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EFFECTS OF 1,3-BIS(2-CHLOROETHYL)-1-NITROSOUREA AND SERA FROM SELECTED PATIENTS ON MAMMALIAN CELL LINES

by

Paula Cosper Vinson

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Physiology and Biophysics in The Graduate School, University of Alabama in Birmingham

Birmingham, Alabama

1974

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CHAPTER I

INTRODUCTION

Cells require many nutrients and other factors to be successfully cultured. Many studies have been done on the various nutritional requirements of cells in culture, but all of the necessary factors have not been identified. At this time there is no completely chemically defined medium that will sustain growth of most cells in culture. The addition of serum to the chemically defined medium is required for most cell lines. It is known that serum is required for most cells to attach to the culture vessel and divide as reviewed by Temin et al. (1972). The precise identification of these serum factors has not been accomplished. It is known that serum from patients with certain diseases such as cystic fibrosis and multiple sclerosis contain factors not present in normal serum. These factors have not been specifically identified, but many studies have been done on their chemical nature. The present study was done to determine the effect of serum from patients with cystic fibrosis and serum, plasma and cultured lymphocytes from patients with multiple sclerosis on certain mammalian cells in culture. These effects were measured by changes in deoxyribonucleic acid (DNA) synthesis rate as reflected by uptake of tritiated thymidine by the cells.

Studies were also done using the anti-tumor agent,

1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). Its effects on DNA synthesis in human skin fibroblasts and rat glial tumor cells in culture were evaluated and its effects on cells derived from human glioma tissue were observed.

A. Multiple Sclerosis

Multiple sclerosis (MS) is a demyelinating disease first described around 1840 by Carswell in England and Cruveilhier in France (Lawyer, 1970). Since that time enormous amounts of literature have been published about the disease but yet many aspects are still unsolved.

Multiple sclerosis is usually manifested between the ages of 20 and 40 years. Presenting clinical symptoms may include complete loss of vision or blurred vision usually due to bulbar or retrobulbar neuritis, diplopia, numbness and tingling in the extremities, weakness of the legs and incoordination. The symptoms usually appear suddenly and slowly disappear over the course of a few weeks or months. It is usually not diagnosed during the first exacerbation. Since there is no definitive diagnosis for the disease it is mainly a matter of eliminating other possible diseases such as hysteria, tumor of the spinal cord, herniated intervertebral disk, cervical osteoarthritis, neurosyphilis, combined system disease, syringomyelia and neoplasma in the posterior fossa of the skull or in the cerebral hemispheres which may present with the same symptoms (Lawyer, 1970).

The brain and spinal cord of a patient with MS appear externally normal, but sections reveal focal areas of discoloration of white matter throughout the neuraxis. The plaques of demyelination are

found in varying locations throughout the nervous system and may involve the gray matter (Lawyer, 1970). The plaques of demyelination may cause lesions not only of motor and sensory pathways in the spinal cord but also autonomic pathways (Johnson and McLellan, 1972).

Multiple sclerosis has a very interesting epidemiology. The disease appears to be more prevalent in areas above latitude 45 degrees north and below 45 degrees south. No specific climate has been correlated with this finding. Immigrants who go from an area of high incidence to an area of low incidence after the age of 15 years carry with them the risk factor of their country of origin, whereas if immigration occurs before the age of 15 years the person would have the same risk of developing MS as the native people of the low incidence area (Dean and Kurtzke, 1970).

There are some racial differences in the incidence of the disease. The people in Japan have a very low incidence of the disease, and there is no well documented case of MS in the African black (Posner, 1972). These differences could be due to environmental factors rather than genetic factors. Family members of a patient with MS have a slightly increased risk of acquiring the disease, but again this may be due to environmental factors rather than genetic ones.

The etiology of MS is still a mystery. Many hypotheses have been made regarding the cause of the disease. In 1963 Baker and coworkers found that there was a relative deficiency in the unsaturated fatty acid content of the brain from patients with MS. This deficiency was found in tissues other than the brain including white blood cells. This work led to the hypothesis that this deficiency might cause the central nervous system to be more sensitive to potentially damaging

factors (Thompson, 1966). Field proposed in 1973 to change the biochemical content of the nervous system in children who showed this deficiency by controlling the amounts of these unsaturated fatty acids in the diet, thus offering prophylactic treatment for the disease.

One of the two main theories about the etiology of MS is that of a viral infection. This theory has many followers and evidence for it is increasing. In 1962 it was found by Adams and Imigawa that the antibody titers to measles virus were higher in sera from patients with MS than in matched controls. Brody et al. (1971) studied antibody titers to 11 viruses and found that in MS patients titers to measles, type C influenza, herpes simplex, parainfluenza 3, mumps and varicella zoster were consistently higher than in carefully matched controls. Antibody titers to adenovirus, parainfluenza 2 and 3, and types A and B were not consistently higher in MS patients than in controls. When each patient was matched with a sibling of the same sex born within three years of the patient, there was no significant difference between antibody titers to any of these viruses suggesting an environmental or familial effect on these responses. Ammitzbøll and Clausen (1972) reported a raised IgG titer to measles virus in blood and cerebrospinal fluid of MS patients, but healthy siblings of the patients had intermediate values. Even though many investigators think that MS is virally induced there are varying theories about how this is accomplished. One such hypothesis is that a subclinical infection during childhood protects against acquiring the disease in later life, and another is that the disease is acquired during childhood but remains dormant until later life when it manifests itself (Dean and Kurtzke, 1970). At least two groups have reported finding whole virus

particles in brain tissue from MS patients (Prineas, 1972; Ter Meulen et al., 1972). These viruses were paramyxovirus-like in appearance. Antibody studies by Ter Meulen et al. (1972) indicated that the agent was a parainfluenza 1 virus.

The other main theory about the etiology of MS is the autoimmune disease theory. This theory led to therapeutic trials of immunosuppressive drugs in treatment of MS, but these were unsuccessful in improving the condition of most patients tested (Neuman and Ziegler, 1972). The theory that MS is an autoimmune disease has led to many attempts to demonstrate humoral or cellular factors directed against nervous system (CNS) components. These studies have produced many conflicting results.

In 1968 Lisak et al. attempted to show the presence of an antiencephalitogen antibody in serum from patients with demyelinative diseases by using direct radioimmunoelectrophoresis but were unsuccessful. Caspary and Chambers, however, in 1970 reported detection of antibody to encephalitogenic basic protein in serum from MS patients as well as from patients with other neurological diseases such as polyneuropathy and motor neuron disease by use of the immune adherence technique.

Dowling et al. (1968) showed a demyelinating factor in serum from MS patients by adding the concentrated whole serum to myelinated neonatal mouse cerebellum cultures and observing the demyelination which usually was complete within 24 hours. The serum was fractionated into the 7S and 19S components, concentrated by vacuum dialysis, and individually added to myelinated cerebellum cultures. The highest demyelinating activity was found in the 7S fraction and this activity

was localized to the IgG class of antibody. In one patient there was demyelinating activity in the IgM portion of the 19S fraction. Demyelinating activity was also found in cerebral spinal fluid (CSF) and brain extracts from MS patients (Kim et al., 1970). Extracts from the normal appearing white matter in MS brain produced levels of demyelination comparable to control white matter, whereas extracts from demyelination plaque, shadow plaque, and periplaque areas of MS brain produced high levels of demyelination. Demyelination with CSF and brain extracts was slower than with serum, but was usually complete within 96 hours. In 1973, Raine et al. published an extensive light and electron microscope study of MS serum induced demyelination using myelinated cultures of embryonic mouse spinal cord. This study showed that the oligodendroglia located in regions of myelinated axons were affected and usually only about one-fourth of the population was affected. It was only rarely that astroglia and neurons were affected.

Similar demyelinating factors were found in serum from animals with experimental allergic encephalomyelitis (EAE) which is a delayed hypersensitivity reaction produced in animals by injection of white matter from the CNS in complete Freund's adjuvant. This autoimmune reaction has been used as the animal model for MS. Studies of the effects of serum from animals with EAE using the myelinated rat cerebellum cultures demonstrated the same demyelination pattern as that observed after the addition of MS serum (Bornstein, 1968; Bornstein, 1963; Bornstein and Iwanami, 1971). As with MS serum the reaction was complement dependent and the demyelinating activity was in the 7S fraction of the serum, the 19S fraction being inactive (Bornstein, 1968). Several investigators have reported demyelination and

subsequent remyelination in vitro after exposure to EAE or MS serum (Bubis and Luse, 1964; Lampert, 1965; Bornstein, 1968; Bornstein and Iwanami, 1971). If the cultures were washed free of the MS or EAE serum and fed normal nutrient medium, they began to remyelinate in about ten days. The new myelin that was formed was incomplete (Bornstein, 1968). It appeared thinner and covered only parts of the axon leaving other portions uncovered. When these remyelinated cultures were fixed and stained with Sudan black B they resembled the so called "shadow plaques" found in MS brain tissue which were considered to represent a partial demyelination (Bornstein, 1968).

Lymph node cells from animals with EAE also caused demyelination of the rat cerebellum cultures (Bornstein and Iwanami, 1971). The effect produced by the lymph node cells took about four days to be fully expressed, whereas serum from the same animals caused changes in only a few hours.

When serum from animals with EAE was added to newly initiated mouse spinal cord cultures the differentiation of oligodendroglia and formation of myelin was completely inhibited (Bornstein and Raine, 1970). Exposure of these cultures to higher concentrations of the EAE serum (25%) for a three week period produced a state of sclerosis where remyelination was scant or absent. The demyelinating factor appeared to be produced only when the animal was sensitized with whole brain tissue. Kies et al. (1973) reported that animals sensitized with myelin basic protein developed EAE, but only two of ten serum samples displayed the demyelinating factor when added to myelinated mouse cerebellum cultures whereas nine of the serum samples from animals sensitized with whole CNS tissue caused demyelination of the cultures.

Glial cells as well as myelin were affected by serum and cells from patients with neurological disease and animals with EAE. Serum from patients with MS and those with old brain lesions was shown to have an observable toxic effect on rat glial cells in culture (Berg and Kallen, 1962a). The effect on rat glial cultures of mononuclear blood cells from MS patients and patients with traumatic encephalopathy who had positive cytotoxic serum reactions was studied by Berg and Kallen (1964). Only the cells from the MS patients had a cytotoxic effect. The authors speculated that the cytotoxic factors could be a result of an immunization process causing the disease or simply a response to degenerated brain tissue. The same gliotoxic effects were seen when serum from animals with EAE was added to rat glial cultures (Berg and Kallen, 1962b).

MS serum was also observed to contain a lymphotoxic factor in about 20% of cases (Stjernholm et al., 1970; van den Noort and Stjernholm, 1971). When serum containing the lymphotoxic factor was added to normal control lymphocytes or lymphocytes from MS patients the RNA synthesis was drastically reduced as measured by uptake of tritiated uridine. The factor was said to be a high molecular weight protein which was stable to heat (56 degrees C. for 30 minutes) and freezing (Stjernholm et al., 1970). The factor appeared and disappeared from the serum and seemed to be associated with acute attacks of the disease (van den Noort and Stjernholm, 1971). Only rarely was the factor found in serum from patients with other neurological diseases. It was theorized that the factor was a host defense reaction against self destruction (Stjernholm et al., 1970). Along the same line Hughes et al. (1968) reported that lymphocytes from patients with acute MS

showed reduced spontaneous transformation in culture when compared to normal controls. When these lymphocytes were exposed to encephalitogenic factor in culture there was a small but definite increase in uptake of tritiated thymidine. The response to encephalitogenic factor was significantly greater in lymphocytes from MS patients than those from normal controls. It was also reported that serum from MS patients would inhibit spontaneous transformation and tritiated thymidine uptake of normal lymphocytes in culture (Knowles et al., 1968). This factor was not found in patients with other neurological diseases.

In mixed leukocyte cultures results showed that leukocytes from some patients with MS have an impaired reaction when stimulated by leukocytes from another patient with MS (Kallen and Nilsson, 1971; Hedberg et al., 1971). This reaction was also seen in patients with rheumatoid arthritis, psoriasis arthropatica, pelvospondylitis, systemic lupus erythematosis, and scleroderma.

Attempts were made by several investigators to demonstrate cellular hypersensitivity in MS patients against CNS antigens. Strandgaard (1970) was unable to show such hypersensitivity by using the leukocyte migration inhibition technique with CSF as the source of antigen. Caspary and Field (1970) used encephalitogenic factor prepared from human brain and basic protein from peripheral nerve as antigens with the same leukocyte migration inhibition technique. They found that all MS patients tested as well as patients with other neurologic diseases showed sensitivity to encephalitogenic factor by decreased migration of donor macrophages. Responses to the basic protein from peripheral nerve were inconsistent in MS patients but generally positive for the other neurologic diseases. Bartfeld and Atoynatan

(1970) using the same system with human cerebral white matter as source of antigen showed the same results. Control lymphocytes when mixed with antigen did not show inhibition of macrophage migration whereas MS lymphocytes did produce inhibition. Supernatant fluids from cultures of MS lymphocytes incubated with brain extract also caused inhibition of macrophage migration when compared to supernatant from control lymphocytes incubated with brain extract (Bartfeld and Atoynatan, 1970).

B. Cystic Fibrosis

Cystic fibrosis (CF) is a common disease of children and young adults of the Caucasian populations. The classic clinical features of the disease are chronic bronchiolar obstruction and infection of the lungs; steatorrhea and azotorrhea, increase in the salinity of the sweat, malnutrition, and growth failure (Lobeck, 1972). The disease was recognized as a clinical entity in 1936 by Fanconi and the biochemical defect remains unknown (Lobeck, 1972).

Two main systems are involved in the disease process -- the gastrointestinal and pulmonary systems. Clinical symptoms and severity vary greatly among patients. In general, few patients die of gastrointestinal complications of the disease, but most succumb to the pulmonary involvement (Shwachman, 1974).

Cystic fibrosis is inherited in an autosomal recessive pattern. The incidence in the Caucasian populations is about one in 1,600-2,500 live births. The disease is very rare in African Negroes and Hapanese (Lobeck, 1972). Heterozygotes show no symptoms of the disease. From the incidence of CF, the heterozygote incidence can be calculated to be about one in 20 or 25.

Many investigators have become interested in cystic fibrosis and its biochemistry. Since the exocrine glands seemed to be involved extensively, a two part study of the parotid saliva in CF was done (Kutscher et al., 1965; Mandel et al., 1965). It was found that an increased parotid secretion rate was not a consistent finding in patients with CF even after stimulation with citric acid (Kutscher, 1965). The electrolyte levels and glycoproteins were measured in CF parotid saliva and compared to normals. The sodium, potassium, chloride, calcium and phosphorus levels in CF saliva had a tendency to be higher than control levels, but no statistically significant differences were found in the mean values except in the case of inorganic phosphorus (Mandel, 1965). There was also no significant difference between glycoproteins in CF saliva and control saliva. Mandel et al. (1967) in another study using parotid and submaxillary saliva found that amylase and lysozyme levels in parotid saliva were not significantly different in CF and control children. In submaxillary saliva they found significant differences in levels of sodium, chloride, phosphorus, calcium and urea between CF patients and controls.

Danes and Bearn in 1968 described the presence of cytoplasmic metachromasia in skin fibroblasts of patients with CF and heterozygous carriers. The metachromatic material was not distributed evenly throughout the cytoplasm as seen in cells from patients with mucopolysaccharidoses, but tended to be clumped in a small area of the cytoplasm. In 1973, Danes reported correction of the metachromasia by growing the metachromatic positive fibroblasts with normal control cells. The cells required intimate contact with the normal cells for correction to occur.

Fitzpatrick et al. (1972) found a substantial increase in serum calcium binding associated with a modified membrane protein electrophoretic pattern of cystic fibrosis red cells. They proposed that perhaps the calcium was bound to an immunoglobulin possibly secreted with mucus which would impair salt and water flow accompanying mucous secretion. This would give rise to increased viscosity of the mucus.

Certain histocompatibility antigens have been linked to various diseases so a study was done to determine if there was any correlation between certain HL-A antigens and CF (Polymenidis, 1973). There was no significant difference in distribution of HL-A antigens in CF patients when compared to normal controls.

Perhaps the most significant recent finding in CF research was the ciliary inhibition factor found in CF serum (Spock et al., 1967). The serum caused dyskinesis in rabbit tracheal cilia which was defined by Spock as disorganization of the regular wave-like appearance of the boundary of the beating cilia. The factor was present in the euglobulin fraction of serum. Bowman et al. (1969) used oyster cilia to detect the serum factor in CF patients and their parents. By fractionating the serum from patients and heterozygotes it was found that the inhibitor migrated with a cationic protein fraction when electrophoresed at pH 8.6 (Bowman et al., 1970). The inhibitor was eluted during gel filtration with a fraction of molecular weight 125,000-200,000 and was fractionated with the immunoglobulin G fraction (Bowman et al., 1970). The same fraction from normal controls did not inhibit oyster ciliary activity.

The oyster ciliary bioassay technique was modified by

Conover et al. (1973a) so that the euglobulin fraction did not have to be concentrated to elicit a response and the positive response time was cut to about 3-6 minutes. This group hypothesized that the ciliary dyskinesis factor was an accumulated cellular product, and that the defect in CF was in production or release of a substance inhibiting the dyskinesis factor.

Doggett et al. (1973) found that they could reverse the dyskinesis factor in serum and parotid secretions in vivo by administering heparin. A subclinical dose of heparin reversed the ciliostasis in eight patients. Ciliostasis returned in 24-48 hours after the heparin was discontinued. It was suggested that since heparin bound and precipitated amylase thus making it inactive, perhaps amylase was the inhibitory factor. A follow up study by Doggett and Harrison (1973) showed reversal of the inhibitory factor in serum and saliva in vitro by heparin. After overnight incubation at 4 degrees C. some of the tubes with saliva and heparin had formed a precipitate. The samples with no precipitate caused dyskinesis of the cilia as expected since the effect of heparin was only transient, but the supernatant from the tubes with the precipitate showed no inhibitory activity. Analysis of the heparin precipitate showed that it was amylase. Adsorption of inhibitory positive samples with antisera to crystalline amylase caused loss of inhibition of the oyster cilia. Purified amylase added to normal serum made it ciliostatic.

In 1972, the ciliary inhibitory factor was found in media from cultured skin fibroblasts from CF patients and their parents (Danes and Bearn, 1972). The media from ametachromatic cells from CF patients could not be distinguished from media from control cells when

added to the oyster cilia. This was taken as evidence for the idea of genetic heterogeneity in cystic fibrosis. Bowman et al. (1973a) fractionated the media from fibroblast cultures derived from CF patient skin biopsies and found the ciliary inhibitory factor present in the same fraction as the inhibitory factor in serum. Radioactive protein studies suggested that the factor was synthesized by the fibroblasts.

Beratis et al. (1973) tested the media from fibroblasts derived from skin of CF patients and carriers on rabbit tracheal cultures and could get a positive response only after incubating the media with IgG. Media from normal fibroblasts did not give a positive response before or after incubation with IgG. If the fibroblast cultures were allowed to become confluent, a positive response from the media was obtained from CF and normal fibroblasts without incubation with IgG. Media from cultured amniotic fluid cells was tested with the same results. Bowman et al. (1973b) tested media from cultured amniotic fluid cells on oyster cilia and obtained positive results from cultures of cells from homozygotes and heterozygotes for CF without having to incubate the media with IgG. Cell free amniotic fluid from homozygote or heterozygote for CF did not produce a positive reaction on rabbit tracheal cultures.

Conover et al. (1973b) studied the ciliary inhibitory factor in long and short term lymphoid lines from CF patients, carriers and normals. Phytohemagglutinin stimulated leukocytes from CF patients and carriers produced the inhibitory factor by 48 hours in culture whereas unstimulated leukocytes did not produce the factor until about 72 hours. Control leukocytes did not produce the factor at all. Cell free media

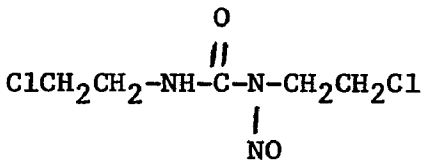
from the long term lymphoid cell lines of CF patients and carriers contained the inhibitory factor, while media from control cultures did not.

The ciliary inhibitory factor was fractionated with the IgG portion of serum but ultrafiltration experiments suggested that the factor was a small molecular weight (1,000-10,000) molecule bound to IgG (Beratis, 1973). Experiments using various immunological techniques indicated that the interaction between oyster cilia and the ciliary inhibitory factor was not a typical antigen-antibody reaction (Herzberg, 1973). Conover et al. (1973a) found that the C3 component of complement was present at much higher levels in CF and carrier serum than in normal control serum. Further experiments have given more evidence that C3a bound to IgG was the ciliary inhibitory factor and the primary genetic defect in CF was a deficiency or defect in the carboxypeptidase-B-like enzyme which inactivates C3a (Conover et al., 1973a). Sera from normal controls were converted to a ciliary inhibitor positive state by addition of epsilon-aminocaproic acid (EACA) which inhibits the carboxypeptidase-B-like enzyme inactivator of C3a. Incubation of the converted sera with carboxypeptidase-B resulted in reversion to the normal state again. Purified human C3a was inactive in causing ciliary dyskinesis in rabbit tracheal cultures until it was incubated with IgG. C3a has a molecular weight of 7,200 which would fall within the 1,000-10,000 range predicted for the inhibitory factor by Beratis et al. (1973).

The present study was done to study the effects on normal human fibroblasts in cell culture by a factor in CF serum. The measurements of uptake of tritiated thymidine by the fibroblasts were used as an indicator of an effect on the DNA synthesis of these cells.

C. 1,3-Bis(2-chloroethyl)-1-nitrosourea

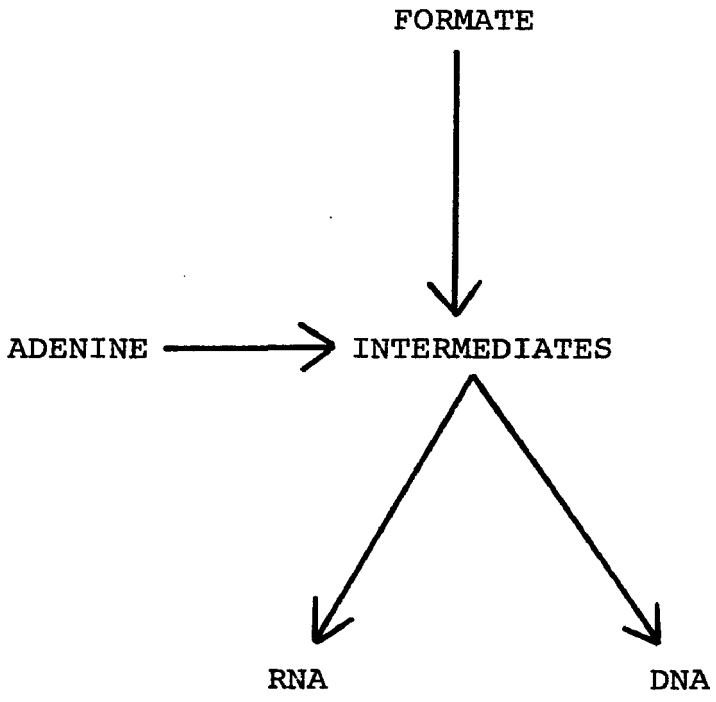
1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) is a relatively new anti-tumor agent with specific properties which make it of possible value in the treatment of brain tumors. The structure of the drug is as follows:



Many studies have been done with the drug, but its exact mechanism of action is still unknown. In 1965, Wheeler and Bowdon published a study of the effects of BCNU upon the synthesis of protein and nucleic acids in vivo and in vitro. They found an inhibition of incorporation of formate-^{14}C into purines of both RNA and DNA. The inhibition of incorporation was slightly greater in DNA than in RNA. In vivo high doses of BCNU also caused inhibition of purine nucleotide synthesis. When the labeled substrate was adenine-8-^{14}C there was an inhibition of incorporation of the label into DNA but not into RNA. These results led to the suggestion that there was interference with the de novo synthesis of purine ribonucleotides and with the conversion of purine ribonucleotides to components of DNA (Figure 1). Protein synthesis was also studied and found to be normal although DNA synthesis was inhibited.

Further studies were done by Wheeler and Bowdon (1968) using cell free systems for evaluation of effects of BCNU on the enzyme, DNA nucleotidyltransferase, and on priming activity of DNA. Cell extracts from L1210 ascites cells were incubated with BCNU and demonstrated

Figure 1. Sites of apparent inhibition by BCNU in purine and DNA synthesis (Wheeler and Bowdon, 1965).


FORMATE
ADENINE
INTERMEDIATES
RNA
DNA

decreased DNA nucleotidyltransferase activity. Incubation of the intact cells with BCNU caused a decrease in DNA synthesis and also a decrease in the DNA nucleotidyltransferase activity in the extract from these cells. The primer activity of the DNA isolated from these cells was not altered. When a lower dose of BCNU was used there was a decrease in DNA synthesis without a decrease in the DNA nucleotidyltransferase activity. This seemed to indicate that inhibition of the transferase enzyme was not the primary cause of inhibition of DNA synthesis. Alteration of the primer activity was not responsible for the inhibition of DNA synthesis, either. It was proposed that the cytotoxic action of BCNU was perhaps due to cleavage of the molecule between the nitrogen atom bearing the nitroso group and the carbonyl group and formation of an alkyl isocyanate. Inhibitory activities similar to BCNU were observed when 2-chloroethyl isocyanate was tested in the same system.

Groth et al. (1971) did studies with BCNU which suggested effects on enzymes involved in 1-carbon transfer reactions in purine synthesis. Inhibition of these enzymes or only one of the enzymes would result in the reduced purine synthesis which was observed.

Chemically, BCNU is related to alkylating agents but the studies cited above seem to indicate another mechanism of action. The drug was most stable at pH 4 in aqueous solution or in petroleum ether and even at these conditions the half life was about 8 hours (Loo et al., 1966). In plasma the half life was reduced to about 20 minutes in vitro and about 15 minutes in vivo. BCNU was highly lipid soluble and passed the blood-brain barrier easily. It appeared in the blood and cerebrospinal fluid simultaneously after IV administration and

concentrations fell at about the same rate in both (Loo et al., 1966).

Barranco and Humphrey (1971) did a study on the effect of BCNU on the cell cycle in Chinese hamster ovary cells. The synchronized cells were observed to be sensitive to BCNU just before the S phase, in early S phase and most sensitive in mid S phase. The drug caused a delay in cell progression in all phases of the cell cycle. The delay was reversible if the drug was removed from the cultures.

BCNU has been used clinically in man on a trial basis. Bone marrow toxicity limits drug dosage and administration (Wilson and Hoshino, 1969). Since BCNU readily passed the blood-brain barrier, it was given to patients with brain tumors. Wilson et al. (1970) reported a series of patients treated for advanced glioblastomas and astrocytomas. Of eighteen patients with glioblastoma, seven were responsive to therapy giving a 39% response rate. There were six patients with astrocytomas and four of these were responsive to BCNU giving a 67% response rate. The toxicity included depression of bone marrow, especially platelets and white blood cells.

A series of 27 patients with brain tumors was reported by Walker and Hurwitz (1970). Two dose schedules were given -- high doses for three successive days or low doses one or three times a week until toxicity appeared. Various types of brain tumors were represented in this series. Of the 27 patients, 14 responded to BCNU. The group receiving intermittent intensive treatment showed better response to the drug. Toxicity was high in both groups.

Koo et al. (1972) studied the relationship between clinical and angiographic findings in patients treated with BCNU for brain tumors. They found no correlation.

BCNU has been tested for effectiveness against various other tumors. De Vita et al. (1965) reported 115 patients with all types of tumors. There were 24 patients who responded to the drug. No particular type of tumor responded more frequently than another, and intense intermittent therapy seemed to produce the best response.

Olshin et al. (1972) reported a series of 25 patients with bronchogenic carcinoma. The drug was given in a low dose once every six weeks for 12 to 18 weeks. There was no clinical response in any of the patients.

The effects of BCNU on advanced breast cancer were reported by Ahmann et al. (1972). Sixteen patients were given intensive intermittent treatment with BCNU. One patient showed clinical response at four weeks of therapy but after eight weeks of therapy the tumor was bigger than the size at beginning of treatment. BCNU was judged clinically ineffective against advanced breast cancer.

BCNU has also been used in combination with cytosine arabinoside. Cell culture studies showed that there was no significant difference between giving the drugs simultaneously or giving cytosine arabinoside alone (Chen and Mealey, 1972). When BCNU was given before cytosine arabinoside the cells were more sensitive to the cytosine arabinoside (Chen and Mealey, 1972). The combination was tried clinically by Kinne and Humphrey (1972) on advanced solid tumors. Only one response was noted in 23 patients when the drugs were given simultaneously.

The present study compares the in vitro effects of BCNU on DNA synthesis in normal human fibroblasts and rat glial tumor cells. The effects of BCNU on human glioma cells in culture were also observed and photographed.

CHAPTER II

MATERIALS AND METHODS

A. Cell Culture Technique

All fibroblast cultures used in these studies involving serum, plasma, and cells from patients were derived from the foreskin of a normal human infant. Cells were maintained in McCoy's 5A medium with 20% fetal calf serum (Grand Island #166-20).

The rat glial tumor cells were obtained from the American Type Culture Collection in Rockville, Maryland (Catalog #CCL-107). The strain was cloned from a rat glial tumor induced by N-nitroso-methylurea. The cells produce S-100 protein which is unique to brain tissue in vertebrates. Their morphology is fibroblast-like and the karyology is diploid (2n=42). The cells were maintained in McCoy's 5A medium with 20% fetal calf serum.

The human glioma cells were initiated from a biopsy of a human glioma obtained courtesy of Dr. Garber Galbraith, Chief of Neurosurgery, University of Alabama in Birmingham. The tissue was minced with foreceps and a scalpel in McCoy's 5A medium with 20% fetal calf serum plus five mg per 100 ml of medium Garamycin (gentamicin sulfate, Schering). Six small pieces were transferred to each of eight 30 ml plastic culture flasks (Falcon #3012) and spaced on the bottom of the flask. The flasks were then inverted and about two ml of

medium added. The flasks containing the explants were incubated overnight at 37 degrees C. in the inverted position and turned the next morning so that the medium flowed around the explants. This aided the attachment of the explants to the culture vessel. The cells were subcultured when adequate numbers of cells were observed around the explant.

Original fibroblast cultures were initiated in 30 ml plastic culture flasks (Falcon). Cells from the frozen ampule of rat glial tumor were subcultured in the small 30 ml plastic culture flasks also. When these cultures formed a monolayer, they were subcultured as described later into larger (250 ml) plastic culture flasks (Falcon #3024). Stock cultures of fibroblasts and rat glial tumor cells were maintained so ample cells were available throughout the study. The medium on the cultures in small flasks was changed twice a week. Each flask received 5 ml of McCoy's 5A medium with 20% fetal calf serum at each change. The medium in the larger flasks was changed once a week with each culture receiving 10 ml of McCoy's 5A medium with 20% fetal calf serum.

All cultures used in this study were subcultured 24 hours before the addition of serum, plasma or cells. To subculture, the medium was removed and the cultures rinsed with 0.25% trypsin solution (Grand Island) to remove any traces of medium which would tend to inactivate the trypsin. After rinsing, 2 ml of trypsin were added to each small 30 ml flask and 5 ml to each large 250 ml flask. The trypsin solution was allowed to remain on the cultures 10-15 minutes or until the cells became detached from the culture flask. The trypsin and cells were then pipetted into a sterile centrifuge tube containing

2 ml of McCoy's 5A medium in each tube. The cell suspensions were then added to the amount of medium needed for the number of new cultures being initiated. The medium and cells were then added to the flasks and each culture was gassed about 20 seconds with 5% CO_2. The cultures were incubated at 37 degrees C. After removal of the old medium, additions of serum, plasma or cells were made approximately 24 hours after subculture by adding the desired component to fresh medium and dividing the mixture among the appropriate cultures.

After the serum, plasma, or cells had been on the cultures for approximately 40 hours the medium was removed and each culture was washed once with Hank's balanced salt solution. The medium was replaced by McCoy's 5A medium containing 2 μCi/ml tritiated thymidine (specific activity - 20 Ci/m mole, New England Nuclear). The tritiated thymidine was left on the cultures four hours to allow uptake of the tritiated thymidine by cells which were synthesizing DNA.

The cultures were harvested after four hours. The medium was removed and each culture rinsed with 0.25% trypsin solution. Two ml of trypsin were added to each culture and left for 10-15 minutes or until all cells were detached from the culture flask. The trypsin and cells from each culture were pipetted into a clean centrifuge tube and centrifuged at 78 x g for five minutes to obtain a cell pellet. The trypsin was decanted and the cells resuspended.

B. Scintillation Counting

To prepare the cell samples for scintillation counting the cells were washed with 5 ml of 0.15 M NaCl, centrifuged and washed

again. The cell pellet obtained after the final wash was dissolved in 0.1 N NaOH by heating for 30 minutes at 37 degrees C. Samples from the cell solution were pipetted into a disposable scintillation vial and 11 ml scintillation fluid added. The scintillation fluid was composed of the following:

1000 ml	Toluene
100 ml	Beckman Bio-Sol Solubilizer (BBS-3)
5.0 g	2,5-diphenyloxazole (PPO)
0.1 g	1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP)

The samples were counted in a Packard Tri-Carb Liquid Scintillation Spectrometer Model 3375. Samples were counted in duplicate.

C. Estimation of Protein

Estimation of protein in the cell solutions was done by the method of Lowry et al. (1951). The sample was pipetted into a test tube and the total volume adjusted to 0.5 ml with distilled water. Standards containing 20 µg, 50 µg, and 100 µg of protein from a 1 mg/ml solution of bovine albumin fraction V (Grand Island) and a blank containing 0.1 N NaOH were prepared and evaluated along with the test samples. An alkaline copper tartrate reagent was prepared by adding two ml of 4% potassium-sodium tartrate solution to two ml of 2% $CuSO_4 \cdot 5H_2O$ and adding 96 ml of 3% Na_2CO_3 in 0.1 N NaOH solution to the mixture. Five ml of this reagent were added to the sample and mixed on a Vortex Genie Mixer. After ten minutes at room temperature 0.5 ml of Folin and Ciocalteau phenol reagent (Harleco) diluted 1:1 with distilled water was rapidly added and mixed on the Vortex mixer. The

tubes were allowed 20 minutes for reaction at room temperature. The optical densities (OD) of the samples were read in a Perkin-Elmer double beam spectrophotometer at 750 nm. The milligrams of protein in the samples were calculated as follows using the 50 µg standard values:

$$\frac{0.05}{\text{OD for 50 µg standard}} = \frac{\text{mg protein in sample}}{\text{OD for test sample}}$$

$$\text{mg protein in sample} = \frac{0.05\ (\text{OD for test sample})}{(\text{OD for 50 µg standard})}$$

D. Leukocyte Culture

Leukocytes used in this study were cultured according to a modified version of the method of Moorhead et al. (1960). For the multiple sclerosis patient study ten ml of venous blood were drawn from each patient. The blood was placed in sterile centrifuge tubes containing sodium heparin (5,000 USP units) (Organon) to prevent clotting and allowed to stand at room temperature for at least three hours to allow separation of the plasma and leukocytes from the red blood cells.

The medium for leukocyte culture was made as follows: To one 100 ml bottle of Medium 199 (Grand Island #115H) were added 20 ml fetal calf serum (Grand Island #614HI) and one ml of a penicillin (10,000 units) and streptomycin (10 mg) mixture (Grand Island). Ten ml of this medium were added to a sterile prescription bottle for each culture.

After separation from the red cells the plasma and leukocytes were separated by centrifugation. The plasma was retained for evaluation on cell cultures and the leukocytes were resuspended in two ml of fetal calf serum. Duplicate cultures were initiated by addition of one

ml of the fetal calf serum-leukocyte suspension to each of two prescription bottles containing ten ml of the medium described above. To each culture was added 0.1 ml pokeweed mitogen (Phytolacca americana, Grand Island) as a mitogenic agent. The cultures were incubated at 37 degrees C. for 48 hours and harvested.

To harvest, the cultures were removed from the incubator, swirled and decanted into a clean centrifuge tube. The cultures were centrifuged for five minutes at 78 x g so that a cell pellet was obtained. The fluid was poured off and the cells resuspended in the remaining fluid.

About two ml of McCoy's 5A medium with 20% fetal calf serum were added and the cells resuspended. The cell suspension was added to ten ml of McCoy's 5A medium with 20% fetal calf serum and divided equally between two fibroblast cultures which had been subcultured the day before. The cells were allowed to remain on the fibroblast cultures for approximately 40 hours at which time the medium was removed and replaced with medium containing tritiated thymidine for uptake studies.

E. Experimental Procedures

1. Multiple Sclerosis (MS)

Samples of blood were obtained from patients with MS in the MS clinic under direction of Dr. S.C. Little, University of Alabama in Birmingham. Dr. Little and Dr. J.D. Bancroft were most cooperative in allowing collection of these samples.

Heparinized ten ml blood samples were obtained from 12 patients with MS who did not appear to have any active progression of

the disease. The red blood cells were allowed to settle and the plasma and white blood cells were separated from them. On three patients the fresh plasma, cultured lymphocytes, and frozen plasma were evaluated for their effect on DNA synthesis in normal human skin fibroblasts and rat glial tumor cells in culture. The frozen plasma and cultured lymphocytes were evaluated on five patients.

The fibroblasts and rat glial tumor cells were subcultured as described above the day before test samples were to be added. The medium was removed from the cultures and fresh medium containing 20% test plasma or 20% serum was added to duplicate cultures for each patient, except in the case of fresh plasma where only one culture per patient was done. The leukocytes were separated from the plasma, re-suspended in fetal calf serum, cultured, and harvested as described previously. The cells were suspended in ten ml McCoy's 5A medium with 20% fetal calf serum and divided between two cultures for each patient.

The medium with the test material was allowed to remain on the cultures for approximately 40 hours. Control cultures in which fresh medium was added at the time of addition of the test material to the test cultures were done at the same time. After about 40 hours the medium was removed from the cultures, and each culture was washed once with Hank's balanced salt solution. Fresh medium containing 2 μCi/ml tritiated thymidine was added to each culture for four hours. After four hours the medium containing tritiated thymidine was removed, the cells harvested and tritiated thymidine uptake determined as described previously. Protein determinations were done as described and the counts per minute per mg protein were calculated.

2. Cystic Fibrosis (CF)

Blood samples were obtained from 16 children with cystic fibrosis in the CF clinic directed by Dr. Ralph Tiller, The Children's Hospital, Birmingham, Alabama. The blood was allowed to clot and the serum removed. Both fresh and frozen sera were evaluated. Fibroblast cultures used in this study were subcultured the day before addition of test serum as described. Medium was removed and fresh medium containing 20% CF serum was added. This medium plus test serum remained on the cultures about 40 hours when it was removed and the cultures washed once with Hank's balanced salt solution. Fresh medium containing two μCi/ml tritiated thymidine was added to each culture for four hours. The cells were then harvested and thymidine uptake determined as previously described. Protein determinations as described were done and counts per minute per mg protein calculated.

3. 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU)

The effects of BCNU on DNA synthesis in fibroblasts and rat glial tumor cells in culture were evaluated. The drug was added in four different doses (5, 10, 25, and 50 μg/ml) and at three different times after subculturing the cells (24, 48, and 72 hours). Source of the drug was a 100 mg vial (Cancer Chemotherapy, NCI, NIH). The drug was dissolved in one ml absolute ethanol and diluted to proper concentrations using Hank's balanced salt solution. The drug was added in the proper amount to medium and divided between duplicate cultures for each dose and time of addition. The final concentration of ethanol ranged from 0.005% for the lowest dose to 0.05% for the highest dose. The medium containing drug remained on the cultures for four hours when it was removed and replaced with medium containing two μCi/ml tritiated

thymidine. After four hours the medium containing tritiated thymidine was removed, cells harvested, and prepared for counting as described previously. Protein determinations were done and counts per minute per mg protein calculated.

Effects of BCNU on human glioma cells and fibroblasts in culture were observed and photographed. The glioma cells and fibroblasts were derived from glioma tissue as described previously. BCNU was added in four doses (10, 20, 50, and 100 μg/ml) to the cultures. Pictures of the same specific area of the culture were taken with a Leica camera through the eye-piece of a Zeiss inverted microscope. The cells were photographed before drug addition, 15 minutes, 30 minutes, 1, 6, 19, and 24 hours after BCNU was added. At 24 hours the medium was removed and fresh medium containing the same dose given the day before was added. Pictures were taken 1, 6, 18, and 24 hours after addition of the second dose.

CHAPTER III

RESULTS

Preliminary studies were done to determine the optimum time of addition of the material to be tested to the various cultures. It was decided to add the test substance 24 hours after subculture of the cells and to leave it on the cultures for approximately 40 hours before adding the medium containing tritiated thymidine. In all tests the medium containing the tritiated thymidine was allowed to remain on the cultures for four hours before harvesting and counting.

Studies were also done to determine the effects of fetal calf serum and human plasma on DNA synthesis in normal human skin fibroblasts and rat glial tumor cells. In Experiments A and B (Table I) varying amounts of fetal calf serum and plasma from one normal individual were added to McCoy's 5A medium and divided between duplicate fibroblast cultures. As described the medium was removed after about 40 hours and replaced with medium containing 2 μCi/ml tritiated thymidine. The cells were harvested after four hours and prepared for scintillation counting. Protein determinations were done and the CPM/mg protein calculated. Experiment A showed a two-fold increase when human plasma and fetal calf serum were added rather than fetal calf serum alone. Experiment B, however, showed similar values for both fetal calf serum alone and human plasma and fetal calf serum.

Table I

THE EFFECTS OF FETAL CALF SERUM AND FRESH HUMAN PLASMA ON DNA SYNTHESIS IN NORMAL HUMAN SKIN FIBROBLASTS*

		TRITIATED THYMIDINE UPTAKE (CPM x 10^5/MG PROTEIN)		
	Sample	Culture I	Culture II	
EXPERIMENT A				
20% Fetal calf serum	1	2.36	4.29	
	2	3.63	3.50	
	Average	2.99	3.89	3.44
20% Human plasma	1	4.48	7.26	
	2	6.27	6.80	
	Average	5.38	7.03	6.21
10% Fetal calf serum + 10% Human plasma	1	8.96	7.91	
	2	6.81	7.65	
	Average	7.88	7.78	7.83
20% Fetal calf serum + 20% Human plasma	1	7.36	7.26	
	2	7.64	9.05	
	Average	7.50	8.15	7.82
No serum	1	1.38	0.88	
	2	1.36	0.89	
	Average	1.37	0.89	1.13
EXPERIMENT B				
20% Fetal calf serum	1	3.28	3.09	
	2	3.41	3.05	
	Average	3.34	3.07	3.20
20% Fetal calf serum + 20% Human plasma	1	4.12	2.90	
	2	4.33	2.02	
	Average	4.22	2.46	3.34

*Initiated from normal male infant foreskin

Table II gives the results for the same experiment done with rat glial tumor cells. The tritiated thymidine uptake for fetal calf serum alone was only slightly higher than the tritiated thymidine uptake for fetal calf serum plus human plasma and human plasma alone.

A. Multiple Sclerosis (MS)

It has been shown that in some patients with multiple sclerosis (MS) a factor was present in the serum which caused demyelination of nerve fibers in cultures of nervous tissue derived from rats or mice (Raine et al., 1973; Kim et al., 1970; Dowling et al., 1968). It is unknown whether this factor causes demyelination by damaging the myelinating cells which are the oligodendrocytes in the central nervous system and Schwann cells in the peripheral nervous system or by directly damaging the myelin. In the present study it was decided to measure the effect of both fresh and frozen plasma and fresh serum from patients with MS on DNA synthesis in normal human skin fibroblasts and rat glial tumor cells. The uptake of tritiated thymidine expressed as counts per minute (CPM) per milligram protein was used as a measure of DNA synthesis. Duplicate counts were done on each culture and these values were averaged. Duplicate cultures were evaluated for each patient and the values for these averaged. Control cultures were done in the same manner for each group of patients and an average value for all control cultures was obtained. A ratio of the CPM/mg protein for the patient values versus the control value was calculated.

Table III shows the effects of fresh plasma from patients with MS on DNA synthesis in skin fibroblasts and Table IV A and B the

Table II

THE EFFECTS OF FETAL CALF SERUM AND HUMAN PLASMA ON DNA SYNTHESIS IN CULTURED RAT GLIAL TUMOR CELLS (CCL-107)*

		TRITIATED THYMIDINE UPTAKE (CPM x 10^5/MG PROTEIN)		
	Sample	Culture I	Culture II	
20% Fetal calf serum	1	14.40	15.19	
	2	16.11	15.61	
	Average	15.26	15.40	15.33
20% Human plasma	1	10.15	15.59	
	2	10.00	12.35	
	Average	10.08	13.97	12.02
10% Fetal calf serum + 10% Human plasma	1	13.82	12.25	
	2	12.80	10.64	
	Average	13.31	11.45	12.38
No serum	1	5.55	9.23	
	2	5.23	8.89	
	Average	5.39	9.06	7.23

*Obtained from the American Type Culture Collection

Table III

THE EFFECTS OF FRESH PLASMA FROM PATIENTS WITH MULTIPLE SCLEROSIS ON DNA SYNTHESIS IN CULTURED NORMAL HUMAN SKIN FIBROBLASTS*

Patient	Sample	TRITIATED THYMIDINE UPTAKE (CPM x 10^5/MG PROTEIN) Culture	Ratio (Patient/Control)
SH	1	6.84	
	2	8.25	$\frac{7.55}{11.82} = 0.64$
	Average	7.55	
MA	1	12.79	
	2	16.88	$\frac{14.83}{11.82} = 1.25$
	Average	14.83	
WO	1	13.98	
	2	10.83	$\frac{11.91}{11.82} = 1.01$
	Average	11.91	
CONTROLS**			
I	1	15.59	
	2	17.15	
	Average	16.37	
II	1	11.18	
	2	9.15	
	Average	10.16	
III	1	7.74	
	2	10.12	
	Average	8.93	
Average			11.82

*Initiated from normal male infant foreskin

**McCoy's 5A medium with 20% fetal calf serum

Table IV

THE EFFECTS OF FROZEN PLASMA FROM PATIENTS WITH MULTIPLE SCLEROSIS ON DNA SYNTHESIS IN CULTURED NORMAL HUMAN SKIN FIBROBLASTS*

Patient	Sample	TRITIATED THYMIDINE UPTAKE (CPM x 10^5/MG PROTEIN) Culture I	Culture II		Ratio (Patient/Control)
EXPERIMENT A					
SH	1	6.17	1.83		$\frac{4.12}{1.36} = 3.03$
	2	6.52	1.94		
	Average	6.34	1.89	4.12	
MA	1	23.36	Lost		$\frac{23.19}{1.36} = 17.09$
	2	23.01	sample		
	Average	23.19		23.19	
WO	1	0.51	1.08		$\frac{0.80}{1.36} = 0.59$
	2	0.52	1.10		
	Average	0.52	1.09	0.80	

CONTROLS**		
I	1	1.33
	2	1.42
	Average	1.37
II	1	1.57
	2	1.65
	Average	1.61
III	1	1.96
	2	1.99
	Average	1.98
IV	1	0.43
	2	0.42
	Average	0.42
V	1	2.04
	2	2.06
	Average	2.05
VI	1	0.69
	2	0.71
	Average	0.70
Average		1.36

*Initiated from normal male infant foreskin

**McCoy's 5A medium with 20% fetal calf serum

Table IV (continued)

Patient	Sample	TRITIATED THYMIDINE UPTAKE (CPM x 10^5/MG PROTEIN) Culture I	Culture II		Ratio (Patient/Control)
EXPERIMENT B					
GO	1	9.23	10.45		$\frac{9.75}{5.45} = 1.79$
	2	9.02	10.31		
	Average	9.12	10.38	9.75	
MU	1	14.72	1.22		$\frac{7.81}{5.45} = 1.43$
	2	14.14	1.14		
	Average	14.43	1.18	7.81	
MC	1	17.34	13.75		$\frac{15.49}{5.45} = 2.84$
	2	17.44	13.41		
	Average	17.39	13.58	15.49	

CONTROLS**			
I	1	11.32	
	2	12.15	
	Average	11.73	
II	1	3.47	
	2	3.75	
	Average	3.61	
III	1	7.71	
	2	7.09	
	Average	7.40	
IV	1	3.65	
	2	3.77	
	Average	3.71	
V	1	2.86	
	2	3.02	
	Average	2.94	
VI	1	3.13	
	2	3.54	
	Average	3.33	
Average			5.45

*Initiated from normal male infant foreskin

**McCoy's 5A medium with 20% fetal calf serum

effects of frozen plasma. In Table IV Experiment A plasma from patient MA seemed to stimulate tritiated thymidine uptake. Fresh plasma from the same patient (Table III) showed only a very slight stimulation of tritiated thymidine uptake. Plasma from five of six patients showed stimulation of tritiated thymidine uptake when added to fibroblast cultures (Table IV) whereas plasma from eight of nine patients showed inhibition of tritiated thymidine uptake when added to rat glial tumor cells in culture (Table V). Table VI gives a summary of the effects of frozen plasma from patients with MS on rat glial tumor cells and normal human skin fibroblasts. The results of the effects of fresh serum from MS patients on DNA synthesis in rat glial tumor cells are shown in Table VII. A summary is given in Table VIII. None of the patients showed an extreme effect on the tritiated thymidine uptake in these cells.

Multiple sclerosis is considered by some investigators perhaps to be an autoimmune disease. Because of this fact it was decided to look at the effects of lymphocytes obtained from patients with MS and cultured using pokeweed to stimulate mitosis on DNA synthesis in normal human skin fibroblasts and rat glial tumor cells in culture. Pokeweed was chosen as the mitogen since it stimulates both thymus and bursa derived lymphocytes. The lymphocytes were cultured for 48 hours, harvested and added to the fibroblast and rat glial tumor cultures. The procedure from that point was identical to that used for evaluation of serum and plasma. Tables IX and X give the results of these studies. Lymphocytes from all patients except one showed stimulation of tritiated thymidine uptake by fibroblasts and rat glial tumor cells. The results are summarized in Table XI.

Table V

THE EFFECTS OF FROZEN PLASMA FROM PATIENTS WITH MULTIPLE SCLEROSIS ON DNA SYNTHESIS IN THE CULTURED RAT GLIAL TUMOR CELLS (CCL-107)*

Patient	Sample	TRITIATED THYMIDINE UPTAKE (CPM x 10^5/MG PROTEIN) Culture I	Culture II		Ratio (Patient/Control)
EXPERIMENT A					
GO	1	1.89	1.63		$\frac{1.77}{1.81} = 0.98$
	2	2.01	1.54		
	Average	1.95	1.58	1.77	
MU	1	2.79	2.67		$\frac{2.70}{1.81} = 1.50$
	2	2.89	2.47		
	Average	2.84	2.57	2.70	
MC	1	1.31	1.32		$\frac{1.45}{1.81} = 0.80$
	2	1.53	1.65		
	Average	1.42	1.48	1.45	

CONTROLS**		
I	1	2.07
	2	1.74
	Average	1.91
II	1	1.32
	2	1.33
	Average	1.32
III	1	3.12
	2	2.31
	Average	2.21
IV	1	2.31
	2	2.62
	Average	2.47
V	1	1.61
	2	1.49
	Average	1.55
VI	1	1.36
	2	1.39
	Average	1.37
Average		1.81

*Obtained from American Type Culture Collection

**McCoy's 5A medium with 20% fetal calf serum

Table V (continued)

Patient	Sample	TRITIATED THYMIDINE UPTAKE (CPM x 10^5/MG PROTEIN) Culture I	Culture II		Ratio (Patient/Control)
EXPERIMENT B					
CA	1	9.23	6.83		$\frac{8.18}{11.94} = 0.69$
	2	9.92	6.75		
	Average	9.58	6.79	8.18	
BE	1	8.13	8.14		$\frac{8.02}{11.94} = 0.67$
	2	7.44	8.38		
	Average	7.78	8.26	8.02	
VI	1	8.35	10.39		$\frac{9.51}{11.94} = 0.80$
	2	9.10	10.18		
	Average	8.73	10.28	9.51	
RE	1	7.29	8.79		$\frac{8.03}{11.94} = 0.67$
	2	7.30	8.74		
	Average	7.30	8.76	8.03	
MI	1	10.63	8.24		$\frac{9.89}{11.94} = 0.83$
	2	11.56	9.14		
	Average	11.10	8.69	9.89	

JE	1	10.63	10.50		$\frac{10.22}{11.94} = 0.86$
	2	10.20	9.53		
	Average	10.42	10.02	10.22	
CONTROLS**					
I	1	8.14			
	2	8.45			
	Average	8.29			
II	1	16.81			
	2	14.37			
	Average	15.59			
Average				11.94	

*Obtained from American Type Culture Collection

**McCoy's 5A medium with 20% fetal calf serum

Table VI

SUMMARY OF THE EFFECTS OF FROZEN PLASMA FROM PATIENTS WITH MULTIPLE SCLEROSIS ON DNA SYNTHESIS IN NORMAL HUMAN SKIN FIBROBLASTS AND RAT GLIAL TUMOR CELLS (CCL-107)*

	TRITIATED THYMIDINE UPTAKE Patient/Control Ratios	
Patient	Fibroblasts	Rat Glial Tumor Cells
SH	3.03	
MA	17.09	
WO	0.59	
GO	1.79	0.98
MU	1.43	1.50
MC	2.84	0.80
CA		0.69
BE		0.67
VI		0.80
RE		0.67
MI		0.83
JE		0.86

*Skin fibroblasts initiated from normal male infant foreskin and rat glial tumor cells obtained from American Type Culture Collection

Table VII

THE EFFECTS OF FRESH SERUM FROM PATIENTS WITH MULTIPLE SCLEROSIS ON DNA SYNTHESIS IN CULTURED RAT GLIAL TUMOR CELLS (CCL-107)*

Patient	Sample	TRITIATED THYMIDINE UPTAKE (CPM x 10^5/MG PROTEIN) Culture I	Culture II		Ratio (Patient/Control)
EXPERIMENT A					
BR	1	4.40	5.61		$\frac{4.87}{3.17} = 1.54$
	2	4.36	5.12		
	Average	4.38	5.37	4.87	$\frac{4.87}{2.53} = 1.93$
FL	1	4.56	4.71		$\frac{4.77}{3.17} = 1.51$
	2	4.91	4.90		
	Average	4.73	4.80	4.77	$\frac{4.77}{2.53} = 1.88$
WH	1	2.72	2.81		$\frac{2.82}{3.17} = 0.89$
	2	2.78	2.97		
	Average	2.75	2.89	2.82	$\frac{2.82}{2.53} = 1.11$
JU	1	4.48	4.29		$\frac{4.46}{3.17} = 1.47$
	2	5.35	4.54		
	Average	4.91	4.41	4.46	$\frac{4.46}{2.53} = 1.76$

CONTROLS

Control A**			
I	1	3.73	
	2	3.59	
	Average	3.66	
II	1	2.85	
	2	2.70	
	Average	2.67	
Average			3.17
Control B***			
I	1	2.64	
	2	2.75	
	Average	2.70	
II	1	2.55	
	2	2.17	
	Average	2.36	
Average			2.53

*Obtained from American Type Culture Collection

**McCoy's 5A medium with 20% fetal calf serum

***McCoy's 5A medium with 20% fetal calf serum and 20% fresh human serum

Table VII (continued)

Patient	Sample	TRITIATED THYMIDINE UPTAKE (CPM x 10^5/MG PROTEIN) Culture I	Culture II		Ratio (Patient/Control)
EXPERIMENT B					
CO	1	8.30	8.84		$\frac{8.45}{7.91} = 1.07$
	2	8.53	8.12		
	Average	8.42	8.48	8.45	
FA	1	9.62	6.18		$\frac{7.07}{7.91} = 0.97$
	2	9.01	6.00		
	Average	9.31	6.09	7.70	

CONTROLS**			
I	1	7.93	
	2	6.96	
	Average	7.44	
II	1	8.52	
	2	8.24	
	Average	8.38	
Average			7.91

*Obtained from American Type Culture Collection

**McCoy's 5A medium with 20% fetal calf serum and 20% fresh human serum

Table VIII

SUMMARY OF THE EFFECTS OF FRESH SERUM FROM PATIENTS WITH MULTIPLE SCLEROSIS ON DNA SYNTHESIS IN CULTURED RAT GLIAL TUMOR CELLS (CCL-107)*

	TRITIATED THYMIDINE UPTAKE Patient/Control Ratios	
Patient	20% Fetal Calf Serum	20% Fetal Calf Serum + 20% Human Serum
BR	1.54	1.93
FL	1.51	1.88
WH	0.89	1.11
JU	1.47	1.76
CO		1.07
FA		0.97

*Obtained from the American Type Culture Collection

Table IX

THE EFFECTS OF CULTURED LYMPHOCYTES FROM PATIENTS WITH MULTIPLE SCLEROSIS ON DNA SYNTHESIS IN CULTURED NORMAL HUMAN SKIN FIBROBLASTS*

Patient	Sample	TRITIATED THYMIDINE UPTAKE (CPM x 10^5/MG PROTEIN) Culture I	Culture II		Ratio (Patient/Control)
EXPERIMENT A					
SH	1	2.53	2.84		$\frac{2.71}{1.36} = 2.00$
	2	2.59	2.88		
	Average	2.56	2.84	2.71	
WO	1	6.64	4.61		$\frac{5.73}{1.36} = 4.22$
	2	6.88	4.79		
	Average	6.76	4.70	5.73	
MA	1	6.98	7.82		$\frac{7.45}{1.36} = 5.49$
	2	7.17	7.81		
	Average	7.08	7.81	7.45	

CONTROLS**			
I		1	1.33
		2	1.42
		Average	1.37
II		1	1.51
		2	1.65
		Average	1.61
III		1	1.96
		2	1.99
		Average	1.98
IV		1	0.43
		2	0.42
		Average	0.43
V		1	2.04
		2	2.06
		Average	2.05
VI		1	0.69
		2	0.71
		Average	0.70
Average			1.36

*Initiated from normal male infant foreskin

**McCoy's 5A medium with 20% fetal calf serum

Table IX (continued)

Patient	Sample	TRITIATED THYMIDINE UPTAKE (CPM x 10^5/MG PROTEIN) Culture I	Culture II		Ratio (Patient/Control)
EXPERIMENT B					
GO	1	4.90	6.27		$\frac{5.65}{5.45} = 1.03$
	2	4.99	6.43		
	Average	4.94	6.35	5.65	
MU	1	6.94	7.16		$\frac{7.06}{5.45} = 1.29$
	2	7.07	7.05		
	Average	7.01	7.10	7.06	
MC	1	4.72	3.79		$\frac{4.23}{5.45} = 0.77$
	2	5.54	3.84		
	Average	4.63	3.82	4.23	

CONTROLS**

I	1	11.32
	2	12.15
	Average	11.73
II	1	3.47
	2	3.75
	Average	3.61
III	1	7.71
	2	7.09
	Average	7.40
IV	1	3.65
	2	3.77
	Average	3.71
V	1	2.86
	2	3.02
	Average	2.94
VI	1	3.13
	2	3.54
	Average	3.33
Average		5.45

*Initiated from normal male infant foreskin

**McCoy's 5A medium with 20% fetal calf serum

Table X

THE EFFECTS OF CULTURED LYMPHOCYTES FROM PATIENTS WITH MULTIPLE SCLEROSIS ON DNA SYNTHESIS IN CULTURED RAT GLIAL TUMOR CELLS (CCL-107)*

Patient	Sample	TRITIATED THYMIDINE UPTAKE (CPM x 10^5/MG PROTEIN) Culture I	Culture II		Ratio (Patient/Control)
GO	1	3.82	3.37		$\frac{3.52}{1.81} = 1.95$
	2	3.58	3.30		
	Average	3.70	3.34	3.52	
MU	1	3.39	1.67		$\frac{2.54}{1.81} = 1.41$
	2	3.57	1.54		
	Average	3.48	1.61	2.54	
MC	1	3.05	1.96		$\frac{2.44}{1.81} = 1.35$
	2	2.43	2.33		
	Average	2.74	2.15	2.44	

CONTROLS**			
I	1	2.07	
	2	1.74	
	Average	1.91	
II	1	1.32	
	2	1.33	
	Average	1.32	
III	1	2.12	
	2	2.31	
	Average	2.21	
IV	1	2.31	
	2	2.62	
	Average	2.47	
V	1	1.61	
	2	1.49	
	Average	1.55	
VI	1	1.36	
	2	1.39	
	Average	1.37	
Average			1.81

*Obtained from American Type Culture Collection

**McCoy's 5A medium with 20% fetal calf serum

Table XI

SUMMARY OF THE EFFECTS OF CULTURED LYMPHOCYTES FROM PATIENTS WITH MULTIPLE SCLEROSIS ON DNA SYNTHESIS IN NORMAL HUMAN SKIN FIBROBLASTS AND RAT GLIAL TUMOR CELLS (CCL-107)*

	TRITIATED THYMIDINE UPTAKE Patient/Control Ratio	
Patient	Fibroblasts	Rat glial tumor cells
SH	2.00	
WO	4.22	
MA	5.49	
GO	1.03	1.95
MU	1.29	1.41
MC	0.77	1.35

*Skin fibroblasts initiated from normal male infant foreskin and rat glial tumor cells obtained from American Type Culture Collection

B. Cystic Fibrosis (CF)

Serum from patients with cystic fibrosis was shown to contain a factor which caused dyskinesis in rabbit trachea or oyster cilia. Research in the area of identification of this factor was reviewed in the introduction. The present study evaluated the effects of both fresh and frozen sera from patients with CF on DNA synthesis in normal human fibroblasts. The effect was measured by uptake of tritiated thymidine expressed as CPM/mg protein. Duplicate samples were counted from each culture and the values averaged. Duplicate cultures were done for each patient and these values averaged. Control cultures were done at the same time and identical procedures used. The average for each patient was compared to the average of the controls.

Table XII shows the results of evaluation of fresh serum from CF patients. Of 12 patients only one (LA in Table VIIA) showed high values for tritiated thymidine uptake. Table XIII gives the results of the effects of frozen serum from patients with CF on DNA synthesis. Of seven patients, three (Table XIII-Experiment A) show a high value for tritiated thymidine uptake and only one (SP - Table XIII-Experiment B) shows an inhibition. Table XIV summarizes the effects of fresh and frozen serum from patients with CF on DNA synthesis in normal human skin fibroblasts.

C. 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU)

BCNU is an anti-tumor agent with properties which allow it to pass the blood-brain barrier. It has been used clinically to treat brain tumors such as gliomas. The present study compares the effects of BCNU on DNA synthesis in normal human fibroblasts and rat glial

Table XII

THE EFFECTS OF FRESH SERUM FROM PATIENTS WITH CYSTIC FIBROSIS ON DNA SYNTHESIS IN CULTURED NORMAL HUMAN SKIN FIBROBLASTS*

Patient	Sample	TRITIATED THYMIDINE UPTAKE (CPM x 10^5/MG PROTEIN) Culture I	Culture II		Ratio (Patient/Control)
EXPERIMENT A					
LA	1	4.90	4.42		$\frac{4.68}{0.88} = 5.32$
	2	4.91	4.48		
	Average	4.91	4.45	4.68	
MC	1	2.33	1.61		$\frac{1.98}{2.18} = 0.91$
	2	2.28	1.69		
	Average	2.31	1.65	1.98	
EB	1	2.38	6.25		$\frac{4.39}{2.18} = 2.02$
	2	2.48	6.44		
	Average	2.43	6.34	4.39	

CONTROLS**

For LA

I	1	0.86	
	2	0.82	
	Average	0.84	
II	1	0.94	
	2	0.90	
	Average	0.92	
Average			0.88

For MC and EB

I	1	1.14	
	2	1.16	
	Average	1.15	
II	1	3.14	
	2	3.26	
	Average	3.20	
Average			2.18

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*Initiated from normal male infant foreskin

**McCoy's 5A medium with 20% fetal calf serum

Table XII (continued)

Patient	Sample	TRITIATED THYMIDINE UPTAKE (CPM x 10^5/MG PROTEIN) Culture I	Culture II		Ratio (Patient/Control)
EXPERIMENT B					
WH	1	3.86	3.56		$\frac{3.84}{2.28} = 1.68$
	2	4.08	3.86		
	Average	3.97	3.71	3.83	
WI	1	5.31	5.70		$\frac{5.69}{2.28} = 2.50$
	2	5.94	5.82		
	Average	5.62	5.76	5.69	
FO	1	1.43	1.51		$\frac{1.46}{2.28} = 0.64$
	2	1.36	1.54		
	Average	1.40	1.53	1.46	

CONTROLS**			
I	1	2.10	
	2	2.20	
	Average	2.15	
II	1	2.63	
	2	2.20	
	Average	2.41	
Average			2.28

*Initiated from normal male infant foreskin

**McCoy's 5A medium with 20% fetal calf serum and 20% fresh human serum

Table XII (continued)

Patient	Sample	TRITIATED THYMIDINE UPTAKE (CPM x 10^5/MG PROTEIN) Culture I	Culture II		Ratio (Patient/Control)
EXPERIMENT C					
DA	1	5.00	6.01		$\frac{6.08}{6.17} = 0.98$
	2	6.55	6.74		
	Average	5.77	6.38	6.08	
EN	1	6.34	7.84		$\frac{7.30}{6.17} = 1.18$
	2	6.73	8.30		
	Average	6.53	8.07	7.30	
MA	1	8.29	7.30		$\frac{7.99}{6.17} = 1.29$
	2	8.60	7.75		
	Average	8.44	7.53	7.99	

CONTROLS**			
I	1	6.21	
	2	6.36	
	Average	6.29	
II	1	6.06	
	2	6.07	
	Average	6.06	
Average			6.17

*Initiated from normal male infant foreskin

**McCoy's 5A medium with 20% fetal calf serum and 20% fresh human serum

Table XII (Continued)

Patient	Sample	TRITIATED THYMIDINE UPTAKE (CPM x 10^5/MG PROTEIN) Culture I	Culture II		Ratio (Patient/Control)
EXPERIMENT D					
PU	1	2.71	2.84		$\frac{2.76}{5.77} = 0.48$
	2	2.81	2.66		
	Average	2.76	2.75	2.76	
GA	1	7.21	2.40		$\frac{4.76}{5.77} = 0.82$
	2	6.94	2.50		
	Average	7.08	2.45	4.76	
SM	1	13.05	12.62		$\frac{12.84}{8.84} = 1.45$
	2	13.66	12.02		
	Average	13.35	12.32	12.84	

CONTROLS**

For PU and GA

I	1	5.91	
	2	6.45	
	Average	6.19	
II	1	5.55	
	2	5.17	
	Average	5.36	
Average			5.77

For SM

I	1	10.29	
	2	10.90	
	Average	10.60	
II	1	6.85	
	2	7.36	
	Average	7.08	
Average			8.84

*Initiated from normal male infant foreskin

**McCoy's 5A medium with 20% fetal calf serum and 20% fresh human serum

Table XIII

THE EFFECTS OF FROZEN SERUM FROM PATIENTS WITH CYSTIC FIBROSIS ON DNA SYNTHESIS IN CULTURED HUMAN SKIN FIBROBLASTS*

Patient	Sample	TRITIATED THYMIDINE UPTAKE (CPM x 10^5/MG PROTEIN) Culture I	Culture II		Ratio (Patient/Control)
EXPERIMENT A					
MC	1	9.79	11.94		$\frac{10.51}{16.11} = 6.52$
	2	9.20	11.11		
	Average	9.49	11.52	10.51	
EB	1	12.65	13.28		$\frac{13.05}{1.61} = 8.10$
	2	13.13	13.12		
	Average	12.89	13.20	13.05	
GB	1	9.97	10.12		$\frac{9.79}{1.61} = 6.07$
	2	9.48	9.57		
	Average	9.73	9.84	9.79	

CONTROLS**			
I	1	2.09	
	2	2.21	
	Average	2.15	
II	1	1.04	
	2	1.10	
	Average	1.07	
Average			1.61

*Initiated from normal male infant foreskin

**McCoy's 5A medium with 20% fetal calf serum

Table XIII (continued)

Patient	Sample	TRITIATED THYMIDINE UPTAKE (CPM x 10^5/MG PROTEIN) Culture I	Culture II		Ratio (Patient/Control)
EXPERIMENT B					
SP	1	2.07	2.11		$\frac{2.10}{6.17} = 0.34$
	2	2.13	2.09		
	Average	2.10	2.10	2.10	
FO	1	8.73	9.21		$\frac{9.26}{8.84} = 1.05$
	2	9.74	9.36		
	Average	9.24	9.28	9.26	
JB	1	3.49	3.14		$\frac{3.40}{2.28} = 1.49$
	2	3.65	3.31		
	Average	3.57	3.23	3.40	
GB	1	1.92	3.29		$\frac{2.54}{2.28} = 1.12$
	2	2.00	2.97		
	Average	1.96	3.13	2.54	

CONTROLS**			
For SP			
I	1	6.21	
	2	6.36	
	Average	6.29	
II	1	6.06	
	2	6.07	
	Average	6.06	
Average			6.17
For FO			
I	1	10.29	
	2	10.90	
	Average	10.60	
II	1	6.85	
	2	7.36	
	Average	7.08	
Average			8.84
For JB and GB			
I	1	2.10	
	2	2.20	
	Average	2.15	
II	1	2.63	
	2	2.20	
	Average	2.41	
Average			2.28

*Initiated from normal male infant foreskin
**McCoy's 5A medium with 20% fetal calf serum and 20% human frozen serum

Table XIV

SUMMARY OF THE EFFECTS OF FRESH AND FROZEN SERUM FROM PATIENTS WITH CYSTIC FIBROSIS ON DNA SYNTHESIS IN HUMAN SKIN FIBROBLASTS*

	TRITIATED THYMIDINE UPTAKE Patient/Control Ratio	
Patient	Fresh	Frozen
LA	5.32	
MC	0.91	6.52
EG	2.02	8.10
WH	1.68	
WI	2.50	
FO	0.64	1.05
DA	0.98	
EN	1.18	
MA	1.29	
PU	0.48	
GA	0.82	
SM	1.45	
GB		6.07 1.12
JB		1.49
SP		0.34

*Initiated from normal male infant foreskin

tumor cells in culture. The effect on DNA synthesis was measured by uptake of tritiated thymidine expressed as CPM/mg protein. BCNU was added to duplicate cultures in four different doses and at three different times after the cells were subcultured. Only one count was done on each culture but values for duplicate cultures were averaged. Control cultures were done at the same time. The inhibition expressed as a per cent was calculated as follows:

$$\frac{\text{(Average control value-Average test value)}}{\text{(Average control value)}} \times 100 = \% \text{ Inhibition}$$

Table XV shows the tritiated thymidine uptake for fibroblasts when BCNU was added 24 hours after subculturing and Table XVI shows the same for rat glial tumor cells. The inhibition of DNA synthesis for the fibroblasts was greater at each dose of BCNU given than inhibition in the rat glial tumor cells. When BCNU was added at 48 hours after subculturing the results showed greater inhibition in the rat glial tumor cells than in the fibroblasts (Tables XVII and XVIII). Tables XIX and XX give the effects on DNA synthesis when BCNU was added 72 hours after subculturing. At the highest dose the fibroblasts appeared more sensitive to the drug than the rat glial tumor cells.

Figures 2-4 show the comparison between fibroblasts and rat glial tumor cells of the uptake of tritiated thymidine after treatment of the cells with BCNU at various times after subculturing the cells.

BCNU was also added to cultures containing human glioma cells and fibroblasts. The effects on these cells were observed and photographed. The drug was added to duplicate cultures in four different doses (10, 20, 50, and 100 µg/ml.). Photographs were made

Table XV

THE EFFECTS OF 1,3-BIS(2-CHLOROETHYL)-1-NITROSOUREA ON CULTURED NORMAL HUMAN SKIN FIBROBLASTS*
(Drug added 24 hours after subculturing)

Dose (µg/ml)	TRITIATED THYMIDINE UPTAKE (CPM x 10^5/MG PROTEIN) Culture I	Culture II	Average	Inhibition (%)
0	3.35	3.09	3.22	
5	2.31	2.90	2.60	19.2
10	3.08	3.25	3.16	1.8
25	0.93	0.97	0.95	70.1
50	0.21	0.25	0.23	92.9

*Initiated from normal male foreskin

Table XVI

THE EFFECTS OF 1,3-BIS(2-CHLOROETHYL)-1-NITROSOUREA ON CULTURED RAT GLIAL TUMOR CELLS (CCL-107)*
(Drug added 24 hours after subculturing)

Dose (μg/ml)	TRITIATED THYMIDINE UPTAKE (CPM x 10^5/MG PROTEIN) Culture I	Culture II	Average	Inhibition (%)
0	12.03	9.04	10.54	
5	9.71	8.88	9.79	7.1
10	9.39	9.60	9.49	9.9
25	5.81	7.33	6.57	37.7
50	1.70	1.64	1.67	84.2

*Obtained from American Type Culture Collection

Table XVII

THE EFFECTS OF 1,3-BIS(2-CHLOROETHYL)-1-NITROSOUREA ON CULTURED NORMAL HUMAN SKIN FIBROBLASTS*
(Drug added 48 hours after subculturing)

Dose (µg/ml)	TRITIATED THYMIDINE UPTAKE (CPM x 10^5/MG PROTEIN) Culture I	Culture II	Average	Inhibition (%)
0	1.82	1.98	1.90	
5	1.81	4.33	3.07	0
10	1.49	1.31	1.40	26.6
25	0.92	2.43	1.68	11.9
50	0.18	1.11	0.64	66.0

*Initiated from normal male infant foreskin

Table XVIII

THE EFFECTS OF 1,3-BIS(2-CHLOROETHYL)-1-NITROSOUREA ON CULTURED RAT GLIAL TUMOR CELLS (CCL-107)*
(Drug added 48 hours after subculturing)

Dose (μg/ml)	TRITIATED THYMIDINE UPTAKE (CPM x 10^5/MG PROTEIN) Culture I	Culture II	Average	Inhibition (%)
0	9.78	8.55	9.17	
5	4.94	6.53	5.73	37.5
10	6.89	4.99	5.94	35.2
25	5.42	8.06	6.74	26.5
50	3.54	2.91	3.23	64.8

*Obtained from American Type Culture Collection

Table XIX

THE EFFECTS OF 1,3-BIS(2-CHLOROETHYL)-1-NITROSOUREA ON CULTURED NORMAL HUMAN SKIN FIBROBLASTS*
(Drug added 72 hours after subculturing)

Dose (μg/ml)	TRITIATED THYMIDINE UPTAKE (CPM x 10^5/MG PROTEIN) Culture I	Culture II	Average	Inhibition (%)
0	0.66	0.54	0.60	
5	0.43	0.41	0.42	30.2
10	0.34	0.31	0.32	46.2
25	0.15	0.15	0.15	75.3
50	0.03	0.03	0.03	94.6

*Initiated from normal male infant foreskin

Table XX

THE EFFECTS OF 1,3-BIS(2-CHLOROETHYL)-1-NITROSOUREA ON CULTURED RAT GLIAL TUMOR CELLS (CCL-107)*
(Drug added 72 hours after subculturing)

Dose (μg/ml)	TRITIATED THYMIDINE UPTAKE (CPM x 10^5/MG PROTEIN) Culture I	Culture II	Average	Inhibition (%)
0	3.82	4.88	4.35	
5	4.09	2.90	3.50	19.7
10	3.56	2.39	2.97	31.7
25	2.09	2.44	2.26	48.0
50	1.36	1.10	1.23	71.7

*Obtained from American Type Culture Collection

Figure 2. The effects of 1,3-bis(2-chloroethyl)-1-nitrosourea on DNA synthesis in cultured fibroblasts and rat glial tumor cells. (Drug added 24 hours after subculturing)

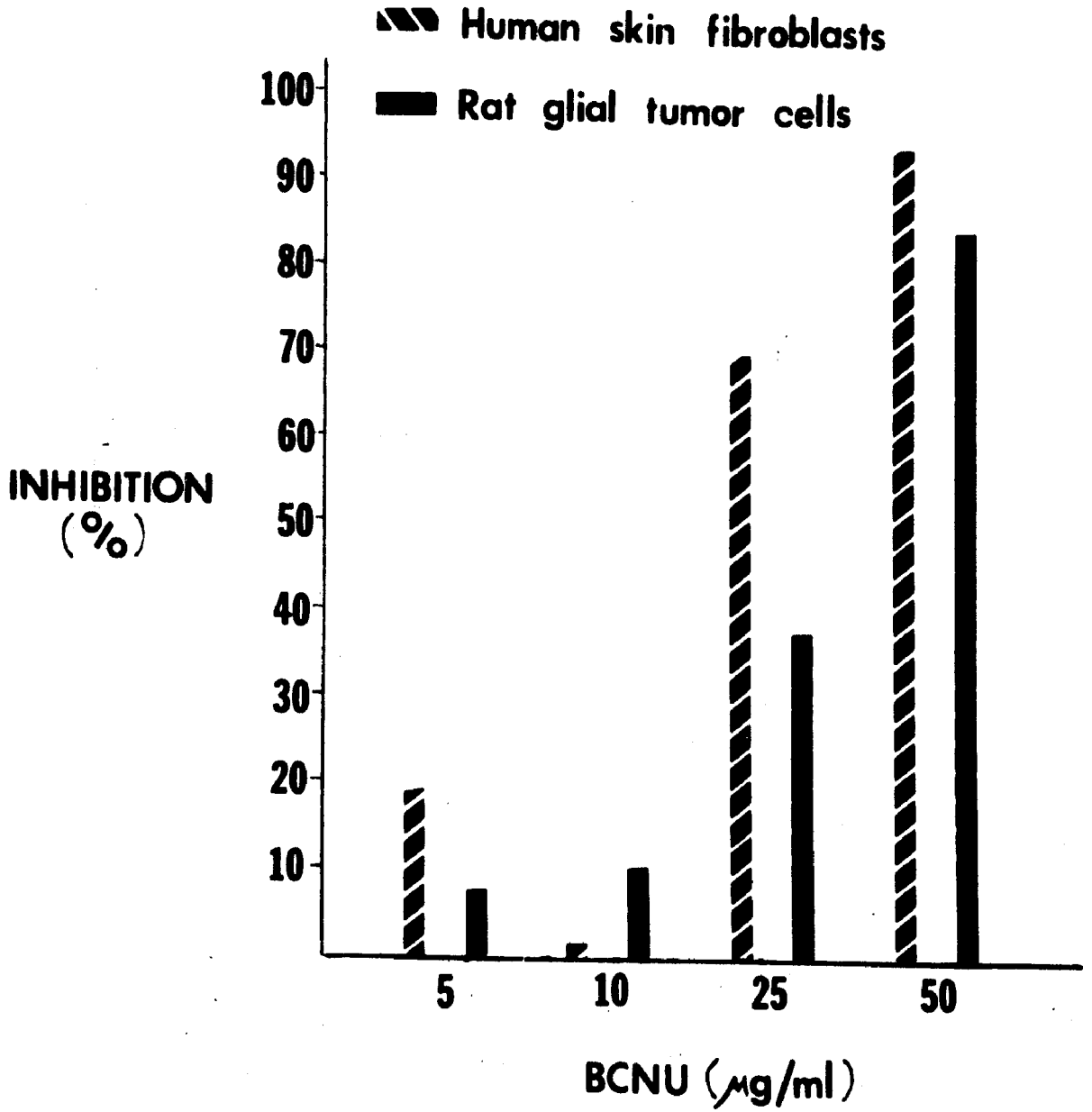

Human skin fibroblasts
Rat glial tumor cells
INHIBITION (%)
100
90
80
70
60
50
40
30
20
10
5
10
25
50
BCNU (μg/ml)

Figure 3. The effects of 1,3-bis(2-chloroethyl)-1-nitrosourea on DNA synthesis in cultured fibroblasts and rat glial tumor cells. (Drug added 48 hours after subculturing)

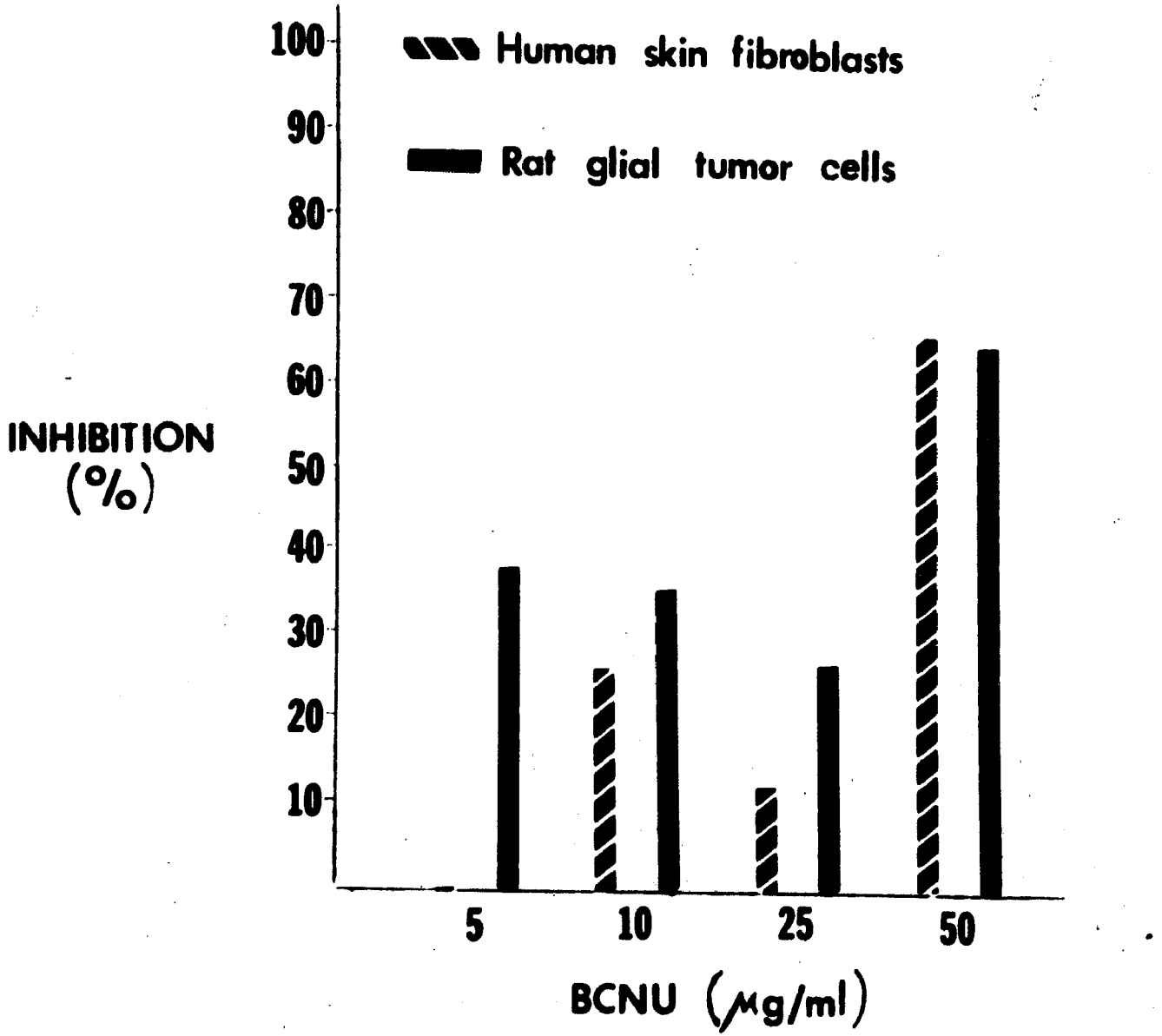
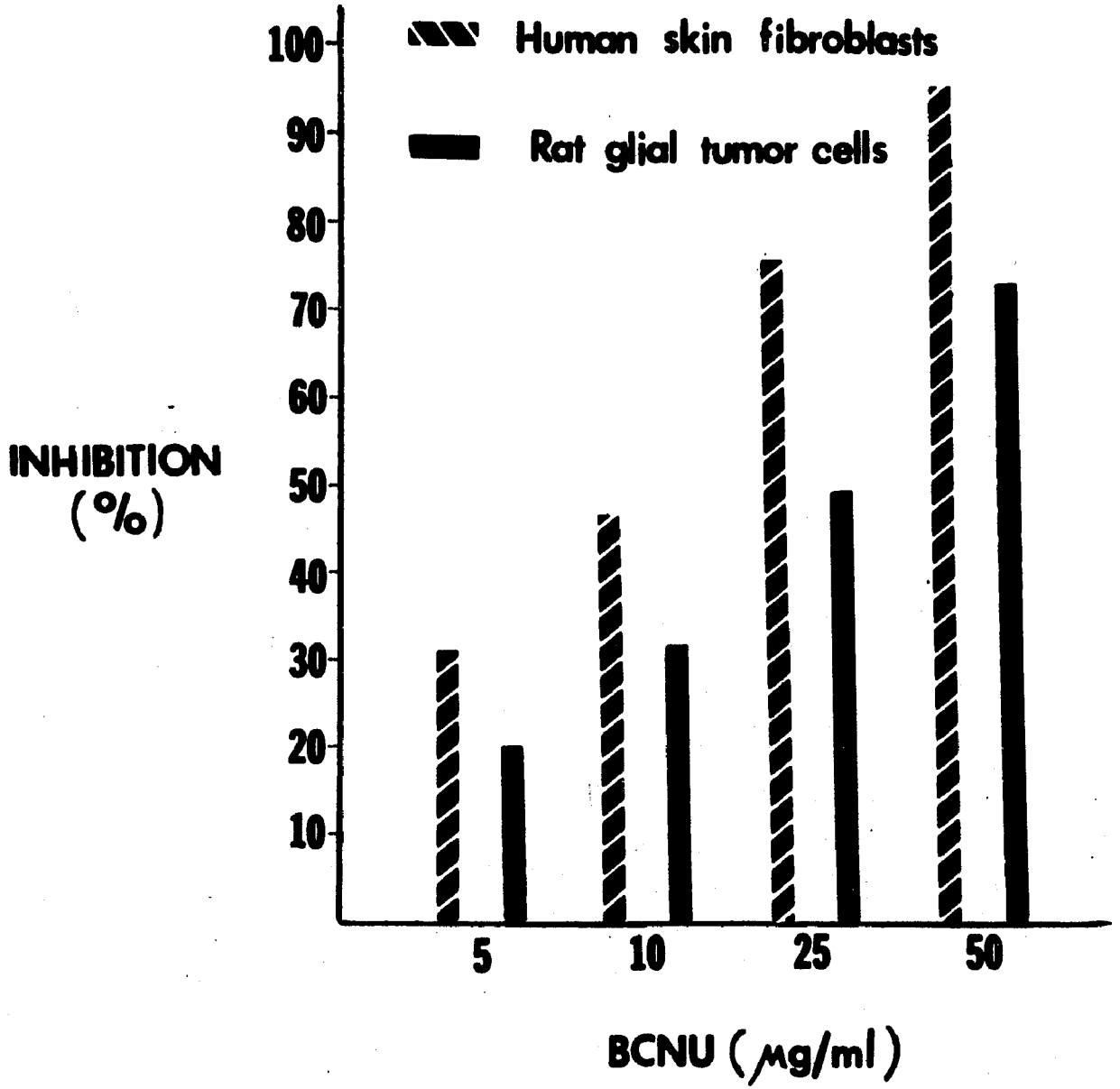
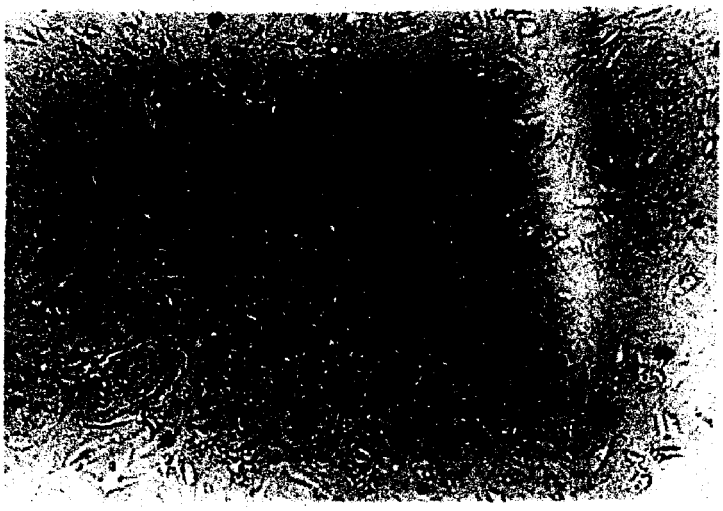

Human skin fibroblasts
Rat glial tumor cells
INHIBITION (%)
100
90
80
70
60
50
40
30
20
10
5
10
25
50
BCNU (μg/ml)

Figure 4. The effects of 1,3-bis(2-chloroethyl)-1-nitrosourea on DNA synthesis in cultured fibroblasts and rat glial tumor cells. (Drug added 72 hours after subculturing)


Human skin fibroblasts
Rat glial tumor cells
INHIBITION (%)
100
90
80
70
60
50
40
30
20
10
5
10
25
50
BCNU (μg/ml)

before drug addition and after 15 minutes, 30 minutes, 1, 6, 19, and 24 hours. After 24 hours an additional dose of BCNU was given in the same amount as the first dose. Pictures were taken at 1, 6, 18, and 24 hours after the second dose. Figures 5-8 show the pictures taken of one culture for each dose before drug addition and 24 hours after the second dose. An effect is not observed for the lowest drug dose (Figure 5) but all other drug doses show changes in the cell morphology (Figures 6-8).

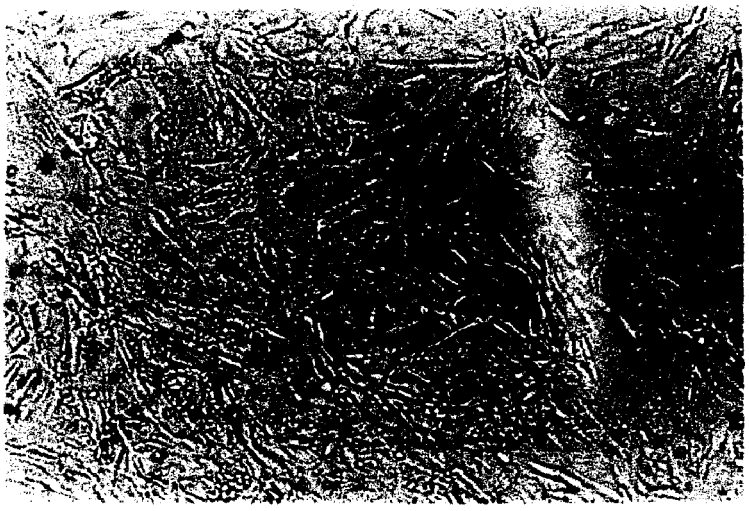
Figure 5. The effects of 10 μg/ml of 1,3-bis(2-chloroethyl)-1-nitrosourea on human glioma cells and fibroblasts in culture.



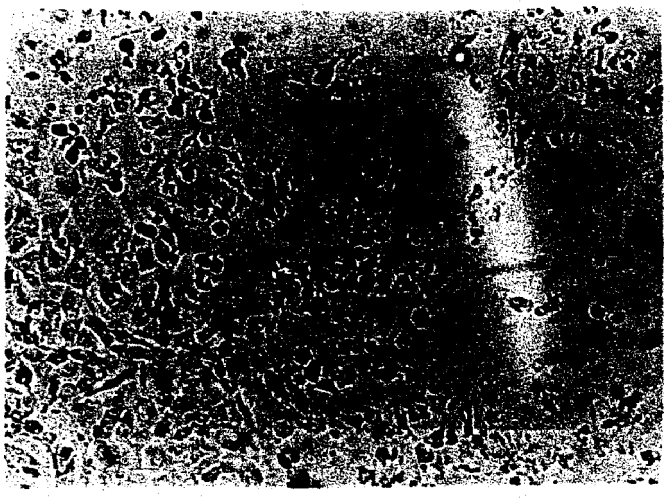
Cells Before Addition of Drug

Cells 24 Hours After Addition of the Second Dose of Drug

Figure 6. The effects of 20 μg/ml of 1,3-bis(2-chloroethyl)-1-nitrosourea on human glioma cells and fibroblasts in culture.

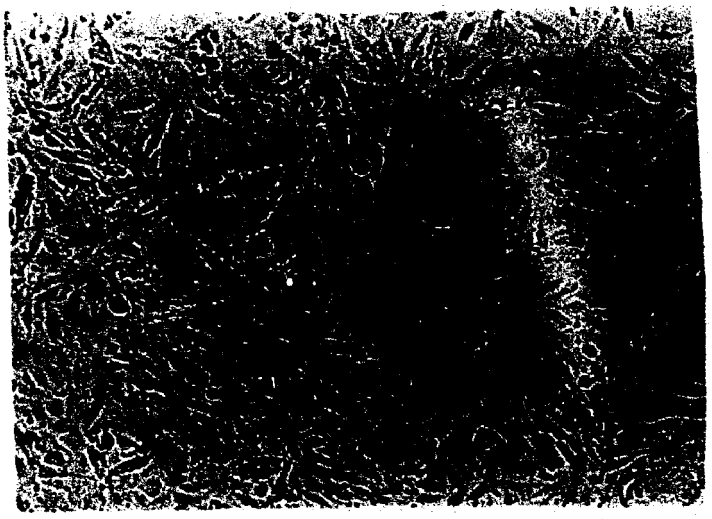


Cells Before Addition of Drug

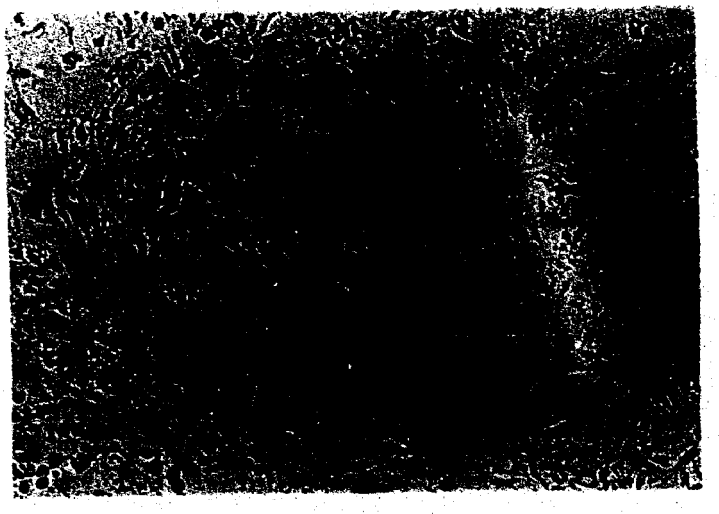


Cells 24 Hours After Addition of the Second Dose of Drug

Figure 7. The effects of 50 µg/ml of 1,3-bis(2-chloroethyl)-1-nitrosourea on human glioma cells and fibroblasts in culture.

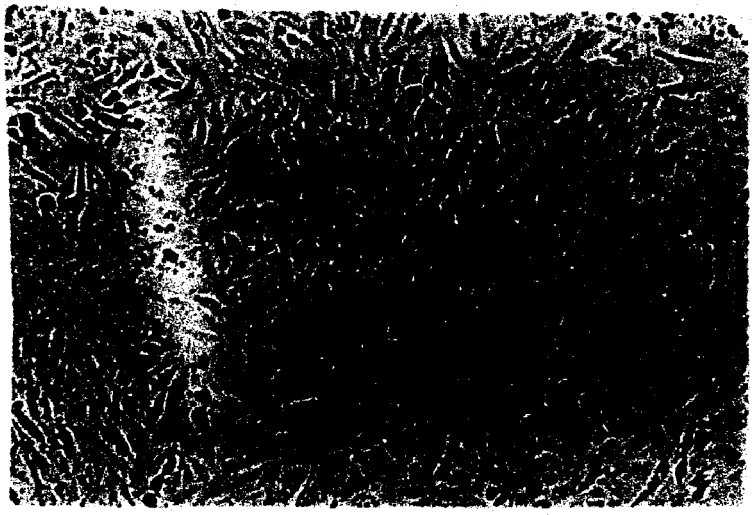


Cells Before Addition of Drug

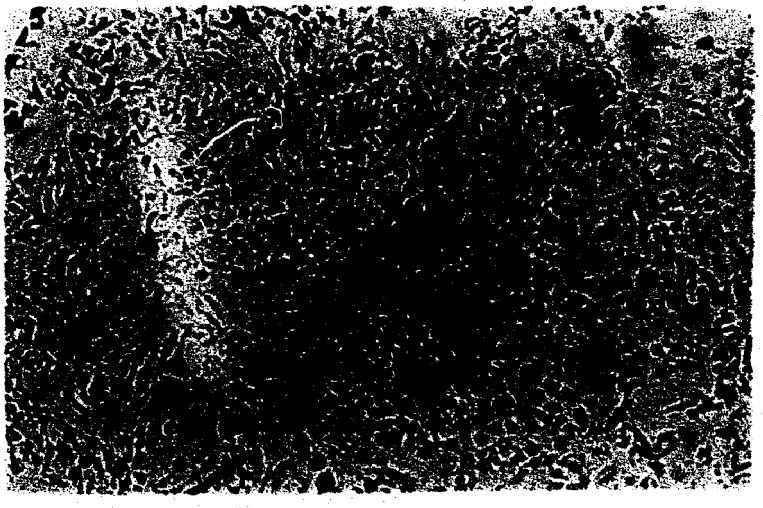


Cells 24 Hours After Addition
of the Second Dose of Drug

Figure 8. The effects of 100 μg/ml of 1,3-bis(2-chloroethyl)-1-nitrosourea on human glioma cells and fibroblasts in culture.



Cells Before Addition of Drug



Cells 24 Hours After Addition
of the Second Dose of Drug

CHAPTER IV

DISCUSSION

Most cells in culture require serum for growth. The specific components of serum which cells require have not been identified. Studies have shown that serum affects many cellular processes including DNA synthesis (Todaro et al., 1965; Yoshikura and Hirokawa, 1968; Temin, 1968), mitosis (Temin, 1969), RNA synthesis (Todaro et al., 1965; Rhode and Ellem, 1968; Yoshikura and Hirokawa, 1968; Yeh and Fisher, 1969; Cunningham and Pardee, 1969) and protein synthesis (Todaro et al., 1965).

A. Multiple Sclerosis

Serum from patients with multiple sclerosis (MS) was shown to contain a factor which causes demyelination in neonatal mouse cerebellum cultures (Dowling et al., 1968; Raine et al., 1973; Kim et al., 1970) and was localized to the IgG fraction of serum (Dowling et al., 1968).

Attempts were made during the present study to grow neonatal mouse cerebellum tissue in culture and obtain myelinated neurons. Growth was obtained and the presence of neurons was demonstrated by staining with thionine. Staining for myelin with Sudan black B failed to show the presence of myelinated axons. It was suggested by Berg and Kallen (1962a) that demyelination perhaps was a result of injury

to the myelin producing cells rather than destruction of the myelin itself. The myelin producing cells are the oligodendrocytes in the central nervous system and Schwann cells in the peripheral nervous system. Therefore after six months with no success in producing myelinated axons in the mouse cerebellum cultures it was decided to evaluate the effects of plasma, serum, and cultured lymphocytes from patients with MS on DNA synthesis in normal human fibroblasts and rat glial tumor cells by measuring uptake of tritiated thymidine. Hopefully if there was injury to these cells it would be reflected by an inhibition of DNA synthesis.

The effects of both fresh and frozen plasma from patients with MS on DNA synthesis were evaluated. The fresh plasma was added to normal human fibroblasts only. The results varied with the plasma from one patient showing stimulation and another suggesting inhibition of tritiated thymidine uptake (Table III). Table VI gives a summary of the data obtained when frozen plasma was evaluated for its effects on DNA synthesis in normal human fibroblasts and rat glial tumor cells. These data suggest slight stimulation of DNA synthesis in fibroblasts by the frozen plasma from patients with MS and an inhibition of the DNA synthesis in the rat glial tumor cells. Only one patient (WO) of six showed an inhibition of DNA synthesis when frozen plasma was added to the fibroblast cultures. Only one patient (MU) of nine showed a stimulation of DNA synthesis when the frozen plasma was added to the cultured rat glial tumor cells.

Fresh serum from patients with MS was evaluated for its effects on DNA synthesis in cultured rat glial tumor cells. The summary of these results is found in Table VIII. The data suggest slight

stimulation of DNA synthesis in cultured rat glial tumor cells by fresh serum from patients with MS. It is interesting to note that the frozen plasma tended to inhibit the DNA synthesis in the rat glial tumor cells and the fresh serum did not.

Since a factor in serum from patients with MS was shown to cause demyelination of axons in culture, it was thought that this factor might cause injury to glial cells which are known to produce myelin (Berg and Kallen, 1962a). Inhibition of DNA synthesis in glial cell cultures could reflect the injury to the glial cells. Findings in this study suggested greater injury was caused by frozen plasma from patients with MS to rat glial tumor cells than to normal human skin fibroblasts. This was indicated by an inhibition of DNA synthesis in rat glial tumor cells and a slight stimulation in fibroblasts. Serum from patients with MS caused a stimulation of DNA synthesis in rat glial tumor cells and thus does not suggest cellular injury in this system. Perhaps the factor is present in such small amounts in serum that concentration or fractionation of the serum would be necessary to show an effect on DNA synthesis.

Because MS was theorized to be an autoimmune disease, the suggestion was made that perhaps the myelinotoxic factors present in serum from MS patients were formed by lymphocytes in the central nervous system (Kim et al., 1970). As a result of this theory it was decided to study the effects of cultured lymphocytes from patients with MS on DNA synthesis in normal human fibroblasts and rat glial tumor cells in culture. The lymphocytes were stimulated with pokeweed mitogen which stimulates both thymus and bursa derived lymphocytes. It was thought that perhaps the stimulation by pokeweed would cause

the bursa derived lymphocytes to produce the factor responsible for demyelination. If the demyelination is a result of injury of the glial cells as suggested by Berg and Kallen (1962a) the factor may cause inhibition of DNA synthesis in glial cells as a result of injury of the cells. The data from this study are summarized in Table XI. The cultured lymphocytes were found to stimulate DNA synthesis in most of the fibroblasts and rat glial tumor cells. If a factor in MS patients is produced by the lymphocytes in the central nervous system which causes demyelination, it is either not produced by cultured lymphocytes from MS patients or does not exert an inhibition on DNA synthesis in this experimental system.

B. Cystic Fibrosis

A factor in the serum from patients with cystic fibrosis (CF) has been described by Spock et al. (1967) which caused dyskinesis in rabbit tracheal cilia and was present in the euglobulin fraction of serum. Other investigators have confirmed these findings (Bowman et al., 1970; Conover et al., 1973a; Doggett et al., 1973). Several hypotheses about the identification of this factor have been proposed. Studies done by Doggett et al. (1973a) suggested that the factor might be amylase. Conover et al. (1973) found the C3 component of complement present in much higher levels in serum from patients with CF than in normal serum. Further studies have led this group to propose that C3a bound to IgG was the ciliary inhibitory factor and the primary genetic defect in CF was a deficiency or defect in the carboxypeptidase-B-like enzyme which inactivates C3a.

Dr. C.A. Schneyer (personal communication) has found that injection of serum from patients with CF intraperitoneally into rats

caused a stimulation of mitosis in the salivary gland. She suggested evaluation of the effects of serum from patients with CF on DNA synthesis in normal human fibroblasts to determine if a stimulation of mitosis could be affected in an in vitro system; human fibroblasts appeared to be a good system for this study.

The effects of both fresh and frozen serum from patients with CF on DNA synthesis in cultured normal human fibroblasts were evaluated. The results are summarized in Table XIV. When fresh serum was evaluated only seven of 12 patients had a patient/control ratio greater than one. The frozen serum gave patient/control ratios greater than one in all patients except one (SP), thus, there was a suggestion of a stimulating effect by frozen serum on DNA synthesis.

Neither definite stimulatory nor inhibitory effects on DNA synthesis by fresh serum from patients with CF were observed consistently in fibroblasts. Slight stimulation was observed by frozen serum. Only in some patients' serum did the CF factor alter DNA synthesis in the fibroblasts evaluated. Consistent stimulation of mitosis was not shown in this cell culture system which may not be comparable to the in vivo studies of mitotic stimulation in rat salivary gland.

C. 1,3-Bis(2-Chloroethyl)-1-Nitrosourea

The anti-tumor agent, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), has specific properties which allow it to easily pass the blood-brain barrier. Because it appears simultaneously in the blood and cerebrospinal fluid, it has been used in the treatment of various brain tumors (Wilson et al., 1970; Walker and Hurwitz, 1970; Koo et al., 1972). Since this drug is being tested for its effectiveness in treatment of gliomas, it was decided to study its effects on DNA synthesis

in an established rat glioma cell line versus the effects on DNA synthesis in normal human fibroblasts. Four different doses of BCNU were used and added at three different times after subculturing the cells. The results of these studies are shown in Tables XV-XX. Figures 2-4 compare the inhibition expressed as percent in fibroblasts versus the rat glial tumor cells. All of the doses of BCNU inhibited DNA synthesis in both the cultured fibroblasts and rat glial tumor cells. The inhibition was observed when the drug was added at 24, 48, and 72 hours after subculturing the cells. Increasing doses of BCNU caused increased reduction in DNA synthesis in both cell types. The DNA synthesis was inhibited to a greater extent in fibroblasts than in the rat glial tumor cells when 50 μg/ml of BCNU was added.

The effects on DNA synthesis in rat glial tumor cells as compared to fibroblasts was used as an evaluation of BCNU, a drug which offers palliative treatment of human brain neoplasms. The synthesis of DNA in fibroblasts was inhibited to a greater extent than DNA synthesis in the rat glial tumor cells. However, variables in this system such as cell cycle times and duration of log phase may not make the above conclusion valid.

There has been some difficulty in establishing a human glioma cell line. None are available through the American Type Culture Collection. An agent which would suppress the growth of fibroblasts and select for the tumor cells would be of great value in establishing tumor cell clones. For this reason BCNU was added to the cultures derived from human glioma tissue and the effects observed and photographed. There was an observable effect on the cells as shown in Figures 5-8 when 20, 50, and 100 μg/ml were added but not when the

lowest dose of 10 µg/ml was added. The pictures show the cells before drug addition and 24 hours after the addition of the second dose. Cell cultures treated with BCNU had not recovered within 48 hours. The morphology of the cells present after 48 hours did not appear to be predominantly fibroblast. A longer period in culture may provide information indicating selection for certain cell types. If BCNU is selective for tumor cells, it could be of great use in selection of cell clones. Some means of differentiating fibroblasts from tumor cells such as differential staining would have to be used.

An evaluation of this particular culture system utilizing tritiated thymidine uptake as compared to mg protein as a measure of DNA synthesis perhaps should be done at this point. The control values as shown in the various tables of results show wide variation. There are also differences between control values for cultures done at different times so that CPM/mg protein cannot be compared from one test to another done at a different time. The only means of comparison is to calculate a patient/control ratio. Because of the inconsistencies among control and test data it is felt that this system is not the ideal system for evaluation of effects on DNA synthesis by serum, plasma, or leukocytes from patients with MS or serum from patients with CF. Perhaps the system could be improved by measuring thymidine uptake in precipitated DNA as compared to µg of DNA present.

CHAPTER V

CONCLUSIONS

A. Multiple Sclerosis (MS)

The present study evaluated the effects of fresh and frozen plasma, cultured lymphocytes and fresh serum from patients with MS on DNA synthesis in normal human fibroblasts and rat glial tumor cells. The results of this study suggested:

1. Slight stimulation of DNA synthesis in fibroblasts by frozen plasma and cultured lymphocytes from patients with MS.
2. Inhibition of DNA synthesis in rat glial tumor cells by frozen plasma from patients with MS.
3. Slight stimulation of DNA synthesis in cultured rat glial tumor cells by cultured lymphocytes and fresh serum from patients with MS.

B. Cystic Fibrosis (CF)

The effects of fresh and frozen serum from patients with CF on DNA synthesis in normal human fibroblasts were also studied. The data from these experiments suggested:

1. A trend for slight stimulation of DNA synthesis in normal human fibroblasts by frozen serum from patients with CF.

2. No consistent effect on DNA synthesis in normal human fibroblasts by fresh serum from patients with CF.

C. 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU)

The anti-tumor agent BCNU was added to normal human fibroblast cultures and rat glial tumor cells in culture at four doses and at three different times after subculture of the cells. The effects on DNA synthesis in these cells were evaluated. The drug was also added to cell cultures derived from human glioma tissue and the effects observed and photographed. From the data obtained in this study it was concluded:

1. All doses of BCNU (5, 10, 25, and 50 µg/ml) inhibited DNA synthesis in cultured human skin fibroblasts and in an established rat glial tumor cell line.
2. DNA synthesis was inhibited in normal human fibroblasts and rat glial tumor cells in culture whether BCNU was added at 24, 48, or 72 hours after subculturing the cells.
3. Increasing doses of BCNU caused increased reduction in DNA synthesis in fibroblasts and rat glial tumor cells in culture.
4. DNA synthesis was inhibited to a greater extent in fibroblasts than in rat glial tumor cells at the 50 µg/ml dose of BCNU.
5. Preliminary studies indicate that BCNU could be useful in selection of tumor cell clones. Further work is necessary.

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