RETROVIRAL P15E-RELATED FACTORS IN HEAD AND NECK CANCER I.B. TAN

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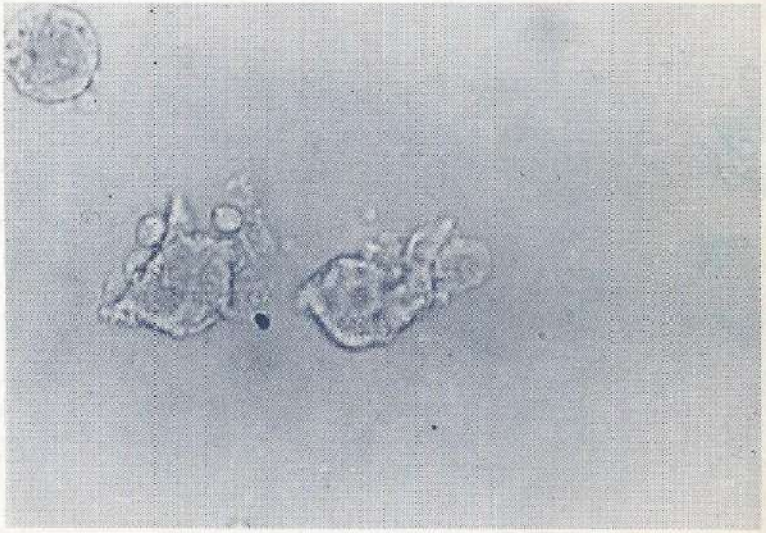

I.B. TAN

VRIJE UNIVERSITEIT TE AMSTERDAM

RETROVIRAL P15E-RELATED FACTORS IN HEAD AND NECK CANCER

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Promotor : prof. dr. G.B. Snow
Copromotores : dr. A.J.M. Balm
dr. H.A. Drexhage
Referent : prof. dr. Ph. Rümke

The investigations reported in this thesis were performed in the Department of Otolaryngology and Head & Neck Surgery (head of the department: Prof. dr. G.B. Snow) and the Pathological institute (head of the department: Prof. dr. C.J.L.M. Meijer) of the Free University Hospital Amsterdam under the supervision of the staff of the laboratory for clinical immunology. The experimental work was done by Mrs M. de Haan-Meulman. The photographs were made by Mr. G.J. Oskam and Mr. J.T. van Veldhuisen.
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aan mijn ouders
aan Geraldine

Abbreviations

AIDS	acquired immune deficiency syndrome
BSA	bovine serum albumin
Con A	concanavalin A
DNFB	dinitro-fluoro-benzene
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
FeLV	feline leukemia virus
FLV	Friend leukemia virus
FMLP	n-formyl-methionyl-leucyl-phenylalanine
H/N ca LMWF's	head and neck cancer derived LMWF's
HRP	horseradish peroxidase
HTLV	human T-cell lymphotropic virus
IL-2	interleukin-2
ip	intraperitoneal
kD	kilo Daltons
LMWF's	low molecular weight factors
Mab's	monoclonal antibodies
MCR	monocyte chemotactic responsiveness
MIF	migration inhibition factor
MLV	Molony leukemia virus
MNL	mononuclear leucocytes
MPS	mononuclear phagocyte system
MuLV	murine leukemia virus
Mw	molecular weight
NBT	nitroblue tetrazolium
NSE	non-specific esterase
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PHA	phytohaemagglutinin A
PWM	pokeweed mitogen
RLA	rat liver araginase
RLV	Rauscher leukemia virus
sc	subcutaneous
S.E.M.	standard error of the mean
SLE	systemic lupus erythematosus
UICC	union internationale contre le cancer

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Contents

Mononuclear phagocytes and head and neck cancer

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, alveolar macrophages and histiocytes) and each of these mononuclear phagocyte types has its own functional and biochemical characteristics (1,2). The Mononuclear Phagocyte System (MPS) plays an important role in host defence, which is indicated by the fact that the mononuclear phagocyte has a variety of functions important in immunity i.e. phagocytosis, antigen presentation, secretion of inflammatory mediators and a cytotoxic capability (3,4).

The importance of the MPS in the defence against neoplastic disease has been hypothesized by many investigators. In 1941 Stern was one of the first who found a depressed macrophage function in patients with cancer. Later a correlation was established between such a depressed function and both severity of disease and lack of response to therapy (5). A relationship between macrophage contents of the tumour and its biological behaviour was demonstrated by Eccles and Alexander (6) using animal tumour models. Similar findings were reported by Russel et al (7). Following these experiments Lauder et al (8) assessed the number of macrophages in 50 cases of human breast carcinomas and they were able to show that there were fewer cases with metastases amongst patients having higher macrophage and plasma cell infiltration. However, others failed to confirm such a positive relationship between macrophage contents of the tumour and prognosis in particular in the case of colon carcinomas (9,10,11). Some have even found an inverse relationship between macrophage content and prognosis studying malignant melanoma (12,13). One of the most important functions of the mononuclear phagocyte necessary for an effective host defence is its capability to migrate into sites of inflammation or malignant growth. It is worthy to note that just this important function has been found disturbed in the malignant state. A defective chemotaxis as measured with the so-called "Boyden chamber" (migration through millipore membranes) was established in patients with several types of carcinomas, such as those of the genito-urinary tract (14), of the gut (15), the lung and the prostate (16). Not only in man, but also in experimental animal tumour models, e.g. mammary carcinomas of C_3H mice, monocyte chemotaxis was found to be disturbed (17,18).

There are only a few reports paying attention to the function of mononuclear phagocytes in head and neck cancer. Berlinger et al (19) described an abnormal low mixed lymphocyte reaction in patients with head and neck cancer. He found an improvement of this function after filtration of the blood lymphoid cell suspensions through columns removing adherent cells; reconstitution of the cultures with the adherent cells usually reintroduced the suppressive effect. Upon microscopical examination of the adherent cell population typical non-specific esterase positive macrophages were detected. The authors concluded from these experiments that macrophages probably play an important role in hampering T-cell immune responsiveness

in head and neck cancer. Cameron et al (20), also studying head and neck cancer patients, reported a defect in tumouricidal capacity of macrophages. Of 30 patients, 29 were incapable of killing tumour cells in vitro. They reasoned that this was due either to a "defect" in the macrophages or to a plasma inhibitory factor circulating in the patients. From our group, Balm et al (21) investigated at an earlier occassion the influence of head and neck cancer on the NBT-dye reduction, the maturation and the migratory capacity of blood monocytes. They were unable to establish an effect on NBT-dye reduction; however a clear impairment of chemotactic responsiveness was evident (using modified Boyden chambers). In addition they were able to establish a clear association between in vitro chemotactic responsiveness of blood monocytes and the numerical presence of macrophages in head and neck cancer specimens.

Evidence for tumour derived factors influencing mononuclear phagocytes

In 1976 Snyderman et al (22) developed an in vivo bioassay to study the effect of the presence of a tumour on the accumulation of macrophages in the peritoneal cavity of mice after intraperitoneal (i.p.) injection of Phytohaemagglutinin A (PHA). By quantifying the number of macrophages in the peritoneal cavity they found a significant inhibition of accumulation when sarcoma cells had been inoculated subcutaneously (s.c.) in the thigh of the animals before. Macrophage accumulation was not only affected by intact tumour cells, but also by cell free extracts and dialysates of the tumour (23). Other investigators have also demonstrated that tumours and tumour derived factors interfere with inflammatory responses or biological processes (24). Relevant to our study is that Norman and Sorkin (25) reported an inhibitory activity on monocyte chemotaxis by Polyoma virus induced tumour cell lines and supernatants of the murine NIH 3T3 cell line. Otu et al (26) described that an inhibitory effect on macrophage migration is hampered by supernatants of cultured Lewis Lung carcinoma cells and by sera of mice bearing such tumours. In 1980 Cianciolo et al (27) took the afore mentioned experiments of Snyderman further by the demonstration that the tumour factors influencing the macrophage accumulation in the peritoneal cavity of mice were of low molecular weight (<25,000 Daltons). They also occurred in the plasma and urine of mammary carcinomas bearing mice. Even tiny amounts of plasma and urine ($4.1O^{-5}$ml) injected into the thighs of normal mice significantly depressed the accumulation of peritoneal macrophages after the i.p. injection of PHA. In the study on monocyte chemotaxis in man it has become known that surgical removal of the tumour restored the chemotactic defect: peripheral monocytes of 10 out of 12 breast cancer patients with depressed chemotactic responsiveness before operation had normal or above normal functions after radical surgery (28). This notion further supported a concept that the defective macrophage chemotaxis could be considered as the result of factors released from the tumour. With regard to head and neck cancer, Balm et al (29) demonstrated a significant inhibition of macrophage accumulation in the peritoneal cavity of mice after injection of low molecular weight factors (LMWF's <25,000 Daltons) isolated from these tumours. In 1981 it was demonstrated by Cianciolo et al (30) that the inhibitory effect on monocyte chemotaxis exerted by the LMWF's of both animal and human cancers could be absorbed by any of three different monoclonal antibodies to a retroviral capsular protein, i.e. P15E. This observation highlights an important relationship between a retroviral component and a probably widely occurring tumour factor of low molecular weight with an influence on monocyte chemotaxis. P15E is the hydrophobic transmembrane protein of the retroviral envelope of several oncogenic murine and feline retroviruses. The protein is synthesized as part of a precursor

molecule with a molecular weight of 80-90.000 Daltons (GP85) (31). GP85 is cleaved into P15E and GP70 during protein maturation (fig.1).

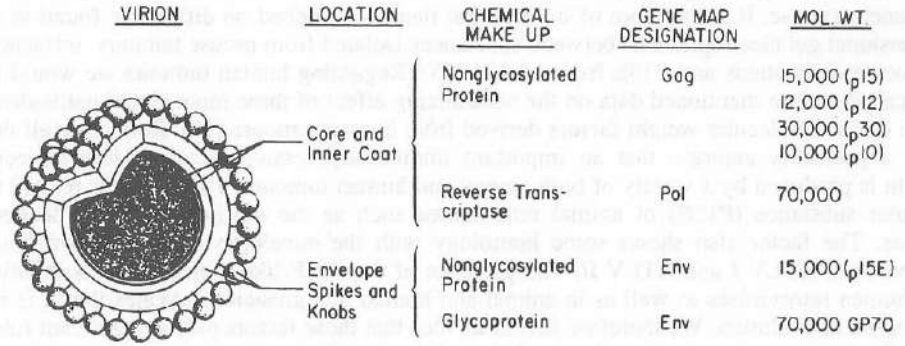

Fig. 1. Scheme of the virion of a retrovirus.

Retroviruses and immunesuppression

Oncogenic retroviruses have been isolated from the tumours of many mammalian species, including mice (32,33,34), hamsters (35), cats (36), cows (37) and primates (38,39,40). It has been shown that such viruses are in many instances not only tumourigenic, but also immunosuppressive. Such properties have been well documented in particular in the case of Murine Leukemia Viruses (MuLV) (41,42). It is for instance known that low molecular weight extracts of certain oncogenic murine leukemia viruses such as Friend Leukemia Virus, (FLV), Molony Leukemia Virus (MLV) and Rauscher leukemia virus (RLV) were able to inhibit macrophage accumulation to chemotactic stimuli in the peritoneal cavity of mice (43) (note the analogy to the afore mentioned tumour factors of low molecular weight, 29). It was also demonstrated by Mathes et al (44,45) that the in vitro blastogenic response to Con A of feline lymphocytes, was inhibited by the purified FeLV envelope protein P15E. Cianciolo et al (30) found that in particular this low molecular P15E, isolated from RLV inhibited the response of human monocytes to chemotactic stimuli in vitro. GP70 had no such effect (30). It is worthy to note that GP70-like molecules have been linked to other disturbances of the immune system, most notably to autoimmune diseases like SLE (46). Several authors were able to demonstrate C type viral antigens in patients with SLE. (47,48).
Although C type antigens were also found in some non-SLE tissues (49), it is possible that certain T-cell tropic viruses may, through depression of T-lymphocyte activities, contribute to SLE (50). Thus a solid body of evidence has accumulated, which suggests that animal retroviruses and retroviral capsular components, are capable of inducing aberrations of the immune response. Noteworthy is also the causal relationship now suspected between the human T-cell lymphotropic virus III (HTLV III) and the Acquired Immuno-Deficiency Syndrome (AIDS), occurring mostly in homosexual men (51,52). The latter syndrome again establishes a relation between retroviruses and immune disturbances. Regarding the other retroviruses playing a role in human pathology - namely HTLV I and HTLV II - a significant homology with P15E

(73%) was found occurring in a 26-ammino acid sequence located in the P21 region of the capsular protein of the human retroviruses (53). As said before the animal retroviruses are particularly known for their effect to induce tumours which produce low molecular weight factors influencing macrophage function (43). It was just a short step to the proposition that these low molecular weight factors were P15E-like and would thus act as the inhibitors of the immune response. It is therefore of interest that there was indeed no difference found in two dimensional gel electrophoresis between substances isolated from mouse tumours influencing monocyte chemotaxis and P15E from MuLV (53). Regarding human tumours we would like to recall the afore mentioned data on the neutralizing effect of three monoclonal antibodies to P15E on low molecular weight factors derived from human tumours (30). Reflecting all these data, a posibility emerges that an important immunosuppressive factor of low molecular weight is produced by a variety of both animal and human tumours. This factor is related to a capsular substance (P15E) of animal retroviruses such as the murine and feline leukemia viruses. The factor also shows some homology with the envelopes of two known human retroviruses, HTLV I and HTLV II. The presence of the P15E-like molecules in both animal and human retroviruses as well as in animal and human malignancies indicates that it is well conserved in evolution. We therefore favour an idea that these factors play a significant role in physiological processes, such as the regulation of the immune response.

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Experimental questions

As has been stipulated in the introduction, a decreased chemotactic responsiveness was found earlier by Balm et al (1) in patients with head and neck carcinomas. The "Boyden chamber" method used in these experiments is time consuming and for that reason not easily applicable in clinical laboratory testing. This has led to the first experimental questions for the present study.

- *Is the newly developed method, the socalled "Polarization assay" (2) suitable for the detection of defects in monocyte chemotactic responsiveness in patients with head and neck cancer? Can a restoration of "polarization" be detected after removal of the tumour?*

These questions are dealt with in chapter 2 .

Low molecular weight factors, derived from a variety of malignancies - including head and neck cancer - are responsible for the defects in chemotactic responsiveness of mononuclear phagocytes. Their effect can be absorbed by monoclonal antibodies to P15E. This notion has led to several experimental questions addressed in this thesis.

- *Can low molecular weight factors isolated from head and neck carcinomas be detected by means of their effect on healthy donor monocytes in a polarization assay; can this effect be neutralized with monoclonal antibodies to P15E?*
- *Can retroviral products as P15E and GP70 be detected in microscopical preparations of head and neck cancers using immuno-histochemical techniques?*
- *Can free P15E like material be detected in the circulation of head and neck cancer patients?*

These questions are dealt with in chapter 3, 4 and 5.

Patients with head and neck cancer not only have chemotactic defects, but also severe disturbances in T-cell mediated immunity. T-cellular defects are not only a feature of head and neck cancer but also of a large variety of tumours, whatever the tissue of origin and species.
Studies arriving at this knowledge have mostly been conducted by assaying Delayed Type Hypersensitivity (DTH) skin reactions, and a variety of tumour factors have been identified suppressing DTH skinreactions.
As an example I want to mention here the experiments of Nelson and Nelson (3). They reported a depression of DTH skin reactions to sheep red blood cells in mice, when fibrosarcoma cells had been mixed with the antigen. This depression was directly proportional to the number of tumourcells used. They found that not only intact fibrosarcoma cells, but also supernatants of cultured human and rat tumour cells were able to induce these effects . Therefore the last experimental question of this thesis is:

- *Are P15E-like low molecular weight factors, present in head and neck cancer and potent in supressing monocyte chemotactic ability also able to suppress DTH skinreactions ?*

This question is dealt with in chapter 6.

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DEFECTIVE MONOCYTE CHEMOTAXIS IN PATIENTS WITH HEAD AND NECK CANCER

Restoration after treatment

I. B. Tan[+], H. A. Drexhage[O], R. J. Scheper[O], B. M. E. Von Blomberg- v.d. Flier[O], M. de Haan-Meulman[+], G. B.Snow[+] and A. J. M. Balm[+].

From the [+]Department of Otolaryngology and Head & Neck Surgery and the [O]Department of Immunopathology, Pathological Institute, Free University Hospital, Amsterdam, the Netherlands.

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Abstract

Monocyte chemotactic responsiveness (MCR), as measured by the cell's capacity to migrate through millipore membranes towards the chemoattractant casein, is impaired in all head and neck cancer patients examined thusfar. Using the "polarization assay", a more rapid and sensitive test system, we tested the MCR in 24 head and neck cancer patients and 31 controls. The outcomes were compared to those of the well established Boyden chamber method. Results correlated well. All head and neck cancer patients showed a seriously depressed monocyte chemotaxis before treatment when tested in the polarization assay. Nine patients were re-examined after surgery and in seven patients the defective MCR was restored. This lends additional support to the concept that tumour derived factors are responsible for the inhibitory effect on monocyte chemotaxis. The potential of the polarization assay for predicting early relapse is discussed.

Introduction

Mononuclear phagocytes are likely to play a role in host resistance to malignancies: the cells have been shown to kill or to inhibit the proliferation of tumour cells in vitro (1) and inverse correlations were found between tumour macrophage infiltration and tendency to metastasize (2). Amongst the many functions of the mononuclear phagocytes, the chemotactic responsiveness has extensively been studied and often found to be defective in patients with a variety of malignancies including squamous cell carcinomas of the head and neck (3,4,5). In these studies monocytes were mostly tested for their capability to migrate towards the chemoattractant casein using Boyden chambers (6). The "Boyden assay" is time consuming and needs specialised equipment. Recently another assay for measuring chemotaxis has been developed, i.e. the "polarization assay" (7). This test is claimed to be rapid, quantitative, reproducible and applicable to the study of even minor disturbances in monocyte migration. In essence the assay determines the rapid change in morphology from a round to a triangular motile configuration ("polarization") under the influence of the chemo-attractant N-Formyl-Methionyl-Leucyl-Phenylalanine (FMLP). Such a morphologic change preceeds the chemotactic response (7). To evaluate the usefulness of this new assay we employed it in a study on 24 head and neck cancer patients. Outcomes of the test were compared to those of the established Boyden chamber method. Patients were tested just prior and several weeks after complete removal of the tumour.

Patients and methods

Patients

Twenty-four patients, 20 males and 4 females, with histologically proven squamous cell carcinomas of the head and neck were studied. The control groups were formed by a group of young (age 20-40 yrs) and aged (57-84 yrs) healthy individuals. The patients data including TNM classification (UICC, 8) are summarized in table 1. All tumour stages were included in our study except disseminated disease. Blood samples were taken prior to treatment, and in 9 patients samples were taken again 2-6 weeks after the tumour had been removed. Informed consent had been obtained.

TABLE 1

SITE	NO.	SEX M/F	AGE YRS	TNM
LARYNX	1	M	74	$T_3N_3M_0$
	2	M	40	$T_1N_0M_0$
	3	M	47	$T_4N_2M_0$
	4	M	56	$T_3N_0M_0$
	5	M	79	$T_3N_2M_0$

SITE	NO.	SEX M/F	AGE YRS	TNM
	6	M	58	$T_1N_0M_0$
	7	M	69	$T_1N_0M_0$
ORAL CAVITY	8	M	69	$T_3N_0M_0$
	9	M	71	$T_2N_0M_0$
	10	M	57	$T_2N_0M_0$
	11	M	75	$T_3N_2M_0$
	12	M	65	$T_2N_1M_0$
	13	M	67	$T_3N_2M_0$
	14	F	50	$T_2N_0M_0$
OROPHARYNX	15	M	65	$T_4N_1M_0$
	16	M	63	$T_1N_0M_0$
	17	M	70	$T_3N_3M_0$
	18	M	80	$T_2N_1M_0$
	19	F	49	$T_2N_0M_0$
	20	M	70	$T_3N_1M_0$
	21	M	54	$T_4N_1M_0$
HYPOPHARYNX	22	M	72	$T_3N_1M_0$
	23	M	78	$T_3N_0M_0$
	24	M	67	$T_4N_1M_0$

Isolation of Human Mononuclear Leucocytes (MNL)

Blood samples were taken by vena puncture and mixed (9:1; v : v) with Trisodium Citrate 2-Hydrate GR (Merck, Darmstadt, Germany). The mononuclear leucocyte (MNL) fraction was isolated by Ficoll Paque density gradient centrifugation (Pharmacia Diagnostics AC, Uppsala, Sweden). After isolation, the cells were washed three times in Phosphate Buffered Saline (PBS), ph 7.4, containing 0.38% Trisodium Citrate 2-Hydrate and 0.5% Bovine Serum Albumin (BSA; Sigma Chemical Company, St. Louis, USA) and counted in a haemocytometer. The number of monocytes was determined in suspension employing the positive staining with Non Specific Esterase (NSE) (9). The percentage of NSE positive cells varied from 5-25%. An enrichment for the monocytes in the Ficoll Paque isolated fraction was obtained by Percoll gradient centrifugation (10). The leucocyte pellet was mixed with a 50% Percoll solution in NaCl (Pharmacia Diagnostics AC, Uppsala, Sweden) and overlayed with 1 ml M 199 (Merck, Darmstadt, Germany). After centrifugation at 1100 G for 15 minutes the interface was collected and washed twice (400 g , 5-10 min.) with M 199 and counted in a haemocytometer. The MNL suspension now contained 40-60% NSE positive cells with a recovery of 45-85% of the number of NSE positive cells originally present in the Ficoll Paque interface.

Polarization assay

0.4 ml of the Percoll purified cellsuspension, containing 0.4 x 10^6 NSE positive cells was added to 12-75 mm polypropylene tubes (Falcon Labware division of Becton, Dickinson & Co. Oxford, California, USA) containing either 0.1 ml of plain M 199 or 0.1 ml of solutions of several concentrations N-Formyl-Methionyl-Leucyl-Phenylalanine (FMLP) range 0.1-100.0 nM. All experiments were carried out in duplicate. The tubes were incubated in a 37°C water bath for 10-20 min. Polarization was stopped by adding 0.5 ml 10% Formaldehyde in 0.05M PBS (ph 7.2). The percentage of polarized cells from each tube was determined by counting 400 cells in a haemocytometer using an ordinary lightmicroscope (magnification 250 x). The test was read "blindly" by at least two persons.
A monocyte was considered "polarized" when one of the following criteria was fullfilled (fig.1):
1. Elongated or triangular shape
2. Broadened Lamellopod
3. Membrane ruffling

The percentages of monocytes polarized was calculated as follows:

$$\frac{\%\ \text{total cells polarized}}{\%\ \text{NSE positive cells}} \times 100\%.$$

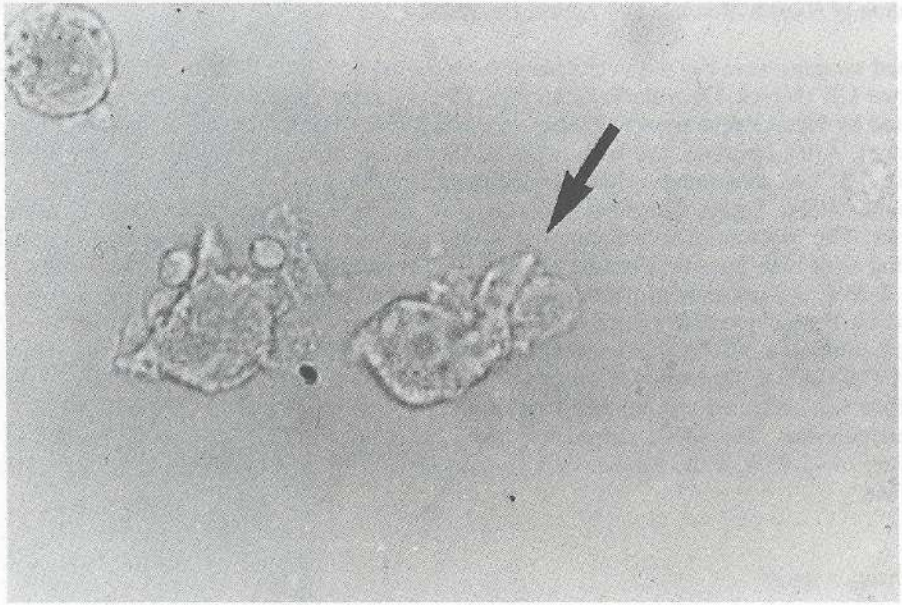

Fig. 1. A polarized monocyte is illustrated. (-->). Note the elongated shape. The cells were suspended in M 199 with 10 nM FMLP, incubated at 37°C for 15 min.

For each sample the percentage of polarized monocytes in the control experiments without the chemo-attractant FMLP was subtracted. It is known that lymphocytes do not exhibit any polarization activity in this assay (7).

Millipore membrane method

Monocyte chemotactic responsiveness towards casein was determined according to the method of Wilkinson (11). Modified Boyden chambers and millipore membranes (Millipore Incorporated, Bedford, Massachussetts, USA) of 12μ pore size were used. Cell suspensions containing 0.2 x 10^6 NSE positive cells per ml were allowed to migrate (60 min, 37°C) into the membrane towards 0.1% casein (Hammerstein, Merck, Darmstadt, Germany) in Gey solution or to plain RPMI solution. The distance of migration into the membranes was recorded, using the leading front method (11) and expressed in micrometer units. The values obtained in the control assay, lacking the chemo-attractant casein, were substracted in each experiment.

Results

Dose response kinetics of the polarization assay; comparison with the Boyden chamber method.

Fig. 2. shows the dose response kinetics of the polarization assay. As can be seen a concentration of 10 nM FMLP induces an optimal polarization of monocytes and this concentration was used all further experiments. To compare the outcomes of the monocyte polarization assay

with those of the millipore membrane method, we performed simultaneous tests on the same preparations of isolated cells. An optimal concentration of 0,1% casein, was used in the millipore membrane method (6). The results (fig. 3.) demonstrate that the outcomes of both assays correlated well: in the 6 cases (•) where the polarization assay was negative (<22%), 5 showed similar negative chemotactic responses in the Boyden assay (<27μM). Healhty individuals (■) showed normal values in both assays. Only 3 patients were tested postoperatively in both assays, the values were normal in the Boyden assay, but marginal in the polarization assay (▲). The overall correlation was thus acceptable. ($p< 0.005$; $r=0.71$; $\chi^2=4.32$, $p< 0.05$).

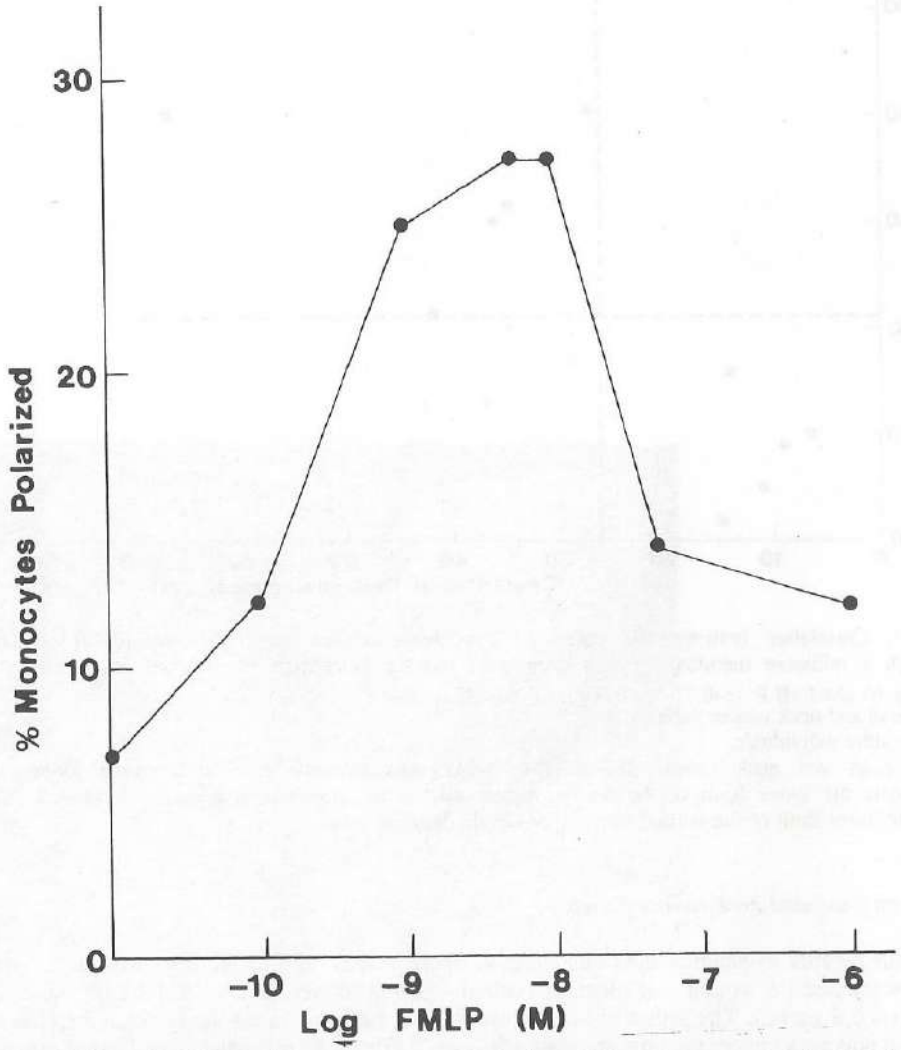

Fig. 2. Ability of FMLP to induce monocyte polarization. Cells were suspended in M 199 at 1 x 10^6 NSE positive cells/ml and incubated at 37°C for 15 min with the indicated concentration of FMLP. A concentration of 10 nM FMLP induces optimal percentages of polarized monocytes.

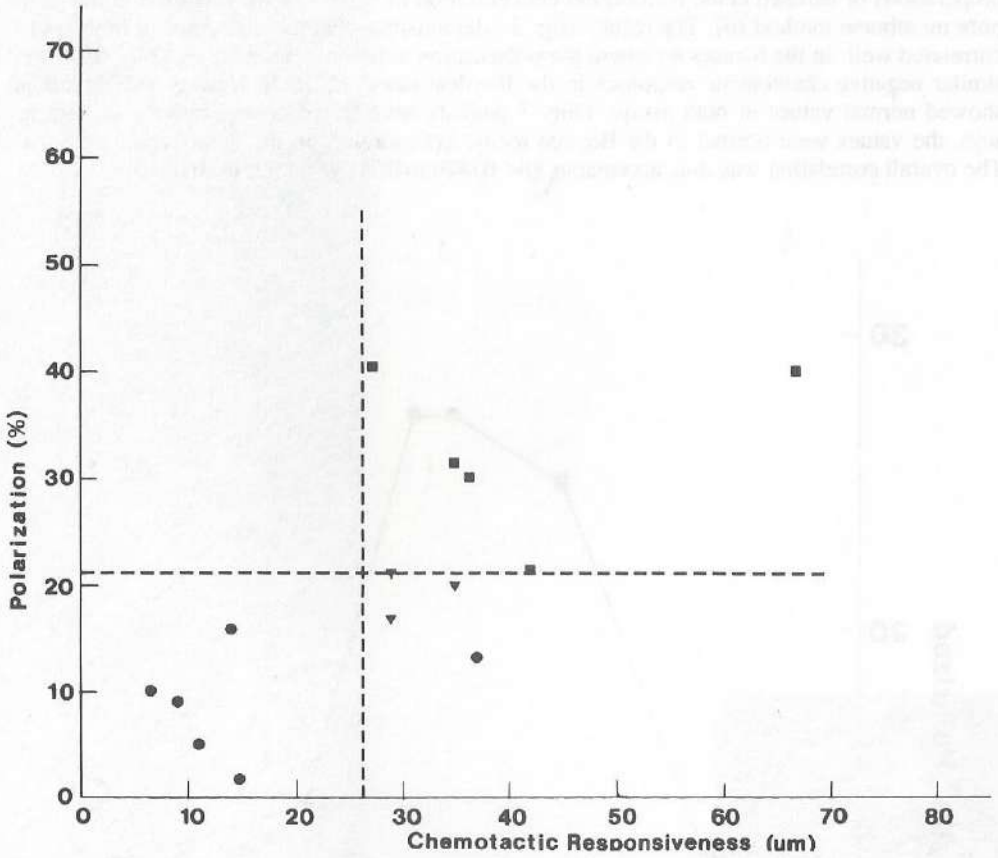

Fig. 3. Correlation between the values of the chemotaxis of monocytes towards 0.1% casein through a millipore membrane (12 μ pore size) and the percentage of polarized monocytes induced by 10 nM FMLP. ($r=0.71$; $p<0.005$), ($\chi^2=4,32$, $p<0.05$)
● = head and neck cancer patients,
■ = healthy individuals,
▲ = head and neck cancer patients 2-6 weeks post operatively. The horizontal dotted line represents the lower limit of the normal values (%) in the polarization assay. The vertical dotted line the lower limit of the normal values (μM) in the Boyden assay.

Data on head and neck cancer patients

With regards to healthy individuals (fig.4), there was no difference between the 2 control groups studied i.e. young and older individuals (young: mean 32% +/- 2.1 s.e.m; old: mean 38% +/- 6.3 s.e.m.). The group of older controls was included in the study since the majority of head and neck cancer patients are above the age of 60 yrs. Percentages of polarized monocytes in the group of head and neck cancer patients were significantly lower as compared to the values of both control groups (mean 12.5% +/- 1.7 s.e.m; $p< 0.001$ Wilcoxon's two sample test). Two to six weeks after surgery the monocyte polarization was re-examined in 9 patients. In all cases the postoperative course was uneventful. In 8 patients the tumour had been radically removed, as established by histological examination of the margins of the surgical specimen. The one positive patient postoperatively received radiotherapy and uptill now (one year follow-up) there is no evidence of local or regional recurrence. Fig.5. illustrates the polarization values before and after surgery. In general there was an improvement, except in two cases. It is worthy to note that the one patient mentioned above without the radical surgical treatment still has a polarization value within the normal range.

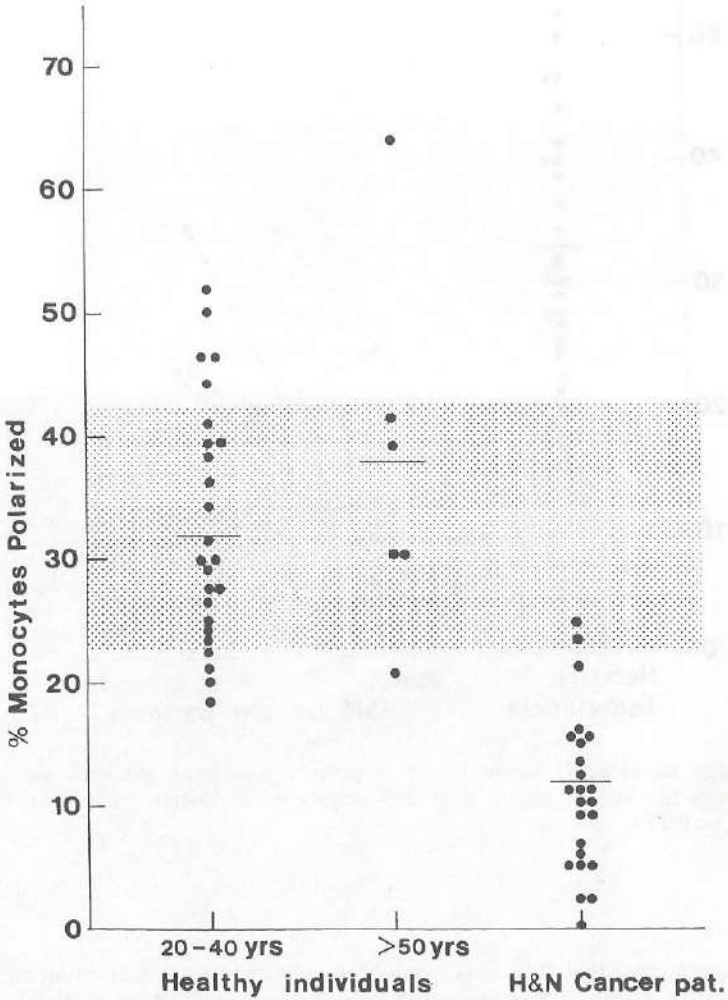

Fig. 4. Polarization of peripheral bloodmonocytes from 24 head and neck carcinoma patients, compared with that of 31 controls.

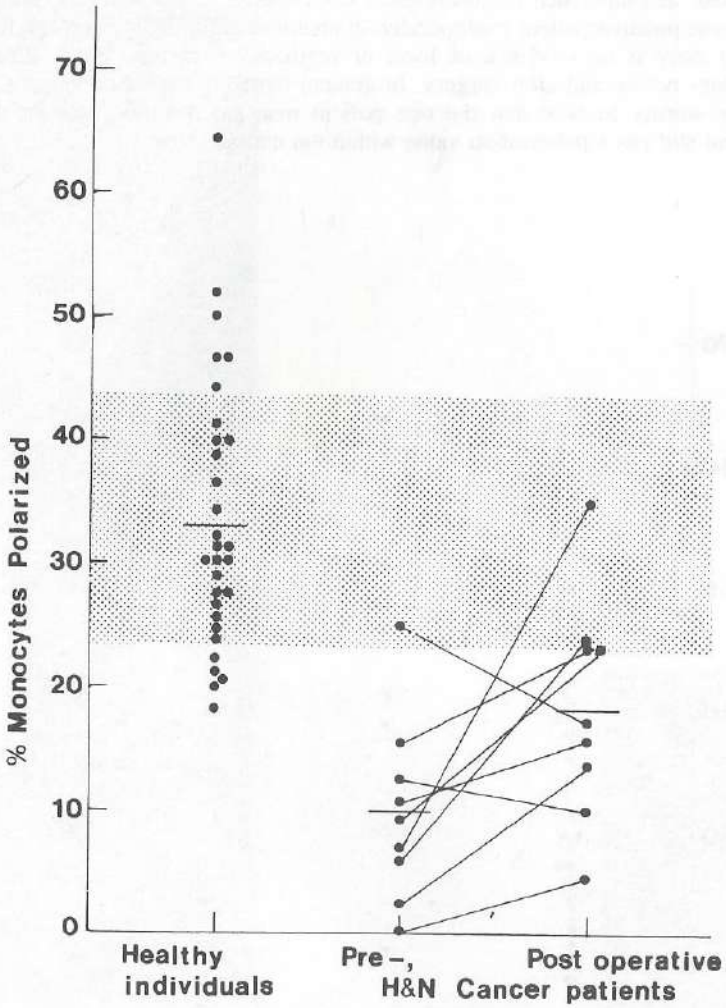

Fig. 5. Percentage of polarized monocytes of 9 patients with head and neck carcinomas. The difference between the activity before and after surgery is statistically significant (Wilcoxon's one sample test; $p < 0.05$).

Discussion

The results demonstrate that the newly introduced polarization assay is an effective tool for the determination of defects in the chemotactic responsiveness of blood mononuclear phagocytes in head and neck cancer patients. An acceptable correlation was found between the data obtained in the polarization assay and those of the well established millipore membrane

method. The polarization assay has however clear technical advantages over the Boyden chamber method. It is quick and easy to perform and it needs no special equipment. This makes the test suitable for more or less routine clinical application. The test may also be of value for therapy evaluation which is indicated by the fact that a complete surgical removal restored in the majority of the patients the outcome of the assay within 2-6 weeks after operation. A prediction of relapse might thus come into reach with this test. The number of operated patients in follow-up is yet too small and the observation period after surgery too short to draw any definitive conclusions as to the value of the assay in the detection of early recurrences. It is worthy to note that 12 patients, 1 - 2 years after radical treatment with no evidence of disease, still show normal MCR values (data not shown). Furthermore it appears worthwhile to investigate monocyte polarization in patients with precancerous lesions to see if the test has any predictive value for the development of overt cancer. Snyderman et al (13,14) were the first to put forward the idea that defective monocyte chemotactic responsiveness depended on the actual presence of neoplastic cells. The surgical removal of breast carcinomas in their studies resulted in the normalization of monocyte chemotactic responsiveness (15). From their and our data it may follow that neoplasms inhibit monocyte locomotion by release of mediators. Pike and Snyderman (16,17) indeed demonstrated that low molecular weight factors (LMWF's) of less than 30,000 Daltons prepared from tumour material were able to inhibit macrophage accumulation into experimentally induced inflammatory reactions. In analogy we found such LMWF's to be present in head and neck carcinomas (18). It is intriguing that these factors are neutralisable by any of three different monoclonal antibodies reactive to P15E (see chapter 3). P15E is a structural component of the envelope of murine and feline retroviruses, which are known for their oncogenic capability (19). A P15E-like material seems present in human head and neck cancer as well and this intriguing finding forms the subject of our ongoing investigations.

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CHAPTER 3

HEAD AND NECK CARCINOMAS CONTAIN IMMUNOSUPPRESSIVE RETROVIRAL P15E-RELATED FACTORS

I. B. Tan[+], H. A. Drexhage[o], R. J. Scheper[o], B. M. E. von Blomberg-v.d.Flier[o], M. de Haan-Meulman[+], G. B. Snow[+] and A. J. M. Balm[+].

From the [+]Department of Otolaryngology and Head & Neck Surgery and the [o]Department of Immunopathology, Pathological Institute, Free University Hospital, Amsterdam, the Netherlands.

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Abstract

Low molecular weight factors, derived from head and neck cancers (H/N ca LMWF's) injected into C_3H mice inhibit the accumulation of macrophages in experimentally induced inflammatory responses. Here we report on the effect of H/N ca LMWF's derived from 14 different head and neck carcinomas on the polarization of healthy donor monocytes. The factors inhibited polarization significantly (a 61.5-94.5% inhibition versus 12.5-29% in cases where LMWF's were derived from healthy oral mucosa). The inhibitory effect exerted by H/N ca LMWF's was neutralisable by absorption with any of 3 different murine monoclonal antibodies (Mab's) or a rabbit polyclonal antibody to the murine retroviral envelope protein P15E. This shows that antigenic material sharing at least 3 epitopes with P15E is present in head and neck cancers and that this material is responsible for the observed defective polarization which probably underlies the earlier described defects in chemotactic responsiveness of patient's monocytes.

Introduction

It has become evident from the work of Snyderman and Cianciolo (1,2, 3) that certain types of tumours are capable of producing factors, inhibiting the chemotaxis of mononuclear phagocytes. Mononuclear phagocytes play a role in the destruction of tumour cells (4,5) and hence this effect of tumour derived factors on the cell's motility may contribute to defects in immune surveillance. The inhibitory factors were detectable in serum and urine of both humans and animals affected by a malignancy, and were of low molecular weight (Mw < 25,000 Daltons). In recent experiments it appeared that the tumour derived LMWF's were physiochemically and anti-genically related to the retroviral capsular protein P15E (6). P15E is known to be immunosuppressive in cat and mice (7,8,9,10,11). At an earlier occasion we described that squamous cell carcinomas of the head and neck produce low molecular weight factors exerting significant inhibitory effects on macrophage accumulation in experimentally induced inflammatory responses, probably via an effect on monocyte chemotactic responsiveness (12). The polarization of monocytes - i.e. the early change from round shape to triangular - in the presence of a chemo-attractant has recently been reported as a reliable measure for moncyte chemotactic responsiveness (13). We have used this phenomenon to establish an easy and rapid assay for the detection of monocyte chemotactic disturbances in head and neck cancer patients (14). These disturbances were indeed found with this assay. In the present report we would like to describe the inhibitory effects of LMWF's isolated from surgical specimens of head and neck cancers on the polarization of healthy donor monocytes. These inhibitory effects exerted by the LMWF's could be neutralized by treating the factors with a panel of monoclonal antibodies reactive to P15E. Our data thus lend additional support to the concept that squamous cell carcinomas of the head and neck produce inhibitors of monocyte chemotaxis, which are antigenically related to the P15E capsular component of oncogenic animal retroviruses.

Materials and methods

Fourteen histologically proven squamous cell carcinomas of the head and neck were studied for the the presence of LMWF. Distribution of cases according to sex, age, site and TNM (UICC) classification (15) are given in table 1.

TABLE 1

SITE	NO.	SEX M/F	AGE yrs	STAGE
LARYNX	01	M	64	$T_2N_1M_0$
	02	M	61	$T_1N_0M_0$
	03	M	61	T_4N_1M
	04	M	75	$T_3N_0M_0$
	05	F	56	$T_4N_0M_0$
ORAL CAVITY	06	M	70	$T_3N_0M_0$

TABLE 1

SITE	NO.	SEX M/F	AGE yrs	STAGE
	07	M	47	$T_2N_1M_0$
	08	F	71	$T_2N_0M_0$
	09	M	48	$T_1N_1M_0$
OROPHARYNX	10	F	64	$T_4N_3M_0$
	11	M	71	$T_4N_2M_0$
	12	M	82	$T_4N_1M_0$
	13	M	71	$T_3N_1M_0$
HYPOPHARYNX	14	M	67	$T_4N_0M_0$

Preparation of low molecular weight factors

Small specimens of tumour material (3 -7 mm^3), obtained from surgical interventions or endoscopic procedures were immediately frozen in liquid nitrogen and stored at - 80°C. The specimens were homogenized by pottering, freezing/thawing and sonification (Sonifier B 12, Branson Sonipower Company) and thereafter centrifugated (10 min at 20,000 G.). Supernatants were subjected to ultrafiltration through Amicon CF 25 Centriflo cones (1800 rpm; molecular weight "cut off point" 25,000 Daltons) resulting in a filtrate with a molecular weight of less than 25,000 Daltons. (see the chapter 3a for further characterization of the factors). The filtrate was brought to a final concentration corresponding to 6.7 mg tumour tissue material (=equivalent to approximately $5x10^5$ cells) per ml RPMI (Gibco, Europe) and stored at -80°C until further use (12). Specimens of healthy oral mucosa and of mammary carcinomas of C_3H mice were prepared similarly and used as negative and positive controls respectively.

Isolation and enrichment of normal human monocytes

Blood samples were taken by venapuncture and mixed (9 : 1, v : v) with 3,8% Trisodium Citrate 2-Hydrate (Merck, Darmstadt, Germany). The mononuclear leucocyte (MNL) fraction was isolated by Ficoll Paque density gradient centrifugation (Pharmacia Diagnostics AC, Uppsala, Sweden). Cells were washed 3x in Phosphate Buffered Saline (PBS), pH 7,4 , containing 0.38 % Tri-Sodium Citrate 2-Hydrate and 0.5% Bovine Serum Albumin (BSA, Sigma Chemical Company, St Louis, USA) and counted in a haemocytometer. The percentage of monocytes present in these suspensions was determined by Non-Specific Esterase staining (NSE) (16) and varied from 5 - 50%. Enrichment of monocytes was obtained by Percoll gradient centrifugation (17). After centrifugation of the original MNL suspension, the pellet was mixed with a Percoll solution in 0.15 M NaCl SD 1.064 (Pharmacia diagnostics AC,

Uppsala, Sweden) and overlaid with 1 ml M199. (Merck, Darmstadt, Germany). After centrifugation (1100G, 15min), cells of the interface were collected, washed twice (400G, 5 - 10 min, M199) and counted. This suspension contained 40 - 60 % NSE positive cells with a recovery of 45 - 85% of the number of monocytes originally present.

Polarization assay (according to the method described by Cianciolo et al 13)

Mononuclear phagocytes obtained from healthy volunteers (aged 20 - 40yrs) were used. 0.4 mls of the mononuclear cell suspension, containing 0.4 x 10^6 NSE positive cells were added to 12 x 75mm polypropylene tubes (Falcon labware, division of Becton, Dickinson & Co, Oxford, California, USA) either containing 0.1 ml M 199 or 0.1 ml N-Formyl-Methionyl-Leucyl-Phenylalanie (FMLP; Sigma); in a final concentration of 10 nM. This FMLP concentration is optimal as has been published at an earlier occasion (14). All experiments were carried out in duplicate. After 15 min incubation (37°C) the polarization process was stopped by adding 0.5 ml 9% Formaldeyde in 0.05 M PBS, ph 7.2 . The percentage of polarized cells (for criteria see below) present in each tube was determined by counting 400 cells in a haemocytometer using light microscopy (magnification 250x). The test was read "blindly" by at least two persons. A cell was classified as "polarized" when one or more of the following criteria were fulfilled:

1. Elongated or triangular shape
2. Broadened Lamellopod
3. Membrane ruffling

The percentage of polarized monocytes was calculated as follows:

$$\frac{\%\ \text{total cells polarized}}{\%\ \text{NSE positive cells}} \times 100\%,$$

since it is known that lymphocytes do not polarize in this assay (13). To obtain the specific value for FMLP induced polarization we subtracted the percentage of olarized monocytes obtained in the control experiment (without the chemo-attractant FMLP) from the value obtained in the FMLP culture.

Inhibition of polarization

The ability of LMWF prepared from head and neck carcinomas (H/N ca LMWF's) and healthy oral mucosa, to inhibit FMLP induced polarization of healthy donor monocytes was determined by incubating (15 min, 37°C) the monocytes ($1x10^6$/ml) either with FMLP alone or with FMLP in combination with LMWF (equivalent to 0.67 mg tissue/ml.). Polarization was stopped and evaluated as described above.

Antibodies

The following antibodies were used in neutralization experiments:

A. 3 murine monoclonal antibodies (Mab's) to retroviral P15E (4F5-IgG2a*, 19F8-IgG2b$^+$, 9E8-IgG2a*).

B. a mouse Mab to GP70-IgG2a* (18), a goat polyclonal antibody to xeno-GP70*, a rabbit polyclonal antibody to xeno-GP 70*.

C. a Mab to GP85 - precursor P15E*.

As a negative control a Mab to rat liver araginase with an IgG2a isotope (identical to the other Mab's) was used. LMWF's, derived from head and neck carcinomas were incubated with these antibodies (4°C overnight, final dilution 1 : 200). The factor-antibody complex was removed via Amicon filtration, whereafter the neutralization procedure was repeated once more.

+). These antibodies were kindly provided by Dr G.J. Cianciolo, Laboratory of Immune Effector Function, Howard Hughes Medical Institute, Division of Rheumatology and Immunology, Department of Medicine, Duke University Medical Center, Durham, NC, USA.

*). These antibodies were kindly provided by Dr. C.J.M. Melief, Central Laboratory of the Blood Transfusion Service, Division of Tumour Biology, Plesmanlaan 121, Amsterdam, The Netherlands.

Results

LMWF-induced-inhibition of the polarization of healthy donor monocytes.

The activity of two LMWF preparations, one derived from a head and neck carcinomas and the other from a specimen of healthy oral mucosa were tested at several concentrations in the monocyte polarization assay. With regard to the H/N ca LMWF's a concentration equivalent to 0.67 mg tumour tissue/ml showed an inhibition of polarization of 90% (fig.1). Such an inhibitory effect was also found when this concentration was diluted $1:10^2$ and $1:10^3$, it rapidly decreased to 60% at a dilution of $1:10^4$ and it was virtually absent when diluted $1:10^5$ and $1:10^6$. With regard to the preparation of healthy oral mucosa a concentration equivalent to 0.67 mg tissue/ml only showed an inhibition of approximately 50 - 60 %. which decreased when diluted $1:10^3$ and over. The shape of these dose - response curves indicates that for the determination of the potency of low molecular weight factors from individual specimens titration studies are prerequisite. For practical purposes we decided to test the low molecular weight factor preparations at one single concentration only and choose a concentration of 0.67 mg/ml since this concentration showed the strongest effects.

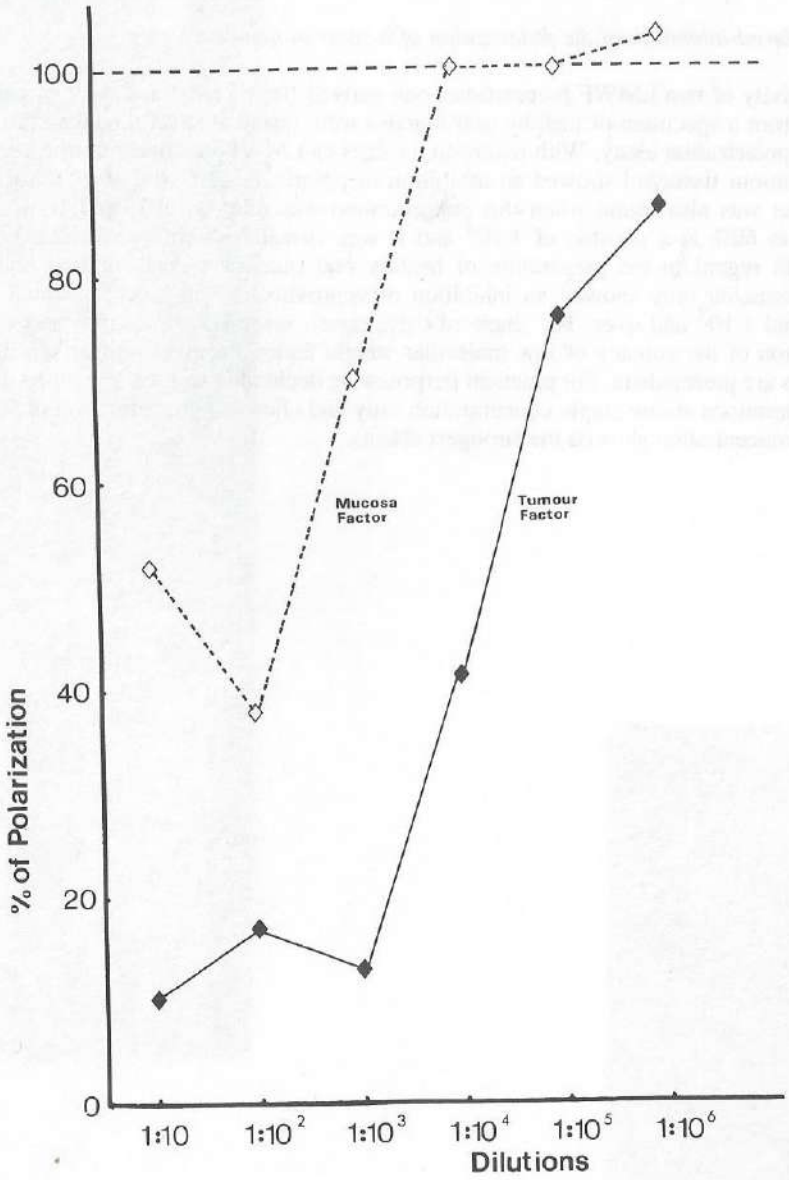

Fig.1. Effect of varying concentrations of low molecular weight factors from head and neck ca's and healthy oral mucosa on the FMLP induced polarization of healthy donor monocytes.
A factor concentration equivalent to 0.67 mg tumour tissue/ml (dilution 1:10) caused a polarization inhibition of 90% whereas an equivalent amount of mucosa factor only reaches inhibition percentages of 50-60%. The inhibition of the mucosa factor was nearly absent at a dilution of $1 : 10^3$, whereas at this concentration still inhibitory effects of the tumour factor could be observed.

Fig.2 shows the effect of 14 H/N ca LMWF's on the polarization of healthy donor monocytes towards 10 nM FMLP tested at the indicated concentration of 0.67mg/ml (see above). All preparations tested significantly inhibited the polarization of healthy donor monocytes (range 61.5-94.5%),whereas LMWF's derived from healthy oral mucosa were only slightly inhibitory at this dose (range 12.5-29%). Note the contrast of inhibition of 50% exerted by the specimen of healthy mucosa tested in fig. 1, the latter factor was actually the strongest inhibitor derived from healthy oral mucosa, ever found by us. Two positive control LMWF preparations from murine mammary adenocarcinomas also exerted a strong inhibitory effect (85% and 87% respectively).

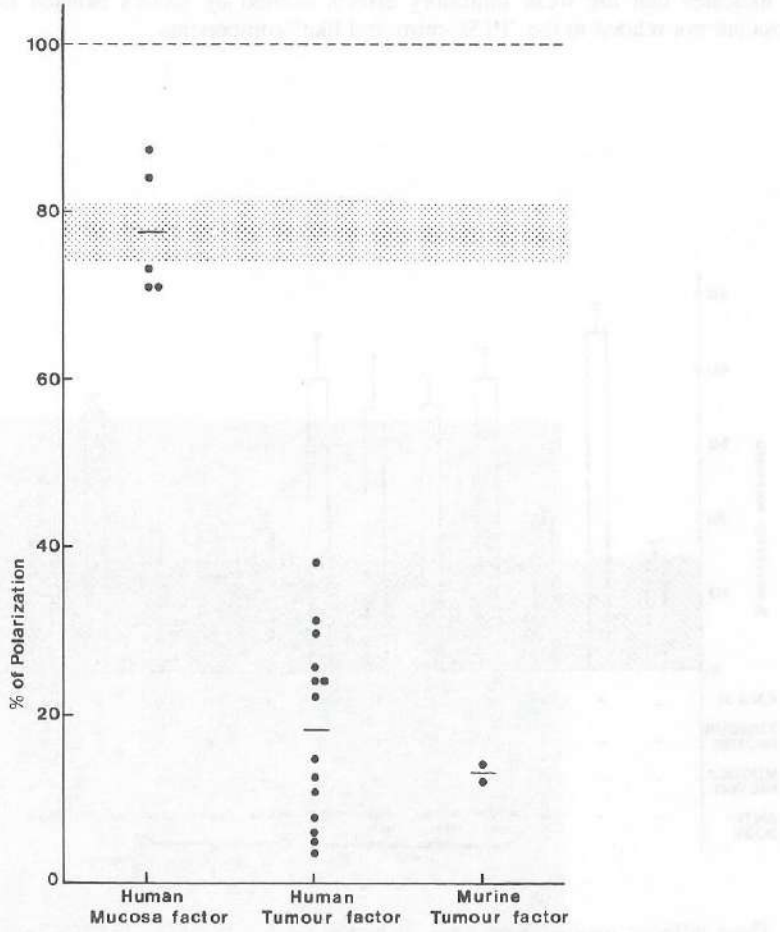

Fig.2. Effect of 14 low molecular weight factors derived from head and neck ca's (H/N ca LMWF's) on the polarization of healthy donor monocytes. All tumour factors tested caused statistically significant inhibition of polarization when compared to effects of factors from healthy oral mucosa. ($P< 0{,}001$; Wilcoxon's two sample test). Factors prepared from spontaneous mammary carcinomas used as positive controls gave similar results as the H/N ca LMWF's.

Neutralization of the LMWF's by Mab's

We were able to neutralize the effect of the H/N ca LMWF's on monocyte polarization by 3 different murine Mab's (4F5, 9E8 and 19F8) and by a rabbit polyclonal antibody, all specific for P15E (fig. 3). The murine monoclonal antibodies react with different epitopes of retroviral P15E(17). The antibodies to P15E were also tested in our assay system alone to see whether they had an intrinsic stimulatory activity on monocyte polarization. Such an effect could not be established (data not shown). Neutralization could also be obtained with the Mab to GP85. The antibodies to GP70, and the monoclonal antibody against rat liver araginase (used as the negative control) lacked any of such neutralising effects (fig.3).The slight inhibition on monocyte polarization exerted by factors prepared from healthy oral mucosa (up to 20 %) could not This indicates that the weak inhibitory effects exerted by factors isolated from healthy oral mucosa are not related to the "P15E-retroviral like" components.

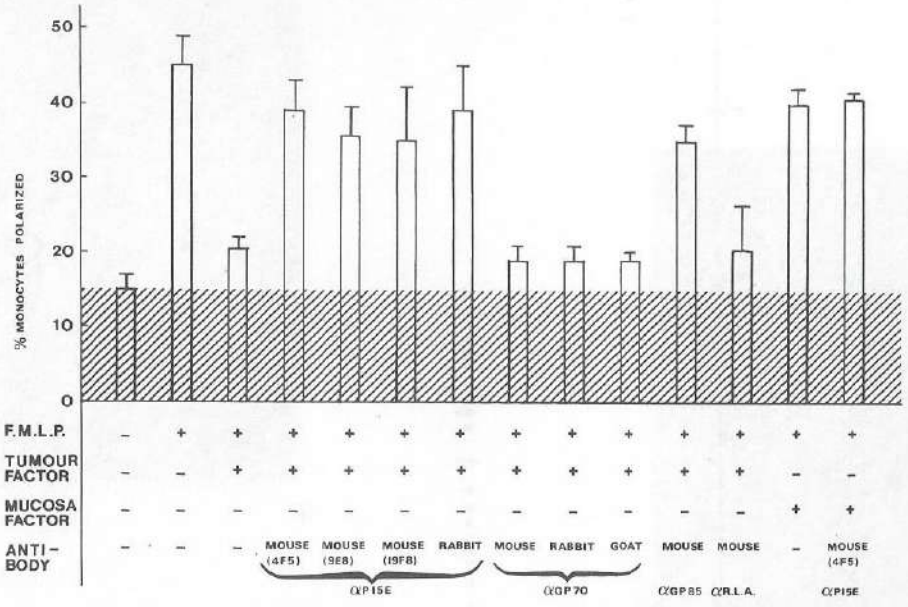

Fig.3. Three different murine monoclonal antibodies (Mab's) to P15E (4F5,9E8 and 19F8), one rabbit polyclonal antibody to P15E and one Mab to GP 85 (dilutions 1 : 200 ; v : v) neutralize the effect of low molecular weight factors from head and neck ca's (H/N ca LMWF's) on the polarization of healthy donor monocytes. Antibodies to GP 70 and Rat Liver Araginase had no such neutralizing effects on the inhibiting activities of the tumour factor. The slight inhibitory effects exerted by mucosa factors were not affected by the anti P15E monoclonal antibody. The bars represent at least 4 experiments with different donors. The S.E.M. is indicated (I-----I).

Discussion

Our studies demonstrate that squamous cell carcinomas of the head and neck contain factors of low molecular weight (<25,000 Daltons) capable of inhibiting monocyte polarization in vitro. This effect of these factors on macrophage motility may underlie the previous described disturbances in the accumulation of macrophages at inflammatory sites: when mice were injected with low molecular weight factors, similar to the ones described in this report, peritoneal inflammatory responses induced by con A were significantly depressed (12). Macrophages are claimed to play a role in the restriction of growth of neoplasms (4,5), and hence our data may be interpreted in the view that tumour cells produce factors in defence to immune attack. The mechanisms whereby the tumour derived factors inhibit macrophage polarization is not clear. Warabi et al (19) suggested that the factors might interfere with the processing of the receptor for chemo-attractants. The LMWF's, studied by us and derived from head and neck malignancies were neutralisable by three monoclonal antibodies reactive to different epitopes of murine retroviral P15E, indicating that the factors share at least three antigenic determinants with a structural component of Murine Leukemia Virus (MuLv). P15E is a hydrophobic transmembrane protein of 15,000 Daltons of the retroviral envelope which is synthesized as part of a precursor molecule with a molecular weight of 80 to 90,000 Daltons. This precursor is cleaved into P15E and GP70 during protein maturation (20). Our experiments showed that monoclonal antibodies reacting with the other cleavage product GP70 did not influence the activity of the H/N ca LMWF. This indicates that the head and neck tumour cells do not produce the complete capsular protein. We now know from recent experiments that P15E related LMWF's are not only produced in head and neck malignancies but also in 87% of cell lines derived from humans with lymphoid neoplasms (U937, K562, SB, MOLT 4, HL60-B11, HSB-2 and CEM) and even by normal lymphocytic blast cells stimulated to proliferate by phyto-haemaglutinin (PHA) (21). This indicates that P15E expression is not a marker for the malignant state. It was hypothesized by Cianciolo et al (20) that the expression of P15E was a more general phenomenon occurring during phases of rapid cellular growth and that the normal role of P15E synthesis could be to downregulate certain immune functions. The recent findings of the inhibition by P15E of the Interleukin 2 (IL-2) production by supernatants of the human histiocytic cell line U937 or human melanoma lines (22,23), in conjunction with the previously demonstrated effects of P15E on monocyte - macrophage functions, (7,11), and the presence of a P15E related antigen in human malignant and mitogen transformed cells (21), support a role for a P15E-like molecule in such immunoregulation. Abnormally regulated expression of P15E by tumour cells may render them more resistant to immune destruction and the process might be similar to the aberrant expression of transforming oncogenes.
Mammalian type retroviruses are known to be leukemogenic in cat and mice, and they are also well known for their immunosuppressive effect: lymphocyte blastogenic responsiveness is affected by both Feline Leukemia Virus (FeLV) and murine Rauscher Leukemia Virus (RLV) (20). Only recently it has become clear that some human T cell leukemias and lymphomas are also related to retrovirusses namely HLTV I and II (24), whereas the severe immune deficiency in AIDS is associated with HTLV III (25,26). Our experiments show that head and neck malignancies produce antigenic material of low molecular weight sharing at least 3 epitopes with the immunosuppressive murine P15E. Since the latter factor shows a structural homology with capsular substances of HTLV I, II and III, a picture emerges of an intriguing relationship between head and neck malignancies, factors related to retrovirusses and immune suppression (27).

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CHAPTER 3a

ADDENDUM

A further characterization of the tumour derived low molecular weight factors, using Amicon Diaflo ultrafiltration and Sephadex gelfiltration

Introduction

Head and neck carcinomas produce low molecular weight factors (H/N ca LMWF's) capable of inhibiting the chemotactic response of mononuclear phagocytes. These factors are antigenically related to the immunosuppressive retroviral envelope protein P15E of the Murine Leukemia Virus (MuLV), since they can be neutralized by three different monoclonal antibodies (Mab's) to this retroviral component (1).
Employing Amicon Diaflo ultrafiltration and Sephadex gelfiltration, we seeked to determine wether the inhibitory factors isolated from head and neck cancer were indeed of the same molecular weight as murine P15E.

Patients and methods

Six specimens of histologically proven squamous cell carcinomas of the head and neck were studied. Patients data are summarized in table 1.
The specimens were homogenized by pottering, freezing/thawing and sonification (Sonifier B12, Branson Sonipower Company). Thereafter they were centrifugated (10 min at 20,000G). One tumour yielded so much material, that we were able to perform our further experiments at a relatively high concentration (preparation A) of 100 mg tumour tissue/ml RPMI (Gibco, Europe). The others were brought to a final concentration of 13 mg tumour tissue/ml RPMI (preparations B to F) (in previous experiments on monocyte polarization, we used a final concentration of 6.7 mg tumour tissue/ml) (1).

TABLE 1.

SITE	NO.	SEX	AGE (yrs)	STAGE
larynx	1(B)	M	67	$T_4N_1M_0$
oropharynx	2 (C)	V	77	$T_3N_2M_0$
hypopharynx	3 (D)	M	58	$T_4N_0M_0$
	4 (E)	V	54	$T_3N_1M_0$
	5 (A)	M	69	$T_2N_1M_0$
stomal recurr.	6 (F)	M	61	-/-

Amicon Diaflo ultrafiltration

The following Diaflo membranes were used: YM 100 (Mw "cut of" point: 100 kD), PM 30 (Mw "cut of" point: 30 kD), PM 10 (Mw "cut of" point: 10kD) and YM 2 (Mw "cut of" point: 1 kD). Ten mls of the preparation A were brought in a "stirring ultrafiltration cell" (Amicon model 12; volume 10 ml; Amicon BV. Oosterhout, Holland) with a pressure of 12 atmosphere (CO_2) to filtrate it through the membrane with the largest pore size (100 kD). The residue was kept apart for further experiments. The filtrate was again subjected to ultrafiltration, but with the membrane with the next largest pore size (30 kD) and so on, and so on. This yielded 4 fractions namely a fraction of 100 kD, one of 30-100 kD, one of 10-30 kD and a fraction of<10 kD. These were tested in the polarization assay as described above in detail (1,2).

Sephadex gelfiltration

An integrated Pharmacia filtration equipment was used (Fraction collector, Frac-100, UV meter, UV-1 opticle unit, a UV-1 control unit, a peristaltic pump P-1 and a recorder).
A column (K9; 60, Pharmacia, Diagnostics, Uppsala, Sweden) with G75 (Sephadex, Fine) appeared to be the most suitable for our purposes (3). The column was calibrated with a panel of calibration proteins purchased from pharmacia namely: Dextran blue (2000 kD), Ovalbumine (45 kD), Chymotripsinogen A (25 kD), Cytochrom C (12.5 kD) and Vitamin B12 (1.355 kD). 0.5 ml of preparation A to F were eluted with 10% Phosphate Buffered Saline (PBS) in 0.9% NaCl in excess (flow rate: 25 ml/hour). Fractions of 0.8 ml were collected and tested in the polarization assay.

Results

Fig. 1. shows the percentage of polarized healthy donor monocytes after incubation with the different filtrates of preparation A, obtained by Amicon Diaflo ultrafiltration. It is evident that the inhibitory activity was present in the fractions of <100 kD (70% inhibition), <30 kD (65% inhibition) and10-30 kD (65% inhibition). This implies that the inhibitory activity of the H/N ca LMWF's is caused by a factor, or factors, with a low molecular weight between 10 and 30 kD. Fig. 2. shows the results of the polarization of healthy donor monocytes after incubation with the different fractions obtained with the Sephadex gelfiltration of preparation A. Only fractions 24 and 25 (with a calculated molecular weight of 12.5+/- 1.5 kD and 16.0+/- 1.5 kD) exerted serious inhibitory effects on monocyte polarization. Similar data were obtained with fractions B to F. (data not shown).

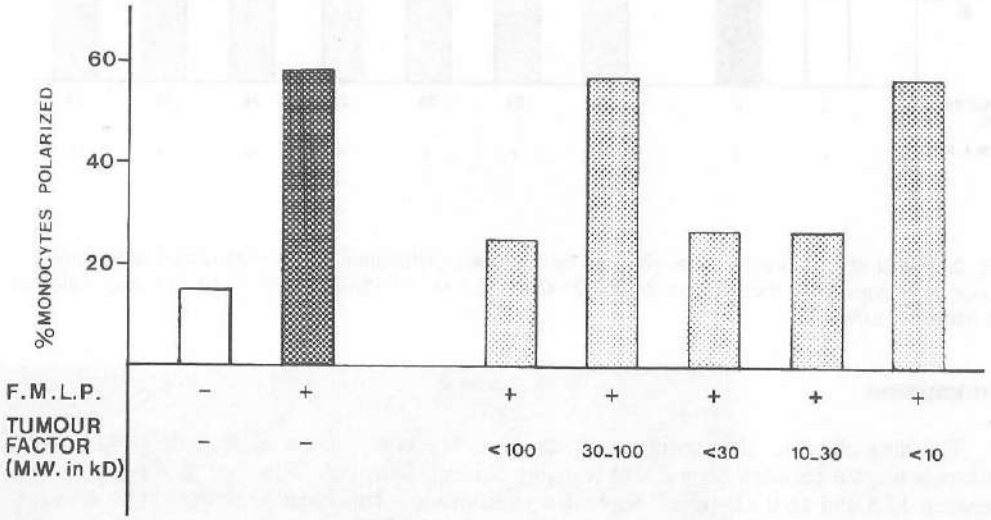

Fig. 1. Effect of the 5 low molecular weight fractions obtained by Amicon Diaflo ultrafiltration on the polarization of healthy donor monocytes. Only the fractions with the molecular weights <100 kD, < 30 kD and 10-30 kD showed an inhibitory effect.

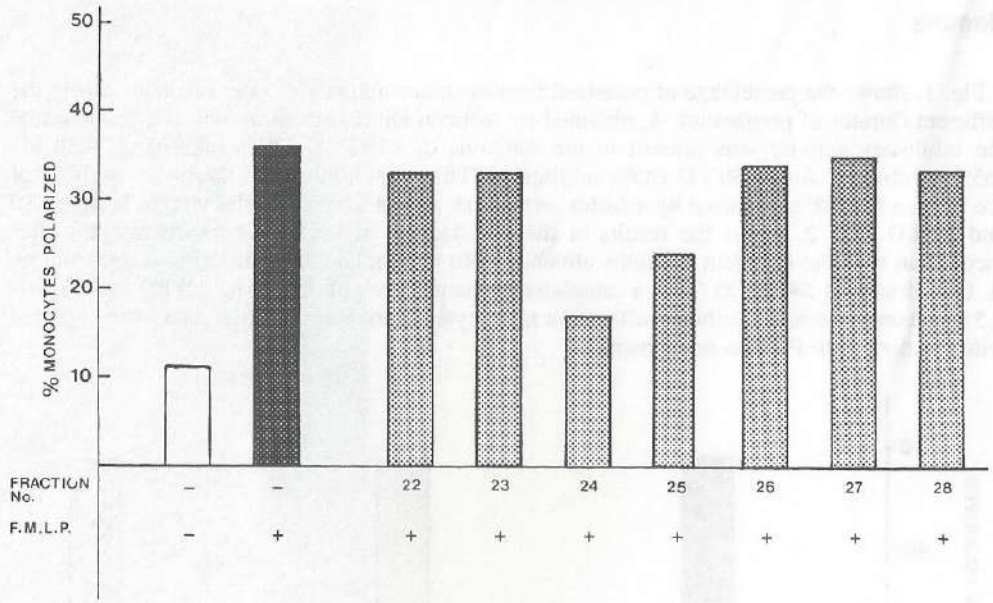

Fig. 2. Effect of 7 elution fractions obtained by Sephadex gelfiltration on the polarization of healthy donor monocytes. Only the fractions 24 and 25 (Mw 12.5 +/- 1.5 kD and 16.0 +/- 1.5 kD resp.) showec an inhibitory effect.

Conclusion

The data obtained, demonstrated that the low molecular weight of the inhibitory tumour factors is located between 10 and 30 kD (using Amicon Diaflo ultrafiltration) and more exactly between 12.5 and 16.0 kD (using Sephadex gelfiltration). This clearly shows that there is not only a antigenic relationship between the H/N ca LMWF's and immunosuppressive murine P15E (see chapter 3), but also that the molecular weight of the factor(s) and the retroviral protein are almost identical.

Aknowledgement: I like to thank Ms. I.M.W. van Hoogstraten for all the experimental work she did on this part of the investigations.

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IMMUNO-HISTOCHEMICAL DETECTION OF RETROVIR. P15E- RELATED MATERIAL IN CARCINOMAS OF THE HEAD AND NECK.

I. B. Tan[+], H. A. Drexhage[o], H. Mullink[o], S.C. Hensen-Logmans[o], M. de Haan-Meulman[+], G. B. Snow[+] and A. J. M. Balm[+].

From [+]the Department of Otolaryngology and Head & Neck Surgery and [o]the Department of Immunopathology, Pathological Institute, Free University Hospital, Amsterdam, the Netherlands.

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Submitted for publication.

Abstract

As has been reported previously, head and neck cancers produce low molecular weight factors (H/N ca LMWF's), capable of inhibiting the chemotactic responsiveness of mononuclear phagocytes. This effect exerted by the factors appeared to be neutralisable by three different monoclonal antibodies specific for P15E, one of the structural envelope proteins of Murine Leukemia Virus (MuLV). This implies that the H/N ca LMWF's show a structural homology with this retroviral envelope protein P15E. Here we would like to report the detection of P15E-like material in head and neck carcinomas, using an indirect immunoperoxidase assay; all 35 head and neck cancers studied gave positive results. Breast, bronchus and ovarium carcinomas were used as controls and17 out of 27 (63%) were also positive. Our studies additionally showed that P15E-like material was not only present in malignant tissues but also in epithelial cells overlaying areas of inflammation. (e.g. delayed type skin reactions, chronic inflammatory bowel disease and gingivitis). Healthy skin, gut and oral mucosa were invariable negative.

This report thus supports that P15E-like molecules can be detected in a variety of malignancies. It additionally underlies the notion that the production of these molecules is not specific for the malignant state.

Introduction

At an earlier occasion we reported that squamous cell carcinomas of the head and neck contain and produce low molecular weight factors (H/N ca LMWF's) exerting an inhibitory effect on the chemotaxis of mononuclear phagocytes (1,2). These factors could be neutralized by antibodies directed to P15E, one of the structural envelope proteins of Murine Leukemia Virus (MulV)(3). This was taken as evidence that the inhibiting factors of low molecular weight, present in head and neck cancers shared a structural homology (of at least 3 epitopes) with this retroviral component. Retroviruses are not only known for their oncogenic properties but also for their potential to inhibit certain immune functions (4,5,6). Illustrative in this respect is the severe Acquired Immune Deficiency Syndrome (AIDS), occurring mostly in homosexual man; this syndrome is nowadays considered to be caused by a retrovirus known as Human T-cell Lymphocytotropic Virus (HTLV III) or Lymph Adenopathy Virus (LAV) (7,8). P15E of murine and feline leukemia viruses is synthezised as part of a precursor molecule of a molecular weight of 80 - 90,000 Daltons (GP85). This precursor is cleaved into P15E and GP70 during protein maturation (9). Over the last decade evidence has emerged that most notably P15E can be held responsible for the immunesuppression (e.g. the depression of monocyte chemotactic responsiveness, lymphocyte blastogenesis and Interleukin-2 (IL-2) production) (10,11), exerted by murine and feline leukemia viruses.

We questioned ourselves, whether P15E related antigens were also detectable by way of immunohistochemistry in malignant cells of head and neck carcinomas. We therefore examined 35 biopsy specimens in an indirect immunoperoxidase assay, using three different monoclonal and one polyclonal antibody to P15E.

Patients and methods

Patients.

Biopsy specimens were obtained from thirty-five patients with histologically proven squamous cell carcinomas of the head and neck. The biopsies were taken from the periphery of the tumour immediately after surgical intervention or during endoscopic procedures. The distribution according site, age, sex and tumour classification (UICC,12), is given in table 1. Breast (n=10), bronchial (n=8) and ovarian (n=9) carcinomas were also included in this study. Control biopsy material was taken from normal skin (n=5), gut (n=2) and oral mucosa (n=11), and also from inflamed skin (n=6, delayed type hypersensitivity skin reactions), gut (n=3, chronic inflammatory bowel disease) and oral mucosa (n=2, gingivitis).

TABLE 1

SITE	NO	SEX	AGE (yrs)	STAGE
larynx	01	M	71	$T_1N_0M_0$
	02	M	66	$T_4N_1M_0$
	03	M	57	$T_1N_1M_0$
	04	M	71	$T_4N_0M_0$
	05	M	49	$T_2N_0M_0$
	06	M	54	$T_2N_0M_0$
	07	M	59	$T_2N_0M_0$
	08	F	41	$T_1N_0M_0$
oral cavity	09	M	54	$T_2N_0M_0$
	10	M	66	$T_2N_1M_0$
	11	F	74	$T_2N_0M_0$
	12	F	64	$T_4N_2M_0$
	13	M	71	$T_3N_1M_0$
	14	M	72	$T_3N_1M_0$
	15	F	73	$T_2N_0M_0$
	16	F	81	$T_4N_3M_0$
	17	M	62	$T_3N_0M_0$
	18	F	76	$T_3N_2M_0$
	19	M	74	$T_3N_0M_0$
	20	M	68	$T_3N_1M_0$
oropharynx	21	M	66	$T_3N_1M_0$
	22	M	53	$T_3N_1M_0$
	23	M	75	$T_3N_1M_0$
	24	M	75	$T_2N_1M_0$
	25	F	68	$T_3N_0M_0$
	26	M	58	$T_3N_1M_0$
hypopharynx	27	F	53	$T_3N_1M_0$
	28	F	75	$T_4N_0M_0$
	29	M	72	$T_4N_1M_0$
	30	M	67	$T_4N_1M_0$
	31	M	73	$T_3N_1M_0$
	32	M	57	$T_4N_0M_0$
	33	M	64	$T_4N_1M_0$
stomal recurr	34	M	60	-
auricle	35	M	75	$T_2N_1M_0$

Immunohistochemistry.

The specimens were frozen in liquid nitrogen (N2) and stored at - 80°C.
Sections of 6 µ thickness were cut from these with a cryostat, air dried for one hour and fixed in ethanol 96% for 10 min. For demonstration of the P15E like material, an indirect (two-step) immunoperoxidase technique (IP), of Delellis and Sternberger (13) was used. The following antibodies figured in the first step;

A. 3 murine monoclonal antibodies (Mab's) to retroviral P15E (4F5-IgG2a+,19F8-IgG2b +, 9E8-IgG2a *.

B. 3 antibodies to GP7O (a mouse Mab to GP70-IgG2a * a goat polyclonal antibody * and a rabbit polyclonal antibody*).

C. A Mab to Rat Liver Araginase with an isotope-specificity identical to two of the above described Mab's. This antibody served as the negative control.

+). Kindly given by Dr G.J. Cianciolo, Laboratory of Immune Effector Function, Howard Hughes Medical Institute, Division of Rheumatology and Immunology, Department of Medicine Duke University Medical Center, Durham, NC, USA.
*). Kindly given by Dr. C.J.M. Melief, Central Laboratory of the Blood Transfusion Service, Division of Tumour biology, Plesmanlaan 121, Amsterdam, The Netherlands.

All the antibodies used were diluted in Phosphate Buffered Saline (PBS), ph.7,4 enriched with 1% Bovine Serum Albumin (BSA), and sections were incubated overnight at room temperature. After incubation the slides were washed in PBS-BSA (3x10min) and thereafter incubated for the second step with either Horseradish Peroxidase (HRP)-conjugated rabbit anti-mouse serum (dil. 1:50), swine- anti-rabbit serum (dil.1:100) or rabbit-anti-goat serum (dil.1:100) (DAKO). After these incubations (30 min 20°C) slides were extensively washed with PBS (3x10 min.). Bound peroxidase was visualized in a staining procedure (5 min, 20°C) with a solution of 3,3-Diamine-Benzidin-Tetra- Hydro-Chloride (5mg DAB in 10 ml Trisbuffer, ph. 7,6 plus one drop 30% H_2O_2). Thereafter sections were rinsed in tapwater, and counterstained with hematoxylin for 30 seconds, dehydrated and mounted in malinol.

Results

All head and neck carcinomas tested in this series (n=35), gave positive results with the antibodies to P15E (table 2). The P15E related material was present in the majority of these tumours in the form of fusiform bands located in the cytoplasm of the malignant cells (fig.1). Not only head and neck carcinomas were found positive for P15E-related material, but also 6 out of the10 adenocarcinomas of the breast, 5 out of the 8 squamous or adenocarcinomas of the bronchus and 6 out of the 9 adenocarcinomas of the ovarium (table 2). This further illustrates the earlier observation of others that the production of the retroviral-like material is not restricted to one particular form of cancer only. Nevertheless the highest prevalence of expression in this series (i.e. 100%) was found in the head and neck malignancies. P15E like material could also be detected in epithelial cells, overlaying inflammatory responses of skin, gut and oral mucosa; and again its pattern of distribution was in spindleform bands (fig.2). Healthy control skin, gut or oral mucosa were invariably negative for P15E-like material.The antibodies against GP70 never gave any positivity in any of the specimens studied. The control Mab to rat liver araginase, having the same isotype as two of the Mab's to P15E (4F5 and 9E8) was also negative in all specimen studied. This excludes a non-specific binding (f.i. via F_C receptors) of the positive P15E reactive Mab's.

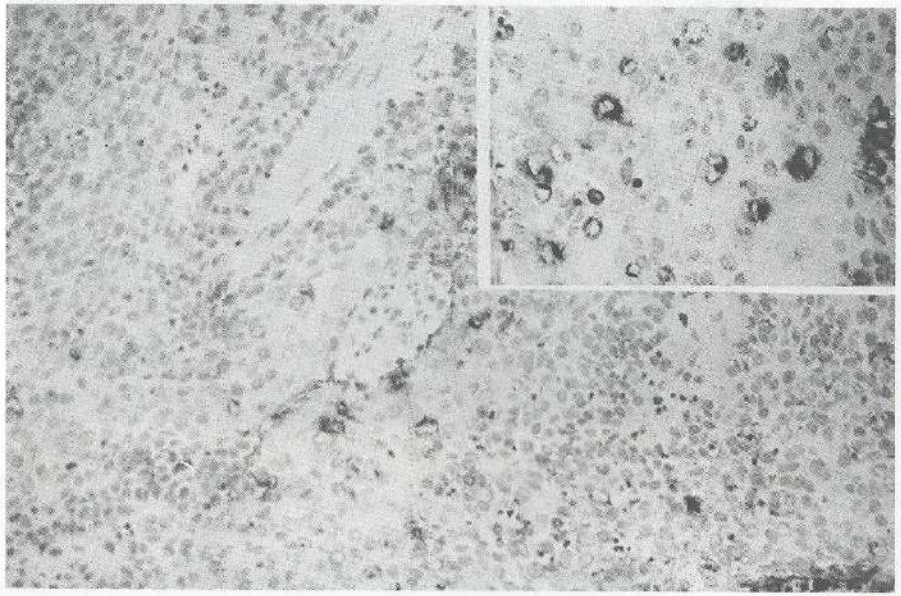

Fig. 1. Indirect peroxidase staining with anti-P15E monoclonal antibodies on frozen sections of a poorly differentiated squamous cell carcinoma of the oral cavity. Scattered tumour cells show a positive reaction whithin their cytoplasm. (magnification: 208x; inlay: 330x)

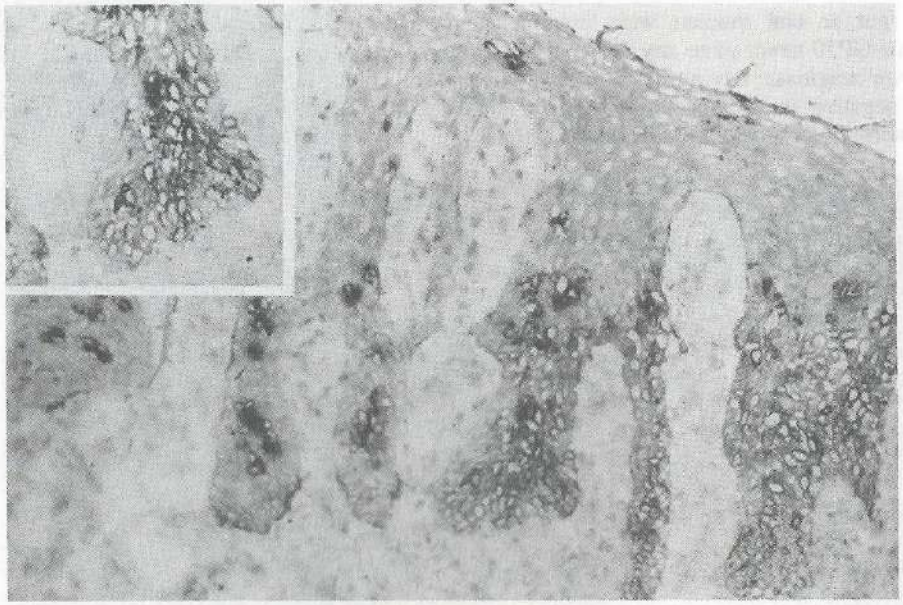

Fig. 2. Indirect peroxidase staining with anti-P15E monoclonal antibodies on a frozen section of non-malignant oral mucosa with chronic inflammation. Within the epithelial surface, scattered parabasal cell groups show a positive reaction within their cytoplasm. (magnification: 208 x; inlay: 330 x).

TABLE 2.

IMMUNOPEROXIDASE LOCALISATION OF P15E-LIKE MATERIAL

TISSUE	POSITIVITY		% POSITIVITY
HEAD AND NECK CARCINOMAS	35/35		100
OTHER CARCINOMAS;			
Breast cancer	6/10		
Lung cancer	5/8	17/27	63
Ovarium cancer	6/9		
INFLAMED TISSUE;			
Gut	3/3		
Skin	5/6	10/11	91
Mucosa	2/2		
NORMAL TISSUE;			
Gut	0/2		
Skin	0/5	0/18	O
Mucosa	0/11		

Discussion

This study shows that P15E like material could easily be detected in all head and neck malignancies studied by way of immuno-histochemistry. The material was also present in about 63% of other malignancies (breast, bronchus and ovarium) and in epithelial cells overlaying inflammation, i.e. delayed type hypersensitivity skin reactions, chronic inflammatory bowel disease, and gingivitis. GP70-like material was not detected in any case, arguing strongly against an active retroviral involvement.

From recent experiments of others as well as from our own experience, it has gradually become clear that P15E-like molecules are not only expressed in malignant epithelial cells, but also in a few lymphoid cell lines, monocytic cell lines (U937, K562, SB, MOLT-4, HL60-BII, HSB-2 and CEM) and normal blood lymphocytes after mitogen stimulation (14). Together with our morphological data, presented here, a picture emerges, which indicates that expression of P15E-like material is a phenomenon not only occurring in malignant situations, but also quite general in benign conditions. A common denominator for P15E expression might be "rapid cell division". This mechanism was as suggested by Cianciolo et al (6). Following this view, P15E-like material could be considered as related to - or even be - one of the numerous polypeptide growth factors like Epidermal Growth Factor (EGF) or Platelet Derived Growth Factor (PDGF), etc. The role of these growth factors in tumorigenesis and their relationship to viral and cellular oncogenes is in the process of being invested (15,16,17). Arguing against a concept of P15E-material being related to growth factors, is the fact that it was not expressed in normal gut epithelial cells, known to have a rapid turn over; and in addition it was found in non-proliferating epithelial cells overlaying inflammatory responses. P15E-like material might therefore rather be involved in immune reactions than in proliferation. The factor is known to depress interleukin-2 production (18) and this indicates - in conjunction with its effect on monocytes (19,20) - an important role of the substance in downregulating the immune response. This effect of P15E-like substances may play a role in the aggravation of malignant disease enabeling metastatic cancer cells to escape from immune destruction, and rendering patients with widespread disease prone to secondary infections.

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CHAPTER 5

THE DETERMINATION OF RETROVIRAL P15E-RELATED FACTORS IN SERA OF PATIENTS WITH HEAD AND NECK CANCER.

I. B. Tan[+], H. A. Drexhage[O], R. J. Scheper[O], B. M. E. von Blomberg- v.d.Flier[O], M. de Haan-Meulman[+], G. B.Snow[+] and A. J. M. Balm[+].

From [+]the Department of Otolaryngology and Head & Neck Surgery and [O]the Department of Immunopathology, Pathological Institute, Free University Hospital, Amsterdam, the Netherlands.

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Abstract

The chemotactic responsiveness of mononuclear phagocytes has often been found defective in patients with malignancies. Also in head and neck cancer patients we previously reported a defective chemotactic responsiveness. Low molecular weight factors (LMWF's) have been isolated from the tumour and can be held responsible for the inhibitory effects on monocyte chemotactic responsiveness. It is an intriguing new finding that these LMWF's can be neutralized by antibodies reactive to P15E (a structural envelope protein of murine leukemia retroviruses; MuLV)

In this report we describe a relatively easy and rapid method for the detection of immunosuppressive P15E-like factors in the sera of patients with head and neck cancer. The test is based on the monocyte polarization assay. Although the number of head and neck cancer patients included in this study is small (n=9), the findings indicate that the test might be of value for clinical application. An early detection of a recurrence after radical therapy of a cancer might come in reach by the finding of a reappearence of the P15E-like factors in the serum of treated patients in follow up.

Introduction

The chemotactic responsiveness of mononuclear phagocytes has extensively been studied in cancerous growth (1,2,3). The last five years we have studied monocyte chemotactic responsiveness in head and neck cancer, and we also found that with this type of tumours this function is defective in such patients. We tested monocyte chemotaxis both with the "Boyden chamber method" as well as with a recently developed "polarization assay" (4,5). In the latter polarization of monocytes is defined as the rapid change in morphology from a round to a triangular motile configuration under the influence of the chemo-attractant N-Formyl-Methionyl-Leucyl-Phenylalanine (FMLP). The test is quick, easy to perform and does not need special laboratory equipment, this in contrast to the Boyden chamber method. The polarization assay may thus become of value for routine clinical application (6).

Cianciolo et al found in 1980 that carcinomas themselves were responsible for the suppression of monocyte chemotactic ability: they were able to isolate low molecular weight factors (LMWF's) from tumours capable of depressing macrophage accumulation at an experimentally induced inflammatory site. (e.g. the mouse peritoneal cavity) (7,8,9). Similar effects of LMWF's derived from head and neck cancers have been reported by us (10).

The LMWF's appeared not only to be active in the in vivo mouse model, but also in the in vitro system of monocyte polarization, e.g. donor monocytes were inhibited in their polarization when incubated in the presence of LMWF's . This inhibiting effect of the factors could be abolished by treating them with antibodies specific for a structural envelope protein of Murine Leukemia Virus (MuLV) e.g. P15E (11,12). This points to a possible relationship of tumour derived factors of low molecular weight influencing monocyte chemotactic responsiveness and a retroviral component, known to be immunosuppressive.

In the present report we sought to determine whether the P15E-related factors were also detectable in the serum of cancer patients. For this purpose we isolated LMWF's from the serum of nine patients with head and neck cancer and studied their effect on in vitro polarization of healthy donor monocytes.

Patients and methods

Patients

Sera of 9 patients with histologically proven squamous cell carcinomas of the head and neck and of 5 healthy controls were studied. The distribution according site , age, sex and TNM classification (UICC,13) is summarized in table 1. Blood samples were taken by venapuncture prior to treatment. Informed consent had been obtained.

TABLE 1

SITE	PATIENTS	SEX M/F	AGE YRS	TNM
LARYNX	1	M	56	$T_3N_0M_0$
	2	M	65	$T_3N_1M_0$
	3	M	74	$T_3N_3M_0$
ORAL CAVITY	4	M	69	$T_3N_0M_0$
	5	M	61	$T_2N_0M_0$
	6	M	59	$T_2N_2M_0$
OROPHARYNX	7	M	80	$T_2N_1M_0$
	8	M	70	$T_3N_1M_0$
STOMAL REC.	9	M	60	-/-

Preparation of LMWF's from patient's sera

Serum was obtained by routine procedures and diluted (1:1; v:v) with physiological saline. Low molecular weight factors were prepared by ultra centrifugation of the diluted sera through Amicon CF 25 Centriflo Cones (molecular weight "cut of point" of 25,000 Daltons). Filtrates were collected and the procedure was repeated once more. The two ultrafiltrates were combined and diluted (1:3; v:v) in physiological saline.

Isolation of monocytes from patient and healthy control blood;

Ten ml blood samples were drawn by venapuncture and immediately mixed (9 : 1, v : v) with 3,8% Trisodium Citrate 2-Hydrate (Merck, Darmstadt, Germany). The Mononuclear Leucocyte (MNL) fraction was isolated by Ficoll Paque density gradient centrifugation. Enrichment for monocytes in this fraction was obtained via further Percoll gradient centrifugation (details have been described at earlier occasions 5). After this enrichment percentages of monocytes defined as Non-Specific Esterase (NSE) positive cells from these suspensions is over 40% (14).

Polarization assay;

0.4 mls of a M199 suspension, containing 0.4 x 10^6 monocytes were added to 12 x 75 mm polypropylene tubes (Falcon Labware, Division of Becton, Dickinson and Co, Oxnard, California, USA) either containing 0.1 ml M199 or 0.1 ml of a N-Formyl-Methionyl-Leucyl-Phenylalanine (FMLP; Sigma) solution in M199 such that the final FMLP concentration

was10 nM. All experiments were carried out in duplicate. After incubation (20 min. 37°C) the polarization process was stopped by the addition of 0.5 ml 10% formaldehyde in 0.05 M PBS, (pH 7.2) to the tubes. The percentage of polarized cells in each tube was determined by counting 400 cells in a haemocytometer using an ordinary light microscope (magnification 250x). The test was read "blindly" by at least two persons.

Monocytes were classified as "polarized" when the following criteria were fulfilled:
1. Elongated or triangular shape
2. Broadened lamellopod
3. Membrane ruffling

The percentage of polarized monocytes was calculated as follows:

$$\frac{\%\ \text{total cells polarized}}{\%\ \text{NSE positive cells}} \times 100.$$

For each sample this value was corrected by subtracting the value found in the control experiment without the chemo-attractant FMLP. The polarization activity of lymphocytes is negligible in this assay (6).

The test for the effect of serum factors on the polarization of healthy donor monocytes

0.4 mls of the monocyte suspension described above, were incubated with the chemoattractant FMLP either alone or in combination with the serum factors in a final concentration of 1:30 (v:v). This concentration appeared to be optimal (data not shown). The assay was stopped and evaluated as described above.

Absorption experiments with a monoclonal antibody to P15E

A monoclonal antibody specific for P15E (4F5, IgG2a isotype, kindly provided by Dr. G.J. Cianciolo, Duke University, Durham, North Carolina, USA) was incubated at a final dilution of 1:200 (4°C, overnight) with serum factors of either head and neck cancer patients or healthy controls. The factor-antibody complex was removed via an Amicon filtration, as described previously (12). The ultrafiltrate was absorbed and filtered once more, and the final filtrate was used in the assay.

Results

The polarization assay with patient monocytes

Fig.1. shows the percentages of patient's peripheral monocytes, capable of changing shape under the influence of the chemoattractant FMLP. It is evident from this figure, that a significant impairment of this function existed in eight out of the nine cancer patients tested: lower percentages of peripheral monocytes were found to polarize under the influence of FMLP as compared to the values we normally found in healthy controls (see previous work, 5). The monocyte fractions of three healthy controls tested in this series were entirely comparable to historical controls (5).

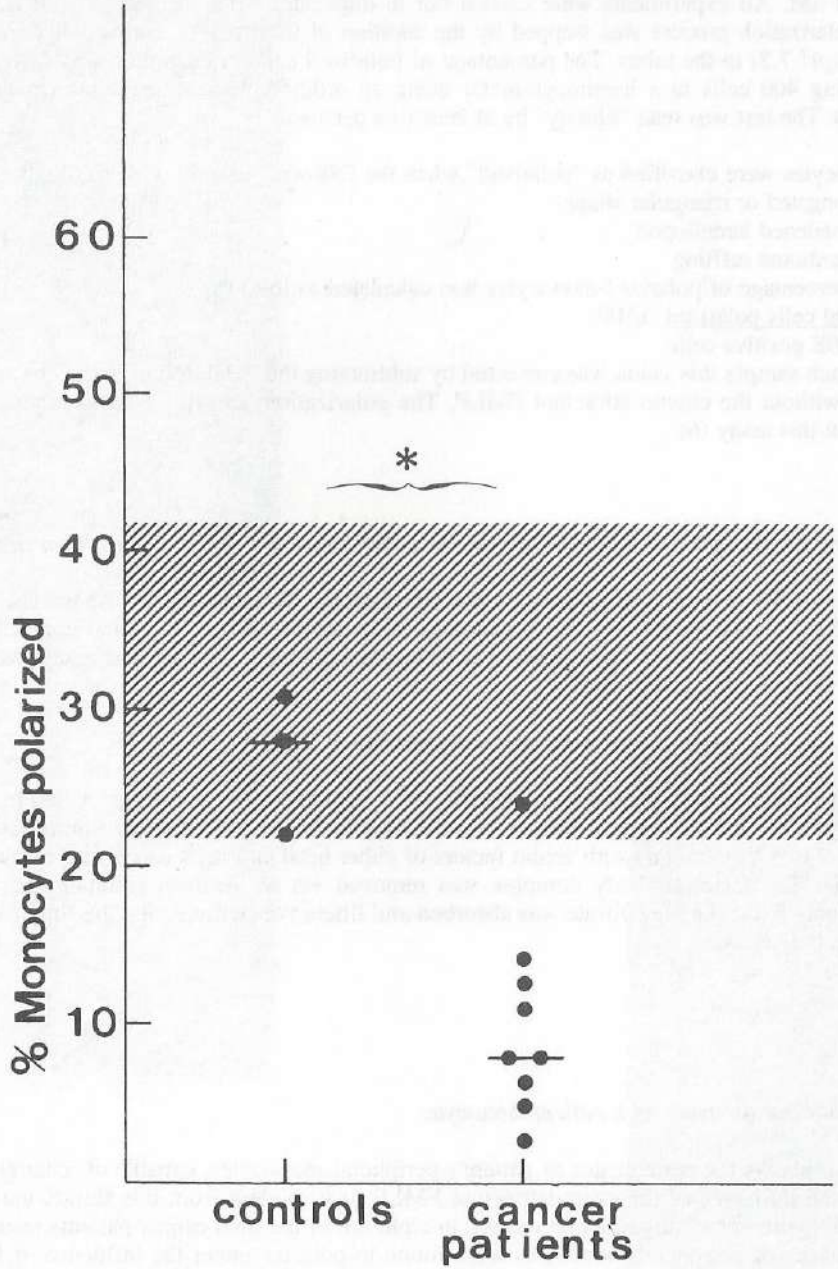

Fig. 1. Polarization of peripheral blood monocytes from 9 head and neck carcinoma patients, compared with that of 3 controls. The hatched area represents the outcomes of earlier series of 30 controls tested previously. * The difference between the two groups tested is statistically significant. (Wilcoxon's two sample test; P< 0,005).

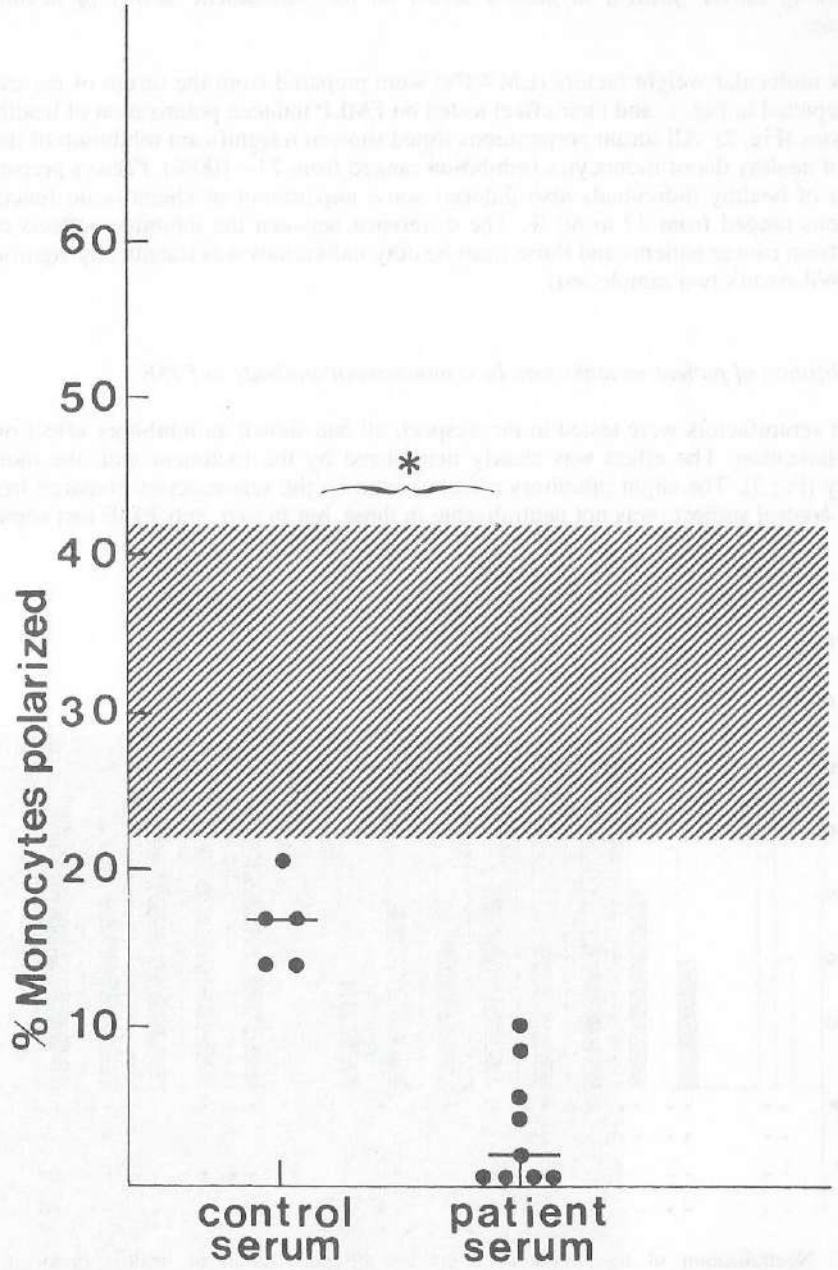

Fig. 2. Polarization of healthy donor monocytes in presence of Low Molecular Weight Factors, derived from the sera of 9 patients with head and neck carcinomas compared with that of 5 healthy individuals. The hatched area represents the monocyte polarization of 30 healthy controls, tested at an earlier occasion. * The difference between the two groups tested is statistically significant. (Wilcoxon's two sample test; P< 0,005).

Influence of LMWF present in patient serum on the chemotactic ability of healthy donor monocytes

Low molecular weight factors (LMWF's) were prepared from the serum of the cancer patients depicted in Fig. 1. and their effect tested on FMLP induced polarization of healthy donor monocytes (Fig. 2). All serum preparations tested showed a significant inhibition of the polarization of healthy donor monocytes (inhibition ranged from 71 - 100%). Factors prepared from the sera of healthy individuals also induced some impairment of chemotactic function, and inhibitions ranged from 37 to 60 %. The difference between the inhibitory effects of serum factors from cancer patients and those from healthy individuals was statistically significant ($P < 0{,}005$; Wilcoxon's two sample test).

Neutralization of patient serumfactors by a monoclonal antibody to P15E

Eight serumfactors were tested in this respect; all had shown an inhibitory effect on monocyte polarization. The effect was clearly neutralized by the treatment with the monoclonal antibody (Fig.3). The slight inhibitory effect exerted by the serumfactors prepared from the 5 healthy control subjects was not neutralisable in three, but in two anti-P15E had some effects (fig.3).

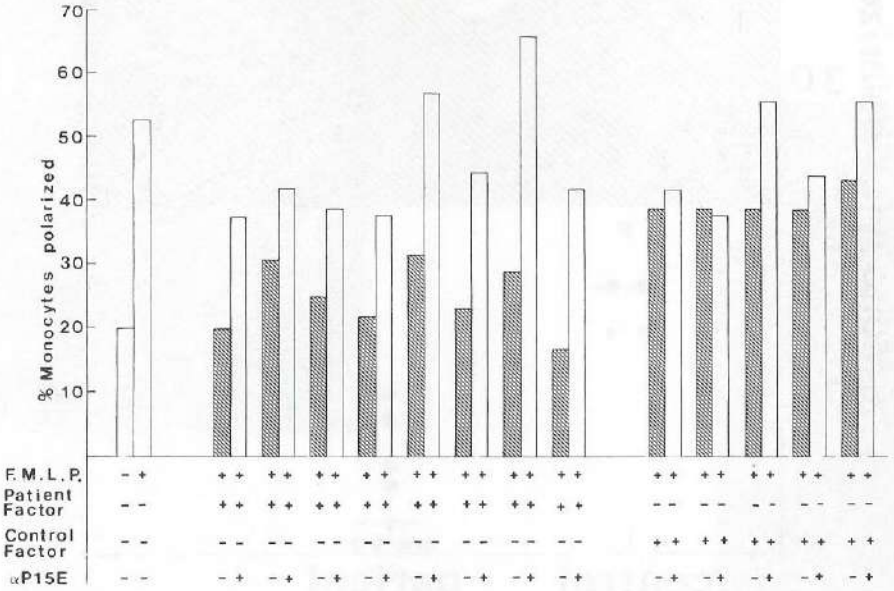

Fig. 3. Neutralization of the inhibitory effect on the polarization of healthy donor monocytes in presence of Low Molecular Weight Factors, derived from the sera of 8 patients with head and neck carcinomas, compared with that of 5 healthy controls. The serumfactors were incubated with a monoclonal antibody specific for P15E (4F5) (final dilution 1:200; 4°C overnight). The slight inhibitory effect exerted by the serum factors prepared from the healthy controls was only neutralisable in two, in the other three cases anti-P15E had no effect.

Discussion

Although the number of sera tested in this study is small, it is conceivable that a considerable proportion of patients with head and neck cancer posess P15E related circulating serumfactors of less than 25,000 Daltons capable of inhibiting monocyte polarization in vitro. The nature and the effect of the circulating factors is entirely comparable to those extractable from the cancers of such patients (12). We are thus probably dealing with serumfactors originating from the tumour, released in circulation and systemically influencing the mononuclear phagocyte system. The fact that the inhibitory effects of both the circulating factors (this study) and the factors extracted from the tumour (12) can be neutralized by antibodies to P15E points to a relationship between cancerous growth and an involvement of a retroviral factor. However the presence of such a factor is not at all tumour-specific: using a morphological detection method of the P15E-like material, we found it present not only in the epithelial cells of several carcinomas, but also in the epithelial cells of 91% of specimens of inflammatory lesions of the skin, gut and oral mucosa (15). The latter observation indicates that the factor might earlier play a role in the regulation of immune responsiveness and can be produced in principle by many epithelia. This might in term explain the fact that it was also detectable in this series of experiments in some of the healthy control sera (be it in low concentrations).

The clear presence of relatively large quantities of the P15E-like material in sera of patients with cancer might turn out to be a useful parameter in clinical practice, in particular in the early detection of recurrent malignant disease after radical surgery or radiotherapy.

However, the presence of the factor in non-malignant situations and in a low concentration in normal healthy serum may hamper such an approach.

Our future research will concentrate on a screening of larger groups of radically treated head and neck cancer patients for the reappearance of the P15E-like material in the serum; we earlier demonstrated that the defective monocyte chemotactic responsiveness returns to normal after complete removal of a head and neck tumour in a period of several weeks (5). Such an approach might be worthwhile since it has been demonstrated that patients with head and neck cancer of stage III and IV (TNM classification UICC,13) are at risk for the development of second primary tumours in the respiratory and upper alimentary tract (16,17). Particularly male patients with cancer of the floor of the mouth and laryngeal cancer seem to be at risk for the development of a second primary tumour (18).

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CHAPTER 6

THE EFFECT OF P15E-RELATED RETROVIRAL MATERIAL, ISOLATED FROM HEAD AND NECK CARCINOMAS ON DELAYED TYPE HYPERSENSITIVITY SKIN REACTIONS IN MICE.

I. B. Tan[+], H. A. Drexhage[o], R. J. Scheper[o], B. M. E. von Blomberg-v.d.Flier[o], M. de Haan-Meulman[+], G. B.Snow[+] and A. J. M. Balm[+].

From [+]the Department of Otolaryngology and Head & Neck Surgery and [o]the Department of Immunopathology, Pathological Institute, Free University Hospital, Amsterdam, the Netherlands.

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Abstract

Low molecular weight factors, derived from head and neck carcinomas (H/N ca LMWF's) exert a strong inhibitory effect on monocyte chemotaxis both in vivo and in vitro. This effect can be neutralised by the treatment of the factors with three different monoclonal antibodies to P15E, one of the structural envelope proteins of Murine Leukemia Virus (MuLV).
In this study we report the effect of the H/N ca LMWF's on the delayed type hypersensitivity responsiveness in mice: the factors significantly inhibited the 24hrs DNFB skin reaction. These effects of the H/N ca LMWF's were again neutralisable by antibodies to P15E. Additional experiments showed that the H/N ca LMWF's had equal suppressive effects on toxic skinreactions to croton oil, and this indicates that the P15E-like H/N ca LMWF's exert their effect by aspecific mechanisms.

Introduction

It is known that mononuclear phagocytes play an important role in tumour resistance (1,2). At earlier occasions we reported the presence of P15E-like low molecular weight factors in head and neck carcinomas (H/N ca LMWF) exerting inhibitory effects on monocyte migration both in vivo and in vitro (3,4,5). These factors may play a role in hampering the immune attack against the tumour.

It is well established that the presence of a tumour influences host defence by an effect on T-cell mediated immune mechanisms (6,7,8). Nelson and Nelson (9,10) reported that all their tumours, whatever species or tissue of origin, produced factors suppressing delayed type hypersensitivity (DTH) skin reactions.

Here we would like to present a study where we document the inhibitory influence of P15E-like H/N ca LMWF's on the swelling of delayed type hypersensitivity (DTH) skin reactions in mice. Skin reactions were elicited by the contact sensitizing agent Dinitro-Fluoro-Benzene (DNFB). The effect of these factors on a toxic skinreaction elicited by croton oil was studied as well.

Materials and methods

Animals

Mice: - Female C_3H mice aged 2-6 months weighing 20-30 grams were used. (TNO, Rijswijk)

Preparations of LMWF

Five patients, 4 males and 1 female, with histologically proven squamous cell carcinomas of the head and neck were included in this study. None of the patients had received prior treatment. Site, age, and tumour classification (UICC, 11) of the malignancies are listed in table 1.

TABLE 1.

SITE	NO	SEX M/F	AGE(YRS)	TNM
LARYNX	1	M	63	$T_2N_0M_0$
ORAL CAVITY	2	M	47	$T_2N_1M_0$
ORAL CAVITY	3	M	66	$T_3N_0M_0$
OROPHARYNX	4	F	63	$T_4N_3M_0$
AURICLE	5	M	90	-/-

Biopsies were taken from the periphery of the tumour (3-7mm^3) immediately after surgical removal of the tumour or during endoscopic procedures. They were frozen in liquid nitrogen and stored at -80^{o}C. For the preparation of the factors, the specimens were subjected to freezing/thawing, and tumour cells were further disrupted by sonification (Sonifier B12, Branson Sonis Power Company) until no intact cells could be detected by light microscopy. Thereafter the suspension was centrifuged for 10 minutes at 20,000 G, the supernatant collected and portions were subjected to ultrafiltration (1800 r.p.m.) through Amicon CF 25 Centriflo Cones (Molecular Weight "cut off point" 25,000 Daltons). The filtrates with a molecular weight of less than 25,000 Daltons were aliquoted and adjusted to a concentration equivalent to 6.7 mg tumour tissue / ml in medium RPMI (Gibco, Europe) and stored at - 80^o C until further use (3). Biopsies from healthy oral mucosa were prepared similarly and used in control experiments.

Sensitization of animals and elicitation of DTH skin reactions

Sensitization was achieved by two daily paintings of 25 micro liter of 0.5% 2,4-Dinitro-1-Fluoro-Benzene (DNFB; Merck, Darmstadt, Germany) in a mixture of acetone and olive oil (4:1) onto the shaved flank of the animal according a treatment schedule introduced by Man Sun Sy (12). Four days after the last sensitizing painting a challenge dose of 20 micro-liter of 0.2% DNFB in acetone/olive oil was applied to the dorsal surface of the ear. The increase in earswelling was used as a parameter of DTH reactivity and measured at 24, 48 and 72 hrs. after the challenge, using a screw micrometer (H.C. Kroeplin GmbH, Schleuchtern, Germany).

To investigate the influence of low molecular weight factors on DTH skin reactivity, the test mice were injected into the thigh with 0.2 ml of the tumour filtrate (6.7 mg/ml.) or 0.2 ml RPMI (control animals), one day prior to challenging. LMWF's prepared from murine mammary carcinomas and healthy oral mucosa were used as positive and negative control factors respectively.

To investigate whether the activities exerted by the H/N ca LMWF's were specific for immune-reactions, we studied their effect on the swelling of an ear reaction elicited by the toxic substance croton oil. Croton oil was applied simultaneously with the DNFB challenge on the opposite ear; in earlier experiments it had been established that an optimal swelling could be achieved using a dose of 20 microliter of 3% croton oil in ethanol (R.J. Scheper, personal communication).

Monoclonal antibodies

Two different monoclonal antibodies (4F5 and 19F8, isotypes resp. IgG2a and IgG2b) reacting with the capsular protein P15E of Murine Leukemia Virus (MuLV) were kindly provided by Dr. G.J. Cianciolo, Duke University, Durham, North Carolina, USA. They were pooled for use in our experiments to obtain an enhanced affinity (13). LMWF's were incubated (overnight, 4^{o}C) with the antibodies in a final dilution of 1:200. The formed factor-antibody complex was filtered through Amicon CF 25 Centriflo Cones (Molecular Weight "cut off point" of 25,000 Daltons) whereafter the procedure was repeated once more. 0.2 mls of the final filtrate were injected into the thigh of the animals as indicated above.

Results

Fig. 1. shows the time-course of the DTH reactivity in mice, as measured at 0, 24, 48 and 72 hrs. The normal pattern of DTH reactivity in the absence of an factor treatment showed more or less equally maximal swellings at 24, 48 and 72 hrs (approximately 80 µM induration). It is further evident from this figure that H/N ca LMWF's inhibited the swelling of the DTH reaction significantly, particularly in the first 24 hrs, when compared to the factors prepared from healthy oral mucosa or the RPMI situations. At 48 and 72 hrs the inhibitory effects of the LMWF's were less outspoken. Table 2. shows that the H/N ca LMWF's were equally potent in suppressing the swelling of the toxic reaction to Croton oil. A smaller increase in ear thickness was measurable again during the first 24 hrs after the treatment with two tumour factors tested in this respect. One of these tumourfactors was tested with a pool of two monoclonal antibodies reactive to P15E; table 3 shows that this resulted in the abolition of the inhibitory effect exerted on both the DTH reactivity and the toxic swelling to croton oil. A control monoclonal antibody with the same isotype as one of the the two P15E reactive monoclonal antibodies (IgG2a), but with a specificity for an unrelated antigen, i.e. Rat Liver Araginase, lacked these neutralizing effects.

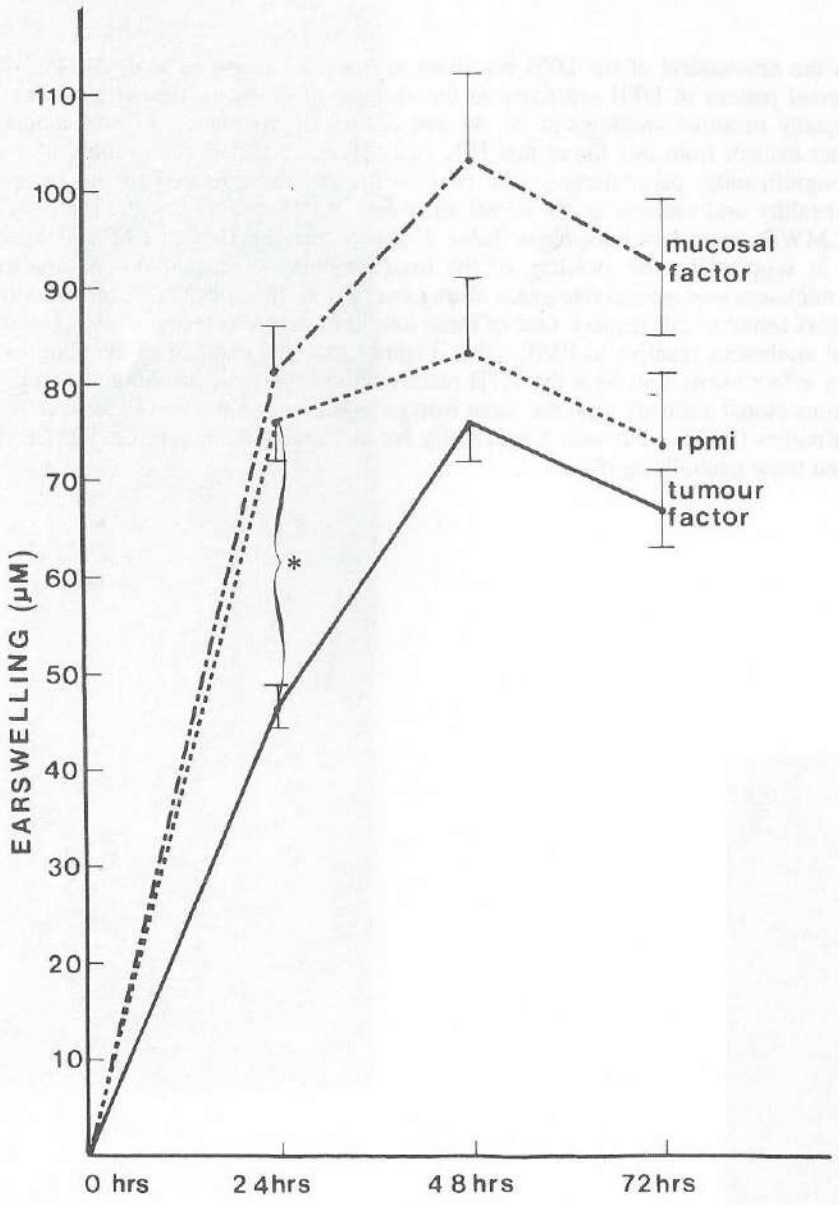

Fig. 1. Time-course of the DTH reactivity to DNFB in mice. One day before the challenge dose was given, mice were injected into the thigh with either 0.2 ml tumour factor, mucosa factor or RPMI. The increase in earswelling was used as a parameter of DTH reactivity and measured 24, 48 and 72 hrs after the challenge, using a screw micrometer. 24 hrs after the challenge dose has been given, mice treated with tumour factors derived from head and neck carcinomas showed a significant decrease in DTH reactivity when compared with the mucosa and RPMI treated mice. (*Wilcoxon's two sample test; P< 0,005).

TABLE 2

EFFECT OF H/N CA AND MUCOSA LMWF ON A DTH REACTION TO DNFB AND ON AN ASPECIFIC INFLAMMATORY RESPONSE TO CROTON OIL

TREATMENT	EARSWELLING IN µM 24 HRS AFTER CHALLENGE (DNFB)	% INHIBITION	EARSWELLING IN µM 24 HRS AFTER CHALLENGE (CROTON OIL)	% INHIBITION
MEDIUM	80+/- 15	-	87 +/- 15	-
TUMOUR FACT.1	27 +/- 15	66	33 +/- 15	62
TUMOUR FACT.2	33 +/- 12	59	23 +/- 12	74
MUCOSA FACT.1	65 +/- 16	19	65 +/- 20	28
MUCOSA FACT.2	63 +/- 06	22	63 +/- 15	28

TABLE 3

THE EFFECT OF P15E SPECIFIC MONOCLONAL ANTIBODIES IN NEUTRALIZING THE EFFECT OF H/N CA LMWF's

TREATMENT	EARSWELLING IN µM. 24 HRS AFTER CHALLENGE	% INHIBITION
MEDIUM	64 +/- 17	-
TUMOUR FACTOR 2	32 +/- 15	50
TUMOUR FACTOR 2 + a.P15E	70 +/- 21	-9
TUMOUR FACTOR 2 + a.RLA	29+/- 13	55

Discussion

Our data obtained in this mouse model show that P15E related H/N ca LMWF's exert a significant inhibitory effect on a T-cell response, i.e. the DTH skin test reactivity to DNFB. The swelling of toxic inflammatory responses to croton oil were equally affected and this shows that the factors do not exert their inhibiting effects solely via immunologically specific mechanisms. Since it is well established that P15E related H/N ca LMWF's have clear influences on monocyte chemotaxis (3,5,14,15) it is likely that a defect in the migratory capacity of particularly these cells may underlie the decreased DTH skintest reactivity.

Patients with squamous cell carcinomas of the head and neck have been described as immunobiologically unique in that they often show abnormalities of the T-cell mediated immunesystem. The majority of the studies arriving at this concept have been conducted by testing DTH skin reactivity (16,17,18,19,20); f.i. it was described that skin reactions were impaired in 36-70% of patients with head and neck cancers and that the skin anergy was more serious in patients with an advanced tumour. Chretien et al (21) also employing the DTH skin test observed a persistent defective cellular immunity in cured squamous cell cancer patients. On this basis they hypothesized that patients who developed these tumours may represent a subpopulation with a genetically determined impaired T-cell mediated immune reactivity. Our data however point to the possibility that the malignant state is associated with an aspecific suppression of the inflammatory responsiveness, which may also affect at least in part immunologically specific reactions. This aspecific suppression is most likely exerted by LMWF's of which we showed at earlier occasions that they are produced by the tumour, that they circulate in the serum, that they inhibit monocyte chemotaxis both in vivo and in vitro, and that they share at least three epitopes with murine P15E (4 , 5,14,15,22). Our absorption studies with the anti-P15E monoclonal antibody reported here lend additional support to this view.

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GENERAL DISCUSSION AND FUTURE PROSPECTS

The relevance of studies on macrophage motility for the diagnosis and prognosis of head and neck cancer.

It is a major clinical problem to distinguish premalignant from malignant lesions in the head and neck region (particularly with regards to squamous cell carcinomas of the larynx). At present a distinction is made on the basis of histological examination, but it is generally accepted that such examination carries a high degree of subjectivity. Three classes of premalignant lesions are distinguished. In the greater part of Europe the classification of Kleinsasser (1,2) is followed (as we use to do):
Class 1. Simple squamous cell hyperplasia.
Class 2. Squamous cell hyperplasia with atypia.
Class 3. Carcinoma in situ.
Each catagory has its own prognostic value: malignant transfor-mation is rarely seen in class 1, it is fairly common in 2 and frequently observed in class 3. The problem with these classification systems lies in a clear distinction between class 2 and 3 and between class 2 and microinvasive squamous cell carcinomas (3,4). This makes a reliable and reproducible classification difficult or even impossible in roughly half the cases. One has attempted to improve the histopathological diagnosis employing objective investigational morphological methods such as DNA quantification, photomorphometric analysis (5) and other quantitative morphological techniques (6). Uptil now these methods are not practical for routine clinical application. The polarization assay as described in this thesis might be of value for such a distinction between premalignant lesions in the head and neck region and carcinoma in situ. In favour at the test we found the polarization assay already disturbed with real minimal head and neck cancerous disease (such as T_1 glottic laryngeal cancer), and high dilutions of tumour derived factors (upto $1:10^4$) caused a significant depression of polarization of healthy donor monocytes. However the LMWF's detected in the test are not specific for malignant disease and controlled studies are prerequisite to establish the usefullness of the test in this respect.

Patients with established head and neck cancers (stages III and IV according to the UICC classification; 7) constitute a therapeutical problem. Failure to eradicate the disease completely was regarded as the major cause of death in patients with advanced squamous cell carcinomas of the head and neck. However, this pattern of failure has changed with the employment of treatment policies consisting of surgery followed by radiation in patients with a high risk for local or regional recurrence (8). Many studies now report an improvement in the eradication of the primary tumour by this type of combined therapy (9,10,11). This is however disappointingly not reflected in a proportionate increase in the 5 years survival. While fewer patients die from uncontrolled disease above the clavicle, more are affected by disseminated disease and second malignant neoplasms such as bronchial carcinoma. In other words a changing pattern is observed regarding the follow-up of these patients: whereas in the past local or regional

recurrence was the major cause of death, nowadays malignant disease at a distant site is becoming increasingly more important. The chance of getting a distant metastasis correlates with the presence of tumour cells in the cervical nodes rather than with the actual T-Stage of the tumour (UICC classification) (12,13): in a recent study carried out in our hospital (14) a higher incidence of distant metastases was found in patients with more than 3 histologically proven tumour positive nodes. The spread of tumourcells beyond the lymphnode capsule appeared to be an additional risk factor. It was found that in the "high risk" group with three positive nodes and/or extranodal involvement over half of the patients developed distant metastases within the next two years. Our hospital plans adjuvant chemotherapy in this group of patients (15). A method that would allow for an early detection of distant micrometastases in this high risk group of patients would be very usefull. Again the polarization assay could be of value since we showed that the polarization of monocytes restores almost completely after radical treatment (16). Relapses might be announced by early re-impairment of the polarization when the test is applied as routine follow-up after surgery or radiation. The detection of P15E-like material in the circulation of such patients might make such a routine screening even more worthwhile as such an assay is more practical to carry out. (The precision seems to be equal and the assay on peripheral blood monocytes of patients must be performed on freshley collected blood, which is a practical problem in routine application). A further advantage of testing the effect of serumfactors is that large numbers of serum samples can be collected and stored until further use in a standardized polarization assay with healthy donor monocytes. It is worthy to note that the group of Cianciolo et al recently introduced an ELISA for the detection of serum P15E-like molecules (personal communication). Such an assay would even be more practical than the above mentioned bio-assays. In the near future we plan to follow our patients with an assay for the serum detection of P15E like molecules and the measurement of patient monocyte polarization. This will enable us to determine whether these tests are of clinical value in head and neck oncologic practice. Caution should be made since the factors do not seem to be specific for the the malignant state and the effect of intercurrent infections and the like, are not known yet.

The possible significance of P15E-like molecules.

It has been described that P15E-like molecules are not only expressed in malignant epithelial cells, but also in malignant lymphoid and monocytic cell lines (U937, K562, SB, MOLT-4, HL60-BII, HSB-2 and CEM) and even in normal blood lymphoid cells after stimulation with mitogens such as PHA, Con A and PWM. P15E like material could not be detected in unstimulated peripheral blood mononuclear cells (17). These observations led to a concept that the production of P15E-like molecules and rapid cell division were closeley associated. The P15E-like molecule could be a product of a gene which is normally unexpressed, but becomes expressed during cell division. Tumour cells may regulate this gene abnormally, as they do for transforming oncogenes (18). This results in an exaggerated production of the immuno- suppressive material; and some view this as the cause of the immune disturbances accompanying malignant disease (19,20,21 22,23,24). Cancer may develop in at least two major stages (fig.1.). The first one involves the complex process of neoplastic transformation relating to the regulation of cell division. This process may require alteration in the function of multiple genes which govern cell growth. This could f.i. be achieved by the activation of oncogenes (18). Such oncogenes have recently been described by Friedman et al (25) in head and neck squamous cell carcinomas, and the authors suggested the involvement of external factors like tabacco and alcohol abusis in this process. Acquisition of key alterations induced by such

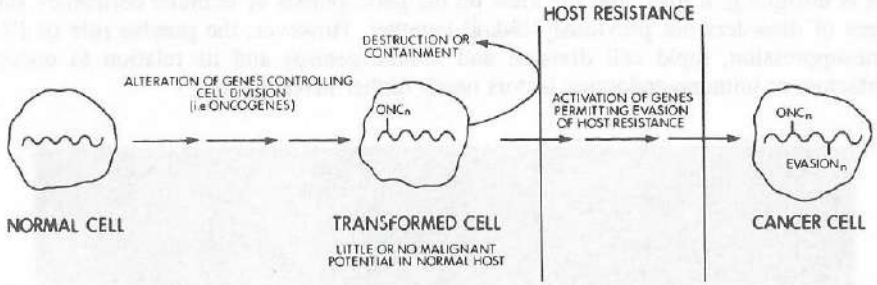

Fig. 1. A scheme of the two stage model for the developement of a clinically apparent neoplasm. (Used with permission from Immunology Today, 1984 vol.5, No.8 p. 242).

genes may lead to a transformed state which is characteristic of tumour cells seen in culture. Such cells, however, may not have the ability to withstand the normal resistance exerted by a healthy immune system. The acquisation of the means to evade host resistance may be a critical second step in the actual outgrowth of tumour cells to an overt malignancy, and the production of P15E-like molecules might be a critical event in this stage. However, the situation is probably much more complex, P15E-like material was not only detected histomorphologically by us in head and neck malignancies, other carcinomas and tumour cell lines, but also in epithelial cells overlaying inflammatory responses. Healthy ephithelia of skin, oral mucosa and gut (the latter also known to have a high turn-over) were negative (26). This implies that the production of P15E-like molecules by human cells is not only linked to rapid cell division, but may be also to other processes related to the guidance of inflammatory responses. It is worthy to note that P15E-like material was recently detected in our laboratory in the serum of 6 selected cases of chronic purulent rhinosinusitis (v.d. Plasche-Boers, unpublished results). These patients were special in that they had impaired DTH skintests and lowered T-cellular MIF production towards antigens of several commensal micro-organisms coloning their respiratory tract (27,28). Moreover these patients showed a disturbed monocyte polarization. Since these particular cases of chronic upper respiratory tract infections were not suffering at all from a malignancy, this observation highlights a notion that P15E- like molecule production might earlier be related to physiologic or pathologic processes of immune (dys)regulation. Following this reasoning and aware of the fact that P15E-like molecules are primarily produced by squamous epithelial cells, we developed a hypothesis that P15E-like molecules may be present and play a role in the thymic micro-environment. Fig. 2 shows that P15E-like material was indeed detectable by way of immunohistochemistry in the thymus of both guinea pig and men. Its distribution pattern was similar to that found earlier in benign and malignant squamous cell epithelia (see chapter 4). Though this observation is intriguing, it needs further confirmation by the isolation of the factors from the thymus and by testing these in the polarization assay. Most relevant is a recent report of Haynes (29) where he describes that Mab's to P19 of HTLV-I also show a reactivity for thymus epithelial cells. It is tempting to speculate that the P15E-like factors (and may be even other retroviral factors)

might be considered as being identical to normal immuno-endocrine polypeptide factors (such as thymic hormones). The enhanced production of P15E-like molecules by epithelial tumour cells would then represent an autonomous aberration of the production of a factor normally expressed under the influence of inflammatory or immune stimuli (30,31). The structural homology between such a presumptive immuno-endocrine factor and the P15E of murine retroviruses is intriguing, it may alter the view on the pathogenisis of immune deficiency states in a variety of disorders not previously linked together. However, the precise role of P15E in immunesuppression, rapid cell division and tumourigenises and its relation to oncogenes, growthfactors or immuno-endocrine factors needs further investigation.

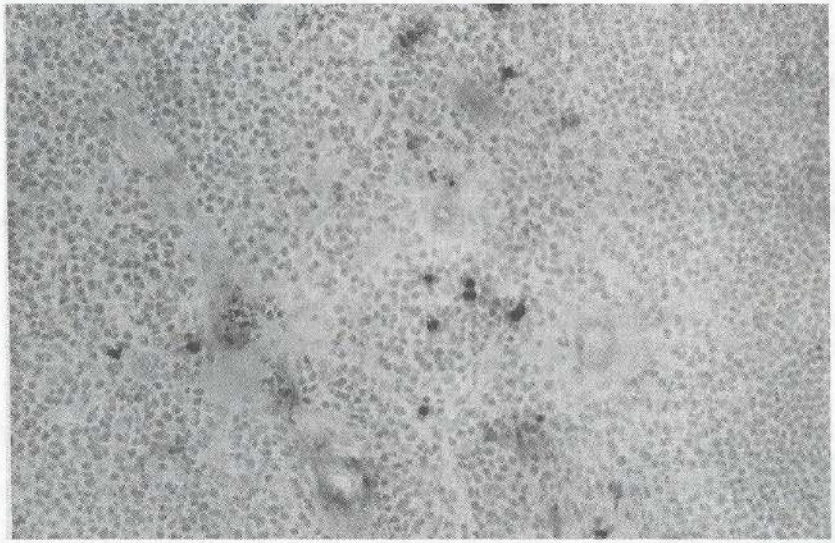

Fig. 2. Indirect immuno-peroxidase staining with a anti-P15E monoclonal antibody on a frozen section of human thymic epithelia. Scattered epthelial cell groups show a positive reaction within their cytoplasm. (magnification: 208x).

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CHAPTER 8

SUMMARY AND CONCLUSIONS

This thesis deals with the influence of head and neck carcinomas and factors of low molecular weight (LMWF's) derived from these tumours on the chemotactic responsiveness of mononuclear phagocytes.

In the introduction a short overview is given of the literature concerning the relationship between head and neck cancer and the function of the mononuclear phagocyte system: the presence of head and neck cancers have been described to induce clear impairments of several of the functions of the mononuclear phagocyte system, most notably chemotaxis. Evidence has been produced of tumour-derived factors as the cause of this impaired function: low molecular weight factors of less than 25,000 Daltons, hampering chemotaxis can be isolated from tumours. The factors were found to be related to P15E. P15E is a structural component of the envelope of animal retroviruses (murine and feline leukemia viruses), and the protein is known to be immuno-suppressive.

This thesis addresses with the following experimental questions:

- *Is a newly developed method, the socalled "Polarization assay" suitable for the detection of defects in monocyte chemotactic responsiveness in patients with head and neck cancer; and can a restoration of "polarization" be detected after removal of the tumour? (chapter 2)*

- *Can low molecular weight factors biochemically isolated from head and neck cancers be detected by means of their effect on healthy donor monocytes in the polarization assay; and can their effect be neutralized with monoclonal antibodies to P15E? (chapter 3)*

- *Can retroviral proteins such as P15E and GP70 be detected in microscopical preparations of head and neck cancers using immunohistochemical techniques? (chapter 4)*

- *Can free P15E-like material be detected in the circulation of head and neck cancer patients? (chapter 5)*

- *Are P15E-like low molecular weight factors, present in head and neck cancers and potent in suppressing monocyte chemotactic ability also able to suppress DTH skinreactions. (chapter 6)*

In chapter 2 the newly developed "polarization assay" for the measurement of monocyte chemotaxis is described as being rapid and more easy to perform as compared to the old fashioned "Boyden chamber" method (migration through millipore membranes). The latter assay was used by us in earlier studies to detect the disturbances in monocyte chemotactic responsiveness. A good correlation was found between the data obtained in both assays ($P<0,005$; $r=0.71$). Using the polarization assay, peripheral monocytes of 24 head and neck cancer patients were tested for their chemotactic ability. All showed a depressed chemotactic respon-

siveness (mean 12,5% +/- 1,7 s.e.m. P<0,001) before operation. Nine patients were tested several weeks after radical surgery and 7 showed a clear restoration of responsiveness. Twelve patients tested 2 years after radical treatment all showed normal monocyte chemotactic responses.

Chapter 3 describes the effect of LMWF's isolated from 14 head and neck cancer specimens on the "polarization" of healthy donor monocytes. All significantly inhibited monocyte polarization when used in a dilution of 1:10 (0.67 mg tumour tissue/ml). Healthy control factors only had normal effects on monocyte polarization, when tested in this dilution. The inhibitory effect of the tumour derived LMWF's could be neutralized by treating the factors with any of 3 different murine monoclonal antibodies to P15E. A rabbit polyclonal antibody was as effective. This again illustrates the relationship between tumour factors and P15E.

Chapter 4 reports the data of an indirect immuno-peroxidase assay, showing the presence of P15E-like material in surgical specimens of head and neck carcinomas. Thirty-five biopsy specimens were included in this study and all gave positive results. The P15E-like material was mostly distributed in the form of fusiform bands in the cytoplasm of malignant epithelial cells. 63% of other carcinomas (breast, bronchus and ovarium) and 91% of epithelia overlaying inflammatory responses in skin, gut and oral mucosa gave positive results as well. Healthy skin, gut and oral mucosa were invariable negative. This indicates that P15E-like material - though probably playing an important role in cancerous disease - does not seem to be specific for the malignant state.

In chapter 5 we describe the detection of P15E-like molecules in the circulation of head and neck cancer patients. Low molecular weight fractions of less than 25,000 Daltons prepared from the sera of nine patients were tested. All showed a significant inhibition of polarization of healthy donor monocytes. This inhibitory activity was neutralisable by a monoclonal antibody reactive to P15E.

Chapter 6 reports a study on the influence of five head and neck cancer derived LMWF's on the expression of delayed type skinreactions. Investigations were caried out in mice sensitized to the contact allergen Dinitro-Fluoro-Benzene (DNFB). The factors were injected 24 hrs prior to skintesting. The LMWF's appeared to inhibit the skintests, particularly at 24 hrs. Toxic skin reactions to croton oil were equally affected and this shows the aspecificity of the suppressive effect exerted by the factors. The monoclonal antibodies to P15E again abolished the effects of the tumour factors.

In conclusion, this study clearly confirms our earlier findings that monocyte chemotactic responsiveness of patients with head and neck carcinomas is defective. Factors antigenically and physicochemically related to murine P15E are produced by the tumour and detectable in the circulation of the patients; these factors can be hold responsible for the suppressive effects. Although the number of patients tested in this study is small, the findings might point to a clinical applicability of the polarization assay; an early detection of tumour growth or recurrences might come in reach by the "titration" of P15E-like molecules in the circulation of patients. The P15E-like molecules, produced by the head and neck cancers are not specific for malignant growth. They were also found in nondividing epithelial cells overlaying inflammatory responses and in thymus epithelial cells. Its precise role and relation to growthfactors, oncogenes, animal retroviruses and thymic hormones needs further clarification.

CHAPTER 9

SAMENVATTING EN CONCLUSIES

Dit proefschrift beschrijft de invloed van hoofd/hals tumoren en van laag moleculaire factoren, afgeleid van deze tumoren, op het chemotactisch vermogen van de mononucleaire phagocyten.

In de introductie wordt een kort overzicht gegeven van de literatuur betreffende de relatie tussen het mononucleaire phagocyten systeem en hoofd/hals tumoren. De aanwezigheid van deze tumoren induceert een duidelijke verstoring van verscheidene functies van de mononucleaire phagocyten en met name van het chemotactisch vermogen van deze cellen.
Er wordt beschreven dat laag moleculaire factoren, afgeleid van tumoren, verantwoordelijk zijn voor deze gestoorde functie. Deze factoren blijken gerelateerd te zijn aan P15E. P15E is een structurele component van het kapsel van dierlijke retrovirussen (leukemie virussen van katten en muizen) en is bekend om zijn immunosuppressieve werking.

Dit proefschrift behandelt de volgende experimentele vragen:

- *Is de onlangs ontwikkelde zgn. "polarisatie assay" bruikbaar voor het meten van verstoringen in het chemotactisch vermogen van monocyten bij patienten met hoofd/hals tumoren? Is er sprake van een herstel van deze functie na radicale behandeling van de tumor?(Hoofdstuk 2)*

- *Is de polarisatie assay met gezonde donor monocyten een bruikbare methode om het effect te testen van laag moleculaire factoren geisoleerd van hoofd/hals tumoren? Kan dit effect geneutraliseerd worden met monoclonale antilichamen gericht tegen P15E?(Hoofdtuk 3)*

- *Kunnen retrovirale producten, zoals P15E en GP70 worden aangetoond in weefselcoupes van hoofd/hals tumoren met behulp van immunohistochemische technieken?(Hoofdstuk 4)*

- *Kan P15E- achtig materiaal worden aangetoond in sera van patienten met hoofd/hals tumoren?(Hoofdstuk 5)*

- *Zijn van hoofd/hals tumoren afgeleide laag moleculaire factoren, die het chemotactisch vermogen van de monocyten kunnen beinvloeden, ook in staat huidtest reacties van het vertraagde type te onderdrukken? (Hoofdstuk 6)*

Hoofdstuk 2 beschrijft de onlangs ontwikkelde "polarisatie assay", die gebruikt wordt voor de meting van het chemotactisch vermogen van de monocyten. Deze methode blijkt sneller en makkelijker uitvoerbaar te zijn dan de eerder gebruikte "Boyden Chamber" methode (migratie door millipore membranen). Deze laatste methode werd door Balm et al gebruikt tijdens vroegere studies naar de chemotaxis van monocyten bij patienten met hoofd/hals tumoren. De gegevens die met de beide bovengenoemde methoden werden verkregen kwamen goed met elkaar overeen ($P<0,005$; $r= 0,71$). Gebruikmakend van de polarisatie assay werden de perifere bloedmonocyten van 24 patienten met hoofd/hals tumoren getest op hun migratie capaciteit. Alle toonden voor de operatie een verminderde chemotactische respons (gemiddeld 12,5% +/- 1,7 s.e.m. $P<0,001$).
9 Patienten werden enkele weken na radicale chirurgie getest. Hiervan werd bij 7 een duidelijke verbetering van de chemotaxie gezien. Bij 12 patienten die twee jaar na radicale therapie werden getest bleek het chemotactisch vermogen weer normaal te zijn.

Hoofdstuk 3 beschrijft het effect van laag moleculaire factoren (molecuul gewicht < 25.000 Daltons), afgeleid van 14 hoofd/hals tumoren op de polarisatie van gezonde donor monocyten. In alle gevallen werd een significante remming van de monocyten polarisatie gevonden bij een verdunning van 1 : 10 (0,67 mg tumor weefsel per ml). Gezonde controle factoren hadden nauwelijks effect op de monocyten polarisatie in deze verdunning. Het remmend effect kon worden geneutraliseerd door de tumor factoren te behandelen met 3 verschillende muizen monoclonale antilichamen gericht tegen P15E. Een konijnen polyclonaal antilichaam gericht tegen P15E had een zelfde neutraliserend effect. Deze bevindingen wijzen wederom op de relatie tussen deze tumor factoren en P15E.

In hoofdstuk 4 wordt met behulp van indirecte immunoperoxidase technieken de aanwezigheid van P15E-achtig materiaal onderzocht in tumor preparaten van hoofd/hals tumoren. Er werden 35 preparaten onderzocht. In alle preparaten kon dit materiaal worden aangetoond. Het bleek hoofdzakelijk gelocaliseerd te zijn in de vorm van ringvormige banden in het cytoplasma van maligne epitheliale cellen. Bij 63% van andere tumoren (mamma, bronchus en ovarium carcinomen) en in 91% van ontstoken weefsel van huid, orale mucosa en darm werd eveneens reactiviteit gevonden in de epitheliale gebieden. In gezond epitheel van huid, mucosa en darm werd geen reactiviteit gevonden. Dit toont aan dat, hoewel het P15E-achtig materiaal een belangrijke rol speelt bij maligniteiten, het niet specifiek blijkt te zijn voor maligne groei.

In hoofdstuk 5 wordt gezocht naar de aanwezigheid van aan P15E gerelateerd materiaal in de circulatie van patienten met hoofd /hals tumoren. Laag moleculaire factoren (molecuulgewicht < 25.000 Daltons), bereid uit de sera van 9 patienten werden getest. Alle toonden een significante remming van de polarisatie van gezonde donor monocyten. Dit remmende effect kon worden geneutraliseerd met een monoclonaal antilichaam gericht tegen P15E.

Hoofdstuk 6 beschrijft een onderzoek naar de invloed van 5 laag moleculaire hoofd/hals tumor factoren op de huidtest reactie van het vertraagde type. Deze experimenten werden uitgevoerd met muizen die gesensibiliseerd waren met het contact allergeen Dinitro-Fluoro-Benzeen (DNFB). De factoren werden 24 uur voor de huidtest geïnjiceerd.
Er werd een significante remming gevonden van de huidreactie, vooral na 24 uur. De toxische huidreactie onder invloed van Croton olie werd op identieke wijze geremd onder invloed van deze tumor factoren. Dit toont aan dat de remmende werking op de huidtest reactie aspecifiek is. Monoclonale antilichamen gericht tegen P15E konden wederom het effect van de tumor factoren neutraliseren.

Concluderend kan gesteld worden dat deze studie onze eerdere bevindingen duidelijk bevestigt en aantoont dat het chemotactisch vermogen van de monocyten van patienten met hoofd/hals tumoren verstoord is. De tumoren produceren laagmoleculaire factoren die zowel antigeen als fysisch/chemisch gerelateerd zijn aan het muizen P15E. Deze factoren, die aantoonbaar zijn in de circulatie, kunnen voor een groot gedeelte voor deze verstoring verantwoordelijk worden gesteld.

Hoewel het in deze studie om een beperkt aantal patienten gaat, wijzen de bevindingen toch mogelijk op een klinische toepasbaarheid van de polarisatie assay; De vroege detectie van maligne groei of recidieven wordt wellicht mogelijk door de "titratie" van P15E-achtig materiaal in het serum van deze groep patienten.

Het P15E-achtig materiaal bleek niet specifiek te zijn voor maligne groei en kon ook worden aangetoond in niet delende cellen van het epitheel bij ontstekings reacties en in epitheliale cellen van de thymus. De precieze rol en de relatie tot groeifactoren, oncogenen, dierlijke retrovirussen en thymus hormonen zal nader onderzocht moeten worden.

9. Euthanasie mag door niemand misbruikt worden, zelfs niet door politici.

10. De combinatie van een geavanceerde microcomputer met een laser-writer, zal in de drukwereld eenzelfde revolutie teweeg brengen als de overgang van conventioneel loodzetten naar offset-technieken.

11. Door gebruik te maken van een personal computer kan de promovendus efficienter werken, zijn geheugen vergroten en kosten besparen. Het eindeloze, onvermijdelijke "knip en plakwerk" blijft echter, zij het nu elektronisch uitgevoerd, even frustrerend.

I.B. Tan 11 april 1986

VRIJE UNIVERSITEIT TE AMSTERDAM

RETROVIRAL P15E-RELATED FACTORS IN HEAD AND NECK CANCER

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor in de geneeskunde
aan de Vrije Universiteit te Amsterdam,
op gezag van de rector magnificus dr. P.J.D. Drenth,
hoogleraar in de faculteit der sociale wetenschappen,
in het openbaar te verdedigen
op vrijdag 11 april 1986 te 13.30 uur
in het hoofdgebouw der universiteit,
De Boelelaan 1105

door

TAN ING BING

geboren te Maastricht

1986
VONK/ZEIST

Promotor : prof. dr. G.B. Snow
Copromotores : dr. A.J.M. Balm
dr. H.A. Drexhage
Referent : prof. dr. Ph. Rümke

STELLINGEN

behorende bij proefschrift:

"Retroviral P15E-related factors in head and neck cancer"

1. De polarisatie assay is een praktische methode om het chemotactisch vermogen van monocyten bij patienten met hoofd- halstumoren te meten.

2. De verstoorde chemotaxie van de monocyten bij patienten met hoofd- hals-tumoren, herstelt na radicale behandeling van de tumor.

3. Het verstoorde chemotactisch vermogen van monocyten van patienten met een tumor in het hoofd- halsgebied, wordt veroorzaakt door factoren, die zowel fysisch, chemisch als antigeen verwant zijn aan het retrovirale kapsel-eiwit P15E.

4. De verminderde huidtest reactiviteit van het vertraagde type bij patienten met een hoofd- halstumor kan voor een groot gedeelte worden toegeschreven aan een niet-specifiek mechanisme.

5. De gunstige resultaten, die zowel dier-experimenteel als klinisch behaald zijn met locale chemotherapie in reeds ver gevorderde tumoren, berusten niet alleen op een direct tumor cytotoxisch effect, maar ook op een selectieve uitschakeling van suppressor-lymphocyten.

6. Gezien het leefpatroon van patienten met hoofd- halstumoren, dient reeds bij het eerste polikliniek bezoek een rookverbod te worden opgelegd, aangezien dit reeds op korte termijn een aanzienlijke daling van het operatie-risico kan bewerkstelligen.

7. Bij een larynxcarcinoom dat de luchtweg in ernstige mate obstrueert, verdient endoscopische tumorreductie met behulp van de CO_2 laser als eerste behandeling de voorkeur boven een laryngectomie à chaud.

8. De diagnose "otitis media acuta" wordt niet alleen gesteld door (hetero) anamnese, lichamelijk onderzoek en inspectie van het trommelvlies, maar vooral door de juiste interpretatie van het trommelvliesbeeld.