IMMUNOHISTOLOGICAL STUDIES IN BASAL CELL CARCINOMA

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"NONMELANOTIC SKIN CANCER AND SOLAR KERATOSES.

The Quiet 20th Century Epidemic"

ROBIN MARKS, 1987.

CONTENTS

CHAPTER 1

INTRODUCTION

1.1. General characteristics of basal cell carcinoma

Basal cell carcinoma (BCC) is the most common cutaneous cancer of epithelial origin in caucasians (1-3). The tumor was first described in 1827 by A. Jacob and named as Jacob's ulcer (4). In 1902, E. Krompecher clearly delineated this tumor from other epithelial tumors (5). Many synonyms have been used for this tumor, e.g. Krompecher's tumor, rodent ulcer, adnexal carcinoma, carcinoid of the skin, basalioma, basal cell epithelioma, Basalzellenkrebs, non-cornifying epithelioma and basal cell carcinoma. Although BCC are slowly growing tumors, a proportion of these tumors depending on their specific clinical and histological features are aggressive in their biological behaviour leading to mutilation and recurrence after therapy (6-8). The occurrence of metastasis is extremely seldom, but has been reported (9,10). Most metastases were located in the regional lymph nodes. If metastasis occurs in the lungs, bones or other internal organs, the prognosis is poor and the average survival time is less than 1 year (11). Since these tumors have the potential for metastasizing and local destruction, they must be considered as cancerous. Therefore, in this thesis they are referred to as basal cell carcinoma.

One of the most important etiological factors in the development of epithelial skin cancer, i.e. basal cell carcinomas and squamous cell carcinomas (SCC) is the intensity and duration of skin exposure to the ultraviolet (UV) fraction of sun light (1). Areas of the body that are exposed to sun light are more prone to develop BCC and SCC than non-exposed areas (1,7). In the case of BCC, about 90% are located in the head and neck region, with a particular predilection for the upper central part of the face (1,2). However, the superficial type BCC occurs mainly on the trunk. BCC can also occur on the limbs, but seldom on handpalms or footsoles (2,12). The incidence of epithelial skin cancer in fair skinned individuals is much higher than in dark skinned individuals (2). This observation is highly suggestive for the role of melanin pigment as a protector against the UV-fraction of sun light. This is illustrated by the observations in albinos of black race in whom the incidence of BCC is higher than in normally pigmented black individuals (13-15). The susceptibility of fair skinned individuals for developing epithelial skin cancers is also related

to their tanning ability. Individuals who sun-burn easily and never tan run a higher risk of developing epithelial skin cancers than individuals who tan easily. In this respect an increase in the incidence of epithelial skin cancer is expected due to several factors. These factors are attributed to the economical wellbeing in the western countries which allows individuals to visit sun-drenched areas of the world more frequently, thereby increasing their exposure to the UV-fraction of sun light. A rapidly diminishing protective ozone layer around the globe due to chloro-fluoro carbons would also indirectly contribute towards an increase in the incidence of epithelial skin cancers in the future. In the USA, each year 500.000 new cases of non-melanoma skin cancers are diagnosed, which represent 50% of all types of cancer in the USA (16). The incidence rate of non-melanoma skin cancer for white Americans in the Southern States of the USA is more than 230/100.000 per year and is about 4 times higher than in the Northern States (17,18). In the central Victoria province of Australia, the incidence rate for non-melanoma skin cancer is even higher - 700/100.000 per year (19). Different ratios of BCC and SCC have been reported depending on the region and population studied; 10:1 (3) 6.5:1 (20) and 1.3:1 (21). Little is known about the exact incidence of BCC and SCC in the Netherlands. It has been reported at an estimate of 15.000 new cases per year (22). At the Academic Hospital Dijkzigt in Rotterdam between 1980 and 1987 some 800 new cases of non-melanoma skin cancer were diagnosed. The ratio of BCC to SCC was 6:1.

The role of the UV-fraction of sun light as an etiological factor is further illustrated by the frequent occurrence of skin cancer in xeroderma pigmentosum, a disorder in which the skin is extremely vulnerable to damage from UV-radiation due to defects in the DNA-repair mechanisms (23). However, there are still some discordent notes on the importance of exposure to UV-radiation as being the single most important causative factor in BCC.

Although, the dorsal side of the hands and the forearms are frequently exposed to sun light, the occurrence of BCC in these areas is relatively infrequent (1). Almost all SCC occur in skin areas which are prominently exposed to sun light while two third of the total BCC are observed in these areas (1,24). Furthermore, solar dosimetry studies of the face did not show a clear correlation between BCC density in a given area and the UV-dose (25). Therefore, there must be additional etiological factors which play a role in the development of BCC. These factors include injury to the skin by X-rays, chronic inflammation, congenital malformations, e.g. nevus sebaceous and exposure to inorganic arsenic (1,2,7). Genetic disorders such as the basal cell nevus syndrome and the Bazex syndrome are characterized by the development of multiple BCC, which may already occur in childhood (26). Recently, it has been shown that some BCC and SCC have a point mutation in the ras-oncogenes. This indicates that activation of ras-oncogenes may be involved in the development of epithelial skin cancer (27). The immune competence of the patient

seems to play a role in preventing the development of BCC and SCC (28). The incidence and the aggressiveness of epithelial skin cancer are increased in organ transplantation patients who receive azathioprine and prednisolone or ciclosporin-A as a maintenance anti-graft rejection therapy (29,30). However, in these chronically immunocompromised patients the ratio of BCC to SCC reverts in favour of SCC. It has also been observed that epithelial skin cancers which developed in patients with chronic lymphocytic leukemia or lymphoma were more aggressive in their behaviour than usual, which may be related to defective cell-mediated immunity in these patients (31).

The clinical forms of BCC are extremely variable (2,3,32). The clinical and histological subtypes of BCC are summarized in Table I.

The early tumors are generally small, pearl-like, raised and rounded areas covered by thin epidermis through which a few teleangiectatic vessels are visible. Other forms include small lichenoid papules, small erythematous plaques, superficial ulcerative papules or keratotic slightly indurated areas. The more advanced tumors resemble the same variety of forms as the early lesions. One of the most common types, the nodular form which is covered by a thinned epidermis will periodically erode and crust and finally result in permanent ulceration. In the literature, the ulcerative BCC is referred to as ulcus rodent and the large ulcerative BCC as ulcus terebrans (2,12,32). Irregularly

Table I. Clinical and histological subclassification of basal cell carcinoma.

Clinical type	Histological type		Growth pattern
Nodular	Non-differentiated	-solid	Non-infiltrative (Nodular)
pigmented		-pigmented	
	Differentiated	-adenoid	
ulcus rodens		-cystic	
Ulcerative		-keratotic	Infiltrative
ulcus terebrans		-basosquamous	
Cicatrizing			
Morphoiec	Morphoiec		
Superficial	Superficial		Multifocal
Fibroepithelioma	Fibroepithelioma		

distributed melanin pigment may also be present in some BCC. These pigmented BCC are sometimes clinically difficult to distinguish from a melanoma maligne. The morphoiec type BCC or sclerodermiform BCC is rather uncommon and resembles a localized scleroderma. In these tumors a dense stromal fibrosis causes a thickened dermal plaque. The margin of the lesion cannot be defined visually but only by palpation (2). Further clinical types are the cicatrizing type which is characterized by a superficial nodular plaque accompanied by clinically apparent central healing- but histologically evident persistent tumor. The superficial type BCC is characterized by a superficial centrifugal growth pattern with a slightly raised margin and a central zone which is covered by scales and crustae. An exceptional type of BCC is the premalignant fibro-epithelial tumor (of Pinkus) which is characterized by a dome-shaped or pendiculated flesh coloured nodule, mainly located in sacral region of the back. The histology is very typical but has the characteristics similar to those of BCC (33).

The broad clinical forms of BCC are also reflected in a variety of histological features (34-36). An obvious relationship between the clinical and the histological features of BCC has been reported (12). Histologically, the most common type of BCC is the undifferentiated solid type (35).The tumor consists of individual nests of tumor cells which are surrounded by fibrous stroma. The tumor cells resemble epidermal basal cells but they differ in cell size and shape and have a large nucleus to cytoplasm ratio. The peripheral cell layer of the tumor nests shows a palisade arrangement, whereas the tumor cells inside lie in a haphazard fashion. The BCC showing differentiation towards sebaceous structures is called cystic, that with eccrine or apocrine differentiation is called adenoid and that with keratinization is called keratotic. A special form is the basosquamous type which shows characteristics of both BCC and SCC.

As far as the origin of BCC is concerned, it is still not clear whether they are derived from skin adnexal organs or from transformed basal cells of the interfollicular epidermis. The observation that histologically, BCC show patterns of differentiation similar to different structures of skin adnexal organs argue for an adnexal origin (34,35). In addition, Kint observed a similarity between hair-cycle growth and BCC (12). However, in many BCC, especially the superficial type BCC, tumor buds show no contact with skin adnexal organs, but are only observed in close contact with the basal cell layer of the epidermis. This observation supports an interfollicular epithelial origin of BCC. Considering these observations it has been suggested that the tumor cells of BCC may arise from undifferentiated epithelial cells that can originate from any point of the epidermis or adnexal organ (12,33).

The mode of growth of a BCC is an important criterium to predict whether it will behave aggressively (6,8,37). The growth patterns of BCC can be classified as the nodular type which is characterized by well-circumscribed nodules with smooth and sharply demarcated border; the infiltrative type which is

characterized by poorly circumscribed tumor nests with irregular edges and often elongated with spikey processes reaching deep into the dermis or even into the subcutis; and the multi-focal type which is characterized by multifocal tumor buds arising from the overlaying epidermis and it mostly exhibits a superficial expansion growth. The infiltrative growth pattern is an important feature for its aggressive behaviour which is reflected in a high recurrence rate after treatment (8,37).

The aggressiveness of BCC has also been correlated with the site of its origin. The location of BCC in the embryological fusion planes show a tendency to invade into the depth (38). Areas with a high recurrence rate are the midface, especially the medial canthus, nasolabial fold and the nosetip, and the postauricular region (38-40). The high recurrence rate of the BCC in these particular areas may not only be due to its growth pattern along the embryological fusion planes, but also on the delay prior to clinical detection and the mode of therapy that is used (7). The current treatment methods include excision, cryosurgery, irradiation and currettage and electrodessication.

1.2. Tumor markers

Products or constituents of tumor cells which differ in antigenicity, distribution or quantity from normal tissue cells can be considered as tumor markers. These markers include carcinoembryonic antigens (CEA), alpha-fetoprotein (AFP), tumor-type associated antigens, mucinous carcinoma-associated antigens and intermediate-sized filaments (cytokeratins). The importance and the value of tumor markers in clinical oncology may be summarized as follows. *a.* The detection of tumor markers in a patient`s serum may be helpful in an early diagnosis. *b.* The diagnosis and the classification of tumor tissue of unknown or disputable origin. *c.* The localisation and the staging of metastases is possible using radiolabeled specific monoclonal antibodies (MoAb) against tumor markers. *d.* Specific monoclonal antibodies raised against tumor markers can be used for targeting chemotherapeutic agents. One group of tumor markers called tumor associated antigens (TAA) are glycoproteins or glycolipids in nature and are mainly present on the surface of tumor cells. TAA represent a wide spectrum of antigens which may evoke anti-tumor immune response which can be assessed using a variety of immunological assays (41,42).

To date, tumor-specific antigens have been isolated only incidentally from spontaneous human tumors despite intensive continuous efforts (42,43,43a). TAA can be divided into oncofetal antigens and tumor-type associated antigens. Oncofetal antigens are present in high amounts in malignant and fetal tissues, but may also be present in very low amounts in normal tissues (43). Several oncofetal antigens have been isolated amongst which the carcinoembryonic antigen (CEA) and alpha-fetoprotein (AFP) have been the most thoroughly investigated (44). The tumor-type associated antigens are antigens which are

present in high amounts in a given type of tumor, but are present in very low amounts (or absent) in benign or normal tissue from which the tumor is plausibly derived.

In recent years, numerous MoAb have been manufactured against oncofetal antigens and tumor-type associated antigens of melanoma, colorectal cancer, prostate cancer and breast cancer (45-47). The proteins of the intermediate-sized filaments form a considerable part of the intracellular matrix. These filaments consist of a number of biochemically and immunologically distinct proteins which are selectively found in different tissues. Neurofilament protein is characteristic for nerve cells, glial fibrilary acidic protein is characteristic for astrocytes, desmin is characteristic for muscle cells, vimentin is characteristic for mesenchymal cells and cytokeratins are characteristic for epithelial cells (48). At present, a large number of specific and sensitive MoAb to these proteins have been developed and are used extensively in diagnostic pathology (47-50).

1.2.1. Expression of different markers in epithelial skin cancer

Little is known on the expression of tumor markers in BCC and SCC. Several

	MoAb	NE	BCC	SCC	Ref.
TAA					
alpha-fetoprotein (AFP)		-	-	(+)	(59)
carcinoembryonic antigen (CEA)		-	-	(+)	(58,61)
?	VM-1	+	+	+	(52)
protein 100, 120	VM-2	+	+	?	(53,54)
sialylated Lewis[a]	C241,C50	-	+	+	(55)
sialosyllactotetraose	C50	-	+	+	(55)
glycoprotein 280, 500	653.40s	-	+	+	(56)
glycoprotein 280, 400	225.28s	-	+	+	(57)
?	HMB-45	-	(+)	-	(60)
Differentiation Antigens (Ag)					
epithelial membrane Ag		+	-	(+)	(62)
basal cell Ag		+	-	?	(63)
upper cytoplasmic Ag		+	-	?	(64)
involucrin		+	-	+	(65,66)
pemphigus Ag		+	-	-	(62)
MHC class I Ag					
HLA-ABC/β_2-microglobulin		+	(+)	(+)	(67-69)

Table II. An overview of the expression of tumor associated antigens (TAA), differentiation antigens (Ag) and class I antigens of the major histocompatibility complex (MHC) in normal epidermis (NE), basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). + = positive; (+) = Weakly positive or incidentally positive; - = negative; ? = unknown.

melanoma-associated antigens have been defined using MoAb (51). Ruiter et al (52) divided the melanoma-associated antigens into three groups, namely differentiation markers, markers associated with certain stages of neoplastic progression and markers associated with cell function. The third group of antigens are involved in immune regulation and belong to the major histocompatibility complex (MHC), class I and class II antigens. Both class I and class II antigens can be expressed on melanoma cells. However, in epithelial skin cancer there are some reports on the presence of TAA which have been detected using polyclonal antisera and MoAb (52-61). The expression of TAA and differentiation antigens in normal epidermis, BCC and SCC are summarized in Table I. The MoAb VM-1 and VM-2 reacted not only with cell membranes of epidermal basal cells but also reacted with BCC (52-54). MoAb VM-2 reacted with a membranous glycoprotein of molecular weight 100-120 kD (53). The MoAb C50 and C241 specific for the carcinoma-associated antigens sialylated Lewis^a and sialosyllactotetraose resprectively, also reacted with BCC (55). The MoAb 653.40s, 225.28s and HMB-45 detecting melanoma-associated antigens did not only react with melanomas, but also with some BCC (56,57,60). The oncofetal antigens CEA and AFP are not expressed in BCC (59,61). Several differentiation antigens, such as epithelial membrane antigen (EMA) (61), pemphigus antigen (62), basal cell layer antigens (63), upper cytoplasmic antigens (64) and involucrin (65,66) are not expressed in BCC and SCC. Furthermore, in BCC β_2-microglobulin and HLA-ABC (class I antigens) are not expressed (67-69).

1.2.2. Cytokeratins

The cytoskeleton of epithelial cells is characterized by the presence of numerous bundles of intermediate-sized filaments (tonofilaments), composed of a complex family of proteins called cytokeratins (K). They consist of 19 distinct polypeptides which are biochemically and immunochemically related and each is coded for by a separate gene (70,71). Cytokeratins can be divided into two groups; type-I cytokeratins which have an acidic isoelectric point and type-II cytokeratins which have a basic isoelectric point (70,72). Cytokeratins can only form heteropolymers, i.e. a type-I cytokeratin must combine with a type-II cytokeratin to form a filament. In each cytokeratin pair the basic member is always about 8 kD heavier than the acidic member (72,73). Moll et al (70) catalogued human cytokeratins and numbered them from 1 to 19. Cytokeratin 1 has the highest molecular weight of 67 kD and the highest isoelectric point and cytokeratin 19 has the lowest molecular weight of 40 kD and a low isoelectric point.

Specific cytokeratin patterns are characteristic of certain epithelial cell types (72). In Figure 1, the distribution of cytokeratins in different epithelial tissues is shown. Different cytokeratin patterns have also been observed in different cell

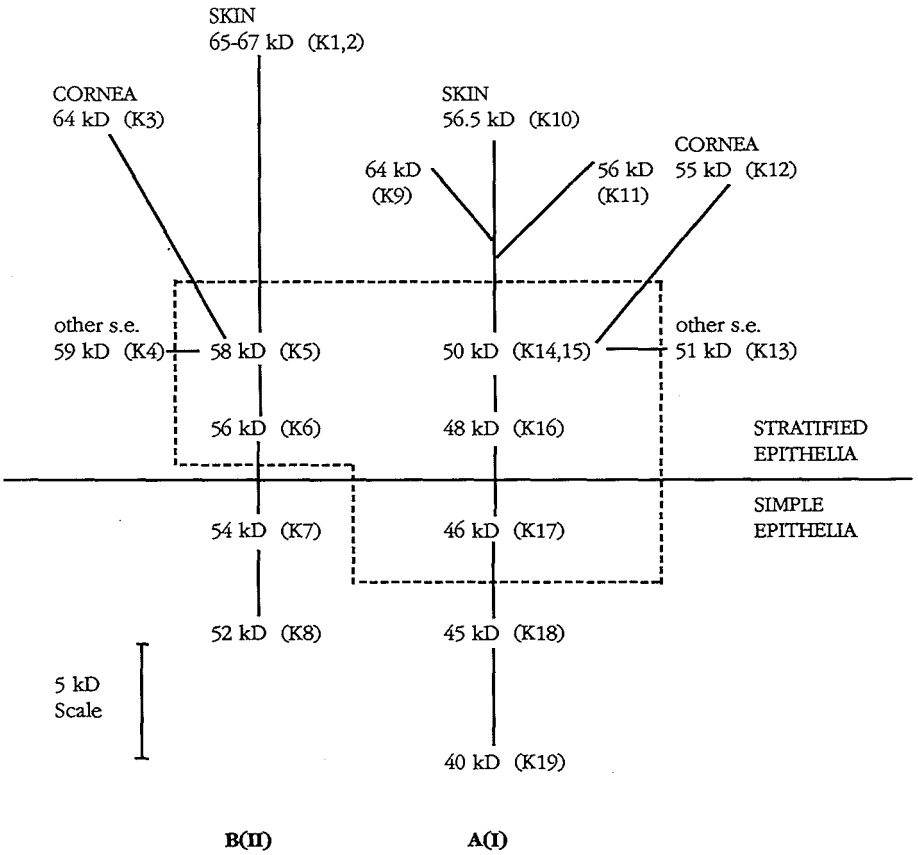

Figure 1. Model of cytokeratin expression proposed by Cooper et al (76).Cytokeratins of the Acidic (type I) and Basic (type II) subfamilies are arranged vertically according to their molecular weight (see the 5 kD scale).Cytokeratins below the horizontal line are mainly expressed by simple epithelia, and those above the line are unique to stratified epithelia (s.e.). Dashed box encloses the cytokeratins commonly expressed by all stratified epithelia in neoplasms, hyperproliferative diseases and in culture.

layers of the epidermis and in different adnexal organs of the skin (62,72,74). In addition, complex cytokeratin patterns have been observed in certain human carcinomas in which the cytokeratin pattern differed from that of the tissue of plausible origin (70,75).

In recent years, a large number of sensitive MoAb to intermediate filaments, especially to specific cytokeratins have been produced and extensively used (47,50,76-82). The use of anti-cytokeratin MoAb has been found very valuable in the differential diagnosis and also allows a further subdivision of carcinomas (48,83).

1.2.2.1. Expression of cytokeratins in basal cell carcinoma

The cytoskeleton of tumor cells in BCC is mainly composed of low molecular weight cytokeratins (84-87). Various amounts of cytokeratins 5, 6, 8, 14, 15 and 17 have been isolated from BCC using two-dimensional gel electrophoresis (70,86).

High molecular weight cytokeratins 1, 2 and 10 are expressed in normal epidermis but are not expressed in BCC (70,84-86). Cytokeratin 17 is present in considerable amounts in BCC and also in the pilosebaceous tract. The absence of cytokeratin 17 in the normal interfollicular epidermis suggests that BCC may originate from pilosebaceous cells. However, the expression of cytokeratin 17 in cultured normal skin and in hyperproliferative diseases such as psoriasis has also been reported (88,89). The detection of cytokeratin 8 in small amounts in some BCC reported by Moll et al is of particular interest (70,86). This low molecular weight cytokeratin belongs to the basic group and is normally expressed in simple epithelia, in early fetal epidermis and is also observed in tumors derived from non-keratinized tissues (75,90). Therefore, it is possible that the expression of cytokeratin 8 is a useful marker for these tumors.

1.3. Tumor immunology

The role of immunology in cancer has a considerable history dating back to the end of the last century. One of the first evidence for the existence of a host`s defence against tumors was reported by Loeb in 1901 (91), who demonstrated the rejection of incompatible tumor grafts. A few years later Ehrlich (92,93) demonstrated that mice with an established tumor transplant often failed to produce an additional tumor when reinoculated with tumor cells. This observation suggested the existence of an immune surveillance system. Twenty years later it was Murphy (94) who noticed that lymphocytes played a role in the resistance to tissue grafts and neoplasia. In the 1940s, the discovery of the genetic basis of histocompatibility, specific immunotolerance and the graft-versus-host (GVH) reaction led to additional knowledge on the immunogenicity of transplanted neoplastic tissues and the host`s immune response (95). The discovery of the existence of so-called tumor associated antigens (TAA), defined as determinants which are present on tumor cells but are either totally absent or present to a limited amount only on normal tissue cells, created a new field of tumor investigations. Furthermore, TAA of some tumors included a subset of cell- surface antigens that were termed tumor-associated transplantation antigens (TATA) which were responsible for evoking an immune response in other animals syngeneic with the animal in which the tumor originated (96).

Since the etiological agents of tumors arising in humans are often unclear, these tumors are classified as spontaneous tumors. In contrast to virus-induced, chemically and/or physically induced tumors, spontaneous tumors usually express no specific tumor antigens.

Clinical observations also support the role of immunologic factors in the defence against neoplasia. Organ transplant recipients who received anti-rejection maintenance dose of immunosuppressants for prolonged periods developed more neoplasia than the normal population (29,30,97-99). The mechanisms of anti-tumor immune response are rather complex and are as yet neither fully elucidated nor understood. However, several subpopulations of T cells, cells of the monocyte-macrophage lineage, Natural Killer (NK) cells, B cells producing specific and natural cytotoxic antibodies and Killer cells have been implicated in the host`s anti-tumor immune response (100,101). Especially NK cells, probably a subpopulation of lymphoid cells are considered to be the first line of defence against tumors (102,103). The recognition and elimination of tumor cells by NK cells is not MHC-restricted.

Since the development of the somatic hybridization technique in 1975 (104), many MoAb have been produced which specifically react against membrane antigens on different immunocompetent cells. In recent years, several studies have been reported on the detailed characterization of the immune infiltrate and its possible role in the defence against different neoplasia (105-111).

1.3.1. Immunology of epithelial skin cancer

At the end of the last century both Unna and Dubreuilh associated the occurrence of skin cancer with the exposure to sun light as a result of investigations of individuals with sun-damaged skin (112,113). Forty years later, experiments in laboratory mice and rats demonstrated that UV-light was responsible for inducing skin cancer (114,115). Moreover, it was shown that UV-light had the capacity to act either as an initiator, or as a promotor, or as a complete carcinogen (116,117). Kripke and co-workers have made important contributions towards the understanding of the effect of UV-light on the immune system, especially in relation to skin cancer (118,122). In trans-plantation studies in syngeneic mice, Kripke showed that UV-induced skin tumors were highly antigenic since tumors transplanted to untreated syngeneic mice were rejected (118). This rejection was unusual because most mouse tumors could be easily transplanted among members of the same inbred mouse strain without rejection. However, in mice where the skin had been exposed to UV-light, the transplanted tumor were not rejected. Furthermore, UV-induced tumors that were implanted in mice whose immune competence was derived from normal donors were rejected, in contrast to mice whose immune competence was derived from UV-light exposed donors. This indicated a systemic effect by UV-light (119,120). T suppressor lymphocytes seemed to be responsible for this tolerance-mechanism (121,122). Recently, evidence has been presented showing that tumor antigens on UV- induced tumors can be divided into tumor-specific transplantation antigens which are targets for the immunological effector cells and a common UV-associated antigen recognized

by T suppressor lymphocytes which are responsible for the inhibition of tumor rejection (123-125). It is not yet clear how and why the UV- associated antigen activates the suppressor cell pathway. It has been shown in studies on contact hypersensitivity that UV-light inactivates Langerhans cells (126,127). This inactivation leads to a direct or an indirect activation of T suppressor lymphocytes. There is evidence that UV-resistant antigen presenting cells, probably Thy-1$^+$ cells, activate the suppressor cell pathway (128,129). Prevention of tumor rejection may be achieved via the same mechanism (130).

The relevance of these findings in human epithelial skin cancer is at present uncertain. It is not known whether the immune system plays as important a role in human epithelial skin cancer as in mouse skin cancer. Whether in humans suppressor cells are activated following exposure to UV-light, thereby allowing the development of epithelial skin cancer, is at present unclear. However, there are several observations suggesting that the immune system plays a role in the pathogenesis of and the defence against skin cancer. A diminished immune responsiveness to dinitrochlorobenzene (DNCB) and standard intradermal antigens in sun-damaged skin as compared to non-sun exposed skin has been reported (131). Lymphocyte stimulation tests using aquous extracts of epithelial skin cancers (e.g. melanoma, SCC and BCC) indicated that the peripheral blood lymphocytes of patients were responsive to their respective tumors (132-134). A depletion in the surface markers of Langerhans cells in human skin after exposure to UV-light has been observed (135). Exposure of human skin to sun light for 1 hour/day for more than 12 consecutive days led to decreased numbers of total circulating T cells and a decrease in the T helper and T suppressor/cytotoxic ($T_H/T_{S/C}$) ratio (136). Similar decreases in $T_H/T_{S/C}$ ratios and delayed hypersensitivity reactions to DNCB have been observed following solarium exposure (137). Furthermore, a decreased natural killer cell activity after exposure to UV-light has been reported (138). Finally, the presence of an inflammatory mononuclear infiltrate consisting mainly of T cells in epithelial skin cancer indicated a cell-mediated immune response. In melanoma (139) and in BCC (140), an association was observed between heavy lymphocytic infiltration and partial regression of the tumors indicating an important role of the immune system in the defence against these tumors.

1.3.2. Immune response in basal cell carcinoma

Immunological studies in patients with BCC have demonstrated several abnormalities. Dellon et al (141) observed a low T cell level in the peripheral blood of patients with BCC of large size (>2cm in diameter) and aggressive growth pattern. A statistically significant decrease in the $T_H/T_{S/C}$ ratio in patients with BCC as compared to healthy controls has been reported (142). The proliferative responses of lymphocytes to common mitogens were decreased in patients with multiple or aggressive BCC, while in patients with BCC without

aggressive growth pattern, the proliferative responses of lymphocytes were normal (143,144). The number of T cells and the lymphocytic proliferative responses decrease with increasing age (145,146). Therefore, the development of BCC in older patients may be influenced by a decreased immune surveillance capacity as a result of the physiological aging process. There are apparently conflicting reports on the lymphocytic responses in BCC (134,147,148). Aquous extracts prepared from BCC cells when incubated with peripheral blood lymphocytes of healthy individuals inhibited their proliferative responses (148). However, incubation of peripheral blood lymphocytes from BCC patients with aquous extracts of BCC increased the lymphocytic proliferative response in 3 out of the 8 cases (134). These contradicting results may be explained by the results of Sheretz et al (147) who noted that only the extracts prepared from BCC with aggressive biological behaviour inhibited the autologous lymphocytic proliferative response while no inhibition occurred using extracts from non-aggressive BCC.

Macrophages may act as anti-tumor effector cells (149,150). In patients with BCC a defective macrophage mobilization has been observed (151,152). It has been suggested that soluble factors released by the tumor cells may inhibit the accumulation of macrophages at the site of such tumors. This inhibition can interfere with tumor antigen-processing and/or tumor cell killing, thus allowing more aggressive tumor growth.

In the last decade, several reports on the local immune response have been published in which attempts were made to elucidate the function of the cellular inflammatory infiltrate surrounding BCC and its possible role in the control of tumor growth (105,153-156). The peritumoral inflammatory infiltrate consisted mainly of T cells (105,153,154). A considerable number of immunoglobulin-bearing cells in the infiltrate were also observed (153,155). Using the E-rosette test and the EAC-rosette test, Viac et al (153) observed a T/B ratio of 4.6 in the inflammatory infiltrate eluted from BCC. Synkowski et al (156), using a panel of MoAb, observed a T/B ratio of 1.0 in situ. These results may suggest a significant role of the humoral response in BCC. However, Eaglstein et al (105) reported that the infiltrate in BCC consisted of 90% T cells and less than 10% B cells, which suggested a minor role of the humoral immune response. Furthermore, to date no circulating antibodies against BCC have been isolated. Therefore, the role of humoral immunity in the defence against BCC is rather doubtful. Little is known about the presence and the role of NK cells in epithelial skin cancer. Recently, Kohchiyama et al reported on the presence of NK cells in SCC (109). They suggested an important role of NK cells in the defence against SCC, but not against BCC.

1.4. Objectives of the study

The main aims of the investigations reported in this thesis were to obtain

detailed insight into the immunopathology of BCC which exhibit a broad spectrum of clinical and histological appearances. Two lines of investigations were followed. The first line concerned a search for useful tumor markers for BCC. Although the histological diagnosis of BCC is uncomplicated, in certain instances in order to establish a diagnosis, it would be advantageous if useful tumor markers for BCC were abailable. One such marker that was investigated was cytokeratin 8 (see § 1.2.2.1).

The second line of investigation concerned the characterization of the inflammatory infiltrate in BCC. Previously reported studies (105,153-156) on the characterization of the immune infiltrate in BCC were inconclusive and controversial, especially concerning the composition of the infiltrate and the role of humoral immunity. Therefore, investigations were undertaken to obtain a detailed insight into the composition of the peritumoral inflammatory infiltrate and its possible role in the defence against not only BCC, but also Bowen`s Disease (carcinoma in situ).

In recent years, several biological response modifiers, e.g. interferons (alpha, beta, gamma), interleukin-2 and tumor necrosis factor with anti-proliferative properties have become abvailable and their therapeutical potential in anti-cancer therapy has received much attention. There is evidence for an immune response, although not adequately functioning, against BCC. Since new approaches to therapy, especially in selected cases of BCC, may be of advantage, a clinical pilot study was undertaken to investigate the efficacy of human recombinant interferon-gamma in patients with BCC.

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CHAPTER 2

The detection of basal cell determinants in human basal cell carcinomas using two different monoclonal antibodies

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ABSTRACT

This report deals with the reaction pattern(s) of two monoclonal antibodies (MoAbs) with normal skin and basal cell carcinomas (BCC). Using indirect immunoperoxidase (IIP) and indirect immunofluorescence (IIF) techniques, MoAb 12 G7 was observed to react with a determinant related to the cell membrane of the epidermal basal cells. In the IIP technique MoAb 12 G7 showed a positive reaction with 32 out of 34 BCC (94%), while in IIF all the 14 BCC that were studied were positive. In most cases only the cells at the periphery of the tumour nests were stained. MoAb 253 B7 reacted with cytoplasmic determinant(s) of the epidermal basal cells both in the IIF as well as in the IIP techniques. Using the IIP technique only 5 out of 34 BCC (15%) showed a positive reaction with this MoAb. Four of the 5 positively staining tumours showed aggressive histological features. Using IIF technique only 2 out of 14 BCC were positive. The results presented in this communication are discussed with regard to the possible expression of selective differentiation and tumor-associated determinant(s) in BCC.

INTRODUCTION

Basal cell carcinoma (BCC) is the most common cancer of epithelial origin although metastasis is fortunately extremely seldom (15, 23, 30). However, a proportion of BCC depending on the specific histologic features are aggressive in their behaviour: local destruction leading to mutilation and recurrence after surgical excision (11, 15, 26). It is well known that in malignant epithelial skin tumours differentiation antigens, such as pemphigus antigen (3), basal cell layer antigens (2), upper cytoplasmic antigens (33), β_2-microglobulin (7, 10, 14, 29) and involucrin cannot be detected (20, 25). As in other neoplastic diseases, in epithelial skin tumours too, there are some reports on the presence of tumour-associated antigens (TAA) recognized by monoclonal antibodies (MoAbs), which form an additional aid for the detection of residual tumour cells in Mohs' surgery (1, 19, 22). Investigations, into the cytokeratin composition of normal and neoplastic epithelium have provided insight into the pathogenesis (16, 17, 18, 31, 32). The exclusive expression of cytokeratin 17 in BCC and pilosebaceous tract and not in the normal interfollicular epidermis has been reported previously by Moll et al. (17). This would suggest that the origin of BCC is related to pilosebaceous cells. However, the expression of keratin 17 in cultured normal skin, conjunctival and esophageal keratinocytes, and in hyperproliferative epidermal diseases such as psoriasis have all been previously reported (4, 16, 27, 31). Therefore as stated by Weiss et al. (31), the expression of keratin 17 is not cell-type specific and its presence in BCC should not be taken as evidence for a pilosebaceous origin of the tumour.

The lack of insight into the pathogenesis of BCC, the spectrum of various

clinical and histological appearances of BCC (15, 30) and the diagnostic problems associated with some histological types of BCC (13, 15) formed the basis of the current investigations. Recently we reported on the reaction patterns of MoAbs 12 G7 and 253 B7 in normal and psoriatic skin (28). In the present study these two MoAbs were used for investigation into basal cell determinant(s) of BCC.

MATERIALS AND METHODS

Preparation of skin samples

Thirty-four specimens of basal cell carcinomas (BCC) and surrounding normal skin were obtained by surgical excision from 29 patients aged 35 to 88 years. The diagnosis of the tumours were confirmed by examination of haematoxylin and eosin (H & E)-stained frozen sections and stained paraffin embedded sections. The BCC were classified in the conventional manner as described previously (12, 15). For the histopathological examination of the skin adjacent to the tumour, the samples were cut in their length in two equal parts. One portion and the edges of the other portion were fixed in formalin and paraffin embedded for histological confirmation of diagnosis and for determination of tumour free margins. The remaining portion was frozen in liquid nitrogen-cooled isopentane and stored in liqued nitrogen. Cryostat sections (5 μm in thickness) were placed on alcohol-cleaned glass slides, air drie dand fixed in acetone for 10 min at room temperature. Sections were air-dried and stained immediately for the indirect immunoperoxydase (IIP) procedure or for the indirect immunofluorescence (IIF) procedure. Normal skin samples were obtained from 10 healthy volunteers and were processed in the same way.

Monoclonal antibodies

MoAb 12 G7 was generated after three intraperitoneal immunizations of BALB/C mice with $1x10^7$ cells from primary culture of human mesothelioma. Spleen cells from immunized mice were hybridized with SP2/0 cells using the somatic hybridization techniques (9). The hybridoma supernatants were prescreened using the standard ELISA technique and cultured mesothelioma cells. They were also prescreened on cryostat skin sections using IIF technique. MoAb 253 B7 was obtained after three intraperitoneal immunizations of BALB/C mice with an extract of total tumour material of human skin squamous cell carcinoma, prepared using an ultra-Turrax homogenizer. Spleen cells from immunized mice were hybridized with P_3 myeloma cells (9). The hybridoma supernatants were prescreened on cryostat sections of normal skin and squamous cell carcinoma using IIP technique. MoAb 253 B7 was subcloned

twice and 10^7 cells were injected intraperitoneally in BALB/C mice. After 10 days the ascites fluid was collected. A titer of 1:1200 was used in the IIP and IIF techniques.The results of prescreening showed that the 2 MoAbs reacted with the basal cell layer of normal epidermis. The 2 MoAbs were produced at the Dept. of Cell Biology and Genetics.

Indirect immunofluorescence test (IIF)

The staining pattern of the epidermis adjacent to the tumour and the epidermis at a distance (>150 μm) was studied in 14 BCC. Dry fixed cryostat sections were preincubated for 30 min with a 1:20 dilution of bovine serum albumin (BSA) to reduce aspecific staining. The sections were then incubated for 60 min at room temperature with one of the MoAbs, rinsed with phosphate-buffered saline (PBS, pH 7.4) and incubated for 30 min with Fluorescein isothiocyanate (FITC) labelled rabbit-antimouse IgG (Dakopatt, Copenhagen, Denmark) at a dilution of 1:50.

The sections were then rinsed with PBS and mounted in glycerol-PBS (9:1) solution. A Leitz Ortholux fluorescence microscope equipped with filters for epi-illumination and narrow band excitation was used for examining the sections.

Indirect immunoperoxidase test (IIP)

The cryostat sections were preincubated with BSA at a dilution of 1:20 for 30 min. The sections were then incubated with one of the MoAbs for 60 min, rinsed in PBS and incubated with rabbit peroxidase-conjugated antimouse IgG at a dilution of 1:50. The peroxidase reaction was developed by incubating the sections with 3,3'-diaminobenzidine (DAB) at a concentration of 0.5 mg/ml and hydrogen peroxide (0.01%) for several minutes at room temperature. Sections were then rinsed in tap water, counterstained with haematoxylin for 1-2 min and rinsed again in tap water. The sections were mounted in Malinol (Chroma-Gesellschaft, Stuttgart). All sera were diluted in PBS, pH 7.4.

In both procedures the controls comprised: antikeratin MoAbs K80 (Sanbio b.v., Uden, The Netherlands) and K92 (Dakopatts, Holland) were used as positive controls (21, 24) and negative controls were performed by omitting either the primary antibody or the rabbit-antimouse immunoglobulin.

RESULTS

Normal skin

In the 10 normal skin samples MoAb 12 G7 reacted consistently with

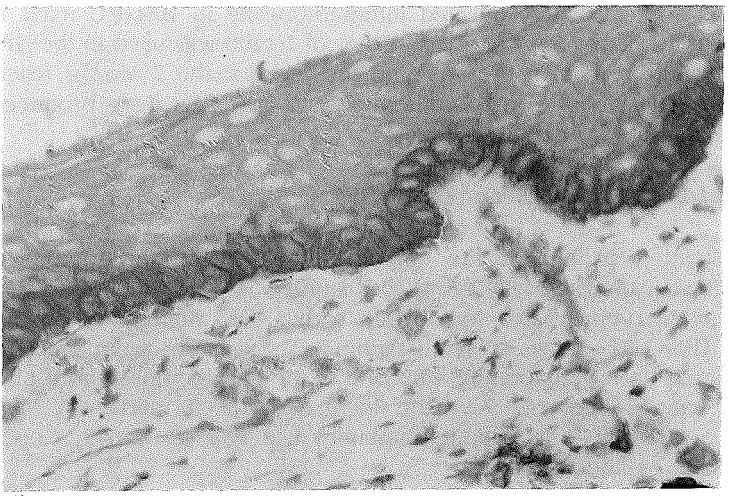

Fig.1.Normal skin cryostat section (5 μm) MoAb 12 G7, IIP technique. ×340. The staining is confined to the membrane region of the epidermal basal cells.

Fig.2. Normal skin cryostat section (5 μm) MoAb 253 B7, IIP technique. ×340. The staining is restricted to the cytoplasm of the epidermal basal cell layer.

membrane related determinant(s) of epidermal basal cells as observed in the IIF and IIP (Fig. 1). MoAb 12 G7 also showed a positive reaction with the outer root sheath of the hair follicles, sebaceous glands and the ducts and the secretory portions of the sweat glands. MoAb 253 B7 showed consistently a diffuse staining of the cytoplasm of the epidermal basal cells (Fig. 2) and also stained the outer root sheath of the hair follicles, sebaceous glands, sweat glands, especially the ducts and the myoepithelial cells of the secretory ducts, smooth muscle fibers of blood vessels and musculi arrector pili.

Basal cell carcinomas (BCC)

Thirty-four BCC from 29 patients were evaluated. Twenty-eight were located in head and neck region, four were in the trunk region and two were located on one of the extremities. The histological subclassification of BCC is summarized in Table I. Table II shows a summary of the reactivity pattern of MoAbs 12 G7 and 253 B7.

Table I Histological subclassification of 34 basal cell carcinomas from 29 patients.

Histological type	Number of tumours
Solid	16
Keratotic	2
Cystic	2
Adenoid	2
Infiltrating	5
Morphoeic	3
Superficial	1
Pigmented	1
Basosquamous	2

Table II. Reactivity patterns of monoclonal antibodies (MoAbs) 12 G7 and 253 B7 in normal skin and basal cell carcinomas

MoAb	NE	Tumour	EPA	EPD	Staining technique
12G7	10/10	32/34	34/34	34/34	IIP
	10/10	14/14	14/14	14/14	IIF
253 B7	10/10	5/34	34/34	34/34	IIP
	10/10	2/14	13/14	14/14	IIF

NE = no. of normal skin biopsies which showed positively staining basal cell layer, EPA = no. of tumours in which the epidermis adjacent to the tumour showed positive staining, EPD = no. of tumours in which the epidermis distant from the tumour showed positive stanining.

MoAb 12 G7: in the IIP, 32 out of 34 BCC (94%) stained positively. The peripheral cell layers of most tumour nests stained strongly, whereas the center of the tumour nest stained either very weakly or none at all (Figs. 3a and 3b). In the IIF all 14 BCC evaluated were positive.

MoAb 253 B7: only 5 out of 34 BCC (15%) stained positively in the IIP. All tumour cells in the tumour nests and strands of tumour cells were stained, except the tumour cells with squamous-like differentiation in the keratotic BCC and in the basosquamous BCC (Fig. 4). The other 29 BCC stained either very weakly or none at all. The 5 positively staining tumours comprised 2 morphoeic type, 1 keratotic type, 1 fibrosing infiltrated type and 1 basosquamous type. Both in the IIF and in the IIP no diminishing staining was observed adjacent to the tumour. The IIF staining pattern is shown in Fig. 5.

Fig. 3a and 3b. Cryostat sections (5 μm) of solid type BCC and infiltrating type BCC respectively, MoAb 12 G7, IIP technique. ×340 and ×135, respectively. Tumour cells at the periphery of the tumour nests are predominantly stained (arrows).

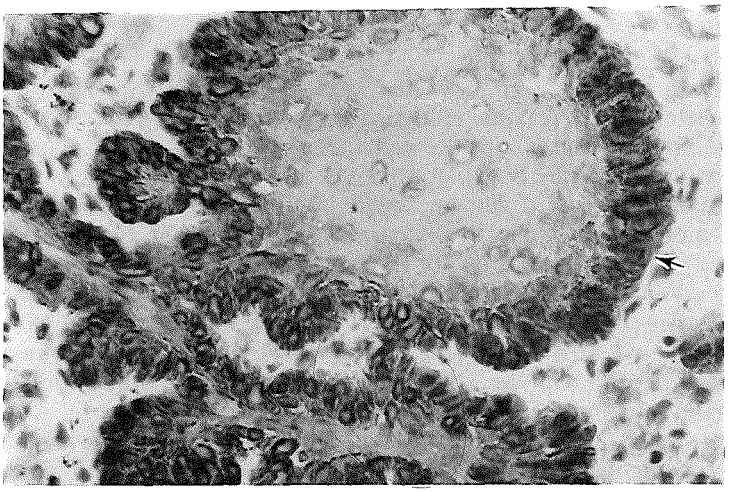

Fig.4. Cryostat section (5 μm) of basosquamous BCC, MoAb 253 B7, IIP technique. ×340. Tumour cells at the periphery stain strongly (arrow) whereas squamous-like cells in the center of the tumour nests remain unstained.

Fig. 5.
Cryostat section (5 μm) of solid type BCC, MoAb 253 B7, IIF technique. ×340. The epidermis nearby the tumour showed a strong staining with an abrupt change to no staining of the adjacent tumour cells (arrows).

DISCUSSION

The results of this report show that MoAb 12 G7 and 253 B7 reacted with different determinants of the epidermal basal cells. MoAb 12 G7 probably reacted with a membrane related determinant and MoAb 253 B7 reacted with a cytoplasm determinant. Both MoAbs did not react exclusively with skin epithelium; 12 G7 reacted with mesothelium and 253 B7 with smooth muscle cells and myoepithelial cells. In the current studies we observed that MoAb 12 G7 reacted positively in 32 out of 34 BCC (94%) and MoAb 253 B7 reacted positively only in 5 out of 34 BCC (15%). There are several reports in the recent literature concerning MoAbs VM-1 (22), VM-2 (19) and PKK-2 (8), which react with normal epidermal basal cells and BCC. Eto et al. (5) described MoAb EKH4 which reacted with the lower 2-3 cell layers of the normal epidermis and upon immunoblotting was found to react predominantly with a 50 KD keratin. MoAb EKH4 also reacted positively with BCC. Each of the above mentioned MoAbs reacted consistently with all the tumour nests of BCC. However, in the present study, using MoAb 12 G7 only the cells at the periphery of the tumour nests were strongly stained, whereas the cells at the center of the tumour nests were either stained very weakly or not at all. In most cases, the tumour cells in the center of the tumour nests showed on light microscopical level aspects of differentiation (elongated cells with an oval pale nucleus). The differentiated tumour cells in keratotic BCC and basosquamous BCC also did not react with MoAb 12 G7. A possible explanation for this observation might be that the tumour cells, which show some degree of differentiation, may have lost this determinant(s) as compared to that observed in suprabasal cell layer of normal epidermis. Another explanation may be that the tumour cells at the center of the tumour nests become less vital and lose some markers due to physiologic factors, such as diminished availability of nutrients. In this respect the findings recently reported by Grimwood et al. (5) are of particular interest. They observed that dividing cells in nodular BCC were mostly located at the periphery of tumour nests. The cells in the center of the tumour nests had lost their capacity to divide and had undergone some type of differentiation similar to normal epithelial cells.

MoAb 253 B7 reacted only with 5 out of 34 BCC (15%). Conspicuously 4 out of 5 positively staining tumours were of aggressive type. The other 29 out of 34 BCC stained either very weakly or not at all. Probably the cytoplasmic determinant is not lost but there is a variation in the quantity of the expression of this determinant in BCC. Whether the quantity of expression of this determinant is a marker for the aggressive behaviour is subject to speculation, especially, since 6 out of 10 tumours with aggressive histological features did not stain positively. The exact nature of the determinants detected by the two MoAbs 12 G7 and 253 B7 is currently under investigation. However, our present results lead to the conclusion that the MoAbs 12 G7 and 253 B7 detect different determinants in

basal epidermal cells and tumour cells of BCC. Therefore, these two MoAbs may be helpful in elucidating the pathogenesis of basal cell carcinomas.

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CHAPTER 3

Absence of cytokeratin 8 and inconsistent expression of cytokeratins 7 and 19 in human basal cell carcinoma

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ABSTRACT

The expression of the low molecular weight cytokeratins (K) 7,8,18, 19 and the high molecular weight cytokeratin 10 in 21 basal cell carcinoma (BCC) was studied using seven different monoclonal antibodies (MoAbs) with specific anticytokeratin activity. MoAbs RCK 105 (anti-K7), RPN 1164 (anti-low molecular weight cytokeratins of basic group), Ks 19.1 (anti-K19) and Cam 5.2 (anti-K8, K18, K19) reacted positively but inconsistently in the BCC that were examined. MoAbs 1166 (anti-K8) and RGE 53 (anti-K18) did not react at all. MoAb RKSE 60 (anti-K10) did not react with the tumor cells. From the results of this study, it can be concluded that cytokeratins 7 and 19 are expressed in BCC (43% and 71%, respectively), whereas cytokeratin 8 is not expressed.

INTRODUCTION

Basal cell carcinomas (BCC) show a wide spectrum of clinical and histological appearances (1-3). As far as the origin of BCC is concerned, it is still not clear whether they are derived from skin adnexal organs or from transformed basal cells of the interfollicular epidermis. The first hypothesis is supported by the observation that histologically BCC can show patterns of differentiation analogous to different structures of skin adnexal organs (2, 4). Moreover, upon biochemical analysis of cytokeratins, Moll et al (5) observed the expression of cytokeratin 17 in BCC and pilosebaceous tract but not in the normal interfollicular epidermis. This would imply that BCC may be derived from pilosebaceous cells. However, the expression of cytokeratin 17 in cultured normal skin and in hyperproliferative diseases such as psoriasis has been reported (6, 8). Therefore, this cytokeratin is not cell-type specific and its presence in BCC cannot be regarded as evidence for a pilosebaceous origin of the tumor. In addition to cytokeratin 17, cytokeratins 5 and 14 are the main constituents of BCC. These two cytokeratins are also expressed in normal interfollicular epidermis and adnexal organs (5, 7). The second hypothesis is supported by the observation that many basal cell carcinomas show no clear contact with skin adnexal organs, e.g. in the superficial type of BCC, tumor buds are only observed in close contact with the basal layer of the epidermis. It is of particular interest that, using two - dimensional gel electrophoresis, cytokeratin 8 was detected in small amounts in some BCC but not in normal epidermis (7). This cytokeratin is normally expressed in simple epithelia and is also observed in tumors derived from nonkeratinized tissues and early fetal epidermis (9, 10).

Recently, since a number of specific monoclonal antibodies against individual cytokeratins have become commercially available, it has become possible to classify and catalogue the distribution of cytokeratins in various dermatological diseases including the disorders of keratinization and epithelial skin tumors.

In this communication, we report on the distribution of the low molecular weight cytokeratins 7, 8, 18, 19 and the high molecular weight cytokeratin 10 in BCC using an indirect immunoperoxidase technique and monoclonal antibodies of specific anti-cytokeratin activity.

MATERIALS AND METHODS

Twenty-one specimens of basal cell carcinoma (BCC) were surgically excised from 20 patients aged 33 to 81 years. The diagnoses of BCC were confirmed by examination of haematoxylin and eosin (H&E) - stained frozen sections and stained paraffin embedded sections. The BCC were classified in the conventional manner as described previously (3, 4). For the histopathological examination of the tumor and the skin adjacent to it, the samples were cut lengthwise in two equal parts. One portion and edges of the other portion were fixed in formalin and paraffin embedded for histological confirmation of diagnosis and for determination of tumor free margins. The remaining portion was frozen in liquid nitrogen-cooled isopentane and stored in liquid nitrogen. Serial cryostat sections, 5 μm thick, were placed on alcohol- cleaned glass slides, air dried and fixed in acetone for 10 min at room temperature and stained using the procedure described in our previous study (11). Briefly, the cryostat sections were preincubated with 5% (W/V) bovine serum albumin in phosphate-buffered saline (PBS, pH 7.4) for 30 min. The sections were then incubated with an optimal dilution of one of the monoclonal antibodies (MoAbs) with anti- cytokeratin reactivity for 60 min. The specificity of the MoAbs is shown in Table I. Sections were then rinsed in PBS and incubated with rabbit peroxidase - conjugated anti - mouse IgG (Dakopatts, Amsterdam, The Netherlands) at a dilution of 1:50. The peroxidase reaction was developed by incubating the sections with 3,3' - diaminobenzidine (Sigma Chemical Co., Amsterdam, The Netherlands) at a concentration of 0.5 mg/ml and 0.01% hydrogen peroxide for 10 min at room temperature. Sections were then rinsed in tap water, counter - stained with haematoxylin for 1-2 min and rinsed again in tap water. The sections were mounted in Malinol (Chroma - Gesellschaft, Stuttgart). The negative controls comprised either the omission of primary antibody or rabbit anti- mouse immunoglobulin.

RESULTS

Twenty-one basal cell carcinomas (BCC) from 20 patients were evaluated. Sixteen were located in the head and neck region, 4 were in the trunk region and 1 was located on one of the upper arms. From the 16 BCC in the head and neck, 4 were located in the temporal region, 3 were located on one of the cheeks, 1 was retroauricular, 2 were paranasal, 2 were on the forehead, 2 were on the nose tip, 1 was on the upper lip and 1 was in the neck. The histological classification of the 21 BCC showed that 8 were solid type, 7 were infiltrating

Table I. Specificity and source of the seven monoclonal antibodies.

Monoclonal antibody	Specificity	Manufacturer
RCK 105**	Anti-cytokeratin 7	Gift, Dr. F.C.S. Ramaekers.
RPN 1166*	Anti-cytokeratin 8	Amersham International PLC, Bucks, England, U.K.
RPN 1164 ***	Anti-low molecular weight cytokeratin of the basic group	Amersham International PLC, Bucks, England, U.K.
Cam 5.2 **	Anti-cytokeratins 8, 18 and 19	Becton-Dickinson, Amersfoort, The Netherlands.
RKSE 60 **	Anti-cytokeratin 10	Euro-Diagnostics, Apeldoorn, The Netherlands.
RGE 53 **	Anti-cytokeratin 18	Euro-Diagnostics, Apeldoorn, The Netherlands.
Ks 19.1**	Anti-cytokeratin 19	ICN-Biomedicals Ltd, Bucks, U.K.

Dilution * = 1:5
** = 1:10
*** = 1:20

Table III. A summary of the reactivity of the seven anti-cytokeratin monoclonal antibodies in 21 basal cell carcinoma.

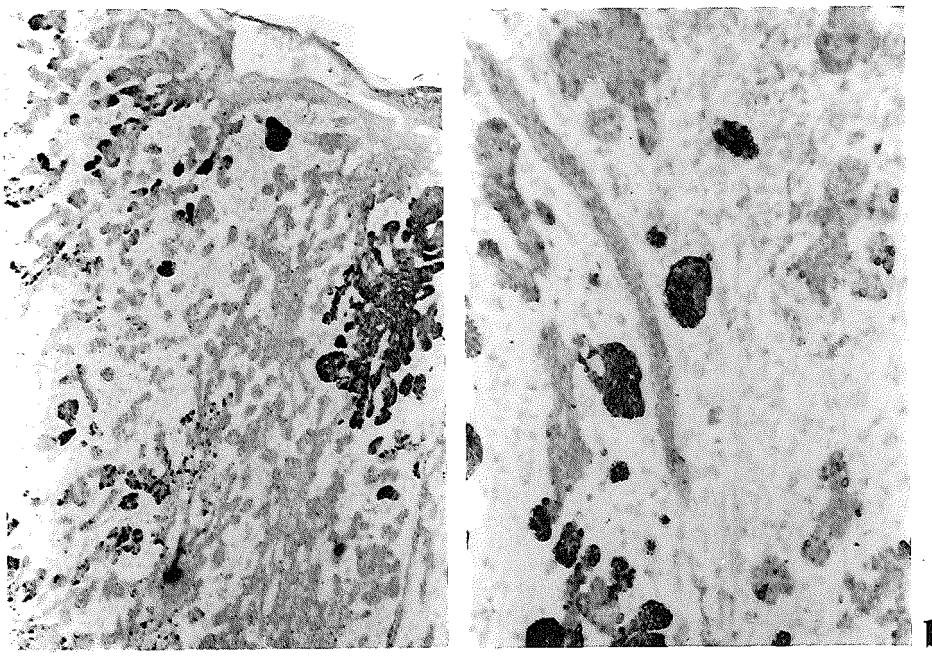
Catagory	RCK 105	RPN 1166	RPN 1164	Cam 5.2	RKSE 60	RGE 53	Ks 19.1
I	12/21(57%)	21/21(100%)	10/21(48%)	5/21(24%)	21/21(100%)	21/21(100%)	6/21(29%)
II	2/21(10%)	0/21(0%)	2/21(10%)	1/21(5%)	0/21(0%)	0/21(0%)	0/21(0%)
III	4/21(19%)	0/21(0%)	3/21(14%)	2/21(10%)	0/21(0%)	0/21(0%)	6/21(29%)
IV	1/21(5%)	0/21(0%)	1/21(5%)	1/21(5%)	0/21(0%)	0/21(0%)	1/21(5%)
V	2/21(10%)	0/21(0%)	5/21(24%)	12/21(57%)	0/21(0%)	0/21(0%)	8/21(38%)

I = None or less than 5% positively staining tumor cells
II = 5-25% positively staining tumor cells
III = 25-50% positively staining tumor cells
IV = 50-75% positively staining tumor cells
V = More than 75% positively staining tumor cells

Table II. Reactivity patterns of monoclonal antibodies against cytokeratins 7, 8, 10, 18 and 19 in 21 basal cell carcinomas.

Case no.	Age (years)	Sex	Location	Histological type	RCK 105	RPN 1166	RPN 1164	Cam 5.2	RKSE 60	RGE53	Ks 19.1
1.	76	M	temporal	solid	0%	0%	0%	0%	0%	0%	25-50%
2.	52	M	pre-sternal	solid	0%	0%	0%	50-75	0%	0%	0%
3.	57	M	temporal	solid	0%	0%	0%	<5%	0%	0%	>75%
4.	52	M	back	solid	25-50%	0%	>75%	>75%	0%	0%	25-50%
5.	63	F	paranasal	solid	0%	0%	0%	0%	0%	0%	25-50%
6.	49	F	upperarm	solid	25-50%	0%	25-50%	>75%	0%	0%	>75%
7.	39	M	temporal	solid	<5%	0%	5-25%	>75%	0%	0%	>75%
8.	33	F	neck	solid	0%	0%	0%	>75%	0%	0%	25-50%
9.	51	M	forehead	infiltrating	25-50%	0%	>75%	>75%	0%	0%	>75%
10.	69	M	retroau-ricular	infiltrating	25-50%	0%	>75%	>75%	0%	0%	>75%
11.	64	M	cheek	infiltrating	>75%	0%	>75%	>75%	0%	0%	>75%
12.	70	F	nose tip	infiltrating	0%	0%	0%	0%	0%	0%	0%
13.	47	F	back	infiltrating	5-25%	0%	5-25%	>75%	0%	0%	>75%
14.	51	M	nose tip	infiltrating	<5%	0%	25-50%	25-50%	0%	0%	25-50%
15.	68	F	temporal	infiltrating	25-50%	0%	50-75%	>75%	0%	0%	>75%
16.	69	F	back	superficial	0%	0%	0%	5-25%	0%	0%	>75%
17.	74	M	cheek	adenoid	0%	0%	<5%	0%	0%	0%	0%
18.	81	F	upper lip	adenoid	0%	0%	0%	>75%	0%	0%	0%
19.	49	F	cheek	morphoeic	0%	0%	0%	25-50%	0%	0%	25-50%
20.	52	M	forehead	morphoeic	>75%	0%	>75%	>75%	0%	0%	50-75%
21.	81	F	paranasal	cystic	5-25%	0%	5-25%	>75%	0%	0%	>75%

The results represent the estimated percentage of positively staining tumor cells in each basal cell carcinoma.

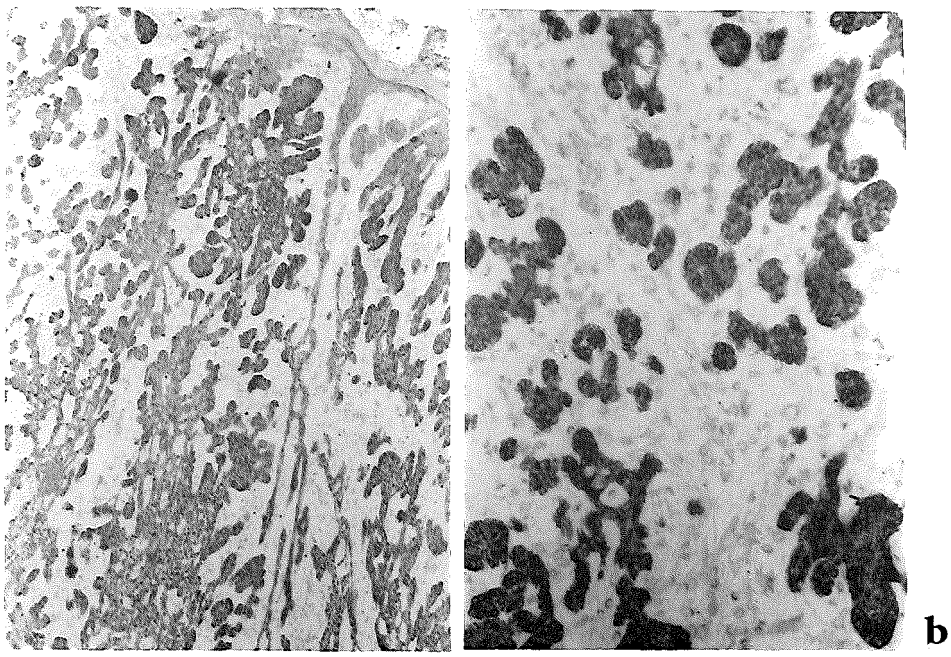


a b

Figure 1a + 1b. Cryostat sections (5µm) of infiltrating type basal cell carcinoma, MoAb RCK 105 (anti-K7), IIP technique, × 135 and ×340 respectively. An inconsistent expression of cytokeratin 7 is seen between and within various tumor nests. The staining pattern of MoAb RPN 1164 (anti-low molecular weight cytokeratins of basic group) was identical.

type, 2 were adenoid type, 1 was superficial type, 1 was cystic type and 2 were morphoeic type.

Table II shows the individual reactivity pattern of the MoAbs RCK 105, RPN 1166, RPN 1164, Cam 5.2, RKSE 60, RGE 53 and Ks 19.1 in each of the 21 BCC. It can be seen that MoAb RCK 105 stained more than 5% of the tumor cells in 9 out of 21 (43%) BCC. An example of the staining pattern is shown in Figure 1a and 1b. In the remaining 12 BCC incidental or no staining of the tumor cells was observed. MoAb RPN 1166 did not stain the tumor cells in any of the 21 BCC that were examined, but sweat glands were stained in all cases. MoAb RPN 1164 stained more than 5% of the tumor cells in 11 out of 21 (52%) BCC. The staining pattern was identical to that observed using MoAb RCK 105. MoAb Cam 5.2 stained more than 5% tumor cells in 16 out of 21 (76%) BCC. It also reacted with the upper cell layer of *stratum spinosum* and *stratum granulosum.* MoAbs RKSE 60 and RGE 53 did not react at all with the tumor cells in any of



a b

Figure 2a + 2b. Cryostat sections (5 μm) of infiltrating type basal cell carcinoma, MoAb Ks 19.1 (anti-K19), IIP technique, ×135 and ×340 respectively. More than 75% of the tumor cells were stained. The reactivity pattern of MoAb Cam 5.2 (anti-K8, K18 and K19) was identical.

the 21 BCC that were examined, but reacted in all cases with the suprabasal cell layer of the epidermis and the sweat glands respectively. MoAb Ks 19.1 stained more than 5% of the tumor cells in 15 out of 21 (71%) BCC. An example of the staining pattern is shown in Figure 2a and 2b.

In Table III, the reactivity patterns of the seven MoAbs are summarized. When the percentages of the positively stained tumor cells are divided into five categories, it can be seen that the reactivity pattern of MoAbs RPN 1164, RCK 105, Cam 5.2 and Ks 19.1 are inconsistent. There was no correlation between the expression of cytokeratins 7 and 19 and the sex or the age of the patients, or the location or the histological type of BCC.

DISCUSSION

Extensive investigations have demonstrated that low molecular weight cytokeratins 7, 8, 18 and 19 which are characteristic for simple epithelia are not expressed in stratified epithelia (7). However, using biochemical techniques, cytokeratins 8 and 19 were demonstrated in esophagus carcinomas and squamous cell carcinoma of the rectal - anal region and cytokeratins 7, 8 and 19 in cloacogenic carcinoma. In addition, a limited amount of cytokeratin 8 was detected in some basal cell carcinomas (BCC) but not in normal human epidermis (7, 9).

The main objective of the investigations presented here was to determine whether cytokeratin 8 could be detected in BCC using an indirect immunoperoxidase technique. The results of this study showed that using MoAb RPN 1166 the expression of cytokeratin 8 was not observed in any of the BCC that were examined. This discrepancy may be due to the differences in the techniques. Alternatively, the epitope that the MoAb RPN 1166 detects in immunoblotting studies is not detected in cryostat sections using immunoperoxidase technique.

Contrary to the results of biochemical studies, in this study cytokeratins 7 and 19 were expressed in BCC. However, their expression was inconsistent between and within various tumor nests of any particular BCC. A correlation between the particular histological type of BCC and the expression of the cytokeratins was not observed. There was no correlation between the expression of cytokeratins 7 and 19 and the location of the tumor, or with the sex and the age of the patients. Recently, it has been reported that cytokeratin 19 was expressed in a BCC showing an eccrine differentiation (12). Bartek et al (13) recently reported on the reactivity of two MoAbs against cytokeratin 19 in human tumors. However, these two MoAbs did not detect cytokeratin 19 in the two BCC that were examined. Their results therefore must be considered as preliminary, since only two BCC were examined.

The expression of cytokeratin 18 was not observed in any of the BCC using MoAb RGE 53, in the present study. This confirms the previous observations by Debus et al (14). Two reports (14, 15) have been published on the presence of low molecular weight cytokeratins in BCC. However, the MoAb 16a used by Knight et al (15) did not react exclusively with cytokeratin 18, but also reacted with cytokeratin 17. The latter cytokeratin is present in a considerable amount in BCC (5). Thomas et al (16) reported on the presence of a 45 Kd cytokeratin in BCC and in the basal cell layer of normal human epidermis. However, their results were based on studies with formalin - fixed tissues. In addition, for preparing antibodies to individual cytokeratins, they used proteins which were extracted from human *stratum corneum*. Since a 45 Kd cytokeratin has not been detected in *stratum corneum* or even in normal epidermis, the specificity of the antiserum for the 45 Kd cytokeratin is uncertain.

The slight differences between the reaction patterns of MoAbs Cam 5.2 and Ks 19.1 in the present study can be explained on the basis of the detection of different epitopes of cytokeratin 19. Since MoAb Cam 5.2 was observed also to react with the upper cell-layers of the *stratum spinosum* and the *stratum granulosum*, it seems likely that this MoAb has additional activity. Similar slight differences between the reaction patterns of MoAbs RPN 1164 and RCK 105 were also observed, which could be explained on the basis of the detection of different epitopes of cytokeratin 7. Alternatively, MoAb RPN 1164 could detect an epitope of cytokeratin 8, which cannot be detected by MoAb RPN 1166 (anti- cytokeratin 8).

The inconsistent expression of cytokeratins 7 and 19 in the present study may be due to the variability in the expression of cytokeratins as a result of the heterogeneity of the tumor cells of BCC. There is evidence that tumors are heterogeneous and that different sub-populations may express different antigens (17). The results of the present study also support the concept that during cellular transformation and tumor development, a switch occurs in favour of the expression of low molecular weight cytokeratins 7 and 19 in BCC. Since low molecular weight cytokeratins are expressed in early fetal skin (10), it is conceivable that only that sub-population of tumor cells that has reverted back to its fetal form expresses these cytokeratins.

At the present time, moreover, the possibility cannot be totally excluded that the inconsistent expression of cytokeratins 7 and 19 observed in the current studies is due to the limited sensitivity of the immunoperoxidase technique. In our previous studies (11, 18), we used the indirect immunofluorescence (IIF) technique and the indirect immunoperoxidase (IIP) technique simultaneously. In those studies the IIP-technique was observed to be more sensitive and reproducible in our hands than the IIF- technique. Indeed, further investigations using a more sensitive technique such as immuno-electron microscopy should elucidate whether the expression of cytokeratins 7 and 19 in BCC can be regarded as suitable markers.

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CHAPTER 4

Immunoelectron microscopic studies on cytokeratins in human basal cell carcinoma

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ABSTRACT

Ultrastructural investigations into the location and the expression of the cytokeratins 7, 8, 10 and 19 were undertaken on ultrathin cryosections of 8 basal cell carcinomas (BCC) using a high resolution immunogold labeling method and five different monoclonal antibodies against specific cytokeratins. The results showed that cytokeratin 10 was expressed only in the differentiated keratinocytes. In contrast to the previously reported biochemical and immunohistochemical studies, in this study cytokeratin 19 was expressed not only in the tumor cells of BCC but also in the normal epidermal keratinocytes. The expression of cytokeratin 7 in BCC could not be confirmed but the lack of expression of cytokeratin 8 was confirmed, excluding its potential role as a specific histopathological marker for BCC.

INTRODUCTION

The intermediate-sized filaments of human epithelial cells are composed of various keratin-like proteins called cytokeratins. They constitute a family of 19 biochemically and immunologically related polypeptides. These polypeptides can be divided into two groups; type-I cytokeratins which have an acidic isoelectric point and type-II cytokeratins which have a basic isoelectric point (1, 2). These cytokeratins can only form heteropolymers, i.e. a type-I cytokeratin must combine with a type-II cytokeratin to form a filament. In each cytokeratin-pair the basic member is always about 8 kilo dalton heavier than the acidic member (2, 3). Specific cytokeratin patterns are characteristic of certain epithelial cell types (1). Moreover, different cytokeratin patterns have also been observed in different cell layers of the epidermis and in different skin adnexal organs (1, 2, 4). Various amounts of cytokeratins 5, 6, 8, 14, 15 and 17 have been isolated from basal cell carcinoma (BCC) using two-dimensional gel electrophoresis (1, 5). The expression of cytokeratin 8 in some BCC which was demonstrated using biochemical techniques is of particular interest, since this cytokeratin is not expressed in normal human epidermis and in skin adnexal organs, but it is expressed in simple epithelia and early fetal skin (1, 6, 7).

Recently, we reported on the distribution of low molecular weight cytokeratins 7, 8, 18, 19 and the high molecular weight cytokeratin 10 in 21 BCC using an indirect immunoperoxidase technique and monoclonal antibodies of specific anti-cytokeratin activity (8). In that study it was observed that cytokeratin 8 was not expressed in any of the 21 BCC that were examined. However, cytokeratins 7 and 19 were expressed inconsistently in 10 of the 21 (48%) BCC.

The aim of the present study was to investigate whether the results of our recent study (8) could be confirmed using immunoelectron microscopy, and to try to resolve the discrepancy concerning the expression of cytokeratin 8 in BCC observed in the previously reported biochemical studies (1, 5) and in our recently reported light microscopy study (8).

MATERIALS AND METHODS

Tissue preparation. Eight 4 mm punch biopsies of basal cell carcinoma (BCC) were obtained from 2 females and 5 males aged 37 to 82 years. The biopsies were cut lengthwise into two equal parts. One part was fixed in formalin and paraffin embedded. Histological confirmation of the diagnosis was obtained by examination of haematoxylin and eosin (H&E) - stained sections. The BCC were classified in the conventional manner as described previously (9, 10). The remaining part was processed using the method described previously (11) with some modifications. Briefly, it was fixed by immersion in 2% paraformaldehyde and kept for 7 days at 4° C. Tissue blocks (< 1 mm^3) were transferred to a conservation fixative solution containing 33.9% (W/V) sucrose and 1% paraformaldehyde. They remained in this solution until ultrathin sections were cut. Two hours prior to sectioning, the tissue blocks were transferred into a 25% (W/V) polyvinyl pyrolidone solution (PVP) containing 0.04 M sodium carbonate and 2.3 M sucrose. All solutions were prepared in 0.1 M phosphate buffer, pH 7.2.

Ultracryotomy. Ultrathin cryosections (gold to blue interference colours) were cut with glass knives using a LKB Cryo-nova ultramicrotome (LKB, The Netherlands) at -90°C to -110°C and transferred to carbon coated formvar filmed 200 mesh copper grids in 2 mm wire loop with a droplet of 2.3 M sucrose. The grids were then transferred to a droplet of 2% (W/V) gelatine solution for a minimum of 5 minutes to remove the sucrose.

Immunogold labeling. All incubations were performed at room temperature and all sera were diluted in phosphate-buffered saline (PBS, pH 7.2). Initially, all grids were rinsed 3 times with a droplet of PBS containing 0.2% (W/V) glycine. The sections were then incubated for 1 hour with undiluted primary monoclonal antibodies (MoAbs) of known specificity as shown in Table I. The sections were then rinsed 3 times for 3 min each in PBS containing 0.2% (W/V) glycine in order to remove the unreacted primary MoAb. Subsequently, the sections were incubated for 20 min with PBS containing normal goat serum followed by a further 1 hour incubation with 10 nm collodial gold-labeled goat-anti mouse antiserum (GAM 10, Auroprobe EM, Janssen life science products, Tilburg, The Netherlands). Excess GAM 10 was removed by 3 rinses of 3 min each with PBS containing 0.2% (W/V) glycine, followed by 3 rinses of 1 min each with distilled water. Sections were positively stained by a 10 minute incubation with uranyl acetate (pH 4.0). Grids were subsequently floated on a 1.5% methyl cellulose solution for a few seconds and scooped up on wire loops of diameter 3-3.5 mm. Access fluid was then removed using a filter paper until gold to blue interference colours developed on the grids. The sections were air-dried and examined in a Zeiss 901 electron microscope operated at 80 KV.

Control sections were treated with rabbit anti-human albumin followed by the 10 nm GAM-gold probe or by the GAM-gold probe only. Background labeling was negligible. Areas of sections showing dark gold grain deposits were regarded as positive for cytokeratins.

The background labeling was very low and was similar to that in the controls in which the omission of the primary antibody resulted in the presence of sporadic or no gold grains.

Table I. Source and specificity of the five monoclonal antibodies.

Monoclonal antibody	Specificity	Manufacturer
RCK 105	Anti-cytokeratin 7	Gift, Dr. F.C.S. Ramaekers.
RPN 1166	Anti-cytokeratin 8	Amersham International PLC, Bucks, England, U.K.
Cam 5.2	Anti-cytokeratins 8, 18 and 19	Becton-Dickinson, Amersfoort, The Netherlands.
RKSE 60	Anti-cytokeratin 10	Euro-Diagnostics, Apeldoorn, The Netherlands.
Ks 19.1	Anti-cytokeratin 19	ICN-Biomedicals Ltd., Bucks, England, U.K.

RESULTS

The 8 basal cell carcinomas (BCC) that were evaluated comprised 5 solid, 1 superficial, 1 adenoid and 1 morphea type. The results of the immunoelectron microscopy are summarized in Table II. It can be seen that MoAb Ks 19.1 reacted with the intermediate filaments in the tumor cells and in the normal epidermal keratinocytes. An identical number of immunogold grains were observed in both cases, indicating the expression of cytokeratin 19 (Figures 1 and 2).

A weak reaction was observed with the intermediate filaments in the tumor cells and in the normal epidermal keratinocytes using MoAb Cam 5.2 (Figure 3). A considerable number of immunogold grains were observed only on the intermediate filaments in the tumor cells of the superficial type BCC.

No reaction at all was observed either in the tumor cells or in the normal epidermal keratinocytes in any of the 8 BCC using MoAb RCK 105 and MoAb RPN 1166, indicating the lack of expression of cytokeratins 7 and 8 respectively.

A strong reaction was observed with the intermediate filaments in the normal epidermal keratinocytes, and especially with those in the upper cell layer using MoAb RKSE 60, indicating the expression of cytokeratin 10 (Figure 4). No reaction was observed with this MoAb either in the tumor cells or the basal cells of the epidermis in any of the 8 BCC that were examined (Figure 5).

Table II. Reactivity patterns of monoclonal antibodies against cytokeratins 7, 8, 10 and 19 in 8 basal cell carcinomas.

		Monoclonal antibodies									
		RCK 105 (a-K7)		RPN 1166 (a-K8)		Cam 5.2 (a-K8, 18, 19)		Ks 19.1 (a-K19)		RKSE 60 (a-K10)	
No.	Histological type	tumor	epid.	tumor	epid.	tumor	epid.	tumor	epid.	tumor	epid.
1.	solid	-	-	-	-	(+)	(+)	++	++	-	++
2.	solid	(+)	-	-	-	(+)	(+)	++	++	-	++
3.	solid	-	-	-	-	(+)	(+)	++	++	-	++
4.	solid	nd	nd	nd	nd	nd	nd	++	++	nd	nd
5.	solid	-	abs	(+)	abs	++	abs	++	abs	-	abs
6.	adenoid	-	-	-	-	(+)	(+)	++	++	-	++
7.	superficial	-	-	-	-	++	++	++	++	-	++
8.	morphea	-	-	-	-	(+)	abs	(+)	(+)	-	(+)

abs = absent
nd = not done
epid. = epidermis
- = no reactivity
(+) = dubious reactivity
++ = positive

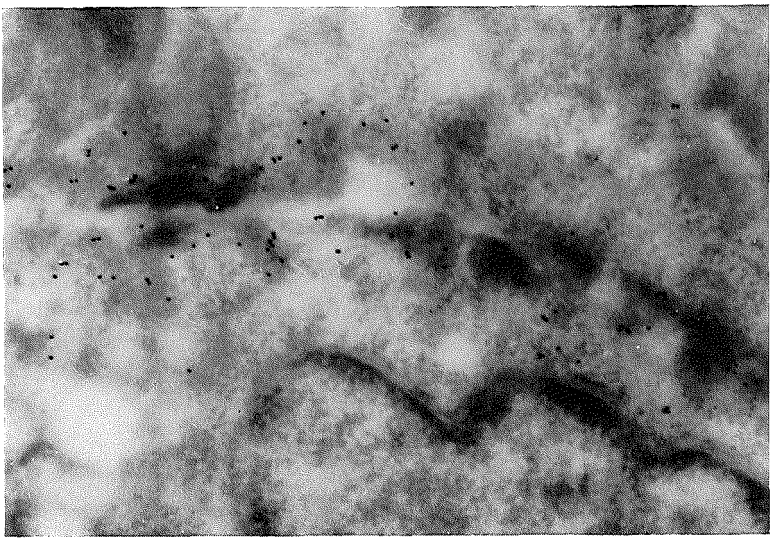

Figure 1. Ultracryotomy section of epidermal keratinocyte labeled for cytokeratin 19. Intense immunogold labeling near the cell membrane is observed (× 60,000).

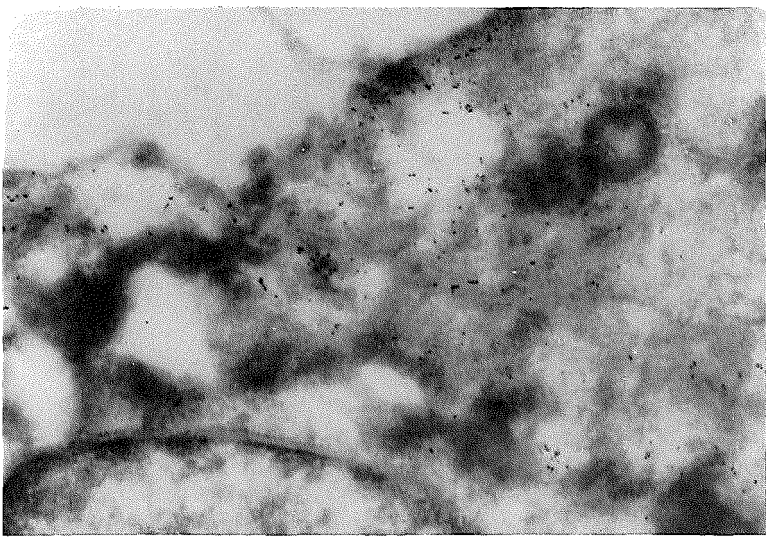

Figure 2. Ultracryotomy section of basal cell carcinoma labeled for cytokeratin 19. There is an intense immunogold labeling (× 60,000).

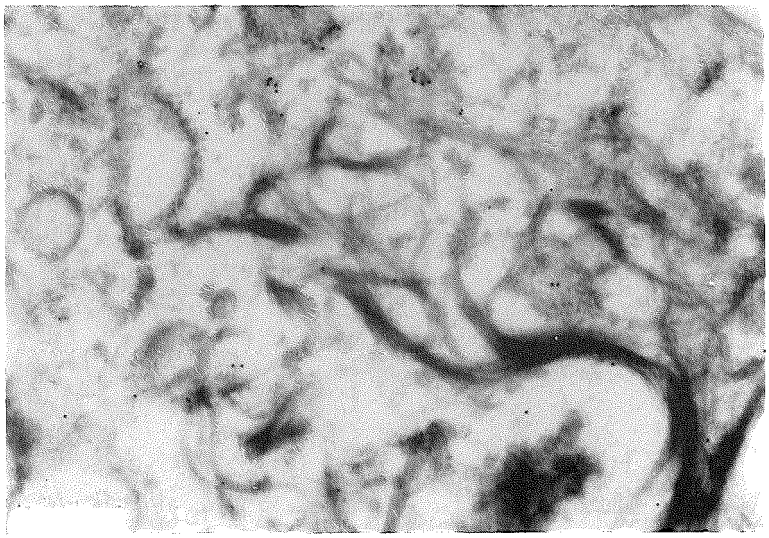

Figure 3. Ultracryotomy section of epidermal keratinocyte labeled for cytokeratins 8, 18 and 19. A weak immunogold labeling of coarse bundles of filaments is present (× 52,000).

Figure 4. Ultracryotomy section of epidermal keratinocyte and corneocyte labeled for cytokeratin 10. A selective intense immunogold labeling is observed in the coarse bundles of filaments and of the amorphous material of stratum corneum (× 60,000).

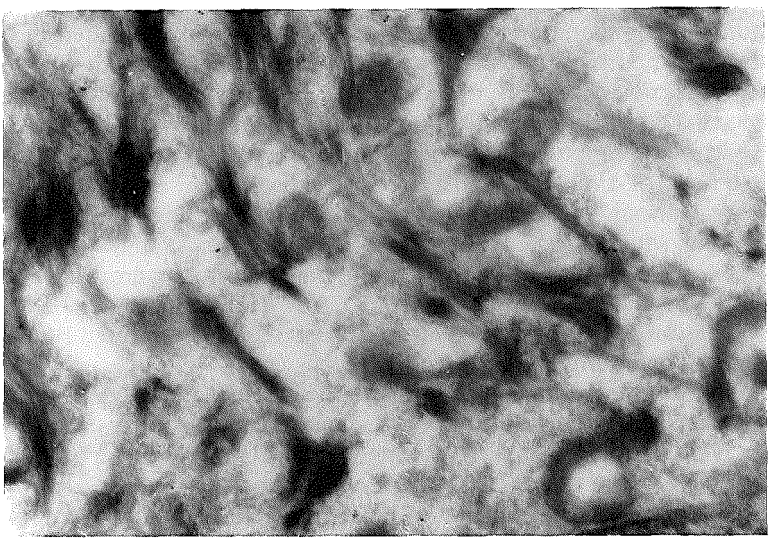

Figure 5. Ultracryotomy section of basal cell carcinoma labeled for cytokeratin 10. Sporadic gold grains are present in the tumor cell (× 60,000).

DISCUSSION

Recently, we reported on the expression of cytokeratins 7 and 19 in basal cell carcinoma (BCC) using an indirect immunoperoxidase technique (8). In that study the expression of these cytokeratins was inconsistent between and within various tumor nests of any particular BCC. Furthermore, cytokeratin 8 was not expressed in any of the 21 BCC that were examined. In that study, MoAb Ks 19.1 reacted with cytokeratin 19 in the tumor cells but did not react with cytokeratins in the epidermal keratinocytes. In the present study, gold grains were observed on the intermediate filaments in the tumor cells and those of the epidermal keratinocytes using MoAb Ks 19.1. In normal skin, used as control, cytokeratin 19 was also expressed. When the MoAb Ks 19.1 was diluted, a reduction in the reactivity with the intermediate filaments in both epidermal keratinocytes and in tumor cells was observed. Although a similar reactivity pattern was observed using MoAb Cam 5.2, the intensity of labeling was lower in most BCC.

A possible explanation for the discrepancy in the observations concerning the expression of cytokeratin 19 in our previous study (8) and this study may be that cytokeratin 19 which may be present in a low amount in the epidermal keratinocytes reacts weakly with MoAb Ks 19.1. This weak reactivity can only be observed after it has been amplified in immunoelectron microscopy. It is

also possible, but highly unlikely, that MoAb Ks 19.1 cross-reacted with structures other than cytokeratins in the epidermal keratinocytes, since this MoAb is claimed by the manufacturer to react exclusively with cytokeratin 19. In any case, our present observation need to be confirmed using other commercially available specific anti-cytokeratin 19 monoclonal antibodies.

It has been reported that low molecular weight cytokeratins which are characteristic of simple epithelia are not expressed in stratified epithelia (1). However, various low molecular weight cytokeratins have been demonstrated in different squamous cell carcinomas derived from stratified epithelia using biochemical techniques (12). Limited amounts of cytokeratin 8 have also been demonstrated biochemically in some BCC (5). However, in the present study, the expression of cytokeratin 8 was not observed in any of the 8 BCC that were examined, confirming our earlier observations (8).

The intermediate filaments in the suprabasal keratinocytes were labeled using MoAb RKSE 60, indicating the expression of cytokeratin 10. No gold grains were observed on the intermediate filaments either in the epidermal basal cells or in the tumor cells. These observations confirm the observations of previous biochemical and immunohistochemical studies (1, 8).

We did not observe gold grains on the intermediate filaments of the tumor cells or the keratinocytes in any of the 8 BCC using MoAb RCK 105. This demonstrated that cytokeratin 7 was not expressed and is in contrast to our previous observations of the light microscopy study in which cytokeratin 7 was expressed in 9 of the 21 (43%) BCC that were examined (8). At present we do not have a satisfactory explanation for this discrepancy. It has been previously reported that fixation procedures may lead to a loss of antigenicity (13). It is possible that the fixation procedure that was used in the present study may have decreased the antigenicity of the cytokeratin, thereby reducing its affinity for MoAb RCK 105.

It has been suggested that the use of other fixation methods, such as fixation in gluteraldehyde and embedding the tissue samples in epoxy resins, also leads to a loss of reactivity (14). The preservation of antigenicity in human epidermis has been demonstrated using the Lowicryl K4M protein A-gold method (15, 16). In our hands, this method was found to be unsuitable for investigating the expression of different cytokeratins in BCC (unpublished observations).

In summary, the results presented here show that cytokeratin 19 was expressed in the tumor cells and the epidermal keratinocytes. However, this observation must be confirmed before any firm conclusions concerning the expression of cytokeratin 19 in BCC can be drawn. Cytokeratin 10 was expressed only in the differentiated epidermal keratinocytes. The expression of cytokeratin 7 in BCC could not be confirmed in this immunoelectron microscopic study, but the lack of expression of cytokeratin 8 in BCC was confirmed, eliminating its possible role as a specific histopathological marker.

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CHAPTER 5

Characterization of the mononuclear infiltrate in basal cell carcinoma: A predominantly T cell-mediated immune response with minor participation of Leu-7$^+$ (natural killer) cells and Leu-14$^+$ (B) cells

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ABSTRACT

We investigated the peritumoral inflammatory infiltrate in 22 basal cell carcinoma (BCC) from 18 patients using a series of monoclonal antibodies. In all the 22 BCC the infiltrate consisted mainly of T cells (55±15%) and only in three cases an invasion of the tumor nests by these cells was observed. The T helper (T_H) subset predominated over the T suppressor/cytotoxic ($T_{S/C}$) subset ($T_H/T_{S/C}$ ratio of 1.9±0.8). In 8 of 22 BCC mild infiltrate was observed with 48±13% T cells and a $T_H/T_{S/C}$ ratio of 1.5±0.6.In 14 of 22 BCC moderate to heavy infiltrate with 59±15% T cells and a $T_H/T_{S/C}$ ratio of 2.0±1.0 was observed. There was a significant difference in the percentage of T cells in BCC with moderate to heavy infiltrate and that in BCC with mild infiltration. The mean percentage of HLA-DR$^+$ cells was 54±11%; Langerhans cells (LC) 4±5%; and Leu-5$^+$ (monocytes and macrophages) 16±11%. Less than 2% Leu-14$^+$ (B) cells were seen in the infiltrate. The mean percentage of Leu-7$^+$ (natural killer) cells was 4±4%, and only 1 of 22 BCC Leu-7$^+$ cells invaded tumor nests, contacting with tumor cells. From these results we concluded that T cells play a major role in the defence against BCC proliferation. The main role of Langerhans cells and Leu-M5$^+$ cells may be that of antigen presentation. B cells and NK cells probably play a minor role in the local defence against BCC proliferation.

Abbreviations:

APC: antigen-presenting cell
BCC: basal cell carcinoma
IL: interleukin
LC: Langerhans cells
MHC: major histocompatibility complex
MoAb: monoclonal antibody
NK cells: natural killer cells
PBS: phosphate-buffered saline
T_H cells: T helper cells
$T_{S/C}$ cells:T suppressor/cytotoxic cells
H&E: haemotoxylin and eosin

INTRODUCTION

Basal cell carcinoma (BCC) is the most common tumor of epithelial origin, although metastasis fortunately is rare [1,2]. In most cases BCC are slowly growing tumors with less tendency of local invasiveness. However, a small proportion of BCC showing the histological picture characteristic of aggressive growth pattern often leads to local destruction [2,3]. In the last decade several

studies have been reported in which attempts have been made to elucidate the function of the inflammatory cellular infiltrate surrounding BCC and their possible role in the control of tumor growth [4-9]. Dellon et al [4] observed a mild lymphocytic infiltrate in situ and a low T cell level in the peripheral blood in BCC of large size (>2cm) and an invasive growth pattern. Several authors observed that the cellular infiltrate in BCC consisted mainly of T cells [6-8]. Also a considerable number of immunoglobulin-bearing cells in the infiltrate surrounding BCC have been demonstrated [5,7]. Using the E-rosette test and the EAC-rosette test, Viac et al [6] observed a T/B ratio of 4.6 in the infiltrated cells eluted from BCC. Recently Synkowsky et al [9], using a panel of monoclonal antibodies (MoAbs), observed a T/B ratio of 1.0 in situ. These results may suggest a significant role of the humoral response in BCC. However, in an earlier study Eaglestein et al [8] reported that the infiltrate in BCC consisted of 90% T cells and less than 10% B cells, which suggested a predominantly T cell-mediated immune response.

It is well known that natural killer (NK) cells are important in the defence against tumors [10,11]. Little is known about the presence and the role of these cells in cutaneous tumors. Recently Kohchiyama et al [12] reported on NK cells in squamous cell carcinoma of the skin. They observed NK cells in contact with carcinoma cells. These authors suggested that NK cells played an important role in the defence against squamous cell carcinoma, whereas their role in BCC is not clear as yet. To obtain further insight into the composition of the immune infiltrate in BCC, and their role especially in tumor defence, we characterized the immunoinfiltrate in surgically excised BCC using a panel of MoAbs.

MATERIALS AND METHODS

Twenty-two specimens of BCC were obtained by surgical excision from eighteen patients aged 31 to 89 years. The diagnosis of BCC was confirmed by examining haematoxylin and eosin (H&E) - stained frozen sections and stained paraffin embedded sections. The BCC were classified in the conventional manner as described previously [2,13]. For histopathological examination of the tumor and the skin adjacent to the tumor, the samples were cut lengthwise into two equal parts. One portion and the edges of the other portion were fixed in formalin and embedded in paraffin for histological confirmation of diagnosis and for determination of tumor-free margins. The remaining portion was frozen in liquid nitrogen- cooled isopentane and stored in liquid nitrogen. Serial cryostat sections (5 µm in thickness) were placed on alcohol-cleaned glass slides, air-dried, and fixed in acetone for 10 min at room temperature and stained for the indirect immunoperoxidase (IIP) procedure as described elsewhere [14]. Briefly, the cryostat sections were preincubated with bovine serum albumin at a dilution of 1:20 in phosphate-buffered saline (PBS, pH 7.4),

for 30 min. The sections were then incubated with an optimal dilution of Leu-2a, Leu-3a, Leu-4, Leu-6, Leu-7 (HNK1), Leu-14, anti-HLA-DR, or Leu-M5 (Becton and Dickinson) for 60 min, rinsed in PBS, and incubated with rabbit peroxidase-conjugated anti-mouse IgG at an optimal dilution. The perixodase reaction was developed by incubating the sections in a mixture of 3,3'-diaminobenzidine (DAB) at a concentration of 0.5 mg/ml and hydrogen peroxide (0.01%) for 10 min at room temperature. Sections were then rinsed in PBS, counterstained with haematoxylin for 1 min, and rinsed again in tap water. The sections were mounted in Malinol (Chroma-Gesellschaft, Stuttgart, Federal Republic of Germany).

The specifity of MoAbs Leu-7 (NK cells) and Leu-14 (B cells) was verified using frozen tissue sections of human lymph nodes. The negative controls comprised the omission of primary antibody and the omission of rabbit anti-mouse immunoglobulin.

The amount of mononuclear infiltrate surrounding the tumor nests was classified into mild, moderate, and heavy. In most cases the infiltrate was examined at three sites. These were the superior (between tumor nests and epidermis), lateral, and inferior border of the tumor. For each antibody the percentage of positive staining cells in the infiltrate was estimated by counting 200 mononuclear cells at ×400 magnification. Only the cells showing membrane staining were counted. For each antibody identical locations in the serial sections were examined. For statistical analysis of the results the Wilcoxon rank sum test was used. A P value <0.05 was considered statistically significant.

RESULTS

The results are summarized in Table I. The 22 BCC we examined comprised 9 solid, 6 infiltrating, 3 superficial, 1 morphea, 1 basosquamous, 1 adenoid, and 1 keratotic type; 10 of the 22 (45%) were smaller than 1 cm in size, 8 (36%) between 1 and 2 cm, and 4 (18%) larger than 2 cm (3 of which belonged to the superficial type). In 8 of the 22 (36%) we observed a mild infiltrate, a moderate infiltrate in 11 (50%), and a heavy infiltrate in 3 (14%). Seven out of 22 BCC (32%) showed ulceration, of which 4 BCC had moderate infiltration, 2 mild, and 1 heavy infiltration. The infiltrate was predominantly situated at the tumor`s lateral and inferior border. In 17 of 22 (77%), either no or only a mild infiltrate was observed between the epidermis and the tumor, and only sporadic infiltration was observed between the different tumor nests. In cases in which either no or only a mild infiltrate was observed at the superior border, and in which a moderate or a heavy infiltrate was observed at the other borders, the results from the superior border were excluded from calculation of the mean infiltrate value. All 3 BCC showing a heavy infiltrate were smaller than 1 cm and demonstrated a histological picture characteristic of an aggressive growth pattern.

Table I: Summary of Phenotypes of Immunoinfiltration in 22 Basal Cell Carcinomas

Case	Histological Type	Degree of Infiltration	Leu-4	Leu-3a	Leu-2a	$T_H/T_{S/C}$	Leu-6	Leu-7	Leu-14	HLA-DR	Leu-M5
1	Solid	Moderate	33	42	13	3.2	2	1	1	62	10
2	Solid	Moderate	60	50	25	2.0	1	1	0	45	20
3a	Solid	Mild	53	30	41	0.7	1	2	0	38	11
4a	Solid	Moderate	70	47	42	1.1	13	9	1	41	9
5	Solid	Mild	64	60	28	2.1	6	3	0	61	31
6	Solid	Mild	34	32	30	1.1	6	4	0	46	10
7	Solid	Mild	43	38	33	1.2	6	9	0	48	11
8	Solid	Mild	25	30	25	1.2	0	0	0	60	22
9a	Solid	Moderate	50	50	15	3.3	1	0	0	75	15
10a	Infiltrative	Mild	50	45	25	1.8	1	0	5	55	15
11	Infiltrative	Heavy	57	48	30	1.6	3	5	1	63	22
12a	Infiltrative	Heavy	74	57	57	1.0	8	15	7	35	9
13	Infiltrative	Mild	59	51	40	1.3	2	3	0	50	13
14a	Infiltrative	Moderate	60	50	28	1.8	5	2	0	65	25
15a	Infiltrative	Moderate	25	22	17	1.3	1	1	1	63	12
16	Superficial	Moderate	60	50	30	1.7	5	1	1	60	1
17	Superficial	Moderate	70	70	20	3.5	2	1	3	50	30
18	Superficial	Moderate	65	60	20	3.0	2	1	0	70	10
19	Morphea	Mild	55	60	25	2.4	3	2	2	50	50
20	Adenoid	Moderate	68	69	28	2.5	4	2	3	60	20
21	Keratotic	Moderate	67	62	50	1.2	4	0	1	53	6
22	Basosquamous	Heavy	73	65	33	2.0	10	3	3	40	5
Mean ± S.D.			55±15	49±13	30±11	1.9±0.8	4±5	4±4	1±2	54±11	16±11

The results represent the mean estimated percentage of positively staining cells examined when possible at the superior, lateral, and inferior border of each tumor.
[a]Ulcerative basal cell carcinoma

Table II: A Comparison of Mean Percentages of Immunocompentent Cells in Basal Cell Carcinomas with Mild Cellular Infiltrate and Moderate-to-Heavy Cellular Infiltrate.

Infiltrate	Leu-4	Leu-3a	Leu-2a	$T_H/T_{S/C}$	Leu-6	Leu-7	Leu-14	HLA-DR	Leu-M5
Mild (no. 8)	48±13	43±13	31±7	1.5±0.6	3±3	3±3	1±2	51±8	20±14
Moderate and heavy (no.14)	59±15	53±13	30±13	2.0±1.0	4±4	3±4	2±2	56±12	14±9
P value	<0.05	NS	NS	NS	NS	NS	NS	NS	NS

The results show a significant increase of Leu-4$^+$ (Pan T) cells in the basal cell carcinoma with moderate-to-heavy infiltrate. NS=not significant.

The mean immunoinfiltration in the 22 BCC consisted of 55±15% Leu-4$^+$ (pan T) cells, 49±13% Leu-3a$^+$ (T_H) cells, 30±11% Leu-2a$^+$ ($T_{S/C}$) cells, 4±5% Leu-6$^+$ cells (LC), 4±4% Leu-7$^+$ (NK) cells, 1±2% Leu-14$^+$(B) Cells, 54±11% HLA-DR$^+$ cells, and 16±11% Leu-M5$^+$ (monocytes/macrophages) cells. In 3 BCC (cases 4, 11, and 22), some tumor nests had been invaded by T_H and $T_{S/C}$ cells. In only 1 of the BCC (case 12) had Leu-7$^+$ cells invaded tumor nests, moving into contact with tumor cells. Leu-6$^+$ and HLA-DR$^+$ cells with a dendritic appearance were sporadically observed in tumor nests. In none of the 22 BCC were HLA-DR$^+$ tumor cells or HLA-DR$^+$ epidermal keratinocytes seen. In the single basosquamous type BCC (case 22), many HLA-DR$^+$ and Leu-6$^+$ cells were observed at the periphery of the tumor nests close to T cells. In all 22 BCC few Leu-14$^+$ (B) cells were observed in the infiltrate. No B cells were observed in any tumor nest.

There was no statistically significant correlation between the different histological types of BCC and the degree or the composition of the cellular infiltrate. There was also no correlation between either the size of the tumors or the presence of ulceration and the degree and composition of the infiltrate. It can be seen in Table II that, as a group, the BCC with moderate-to-heavy infiltrate differed in the composition of their immunocompetent cells from the composition found in those BCC with mild infiltrate. The percentage of Leu-4$^+$ (PanT) cells was significantly higher in the first group (59%) than in the latter (48%). There was no significant difference between T_H cells, $T_{S/C}$ cells, $T_H/T_{S/C}$ ratio, LC, NK cells, B cells, HLA-DR$^+$ cells, and monocytes/macrophages in these two groups of BCC.

DISCUSSION

The results show that the cellular infiltrate in the 22 BCC examined consists predominantly of T cells (55±15%). These results agree with those of other studies [5-8,15]. In studies using a panel of MoAbs for examining the cellular infiltrate in BCC, Synkowski et al [9] found 50%-75% T cells with a $T_H/T_{S/C}$ ratio of 1.0, while Ernst et al [16] observed 75% T cells with a $T_H/T_{S/C}$ ratio of 1.4. In most cases (20 of 22), we observed that in the infiltrate the T_H subset exceeded the $T_{S/C}$ subset (mean $T_H/T_{S/C}$ ratio 1.9±0.8). Eaglstein et al [8] also reported a larger proportion of T helper/inducer cells with a $T_H/T_{S/C}$ ratio of 2.2, although more than 90% pan T cells were observed. This variation in the results between the different studies can be explained on the basis of the use of semi-quantitative methods, the sensitivity of the immunohistochemical technique, and the use of MoAbs obtained from different manufacturers. However, Synkowski et al [9] observed no difference in the composition of the cellular infiltrate using the same MoAbs (OKT-sera and Leu-sera) simultaneously.

In the group of BCC with moderate-to-heavy infiltrate, the mean percentage of T cells was significantly increased compared to the mean percentage of T

cells in BCC with mild infiltration. Although statistically not significant, the T_H subset and the $T_H/T_{S/C}$ ratio in the first group showed a tendency to increase compared to the latter group. Usually most of the infiltrate was localized at the lateral and inferior border of the tumor. Since there was no dense infiltrate adjacent to the epidermis and no inflammatory cells invaded the epidermis, it can be assumed that the concentration of lymphokines and γ-interferon in the epidermis was too low for initiating the production of class II antigens on keratinocytes. In the present study, class II antigens were also not detected on the tumor cells in any of the 22 BCC. In cases in which the peritumoral infiltrate was heavy, it is possible that although enough lymphokines may be present, the tumor cells were unable to produce class II antigens. Since BCC are very slow growing tumors that remain localized, it is not likely that T cells are activated anywhere but the skin. A decrease in the expression of β_2-microglobulin and class I antigens in BCC has also been reported [17-19]. Since the expression of MHC class I or class II antigens on the membrane of the antigen-presenting cell (APC) is necessary for the activation of cytotoxic T cells, or of T helper cells, respectively, it is conceivable that tumor cells may secrete IL-1 or other chemotactic factors, which may lead to a nonspecific activation of T cells in the peritumoral infiltrate. In only 3 BCC did cells of the T_H and $T_{S/C}$ subsets invade some tumor nests, where they appear in contact with tumor cells, which suggests a specific T cell-mediated defence against BCC proliferation. It is well known that a part of Leu-$2a^+$ cells can act as cytotoxic cells [20,21]. The cytolytic capacity of T helper cells has also been reported [22-24]. Cell-mediated immune killing was also implicated in BCC showing spontaneous regression [25]. Recently Greenway et al [26] reported complete cure in eight patients with primary BCC who were treated with recombinant alpha-2 interferon. Biopsy examination 2 months after treatment showed no tumor cells, but mononuclear infiltration was present. Based on these two studies [25,26], one may speculate on the existence of a cell-mediated immune killing that leads to tumor regression.

In most tumor nests, we observed LC only sporadically, whereas in the surrounding infiltrate the mean percentage was 4±5%. There was no correlation between the number of LC and the number of T cells. Recently Smolle et al [27] reported that the number of LC does not influence the extent of the antitumor immune response. The cells of macrophage/monocyte lineage can kill tumor cells nonspecifically, induce killing via antitumor antibodies, or can act as an APC or indirectly stimulate neoplastic proliferation by secreting growth factors [28]. We observed 16±11% Leu-$M5^+$ cells in the infiltrate. It is conceivable that LC and a proportion of the monocytes/macrophages in the infiltrate surrounding the BCC bind tumor products that may induce T cell activation by release of interleukin-1 (IL-1). The activated T cells will cause proliferation of T cells by the release of IL-2.

We observed less than 2% B cells in the infiltrate. A similar outcome was also

obtained in 10 BCC using MoAb Leu-12, which reacts with B cells exhibiting kappa or lambda light chains. Eaglstein et al [8] and Ernst et al [16] reported less than 10% B cells in BCC. However, it cannot be excluded that the MoAbs used to identify B cells do not detect all B cells in the infiltrate, especially mature B cells or plasma cells. In the H&E sections of the 22 examined BCC the percentage of plasma cells ranged from 0% to 10%. Several authors have previously demonstrated a considerable number of immunoglobulin bearing cells in the cellular infiltrate surrounding BCC [5-7]. Synkowski et al [9] reported that 50%-70% of the cellular infiltrate surrounding BCC consisted of B cells identified by MoAb Leu-10. Both these authors [9] as well as the manufacturer (Becton and Dickinson) mentioned that Leu-10 has a low specificity (reacts also with monocytes, macrophages, and T cells). Nevertheless, Synkowski et al [9] concluded that humoral immunity may play an important role in controlling BCC proliferation. In our study, the low percentage of B cells would suggest a limited production of antitumor antibodies. However, these antibodies were not measured. In addition, a systemic production of anti-BCC antibodies cannot be totally excluded.

NK cells, probably a subpopulation of lymphoid cells, are histologically equal to large granular lymphocytes, and considered to be the first line of defence against tumors [10,11]. Most Leu-7$^+$ (NK) cells lack T cell markers; however, a small proportion of NK cells can express these markers [29,30]. NK cells have been observed in squamous cell carcinoma, follicular lymphoma, Hodgkin`s lymphoma, and hepatocellular carcinoma, where they frequently appeared in contact with tumor cells [12,31,32]. We observed 4±4% Leu-7$^+$ cells in the cellular infiltrate surrounding BCC. In only 1 of 22 BCC (case 12) 15% Leu-7$^+$ cells were observed, and some NK cells had invaded the tumor nests. No NK cells were observed either invading or in the tumor nests of the other 21 BCC. This would plead for a minimal role of NK cells in the defence against BCC proliferation. Similar observations have been made in BCC in a study cited in the footnote by Kohchiyama et al [12].

In conclusion, the results of this immunohistological study suggest a predominantly T cell-mediated immune response, with a minor participation of NK cells and B cells in defence against BCC. For further understanding of the exact function of T cells, the number of activated T cells exhibiting IL-2 receptor (anti-Tac) and the subclassification of the T helper subset in helper inducer and helper suppressor (4B4 and 2H4) will be of much value and is currently being investigated.

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HNK-1 Antigen Is not Specific for Natural Killer Cells*

To the Editor:

we read with great interest the article by Habets et al, published in the March 1988 issue [1]. The peritumoral inflammatory infiltrate of basal cell carcinoma (BCC) has been investigated by using a series of monoclonal antibodies. The authors provided evidence for a minor participation of natural killer (NK) cells, in defense against BCC, on the basis of the low percentage of Leu-7 (HNK-1)-positive cells in the infiltrate. Unfortunately the HNK-1 antigen, isolated by Abo and Balch in 1981 [2], cannot be considered specific for NK cells. Rather, the HNK-1 antigen is coexpressed by most CD8-positive/CD11b-positive suppressor cells [3]. Indeed, Phillips and Babcock identified a new antigen in 1983 [4], named NKP-15 (CD16), considered to be specific for essentially all human NK cells. A number of findings demonstrate that CD16 antigen is specific for NK cells, while the HNK-1 antigen is not. First, functional studies from Lanier et al [5] and Abo et al [6] have well established that CD16-positive cells display high levels of NK activity, while HNK-1-positive/CD16-negative cells possess low levels of cytotoxic cell function. On the other hand, CD16-positive/HNK-1-negative cells, when stimulated with interleukin-2 [7] or with NK-sensitive tumor cells K-562 [8], markedly augment their NK cell activity, while stimulated HNK-1-positive/CD16-negative cells never display higher NK cell function. Moreover, CD16-positive cells are able to phagocytize AET-sheep red blood cells (SRBC), whereas HNK-1-positive/CD16-negative cells never display phagocytic capability for AET-SRBC [9]. As for morphologic characteristics, CD16-positive cells show significant ultrastructural differences in comparison to HNK-1-positive/CD16- negative cells [10,11], as previously demonstrated by using a peroxidase-colloidal gold double labeling in immunoelectron microscopy [12]. Ultrastructural differences were confirmed by morphometric investigations [13]. Finally, a new antigen, named NKH-1 (Leu-19), which is expressed on the whole non-major histocompatibility complex-restricted cytotoxic cell population, comprising NK cells has recently been described [14,15].

In conclusion, the HNK-1-positive/CD16-negative cell subset seems to be distinct from the CD16-positive NK cell population. As a result, immunohistochemical studies performed by Werner Habets et al cannot rule out the possibility that NK cells play a role in the defense against BCC. This issue could be resolved by further investigations dealing with CD16 and NKH-1 antigens.

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REPLY.

The authors acknowledge Dr. Manara's comments regarding our recent publication [1]. Indeed, as stated in our article we did not totally exclude the participation of leu 7 (HNK-1) positive cells in the defense against basal cell carcinoma (BCC). In a recent publication Kohchiyama et al [2], using leu 7 (HNK-1) monoclonal antibody, also concluded that the cellular cytotoxicity mediated by NK cells may not be the main defense against BCC. Although HNK-1 antigen may be coexpressed by most leu 2a (CD-8) positive suppressor cells, our results showed only 4±4% leu 7 (HNK-1) positive cells, whereas the mean percentage of leu 2a (CD-8) positive suppressor cells was 30±11%. Thus, despite its broader specificity, the leu 7 (HNK-1) antibody fails to react with the majority of Leu 2a (CD-8) positive suppressor cells. We agree that CD-16 antigen is specific for NK cells, but in our recent investigations, using anti-leu 11b (CD-16) and anti-leu 7 (HNK-1) monoclonal antibodies simultaneously, we observed identical percentages of positively reacting cells in the peritumoral inflammatory infiltrate in BCC. We intend to extend these studies using both these monoclonal antibodies in a double labeling technique in order to verify our observations. Further investigations dealing with CD-16 and the newly described NKH-1 (Leu-19) antigens would also be useful in elucidating the participation of NK cells in the defense against BCC.

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CHAPTER 6

An absence of human leukocyte antigen-DR and a decreased expression of β_2-microglobulin on tumor cells of basal cell carcinoma: No influence on the peritumoral immune infiltrate

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ABSTRACT

The expression of human leukocyte antigen-DR (HLA-DR) and β_2-microglobulin on the tumor cells and their correlation (if any) to the degree and the composition of the peritumoral mononuclear infiltrate were studied in 37 basal cell carcinomas (BCC) from 32 patients using an indirect immunoperoxidase technique. In 36 out of 37 BCC (97%) there was no expression of HLA-DR of tumor cells of BCC. In 13 out of 37 BCC (35%) β_2-microglobulin was expressed on the tumor cells. In only 6 out of these 13 BCC both a diffuse cytoplasmic as well as a membrane staining was observed; in the other 7 BCC only a diffuse cytoplasmic staining was observed.In all the 37 BCC there was membrane staining for β_2-microglobulin in the normal epidermis. The intensity of staining in the normal epidermis was always stronger than that in the tumor nests. There was a varying degree of peritumoral immune infiltrate in all BCC. It comprised mainly of T cells (mean percentage 57±15). In the group of BCC with moderate to heavy infiltrate the mean percentage of T cells was 63±13, which was significantly higher than the mean percentage of T cells (46±14%) in the group of BCC with a mild infiltrate. This difference was mainly due to an increase in T helper cells. The absence of HLA-DR or the decreased expression of β_2-microglobulin neither correlated with the degree nor the composition of the peritumoral infiltrate. From these results it can be concluded that despite the absence of HLA-DR and a decreased expression of β_2-microglobulin on tumor cells of BCC, a T cell-mediated immune response against BCC proliferation is evoked.

INTRODUCTION

Basal cell carcinomas (BCC) are the most common cutaneous cancers of epithelial origin in humans. A proportion of BCC depending on the specific histological features are aggressive in their growth pattern causing local destruction that leads to mutilation and recurrence after surgery (1-3). There is a varying degree of mononuclear cell infiltration in BCC. Several studies have been undertaken to elucidate the function of the peritumoral mononuclear infiltrate and its possible role in the control of tumor growth (4-9). In our recent study (10), it was demonstrated that the peritumoral infiltrate in BCC consisted mainly of T cells, suggesting a predominantly T cell-mediated immune response in these tumors. As to the role of T cells, it is as yet uncertain whether they are specifically or non-specifically activated against tumor cells (10). For a specifically activated T cell-mediated immune response against tumor cells, it is necessary that class I and class II antigens of the major histocompatibility complex (MHC) be expressed on tumor cells (ll). An absence of β_2-microglobulin and HLA class I antigens in BCC has been reported (12-16), but in three of these studies (12, 15, 16), β_2-microglobulin and HLA-ABC were expressed to a varying degree in a proportion of the BCC. Little is known concerning the expression of class II antigens (HLA-DR) on tumor cells in BCC.

The aim of our study was to investigate the expression of β_2-microglobulin and HLA-DR on tumor cells of BCC and whether their expression had any influence on the degree and the composition of the peritumoral inflammatory infiltrate. Our results fail to support those reported recently in this *Journal* by Kohchiyama et al (17) who observed the expression of HLA-DR in 5 out of 8 BCC. In the present study, HLA-DR was not expressed on the tumor cells in 36 out of 37 BCC that were examined. There was a weak expression of HLA-DR in the remaining BCC.

METHODS

Samples

Thirty-seven specimens of basal cell carcinoma (BCC) were excised from 32 patients aged 31 to 89 years (mean 63 years). The diagnoses of BCC were confirmed by examination of haematoxylin and eosin (H&E)-stained frozen sections and stained paraffin-embedded sections.

The BCC were classified in the conventional manner as described previously (2,18). For the histopathological examination of the tumor and the skin adjacent to it, the samples were cut lengthwise into two equal parts. One portion and the edges of the other portion were fixed in formalin and paraffin-embedded for histological confirmation of the diagnosis and for determination of tumor-free margins. The remaining portion was frozen in liquid nitrogen-cooled isopentane and stored in liquid nitrogen.

Indirect immunoperoxidase test (IIP)

Serial cryostat sections, 5 μm thick, were placed on alcohol-cleaned glass slides, air-dried, fixed in acetone for 10 min at room temperature, and stained using the procedure described in our previous studies (10,19). Briefly, the cryostat sections were preincubated with bovin serum albumin at a dilution of 1:20 in phosphate-buffered saline (PBS, pH 7.4) for 30 min. The sections were then incubated with an optimal dilution of one of the monoclonal antibodies Leu-4, Leu-3a, Leu-2a, anti-HLA-DR or β_2-microglobulin (Becton and Dickinson) for 60 min, rinsed in PBS and incubated with rabbit peroxidase-conjugated anti-mouse IgG (Dakopatts, Amsterdam) at a dilution of 1:50. The peroxidase reaction was developed by incubating the sections with 3,3'-diaminobenzidine (Sigma chemical Co., St. Louis, MO) at a concentration of 0.5 mg/ml and 0.01% hydrogen peroxide for 10 min at room temperature. Sections were then rinsed in tap water, counterstained with haematoxylin for 1 to 2 min and rinsed again in tap water. The sections were mounted in Malinol (Chroma-Gesellschaft, Stuttgart). The negative controls comprised either the omission of primary antibody or rabbit anti-mouse immunoglobulin.

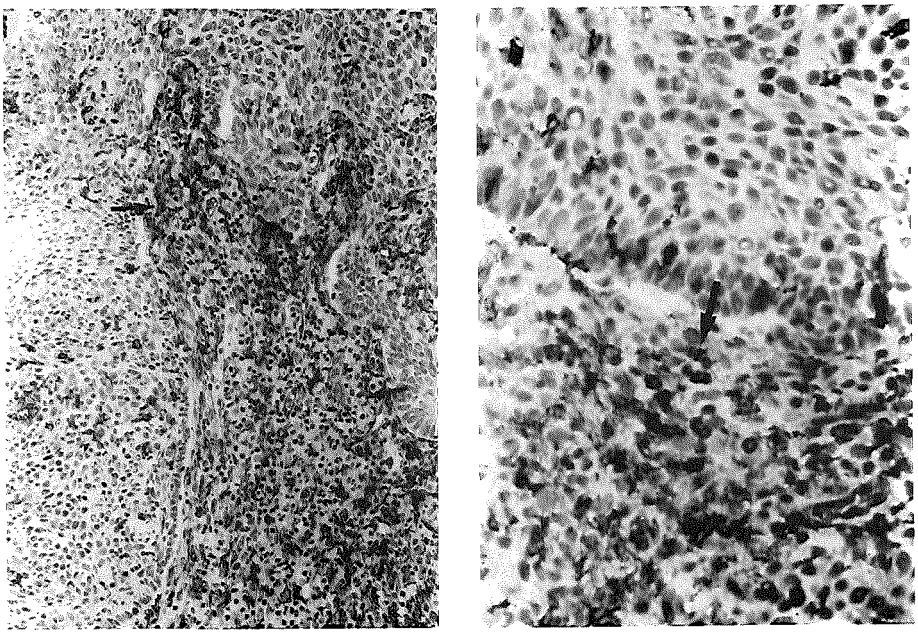
The peritumoral mononuclear infiltrate was graded as mild, moderate, or heavy as reported previously (10). For each antibody the percentage of positive staining cells in the infiltrate was determined by counting 200 mononuclear cells at ×400 magnification. The number of tumor cells that stained for HLA-DR or β_2-microglobulin was estimated in the whole section and expressed as a percentage. For statistical analysis of the results the Wilcoxon rank sum test was used. A p value of < 0.05 was considered as statistically significant.

RESULTS

The 37 BCC we examined comprised 16 solid, 3 superficial, 3 adenoid, 1 keratotic, 1 cystic, 9 infiltrating, 2 morphoeic and 2 basosquamous types. The latter 3 have histological features that are characteristic of an agressive growth pattern. The tumor size of the 37 BCC ranged from 0.4 to 4 cm and 13 of them showed ulceration. Twenty-seven BCC were located in the face, 3 in the neck, 6 on the trunk and 1 on the arm. There was no correlation between the size of the BCC, the presence of ulceration or the aggressiveness and the degree or the composition of the peritumoral infiltrate.

In none of the 36 BCC HLA-DR was expressed on tumor cells. In the remaining basosquamous type BCC with a heavy peritumoral infiltrate, the tumor cells showed a weak membrane staining for HLA-DR. In 5 of 37 BCC, it was observed that Leu-4$^+$ (pan T) cells and HLA-DR$^+$ cells invaded some tumor nests and were in close contact with the tumor cells. However, in these cases HLA-DR was not expressed on the tumor cells. An example of the reactivity pattern of MoAb HLA-DR is shown in Figure 1. In all BCC HLA-DR$^+$ cells with a dendritic appearance were sporadically observed in tumor nests. In 35 of 37 BCC, keratinocytes in the normal epidermis did not express HLA-DR. However, in the remaining 2 cases with a heavy infiltrate between the tumor nests and the epidermis, the epidermis was focally stained. In 13 of 37 BCC (35%) β_2-microglobulin was expressed on the tumor cells. The estimated percentage of positively stained tumor cells ranged from 5% to 80% (mean 60%). Seven of the 13 positively stained tumors only showed a diffuse cytoplasmic staining, whereas the other 6 BCC showed both a membrane as well as a diffuse cytoplasmic staining. An example of the latter staining pattern is shown in Figure 2. In all BCC, the intensity of the staining in the normal epidermis was stronger than that in the tumor cells. The keratinocytes in the normal epidermis only showed a membrane staining. There was no correlation between the expression of β_2-microglobulin in BCC and the histological type of BCC or the degree of peritumoral immune infiltrate as shown in Table I. However, it was striking that 4 of 6 BCC that showed a diffuse cytoplasmic as well as a membrane staining for β_2-microglobulin were of the infiltrating type BCC.

In 14 of 37 BCC (38%) a mild peritumoral infiltrate was observed, and in 23 of 37 BCC (62%) a moderate to heavy infiltrate was observed. It can be seen in



Figures 1a and 1b: Cryostat section (5 μm) of solid type BCC showing a heavy HLA-DR$^+$ peritumoral infiltrate (arrows). Tumor cells do not stain. (MoAb HLA-DR, IIP technique, × 135 and × 340, respectively.)

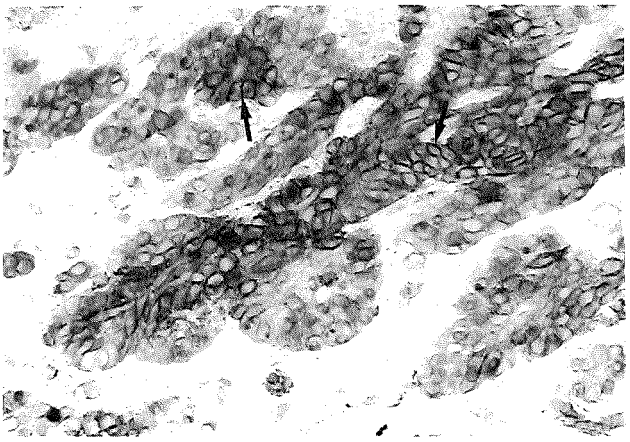


Figure 2: Cryostat section (5 μm) of solid type BCC. Membrane staining of most of the tumor cells (arrows).(MoAb β_2-microglobulin, IIP-technique, × 340.)

Table II, that the mean percentages of T cells and HLA-DR$^+$ cells in all the 37 BCC were 57±15 and 57±11, respectively. The mean percentages of T helper cells and T suppressor/cytotoxic cells were 49±14 and 28±13, respectively. The mean percentage of T cells in the group of BCC with moderate-to-heavy peritumoral infiltrate (63±13%) was significantly higher ($p < 0.01$) than in the group with mild infiltrate (46±14%). The mean percentage of T helper cells was also significantly higher ($p < 0.05$) in the group of BCC with moderate-to-heavy peritumoral infiltrate (55±12%) than in the group with mild infiltrate (43±13%).There was no significant difference in mean percentages of HLA-DR$^+$ cells and T suppressor/cytotoxic cells in both groups of BCC. There was also no correlation between the expression of β_2-microglobulin in BCC and the mean percentages of T cells, T helper cells, T suppressor/cytotoxic cells, or of HLA-DR$^+$ cells in the peritumoral infiltrate.

Table I: Expression of β_2-microglobulin in basal cell carcinoma (BCC) and its relationship to histological characteristics and degree of peritumoral immunoinfiltrate.

	Number and percentage of BCC expressing β_2-microglobulin	
Total numbers of BCC	13/37	(35%)
Group I	9/24	(37%)
Group II	4/13	(31%)
Group III	5/14	(36%)
Group IV	8/23	(35%)

Group I = BCC showing benign histological characteristics
Group II = BCC showing aggressive histological characteristics
Group III = BCC with mild peritumoral infiltrate
Group IV = BCC with moderate-to-heavy peritumoral infiltrate

Table II: Comparison of mean percentages of T cells, T helper cells, T suppressor/cytotoxic cells and HDL-DR$^+$ cells in the peritumoral infiltrate of various groups of basal cell carcinoma (BCC).

Basal Cell Carcinoma (BCC)		Leu 4 (pan T) cells (mean percentage ±S.D.)	Leu 3a (T helper) cells (mean percentage ±S.D.)	Leu 2a (T suppressor/ cytotoxic) cells (mean percentage ±S.D.)	HLA-DR (mean percentage ±S.D.)
All BCC	(n=37)	57±15 (range 15%-74%)	49±14 (range 20%-70%)	28±13 (range 5%-60%)	57±11 (range 35%-75%)
Group I	(n=14)	46±14 (range 15%-64%)	43±13 (range 20%-60%)	35±15 (range 10%-41%)	52± 8 (range 38%-61%)
Group II	(n=23)	63±13 (range 25%-74%)	55±12 (range 22%-70%)	29±14 (range 5%-60%)	60±12 (range 35%-75%)
p-value		$p < 0.01$	$p < 0.05$	N.S.	N.S.
Group III	(n=13)	61±12 (range 25%-70%)	48±12 (range 29%-69%)	23± 6 (range 13%-30%)	57± 9 (range 40%-70%)
Group IV	(n=24)	56±15 (range 15%-74%)	53±14 (range 20%-70%)	30±15 (range 5%-60%)	57±11 (range 35%-75%)
p-value		N.S.	N.S.	N.S.	N.S.

Group I = BCC with mild infiltrate; group II = BCC with moderate-to-heavy infiltrate; group III = BCC that express β_2-microglobulin; group IV = BCC that do not express β_2-microglobulin.
There is a significant difference between pan T cells ($p < 0.01$) in groups I and II. T helper cells also show a significant difference ($p < 0.05$) in these two groups. There was no difference between T suppressor/cytotoxic cells or HLA-DR$^+$ cells, respectively in groups I en II. There was no difference between pan T cells, T helper cells, T suppressor/cytotoxic cells or HLA-DR$^+$ cells, in groups III and IV.
N.S. = not significant.
S.D.= Standard deviation.

DISCUSSION

The results of this study show that the tumor cells of basal cell carcinomas (BCC) do not express HLA-DR antigens. Several studies reporting the peritumoral infiltrate in BCC have been published (9, 20-22). In those studies monoclonal antibodies against HLA-DR were also used. In none of those studies did the authors report on the expression of HLA-DR on tumor cells in BCC. However, Natali et al (15, 23), using 2 different monoclonal antibodies observed the expression of HLA-DR on tumor cells in 1 of 13 BCC in one study (15) and in 2 of 3 BCC in a separate study (23). Furthermore, Kohchiyama et al (17) recently reported in this *Journal* on the expression of HLA-DR in BCC. They observed the expression of HLA-DR on tumor cells in 5 of 8 BCC. The tumor cells located at the periphery of the tumor nests were predominantly stained with monoclonal antibody (MoAb) OKIa1. However, as shown in their article, the intensity of staining of the tumor cells for HLA-DR was weak especially as compared to the intensity of staining of the peritumoral mononuclear infiltrate cells. Moreover, in our experience, it is rather difficult to distinguish between the infiltrating cells and the tumor cells which are HLA-DR positive in those BCC in which the mononuclear inflammatory cells invaded the tumor nests. The possible explanations for the contradictions between their results and the results of the present study may be due to the use of different immunohistological techniques, the use of monoclonal antibodies obtained from different manufactures and the differences in the interpretation of the staining patterns.

In the present study, β_2-microglobulin was not expressed in 24 of the 37 (65%) BCC that were examined. In the other 13 (35%) β_2-microglobulin was expressed. However, in these cases the mean percentage of stained tumor cells was 60% (range 5% to 80%), but the intensity of staining was always lower than that of the normal epidermis in the same cryostat section. In 6 of these 13 BCC, in addition to a diffuse cytoplasmic staining, a membrane staining of the tumor cells was also observed. Both the total absence of β_2-microglobulin on tumor cells and the lower intensity of staining are indicative of a decreased expression of β_2-microglobulin in BCC. These results are in agreement with the results of other studies (12,15,16).

There have been several conflicting reports regarding the expression of the heavy chain of the major histocompatibility complex (MHC), class I antigens in BCC (14-16, 24). Mauduit et al (14) observed that in BCC the HLA-heavy chain molecule was expressed without the expression of HLA-light chain (β_2-microglobulin). However, other authors reported both an absence and a reduction in the intensity of staining for HLA-ABC and for β_2-microglobulin in most BCC (15,16,24). An absence of class I antigens in malignant tumors of other tissues such as breast, colon, lung, endothelium has been observed (25). It is well known that expression of class I antigens on tumors is necessary for associative

recognition of tumor antigens by cytotoxic T cells (11). However, in the present study, there was no correlation between the expression of β_2-microglobulin on the tumor cells and the mean percentage of Leu 4^+ cells, the mean percentage of Leu $3a^+$ cells, the mean percentage of Leu $2a^+$ cells, or the mean percentage of HLA-DR^+ cells in the peritumoral infiltrate, including the T cells invading the tumor nests. This is similar to the observations in a breast cancer study in which such a correlation could not be established (25). In contrast to these observations in melanoma and in melanocytic nevi, the degree of mononuclear inflammatory infiltrate was correlated to the expression of class I antigens on these tumor cells (26). In the present study we observed that an increase of peritumoral mononuclear infiltration cells was caused by an increase of T cells. This increase was mainly due to an increase in T helper cells. Although, class II antigens were not expressed on the tumor cells of BCC, there was an increase in T helper cells. Specific activation of T helper cells against tumor antigens on tumor cells can only occur in associative expression of class II antigens. Therefore, it is conceivable that tumor products binding to antigen-presenting cells such as Langerhans cells and macrophages, which were present in the peritumoral infiltrate as observed in our previous study (10), could evoke a specific T cell-mediated response. Alternatively, non-specific activation of T cells by interleukin-1 and other chemotactic factors secreted by tumor cells cannot be totally excluded.

In conclusion, the results presented here suggest that although the tumor cells of BCC do not express HLA-DR and in a majority of cases do not express β_2-microglobulin, the presence and the degree of peritumoral immunoinfiltrate are not affected. Moreover, a T cell-mediated immune response against BCC proliferation is not influenced.

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CHAPTER 7

Characterization of the mononuclear infiltrate in Bowen's disease (squamous cell carcinoma in situ); Evidence for a T cell-mediated anti-tumor immune response.

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ABSTRACT

We investigated the dermal inflammatory infiltrate and the expression of HLA-DR and β_2-microglobulin on the tumor cells in 8 Bowen's disease (BD) using a series of monoclonal antibodies. The inflammatory infiltrate was classified as mild, moderate or heavy. The infiltrate in all cases consisted mainly of T cells (55±21%) and where the T helper (T_H) subset predominated over the T suppressor/cytotoxic ($T_{S/C}$) subset ($T_H/T_{S/C}$ ratio of 2.4±1.0). The mean percentage of HLA-DR positive cells was 58±18%, Langerhans cells (LC) 4±1% and Leu-M5 positive cells (monocytes/macrophages) 9±2%. The mean percentage of B cells and natural killer (NK) cells was 4±5% and 1±2% respectively. B cells and NK cells did not invade any of the tumors. In the 5 BD with moderate or heavy infiltrate, T_H cells, $T_{S/C}$ cells and Leu-M5 positive cells did invade the tumor. In the tumor area where there was invasion, the number of LC was increased and HLA-DR was expressed on the tumor cells. β_2-microglobulin was generally expressed on the tumor cells of BD. We concluded that there is evidence for a T cell-mediated anti-tumor immune response which may account for the infrequent invasive growth in BD.

INTRODUCTION

Bowen's disease (BD) is an uncommon intraepithelial cancer of the skin. Inorganic arsenic and exposure to sun light are considered as possible etiological factors. Although invasive growth is rare, at least 5% of the lesions are known to invade the underlying dermis (Maize et al. 1979). In these cases, more than one third of the patients will develop metastasis. In contrast to BD, solar keratosis in which 10% to 15% of the lesions develop into invading squamous cell carcinoma, no metastasis has been observed (Graham et al. 1966).

In most BD, a moderate amount of mononuclear inflammatory infiltrate is observed in the upper dermis (Lever et al. 1983).It has been reported that the inflammatory infiltrate in other types of skin neoplasia such as basal cell carcinomas, squamous cell carcinomas and melanoma consists mainly of T cells (Eaglstein et al. 1982; Ruiter et al. 1982; Kohchiyama et al. 1986; Ralfkiaer et al. 1987; Habets et al. 1988). We recently reported (Habets et al. 1988) that the defence against basal cell carcinomas is predominantly T cell-mediated with minor participation of NK cells and B cells. In contrast to basal cell carcinomas, in squamous cell carcinomas not only T cells but also B cells and NK cells seem to play a role in the local defence (Kohchiyama et al. 1986). To date, the inflammatory infiltrate in BD has not been characterized. Since only about 5% of the BD will invade the dermis it would be interesting to know whether the inflammatory infiltrate plays a role in any defence against invasive growth. To obtain insight into the composition of this inflammatory infiltrate and its role in

tumor defence, we typed this infiltrate and investigated the expression of β_2-microglobulin and HLA-DR on the tumor cells.

MATERIALS AND METHODS

Six 4 mm biopsy specimens and 2 surgically excised specimens of BD were obtained from 5 patients aged 42-74 years. The location of the 8 BD that were examined was as follows. Three were located in the head and neck region, 2 were located on the arms and 3 were located in the trunk region. For histological examination of the tumor, the samples were cut in two equal parts. One part was fixed in formalin and paraffin embedded. The diagnosis of BD was confirmed by examining haematoxylin and eosin (H&E)-stained sections. The remaining part was frozen in liquid nitrogen-cooled isopentane and stored in liquid nitrogen. Three serial cryostat sections (5 μm in thickness) were placed on each alcohol-cleaned glass slide, air dried and fixed in acetone for 10 min at room temperature and stained using the indirect immunoperoxidase (IIP) procedure described in our previous study (Habets et al. 1988). Briefly, the cryostat sections were preincubated with 5% (w/v) bovine serum albumin in phosphate- buffered saline (PBS, pH 7.4) for 30 min. The sections were then incubated with an optimal dilution of the monoclonal antibody (MoAb) Leu-2a for T suppressor/cytotoxic ($T_{S/C}$) cells, Leu-3a for T helper (T_H) subset, Leu-4 for all T cells (pan T), Leu-6 for Langerhans cells (LC), Leu-7 (HNK1) for natural killer (NK) cells, Leu-14 for B cells, Leu-M5 for monocytes/macrophages, anti-HLA-DR and anti-β_2-microglobulin (Becton & Dickinson) for 60 min, rinsed in PBS and incubated with rabbit peroxidase- conjugated anti-mouse IgG or in the case of Leu-7 with rabbit peroxidase conjugated anti-mouse IgM at an optimal dilution. The peroxidase reaction was developed by incubating the sections with 3,3'-diaminobenzidine (DAB) at a concentration of 0.5 mg/ml and hydrogen peroxide (0.01%) for 10 min at room temperature. Sections were then rinsed in PBS, counterstained with haematoxylin for 1 min and rinsed again in tap water. The sections were mounted in Malinol (Chroma-Gesellschaft, Stuttgart).

The specificity of MoAbs Leu-7 and Leu-14 was verified using frozen tissue sections of human lymph nodes. The negative controls comprised the use of an irrevelant MoAb, the omission of primary antibody and the omission of rabbit anti-mouse immunoglobulin.

The criteria for the classification of the dermal mononuclear infiltrate were based on the estimation of the total number of infiltrating cells. In each case the infiltrate was examined in 3 serial sections on the same glass slide. Depending on the total number of infiltrating cells in each case, the infiltrate was graded as mild (a low number), as heavy (a high number) and as moderate when the total number of infiltrating cells was between the other two categories. For each monoclonal antibody the percentage of stained cells in the infiltrate was

estimated by counting 200 mononuclear cells at ×400 magnification. Only the cells showing membrane staining were counted. For each antibody identical locations in the serial sections were examined.

RESULTS

The composition of the inflammatory infiltrate is summarized in Table I. In 3 of the 8 BD, we observed a mild inflammatory infiltrate, a moderate infiltrate in 3 and a heavy infiltrate in 2. In the latter 5 BD, mononuclear infiltrate was also observed in the lower part of the tumor. The mean composition of the infiltrate in 8 BD was 55±21% Leu-4 positive (pan T) cells, 50±19% Leu-3a positive (T_H) cells, 23±11% Leu-2a positive ($T_{S/C}$) cells, 4±1% Leu-6 positive cells (LC), 1±2% Leu-7 positive (NK) cells, 4±5% Leu-14 positive (B) cells, 9±2% Leu- M5 positive cells (monocytes/macrophages) and 58±18% HLA-DR positive cells. The $T_H/T_{S/C}$ ratio was 2.5±1.0. The composition of the mononuclear cells that invaded the tumor is shown for T cells (Fig. 1), T_H and $T_{S/C}$ cells (Fig. 2) and macrophages (Fig. 3). No B cells or NK cells were observed in the tumors. In the 5 cases with moderate and heavy infiltrate there was an increase in LC in the lower part of the tumor in the region where the T cells and macrophages invaded the tumor (Fig. 4) as compared to the marginal epidermis. In addition in that area, the tumor cells expressed HLA-DR (Fig. 5).

β_2-microglobulin was expressed on the tumor cells of all the 8 BD. However, in 3 cases (cases 5, 6 and 8) groups of tumor cells were either stained very weakly or not at all, but the tumor cells which were in contact with the immunocompetent cells present in the lower part of the BD were always stained.

Table I:
Summary of phenotypes of infiltration and expression of β_2-Microglobulin (β_2-MG) and HLA-DR on tumor cells in 8 Bowen's Disease.

Case	Degree of infiltration	Leu-4	Leu-3a	Leu-2a	$T_H/T_{S/C}$	Leu-6	Leu-7	Leu-14	Leu-M5	HLA-DR	Expression on tumor cells β_2-MG	HLA-DR
1.	mild	20	20	5	4.0	5	0	0	5	25	+	-
2.	mild	40	40	10	4.0	5	1	0	5	40	+	-
3.	mild	50	40	20	2.0	2	1	0	10	75	+	-
4.*	moderate	80	60	30	2.0	5	1	4	10	70	+	(+)
5.*	moderate	40	35	25	1.4	5	1	7	10	60	+	(+)
6.*	moderate	60	70	35	2.0	3	0	2	8	60	+	(+)
7.*	heavy	80	70	35	2.0	5	5	15	10	60	+	(+)
8.*	heavy	70	65	25	2.6	2	1	3	10	76	+	(+)
Mean±S.D.		55±21	50±19	23±11	2.5±1.0	4±1	1±2	4±5	9±2	58±18		

The results represent the mean estimated percentage of cells stained in the upper dermis.
* = Invasion of tumor by immunocompetent cells
\+ = Membrane staining of tumor cells
(+) = Membrane staining of tumor cells in the lower part of the tumor close to the dense dermal infiltrate.
\- = No staining

Figure 1:
Cryostat section (5µm) of Bowen's Disease showing a heavy dermal infiltrate with more than 75% Leu-4 positive (pan T) cells. MoAb Leu-4, IIP technique, ×55. T cells invade the tumor (arrows)

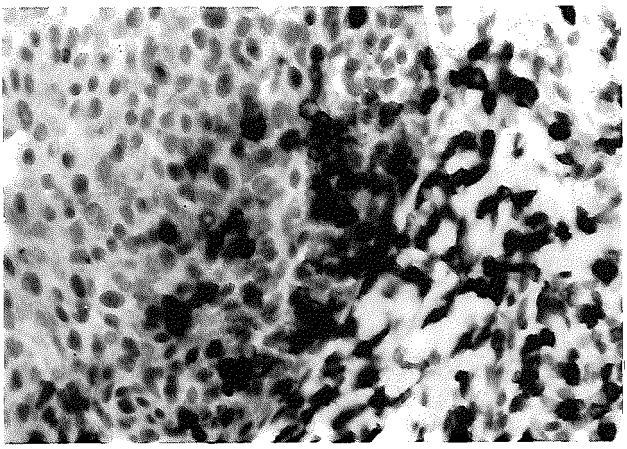

Figure 2:
Cryostat section (5µm) of Bowen's Disease showing Leu-2a positive cells (suppressor/ cytotoxic T cells) invading the tumor and in contact with tumor cells. MoAb Leu-2a, IIP technique, ×340.

Figure 3
Cryostat section (5μm) of Bowen's Disease showing Leu-M5 positive cells (monocytes/-macrophages) in the infiltrate. A few stained cells are seen invading and in contact with the tumor cells (arrow). MoAb Leu-M5, IIP technique, ×340.

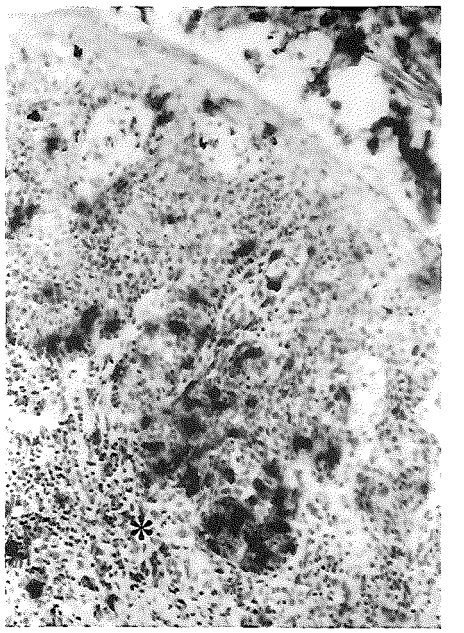

Figure 4:
Cryostat section (5 μm) of Bowen's Disease showing an accumulation of Leu-6 positive cells (Langerhans cells) in the lower part of the tumor. MoAb Leu-6, IIP technique, ×135. Note the dense dermal infiltrate (asterisk).

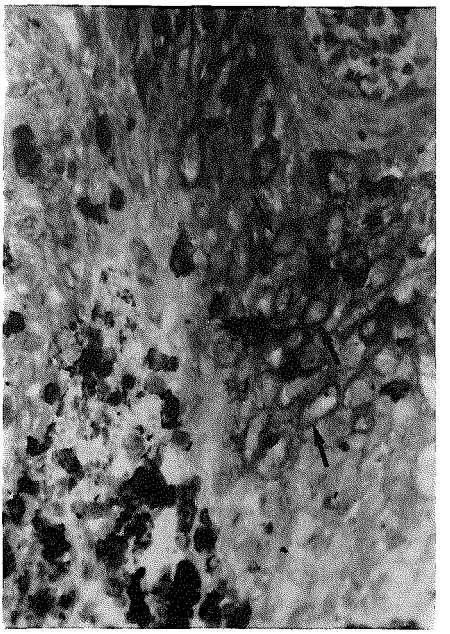

Figure 5: Cryostat section (5 μm) of Bowen's Disease showing HLA-DR positive tumor cells (arrows). HLA-DR positive infiltrate cells are seen both in the dermal infiltrate and in the tumor. MoAb anti-HLA-DR, IIP technique, × 340.

DISCUSSION

The results show that the inflammatory infiltrate in BD consisted predominantly of T cells (55±21%). This corroborates the results of the infiltration studies in other skin tumors (Eaglstein et al. 1982; Ruiter et al. 1982; Kohchiyama et al. 1986; Ralfkiaer et al. 1987; Habets et al 1988). In all the BD we examined, the T_H subset predominated the $T_{S/C}$ subset (mean $T_H/T_{S/C}$ ratio of 2.4±1.0). In basal cell carcinomas the T_H subset also predominates the $T_{S/C}$ subset (Eaglstein et al. 1982; Habets et al. 1988), whereas in melanomas the T_H subset generally equals the $T_{S/C}$ subset (Poppema et al. 1983; Ralfkiaer et al. 1987). In squamous cell carcinomas the $T_H/T_{S/C}$ ratio is reversed in favour of the $T_{S/C}$ subset (Kohchiyama et al. 1986).

In the 5 BD (cases 4-8) with moderate or heavy infiltrate, T_H as well as $T_{S/C}$ cells invaded the tumor and were observed to be in contact with the tumor cells. At the site of invasion, the tumor cells were observed to be HLA-DR positive and the number of Langerhans cells (LC) in the same area was increased as compared to the rest of the tumor. In the remaining 3 BD (cases 1-3) with a mild infiltrate, the tumor was not invaded either by T_H or by $T_{S/C}$ cells and HLA-DR was not observed on the tumor cells. This suggests that the expression of HLA-DR in BD may be dependent on whether or not there is an invasion of the tumor by T cells. However, a larger number of patients must be investigated to confirm this observation. The expression of HLA-DR on the tumor cells has been observed in melanoma (Ruiter et al. 1982; Brocker et al. 1984) and colorectal cancer (Daar et al. 1983). In both these types of tumors, there was no correlation between the degree of mononuclear infiltrate and the expression of HLA-DR on the tumor cells. In basal cell carcinomas and squamous cell carcinomas HLA-DR was not expressed on the tumor cells (Kohchiyama et al. 1986; Habets et al. 1988, 1989). In a recent study by Kohchiyama et al. (1987), HLA-DR was expressed by basal cell carcinoma but these results have not yet been confirmed. The results of our recent study (Habets et al. 1989) contradict their findings. In the present study β_2-microglobulin was expressed on the tumor cells of all BD, but in three cases there were also groups of tumor cells that either stained weakly or not at all. A total or a partial absence of β_2-microglobulin on the tumor cells in BD has been reported (Turbitt et al. 1981; Mauduit et al. 1983; Hua et al. 1985).

In those BD where the major histocompatibility complex (MHC) class I and class II antigens are expressed on the tumor cells, activation of T_H and $T_{S/C}$ subsets may occur leading to a T cell-mediated anti-tumor response. The tumor antigens presented in conjunction with HLA-DR will be recognized by T_H cells. Subsequently, activation and proliferation of T cells occur by release of lymphokines (interleukin-l, interleukin-2 and interferon-gamma). Since HLA-DR positive tumor cells were not observed in BD with a mild inflammatory infiltrate, it seems unlikely that the expression of HLA-DR on the tumor cells preceeds the

activation and proliferation of the immune infiltrate. In the BD with a moderate or heavy infiltrate, we observed an accumulution of LC in the lower part of the tumor close to the dense dermal infiltrate. Since not only activated T cells but also LC may produce interferon-gamma (Knop et al. 1988), it is likely that the production of interferon-gamma is high enough to induce HLA- DR expression on the tumor cells.

The anti-tumor activity of macrophages may consist of non- specific killing of tumor cells, killing of tumor cells byanti-tumor antibodies via antibody-dependent cell-mediated cytotoxicity (ADCC) or even act as antigen-presenting cells (APC) for tumor antigens (Mantovani et al. 1985; Hamilton et al. 1987). We observed 9±2% Leu-M5 positive cells some of which invaded the tumor. Upon closer careful examination of the sections, HLA-DR positive large non-dendritic cells were observed indicating the presence of activated macrophages in contact with the tumor cells. These observations suggest that macrophages act as anti-tumor effector cells.

We observed 4% B cells in the infiltrate. However, in 2 of the 8 BD (cases 5 and 7) we observed 7% and 15% B cells which suggest a possible local production of anti-tumor antibodies. Since in these cases activated macrophages were observed in contact with the tumor cells, it is possible that tumor killing via ADCC may also occur. Kohchiyama et al. (1986) reported that in most squamous cell carcinomas, a considerable number of B cells were present which suggested that B cells played a role in the local anti-tumor response. In other cutaneous tumors such as basal cell carcinomas (Eaglstein et al. 1982; Habets et al. 1988) and melanomas (Ruiter et al. 1982; Ralfkiaer et al. 1987), the number of B cells in the infiltrate was very limited, indicating a minor role of B cells.

NK cells are considered to be the first line of defence against tumors (Herberman et al. 1979, 1981). In the present as well as our previous study on basal cell carcinoma (Habets et al. 1988), a minimal number of NK cells were observed. This suggested a limited role of NK cells in the local defence against these tumors. A low number of NK cells have also been reported in melanoma (Ralfkiaer et al. 1987). Since the number of NK cells in most tumors in situ is limited, it has been proposed that their main role may be that of eliminating micro-metastases (Vose et al. 1985).

In conclusion, our results support a T cell-mediated anti-tumor response in BD in which the Langerhans cells and the cells of macrophage/monocyte lineage also play a supportive role. B cells and NK cells seem to play a minor role or no role at all in the local defence against BD. This predominantly T cell-mediated immune response may at least in a proportion of BD be directly or indirectly responsible for the low incidence of the development of BD into invasive carcinoma.

ACKNOWLEDGEMENTS

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CHAPTER 8

Intralesional treatment of basal cell carcinoma with low-dose recombinant interferon gamma.

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ABSTRACT

In this pilot trial, the efficacy of low dose human recombinant interferon gamma (rIFN–γ) as an outpatient therapy was investigated in 7 patients. Each patient had a biopsy-confirmed basal cell carcinoma (BCC) of the nodular type. Each BCC was injected intralesionally twice weekly for 4 weeks (8 total injections) with $2x10^5$ IU (0.1 ml) of rIFN–γ per injection (total dose $1.6x10^6$ IU). All patients were evaluated for tumor response and toxicity with a follow-up of 8 weeks after the completion of the therapy. Clinical responses measured during therapy showed that although there was a flattening of the lesions in all cases, there was no reduction in the size of the lesions in any case. Histopathological examination of the biopsies obtained 8 weeks after the completion of the therapy confirmed the presence of tumor in all cases. This demonstrated that low-dose intralesional therapy had no antitumor effect. The therapy was safe and was well tolerated with minimal toxic effects by all patients. The lack of antitumor effect in the present pilot trial may be due to the low-dose that was used but is in contrast to the successful intralesional therapy of BCC with recombinant alpha-2 interferon.

INTRODUCTION

Basal cell carcinomas (BCC) are the most common cutaneous cancers of epithelial origin in man. Fortunately, metastases are extremely rare (1). However, a proportion of BCC depending on the specific histological features are aggressive in their growth pattern causing local destruction which leads to mutilation and recurrence after surgery (2). Various clinical and histologic forms of basal cell carcinoma include the nodular-ulcerative, superficial, pigmented, morphea-like and fibroepitheliomatous types (3).

The current treatment methods include excision, cryosurgery, irradiation, and currettage and electrodesiccation with high cure rates of around 95% (4). Some primary BCC depending on their location and histopathology and recurrent BCC may respond best to Mohs' surgery. Cure rates via Mohs' surgery approach 99% for all primary BCC and 96% to 98% for recurrent BCC (5). However, all the above mentioned therapies, especially irradiation can cause undesirable side effects. Although the cure rates of BCC using surgical techniques are high, non-surgical approaches to BCC therapy in selected cases is not only desirable but may offer advantages especially in cases of multiple BCC, recurrent BCC and BCC that are difficult to operate due to their location.

In recent years, much attention has been focused on interferons as promising therapeutic agents in the treatment of skin diseases (7). To date, several clinical studies have been reported in which intralesional administration of interferon-α resulted in the successful treatment of various skin diseases such as BCC (6,8),

actinic keratoses (9), and condylomata acuminata (10). Interferon–γ has been used to treat patients with lepromatous leprosy (11).

Although, interferon–γ is a well-characterized macrophage-activating factor and a potent immuno-modulating agent, its physiologic function and therapeutic potential are as yet undefined in patients with BCC. Since there was no previous experience in using recombinant interferon gamma (rIFN–γ) for the treatment of BCC, a conservative approach similar to that used previously to treat lepromatous leprosy patients was chosen (11). The main objectives of the investigations described in this pilot clinical study were to determine the efficacy of twice weekly intralesional treatment with low-dose rIFN–γ in outpatients with histologically confirmed primary BCC of the nodular type and to assess whether rIFN–γ administered according to this treatment regimen was safe and well tolerated (minimal side effects).

MATERIALS AND METHODS

Patients

Two female and 5 male patients aged 55-75 years (mean 64 years), each with a biopsy-confirmed primary basal cell carcinoma (BCC) of the nodular type took part in this pilot trial. The size of the BCC varied form 6x8 mm to 14x22 mm and were located as follows: forehead/temporal region(3), leg (1), ear (1), eyebrow (1) and cheek (1). Four mm punch biopsies were taken prior to the start of the therapy and the diagnosis of BCC was confirmed by histopathological examination of haematoxylin and eosin (H&E)- stained sections. All patients were healthy and their lesion which was to be injected was considered treatable by an alternative therapy. None of the patients had received radio-, chemo-, or immunotherapy prior to rIFN–γ therapy. Patients were aware of their diagnosis and all available therapies but chose to receive rIFN–γ as an outpatient therapy. All patients underwent a complete history and thorough physical examination before the start of the therapy. The lesion which was to be treated was measured, photographed and its location documented. All patients were informed on the nature of the pilot trial, its possible hazards and of their right to withdraw at any time from the study without prejudice and without jeopardy to their future medical treatment. They all agreed to cooperate in all aspects of the trial and provided informed written consent.

Laboratory tests

Pretherapy tests included a total blood count with differential white blood cell count, complete physical examination (including body weight, performance status and vital signs), a chemistry profile including liver and renal function tests and a urine analysis. The entire haematology and chemistry tests were

repeated 2 weeks and 4 weeks after the start of the therapy. A complete physical examination was repeated 8 weeks after the therapy was completed.

Treatment

All patients were treated on an outpatient basis at the department of Dermato-Venereology, academic hospital Dijkzigt, Erasmus University, Rotterdam. They were treated with the human recombinant interferon gamma (rIFN–γ) supplied as a sterile lyophilized powder (specific activity: $2x10^7$ IU/mg protein) in 0.5 mg vials by Boehringer Ingelheim b.v., Alkmaar, The Netherlands. The vials were stored at 2°-8° C. Each 0.5 mg vial was reconstituted with 1 ml sterile water. This solution (concentration 0.5 mg/ml) was diluted 5 fold with sterile physiological saline to correspond to 10 µg (0.1 ml) dose of rIFN–γ. Reconstitution was accomplished by gentle inversion of the vial and each vial was used only once. Each lesion was injected with 0.1 ml ($2x10^5$ IU) rIFN–γ via a 30-gauge needle on a tuberculin syringe. The needle was inserted tangentially into the center of the lesion assuring that the content of the syringe was delivered intralesionally. This was repeated twice weekly (Mondays and Thursdays) for 4 weeks. Each lesion was injected with a total of $1.6x10^6$ IU ($2x10^5$ IU x 8 doses). During therapy, the patients were evaluated for therapeutic response and toxic side effects at weekly intervals for 4 weeks and one and two months after the therapy was completed.

Therapeutic response criteria

The following criteria were used to evaluate the degree of therapeutic response. Clinical healing of all lesions was regarded as complete response. A partial response was defined as a 50% or greater reduction in the size (product of perpendicular diameters) of the lesion. No response was defined as no change in the lesion qualifying as a partial response or disease progression. An increase of 25% or greater in the size of the lesion was considered as progressive disease.

Therapeutic responses were measured during the therapy and follow-up by evaluating the size and flattening of the lesion. Four mm punch biopsies were taken from each lesion before, after and 8 weeks after the completion of the therapy.Histological confirmation for the presence of the tumor was obtained by examining haematoxylin and eosin (H&E)-stained paraffin embedded sections.

RESULTS

The particulars of the seven patients are summarized in Table I. Clinical responses measured during therapy showed that although there was a flattening

Table I. The characteristics of the seven patients.

Patient No.	Age (yr)	Sex	Type of BCC	Location of BCC	Pretreatment lesion size (mm)	Posttreatment lesion size (mm) 0 week	8 weeks
1.	55	M	Nodular	Forehead/temporal region	12 x 13	14 x 15	11 x 13
2.	75	F	Nodular	Leg	6 x 8	6 x 8	4 x 9
3.	56	F	Nodular	Eyebrow	6 x 8	5 x 8	4 x 7
4.	63	M	Nodular [a]	Cheek	11 x 17	8 x 14	9 x 18
5.	70	M	Nodular	Forehead/temporal region	14 x 22	16 x 24	16 x 22
6.	72	M	Nodular	Forehead/temporal region	7 x 12	13 x 15	12 x 15
7.	60	M	Nodular	Ear	7 x 9	8 x 8	8 x 9

[a] *Ulceration present*
During the therapy a flattening of all lesions was observed. Tumor present in all follow-up excisional biopsies.
All tumors were excised 8 weeks after the completion of the therapy.

of the lesions in all seven cases, there was no reduction in the size of the lesions in any case. An example of the nodular basal cell carcinoma lesion before, after and 8 weeks after the completion of the therapy is shown in Figures 1, 2 and 3 respectively.

Histopathological examination of the biopsies taken from the lesions 8 weeks after the completion of the therapy confirmed the presence of tumor in all cases. An example of such a lesion before and 8 weeks after the completion of the therapy is shown in Figures 4 and 5 respectively. In Figure 5 it can be seen that there is an increase in the degree of peritumoral inflammatory infiltrate after the therapy.

A summary of the toxic side effects is depicted in Table II. Fatigue and slight fever were observed in patients 2, 3 and 4 after the first injection. After the second injection, patient 2 had a slight fever and headache whereas patient 3 had only a slight fever. No toxic side effects were observed in patients 1, 5, 6 and 7. All the toxic effects were mild and transient and all the 7 patients were able to complete the therapy.

No laboratory abnormalities were observed in the haematological profile, liver and renal function tests or urine analysis in any patient during or after 4 weeks of the therapy. A complete physical examination of all patients 8 weeks after the completion of the therapy showed them to be in good health.

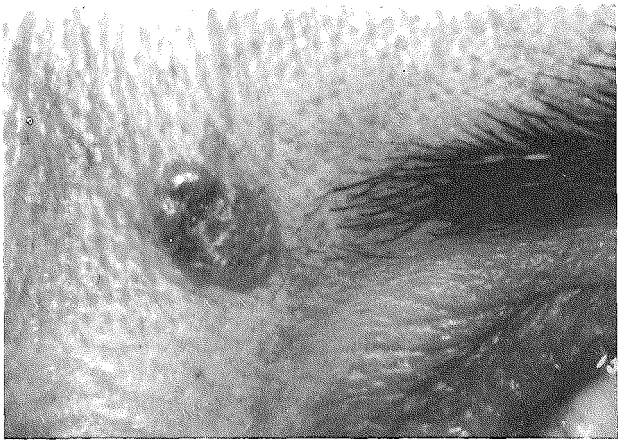

Figure 1:
Nodular basal cell carcinoma lesion in the eyebrow region of patient 3 before therapy.

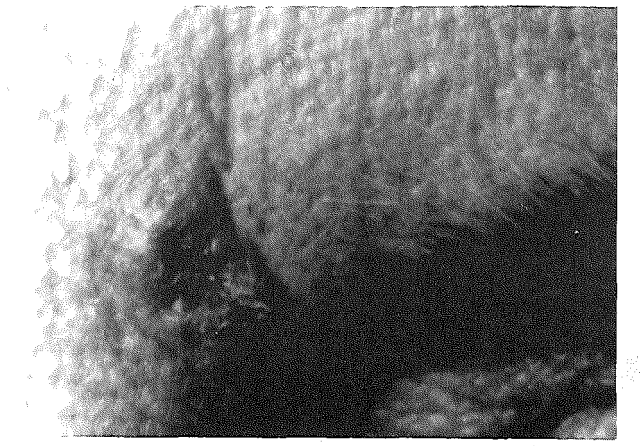

Figure 2:
Nodular basal cell carcinoma lesion in the eyebrow region of patient 3 after receiving 8 interferon injections.

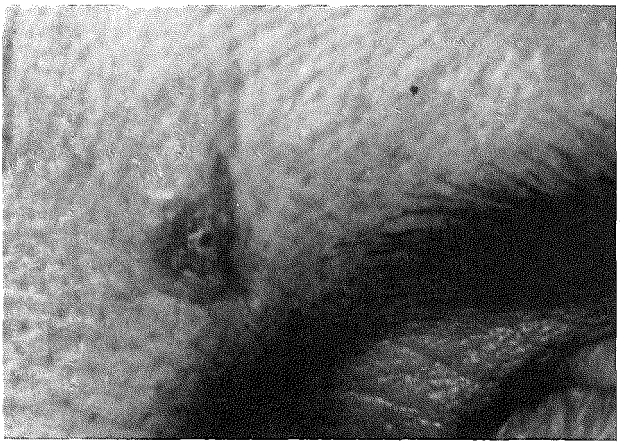

Figure 3:
Nodular basal cell carcinoma lesion in the eyebrow region of patient 3 after a follow-up of 8 weeks posttreatment.

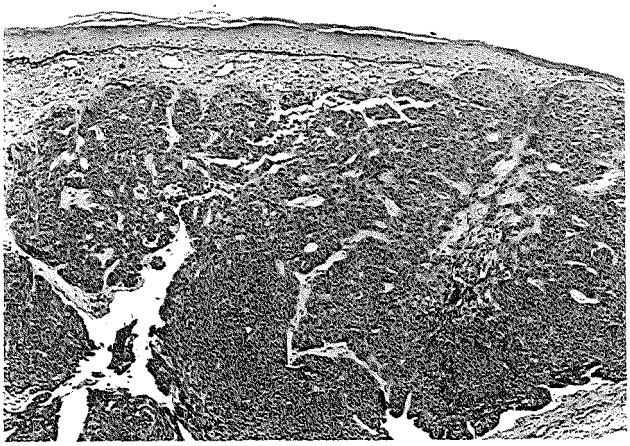

Figure 4:
Photomicrograph of pretreatment biopsy specimen showing nodular basal cell carcinoma. (Hematoxylin-eosin stain; ×60).

Figure 5: Photomicrograph of biopsy specimen of the same nodular basal cell carcinoma after a follow-up of 8 weeks posttreatment. Note the dense peritumoral inflammatory infiltrate (arrows). (Hematoxylin-eosin stain; ×60).

Tabel II. Toxic side effects during treatment[a]

Patient no.	1	2	3	4	5	6	7	Total
Fever (after first dose)		+	+	+				3
Fever (after other doses)			+					1
Malaise								0
Itching (at site)								0
Light headedness								0
Pain (at site)								0
Headache		+						1
Fatigue		+	+	+				3
Perspiration		+						1
Muscle aches								0
Chills								0

[a]All the toxic effects were mild and transient.

DISCUSSION

The therapeutical potential of human recombinant interferon gamma (rIFN-γ) has received considerable attention. It is only recently that human rIFN-γ has become available in sufficient quantities to evaluate its use as an anticancer agent. A number of clinical studies have recently been reported in which rIFN-γ was used as an anticancer agent to treat patients with malignant solid tumors (12, 13, 14), rheumatoid arthritis (15) and skin diseases such as psoriasis (16, 17) and lepromatous leprosy (11). No clinical studies have been reported on the use of rIFN-γ in basal cell carcinoma (BCC). Recently, Greenway et al (6) evaluated the effectiveness of intralesionally injected alpha-2 interferon in 8 patients with BCC. Their results showed that the therapy led to the complete cure of BCC and had mild side effects.

In the pilot clinical trial reported here, the clinical efficacy of low-dose rIFN-γ therapy was evaluated in seven patients with biopsy-confirmed nodular type BCC. Since no guidelines were available for the treatment of BCC with rIFN-γ, a conservative approach was adopted. The choice of dose and the route of rIFN-γ administration were aimed at minimizing the dose-related toxic side effects and were similar to those used in a previous study by Nathan and coworkers to treat 6 patients with lepromatous leprosy (11). In the present study, each patient received a total of 1.6×10^6 IU rIFN-γ administered intralesionally on an outpatient basis.

The results of this pilot trial clearly showed that intralesional therapy with low-dose rIFN-γ was ineffective. No antitumor effect was observed since there

was no reduction in the size of the lesions during or after the completion of the therapy. However, a flattening of the lesions was noted in all 7 patients during the therapy. The presence of tumor was also confirmed by histopathological examination of biopsies taken 8 weeks after the completion of the therapy.

The clinical ineffectiveness of rIFN-γ therapy reported here is in marked contrast to the highly effective alpha-2 interferon therapy reported by Greenway et al (6). In their study, although the type of BCC (superficial or nodular) and the route of administration (intralesional) of alpha-2 interferon were identical to that of the present study, a much higher dose (about 8 fold) of alpha-2 interferon was used. However, this marked difference in the total dose used in the two studies does not explain the opposite therapeutic effects on the same type of BCC. It is noteworthy, that although the low-dose rIFN-γ that was used in the present study showed no antitumor effect did show immunomodulatory effects (Tank et al. manuscript in preparation).

It is also possible that the low-dose which was used for therapy in the present study was insufficient to boost the natural killer (NK) cell activity, to induce the release of interleukin-2 required for T cell activation, and to induce the expression of the major histocompatibility complex class II (HLA-DR) antigens on the tumor cells of BCC. Normally, the tumor cells of BCC do not express HLA-DR antigens (18). The expression of HLA-DR antigens is essential for a specific T cell-mediated antitumor immune response (19). However, it was recently reported by Schulze et al (16) that rIFN-γ failed to induce the expression of HLA-DR and of β_2-microglobulin in BCC. This may explain the observed lack of antitumor effect reported here.

In psoriasis, the therapeutic effects of recombinant interferons alpha and gamma seem to be the reverse of those observed in BCC. Intramuscular administration of rIFN-γ to patients with chronic plaque-type psoriasis resulted in a marginal therapeutic effect (17), whereas the administration of interferon alpha was reported to cause exacerbation of psoriasis (20). Therefore, it seems very likely that the clinical therapeutic effects of recombinant interferons alpha and gamma in BCC and psoriasis are inherent to the type of interferon that is used for therapy.

Although the results of this pilot clinical trial failed to demonstrate the therapeutic effectiveness of low-dose rIFN-γ in BCC, the intralesional therapy on an outpatient basis was safe and was well tolerated with minimal side effects.

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CHAPTER 9

General Discussion and Summary

Although considerable literature exists on various aspects of basal cell carcinoma (BCC), there are controversies concerning the immuno-histopathology of BCC which not only exhibit a broad spectrum of clinical manifestations, but also of histological appearances. In order to obtain a detailed insight into the immuno-histopathology of BCC, two different investigational approaches were persued. The investigations described in the first half of this thesis (Chapters 2, 3 and 4) were performed in an attempt to investigate whether useful tumor markers were present in BCC. The presence of such markers would be of value in diagnostic histopathology. The investigations described in the second half (Chapters 5, 6 and 7) were mainly focused on the detailed characterization of the inflammatory infiltrate in BCC and Bowen`s Disease (BD) and its role in the defence against these tumors. Investigations into the therapeutic efficacy of an immunomodulatory agent in BCC are described in Chapter 8.

The investigations described in Chapter 2 were undertaken using two monoclonal antibodies (MoAb) which were observed to react with basal cell determinants of normal epidermis. Indirect immunoperoxidase and indirect immunofluorescence techniques were used. The results showed that the two MoAb reacted with different determinants of the epidermal basal cells and BCC tumor cells. Initially, it was believed that one of the Moab had anti- cytokeratin activity. However, upon detailed immunoblotting studies it was observed to lack this activity. At the same time, a number of specific MoAb against specific cytokeratins had become commercially available and it was possible to classify and catalogue the distribution of cytokeratins in various dermatological diseases. As a result of earlier biochemical studies, Moll et al (1,2) had reported that cytokeratin 8 was expressed in some BCC. Therefore, in the studies described in Chapter 3, the distribution of the low molecular weight cytokeratins 7, 8, 18, 19 and the high molecular weight cytokeratin 10 were investigated in BCC using an indirect immunoperoxidase technique and MoAb of specific anti-cytokeratin activity in an attempt to establish whether cytokeratin 8 was expressed in BCC and whether it could be a useful histopathological marker for these tumors. The results showed that cytokeratins 7 and 19 were expressed inconsistently between and within various BCC, whereas cytokeratin 8 was not expressed in any of the BCC that were examined. In order to resolve the discrepancy between the observations of the immuno-histological and biochemical studies, immunoelectron microscopic investigations described in Chapter 4 were conducted. The results failed to confirm the expression of cytokeratin 7 in BCC but the lack of expression of cytokeratin 8 was confirmed in BCC excluding its possible role as a useful histopathological marker. These results also failed to corroborate the results of earlier biochemical studies (1,2).

Previous studies (3-7) on the characterization of the inflammatory infiltrate in BCC were contradictory, especially with regards to its cellular composition and the role of humoral immunity. Furthermore, the presence of NK cells in BCC was not investigated. The detailed characterization of the immune infiltrate in surgically excised BCC is described in Chapter 5. The expression of β_2-microglobulin and the human leukocyte antigen-DR (HLA-DR) on the tumor cells of BCC and their influence on the degree and composition of the peritumoral immune infiltrate is described in Chapter 6. The results showed that the peritumoral infiltrate consisted predominantly of T cells suggesting that these cells played a major role in the defence against BCC. The low number of B cells and NK cells in the infiltrate indicated that these either played a minor role or no role at all in the defence against BCC. The limited involvement of these cells against BCC proliferation was also simultaneously confirmed by Kohchiyama et al (8). The absence of HLA-DR and the decreased expression of β_2-microglobulin on the tumor cells of BCC did not influence the degree or the composition of the peritumoral infiltrate. These results also failed to corroborate the results reported earlier (8). The composition of the infiltrate in SCC differed from that observed in BCC (9). In SCC, not only T cells, but also B cells and NK cells seem to play a role in the defence against tumor proliferation. However, the inflammatory infiltrate in carcinoma in situ, namely Bowen's Disease (BD) has not been previously characterized. Since only a low number of carcinoma in situ ever invade the dermis, an insight was obtained into whether the inflammatory infiltrate played a role in preventing invasive growth and whether its composition differed from that observed in BCC and SCC. In the studies described in Chapter 7, this infiltrate was characterized and the expression of β_2-microglobulin and HLA-DR on the tumor cells of BD was investigated. The results showed that the infiltrate consisted predominantly of T cells which suggested a T cell-mediated anti- tumor response which may be at least in a proportion of BD directly or indirectly responsible for the low incidence of the development of BD into invasive carcinoma. Langerhans cells and cells of macrophage/monocyte lineage also played a supportive role. HLA-DR was expressed on tumor cells only in those cases where T cells invaded the tumor. However, a larger number of tumors must be evaluated prior to drawing any firm conclusions.

In recent years, the therapeutic potential of biological response modifiers such as interferons has received much attention in cancer (10). The production of these agents in adequate amounts via the recombinant-DNA technology has permitted their clinical evaluation as anti-cancer drugs. Recently, two studies were reported in which human recombinant interferon-alpha 2a (11) and -alpha 2b (12) were administered intralesionally to patients with BCC. The results of these two clinical trials were contradictory in that interferon-alpha 2a was observed to have no anti-tumor effect, whereas interferon-alpha 2b was shown

to be effective. Although interferon gamma is a potent immunomodulatory agent, its therapeutic potential in BCC had not been investigated. Therefore, the pilot clinical study described in Chapter 8 was undertaken. There was no anti-tumor response in any case after intralesional therapy with low-dose human recombinant interferon gamma in BCC. This failure could have been due to the low dose that had been used. However, the final assessment concerning the therapeutic value of this drug has to await further clinical investigations.

Concluding remarks

One of the commonest epithelial cancers in caucasians is BCC. At present it is expected that the incidence of BCC will increase in the future and as a result the morbidity of this disease in the western population is also expected to increase. Considering this aspect, the investigations reported in this thesis contribute towards a clearer insight into the immunohistological aspects of BCC which may form the basis for further research and may contribute towards the development of new effective therapeutic modalities.

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CHAPTER 10

Samenvatting

Het basaalcelcarcinoom (BCC) is de meest frequent voorkomende huidmaligniteit bij het blanke ras. Zowel klinisch als histologisch kan het BCC zich op verschillende wijzen manifesteren. Hoewel over de diverse aspecten van het BCC veel gepubliceerd is, bestaan er ten aanzien van de immuno-histopathologie tegenstrijdige bevindingen.

Hoofdstuk 1 geeft een inleiding in het onderwerp en een overzicht van de diverse aspecten van het BCC.

Om een gedetailleerd inzicht te krijgen in de immuno-histopathologie van het BCC werden twee verschillende onderzoekslijnen gevolgd. In de eerste helft van het proefschrift (Hfdst. 2, 3 en 4) wordt het onderzoek beschreven dat tot doel had bruikbare tumor markers voor BCC op te sporen. Het gebruik van deze markers is van belang voor de diagnostiek in de klinische pathologie.

In hoofdstuk 2 worden de resultaten beschreven van onderzoek met twee monoclonale antilichamen die reageren met verschillende determinanten van de epidermale basale cellen. Hierbij werd aangetoond dat een van deze monoclonale antilichamen (MoAb 12G7) reageerde met een membraan gebonden determinant van zowel de basale cellen van de normale epidermis als van de tumorcellen van het BCC. Het andere monoclonale antilichaam (253B7) reageerde met een cytoplasmatische determinant van de normale epidermale basale cellen en in 15% van de onderzochte BCC met een cytoplasmatische determinant van de tumorcellen. Er werd aanvankelijk verondersteld dat de cytoplasmatische determinant een cytokeratine was. Met behulp van immunoblotting experimenten vertoonde dit monoclonale antilichaam echter geen anti-cytokeratine activiteit.

Inmiddels kwamen diverse monoclonale antilichamen met specifieke anti-cytokeratine activiteit commercieel beschikbaar, zodat in een volgende studie de expressie van de cytokeratines 7, 8, 18 en 19 met een laag moleculair gewicht en cytokeratine 10 met een hoog moleculair gewicht in het BCC bestudeerd kon worden (Hoofdstuk 3). Met name het onderzoek naar de expressie van cytokeratine 8 in het BCC was van belang, daar in het verleden uit biochemisch onderzoek is gebleken dat cytokeratine 8 in sommige BCC voorkomt. In geen van de 21 onderzochte BCC kwam cytokeratine 8 tot expressie; wel werden de cytokeratines 7 en 19 aangetoond. In een daaropvolgend onderzoek werd met behulp van een immuno-electronenmicroscopische techniek getracht de bestaande discrepantie tussen de immunohistologische en biochemische bevindingen op te heffen (Hoofdstuk 4). Ook in deze studie kon cytokeratine 8 niet worden aangetoond in BCC. Hieruit werd geconcludeerd dat cytokeratine 8 niet kan dienen als een bruikbare histopathologische tumor marker voor het BCC.

De onderzoekingen in de tweede helft van het proefschrift (Hfdst. 5, 6 en 7)

hadden tot doel het ontstekingsinfiltraat in het BCC en in het intra-epidermale carcinoom (Morbus Bowen) immunohistologisch te typeren en de mogelijke rol van dit infiltraat ten aanzien van de tumorafweer te bestuderen. Hoewel in het verleden diverse onderzoekingen zijn verricht naar de karakterisering van het ontstekingsinfiltraat in het BCC, waren de resultaten niet eensluidend, vooral niet ten aanzien van de samenstelling van het ontstekingsinfiltraat en de rol van de humorale immuniteit. Bovendien was de aanwezigheid van NK-cellen in het BCC niet onderzocht. Uit de in hoofdstuk 5 beschreven resultaten blijkt dat het ontstekingsinfiltraat in het BCC voornamelijk uit T-cellen bestaat. B-cellen en NK-cellen zijn slechts in een gering aantal aanwezig. Hieruit kan geconcludeerd worden dat T-cellen een belangrijke rol spelen in de tumorafweer, terwijl de rol van B-cellen en NK-cellen waarschijnlijk beperkt is. Verder werd aangetoond dat het humaan leucocyten antigeen DR (HLA-DR) niet tot expressie komt en dat β_2-microglobuline slechts in een aantal gevallen tot expressie komt op de tumorcellen van het BCC (Hoofdstuk 6). Noch de mate van het ontstekingsinfiltraat noch de samenstelling van het infiltraat werd hierdoor beinvloed. Bij plaveiselcelcarcinomen van de huid blijken behalve T-cellen ook B-cellen en NK-cellen een rol te spelen in de tumorafweer. Daar over de compositie van het ontstekingsinfiltraat en zijn rol in de preventie van invasieve groei van het intra-epidermale carcinoom (Morbus Bowen) niets bekend was, werd het infilraat in deze tumor immunohistologisch getypeerd en de expressie van HLA-DR en β_2-microglobuline op tumorcellen onderzocht (Hoofdstuk 7). Het infiltraat bestond voornamelijk uit T-cellen. Bij meer dan de helft van de onderzochte intra-epidermale carcinomen werd geobserveerd dat T-cellen de tumor binnendrongen en dat de tumorcellen positief waren voor HLA-DR. Tevens bestond er een toename van Langerhans cellen en macrophagen. Deze bevindingen leidden tot de veronderstelling dat de T-cel gemedieerde tumorafweer direct of indirect ertoe bijdraagt, dat de overgang van het intra-epidermale carcinoom naar een invasief groeiend carcinoom wordt voorkomen. Om definitieve conclusies te kunnen trekken dient echter een groter aantal tumoren onderzocht te worden.

De laatste jaren wordt veel aandacht besteed aan de therapeutische waarde van zogenaamde "biological response modifiers", zoals interferonen, bij kanker. Dankzij de recombinant DNA-techniek kan voldoende van deze stoffen geproduceerd worden. Dit heeft o.a. geleid tot klinisch onderzoek bij de behandeling van diverse soorten kanker. In hoofdstuk 8 worden de resultaten beschreven van een klinische studie naar het effect van humaan recombinant interferon-gamma, een potent immuno-modulerend agens, dat intralesionaal in een lage dosis toegediend werd bij patiënten met een BCC. Bij geen van de

patiënten werd een verbetering waargenomen. Voordat echter een eindoordeel kan worden gegeven over de therapeutische waarde van interferon-gamma dient er verder klinisch onderzoek verricht te worden.

De incidentie van het BCC, de meest voorkomende vorm van huidkanker, zal naar verwachting in de westerse wereld toenemen. Deze ontwikkeling in ogenschouw nemend vormen de bevindingen in dit proefschrift een basis voor verder onderzoek en dragen zij bij tot een beter begrip van de immunohistologie van het BCC. Tevens kan dit proefschrift een bijdrage leveren aan de ontwikkeling van nieuwe therapeutische modaliteiten.

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CURRICULUM VITAE

De auteur van dit proefschrift werd op 31 maart 1953 te Heerlen geboren. Hij behaalde in 1972 het diploma HBS-B aan het St.Janscollege te Hoensbroek. In hetzelfde jaar begon hij met de studie Biologie aan de R.U. te Utrecht en in 1976 werd het kandidaatsexamen B5* behaald. In 1977 werd met de studie Geneeskunde gestart aan voornoemde universiteit.

In 1979 werd het doctoraal examen Biologie behaald met als hoofdvak Antropobiologie, als bijvakken Immuno-Haematologie en Biohistorie en als nevenvak Klinische Immunologie. In 1983 behaalde hij het artsexamen en begon direct hierna met de specialisatie tot dermato-venereoloog op de afdeling Dermatologie en Venereologie van het Academisch Ziekenhuis Dijkzigt Rotterdam (Hoofden: Prof. Dr. Th. van Joost en Prof.Dr. E. Stolz). Op deze afdeling werd in 1984 begonnen met het in dit proefschrift beschreven onderzoek. In 1987 vond registratie plaats als dermato-venereoloog.

Sinds 1 mei 1988 oefent hij de dermatologische praktijk uit in Middelburg en Vlissingen, in associatief verband met I. Vermeiden, en is hij als staflid verbonden aan de Stichting Streekziekenhuis Walcheren.