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Squamous cell carcinoma: infiltrating monocyte/macrophage subpopulations express functional mature phenotype

C. Neuchrist[1], M. Grasl[2], O. Scheiner[1], H. Lassmann[3], K. Ehrenberger[1] & D. Kraft[1]

[1]*Institute of General and Experimental Pathology, Währingerstrasse 13, 1090 Vienna;* [2]*Department of Otolaryngology, and* [3]*Institute of Neurology, University of Vienna, Vienna, Austria.*

Summary Biopsies from 26 patients with advanced stage squamous cell carcinoma of the head and neck were investigated to determine the intensity of the inflammatory cellular infiltrate and the expression of leucocyte antigens. Mononuclear cell infiltration varied considerably between the individual patients and also within the tumour. Tumour-infiltrating cells consisted mainly of T lymphocytes and monocytes (Mo)/macrophages (MΦ). Staining procedure with monoclonal antibodies (moabs) against Mo/MΦ revealed different clusters of antigen expression: (1) moabs 27E10 and a-CD35 detected a subgroup of Mo/MΦ with particular staining of perivasal Mo; (2) moab a-CD1 stained preferentially cells in tumour cell clusters; (3) moabs that reacted with cells of either typical MΦ or dendritic morphology throughout the tumour-tissue: a-Fcγ receptor I–III, a-class II antigens, a-CD4, Rm3/1, a-CD36 and 25F9. Thus, the majority of tumour-infiltrating mononuclear phagocytes were found to possess a rather mature phenotype. The number of Mo/MΦ with mature phenotype within the tumours correlated with T lymphocyte infiltration in the tissue.

Tumour growth is influenced by cells and/or products of the tumour's microenvironment. These influences are mainly due to cells of the defence system (Böheim *et al.*, 1987; Kopper & Lapis, 1985; Zeromski *et al.*, 1986; Kabawat *et al.*, 1983). *In vitro* assays have demonstrated a significant role for T cells, natural killer (NK) and lymphokine-activated killer (LAK) cells in killing and lysing of neoplastic cells (Patek & Collins, 1988; Strohme *et al.*, 1987; Whiteside *et al.*, 1988; Vinzenz & Micksche, 1987; Gottlinger *et al.*, 1985). Activated macrophages (MΦ), also, can bind to and destroy neoplastic cells *in vitro* and *in vivo* (Fidler & Schroit, 1984; Fidler & Schroit, 1988; Kopper & Lapis, 1985). Furthermore, MΦ, via cytokines, are capable of enhancing lymphocyte-mediated immunity (Bentzen, 1988). On the other hand, depending on their localisation in tissue, MΦ may enhance rather than inhibit tumour growth, possible by triggering local immunosuppression (Gronberg *et al.*, 1989; Kopper & Lapis, 1985; Yamanaka *et al.*, 1988; Kronke, 1988). Considering this bimodal role of MΦ in immunesurveillance of tumours *in vitro* we focused our studies on the phenotype and surface receptor expression of MΦ, which could be involved in local immunesurveillance of malignant growths.

Patients and methods

Patients

The study group consisted of 28 patients with histologically confirmed diagnosis of a squamous cell carcinoma of the upper gastro-intestinal and respiratory tract with varying degree of differentiation (Table I). All patients were in an advanced stage of their disease, graded T_3 and T_4 according to the UICC classification of 1987 (Hermanek *et al.*, 1987); 14 patients had evidence of regional metastases, none had distant metastases at the time of surgery. The patients' (25 males, three females) ages ranged from 35 to 71 years (median 56.5). The patients had not received chemotherapy, radiotherapy or previous surgery.

Preparation of tissue sections

Excised tissue blocks, consisting macroscopically of tumour taken from the primary tumour focus, were snap-frozen in liquid-nitrogen cooled methylbutan and stored under liquid nitrogen until use. Serial cryostat sections (6 μm), mounted on glass slides were air-dried for 2 h and fixed at room temperature for 5 min in acetone, then for 5 min in chloroform and finally for 5 min in acetone. The rest of the tumour was fixed in 40% formaldehyde and embedded in paraffin; sections were stained with haematoxilin-eosin for diagnosis and grading at the Department of Pathology of the University of Vienna.

Immunocytochemistry

Source and specificity of monoclonal antibodies (moabs) are listed in Table II. Appropriate dilutions were determined in preliminary experiments. Immunostaining was performed with an indirect peroxidase system as described in detail earlier (Köller *et al.*, 1986).

Double immunostaining

To distinguish between $CD4^+$ MΦ and other $CD4^+$ cells or between $CD16^+$ granulocytes and other $CD16^+$ cells, a double immunohistochemical staining was employed. First, moabs

Table I Patient details

Patient	*TU localisation*	*TNM*	*TU histological grading*
1	oropharynx	T_3N_3	high
2		T_4N_2	high
3		T_4N_3	high
4		T_4N_2	poor
5		T_4N_0	high
6		T_3N_0	poor
7		T_3N_0	high
8	hypopharynx	T_4N_3	high
9		T_4N_3	moderate
10		T_4N_2	poor
11		T_4N_2	high
12		T_3N_0	high
13		T_3N_3	high
14		T_4N_0	high
15		T_4N_0	moderate
16		T_4N_2	poor
17		T_4N_2	poor–moderate
18		T_4N_1	moderate–high
19		T_4N_3	poor
20		T_3N_0	poor
21	larynx	T_4N_0	high
22		T_4N_0	high
23		T_3N_3	moderate
24		T_4N_2	moderate
25		T_4N_2	high
26		T_4N_0	moderate

Correspondence: D. Kraft.
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Table II Monoclonal antibodies

Antibody	*Subclass*	*Dilution*	*Specificity*	*Source*
Anti Leu4	IgG1	1:50	all T cells, CD3	BD (Ledbetter *et al.*, 1981)
Anti Leu3a + b	IgG1	1:3	T helper/inducer, CD4	BD (Wood *et al.*, 1983)
Anti Leu2a	IgG1	1:50	T cytotoxic/suppressor, CD8	BD (Evans *et al.*, 1981)
Anti Leu14	IgG2b	1:3	B cells, CD 22	BD (Leukocyte typing II)
VEP 9	IgM	1:1000	granulocytes, CD15	(Gooi *et al.*, 1983)
Anti HLA-DR	IgG2a	1:100	B cells, Mo/MΦ	BD (Lampson & Levy, 1980)
Anti HLA-DQ	IgG1	1:100	B cells, Mo/MΦ	BD (Chen *et al.*, 1984)
Anti HLA-DP	IgG1	1:100	B cells, Mo/MΦ	BD (Chen *et al.*, 1984)
Vim-12	IgG1	1:100	Mo/MΦ, granulocytes activated T cells, CD11b (CR3)	(Knapp *et al.*, 1984)
Anti-CR1	IgG1	1:200	dendritic reticulum cells, B cells, subset of Mo/MΦ, red cells, granulocytes, CD35	Dako (Gerdes *et al.*, 1982)
3-2	IgG1	1:1000	FcγR I, CD64	Medarex (Graziano & Fanger, 1987)
4-3	IgG2b	1:1000	FcγR II, Mo/MΦ, granulocytes, B cells, endothelium, CD32	Medarex (Looney *et al.*, 1986)
BW209	IgG1	1:100	FcγRIII, granulocytes, NK cells, CD16	Behring (Simmons & Seed, 1988)
Anti Leu6	IgG2b	1:100	60-90% thymocytes, Langerhans cells in the skin, CD1	BD (Wood *et al.*, 1983
27E10	IgG1	1:25	Subpopulation of Mo/MΦ, granulocytes	(Zwadlo *et al.*, 1986)
OKM5	IgG1	1:50	Mo, thrombocytes, CD36	Ortho
25F9	IgG1	1:25	mature MΦ	(Zwadlo *et al.*, 1985)
RM3/1	IgG1	1:100	mature MΦ	(Zwadlo *et al.*, 1987)

Leu4 (a-CD3) or VEP9 (a-CD15) were used as described before and staining procedure continued with the respective second moab (Leu3a = a-CD4 or BW209 = a-CD16) using an alkaline phosphatase/anti-alkaline phosphatase technique (Cordell *et al.*, 1984). Controls consisted of irrelevant antibodies of the IgG1 (BIP-1) and IgM (SQ4F3) isotypes respectively. Both control moabs reacted with *Bet v* I, the major allergen from birch pollen (Jarolim *et al.*, 1989).

Evaluation of tumour infiltrating cells

Cells were counted in five representative areas in a square lattice with 100 squares sized 0.04 × 0.04 mm; final magnification × 400. Positive cells were expressed as absolute numbers of immunostained cells per mm^2. Statistical correlation was performed by linear regression analysis and as the values were unevenly distributed, a non parametric test was used (Mann-Whitney U test) as a two group test.

Results

Clinical histopathology

The tumours were graded histologically into 15 cases of highly, five cases of moderately and eight cases of poorly differentiated squamous cell carcinomas. Mononuclear cell infiltration of the tumours was found to vary considerably between the individual patients and also within each tumour.

T cells, defined by a-CD3, a-CD4 and a-CD8 moabs and MΦ, stained by MΦ-markers (Table II) were located predominantly in the tumour stroma. Variable numbers of T cells (58-1017 mm^{-2}) and particularly MΦ (197-1397 mm^{-2}) were found, some of which had infiltrated also into the tumour cell clusters. Quantitation of tumour-infiltrating cells in 22 patients revealed marked T cell infiltration and concomitantly high numbers of MΦ (Figure 1).

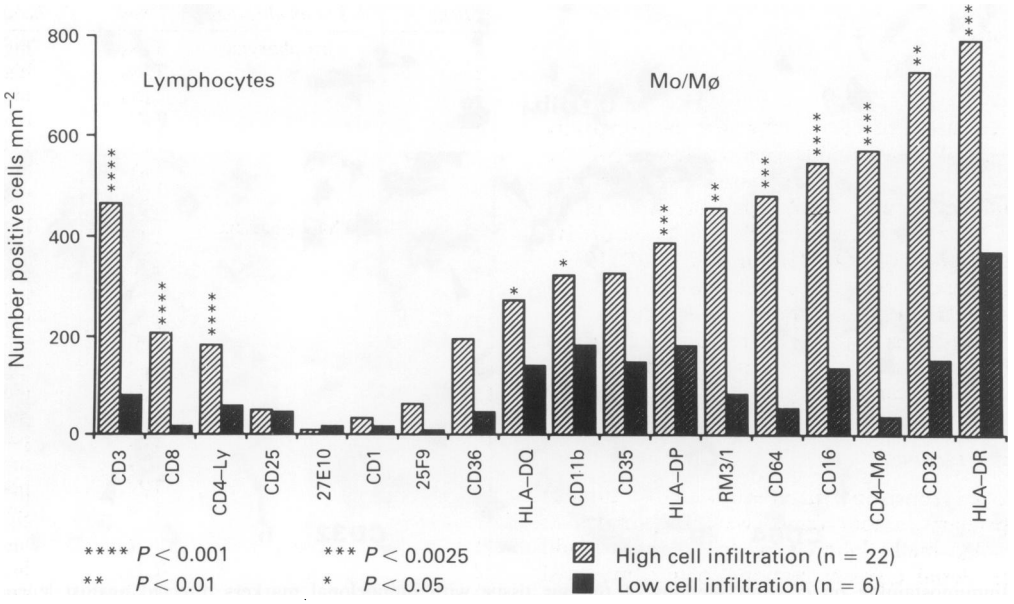

Figure 1 Leucocyte surface antigen-expression on tumour-infiltrating cells: Columns show median values: high T cell infiltration and strong expression of HLA-DR, HLA-DP, FcγRI (CD64), II (CD32), III (CD16), CD4 and Rm3/1 in 22 patients. In contrast, significant fewer MΦ of patients with low T cell infiltration expressed MΦ-antigens characteristic for mature and activated MΦ.

Marker for Mo/MΦ subpopulations

Staining by moabs directed against the three FCγ receptors (Figure 2f,g,h), CD4 (Figure 2c), HLA-DR (Figure 4a), HLA-DP and the Rm3/1 (Figure 2e) antigens respectively, was found on most MΦ present in the specimen studied (500–1400 cells mm^{-2}). Antibodies of this cluster also correlated significantly with each other (Table III). The number of MΦ expressing complement receptors CR1 (CD35) and CR3 (CD11b) varied considerably, forming thus an intermediate group with respect to antigen expression. Moabs a-CD1, a-CD36, a-HLA-DQ, 25F9 and 27E10 recognised only subgroups of Mo/MΦ and were found on about 25–40% of MΦ, defined by HLA-DR and a-Fcγ receptor I–III moabs (Figure 3). Out of these moabs, only a-CD11b correlated significantly with the cluster of moabs mentioned above (Table III). The other moabs (a-CD1, a-CD35, a-CD36, a-HLA-DQ, 25F9, 27E10) formed a different group showing strong correlation between CD36 and CD35 ($r = 0.772$ $P < 0.001$). Moabs 27E10 and a-CD36 particularly stained perivascular cells with monocyte morphology (Figure 3b,d). Anti-CD1, which recognises an epitope on Langerhans cells of the skin, in most cases stained only few cells in tumour specimens, which – unlike T cells or other MΦ – were found mainly within solid tumour nests (Figure 3a).

In six patients, only small numbers (58–155 mm^{-2}) of $CD3^+$ lymphocytes were present in the tumour tissue, and in these cases also the number of MΦ was low (169–502 mm^{-2}). The differences in the MΦ-marker expression between these six and the other 22 patients were significant with respect to moabs a-CD4, a-Fcγ receptors I, II, III, a-HLA-DR and Rm3/1. Only minor or no significant differences were seen with the other Mo/MΦ moabs 27E10, a-CD1, 25F9, a-CD36, a-CD35, a-HLA-DQ and a-CD11b (Figure 1).

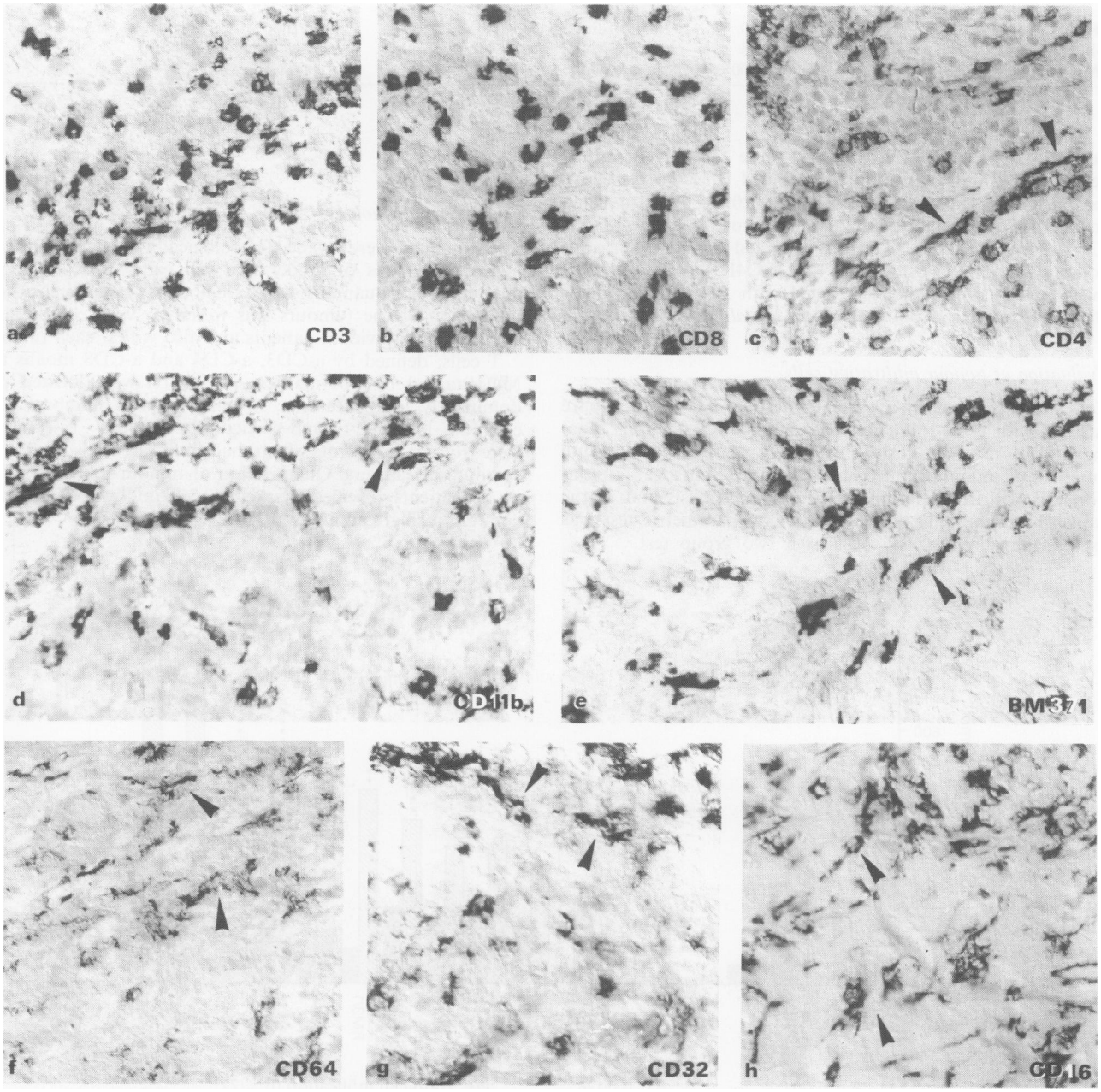

Figure 2 Immunostaining on cryostat sections of tumour tissue with monoclonal markers directed against leucocyte surface antigens. **a–h**, × 244.4, interference contrast, counterstained with nuclear fast red. **a**, Section stained with a-CD3 (T lymphocytes). **b**, Adjacent section stained with a-CD8. **c**, Area similar to that in **a** and **b** stained with a-CD4. **d**, Cells stained with a-CD11b (CR3). **e**, Cells stained with moab Rm3/1. Cells stained with a-CD64 (FcγRI, **f**), a-CD32 (FcγRII, **g**) and a-CD16 (FcγRIII, **h**). Note the dendritic morphology found of positive cells in **c–h** (►).

Table III Correlations of antigen expression

moabs	*CD4-MΦ*	*CD64*	*CD32*	*CD16*	*CD3*
CD4-MΦ	1	0.584**	0.640**	0.793***	0.718***
CD64	0.584**	1	0.696***	0.696***	0.507*
CD32	0.640**	0.883***	1	0.803***	0.414*
CD16	0.793***	0.696***	0.803***	1	0.538**
CD3	0.718***	0.507*	0.414*	0.538**	1
HLA-DR	0.438*	0.518*	0.520*	0.511**	0.383*
HLA-DP	0.383*	0.534*	0.615**	0.591**	0.437*
Rm3/1	$0.202^{n.s.}$	0.771***	0.665***	0.479*	$0.113^{n.s.}$
CD1	0.576**	$0.286^{n.s.}$	0.452*	0.602**	$0.185^{n.s.}$
25F9	$0.346^{n.s.}$	$0.244^{n.s.}$	$0.158^{n.s.}$	0.404*	$0.070^{n.s.}$
CD11b	$0.310^{n.s.}$	$0.228^{n.s.}$	$0.292^{n.s.}$	0.468*	$0.303^{n.s.}$

***$P<0.001$; ** $P<0.01$; *$P<0.05$. n.s. = not significant.

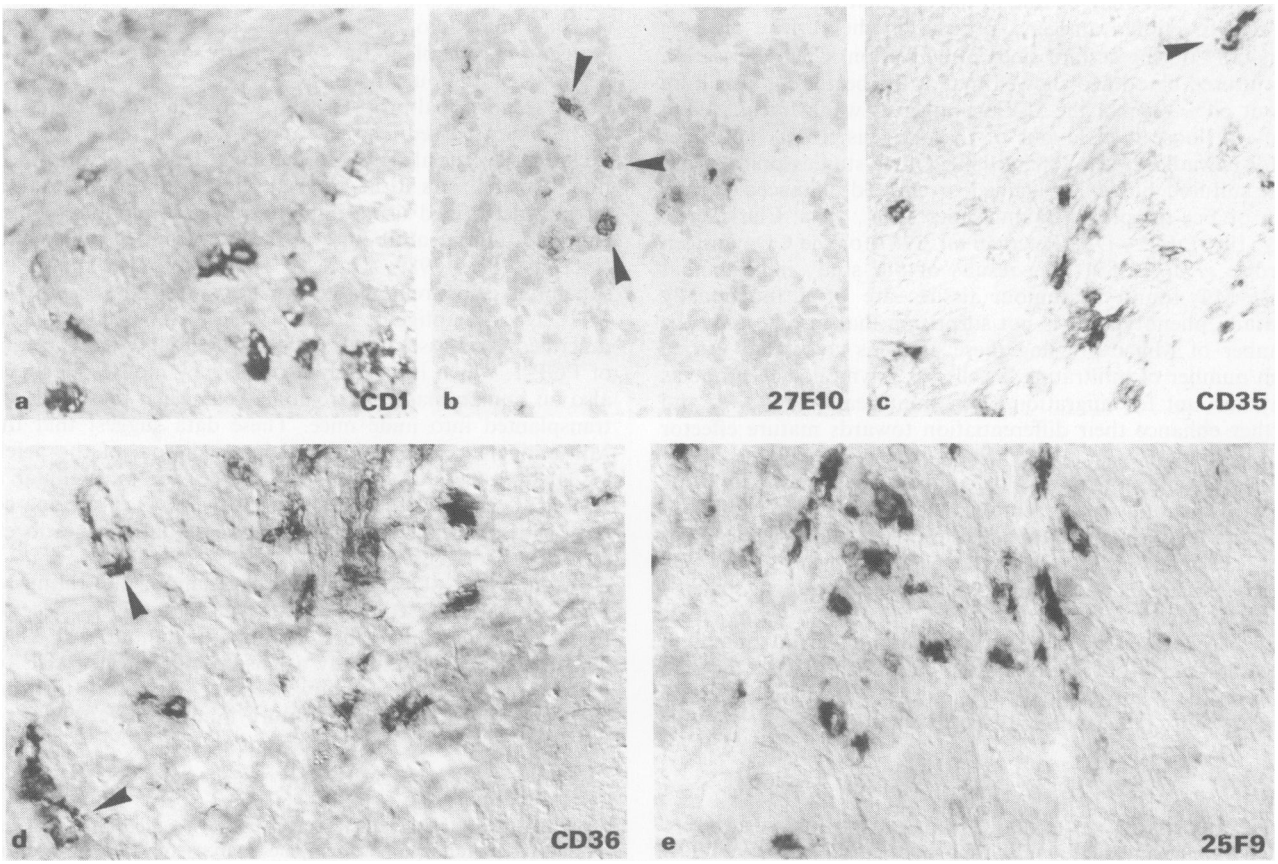

Figure 3 Distribution of Mo/MΦ markers in tumour tissue. **a**–**e**, Cryostat sections, × 249.6, interference contrast, counterstained with nuclear fast red. **a**, Cells stained with a-CD1 (Langerhans cells of the skin). Positive cells are found rather in the solid tumour than in the stroma. **b**, Section stained with moab 27E10 (subpopulation monocytes). Only few cells with monocyte-like appearance are labelled. **c**, Similarly, only few cells are detected by a-CD35 (CR1), similar in shape to that seen in **b**. **d**, Cells stained with a-CD36 (Mo/MΦ). Note the perivascular position of positive cells as found in **b** (►). **e**, 25F9$^+$cells (mature MΦ).

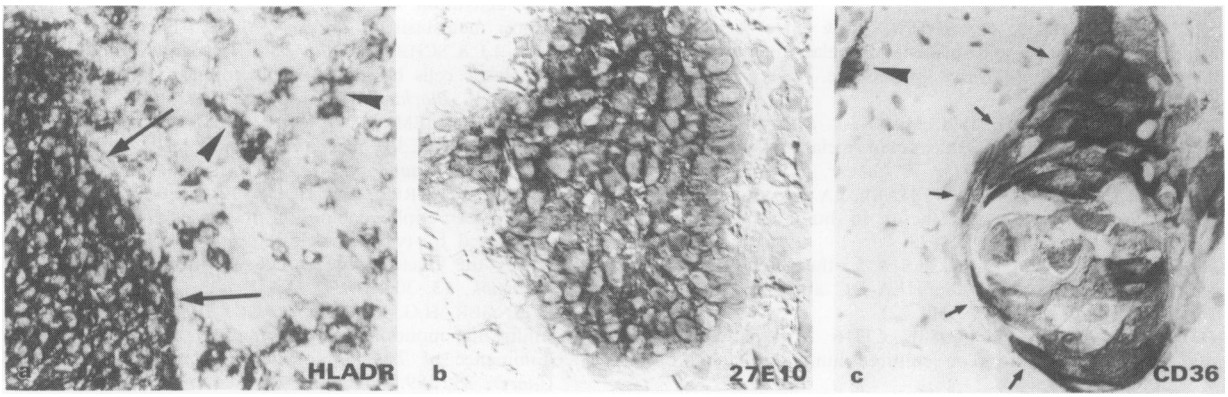

Figure 4 Expression of Mo/MΦ markers on tumour cells. **a**–**c**, × 234, interference contrast, counterstained with nuclear fast red. **a**, Cells with MΦ-morphology (►) but also tumour cells (→) stain positive with a-HLA-DR. **b**, Tumour cells show reactivity with 27E10 (Mo). **c**, Tumour cells, forming a horny pearl, stain positively for a-CD36 (Mo/MΦ) (→). In the upper left corner a CD36$^+$ MΦ

Independently from the histological grading of the whole tumour, in 11 cases the tumour cells expressed class II antigens (Figure 4a). In 13 specimens, tumour cells forming more differentiated parts – like horny pearls or stratified epithelium – reacted with moab 27E10 and a-CD36 (Figure 4*b*,*c*).

Low numbers of B cells ($CD22^+$ cells) and granulocytes ($CD15^+$ cells) were detected in the tissues studied, the latter cells being found predominantly in necrotic areas (data not shown).

Discussion

The highly divergent results on the numbers of tumour infiltrating Mo/MΦ may be due to the fact that these cells mainly express functional antigens, present on the surface of these cells only during certain steps of activation. Characterisation of cultured blood Mo showed that at the beginning of culture about 60% express the 27E10-antigen (Zwadlo *et al.*, 1986) and will loose it in favour of the antigens Rm3/1 and later 25F9 (Zwadlo *et al.*, 1985; 1987). Other studies pointed out that cultured blood Mo gained or showed enhanced expression of Fcγ-receptors (Baumgartner *et al.*, 1988; Clarkson & Ory, 1988), HLA-DR (Peters *et al.*, 1987) or the CD4 antigen (Crowe *et al.*, 1987). The results of our study indicate that most MΦ found in tumour tissues are of a functionally 'mature' phenotype. It is not surprising that in our study the number of MΦ expressing these antigens correlated with a high number of infiltrating T cells, as T lymphocyte products may account for migration of mononuclear phagocytes and further enhance their differentiation towards mature effector cells (Burchett *et al.*, 1988; Makovsky *et al.*, 1988).

It is certainly of interest to compare the tumour infiltrate seen in squamous cell carcinomas with the infiltrate found in tumours and inflammatory tissues of other origin. Other authors found that in malignant melanomas and gastric carcinomas $27E10^+$ and $25F9^+$ macrophages were associated with tumour progression (Bröcker *et al.*, 1987; Heidl *et al.*, 1987; Bröcker *et al.*, 1988). In acute gingival inflammation the dominant Mo/MΦ population carried the 27E10 antigen, whereas the numbers of Rm3/1 and 25F9 positive cells were low (Zwadlo *et al.*, 1985, 1986, 1987). Furthermore, double staining revealed that all three markers labelled distinct, non overlapping MΦ subpopulations. In contrast, in chronic inflammatory processes high numbers of Rm3/1 and 25F9 positive cells were found, some of these cells carrying both antigens simultaneously (Zwadlo *et al.*, 1987). Our present results with the tumour tissue thus resembles the pattern of Mo/MΦ infiltration found in chronic inflammatory lesions.

In spite of the marked inflammatory reaction within the tumours of most patients, we could not correlate the number of T lymphocytes with histological grading or tumour differentiation. This finding makes a major influence of tumour malignancy on the number and composition of inflammatory infiltrates unlikely, although subtle differences may have escaped detection because of the limited number of patients and tumour samples investigated. Furthermore, functional defects of immunocompetent cells or the lack of specific immunological responses against putative tumour antigens may explain the ineffectivity of the infiltration to destroy the tumour tissue.

Thus, functional properties of tumour infiltrating cells have to be characterised *in vitro* and *in situ* in more detail, by excluding a crucial defect in cytokine and cytotoxin production. This appears to be especially important, since trials are already performed using immune response modifiers or specific anti-tumour antibodies in the treatment of malignancies (Ozawa *et al.*, 1989; Mace *et al.*, 1988). The finding that squamous cell carcinoma cells may express MΦ markers (as MHC class II antigens, 27E10, CD36 in this study) was also described by Russel *et al.* (1988), who found the expression of PGP-1, which is strongly expressed by mouse phagocytes, also on human bladder carcinoma cells when these cells were transplanted into nude mice. These data suggest that these MΦ antigens could be induced by mediators of the defence system to generate additional molecules for intercellular adhesion. It seems further likely that squamous carcinoma cells only with a certain differention-grade would be able to express these antigens.

In our study, it is shown that the tumour-infiltrating Mo/MΦ population is equipped with surface antigens necessary for enhanced cellular interaction and tumour cell killing. These MΦ could eventually act as specific effector cells, e.g. when a monoclonal antibody is added – which in turn gives them an important role in future immunotherapy.

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