Clin. exp. Immunol. (1980) 39, 395–402.

Immunobiology of primary intracranial tumours

III. DEMONSTRATION OF A QUALITATIVE LYMPHOCYTE ABNORMALITY IN PATIENTS WITH PRIMARY BRAIN TUMOURS

T. L. ROSZMAN & W. H. BROOKS *Department of Pathology, Division of Experimental Pathology and Division of Neurosurgery, College of Medicine, University of Kentucky, Lexington, Kentucky, USA*

(*Accepted for publication* 13 *July* 1979)

SUMMARY

Peripheral blood lymphocytes obtained from patients with primary intracranial tumours respond poorly when stimulated with phytohaemagglutinin (PHA) as compared to lymphocytes from control subjects. This defect could not be corrected by employing purified thymus-derived lymphocytes (T cells) obtained from the peripheral blood of these patients. Moreover, neither increasing the number of lymphocytes placed in culture nor the duration of culture (3–6 days) corrected the defect. Preincubation of these lymphocytes for 24 hr in media supplemented with human AB sera followed by stimulation with PHA did not result in an increase in blast transformation when compared to cells which were not preincubated. However, when the percentage of sheep red blood cell rosetting lymphocytes was determined in a similar type of experiment a marked decrease in the ability of lymphocytes from patients with brain tumours to form rosettes was noted. Quantitation of the number of L-PHA binding sites on lymphocytes from patients with brain tumors revealed that these lymphocytes had approximately twice as many receptor sites per cell as did control lymphocytes suggesting either membrane alterations or changes in lymphocyte subpopulations.

INTRODUCTION

Patients harbouring malignant central nervous system neoplasia have impaired cell-mediated immunity as evidenced by cutaneous anergy (Brooks *et al.*, 1972; Mahaley *et al.*, 1977), diminished numbers of lymphocytes (Brooks, Roszman & Rogers, 1976; Brooks *et al.*, 1977), and impaired lymphocyte blastogenic responsiveness (Brooks *et al.*, 1972; Brooks, Caldwell & Mortara, 1974). Previously, we have demonstrated that the observed impaired cellular reactiveness may in part be ascribed to the presence of autochthonous humoral factors capable of abrogating both autologous and homologous, normal lymphocyte blastogenesis (Brooks *et al.*, 1972; 1974). Nevertheless, although activation of lymphocytes obtained from patients with brain tumours is somewhat improved when cultured with normal sera, the response remains significantly less than expected. Therefore, other explanations, in addition to circulating humoral factors, must be sought to explain the presence of impaired cell-mediated immune responsiveness in patients with primary brain tumours.

The present report confirms and extends our previous observations and demonstrates that the characteristics of specific receptors on the surface of peripheral blood lymphocytes obtained from these patients are not normal. These modulations may contribute to the routinely observed impaired host immunocompetence of patients with primary cerebral tumours.

Correspondence: Dr T. L. Roszman, Division of Experimental Pathology, College of Medicine, University of Kentucky, Lexington, Kentucky 40536, USA.

0099-9104/80/0200-0395 $02.00

MATERIALS AND METHODS

Patient population. Patients in this study were diagnosed as having either glioblastoma multiforme or anaplastic astrocytomas. 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 the study. Both men and women were included ranging in age from 26 to 68 years (median 49). Healthy hospital employees, men and women, served as normal controls; ages ranging from 21 to 65 years (median 45).

Lymphocyte preparation. Heparinized venous blood was separated on a Ficoll–Hypaque gradient (Böyum, 1968). After centrifugation at 400 *g* for 35 min, the lymphocyte layer was separated, washed twice in Eagle's medium and adjusted to the desired concentration. From 80 to 90% of these cells were lymphocytes while 10 to 20% were determined to be monocytes as assessed by non-specific esterase stain (Yam, Li & Crosby, 1971).

Enumeration and isolation of sheep red blood cell (SRBC) rosette forming cells (E-RFC). The percentage of E-RFC in the lymphocyte suspension was determined as previously described (Brooks *et al.*, 1977). The method of Jondal (Jondal, Holm & Wigzell, 1972) was employed to isolate E-RFC. Briefly, equal volumes of lymphocytes (1×10^7/ml) and 2·5% neuraminidase-treated SRBC were mixed and SRBC absorbed foetal calf serum added to attain a final concentration of 20%. The mixture was incubated at 37°C for 15 min, centrifuged at 100 *g* for 5 min and incubated at 4°C for 2 hr, and separated on a Ficoll–Hypaque gradient. The pellet containing the rosettes was treated with 0·83% NH_4Cl to lyse the SRBC, the lymphocytes washed and counted. Less than 3% of these lymphocytes were B cells and less than 1% were monocytes.

Cell cultures. Lymphocytes were suspended in Eagle's medium containing 10% pooled human AB serum, vitamins, non-essential amino acids, glutamine, penicillin and streptomycin. The cells were dispensed in 0·2 ml volumes in microtitre plates at a final concentration of 2×10^5 lymphocytes per well unless otherwise stated. The cell cultures were stimulated with various concentrations of phytohaemagglutinin (PHA-P, Difco Laboratories, Detroit, Michigan) in a volume of 10 μl and incubated at 37°C in an atmosphere of 5% CO_2–95% air. The cultures were incubated for 54 hr with 0·5 μc of ^{3}H-thymidine, New England Nuclear, Boston, Massachussetts (specific activity 6·7 Ci/mm) added 18 hr before termination. The cells were harvested with multiple automatic sample harvester and the radioactivity determined by scintillation counting.

Lymphocyte binding studies. Iodination of leucoagglutinating phytohaemagglutinin (L-PHA, Pharmacia, Piscataway, New Jersey) with ^{125}I (Amersham Searle Corporation, Arlington Heights, Illinois) was performed by the chloramine T method (McConahy & Dixon, 1969). The method of Boldt, Skinner & Kornfeld (1972) was employed for binding ^{125}I lectin to lymphocytes. Briefly, binding studies were performed in 13 × 100 mm disposable glass tubes previously pre-soaked in 5 mg/ml bovine serum albumin. Lymphocytes at 2×10^6/0·4 ml in Eagle's medium containing 1 mg bovine serum albumin were incubated at 25°C for 45 min with various concentrations of ^{125}I PHA. Parallel incubation mixtures without cells were carried out to correct for non-specific binding to the glass tubes. After incubation the cells were washed twice in 5 ml of 0·9% NaCl–0·01 M $NaHCO_3$ and the pellets counted in a gamma counter. The data was plotted according to the method of Steck & Wallach (1965) and the number of binding sites calculated.

RESULTS

Responsiveness of PBL from patients with brain tumours to PHA

The responsiveness of PBL from normal individuals and patients with brain tumours to various concentrations of PHA are compared in Fig. 1. At all concentrations of PHA the response of PBL from normal individuals was 3 to 3·7 times greater than that of PBL obtained from patients with brain tumours. This difference cannot be attributed to a preferential loss of cell viability in cultures containing PBL from patients with brain tumours since these cultures exhibited a viability (90% or greater) similar to those of normal PBL. Thus, other explanations were sought for the observation of diminished mitogenic responsiveness in brain tumour patients.

Temporal responsiveness of PBL to PHA

Lymphocytes obtained from normal individuals and patients with brain tumours were stimulated with PHA and cultured for various lengths of time. The data depicted in Fig. 2 demonstrates that lymphocytes from patients with brain tumours continue to be less reactive to PHA on later days of culture.

Effect of lymphocyte cell concentration on PHA reactivity

In an attempt to correct the defect in mitogen reactivity of lymphocytes from patients with primary intracranial tumours, various numbers of these lymphocytes were stimulated with an optimal concentration of PHA. The results presented in Fig. 3 demonstrate that the optimal response was obtained with 2×10^5 lymphocytes per well for both patients and controls.

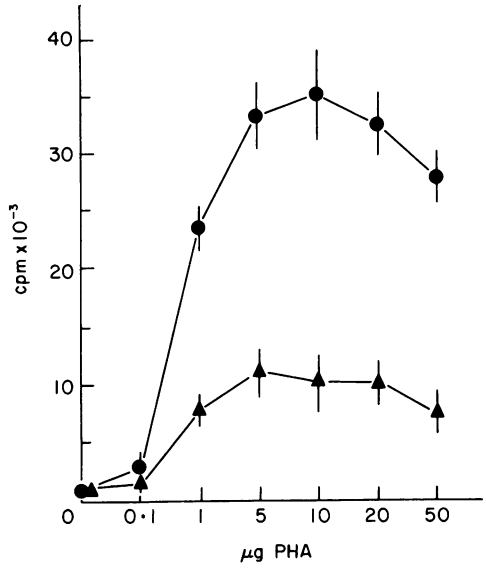

FIG. 1. Response of peripheral blood lymphocytes from control subjects (●——●, $n = 31$) and patients with brain tumours (▲——▲, $n = 26$) to PHA.

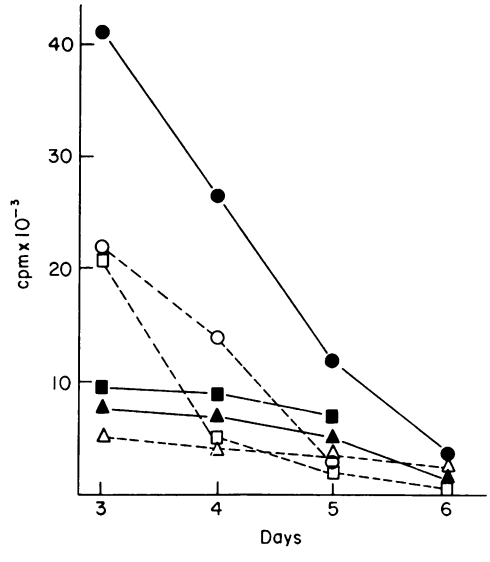

FIG. 2. Temporal response of peripheral blood lymphocytes from control subjects (●——●) and brain tumour patients (■——■, ▲——▲, □ - - - □, △ - - - △, ○ - - - ○) to PHA (10 μg).

Responsiveness to PHA of purified T cells from patients with brain tumours

Because PHA is mitogenic for T cells (Graves & Janossy, 1972), one explanation for the observed diminished blastogenesis may be the T-lymphopenia of patients with primary intracranial tumours (Brooks *et al.*, 1976; 1977). To test this hypothesis, 'purified' T cells were obtained from the peripheral blood of patients by SRBC rosetting and their responsiveness to various concentrations of PHA assessed (Fig. 4). Unseparated PBL responded as well as the purified population to various concentrations of PHA. Similar results were obtained with PBL obtained from control (data not shown). Therefore, in addition to the absence of correlation between percent E-RFC and PHA induced responsiveness, the activation of these purified T cells remained less than normal.

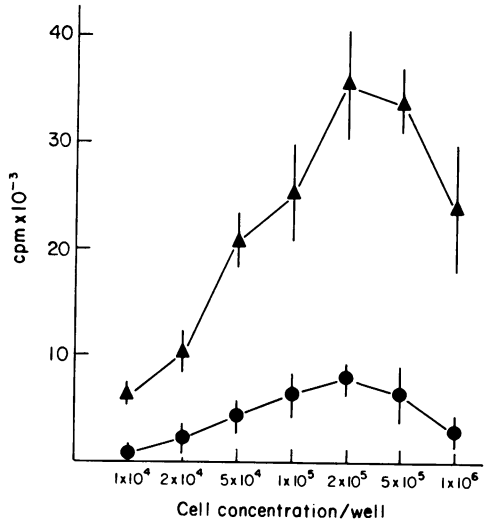

FIG. 3. Effect of lymphocyte cell concentration on their responsiveness to PHA (10 μg). Controls (△——△, $n = 6$); brain tumour patients (●——●, $n = 7$).

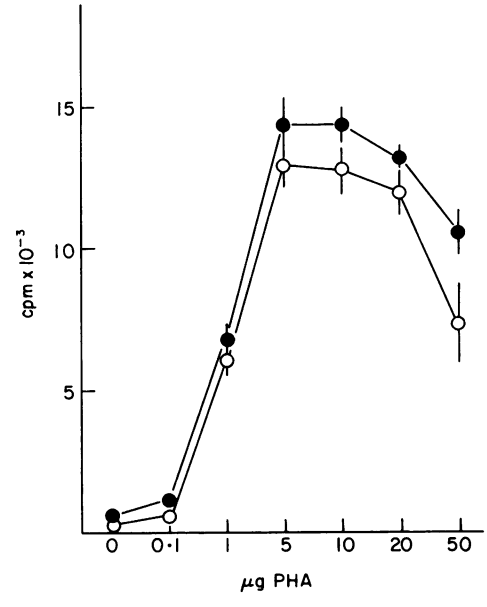

FIG. 4. Response of unseparated peripheral blood lymphocytes (●——●, $n = 6$) and purified T cells (○——○, $n = 6$) from patients with brain tumours to PHA (10 μg).

Effect of preincubating PBL on their responsiveness to PHA and ability to form E-rosettes

Previously, we have observed that the sera obtained from patients with brain tumours is capable of abrogating both autologous and homologous, normal lymphocyte responsiveness (Brooks *et al.*, 1972; 1974). Therefore, impaired blastogenesis may result from the masking of surface membrane receptors thereby preventing lectin binding and subsequent blastogenesis. This set of experiments was designed to test this hypothesis by preincubating PBL of brain tumour patients to elute any adherent humoral factors and thus theoretically improve responsiveness.

Peripheral blood lymphocytes from patients with brain tumours and control subjects were preincubated for 24 hr at 37°C and their reactivity to PHA determined. The responsiveness of PBL from brain tumour

patients does not change after preincubation when stimulated with from 1–50 μg of PHA but does increase approximately three-fold at 0·1 μg PHA, a suboptimal dose of PHA (Fig. 5). No change was noted in the responsiveness of PBL from control subjects after incubation with again the exception of 0·1 μg PHA where a two-fold increase was noted.

Previously, we have found that the E-rosetting ability of lymphocytes obtained from normal subjects is transitorily lost upon preincubation but reappears and persists after 2 hr or more of continued incubation (Roszman, Brooks & Muse, 1977).

Similar preincubation experiments were performed with lymphocytes obtained from patients with brain tumours (Fig. 6). The results demonstrate that the percentage of E-RFC in PBL suspensions obtained from patients with brain tumours decreased about 60% after preincubation regardless of the serum used to supplement the medium (Fig. 6). Furthermore, improvement of E-rosetting ability did not occur with continued incubation. A much smaller decrease in the rosetting ability of PBL from normal subjects was observed as previously reported.

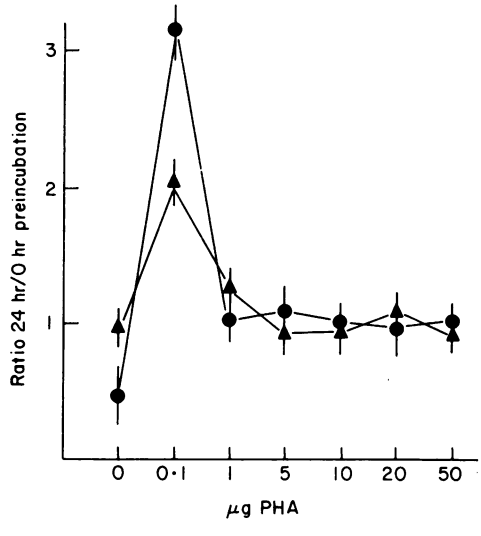

FIG. 5. Effect of preincubating peripheral blood lymphocytes obtained from control subjects (▲——▲, $n = 7$) and brain tumour patients (●——●, $n = 6$) on their ability to respond to PHA.

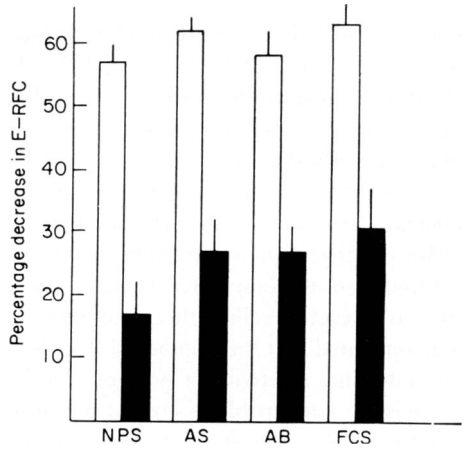

FIG. 6. Effect of preincubating peripheral lymphocytes obtained from control subjects (■, $n = 10$) and brain tumour patients (□, $n = 8$) on their ability to form E-rosettes.

TABLE 1. Number of L-PHA receptors on lymphocytes from patients with brain tumours

Cell source	Sites/cell × 10^5
Experiment 1	
Patient J.R.	31·1
Patient B.B.	11·8
Patient L.D.	18·8
Control 1	7·2
Control 2	9·4
Experiment 2	
Patient H.H.	9·4
Patient B.B.	15·7
Patient F.M.	18·8
Control 1	6·7
Control 2	7·3
Experiment 3	
Patient T.P.	10·4
Patient J.D.	6·7
Control 1	5·9

* Sites per cell were determined by plotting the data obtained from binding experiments according to the method of Steck & Wallach (1965).

Number of PHA Binding Receptors on PBL

Therefore, to access cell-surface characteristics and to determine further the relationship of these properties to lymphocyte activation, the number of PHA binding sites on PBL obtained from control subjects and patients with brain tumours was determined and compared. The results presented in Table 1 demonstrate that lymphocytes from brain tumour patients have approximately twice as many receptors per cell for PHA than do lymphocytes from control subjects.

DISCUSSION

Initial experiments in patients with primary brain tumours demonstrated broad suppression of general host immunocompetence (Brooks *et al.*, 1972; Mahaley *et al.*, 1977; Brooks *et al.*, 1974). Sequential evaluation of these patients indicates that alterations in immunological responsiveness can be correlated with the presence and size of the intracranial neoplasm and, furthermore, these fluctuations may be useful in detecting pre-clinical recurrence (unpublished observation).

The current study represents our continuing efforts to explore various hypotheses that can be evoked to explain this observed phenomenon. In view of the evidence that T cells are mediators of cellular immunity, one attractive explanation is that a quantitative diminution of T cells exists in the peripheral blood of patients with primary tumours of the brain. Accordingly, impaired immunological responsiveness results from a lack of potentially reactive cells rather than alterations in their functional abilities. Although we have previously demonstrated that the number of T cells is abnormally low (Brooks *et al.*, 1976; 1977), comparison of the mitogenic reactivity of purified T cells to unfractionated lymphocytes from patients indicates these T cells are not capable of normal responsiveness.

Alterations in functional qualities of a lymphocyte imply either an intrinsic defect within the cell or in the surface membrane rendering it incapable of normal activation, or that surface receptors are 'masked,' thus preventing proper signalling for responsiveness. Support for the latter proposition is gained from

evidence indicating that brain cells and thymocytes share certain membrane determinants (Graves & Brown, 1974). Although the central nervous system is considered an immunologically privileged site, malignant degeneration of normal brain cells is associated with loss of the normal blood–brain barrier (Long, 1970), thus allowing previously 'hidden' antigens, normal and neoplastic, access to the host's immunological network, and vice versa. Therefore, antibody may be synthesized against not only tumour associated antigens but normal brain antigens, and, in turn, shared antigens found on T lymphocytes. Previously, we have demonstrated that sera collected from these patients abrogates autologous and normal, homologous *in vitro* correlates of cellular immunity (Brooks *et al.*, 1972). Thus, at least one explanation for depressed host immunity in patients with primary brain tumours may be ascribed to the presence of humoral suppressor factors. The nature and characterization of these factors is currently under investigation and remains to be fully elucidated.

In the present study we have demonstrated the presence of a quantitative functional defect in lymphocytes obtained from patients harbouring primary intracranial tumours which, in addition to the presence of humoral factors, contributes to the impairment of general host immunocompetence. First, the receptor for sheep erythrocytes characteristically found upon T lymphocytes fails to be re-expressed on those cells obtained from patients with brain tumours. Secondly, the peripheral blood lymphocytes of these patients bind twice the amount of lectin that is bound to L-PHA receptors of normal lymphocytes. Paradoxically, the mitogenic induced activation is significantly less. These data, taken together, indicate that lymphocytes obtained from brain tumour patients are capable of binding lectin, indeed have more lectin receptors, yet the fluidity of the cell membrane is such that extreme modulations must occur which apparently result in impaired *in vitro* cellular responsiveness.

Although the precise characterization of the functional defect remains to be elucidated, several testable hypotheses may be suggested. Recently, it has been shown that those lymphocytes which bind 'medium' amounts of lectin are responsible for PHA induced blastogenesis (Callard & Basten, 1978). Cells binding more or less lectin are poorly responsive. This observation suggests that PHA induced activation detects only a subpopulation of lymphocytes and, furthermore, a selective loss of these mitogenically responsive cells may occur in brain tumour patients. One explanation of diminished mitogenicity, therefore, may be a selective loss of that subpopulation of lymphocytes that is activated by PHA.

The present study utilized patients prior to surgical and/or medical manipulation, yet the effects of endogenous hormones upon qualitative and quantitative aspects of the human immune system is relatively unknown. Recently, a lymphocyte membrane receptor for insulin has been detected and has been shown to be an index for cellular activation (Helderman & Strom, 1978). Additionally, this receptor has been demonstrated to accept and bind lectin (Brown & Hunt, 1978). The presence of excessive lectin receptors present on lymphocytes obtained from brain tumour patients may reflect excessive and continual activation which, rather than exhibited as enhanced reactivity, may result in diminished mitogenic responsiveness. More importantly, subtle changes in the normal–neural–endocrine–immune network may occur in all patients with neurological disorders which dramatically influence immunological responsiveness as presently determined via qualitative and/or quantitative alterations in lymphocyte subpopulations.

Rather than a selective loss of PHA responsive lymphocyte subpopulations, endogenous hormonal modulation, or excessively activated cells, the present results may indicate the expansion of an otherwise normally present subpopulation of cells which bind large amounts of lectin and are capable of modulating the responsiveness of other lymphocytes. The presence of suppressor cells has been shown in the peripheral blood of normal subjects (Gershon, 1974). In addition to regulating cellular reactivity this subpopulation of T lymphocytes is maximally responsive at suboptimal concentrations of mitogen (Moretta *et al.*, 1977). Thus, the observed significant increase in mitogenic responsiveness at the lowest concentrations of PHA may indicate the presence of suppressor cells in patients with primary brain tumours which modulate general host immune responsiveness. Such cells have been demonstrated in an experimental animal brain tumour model as well as an experimental allergic encephalitis resistant model (Roszman *et al.*, 1978; Welch, Swierkosz & Swamborg, 1978). Their presence in increased number and/or activity may serve an important function by preventing allergic encephalitis in patients with

brain tumours sensitized to normal and malignant brain antigens in addition to non-specifically abrogating immunocompetence.

In addition to demonstrating the presence of a qualitative functional abnormality of peripheral blood lymphocytes obtained from patients with central nervous system neoplasia and indicating avenues of continued exploration of impaired immune responsiveness, this study underscores the extreme caution of interpreting mitogenic activation as an index of antigenic induced lymphocyte activation and, furthermore, indicates that elucidation of lymphocyte plasma membrane events are crucial to our understanding of normal immunological responsiveness as well as that of patients with cancer.

The authors would like to thank Ms Kathy Keaton, Francis Bobbitt and Marty Salyer for their technical assistance.

This work was supported in part by American Cancer Society Grant No. IM-92, National Cancer Institute Grant No. CA-18234 and Veterans Administration Project No. 596-1096-01.

REFERENCES

BOLDT, D., SKINNER, A.M. & KORNFELD, S. (1972) Studies of two subpopulations of human lymphocytes differing in responsiveness to concanavalin A. *J. clin. Invest.* **51**, 3225.

BÖYUM, A. (1968) Separation of leukocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest.* **21**, Suppl. 97, 1.

BROOKS, W.H., CALDWELL, H.D. & MORTARA, R.H. (1974) Immune responses in patients with gliomas. *Surg. Neurol.* **2**, 419.

BROOKS, W.H., NETSKY, M.G., HORWITZ, D.A. & NORMANSELL, D.E. (1972) Cell-mediated immunity in patients with primary brain tumors. *J. exp. Med.* **136**, 1631.

BROOKS, W.H., ROSZMAN, T.L., MAHALEY, M.S. & WOOSLEY, R. (1977) Immunobiology of primary intracranial tumors. II. Analysis of lymphocyte subpopulations in patients with primary brain tumours. *Clin. exp. Immunol.* **29**, 61.

BROOKS, W.H., ROSZMAN, T.L. & ROGERS, A.S. (1976) Impairment of rosette-forming T lymphocytes in patients with primary intracranial tumours. *Cancer*, **37**, 1860.

BROWN, J.C. & HUNT, R.C. (1978) Lectins. *Internal review of cytology*, **52**, 277.

CALLARD, R.E. & BASTEN, A. (1978) Identification of T cell subpopulations binding phytohemagglutinin: functional characteristics. *Eur. J. Immunol.* **8**, 247.

GERSHON, R.K. (1974) T cell control of antibody production. *Contemp. Top. Immunol.* **3**, 1.

GRAVES, M.F. & BROWN, G. (1974) Antigenic correlation between brain and thymus. *Lancet*, i, 455.

GREAVES, M. & JANOSSY, G. (1972) Elicitation of selective T and B lymphocyte responses by cell surface binding ligands. *Transplant. Rev.* **11**, 87.

HELDERMAN, H.J. & STROM, T.B. (1978) Specific insulin binding site on T and B lymphocytes as a marker of cell activation. *Nature*, **274**, 62.

JONDAL, M., HOLM, G. & WIGZELL, H. (1972) Surface markers on human T and B lymphocytes. I. A large population of lymphocytes forming non-immune rosettes with sheep red blood cells. *J. exp. Med.* **136**, 207.

LONG, D.M. (1970) Capillary ultrastructure and the blood–brain barrier in human malignant brain tumors. *J. Neurosurg.* **32**, 127.

MAHALEY, M.S., BROOKS, W.H., BIGNER, D.D. & ROSZMAN, T.L. (1977) Immunobiology of primary intracranial tumors. I. Studies of the cellular and humoral general immune competence of brain tumor patients. *J. Neurosurg.* **46**, 467.

MCCONAHEY, P.J. & DIXON, F.J. (1969) A method of trace iodination of proteins for immunologic studies. *Int. Arch. Allergy Appl. Immunol.* **29**, 185.

MORETTA, L., WEBB, S.R., GROSS, C.E., LYDYARD, P.M. & COOPER, M.D. (1977) Functional analysis of two human T cell subpopulations: help and suppression of B cell responses by T cells bearing receptors for IgM or IgG. *J. exp. Med.* **146**, 184.

ROSZMAN, T.L., BROOKS, W.H., MARKESBURY, W.R. & BIGNER, D.D. (1978) General immunocompetence of rats bearing avian sarcoma virus-induced intracranial tumors. *Cancer Res.* **38**, 74.

ROSZMAN, T.L., BROOKS, W.H. & MUSE, K.N. (1977) Effects of preincubating human peripheral blood lymphocytes on their ability of bind sheep red blood cells. *J. Immunol. Methods*, **14**, 93.

STECK, T.L. & WALLACH, D.F.H. (1965) The binding of kidney-bean phytohemagglutinin BT Ehrlich ascites carcinoma. *Biochem. Biophys. Acta*, **95**, 510.

WELCH, A.M., SWIERKOSZ, J.E. & SWAMBORG, R.H. (1978) Regulation of self tolerance in experimental allergic encephalomyelitis. I. Differences between lymph node and spleen suppressor cells. *J. Immunol.* **121**, 1701.

YAM, L.T., LI, C.Y. & CROSBY, W.H. (1971) Cytochemical identification of monocytes and granulocytes. *Am. J. clin. Pathol.* **55**, 283.