THE PROBLEMS OF IMMUNOCOMPETENCE IN BRAIN TUMOR PATIENTS

by

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DEDICATION

To My Wife, Yuko

and

My Parents

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ABBREVIATIONS

CNS	Central Nervous System
BBB	Blood-Brain Barrier
CSF	Cerebrospinal Fluid
PHA	Phytohemagglutinin
Con A	Concanavalin A
PWM	Pokeweed Mitogen
RPMI 1640	Roswell Park Memorial Institute Medium 1640
T cells	Thymus-derived Lymphocytes
B cells	"Bursa-equivalent" Lymphocytes
IgG, A, M	Immunoglobulin G, A, M
^{3}H-thymidine	Tritiated Thymidine
DNCB	Dinitrochlorobenzene

INTRODUCTION

1. Immunology of the Central Nervous System

The anatomy and physiology of the immune system in the central nervous system (CNS) differ from those in other tissues. It is generally considered that the CNS contains no lymphatics and the presence of the blood-brain barrier (BBB) makes the CNS an immunologically unique organ. The anatomical structure of the BBB lies in the endothelium of brain capillaries and tight junctions between the endothelial cells (1). Tight junctions are generally not present in other organs of the body. This barrier prevents the entry of antibodies and effector cells into the brain parenchyma. It is because of the presence of this barrier and the absence of lymphatics within the CNS that the CNS has been considered an immunologically privileged site. Before discussing CNS tumor immunology, it seems necessary that immunology of the central nervous system should be briefly summarized.

Immunological aspects of the CNS were first evaluated by Shirai in 1921 (2) and further investigated by Murphy and Sturm in 1923 (3). They demonstrated that tumors transplanted to the brain would often grow although the same tumor transplanted subcutaneously was rejected. These observations were further elaborated and extended by Medawar (1948) (4). He found that if a fragment of foreign skin was implanted into the brain of a rabbit it was not rejected, but that if a rabbit was initially sensitized with a skin graft, it was rejected. He concluded that antigens within the brain could not activate the afferent limb of the cellular immune response although they could activate the efferent

response in a sensitized host. Subsequently, similar results were reported by Greene (5, 6) and others (7, 8) who appeared to substantiate Medawar's conclusion. These phenomena led to the concept that the brain is "an immunologically privileged site". Scheinberg et al.(1964) (9-11), however, showed that chemically induced ependymoblastoma obtained from inbred mice could be successfully transplanted intracerebrally 100% of the time into syngenic mice but only 10 to 35% of the time into allogenic mice. It was also found that the intracerebrally transplantable tumor was capable of eliciting first set rejection in allogenic hosts and second set rejection of either subcutaneous or intracerebral tumor implants in syngenic hosts. This second set reaction could also be elicited by primary subcutaneous transplant or prior immunization with ependymoblastoma cells incorporated in complete Freund's adjuvant. These studies indicated that the brain was not a completely but a partially immunologically privileged site in that the brain appeared to have some competence in evoking an immune response to some extent.

In any event, the limited immunological system which does exist in the CNS is important for the understanding of various CNS disorders including brain tumors.

2. Evidence of Immune Response to Brain Tumors

1) Cellular Immune Response:

a. Specific Lymphocytotoxity in Vitro:

Specific lymphocytotoxity directed against glioma cells has been demonstrated *in vitro* by a number of investigators. Ciembroniewicz and Kolar (12), one of the early workers who investigated lymphocyte-mediated cytotoxity in patients with primary intracranial tumors in the late 1960's, observed blast-like transformation and the clustering of lymphocytes around autologous and allogenic glioblastoma cells after 72 hours of their coculture. These observations were interpreted as evidence for the sensitization of peripheral lymphocytes by the presence of the brain tumor cells. Similar observations were made by Eggers (13) in 1971 when he evaluated host cellular immune response to glioma in autoradiographic studies. He found that 6 out of 6 gliomas stimulated the aggregation of autologous lymphocytes around the tumor cells in culture. These results suggested that gliomas could spontaneously activate the afferent limb of the cellular immune response.

Levy *et al.* (1972) (14) investigated the specificity of lymphocyte-mediated *in vitro* cytotoxic reactions by utilizing Hellström's microcytotoxicity assay. They demonstrated that peripheral blood lymphocytes from primary intracranial tumors (both gliomas and meningioma), regardless of the degree of anaplasia, were specifically cytotoxic to both autologous and allogenic tumor cells *in vitro*. It was also found that lymphocytes from one of the glioblastoma patients were cytotoxic not only to autologous but also to allogenic tumor cells and normal glial cells, but not to the normal fibroblasts. It was felt from these studies that the lymphocytotoxicity seemed to be tumor specific. This specificity was further affirmed by the same author (15) in 1978 by showing that the lymphocytotoxicity could be abolished by prior absorption of the lymphocytes on appropriate cell monolayers.

It is of particular interest that Wahlström et al. (1973) (16) demonstrated cell-mediated cytotoxicity against autologous glioma cells, normal glial cells and fetal glial cells by using ^{51}Cr release assays. Contrary to Levy's observations, little or no cytotoxity against low grade gliomas and meningiomas was observed. Kumar et al. (1973) (17) also studied lymphocytotoxicity in astrocytomas, ependymomas and medulloblastomas to see if tumor antigens are common to all the glial cells. They found that lymphocytes from astrocytoma patients were 90% cytotoxic to autologous target cells and 68.9% cytotoxic to allogenic tumor cells, and autologous and allogenic lymphocytes from patients with medulloblastoma and ependymoma showed similar but less marked lymphocytotoxicity. There was minimal lymphocytotoxicity in combinations in which allogenic lymphocytes were used which were taken from patients whose tumors were histologically different from target cells. They concluded that the lymphocytotoxity was specifically directed against the neoplastic component of the tumor.

Specific cell-mediated anti-glioma activity has also been detected by Sheikh et al. (1979) (18) using the leukocyte adherence inhibition assay. However, Woosley et al. (1977) (19) demonstrated a significant cytotoxic response to patient effector cells in a glioblastoma in only seven out of 36 cases, suggesting that cytotoxicity could not be demonstrated with lymphocytes from all glioma patients.

Quite recently Rainbird et al. (1981) (20) studied the effects of peripheral blood lymphocytes from patients with glioma on glioma cells grown in culture using an in vitro microcytotoxicity test. They confirmed the previous reports. It was found that lymphocytes from 12 of the 20 patients studied showed significant cytotoxic activity against

their own tumor cells. Four of them reacted against allogeneic glioma cells and 4 also showed some reactivity against fetal brain cells. In this regard, they were unable to substantiate Levy's observation that the lymphocyte-mediated response detected in glioma patients was directed against a glioma-specific tumor antigen(s).

Regardless of whether or not lymphocytotoxicity is tumor specific, it is important that lymphocytes could be sensitized to tumor cells and acquire cytotoxicity against them.

b. Lymphocyte Infiltration in Vivo:

There is also in vivo evidence suggesting that lymphoctyes seem to play an important role in the interaction between brain tumor tissue and the host immune system. As described earlier in the section: "Immunology of the Central Nervous System," the brain has been regarded as an immunologically privileged site because it lacks a conventional lymphatic system and has a blood-brain barrier. There is considered to be no entry of circulating lymphocytes into the normal brain, but Konigsmark and Sidman (1963) (21) and subsequently Roessmann and Friede (1968) (22) using autoradiographic techniques showed significant numbers of labelled bone marrow-derived cells or blood monocytes in the damaged brain.

Ridley and Cavanagh (1971) (23) reported an overall 58% incidence of lymphocyte infiltrations in autopsied material from glioma patients. Although the possibility that cellular infiltration simply results from a response to necrosis within the tumor tissue remains to be determined, it was suggested that the lymphocytic response may represent the in vivo example of host immune reaction to brain tumors.

Since this initial report in 1971, a number of investigators have investigated correlation between the degree of lymphocytic infiltrations and prognosis of the brain tumor patients (24-26). One of the most elaborate studies regarding the association between lymphocyte invasion and survival of brain tumor patients was done by Brooks et al.(1978) (26). They found that approximately one half of glioblastoma multiforme and nearly one fourth of anaplastic astrocytoma biopsies showed variable degrees of lymphocytic perivascular infiltration. Furthermore, lymphocyte invasion of malignant glioma was clearly associated with length of survival. It was concluded that patients with malignant gliomas containing lymphocytic perivascular infiltration had better prognosis than those without lymphocyte infiltration, living up to 4 months longer.

Further characterization of these lymphocyte infiltrations has been recently carried out. Stavrou et al. (1977) (27), using tissue sections and a fluorescein isothiocyanate-labelled antiserum raised against human T lymphocytes, found thymus-derived lymphocytes in the perivascular lymphocyte infiltration present in some of the gliomas examined. This was further extended and confirmed by Phillips et al. (1982) (28) who showed T and B lymphocytes and their subsets in 5 out of 14 glioma suspensions using rosetting reactions. Furthermore, macrophages have been recently described in both experimental and human brain tumors by Morantz et al. (1979) (29, 30) and by others (31, 32).

Although the precise function(s) of these lymphoreticular cells, and in particular their anti-tumor role in man, is still poorly defined, Rainbird et al. (1981) (20) observed recently that 2 of the 3 patients in their series who showed perivascular cuffing gave a positive

lymphocytotoxic result, suggesting an association between in vitro cytotocity and the degree of lymphocytic infiltration.

2) Humoral Immune Response

It has been shown by a number of investigators that human glioma cells possess specific antigenic determinants on their surface.

Trouillas (33) in 1971 was able to produce anti-glioblastoma antibody in autologous serum by intradermally injecting 14 patients with emulsion of their own tumor extract and Freund's complete adjuvant. The antibody was specific for tumor but was not present prior to immunization. It was also found that anti-glioblastoma sera reacted positively with fetal brain extracts, suggesting that glial tumors possess a fetal antigen.

Lim et al. (1972) (34) demonstrated tumor specific antigens in an established rat astrocytoma cell line using allogeneic anti-serum.

Coakam (1974) (35) immunized a rabbit with tissue culture cells from a human astrocytoma. The resultant anti-serum after extensive absorption (including absorption with normal but not autologous brain) was cytotoxic to the astrocytoma cells in the presence of guinea pig complement. The cytotoxic effect was lost when the serum was absorbed with cells from any of seven different astrocytomas.

Wahlström (1974) (36) also reported tumor-specific membrane antigens in established cell lines from gliomas. A rabbit was immunized with cells from a glioblastoma with Freund's adjuvant. The serum was absorbed with various tissues including fetal brain but not normal adult brain.

The immunofluorescent studies showed that the resultant serum stained glioblastoma but no other neoplastic or normal tissue culture cells.

Levy et al. (1978) (15) defined two antigens on the surface of glioma cells. One was found on cells from all glial tumors, and was called the common glioma antigen (CGA). The other was expressed on anaplastic gliomas, melanomas, and fetal glial cells, but not on well-differentiated gliomas, normal adult glial cells, fetal fibroblasts or other tumors.

That gliomas have tumor-specific antigens has also been currently suggested by many investigators utilizing recently developed hybridoma technology (37-42). Most recently Wikstrand and Bigner (1982) (42) showed that 2 anti-human fetal brain monoclonal antibodies recognized determinants which were predominantly associated with human gliomas but which were also expressed on other tumors of neuroectodermal origin, second-trimester fetal tissue and some lymphoid cells.

There is also some evidence suggesting that brain antigens shed into systemic blood circulation to produce immune complexes. Martin-Archard et al. (1980) (43) showed that the level of circulating immune complexes detectable by C1q binding was abnormally high in 28% of patients with high-grade gliomas and only 14% of patients with low grade gliomas.

In summary, these results suggest that human glial tumors possess specific antigenic determinants on their surface capable of producing antibody. It is also suggested that antigens are shed from brain into systemic blood circulation and evoke humoral immune response in brain tumors.

3. General Immunocompetence in Brain Tumor Patients

Several recent studies have demonstrated depressed cell-mediated immunity in patients with brain tumors (44-56). This impaired cellular immunity has been identified by *in vivo* skin test reactivity to a variety of antigens, by a decrease in the number of T cells in the circulating lymphocyte population, or by decreased *in vitro* responsiveness of lymphocytes to mitogens and/or antigens. Brooks *et al.* (1972) (44), one of the early pioneers in this field of study, reported depressed cell-mediated immunity among a series of 23 patients with both benign and malignant intracranial tumors. Cell mediated reactivity in their study was evaluated both by *in vivo* skin test reactivity including the use of dinitrochlorobenzene (DNCB) and by *in vitro* lymphocyte responsiveness to the mitogen phytohemagglutinin (PHA). Of these 23 patients, 11 demonstrated evidence of anergy. This group included 3 patients with glioblastoma, 1 astrocytoma, 2 meningiomas, 2 neurinomas and 3 other miscellaneous tumors. They also demonstrated that the IgG fraction of serum from patients with glioblastoma inhibited ^{3}H-thymidine incorporation in 1) PHA stimulated lymphocyte culture, 2) mixed tumor lymphocyte culture, and 3) one-way mixed lymphocyte culture. The potency of inhibition was found to be proportional to the degree of skin test anergy in the patient from whom the serum was obtained. These results were confirmed in studies by Thomas *et al.* (1975) (46), Young *et al.* (1976) (47, 49), Mahaley *et al.* (1977) (50), Matsukado and Uemura (1977) (51) and more recently by Gerosa *et al.* (1980) (52, 53) and Roszman and Brooks (1980) (54).

To date the pathogenesis of this depressed cell-mediated immunity is unknown. A number of hypotheses have been postulated to explain the underlying mechanism(s) whereby the impaired cellular immunity occurs in patients with brain tumors.

The most widely studied and documented mechanism is that involving soluble humoral factors present in the serum of brain tumor patients. Such serum blocking factor(s) (antibody, antigen-antibody complex, or serum protein such as α-globulin) was demonstrated to interfere with in vitro lymphocytotoxicity assays in an immunologically specific manner, as well as to block non-specifically lymphocyte blastogenesis as evaluated by ^{3}H-thymidine incorporation. Eggers (1971) (13), one of the early workers who studied serum blocking factors in brain tumors, observed that autologous serum blocked the aggregation around tumor cells of the lymphocytes from systemic malignancies but not from glial tumors. Levy et al. (1972) (14) suggested that IgG serum blocking factor was present in some glioma patients in the early postoperative stage.

Kumar et al. (1973) (17) also found that two of nine medulloblastoma patients and one of four astrocytoma patients had blocking activity as detected in microcytotoxicity assay. It was also found that this blocking activity was tumor specific since the serum blocked lymphocytotoxicity when both the target tumor cells and lymphocytes were derived from patients with histologically the same type of tumors.

Characterization and isolation of this blocking factor(s) were extensively studied by Brooks et al. in 1972 (44). They demonstrated that mitogen-induced lymphocyte blastogenesis was inhibited with the IgG fraction from the serum of patients with glioblastoma. They also found that this blocking effect was not specific to lymphocytes from brain tumor patients. This is in contrast to the observations made by Levy et al. (14, 57) and subsequently by Kumar et al. (17) that serum blocking factor(s) was immunologically tumor specific. It is not at all clear how serum blocking factor(s) really work in modulating lymphocyte function,

but it has been suggested that normal surface receptors on the lymphocytes are "masked" by the blocking factor(s) so that proper signalling for lymphocyte activation is prevented.

In contrast to so-called "extrinsic" mechanism(s) where the serum blocking factor(s) appears to play a major role as an immunomodulator, there is also some evidence suggesting that patient lymphocytes themselves are "intrinsically" abnormal. The surface receptors on the lymphocytes from brain tumor patients appear to be qualitatively different from those of normal lymphocytes (54, 58). Furthermore, recent studies (59, 60) regarding the presence of circulating T lymphocytes provide evidence that there is an actual quantitative decrease in the number of T lymphocytes which are a major force in tumor destruction. Indeed, Mahaley (50) reported that preoperative lymphocyte counts were most reduced in patients with glioblastoma and slightly reduced in patients with less malignant lesions as well.

Another example of mechanism explaining impaired lymphocyte function is related to the presence of specific suppressor cells (T lymphocytes or monocytes) in the peripheral blood from brain tumor patients. Braun et al. (1982) (61) reported that the depressed level of mitogenic response in lymphocytes was associated with significantly increased levels of glass-adherent immunoregulatory cell activity, alone or in conjunction with decrease in T cells. Wood and Morantz (1982) (62) also reported that in most patients with brain tumors, depressed immunocompetence was related to monocytosis and that monocytes functioned as suppressor cells. The same authors further extended their studies and suggested that there was a possible link between the suppressor cell activity and humoral factors present in the serum of brain tumor patients. They postulated

that circulating, soluble blocking factor(s) were bound to the surface of Fc receptor-positive cells, including monocytes, and were then activated to suppress lymphocyte function.

4. Rationale for the Present Studies

The primary objectives of the current investigations were three-fold. First, the studies were designed to document the presence of anergy in a brain tumor population as evidenced by lymphocyte hyporesponsiveness to mitogens, and a search was made to define the possible causes for such defective responsiveness. Secondly, the role of anti-convulsant therapy in anergy was investigated since in previous studies, with the single exception of Roszman and Brooks (1980) (54), no mention of the use of anti-convulsants was made. In vitro studies were therefore conducted evaluating the effects of phenytoin, barbiturates (thiopental, pentobarbital and phenobarbital), and steroids on three different parameters of mitogen induced lymphocyte activation (^{3}H-thymidine incorporation, cell size distribution, and cellular proliferation). Thirdly, the effects of tumor cyst fluid and cerebrospinal fluid (CSF) from brain tumor patients upon mitogen induced normal lymphocyte activation in vitro was examined. The presence of soluble immunosuppressive factors in ascites has been recently described in patients with advanced systemic malignancies which metastasized to the peritoneum (63-70). Similar suppressive factors were also detected and characterized from tumor tissue extracts (71-76) and tissue culture media (77-81). McKeever et al. (1981) (82) recently evaluated several proteins produced and released by cultured human astrocytoma cells to see if they

could function as immunoregulators. The present studies were therefore designed to evaluate the hypothesis that brain tumor cyst fluid and CSF contain locally produced immunosuppressive factors that then might be released by the tumor into serum and/or CSF.

MATERIALS AND METHODS

1. Basic Considerations for Methodology

1) Ficoll-Hypaque Centrifugation

The isolation of lymphocytes from blood involves separating them from erythrocytes, platelets, and other leukocytes. Several methods have been described including dextran sedimentation and plasmagel sedimentation (83). One of the most elaborate methods was described by Boyum in 1964 (84) for separating lymphocytes from human blood and has been used successfully with blood from other species. Other authors (85, 86), using slight modifications of Boyum's original procedure, have emphasized that the Ficoll-sodium metrizoate centrifugation procedure is an easy, one-step, rapid, and reproducible method for the preparation of viable lymphocytes in high yield. Sodium diatrizoate has been successfully substituted for sodium metrizoate in this procedure by numerous workers (48, 49, 54, 87). In the present studies a Ficoll-Hypaque gradient was prepared consisting of 10 parts of 34% sodium diatrizoate (Hypaque Sodium: Winthrop Laboratories, New York, New York) and 25 parts of 9% (w/v) Ficoll 400 (Pharmacia, Piscataway, New Jersey)

in distilled water to produce a solution of density 1.064 g/ml. This separation mixture is sterilized through a 0.2 μm filter (Nalge Company, Rochester, New York) before use. The preliminary experiments showed that the separation mixture described above was superior to any other combination of mixtures in isolating lymphocytes from other cellular components of peripheral blood in terms of purity and yield. Thus, Ficoll-Hypaque solution adjusted with the ratio of 2.5:1 was successfully used throughout the present studies.

2) Counting Lymphocytes

Cell counts were determined on a ZBI Coulter Counter (Coulter Electronics, Inc., Hialeah, Florida) (88, 89). For Coulter counting, original lymphocyte suspensions in RPMI 1640 culture medium (M.A. Bioproducts, Walkersville, Maryland) were diluted 1:500 in isotonic solution (Isoton: Coulter Electronics, Inc., Hialeah, Florida). The Coulter Counter settings were as follows: Amplification - 1/4; 1/aperture current - 1/2; Lower threshold - 9; and Upper threshold - 100. With these settings, the lower threshold was designed to exclude cells with a diameter (volume) of less than 6.8 μm (148 μm^3). Thus lymphocyte suspensions which were contaminated with erythrocytes could be counted without significant interference by the latter. More accurate counts for mononuclear cells were accomplished on a ZBI Coulter Counter by adding Zap-Oglobin II (Coulter Diagnostic, Hialeah, Florida) to lyse the cell membrane so that only nucleated cells were counted (90, 91). To count nucleated cells, the Coulter Counter settings were as follows:

Amplification - 1/4; 1/aperture current - 1/2; Lower threshold - 2; and Upper threshold - 27.

3) Mitogens

In the present studies, three common mitogens were used for stimulating lymphocytes to undergo blastogenesis: phytohemagglutinin (PHA), concanavalin A (Con A), and pokeweed mitogen (PWM). PHA and Con A have been termed T cell mitogens in that 80-90% of the induced blast cells derive from T cells whereas the remainder are of B cell origin (92). In contrast, roughly 50% of PWM-induced blasts are T cells and 50% are of B cell origin (92). Since the blastogenic responses to PHA, Con A and PWM are dependent on the concentrations of mitogens used, the dose response curves were established to determine the optimal stimulant concentration for each of these mitogens. The preliminary experiments showed that the optimal concentrations for blastogenic responses with PHA, Con A and PWM were approximately 0.5, 25, and 2.5 μg/ml respectively as final concentrations in a culture system. It must be remembered, however, that the dose response curve may vary from individual to individual. It was also noted from the preliminary experiments that there were significant differences in lymphocyte blastogenic responses induced by mitogens produced by various manufacturers. A search was made for the best mitogens (in terms of mitogenic activity), and as a result, PHA was purchased from Wellcome Reagents Limited, Beckenham, England, Con A from Pharmacia Fine Chemicals, Piscataway, New Jersey, and PWM from Gibco Laboratories, Grand Island, New York. Mitogens were dissolved in sterile normal saline to give stock concentrations of 100 μg/ml for PHA

and 1 mg/ml for both Con A and PWM. PHA and PWM were stored in aliquots at -70°C and Con A at 4°C according to the manufacturers' instruction.

4) Cell Culture in General

a. Culture Medium and Serum Used

Previous reports have shown that successful long-term culture of human leukocytes and lympho-reticular cells were performed with Roswell Park Memorial Institute 1640 culture medium (RPMI 1640) (93, 94). Therefore, RPMI 1640 was chosen in the present studies as an adequate culture medium providing nutritional support for lymphocyte culture. RPMI 1640 was purchased from M.A. Bioproducts, Walkersville, Massachusetts and supplemented with L-glutamine (2mM/ml), penicillin (200 units/ml), streptomycin (200 µg/ml) and gentamicin (10 µg/ml). It is generally considered that the addition of L-glutamine may be beneficial if the medium is to be utilized over longer intervals (95). Antibiotics were used for minimizing contamination although strict sterile techniques were used in a laminar flow hood (Biogard Hood: The Baker Company, Inc., Sanford, Maine).

Pooled human serum was obtained from healthy normal donors (various blood types), heated at 56° C for 30 minutes to inactivate complement, and stored at -70° C until use. Other investigators use either fetal calf serum or pooled human AB serum; however, the preliminary experiments showed that lymphocytes cultured in pooled human serum responded far better than those cultured in fetal calf serum or pooled human AB serum.

The ^{3}H-thymidine incorporation of normal control lymphocytes in the presence of normal autologous serum versus normal pooled human serum resulted in no statistically significant difference. Pooled human serum was therefore successfully used as a control serum throughout the present investigation.

b. Culture Technique

Two different techniques were used for lymphocyte culture in the present studies: 1) "microculture" and 2) "macroculture" techniques (95). Short term lymphocyte cultures in microtiter plates are referred to here as microculture techniques. They are more economical in use of tissue-culture materials and require less labor than macrocultures which will be discussed later on. They also make it relatively easy to assess DNA synthesis by lymphocytes through quantitative assays of incorporation of radioisotope precursors. Microtiter plates with 96 round-bottomed wells were purchased from Dynatech Laboratories, Alexandria, Virginia. For basic microculture studies, each well in a microtiter plate received 200 µl of complete medium containing 5 x 10^4 cells, 20 µl of various mitogens, and 20 µl of heat-inactivated pooled human serum (serum concentration was therefore 8%). Automatic pipettes with disposable sterile plastic tips (Medical Laboratory Automation, Inc., Mount Vernon, New York) were used to dispense the cell suspension and stimulants. They were then covered with sterile plastic lids (Falcon, Oxnard, California) and incubated.

Another approach to the quantification of lymphocyte activation is the measurement of increases in cell volume and total cell number in response to a mitogenic stimulus. Since these measurements require a greater number of cells for assay, short term lymphocyte cultures were performed in tubes simultaneously with microcultures. They are designated

here as "macroculture" techniques. To each sterile tube (17 x 100 mm, polypropylene culture tube; Falcon, Oxnard, California), generally 1.0 x 10^6 cells were added in 1.6 ml of RPMI 1640 culture medium with 0.2 ml of PHA and 0.2 ml of heat-inactivated pooled human serum (serum concentration was therfore 10%). Culture tubes were then capped and incubated.

c. Incubation Procedure

A vitrotherm CO_2 incubator with automatic CO_2 control monitor system (New Brunswick Scientific, Edison, New Jersey) was used for incubating lymphocyte cultures. It provided stable temperature and CO_2 regulations. The temperature was automatically adjusted to 37°C and a humidified atmosphere of 5% CO_2 and 95% air was maintained in a stable fashion. Incubation of cells in a humidified atmosphere of 5% CO_2 and 95% air is important for optimal cellular growth since the presence of CO_2 maintains the bicarbonate-buffered culture medium at the proper physiological range of pH.

Cell cultures were incubated under the conditions specified above for a total of 90 hours, after which time the cells were harvested. Eighteen hours before harvesting the microcultures, 1.0 μCi of ^{3}H-thymidine (specific activity 6.7 Ci/mmole: New England Nuclear, Boston, Massachusetts) was added to each well in a volume of 20 μl of Hanks' Balanced Salt Solution (Gibco Laboratories, Grand Island, New York). The microtiter plates were then reincubated.

d. Harvesting Cultures

Harvesting microplate cultures was performed with an automatic multiple sample harvester (M.A. Bioproducts, Walkersville, Maryland) (95), which aspirated and washed the cells in each well with distilled water onto glass fiber filters (Microbiological Associates, Walkersville, Massachusetts). This method resulted in more reproducible data and saved time and effort in comparison with other approaches. The filters retained predominantly macromolecules (DNA) with incorporated radioisotope (^3H) since the cells were broken up on the filter and the unincorporated radiolabeled precursor was washed out. The filter discs were placed in scintillation vials (Wheaton Scientific, Millville, New Jersey), and 3.0 ml of scintillation fluid (Aquasol; New England Nuclear, Boston, Massachusetts) were added to each vial. It was shown from the preliminary studies that the elution of radioactivity in terms of cpm seemed to be stabilized 24 hours after the addition of the scintillation fluid and that there was little difference in cpm between countings taken at various periods after 24 hours. Thus in practice, radioactivity of the filters could be measured in a liquid scintillation counter (Beckman, Palo Alto, California) just after 24 hours of processing in a scintillation cocktail. ^3H-thymidine incorporation was quantitated as the count of ^3H per minute (cpm) per filter disc.

With regard to harvesting macrocultures, lymphocyte suspensions in tubes were diluted 1:20 in isotonic solution (Isoton) following 90 hours of incubation and subjected to analysis of cell size distribution and cellular proliferation which will be discussed in detail in the next section.

5) Assessment of Mitogen-induced Lymphocyte Activation

Assays for *in vitro* lymphocyte responsiveness to mitogens were done using three parameters: 1) ^{3}H-thymidine incorporation into DNA; 2) cell size distribution; and 3) cellular proliferation.

a. ^{3}H-thymidine Incorporation into DNA

^{3}H-thymidine incorporation into DNA was evaluated using a microtiter culture system. Since microcultures were performed in triplicate, the arithmetic mean and standard error of the mean (SEM) of the counts per minute (cpm) of the samples were determined through computer data analysis. Some authors correct the cpm for both degree of quenching and efficiency of their counters and express their results in disintegrations per minute (dpm). In the preliminary experiments a quench curve was established by counting commercial standards (Packard Instrument Co., Downers Grove, Illinois) in a liquid scintillation counter (Beckman Model LS7000, Palo Alto, California) and monitoring for quench by H-Number. The H-Number is a measure of the pulse-height response for the Compton edge of the pulse-height distribution from the Compton electrons produced in the solution by the interaction between the external gamma ray (^{137}Cs) and sample vials. It was found on the basis of the ^{3}H quench curve in this experimental design that the H Number varied little from experiment to experiment: the efficiency was fairly constant, approximately 55% with the maximum counting efficiency being 62%. When, as is usually the case, the degree of quenching of samples within a given experiment as monitored by H-Number is similar and therefore counting efficiency constant, it is reasonable to assume from the equation, cpm/dpm x 100 = counting

efficiency (%), that ^{3}H-thymidine incorporation into DNA can be expressed by cpm instead of dpm.

b. Cell Size Distribution and Cellular Proliferation

Cell size distribution analysis and cellular proliferation were evaluated on a ZBI Coulter Counter and Coulter Channelyzer using macroculture techniques after 90 hours of incubation at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

The Coulter Counter and Coulter Channelyzer were set as follows to evaluate each experimental culture for cell size distribution: Amplification - ¼; 1/aperture current - ½; Lower threshold - 20; Upper threshold - 100; Base channel threshold - 20; Window width - 100; Count range - 1K; and Count control - stop at full scale. With these settings, the preliminary studies indicated that the vast majority (approximately 80-90%) of unstimulated normal human lymphocytes were observed between channels 1 and 19. Channel 1 corresponds to a cell diameter of 8.71 μ, channel 20 to a diameter of 10.80 μ, and channel 100 to a cell diameter of 15.58 μ. Channel 20 was arbitrarily selected as the upper normal limit of volume for unstimulated normal human lymphocytes. Thus, activated lymphocytes were defined as those cells assigned to channels 20-100 on the Coulter Channelyzer after 90 hours of incubation with PHA. The number of cells between channels 1 and 19 and those between channels 20 and 99 were integrated separately and the percentage of cells above channel 20 (defined as % activated lymphocytes) was calculated for each experimental culture.

With regard to evaluation of cellular proliferation, accurate counts for mononuclear cells were accomplished on a ZBI Coulter Counter by adding Zap-Oglobin II (Coulter Diagnostic, Hialeah, Florida) to lyse the cell membrane so that only nucleated cells were counted. To count cells, the Coulter Counter settings were as follows: Amplification - ¼; 1/aperture current - ½; Lower threshold - 2; and Upper threshold - 27. Cellular proliferation was calculated by averaging multiple counts for each culture sample and calculating a proliferation ratio by dividing the number of cells in each experimental culture by the cell number in the control containing no mitogen.

2. Patient Population and Preparation of Samples

1) Lymphocyte Donors (Patients and Normal Volunteers)

The characteristics of the 22 patients with benign or malignant intra-cranial tumors who were studied for cellular immune responsiveness are shown in Table 1. These patients were at the University of Texas Health Science Center at Dallas and the Veterans Administration Medical Center at Dallas. There were 12 men and 10 women, ranging in age from 15 to 65 years. In addition, volunteers with similar sex distribution, an age range from 20 to 35, and determined to be clinically normal were used as a control group (though not exactly paralelling the age distribution of the experimental group).

2) Preparation of Test Fluid (Cyst Fluid, CSF and Serum)

Tumor cyst fluid, cerebrospinal fluid (CSF) and serum were obtained at the Oregon Health Sciences University and Veterans Administration Medical Center at Portland from eight patients with primary intracranial neoplasms (1 astrocytoma, 5 glioblastoma multiforme, 1 medulloblastoma, and 1 microglioma). The test fluids were centrifuged at 500 x g for 10 minutes, filtered through a 0.2 μm filter (Gelman Sciences, Inc., Ann Arbor, Michigan), and stored at -70°C. Sera from patients were heat-inactivated at 56°C for 30 minutes.

3) Isolation and Purification of Peripheral Blood Lymphocytes

30-50 ml of peripheral blood was obtained with heparinized blood collection tubes (Becton-Dickinson, Rutherford, New Jersey) from either patients or normal volunteers, and diluted 1:3 with 0.9% normal saline. Aliquots (40 ml) of the blood-saline mixture were placed in 50 ml polypropylene conical tubes (Falcon, Oxnard, California), and Ficoll-Hypaque mixture (10 ml) specified earlier was layered below the blood-saline mixture using a long spinal needle. After centrifugation at 200 x g for 45 minutes at room temperature, the cloudy lymphocyte rich layer at the plasma-Ficoll-Hypaque interface was removed by aspiration with a Pasteur pipette and washed three times in sterile normal saline. The cells were resuspended in RPMI 1640 culture medium (M.A. Bioproducts, Walkersville, Maryland) supplemented with L-glutamine (2 mM/ml), penicillin (200 units/ml), streptomycin (200 μg/ml), and gentamicin (10 μg/ml). This is hereafter referred to as complete RPMI 1640 culture medium. The cells were then counted on a ZBI Coulter Counter (Coulter Electronics, Hialeah, Florida) and the cellular morphology of the

purified lymphocyte suspension was evaluated by examining Wright-stained smears and viability was determined by the trypan blue dye exclusion technique (96). The final cell population was 98% mononuclear in character and approximately 90% of the cells were viable.

4. Short-term Lymphocyte Culture in Microtiter Plates ("Microculture Technique")

1) Studies on Cellular Responsiveness of Brain Tumor Patients and the Effects of Various Drugs on Mitogen-induced Normal Lymphocyte Activation

Mitogen-induced DNA synthesis was measured by tritiated thymidine incorporation into lymphocytes in a microtiter culture system. Cells (5 x 10^4 per micro-well) were cultured in round-bottomed microplates (Dynatech Laboratories, Alexandria, Virginia) using complete culture medium (0.2 ml), with 20 µl of heat inactivated serum (patient or pooled normal). Either 20 µl of phytohemagglutinin (PHA: Wellcome Reagents Limited, Beckenham, England, 5 µg/ml) or 20 µl of pokeweed mitogen (PWM: Gibco Laboratories, Grand Island, New York, 25 µg/ml) was added; the final volume in each well was 240 µl. Cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air for 72 hours. Then 1.0 µCi of ^{3}H-thymidine (6.7 Ci/mmole, New England Nuclear, Boston, Massachusetts) was added to each well. Eighteen hours later the cells were harvested on glass fiber filters (Microbiological Associates, Walkersville, Maryland) and the unincorporated radiolabeled precursor was removed by washing with an automatic multiple sample harvester (M.A.

Bioproducts, Walkersville, Maryland). Radioactivity was measured in a liquid scintillation counter (Beckman Model LS-150, Palo Alto, California). Responsiveness was quantitated as mean count of ^{3}H per minute (cpm) per filter disc, where the mean was that of the triplicate assay.

To establish a criterion of hyporesponsiveness the mean ^{3}H-thymidine incorporation of all the cultures of lymphocytes from normal volunteers was calculated and the standard deviation determined. Marked hyporesponsiveness was defined as ^{3}H-thymidine incorporation into lymphocytes that was more than 2 standard deviations less than the mean incorporation into lymphocytes from the 22 normal controls. Moderate hyporesponsiveness was defined as ^{3}H-thymidine incorporation that was more than 1 standard deviation below the mean control value.

To evaluate the effects of phenytoin, barbiturates or dexamethasone on mitogen-induced blastogenesis, various concentrations (0.05-1000 μg/ml in final concentration) of either phenytoin (Parke-Davis, Morris Plains, New Jersey), barbiturates (thiopental, pentobarbital: Abbott Laboratories, North Chicago, Illinois; phenobarbital: Elkins-Sinn, Inc., Cherry Hill, New Jersey), or dexamethasone (Merck, Sharp and Dohme, West Point, Pennsylvania) were added to each culture.

2) Studies on the Presence of Immunosuppressive Factor(s) in Tumor Cyst Fluid

Mononuclear cells (5×10^{4}) in 200 μl of complete RPMI 1640 culture medium containing 10% (v/v) heat-inactivated (at 56°C for 30 minutes) pooled human serum were cultured in triplicate using round-bottom

microtiter plates (Dynatech Laboratories, Alexandria, Virginia). An optimal concentration of either phytohemagglutinin (PHA: 5.0 µg/ml, Wellcome Reagents Limited, Beckenham, England), concanavalin A (Con A: 250 µg/ml, Pharmacia Fine Chemicals, Piscataway, New Jersey), or pokeweed mitogen (PWM: 25 µg/ml, Gibco Laboratories, Grand Island, New York) were added in a volume of 20 µl. In addition, tumor cyst fluid, cerebrospinal fluid (CSF), or patients' sera were tested for their ability to inhibit lymphocyte proliferation in response to the mitogens by adding 40 µl of the test fluids to triplicate wells of the microtiter plates. Control responses represented triplicate tests to which an additional 40 µl of normal pooled human serum, normal CSF (obtained at myelography for lumbar disc disease) or 40 µl of normal saline were added in addition to the standard concentration of pooled human serum (10% v/v) and other nutrients. In titrating the inhibitory activity of the test fluids, a range of volumes of the tumor cyst fluid, CSF, or patients' sera were added (40, 30, 20, 10 µl/well) at the initiation of the culture with the volumes being equalized by addition of 0.9% sterile normal saline. The final volume in each well was 260 µl. Cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air for 72 hours. Then 1.0 µCi of ^{3}H-thymidine (New England Nuclear, Boston, Massachusetts: specific activity 6.7 Ci/mmole) was added to each well. At 90 hours the cells were harvested on glass fiber filters (Microbiological Associates, Walkersville, Maryland) with an automatic multiple sample harvester (M.A. Bioproducts, Walkersville, Maryland). The filter paper was processed in a cocktail for liquid scintillation counting (Aquasol: New England Nuclear, Boston, Masschusetts) and then radioactivity was measured in a liquid scintillation counter (Beckman

Model LS7000, Palo Alto, California). Tritiated thymidine incorporation into DNA was quantitated as the mean count of ^{3}H per minute (cpm) per filter disc, where the mean was that of the triplicate assay. Where the average cpm from any well deviated by more than 50% of the median value of the three wells, that value was discarded.

5. Short-term Lymphocyte Culture in Tubes ("Macroculture Technique")

1) Studies of Drug Effects on Mitogen-induced Normal Lymphocyte Activation

Another approach to the quantification of lymphocyte activation was the measurement of cellular proliferation and cell volume in response to a mitogenic stimulus. Since these measurements require a greater number of cells for assay, the studies were carried out in a tube culture system. To each sterile tube (17 x 100 mm polypropylene culture tubes, Falcon, Oxnard, California), 4.0×10^5 cells were added in 1.6 ml of complete RPMI 1640 medium containing 0.2 ml of heat-inactivated pooled normal human serum (serum diluted 1:1 with 0.9% NaCl). PHA (0.5 μg/ml) and various concentrations of either phenytoin, barbiturates, or dexamethasone were then added to a final volume of 2 ml. The pH of each culture before and after incubation was determined to be 7.2-7.6.

2) Studies on the Presence of Immunosuppressive Factor(s) in Tumor Cyst Fluid

Mononuclear cells (1.0×10^6) were suspended in 1.6 ml of complete RPMI 1640 culture medium containing 10% (v/v) heat-inactivated pooled normal human serum and the suspension was placed in sterile tubes (17 x 100 mm polypropylene culture tubes, Falcon, Oxnard, California). PHA (0.2 ml, 5 μg/ml) and then test fluids (tumor cyst fluid, serum, or CSF) were added at various volumes (0.2, 0.15, 0.1, 0.05 ml/tube). The final volume (2.0 ml) was equalized by addition of 0.9% sterile normal saline. Control cultures containing normal pooled human serum or normal CSF instead of tumor cyst fluid or patient CSF were also prepared as in the microtiter cultures. Cell size distribution as well as cellular proliferation was evaluated on a ZBI Coulter Counter and Coulter Channelyzer after 90 hours of incubation at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Evaluations of cell size distribution and cellular proliferation were described earlier in the section "Basic Considerations for Methodology".

6. Characterization of Tumor Cyst Fluid and CSF from Patients with Brain Tumors

1) Analysis of Protein Content and Immunoglobulins and Measurement of Phenytoin Concentration in Tumor Cyst Fluid

Tumor cyst fluid from four brain tumor patients was analyzed for total protein content and its composition using standard Biuret test and protein electrophoresis. Quantitative measurements of IgG, IgA and IgM of cyst fluid and CSF were evaluated by laser nephelometry (Laser Immunoglobulin Kits: Calbiochem-Behring Corp., La Jolla, California)

(97). The phenytoin level of cyst fluid was measured by enzyme immunoassay (EMIT Phenytoin Assay: Syva Corp., Palo Alto, California) (98) at the time the cyst fluid was evaluated. The lower limits of sensitivity of these assays are 0.1 mg/dl for immunoglobulins and 2.5 μg/ml for phenytoin. These evaluations were performed in the clinical laboratory at the Veterans Administration Hospital, Portland, Oregon.

2) Dialysis of Tumor Cyst Fluid

To determine if any of the inhibitory factor(s) were dialyzable, tumor cyst fluid (1.0 ml) was dialyzed against several changes of 0.9% normal saline during a 24 hour period at 4°C. Dialysis tubing (VWR Scientific Inc., Seattle, Washington) has a 24 Å pore radius and allows the passage of molecules of less than 12,000-14,000 daltons. The dialysates were sterilized through a 0.2 μm filter (Gelman Sciences, Inc., Ann Arbor, Michigan). Normal lymphocyte activation in response to mitogens was evaluated as described earlier using both original and dialyzed cyst fluid to see if there is any change in its inhibitory effect on lymphocyte function.

3) IgG Depletion of Tumor Cyst Fluid

Protein A, a constituent of the cell wall of Staphylococcus aureus Cowans 1, is known to bind specifically with the Fc region of IgG and related molecules (99). Therefore, Protein A was used to selectively remove IgG from the tumor cyst fluid to evaluate what role IgG may play as an inhibitory factor on lymphocyte function. Protein A Sepharose 4B

(Pharmacia Fine Chemicals, Piscataway, New Jersey) was suspended in 0.1 M phosphate buffer (pH 7.0). Binding capacity of Protein A - Sepharose 4B is 20 mg IgG/ml of gel. The Protein A gel suspension (1.0 ml) was mixed with 1.0 ml of tumor cyst fluid at 20°C for 30 minutes. After centrifugation at 500 x g for 15 minutes at room temperature, the supernatant was removed and assayed for IgG content by laser nephelometry. Plain Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, New Jersey) was simultaneously used as a control under the same conditions in order to examine how much non-specific binding of IgG to Sepharose 4B would take place. After dialysis against a 0.1 M phosphate buffer solution, IgG-depleted cyst fluid was sterilized through a 0.2 μm filter (Gelman Sciences Inc., Ann Arbor, Michigan). Normal lymphocyte activation in response to mitogens was evaluated as described earlier using both control (exposed to Sepharose 4B) and IgG-depleted (exposed to Protein A-Sepharose 4B) cyst fluid to see if there was any change in the inhibitory effect on lymphocyte function.

RESULTS

1. Studies on in Vitro Lymphocyte Responsiveness in Brain Tumor Patients.

Responsiveness of lymphocytes to PHA (T cell mitogen) and PWM (B and T cell mitogen) was evaluated in 22 patients with brain tumors and in 22 normal individuals (Table 1 and Figure 1). Lymphocyte blastogenic responses were evaluated on the basis of ^{3}H-thymidine incorporation into DNA in response to optimal stimulation of the mitogens following a total

of 90 hours of incubation. In response to at least one of the two mitogens used in these studies, lymphocytes obtained from eight of twenty-two brain tumor patients showed markedly depressed ^{3}H-thymidine incorporation in the presence of autologous serum (i.e. more than 2 S.D. from the mean response of the control cells). The hyporesponsiveness was more apparent with PHA than with PWM. Lymphocytes obtained from the 22 control individuals similarly cultured with autologous serum displayed no such depression.

Lymphocytes from 13 of 22 tumor patients demonstrated moderate hyporesponsiveness when it was defined as ^{3}H-thymidine incorporation more than 1 standard deviation below the mean level for 22 normal individuals. Blastogenesis in response to mitogens remained depressed in 12 of 13 lymphocyte cultures from anergic patients even when normal human serum was used in place of autologous serum. It is noteworthy that patients whose lymphocytes demonstrated moderate hyporesponsiveness (as measured by ^{3}H-thymidine incorporation) also had evident a second parameter of diminished activity, reduced blastogenesis (i.e. increase in cell volume), as monitored by the Coulter Channelyzer (Figure 2). This suggested that an intrinsic lymphocyte abnormality was responsible for the depressed mitogen response in brain tumor patients.

To evaluate for the presence of serum factors, normal and tumor patient lymphocytes were cultured with mitogen (PHA) in the presence of either pooled normal human serum or tumor patient serum (Table 2). Lymphocyte responsiveness, as measured by ^{3}H-thymidine incorporation, was compared between cultures containing normal human serum and those containing tumor patient serum using the Student t test for paired observations. Pooled normal human serum as compared with autologous

patient serum significantly ($p<0.05$) increased tumor patient lymphocyte responsiveness in 9 of 22 tumor patients. Seven of the nine cell cultures (from the tumor patients) in which pooled human serum significantly improved responsiveness were derived from the markedly hyporesponsive patients (Tables 1 and 2).

Subsequently, normal lymphocytes were used instead of patient lymphocytes and the effects of pooled normal human serum were compared with those of tumor patient serum. In those cultures, 11 of 22 tumor patient sera significantly decreased normal lymphocyte responsiveness. Eight of the eleven suppressive sera were derived from hyporesponsive tumor patients (Tables 1 and 2). Thus, serum factors which suppress both tumor patient and normal lymphocyte activation were present in the serum of the majority of hyporesponsive tumor patients. These results indicate that suppressive serum factors may also play a role as one of the factors responsible for anergy in brain tumor patients.

The present series (defined in Table 1) included 11 patients with glioblastoma, 4 patients with low grade astrocytoma, 2 patients with benign primary tumors and 5 with miscellaneous brain tumors. Since only about a half of the brain tumor patients in the series were hyporesponsive by the defined criteria, a search was made for the variable(s) that could effect this distribution. As shown in Table 1, the degree of anergy did not correlate with cell type or degree of anaplasia. Although it was greatest in patients with glioblastoma as compared to patients with low grade gliomas, 2 patients with decreased mitogenic responsiveness had benign tumors and 2 other patients had low grade astrocytomas. There was no correlation between the age of the patient and the presence of anergy. Investigation therefore continued

into evaluation of possible intrinsic lymphocyte defects as well as identification of immunosuppressive factors (including anticonvulsants, corticosteroids, etc.) which may be responsible for the *in vitro* hyporesponsiveness of patients' lymphocytes to mitogens.

2. Studies of the Effects of Pharmacological Factors on Mitogen-induced Normal Lymphocyte Activation (Anticonvulsants, Steroids, and Barbiturates)

1) Evaluation of ^{3}H-thymidine Incorporation

In reviewing the clinical data in patients with brain tumors, two types of pharmacologic agents were documented to be in use (Table 1); the first was corticosteroids, primarily dexamethasone, and the second was an anti-convulsant medication, usually phenytoin and/or phenobarbital. Those medications are universally used for the management of brain tumor patients. Preliminary studies demonstrated that both phenytoin and possibly phenobarbital were capable of suppressing mitogen-induced ^{3}H-thymidine incorporation of lymphocytes. *In vitro* dose response curves for both of these agents were performed, individually and in combination, and are shown in Figure 3A. Since the therapeutic range for phenytoin is generally between 10 and 20 μg/ml, it is clear that even these levels are associated with a mild suppression of lymphocyte responsiveness. The steep character of the dose response curve emphasizes the potential for suppression by phenytoin. Similar studies were performed with phenobarbital. At the usual anti-convulsant upper therapeutic range of 50 μg/ml or greater, mild inhibition of PHA-induced lymphocyte activation

was seen. In addition, the suppression of lymphocyte function by phenytoin was augmented by the addition of phenobarbital (Fig. 3A).

Since steroids are commonly used in patients with brain tumors and were a potential factor in the hyporesponsiveness in the patients (see Table 1), the effects of dexamethasone on mitogen responsiveness were evaluated. Figure 3B shows significant inhibition of ^{3}H-thymidine incorporation in PHA-stimulated normal human lymphocytes cultured in the presence of dexamethasone. The serum level of dexamethasone concentration 1 hour after a single oral dose of 6 mg is about 0.05 μg/ml (Merck, Sharp and Dohme and Co., unpublished data). In vitro this concentration produces a 50% inhibition of lymphocyte responsiveness to optimal mitogenic stimulation.

The investigation was further continued to examine the effects of three different classes of barbiturates on normal human lymphocyte function. The incorporation of ^{3}H-thymidine into normal human lymphocytes in response to PHA was also affected by the presence of each of the barbiturates studied (Figure 4). Mitogen-induced lymphocyte ^{3}H-thymidine incorporation was reduced by each, but was most affected by the presence of the lipid-soluble short-acting barbiturate, thiopental. Lymphocyte activation was inhibited by 50% when the thiopental concentration was 40-50 μg/ml. Phenobarbital required a concentration of 250 μg/ml to suppress lymphocyte blastogenesis to this same degree. The intermediate acting barbiturate, pentobarbital, was also intermediate in its ability to inhibit blastogenesis. That is, 50% inhibition of lymphocyte activation required a drug level in the culture medium of 150 μg/ml.

2) Evaluation of Cell Size Distribution and Cellular Proliferation

Another measure of lymphocyte activation was based on the fact that stimulated lymphocytes enlarge or undergo blast transformation in response to mitogenic stimuli. Normal lymphocytes after 72 hours of culture remain small and homogeneous and 80% are assigned to channels 1-20 of the Coulter Channelyzer. Following similar culture in the presence of PHA, the evidence of blastogenesis was clearly expressed by a shift in the distribution of lymphocytes to cells of large volume (channels 20-99 of the Coulter Channelyzer). In the presence of phenytoin (20 μg/ml or greater) (Figure 5A) or with dexamethasone (0.05 μg/ml or greater) (Figure 5B), the shift in cell volume in response to PHA was suppressed. Barbiturates also suppressed this mitogen-induced change in cell volume (see Table 3 and Figure 6). The suppression was most extensive at any given dose when thiopental (see Table 3 and Figure 6A) was used. Thiopental actively suppressed lymphocyte responsiveness at a final concentration of 50 μg/ml (Figure 6A). All three barbiturates markedly suppressed the expected mitogen-induced increase in cell volume when tested at 500 μg/ml (Figure 6).

Lymphocyte responsiveness was also evaluated in terms of a cellular proliferative index where the result of the DNA synthetic process was quantitated in terms of an increase in cell numbers. The effects of varying concentrations of phenytoin, dexamethasone, and barbiturates on lymphocyte proliferation in response to PHA were studied. As shown in Table 4, phenytoin at concentrations over 20 μg/ml or dexamethasone at concentrations over 0.05 μg/ml inhibited mitogen-induced lymphocyte proliferation.

Suppression of proliferation was also seen with each of the barbiturates (Table 5) and the pattern of suppression was clearly dose related. Again, thiopental produced the greatest degree of suppression. It is of interest that this parameter of lymphocyte activation did not recognize differences between the effects of phenobarbital and pentobarbital, as was evident when ^{3}H-thymidine incorporation was the measure of stimulation.

Routine culture medium contains 5% serum, providing a low protein concentration in vitro relative to that in vivo. To evaluate the possible protective effect of protein binding upon the inhibitory effect of phenytoin, steroid, and barbiturates on mitogen-induced lymphocyte activation, studies were carried out with cells cultured in the presence of increasing concentrations of serum.

The studies described above were repeated using culture medium which was enriched with either 10 or 20% pooled normal human serum. The results of these studies indicate that increased protein concentrations decrease the lymphocyte suppression induced with phenytoin (Figure 7), whereas protein concentration has little effect with regard to phenobarbital and dexamethasone. This was further examined in more extensive investigations by adding human serum albumin in a culture system in addition to 20% pooled normal human serum. With increasing concentration of human serum albumin, the percentage of inhibition of PHA-induced ^{3}H-thymidine uptake by phenytoin increased. The increased albumin concentration did not appear to have protective effects against the phenytoin inhibition on lymphocyte activation.

However, as shown in Figure 8, increased serum concentrations did have some protective effects against the barbiturate suppression.

Protein binding appeared to be more of a factor with thiopental than with the other two barbiturates (Figure 8). With all three barbiturates little difference was seen when the protein concentration was increased beyond 10%, suggesting that protein binding has only a small effect on barbiturate suppression of mitogen-induced lymphocyte activation.

3. Studies of the Effects of Brain Tumor Cyst Fluid, CSF, and Serum on Mitogen-induced Lymphocyte Activation.

1) The Composition of Brain Tumor Cyst Fluid

An aliquot of brain tumor cyst fluid from each patient was subjected to protein electrophoresis (Table 6). The total protein content and composition of tumor cyst fluid are similar to, but quantitatively less than, those of serum. Similarly the globulin content of tumor cyst is similar to that of serum. Thus since cyst fluid protein content is much more like serum than CSF, normal pooled human serum was used instead of cyst fluid as a control throughout these studies. In these control cultures the serum content was 18-23% instead of 8%. The increased serum content of these control cultures did not affect DNA synthesis (Figure 9), blastogenesis, or lymphocyte proliferation. Thus, the effects of cyst fluid on lymphocyte proliferation are due to the presence of a factor other than normal serum protein.

Quantitative measurements of immunoglobulins IgG, IgA and IgM in cyst fluid and CSF were also performed (Table 7). These immunoglobulins were found present in tumor cyst fluid in relatively high quantities as compared to the levels in CSF.

With regard to phenytoin concentration in cyst fluid, three different samples of cyst fluid were measured. Among them, the highest level was 13.0 μg/ml which corresponds to a final concentration of 2.0 μg/ml in each microtiter well and 1.3 μg/ml in the tube culture system. On the basis of previous in vitro studies, these levels are too low for phenytoin to be the suppressive factor.

2) Evaluation of ^{3}H-thymidine Incorporation

Tables 8, 9, and 10 show the effects of tumor cyst fluid, CSF, and serum on ^{3}H-thymidine incorporation into DNA of mitogen-stimulated normal human lymphocyte cell cultures. The data are expressed in terms of mean incorporation of ^{3}H per minute (cpm) $\pm$ standard error of mean (SEM) in the presence or absence of test fluid. The suppression of ^{3}H-thymidine incorporation ranged from 63% to 94% in the presence of cyst fluid in 5 of 6 cases when compared to the controls containing heat-inactivated pooled human serum instead of tumor cyst fluid. The inhibitory activity of cyst fluid was dose dependent (Figure 10A); that is, the suppression of mitogen-induced lymphocyte activation by cyst fluid increased in direct proportion to the concentration of the cyst fluid present in microtiter wells.

The responsiveness of normal lymphocytes to the various mitogens (PHA, Con A and PWM) in the presence of cyst fluid varied. The cyst fluid from one of the patients (E.J.) markedly suppressed lymphocyte activation to all the mitogens (PHA, Con A and PWM) (Figure 9). In all the other patients, the effects of tumor cyst fluid were less apparent with Con A and PWM than with PHA.

In contrast to cyst fluid, CSF from brain tumor patients had little effect on mitogen-induced normal lymphocyte activation (Table 9, Figures 10B and 11). Serum from brain tumor patients had an inhibitory activity on mitogen-induced lymphocyte activation that was between the inhibitory activities of cyst fluid and CSF (Figures 10C and 11). Dose response studies of the inhibitory effects of serum showed similar trends as with cyst fluid obtained from the same patient, even though the effects were much more prominent with cyst fluid (Figure 12).

The possibility that inhibitory factors might bind mitogens rather than affecting lymphocyte function directly was also examined. Mitogens were added at various concentrations to give both optimal and suboptimal stimulation. Figure 13 shows that the entire response to PHA can be completely suppressed by the presence of tumor cyst fluid and the pattern of the PHA dose-response curve is exactly identical in the presence or absence of cyst fluid. A similar trend was observed in dose response curves with Con A and PWM. This indicates that cyst fluid affects lymphocytes directly rather than by binding to (inactivating) mitogen. Heat stability of inhibitory factors was also evaluated (results not shown). The inhibitory effect of tumor cyst fluid was not changed by heat treatment at 56°C for 30 minutes.

3) Evaluation of Cell Size Distribution

Cell distribution analysis was performed on the Coulter Channelyzer with the settings described in "Materials and Methods" using cells cultured in tubes rather than microtiter plates. As shown in Table 11, approximately 75% of normal lymphocytes following 90 hours of incubation

in the presence of PHA were found between channels 20 and 100, corresponding to a cell diameter of between 10.80μ and 15.58μ. The dose-dependent suppression by brain tumor cyst fluid of lymphocyte activation was observed as in the studies of DNA synthesis described above. This correlation of the effects of cyst fluid on blastogenesis (i.e. increase in cell size) and DNA synthesis is well illustrated in Figures 9 and 14. Increasing volumes of cyst fluid inhibited blastogenesis just as it did DNA synthesis. For example, 10% cyst fluid (v/v) decreased the percentage of activated lymphocytes (as defined in "Methods") from 73.3% to 50.5%, whereas ^{3}H-thymidine incorporation decreased from 75,000 to 24,000 cpm with 10% tumor cyst fluid in the culture medium.

4) Evaluation of Cellular Proliferation

An increase in cell number, designated here as cellular proliferation, is another measure of lymphocyte activation. A proliferation ratio, as defined in "Methods", was calculated by dividing the cell number in each experimental culture by the number of cells in control culture which contained no PHA. As shown in Table 12, the proliferation ratio for control cultures containing PHA but no test fluid (tumor cyst fluid or patient CSF) was found to be approximately 2.5. Three out of four experimental cultures which contained a range of volumes of cyst fluid demonstrated dose-related suppression of lymphocyte proliferation.

Cyst fluid from one patient (E.J.) produced the greatest degree of suppression of lymphocyte activation, whereas in another patient (S.S.)

there was no evidence of suppressed cellular proliferation as measured by the proliferation ratio. Again the dose-related inhibitory activity of cyst fluid on lymphocyte proliferation as evidenced by this method (Table 12) correlated with the results from the assays of the other two measures of lymphocyte activation, i.e. DNA synthesis and blastogenesis. Similarly, as with the other two parameters of lymphocyte activation, CSF from brain tumor patients did not appear to affect mitogen-induced lymphocyte proliferation, in contrast to the marked inhibitory effect of cyst fluid (Table 12).

5). Effects of Dialysis and IgG Depletion of Cyst Fluid on Mitogen Induced Lymphocyte Activation

Cyst fluids (1.0 ml) from three patients with brain tumors were dialyzed against several changes of 0.9% normal saline (total 3 liters) for 24 hours at 4°C and then tested for their ability to inhibit the PHA response of normal lymphocytes. The results are shown in Table 13. Dialysis of the cyst fluid did not result in significant loss of inhibitory activity compared to that of the original, untreated fluid. IgG-depletion of cyst fluid by Protein A-Sepharose 4B, however, resulted in a minimal loss of inhibitory activity. As shown in Table 14, approximately 98% of IgG removal was achieved in each experiment and yet IgG-depleted cyst fluid still inhibited 77% of mitogen-induced lymphocyte activation in comparison to 86% suppression with cyst fluid exposed to Sepharose B to which no Protein A was bound. It is therefore unlikely that the IgG fraction of cyst fluid (with possible exception of IgG_3) is responsible for inhibition of lymphocyte activity.

DISCUSSION

The present studies confirmed the observations of others (44-56), that systemic anergy, defined as reduced lymphocyte responsiveness to a stimulus, could be identified in patients with brain tumors. Two factors contribute to this hyporesponsiveness, serum factors and intrinsic lymphocyte dysfunction. In a series of patients with both benign and malignant brain tumors approximately 60% of the patients were shown to be hyporesponsive to PHA, primarily a T cell mitogen, and/or PWM, a T and B cell mitogen (92). Induced lymphocyte responsiveness was evaluated by the parameters of uptake of radiolabeled precursors of DNA, increase in cell size and an increase in cell number. As has been shown by others (44-56), the decreased lymphocyte responsiveness primarily involves the T cell population. This may explain why hyporesponsiveness was more apparent in the current studies with a pure T-cell mitogen (PHA) than with a mitogen which affects both T and B cells (PWM).

One new contribution of the current study is the implication that agents commonly used in the management of patients with brain tumors may be a significant basis for the anergy that has been observed. The presence of serum factors found in the majority of hyporesponsive patients which were able to suppress both normal and tumor patient lymphocyte activation is certainly consistent with such a hypothesis. The anticonvulsants phenytoin and possibly phenobarbital in therapeutic concentrations were shown to have a suppressive effect on *in vitro* lymphocyte responsiveness.

The well-known suppressive effect of dexamethasone was also observed. The attenuation of lymphocyte function is known to disappear

rapidly four to five hours after each oral dose of steroid (100), and this decay curve may explain the limited incidence of anergy in those members of the patient population on steroids. In view of the present in vitro studies detailing depressed lymphocyte function in cells exposed to dexamethasone, it is of interest that eleven patients whose lymphocytes were hyporesponsive in this series were on steroids. It must be emphasized, however, that four other patients were also on steroids, but had no evidence of impaired lymphocyte activation, including one patient who was receiving 96 mg of dexamethasone per day.

Twenty of the twenty-two patients were on therapeutic doses of phenytoin and six were on therapeutic doses of phenobarbital (Table 1). Unfortunately, we do not have correlative serum levels for these drugs at the time the lymphocytes were obtained from the patients for studies. Although the relative or absolute role played by these drugs in the identifiable hyporesponsiveness is not clear, the anergic patients in our series were all on one or more of these drugs. A review of the previously reported cases is noteworthy for the omission of details concerning the presence and/or dosage of anti-convulsants and other drugs in the patients described as anergic (44-56). Phenytoin and carbamazepine have been clearly implicated by Sorrell and Forbes both in vitro and in vivo in depressing cellular and humoral immune responses in thirty-eight of sixty-three patients with epilepsy (101). The onset of the immune dysfunction correlated with initiation of anti-convulsant therapy in their report. They were unable to show a similar effect for phenobarbital. The effect of anti-convulsants on the immune system has been reviewed recently (102).

That other barbiturates may have a potentially more important effect than phenobarbital is also suggested by the present studies noting that barbiturates varied as to their potency in suppressing an immunologic response. For instance, thiopental, a commonly used intravenous anesthetic in patients undergoing craniotomy for tumors, was five-fold more potent than phenobarbital in suppressing lymphocyte response. Indeed, at therapeutic concentrations, this barbiturate is highly suppressive, as is pentobarbital (103), indicating that lymphocyte function in brain tumor patients probably should not be evaluated in the peri-operative period. It is also of particular interest in this regard that a significant increase in metastatic spread and progression was observed in animal models when the anesthetic drugs, including thiopental, were used (104).

In order to evaluate the possibility that anergy seen in brain tumor patients may also relate in part directly to the presence of the tumor, the current studies were designed to investigate the effects of tumor cyst fluid and cerebrospinal fluid (CSF) from brain tumor patients upon mitogen-induced normal lymphocyte activation. An in vitro culture system was used to evaluate the hypothesis that such fluids contain locally produced immunosuppressive factors which then might be released by the tumor into serum and/or CSF.

In the present studies, brain tumor cyst fluid markedly suppressed mitogen-induced lymphocyte activation, in contrast to CSF from brain tumor patients which demonstrated little effect. Matsukado et al. (51) also were not able to detect any "blocking" activity in CSF. Serum from brain tumor patients suppressed lymphocyte activation but to a much lesser extent than cyst fluid. Brain tumor cyst fluid not only

suppressed the incorporation of a radiolabeled pyrimidine nucleoside (^{3}H-thymidine) into DNA in cultured lymphocytes, but also inhibited the process of blastogenesis (increase in cell volume) and cell division/cell proliferation. The suppression of lymphocyte activation induced by the cyst fluid was dose-related, and not caused by the lack of supporting nutrients in the culture system because both pooled normal human serum and cyst fluid were added. No suppression of lymphocyte activation was observed in the presence of normal pooled human serum.

The total protein, albumin, and immunoglobulin concentrations in cyst fluid were found to resemble those of normal human serum; however, normal serum had no suppressive activity and serum from brain tumor patients was much less suppressive than brain tumor cyst fluid. It is also clear from the current studies that the suppression of mitogen-induced lymphocyte activation results from the direct interaction between a factor(s) in cyst fluid and lymphocytes, rather than binding of the suppressive factor(s) in cyst fluid to PHA. These results suggest that the immunosuppressive factors may be produced and secreted by the tumor cells and released into both tumor cyst fluid and subsequently the systemic circulation. The gradient of immunosuppressive activity of tumor cyst fluid and serum suggests that at least some of the previously reported serum "blocking" factors (44, 45, 47, 49) may originate directly from tumor. An alternative explanation is that the immunosuppressive factor(s) may be produced elsewhere and sequestered in tumor cyst fluid since the major protein content of brain tumor cyst fluid may be a transudate from serum (105). Interestingly, only minimal immunosuppressive factors were detected in CSF.

The present studies also demonstrated that mitogen activation induced by Con A and PWM in the presence of tumor cyst fluid was less affected than the PHA-induced lymphocyte response. This could indicate that the suppressor factor(s) present in cyst fluid competitively bind to PHA receptors but not Con A or PWM receptors on the surface of peripheral T-cells. PHA and Con A are both T-cell mitogens but can activate different sub-populations of T-cells whereas PWM is a mixed T- and B-cell mitogen (92). Thus the suppressive factor(s) could be preventing activation of a sub-population of T-cells that are normally activated by PHA but not by Con A or PWM.

With regard to the preliminary characterization of the suppressive factor(s) present in tumor cyst fluid, the suppressive factor(s) does not seem to be dialyzable and therefore has a molecular weight of greater than 12,000. On the basis of the studies with Protein A which removed IgG from cyst fluid, it is unlikely that the IgG fraction of tumor cyst fluid is directly involved in immunosuppression of normal lymphocyte function by cyst fluid. This result is contrary to the previous observation made by Brooks et al. (44, 45), who demonstrated that mitogen-induced lymphocyte blastogenesis was inhibited with the IgG fraction from the serum of patients with glioblastoma. They used DEAE column chromatography and felt that the eluates contained only IgG, as evaluated by both immunoelectrophoresis and immunodiffusion. It is possible, however, that the eluates might have also contained other proteins unable to be detected by such methods. The Protein A used in the current studies reacts in a highly specific fashion with the Fc region of IgG molecules from many species (99). The present study showed that approximately 98% of IgG was removed from the original tumor cyst

fluid and that such IgG depletion resulted in a minimal change with regard to the inhibitory activity of cyst fluid on mitogen-induced normal lymphocyte activation. Further characterization of the immunosuppressive factor(s) is needed since it may give significant insight into understanding the impaired cellular immunity in patients with brain tumors.

SUMMARY AND CONCLUSION

The responsiveness of lymphocytes obtained from patients with brain tumors to *in vitro* stimulation with mitogenic lectins was examined. The previously reported finding that some patients with brain tumors have depressed cellular immunity was confirmed. The present studies suggest that the defect is in part attributable to intrinsic abnormality of lymphocytes and in part to the presence of serum factors. The depressed cellular immunity in brain tumor patients does not appear to have clinical sequalae. The present studies also showed that significant depression of *in vitro* lymphocyte responsiveness occurred with exposure to two anti-convulsant agents (phenytoin and phenobarbital) and dexamethasone, suggesting that the depressed cellular immunity may in part be iatrogenic secondary to such drugs as phenytoin. It was also found in the current studies that brain tumor cyst fluid contains immunosuppressive factors, suggesting that brain tumor cells may locally produce suppressive factor(s) capable of inhibiting lymphocyte activation.

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Table 1

Brain Tumor Patient Characteristics and Lymphocyte Mitogen Responsiveness

Patient (age/sex)	Diagnosis	Decadron (mg/day)	Anti-Convulsants*	Mean ^{3}H-thymidine incorporation (cpm) PHA	PWM
65/M	Glioblastoma	96	PH,B	517 (A)†	1543 (A)
40/F	Glioblastoma	96	PH,B	40180 (N)	9773 (N)
24/M	Glioblastoma	64	PH,B	1550 (A)	2160 (A)
54/F	Glioblastoma	16	C,B	12073 (A)	3640 (M)
29/M	Glioblastoma	8	PH	67810 (N)	18680 (N)
20/F	Glioblastoma	0	PH	21777 (M)	- (-)
52/F	Glioblastoma	0	PH	145060 (N)	25323 (N)
39/F	Glioblastoma	0	PH	43280 (N)	9363 (N)
16/F	Glioblastoma	0	PH,C	42750 (N)	6230 (M)
29/M	Glioblastoma	0	PH,B	46990 (N)	10103 (N)
34/F	Glioblastoma	16	PH,C,V	78380 (N)	10213 (N)
34/M	Astrocytoma (Grade II)	8	PH	17467 (M)	- (-)
52/F	Astrocytoma (Grade II)	16	PH	46303 (N)	8570 (N)
22/M	Astrocytoma (Grade II)	0	PH	47583 (N)	8113 (N)
15/M	Astrocytoma (Grade I)	4	PH,PR	34267 (M)	8350 (N)
42/F	Microglioma	0	PH	44377 (N)	9573 (N)
63/M	Metastasis	16	No	9490 (A)	8210 (N)
53/M	Metastasis	16	PH,B	13873 (A)	- (-)
55/M	Metastasis	16	PH	23287 (M)	8903 (N)
35/M	Pineoblastoma	16	PH	10630 (A)	4870 (M)
45/F	Meningioma	96	PH	7747 (A)	5033 (M)
62/M	Pituitary adenoma	40	PH	13025 (A)	7093 (M)

* PH: Phenytoin (300-400 mg/day); B: Phenobarbital (60-120 mg/day); C: Carbamazepine (200-600 mg/day); V: Valproic acid (500 mg/day); PR: Primidone (500 mg/day); No: No anti-convulsants

Ψ The response (CPM) of tumor patients' lymphocytes, cultured in triplicate with PHA or PWM in autologous serum.

† A: Marked Hyporesponsiveness (i.e. anergy); M: Moderate Hyporesponsiveness; N: Normal Mitogen Response.

Marked hyporesponsiveness as defined in "Methods" was a mitogenic response (i.e., ^{3}H-thymidine incorporation) which was at least 2 standard deviations from the mean of 22 control cultures (i.e., normal lymphocytes cultured in the presence of normal pooled human serum and mitogen); moderate hyporesponsiveness is a mitogenic response more than 1 standard deviation from the mean of 22 control cultures.

Table 2

Brain Tumor and Normal Lymphocyte Mitogenic (PHA) Responses*
in the Presence of Pooled Normal Human Serum or Tumor Patient Serum

Patient (Age/Sex)	Mean Tumor Patient Lymphocyte Responsiveness: Normal Serum	Mean Tumor Patient Lymphocyte Responsiveness: Patient Serum	t Stat.** (p value)	Mean Normal Lymphocyte Responsiveness: Normal Serum	Mean Normal Lymphocyte Responsiveness: Patient Serum	t Stat.** (p value)
65/M	5053	517	≦0.005	58180	7923	≦0.005
40/F	57830	40180	≦0.02	87193	73430	≦0.05
24/M	7803	1550	≦0.005	32447	9797	≦0.001
54/F	17267	12073	≦0.005	74200	40857	≦0.05
29/M	63177	67810	N.S.	88767	107580	N.S.
20/F	27753	21777	N.S.	63790	50847	≦0.025
52/F	122293	145060	≦0.02	100723	109307	N.S.
39/F	55867	43280	≦0.005	64330	32483	≦0.01
16/F	36677	42750	≦0.025	42090	43577	N.S.
29/M	57343	46990	N.S.	60657	56303	N.S.
34/F	64840	78380	N.S.	-	-	-
34/M	24583	17467	≦0.05	43297	39650	N.S.
52/F	44660	46303	N.S.	72770	44440	≦0.01
22/M	42970	47583	N.S.	72790	69530	N.S.
15/M	31057	34267	N.S.	42090	45553	N.S.
42/F	52687	44377	N.S.	50817	45123	N.S.
63/M	11993	9490	N.S.	96917	78613	≦0.025
53/M	16613	13873	N.S.	46737	53087	N.S.
55/M	35973	23287	≦0.025	32447	27843	≦0.05
35/M	12243	10630	N.S.	29760	22140	N.S.
45/F	16300	7747	≦0.01	47627	25150	≦0.01
62/M	50197	13025	≦0.01	101810	23407	≦0.001
Mean	38872	34928		62389	47935	
SEM	±5698	±6919		±5024	±6006	

*The response (cpm) of lymphocytes, (normal or from tumor patients) cultured in triplicate with PHA in pooled normal human serum or brain tumor patient serum.

**The t statistic for 2 means was used to compare the mean ^{3}H-thymidine incorporation (cpm) in the presence of pooled normal human serum-vs-serum from brain tumor patients. N.S. indicates that the p value was not significant ($p \geq 0.05$).

Table 3

Effects of Barbiturates on Mitogen Induced Increases in Normal Human Lymphocyte Cell Size

	% Activated Lymphocytes*
Control	
No PHA	20.9 ± 2.2
PHA/No Drug	67.8 ± 2.4
Thiopental Sodium	
50 µg	39.3 ± 4.5
100 µg	23.0 ± 1.5
500 µg	24.2 ± 5.5
Pentobarbital Sodium	
50 µg	60.9 ± 3.0
100 µg	59 ± 3.9
500 µg	25.4 ± 4.4
Phenobarbital Sodium	
50 µg	64.2 ± 3.4
100 µg	59.8 ± 4.9
500 µg	27.9 ± 6.2

*Activated lymphocytes, as defined in "Methods", are those cells assigned to channels 20-100 on the Counter Channelyzer.

Table 4

Effect of Phenytoin or Dexamethasone on Mitogen Stimulated Lymphocyte Proliferation

	Proliferation Ratio*
Control (n = 5)	
No PHA	1.0
PHA/No Drug	1.86 ± 0.13
Phenytoin (n = 5)	
5 μg	1.90 ± 0.11
20 μg	1.84 ± 0.12
50 μg	1.35 ± 0.09
100 μg	1.07 ± 0
Dexamethasone (n = 3)	
0.05 μg	1.49 ± 0.33
0.10 μg	1.37 ± 0.28
0.40 μg	1.25 ± 0.15
0.80 μg	1.25 ± 0.24

*The proliferation ratio was calculated by dividing the number of cells in each culture by the number of cells in the control culture which contained no PHA.

Table 5

Effect of Barbiturates on Mitogen Stimulated Lymphocyte Proliferation

	Proliferation Ratio*
Control	
No PHA	1.0
PHA/No Drug	2.12 ± 0.07
Thiopental Sodium	
50 µg	1.52 ± 0.24
100 µg	1.38 ± 0.07
500 µg	0.83 ± 0.20
Pentobarbital Sodium	
50 µg	1.98 ± 0.10
100 µg	1.80 ± 0.09
500 µg	1.13 ± 0.11
Pentobarbital Sodium	
50 µg	1.97 ± 0.13
100 µg	1.85 ± 0.12
500 µg	1.17 ± 0.10

*The proliferation ratio was calculated by dividing the cell number in each experimental culture by the number of cells in the control culture which contained no PHA.

Table 6

Brain Tumor Cyst Fluid
Protein Electrophoresis (g/dl)[a]

Patient	Diagnosis	Total Protein	Albumin	Globulin α1	α2	β	γ
SB	Astrocytoma (Grade II)	5.40	3.96	0.14	0.42	0.49	0.39
EJ	Glioblastoma	5.90	3.78	0.24	0.73	0.40	0.75
CG	Glioblastoma	3.90	2.61	0.17	0.32	0.56	0.24
GJ	Microglioma	5.30	3.32	0.21	0.24	0.52	1.01
PHS[b]		6.90	4.24	0.20	0.65	0.85	0.96

a. Cyst fluid was collected at the time of surgery, centrifuged, filtered through a 0.2 μm filter and stored at -70° C.

b. Pooled Human Serum.

Table 7

Immunoglobulin Levels in Cyst Fluid & CSF from Brain Tumor Patients (mg/dl)[a]

Test Fluid	Patient	Diagnosis	IgG	IgA	IgM
Cyst Fluid	SB	Astrocytoma (Grade II)	548	142	52
	EJ	Glioblastoma	54	101	27
	CG	Glioblastoma	297	65	33
CSF	DL	Glioblastoma	<0.2	<0.2	<0.1
	CR	Glioblastoma	7.0	0.4	0.3
	CG	Glioblastoma	<0.2	<0.2	<0.1
	JT	Medulloblastoma	<0.2	<0.2	<0.1
	GJ	Microglioma	4.7	0.7	<0.1
	PY	Hydromyelia (Control)	<0.2	0.4	<0.1
PHS[b]			1331	219	196

a. Cyst fluid and CSF were centrifuged, filtered through a 0.2 μm filter and stored at -70° C.

b. Pooled Human Serum.

Table 8

Effect of Tumor Cyst Fluid From Brain Tumor Patients on Mitogen-induced Normal Lymphocyte Activation

				^{3}H-thymidine incorporation into DNA (cpm)		
Patient	Age/ Sex	Diagnosis	Mitogen[a]	Control Response[b]	Response in presence of cyst fluid[c]	% supp.[d]
SB	34/M	Astrocytoma (Grade II)	PHA	70560±4066[e]	14306±970	79.7*
			Con A	39753±1403	37621±2637	5.4
			PWM	29374±730	25496±752	13.2*
SS	20/F	Glioblastoma	PHA	71389±5167	90459±2872	None
			Con A	56955±1493	40149±1062	29.5*
			PWM	29435±1248	29187±1327	0.8
EJ	60/M	Glioblastoma	PHA	76957±414	4614±55	94.0*
			Con A	47045±2559	4531±500	90.4*
			PWM	25034±359	834±134	96.7*
CR	30/F	Glioblastoma	PHA	77906±4782	4692±483	94.0*
			Con A	54046±3316	48037±1544	11.1
			PWM	26806±1277	29095±1773	None
CG	20/F	Glioblastoma	PHA	75447±1448	13726±454	81.8*
			Con A	56040±1819	43947±1994	21.6*
			PWM	26887±1014	19858±869	26.1*
GJ	42/F	Microglioma	PHA	73804±5704	27606±291	62.6*
			Con A	46677±789	41708±4063	10.7
			PWM	23549±757	19871±998	15.6*

a. PHA: phytohemagglutinin
Con A: concanavalin A
PWM: pokeweed mitogen

b. The control response represents the mean of triplicate cultures to which 40 µl of pooled human serum was added instead of cyst fluid.

c. In experimental cultures done in triplicate 40 µl of cyst fluid was added to each culture.

d. % suppression =

$$\frac{\text{Control Response (cpm)} - \text{Experimental Response (cpm)}}{\text{Control Response (cpm)}} \times 100$$

e. Mean ± SEM in triplicate assay.

* Statistically significant suppression ($P < 0.05$) using Student's t test.

Table 9

Effect of CSF from Brain Tumor Patients
on Mitogen-induced Normal Lymphocyte Activation

				^{3}H-thymidine incorporation into DNA (cpm)		
Patient	Age/ Sex	Diagnosis	Mitogen[a]	Control Response[b]	Response in presence of cyst fluid[c]	% supp.[d]
DL	39/F	Glioblastoma	PHA	124386±7843[e]	92606±7761	25.6*
			Con A	64991±1308	56920±529	12.4*
			PWM	18398±603	14512±416	21.1*
CR	30/F	Glioblastoma	PHA	138717±2230	129898±3770	6.4
			Con A	73186±1035	73324±3001	None
			PWM	20799±771	19434±1427	6.6
CG	20/F	Glioblastoma	PHA	131751±4397	89127±3767	32.4*
			Con A	72022±3173	67290±3552	6.6
			PWM	25638±1346	25530±1351	0.4
JT	13/M	Medulloblastoma	PHA	80288±6057	75322±2712	6.2
			Con A	65588±2431	72368±949	None
			PWM	15711±299	12719±513	19.0*
GJ	42/F	Microglioma	PHA	90243±3376	82378±3125	8.7
			Con A	68811±1245	64502±1573	6.3
			PWM	16861±1309	17614±955	None

a. PHA: phytohemagglutinin
Con A concanavalin A
PWM: pokeweed mitogen

b. The control response represents the mean of triplicate cultures to which 40 µl of normal saline was added instead of CSF.

c. In experimental cultures done in triplicate 40 µl of CSF was added to each culture.

d. % suppression =

$$\frac{\text{Control Response (cpm)} - \text{Experimental Response (cpm)}}{\text{Control Response (cpm)}} \times 100$$

e. Mean ± SEM in triplicate assay

* Statistically significant suppression ($P < 0.05$) using the Student's t test.

Table 10

Effect of Serum from Brain Tumor Patients
on Mitogen-induced Normal Lymphocyte Activation

				^{3}H-thymidine incorporation into DNA (cpm)		
Patient	Age/ Sex	Diagnosis	Mitogen[a]	Control Response[b]	Response in presence of cyst fluid[c]	% supp.[d]
SS	20/F	Glioblastoma	PHA	94773±3066[e]	88709±9996	6.4
			Con A	48065±3823	38597±4804	19.7
			PWM	30497±1134	26044±1401	14.6*
DL	39/F	Glioblastoma	PHA	80071±4908	20646±950	74.2*
			Con A	47647±1575	21353±925	55.2*
			PWM	24840±1324	11269±242	54.6*
CR	30/F	Glioblastoma	PHA	89465±1213	35885±1175	59.9*
			Con A	47380±2500	48143±785	None
			PWM	26391±536	23139±513	12.3*
CG	20/F	Glioblastoma	PHA	75447±1448	48172±3384	36.2*
			Con A	56040±1819	28985±1919	48.3*
			PWM	26887±1014	24427±1509	9.2
GJ	42/F	Microglioma	PHA	94082±6214	68478±6416	27.2*
			Con A	58959±2548	35413±440	39.9*
			PWM	29281±397	21306±1274	27.2*

a. PHA: phytohemagglutinin
Con A: concanavalin A
PWM: pokeweed mitogen

b. The control response represents the mean of triplicate cultures to which 40 µl of normal pooled human serum was added instead of patient serum.

c. In experimental cultures done in triplicate, 40 µl of patient serum was added to each culture.

d. % suppression =

$$\frac{\text{Control Response (cpm)} - \text{Experimental Response (cpm)}}{\text{Control Response (cpm)}} \times 100$$

e. Mean ± SEM in triplicate assay.

* Statistically significant suppression ($P < 0.05$) using the Student's t test.

Table 11

Effect of Cyst Fluid and CSF on Mitogen-induced Increases in Normal Human Lymphocyte Cell Size.

	% Activated Lymphocytes[a]
Control Response:	
No PHA	9.6±0.5[c] (n=8)
PHA + 0.2 ml NS[b]	75.8±1.2[c] (n=8)
PHA + 0.2 ml PHS[b]	73.3±2.8[c] (n=8)

% Activated lymphocytes in the presence of cyst fluid:[d]

	Volume of cyst fluid added			
Patient	0.05 ml	0.1 ml	0.15 ml	0.2 ml
SS	--	73.1	--	64.0
SB	62.1	52.0	43.3	40.7
EJ	60.9	51.5	47.9	44.6
GJ	63.0	59.2	51.2	52.7

% Activated lymphocytes in the presence of CSF:[d]

	Volume of CSF added			
Patient	0.05 ml	0.1 ml	0.15 ml	0.2 ml
DL	78.3	78.0	77.4	76.7
CR	77.1	79.2	80.9	81.3
JT	79.6	79.7	79.0	80.0
GJ	78.8	78.2	79.5	80.1

a. Activated Lymphocytes, as defined in "Methods", are those cells assigned to channels 20-100 on the coulter channelyzer. The percentage of cells above channel 20 is referred to as: % Activated Lymphocytes.

b. NS: Normal Saline + complete culture medium
PHS: Pooled Human Serum + complete culture medium

c. Mean ± SEM

d. Mean values of % Activated Lymphocytes from duplicate experimental cultures.

Table 12

Effect of Cyst Fluid and CSF on Mitogen-stimulated Lymphocyte Proliferation

Control Response	Proliferation Ratio[a]
No PHA	1.0
PHA + 0.2 ml NS[b]	2.41±0.13[c] (n=8)
PHA + 0.2 ml PHS[b]	2.50±0.19[c] (n=8)

Proliferation ratio in the presence of cyst fluid:[d]

	Volume of cyst fluid added			
Patient	0.05 ml	0.1 ml	0.15 ml	0.2 ml
SS	---	2.67	---	2.67
SB	2.67	2.16	1.87	1.57
EJ	1.77	1.35	1.16	1.03
GJ	2.13	2.04	1.79	1.83

Proliferation ratio in the presence of CSF:[d]

	Volume of CSF added			
Patient	0.05 ml	0.1 ml	0.15 ml	0.2 ml
DL	2.22	2.14	2.03	2.01
CR	1.79	1.97	2.08	2.09
JT	2.52	2.46	2.71	2.46
GJ	2.54	2.51	2.61	2.65

a. The proliferation ratio was calculated by dividing the cell number in each experimetnal culture by the number of cells in the control culture which contained no PHA.

b. NS: Normal Saline + complete culture medium
PHS: Pooled Human Serum + complete culture medium

c. Mean ± SEM

d. Mean values of % Activated Lymphocytes from duplicate experimental cultures.

Table 13

Effect of Dialyzed Cyst Fluid on PHA Induced Normal Lymphocyte Activation

	^{3}H-thymidine incorporation into DNA (cpm)		
Patient	Control Response[a]	Response in the presence of original cyst fluid[b]	Response in the presence of dialyzed cyst fluid[b]
EJ	96993±1969[c]	30003±1508	35328±1138
CG	91404±4753	64421±1879	56893±3075
SB	91404±4753	16170±4437	10639±1188
Overall % suppression[d]		60.3±15.9	63.2±14.6

a. The control response represents the mean of triplicate cultures to which 40 µl of pooled human serum were added instead of cyst fluid.

b. In experimental cultures done in triplicate 40 µl of either original or dialyzed cyst fluid were added to each culture.

c. Mean ± SEM in triplicate assay.

d. % suppression =

$$\frac{\text{Control Response (cpm)} - \text{Experimental Response (cpm)}}{\text{Control Response (cpm)}} \times 100$$

Table 14

Effect of IgG Depleted Cyst Fluid on PHA Induced Normal Lymphocyte Activation

	^{3}H-thymidine incorporation into DNA (cpm)		
Patient	Control Response[a]	Response in the presence of original cyst fluid[b]	Response in the presence of treated cyst fluid[b]
EJ	76776±4695[c]	11908±2404	16161±1537
CG	70236±4358	14381±846 (297)[d]	24859±987 (5)
SB	94929±7691	5513±620 (548)	11130±792 (10)
Overall % suppression[e]		86.1±4.3	77.1±7.1

a. The control response represents the mean of triplicate cultures to which 40 µl of pooled human serum were added instead of cyst fluid.

b. In experimental cultures done in triplicate 40 µl of either original or IgG depleted cyst fluid were added to each culture.

c. Mean ± SEM in triplicate assay

d. Number in parentheses is the IgG level in cyst fluid (mg/dl).

e. % suppression =

$$\frac{\text{Control Response (cpm)} - \text{Experimental Response (cpm)}}{\text{Control Response (cpm)}} \times 100$$

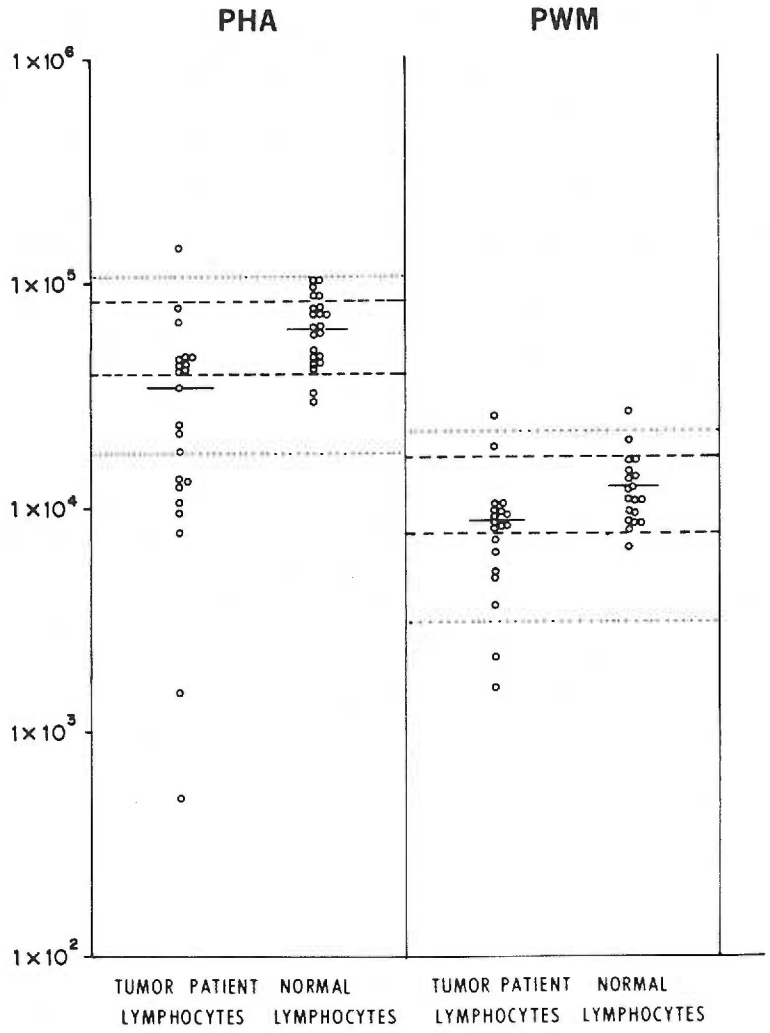

Fig. 1. ^{3}H-thymidine incorporation of normal and tumor patient lymphocytes in response to PHA and PWM. Horizontal bars indicate mean ^{3}H-thymidine incorporation for each group. Dashed line indicates 1 standard deviation from the mean level of incorporation of normal lymphocytes. Dotted line indicates 2 standard deviations from that mean.

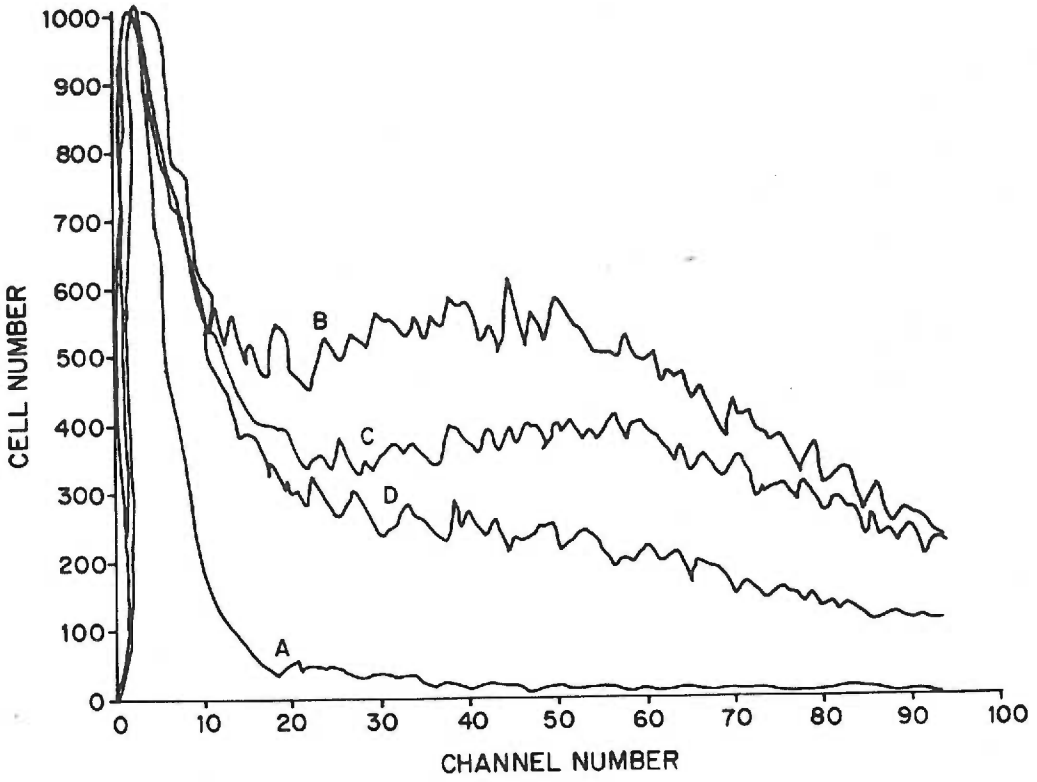

Fig. 2. Lack of normal mitogen (PHA) induced increase in cell volume in lymphocytes from a brain tumor patient. Curve A: Normal lymphocytes (4×10^5 cells) cultured in normal pooled human serum (PHS) without PHA: Curve B: Normal lymphocytes (4×10^5 cells) cultured in PHS and PHA: Curve C: Normal human lymphocytes (2×10^5 cells) + tumor patient lymphocytes (2×10^5 cells) cultured in PHS + PHA; Curve D: tumor patient lymphocytes (4×10^5 cells) cultured in PHS + PHA.

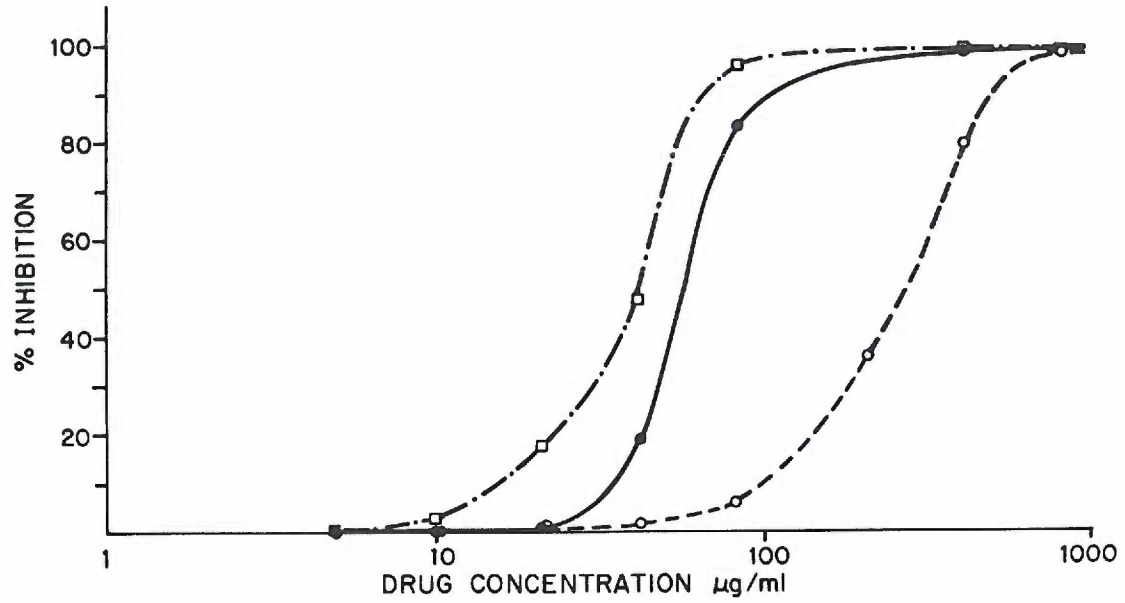

Fig. 3A. Inhibition of ^{3}H-thymidine incorporation of PHA stimulated normal human lymphocytes cultured in the presence of phenytoin (closed circle) phenobarbital (open circle) or both drugs (open square). The percent inhibition was calculated by dividing the incorporation of radiolabel in cultures containing drug by the amount of incorporation in drug free cultures. When both phenytoin and phenobarbital was added in equal amounts, the percent inhibition of ^{3}H-thymidine incorporation was augmented.

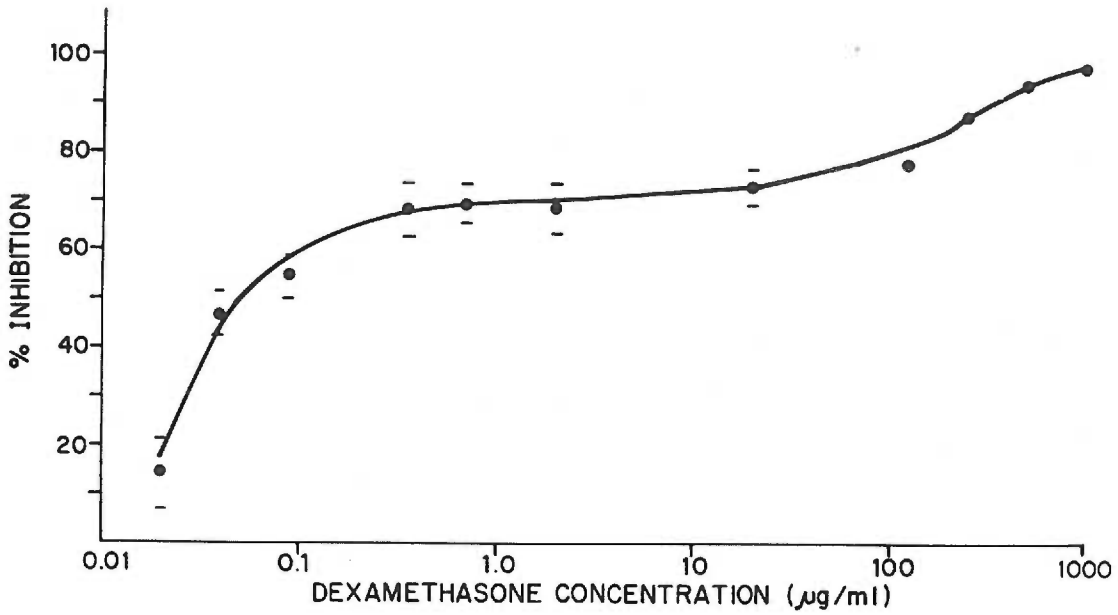

Fig. 3B. Inhibition of ^{3}H-thymidine incorporation of PHA stimulated normal human lymphocytes cultured in the presence of dexamethasone. The percent inhibition was calculated by dividing the incorporation of radiolabel in cultures containing drug by the amount of incorporation in drug free cultures.

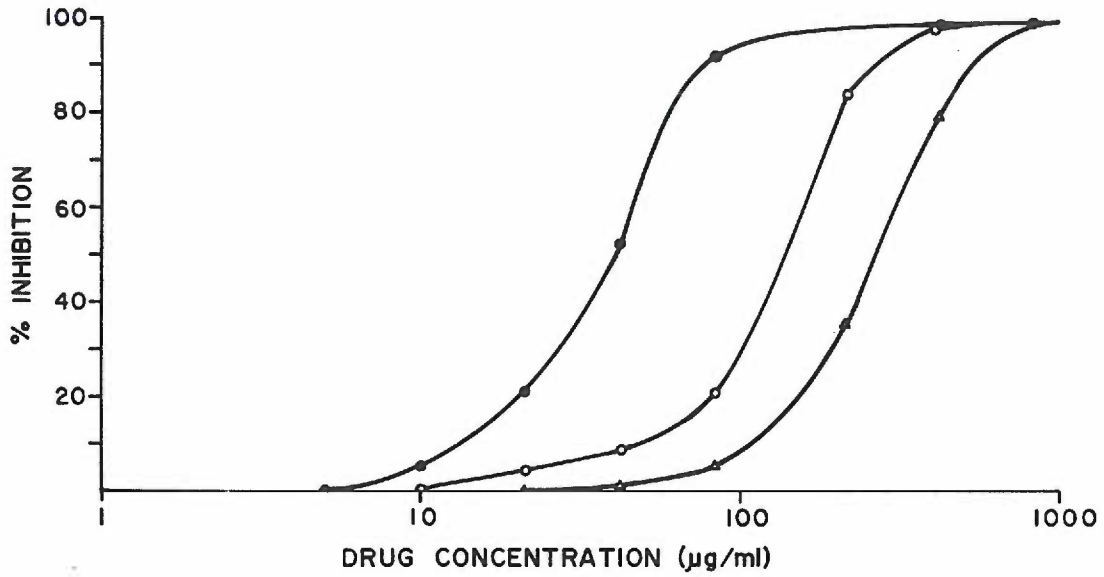

Fig. 4. Inhibition of mitogen stimulated ^{3}H-thymidine incorporation into normal human lymphocytes by thiopental (closed circles), pentobarbital (open circles) and phenobarbital (open triangles). The percent inhibition was calculated by dividing the incorporation of radiolabel in cultures containing drug by the amount of incorporation in drug free cultures.

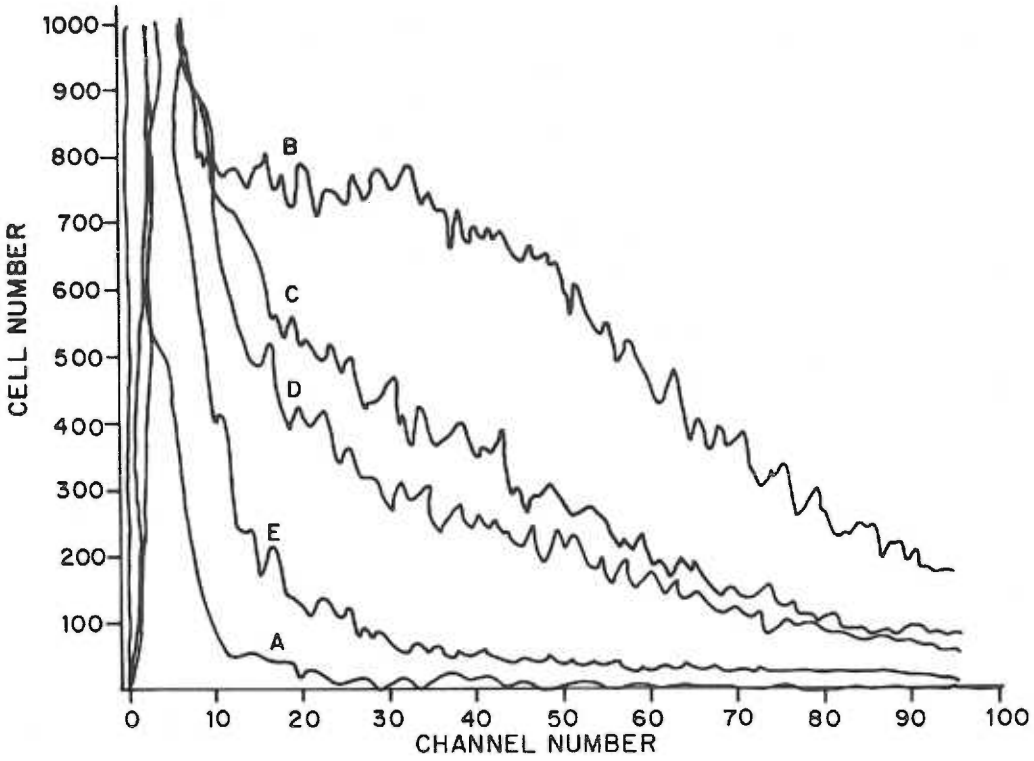

Fig. 5A. Inhibition of the mitogen induced increase in cell volume of normal human lymphocytes cultured in the presence of phenytoin.

Curve A: No PHA, no phenytoin; Curve B: PHA, no phenytoin; Curve C: PHA, 5 μg/ml phenytoin; Curve D: PHA, 20 μg,ml phenytoin; Curve E: PHA, 50 μg/ml phenytoin.

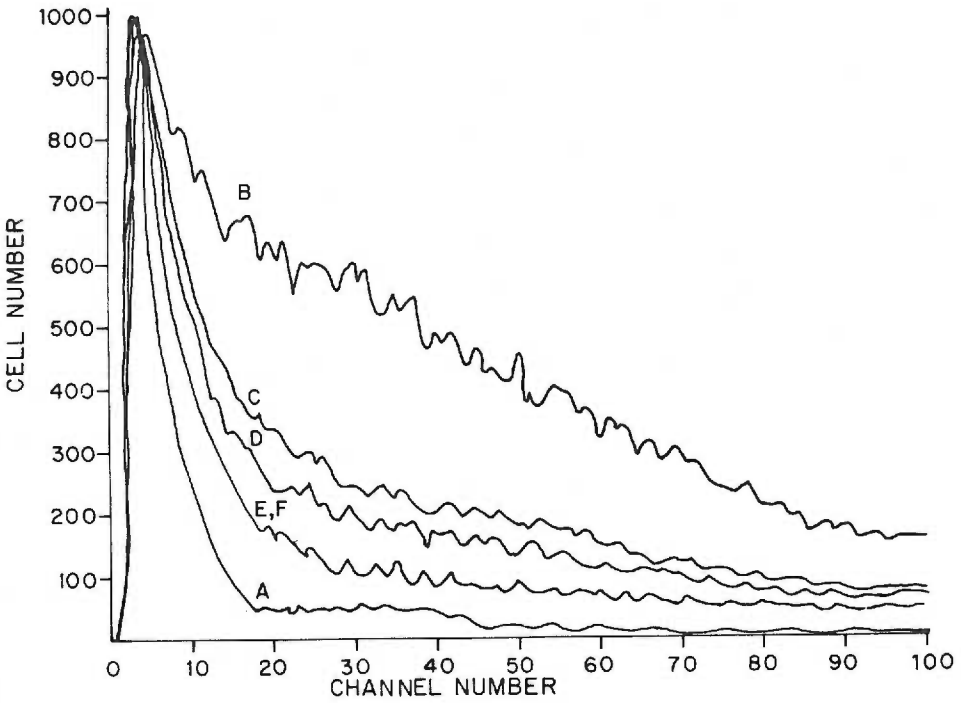

Fig. 5B. Inhibition of mitogen induced increase in cell volume of normal human lymphocytes cultured in the presence of dexamethasone.

Curve A: No PHA, no dexamethasone; Curve B: PHA, no dexamethasone; Curve C: PHA, 0.05 μg/ml dexamethasone; Curve D: PHA, 0.10 μg/ml dexamethasone; Curve F: PHA, 0.80 μg/ml dexamethasone.

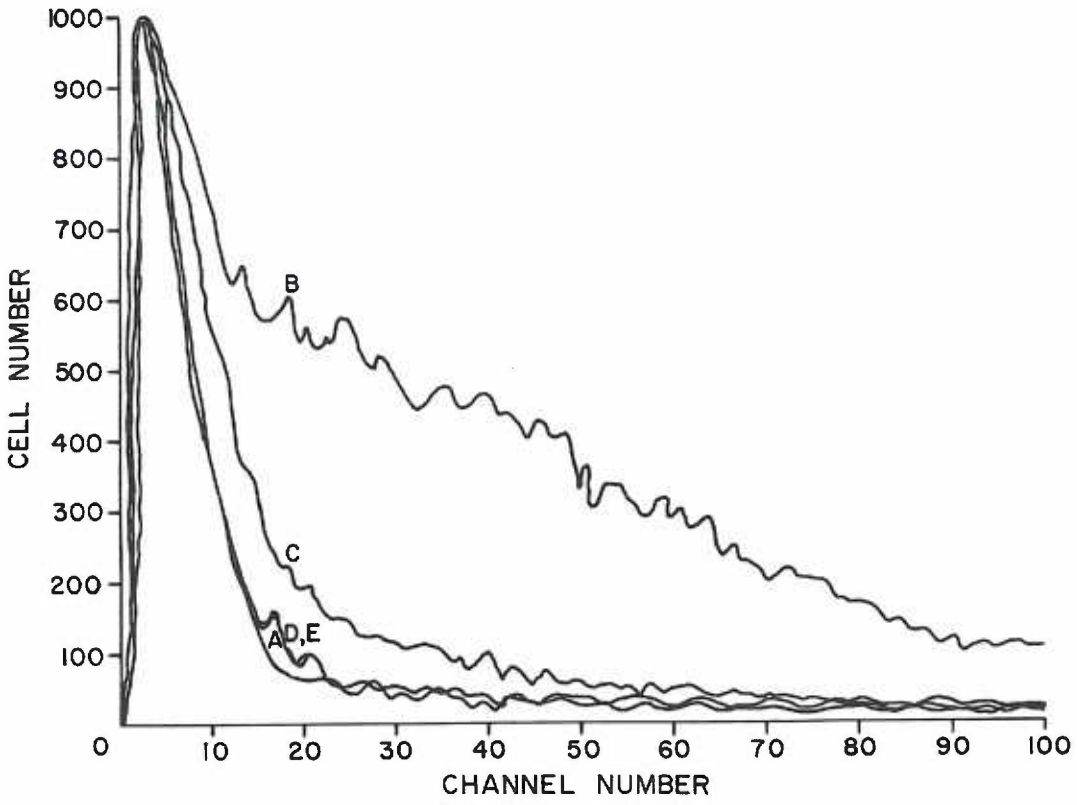

Fig. 6A. Effect of barbiturates on mitogen stimulated increases in normal human lymphocyte cell volume.

The effect of thiopental (Curve A: Control-no PHA, no barbiturate; Curve B: Control-PHA, no barbiturate; Curve C: PHA, thiopental 50 μg/ml; Curve D: PHA, thiopental 100 μg/ml; Curve E: PHA, thiopental 500 μg/ml).

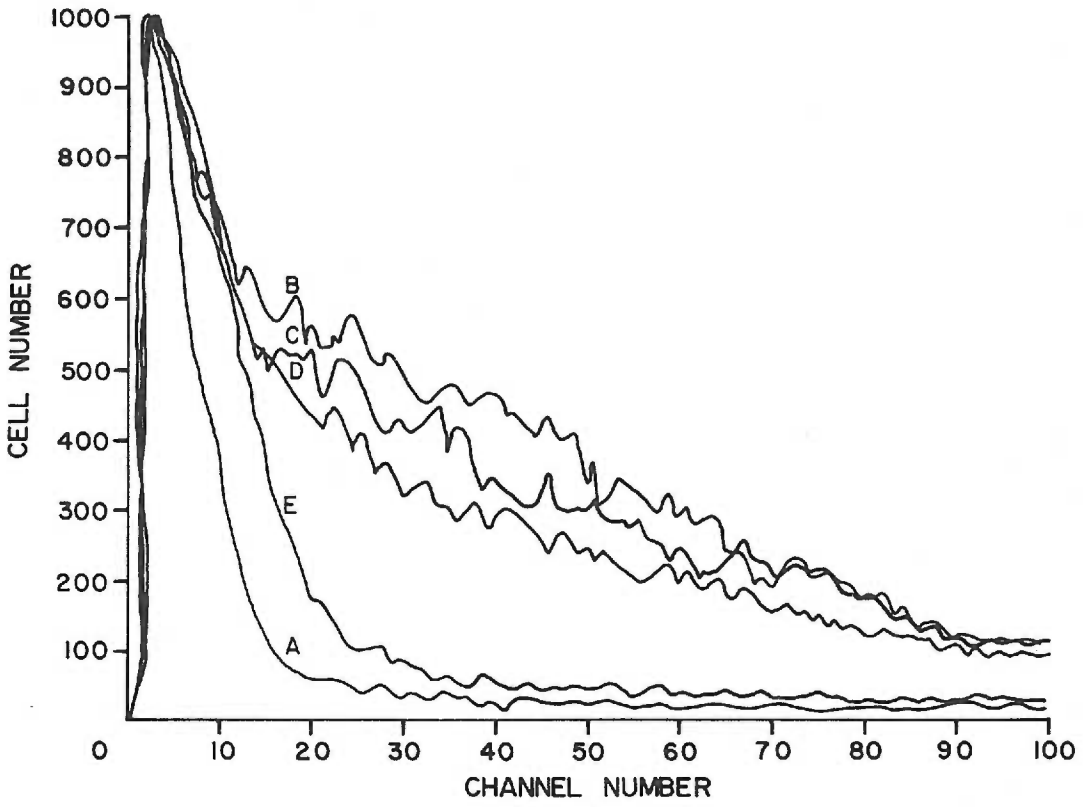

Fig. 6B. Effect of barbiturates on mitogen stimulated increases in normal human lymphocyte cell volume.

The effect of pentobarbital (Curve A: Control-no PHA, no barbiturate; Curve B: Control-PHA, no barbiturate; Curve C: PHA, pentobarbital 50 µg/ml; Curve D: PHA, pentobarbital 100 µg/ml, Curve E: PHA, pentobarbital 500 µg/ml.

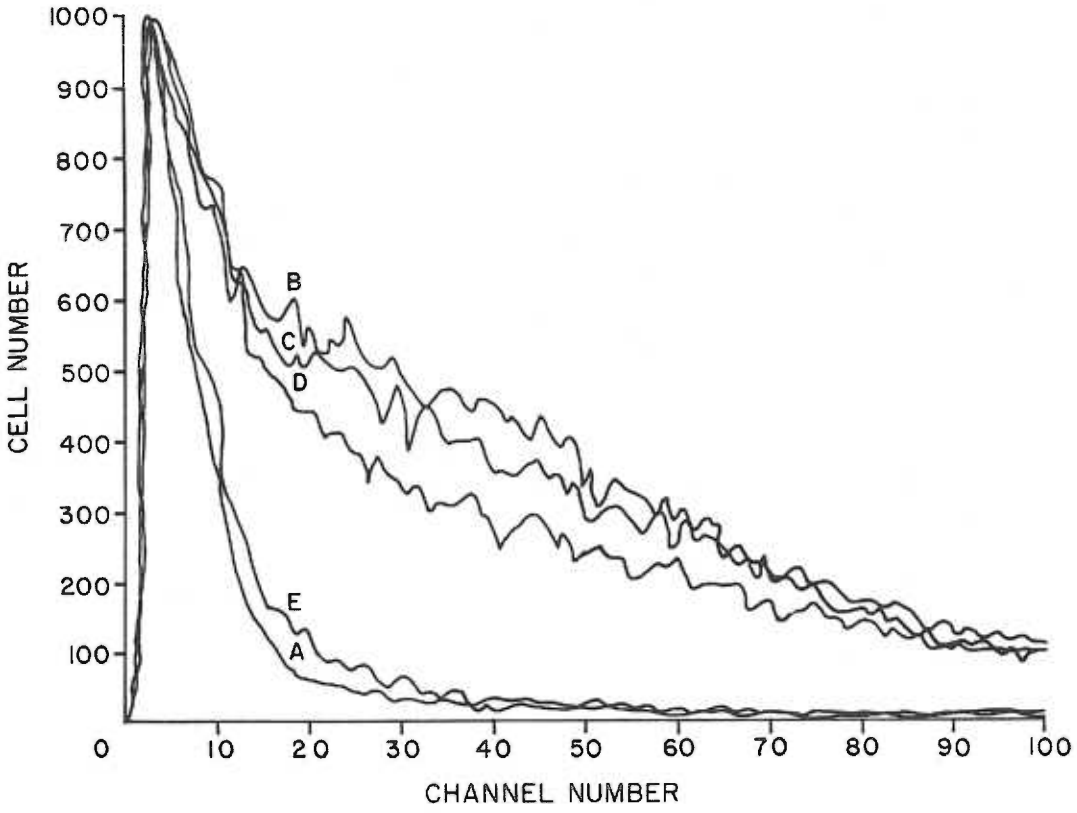

Fig. 6C. Effect of barbiturates on mitogen stimulated increases in normal human lymphocyte cell volume.

The effect of phenobarbital (Curve A: Control-no PHA, no barbiturate; Curve B: Control-PHA, no barbiturate; Curve C: PHA, phenobarbital 50 μg/ml, Curve D: PHA, phenobarbital 100 μg/ml, Curve E: PHA, phenobarbital 500 μg/ml).

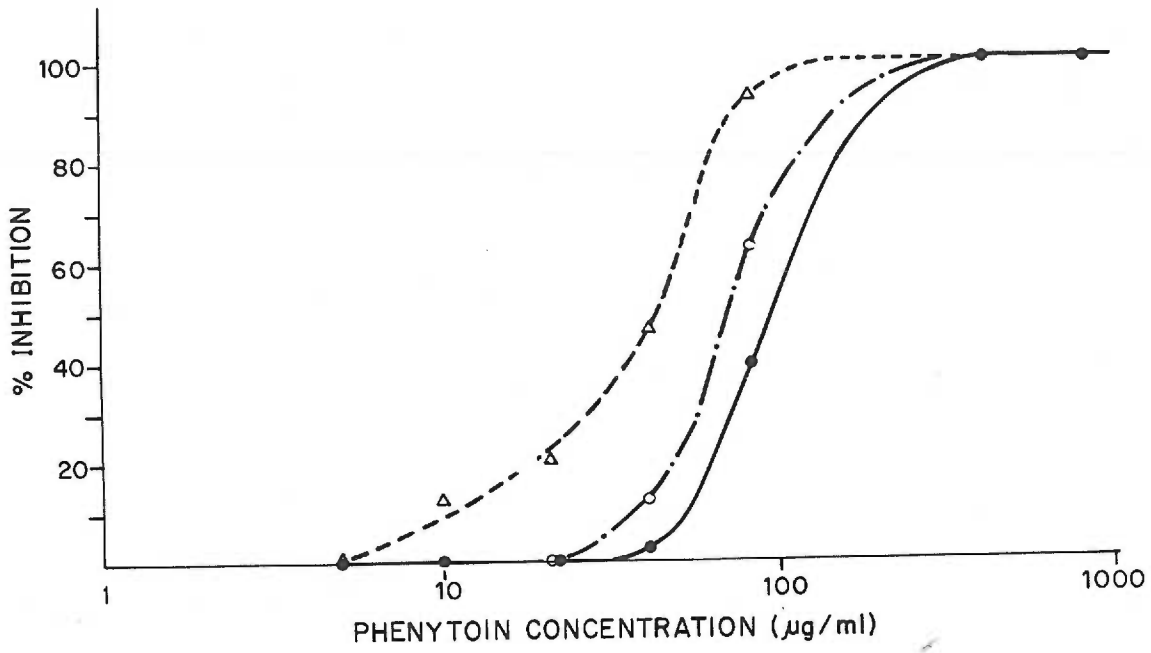

Fig. 7. Effect of serum concentration (5% serum-open triangle; 10% serum-open circle; 20% serum-closed circle) on phenytoin induced suppression of mitogen induced blastogenesis. Percent inhibition was calculated by dividing the incorporation of radiolabel in cultures containing drug by the amount of incorporation in drug free cultures.

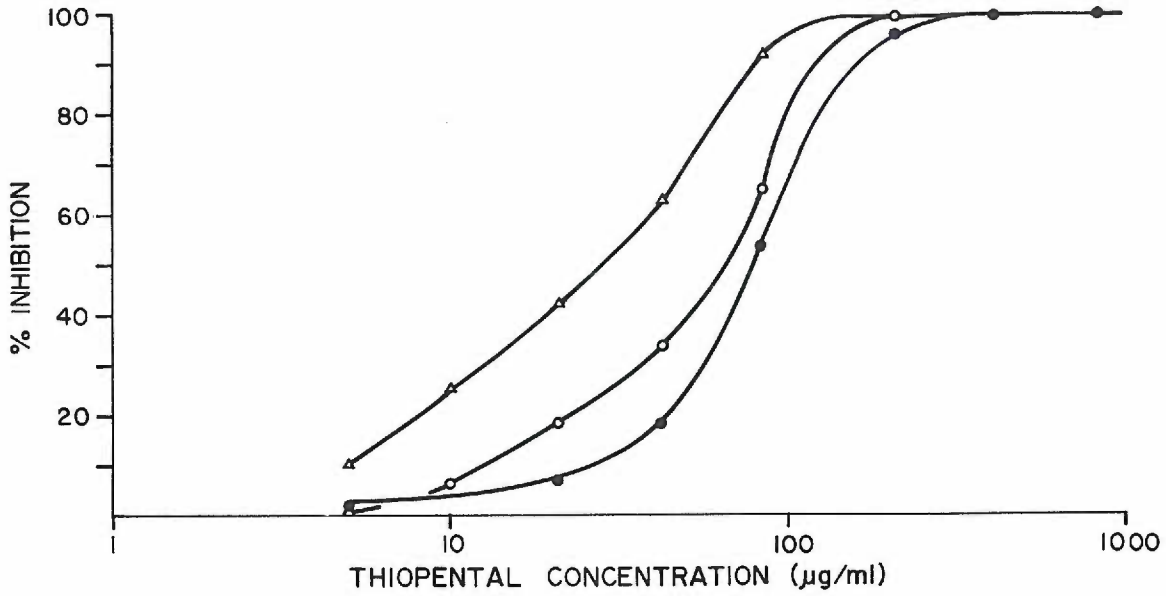

Fig. 8A. Effect of protein concentration on thiopental inhibition of mitogen stimulated ^{3}H-thymidine incorporation. Each microtiter well contained complete medium, mitogen (PHA), barbiturate and varying concentrations of pooled human serum (Open triangles: 5% pooled human serum; Open circles: 10% pooled human serum; Closed solid circles: 20% pooled human serum).

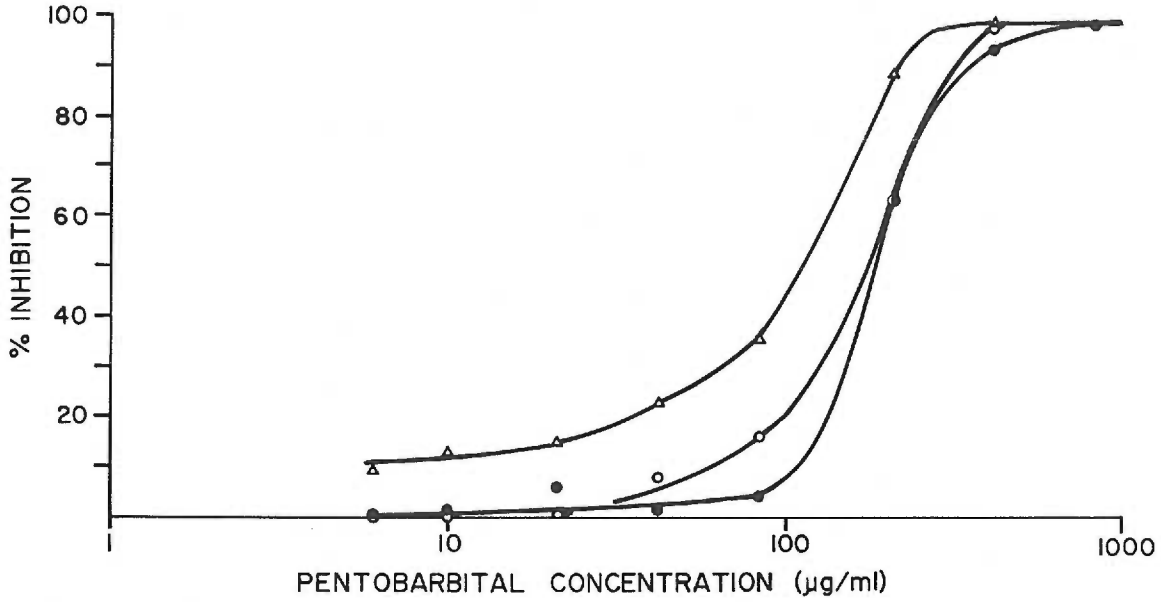

Fig. 8B. Effect of protein concentration on pentobarbital inhibition of mitogen stimulated ^{3}H-thymidine incorporation. Each microtiter well contained complete medium, mitogen (PHA), barbiturate and varying concentrations of pooled human serum (Open triangles: 5% pooled human serum; Open circles: 10% pooled human serum; Closed solid circles: 20% pooled human serum).

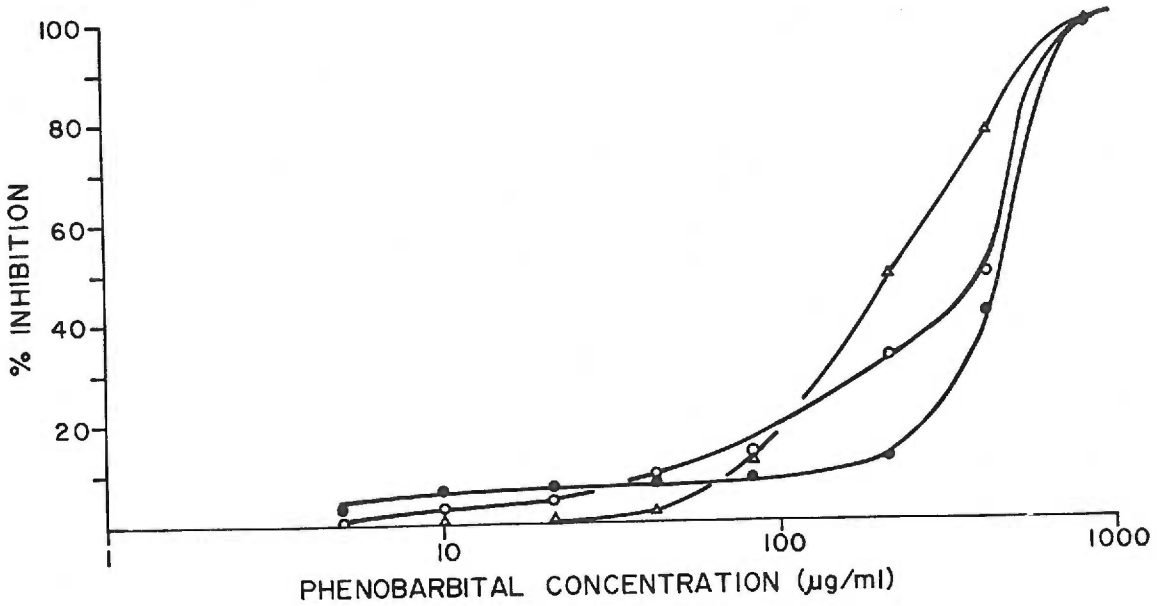

Fig. 8C. Effect of protein concentration on phenobarbital inhibition of mitogen stimulated ^{3}H-thymidine incorporation. Each microtiter well contained complete medium, mitogen (PHA), barbiturate and varying concentrations of pooled human serum (Open triangles: 5% pooled human serum; Open circles: 10% pooled human serum; Closed solid circles: 20% pooled human serum).

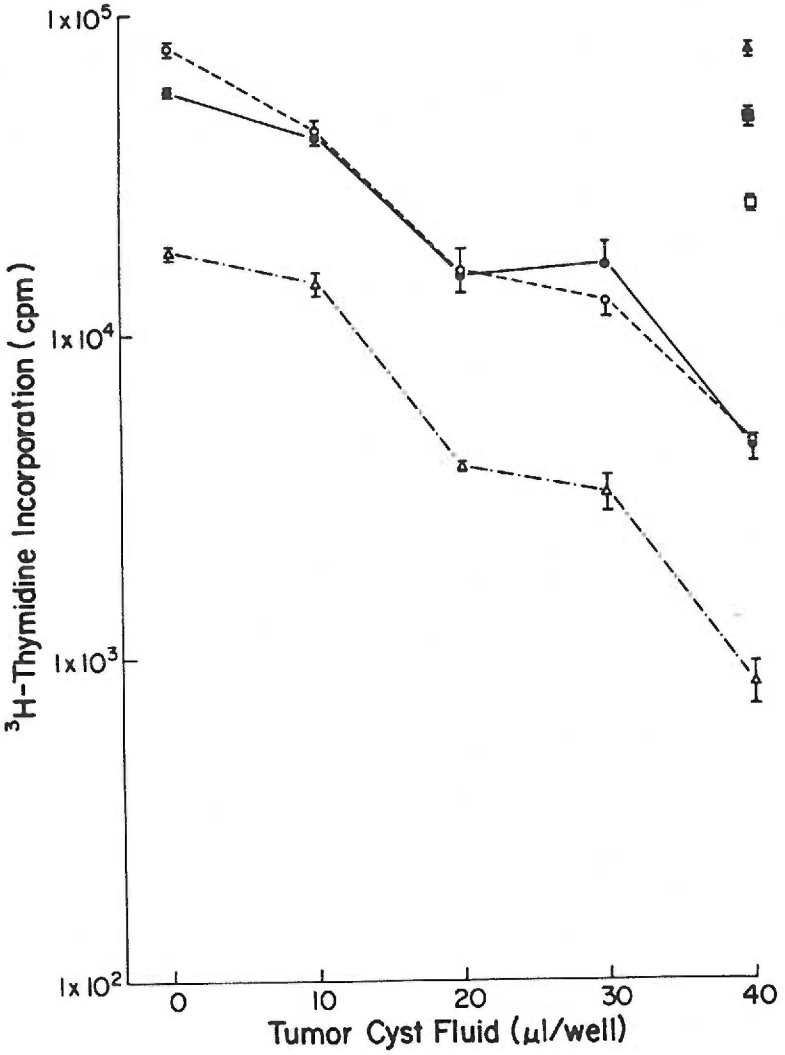

Fig. 9 Suppression of normal lymphocyte ^{3}H-thymidine incorporation by tumor cyst fluid from a glioblastoma patient (patient E.J.)

o--------o (open circles): PHA response
●————● (closed circles): Con A response
Δ-·-·-·-·Δ (open triangles): PWM response
▲ (closed triangle): PHA + 40 µl of PHS
■ (closed square): Con A + 40 µl of PHS
□ (open square): PWM + 40 µl of PHS

Data are presented as the mean cpm ± S.E.M. PHS indicates pooled human serum. Control cultures which received no mitogen incorporated between 180-210 cpm of ^{3}H-thymidine.

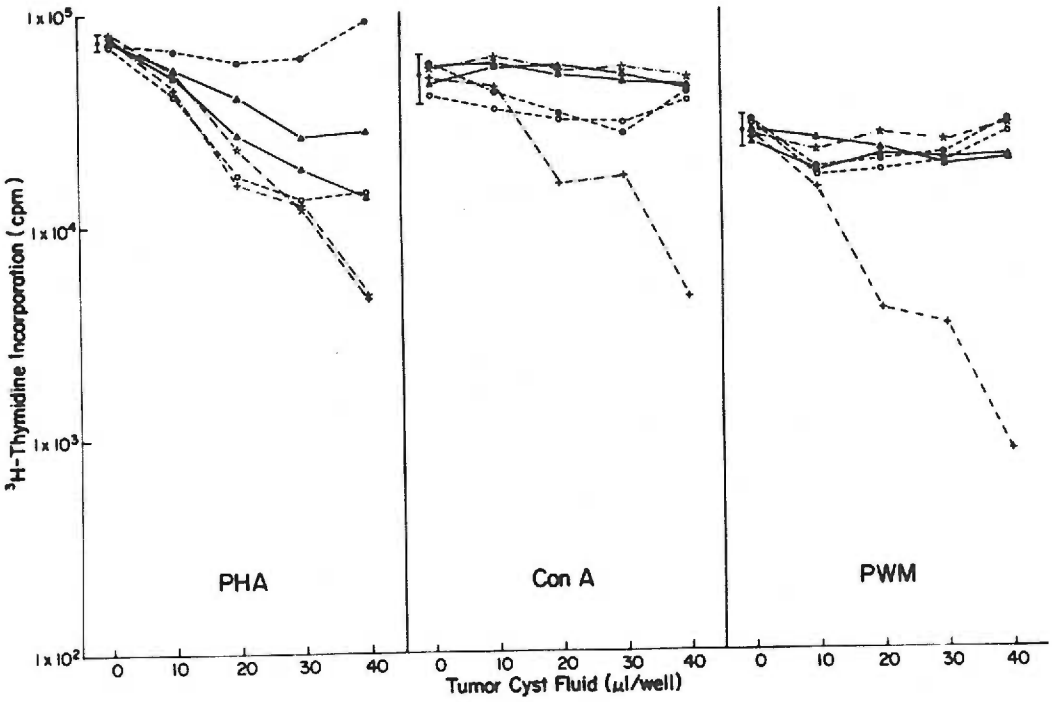

Fig. 10A Effect of tumor cyst fluid from six brain tumor patients on mitogen-induced normal lymphocyte activation.

●---------● (closed circles): patient SS (glioblastoma)
o---------o (open circles): patient SB (astrocytoma II)
▲———————▲ (closed triangles): patient CG (glioblastoma)
Δ———————Δ (open triangles): patient GJ (microglioma)
-·-·-·-· (asterisk): patient CR (glioblastoma)
+·-·-·-·-+ (cross): patient EJ (glioblastoma)

The vertical bars indicate the mean values (cpm) ± 2 S.D. Control assays received 40 µl of pooled human serum instead of cyst fluid.

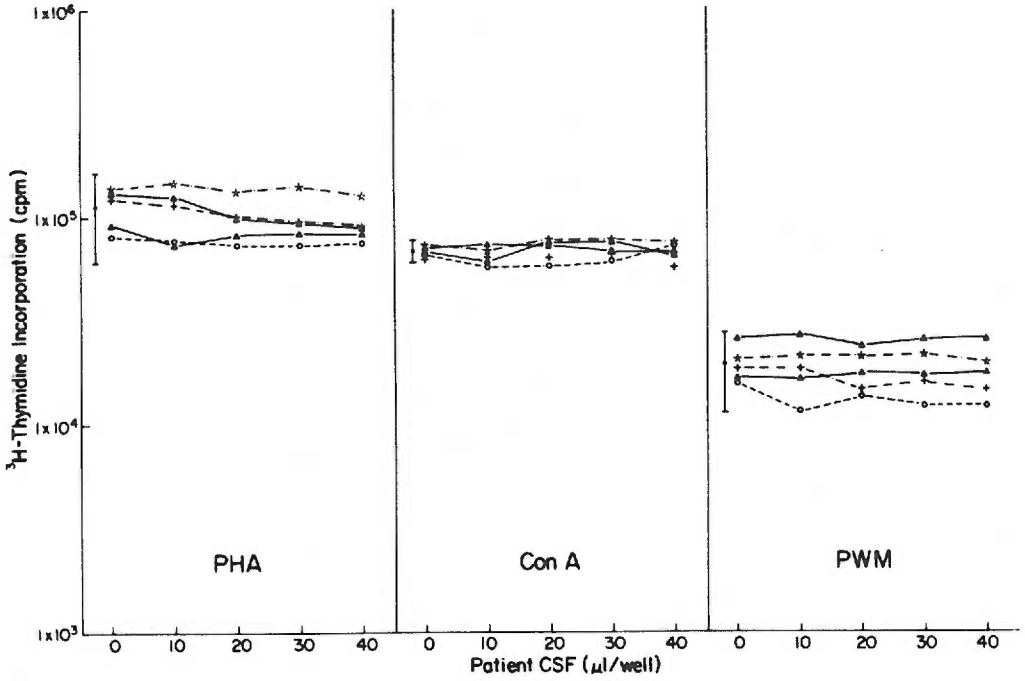

Fig. 10B Effect of CSF from five brain tumor patients on mitogen-induced normal lymphocyte activation.

o---------o (open circles): patient JT (medulloblastoma)
▲———————▲ (closed triangles): patient CG (glioblastoma)
Δ———————Δ (open triangles): patient GJ (microglioma)
-·-·-·-· (asterisk): patient CR (glioblastoma)
+ ·-·-·-·+ (cross): patient DL (glioblastoma)

The vertical bars indicate the mean value (cpm) ± 2 S.D. Control assays received 40 µl of 0.9% NaCl saline instead of CSF.

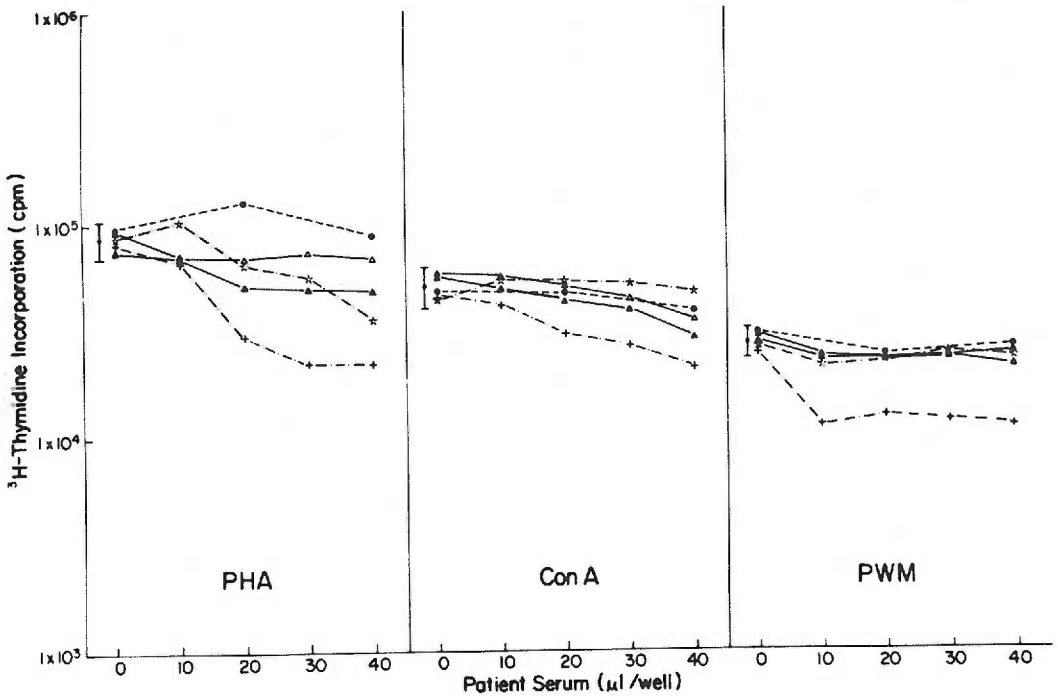

Fig. 10C Effect of serum from five brain tumor patients on mitogen-induced normal lymphocyte activation.

●--------● (closed circles): patient SS (glioblastoma)
Δ————Δ (open triangles): patient GJ (microglioma)
▲————▲ (closed triangles): patient CG (glioblastoma)
-.-.-.-. (asterisk): patient DL (glioblastoma)
+-.-.-.-.-+ (cross): patient CR (glioblastoma)

The vertical bar indicates the mean value (cpm) ± 2 S.D. Control assays received 40 µl of normal pooled human serum instead of patient serum.

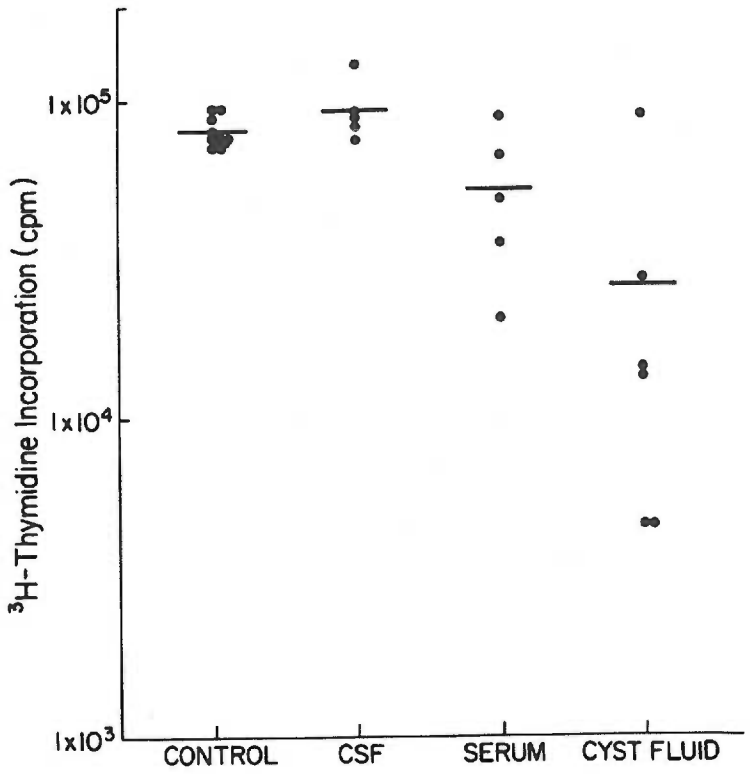

Fig. 11 PHA-stimulated ^{3}H-thymidine incorporation of normal lymphocytes in the presence of cyst fluid, CSF and serum from brain tumor patients. Horizontal bars indicate mean cpm for each group. In control cultures 40 µl of normal pooled human serum were added in each well. Each experimental culture received either cyst fluid, CSF or serum (40 µl). Statistical analysis of data using unpaired Student's t test shows that both serum and cyst fluid, but not CSF, are statistically different from control ($P<0.01$).

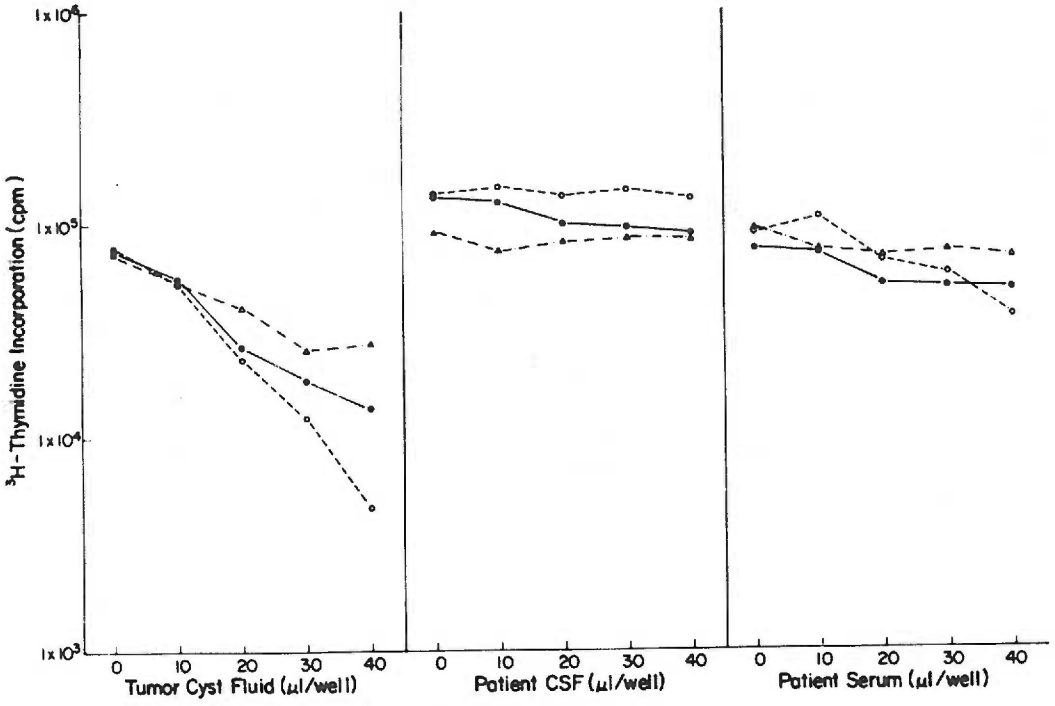

Fig. 12 Comparison of the effects of tumor cyst fluid, CSF and serum simultaneously obtained from the same patient on PHA induced normal lymphocyte activation as measured by ^{3}H-thymidine incorporation.
o--------o (open circles): patient CR (glioblastoma)
●————● (closed circles): patient CG (glioblastoma)
Δ·-·-·-·-Δ (open triangles): patient GJ (microglioma)

Control cultures received 40 µl of pooled human serum instead of either cyst fluid or serum. As a control for cultures receiving CSF from brain tumor patients 40 µl of saline (0.9%) or normal CSF was added.

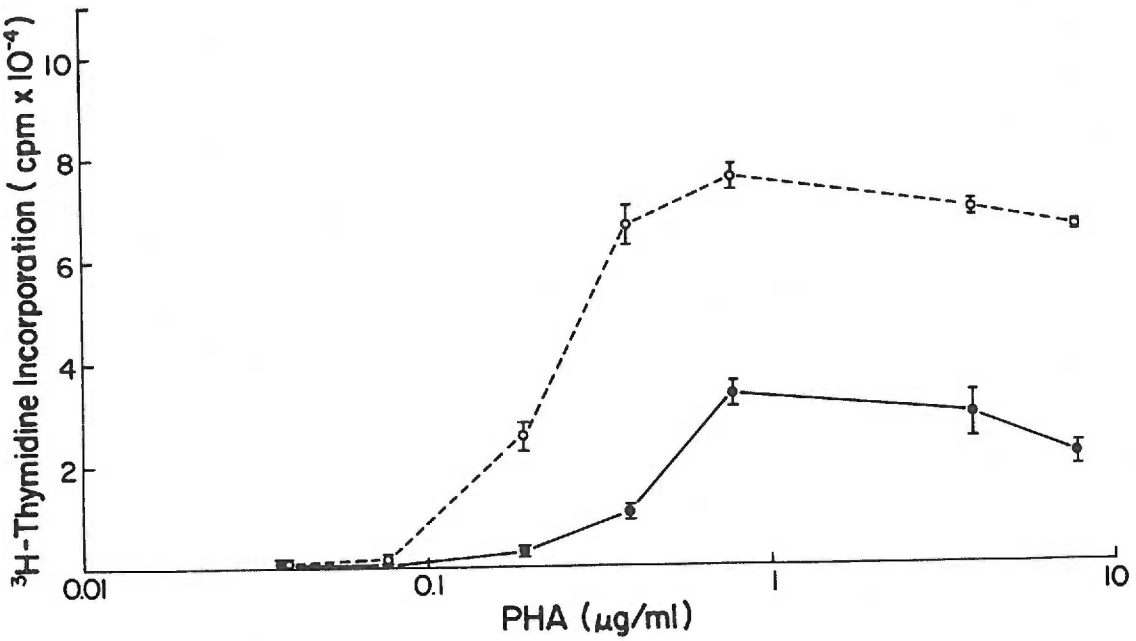

Fig. 13 Effect of PHA concentration on the inhibition by tumor cyst fluid of normal lymphocyte activation. Dashed line indicates the control lymphocyte culture to which 40 µl pooled human serum was added instead of cyst fluid. Solid line indicates experimental culture to which 40 µl of cyst fluid from one of the patients (EJ) was added. PHA was added at various concentrations to give both suboptimal and optimal stimulation. Data are presented as the mean cpm ± SEM.

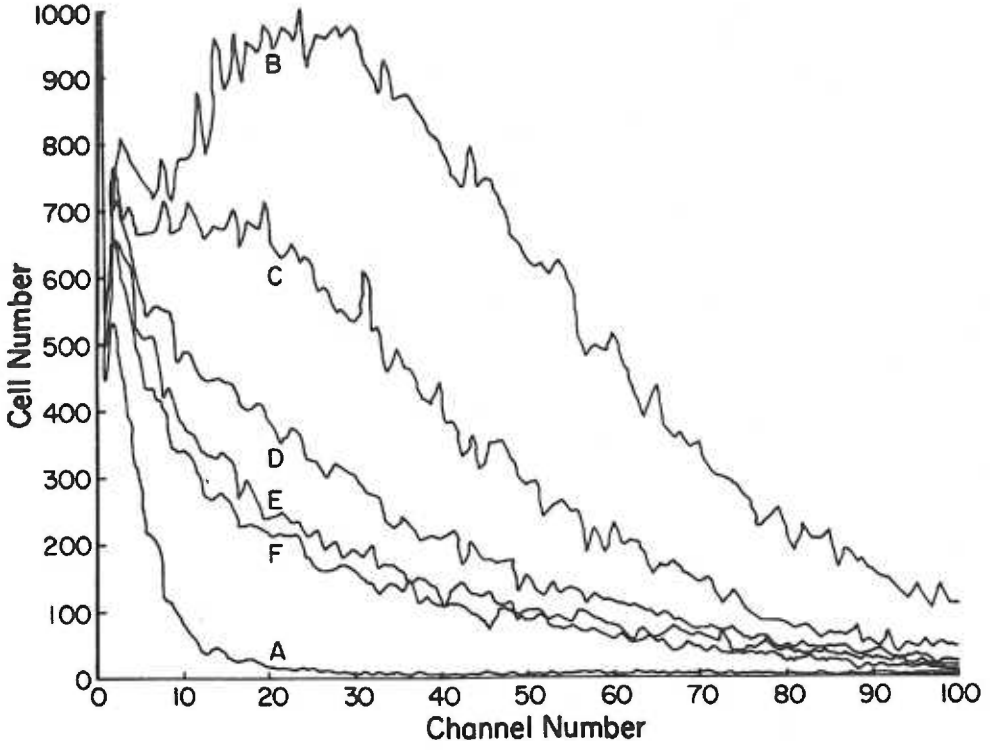

Fig. 14 Effect of glioblastoma tumor cyst fluid on mitogen-induced increase in normal lymphocyte cell size. The cyst fluid was obtained from patient E.J. Channel number as indicated in "Methods" is a measure of cell size.

Curve A: Control + no PHA, no cyst fluid
Curve B: Control + PHA + no cyst fluid
Curve C: PHA + cyst fluid (0.05 ml/tube)
Curve D: PHA + cyst fluid (0.10 ml/tube)
Curve E: PHA + cyst fluid (0.15 ml/tube)
Curve F: PHA + cyst fluid (0.20 ml/tube)