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

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Summary. We investigated the dermal inflammatory infiltrate and the expression of HLA-DR and β_2-microglobulin on the tumour 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 ($55 \pm 21\%$) 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 $58 \pm 18\%$, Langerhans cells (LC) $4 \pm 1\%$ and Leu-M5 positive cells (monocytes/macrophages) $9 \pm 2\%$. The mean percentage of B cells and natural killer (NK) cells was $4 \pm 5\%$ and $1 \pm 2\%$ respectively. B cells and NK cells did not invade any of the tumours. In the 5 BD with moderate or heavy infiltrate, T_H cells, $T_{s/c}$ cells and Leu-M5 positive cells did invade the tumour. In the tumour area where there was invasion, the number of LC was increased and HLA-DR was expressed on the cells. β_2-microglobulin was generally expressed on the tumour cells of BD. We concluded that there is evidence for a T cell-mediated anti-tumour immune response which may account for the infrequent invasive growth in BD.

Key words: Bowen's Disease – Inflammatory Infiltrate

Introduction

Bowen's disease (BD) is an uncommon intraepithelial cancer of the skin. Inorganic arsenic and exposure to sun light are considered as possible aetiological factors. Although invasive growth is rare, at least 5% of the lesions are known to invade the underlying dermis (Maize 1979). In these cases, more than one third of the patients will develop metastasis. In contrast to BD, in solar keratosis, in which 10% to 15% of the lesions develop into invading squamous cell carcinoma, metastasis has not been observed (Graham et al. 1966).

In most BD, a moderate 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 carcinoma 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 tumour defence we typed this infiltrate and investigated the expression of β_2-microglobulin and HLA-DR on tumour 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 head and neck 3, arms 2 and trunk region 3. For histological examination of the tumour the samples were cut in two equal parts. One was fixed in formalin and paraffin embedded and the diagnosis

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of BD 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 1. 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 tumour. The mean composition of the infiltrate in the 8 BD was $55\pm21\%$ Leu-4 positive (pan *T*) cells, $50\pm19\%$ Leu-3a positive (T_H) cells, $23\pm11\%$ Leu-2a positive ($T_{s/c}$) cells, $4\pm1\%$ Leu-6 positive cells (LC), $1\pm2\%$ Leu-7 positive (NK) cells, $4\pm5\%$ Leu-14 positive (B) cells, $9\pm2\%$ Leu-M5 positive cells (monocytes/macrophages) and $58\pm18\%$ 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 tumour 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 tumours. In the 5 cases with moderate and heavy infiltrate there was an increase in LC in the lower part of the tumour in the region where the *T* cells and macrophages invaded (Fig. 4) when compared with the marginal epidermis. In addition, in that area the tumour cells expressed HLA-DR (Fig. 5).

β_2-microglobulin was expressed on the tumour cells of all the 8 BD. However, in 3 cases (cases 5,

Table 1. Summary of phenotypes of infiltration and expression of β_2-Microglobulin (β_2-MG) and HLA-DR on tumour 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 tumour 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.[a]	moderate	80	60	30	2.0	5	1	4	10	70	+	(+)
5.[a]	moderate	40	35	25	1.4	5	1	7	10	60	+	(+)
6.[a]	moderate	60	70	35	2.0	3	0	2	8	60	+	(+)
7.[a]	heavy	80	70	35	2.0	5	5	15	10	60	+	(+)
8.[a]	heavy	70	65	25	2.6	2	1	3	10	76	+	(+)
Mean±SD		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.

[a] Invasion of tumour by immunocompetent cells

\+ = Membrane staining of tumour cells

(+) = Membrane staining of tumour cells in the lower part of the tumour close to the dense dermal infiltrate

− = No staining

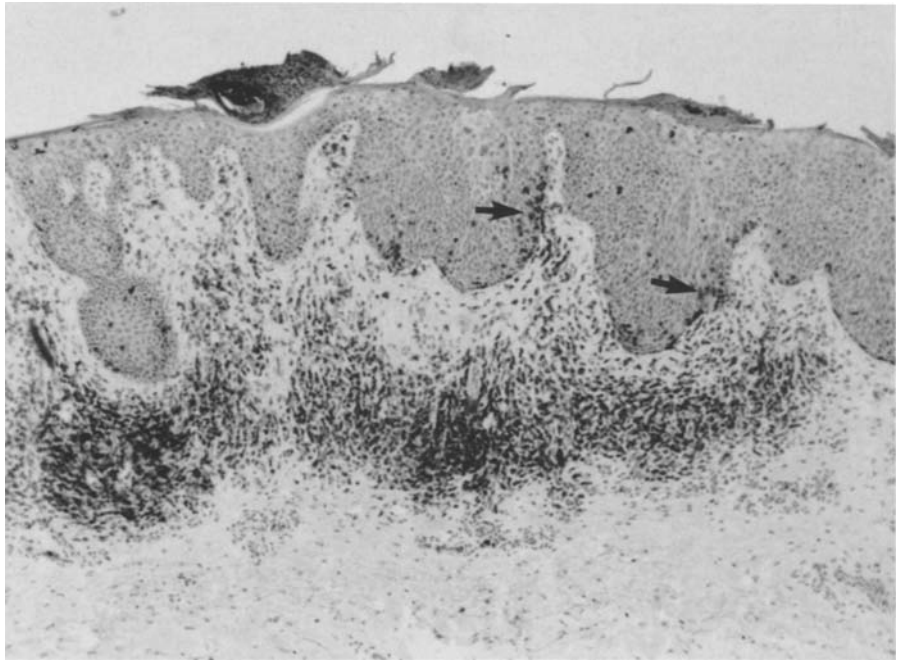


Fig. 1. Cryostat section (5 μm) of Bowen's Disease showing a heavy dermal infiltrate with more than 75% Leu-4 positive (pan T) cells. MoAb Leu-4, IIP technique, ×55. *T* cells invade the tumour (*arrows*)

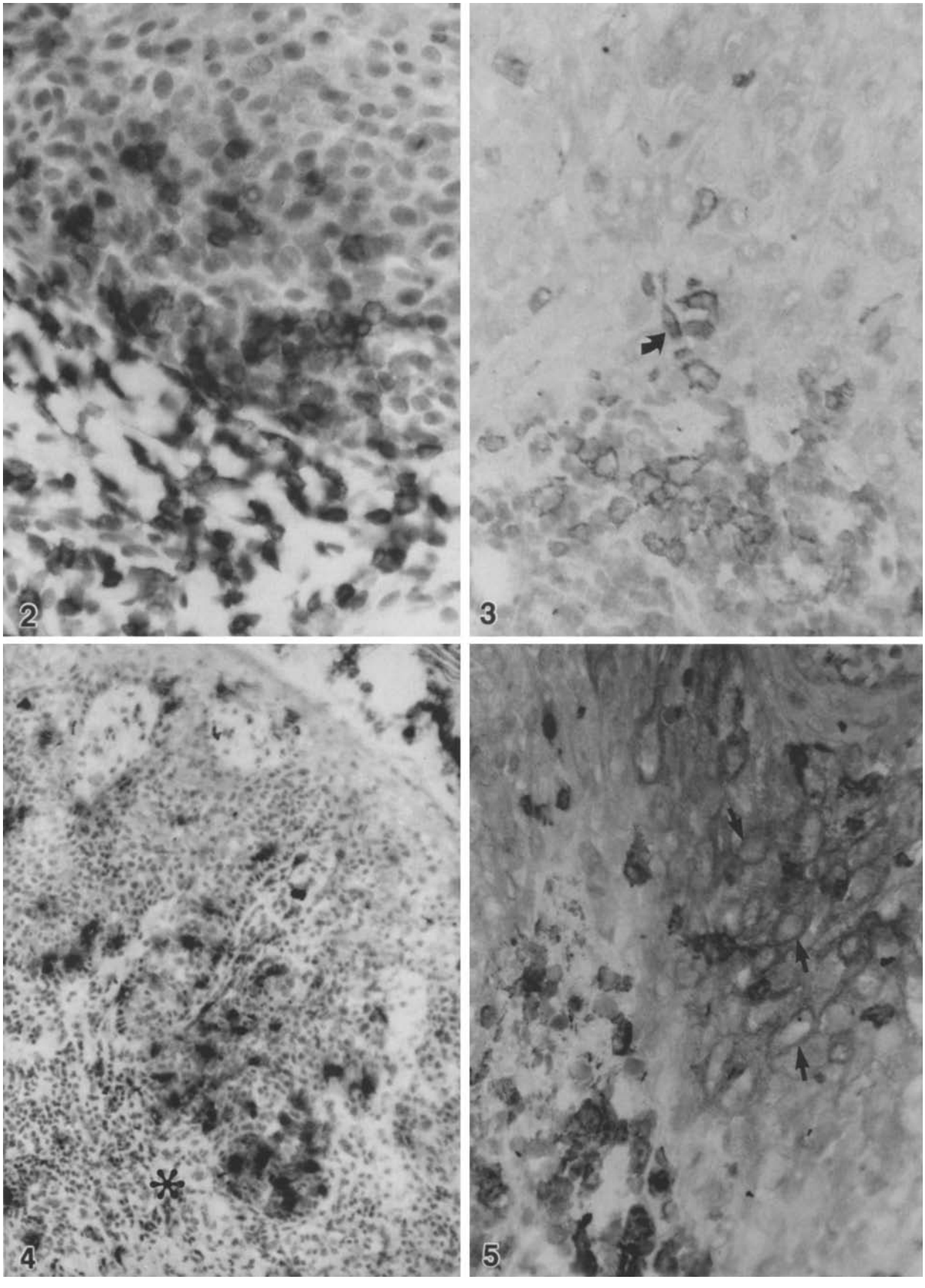
6 and 8) groups of tumour cells were either stained very weakly or not at all, but those cells which were in contact with the immunocompetent cells present in lower part of the BD were always stained.

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 tumours (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 a moderate or heavy infiltrate, T_H and $T_{s/c}$ cells invaded the tumour and were observed to be in contact with the tumour cells. At the site of invasion, the cells were observed to be HLA-DR positive and the number of Langerhans cells (LC) in the same area was increased when compared with the rest of the tumour. In the remaining 3 BD (cases 1–3) with a mild infiltrate, the tumour was not invaded either by T_H or by $T_{s/c}$ cells and HLA-DR was not observed on the cells. This suggests that the expression of HLA-DR in BD may be dependent on whether or not there is an invasion of the tumour by *T* cells. However, a larger number of patients must be investigated to confirm this observation. The expression of HLA-DR on the tumour cells has been observed in melanoma (Ruiter et al. 1982; Brocker et al. 1984) and colorectal cancer (Daar et al. 1983). In neither of these tumours was there a correlation between the degree of mononuclear infiltrate and the expression of HLA-DR on the tumour cells. In basal cell carcinomas and squamous cell carcinomas HLA-DR was not expressed on the tumour 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 study (Habets et al. 1989) contradict their findings. In the present study β_2-microglobulin was expressed on the tumour 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 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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expressed on the tumour cells, activation of T_H and $T_{s/c}$ subsets may occur leading to a T cell-mediated anti-tumour response. The tumour 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-1, interleukin-2 and interferon-gamma). Since HLA-DR positive tumour cells were not observed in BD with a mild inflammatory infiltrate, it seems unlikely that the expression of HLA-DR on the tumour cells preceeds the activation and proliferation of the immune infiltrate. In the BD with a moderate or heavy infiltrate, we observed an accumulation of LC in the lower part of the tumour 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 tumour cells.

The anti-tumour activity of macrophages includes non-specific killing of tumour cells, killing by anti-tumour antibodies via antibody-dependent cell-mediated cytotoxicity (ADCC) or acting as antigen-presenting cells (APC) for tumour antigens (Mantovani et al. 1985; Hamilton et al. 1987). We observed $9 \pm 2\%$ Leu-M5 positive cells some of which invaded the tumour. 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 tumour cells. These observations suggest that macrophages act as anti-tumour 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-tumour antibodies. Since activated macrophages were observed in contact with the tumour cells, in these cases it is possible that tumour 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, suggesting that B cells played a role in the local anti-tumour response. In other cutaneous tumours 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.

NK cells are considered to be the first line of defence against tumours (Herberman et al. 1979, 1981). In this and in our previous study on basal cell carcinoma (Habets et al. 1988) a minimal number of NK cells were observed. This suggests a limited role for NK cells in local defence. A low number of NK cells have also been reported in melanoma (Ralfkiaer et al. 1987) and since the number of NK cells in most tumours 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-tumour response in BD in which the Langerhans cells and the cells of macrophage/monocyte lineage 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 be directly or indirectly responsible for the low incidence of the development of BD into invasive carcinoma at least in a proportion of BD.

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

Fig. 3. Cryostat section (5 μm) 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 tumour cells (*arrow*). MoAb Leu-M5, IIP technique, × 340

Fig. 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 tumour. MoAb Leu-6, IIP technique, × 135. Note the dense dermal infiltrate (*asterisk*)

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

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