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Immunobiology of primary intracranial tumours

II. ANALYSIS OF LYMPHOCYTE SUBPOPULATIONS IN PATIENTS WITH PRIMARY BRAIN TUMOURS

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SUMMARY

Circulating peripheral blood lymphocyte subpopulations were analysed in patients with primary intracranial neoplasia. Patients with tumours of glial origin demonstrated a significant depletion of E-rosetting lymphocytes whereas the quantitative lymphocyte profiles of patients with non-glial brain tumours were normal. The number of immunoglobulin and Fc receptor-bearing cells was not significantly altered in any group of patients: however, the EAC-RFC subpopulation was increased in those with malignant gliomas. Two hypotheses are suggested to explain these observations: first, the presence of cross-reacting antibody between T cells and brain (glial) cells; and secondly, the proliferation of EAC-RFC in response to malignant degeneration.

INTRODUCTION

Although temporal inter-relationships are speculative, it is apparent from numerous clinical studies that a link exists between host-immunity and cancer. Furthermore, evidence suggests that immunological reactivity associated with malignant neoplasia inversely parallels the extent of the disease. Thus, patients with widely disseminated cancer are immunologically less responsive than those with localized disease.

Patients with primary intracranial tumours are among the best-suited for such studies because these neoplasms rarely metastasize and generally the patient is not as debilitated as those with metastatic disease. These patients, therefore, may be considered to have localized neoplasia throughout the course of their disease. Previously it has been demonstrated that brain-tumour patients have impaired cellular responsiveness to mitogens, depressed cutaneous reactivity to common antigens, and inability to become sensitized to dinitrochlorobenzene or keyhole limpet haemocyanian (Brooks *et al.*, 1972; Brooks, Caldwell & Mortara, 1974; Thomas, Lannigan & Behan, 1975; Young, Sakalas & Kaplan, 1976; Mahaley *et al.*, 1976).

In addition to blast transformation with exposure to mitogens, thymus-derived lymphocytes (T cells) and bone marrow-derived lymphocytes (B cells) can be characterized by identification of specific membrane markers. Human T cells form rosettes with unsensitized sheep red blood cells (E-RFC) (Jondal, Holm & Wigzell, 1972) while B lymphocytes have membrane-bound immunoglobulin (Ig) (Brown & Greaves, 1974). A presumed subpopulation of B cells have surface-bound receptors for complement and form rosettes with sensitized sheep erythrocytes (EAC-RFC) (Ross & Polley, 1975). Other subpopulations of lymphocytes have been shown to have receptors for the Fc portion of immunoglobulin, however it is not clear whether this is a B- or T-cell property (Dickler & Kunkel, 1972; Froland, Wisloff & Michaelson, 1974). These techniques afford an opportunity to quantitatively evaluate the cellular elements of immune responsiveness.

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In the present study, peripheral blood lymphocytes obtained from patients with primary intracranial tumours were isolated and enumerated according to their specific cell-surface markers. The number of T cells in patients with primary intracranial tumours of glial origin was less than that observed in normal subjects, patients with non-neoplastic CNS disease, and in patients with intracranial non-glial neoplasms. Interestingly, the lymphocyte subpopulation with receptors for complement was increased only in those patients with malignant brain tumours.

MATERIALS AND METHODS

Subject population. Patients included in the present study were grouped according to histological verification of the primary intracranial tumour (Table 1). Those with metastatic neoplasia were excluded. All patients had symptoms and signs of active and progressive tumour growth. No patient was receiving radiation therapy, corticosteroids, chemotherapy, or phenytoin at the time of this study. Controls and patients had not received any anaesthetic other than local for at least 6 weeks prior to study. Both men and women were included; ages ranging from 26–68 years (median 49). 115 healthy hospital employees, men and women, served as normal controls; ages ranging from 21–65 years (median 45). Thirty-five hospitalized patients with non-neoplastic central nervous system disorders, e.g. stroke and head injury, were also studied as controls. The ages of this group ranged from 22–74 (median 56).

TABLE 1. Patient groups

Group	Tumour
1	Glioblastoma (11)
2	Anaplastic astrocytoma (11)
3	Non-malignant glioma (11)
4	Meningioma and schwanoma (15)

Lymphocyte preparation. Heparinized (10 u/ml) venous blood was layered on a 9% Ficoll–34% Hypaque gradient according to a modification of the technique described by Böyum (1968). After centrifugation at 400 *g* for 35 min, the lymphocyte layer was carefully aspirated, washed twice in RMPI 1640 and adjusted to the desired cell concentration. Contamination with macrophages measured by latex-bead ingestion ranged from 2–4%. Polymorphonuclear contamination was less than 1% when measured by conventional staining techniques.

Assay for sheep erythrocyte binding lymphocytes (E-RFC). A modified method as described by Jondal *et al.* (1972) was employed to determine the percentage of E-RFC. Briefly, 2×10^6 lymphocytes in 0·2 ml of media were mixed with a 0·2 ml of a 0·5% suspension of sheep erythrocytes (SRBC) and incubated at 37°C for 15 min. The mixture was centrifuged at 50 *g* for 5 min and incubated for 2 hr at 4°C. The cells were gently resuspended and coverslip slides prepared. Lymphocytes with more than three rosetting SRBC were considered E-RFC.

Determination of membrane-bound Ig. 2×10^6 lymphocytes in 0·1 ml were stained with a 1:5 dilution of fluorescein-conjugated polyvalent goat anti-human immunoglobulin serum (Lot No. 8660, Cappel Lab. Inc., Downingtown, Pennsylvania). The cells were incubated at 4°C for 30 min, washed with media containing 0·02% sodium azide and 5% foetal calf serum, and coverslip slides prepared. These preparations were examined using a Leitz Orthoplan microscope equipped with KP490 interference filter and a K530 barrier field with a Ploem illuminator. Cells with a scanty cytoplasm, diameter no more than 2/3 that of typical monocytes and circumferential fluorescence were considered Ig-bearing (B cell) lymphocytes.

Determination of complement receptor-bearing lymphocytes (EAC-RFC). A modified method as described by Shevach, Herberman & Frank (1972) was used to determine the percentage of EAC lymphocytes. Briefly, 0·4 ml of lymphocytes (2×10^6) were mixed with 0·4 ml of SRBC previously sensitized with rabbit anti-sheep 19S antibody and complement obtained from C57BL C5-deficient mice (Jackson Laboratories, Bar Harbor, Maine). The mixture was centrifuged at 50 *g* for 2 min and subsequently incubated at 37°C for 30 min. To facilitate lymphocyte and monocyte differentiations euchrysin 3RX (Lot No. 7101, Chroma-Gesellschaft, Schmid and Co., Stuttgart-Unterterturkheim, W. Germany) was added to the suspensions prior to coverslip slide preparation. Utilizing simultaneous u.v. and light microscopy, lymphocytes with more than three attached SRBC were designated EAC-RFC.

Determination of Fc-binding lymphocytes (EA-RFC). The percentage of lymphocytes able to bind human erythrocytes (HRBC), previously sensitized with antibody directed against the CD Rh antigen, was determined by the method of Froland *et al.* (1974). Briefly, Rh^+ HRBC (R_1R_1) were sensitized with heat-inactivated anti-Ripley CD serum and mixed with 0·2 ml (2×10^6) purified lymphocytes. This mixture was centrifuged at 200 *g* for 2 min and incubated at room temperature for 15–30 min. The mixture was gently resuspended and coverslip slides prepared for counting. Lymphocytes binding more than three HRBC were considered EA-RFC.

RESULTS

Two significant findings are made evident by comparing the percentages of the various lymphocyte subpopulations in brain-tumour patients and normal controls (Table 2). First, the percentage of E-RFC found in patients with primary glial tumours (groups 1, 2 and 3) is significantly less than that observed in either normal or patient controls, or those patients with primary non-glial intracranial tumours (group 4). Secondly, those patients with histologically malignant glial tumours (groups 1 and 2) had a marked increase in EAC-RFC while those patients with benign non-glial neoplasia (group 4) were not significantly different from normals. The differences in the percentage composition of Ig-bearing and Fc receptor-bearing lymphocytes among all groups were not statistically significant.

TABLE 2. Comparison of lymphocyte subpopulation percentages in peripheral blood of normal subjects and patient groups

Patient groups	E-RFC	Ig	EAC	EA
Control				
Normal	64±6*	17±3	16±5	20±8
Patient	61±6	18±5	14±4	—
Group 1	39±15†	17±8	30±12†	25±9
Group 2	49±18†	15±9	29±13†	28±13
Group 3	55±8†	15±5	18±4	21±9
Group 4	60±10	16±7	18±8	20±8

* s.e.m.
† $P<0{\cdot}05$, multiple comparisons using rank sums (Dunn, 1964).

The absolute number of the various subpopulations is shown in Table 3. Examination of these data indicates a significant lymphopenia in patients with glial tumours but not in those with intracranial non-glial tumours. Thus, patients in group 1 have a 37% reduction in the number of circulating lymphocytes; group 2 show a 24% reduction; and group 3 a 20% reduction.

Comparison of the observed values for each lymphocyte subpopulation with the expected value (absolute value of each subpopulation in normals adjusted for the percent lymphopenia in each tumour group) is shown in Table 4. Inspection of these data reveals a marked decrease in E-RFC beyond that expected in patients with glial tumours. Although the decrease in E-RFC was found in all these patients, it was most evident in those with malignant tumours (groups 1 and 2). There was no significant difference

TABLE 3. Comparison of absolute number of lymphocyte subpopulations in peripheral blood of normal subjects and patient groups

Patient groups	Lymphocyte count (cells/mm^3)	E-RFC	Ig	EAC	EA
Control					
Normal	1700±210*	1127±102	292±63	284±92	366±132
Patient	2070±272	1263±215	373±56	290±35	—
Group 1	1079±298†	434±230†	175±75†	339±168	303±169
Group 2	1296±532†	690±44†	190±97†	366±176	404±325
Group 3	1361±209†	754±168†	207±76	242±80	321±179
Group 4	1599±363	955±227	262±147	362±231	316±129

* s.e.m.
† $P<0{\cdot}05$ (see Table 2).

TABLE 4. Comparison of expected and observed numbers of lymphocyte subpopulations of brain-tumour patients*

Lymphocyte subpopulations	Group 1		Group 2		Group 3	
	Expected	Observed	Expected	Observed	Expected	Observed
E	710	434	857	690	902	754
Ig	184	175	222	190	234	207
EAC	179	339	216	366	227	242
EA	231	303	278	404	293	321

* [Absolute value of each lymphocyte subpopulation in normals (E, Ig, etc.)] × [100 − per cent lymphopenia in each tumour group] ÷ 100.

in the results obtained from patients studied pre-operatively compared to those obtained from patients with tumour recurrence.

Examination of the percentage of EAC-RFC indicates higher values in patients of groups 1 and 2 when compared to the control groups (Table 2). However, when the absolute number of EAC cells in these groups is compared to the values obtained from control subjects there was no significant difference (Table 3). Computation of the expected values for the absolute number of EAC-RFC in group 1, taking into consideration the lymphopenia, indicates there should be 179 EAC cells/mm^3; however, 339 cells/mm^3 were detected. A similar discrepancy was detected in those patients comprising group 2 (Table 4). Thus, it is apparent that there is almost a two-fold increase in the number of circulating EAC lymphocytes in these patients. There was no significant difference in the expected and observed values for Ig-bearing and Fc receptor-bearing lymphocytes among any group.

DISCUSSION

Previous investigations have demonstrated that patients with primary intracranial tumours have impaired delayed-type hypersensitivity and depression of various *in vitro* correlates of cellular immunity (Brooks *et al.*, 1972, 1974; Mahaley *et al.*, 1976). Additional studies indicate this suppression apparently is serum-mediated and correlates with the presence and growth of the autochthonous neoplasm (Brooks *et al.*, 1974; Young *et al.*, 1976). The present studies provide further evidence that the immune status of these patients is not normal.

Although patients with glial tumours were lymphopenic, the loss of cells within the individual lymphocyte subpopulations was not quantitatively identical. A selective depletion of E-RFC (T cells) was found in these patients, whereas the number of Ig-bearing lymphocytes was that expected when correcting for the lymphopenia. Furthermore, the decrease in E-RFC was most evident in those patients with malignant glial neoplasia. The quantitative immunological profiles of those patients with intracranial tumours of non-glial origin (group 4) were normal. Thus, it can be suggested that the observed alterations are related to tumours of glial origin, particularly those with malignant degeneration.

Two important questions should be considered. First, what is the aetiology of the depletion of E-rosetting cells; and secondly, why are the EAC-RFC concomitantly increased? Although the brain may be considered as a 'partial immunologically privileged site' (Scheinberg *et al.*, 1965), in the course of neoplastic changes the blood–brain barrier is lost (Long, 1970), which allows the escape of tumour-associated and normal brain antigens as well as entrance to the brain of components of the immune system. Therefore, when normal neural cells acquire tumour antigens antibody may be synthesized against both tumour antigens and normal membrane determinants. Because brain and T lymphocytes share certain membrane determinants (Golub, 1971), some of these antibodies or antigen–antibody complexes may crossreact with determinants on the lymphocyte surface thus modulating certain

membrane properties of these cells. The presence of such circulating crossreacting antibody or complex could account for the T-cell lymphopenia in patients with intracranial tumours of neural origin and not in those with similarly located non-glial tumours. The observation that the receptor for the sheep erythrocyte and T cell-specific antigens may be identical, or at least linked, adds further support to this hypothesis (Owen & Fanger, 1974).

The observation that the depression of E-RFC was most significant in those patients with malignant gliomas suggests an alternative explanation. It has been proposed that non-immunological modulation of host immune responsiveness is due to tumour-cell products (chalones) rather than humoral suppressor or blocking factors (Ranney, 1975). Similar factors have been found to be associated with malignant neural tumours (Osther, Hojgaard & Dybkjaer, 1974). Thus, the decrease in detectable E-RFC in patients with malignant neural tumours may more accurately reflect a non-immunological 'blocking' rather than a real depletion. Support for this hypothesis is gained from previous investigations which indicate that the lymphocyte mitogenic responsiveness of these patients is normal when cultured in normal sera (Brooks *et al.*, 1972; Young *et al.*, 1976).

The finding that EAC-RFC are increased in patients with malignant intracranial neoplasia, as well as those with malignant lung cancer (Anthony *et al.*, 1975), may be more indicative of host responsiveness to malignancy rather than specific alteration of these subpopulations peculiar to patients harboring brain tumours. Circulating lymphocytes which lack conventional T- and B-cell surface markers but possess EAC receptors (null cells) have been shown to be effector cells of non-specific antibody-dependent cellular cytotoxicity (MacDermott, Chess & Schlossman, 1975). Additional studies suggest that this 'null' subpopulation contains immunoglobulin-producing cells capable of elaborating quantitatively more immunoglobulin than mature B cells (Chess *et al.*, 1975). These data, taken together, suggest the possibility that the proliferation of EAC-RFC is a response to tumour-associated antigenic stimulation. Proliferation of this subpopulation would, therefore, subsequently increase both effector cell and possibly cytotoxic antibody activity which in turn would be beneficial to the host.

The interrelation between T cells and null cells, both effector cells, is speculative. The recent evidence that a subpopulation of circulating lymphocytes possess both surface markers (Chiao, Pantic & Good, 1975; Arnaiz-Villena & Hay, 1975) suggests the possibility that one receptor may be lost or 'hidden' whereas another receptor on the same cell is readily detectable. Thus, this lymphocyte subpopulation is not deleted from the circulation but cannot be identified as belonging to a particular subpopulation. The EAC-RFC accumulation also may reflect stimulation of B cells, when the thymus-dependent system is functionally deficient in an effort to maintain an effective immune system.

In summary, the immunological significance of these observations in patients with primary brain tumours remains to be elucidated. Interestingly, a decrease in E-RFC and an increase in EAC cells has been reported in patients with active multiple sclerosis (Oger *et al.*, 1975). This observation tends to support the idea that the immune response of patients with disease processes affecting the central nervous system is characteristically modified by interactions of the immune system with cells of the brain and/or their products. Long-term sequential studies are now in progress to correlate the observed alterations of the quantitative immunological profile of brain-tumour patients with their clinical course in an effort to determine whether immunotherapy is a viable possibility.

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