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ALTERATION OF LYMPHOCYTE RESPONSE
USING MEMBRANE ACTIVE COMPOUNDS

DISSERTATION
Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By
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The Ohio State University
1973
ACKNOWLEDGEMENTS

I would like to dedicate this dissertation to a good friend who should have reached this point long before I, but because of circumstances was unable to attain his goal before his death, Bob Holmes.

I would also like to express my appreciation to Ann Barker for her unending moral support. I would like to express to Dr. Rheins my gratitude for his literary guidance.

Finally, I would like to thank all of the other graduate students for making this experience fairly enjoyable. My special thanks to John Rice and Jeff Rossio for their late night scientific discussions.

The remainder of my gratitude (if you can parcel such a thing) goes to my parents and India and to all of the others that have influenced my personal life with love.
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Major Field: Microbiology


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INTRODUCTION

All critical interactions occurring between the mammalian cell and the external milieu are mediated through the plasma membrane. As the result of a disease process the cell membrane may be sufficiently altered so as to reduce the functional response of the cell. The phenomenon of mutation of membrane integrity through neoplasia probably accounts for a portion of the broad spectrum of abnormal processes observed in malignancies. Certainly such aberrations as loss of contact inhibition noted for cells of solid tumor origin and the depressed responsiveness of leukemic lymphocytes to stimulation by a variety of plant mitogens are direct results of plasmallemal changes. Membrane modification of malignant cells both in vivo and in vitro has been aimed toward either detrimentally effecting the malignant cell or manipulation of the surface configuration of the neoplastic cell to confer greater antigenic distinction from the host tissue of its origin. Because of the immunological implications of antigenic modulation at the membrane level, the lymphomatous proliferative diseases are of particular interest. This is attributable to the fact that in lymphocyte neoplasias the pivotal cell type involved in a great
majority of immunologic functions, namely the lymphocyte, is involved also in the malignant proliferation as well.

Chronic lymphocytic leukemia is a lymphoproliferative disease in which the individual malignant cell seems to be largely inactive at the membrane level and may give rise to a chemotherapeutically resistant clone of cells (1, 2, 3, and 4). Patients with this disease manifest a depressed immunologic capability and their peripheral lymphocytes from demonstrate a quantifiable decrease in reactivity to the plant mitogen, phytohemagglutinin (5 and 6), as well as a severe curtailment of the capacity to produce interferon (7 and 8). Based on data concerning this inactivity it appeared proper to attempt to alter the cellular immune responsiveness of the leukemic cell by manipulation of the cell membrane.

Accordingly, this investigation was undertaken to examine the in vitro effectiveness of four compounds, all of which have been used clinically in man, on the mutability of the plasma membranes of normal and leukemic peripheral lymphocytes. The drugs tested included dimethyl sulfoxide (a surface perturbant and cryopreservative), neuraminidase (an enzyme specific for sialomucin residues), iodoacetate (a sulfhydryl inhibitor) and cyclophosphamide (an alkylating agent). These drugs were employed either singly or in combination to treat test lymphocytes in vitro, using the measurement of incorporation of tritiated thymidine and the
production of interferon as indices of cellular membrane activity. From the data, several hypotheses are offered concerning the configuration and function of the leukemic cell membrane, particularly in conjunction with the fluid mosaic model of membrane structure presented by Singer and Nicolson (9 and 10).
REVIEW OF THE LITERATURE

Chronic lymphocytic leukemia (CLL) in man has been defined traditionally as a generalized, progressive, and self-perpetuating proliferative abnormality of the lymphoid tissues affecting the small lymphocytes particularly (11). The major signs of the disease are enlarged lymph nodes, splenomegaly and the presence of abnormal lymphocytes in the circulating blood. As the disease progresses, these lymphocytes infiltrate the bone marrow, spleen, liver and skin, and into the walls of the respiratory and gastrointestinal tracts. Hypogammaglobulinemia is characteristically present in late stages of the disease.

Genetically there seems to be some basis for the familial transmission or incidence of CLL. In all studies, familial CLL tends to occur in closely related persons, siblings, parents, children and first cousins, but otherwise there is no clear cut pattern of inheritance. Consanguinity does not appear to be a factor. There is also no evidence of characteristic chromosomal abnormalities.

There is a strong age link in the incidence of CLL, inasmuch that 90 percent of patients with CLL are over 50 years of age and nearly two-thirds are over 60 (12). The
link may be the reduction in the immunologic competence associated with aging as evidenced by a decrease in immunoglobulin concentration, reduced phagocytic efficiency and impairment of lymphocyte transformation (5 and 6).

CLL also has the greatest sex disparity of all of the leukemias. The male-to-female ratio in CLL is more than 2 to 1. No satisfactory explanation for this is forthcoming and the role of sex hormones in the incidence of this disease has not been elucidated.

The earliest clinical manifestations of the disease are general fatigue, reduced tolerance to exercise and often enlargement of the superficial lymph nodes and/or splenomegaly. As the disease progresses the splenomegaly increases and hepatomegaly occurs. Lymphoid tissue may develop in unusual areas such as the skin, scalp, pharynx, abnormally in the walls of the gastrointestinal tract and in abdominal muscle. Symptoms in the advanced stage include severe fatigue, recurrent or persistent infection, ease of bruising, fever and increased bone tenderness. The average life span of patients from initial diagnosis to death is 4 to 5 years if treatment is not begun.

The early efforts at treatment of CLL involved the use of local ionizing radiation. However, in a protracted disease such as CLL the bone marrow depression attendant with radiation therapy often contributed to a more rapidly fatal disease than did failure to institute any management.
Subsequent use of the radiomimetic derivatives of nitrogen mustard, namely chlorambucil and cyclophosphamide, have greatly improved survival times with 40% of the patients surviving more than 9 years.

A prognosis for CLL patients then, indicates an expected life span of five years following diagnosis with over one-third of those patients surviving for 10 years. However as the course of CLL evolves, the stage is eventually reached, unless another illness supervenes (as it does in perhaps half of all patients) when increasing cellular immaturity, bone pain, fever, neutropenia and thrombocytopenia, and progressive anemia develop all of which are refractory to therapy. This final phase develops gradually over a period of 4 to 6 months and up to 1 to 2 years, and patients succumb to complications of uncontrolled disease, inanition, intercurrent infection, anemia or bleeding (11 and 12).

Accumulated data involving the clinical manifestations of CLL coupled with recent studies of leukocyte kinetics and in vitro lymphocyte responses lend credence to the theory that CLL is a monoclonal disorder probably of B-lymphocyte (bone-marrow derived) origin.

CLL in man is characterized by an increase in the absolute numbers of small lymphocytes in peripheral blood, usually above 15,000/cubic millimeter. This increase is accompanied by enlargement of the lymph nodes and the spleen, due to infiltration and accumulation of lymphocytes.
Underlying immunoglobulin deficiencies and impaired blastogenic responsiveness of CLL lymphocytes to phytohemagglutinin (PHA) have been suggested as also being characteristic of the disease (13).

Experimental evidence (14 and 15) is consistent with the observation that the disease reflects an accumulation of recirculating lymphocytes. Despite the predominance of small long-lived lymphocytes, there is, as is in the normal individual, a small population of more rapidly proliferating lymphocytes (14). Thus the lymphocyte population of CLL appears to be composed of two distinct classes of morphologically similar cells (16). These two classes have been separated by means of polystyrene bead column elution (17). The pre-column cell population, composed of both leukemic and normal lymphocytes, showed impaired PHA transformation in vitro and ultrasensitivity to the cytocidal action of colchicine (18). The eluted population exhibited near normal response in all of the tests, with the colchicine-ultrasensitive leukemic lymphocytes adhering to the column. Experiments involving radioactive labelling (tritiated thymidine) of blood lymphocytes from CLL patients revealed that very few labelled lymphocytes appeared in the bone marrow but preferentially localized in the vascular spaces of the lymph nodes (19). This inability of the leukemic leukocyte to enter the lymph system is further demonstrated by the fact that thoracic duct lymphocytes were found to have
normal in vitro response to both PHA and to bacterial antigen, while peripheral blood lymphocytes had impaired responses to both (20).

Increased accumulation of these unreactive lymphocytes late in the course of the disease may account for the abnormally low levels of gamma globulin in CLL patients (21), as well as the noted failures in the immune surveillance system. In this regard, hypogammaglobulinemia is frequently associated with CLL (22 and 23) and may be directly related to the duration of the disease, with those patients surviving more than 5 years demonstrating very low gamma globulin levels (24). Other manifestations of immune imbalance include a suppression in the incidence of allergic manifestations in patients with CLL and an accompanying decrease in the level of IgE (25). The lowered levels of protective antibody probably account for the severely depressed resistance of CLL patients to various bacterial, mycotic and viral pathogens (26). These known immunologic deficiencies also may promote, or in some way contribute to, the three to four-fold increase in superimposed solid cancers and the 8 to 13 fold increase in skin cancers observed in terminating CLL patients (27 and 28).

There seems to be no impairment of delayed type skin reactivity in these patients as evidenced by anticipated reactions to previously encountered antigens such as tubercle bacilli and Histoplasma capsulatum (29). Patients with CLL
are unable, however, to become sensitized to new antigens such as 2,4-dinitro-1-fluorobenzene (30), but may become sensitized to 2,4 dinitrochlorobenzene by passive cellular transfer (31).

During the past 3 years it has become increasingly evident that the lymphoproliferative diseases (e.g., CLL) can be more accurately classified by which sub-population(s) of lymphocyte is(are) involved. With the elucidation of the existence, function and characteristics of the B-lymphocyte (bone marrow-derived or -dependent) and T-lymphocyte (thymus-derived or -dependent) it is now possible to better examine the neoplastic cell of the CLL patient.

The origin of lymphocytes bearing surface immunoglobulins has been determined in animals and these cells appear to belong to the bone-marrow-derived or dependent population of lymphocytes (32 and 33), whereas cells with little or no detectable surface immunoglobulin are associated with the thymus-derived or dependent population (34). When lymphocytes isolated from the peripheral blood of CLL patients are treated with fluorescein-conjugated antisera, the presence of cell surface IgM is demonstrated (35 and 36). These lymphocytes also exhibit an immunoglobulin receptor specific for antigen-antibody-complement complexes. These receptors are found exclusively on the CLL B-cell and not on the T-lymphocyte (37). Light chain analysis of the surface IgM indicate that these leukemic cells bear either kappa or
lambda determinants, but not both, suggesting the clonal nature of this neoplasm (35 and 38). Apparently the presence of surface immunoglobulin imparts an adhesive quality to the CLL lymphocyte as reported in the earlier polystyrene bead experiments (18).

There is also a functional disparity between normal B and T cells in the blastogenic responsiveness to several plant mitogens. Using the mouse spleen as a source of both B and T cells it has been shown that B cells respond well to pokeweed mitogen (PWM), but poorly, if at all, to phytohemagglutinin (PHA). This same pattern of responsiveness generally holds true for peripheral lymphocytes isolated from patients with CLL. Findings indicate that although PWM stimulates optimal cell transformation in cultures of CLL lymphocytes, the percentage of cells transformed is abnormally low when compared with the number of cells transformed in cultures of normal cells (39).

The response of the CLL lymphocyte to (PHA) has been examined from the aspects of onset, duration and degree of responsiveness. Many researchers have reported that the CLL peripheral lymphocyte has a delayed but not an absent response to PHA. It appears that the blastogenic response of the CLL lymphocyte (as measured by morphological changes and thymidine incorporation) when measured after 7 days exposure to PHA is equivalent to the maximal response observed in cultures of normal lymphocytes after 3 days of mitogen
exposure (39 and 40).

The gross morphological changes attendant with a blast response to PHA depend on an intricate series of enzymatic pathways leading to DNA replication, RNA transcription and to eventual protein synthesis. In Table 1 is outlined the alterations in lactate dehydrogenase, thymidine salvage pathway, pentose phosphate shunt activity and RNA synthesis. Enzyme studies are, at this time, inconclusive due to the lack of corroborative data. However, examination of membrane surface configuration and activity may be more fruitful in that this is the area of initial cellular interactions and therefore any alteration may be examined on a cellular as opposed to macromolecular level.

*In vivo* unresponsiveness of a leukemic patient to anti-leukemic chemotherapeutic agents ultimately can be attributed to the resistance of the individual neoplastic cell to the action of the particular drug in question. This resistance may be due to: 1) clonal selection of drug-resistant mutant cells with the enzymatic capacity to inhibit drug function, 2) alterations of the plasma membrane resulting in a decrease in membrane permeability, or 3) a combination of the above.

A compact clone from the diploid murine leukemia cell line, L5178Y, was found to mutate *in vivo* to a level of significant resistance to the action of methotrexate (MTX), cytosine arabinoside (Ara-C) or 5-iododeoxyuridine (IUDR).
### TABLE 1

**SUMMARY OF COMPARATIVE CHARACTERISTICS OF THE CLL LYMPHOCYTE vs THE NORMAL LYMPHOCYTE**

<table>
<thead>
<tr>
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<th>NORMAL LYMPHOCYTE</th>
<th>CLL LYMPHOCYTE</th>
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<tr>
<td><strong>Morphology (E.M. &amp; Light)</strong></td>
<td>Small Lymphocyte, Dense Nuclear Material, Nucleoli</td>
<td>Small Lymphocyte, Slightly Less Dense Chromatin, Nucleoli</td>
</tr>
<tr>
<td><strong>Polystyrene Bead Adherence</strong></td>
<td>Non-Adherent</td>
<td>Adherent</td>
</tr>
<tr>
<td><strong>PHA Transformation</strong></td>
<td>3 Days</td>
<td>7 Days</td>
</tr>
<tr>
<td>(Optimal Thymidine Incorporation)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Colchicine Sensitivity</strong></td>
<td>Non-Sensitive</td>
<td>Ultrasensitive</td>
</tr>
<tr>
<td><strong>Lymph Passage</strong></td>
<td>Free Movement</td>
<td>Blocked at Node Barrier</td>
</tr>
<tr>
<td><strong>Immunoglobulin Coating</strong></td>
<td>T-Cell Non-Coated</td>
<td>IgM Surface Globulin Only, Either Kappa or Lambda Light Chain But Not Both</td>
</tr>
<tr>
<td></td>
<td>B-Cell IgM or IgG, Either Kappa or Lambda Light Chain or Both</td>
<td></td>
</tr>
<tr>
<td><strong>PWM Transformation</strong></td>
<td>T-Cell No Effect</td>
<td>Very Low Level of Transformed Cells</td>
</tr>
<tr>
<td>(Optimal Thymidine Incorporation)</td>
<td>B-Cell 3 Days</td>
<td></td>
</tr>
<tr>
<td><strong>RNA</strong></td>
<td>Stable Single Strand</td>
<td>Single Strand, Unstable Rapidly Labelled With Tritiated Uridine</td>
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<td>Interferon Induction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A) Sendai Virus Inducer</td>
<td>In Excess of 1000 Units</td>
<td>Non-Detectable</td>
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<tr>
<td>B) Tilorone HCl Inducer</td>
<td>Peak 24 Hours (Low Level)</td>
<td>Peak Comparable to Normal But Delayed to 48 Hours</td>
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<td>Beta-Glucuronidase Activity Following PHA Stimulation</td>
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<td>Totally Absent</td>
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<td>Acid Phosphatase Synthesis Following PHA Stimulation</td>
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<td>Below Normal</td>
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<td>Glycogen Storage</td>
<td>Normal</td>
<td>Above Normal</td>
</tr>
<tr>
<td>Hexose Monophosphate Shunt Activity</td>
<td>Normal</td>
<td>Below Normal</td>
</tr>
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<td>Thymidine Kinase Activity Following PHA Stimulation</td>
<td>Normal</td>
<td>No Activity in CLL Cells That Do Not Respond to PHA at Three Days</td>
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<td>Significant Increase at 24 Hours</td>
<td>Increase Varies Inversely With Height of Peripheral White Blood Cell Counts</td>
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In DBA/2-J mice, mutation of leukemic L5178Y lymphoblasts occurred without exposure to the drug. The drug functioned as a selective agent and rapidly brought about an increase in the resistant population (41, 42, 43 and 44). In this instance, the cells were resistant because of altered enzymatic action which cancelled the effective site of action of each drug. Clones resistant to MTX were shown to be resistant by virtue of an increase in the level of folic acid reductase (43). Resistance to Ara-C was associated with a decrease in deoxycytidine kinase activity (45), while resistance to IUDR resulted from a partial or extensive loss of thymidine kinase activity.

These biochemical data provide satisfactory evidence for the alteration of 6 of 7 available genetic markers. These murine leukemic cell lines obtained directly from the host reproduce in culture with a high cloning efficiency, a finding which establishes that the leukemic cells recovered from culture indeed represent those present in ascitic fluid of the host. This acquisition of drug-resistant cells (and a presumptive increase in disease symptoms) would seem to depend on the regimen of chemotherapeutic treatment (i.e., dosage, duration of treatment, type of agent, route of application, etc.). However, inherent drug resistance is not uncommon and, as such, is not wholly explained by genetic selection.

There are physiologic barriers involved in drug uptake
such as drug-surface membrane relationships which are related to earlier events in drug-cell interactions. The most striking example of the role of permeability barriers as determinants of drug responsiveness is found with MTX. The capacity of freshly isolated cells for MTX uptake was found to be related to drug responsiveness in both animal (46) and human (47) leukemia cells. Levels of the drug-sensitive enzyme, dihydrofolate reductase, were increased in examples of drug-induced resistance (48), but not in inherent drug resistance (49). Among the normal cell types, granulocytes and platelets had a high capacity for MTX uptake while normal lymphocytes and erythrocytes were relatively resistant. Although the measurement of drug uptake in vitro can serve to predict drug responsiveness for both human and murine leukemias, it is noteworthy that drug accumulation by the most responsive human cell type was slower than was the drug accumulation for the cultured murine leukemia cells.

Other examples of permeability barriers to drug absorption have been implicated in the development of resistance to methylglyoxal-bis-guanhydrozone (50), to antinomycin D (51), to certain terephthalalalides (52) and to daunomycin (53). For the latter 3 agents, such barriers could only be demonstrated in vivo or in cells undergoing mitosis in culture.

In a related study the alkylating agents, busulfan and nitrogen mustard, readily penetrated both drug-resistant and
drug-sensitive murine leukemia cells (54). These two agents readily inhibited nucleic acid synthesis in vitro by both drug-resistant and drug-sensitive murine cell types.

Extensive clinical investigations in human patients involving the use of two related alkylating agents (cyclophosphamide and chlorambucil) in the therapy of chronic lymphocytic leukemia (CLL), indicate a very real difference in the cell reactivity of human cell types as compared with the murine system. There exists a significant percentage of individuals who readily develop drug non-responsiveness as well as those who are inherently resistant to these drugs (55, 56, 57 and 58). Both cyclophosphamide and chlorambucil are nitrogen mustard derivatives with the overall intracellular action of blocking RNA, and eventually DNA and protein synthesis. These alkylating agents are radiomimetic drugs with chlorambucil having chiefly the lymphoid effects of X-radiation and cyclophosphamide a somewhat broader mode of action (59). Intracellular pharmacologic mechanisms are not clearly understood for either of these drugs (60). The basis of both types of drug resistance noted in human CLL is unknown, but is suggestive of the mutated clonal cells, or surface membrane altered neoplastic cells described for the murine L5178Y system.

Treatment of CLL lymphocytes in vitro with autologous sera demonstrated that the delay in PHA response was not due to serum factors blocking the available surface receptors
for PHA (61). Rather, by using $^{131}$I labelled PHA it was determined that CLL lymphocytes were able to bind only 1/3 to 1/2 as much PHA to the surface membrane as could normal lymphocytes (62). The decreased number of PHA receptor sites is probably just one reflection of the altered cell surface of the leukemic lymphocytes. The electro-kinetic behavior of these lymphocytes at alkaline pH also suggests the presence of surface amino and sulfhydryl groups (63). Moreover, the finding that the ABH blood group antigens have markedly decreased or even absent reactivity in CLL lymphocytes as compared with normal lymphocytes (64) is consistent with previous cell surface data.

In this view, many studies have been concerned with the effects of deliberate alteration of the plasma membranes of tumor cells. Several compounds with a wide range of activities have been used to alter the reactivity of neoplastic cells. Among these are included the enzyme neuraminidase, the sulfhydryl inhibitor iodoacetate and the hydration active compound dimethyl sulfoxide.

1. Neuraminidase (NASE) - Through the use of the enzyme neuraminidase (N-acetyl neuraminate glycohydrolase) (65) several investigators have demonstrated that sialic acids (a collective term for N-acetyl, N-glycolyl and N,D-diacetyl-neuraminic acid) occur at the surfaces of Ehrlich ascites tumor cells (66 and 67), of red blood cells (68 and 69) and of normal and malignant rat liver cells
Sialic acids usually are an integral component of the carbohydrate prosthetic groups of glycoproteins (71, 72 and 73) and of acid mucins (72), and it may well be that the primary function of the sialic acid molecule is to confer structural rigidity to glycoproteins (74). Some evidence for this has been provided by Weiss (75) who has shown that the removal of sialic acid residues from the surfaces of sarcoma 37 cells increased the overall cellular deformability. Cell electrophoretic studies have detected sialic acids in many other cell types (76). These studies also have revealed that the increased negative mobilities of some transformed cell lines (77) and of RPMI #41 cells (derived from human osteogenic sarcoma) in mitotic peak phase (78) are attributable to an increased amount of cell surface sialic acids. It also was found that the increased electrophoretic mobility of hamster kidney fibroblasts transformed by polyoma virus was reduced to the level of mobility of normal kidney cells following NASE treatment (79).

As an outgrowth of these investigations a number of workers (80 and 81) have employed NASE as one approach to specific active immunotherapy in animal models. The rationale for this rests on the presumption that sialomucins act as barriers to the expression or exposure of tumor antigens, either by increasing membrane rigidity or by actively masking such antigens. NASE is reported to alter the antigenicity of a variety of animal tumor cells and, more
recently, of cultured human lymphoid cells (81).

The presence of sialomucins in cell membranes may influence the initial phase reaction in mitogenic transformation which is a specific receptor-mitogen interaction in which PHA combines with surface glycopeptide structures (82, 83, 84 and 85). Adler et al reported that following the receptor-binding phase, PHA must be rapidly internalized (approximately 10 - 20 minutes following initial exposure) by normal lymphocytes before transformation may occur. NASE treatment of normal cells was shown to significantly increase the binding of PHA to the lymphocyte membrane. This binding however, blocked rapid internalization of PHA and therefore abrogated early transformation. Prolonged incubation of the cells (to 72 hours) was necessary for initiation of active membrane turnover, after which time the cells internalized the bound PHA and underwent transformation.

2. Iodoacetate - Iodoacetic acid and iodoacetamide have been used in a variety of protein alkylations. Most commonly, initial modification is observed as an effect on cysteine, methionine, or histidine, but selective alkylations of amino (86, 87 and 88) and of carboxyl groups (86, 89, 90 and 91) have also been reported. Iodoacetate has been suggested as inhibiting the rebonding of disulfide bonds in proteins and thereby inhibiting the return of proteins to their native conformation (White). Since alterations in electrophoretic mobilities and membrane charge indicate that
many (if not all) neoplastic cells exhibit an increase in sulfhydryl groups (SH) at the membrane level, Knock (92 and 93) has suggested that iodoacetate reacts more readily with neoplastic cells due to this increased availability of SH groups, decreased activation energies and increased concentrations of mitotic proteins. The surface configurational changes induced by iodoacetate therefore have prompted the examination of its potential as a chemotherapeutic agent. Several workers have demonstrated immunoprophylaxis in animals treated with tumor cells previously exposed to iodoacetate. These animals survived better when subsequently challenged with viable syngeneic tumor cells, than did the control groups (94, 95, 96 and 97). Young AkR mice, which eventually demonstrate a spontaneous fatal lymphocytic leukemia, were shown to have significant increases in the total numbers of mature splenic lymphoid cells which were responsive to sheep red blood cells as a test antigen, following stimulation with iodoacetate treated tumor cells (98). Also some evidence of increased survival was noted in groups of AKR mice which had received several spaced injections of this agent (98 and 99).

3. Dimethyl sulfoxide - Membrane alteration is possible through the use of another more chemically defined organic compound, namely dimethyl sulfoxide (DMSO). DMSO is a derivative of lignin with exceptional solvent properties. DMSO is a dipolar aprotic solvent which is miscible with
water, lipoids and organic solvents (100). Initial studies with plants revealed that the chemical exerted a profound effect on biological membranes altering their natural selectivity. DMSO appears to be extremely effective in modifying the configuration of proteins and this change is apparently reversible after the removal of the DMSO (101). This total reversibility also has been demonstrated with several other enzyme proteins (102). The effectiveness of DMSO, in this regard, appears to be related to its size and capacity to substitute for, or bind, water, in addition to effecting other hydrogen bonded structures (103). Because of these properties, membranes treated with DMSO are rendered porous to compounds generally considered to be non-dialyzable and the penetration of normally dialyzable ions and compounds is increased (104). DMSO also has the properties of a radio-protective compound (105) and is used as a cryopreservative for the storage of viable cells at liquid nitrogen temperatures (106).

Extensive clinical research (107) initially permitted the use of DMSO in humans as a carrier base for topical medicines by increasing the penetration of the drugs through the keratinized layers of the skin. However several reports of opthalmalogic complications in dogs has prevented further use of the drug at this time. Currently, however, DMSO is being employed extensively to study in human cells in culture and in animal systems. In vitro, DMSO permits the
differentiation between blood lymphocytes from patients with CLL and cells from normal donors by exerting a greater cytocidal effect on the leukemic cells (108). A similar increase in cytocidal effect was noted by these workers in the application of DMSO to cells from the thymuses of AKR mice with lymphomas as compared to lymphocytes obtained from normal mice. The mode of action of DMSO as a cytocidal agent is unknown, but is probably closely linked to its membrane activity.

The foregoing review was not intended as an exhaustive effort of searching the literature concerning chronic lymphocytic leukemia, but rather as a pertinent overview of the disease and of several parameters of the individual lymphocyte which were of particular interest.
MATERIALS AND METHODS

Lymphocyte Donors

(A) Chronic Lymphocytic Leukemia Patients - J.Y., a male caucasian was admitted to University Hospital (The Ohio State University, Columbus, Ohio) on January 7, 1971. At the time of admittance the patient was 59 years of age and complained of back pain and general fatigue. He presented a history of urinary problems and migrane headaches. Both parents and one sister had died of coronary disease, another sister had succumbed of malignant lymphoma. Initial examination revealed lymphocyte infiltrated petichiae of the buccal mucosa and swelling of the cervical, axillary and inguinal lymph nodes. The liver and spleen were enlarged and palpable, and some bone tenderness was noted at the sternum. Laboratory reports indicated a peripheral white blood cell (WBC) count of 127,000 cells per cubic millimeter (consisting of 98% lymphocytes), a low hematocrit, elevated uric acid and a general decrease in gammaglobulins. Several atypical polyclonal IgG types were noted. The patient was diagnosed as having chronic lymphocytic leukemia of the lymphosarcoma type. A chemotherapy regimen of
cyclophosphamide ("Cytoxan"), vincristine and prednisone of prescribed dosage was initiated. No hematologic improvement was noted and the patient was placed on mechanical depletion therapy on March 2, 1971.

W. W., a male caucasian, age 62 years was admitted to The University Hospital on January 9, 1971. Chief complaints included general malaise and urinary tract problems. Initial examination revealed numerous macropapular lesions on the trunk and extremities and enlarged lymph nodes in the axillary, cervical and inguinal regions. The spleen was enlarged and palpable, and there was severe infiltration of the prostate gland. The peripheral WBC count was 119,000 cells (90% small lymphocytes) per cubic mm. Bone marrow aspiration showed the marrow densely packed with small lymphocytes and some "sarcoma" type cells. The patient was placed on chlorambucil and prednisone therapy for 3 months, after which time no improvement was noted and therapy was curtailed. On April 25 the patient was placed on the blood cell separator for supportive therapy by mechanical depletion.

C.K., a male caucasian age 56, was admitted to The University Hospital on November 20, 1970, following an upper respiratory episode of unusual severity. Upon initial examination the patient demonstrated tenderness of the sternum and enlarged lymph nodes in the axillary, cervical and inguinal regions. A mild hepatomegally (18 cm laterally
and 10 cm vertically) was noted but no splenomegally was evident. The peripheral WBC count was 180,000 cells per cubic mm, of which 98% were small lymphocytes. Aspiration of the bone marrow produced but little fluid which was densely packed with lymphocytes. The patient was placed on chlorambucil and cyclophosphamide therapy but showed no signs of improvement. A arterial-venous shunt was performed on the right wrist and the patient was placed on mechanical depletion therapy on March 27, 1971. The patient died on November 13, 1971 and the autopsy revealed lymphocyte infiltration and hemorrhage in the kidneys, liver and spleen.

(B) Normal Donors - Normal donors consisted of two laboratory personnel and one relative of a hospital patient, all of whom were male caucasians ranging in age from 24 to 29 years. All three donors were in good health with a normal peripheral WBC range of 6,000 to 8,000 per cubic mm. Differential counts for lymphocytes, polymorphonuclear neutrophils, monocytes, basophils and eosinophils also were within normal ranges. Two of the donors were placed on the blood cell separator for WBC extraction, and the remaining donor was bled via the cephalic vein.

Lymphocyte Culture Media and Solutions

The standard tissue culture fluid (TCF) used for all experimentation was RPMI - 1640 + 10% fetal bovine serum.
The medium was obtained from Grand Island Biological Company as foil lined packets of dehydrated powder. The powder from one package was reconstituted with 900 ml of double distilled demineralized water (supplied by The Ohio State University Lab Stores) and 100 ml fetal bovine serum (Flow laboratories Lot #455555 and screened for bovine viruses and mycoplasma). The pH was adjusted by the addition of 2.0 grams of sodium bicarbonate per liter of medium. The complete medium containing the calf serum was filter sterilized by positive pressure through a Millipore unit using (in the order of medium flow) a Seitz prefilter, a 0.45 micron Millipore filter and a 0.22 micron Millipore filter. The medium was stored in gas tight glass bottles at 4°C until needed. Twenty-four hours prior to use, the medium was incubated at room temperature to determine sterility. Immediately prior to use, penicillin and Mycostatin (nystatin) were added at concentrations of 100 units and 20 milligrams per ml respectively.

Ficoll-hypaque density gradient lymphocyte extraction technique was modified from that described by Bouyam (109). Approximately 9 gm Ficoll (Sigma Chemical Co. molecular weight of 400,000 daltons) was dissolved in 91.0 ml of double distilled demineralized water containing 0.85 gm sodium chloride. This solution was adjusted to a buoyant density of 1.0235 gm/cubic cm by the further addition of either powdered Ficoll or saline. Hypaque solution was
prepared by diluting Hypaque-M (75% solution, a brand of sodium and meglumine diatrozoates marketed by Winthrop Laboratories, New York, N.Y.) to an approximate 34% in saline. This solution was adjusted to a final density of 1.2000 gm/cubic cm by the further addition of either stock hypaque or saline. These two stock solutions of Ficoll and hypaque were mixed in a proportion of 24 parts Ficoll to 10 parts hypaque. The final working separation fluid was adjusted to a density of 1.0770 gm/cubic cm, filter-sterilized through a 0.45 micron Millipore 'in line' filter and stored at room temperature in gas tight brown glass containers.

**Lymphocyte Removal, Separation and Culture**

The CLL lymphocytes were removed and utilized for culture only during a period in which the patient's peripheral WBC count had been stabilized between 35,000 and 40,000 WBC's per cubic millimeter. This was in accordance with the observation that the PHA response varies in an individual patient as the peripheral count increases or decreases (110).

Lymphocytes were removed by mechanical depletion using the "Celltrifuge" (American Instrument Co.) blood cell separator. The donor was catheterized in the cephalic veins of both arms (except in the instance of the arterial-venous shunt) and connected to the zonal centrifuge of the separator. Approximately 250.0 ml of heparinized saline was added
to the blood volume via the "recipient" arm while the same amount was drawn out of the body through the "donor" vessel of the opposite arm. The blood entered a continuous flow centrifuge bowl spinning at 500 RPM and the "buffy coat" band was drawn out by a peristaltic pump. Red cells, platelets and plasma were returned to the donor while the WBC's were pumped into a plastic, 300.0 ml capacity TA-5 transfer pack (Fenwal) containing 10.0 ml of citrated dextrose solution (ACD-A).

WBC's were removed from the bag by sterile aspiration with a 20.0 ml syringe fitted with an 18 gauge needle. A sample of the WBC enriched suspension was diluted and counted on a hemacytometer. The suspension was also smeared, air dried and fixed by Wright's - Giemsa stain (111) for performing differential counts. Throughout the preparation procedure the cells were retained in an open vessel at a temperature of 37°C and in an atmosphere of 5% carbon dioxide.

The initial cell suspension was diluted 1:8 with TCF as the diluent. The resulting diluted cell suspension was layered over a Ficoll - hypaque cushion (buoyant density = 1.077 gm/cubic cm) in a proportion of 8 parts cell suspension to 3 parts Ficoll - hypaque, in a sterile 40 ml capacity, thick walled, screw capped, conical glass centrifuge tube. The tubes were centrifuged at 1300 g for 8 min at room temperature in an I.E.C. clinical centrifuge. The mononuclear cell band was removed by aspiration with a
sterile 10 ml pipette along with approximately 2.0 cm of accompanying fluid above and below the cell band. This suspension was diluted with two volumes of TCF and recentrifuged at 800 g for 10 min in order to pellet the cells. The cells were resuspended in 10.0 ml of TCF and centrifuged to wash free any remaining gradient fluid. The cell pellet again was resuspended in 10.0 ml of TCF and the cells were evenly dispersed by vigorous pipette agitation. A sample of the cell suspension was smeared, air dried and stained with Wrights - Giemsa to determine the percentage of contaminating PMN's and monocytes in the final cell preparation. The cell suspension was diluted 1:100 (0.1 ml cell suspension in a previously prepared 9.9 ml blank of Hank's Balanced Salt Solution) and 4 drops of 0.5% Trypan Blue were added to the diluted cell suspension. This was allowed to stand at room temperature for 5 min after which a sample was withdrawn and placed on a hemacytometer. Those cells which retained the dye, Trypan Blue, were considered non-viable and those cells which excluded the dye were adjudged viable.

The original cell suspension was adjusted to $1.0 \times 10^6$ viable lymphocytes per ml using TCF as a diluent. One ml (or $1.0 \times 10^6$ viable lymphocytes) of this suspension was placed in a pre-cleaned 16 x 125 mm glass culture tube fitted with a metal SS cap to allow free gas exchange. The cultures were incubated at 37°C in an atmosphere of 5% CO$_2$ prior to, and during, experimentation.
Membrane Active Compounds and Chemotherapeutic Agent

(1) Cyclophosphamide was obtained from the U.S. government under the auspices of "Cancer Chemotherapy," National Cancer Institute (National Institutes of Health). Cyclophosphamide ('Cytoxan' brand, Mead Johnson Laboratories) was supplied as a sterile solution of 100 milligrams of drug without carrier suspended in 1.0 ml of 0.85% sodium chloride in distilled water. The working stock solution was diluted with sterile RPMI-1640 medium without calf serum to a concentration of 100 micrograms cyclophosphamide per ml. This solution was aliquoted to 2.0 ml quantities in sterile ampoules and stored at -20°C until needed.

(2) Iodoacetate was procured as the crystal "Iodoacetic Acid Sodium Salt (Iodide Free)" from Matheson, Coleman and Bell division of Matheson Company Inc. with the molecular formula ICH₂CO₂Na and a molecular weight of 207.94. The stock crystals were stored at 4°C and dissolved to either 10⁻³, 10⁻⁴ or 10⁻⁵ molar quantities in 200 ml of RPMI-1640 medium without calf serum. Each working solution was stored in a glass gas tight bottle at 4°C.

(3) Dimethyl sulfoxide was obtained from Fisher Scientific Co. (Fair Lawn, New Jersey) as a liquid in a one pint brown glass container: molecular formula (CH₃)₂SO with a molecular weight of 78.13, specific gravity at 25°C was 1.1006 gm/cm³, refractive index 1.476 and melting point
18.3°C. For experimental purposes, DMSO was used directly from this stock.

(4) Neuraminidase (Vibrio cholerae - Lot #201315, Calbiochem, San Diego, California) was purchased as 1.0 ml of fluid containing 500 units of activity. The enzyme was stored at 4°C and diluted in cold RPMI-1640 medium without calf serum immediately prior to use.

(5) Phytohemagglutinin-P (Control #5555583, Difco Laboratories, Detroit, Michigan) was provided as a dehydrated powder and stored at 4°C. The powder was rehydrated with 5.0 ml RPMI-1640 medium without calf serum at the time of use. This stock solution of PHA-P was diluted in TCF to a concentration of 5.0 microliters per ml of TCF for addition to cultures.

Standard Experimental Technique

Immediately following the preparation of lymphocyte cultures as previously described, both normal and leukemic cultures were treated with various regimens of drugs, individually and in combinations. Two sets of quadruplicate cultures were prepared for each experimental group, one for measurement of tritiated thymidine incorporation and the other for interferon assay. The drugs were either held in the culture medium for the duration of the experiment, or removed by repeated washing following a recommended incubation time as dictated by the experimental design.
Control cultures were included for each test group. These were arranged in quadruplicate and incubated and processed simultaneously with the experimental cultures. These controls were as follows:

1. Medium control - 3.0 ml TCF
2. Cell control - 3.0 ml TCF with 1.0 x 10⁶ lymphocytes
3. Drug control - 3.0 ml TCF containing only the concentration of drug being tested
4. PHA-P control - 3.0 ml TCF with 5.0 microliters of PHA-P

All cultures were incubated at 37°C in a 5% CO₂ atmosphere for the course of the experiment.

Thymidine Incorporation (Tritium Labelled)

Twenty-four hours prior to termination of the experiment, each culture was pulsed with 1 microCurie (uCi) of tritiated thymidine (supplied as 1.0 milliCurie of thymidine-methyl ³H contained in 1.0 ml of a sterile aqueous solution, Batch #102, activity 5 Curies/millimole, by Amersham/Searle Corporation, Arlington Heights, Illinois). The tritiated thymidine was diluted to 10 uCi per ml with RPMI-1640 medium, without calf serum, as a diluent and stored at 4°C in a foil lined container. After the 24 hour pulse, the samples were processed by the following method which was slightly modified from that described by Caspary and Hughes (112). The samples were centrifuged at 800g for
10 min, the supernates discarded, and the cells resuspended in 2.0 ml of cold TCF and recentrifuged. This process was repeated twice. DNA and protein were precipitated with 2.0 ml ice-cold trichloroacetic acid (TCA) after the addition of two drops of calf serum. Following centrifugation (800g for 10 min) and removal of supernates, the precipitates again were washed with 2.0 ml TCA. Finally, the precipitate pellets were dehydrated by twice washing with 1.0 ml aliquots of cold absolute ethanol, and then dissolved in 0.5 ml NCS (Tissue solubalizer - Amersham/Searle Corp.). The tubes were parafilm sealed and stored at 37°C for 18 hours for solubilization. The dissolved products were transferred to glass counting vials, using three 5 ml quantities of toluene scintillator per vial. The toluene scintillator consisted of 4.0 grams 2,5-diphenyl oxazole (PPO) and 0.25 gram 1,4-bis-2-(5-phenyl oxazolyl)-benzene (POPOP) contained in one liter of "Reagent Grade" toluene (Fisher Scientific). The tritium label was counted in borosilicate glass vials containing a total volume of 15.5 ml fluid using a Packard Tricarb Scintillation Spectrometer model #3375. The samples were dark adapted for 24 hours at 4°C prior to counting. Samples were routinely counted for 10 min at a gain setting of 55% with a window of 55 - 1000. Standard Quench curves were determined by Automatic Standardization (AES) utilizing a built-in gamma source of americium-241 and radium-226 and water as a source of quenching (113). The AES ratios for
the scintillator ranged from 7246 to 7500 for all samples tested. Due to the close approximation of these values when superimposed on a standard quench curve, the data were reported as counts per minute (CPM) rather than the more absolute values of disintegrations per minute (DPM).

**Interferon Assays**

Interferon assays were performed using a cytopathic effect (CPE) inhibition modification of the plaque inhibition system described by Merigan (114). Samples from a parallel set of lymphocyte cultures for each experimental and control group were quick frozen in acetone-dry ice and stored at -70°C. Prior to assay, the samples were thawed at room temperature and the pH lowered to pH 2.0 for incubation at 4°C for 24 hours. Spot samples previously assayed and proven to have antiviral activity were selected from each group for reaction with 0.25% Trypsin and overnight dialysis against a 10-fold excess volume of distilled water and again examined for antiviral activity to aid in the identification of the compound as interferon.

Primary target cell monolayers of human foreskin fibroblasts (H-F cells) were prepared from tissue isolates obtained fresh weekly from The Ohio State University Hospital, Division of Neonatology. Foreskins were stored in Hank's Modified Minimum Essential Medium + 10% calf serum (MEM + 10) (GIBCO and FLOW Labs respectively) with 200 units
of penicillin per ml and 40 milligrams of Mycostatin added per ml of culture medium. The tissue was macerated with blunted scissors, washed and trypsinized in 0.25% Trypsin at 37°C with constant agitation. The detached cells were recovered by centrifugation, resuspended in MEM + 10 and planted in gas tight 250 ml plastic flasks (Falcon Plastics #3024). When the fibroblast monolayers reached confluency (approximately 14 - 21 days) the cell layers were treated with 3 ml of 0.25% Trypsin for 30 min at 37°C and then 17 ml of MEM + 10 was added to stop the reaction. The cell containing fluids were pipetted vigorously to disperse any cell clumps and the cells passaged in a one to two volume split, into 250 ml Falcon plastic flasks. The cell monolayers were maintained in this manner until needed for the titrations.

Vesicular Stomatitis Virus (VSV), was from the culture collection of the Department of Microbiology, The Ohio State University and was propagated in monolayer cultures of secondary mouse L-cells. Infected monolayers were frozen-thawed twice to effect viral release and clarified by centrifugation at 8000 rpm for 30 min in the RC-2B centrifuge at 4°C. The virus was dispersed by sonication, passed thru a 450 mu filter, quick frozen in acetone-dry ice and stored in liquid nitrogen. VSV was titrated in tube cultures of H-F cells with the appearance of CPE serving as an endpoint. The Spearman-Kaerber method (115) was used to determine the
TCID$_{50}$ (50% tissue culture infectious dose).

Micro-system interferon assays were adapted from that described by Barker et al (116). Assays were performed in 24 hour monolayer cultures of H-F cells, grown in Micro Tissue Culture II plates purchased from Falcon Plastics, Division of Bioquest, Los Angeles, California. Microtiter pipettes which deliver 0.05 ml were used to quantitate fluids in this assay system. Samples were acidified for 24 hours, neutralized, and serially diluted in TCF. Two-tenths ml of each dilution was placed in duplicate on 24 hour monolayers and the cultures incubated at 37°C in an atmosphere of 5% CO$_2$ for 24 hours. The monolayers were washed once with Hank's Balanced Salt Solution and challenged with a standard dose of VSV (300 TCID$_{50}$) contained in 0.2 ml of TCF. This dosage of VSV produced 100% CPE in control cultures in 24 hours. Cultures were incubated as before for an additional 24 hours and then examined microscopically at that time. Ten representative fields were counted to determine the extent of CPE. The interferon titer was expressed as the reciprocal of the dilution of test material, 0.2 ml of which protected 50% of the cells in a culture from CPE at a time when control cultures exhibited 100% CPE.
RESULTS

Individual Drug Regimens

A number of workers (N.B. - Table #1) have described the almost totally non-reactive character of the CLL lymphocyte. One of the major reasons offered for this lack of reactivity is the altered nature of the cell membrane (62, 63 and 64), a fact which may account for some of the clinical manifestations observed in the disease (e.g. hypogammaglobuleniemia and blockage of lymphocyte transport at lymph barriers). Most certainly the modified cell membrane accounts for many of the phenomena seen in vitro, among which are included a decreased and delayed response to stimulation with PHA (as measured by incorporation of tritiated thymidine) and a decreased, if not absent capacity to produce interferon. Both of these in vitro correlatens of cell-mediated events are initiated through a primary reaction at the cell membrane level. The response to PHA involves binding of the phytomitogen to glycoprotein receptors on the cell membrane (117). A cell membrane interaction also is a principal non-specific occurrence in the initiation of interferon production following exposure of lymphocytes.
to PHA (118). It would follow that any additional alteration of the cell membrane would result in either an increase or decrease in one or both of these measurements.

Accordingly, CLL lymphocytes were tested for the incorporation of tritiated thymidine and interferon production following treatment with the non-specific membrane modifier, dimethyl sulfoxide. DMSO has been described as a dipolar aprotic solvent which replaces water in molecular hydration sheaths (especially those of proteins) and thereby increases membrane permeabilities by decreasing Zeta potentials (101 and 102). In keeping with the suggestion of Shrek, et al. (108), both normal and CLL lymphocytes were treated with varying concentrations of DMSO, ranging from 0.1% to 2.0% per volume of TCF. Cell viability determinations using the trypan blue exclusion technique confirmed these authors' observation that 2.0% DMSO is preferentially toxic to CLL lymphocytes as compared with normal lymphocytes. Viability counts of lymphocytes, both normal and leukemic, indicated that DMSO had no preferential cytotoxic effect up to and including a concentration of 1.0% (viability counts for normal and leukemic cells were in excess of 86% at 7 days for DMSO concentrations of 0.1%, 0.25%, 0.5% and 1.0%).

Following these preliminary observations, lymphocytes from both normal and leukemic donors were placed in culture as previously described and examined for incorporation of tritiated thymidine and interferon production following
stimulation with PHA in a medium containing varying concentrations of DMSO and according to the following schema.

Normal lymphocytes were cultured in TCF containing 0.1%, 0.25%, 0.5%, 1.0% and 2.0% DMSO by volume for periods of 1, 3, and 7 days. The cultures were pulsed with tritiated thymidine on days 0, 2 and 6 in order to allow 24 hours for the incorporation of the labelled nucleotide into the DNA of cells undergoing replication. The graphs in Figure 1 (N.B. the expanded CPM scale to allow for better visualization of graphic material) indicate that the amount of radioactive nucleotide incorporated in unstimulated normal lymphocytes was constant, regardless of the concentration of DMSO employed. Statistical analysis of these values, using Student's 't' test as a method of excluding paired samples from a Gaussian distribution on a confidence limit basis, revealed that none of these values differed significantly from the values observed in the untreated cell control cultures (the lowest p-value being greater than 0.30). Therefore DMSO did not appear to induce a non-specific uptake of the labelled thymidine.

Assay for the presence of interferon in the supernatant fluid of identical normal cultures in parallel with the tritium labelled cultures showed no detectable amount of anti-viral activity in any of the test samples.

Normal lymphocyte cultures treated with DMSO in a fashion identical to that described above were stimulated
Figure 1. Tritiated thymidine incorporation in cultures of normal human lymphocytes treated with varying doses of dimethyl sulfoxide.
by the addition of 5 microliters per culture of the non-specific mitogen, PHA. As presented in Figure 2, the DMSO treated normal cells were stimulated to incorporate from 3 to 4 fold the amount of tritiated thymidine routinely reported to be incorporated by untreated normal lymphocytes exposed to PHA (39 and 40). Mean counts per minute for untreated, PHA stimulated normal cells (8 samples-duplicate experiments) were 33,351 after three days of culture. Similar cultures but containing 0.5% DMSO, exhibited counts per minute in excess of 110,000. This same pattern held for all of the concentrations of DMSO tested, in which the test samples gave significantly higher counts (p less than 0.01 for all value comparisons) than did the untreated but PHA stimulated controls.

Assay for interferon production in the DMSO treated, PHA stimulated normal cells exhibited a pattern of increased release (Figure 3). Samples of the supernates taken at 1, 3, and 7 days from duplicate cultures to those described above, showed a one to two fold increase over values of control cultures. Those samples exhibiting antiviral activity were tested further in order to confirm the compound as interferon. The samples which were treated to pH 2.0 for 24 hours at 4°C and then neutralized, retained antiviral activity (119). Similar samples when exposed to 200 micrograms per ml of Trypsin for 15 minutes at 37°C lost all antiviral activity (120), however heat treatment at 44°C
Figure 2. Tritiated thymidine incorporation in cultures of normal human lymphocytes treated simultaneously with varying doses of dimethyl sulfoxide and a constant quantity of phytohemagglutinin.
Figure 3. Interferon production in cultures of normal human lymphocytes treated simultaneously with varying doses of dimethyl sulfoxide and a constant quantity of phytohemagglutinin.
for one half hour did not adversely affect antiviral activity, whereas heating at 60°C for the same period destroyed that activity (121).

Exposure of cultures of lymphocytes from patients with CLL to DMSO in concentrations of 0.1%, 0.25%, 0.5%, 1.0% and 2.0% resulted in a response similar to that described for the normal cell cultures (Figure 4). CLL lymphocytes treated only with DMSO for periods of 1, 3 and 7 days incorporated labelled thymidine at a rate statistically identical to that observed for the untreated leukemic cells. The only exceptions were those cultures treated with 2.0% DMSO which, as previously noted, was due to loss of cell viability (N.B. expanded CPM scale necessary for graphic representation of data). There was no indication of interferon production or release in any of the test samples assayed within this group.

CLL lymphocytes simultaneously treated with DMSO and stimulated with PHA showed a 6 to 6.5 fold increase in tritiated thymidine incorporation at three days. (Figure 5) It should be recalled that the normal pattern reported and reproduced for PHA stimulation of CLL lymphocytes is one of a delayed response in which the cells maximally incorporate nucleotide within 5 to 7 days. However, cells cultured in DMSO responded maximally at three days, a pattern similar to that noted for normal lymphocytes, with the stimulated cultures attaining over 13,000 CPM as compared with 2,000 CPM
Figure 4. Tritiated thymidine incorporation in cultures of CLL lymphocytes treated with varying doses of dimethyl sulfoxide.
Figure 5. Tritiated thymidine incorporation in cultures of CLL lymphocytes treated simultaneously with varying doses of dimethyl sulfoxide and a constant quantity of phytohemagglutinin.
for the untreated cells. Again, the 2.0% concentration of DMSO was toxic to CLL lymphocyte cultures and abrogated all response. Cell responses at one and seven days of culture did not differ significantly from the response of the control cultures.

Supernates from DMSO-treated, PHA stimulated cultures of CLL lymphocytes demonstrated a consistent but modest increase in interferon production, as may be noted in Figure 6. Samples possessing antiviral activity were treated further and the biological and physical properties of interferon were confirmed in the usual manner.

Other experiments were performed with both normal and CLL lymphocyte cultures, in which DMSO was applied for one hour and then removed from the cultures by three vigorous washings with fresh TCF. Following this, the cultures were prepared as for that described for the normal and leukemic cells in the previous groups. CPM values for normal and CLL lymphocytes at all test periods and over the range of DMSO concentrations showed that removal of DMSO permitted all counts to return to values which were essentially those recorded for untreated cultures.

Iodoacetate, it may be recalled, is a powerful sulfhydryl inhibitor which inhibits the rebonding of the disulfide bonds of proteins on the surfaces of cells (92). Knock (93) has suggested that leukemic cells react more strongly with iodoacetate due to an increased availability of SH groups.
Figure 6. Interferon production in cultures of CLL lymphocytes treated simultaneously with varying doses of dimethyl sulfoxide and a constant quantity of phytohemagglutinin.
The lymphocyte of the CLL patient not only has more available SH groups, but also a relatively heavy coating of Immunoglobulin light chain receptors (35) of a proteinaceous character. Because of this possible masking, treatment of leukemic cells *in vitro* with iodoacetate does not decrease the number of viable cells. However, it does alter protein rebonding capacity and therefore alters the cell surface and the cellular response to surface phenomena involving membrane receptors. Therefore, normal and leukemic lymphocytes were treated with varying doses of iodoacetate and incubated for specified periods with each drug dose to determine the effect of iodoacetate upon cell reactivity to PHA. From Table 3 it is apparent that the treatment of lymphocytes with $10^{-3}$ molar iodoacetate resulted in a total abolition of blast response and interferon production in both normal and leukemic cell cultures. Viability counts performed on these cells indicated at least a 60% viability in all cultures after treatment with iodoacetate. These cells were cultured in iodoacetate containing RMPI-1640 without calf serum for the periods expressed, and then freed of the drug by three washings with fresh TCF. The cells then were resuspended in fresh TCF, either with or without PHA and cultured for 7 days under normal culture conditions.

Normal and CLL lymphocytes cultured under identical conditions then were treated with $10^{-4}$ molar iodoacetate for initial exposures of 10 and 30 minutes (Table 4).
TABLE 3
INCORPORATION OF TRITIATED THYMIDINE AND INTERFERON PRODUCTION BY NORMAL AND LEUKEMIC LYMPHOCYTES FOLLOWING TREATMENT WITH $10^{-3}$ MOLAR IODOACETATE (WITH AND WITHOUT PHYTOHEMAGGLUTININ)

<table>
<thead>
<tr>
<th>TEST CULTURE</th>
<th>NORMAL</th>
<th>LEUKEMIC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEAN CPM$^A$</td>
<td>INTERFERON$^B$</td>
</tr>
<tr>
<td>Iodoacetate - 10 Min</td>
<td>44 ± 8</td>
<td>4</td>
</tr>
<tr>
<td>Iodoacetate + PHA - 10 Min$^C$</td>
<td>92 ± 27</td>
<td>4</td>
</tr>
<tr>
<td>Iodoacetate - 20 Min</td>
<td>40 ± 6</td>
<td>4</td>
</tr>
<tr>
<td>Iodoacetate + PHA - 20 Min</td>
<td>79 ± 8</td>
<td>4</td>
</tr>
<tr>
<td>Iodoacetate - 30 Min</td>
<td>35 ± 11</td>
<td>4</td>
</tr>
<tr>
<td>Iodoacetate + PHA - 30 Min</td>
<td>84 ± 11</td>
<td>4</td>
</tr>
</tbody>
</table>

$^A$Mean counts per minute for 8 samples (duplicate experiments).

$^B$Titer expressed as the reciprocal of the dilution of test material, 0.2 ml of which protected 50% of the cells.

$^C$Initial exposure time to iodoacetate, followed by three washes and addition of PHA.
TABLE 4
INCORPORATION OF TRITIATED THYMIDINE AND INTERFERON PRODUCTION BY NORMAL AND LEUKEMIC LYMPHOCYTES FOLLOWING TREATMENT WITH $10^{-4}$ MOLAR IODOACETATE (WITH AND WITHOUT PHYTOHEMAGGLUTININ)

<table>
<thead>
<tr>
<th>TEST CULTURE</th>
<th>NORMAL</th>
<th></th>
<th>LEUKEMIC</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEAN CPM$^A$</td>
<td>INTERFERON$^B$</td>
<td>MEAN CPM</td>
<td>INTERFERON $^B$</td>
</tr>
<tr>
<td>Cell Control</td>
<td>8909 ± 54</td>
<td>4</td>
<td>2499 ± 217</td>
<td>4</td>
</tr>
<tr>
<td>Cell Control + PHA</td>
<td>246764 ± 6848</td>
<td>256</td>
<td>5621 ± 129</td>
<td>32</td>
</tr>
<tr>
<td>Iodoacetate - 10 Min</td>
<td>56 ± 6</td>
<td>4</td>
<td>46 ± 6</td>
<td>4</td>
</tr>
<tr>
<td>Iodoacetate + PHA - 10 Min$^C$</td>
<td>176 ± 6</td>
<td>4</td>
<td>41 ± 10</td>
<td>4</td>
</tr>
<tr>
<td>Iodoacetate - 30 Min</td>
<td>42 ± 3</td>
<td>4</td>
<td>38 ± 6</td>
<td>4</td>
</tr>
<tr>
<td>Iodoacetate + PHA - 30 Min$^C$</td>
<td>140 ± 17</td>
<td>4</td>
<td>68 ± 8</td>
<td>4</td>
</tr>
</tbody>
</table>

$^A$Mean counts per minute for 8 samples (duplicate experiments).

$^B$Titer expressed as the reciprocal of the dilution of test material, 0.2 ml of which protected 50% of the cells.

$^C$Initial exposure time to iodoacetate, followed by three washes and addition of PHA.
Incorporation of tritiated thymidine was not significantly increased above background for either the normal or the leukemic cell cultures. No interferon production was noted in any of the cultures from either cell source.

Conversely, normal cells treated with $10^{-5}$ molar iodoacetate (Table 5) retain approximately half (at 10 minutes) of the thymidine incorporation rate as compared with untreated normal cells. These iodoacetate treated normal cells also produced interferon but at depressed levels following stimulation with PHA. To the contrary, CLL lymphocytes treated with $10^{-5}$ molar iodoacetate neither incorporated labelled thymidine nor produced interferon.

As previously cited, neuraminidase probably functions by altering cell membrane characteristics through the removal of sialomucins present in the matrix of the membrane (75). This sialic acid digestion increases cellular deformability and mobilization or unmasking of antigenic sites and membrane receptors. NASE has been shown to induce modulation of PHA receptors and to essentially "freeze" these glycoprotein receptors until they are regenerated during the period of membrane turnover (117). The effect of neuraminidase on sialic acid residues in membranes has been widely studied in many systems. Holland has reported the use of NASE extensively in the treatment of leukemic cells for immunoprophylaxis in vivo (80). It would seem pertinent then to examine CLL lymphocytes, which have been shown to
TABLE 5

INCORPORATION OF TRITIATED THYMIDINE AND INTERFERON PRODUCTION BY NORMAL AND LEUKEMIC LYMPHOCYTES FOLLOWING TREATMENT WITH $10^{-5}$ MOLAR IODOACETATE (WITH AND WITHOUT PHYTOHEMAGGLUTININ)

<table>
<thead>
<tr>
<th>TEST CULTURE</th>
<th>NORMAL</th>
<th></th>
<th>LEUKEMIC</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEAN CPM$^A$</td>
<td>INTERFERON$^B$</td>
<td>MEAN CPM</td>
<td>INTERFERON</td>
</tr>
<tr>
<td>Cell Control</td>
<td>8909 ± 54</td>
<td>4</td>
<td>2499 ± 217</td>
<td>4</td>
</tr>
<tr>
<td>Cell Control + PHA</td>
<td>246764 ± 6848</td>
<td>256</td>
<td>5621 ± 129</td>
<td>4</td>
</tr>
<tr>
<td>Iodoacetate - 10 Min</td>
<td>5315 ± 329</td>
<td>4</td>
<td>85 ± 3</td>
<td>4</td>
</tr>
<tr>
<td>Iodoacetate + PHA - 10 Min$^C$</td>
<td>94327 ± 4340</td>
<td>64</td>
<td>104 ± 9</td>
<td>4</td>
</tr>
<tr>
<td>Iodoacetate - 30 Min</td>
<td>2470 ± 129</td>
<td>4</td>
<td>63 ± 7</td>
<td>4</td>
</tr>
<tr>
<td>Iodoacetate + PHA - 30 Min</td>
<td>54477 ± 4490</td>
<td>64</td>
<td>84 ± 8</td>
<td>4</td>
</tr>
</tbody>
</table>

$^A$Mean counts per minute for 8 samples (duplicate experiments).

$^B$Titer expressed as the reciprocal of the dilution of the test material, 0.2 ml of which protected 50% of the cells.

$^C$Initial exposure time to iodoacetate, followed by three washes and addition of PHA.
have increased sialomucins in the cell membrane, with NASE as a method of mobilizing PHA receptors.

Accordingly, normal and CLL lymphocytes were treated with NASE at a recommended dosage of 5 units per 1.0 x 10^6 cells, for periods of 30 and 60 minutes (80). The enzyme was washed from the cultures by repeated centrifugation, and the cells resuspended in either fresh TCF or TCF containing PHA. In keeping with the reports of others (122), the incorporation of labelled thymidine was found to be decreased in the cultures of normal lymphocytes with a concomitant decrease in interferon production (Table 6). However, CLL lymphocyte cultures treated with the same protocol demonstrated an opposite trend, with the rate of thymidine incorporation and interferon production increasing as the exposure of the cell cultures to the enzyme was increased.

Treatment Regimens involving Drugs in Combination

In view of the foregoing data involving the use of compounds which alter cell membranes either in a specific (NASE and iodoacetate) or non-specific (DMSO) manner, it appeared that meaningful information might be obtained regarding membrane and the associated cellular reactivity by utilizing combinations of these compounds in the treatment of test cells.

Normal and leukemic lymphocyte cultures were treated with iodoacetate and DMSO as outlined in Table 7 and 8.
TABLE 6

INCORPORATION OF TRITIATED THYMIDINE AND INTERFERON PRODUCTION BY NORMAL AND LEUKEMIC LYMPHOCYTES FOLLOWING TREATMENT WITH NEURAMINIDASE (WITH AND WITHOUT PHYTOHEMAGGLUTININ)

<table>
<thead>
<tr>
<th>TEST CULTURE</th>
<th>NORMAL</th>
<th></th>
<th>LEUKEMIC</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEAN CPM</td>
<td>INTERFERON</td>
<td>MEAN CPM</td>
<td>INTERFERON</td>
</tr>
<tr>
<td>Cell Control</td>
<td>8909 ± 54</td>
<td>4</td>
<td>1549 ± 49</td>
<td>4</td>
</tr>
<tr>
<td>Cell Control + PHA</td>
<td>246764 ± 6848</td>
<td>256</td>
<td>3105 ± 106</td>
<td>16</td>
</tr>
<tr>
<td>Neuraminidase - 30 MinC</td>
<td>998 ± 66</td>
<td>4</td>
<td>2330 ± 231</td>
<td>4</td>
</tr>
<tr>
<td>Neuraminidase + PHA - 30 Min</td>
<td>187349 ± 7226</td>
<td>64</td>
<td>6435 ± 456</td>
<td>32</td>
</tr>
<tr>
<td>Neuraminidase - 60 Min</td>
<td>7686 ± 326</td>
<td>4</td>
<td>4623 ± 503</td>
<td>4</td>
</tr>
<tr>
<td>Neuraminidase + PHA - 60 Min</td>
<td>12697 ± 132</td>
<td>32</td>
<td>10088 ± 233</td>
<td>64</td>
</tr>
</tbody>
</table>

A Mean counts per minute for 8 samples (duplicate experiments).
B Titer expressed as the reciprocal of the dilution of test material, 0.2 ml of which protected 50% of the cells.
C Cells were treated with neuraminidase, washed three times and resuspended in fresh medium.
TABLE 7

INTEGRATION OF Tritiated Thymidine AND INTERFERON PRODUCTION BY NORMAL AND LEUKEMIC LYMPHOCYTES FOLLOWING TREATMENT WITH $10^{-3}$ MOLAR IODOACETATE AND EITHER 0.25% OR 0.5% DIMETHYL SULFOXIDE (WITH AND WITHOUT PHYTOHEMAGGLUTININ)

<table>
<thead>
<tr>
<th>TEST CULTURE</th>
<th>NORMAL MEAN CPM</th>
<th>INTERFERON</th>
<th>LEUKEMIC MEAN CPM</th>
<th>INTERFERON</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Control</td>
<td>8909 + 54</td>
<td>4</td>
<td>2499 + 217</td>
<td>4</td>
</tr>
<tr>
<td>Cell Control + PHA</td>
<td>246764 + 6948</td>
<td>256</td>
<td>5621 + 129</td>
<td>32</td>
</tr>
<tr>
<td>Iodoacetate + 0.25% DMSO</td>
<td>52 + 8</td>
<td>4</td>
<td>46 + 5</td>
<td>4</td>
</tr>
<tr>
<td>Iodoacetate + PHA + 0.25% DMSO</td>
<td>96 + 7</td>
<td>4</td>
<td>58 + 11</td>
<td>4</td>
</tr>
<tr>
<td>Iodoacetate + 0.5% DMSO</td>
<td>71 + 9</td>
<td>4</td>
<td>21 + 2</td>
<td>4</td>
</tr>
<tr>
<td>Iodoacetate + PHA + 0.5% DMSO</td>
<td>93 + 1</td>
<td>4</td>
<td>75 + 8</td>
<td>4</td>
</tr>
</tbody>
</table>

A Mean counts per minute for 8 samples (duplicate experiments).

B Titer expressed as the reciprocal of the dilution of test material, 0.2 ml of which protected 50% of the cells.

C Cells were treated with iodoacetate for 30 min, washed three times and resuspended in medium containing DMSO or DMSO + PHA.
TABLE 8

INCORPORATION OF TRITIATED THYMIDINE AND INTERFERON PRODUCTION BY NORMAL AND LEUKEMIC LYMPHOCYTES FOLLOWING TREATMENT WITH 10^{-5} MOLAR IODOACETATE AND EITHER 0.25% OR 0.5% DIMETHYL SULFOXIDE (WITH AND WITHOUT PHYTOHEMAGGLUTININ)

<table>
<thead>
<tr>
<th>TEST CULTURE</th>
<th>NORMAL MEAN CPM(^A)</th>
<th>NORMAL INTERFERON(^B)</th>
<th>LEUKEMIC MEAN CPM</th>
<th>LEUKEMIC INTERFERON</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Control</td>
<td>8909 ± 54</td>
<td>4</td>
<td>2499 ± 217</td>
<td>4</td>
</tr>
<tr>
<td>Cell Control + PHA</td>
<td>246764 ± 6848</td>
<td>256</td>
<td>5621 ± 129</td>
<td>32</td>
</tr>
<tr>
<td>Iodoacetate + 0.25% DMSO(^C)</td>
<td>4199 ± 96</td>
<td>4</td>
<td>112 ± 12</td>
<td>4</td>
</tr>
<tr>
<td>Iodoacetate + PHA + 0.25% DMSO</td>
<td>40561 ± 1577</td>
<td>64</td>
<td>173 ± 25</td>
<td>4</td>
</tr>
<tr>
<td>Iodoacetate + 0.5% DMSO</td>
<td>5483 ± 207</td>
<td>4</td>
<td>177 ± 21</td>
<td>4</td>
</tr>
<tr>
<td>Iodoacetate + PHA + 0.5% DMSO</td>
<td>55080 ± 3855</td>
<td>64</td>
<td>118 ± 39</td>
<td>4</td>
</tr>
</tbody>
</table>

\(A\) Mean counts per minute for 8 samples (duplicate experiments).

\(B\) Titer expressed as the reciprocal of the dilution of test material, 0.2 ml of which protected 50% of the cells.

\(C\) Cells were treated with iodoacetate for 30 min, washed three times and resuspended in medium containing DMSO or DMSO + PHA.
The lymphocytes were admixed with either $10^{-3}$ or $10^{-5}$ molar iodoacetate for 30 minutes in a medium devoid of DMSO. The cells then were thrice washed and resuspended in medium containing either DMSO, or DMSO plus PHA. From Table 7 it may be seen that the $10^{-3}$ molar concentration of iodoacetate negated all responsiveness in both the normal and the leukemic cell cultures. However, exposure to $10^{-5}$ molar concentration of this agent permitted normal cultures to return to some level of reactivity after being subsequently treated with DMSO. This level of activity did not differ significantly from the level of incorporation reported in Table 5, in which cells were treated only with $10^{-5}$ molar iodoacetate. The CLL lymphocytes remained inactive after treatment with this latter concentration of iodoacetate.

In parallel experiments, normal and leukemic lymphocytes were treated simultaneously with $10^{-5}$ molar iodoacetate and DMSO, washed and resuspended in DMSO-containing medium. The simultaneous exposure to both compounds completely eliminated thymidine uptake and interferon production in both the normal and the leukemic cells.

Assuming that NASE increases membrane deformability and fluidity, the application of DMSO in conjunction with this enzyme should result in an enhancement of this effect. Indeed, treatment of normal and leukemic lymphocytes with NASE followed by washing and resuspension in DMSO or DMSO plus PHA containing medium increased labelled thymidine
incorporation in both types of cultures to the highest values observed in any of the test situations (Table 9). Interferon titers remained unchanged in the normal cell cultures, but increased two-fold in the supernates from cultures of leukemic cells. The duration of exposure to the enzyme, whether 30 or 60 minutes, had no significant effect on the level of reactivity.

Recalling that all of the CLL patients included in this study were resistant to maintainence doses of cyclophosphamide, it seemed relevant to examine the effect of one of the membrane-modifying compounds on the action of this drug in vitro. Although the cytocidal action of cyclophosphamide has not been completely determined it appears that in vivo the compound must be processed through the liver which in turn produces an active chemical intermediate (59). However, the unactivated compound itself is cytotoxic in vitro if administered in sufficient concentrations. Assuming the possibility that DMSO could enhance the effect of the compound in vitro, it most certainly would suggest additional serious investigation.

Varying concentrations of cyclophosphamide (10, 50 and 100 micrograms/ml) were added to the suspending medium of normal and leukemic cells for three days and the rate of incorporation of labelled nucleotide was recorded as an index of cell reactivity.

Cyclophosphamide was less effective in eliminating
TABLE 9

INCORPORATION OF TRITIATED THYMIDINE AND INTERFERON PRODUCTION BY NORMAL AND LEUKEMIC LYMPHOCYTES FOLLOWING TREATMENT WITH CYCLOPHOSPHAMIDE

<table>
<thead>
<tr>
<th>TEST CULTURE</th>
<th>NORMAL</th>
<th>LEUKEMIC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEAN CPM(^a)</td>
<td>INTERFERON(^b)</td>
</tr>
<tr>
<td>Cell Control</td>
<td>8909 ± 54</td>
<td>4</td>
</tr>
<tr>
<td>Cell Control + PHA</td>
<td>246764 ± 6848</td>
<td>256</td>
</tr>
<tr>
<td>Cyclophosphamide(^c) -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 Micrograms</td>
<td>468 ± 39</td>
<td>4</td>
</tr>
<tr>
<td>10 Micrograms + PHA</td>
<td>9856 ± 245</td>
<td>16</td>
</tr>
<tr>
<td>50 Micrograms</td>
<td>75 ± 9</td>
<td>4</td>
</tr>
<tr>
<td>50 Micrograms + PHA</td>
<td>45 ± 3</td>
<td>4</td>
</tr>
<tr>
<td>100 Micrograms</td>
<td>65 ± 13</td>
<td>4</td>
</tr>
<tr>
<td>100 Micrograms + PHA</td>
<td>81 ± 8</td>
<td>4</td>
</tr>
</tbody>
</table>

\(^a\)Mean counts per minute for 8 samples (duplicate experiments).

\(^b\)Titer expressed as the reciprocal of the dilution of test material, 0.2 ml of which protected 50% of the cells.

\(^c\)Cyclophosphamide remained in culture for the first 24 hours of culture, the cells then were washed three times and resuspended in fresh medium.
responses in CLL lymphocyte cultures than in normal cell cultures (Table 9). This is probably related to the lowered metabolic index of the leukemic cell. As anticipated, the addition of DMSO to the medium reduced all counts to background levels (Table 10). Viability counts performed on cultures of cells treated with cyclophosphamide and DMSO confirmed the fact that cell viability had dropped below 10%. Therefore, tentatively it is assumed that treatment with DMSO increased the penetration of the cyclophosphamide into the cell.

It was thought that the relationship of the functions of iodoacetate and NASE at the molecular level might provide some insight into the nature of the alteration of the cell membrane of the CLL cell. Neuraminidase would appear to increase membrane mobility by the cleaving of sialomucin bridges, whereas iodoacetate may function to alter the more superficial protein moieties of the cell (as well as some penetration and complexing of mitotic proteins). Thus, sequential treatment with these compounds may tend to cancel (or possibly enhance) the effect of one another depending on the molecular configurations of the membrane under examination.

To test this normal and leukemic cells were treated with a regimen of $10^{-3}$ molar iodoacetate and 5 units of NASE per $1.0 \times 10^6$ cells in the sequence outlined in Table 12. It was noted that the response of normal cells was not
INCORPORATION OF TRITIATED THYMIDINE AND INTERFERON PRODUCTION BY NORMAL AND LEUKEMIC LYMPHOCYTES FOLLOWING TREATMENT WITH NEURAMINIDASE AND DIMETHYL SULFOXIDE (WITH AND WITHOUT PHYTOHEMAGGLUTININ)

<table>
<thead>
<tr>
<th>TEST CULTURE</th>
<th>NORMAL MEAN CPM</th>
<th>NORMAL INTERFERON</th>
<th>LEUKEMIC MEAN CPM</th>
<th>LEUKEMIC INTERFERON</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Control</td>
<td>8909 ± 54</td>
<td>4</td>
<td>1549 ± 49</td>
<td>4</td>
</tr>
<tr>
<td>Cell Control + PHA</td>
<td>246764 ± 6848</td>
<td>256</td>
<td>3105 ± 106</td>
<td>16</td>
</tr>
<tr>
<td>Neuraminidase - 60 Min 0.25% DMSO</td>
<td>21227 ± 268</td>
<td>4</td>
<td>5995 ± 105</td>
<td>4</td>
</tr>
<tr>
<td>Neuraminidase - 60 Min 0.25% DMSO + PHA</td>
<td>297622 ± 888</td>
<td>256</td>
<td>11100 ± 1204</td>
<td>64</td>
</tr>
<tr>
<td>Neuraminidase - 60 Min 0.5% DMSO</td>
<td>19783 ± 801</td>
<td>4</td>
<td>5049 ± 50</td>
<td>4</td>
</tr>
<tr>
<td>Neuraminidase - 60 Min 0.5% DMSO + PHA</td>
<td>290280 ± 3850</td>
<td>256</td>
<td>11826 ± 1832</td>
<td>64</td>
</tr>
</tbody>
</table>

A Mean counts per minute for 8 samples (duplicate experiments).

B Titer expressed as the reciprocal of the dilution of test material, 0.2 ml of which protected 50% of the cells.

C Cells were treated with neuraminidase, washed three times and resuspended in medium containing either DMSO or DMSO + PHA.
TABLE 11
INCORPORATION OF TRITIATED THYMIDINE AND INTERFERON PRODUCTION BY NORMAL AND LEUKEMIC LYMPHOCYTES FOLLOWING TREATMENT WITH CYCLOPHOSPHAMIDE AND DIMETHYL SULFOXIDE (WITH AND WITHOUT PHYTOHEMAGGLUTININ)

<table>
<thead>
<tr>
<th>TEST CULTURE</th>
<th>NORMAL MEAN CPM&lt;sup&gt;A&lt;/sup&gt;</th>
<th>NORMAL INTERFERON&lt;sup&gt;B&lt;/sup&gt;</th>
<th>LEUKEMIC MEAN CPM</th>
<th>LEUKEMIC INTERFERON</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophosphamide - DMSO&lt;sup&gt;C&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 micrograms - 0.5%</td>
<td>37 ± 5</td>
<td>4</td>
<td>86 ± 12</td>
<td>4</td>
</tr>
<tr>
<td>10 micrograms - 0.5% (PHA)</td>
<td>85 ± 9</td>
<td>4</td>
<td>64 ± 7</td>
<td>4</td>
</tr>
<tr>
<td>50 micrograms - 0.5%</td>
<td>46 ± 2</td>
<td>4</td>
<td>59 ± 18</td>
<td>4</td>
</tr>
<tr>
<td>50 micrograms - 0.5% (PHA)</td>
<td>30 ± 8</td>
<td>4</td>
<td>4 ± 12</td>
<td>4</td>
</tr>
<tr>
<td>100 micrograms - 0.5%</td>
<td>46 ± 14</td>
<td>4</td>
<td>26 ± 6</td>
<td>4</td>
</tr>
<tr>
<td>100 micrograms - 0.5% (PHA)</td>
<td>64 ± 7</td>
<td>4</td>
<td>52 ± 3</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>A</sup>Mean counts per minute for 8 samples (duplicate experiments).

<sup>B</sup>Titer expressed as the reciprocal of the dilution of test material, 0.2 ml of which protected 50% of the cells.

<sup>C</sup>Cyclophosphamide remained in culture for the first 24 hours in medium containing dimethyl sulfoxide, the cells were washed and resuspended in fresh medium containing dimethyl sulfoxide but no cyclophosphamide.
<table>
<thead>
<tr>
<th>Test Culture</th>
<th>Normal</th>
<th>Leukemic</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean CPM&lt;sup&gt;A&lt;/sup&gt;</td>
<td>Interferon&lt;sup&gt;B&lt;/sup&gt;</td>
<td>Mean CPM</td>
<td>Interferon</td>
</tr>
<tr>
<td>Iodoacetate + Neuraminidase (30 Min&lt;sup&gt;C&lt;/sup&gt;)</td>
<td>55 ± 3</td>
<td>4</td>
<td>62 ± 11</td>
<td>4</td>
</tr>
<tr>
<td>Iodoacetate + PHA Neuraminidase (30 Min)</td>
<td>83 ± 4</td>
<td>4</td>
<td>230 ± 26</td>
<td>4</td>
</tr>
<tr>
<td>Iodoacetate * Neuraminidase (60 Min)</td>
<td>98 ± 4</td>
<td>4</td>
<td>429 ± 27</td>
<td>4</td>
</tr>
<tr>
<td>Iodoacetate + PHA Neuraminidase (60 Min)</td>
<td>114 ± 12</td>
<td>4</td>
<td>2327 ± 272</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>A</sup>Mean counts per minute for 8 samples (duplicate experiments).

<sup>B</sup>Titer expressed as the reciprocal of the dilution of test material, 0.2 ml of which protected 50% of the cells.

<sup>C</sup>Cells were treated with iodoacetate for 30 min, washed three times, retreated with neuraminidase, again washed three times and resuspended in fresh medium.
changed significantly, while CLL cells incorporated increasing quantities of thymidine with increases in exposure to NASE.

Normal cells treated with $10^{-5}$ molar iodoacetate and NASE according to the same protocol retained some reactivity after the 60 minute exposure to the enzyme (Table 13). Leukemic cells treated similarly demonstrated total reactivity of incorporation after a 60 minute exposure to NASE, but produced only one-half the level of interferon.

**Treatment Regimen Involving Three Drugs**

Obviously the interaction of three drugs, none of which has been totally defined as to pharmacologic action, with the membrane of a cell will result in some interesting, but difficult to interpret data. In another sense, however, the data obtained may suggest a trend which might provide useful information. For this purpose, normal and leukemic cell cultures were treated with a sequential regimen of optimal drug concentrations (i.e. $10^{-3}$ molar iodoacetate, 5 units of NASE and 0.5% DMSO in that order). The results indicated that in cultures of leukemic cells the addition of DMSO enhanced the increase in reactivity previously noted in cultures of cells which had been initially treated with iodoacetate and subsequently exposed to NASE (Table 12).
<table>
<thead>
<tr>
<th>TEST CULTURE</th>
<th>NORMAL MEAN CPM&lt;sup&gt;A&lt;/sup&gt;</th>
<th>NORMAL INTERFERON&lt;sup&gt;B&lt;/sup&gt;</th>
<th>LEUKEMIC MEAN CPM</th>
<th>LEUKEMIC INTERFERON</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodoacetate + Neuraminidase (30 Min&lt;sup&gt;C&lt;/sup&gt;)</td>
<td>173 ± 17</td>
<td>4</td>
<td>2205 ± 105</td>
<td>4</td>
</tr>
<tr>
<td>Iodoacetate + PHA Neuraminidase (30 Min)</td>
<td>289 ± 33</td>
<td>4</td>
<td>4311 ± 279</td>
<td>16</td>
</tr>
<tr>
<td>Iodoacetate + Neuraminidase (60 Min)</td>
<td>495 ± 6</td>
<td>4</td>
<td>2403 ± 58</td>
<td>4</td>
</tr>
<tr>
<td>Iodoacetate + PHA Neuraminidase (60 Min)</td>
<td>9595 ± 641</td>
<td>4</td>
<td>5336 ± 313</td>
<td>16</td>
</tr>
</tbody>
</table>

<sup>A</sup> Mean counts per minute for 8 samples (duplicate experiments).

<sup>B</sup> Titers expressed as the reciprocal of the dilution of test material, 0.2 ml of which protected 50% of the cells.

<sup>C</sup> Cells were treated with iodoacetate for 30 min, washed three times, treated with neuraminidase, again washed three times and resuspended in fresh medium.
DISCUSSION

Relevant to the discussion of the data presented herein would be a brief reemphasis of some of the characteristics of the CLL lymphocyte.

Grossly, the CLL lymphocyte is indistinguishible from the normal peripheral lymphocyte, even at the level of electron microscopic examination. However, the leukemic cell is notably impaired functionally. For example, an \textit{in vivo} dysfunction such as blockage at lymph barriers (19) may be due to membrane incompatibility between the lymphocyte and the stromal elements of the lymph node and spleen. Extensive comparison of normal and leukemic cells \textit{in vitro} has revealed other significant differences, among these are adherence to polystyrene bead columns (17), ultrasensitivity to colchicine (18), reduction in some cyclic AMP triggered mechanisms (Table 1), and increased sialic acid content (63). All of the above focus on the abnormal qualities of the cell membrane. Recalling the previous considerations of the mechanism of labelled thymidine incorporation and interferon induction following PHA stimulation, these cellular reactions also may be classed as membrane phenomena, at
least in the initial phases. Presumably, any further alteration of the membrane should result in changes in these cellular manifestations. These changes would be due to either a non-specific increase or to a decrease in membrane permeability, or to a qualitative or quantitative change in membrane bound receptors.

The interferon mechanism, which is generally considered a facet of the cellular immune response, exhibited a pattern of trends similar to those observed for the thymidine incorporation data. However, the uptake of thymidine by lymphocytes seemed to be a more sensitive index of cellular reactivity. Only in situations in which there was a considerable variation in the rate of nucleotide incorporation was there any change in the interferon titer. The interferon titers observed in supernates from cultures of leukemic cells were not in themselves significant (the maximal titer observed for all experimental CLL cultures was 64) but the trend represented was of some significance. Therefore, much of the data is expressed in terms of the tritium incorporation results with the general comments such as "cellular reactivity" referring to both measurements.

The addition of DMSO to cultures of normal lymphocytes did not increase the penetration of labelled thymidine into the interior of the cell, nor did it result in the triggering of DNA replication as measured by uptake of the tritium label (Figure 1). The further exposure of normal lympho-
cytes to PHA in the presence of DMSO elicited an amplified response which was due to increased internalization of PHA, either by simple enhancement of penetration or, more realistically, to an increased mobility of membrane receptors in the presence of DMSO resulting in a higher binding affinity for the mitogen (Figure 2). These data are supported by the findings of Adler, et al. who reported that PHA became bound to the surface of normal lymphocytes and then became internalized through a pinocytic phenomenon (117). Obviously, membrane fluidity would be increased by the presence of DMSO through the reduction of hydrostatic forces between molecules, and thereby would optimize a membrane manipulative process such as pinocytosis.

As previously mentioned, CLL lymphocytes were preferentially inactivated by a dose of DMSO, including, and in excess of, 2.0% by volume. This preferential cytotoxicity was probably linked to an exaggerated membrane response. Since the membrane of the CLL lymphocyte is highly restrictive to receptor migration, mitogen binding and pinocytosis because of an increase in hydrophilic moieties in the membrane (e.g. sialic acids and monoclonal globulins), it would be more definitive to examine the consequences of lowered concentrations of DMSO on the ability of these cells to bind and internalize PHA. From the data (Figure 4), it is apparent that DMSO had no detectable toxic effects upon leukemic cells up to a concentration of 2.0% as was true for
normal cells (the unusual reaction of leukemic cells at 2.0% DMSO being previously noted). CLL Lymphocytes also had a delayed and lowered response to PHA which was reasonable upon considering the nature of the membrane. However, cultures of CLL lymphocytes in which DMSO was present for the duration of the exposure of the cells to PHA, showed a significantly enhanced level of stimulation (Figure 5). Again this may be explained by increased internalization of PHA through an augmentation of the pinocytic process. There is, however, a more detailed but related explanation based on this fluid mosaic model of membrane structure presented by Singer and Nicolson (9 and 10). It is their contention that the plasma membrane of the mammalian cell is a fluid mosaic structure in which hydrophobic and hydrophilic forces are the ruling factors governing molecular configuration. Phospholipids presumably are arranged in a discontinuous bilayer in which integral proteins are embedded, with the non-polar amino acid residues in contact with phospholipid and ionic amino acid residues protruding from the layer to contact the external aqueous environment. The integral proteins described are probably associated with glyco- or phospholipids and thereby correspond in nature to the glycoprotein surface receptors for PHA. According to the "capping" phenomenon described by Karnovsky, these membrane receptors must migrate and cluster to bind mitogen and become pinocytically internalized (123). The presence of
DMSO would enhance the migration of receptors by enhancing membrane fluidity. Predictably, this enhancement should be more pronounced in CLL cells than in normal cells because of the increased rigidity of the leukemic plasma membrane. Indeed, the CLL lymphocytes responded with a 6 to 6.5 fold increase in thymidine incorporation over untreated (with DMSO) leukemic cells as compared to a 3 to 3.5 fold increase for normal cells (Figures 5 and 2, respectively).

The results of a more specific approach to membrane alteration using NASE should, in light of the above be reasonably predictable. Normal cells which received NASE treatment, followed by PHA stimulation, responded in a manner similar to that previously reported (122). The investigations here indicated that NASE increased binding of PHA, but inhibited the internalization of the bound mitogen, probably by the inhibition of receptor aggregation and pinocytosis. As shown in Table 6, the response (both incorporation of tritiated thymidine and interferon production) of normal cells decreased with increasing exposure to NASE. Contrarily, the CLL lymphocyte increased in responsiveness with increasing exposure to the enzyme. Evidently the process of internalization of bound PHA was not inhibited, but was enhanced by removal of the stearic or bond hindrances offered by the abnormal abundance of membrane sialomucins. It was possible also, that NASE initiated a period of membrane turnover in which receptors were regenerated and then
"capping" could then occur. Therefore, compounds which ef-
ected either the matrix of the plasma membrane (NASE) or
the interaction of the receptors among themselves and the
matrix (DMSO) could alter the response of the cell to PHA.

By treating cells with iodoacetate it was possible to
alter the receptors themselves by inhibiting sulfhydryl
bonding within the protein structure of the receptor. Re-
cent reports indicate that the accepted clinical dosage of
iodoacetate (10^{-3} molar) used for the treatment of neoplas-
tic cells not only complexes surface receptors, but also
inhibits bonding in early mitotic proteins (92). This phe-
nomenon also was observed in the data presented here, in
which 10^{-3} and 10^{-4} molar quantities of iodoacetate abro-
gated all responses in both normal and leukemic cells
(Tables 3 and 4). However, normal lymphocytes treated with
10^{-5} molar iodoacetate retained approximately one third of
the CPM's noted for untreated cultures (Table 5). Ob-
viously, the compound was not rebonding early mitotic pro-
teins or DNA replication would not have been initiated and
thymidine incorporation would not have occurred at any lev-
el. Presumably, the effect was on the membrane receptor.
Normal lymphocytes can regenerate complexed or non-func-
tional receptors for mitogens through a period of membrane
turnover (123). Apparently this process occurs also when a
receptor has been damaged by iodoacetate and the cell re-
gains at least some reactivity, although that reactivity
was reduced proportionally with increasing drug exposure time. CLL lymphocytes treated in the same manner failed to respond to any significant degree (Table 5). The failure of the leukemic cells to incorporate labelled thymidine after treatment with iodoacetate will be discussed in conjunction with a treatment regimen using iodoacetate and NASE.

Modalities of treatment in which drugs were used in combination strengthens the conceptualization of receptor availability and mobility at the surface of leukemic cells. By treating normal lymphocytes with $10^{-5}$ molar iodoacetate incorporated in medium containing DMSO it was possible to eliminate the entire PHA response, probably by allowing penetration of the iodoacetate into the cell and the resultant complexing of the drug with internal proteins necessary for the initiation of DNA replication. Treatment of normal cells using, $10^{-5}$ molar iodoacetate and DMSO in sequence as outlined in Table 8 indicated that there may be some penetration of residual SH inhibitor. The sample entitled "Iodoacetate + PHA + 0.25% DMSO" has counts approximately one half those recorded for the similar culture containing only iodoacetate and PHA. The addition of DMSO to the medium of cultures of leukemic cells treated with iodoacetate caused no reversal of the drug abrogated response previously noted. However, when normal cells were treated with a sequential regimen of $10^{-5}$ molar iodoacetate and either a 30 or 60 minute exposure to NASE the response
retardation effect noted for iodoacetate or NASE, alone was exaggerated (Table 13). This synergistic effect would suggest that the inhibition of the formation of disulfide bonds on the protein portion of the receptor and the removal of sialomucin residues strongly inhibited membrane turnover, possibly by weakening the membrane to an extent that the actual integrity of the cell itself was involved. This tendency was reversed in the sequential treatment of leukemic cells with iodoacetate followed by NASE (Table 13). It would seem then that the NASE might function in the exposure of "cryptic" antigens or receptors for mitogen which have not been affected by previous iodoacetate treatment. Singer has expressed a concept which involved the exposure of "cryptic" antigens or receptors on malignant cells by treatment with proteolytic enzymes, as a consequence of the fluid-mosaic model. He hypothesized that mild proteolysis would release polar peptides from the outer surface of integral proteins, thus resulting in these proteins being functionally more hydrophobic due to the overbalance of remaining hydrophobic components. The outcome of this reaction would be the clustering of receptors on the surface of the enzyme-treated cell, and an increase in the functional ability of these sites to bind complementary structures (e.g. agglutinins). NASE may perform a similar function on the sialomucins of the leukemic cells thereby allowing the aggregation of receptors untouched by the SH inhibitor.
Additionally, the effect of DMSO and NASE applied simultaneously as shown in Table 10 supports the concept of increased cellular response with increased membrane mutability. Normal lymphocytes showed a trend (not statistically significant) toward higher degrees of incorporation following such treatments, whereas leukemic cells exhibited an increase which was greater than three-fold in magnitude. However, when the cells (either normal or leukemic) were initially treated with $10^{-3}$ molar iodoacetate and followed with treatment by NASE and DMSO (applied simultaneously) the functional impairment to receptors and to mitotic proteins was too great to be compensated for by the stimulatory action of NASE and DMSO in combination.

In conclusion, data presented here suggests that it is feasible to alter the \textit{in vitro} responses of not only normal lymphocytes but also leukemic lymphocytes by modification of the cell membranes. By enzymatic and solvent application it was possible to enhance the functional responses of lymphocytes. Enhancement of functional responses, especially those responses related to immunologic function, is of importance in a disease in which immunocompetence is known to be greatly impaired. The nature of this impairment was shown to reside at a cellular level, with a major portion of the alterations appearing in the plasma membrane. Even though the CLL lymphocyte is a cell of monoclonal origin and appears functionally to be an end cell (in the sense of a
line of cellular maturation) it was stimulatable. The altered phenotypic expression of a mutated genotype, as seen in the plasma membrane of the leukemic cell, is not therefore an "on" or "off" control point, but rather a reasonably deformable structure.

The mechanisms by which the alterations of cell function were performed may be of some importance clinically. DMSO, which was used extensively in humans prior to 1968, not only preferentially inactivated CLL lymphocytes as compared with normal cells, but it also potentiated the effectiveness of drugs now in clinical use (as demonstrated in Table II which shows an increase in the cytotoxicity of cyclophosphamide for cultures of CLL cells when those cells were incubated with both the alkylating agent and DMSO). Recalling that the CLL patient often succumbs to viral or bacterial infections, a boosting of interferon levels also might prove to be a critical factor in the lowering of mortalities from superimposed infections. In focusing on the effects of NASE on leukemic cells it may be hypothesized that the enzyme alters the antigenicity of tumor cells by modulation of surface receptors. The promise for the effectiveness of NASE as a therapeutic agent in patients is somewhat dubious since the enzyme appears to induce relatively active episodes of membrane turnover in leukemic cells. This release of membrane fragments would act in a fashion similar to that described by Baldwin and block receptor sites on
normal sensitized lymphocytes and thus block cytotoxic removal of neoplastic cells. This antigen or receptor shedding and consequent lymphocyte blockage could account for the recently reported findings that NASE treatment actually may enhance the establishment of tumor cells in vivo (121).

Iodoacetate may prove to be one of the most useful compounds in the treatment of some neoplastic diseases. This SH inhibitor not only appears to have a preferential affinity for leukemic cells as opposed to normal cells (N.B. Table 5), but also apparently inhibits membrane modulation and turnover.

The evidence presented here would indicate that a further in vivo study of the synergistic action of these compounds would contribute to our knowledge concerning the primary unit of the neoplastic process, namely the malignant cell.
SUMMARY

1. Peripheral lymphocytes from patients with chronic lymphocytic leukemia display a delayed and lowered response to stimulation by phytohemagglutinin as compared with the lymphocytes from normal donors. This response was determined by measuring the incorporation of tritiated thymidine. Interferon production also was quantitated and paralleled the PHA responses noted.

2. The addition of dimethyl sulfoxide in concentrations of 0.1%, 0.25%, 0.5%, 1.0% and 2.0% per volume to the culture fluid of normal cell cultures which had been stimulated with PHA elicited a 3 to 4-fold increase in the rate of labelled thymidine incorporation at the 3 day test period.

3. The addition of 2.0% dimethyl sulfoxide proved to be preferentially cytotoxic to cultures of leukemic cells after 48 hours as compared with the virtual absence cytotoxicity for cultures of normal lymphocytes.

4. The addition of 0.1%, 0.25%, 0.5% and 1.0% DMSO to cultures of leukemic cells stimulated with PHA as were normal cell cultures resulted in a 6-6.5 fold increase in the rate of tritiated thymidine uptake at three days, and
a three fold increase in interferon titers.
5. Pre-incubation of normal lymphocytes with $10^{-3}$ and $10^{-4}$ molar concentrations of iodoacetate negated all response to PHA while some responsiveness was retained at the treatment level of $10^{-5}$ molar iodoacetate. Leukemic cells remained non-reactive at all concentrations of iodoacetate tested.
6. Normal and leukemic lymphocytes showed no improved response to PHA stimulation following iodoacetate treatment if the cells had been post-treated with DMSO.
7. Leukemic cells simultaneously exposed to DMSO and to cyclophosphamide incorporated the nucleotide at a much lower rate, indicating an enhanced penetration of the alkylating agent.
8. Leukemic lymphocytes treated with neuraminidase demonstrated a 4-fold increase in the rate of thymidine incorporation as compared with untreated control cell cultures. Addition of DMSO to NASE-treated leukemic cells increased the rate of incorporation to 5-fold.
9. Exposure of leukemic cells, pre-treated with iodoacetate, to NASE and to DMSO partially reversed the detrimental effects of the sulfhydryl inhibitor as reflected in stimulatability by PHA.
BIBLIOGRAPHY


