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Monocyte subpopulations in human gliomas: expression of Fc and complement receptors and correlation with tumor proliferation*

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Summary. Cryostat sections of 12 gliomas and of 3 peritumoral brain tissue samples were investigated for mononuclear cell infiltration by immunohistochemistry, concentrating on cells expressing monocyte/macrophage markers. Only low numbers of T cells were detected in the tumors, whereas in average 20%–30% of all cells present in the samples were recognized by various macrophage markers. These cells carried surface epitopes with known function, like Fc-γ (Fcg) and complement receptors. Microglial cells, in comparison to typical debris laden macrophages, were only recognized by a restricted panel of macrophages markers (anti-Fcg receptors 1, 2, 3, complement receptor CR3, HLA DR, common leucocyte antigen CD45 and the monocyte marker RM3/1). In peritumoral tissue mainly dendritic, microglia-like cells were present, which revealed decreased expression of antigens CD4, RM3/1 and Fcg receptors in comparison to those in gliomas. A significant positive correlation was found between the number of RM3/1 or CR3 (CD11b)-positive cells and the proliferation rate of the tumors as documented by the number of bromodeoxyuridine-positive or Ki-67$^+$ cells.

Key words: Glioma – Macrophages – Microglia – Fc-receptors – Complement receptors

Immunotherapy has recently been suggested as an attractive new strategy for treating brain tumors [6, 8, 10]. However, the mechanisms of immune surveillance of malignomas, especially of brain tumors, are at present poorly understood. In addition to specific recognition of tumor-associated antigens, an effective immune-mediated destruction of tumor cells requires the presence of activated effector cells.

Immunocytochemical studies revealed the presence of T lymphocytes [44], B lymphocytes [54] and suppressor/cytotoxic T cells [23, 28, 29, 45, 51] in human gliomas. Furthermore, a significant proportion of infiltrating mononuclear cells was identified as macrophages [20, 26, 28, 34, 38], numbering up to 41% of total cells in short-time glioblastoma cultures [26, 53].

However, highly divergent results on the numbers of macrophages in gliomas have been reported. This may be partly due to problems in the selection of appropriate macrophage markers. In contrast to most T and B cell markers, antibodies directed against cells of the monocyte/macrophage lineage in general recognize epitopes, which are expressed on the surface of the cells only during certain steps of activation and, thus, only detect a proportion of total macrophages [11, 21, 49, 56].

In this present study we used a panel of monoclonal antibodies, directed against different monocyte/macrophage antigens, to determine the total number of this cell population in gliomas of different malignancy, to elucidate some of their functional properties and to evaluate a possible correlation between the proliferation of the tumor and the infiltration by mononuclear cells.

Materials and methods

Tissue specimens

Fresh tissue specimens were obtained at craniotomy from 15 patients undergoing tumor resection. The material included 12 gliomas and 3 samples of peritumoral brain tissue (Table 1). The peritumoral brain tissue was obtained from cases with pilocytic astrocytoma (case 13), glioblastoma (case 14) and metastatic melanoma (case 15). In all samples the peritumoral tissue was several centimeters apart from the tumors and did not contain tumor tissue, detectable by light microscopy. The tissue was embedded in OCT (Tissue Tek), snap frozen and stored at −70° C until use. The tissue was cut at 7 μm in a cryostat, dried for 30 min at room temperature, immersed in acetone for 10 min and then dried again for 20 min. Dried sections

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Table 1. Summary of clinicopathologic data

Patients			Neuropathological diagnosis	Inflammation[a]	Necrosis[b]	Comments
No.	Age	Sex				
1	5	m	Rec. pilocytic astrocytoma	+	−	
2	63	m	Astrocytoma	+	−	
3	38	m	Astrocytoma	+	−	
4	64	f	Glioblastoma	+	++	
5	72	f	Glioblastoma	+	+	
6	63	m	Glioblastoma	+	+	
7	55	m	Glioblastoma	+	++	
8	56	m	Glioblastoma	+	++	
9	34	f	Rec. astrocytoma	++	++	Radiation damage
10	64	m	Rec. glioblastoma	++	+	
11	28	f	Rec. glioblastoma	+	+	
12	59	f	Rec. glioblastoma	++	+	
13	5	m	Peritumoral tissue	−	−	
14	74	m	Peritumoral tissue	−	−	
15	26	m	Peritumoral tissue	−	−	

No: patient number; m: male; f: female; rec: recurrent;
[a] + = few perivascular infiltrates; ++ = pronounced inflammation
[b] + = small, focal; ++ = large, confluent

Table 2. Monoclonal antibodies

Antibodies	CD	Subclass	Specificity	Source/ eference
Leu-4	CD3	IgG1	All T cells	BD
Leu-3a + b	CD4	IgG1	T helper	BD
Leu-2a	CD8	IgG1	T suppressor	BD
3-2	CD64	IgG1	Fcg 1	Medarex [17]
4-3	CD32	IgG2b	Fcg 2	Medarex [24]
BW209	CD16	IgG1	Fcg 3	[43]
a-CR1	CD35	IgG1	CR 1	Dako
Vim12	CD11b	IgG1	CR 3	[22]
RM3/1	–	IgG1	MO	[58]
25F9	–	IgG1	Mature MO	[57]
Leu-M3	CD14	IgG2b	Granuloc. MO	BD
OKM5	CD36	IgG1	MO thrombc.	Ortho
27E10	–	IgG1	Early MO	[57]
a-Hle	CD45	IgG1	CLA	BD
a-HLA-DR		IgG2a	Class II	BD
Ki-67	–	IgG1	Growth fraction	Dako
a-BrdUrd	–	IgG1	S phase cells	BD

BD: Becton Dickinson; CR: complement receptor; Fcg: Fc-gamma receptor; MO: monocytes; granuloc: granulocytes; thromboc: thrombocytes; CLA: common leucocyte antigen; Class II: class II major histocompatibility antigen

were placed in a tightly closed container and stored at −70°C until staining. Other parts of the tumor were incubated for 60 min with bromodeoxyuridine (BrdUrd), fixed in alcohol, embedded in paraffin, cut, and immunostained with anti-BrdUrd monoclonal antibody as previously described [27].

Immunocytochemistry

After thawing, the sections were shortly refixed with chloroform (100%; 5 min) and acetone (100%, 5 min). Immunostaining was performed with an alkaline phosphatase/anti-alkaline phosphatase (APAAP) technique as described in detail earlier [50]. The primary monoclonal antibodies used in this study are listed in Table 2. For nuclear counterstaining nuclear fast red was used.

To distinguish $CD4^+$ T cells from $CD4^+$ monocytes/macrophages, a double-immunocytochemical staining technique was employed. First anti-Leu-4 (anti-CD3) was used to stain T cells with a peroxidase/anti-peroxidase technique; the reaction was visualized with diaminobenzidine. In the second step, $CD4^+$ cells were stained with anti-Leu-3ab antibody; the reaction was visualized by an APAAP method with fast blue as chromogen as described above. This staining resulted in a brown or brown/blue mixed color for T lymphocytes and pure blue color for $CD4^+$ monocytes/macrophages.

Ki-67 and BrdUrd immunostaining was performed as described previously [27]. For control, primary monoclonal antibodies were omitted and irrelevant antibodies of the same IgG subclass were used.

Quantitative evaluation of immunostained cells

The immunostained mononuclear infiltrate in representative areas, omitting areas of necrosis, was counted on serial sections in ten randomly selected high-power fields at a magnification of ×400 for each marker. Care was taken that within the serial sections comparable areas were quantified for the different markers. Each field was delineated by means of a morphometric eye-piece graticule, covering an area of 0.06 mm^2. Labelled cells were expressed either as percentage of total cells (all cells present in the areas screened quantitatively) or as absolute numbers of immunostained cells per mm^2. Determination of Ki-67 and BrdUrd labeling indices (LIs) was made as detailed previously [27]. Statistical correlation was performed by linear regression analysis.

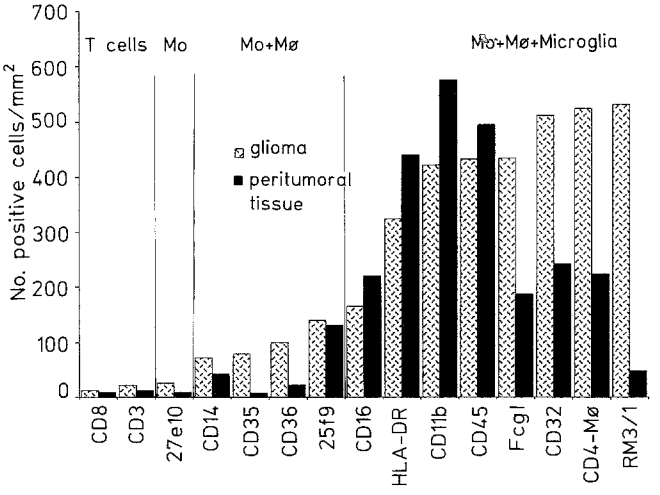

Fig. 1. Quantitative evaluation of immunostained cells in gliomas (*dashed columns*) and in peritumoral tissue (*dark columns*). The columns represent the average number of cells/mm^2 found in all glioma or peritumoral tissue samples. Within the monocyte markers three different marker profiles can be found: (i) markers, which exclusively recognize perivascular monocytes (27E10); (ii) markers, which recognize monocytes as well as classical macrophages but not microglia (CD14, CD35, CD36 and 25F9); and (iii) markers, which are additionally expressed on microglia. With the exception of CD16, HLA-DR, CD11b and CD45 macrophage markers recognize more cells in the gliomas compared to the peritumoral tissue

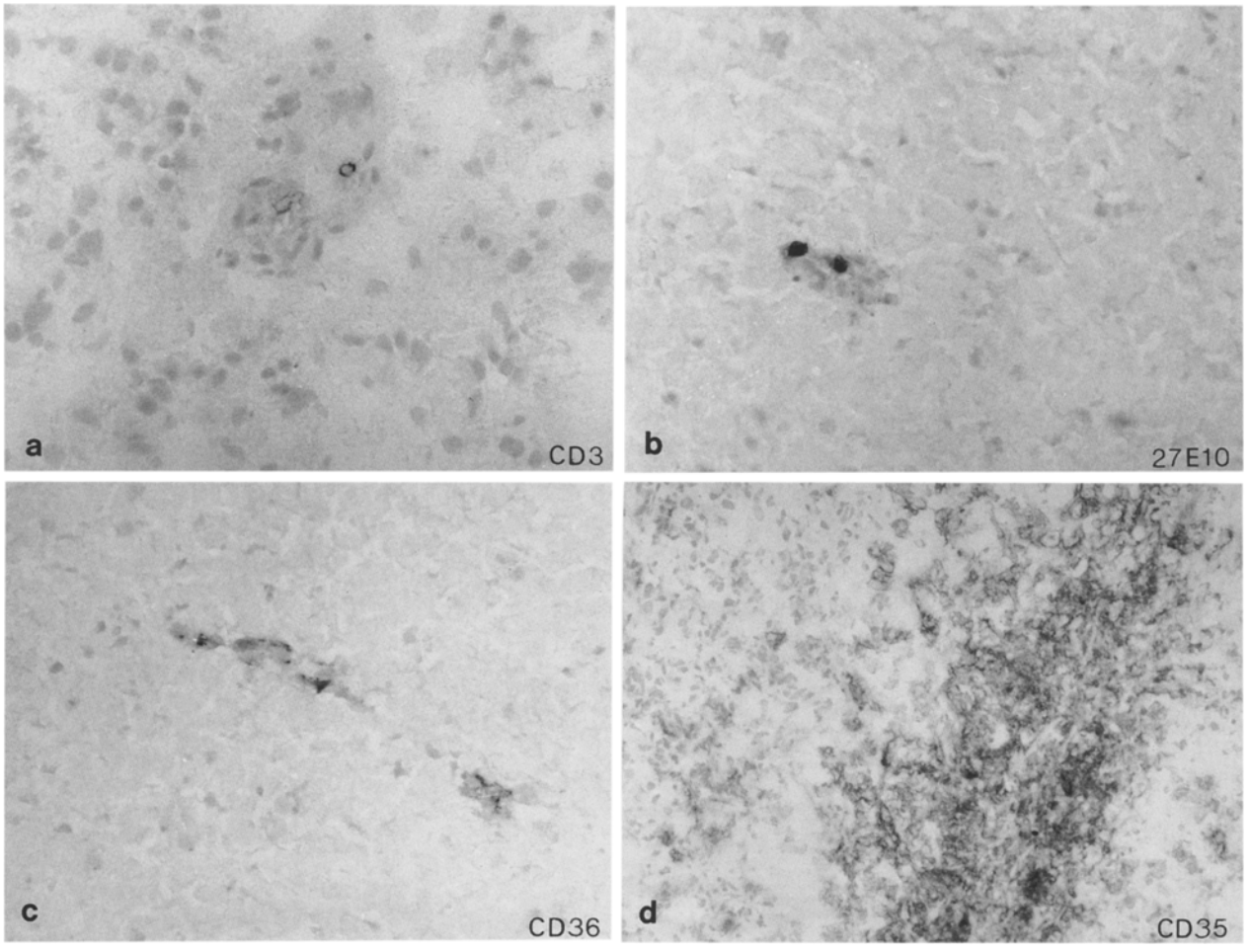

Fig. 2.a Two perivascular T cells in a recurrent glioblastoma (case 12) stained by anti-CD3. **b** Perivascular monocytes labelled with 27E10 in case 11 (recurrent glioblastoma). **c** Perivascular macrophages stained by anti-CD36 in recurrent astrocytoma (case 10). **d** Macrophages in a glioblastoma (case 9) labelled with anti-CD35 (CR 1 complement receptor); diffuse staining is present in the area of necrosis (*right*). **a**–**c** ×260; **d** ×160

Results

T lymphocytes, as defined with antibodies against CD3 and CD8 were present in all samples of human gliomas (Figs. 1, 2a). The percentage and the absolute number of T cells within the tumor tissue was low (Fig. 1) and variable from case to case. No significant correlation between T cell infiltration and histological grading of gliomas or proliferation rate, as defined by Ki-67 or BrdUrd LIs, was found.

When using macrophages antibodies on the same tissue samples, a variable number of immunostained cells was present. In average, the highest numbers were found with antibodies against Fc receptors (CD64, CD32, CD16), CD4 antigens, common leucocyte antigen (CLA; CD45), HLA-DR, complement receptor 3 (CR3; CD 11b) and the macrophage marker RM3/1 (Fig. 1). Much lower cell numbers were found with other macrophage antibodies like anti-CD14, anti-CD35, anti-CD36, 25F9 and 27E10. The number of granulocytes in the tumors, as defined by morphological criteria, was negligible.

Morphologically, three types of labelled cells could be distinguished (Figs. 1–4). The first type consisted of round to oval cells, mainly located in perivascular position and devoid of phagocytic granules (Fig. 2b). Although such cells were stained with all anti-macrophage antibodies used, they represented the only cell type recognized by the 27E10 marker (Fig. 2b). The second type was the classical debris-laden macrophage, located either in the perivascular space or diffusely dispersed in the tumor tissue (Fig. 3a–f). This cell type was recognized by most antibodies used, with the exception of 27E10. OKM 5 (CD36) also stained mainly perivascular cells (Fig. 2c), labeling tissue macrophages weakly and inconsistently. The third population consisted of microglia-like, dendritic cells. They were exclusively located in the tissue, not present in the perivascular space, and showed a stellate, dendritic appearance, with small, rod-shaped nuclei and slender, frequently bipolar, ramifying cell processes. These cells mainly expressed Fc receptors (CD64, CD32, CD16; Fig. 4a, b), CD4 (Fig. 4d), CR3 (CD11b; Fig. 4c), HLA-DR, CLA (CD45; Fig. 4f) and in variable incidence RM3/1 (Fig. 4e), but were unstained by other macrophage antibodies.

Areas of tumor necrosis were frequently present in samples from malignant gliomas. In all necrotic areas an

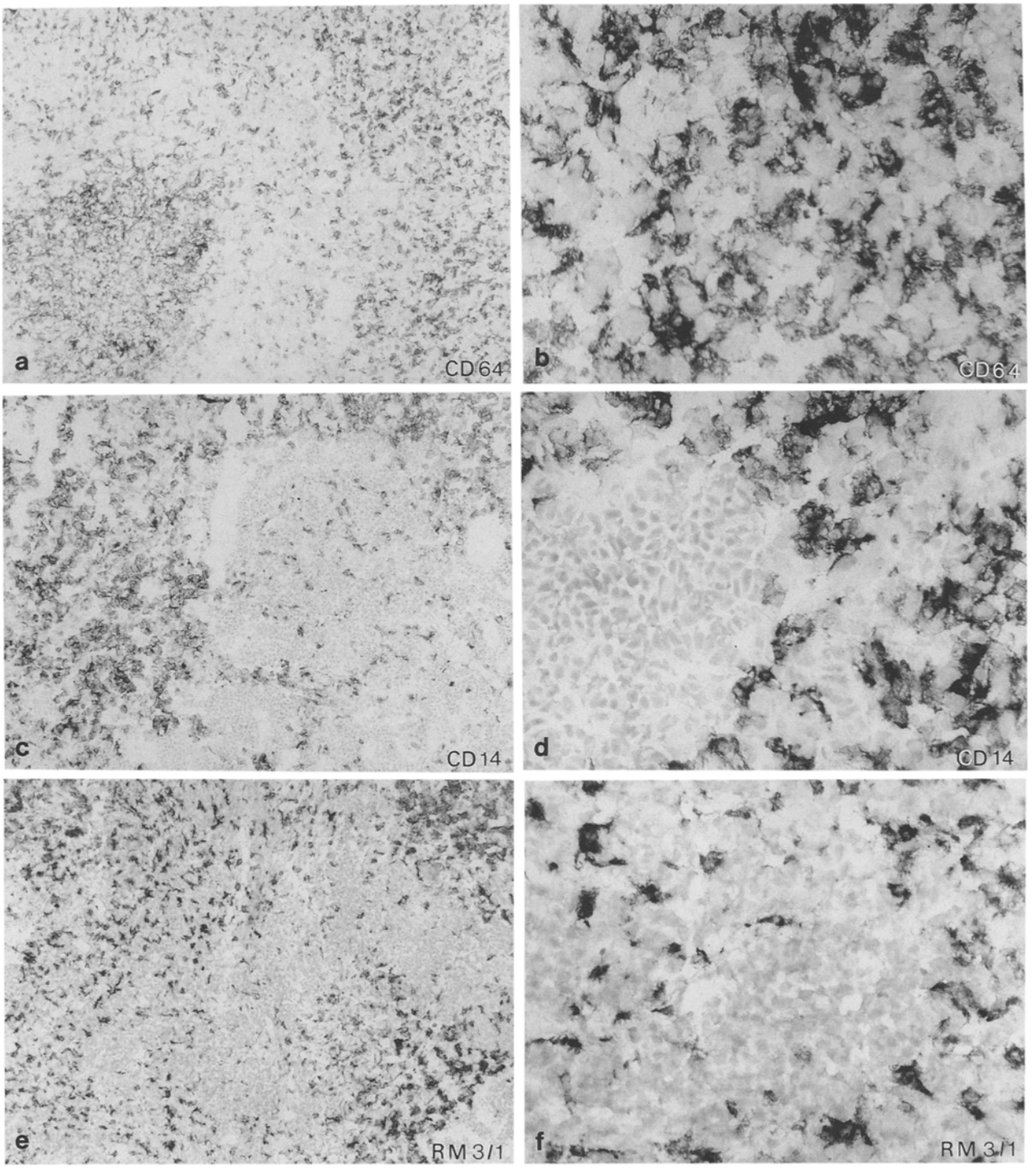

Fig. 3a–f. Recurrent glioblastoma (case 10). Macrophages in the tumor tissue are labelled by antibodies against CD64 (**a, b**) and the macrophage antigens CD14 (Leu-M3; **c, d**) and RM3/1 (**e, f**). **a, c, e** ×65; **b, d, f** ×260

intense, mostly diffuse staining was found with antibodies against Fc and complement receptors (Figs. 2d, 3a). Identification and counting of individual cells was not possible there. With the other markers individual macrophages were labelled within the necrotic areas. In average, the percentage of monocytes/macrophages within gliomas of different malignancy ranged between 20% and 34% of the total cell population. In some glioblastomas, however, more than 50% of the total cell population carried one or more monocyte antigens (Table 3).

Although we did not find a significant difference in the macrophage/microglia infiltration between tumors of different histological grading (Table 3), statistical analysis of the quantitative data revealed a significant positive correlation between the number of RM3/1- or CR3

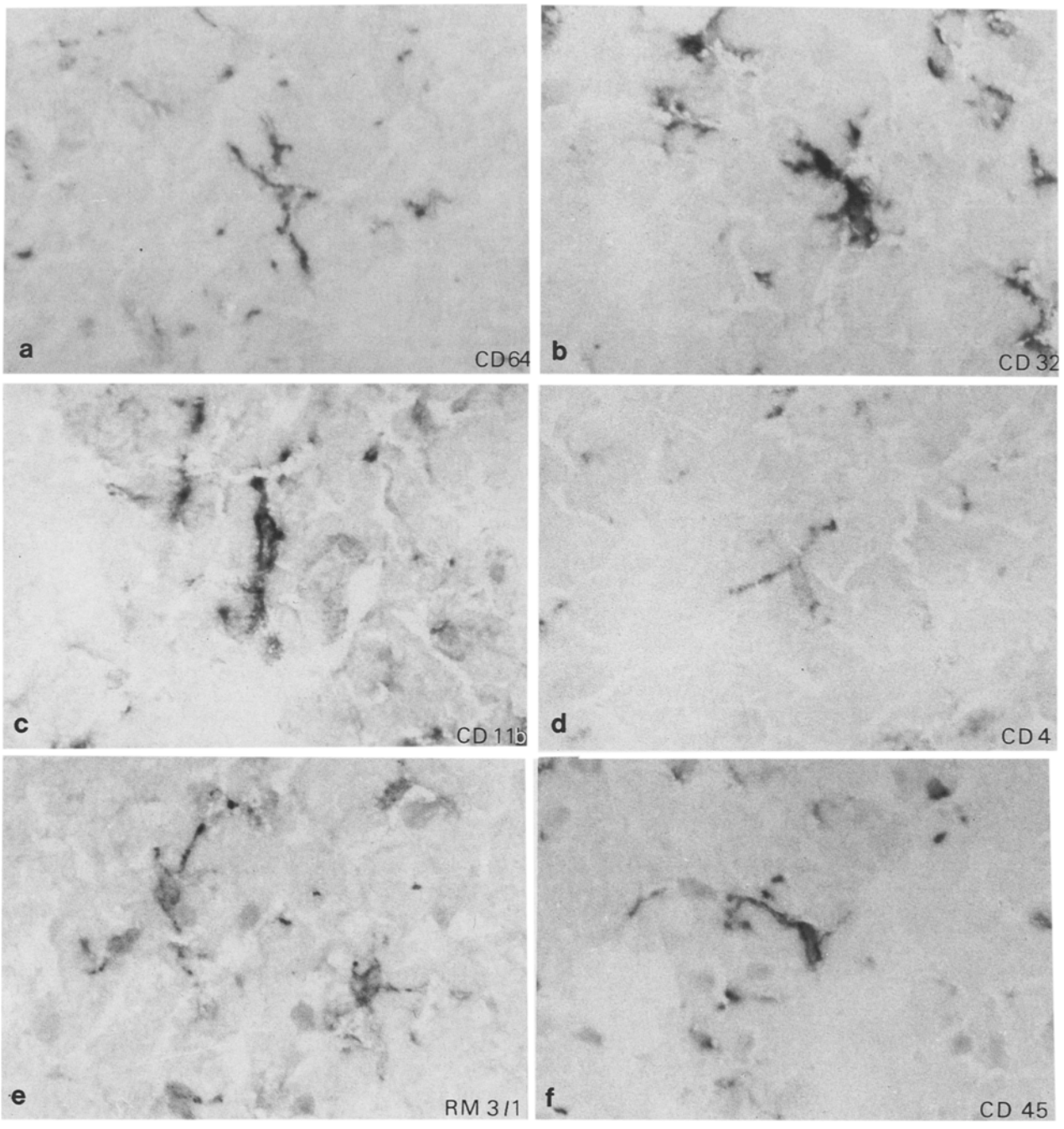

Fig. 4. Recurrent astrocytoma (case 9); dendritic, microglia like cells within the tumor tissue are stained with antibodies against Fc-gamma receptor 1 (CD64; **a**), Fc-gamma receptor 2 (CD32; **b**), CR3 complement receptor (CD11b; **c**), CD4 (**d**), with the macrophage marker RM3/1 (**e**) and with antibodies against the common leucocyte antigen CD45 (**f**). **a**–**f** × 520

(CD11b)-positive cells and the number of BrdUrd- or Ki-67-positive cells in the same tumor (Fig. 5).

In addition, a significant correlation ($P < 0.05$) was found between the number of T lymphocytes in the lesions (defined by CD3 and CD8) and the number of macrophages labelled with 27E10, RM3-1 and anti-CD14, and between the number of $CD4^+$ cells and HLA-DR expression.

In peritumoral, "normal" CNS tissue, a number of cells, similar to that in gliomas, was stained with antibodies against CD11b, CD45 and HLA-DR (Fig. 1). They resembled dendritic microglia-like cells. Only few perivascular monocytes/macrophages were found in peritumoral tissue.

Discussion

A major aim of this study was to find antibodies which identify the monocyte/macrophage population in gliomas as completely as possible.

Table 3. Mean percentage of macrophages in gliomas of different malignancy[a]

Tumor type	CR3 (CD11b)	Fcg 1 (CD 64)	Fcg 2 (CD 32)
Astrocytoma I,II	8.8	25.8	26.4
($n=4$)	(3.9–20.8)	(6.6–39.8)	(5.9–47.1)
Glioblastoma	10.4	34.6	32.7
($n=5$)	(2.5–31.3)	(21.8–60.6)	(24.1–55.5)
Recurrent GB	14.4	20.6	27.2
($n=3$)	(11.9–17.6)	(12.9–24.2)	(24.0–30.4)

n: number of cases

[a] The number reflect the percentage of immunostained cells in relation to total cells in the tumor tissue. The value in parentheses represent the minimal and maximal counts in the investigated tumors

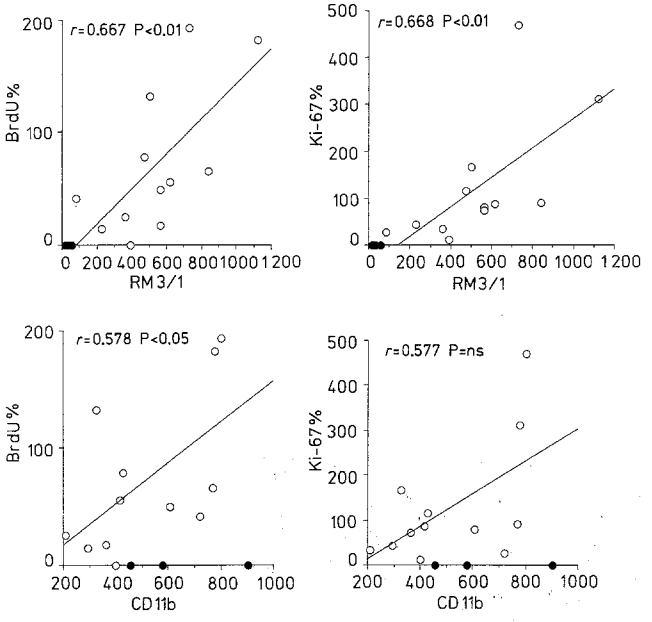

Fig. 5. Correlation between tumor proliferation (percentage of Ki-67 or bromodeoxyuridine-positive cells) and macrophage infiltration (number of CD11b- and RM3/1-positive cells/mm^2); the *open circles* represent values from tumor tissue, the *black circles* from peritumoral tissue. In the peritumoral tissue "microglia" expresses CD11b but not the RM3/1 antigen

This is difficult in the CNS since microglia can only be identified by a fraction of monocyte/macrophage antibodies. We, therefore, concentrated on markers which are known to be expressed in microglia (CD4 and CR3 [21, 32, 37], Fc receptors [26, 33] and HLA-DR [1, 25]. It is, thus, not surprising that, in our present study, these markers revealed the highest numbers of immunostained cells in gliomas. We found all three Fcg receptors, CR3 complement receptor, HLA-DR, CD45, and the macrophage marker RM3/1 to be expressed on cells morphologically consistent with microglia. Interestingly, Fcg 1 and 2, CD4 and the RM3/1 antigen were found mainly on microglia in the tumor tissue, but to a much lower extent in peritumoral tissue. This indicates that the expression of these antigens may depend on the activation of microglia in pathological conditions, similarly as described for CD11b in experimental conditions [16]. Besides of the expression of monocyte/macrophage antigens, microglia cells have been shown to mediate phagocytosis, antigen presentation and tumor cytotoxicity in vitro [15] and may differentiate to phagocytic cells in vivo under certain pathological conditions [46, 47]. All these lines of evidence support the concept that microglia is derived from the monocyte/macrophage lineage. However, microglia cells do not express all, but only some monocytic antigens (CD4, Fcg receptors, CR3, HLA-DR and CD45) which are functionally important but not completely macrophage specific. It thus, remains still unresolved whether microglial cells represent a special subpopulation of monocytes/macrophages or a different cell lineage with, however, similar functional properties.

Although antigens like CD4 or HLA-DR can also be expressed by neoplastic glia cells in vitro [7] and in vivo [5, 35], an unequivocal identification of immunostained glioma cells in situ is difficult at the light microscopic level and requires more detailed ultrastructural double-labelling studies. However, the similar distribution and numbers of cells expressing CD/11b, CD16, CD32, CD64, CD4 and CD45, indicates that CD4 and HLA-DR expression of glioma cells is, if present at all, quantitatively minor.

The high number of cells with surface epitopes similar to macrophages within gliomas may represent an unspecific reaction of the CNS to injury. Alternatively, glioma cells may directly influence macrophage proliferation and differentiation in the tumors. In fact, both immune-stimulatory as well as-inhibitory factors can be produced by glioma cells and normal astrocytes in vitro. They include interleukin-1 [13], interleukin-3, a potent macrophage growth factor [14], prostaglandin E [12] and a glioma-derived suppressor factor [39]. Furthermore, a new chemoattractant factor for monocytes, different from known cytokines, has recently been described to be produced by human glioma cells [55].

Several previous studies tried to find a correlation between inflammatory infiltration of gliomas and their histological appearance, malignancy or prognosis. Early studies pointed out the high incidence of lymphocytic infiltration in malignant tumors [2, 36, 41, 48]. However, the prognostic significance of inflammatory infiltration of the tumors has remained controversial [3, 4, 9, 30, 40, 45]. Since in our study the total number of low-grade astrocytomas was relatively low for a useful comparison with inflammatory infiltration in glioblastomas, we correlated the lymphocytic and monocytic infiltration in the tumors with their proliferation rate, measured by the number of Ki-67 and BrdUrd labelled cells. We found no correlation between lymphocyte infiltration and tumor proliferation. However, there was a significant positive correlation between the number of monocytic cells, defined by RM3/1 and CD11b, and the proliferation rate of the tumors. This finding extends earlier work by Rossi [38], who reported that low-grade astrocytomas had a lower average macrophage infiltrate than malignant astrocytomas.

Macrophages are major effector cells, mediating immunological damage in inflammatory conditions. Such immune-mediated damage may be antigen specific or

occur in an antigen-unspecific "bystander" reaction. In both conditions, macrophages can induce cell damage via oxygen radicals and arachidonic acid metabolites [18, 19], monokines [42] or proteolytic enzymes [52]. In fact, tumor cytotoxicity and tumor necrosis factor production has been described for activated microglial cells in vitro [15]. In the case of antibody-mediated immunological damage, macrophages play an important role in tissue destruction, provided they are activated and express surface receptors like Fc receptors and complement receptors [31]. Our study shows that the monocyte/macrophage/microglia population in human gliomas is armed with the respective surface receptors for antibody- and complement-mediated cytotoxic actions. This important prerequisite offers an attractive perspective for immunotherapy with antibodies.

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