activity of the peripheral blood monocyte. NBT acts as a hydrogen acceptor in this test, which on reduction by products of the pentose shunt forms coloured formazan. The test was formally in use as a clinical assay to indicate the presence of pyogenic infections by examining peripheral blood neutrophils (Park et al, 1968). At present this test is of little clinical importance due to the high incidence of false positive results (Segal, 1974) (Fig.5).

C. Maturation.

Maturation of monocytes into spready, adhered macrophages is induced in vitro by culturing peripheral blood monocytes under standard conditions in microtiterplates during a 7 days period according to techniques described by Currie and Hedley (1977). The attached and spread cells - the macrophages - are

identified by staining them with crystal violet. The number of monocytes becoming such cells (the "macrophage precursors") will give another indication for the well functioning of mononuclear phagocytes.

The assays for chemotaxis, NBT-dye reduction and maturation only give a general impression of the ability of peripheral mononuclear phagocytes to act in host defence. Detailed description of the test methods are given in the material and method sections of chapter VI

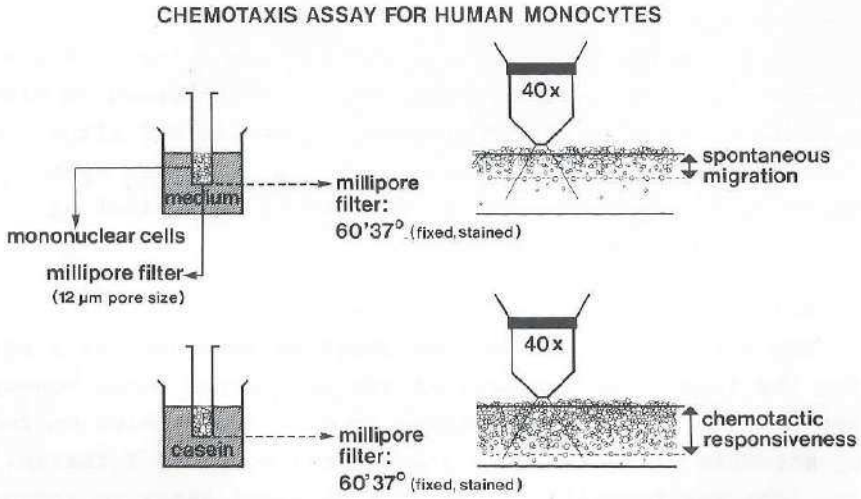


Fig. 4. Schematic representation of monocyte chemotaxis in vitro using Boyden chambers (left). In this technique, the migration of cells across the millipore filter towards a chemotactic substance (casein) is quantified by counting the number of cells which traverse the filter in a certain time (Wilkinson, 1974). The depth where only two monocytes are seen in a x 40 microscopic field, is taken as the distance of migration (leading front method - Zigmond and Hirsch, 1973).

NBT-DYE REDUCTION

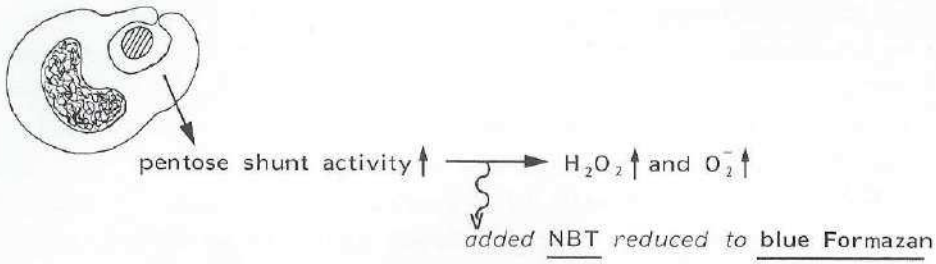


Fig. 5. Schematic representation of nitrobluetetrazolium (NBT) dye-reduction by human monocytes. NBT is a clear yellow,water soluble compound that on reduction forms the precipitate formazan, a deep blue dye, which can be easily measured spectrophotometrically (Alföldy and Lemmel, 1979). This forms the basis of an indirect measurement of pentose shunt activity.

MONOCYTE MATURATION

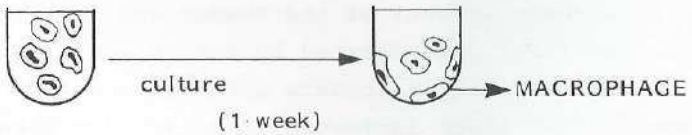


Fig. 6. Schematic representation of monocyte maturation. A mononuclear leucocyte suspension containing lymphocytes and monocytes after separation on a ficoll-isopaque gradient is maintained in culture for 7 days under standard conditions. After removing the supernatant with nonadherent cells the remaining attached cells are stained with crystal violet and considered as "Macrophages" (Currie and Hedley, 1977).

2. Mononuclear phagocytes and cancer.

The importance of the reticulo-endothelial system (RES), which nowadays is known as the mononuclear phagocyte system (MPS), in the defence against neoplastic disease was stressed by Stern as early as 1941. He noted a depressed function of the RES in cancer patients and was able to correlate such as depressed function with both severity of disease and lack of clinical response to therapy. Seven years later Stern (1948) reported that inbred strains of mice known to have a high incidence of cancer also showed depressed activity of the RES when compared to low cancer strains. In 1959 Old et al demonstrated that mice with chronic BCG infections which as a consequence showed an enhanced function of the RES also had an increased resistance to neoplastic growth compared to normal animals. Similar results were also reported by Zbar et al (1970) in guineapigs with BCG infection and mice with toxoplasma infection (Hibbs et al, 1971). An in vitro analogue of this phenomenon is the activation of peritoneal macrophages from mice chronically infected with BCG or toxoplasma gondii, which selectively kill syngeneic, allogeneic or xenogeneic tumour cells in vitro by non-phagocytic mechanisms (Hibbs et al, 1972; Meltzer et al, 1975).

Number of macrophages in tumours: A direct relationship between macrophage content of the tumour and its biological behaviour was first demonstrated by Eccles and Alexander (1974) who found that rat fibrosarcomata with the highest content of macrophages had a lower incidence of metastases than those with a low macrophage content. Similarly Russell et al (1976) reported that spontaneously regressing Moloney sarcomas had more macrophages per gram tumour tissue than advancing sarcomas. In 1977 Lauder et al assessed the number of macrophages in 50 cases of breast carcinoma and described significantly fewer cases with metastases among those with high macrophage and plasma cell scores.

Such a positive correlation between macrophage content and tumour prognosis was also made by Gauci and Alexander (1975). Other authors could not confirm such relationships (Evans and Lawler, 1980; Talmadge et al, 1981). A more direct evidence for the regularoty role of macrophages in the growth and metastasis of tumours was described by Wood and Gillespie (1975) who found signigicantly shortened survival times in mice injected with macrophage-depleted-tumour-cell-suspensions.

Chemotaxis: One of the most important characteristics of macrophages which is necessary for an effective host defence is their capacity to migrate into sites of malignant growth. There is now an increasing body of evidence concerning defects in just that important function of the mononuclear phagocyte in cases of malignancy. Defective chemotaxis has been found in patients with different carcinomas like genito-urinary tract neoplasms (Hausman et al, 1975), other forms of cancer e.g. melanoma, adenocarcinoma of the gut (Boetcher and Leonard, 1974), cancer of the lung and prostate (Kjeldsberg and Pay, 1978) and in mice given tumour implants (Snyderman et al. 1975; Stevenson and Meltzer, 1975). It is of great interest that these abnormalities in monocyte chemotactic responsiveness in cancer patients revert to normal upon surgical removal of the tumour (Snyderman and Stahl, 1975; Snyderman et al, 1977; 1978). This restoration of chemotaxis has been interpreted as implying that neoplasms themselves are capable of causing abnormal monocyte chemotaxis. In laboratory animals macrophage accumulation in vivo in response to an inflammatory stimulus was indeed markedly depressed by the administration of tumour cells as was the in vitro chemotactic activity of peritoneal macrophages (Snyderman et al, 1976; 1978; Stevenson and Meltzer, 1976; Normann and Sorkin, 1977). Further studies on tumour mediated effects on monocyte chemotaxis revealed that murine neoplastic cells were found to contain an extremely potent low

molecular weight factor capable of depressing macrophage accumulation in vivo and chemotaxis in vitro (Snyderman and Pike, 1976; Pike and Snyderman, 1976). Supernatants of mouse, rat and human fibrosarcoma cells also contained products between 10^3 and 10^4 Daltons, inhibiting both spontaneous macrophage migration and chemotaxis in vitro and also depressed delayed hypersensitivity skin reactions in mice (Nelson and Nelson, 1978). In addition factors in the supernatant of Lewis lung carcinoma (a murine tumour cell line) and in the sera of tumour hosts were found to inhibit the migration of macrophage towards casein (Otu et al, 1977). The macrophage mediated antibacterial resistance was also affected by a serum factor of less than 12.000 daltons obtained from tumour bearing mice (North et al, 1976).

Cytotoxicity: Once at the tumour site macrophages may exert cytotoxic activities against tumour cells. Evans and Alexander (1976) suggested that these processes occur primarily extracellularly and that phagocytosis, if it occurs at all, is a late event that follows death and desintegration of malignant cells. Earlier (1970) these authors reported that in some instances macrophage cytotoxicity is antigen specific: in such cases it is directed against syngeneic tumours by virtue of the recognition of tumour specific transplantation antigens (TSTA) present on the surface of malignant cells. They isolated macrophages from the peritoneal cavity of mice which had been immunized against syngeneic tumours and demonstrated in vitro antigen specific cytotoxicity. Specific cytotoxicity to other malignancies such as allogeneic lymphoma or hepatoma cells was subsequently demonstrated by several other authors including in the Netherlands den Otter et al (1972) and van Loveren and den Otter (1974). It is highly probable that antigen specific macrophage cytotoxicity is dependent on co-operation with sensitized lymphocytes, because humoral factors derived from lymphocytes previously sensitized to tumour antigens, are able to arm macrophages in an immunologically specific way (Evans and Alexander, 1972).

Besides antigen specific cytotoxicity human mononuclear phagocytes may show natural non-specific cytotoxicity to both allogeneic (Vose, 1978) and xenogeneic tumour cells (Mantovani et al, 1979a; 1979b). The cells display the same natural cytotoxicity regardless from which organ they originate (Mantovani et al, 1980), though human alveolar macrophages are only cytostatic (Bordignon et al, 1980). Murine macrophages must be activated by BCG or other biological substances to become cytotoxic in a non-specific way (Olivotto and Bomford, 1974; Hibbs, 1974). There is clinical evidence that natural non-specific cytotoxicity is markedly enhanced by the appropriate administration of corynebacterium parvum or BCG (Scott, 1974; Cleveland et al, 1974). Although the process of cytotoxicity itself is difficult to understand, Adams (1982) reported that it is mediated by cytolytic proteases released by macrophages stimulated by a lymphokine (MAF=macrophage activating factor) in combination with endotoxin. This protease effect was synergistically potentiated by H_2O_2 (Adams et al, 1981).

It is also difficult to relate the various cytotoxic activities found in vitro to host protection in vivo. To elucidate this discrepancy Russell and McIntosh (1977) made a comparison between the in vitro cytotoxicity and the behaviour of the tumour. They found that macrophages isolated from regressing Moloney sarcomas exhibit a stronger cytotoxicity than those recovered from progressing sarcomas. Reduced efficiency of tumour cell killing in progressively growing sarcomas was also demonstrated by Taniyama and Holden (1979).

Other functions: In contrast to the defective chemotaxis of human monocytes in tumour bearing hosts, assays for the lysis of antibody coated red cells showed significantly greater lytic activity of monocytes in melanoma patients (Nyholm and Currie, 1978). Hedley and Currie (1978) investigated the NBT-dye reduction of peripheral monocytes in melanoma patients and reported an increased NBT-dye reduction - after latex stimulation - in patients with micrometastatic disease, whereas

in overt dissiminated cases this function was depressed. The increased expression of monocyte receptors for IgG (Fc receptors), which may play a regulatory role in the immune response, also indicate a form of cellular activation in patients with solid tumours (Rhodes, 1977). On the other hand the in vitro maturation of peripheral monocytes in melanoma patients (Currie and Hedley, 1977) and primary breast cancer (Taylor and Currie, 1977) was found to be depressed. Another defect of monocyte function in cancer patients was reported by Kleinerman et al (1980). They found a depressed spontaneous monocyte mediated cytotoxicity which was restored during chemotherapy.

From the previous chapter it may be evident that the significance of mononuclear phagocytes in cancer immunology is not fully understood: some functions of these cells are depressed, for instance chemotaxis, maturation capacity and NBT-dye reduction (in dissiminated disease) whereas others are increased as for instance erythrolysis and expression of Fc receptors on the cell membranes. Functional aspects of these cells in cancer patients therefore need further study.

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CHAPTER IV

IMMUNOLOGICAL ASPECTS OF HEAD AND NECK CANCER

1. Evidence for tumour associated antigens (TAA).

A somewhat tentative summary of the existing literature based on available evidence would conclude that once a tumour is established mononuclear phagocytes could be instrumental in limiting the dissimination of disease. This will require a specific recognition of TAA's. In the next chapter the presence of these - in some cases probably virus induced - antigens in head and neck cancer will be discussed.

The discovery of Epstein et al in 1964 that lymphoid cells of Burkitt's lymphoma contain a herpes virus was some years later followed by a report of Old and associates (1966), who for the first time observed the presence of precipitating antibodies to EBV-related antigens in patients with nasopharyngeal carcinoma. Subsequent reports have demonstrated the presence of high titers of antibodies to EBV antigens in the sera of nasopharyngeal carcinoma patients throughout the world (Henle et al, 1970; 1971; de Schrijver et al, 1972). The specificity of these EBV associated antigens is such that high antibody titers to viral capsid antigens (VCA)[1] and early antigen (EA)[1] in the serum IgA fraction are usually associated

1) VCA is localized in the viral capsid of EBV and develops at the end of the viral cycle. The presence of antibodies against VCA is indicative of an infection with EBV.

EA develops in the early phase of the viral cycle and antibodies against it arise immediately after an infection with EBV. A high titer is indicative of an acute infection. In case of Burkitt's lymphoma or nasopharyngeal carcinoma, it indicates the growth of EBV containing tumour cells (Hilgers, 1977).

with nasopharyngeal carcinoma (Henle and Henle, 1976). Wara et al (1975) and Coates et al (1978a) also found a positive correlation in the presence of nasopharyngeal carcinoma and the level of EBV antibodies of the IgA class. Decreasing antibody titers were found to be correlated with clinical remission. A direct relationship between nasopharyngeal carcinoma and EBV was made when the virus was detected in biopsy specimens (Zurhausen et al, 1970; Nonoyama et al, 1973).

High titers of EBV specific IgA serum antibodies and antibodies to EA are uncommon in patients with neoplasms in other regions of the head and neck and in the normal population (de Schrijver et al, 1972; Henle and Henle, 1976). When these EBV antibodies are detected in patients who do not have nasopharyngeal carcinoma, titers are generally much lower.

In a study of EBV specific IgA serum antibodies in non-American patients with nasopharyngeal carcinoma, these antibodies were present, often at high titers, in 93% of the untreated patients but less than 5% of patients with other untreated head and neck carcinomas and normal controls had VCA specific IgA (Henle and Henle, 1976). This is consistent with the findings of Sessions et al (1974) who neither could demonstrate antibodies to EBV in head and neck carcinomas such as hypopharynx and larynx carcinoma. Only in tonsillar carcinoma EBV antibodies have been reported (Vonka et al, 1976). Henle and Henle (1976) also demonstrated a positive correlation between levels of EBV specific IgA and the chance of recurrence. In 1978 (b) Coates et al reported the development of nasopharyngeal carcinoma in one out of 11 patients who showed raised antibody titers to EBV.

Raised antibody titers to EA were also found to be indicative of active untreated or recurrent nasopharyngeal carcinoma (Coates et al, 1978a). These authors therefore suggest that the assessment of EBV antibody titers is of considerable value in diagnosis, therapeutic evaluation and prognosis of patients with nasopharyngeal carcinoma.

Though other cancers in the head and neck are not related

to EBV (Sessions et al, 1974; Henle and Henle, 1976) an association with other viruses has been described. Hollinshead et al (1973) showed an 89% incidence of antibodies to herpes simplex virus (HSV) in patients with laryngeal cancer and in 62% of a series of patients with squamous cell carcinoma at other head and neck sites. In contrast they found those antibodies to be present in only 6% of normal individuals and 8% of patients with non-squamous malignancies. In 1976 (a) Silverman et al reported a similar high incidence of herpes virus associated antigens in 89% of patients with squamous cell carcinoma of the head and neck and also found that 90% of the patients who were cured still persisted in showing antibodies to the virus. Ibrahim et al (1979) discovered that antisera raised to TAA of invasive cervical cancer also reacted with sera of head and neck cancer patients, indicating the presence of circulating antigens in these patients, which share determinants with TAA of cervical cancer. Cancer of the lip and cervix are both prevalent in transplanted patients (Harris and Penn, 1981) and one could hypothesize that a single type of virus is related to their aetiology.

The detection of TAA in head and neck cancer patients is still a matter of ongoing research. An assay system which is able to detect minute amounts of circulating TAA will not only be suitable for screening purposes but also for judging the effectiveness of therapy. In 1979 Krause and Arbor tried to prove the existance of specific TAA using the leucocyte migration inhibition (LMI) assay. This test is based on the property of T-lymphocytes (footnote 1, p.51) to release pharmacologically active substances when stimulated by specific antigen. These substances are called lymphokines. The best known lymphokines include macrophage migration inhibitory factor (MIF), macrophage activating factor (MAF), macrophage chemotactic factor, blastogenic factor and interferon. Krause and Arbor mentioned that LMI to autologous tumour extracts may range from 22% to 100% inhibition of migration (median 65%). Sixteen of the 28 patients tested by them demonstrated greater

than 32% inhibition and in 12 it was even greater than 52%. These data indicate that the tumour extract used did contain specific TAA though the level of antigenic activity may vary considerably. Antigenic cross reactivity was achieved in only two of the cancer patients, using a pool of all available extracts, indicating that practically none of the single extracts contained shared antigens. The capability of sensitized lymphocytes to release lymphokines was also used by Kessler et al (1981) again to demonstrate the existance of TAA. Based on the observation of Field and Caspary (1970) that TAA share antigenic determinants with alkaline myelin proteins (human encephalotogenic protein (HEP), they were able to trigger lymphocytes with this protein in vitro and subsequently observed a diminished migration of macrophages in an electric field, caused by the release of lymphokines (macrophage-electrophoretic-mobility (MEM)-test). Compared to a control population in 89.3% of the patients lymphocytes specifically sensitized to HEP could be demonstrated by these authors. Negative findings in control patients with non-malignant tumours suggest that T-cell sensitization to TAA was detected in their assay-system.

Another demonstration of the presence of TAA has been described by Zenner and Herrmann (1979) who showed that sera in 47 out of 50 patients had antibody activity against their own malignancies. Of these 20% possessed antibodies reacting with cultured larynx carcinoma cells. Moreover, lymphocytes isolated from these patients were able to lyse these carcinoma cells. Similar observations were made by Mang et al (1981) who detected antitumour antibodies in 48 patients with tonsillar carcinoma. These antibodies could also be used to detect malignant cells in swabs from the oral cavity by surface fluorescence. Positive cells appeared to be present in 5 patients between 3 and 6 months before actual recurrence and they were continuously present during the active stage of the disease. The main fraction of antibodies was of the IgG class. The antibodies reacted in a similar fashion with allogeneic tonsillar carcinomas, indicating a reaction specific for this kind of tumour. A reaction with other allogeneic tumour cells including

bladder and kidney carcinoma could not be detected.

After culture techniques for human malignant larynx carcinoma cells became available (Zenner et al, 1979) monoclonal antibodies against surface antigens could be raised and tested. Zenner (1981) immunized BALB/C mice with a pure suspension of cultured larynx carcinoma cells (HLaC 78) and isolated the antibody producing spleen-cells. Fusion of these cells with myeloma cells resulted in antibody-secreting hybrid cells. These hybridomas were cloned and monoclonal antibodies produced. One clone was isolated producing antibodies which were able to identify various malignant target cells including two laryngeal carcinomas and two salivary gland carcinomas. In contrast this monoclonal antibody did not bind to various normal human cell lines such as fibrocytes and lymphocytes, nor did it bind to cells of non-human origin. These experiments represent a new and very promising approach towards identifying and isolating TAA's.

Krause et al (1981) showed that tumour cell lines in culture may maintain the features of the original tumour cells as measured by histological and electron microscopical techniques. Another parameter for characterizing cell lines in culture is the maintenance of specific cell surface antigens. Therefore serologic probes including antisera to histocompatibility antigens, blood group antigens and auto-immune sera from patients with pemphigus and pemphigoid have been used. A continued in vitro expression of pemphigus antigen was demonstrated on squamous cell carcinoma cell lines by them. This antigen was not identified on any of a variety of non-squamous carcinoma cell lines.

The presence of tumour markers such as CEA have also been examined for their effectiveness in therapeutical evaluation of head and neck cancer patients. Thus far these antigens have not been proven to be useful (Silverman et al, 1976b; Grossenbacher, 1979).

In this chapter attention has been paid to TAA in head and neck cancers. Data from serological studies on EBV-antigens

disclose a probable viral induction in case of nasopharyngeal carcinoma. Antigens associated with, for instance, cultured larynx carcinoma cells, have provided data that other head and neck carcinomas may also be antigenic.

2. The histology of lymphnodes draining the tumour and the leucocyte infiltration of the tumour.

The head and neck area seems somehow to be immunologically unique. The head and neck is characterized by the presence of a chain of lymphoid organs (adenoid, lingual and palatine tonsils and a whole range of draining lymphnodes), which are constantly exposed to environmental agents that may have stimulatory or suppressive effects on the immune system. Little is known about the contribution of these processes on immunological surveillance.

In a double blind, retrospective study Berlinger et al, (1975) microscopically examined sections of regional lymph nodes of head and neck squamous cell carcinomas to assess the immune responsiveness of the patient. A total of 84 patients were entered into their study. They found a significantly higher 5-year survival in patients with "stimulated" regional lymph nodes (81%) in comparison to patients with "unstimulated" lymph nodes (44%). They defined a "stimulated" lymphnode as having active germinal centres whereas in "unstimulated" lymph nodes germinal centres were absent. None of the 5 patients whose lymph nodes showed lymphocyte depletion survived longer than 5 years.

Saxon and Portis (1977) studied lymphoid subpopulation changes in regional lymphnodes draining cancer areas. They found an increase in B-lymphocytes and a general decrease

in T-lymphocytes[1)]. These changes in the composition of subpopulations was most marked in the first draining stations whereas more distal nodes showed a normalized pattern of distribution.

Lymphocytes infiltrating the tumour area may also contribute to the production of specific antibodies. Koneval et al (1977) demonstrated immunoglobulin synthesis in squamous cell carcinoma of the head and neck region itself. They employed fluorescence techniques on cryostat sections and found that primary IgG and also lesser amounts of IgM and IgA were produced and bound to cancer cells. Epithelial tissues of the head and neck region in tumour free controls were negative. These findings are consistent with those of Mang et al (1981) who also found antibodies of the IgG class bound to carcinoma cells.

Sala and Ferlito (1976) also studied the presence of immune competent cells in stroma of larynx carcinoma in a series of

1) Immune responsiveness to antigens in sensitized individuals is classified firstly as the responsiveness mediated by antibodies and secondly as the responsiveness mediated by antigen-specific lymphocytes. Antibodies are produced by plasmacells which derive from B-cells. The study of cell mediated immunity has particularly gained by the study of T-cells and T-cell subpopulations in man using membrane characteristics. T-lymphocytes are able to bind sheep erythrocytes to form rosette shaped clusters (E-rosettes). They are virtually non reactive with fluorescein-labeled antisera to human immunoglobulins. B-cells on the other hand react with anti-Ig sera, thus showing the exposition of Ig molecules on their cytomembranes. B-cells also form rosettes but only with igM antibody coated erythrocytes in the presence of complement (EAC rosettes). It is also possible to demonstrate a receptor for the Fc portion of the Ig molecule on some lymphocytes by their capacity to form rosettes with heterologous erythrocytes primed with IgG antibodies (EA rosettes). EAC and EA rosette techniques can not be regarded as specific for B-cells, other than in exhaustively purified lymphocyte populations, because monocytes and polymorphonuclear leucocytes also possess Fc receptors.

104 patients. Prognostic evaluation was based upon histologic grading and morphological evidence of a host immune response, the latter judged by the presence and degree of lymphocytic and plasmacellular infiltration in the tumour stroma. The degree of lymphoplasmocellular infiltration was found to be correlated with the five year survival rate. In their study the survival rate increased with increasing intensity of cellular response within each histological class of tumour differentiation. Bennett et al (1971) carried out a study on 84 patients with squamous cell carcinoma of the larynx and hypopharynx and noted that survival was longer for the well differentiated tumours and for those displaying dense lymphocyte and plasmacellular infiltration.Marked germinal centre hyperplasia in the regional lymph-nodes was also found by them to be a favourable sign in tumour prognosis. Similar observations were made by Zechner (1975).

3. Skin tests

Studies of immune reactivity in head and neck cancer patients have been conducted especially with assays that evaluate delayed skin test reactivity to primary antigens. The immune response to the primary antigen 2.4 dinitrochloro-1-benzene (DNCB) has most frequently been used in this respect[1)]. Over 95% of normal subjects are able to develop a delayed skin response when challenged a fortnight after sensitization (Eilber and Morton, 1970), but in contrast in several studies a lower percentage of patients with malignancies of various

1) Probably the most simple way to evaluate T-cell bound reactivity is to measure skin delayed hypersensitivity to the contact allergic agent DNCB. The DNCB skin test evaluates both the afferent and efferent limb of the immune response: firstly the ability to become sensitive and secondly to respond when rechallenged by the antigen. For this type of reaction the co-operation of macrophages and T-cells is necessary. The major disadvantage of the DNCB skintest is that a 7-10 days period is required for sensitization and another 2 days to read the challenge response of erythema and induration.

histologies have shown to react. (Chrétien et al, 1973a). In the majority of studies patients are listed as either positive or negative to a challenged dose and we found this to hamper comparibility between data from these studies since challenge doses vary among the different reports (Bates et al, 1979). Nevertheless the DNCB skin test provides a suitable tool to determine whether a cancer patient is able to mount a delayed type immune response to a newly introduced antigen. Patients with carcinomas of the head and neck were found to have serious impairments of cell mediated immunity as demonstrated by a frequent unresponsiveness to DNCB.

Lundy et al (1974) found the progressive loss of delayed skin reactivity to DNCB to be related to the extent of the disease as classified by TNM system. Mandel and Kiehn (1974) reported similar observations. It is also of interest to know that head and neck cancer patients with localized disease more often show negative DNCB skin tests than can be expected in comparison with patients with other types of localized cancer at other sites (Pinsky et al, 1971).

One of the major purposes of studying delayed skin test reactivity in cancer patients was to see whether this reactivity had any prognostic value. Studying 120 patients with head and neck neoplasms Eilber et al (1974) found that the depression in skin test reactivity correlated with the extent of the disease in that patients with inoperable cancer had a 84% incidence of anergy. Maisel and Ogura (1976) followed patients for up to two years after operation and they found that 91% of their DNCB positive patients remained tumour free throughout this period, but this was true for only 55% of their negative patients. However, recent work of Bier and Nicklish (1981) could not confirm the prognostic value of DNCB testing. The results obtained with the DNCB test in different studies are summarized in table I.

4. Quantitation of lymphocyte subpopulations and lymphocyte reactivity in vitro.

Immune derangements in head and neck cancer patients have also been studied by quantifying the number of lymphocyte subpopulations. T-lymphocytes are mostly identified by their ability to form rosettes with sheep red blood cells, whereas B-lymphocytes are usually identified by demonstrating the presence of immunoglobulin on their surface membrane, employing immuno-fluorescent techniques, or by their ability to form rosettes with sheep red blood cells coated with antibody and complement (EAC-rosettes) (Roitt, 1975).

The ability of lymphocytes to respond in vitro to substances such as phytohaemagglutinin A (PHA), concanavelin A (ConA) or pokeweed mitogen (PWM) with a mitogenic reaction (Turk, 1978) has also frequently been used in head and neck cancer to assess the competence of patients's immune system[1)].

A decrease in the number of T-lymphocytes has frequently been observed in patients with carcinomas of the head and neck (Table II). Impaired in vitro responsiveness to PHA, ConA and PWM have also been reported in detail in these types

1) In contact with an antigen specific lymphocytes become triggered and a complicated series of biochemical and physical changes takes place and leads to mitosis. These changes of blast transformation can be detected histologically or by measuring the enhanced incorporation of radio-labeled nucleotides, such as ^{3}H-thymidine. Much use has also been made of various material which are not antigens in the proper sense of the word and which cause blastogenic transformation of lymphocytes. These materials are called mitogens. Measurement of transformation induced in this manner is not in any way immunologically specific, but gives a good measure of an important series of cellular activities. Different mitogens are active against T- and B-cells. ConA and PHA trigger T-lymphocytes, whereas PWM acts on both T- and B-lymphocytes. The MLC falls in the same group of tests, measuring the capacity of lymphocytes to recognize foreign antigens on allogeneic lymphocytes and to respond with a blastogenic response.

of malignancies and the derangements seemed more pronounced when compared to malignancies at other sites (Lichtenstein et al, 1980). The predictive value of these in vitro tests is not always clear. In a study of Hilal et al (1977) on 183 patients, T-cell function tended to be more suppressed with progression of disease but none of the in vitro lymphocyte stimulation tests could be correlated with disease recurrence. Recently Bier and Nicklish (1981) also demonstrated that the in vitro blastogenic responsiveness of lymphocytes was not suitable to indicate prognosis in head and neck cancer patients. In contrast Ryan et al (1980) and Jenkins et al (1980) recently found that PHA and ConA induced blastogenesis was often depressed in cancer patients in whom clinically apparent recurrences developed.

Interestingly enough defects of cell mediated immune functions have been detected in individuals with severe alcohol abuse (Lundy et al, 1975). This makes it sometimes difficult to interpret data on immune function in head and neck cancer patients since epidemiologic observations have shown a high alcohol consumption in patients with oral cancer (Hakulinen et al, 1974). The authors suggested a possible relationship between alcohol mediated immune dysfunction and oral cancer. In a prospective study on 53 patients with head and neck cancer Brookes and Clifford (1981) found a highly significant positive correlation between nutritional status and delayed skin test reactivity to DNCB and blood lymphocyte counts. They suggest that nutrition deficiency is of major prognostic value in patients with head and neck cancer and that defects in immune competence are secondary phenomena.

From the data listed it appears that cell mediated immunity is seriously affected in head and neck cancer patients. Cell mediated immunity defects being defined as frequent unresponsiveness to DNCB (table I), low T-cell numbers (table II) and diminished blastogenic responsiveness of lymphocytes (table III). In predicting prognosis these tests seem to be more

confusing than helpful. Certainly the clinical staging system is more accurate in predicting prognosis than immunologic assessment alone. Only the DNCB test might be of some value as an additional prognostic test. The lack of homogenicity and the influence of other factors as age, nutritional status and tumour site itself makes it difficult to draw final conclusions.

3. The role of the mononuclear phagocyte.

Although a lot of studies have paid attention to the reactivity of lymphocytes in head and neck cancer patients only few reports deal with the function of the mononuclear phagocyte. Berlinger et al (1978) described an abnormal low mixed lymphocyte culture (MLC) responsiveness (see footnote 1), p.58) in 54 patients with epidermoid head and neck cancer. When patients cells were filtered through special columns adherent cells were selectively removed and this subsequently improved in 56% of the deficient individuals the in vitro lymphocyte responsiveness. Reconstituting the cultures with the adherent cells usually reintroduced the suppressive effect. Microscopical examination revealed a typical macrophage morphology of the adherent cells and histochemically these cells were non-specific esterase positive, indicating a role for macrophages in hampering immune responsiveness in head and neck cancer patients.

From the data mentioned it is clear that the mononuclear phagocyte could be of importance in head and neck cancer. This forms the basis of our experimental questions and the studies reported in this thesis.

TABLE I

Summary of reported DNCB reactivity in head and neck cancer patients and the relation with tumour stage and prognosis

Authors	No. patients	DNCB anergy (% of cases)	Positive correlation tumour stage	Prognostic value
Pinsky et al, 1971	*	61	--	pos.
Catalona and Chrétien, 1973	19	42	--	--
Maisel and Ogura, 1973	63	52	--	pos.
Twomey et al, 1974	44	27	--	pos.
Lundy et al, 1974	47	47.6	yes	pos. [1]
Silber et al, 1974	120	50	yes	pos.
Parker et al, 1975	22	85	--	--
Olivari et al, 1976	100	48	yes	--
Maisel and Ogura, 1976	72	56	--	pos.
Hilal et al, 1977	126	45	yes	pos. [2]
Mandel and Kiehn, 1976	56	53	yes	pos.
Gilbert et al, 1978	85	31	--	none
Papenhausen et al, 1979	36	67	yes	--
Sier and Nicklisch, 1981	30	42	none	none

* A total number of 180 patients was investigated. The exact number of head and neck patients is not mentioned in their report.

1) only in localized disease

2) only in stage I and II.

TABLE II

SUMMARY OF REPORTED NUMBERS OF BLOOD LYMPHOCYTES AND LYMPHOCYTE SUBPOPULATIONS IN HEAD AND NECK CANCER PATIENTS

		Lymphocytes		T-cells				B-cells	
		number/mm^3 (mean ± SD)		number/mm^3 (mean ± SD)		Percentage		number/mm^3 (mean ± SD)	
Authors	No.pat.	controls	patients	Controls	patients	Controls	patients	Controls	Patients
Wanebo et al, 1975	137			1875	1283*			450	187*
Olkowski and Wilkins, 1975	46			1460 ± 54.8	876 ± 69*	74.9 ± 0.6	51.4 ± 2.13*		
Potvin et al, 1975	22	2062 ± 44[5]	1793 ± 132						
Eastham et al, 1976	38					69 ± 8	50 ± 13.7*		
Jenkins et al, 1976	52	2915 ± 102	2354 ± 107*	1478 ± 34	1041 ± 82*	71.5 ± 0.5	62.5 ± 2.0*		
Deegan and Coulthard, 1977	26	2084 ± 874	2043 ± 1145	1432 ± 602	1192 ± 731[1]	68.5 ± 6.0	57.6 ± 10.4*		
Deegan et al, 1977	37	1589 ± 155[2]	1989 ± 113	1035 ± 106[2]	1117 ± 76	64.4 ± 1.7[3]	55.9 ± 1.9*		
Papenhausen et al, 1979	24	2360 ± 266	2120 ± 205	1750 ± 179	1450 ± 169*	73.8 ± 2.3	68.2 ± 2.4*		
Lichtenstein et al, 1980	18	2088 ± 745	2060 ± 863[3]	1404 ± 506	1155 ± 431[3]	67 ± 8.0	60 ± 9.6[3]*		
			1357 ± 844[4]*		770 ± 268[4]*		66 ± 6.8[4]		
Bier and Nicklisch, 1981	30			1065	699*	52	39		

* Significantly different from controls

1) $0,05 < p < 0,10$

2) controls > 50 yrs.

3) localized disease

4) advanced disease

5) SEM

TABLE III

Summary of reported blastogenic responsiveness to several mitogens in head and neck cancer patients

		PHA		ConA		PWM	
Authors	no.pat.	Controls	Patients	Controls	Patients	Controls	Patients
Catalona et al, 1973	20	16% 1)	45% 1)*				
Chrétien et al, 1973b	32	15% 1)	50% 1)*				
Wanebo et al, 1975	48	0% 1)	40% 1)*				=
Jenkins et al, 1976	53		19% 2)*		26% 2)*		22% 2)*
Olivari et al, 1976	95		45% 1)*				
Hilal et al, 1977	112		45% 1)*		28% 1)		25% 1)
Deegan et al, 1977	29	3)	29% 2)*				
Papenhausen et al, 1979	24		38% 2)*				
Lichtenstein et al, 1980	18		57% 2)*				
Bier and Nicklish, 1981	30		34% 2)*		37% 2)*		19% 2)

* : significantly different from controls

= : not different from controls

1) : percentage of individuals with depressed blastogenic responses

2) : percentage of blastogenesis depression compared with controls

3) : controls > 50 yrs.

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CHAPTER V

EXPERIMENTAL QUESTIONS

It has become clear from the previous chapters that the immune system has a role to play in host defence against neoplasia. Previous studies on immune reactivity in head and neck cancer have focused on delayed type skin test reactivity, T-cell numbers and T-cell functions (p.54). Uptil now little is known on the function of the MPS in this type of cancer, though the data presented in chapter III concerning mononuclear phagocytes and cancer in general indicate an extremely complicated, but important relationship. This has led to our first experimental question:

I. *Are functions of the peripheral blood monocyte such as chemotaxis, NBT-dye reduction and maturation capacity influenced by the presence of a malignancy in the head and neck region ?*

This question is dealt with in chapter VI. We are aware that the information obtained holds only true for isolated monocytes in vitro. In an attempt to study infiltrated mononuclear phagocytes we identified such cells in microscopical sections of the tumour area, which were enzyme-histochemically stained for acid phosphatase. The number and the ultrastrucure of the macrophages infiltrating the tumour area was studied and correlated with the outcome of the in vitro tests on isolated blood monocytes. Data of these correlations are reported in chapter VII.

In agreement with reports of other investigators (p.33) we were able to show a depressed monocyte chemotactic responsiveness in vitro in head and neck cancer patients (p.78). This impairment of function has been ascribed to low molecular weight factors derived from the tumour (Snyderman et al, p.33). This notion has led to another experimental question:

II. Can low molecular weight factors be isolated from head and neck cancers which influence the migratory capacity of mononuclear phagocytes?

This question is dealt with in chapter VIII

CHAPTER VI

MONONUCLEAR PHAGOCYTE FUNCTION IN HEAD AND NECK CANCER
NBT-dye reduction, maturation and migration of peripheral blood monocytes.

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SUMMARY

The number of blood monocytes, their ability to mature into macrophages, their NBT-dye reduction capacity and their chemotactic responsiveness were studied in 29 patients with squamous cell carcinoma of the larynx, 12 patients with squamous cell carcinoma at other sites within the head and neck and two groups of male controls, one of 29.7 yrs. ± 7.2 (SD) and the other of 71.4 yrs. ± 6.8 (SD). An age dependency of monocyte function in healthy individuals was established. Increased numbers of monocytes per ml blood were found in the older men. These cells showed an enhanced chemotactic responsiveness. Their maturation capacity was decreased.

A clear impairment of chemotactic responsiveness was found in the two groups of carcinoma patients. The number of monocytes was only marginally affected. The maturation was found to be enhanced. An impaired recruitment of mononuclear phagocytes at the site of malignant growth due to diminished migratory capacities might be important for failing immunological surveillance.

INTRODUCTION

Decreased numbers of T-cells, impaired mitogenic responsiveness of these cells and decreased ability to become sensitized to dinitrochlorobenzene (DNCB) have all been described in cancer patients (Quan and Burtin, 1978; Patt et al, 1978). These cellular deficiencies might be of importance in the pathogenesis of malignant disease as T-cell dependent immune mechanisms are known to play a role in tumour cell destruction. Apart from T-lymphocytes, cells of the mononuclear phagocyte series are also affected in the tumour bearing host. A considerable body of evidence shows that macrophages can recognize and kill malignant cells. (Hibbs et al, 1972; Currie, 1978). Especially their influx into neoplasms correlates with tumour cell destruction and decreased incidence of metastases (Evans, 1972; Eccles and Alexander, 1974; Lauder et al, 1977). Moreover cancer immunotherapy with BCG or C Parvum interferes with the functions of the mononuclear phagocyte system, again indicating its importance (Scheinberg et al, 1978; Hedley et al, 1979).

Clinical studies on the function of the mononuclear phagocyte system have been performed in various malignant diseases. Monocyte activity as measured by NBT-dye reduction is abnormal in patients with malignant melanoma, being enhanced when metastases are clinically undetectable but suppressed in advanced disease (Hedley and Currie, 1978). A fairly consistent picture has emerged regarding the suppressed migration and chemotaxis of monocytes in various malignancies (Boetcher and Leonard, 1974; Hausman et al, 1975; Snyderman and Stahl, 1975; Hausman and Brosman, 1976; Kjeldsberg and Pay, 1978 and Snyderman et al, 1978). Impaired monocyte maturation (Currie and Hedley, 1977; Taylor and Currie, 1979) has been demonstrated in cancer of the breast and in malignant melanoma.

Cancers of the head and neck are somewhat peculiar in that the T-cell functions are already affected in early stages of the disease (Wanebo et al, 1978; Lichtenstein et al. 1980).

Data on the functions of the mononuclear phagocyte system in this type of cancer are scarce if not at all absent. Therefore, we have studied the monocyte-macrophage series in 41 patients with a squamous cell carcinoma of the head and neck.

The number of blood monocytes, the ability of monocytes to become macrophages (Currie and Hedley, 1977), their NBT-dye reduction capacity (Hedley and Currie, 1978) and their chemotactic responsiveness (Zigmond and Hirsch, 1973) are reported.

PATIENTS AND METHODS

Patients - Forty-one male patients with histologically proven squamous cell carcinoma of the head and neck - including all tumour stages - were studied. The mean age was 64.1 yrs, range 38-77. Twenty-nine patients had a carcinoma of the larynx, six of the oropharynx and six of the oral cavity. (Table I). Patients with overt dissiminated disease were excluded from our studies. Informed consent had been obtained. A group of 27 healthy male individuals (laboratory staff and hospital personel; mean age 29.7 yrs., range 23-30) and a group of 19 old males (pre-operative catarrhact patients; mean age 71.4, range 53-81) were studied as controls.

Number of peripheral monocytes - The number of peripheral monocytes was calculated from the number of leucocytes per ml blood and the results of a differential blood smear count.

Mononuclear cell suspension(MNC) - MNC suspensions were isolated from 50 ml defibrinated blood on a ficoll-isopaque gradient according to Böyum (1968). The cells were washed x 3 and adjusted to 4 x 10^6/ml in serum free RPMI (Gibco, Glasgow). Percentages of monocytes were determined in suspension by a non-specific esterase (NSE) staining technique as described by Mullink et al (1979).

TABLE I.

Distribution of cases according to site and TNM classification.

Site	Stage	No.patients
Larynx	T_1	9
	T_2	7
	T_3	12
	T_4	1
	N_0	22
	N_+	7
Oral cavity	T_1	1
	T_2	2
	T_3	0
	T_4	3
	N_0	4
	N_+	2
Oropharynx	T_1	0
	T_2	0
	T_3	0
	T_4	6
	N_0	3
	N_+	3

Monocyte NBT-dye reduction - The capacity of monocytes to reduce nitro-blue tetrazolium dye into blue coloured formazan was measured by a quantitative assay described by Hedley and Currie (1978). The rate of dye reduction is an indirect measure of the pentose shunt activity, the enhancement of which is associated with monocyte peroxide formation.

Mononuclear cells were pre-incubated for 15 min. in the presence or absence of latex polystyrene particles, which acted as a phagocytic stimulus, and with a solution of 0,025% NBT (BDH, Chemicals Ltd., Poole, England).

After 60 min. incubation with NBT the reaction was stopped by acidifying the mixture, the cells were washed and thereafter the produced formazan was extracted using dioxan at 70^{0}C. Optical density at 520 nm was measured (Spectrophotometer, Eppendorf 1101 M. Hamburg, Germany) and by reference to a standardization curve, the amount of NBT reduced per NSE positive cell both resting and after latex pre-incubation, was calculated.

Monocyte maturation - This method examines the ability of monocytes to mature into macrophages when cultured in 50% fresh autologous serum at 37^{o}C for 7 days in the wells of 3040 (Falcon Plastics) microplates. The assay has been described in detail by Currie and Hedley (1977). The macrophage nuclei were detached and stained using a solution of 0,1 M citric acid plus 1 : 2000 crystal violet and counted in a haemocytometer. Results were expressed as the percentage of monocytes (NSE positive cells) capable of maturing into macrophages.

Migration - Monocyte spontaneous migration and chemotactic responsiveness were determined according to the method of Wilkinson (1974) using modified Boyden chambers and millipore membranes (Millipore Inc., Bedford, Massachusetts, U.S.A.) of 12 m pore size. Cellsuspensions, containing 10^{6} NSE positive cells per ml, were allowed to migrate for 60 min. at 37^{o}C into the membranes towards an RPMI 1640 solution or towards 0,1% casein (Hammersten, Merck, Darmstadt, Germany) in Gey solution. The distance of migration into the membranes

was recorded using the leading front method as described by Zigmond and Hirsch (1973). All tests were carried out before any form of therapy was given.

Statistical analysis was performed using Wilcoxon's two sample test.

RESULTS

With regard to the number of peripheral monocytes no statistical significant differences could be obtained between the tumour bearing patients and their respective controls, i.e. the older healthy males of 71.4 yrs. ± 6.8 (SD). (Fig 1.)

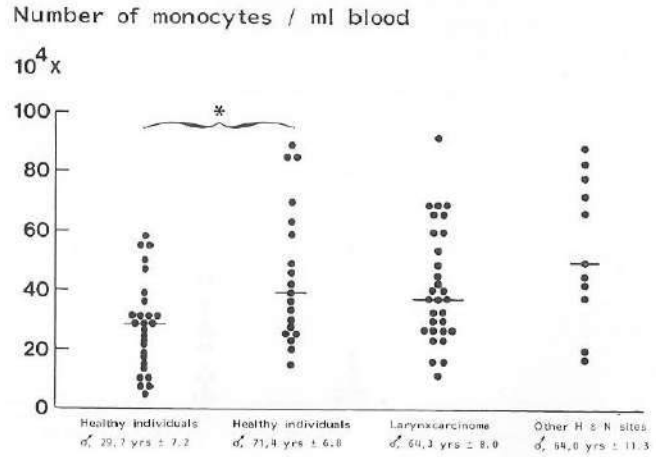

Fig. 1. The number of monocytes per ml blood in healthy male individuals, patients with carcinoma of the larynx and patients with other head and neck carcinomas. * A statistical significant difference exists between these two groups of values, $p < 0,05$, Wilcoxon's two sample test. — = median.

A comparison with this group of healthy individuals of this age was necessary because this group showed significantly more monocytes per ml blood than the younger age group.

The results obtained with the assay measuring the percentage of blood monocytes capable of transforming into active macrophages is shown in Fig. 2. The monocytes of the older healthy males showed a clear impairment of their ability to develop into macrophages. This impairment was not found in the two groups of carcinoma patients. This indicates the possibility of a stimulation of the monocyte-macrophage series in these types of carcinomas.

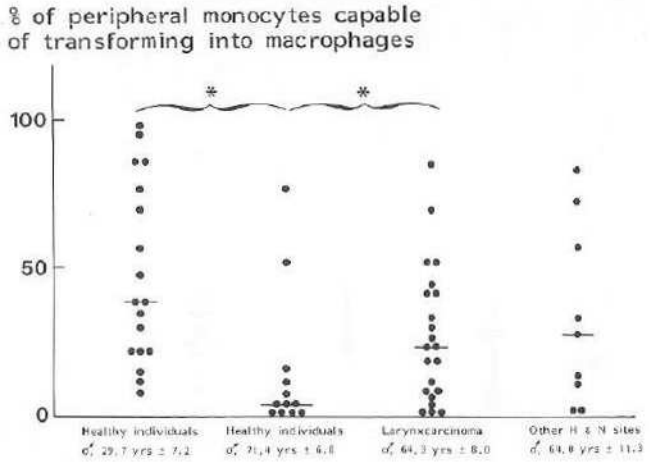

Fig. 2. The percentage of peripheral monocytes capable of transforming into macrophages in healthy male individuals, patients with carcinoma of the larynx and patients with other head and neck carcinomas. * A statistical significant difference exists between these two groups of values, $p < 0,05$, Wilcoxon's two sample test. — = median.

The NBT-dye reduction capacity of monocytes, both normally and during the uptake of latex particles did not reveal any significant difference between cancer patients and their respective controls. Age dependency was also not established.

The spontaneous migration and chemotactic responsiveness were found to be clearly affected in tumour patients (Fig. 3a and Fig. 3b).

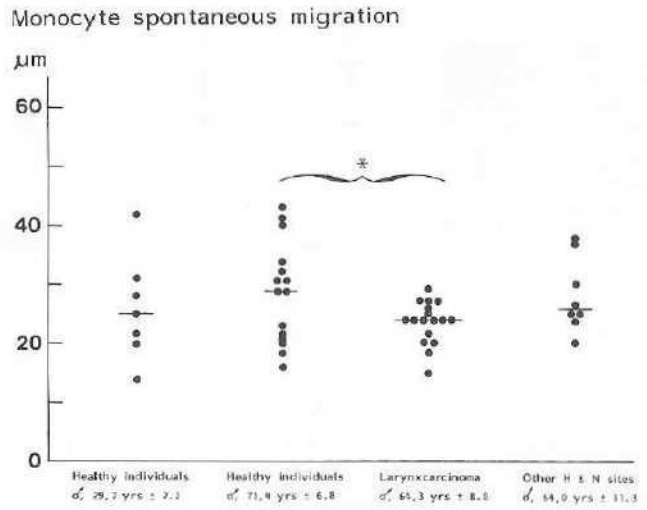

Fig. 3a. Monocyte spontaneous migration in healthy male individuals, patients with carcinoma of the larynx and patients with other head and neck carcinomas. * A statistical significant difference exists between these two groups of values, $p < 0,05$, Wilcoxon's two sample test. — = median.

The spontaneous migration was impaired in larynxcarcinoma patients but not in patients with other carcinomas of the head and neck. The chemotactic responsiveness of monocytes was significantly diminished in both groups of patients, but especially in cancers other than the larynx. Furthermore

a marked age dependency was found in monocyte chemotactic responsiveness: enhanced reactivity was seen with increasing age (71.4 yrs. ± 6.8).

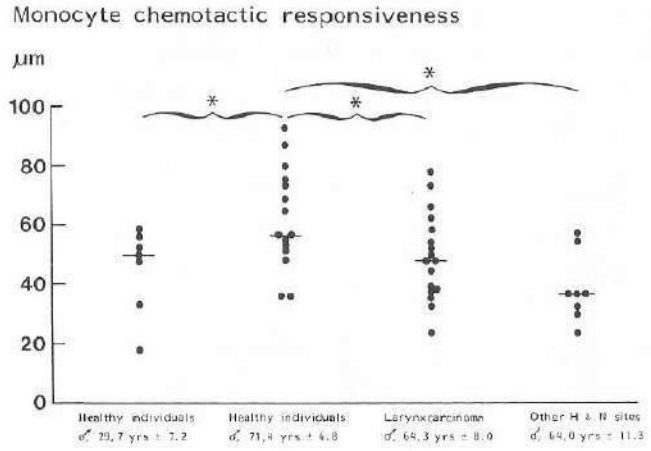

Fig.3b. Monocyte chemotactic responsiveness in healthy male individuals, patients with carcinoma of the larynx and patients with other head and neck carcinomas. * A statistical significant difference exists between these two groups of values, $p < 0.05$, Wilcoxon's two sample test. — = median.

DISCUSSION

Our data show a remarkable age dependency of monocyte function in healthy individuals. Increased numbers of monocytes per ml blood were found in older men and these cells showed an enhanced chemotactic responsiveness. However, their capacity to transform into adherent macrophages was decreased. Age dependent alterations in monocyte-macrophage function have scarcely been reported, but it is known that efficiency of antigen processing, which is a mononuclear-phagocyte function, is reduced by ten fold in old mice. (Price and Makinodan, 1972). Other changes in immune function as a

result of ageing are well established, for instance the changes in the immunoglobulin repertoire and in T-cell function (Radl et al, 1975). It is tempting to speculate that age dependent alterations in monocyte function play an important role in failing immunological surveillance.

All our cancer patients studied belonged to the older age groups and were therefore compared to the controls of 53 to 81 yrs. A decreased chemotactic responsiveness was found and this is comparable to data reported in patients with genito-urinary tract carcinoma (Hausman et al, 1975) and carcinoma of the breast (Snyderman et al, 1978). Low molecular factors derived from the tumour induce this phenomenon and such factors have been isolated (Rhodes, 1980).

Our patients with larynx carcinoma had normal numbers of peripheral monocytes. This is in contrast to findings reported by Barrett (1970) of increased numbers of monocytes in malignancies as carcinoma of the breast and gastro-intestinal tract. A similar increase in monocyte numbers might be apparent in our group of carcinomas other than the larynx, although these results did not reach statistical significance. In general a carcinoma of the larynx is a small malignancy in comparison to adenocarcinoma of the breast, gastro-intestinal tract or non-larynx carcinomas of the head and neck. If tumour derived factors play also a part in influencing the numbers of peripheral monocytes (Rhodes, 1980) the relative low tumour burden might explain our negative findings in patients with carcinoma of the larynx.

In malignant melanoma and cancer of the breast a decrease in the number of monocytes capable of maturing into macrophages was reported by Currie and Hedley (1977) and Taylor and Currie (1979). In comparison to the older controls - which showed very low numbers of "macrophage precursors" - all our patients studied showed an enhanced maturation.

In general our data indicate that in malignant disease one function of the peripheral monocyte might be enhanced, whereas another is decreased. However, the impaired recruitment of

mononuclear phagocytes at the site of a small malignant growth due to defective chemotaxis could be very crucial for failing immunological surveillance, because it overrules the enhancement of other functions of the cell as for instance maturation capacity. The determination of the activity of mononuclear phagocytes infiltrating the tumour area might therefore be more relevant than a study of peripheral blood monocytes. A quantitative morphological analysis of the number and cytochemistry of mononuclear phagocytes in and around cancers of the head and neck will form the subject of our next report.

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We gratefully acknowledge the technical assistance of Rita Sluyter, Diederik van Romondt, Marjan Kokjé-Kleingeld and Stefan van der Hoek.

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THE ULTRASTRUCTURE OF PERIPHERAL BLOOD MONOCYTES IN HEAD AND NECK CANCER PATIENTS

Studies of monocyte function in cancer patients have revealed a variety of abnormalities (Rhodes, 1980). In our studies on head and neck cancer patients we also found a decrease in chemotactic responsiveness of peripheral blood monocytes, whereas the monocyte maturation capacity appeared to be enhanced. Therefore we thought it interesting to investigate whether monocytes of head and neck cancer patients exhibit special ultrastructural features as a reflection of these functional changes. An ultrastructural morphological study was therefore undertaken obtaining monocytes from three healthy individuals and five patients with head and neck cancer. The nucleus/ cytoplasma ratio was measured and calculated as well as the number of mitochondria and the number of lysosomes per cell. We hoped that these parameters could be a reflection of the cell's ability to mature into a macrophage. Our fixation techniques did not permit a study to the cytoskeleton which is assumably correlated with migratory capacities of the cell.

Patients and techniques - Five patients with squamous cell carcinoma of the head and neck were studied. Cells of laboratory staff and hospital personel were used as controls. Mononuclear cell suspensions (MNC) were isolated from 50 ml defibrinated blood on a ficoll isopaque gradient according to Böyum (1968). After centrifugation mononuclear leucocytes were fixed in 2% glutaraldehyde in 0,1 M phosphate buffer at $4^{\circ}C$, postfixation was in 1% OsO_4 in S-collidine buffer at $4^{\circ}C$. Thereafter the cells were dehydrated through graded ethanol and embedded in Epon-812. Sections were cut on a LKB-Ultrotome III and IV, mounted on copper grids, stained with uranyl acetate and lead citrate and examined in a Zeiss-109 electron microscope.

Statistical analysis was performed using Wilcoxon's two sample test.

Results - The nucleus/cytoplasma ratio of the cells ranged from 0.3-0.8 in the two investigated groups (Fig.1). In Fig. 2 the number of mitochondria per monocyte is shown. The absolute number of mitochondria in both groups is within normal limits. (14.1 ± 1.0; Roos et al, 1980). The aspect of mitochondria in patients appeared to be normal and no swelling of mitochondria was seen. Fig. 3 shows the number of lysosomes per monocyte in both the groups of controls and patients. The number of lysosomes varied considerably. A statistical difference between patients and controls could not been shown in any of these parameters.

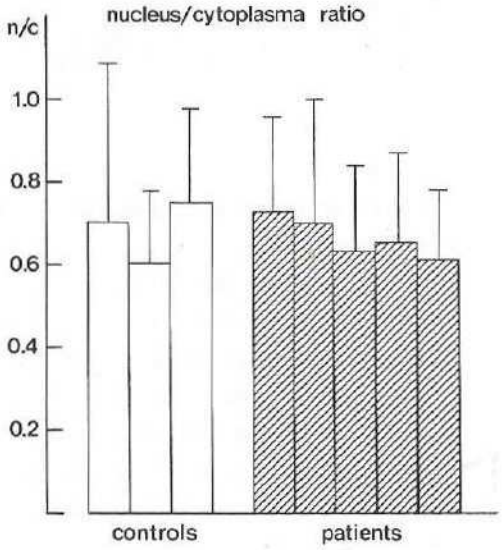

Fig. 1. Nucleus cytoplasma ratio per monocyte (± SD; n=30). No significant differences were found between the two investigated groups. Wilcoxon's two sample test $p > 0,05$.

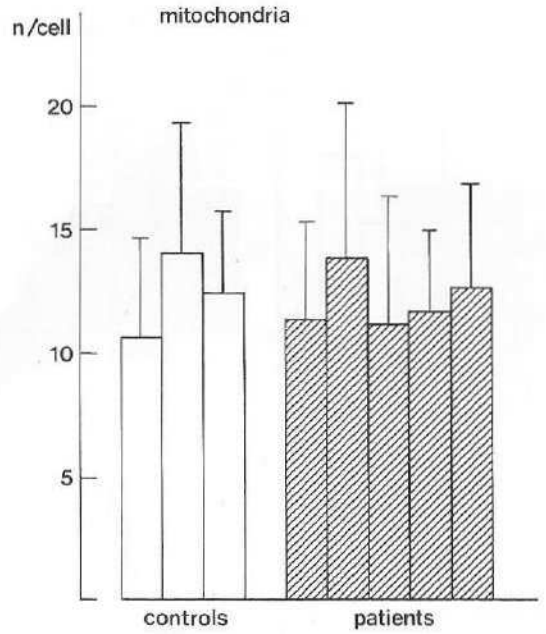

Fig. 2. The number of mitochondria per monocyte (± SD; n=30). No significant differences were found between the two investigated groups. Wilcoxon's two sample test $p > 0,05$

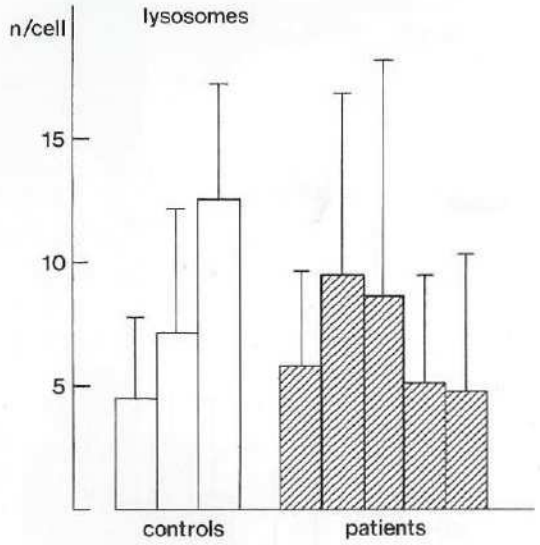

Fig. 3. The number of lysosomes per monocyte (± SD; n=30). No significant differences were found between the two investigated groups. Wilcoxon's two sample test $p > 0,05$.

In conclusion - our data show that the nucleus/cytoplasma ratio, the number of mitochondria and the number of lysosomes of patients with head and neck cancer is not different from the values found in healthy individuals. In monocytes of melanoma patients Tashiro et al (1980) found an increase in the number of lysosomes, particularly after administration of BCG. We could not confirm their observations in head and neck cancer, although our study did not include a group of immune stimulated patients.

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CHAPTER VII

MONONUCLEAR PHAGOCYTE FUNCTION IN HEAD AND NECK CANCER

The chemotactic responsiveness of blood monocytes in correlation to the histological grade of the tumour and the infiltration of these cells into the tumour area.

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SUMMARY

The chemotactic responsiveness of peripheral monocytes and the acid phosphatase activity of tumour infiltrating macrophages as well as the ultrastructural appearance were studied in 40 patients with squamous cell carcinoma of the head and neck.

The chemotactic responsiveness was found to be decreased in carcinoma patients and this value appeared to be positively correlated in individual patients with the number of tumour infiltrating macrophages as well as with the histological grade of the tumour: patients with poorly differentiated malignancies showed impaired monocyte chemotactic responsiveness and low numbers of tumour infiltrating macrophages.

Macrophages present in the parenchyma of the tumour showed a weak and diffuse pattern of acid phosphatase reactivity. The acid phosphatase activity of stromal macrophages was much stronger and distributed in foci. Electron microscopy of the parenchymal macrophages revealed low numbers of lysosomes and the presence of tumour cell debris in the cytoplasm of the cell without any sign of a surrounding phagosomal membrane. Together with the weak cytochemical reactivity this probably indicates the poor functional state of the phagocyte when infiltrated in the parenchyma of the tumour.

Low molecular weight factors derived from the tumour are known to decrease chemotactic responsiveness of peripheral monocytes.

The poor functional state of the macrophages infiltrated within tumour parenchyma might be explained by assuming that a high concentration of such factors in the near vicinity of malignant cells causes toxic effects in macrophages.

INTRODUCTION

Mononuclear phagocytes are important effector cells in host defence against neoplasia (Hibbs et al, 1978; Russell et al, 1980) since they are known to exert cytostatic and cytotoxic effects on tumour cells. Both human peripheral monocytes and macrophages from sites of malignant growth have been shown to exert these effects against allogeneic and xenogeneic tumour cells (Vose, 1978; Mantovani et al, 1979a; Mantovani et al, 1979b). Malignant tumours vary in their content of macrophages and the degree of infiltration may be positively correlated with prognosis (Eccles and Alexander, 1974; Gauci and Alexander, 1975; Wood and Gillespie, 1975; Lauder et al, 1977). It is therefore of interest that it has been demonstrated that in malignant disease, peripheral monocytes show several defects in function: a defective maturation capacity (Currie and Hedley, 1977; Taylor and Currie, 1979), a diminished NBT-dye reduction (Hedley and Currie, 1978) and a depressed spontaneous monocyte mediated cytotoxicity (Kleinerman et al, 1980). Monocyte chemotactic responsiveness was especially found to be markedly affected by tumour growth (Boetcher and Leonard, 1974; Snyderman and Stahl, 1975; Hausman and Brosman, 1976; Snyderman et al, 1977; 1978). It is arguable that this defect is a key phenomenon in poor prognosis since this function regulates the number of macrophages present in the tumour region.

To test this assumption we have studied the number of macrophages present in head and neck malignancies in correlation with monocyte chemotactic responsiveness. The study was carried out on carcinomas of the head and neck.

This report demonstrates that the degree of impairment of peripheral monocyte chemotactic responsiveness was associated with a decrease in number of macrophages per unit tumour-area and that this also correlated with the histological grade of the tumour.

PATIENTS AND METHODS

Patients - Forty male patients with histologically proven squamous cell carcinoma of the head and neck were studied. Mean age 62.8 years ± 12.5 (SD). A group of 15 males (mean age 69.4 ± 5.8 (SD)), 7 patients with acute rhinitis/sinusitis (mean age 35.4 ± 7.8 (SD) and 14 patients with chronic rhinitis/sinusitis (mean age 42.8 ± 6.4 (SD)) were studied as controls. The distribution of carcinomas is as indicated in Table I. All tumour stages were included except overt dissiminated disease. Two histological grades were assigned to the primary tumour based on their capacity to form epithelial pearls: well differentiated (epithelial pearls present) and poorly differentiated (epithelial pearls absent). This classification has proven to be simple and of value for prognosis (Wahi et al, 1971) and is in general use in our hospital.

Enzyme histochemistry of tumour biopsy material - Biopsies (3-7 mm^3) from the periphery of the tumour were taken immediately after surgical removal and they were fixed (2-3 hrs.) in acetic sublimate formalin (Lillie and Fullmer, 1976), washed and kept for 2-6 hrs. in 70% ethanol. Thereafter the biopsies were dehydrated by graded ethanol and toluene (2 hrs. 4°C), put in melted paraffin wax (2 hrs., mpt 45°C), embedded and sectioned at 5 µ .

Paraffin was removed by xylene, after which the sections were rehydrated in ethanol to water. They were then put in Lugol's iodine solution and sodium thiosulphate (5 and 3 min,) followed by washings in distilled water. Acid phosphatase staining was performed by a modification of the method of Burstone (1962): 10 mg naphtol AS-BI phosphate (Sigma) was dissolved in 5 ml 0,01 M $NaHCO_3$. This solution was mixed with a solution of 25 mg Fast Blue B (Sigma) in 10 ml of 0,2 M sodium acetate buffer (pH 5.0), made up to 20 ml and filtered. Drops of this solution were placed on the sections and incubated for 2.5. hrs. at 37°C. The preparations were counterstained with nuclear fast red and mounted in Depex (Gurr). To establish the specifi-

TABLE I.

Distribution of studied cases according to their sites and TNM classification.

Site	Stage	N_0 patients
Larynx	T_1	5
	T_2	3
	T_3	8
	T_4	3
	N_0	17
	N_+	2
Oral Cavity	T_1	1
	T_2	3
	T_3	
	T_4	6
	N_0	6
	N_+	4
Oropharynx	T_1	
	T_2	1
	T_3	5
	T_4	
	N_0	2
	N_+	4
Nasopharynx	-	2
External auditory meatus	-	3

city of the acid phosphatase staining technique and to measure the temperature dependent loss of enzyme, sections of human tonsils were treated similarly, but with incubation at several temperatures. It appeared that embedding of the tissue at 45°C gave optimal results. Microscopical sections of 5 benign nasal polyps were also stained for acid phosphatase.

Measurements of the number of tumour infiltrating macrophages - Tumour infiltrating macrophages were identified by their intra-cytoplasmic acid phosphatase activity. The number of infiltrated acid phosphatase positive mononuclear cells was established semiquantitatively namely ± = 1-5 acid phosphatase positive cells per x 40 microscopic field, + = 5-15 acid phosphatase positive cells per x 40 microscopic field and ++ = 15-25 acid phosphatase positive cells per x 40 microscopic field.

Electron microscopy of tumour biopsy material - Small cubes (1-2 mm^3) were cut from the biopsy and fixed in 2% glutaraldehyde in 0,1 M phosphate buffer at 4°C, post-fixation was in 1% OsO_4 in S-collidine buffer at 4°C. Thereafter the tissue was dehydrated through graded ethanol and embedded in Epon-812. Sections were cut on an LKB-Ultrotome III and IV, mounted on copper grids, stained with uranyl acetate and lead citrate and examined in a Zeiss-109 electron microscope.

Monocyte chemotactic responsiveness - Mononuclear cell suspensions were isolated from defibrinated blood on a ficoll-isopaque gradient (Böyum, 1968) and the percentage of monocytes present in the isolate was determined by non-specific esterase (NSE) staining (Mullink et al, 1979). Monocyte migration and chemotactic responsiveness towards casein was determined according to the method of Wilkinson (1974). Modified Boyden chambers and millipore membranes (Millipore Inc. Bedford,Massachusetts, USA) of 12 μ pore size were used. Cell suspensions containing 10^6 positive cells per ml were allowed to migrate for 60 min. at 37°C into membranes towards a RPMI solution or towards 0,1% casein (Hammersten, Merck, Darmstadt, Germany) in Gey solution. The distance of migration into the membranes was recorded using the leading front method as described by Zigmond and Hirsch (1973).

Statistical analysis - Data were analysed using Wilcoxon's two sample test.

RESULTS

Acid phosphatase positive macrophages were found throughout the tumour area being present in areas of malignant epithelial cells (parenchymal macrophages) as well as in the supporting connective tissue of the tumour (stromal macrophages). It is of interest that the cytochemical reactivity of the phagocytes in these two areas was different, see Fig. 1.

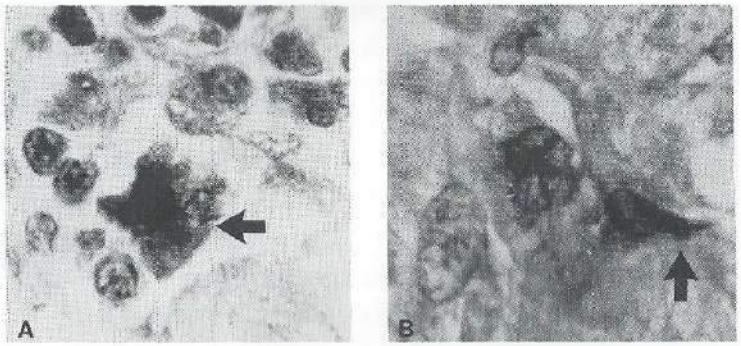

Fig.1. Acid phosphatase positive macrophages (arrows). Stromal macrophages (Fig. 1a) show a strong acid phosphatase activity distributed in spots. Parenchymal macrophages (Fig. 1b) show weak diffuse staining.

It is clear from the photographs shown in this figure that the stromal cells displayed a strong acid phosphatase reactivity which was distributed in foci, whereas the parenchymal cells showed only diffuse weak staining. The acid phosphatase activity in microscopical sections of benign nasal polyps was almost negligable. Electron microscopy of these cells revealed that the stromal phagocytes had an intact lysosomal apparatus (Fig. 2a) but that the number of lysosomes per cell was relatively small in comparison to ordinary histiocytes found in

mucosal membranes. In parenchymal phagocytes very few lysosomes were found and the cells showed an impaired function of the phagosome-lysosome apparatus: though cytophagocytosis and phagocytosis of cell debris had taken place, lysosomal digestion was not found. In some instances phagocytosed material was found in the cytoplasm of the cell without any evidence of surrounding membrane structures (Fig.2b). This ultrastructural appearance of defective digestive function is in agreement with the cytochemical findings of weak acid phosphatase staining. The poor state of the parenchymal phagocytes was also demonstrated by the presence of swollen mitochondria (Sandritter and Wartman, 1972). Surrounding cells did not exhibit this sign of a negative energy balance.

Fig. 3. shows the correlation between the number of tumour-area infiltrating macrophages, both stromal and parenchymal, and values obtained in the chemotaxis assay on peripheral monocytes in 14 patients. A positive correlation was found ($r = 0{,}055$; $p < 0{,}01$), indicating an association between chemotaxis of peripheral monocytes and tumour-area infiltration. In a further analysis of the data it appeared to us that monocyte chemotactic responsiveness was most impaired in patients with poorly differentiated carcinomas. Poorly differentiated tumours were defined as epithelial malignancies showing no keratin pearl formation, whereas well differentiated tumours still possessed this sign of maturation of squamous epithelial cells (Wahi et al, 1971). A significant difference in monocyte chemotactic responsiveness is evident from fig. 4. between tumours thus classified. This impairment of migratory function in poorly differentiated carcinomas is even more striking when one compares it with monocyte chemotaxis in patients with other chronic stimuli of the upper respiratory tract such as acute and chronic sinusitis including polyp formation.

The lower chemotactic responsiveness probably leads to an impaired infiltration of macrophages: Fig. 5 shows that less macrophages, both stromal and parenchymal are present

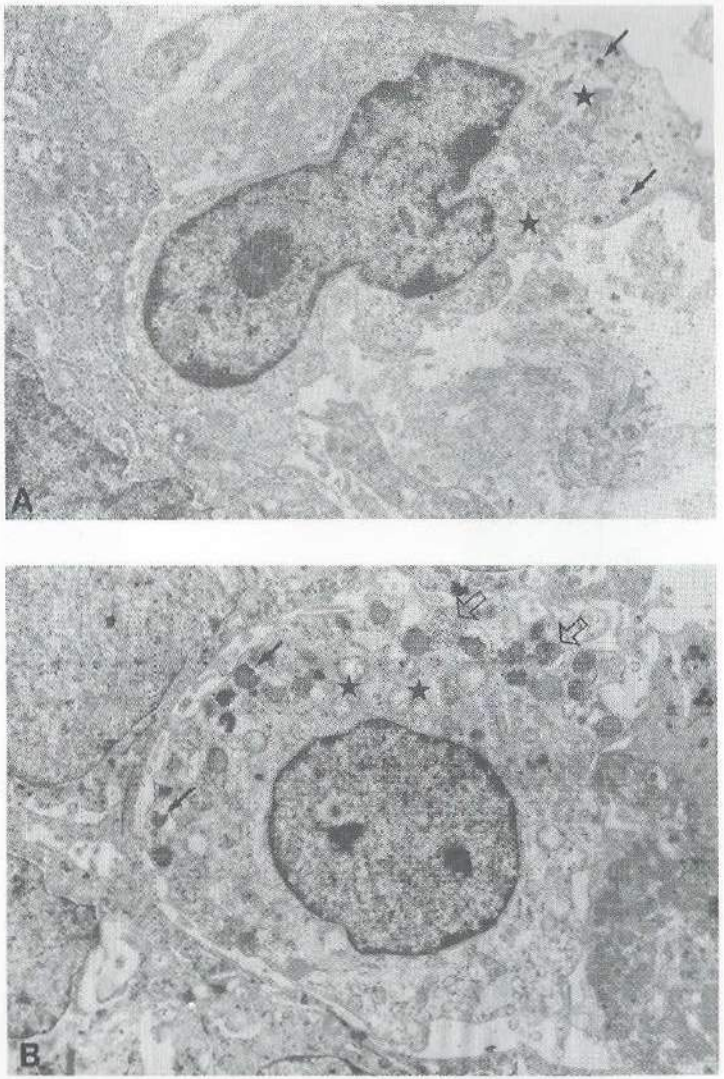

Fig.2. Electronmicroscopy of a stromal macrophage in contact with a tumour cell (Fig. 2a) and a parenchymal macrophage surrounded by tumour cells (Fig. 2b). The stromal macrophage has a normal appearance, although the number of lysosomes (◗) is relatively small. The parenchymal macrophage has only very few lysosomes and contains phagocytosed material (◇) sometimes laying in the cytoplasm of the cell, without any sign of a phagosomal membrane. Mitochondria (★) are swollen.

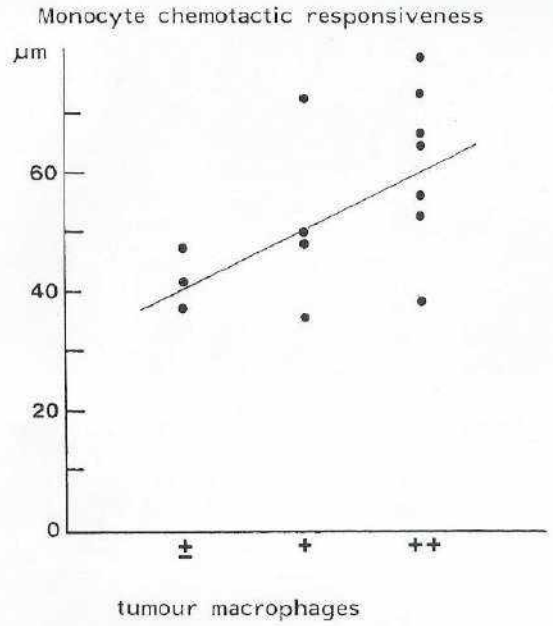

Fig. 3. Correlation between the values of the chemotactic responsiveness of peripheral monocytes (●) and the number of macrophages infiltrating the tumour area (± = 1-5 acid phosphatase positive cells per x 40 microscopic field; + = 5-15 acid phosphatase positive cells per x 40 microscopic field; ++ = 15-25 acid phosphatase positive cells per x 40 microscopic field). r = 0,55; $p < 0,01$.

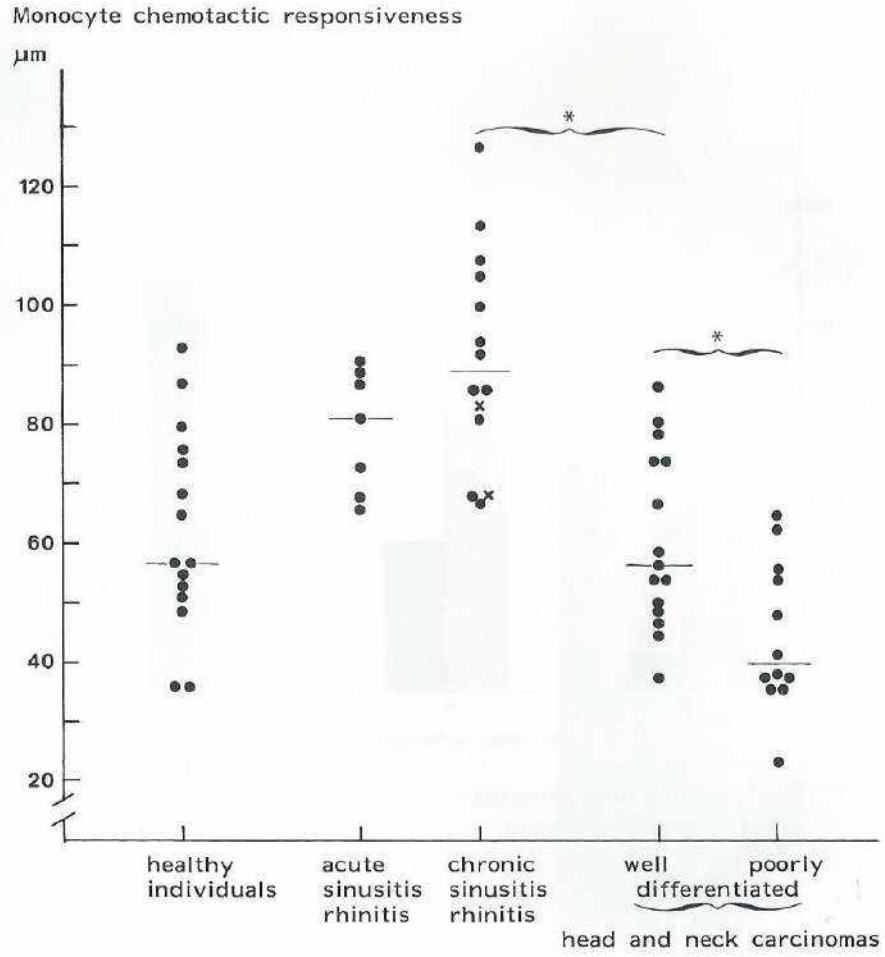

Fig. 4. The chemotactic responsiveness of peripheral monocytes in healthy individuals, patients with acute and chronic sinusitis and patients with well and poorly differentiated head and neck carcinoma.
× Patients with benign nasal polyps.
* A statistical significant difference exists between these two groups of patients ($p < 0,05$, Wilcoxon's two sample test) — = median.

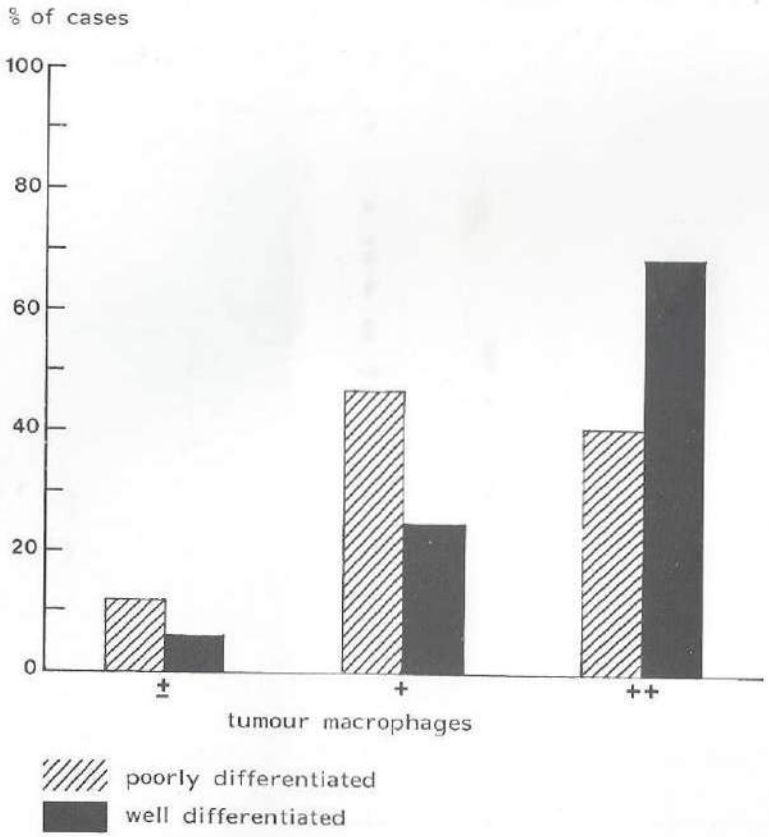

Fig. 5. The number of acid phosphatase positive macrophages per unit tumour area in well (black bar) and poorly (shaded bar) differentiated tumours (± = 1-5 acid phosphatase positive cells per x 40 microscopic field, + = 5-15 acid phosphatase positive cells per x 40 microscopic field and ++ = 15-25 acid phosphatase positive cells per x 40 microscopic field).

in tumours with a low grade of differentiation in comparison to well differentiated tumours.

No difference in chemotactic responsiveness and tumour-area infiltration was found between malignancies of different stage.

DISCUSSION

This study on patients with tumours of the head and neck shows a clear association between the in vitro chemotactic responsiveness of blood monocytes and the infiltration of these cells into an area of malignancy. In patients with poorly differentiated tumours the chemotactic responsiveness was markedly affected and low numbers of acid phosphatase positive cells per unit tumour area were found. Similar findings have been reported by Abraham and Barholt (1978) studying dimethylhydrazine induced colon carcinomas in rats. They also found less macrophages with low enzyme activity when tumours were poorly differentiated. However, in a study on human breast cancer (Lauder et al, 1977) these correlations could not be confirmed.

In mice Snyderman and Pike (1976) have shown that defects in monocyte chemotactic responsiveness and consequently poor tumour infiltration are due to low molecular weight factors derived from the tumour. Other investigators (Otu et al, 1977; Nelson and Nelson, 1978) confirmed these data. Cianciolo and Snyderman (1981) suggest a viral origin for these factors. If tumour derived low molecular weight factors play a part, our findings in the group of poorly differentiated tumours would indicate that these tumours in particular are involved in this phenomenon. At present we have shown that these factors depressing monocyte chemotaxis can be isolated from head and neck malignancies and that they have stronger potency when isolated from poorly differentiated carcinomas. The data of these experiments will be published separately (Balm et al, subm. for publ.).

The ultrastructural appearance e.g. the absence of lysosomal digestion and the mitochondrial swelling, and the weak acid phosphatase staining of the parenchymal mononuclear phagocytes indicate their poor function of state. May be malignant cell derived factors similar to factors influencing chemotaxis play a part in this toxic phenomenon as well, but whatever the mechanisms may be, our data indicate that values obtained in functional tests on peripheral monocytes can not directly be used as indicating the functional state of macrophages in the tumour area itself. These macrophages are, at least in head and neck malignancies, in a much poorer state than peripheral monocytes, with regard to their phagocytic capacity.

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CHAPTER VIII

MONONUCLEAR PHAGOCYTE FUNCTION IN HEAD AND NECK CANCER

Depression of murine macrophage accumulation by low molecular weight factors derived from head and neck carcinomas.

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SUMMARY

In earlier experiments chemotactic responsiveness of peripheral blood monocytes obtained from patients with head and neck cancers was found to be markedly depressed. In an attempt to attribute this defect in migration to an influence excited by low molecular weight factors, of less than 25.000 daltons, derived from the tumour, Amicon filtrates of head and neck cancer cells were administered subcutaneously to C3H mice 24 hrs. before the intraperitoneal (i.p.) injection of Concanavelin A (ConA). Subsequent macrophage accumulation into the peritoneal cavity was quantified. A clear inhibition of macrophage infiltration was found, particularly when filtrates of poorly differentiated tumours were used. Injection of filtrates from healthy oral mucosa were negative, whereas mouse mammary carcinoma filtrates strongly inhibited accumulation.

INTRODUCTION

The number of macrophages within neoplasms has been found to be inversely related to the tumour's metastatic potential (Eccles and Alexander, 1974; Lauder et al, 1977; Gauci and Alexander, 1975) and few macrophages are present in progressively growing tumours (Russell et al, 1976). The local accumulation of macrophages at sites of neoplasmic growth might therefore be a critical event in immunologically mediated tumour cell destruction. Hence it is of interest that tumour patients show impaired monocyte chemotactic responsiveness (Boetcher and Leonard, 1974; Hausman et al, 1975; Kjeldsberg and Pay, 1978). Surgical removal of the tumour often results in a normalisation of this function (Snyderman and Stahl, 1975; Snyderman et al, 1977; 1978) indicating that neoplasms themselves inhibit monocyte chemotaxis by for instance releasing inhibiting factors. In laboratory animals macrophage infiltration into the peritoneal cavity was indeed markedly depressed after administration of a low molecular weight factor derived from experimentally induced animal tumours (Snyderman et al, 1976; Snyderman et al, 1978). The in vitro chemotactic responsiveness was also found to be affected by this factor (Stevenson and Meltzer, 1976; Normann and Sorkin, 1976).

In our previous studies involving head and neck cancer patients an impaired chemotactic responsiveness of peripheral monocytes was also established (Balm et al, 1982a). This impairment of function was found to be correlated to the histological grade of the existing tumour: head and neck cancers with poorly differentiated cells show stronger impairment of in vitro monocyte chemotaxis than well differentiated tumours (Balm et al, 1982b).

In an attempt to attribute these defects in migration to low molecular weight factors derived from head and neck malignancies, we studied the influence of filtrates obtained from tumours of 20 patients with squamous cell carcinoma of the head and neck region. This report deals with the effect

of the administration of low molecular weight factors on macrophage accumulation in mice peritoneal cavities in response to ConA.

PATIENTS AND METHODS

Patients - Twenty patients - 14 males and 6 females - with histologically proven squamous cell carcinoma of the head and neck were studied. Ages ranged from 42-82 years for males and from 50-79 years for females. Informed consent had been obtained. None of the patients had received prior treatment. The site, the stage (TNM) and the histological grade of the malignancies is indicated in Table I. Two histological grades were assigned to the primary tumour based on their capacity to form epithelial pearls: well differentiated (epithelial pearls present) and poorly differentiated (epithelial pearls absent). This classification has proven to be simple and of prognostic value (Wahi et al, 1971) and is in general use in our hospital.

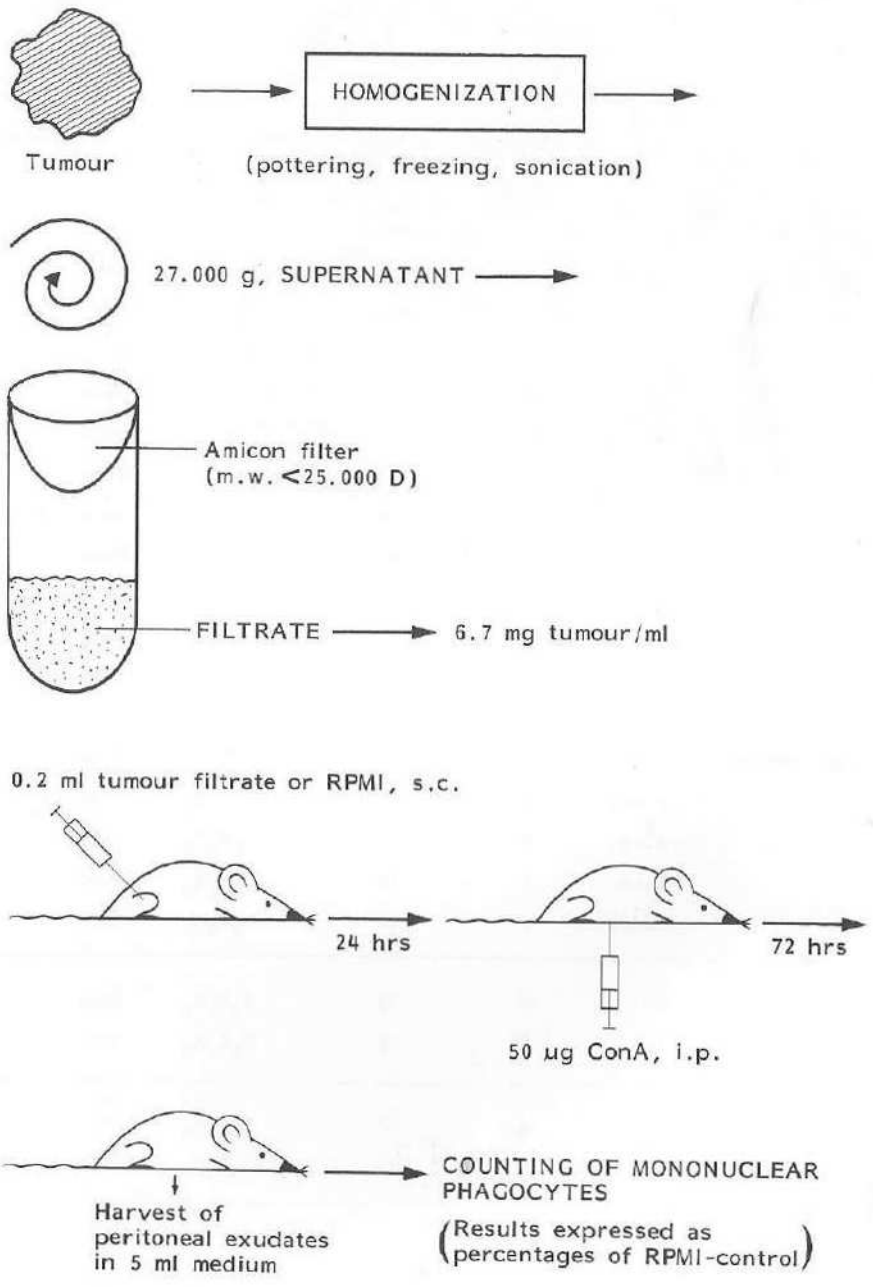
Preparation of tumours and normal filtrates - Biopsies (3-7 mm^3) from the periphery of the tumour were taken immediately after surgical intervention or during endoscopic procedures, frozen in N_2 and stored in - 80°C. After thawing tumour cells were disrupted by sonication (Sonifier B 12, Branson Sonis-Power Company) until no intact cells could be detected by light microscopy. The suspension was then centrifuged for 10 min. at 20.000 g, the supernatant collected and portions were subjected to ultrafiltration by centrifugation (1800 rpm) through Amicon CF 25 Centriflo cones (molecular weight cut of 25.000 Daltons). The filtrate with a molecular weight of less than 25.000 Daltons was aliquoted, suspended to a concentration of 6.7 mg tumour tissue/ml in medium RPMI (Gibco, Europe, batch no. U 816202) and stored at - 80°C until use. Biopsies from spontaneous mammary carcinomas in C3H mice and healthy oral mucosa were similarly prepared and used for respectively positive and negative control experiments.

TABLE I.

Distribution of cases according to site, sex, age, TNM classification and histological grade of the tumours.

Site	Patients	Sex (m/f)	Age (yrs.)	Stage	Differentiation
LARYNX	v.T.	m	59	$T_4N_0M_0$	Poor
	P.	m	42	$T_4N_0M_0$	Well
	v.D.	m	59	$T_3N_0M_0$	Well
	K.	m	76	$T_2N_0M_0$	Well
	v.D.	m	82	$T_4N_0M_0$	Poor
	d.L.	m	67	$T_1N_0M_0$	Poor
	V.	m	50	$T_4N_1M_0$	Well
	B.	m	65	$T_1N_0M_0$	Poor
	R.	m	55	$T_1N_0M_0$	Well
	M.	f	50	$T_3N_1M_0$	Well
	H.-B.	f	71	$T_1N_0M_0$	Poor
ORAL CAVITY	K.	m	68	$T_3N_0M_0$	Poor
	S.-K.	f	71	$T_2N_0M_0$	Poor
	H.-G.	f	71	$T_2N_0M_0$	Well
	S.-G.	f	79	$T_3N_1M_0$	Poor
	v.d.H.	f	71	$T_2N_0M_0$	Well
OROPHARYNX	B.	m	71	$T_3N_1M_0$	Poor
	V.	m	71	$T_4N_2M_0$	Poor
HYPOPHARYNX	T.	m	77	$T_3N_3M_0$	Poor
	V.	m	62	$T_3N_0M_0$	Poor

SCHEME OF BIO-ASSAY



Mice - Female C3H mice aged 5-7 weeks, weight 20-30 grams were obtained from TNO, Rijswijk

Quantification of macrophage accumulation in vivo - Mice were subcutaneously (s.c.) injected in the thigh with 0,2 ml of tissue filtrate or with 0,2 ml RPMI as a control. 24 hrs. later an intraperitoneal (i.p.) injection of 2 ml ConA (Sigma 25 µg/ml of saline) was given. At 72 hrs. thereafter the mice were sacrificed by ether narcosis and the peritoneal cavities were washed vigorously with 5 ml Ca 2+ and Mg 2+ free Hank's balanced salt solution, containing 1 mM EDTA. The 5 ml of medium containing cells was immediately withdrawn and resuspended in a Falcon tube. The total number of cells present in the individual peritoneal cavities was quantified using a haemocytometer. Individual differential white blood cell counts were performed with the aid of 0,05% Turk's solution. All experiments were carried out in triplicate. Macrophage accumulation in tissue filtrate injected mice is expressed as a percentage of the macrophage accumulation in control-RPMI-injected mice (Scheme 1).

Statistical analysis - Data were analysed using Wilcoxon's two sample test.

RESULTS

Initially an attempt was made to establish the most appropriate inflammatory stimulus in order to test the suppressive effects of tumour filtrates. Fig. 1 shows the effects of the i.p. injection of two different agents, phytohaemagglutinin A (PHA) and Concanavelin A (ConA) on the infiltration of macrophages into the peritoneal cavity. In our experiments 50 µg PHA had practically no effect on the macrophage infiltration, but 50 µg ConA appeared to be the agent of choice, giving rise to an approximately 3 fold increase in peritoneal macrophages, both at 48 hrs. and 72 hrs. after i.p. injection. Low molecular weight factors of murine mammary carcinoma given subcutaneously 24 hrs. before

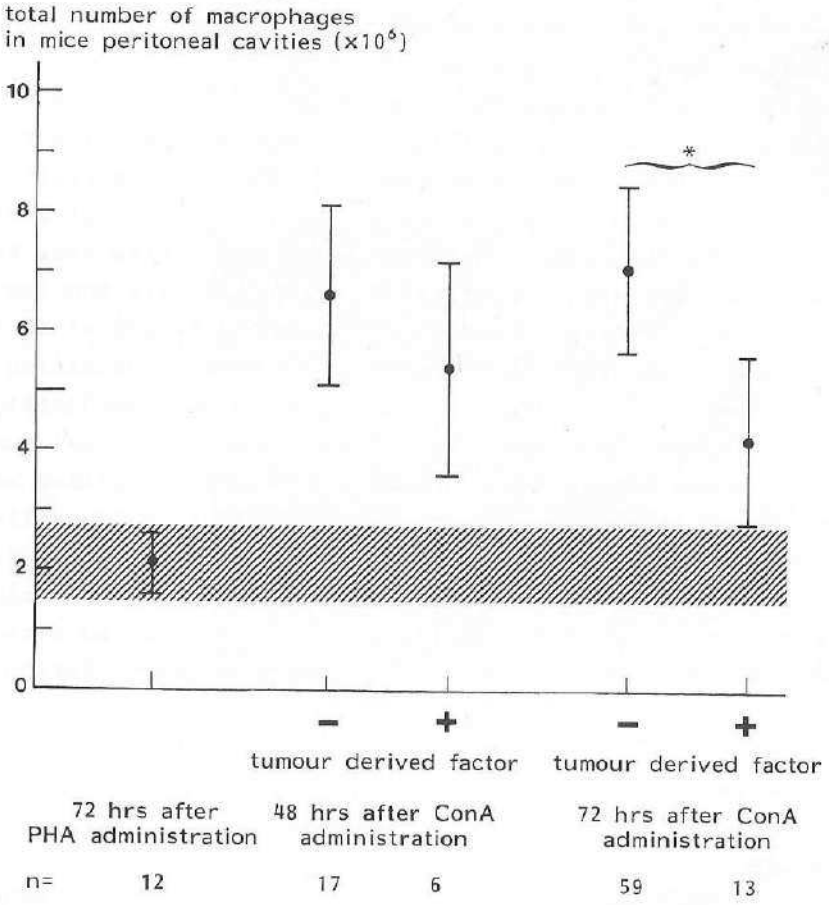


Fig. 1. Kinetics of macrophage accumulation in the peritoneal cavities of C3H mice. The effects of two inflammatory agents were compared: 50 µg PHA and 50 µg ConA. 48 hrs. and 72 hrs. after i.p. injection, peritoneal cavities were lavaged and the total number of macrophages counted. The indicated values represent the mean ± SD of groups of animals treated similarly. The hatched area represents the number of macrophages (x 10^6) normally present in the peritoneal cavity (n=24). This figure also shows the effects on ConA induced macrophage accumulation by subcutaneous injection of filtrates obtained from murine mammary carcinomas (1.3 mg tumour tissue) given 24 hrs. before the i.p. inflammatory stimulus.

* A statistical significant difference exists between these two groups of values ($p < 0,05$, Wilcoxon's two sample test.

— = median).

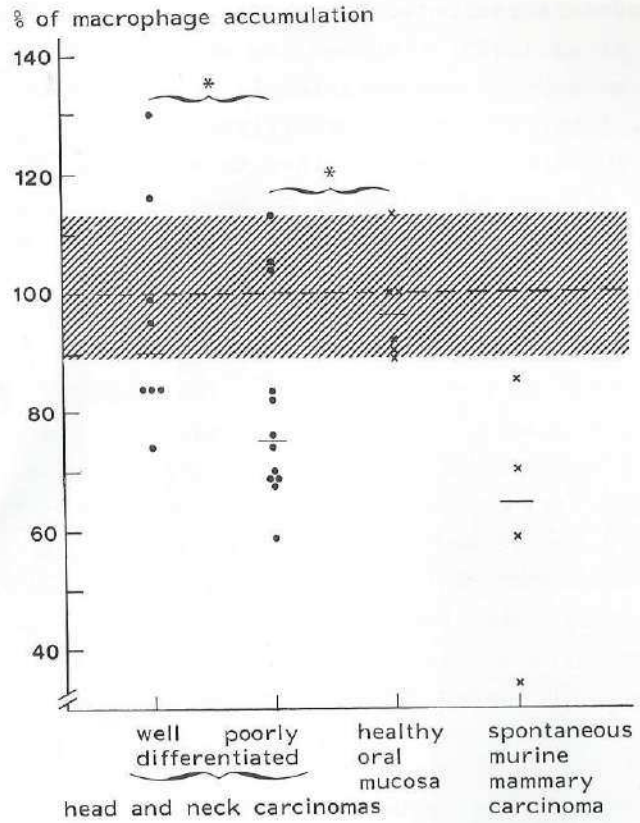


Fig. 2. Effect on peritoneal macrophage accumulation of i.p. injection of low molecular weight factors obtained from well and poorly differentiated head and neck carcinomas (1.3 mg tumour tissue). Factors from healthy oral mucosa (x) and spontaneous murine mammary carcinoma (x) were used as negative and positive controls respectively. Counting of macrophage accumulation took place 72 hrs. after i.p. injection of 50 µg ConA, which was used as inflammatory stimulus and 96 hrs. after i.p. administration of the tumour filtrates. The 100% value at the vertical axis represents the peritoneal macrophage accumulation in the absence of low molecular weight factors (i.p. injection of culture fluid only).

* A statistical significant difference exists between these two groups of values, $p < 0,05$, Wilcoxon's two sample test.

— = median.

the i.p. administration of 50 µg ConA - a scheme advised by Snyderman et al.(1980) - showed the most marked inhibitory effect on macrophage accumulation in an inflammatory response at 72 hrs. After 48 hrs. no significant decrease could be observed. Therefore is was decided to assess the possible inhibitory effects of filtrates of head and neck carcinomas in a ConA induced i.p. inflammatory response at 72 hrs. after initiation.

Fig. 2 shows the results. Filtrates of segments of healthy oral mucosa obtained during reconstructive surgery of non-carcinoma patients were used as negative controls. These filtrates had indeed no influence on macrophage accumulation, whereas filtrates of murine mammary carcinoma used as positive controls gave, as expected, an inhibition of infiltration ranging from 15-65% in respective experiments. It is clear from the data shown that not all tumours exhibited a suppressive action on macrophage accumulation, two of the filtrates even produced values which indicate a slight stimulation of accumulation. These two were found in the group of well differentiated malignancies. It is also noteworthy that in this group the other filtrates induced only slight inhibitory effects. Marked suppression of accumulation was found with extracts of poorly differentiated malignancies; in 12 cases 9 showed inhibition of macrophage accumulation of more than 15% thus reaching values found with filtrates of murine mammary carcinoma, but beyond the range of the negative oral mucosa controls. Consequently a significant difference emerged between values found with poorly and well differentiated carcinomas.

DISCUSSION

Our data obtained in a mouse model show a significant inhibition of i.p. accumulation of exudate macrophages following subcutaneous administration of low molecular weight factors derived from human squamous cell carcinoma of the head and neck. The normal macrophage accumulation under the

influence of filtrates derived from healthy oral mucosa, indicate that inhibitory effects of these cancer filtrates can not be ascribed to epithelial factors normally present. The biochemical identity of these low molecular weight factors excerting inhibitory effects has not yet been ascertained, though several attempts have been made. In independent studies the following characterizations have been made: a low molecular weight oligopeptide designated "anti-kinin" because of its antagonistic action to bradykinin, with which it shares certain chemical characteristics (Stahl et al, 1977); a glycopeptide associated with an RNA fragment on the basis of enzyme susceptibility (Nelson and Nelson, 1978);and a lipid like factor insensitive to pronase and ribonuclease, soluable in organic and aqueous media and possessing ionazable groups(Cheung et al, 1979). Conclusions drawn regarding the molecular weight of suppressive factors must take into account the probability of carrier peptides being associated with much smaller active molecules. Probably of great importance is the recent report of Cianciolo and Snyderman (1981) that monoclonal antibodies directed against the P 15 (E) component of the type C retroviruses neutralize the effect of low molecular weight factors derived from experimental mouse tumours. In their opinion this might indicate a viral origin for these factors. If our suppressing factors derived from poorly differentiated human head and neck malignancies are similar to those derived from mouse experimental tumours, they might also be of viral origin. Proof of this hypothesis in experiments neutralizing the effects of these factors with monoclonal antibodies directed against viruses or viral components may lend additional support to the concept of viral involvement in the pathogenesis of head and neck malignancies.

In our experiments a stimulatory effect of macrophage accumulation was exerted by some of the low molecular weight factors derived from two well differentiated malignancies. Similar stimulatory effects on macrophage function by tumour derived factors have earlier been reported by Otu et al (1977) and

North et al (1976). These authors studied other functions of the mononuclear phagocyte e.g. carbon clearance, colony formation and antibacterial resistance. In mice bearing newly transplanted tumours at first depression was found, later followed by enhanced functions, which gradually fell once again below normal.

In spite of two cases in which macrophage accumulation appeared to be enhanced the majority of our patients had tumours by which macrophage accumulation was evidently suppressed and our findings are therefore in agreement with previous reports touching upon this field: Normann and Sorkin (1976), Pike and Snyderman (1976), Snyderman and Pike (1976), Stevenson and Meltzer (1976), Cianciolo et al (1980) all of whom found that neoplasms of mice, rats and guineapigs contained factors which are potent inhibitors of macrophage infiltration. These investigators postulate that these factors must have important implications for host defence against cancer, indirectly influencing the growth and metastatic capacity of tumours. It is therefore of interest that we were only able to demonstrate these factors in poorly differentiated head and neck cancers, which are known to have a poor prognosis.

In a previous study we showed that peripheral monocytes of patients with poorly differentiated tumours (Balm et al, 1982b) showed in comparison to patients with well differentiated malignancies lower monocyte chemotactic responsiveness and less macrophage infiltration of the tumour area. These findings can now be explained by results reported in this paper, which indicate that especially these tumours produce low molecular weight factors, depressing monocyte chemotaxis.

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CHAPTER IX

SUMMARY AND CONCLUSIONS

This thesis deals with the function and infiltrative capacity of mononuclear phagocytes in head and neck cancer. The main objectives were to assess several in vitro functions of peripheral monocytes in patients suffering from these carcinomas such as: a) pentose shunt activity, which is coupled to digestion of foreign material. b) the ability of monocytes to mature into macrophages and c) their chemotactic activity. In addition to these in vitro tests on peripheral monocytes, the number of tumour infiltrating macrophages as well as their enzyme histochemistry and ultrastrucure were studied and related to the in vitro functions of blood monocytes.

Chapter I and chapter II give a short overview on the literature concerning immunological aspects of cancer in general. After some historical notes the concept of immunological surveillance and immunestimulation are discussed as well as the role of TAA in immunological surveillance.

The hypothesis of this surveillance forms the basis for the assumption that a proper host defence against neoplasia may exist in healthy individuals. It has been difficult to demonstrate TAA in clinical conditions and this may constitute evidence against a surveillance theory. However, the lack of clearcut immunogenicity of human neoplasms may be the very cause for the occurrence of them and indeed may be just evidence in favour of the hypothesis.

In literature immunotherapeutic trials have been reported with mostly negative outcome. They have given rise to doubts that immunotherapy has a real role to play in clinical medicine, but the beneficial effects claimed in some studies including one of our group, indicate that the immunesystem is probably seriously involved in host defence against neoplasia. But understanding of its exact relationship to the growth of tumours

needs to be clarified further to make a more appropriate immunotherapeutical intervention possible.

The detection of TAA employing transplantation techniques, which had been proven useful in animal models, can not be applied in human cancers, but it has been possible to demonstrate tumour specific antigens which are normally present during fetal and embryonic life. In the process of malignant transformation the synthesis of these antigens is probably reinitiated. CEA might be of importance in the assessment of recurrency, especially in tumours of the intestinal tract. AFP is another fetal antigen of which it is claimed to be tumour associated. The antigen is found in a majority of sera of patients with hepatic or testicular carcinoma. The presence of specific antibodies against antigens of EBV in cases of Burkitt's lymphoma and nasopharyngeal carcinoma suggests a viral relationship in these tumours.

Chapter III deals with the relation between the MPS and cancer. Firstly some functional characteristics of these cells are described emphasizing their importance in the uptake of foreign material and the regulation of the immune response. Secondly their importance in host defence against neoplasia is discussed. It is of interest that some functions of these cells such as chemotaxis have been found to be inhibited by low molecular weight factors derived from tumours and that this may counteract their effectiveness at the site of the malignant growth especially in more widespread disease.

In chapter IV the possible viral relationship of some head and neck cancers is reported and as already said EBV has been suggested to be one of the positive agents in nasopharyngeal carcinoma.

Uptill now immunereactivity in head and neck cancer patients has been studied particularly focussing on T-cell function, as the assessment of the number of T-cells and T-cell subpopulations, their blastogenic capacity and the evaluation of delayed skin-test reactivity. Defects of T-lymphocyte reactivity have been established particularly in early stages of disease and this is

relatively unique for this type of cancer.

The results of our studies on the function of the MPS in head and neck cancer are given in chapter VI, VII and VIII.

In chapter VI results are described of the NBT-dye reduction, the maturation capacity and the chemotactic responsiveness of peripheral blood monocytes in head and neck cancer patients and their respective controls. These cellular functional activities appeared to be age-related which demonstrates the necessity of using age-matched healthy controls. In doing so a clear impairment of chemotactic responsiveness was found in the group of carcinoma patients; the maturation capacity however, was found to be enhanced. The results of the NBT-dye reduction assay were not different from those of the controls.

Defects in migratory capacity of mononuclear phagocytes may lead to an impaired recruitment of these cells at the neoplastic site, the latter obviously being a major factor in host defence, overruling other functional enhancements of the cells.

Chapter VII deals with the chemotactic responsiveness of peripheral blood monocytes in relation to the presence of these cells at the tumour site and their ultrastructural and cytochemical aspects. It appeared that the chemotactic responsiveness was most markedly depressed in patients with poorly differentiated malignancies. Two types of macrophages could be distinguished in the tumour area: a) one type with strong acid phosphatase activity, which could be found in the stromal tissue surrounding the malignant epithelial cells and b) another type with diffuse enzyme activity which was found in between the epithelial cells. Electron microscopy revealed that the latter cells contained low numbers of lysosomes and tumour cell-debris in the cytoplasm without any sign of a surrounding phagosomal membrane. Both ultrastructure and cytochemistry probably indicate a poor functional state of macrophages in the near vicinity of tumour cells.

The number of macrophages present at the tumour site correlated with the values found in the chemotaxis assay and consequently poorly differentiated tumours showed low chemotactic values and possessed low numbers of infiltrating macrophages. Low molecular weight factors derived from tumour cells are suggested to play a part.

Chapter VIII described the influences of such factors especially in relation to the histological grade of their tumour of origin. These factors were assessed in a migration model using the peritoneal cavity of the C3H mouse. ConA was used as a migration inducing agent and the capacity of the tumour factors to counteract the action of ConA was tested.

It appeared that when factors were derived from poorly differentiated tumours, the most significant inhibition of macrophage migration was found. These date strongly suggest that head and neck malignancies and in particular poorly differentiated ones produce low molecular weight factors influencing the chemotaxis of monocytes resulting in a poor infiltration of macrophages into these malignancies, possibly with corresponding prognostic implications.

It is of importance that new data on the nature of these low melocular weight factors have been put forward by Cianciolo and Snyderman (1981). They reported the neutralization of these factors by a monoclonal antibody, also reacting with the P(15) E component of the type C retrovirus, indicating a new approach to the study of the role of viruses in malignancy.

We have the impression that such monoclonal antibodies directed against virus products influencing migration patterns of mononuclear phagocytes,may be of increasing importance in the diagnosis and therapy of malignancies.

Reference

Cianciolo, G.J. and Snyderman, R (1981). Characterization of an inhibitor of monocyte function in effusions of cancer patients. In: Lymphokines and thymic hormones: their potential utilization in cancer therapeutics. (Eds. A.L.Goldstein and M.A. Chiligos) Raven Press, New York, p. 205.

HOOFDSTUK X

SAMENVATTING EN CONCLUSIES

Dit proefschrift beschrijft de resultaten van het onderzoek naar de functionele eigenschappen en infiltratieve capaciteit van mononucleaire phagocyten bij patienten met een hoofd-hals tumor. De volgende parameters werden in vitro getest om een indruk te krijgen over het functioneren van de perifere monocyten bij deze carcinoompatienten: a) de pentose shunt activiteit, die gekoppeld is aan de verteringsactiviteit van de cellen, b) het vermogen van monocyten om uit te rijpen tot macrophagen en c) hun chemotactische activiteit.

Ook werd het aantal in de tumor geinfiltreerde macrophagen bestudeerd alsmede hun enzymhistochemie en ulstrastructuur, welke werden gerelateerd aan de beschreven in vitro functies van de perifere monocyt.

Hoofdstuk I en hoofdstuk II zijn bedoeld als een algemene inleiding in de immunologische aspecten van maligne gezwelgroei. Nadat enkele historische aspecten zijn toegelicht, wordt het concept van de immuunbewaking, de immuunstimulatie en het voorkomen van tumor-geassocieerde-antigenen nader toegelicht.

Het concept van de immuunbewaking is gebaseerd op de aanwezigheid van tumor-geassocieerde-antigenen, alhoewel deze bij de mens moeilijk zijn aan te tonen. In experimentele diermodellen heeft men de beschikking over transplantatie technieken waardoor men de aanwezigheid van tumor specifieke antigenen eenvoudig kan vastleggen.

Bij de mens is het met behulp van radio immuno-assays gelukt ook enkele tumor geassocieerde antigenen aan te tonen, welke normaal gesproken tijdens het foetale en embryonale leven aanwezig zijn, doch tijdens het proces van de maligne celtransformatie blijkbaar weer gesynthetiseerd worden. Twee voorbeelden hiervan zijn het CEA en het AFP. Een stijging van een CEA titer is van belang gebleken bij tumorrecidieven van de tractus gastrointestinalis, maar is minder specifiek dan

het AFP dat in de meerderheid van sera bij primair levercarcinoom en testiscarcinoom gevonden wordt.

De aanwezigheid van hoge titers antilichamen tegen de antigenen van het EBV bij patienten met een Burkitt's lymphoma of een nasopharynxcarcinoom kunnen wijzen op het bestaan van tumor geassocieerde antigenen die nauw verwant zijn met virussen of virusprodukten.

Na een hausse van publicaties die gunstige resultaten vermeldden van immunotherapie - zelfs bij uitgebreide tumoren - worden recentelijk meer negatieve resultaten beschreven. Dit heeft aanleiding gegeven tot enige twijfel of immunotherapie wel een reële rol zal kunnen spelen binnen het behandelingsplan voor tumoren. Echter, de gunstige effecten die in sommige studies beschreven worden, waaronder één van ons, wijzen er op dat het immuunsysteem wel degelijk betrokken kan zijn bij de afweer van de gastheer tegen tumoren en dat een verdere evaluatie vereist is.

In hoofdstuk III wordt de relatie beschreven tussen het MPS en maligne tumorgroei. Eerst worden de belangrijkste functionele karakteristieken van deze cellen genoemd, waarbij de nadruk wordt gelegd op het belang in de regulatie van de immuunreactie. Ofschoon deze functie van groot belang is in de afweer van de gastheer tegen tumoren door de inductie van antigeen specifieke T- of B-cellen, de mononucleaire phagocyten vormen ook zelf een belangrijk element in de afweer van de gastheer, omdat zij als directe effector cel een cytotoxische activiteit kunnen ontplooien.

In hoofdstuk IV wordt de mogelijke rol van virussen bij het ontstaan van hoofd-hals tumoren beschreven. Vooral wordt de nadruk gelegd op de relatie tussen het nasopharynxcarcinoom en het EBV, waarvan wordt vermoed dat dit virus belangrijk is voor het ontstaan van dit type carcinoom.

Tot nu toe is de immuunreactiviteit van patienten met een hoofd-hals tumor voornamelijk bestudeerd aan de hand van de T-cel reactiviteit, uitgedrukt in het aantal T-cellen in het

perifere bloed, de blastogene activiteit van deze cellen en de vertraagde huidtest reactiviteit. Dikwijls wordt een vermindering in de T-cel activiteit gevonden, vooral in vroege stadia van de ziekte en dit is betrekkelijk uniek voor dit type tumor.

De resultaten van onze studies naar de functie van het MPS bij patienten met een hoofd-hals tumor worden beschreven in hoofdstuk VI, VII en VIII.

In hoofdstuk VI worden de resultaten beschreven van de reductie van NBT tot formazan, de rijpingscapaciteit en de chemotaxie van perifere bloedmonocyten bij patienten met een hoofd-hals tumor en de daarbij behorende controle groepen. De functionele activiteit van monocyten bleek in belangrijke mate leeftijds afhankelijk te zijn en toonde aan dat het gebruik van een gezonde controle groep van dezelfde leeftijd een conditio sine qua non is. Binnen de groep van de carcinoompatienten werd een duidelijke verlaagde chemotactische activiteit van monocyten gevonden, terwijl de rijpingscapaciteit van deze cellen hoog bleek te zijn. In de resultaten van de NBT reductie test konden geen verschillen aangetoond worden tussen de cellen van de carcinoompatienten en die der controle personen.

Defecten in migratie activiteit van mononucleaire phagocyten zouden kunnen leiden tot een verminderde infiltratie van deze cellen in de tumor, hetgeen een belangrijke negatieve factor zou kunnen zijn binnen de afweer van de gastheer. Andere verhoogde functies van de cel zouden door een dergelijke verminderde chemotaxie overheerst kunnen worden.

Hoofdstuk VII behandelt de chemotaxis van perifere monocyten met betrekking tot de aanwezigheid van deze cellen in de tumor en hun ultrastructurele alsmede hun cytochemische aspecten. De chemotaxis bleek het meest verlaagd te zijn bij patienten met een slecht gedifferentieerde tumor. Twee typen, of liever twee stadia, konden bij enzymhistochemisch onderzoek worden onderscheiden binnen de tumor geinfiltreerde macrophagen: a) één met

een sterke zure fosfatase activiteit, die vooral gevonden werd in het omgevende stroma en b) een andere met een zwakke diffuse enzymactiviteit die vooral tussen de maligne epitheelcellen werd gevonden.

Electronen microscopisch onderzoek toonde aan dat de cellen met de lage enzymactiviteit ook een gering aantal lysosomen bevatte en tumorcel debris in het cytoplasma, zonder de aanwezigheid van een omgevend phagosoom membraan.

Zowel de ultrastructurele beelden als de cytochemische aspecten van deze cellen doen vermoeden dat er sprake is van een slechte functionele toestand van de macrophagen wanneer zij zich tussen, of in de directe nabijheid van de tumorcellen bevinden.

Er bestond een duidelijke correlatie tussen het aantal macrophagen in de tumor en de chemotactische activiteit van monocyten in vitro. Bij gevolg werd in geval van slecht gedifferentiëerde tumoren een lage chemotactische activiteit gevonden en werden in slecht gedifferentiëerde tumoren minder macrophagen gevonden. Er wordt verondersteld dat laag moleculaire factoren afkomstig van tumorcellen hierbij een rol spelen.

In hoofdstuk VIII wordt beschreven in hoeverre laag moleculaire factoren afkomstig van hoofd-hals tumoren van invloed kunnen zijn op de migratie van mononucleaire phagocyten. Deze factoren werden aangetoond in een proefopstelling, waarin de migratie werd bestudeerd van macrophagen naar de peritoneaal holte van C3H muizen en ConA fungeerde hierbij als migratie inductor. Na subcutaan inspuiten van de laag moleculaire factoren werd nagegaan in hoeverre zij de migratie beinvloedden.

Wanneer deze stoffen geïsoleerd waren uit slecht gedifferentiëerde tumoren bleek dat de migratie inhibitie van macrophagen significant hoger lag dan wanneer deze factoren afkomstig waren van goed gedifferentiëerde tumoren. Hieruit werd geconcludeerd dat vooral hoofd-hals tumoren met een lage differentiatiegraad laag moleculaire stoffen produceren, die de chemotaxis van monocyten meer pregnant kunnen beinvloeden. Dit zou een verklaring kunnen vormen voor de slechte infiltratie van macrophagen

in slecht gedifferentiëerde tumoren met alle mogelijke gevolgen voor de prognose.

Cianciolo en Snyderman (1981) beschreven onlangs dat het effect van deze laag moleculaire stoffen geneutraliseerd kan worden door een monoclonale antistof, gericht tegen de P(15)E component van het type C retrovirus. Dit duidt een nieuwe richting aan in de studie naar de rol van virussen en/of virusprodukten bij maligniteiten.

Wij kunnen ons niet aan de indruk onttrekken dat monoclonale antistoffen gericht tegen virus of virusprodukten die de migratie van mononucleaire phagocyten naar tumoren kunnen beinvloeden, van groot belang kunnen zijn bij de diagnose, de prognose en de behandeling van maligniteiten.

LITERATUUR

Cianciolo, G.J. en Snyderman, R. (1981). Characterization and inhibitor of monocyte function in effusions of cancer patients. In: lymphokines and thymic hormones: their potential utilization in cancer therapeutics. (Eds. A.L.Goldstein and M.A.Chiligos). Raven Press, New York, p. 205.