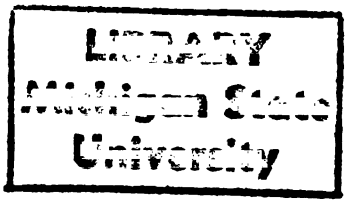

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CHANGES IN THE SURFACE CHARGE OF MOUSE SPLEEN LYMPHOCYTES DUE TO THE S-180J TUMOR

presented by

MARK FREDERICK DESROSIERS

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Major professor

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CHANGES IN THE SURFACE CHARGE OF MOUSE SPLEEN LYMPHOCYTES DUE TO THE S-180J TUMOR

By

Mark Frederick Desrosiers

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ABSTRACT

CHANGES IN THE SURFACE CHARGE OF MOUSE
SPLEEN LYMPHOCYTES DUE TO THE S-180J TUMOR

By

Mark Frederick Desrosiers

The technique of microelectrophoresis was used to investigate the behavior of the outer surface of the membrane of spleen lymphocytes from female ICR mice when the immune system was stressed by the introduction of the S-180J tumor. The two major changes in spleen lymphocytes after injection of the tumor were an increase in the electrophoretic mobility of the B lymphocytes and the appearance of surface nucleic acids on both T and B lymphocytes.

The normal mobility distribution of mouse spleen lymphocytes was bimodal and was altered by the presence of the S-180J tumor. The mobility of the B lymphocytes increased with time after the inoculation of the tumor. This increase in the mobility of the B lymphocytes occurred after the initial injection of the tumor and after a second injection of the tumor into mice that were previously cured of the tumor by cisplatin. In a group of mice who survived the tumor after the second injection, the mobility of the B lymphocytes did not increase.

Mark Frederick Desrosiers

Nucleic acids were not found on the surface of normal T and B lymphocytes, but were found on both T and B lymphocytes after the injection of the S-180J tumor into the mice.

The implications of the shift in mobility of the B lymphocytes and the presence of the surface nucleic acids is discussed in terms of the electrostatic forces involved for cell-cell contact. The presence of nucleic acids may facilitate the cell-cell adhesion due to the likelihood of a mosaic distribution of the surface nucleic acids on the surface of the lymphocytes. The increase in the surface charge of the B lymphocytes after the injection of the S-180J tumor increases the close range (less than 15 Angstroms) electrostatic attractive force, but also increases the long range repulsive forces between cells.

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INTRODUCTION

The purpose of this research is to investigate and attempt to elucidate the role that cell surface nucleic acids on spleen lymphocytes might play in the immune response. Surface nucleic acids have been reported to be involved in the transfer of information for the synthesis of antibodies, cell recognition, tumor immunity, lymphokine induction, macrophage activation, and immunosuppression (1). The scope of this thesis is to investigate the changes in the surface charge and the presence of membrane associated nucleic acids on the spleen cells in a mouse stressed with a tumor.

The surface charge of a cell is the sum of the ionized groups that exist at the cell surface. These ionized groups can be identified by effecters which will specifically alter the charge on the membrane. The effecter can either remove the group from the membrane or alter the degree of ionization of the group. Microelectrophoresis measures this surface charge by measuring the motion of the cell in an electric field. Microelectrophoresis enables the detection of small quantities of a ionized group on the outer surface of a membrane. Changes in the net electrokinetic charge of as little as 10^6 electron charges per cell are detectable (2).

10^6 electron charges correspond to the removal of 6×10^{-16} grams of N-acetylneuraminic acid by neuraminidase.

In this study the technique of microelectrophoresis is used to detect the changes that occur on the surface of the spleen lymphocytes when the animal's immune system is challenged with a tumor. It was postulated that the effect of the tumor would be to lower the surface charge on the lymphocytes. This lower surface charge should reduce the electrostatic repulsion barrier hindering cell to cell contact and thus facilitate interaction between lymphoid cells. Cell surface associated nucleic acids have been implicated in the function of the immune system, but proof of direct involvement of nucleic acids is not available. It is not definitely known as to when membrane associated nucleic acids are expressed on the outer surface of lymphocytes. In this research project, I found that the presence of membrane associated nucleic acids depends on the degree of stimulation of the immune system. The presence of membrane associated nucleic acids on the T and B lymphocytes is usually expressed at the same time.

In this dissertation I describe how microelectrophoresis was used to discern the changes in the electrokinetic surface charge and the presence of surface nucleic acids due to stimulation of the immune system with a tumor.

LITERATURE REVIEW

The Literature Review is divided into two parts. The first part discusses the theory and application of microelectrophoresis to investigate the surface charge of the plasma membrane of lymphocytes. The second part covers the literature involved with membrane associated nucleic acids on lymphocytes.

I. Microelectrophoresis

The plasma membrane is the cell's interface with the environment. Through this interface all constituents necessary for the life of the cell must pass. This interface is a dynamic system and contains such diverse components as proteins, lipids, polysaccharides, and nucleic acids. These components differ in their degree of hydration, ion adsorption, and ionization. Microelectrophoresis is a technique that detects these differences and can yield information about the outer surface of the membrane.

Electrophoresis examines the motion of charged particles relative to the supporting liquid due to force from an applied electric field. Microelectrophoresis is the

branch of electrophoresis that studies the electrophoresis of whole cells or organelles. Microelectrophoresis is used to investigate three types of phenomena associated with surfaces (3):

1. Colloid stability,
2. Ion adsorption, and,
3. Characterization of the particle surface.

All three forms of electrophoresis have been used to investigate biological cells.

Cell surfaces under normal conditions of pH and ionicity are negatively charged. Thus when a cell is suspended in an ionic media and subjected to an applied electric field, it will migrate to the positive electrode. This negatively charged surface is not neutralized by cations from the media due to the finite size of the cations and the hydration of the membrane and cations. The structure of the ionic environment within the interface between the membrane and the media is called a diffuse double layer.

The diffuse double layer develops from the unequal distribution of the ions between the cell surface and the media. This charged interface is created by two forces, the ionization of the acidic and basic groups exposed to the solution and the unequal ion adsorption at the interface. However, the kinetic energy of the molecules due to random thermal energy tends to counteract the formation of a ordered structure of ions at the interface. The electric double layer is an equilibrium state between the random

thermal disordering and the electric double layer structuring of the ions at the interface.

As a cell is moved through the media under the influence of an external electric field, a thin layer of the solvent molecules and ions remain associated with the cell's outer surface. This boundary between the cell-associated solvent molecules and the bulk solvent is called the slip plane. The electric potential at the slip plane is the zeta potential. The zeta potential is the effective potential of the cell arising from its surface charge that is measured by microelectrophoresis.

Microelectrophoresis provides the electrophoretic mobility of cells from which the zeta potential is determined. The relationship between electrophoretic mobility and the zeta potential is:

$$u = \frac{Z * D}{(4 * pi * v)}$$

where u is the electrophoretic mobility; Z is the zeta potential; D is the dielectric constant of the solution; and v is the viscosity of the solution. Since the electrophoretic mobility is directly proporational to the zeta potential, anything that influences the zeta potential (or the electric double layer) directly affects the mobility. Factors that influence the double layer are;

1. permeability of the membrane to ions,
2. surface conduction along the cell membrane (4, 5),
3. ion exclusion from the interface due to the hydration size of the ions (6),
4. surface pH and dielectric strength not being constant but depending on the distance from the cells surface,

5. the nonsymmetric shape of the double layer around a moving cell (6).

The charge density on the cell can be determined from measurements of the zeta potential:

$$q = \left(\frac{2npkT}{pi}\right)^{\emptyset.5} * \sinh\left(\frac{zeZ}{2kT}\right)$$

where q is the charge density (esu/cm^2); n is the concentration of ions in solution; e is the electronic charge; k is the Boltzmann constant; p is the permittivity of the solvent; T is the temperature in Kelvin; Z is the zeta potential; and z is the valence of the ions in solution.

To provide information about the membrane surface components utilizing microelectrophoresis, the components in question must contribute to the surface charge of the membrane. Factors that have been used to alter the membrane surface charge by effecting only specific components are enzymes and chemicals. Enzymes have been used to investigate the membranes of different types of cells. Sialidase, beta-glucosidase, sulfatase, alpha-maltose, beta-galactosidase, and lecithinase have been used to explore the differences between T and B lymphocytes (7). RNase, neuraminidase and trypsin have been used to examine hepatoma cells (8). Ehrlich ascites tumor cells have been examined using both endonucleases and exonucleases (9) to detect the presence of RNA and identify which terminus of the RNA molecule is exposed on the membrane. Chemical agents used to detect the presence of amino groups on the Ehrlich

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ascites cell outer membrane include citraconic anhydride and 2, 3-dimethylmaleic anhydride (10).

II. The Presence of Membrane-Associated Nucleic Acids on Lymphocytes

Lymphocytes synthesize and transport unique sequences of DNA and RNA to the cytoplasmic membrane where they can either remain attached to the membrane or be released into the surrounding media. The function of these nucleic acids is presently unknown although many hypotheses have been presented for their function (11, 12). In this section I will cover the literature concerned with DNA and RNA present on the surfaces of lymphocytes.

A. DNA Associated With The Plasma Membrane

One of the distinguishing characteristics of membrane-associated DNA, DNA)m, from both nuclear and mitochondrial DNA is the timing of its synthesis. By using synchronous cultures of human lymphocytes, Hall and associates established that the synthesis of DNA)m occurred in the nucleus during the S phase of the cell cycle (13).

DNA)m can be distinguished from chromosomal and mitochondrial DNA by its size, configuration, genetic complexity, and labeling properties with radioactive precursors (14, 15). It comprises about 2% of the total cellular DNA (14). DNA)m is a linear double stranded molecule (11, 15). The molecular weight of DNA)m ranges from 3.5×10^5 to 3.7×10^6

daltons (15). The average length of DNA)m is 1.75 microns with the range being from Ø.2 to 4.7 microns with a sedimentation coefficient of 16.6. Results from radioactive labelling experiments with ^{3}H-thymidine indicate that DNA)m has a several fold lower specific activity and shorter labeling times than nuclear DNA. Anker and associates (15) carried out nearest neighbor analysis using phosphorylated precursors for DNA synthesis and found that the DNA)m was labeled along the entire length of the chain ruling out the possibility of a terminal transferase system which would merely attach a nucleotide to the end of a chain.

The genetic complexity of DNA)m differs from both chromosomal and mitochondrial DNA. DNA)m reassociates from the denatured state as two fractions, whose rates of reassociation differ by a factor of forty. The first fraction comprises 7Ø% of the total DNA)m. It reassociates rapidly and is consists of repeated sequences that are homologous with 4% of the repeated sequences of the nuclear DNA (14). The remaining 3Ø% of the DNA)m is the slow reassociating fraction. It is composed of unique sequences of DNA)m and appears to be homologous with 11% of the nuclear DNA. These unique sequences are similar between different donors (16).

The transport of DNA)m from the nucleus to the membrane can be inhibited by rifampicin and this inhibition is not dependent on new DNA synthesis or the overall synthesis of proteins and RNA (17). Rifampicin is known for inhibiting bacterial DNA dependent RNA polymerases and for not

inhibiting the RNA polymerases in eukaryotic cells except at high drug concentration levels (18). It acts by interacting with the RNA polymerase to prevent reversible binding of the DNA to the RNA polymerase. Rifampicin has also shown selective cytotoxicity for human leukemic lymphocytes and for inhibiting reverse transcriptase in RNA transforming virus. Other inhibitors of macromolecular synthesis including cytosine arabinoside, fluorodeoxyuridine, ethidium bromide, and hydroxyurea had no effect on the transport of preformed DNA)m from the nucleus to the cytoplasmic membrane.

DNA)m on the membrane exits in an equilibrium state between its loss to the media and its replacement. Anker and associates (11, 15) found that cultured human lymphocytes released DNA into the culture media in the absence of stimulation from plant lectins. Tonsil lymphocytes also show a high spontaneous rate of DNA release independent of stimulation with phytohemagglutinin. A homeostatic mechanism controls the release of DNA from the lymphocytes into the culture media. The regulatory mechanism stimulates the lymphocytes to release DNA)m into the media until the concentration of DNA in the media is approximately 2% of cellular DNA. If the media is replaced, the cells again add DNA to the media. If fresh media is mixed with DNA)m isolated from used media and then added to cells, then no further DNA)m is released into the media. The amount of DNA found in the supernatant of centrifuged lymphocytes did not

increase when the lymphocytes were 100% dead compared to 2% of the lymphocytes being dead.

There are factors that affect the attachment of the DNA)m to the membrane. Distelhorst and associates (19) investigated the attachment of DNA)m from phytohemagglutinin stimulated human lymphocytes. Small amounts of trypsin such as one microgram per milliliter were sufficient to release DNA)m from the surface membrane into the media. The release of DNA)m by trypsin was reversibly inhibited by prolonged incubation with dibutyryl-cyclic AMP implicating an active regulatory mechanism controlling the attachment of DNA)m. Compounds that did not influence the trypsin induced DNA)m release or cause DNA)m release directly were: leukoagglutinating phytohemagglutinin, erythroagglutinating phytohemagglutinin, concanavalin A, A23187 calcium ionophore, valinomycin, vincristine, colchicine, and cytochalasin B.

The release of DNA)m from lymphocytes was also dependent on the interaction of the different types of lymphocytes. Boldt and associates (20) examined the DNA that was excreted from human lymphocytes after stimulation with plant mitogens. When compared to the amount of DNA excreted by unseparated lymphocytes, both macrophage-depleted cells and B cells excreted low levels of DNA and exhibited low levels of DNA synthesis with stimulation. Reconstitution of the macrophage-depleted cells with macrophages and reconstitution of B lymphocytes with T lymphocytes increased their levels of DNA excretion and synthesis. This implies

synergistic interaction between subpopulations of lymphocytes in both the synthesis and release of DNA)m.

The DNA)m from lymphocytes can be exchanged with other cells. Politis and associates (21) examined autoradiographs of rosettes of human lymphocytes after stimulation with phytohemagglutinin. Seventy percent of the lymphocytes formed rosettes and only the rosette forming lymphocytes registered new DNA synthesis. Closer examination of the rosettes showed that the radioactive label was almost completely transferred to the red blood cells from the lymphocytes. Rogers and Kerstiens (22) investigated the fate of the DNA that is synthesized in response to phytohemagglutinin (PHA) stimulation of human lymphocytes in culture. They discovered that trypsin-released DNA)m accumulated on the membrane of the lymphocytes and was actively capped. The lymphocytes also reportedly formed caps with trypsin-released DNA)m isolated from other lymphocytes, but did not form caps with purified whole lymphocyte DNA.

The function of lymphocyte DNA)m has been laboriously debated, but its presence on spleen lymphocytes can be associated with a suppressor population in AKR mice. Russell and Golub (23) found a suppressor cell population that expresses membrane associated DNA. They discovered that leukemic AKR mouse spleen cells will suppress the normal AKR antisheep erythrocyte antibody responses in vitro. But this suppression could be abrogated if the leukemic spleen cells were first treated with DNase I. The

DNase I treatment did not affect the cell's ability to synthesize DNA. Other enzymes such as, pronase, trypsin and RNase had no effect upon the suppression ability of the leukemic spleen cells. They concluded that there is a population of suppressor spleen cells which requires DNA to be present on the cell surface. Russell and Golub (24) have characterized four subpopulations of spleen cells from leukemic AKR mice on the basis of the presence of membrane surface DNA and I-J antibody. They found that the DNA surface positive, DNA^+, cells are T cell suppressors. Of the DNA^+ cells, the $I\text{-}J^+$ cells are the most potent suppressors and constituted 15% of the total spleen cells and their suppressive ability could be abrogated by treatment with DNase I. The $I\text{-}J^-$ cells constituted 2% of the total spleen cells and are less potent suppressors than the $I\text{-}J^+$, but their suppressive ability is not sensitive to DNase. The $DNA^-/I\text{-}J^+$ subpopulation are poor suppressor cells and are 10% of the total spleen population. The $DNA^-/I\text{-}J^-$ cells show no suppressive ability. By mixing the different subpopulations it was discovered that the cells could act synergistically to give enhanced suppression. Hollinshead and Kundin (25) investigated the suppression of cell-mediated immune responses in leukemia to tumor associated antigens. They discovered that nucleoproteins isolated from the membranes of acute leukemic cells prevented the induction of the cell-mediated immune response to tumor associated antigens. DNase treatment of the nucleoproteins

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abrogated the suppression of the cell mediated immune response to the tumor associated antigens.

Other roles suggested for the DNA)m include the release of excess DNA when the cells are transforming from a proliferative state to a resting state (26). Information transfer is also another role proposed for DNA)m, since the amount of unique sequences in DNA)m, about 10^8 nucleotide pairs, is at least equivalent to 20 times the total informational content in the E. coli genome (14).

B. RNA Associated With the Plasma Membrane

RNA has also been reported associated with the plasma membrane. Usually it is labeled an artifact arising from the isolation procedure. In this section we will cover the literature concerning RNA associated with plasma membranes.

Ribonuclease susceptible anionic groups have been found on many types of cells (27). In human cells, RNA has been found on lymphocytes and polymorphs, but not on erythrocytes, monocytes, acute or chronic lymphocytic leukemic cells and platelets. Cultured human cells showing surface RNA include RPMI #41 cells and normal lymphoid cultured cells. Cultured Ehrlich ascites tumor cells, L1210 cells, S37 mouse tumor cells, and S-180J mouse tumor cells have been shown to have RNA associated with their membranes.

Glick (28) has studied the RNA associated with the surface membranes of mouse L cells, a cultured fibroblast line. The base composition and sedimentation properties

analyzed from sucrose gradients deviated little between RNA)m and cytoplasmic RNA. From measurements of the rate of ^{3}H-uridine incorporation into RNA, RNA)m has a higher specific activity than cytoplasmic RNA inferring a faster rate of synthesis for the RNA)m. By examining the amount of radioactivity from ^{3}H-uridine incorporated into the trichloroacetic acid-precipitable material from the membranes with time, a rapid initial uptake of radioactivity was measured. At 5 minutes 54% of the total radioactivity was found in the acid-insoluble membrane material and it rose to 73% by 30 minutes. The percentage of radioactivity measured in the ribosomes at five minutes was 17% of the total and it rose to 55% within 30 minutes. This rapid early synthesis was not inhibited by concentrations of actinomycin D which would inhibit synthesis of nuclear and cytoplasmic RNA. A third area where the RNA)m differed from cytoplasmic RNA is that RNA)m proved to be less sensitive to RNase degradation. These three differences indicate that even though RNA)m is structurally and chemically similar to cytoplasmic RNA, it appears to be metabolically distinct in its more rapid synthesis and resistance to the actions of RNase and actinomycin D.

Stroun and associates (29) studied the RNA component of a nucleoprotein complex released from cultured human lymphocytes. The RNA component sediments between 2s and 4s depending on the extraction procedure used to isolate the RNA. The RNA has the same density as cellular RNA, but is more

heavily methylated. The RNA is single stranded. It is released as part of a particle that contains DNA and glycoprotein. The RNA is not extracted from the nucleoprotein complex with the usual phenol procedure. It seems too tightly associated with the DNA, possibly hydrogen-bonded, and is not RNase accessible until extracted. It is also tightly associated with a glycoprotein because amalyase digestion allowed 70% of the RNA to become RNase sensitive.

RNA)m is not loosely bound to the plasma membrane. With RPMI #41 cells the amount of RNA detected on the surface decreased little after 14 washes (30). In L1210 cells the surface RNA is not closely associated with the sialic acid on the membrane. The enzymes RNase and neuraminidase function independently in reducing the electrophoretic mobility.

The amount of RNA reported on the surface of lymphoid cells varies among the different types of cells and among laboratories. Mayhew (31) reports a 12.6%, 9.0% and 7.3% decrease in surface charge of human lymphocytes, polymorphs, and monocytes respectively on incubation with RNase; and with mouse thymus cells and macrophages they found 6.7% and 8.6% reductions.

There are two theories on the cellular control of the RNA)m. Stroun (29) describes a homeostatic mechanism that controls the release of a nucleoprotein complex into the culture media. This regulatory release mechanism also controls the release of DNA from the lymphocytes (29). The RNA

is tightly bound with both DNA and glycoprotein to form a nucleoprotein complex. Others researchers describe RNA)m as being associated with the mitotic index and the growth rate of the cells (32).

The amount of membrane RNA on RPMI #41 cells was found to be related to the growth rate (32). When the cells were growing rapidly there was more RNA at the cell periphery. Using synchronous cultures of RPMI #41 cells, Mayhew (32) measured the presence of RNA on surface at different points on the cell cycle. The reduction in electrophoretic mobility due to RNase digestion was 30% in the S phase, 34% in G1, 38% in G2, and 39% during interphase. However rapid growth rate in a population of cells did not guarantee that RNA is present on the surface of the cells.

Stimulation of a mouse's immune system with tetanus toxoid caused an increase in the detectable surface RNA (33). The reduction of electrophoretic mobility of lymph node cells due to RNase incubation increased from 4.8% for unstimulated lymph node cells to 13.3% for stimulated lymph node cells.

Bennett and associates (34) examined the effect of syngeneic and allogeneic transplantation of spleen cells into irradiated mice. There was no significant difference between RNase A and RNase T_1 on the mobility of the cells inferring that separation of the RNA molecule by digestion of RNA between the pyrimidine or guanosine linkages made no detectable electrokinetic difference. RNase had little

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effect on the electrophoretic mobility of normal mouse lymph node cells, but the syngeneic and allogeneic spleen cell chimeras showed a 9.2% and 13.6% decrease in mobility respectively four days after transplantation and decreases of 10.3% and 18.0% respectively six days after transplantation. The difference between the syngeneic and allogeneic chimeras electrophoretic mobilities was not sufficient to draw conclusions about any role that the surface RNA might play and left open the question that the difference between the chimeras and normal mice could be due to a higher rate of cellular proliferation, RNA synthesis, or the relative immaturity of the cells in the chimeras.

The idea that the RNA passes information between cells is the major proposed function for RNA)m. Whether the surface RNA on lymphocytes serves any specific function has not been determined. It has been found to stimulate in vitro DNA synthesis (1). In a purely theoretical framework, Dickson (35) proposed that surface RNA provides a line of communication between the macromolecules on the cell surface and the genome. There is strong evidence for RNA being passed from one cell to another (1), and RNA is involved in the transfer of information in specifying the production of an antibody to a particular antigen (36). It has been shown that macrophages pass an antigen-RNA complex to lymphocytes during the chain of events that lead to antibody production. In vivo experiments have shown transfer of tumor immunity by RNA from lymphocytes isolated from tumor bearing animals.

Double stranded RNA at low concentrations can induce interferon production in mammalian cells. Other researchers, however, believe that the RNA serves as an adjuvant rather than as an information transferring package.

C. Serum Nuclease Activity and Nucleic Acid Binding Proteins

Nucleases have been found in human serum. Examination of the serum RNase levels showed an elevation of 68% of serum RNase in patients with malignant tumors, 24% increase in patients with benign tumors and a 38% increase in smokers (37). The elevated levels of RNase found in these patients was due to a generalized nonspecific increase in serum RNase and not to new species of RNase appearing in the serum. Herriott and associates (38) separated blood fractions to identify the source of the blood nucleases. They found that DNase was actively concentrated in the platelet fraction and a DNase inhibitor could be found primarily in the white blood cells. RNase activity was found in the white blood cell fraction and an RNase inhibitor was discovered in the red blood cell fraction.

There are three types of DNA-binding particles found in the serum of patients with malignant diseases. One DNA binding particle, C3DP, is a degradation product of complement component C3 and is elevated in the sera of individuals with malignant diseases (39). Another DNA binding protein, 64DP, has been purified from human serum (40). 64DP has a

molecular weight of 64,000 daltons. Its levels in serum increase by a factor of four in untreated malignant diseased patients. 64DP is not antigenic with carcinoembryonic antigen, alpha-fetoprotein, C3, or C3DP human complement binding DNA. Malignant disease-associated DNA-binding (MAD-2) was found to be elevated both in patients with malignant diseases and pregnant women when compared with patients with nonmalignant diseases (41). Antisera directed against human cold insoluble globulin would cross react with MAD-2.

Nucleic acids bind to the plasma membrane of cells. Experiments with Ehrlich ascites cells showed that the binding of nucleic acids to the outer layers of the cells are enhanced by such carcinogens as UV light, chemical mutagens, and ultimate carcinogens (42). The nucleic acid binding was not influenced by precarcinogens (require enzyme activation), nonultimate carcinogens (carcinogenic when applied with tumor-promoting agents) or tumor-promoting agents. But when precarcinogens were activated by addition of liver extracts into their active form, the binding of nucleic acids was enhanced.

The autoimmune disease, Systemic Lupus Erythematosus, is characterized by the production of large quantities of autoantibodies, generalized B cell hyperactivity and impaired T cell function. The disease is known for its high level of DNA binding antibody. Another antigen distinguishing SLE is small ribonucleoprotein particles (RNP) (43). The RNA in these RNP originates from both the nucleus

and the cytoplasm. Antibodies to the suppressor T cells have been found in the serum of SLE patients (44). SLE is postulated to be due to a defect in the host's suppressor T cells which results in autoantibodies to the suppressor T cells setting up a positive feedback mechanism. The existence of anticoagulants from Lupus patients that recognize hexagonal but not lamellar phospholipids (45) suggest that autoantibodies may recognize the hexagonal phospholipid structures on cells in the immune system.

The existence of factors in the serum that bind to double stranded DNA is also found in seropositive rheumatoid arthritis (46). The binding factors are not specific antibodies as in SLE, but are a complex of low avidity interactions involving IgG, rheumatoid factor, low density lipoproteins and DNA.

MATERIALS AND METHODS

I. Materials

A. Buffers

Three buffers were used in the preparation of the cells for electrophoresis. Saline (Ø.154 Molar NaCl) with Heparin (2Ø IU/ml) was used for the isolation of red blood cells while just saline was used for the electrophoresis of the red blood cells. The other buffers were used to collect and maintain the spleen lymphocytes and are based on Balanced Salt Solution, BSS (47). Standard BSS consists of 5.6 mM Dextrose, Ø.44 mM KH_2PO_4, 1.34 mM Na_2HPO_4, 1.27 mM $CaCl_2(2H_2O)$, 5.36 mM KCl, 137 mM NaCl, 1.Ø9 mM $MgCl_2$ and Ø.81 mM $MgSO_4(7H_2O)$. For the nylon wool incubations, BSS was supplemented with 5% fetal calf serum. The electrophoresis buffer is based on BSS, but the ionic strength of the buffer was reduced by lowering the concentration of sodium chloride to 64.4mM and adding 154 mM Sorbitol to maintain the osmotic pressure. 14.3 mM Hepes was added to increase the buffering capacity of the electrophoresis buffer. The pH of all buffers was adjusted to 7.3.

The spleen lymphocytes and red blood cells were from female ICR random bred mice from Harlan Animal Supply. The weight of the ICR mice was approximately 20 grams at the start of the each experiment. They were fed (Wayne Lab Blox for mice) and watered ad libitum.

The mouse S-180J tumor was grown in ascitic form in the peritoneal cavity of the ICR mouse. The tumor was obtained from the Sloan Kettering Institute. The tumor was transferred weekly by injecting 1 x 10^6 S-180J tumor cells in saline into the peritoneal cavity of a new group of mice. The tumor cells were isolated by washing the tumor cells out of the peritoneal cavity with saline.

B. Enzymes, Drugs and Antibodies

Two enzymes were used in this study. Deoxyribonuclease I, type DN-EP and lot 24C-2460, was from Sigma Chemical Company. The ribonuclease was from Miles Laboratories and was of lot 7057. The concentration of the nucleases in the enzyme digestions was 0.2 mg and 0.4 mg per sample in 2 ml BSS for DNase and RNase respectively. The activity of the DNase was 280,000 Kunitz (one Kunitz will cause an increase in the absorbence at 260 nm of 0.001 per minute per ml of reaction at 25°C at pH 5.0) per sample.

Cis-dichlorodiammineplatinum(II) was used to cure the mice of the S-180J tumor. It was injected into the peritoneal cavity of the mouse twice, at one and at four days after the injection of the tumor. The platinum compound was

suspended in saline and injected at 7 milligrams platinum compound per kilogram weight of mouse.

The percentage of B and T splenic cells in a sample was determined by immunofluorescence for the antigens Thy 1.2 and IgG. The buffer used was phosphate buffered saline (PBS). Nylon wool columns were used to separate the spleen cells into T and B enriched populations. The two samples of spleen cells were from normal healthy mice and from mice which were injected with the S-180J tumor 14 days previously. Each sample consisted of 7 spleens pooled together. The monoclonal antibody, anti-mouse Thy 1.2, was from Becton Dickinson and of clone 30-1112, lot D0404, and biotin-conjugated. The fluorescein avidin was from Vector Laboratories, lot 10430. The biotinylated antibody to mouse IgG was also from Vector Laboratories and was of lot 41205.

C. Equipment

The electrophoresis equipment consisted of two major parts. The first part was the electrophoresis equipment and the second part consisted of a computer set up for data collection.

The electrophoresis equipment consisted of the electrophoresis chamber, a microscope for viewing the cells and glass assemblies for controlling the flow of the cell suspensions and buffers through the electrophoresis chamber. To view the cells a Nikon MS inverted microscope utilizing dark field phase contrast at 200-fold magnification was

used. The chamber and its controls consisted of three parts. The chamber, a Northrop-Kuntiz design, was rectangular with internal dimensions of 586 microns deep and 12 millimeters wide and was constructed with parallel optical flats. The temperature of the chamber was controlled by thermostatted water circulating around the chamber. Two side glass assemblies connected to the chamber used a system of stopcocks to control the flow of the buffers, cells and electric current through the chamber. Ag/AgCl electrodes powered by a Lamba DC power supply provided the electric field. A television monitor and camera was used to observe the cells. The cells were introduced into the chamber through a fine polyethylene tube of internal diameter of Ø.58 microns.

The data collection equipment consisted of a Timex/-Sinclair computer, a digital multimeter, and a VOTEM from Down East Computers. The digital multimeter was used to monitor the current through the chamber. The VOTEM was an analog to digital, voltage to frequency converter that was directly interfaced with the computer providing for constant measurement of the applied electric field across the Ag/AgCl electrodes. Time measurements were calculated and collected by the computer.

D. Polyacrylamide Gel Electrophoresis

The buffers and the gels used in sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels are described. The running

gel was a 10% polyacrylamide gel and consisted of 8.0 mg ammonium persulfate, 12.5 ml H_2O, 7.5 ml electrophoresis buffer, 7.5 ul N,N,N',N'-tetramethylenediamine (TEMED), and 8.0 ml 30% Acrylamide solution. The stacking gel was a 5% polyacrylamide gel and consisted of 1.67 ml of a 30% Acrylamide solution, 2.5 ml buffer (0.5 M Tris, pH 6.8, 0.4% SDS), 2.5 ul TEMED, 2.5 mg NH_4SO_4, and 5.83 ml double distilled water. The solutions were electrophoresis buffer, overlay solution and a sample preparation buffer. The electrophoresis buffer contained 0.1 M Tris (pH 8.3), 0.768 M glycine, and 0.4% SDS. The electrophoresis buffer was diluted 4 times for electrophoresis. The overlay solution contained 0.1% SDS, 0.15% NH_4SO_4, and 0.05% TEMED. The sample preparation buffer contained 0.125M Tris (pH 6.8), 0.002% bromphenol blue, 4% SDS, 20% glycerol, and 10% mercaptoethanol.

The Coomassie blue staining solutions included the staining solution and the destaining solution. The staining solution consisted of 0.1% Coomassie blue in 50% trichoroacetic acid (TCA). The destaining solution consisted of 20 ml glacial acetic acid, 100 ml methanol, and 100 ml double distilled water.

The solutions for the gluteraldhyde silver stain are the silver stain and the developer solution. The silver stain contained 1.4 ml fresh NH_4OH, 21.0 ml of 0.36% NaOH, 4.0 ml of 19.4% $AgNO_3$, and add double distilled water to 100 ml total volume. The developer solution consisted of 20 ml

methanol, 10 mg citric acid, 100 ul formaldehyde, and 180 ml double distilled water.

II. METHODS

A. Procedures for Isolation of Cells

The spleen lymphocytes were obtained from ICR mice. The procedures for the isolation of the spleen cells, for the removal of red blood cells, and for the separation of the spleen cells on a nylon wool column were from Mishell and Shiigi (47) with slight modifications. The mice were killed by cervical dislocation. The spleen was aseptically removed and placed into cold BSS. Cold BSS was injected into the spleen capsule to disrupt the tissue. The tissue was then teased apart and the resulting cell clumps were forced through 22 and 26 gauge needles to form a cellular suspension. The red blood cells present in the spleen suspension were removed by hypotonic shock treatment which consists of briefly exposing the cells to low osmotic solution lysing the red blood cells followed by adjustment of the solution to isotonicity. The resulting cell suspension was filtered though a loose cotton plug to remove red blood cell membrane aggregates. T and B lymphocytes were separated by exploiting the adherence of B lymphocytes to nylon wool fiber. The spleen lymphocytes were incubated in 5% fetal calf serum supplemented BSS on a nylon wool column (Fenwal Laboratories) for 45 min. at 37^{o}C in a 5% CO_2 incu-

bator. The nuclease treatments consisted of incubating the cells for 30 min. with the appropriate enzyme at 37°C in BSS in a 5% CO_2 atmosphere with gentle agitation to keep the cells suspended. The cells were stored in BSS at 4°C while awaiting microelectrophoresis.

The mouse red blood cells used for the standardization of the electrophoresis system were obtained by cardiac puncture. The blood was mixed with 2 IU units of heparin (Sigma Chemical) per ml of blood to prevent coagulation. The blood was washed 3 times in saline and stored at 4°C.

B. Electrophoresis Procedure

The electrophoresis procedure consists of several steps. The first steps were preparatory. A circulating water bath controlled the temperature of the chamber at 25.0°C. The chamber constant was computed by filling the chamber with 0.100 M KCl and calculating the resistance due to the chamber's dimensions from simultaneous measurements of the voltage and current. The calculation of the chamber constant was adjusted for the loading of the circuit by the VOTEM and the formula can be found in line 951 of the program listed in Appendix I. The conductance of 0.100M KCl solution at 25.0°C is 0.01288 /(ohm-centimeter). The KCl solution was rinsed out of the chamber with distilled water. The electrophoresis buffer was then admitted into the chamber. The microscope was focused at the stationary level inside the chamber.

At this point the chamber was ready for electrophoresis and the sample was introduced into the chamber. Once the sample was in the chamber and the system was ready, the velocity of the individual cells was measured. This was achieved by timing how long it took a cell to travel 50 microns under the influence of the applied electric field. Measurements in both directions (positive and negative polarity) were taken and were averaged to remove the effects of any drift in the chamber. This was repeated until the desired number of cells had been studied. A grid was inserted into the eyepiece of the microscope to provide the 50 micron divisions. Only cells in sharp focus (residing in the stationary plane level) were chosen for measurement. Normally, 200 to 300 cells were measured from a sample of 10^8 cells.

C. Computer Analysis of The Data

The computer collected the data to calculate the electrophoretic mobility of the cells. Electrophoretic mobility is defined as the velocity of cells due to an applied electric field. The time measurements were derived from changes in two registers in the computer. The two registers combine to form a 16 bit number that was decremented each time the television screen was refreshed. This refreshing action occurred 60 times a second. The formula for converting the values in registers to seconds is in line 235 of the program listed in Appendix I.

The electric field is equal to the voltage / length at the cell's position. The equation for this is

$$E = \frac{V}{(A * Kc)}$$

where V is the applied voltage, A is the cross-sectional area of the chamber and Kc is the cell constant of the chamber. The applied voltage was read by the VOTEM and given directly to the computer. The cross sectional area of the chamber was 0.0703 square centimeters.

D. Polyacrylamide Gel Electrophoresis and Staining

The technique follows the procedure outlined in (48). The electrophoresis apparatus was assembled and made ready for the gel. The running gel solution was slowly pipetted to within 1 cm below the comb. Butanol was pipetted on top of the gel to prevent drying while the gel polymerized overnight. After 24 hours the butanol was removed and the stacking gel was carefully layered on top of the running gel to the top of the teeth of the comb. The stacking gel was allowed to polymerize at least 15 minutes before the comb was removed. The apparatus was placed into the running tank with the lower electrophoresis buffer in the lower reservoir. The samples include both DNase and RNase at both 3 ug/ml and 150 ug/ml. A standard molecular weight marker (Sigma Chemical Company, MW-SDS-200) was also prepared. The samples were diluted 1:1 with the sample buffer and boiled two minutes. The comb created 20 wells. Four wells were

filled with the standard marker. The DNase and RNase samples were divided with 5, 10, 15, and 20 ul of each sample placed into a separate well and overlay with running buffer. The upper reservoir was then filled with electrophoresis buffer. Cooling water circulating through a jacket was used to remove the heat. The current was set at 12.5 ma for the first hour and then was set at 30 ma until the bromphenol blue approached the bottom of the gel (approximately 3 hours). After the gel had run it was placed in the appropriate staining solution.

The procedure for staining a SDS-PAGE gel with Coomassie blue was to place the gel into a glass tray containing Coomassie blue staining solution overnight (12 hours) which was continuously gently shaken. The staining solution was pipetted off and the gel was washed with destaining solution (15 minutes per wash with gentle shaking) until no color was present in the destaining solution. The gel was then dried and mounted.

The procedure for staining the SDS-PAGE gel with the silver stain is fixation in a solution of 10% acetic acid and 25% isopropanol overnight (12 hours). The gel was then washed two times with 7.5% acetic acid within 30 minutes and 200 ml of solution per wash. The gel was then soaked in 10% unbuffered gluteraldhyde for 30 minutes with gentle shaking. The gel was then wash with double distilled water every 15 minutes for two hours. The staining solution was added to the gel for 10 minutes. The gel was then quickly rinsed 4

times with double distilled water. The developer was added to the gel for 12 minutes. The gel was then rinsed with double distilled water approximately 8 times and then soaked overnight in water. The gel was then dried.

E. Immunofluorescence of Surface Antigens

The procedure used to label the cells is described. The controls for the labeling were cells and cells plus avidin. Viability determinations and cell counts were done first. The cells were centrifuged to a pellet in PBS. A 100 ul aliquot of an 5 ug Ab/ml saline was added to the pellet and incubated on ice for 30 minutes after mixing. The cells were then washed once (1400 rpm, cold tube carriers, 5 minutes) in PBS. The tubes were blotted dry and 100 ul of fluoresceinated avidin (1:100 dilution) was added to the pellet and incubated for 30 minutes on ice. After incubation with avidin, the cells were washed three times with PBS and then resuspended at approximately 2 x 10^6 cells per ml. The amount of fluorescence per sample was determined in Ortho 58 fluorescent cell sorter and detector.

RESULTS

I. Microelectrophoresis Mobility Distributions of Spleen Lymphocytes

In this section I will examine the microelectrophoresis mobility distributions of spleen lymphocytes. First we will describe the normal spleen cell mobility distribution to establish a baseline. Then we will discuss the changes in the mobility distributions which occur during the growth of a tumor. To compare the differences between a primary response to the tumor and a secondary response, we examined spleens from mice that were cured of their tumors and then reinjected with the tumor. Also we examined splenic lymphocytes from mice that survived after a second injection of the tumor.

A. Microelectrophoresis of Pooled Splenic Lymphocytes From Normal Mice

Figure 1 is a representative histogram of the microelectrophoretic mobility ((um/sec)/(volt/cm)) of spleen cells from normal, healthy ICR mice. Because of the bimodal distribution, statistics based on a single normal

probability distribution fail to accurately describe the data. Statistical treatment of the two peaks as normal distributions also failed to describe the data accurately. The observed level of significance for the Lilliefors test for normality on single peaks is greater than Ø.25 for 1Ø peaks. For this reason, nonparametric statistical methods were used to measure the differences between mobility distribution. The two nonparametric methods used to examine the data were the Smirnov's and Chi-squared methods. These statistical methods and a description of observed levels of significance are described further in Appendix II.

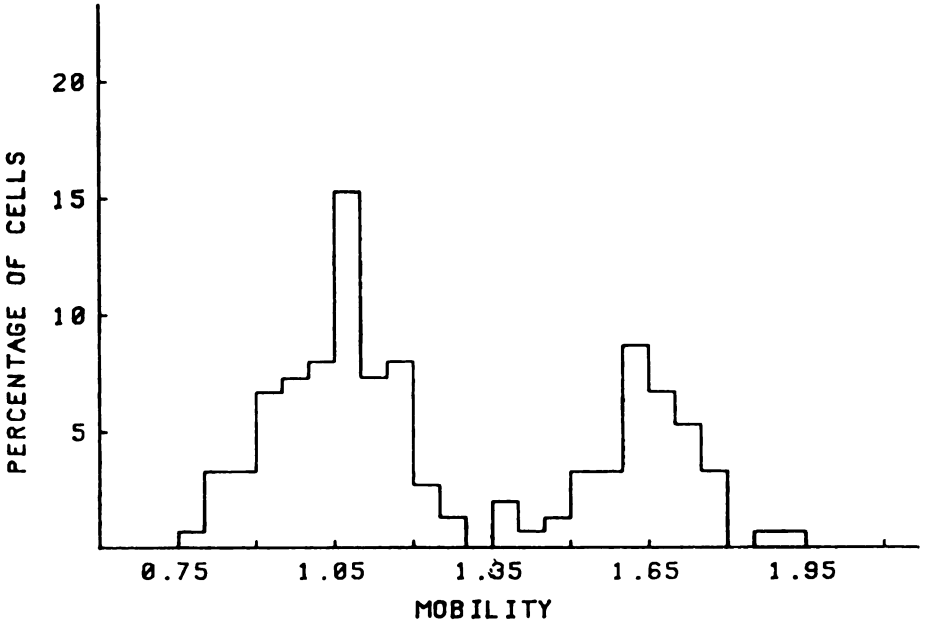

Figure 1. A Mobility Histogram of Normal Spleen Cells.

First the mobility patterns of five samples were examined to determine if absolute mobility comparisons were feasible. Microelectrophoretic mobility patterns of 5 sets of spleen cells from five normal mice is shown in Figure 2. The Chi-squared test on the distributions of the five samples yielded an observed level of significance, P, of less than Ø.ØØ1 for the five distributions being identical. Closer examination of Figure 2 shows that not all of the distributions are so different. Figure 2.2 and Figure 2.4 are similar with the statistical tests yielding P values greater than Ø.25 implying that the variation in the mobility distributions is enough to prevent absolute mobility comparisons, and does not permit the cumulative adding of data from different days.

However when two electrophoretic mobility runs are made from the same sample, (see Figure 3 and Table 1), the statistical tests indicate that the two samples are identical. Table 1 gives the observed levels of significance for 6 trials where a population of cells was separated into two samples and electrophoresis was done on each half separately. Figure 3 illustrates an overlay of two nylon wool effluent distributions which are both from the same population, but which was measured separately.

Figure 2. A Set of Five Mobility Distributions of Normal Spleen Cells. The Ordinate is the Percentage of Cells and the Abscissa is the Mobility in (um/sec)/(volt/cm).

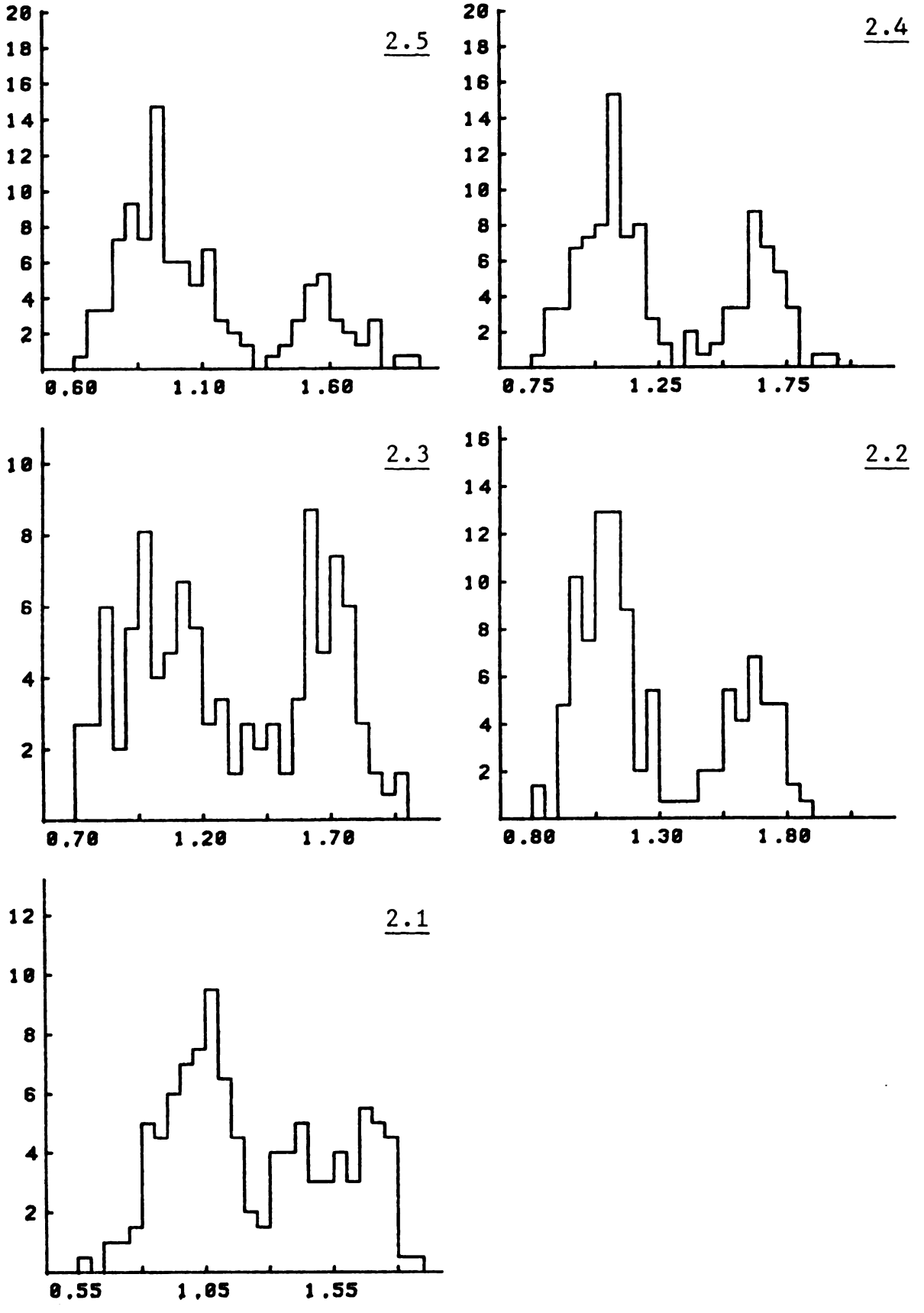
2.5
20
18
16
14
12
10
8
6
4
2
0.60
1.10
1.60
2.4
20
18
16
14
12
10
8
6
4
2
0.75
1.25
1.75
2.3
10
8
6
4
2
0.70
1.20
1.70
2.2
16
14
12
10
8
6
4
2
0.80
1.30
1.80
2.1
12
10
8
6
4
2
0.55
1.05
1.55

Figure 2.

Table 1. Testing the closeness of two samples from the same cell Pool. P values are given for Smirnov's Two sided test and Chi-squared test.

Experiment Number	Observed Level of Significance Smirnov	Chi-squared
1	>0.20	0.16
2	>0.20	>0.25
3	>0.20	>0.25
4	>0.20	>0.25
5	>0.20	0.22
6	>0.20	>0.25

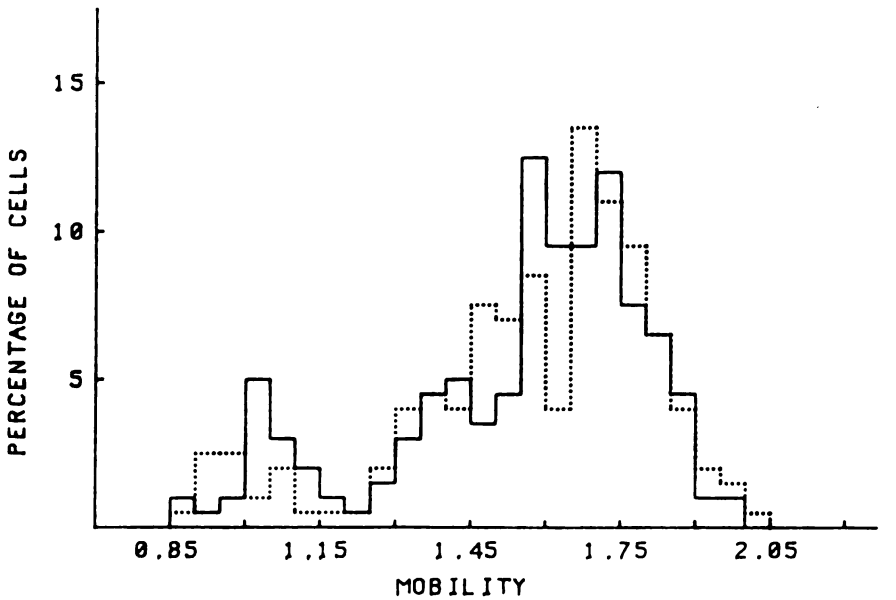

Figure 3. A Comparison of Two Mobility Distributions from the same T Cell Sample.

B. Microelectrophoresis of Pooled Spleen Lymphocytes After Separation by Nylon Wool Columns.

Nylon wool columns are routinely used to separate spleen lymphocytes into T and B lymphocyte populations. Appendix III covers the general properties of lymphocytes and discusses the markers used to distinguish T and B lymphocytes. In this section we will examine the electrophoretic mobility distribution of normal splenic lymphocytes after separation by nylon wool columns. The basis of the separation of lymphocytes by nylon wool columns is empirical, owing to observation and not to any specifically known surface feature of the type of lymphocyte. B cells, plasma cells and some accessory cells preferentially adhere to the column, while T cells pass through the column.

The electrophoresis distribution of effluent cells (T lymphocytes) from a nylon wool column separation is shown in Figure 4. The lymphocytes are predominantly of high microelectrophoretic mobility. The mobility of the main peak correlates with the high mobility peak seen in the precolumn mobility distributions. These lymphocytes are presumed to be primarily T lymphocytes. To check this assumption, immunofluorescence using avidin and biotin-conjugated anti-Thy 1.2 antigen showed an enrichment from 37.4% fluorescent cells in the precolumn splenic lymphocytes to 60.7% fluorescent cells in the effluent.

The adherent cells of the nylon wool column (called B lymphocytes from here on) are released from the column with

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a cold saline rinse. A microelectrophoretic mobility histogram of the nylon wool adherent cells is seen in Figure 5. The histogram consists primarily of a low mobility peak with a trailing edge into the high mobility region. The percentage of spleen cells bearing surface IgG increased from 20.4% in the precolumn cell suspension to 25% of the cells in the cold saline rinse of the column.

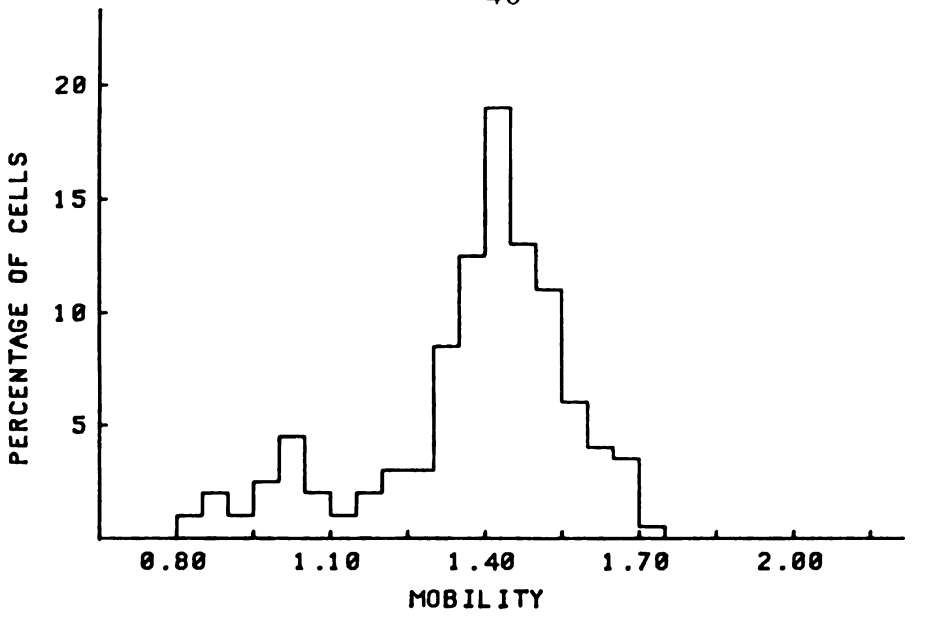

Figure 4. A Mobility Distribution of T Lymphocytes.

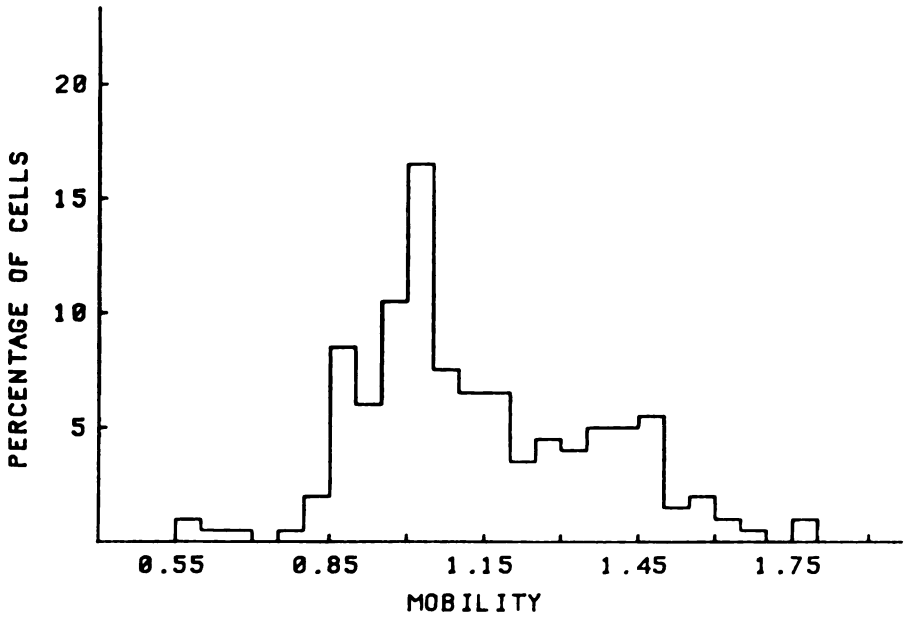

Figure 5. A Mobility Distribution of B Lymphocytes.

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II. Changes in the Mobility of Spleen Lymphocytes After Tumor Inoculation

In this section we will examine the changes in the mobility distribution of ICR mouse splenic lymphocytes following inoculation with the S-180J tumor.

A. Injection of Dead Tumor Cells Into The Mice

To insure that any effects that the tumor might have on the mobility of the splenic lymphocytes would be dependent on the live tumor, 1 x 10^6 dead tumor cells were injected into mice. The tumor cells were killed by heating the cells to 56^{o}C for 20 minutes. The viability of the tumor cells after heating was determined by trypan blue staining to be zero percent on examining 500 cells. At various times after the injection, spleen cells were harvested and examined electrophoretically. The results are illustrated in Figure 6.

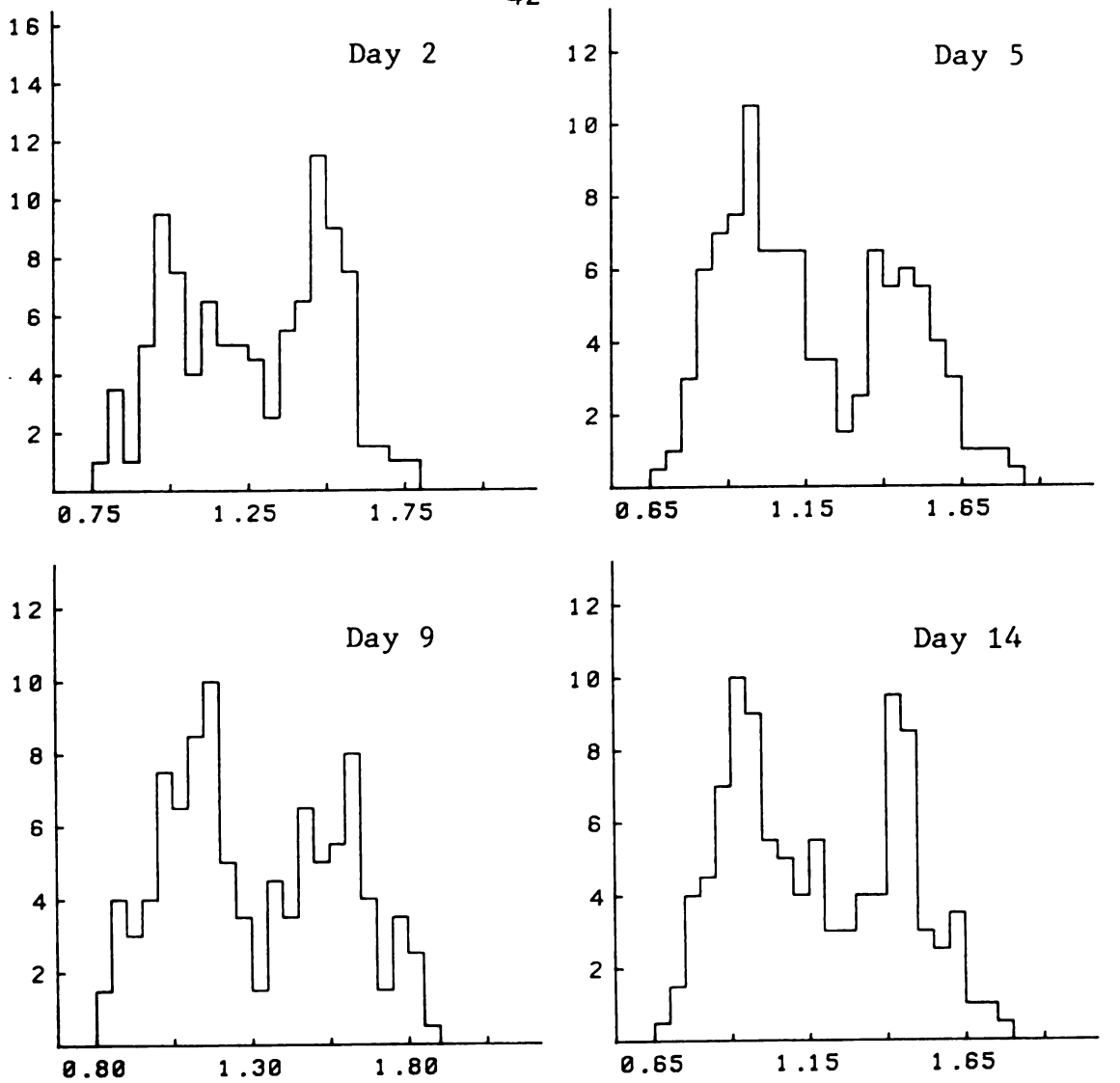

Figure 6. Mobility Distribution of Spleen Lymphocytes after the Injection of 1 x 10^6 Dead S-180J Tumor Cells into the Mice.

B. Primary Inoculation

The mobility distribution of splenic lymphocytes are affected by the injection of the tumor into the peritoneal cavity of the ICR mice. Figure 7 illustrates the changes in the electrophoretic mobility distributions with time following the injection of the tumor. Table 2 shows the increase in the mean mobility with time following the injection of the tumor. The Smirnov multiple sample test and Chi-squared test both give an observed level of significance less than Ø.ØØ1 (alternate hypothesis is accepted that the difference between the distributions is significant enough not to be due to chance) for the distributions remaining the same with time. Day 2 as seen in Figure 7, is similar to the mobility distributions seen previously for the normal mice. By day 5, the percentage of low mobility cells has decreased as reflected by the increase in the sample mean. A bimodal pattern is still discernible. By day 9 the electrophoretic distribution is no longer bimodal and on day 13 it is seen to resolve itself into one peak at mobility of 1.5. The decrease in the percentage of the low mobility cells (cells with mobility less than 1.2) is given in Table 3.

Table 2. The increase in the mean electrophoretic mobility following the injection of the tumor into the mice.

Days after Inoculation	Mean Mobility (Standard Deviation) Exp. 1	Exp. 2
Ø	1.35 (Ø.33)	1.35 (Ø.33)
2	1.14 (Ø.3Ø)[1]	1.24 (Ø.28)[2]
4		1.33 (Ø.28)[2]
5	1.3Ø (Ø.33)[2]	
6		1.48 (Ø.36)
8		1.45 (Ø.33)
9	1.46 (Ø.27)	
1Ø		1.68 (Ø.24)
13	1.43 (Ø.2Ø)	

Note: [1] The means differed significantly from Day Ø at the Ø.Ø1 level unless noted otherwise.
[2] The mean does not differ from the Day Ø mean at the the Ø.Ø1 level.

Table 3. The loss of low mobility spleen cells with time after mice were inoculated with tumor.

DAY	Percentage of low mobility cells Exp. #1	Exp. #2
Ø	63.7 (8.4)	
2	59.Ø	51.Ø
4		34.5
5	38.Ø	
6		25.5
8		26.Ø
9	21.Ø	
1Ø		4.Ø
13	12.6	

Notes: DAY refers to the number of days after the mice were inoculated with the S18ØJ tumor. Low mobility cells refers to cells with mobilities less than 1.2. Day Ø is the average of 5 normal samples.

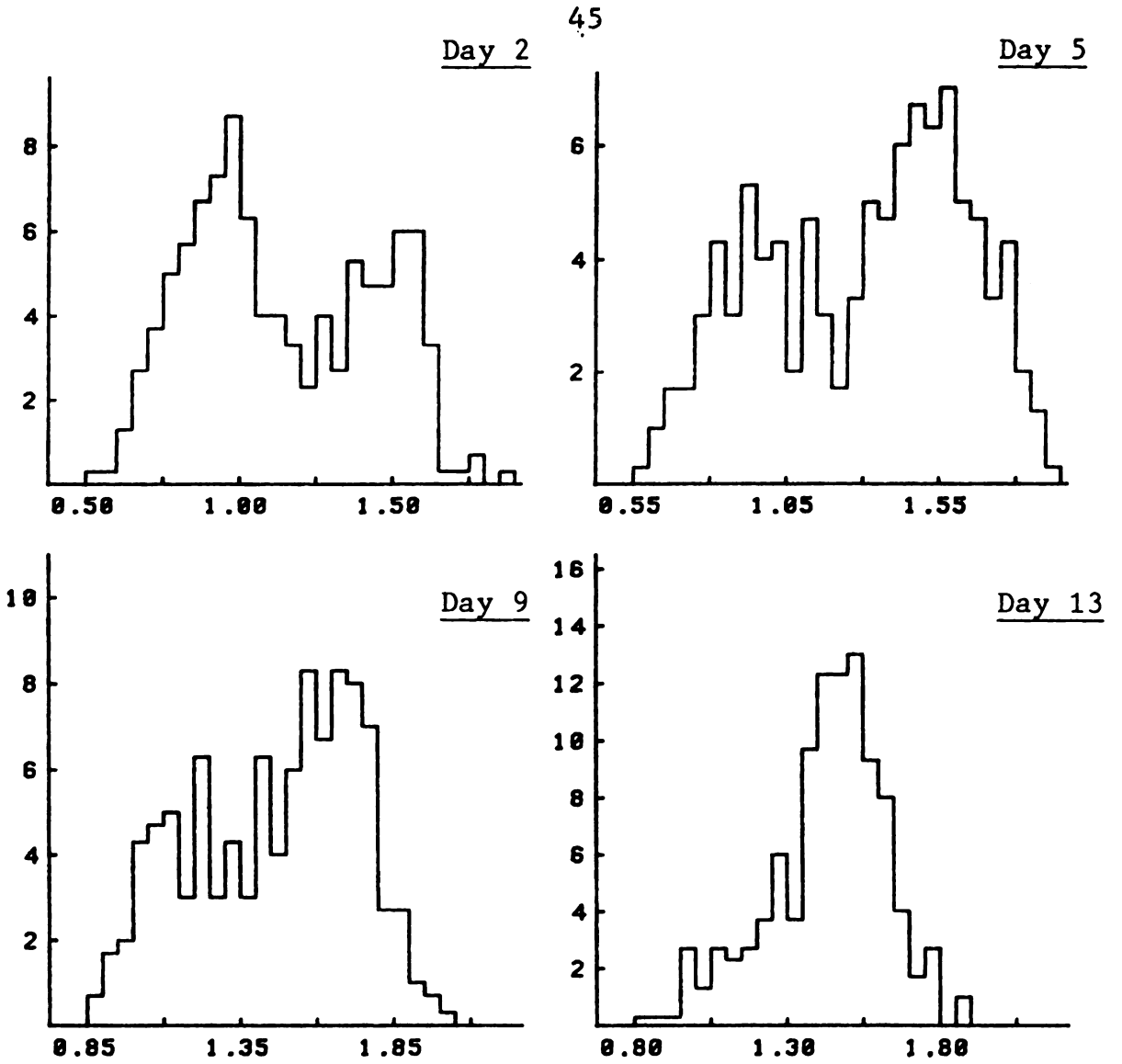

Figure 7. The Mobility Distributions of Spleen Cells after S-180J Inoculation.

To investigate further the effect of the presence of the tumor in the mouse on the mobility of the spleen lymphocytes, the spleen lymphocytes were separated by nylon wool columns. Figure 8 gives the electrophoretic distributions of the nylon wool effluent spleen lymphocytes with time after the tumor inoculation. The distributions vary as expected from the whole spleen data and are not constant with time ($P < 0.001$) overall, but days 4 and 6 are similar with $P = 0.04$. The rise in mobility is seen in Table 4. The width of the distributions as measured by the standard deviation also increases with time. The nylon wool column enriched the percentage of spleen cells expressing Thy 1.2 (as measured by immunofluorescence) from 16.2% to 28.1% for spleen cells from mice afflicted with the tumor 14 days.

But the real effect of the tumor upon the electrophoretic mobility distribution of the spleen cells is seen in nylon adherent cells. Figure 9 is an overlay of the nylon wool effluent and adherent cells at 9 days after the injection of the tumor. From Figure 9 one can see that it is the mobility of the nylon wool adherent cells that rises and it is not their disappearance that leads to the increase in the overall mobility. Also 44.5% of the nylon wool adherent splenic cells expressed surface IgG as evidenced by fluorescence implying that the B lymphocytes are still present in the spleen and have not disappeared. The two histograms of the T and B splenic cells overlap significantly. Simple descriptions of B cells as low mobility cells and T cells as

high mobility cells does not accurately describe the situation when the presence of a tumor in the mouse can increase the mobility of the B cells to that of the T cells.

Table 4. Changes in the mean electrophoretic mobility of nylon wool separated spleen cells with time after the inoculation of the tumor into the mouse. The units of mobility are (microns/sec)/(volt/cm) with the standard deviation in parenthesis.

Day	T lymphocytes	B lymphocytes
Ø	1.38 (Ø.19)	1.14 (Ø.23)
2	1.4Ø (Ø.26)	
4	1.55 (Ø.25)[1]	
6	1.53 (Ø.25)[1]	
8	1.46 (Ø.25)[1]	
9	1.46 (Ø.27)[1]	1.27 (Ø.19)[1]

Note: [1] Mobility values are significantly different at the the Ø.Ø1 level.

Figure 8. The Mobility Distribution of T Lymphocytes after Tumor Inoculation.

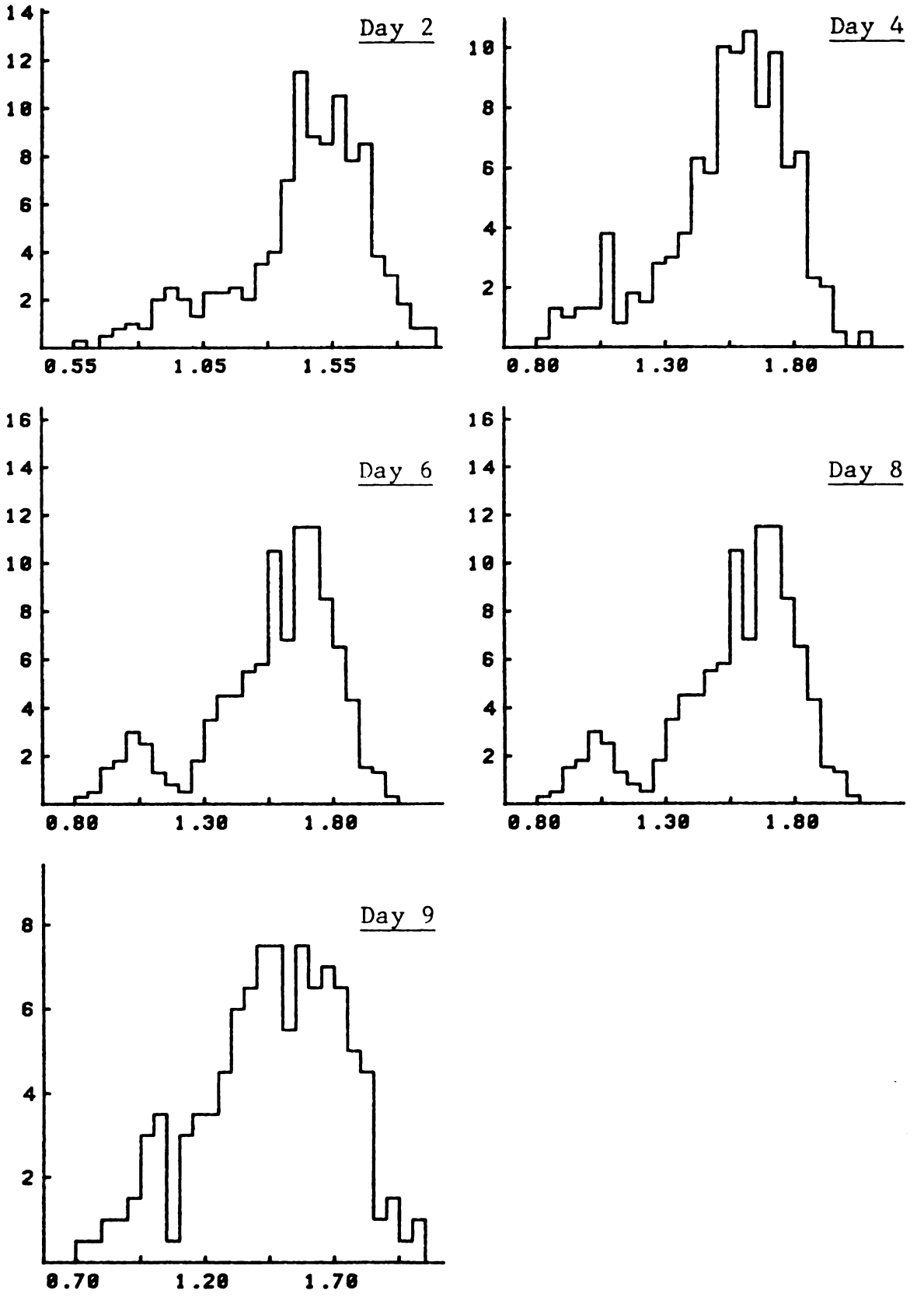
Day 2
14
12
10
8
6
4
2
0.55
1.05
1.55
Day 4
10
8
6
4
2
0.80
1.30
1.80
Day 6
16
14
12
10
8
6
4
2
0.80
1.30
1.80
Day 8
16
14
12
10
8
6
4
2
0.80
1.30
1.80
Day 9
8
6
4
2
0.70
1.20
1.70

Figure 8.

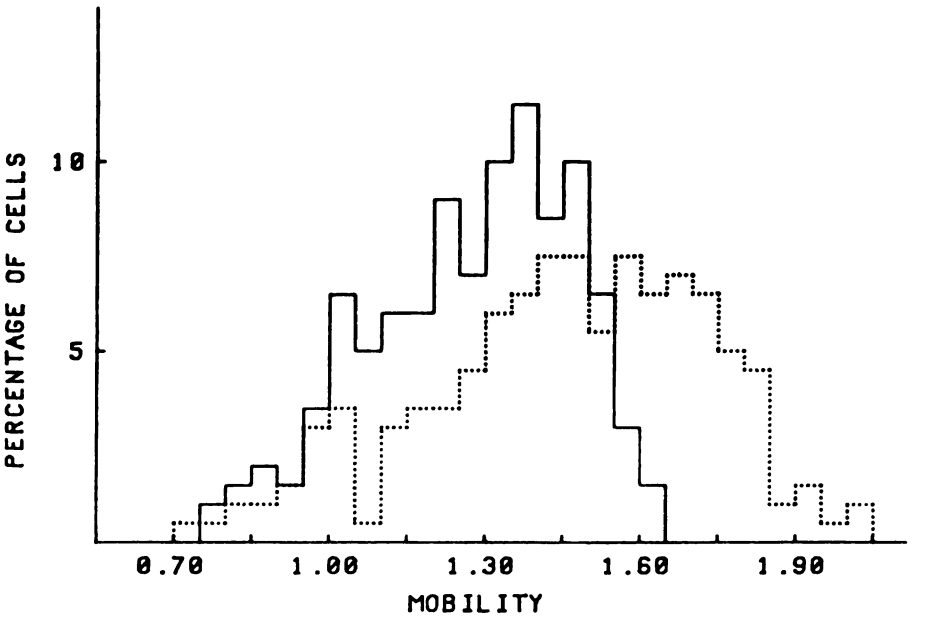

Figure 9. The Mobility Distribution of B (solid) and T (dotted) Lymphocytes.

C. The Changes in the Electrophoretic Mobility Distributions of Spleen Lymphocytes After a Second Injection of the Tumor

The secondary immune reaction to a tumor is different from a primary reaction. In a secondary immune response the immune system has already been exposed to the tumor specific antigens. We have already seen that the mobility of the splenic B cells changed after the injection of the tumor into the mice. To examine if the timing of the increase of mobility of the B cells would change with a second injection of the tumor, mice previously given the S-180J tumor were cured of the tumor by cis-platin injections. These mice were then reinoculated with the tumor and their splenic lymphocytes were removed at various times to study their electrophoretic behavior. We examined both unseparated and nylon wool separated spleen lymphocytes.

The time development of the unseparated splenic lymphocytes from the reinoculation of the S180J tumor is shown in Figure 10. The mean mobility and the percentage of cells with mobilities less than 1.2 are listed in Table 5. There is little change with time of the mean mobility or the variance. The percentage of low mobility cells (mobility < 1.2) decreases from 56% to 47%. This contrasts with the primary response where the decrease in the percentage of low mobility cells is much greater (Table 3).

Figure 1Ø. The Mobility Distribution of Unseparated Spleen Cells after a Reinoculation with the Tumor.

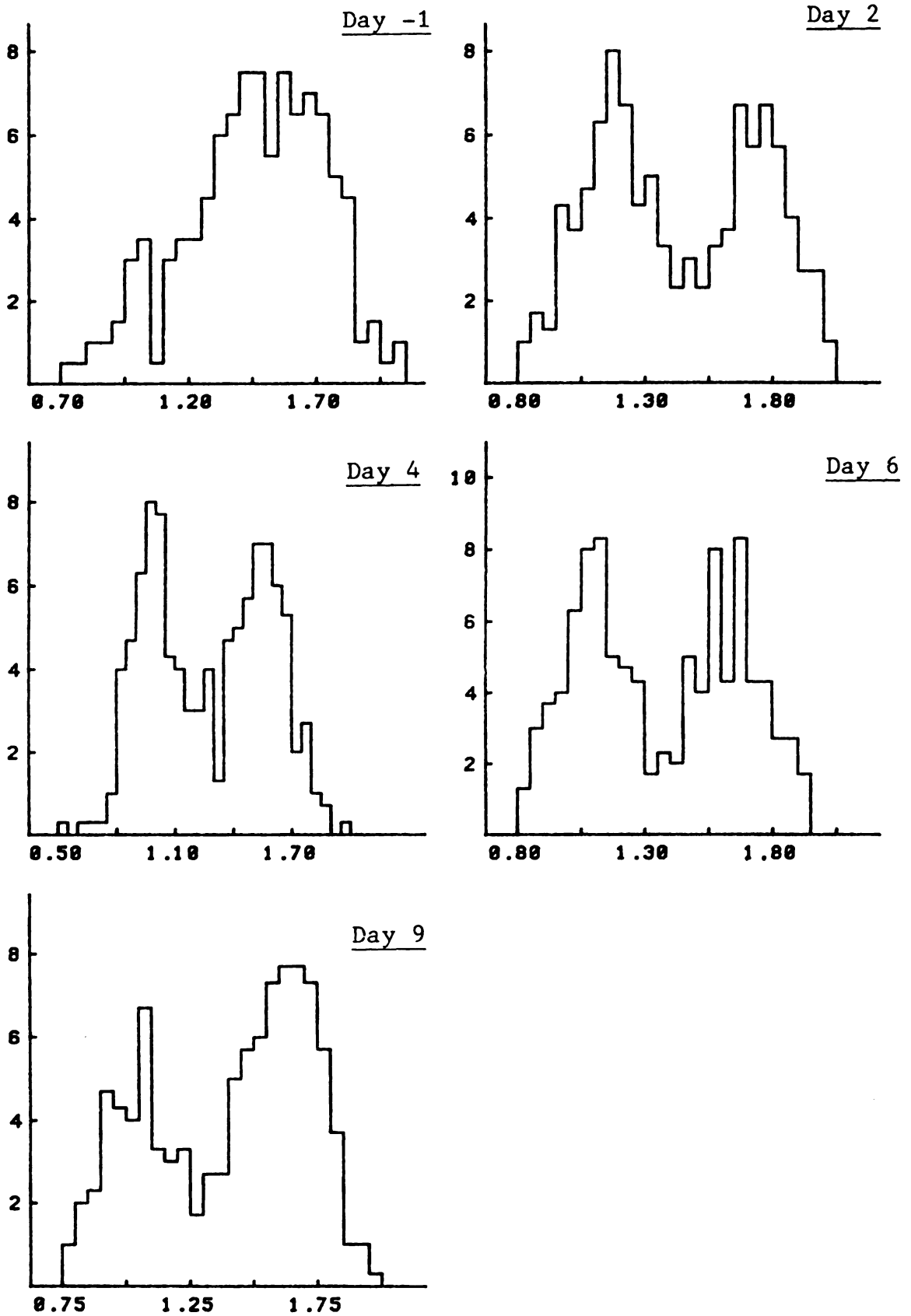
Day -1
8
6
4
2
0.70
1.20
1.70
Day 2
8
6
4
2
0.80
1.30
1.80
Day 4
8
6
4
2
0.50
1.10
1.70
Day 6
10
8
6
4
2
0.80
1.30
1.80
Day 9
8
6
4
2
0.75
1.25
1.75

Figure 10.

Table 5. Mean and standard deviation of spleen lymphocytes and percentage of low mobility cells (reinoculated tumor into previously cured mice).

DAY	Mean Mobility	Percentage of low Mobility cells
-1	1.37 (Ø.3Ø)[1]	56.Ø[1]
2	1.44 (Ø.32)[1]	46.5[1]
4	1.28 (Ø.31)[1]	66.5[1]
6	1.36 (Ø.31)	59.5
9	1.4Ø (Ø.31)	47.Ø

Notes: [1] values are significantly different from Day -1 at the Ø.Ø1 level.
DAY refers to the number of days after the mice were inoculated with the S18ØJ tumor. Low mobility cells refers to cells with mobilities less than 1.2.

Examination of nylon wool effluent cells (Figure 11) from mice reinoculated with the tumor into the previously cured mice shows that there is an immediate increase in the low mobility population followed by a gradual decrease that lasts until the animals die. Figure 11 gives the histograms of the electrophoretic mobility with time after the second inoculation for the nylon wool column effluent cells. Table 6 lists the means of the effluent cells and the percentage of low mobility cells. The mobility falls on day 2 following the second inoculation and the percentage of B cells rises to 62% over the original level of 16%. By day 5, the percentage of low mobility cells, means, and the distributions do not differ with a level of significance of Ø.Ø1.

Figure 11. The Mobility Distribution of T Lymphocytes after Reinoculation with the Tumor.

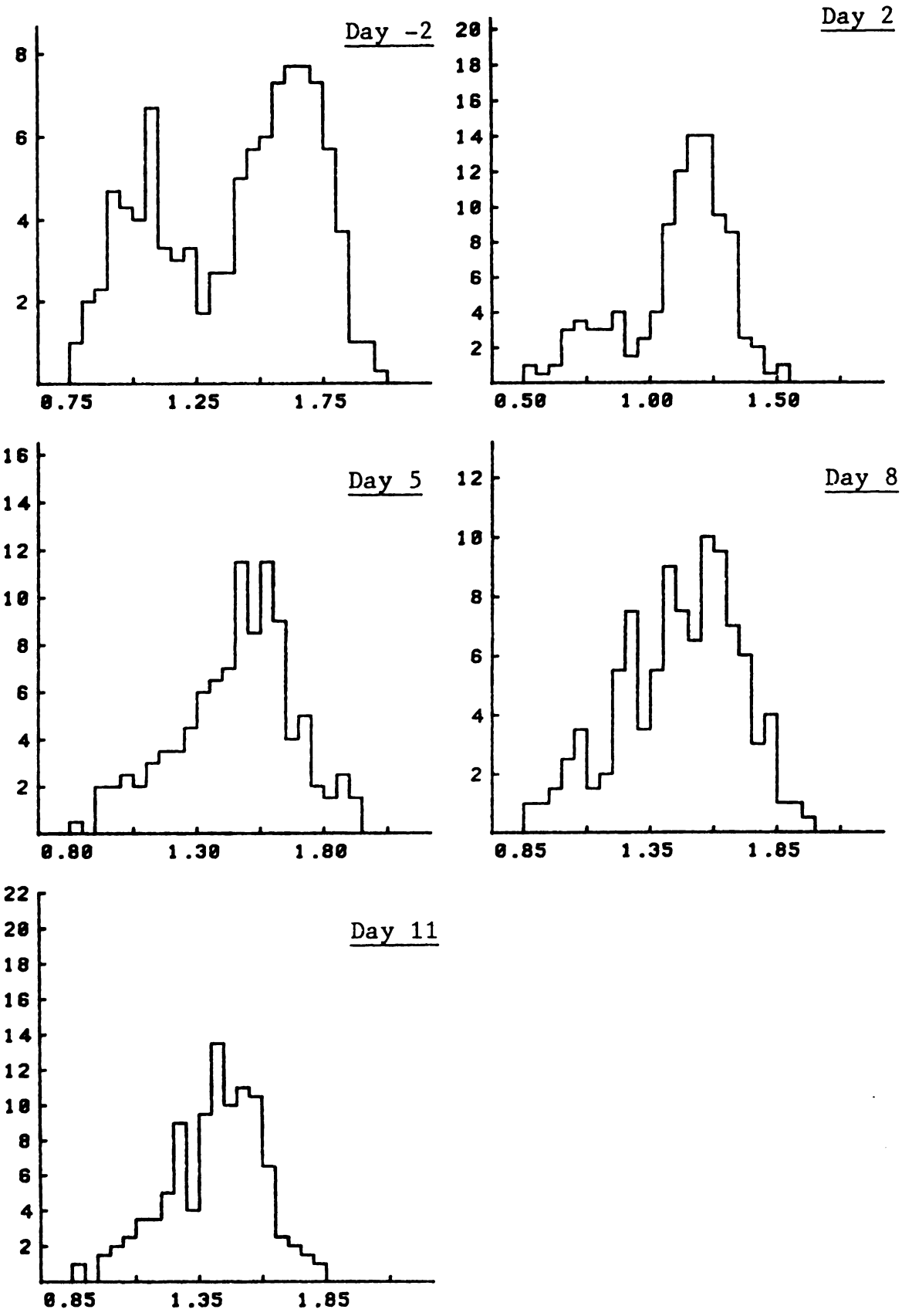
Day -2
8
6
4
2
0.75
1.25
1.75
Day 2
20
18
16
14
12
10
8
6
4
2
0.50
1.00
1.50
Day 5
16
14
12
10
8
6
4
2
0.80
1.30
1.80
Day 8
12
10
8
6
4
2
0.85
1.35
1.85
Day 11
22
20
18
16
14
12
10
8
6
4
2
0.85
1.35
1.85

Figure 11.

Table 6. The mean mobility of the T lymphocytes from mice subjected to a second injection of tumor after being cured of the tumor previously.

DAY	Mean Mobility Exp. #1	Exp. #2	Percentage of Low Mobility Cells
-2		1.38 (Ø.19)	16.Ø
-1	1.4Ø (Ø.33)		23.5
2		1.11 (Ø.21)[1]	62.Ø
2	1.36 (Ø.21)		15.Ø
5		1.45 (Ø.24)[1]	15.5
6	1.58 (Ø.33)[1]		14.Ø
8		1.47 (Ø.24)[1]	13.Ø
9	1.58 (Ø.26)[1]		8.5
11		1.41 (Ø.19)	14.Ø

Notes: [1] The mobility values differ significantly ($P < Ø.Ø1$) from the day before the injection.
The standard deviation is parenthesis beside the mobility value. Low mobility cells refers to cells with a mobility less than 1.2 (um/sec)/(volt/cm). Day is referenced as Day Ø for the second injection of the tumor.

The main effect of the second inoculation upon the B lymphocytes is the shift of the main peak of the cells from a mobility of 1.Ø to a mobility of 1.5. The nylon wool adherent cells from reinoculated mice show that there is an immediate increase in the low mobility population followed by a gradual decrease that lasts until the animals die. Table 7 contains the list of the percentage of low mobility cells with time. If the mobility is graphed against the percentage of low mobility cells the mobilities correlate inversely with this decrease in the percentage of the B lymphocytes with a correlation coefficient of $r = -Ø.962$. Figure 12 contains the histograms of the electrophoretic mobility. The mobility of the main peak before the second

inoculation is about 1.Ø. By 11 days after the second inoculation the mobility of the main main peak is about 1.5, with almost no cells at a mobility of 1.Ø.

Table 7. The mean mobility of the B lymphocytes from mice subjected to a second injection of tumor after being cured of the tumor previously.

DAY	Mean Mobility Exp. #1	Exp. #2	Percentage of Low Mobility Cells
-2		1.14 (Ø.23)	66.5
-1	1.27 (Ø.34)		55.5
2	Ø.99 (Ø.28)		74.5
2		1.11 (Ø.21)[1]	72.Ø
5		1.23 (Ø.25)[1]	5Ø.Ø
6	1.27 (Ø.35)		5Ø.Ø
8		1.41 (Ø.27)[1]	23.5
9	1.42 (Ø.24)[1]		18.Ø
11		1.46 (Ø.18)[1]	8.5

Notes: [1] The mobility values differ significantly ($P < Ø.Ø1$) from the day before the injection.
The standard deviation is parenthesis beside the mobility value. Low mobility cells refers to cells with a mobility less than 1.2 (um/sec)/(volt/cm). Day is referenced as Day Ø for the second injection of the tumor.

Figure 12. The Mobility Distribution of B Lymphocytes after a Reinoculation with the Tumor.

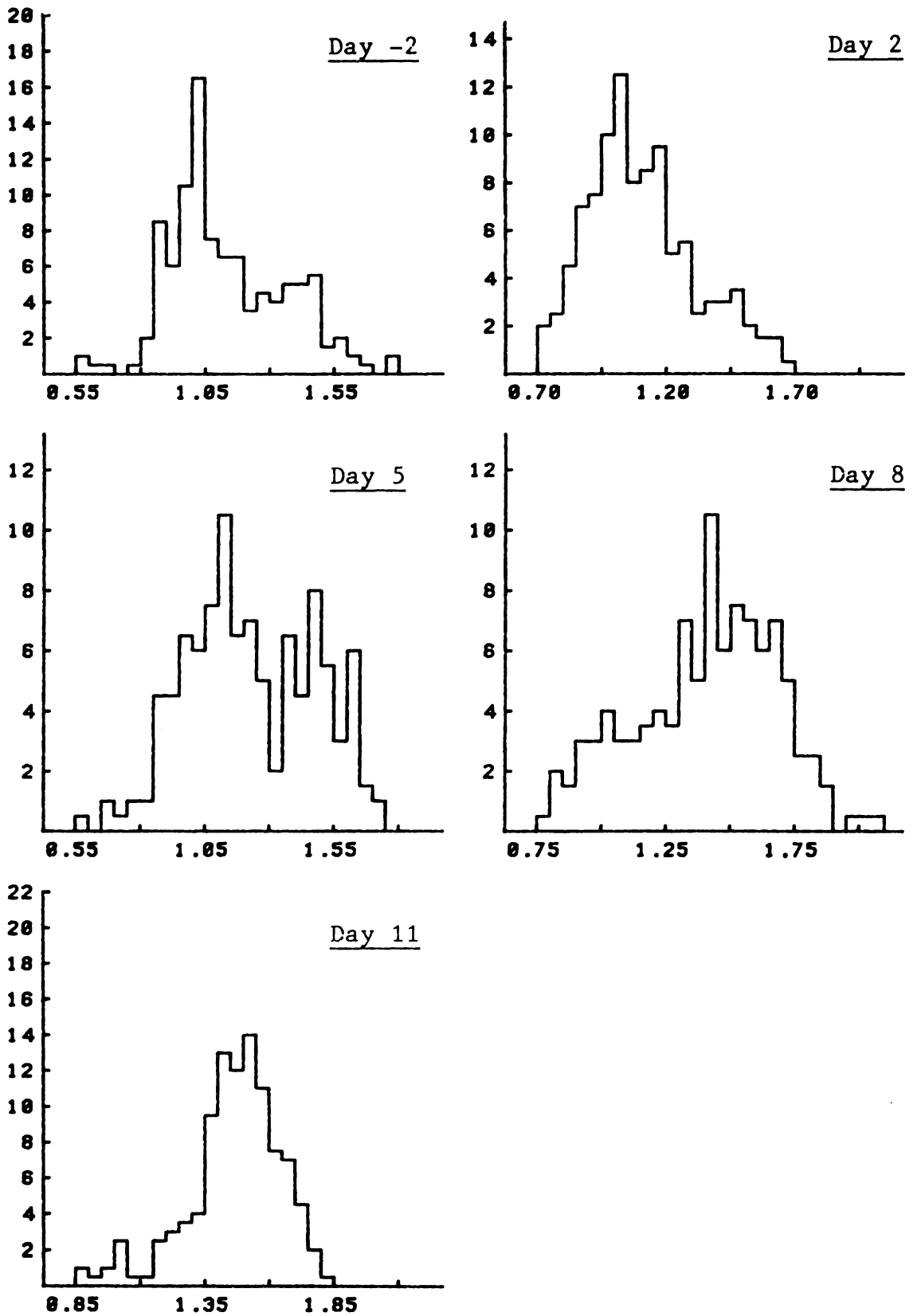
Day -2
20
18
16
14
12
10
8
6
4
2
0.55
1.05
1.55
Day 2
14
12
10
8
6
4
2
0.70
1.20
1.70
Day 5
12
10
8
6
4
2
0.55
1.05
1.55
Day 8
12
10
8
6
4
2
0.75
1.25
1.75
Day 11
22
20
18
16
14
12
10
8
6
4
2
0.85
1.35
1.85

Figure 12.

D. The Mobility Distribution of Spleen Cells From Mice That Were Resistant to the Second Injection of the Tumor

After the second inoculation of tumor cells into the mice, there were some mice which showed no signs of the tumor past the expected time of death based on the number of cells injected into the mice. The mobility histograms of the splenic cells appeared similar to the mobility histogram of splenic cells from normal mice. Figures 13 and 14 are histograms of spleen T and B lymphocytes respectively from such resistant mice taken 34 days after the second injection. Figures 15 and 16 are unseparated spleen lymphocytes from resistant mice at days 22 and 11 respectively. Both show the bimodal curve which is absent in histograms of splenic cells from mice with the S-180J tumor after 8 days. The normal life expectancy of ICR mice injected with 1×10^6 tumor cells is 14 to 18 days. The mobility histograms of the splenic lymphocytes from these mice free of the tumor after the second injection do not show the increase in the mobility of the B cells that was seen in the spleen cells from mice after day 8 of the tumor.

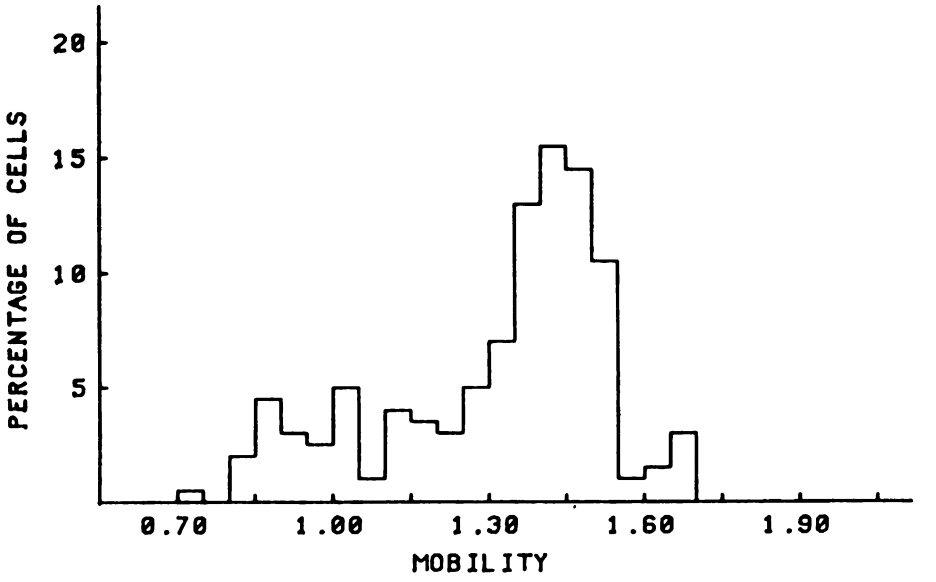

Figure 13. The Mobility Distribution of T Lymphocytes from Survivor Mice.

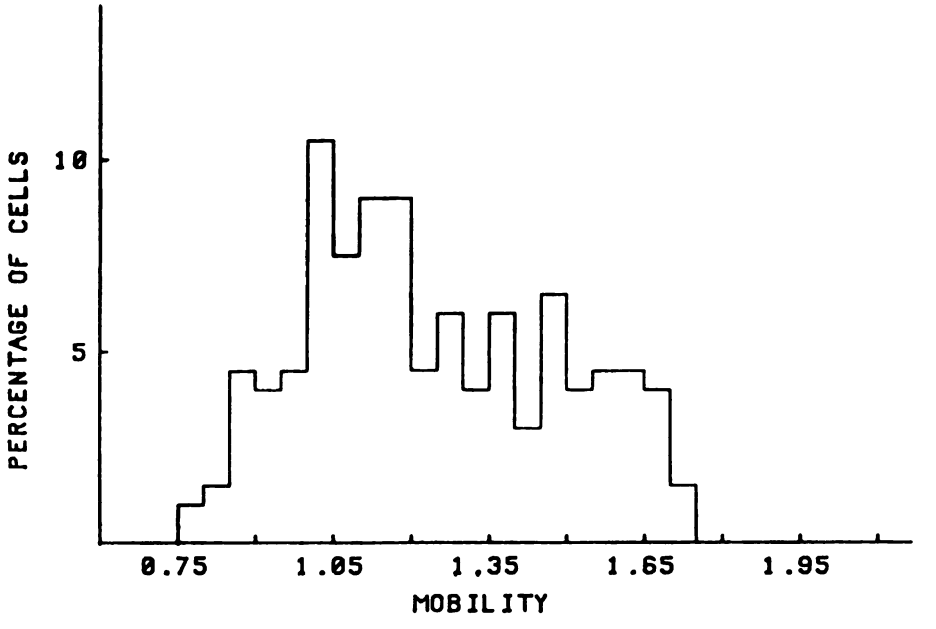

Figure 14. The Mobility Distribution of B Lymphocytes from Survivor Mice.

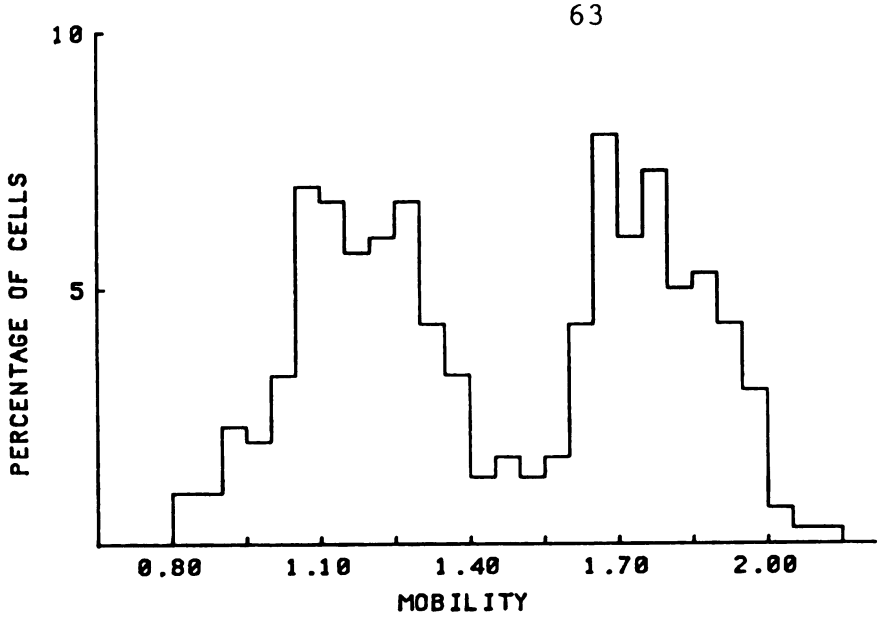

Figure 15. The Mobility Distribution of Spleen Cells from Tumor-resistant Mice 22 days after the Second Injection of the Tumor.

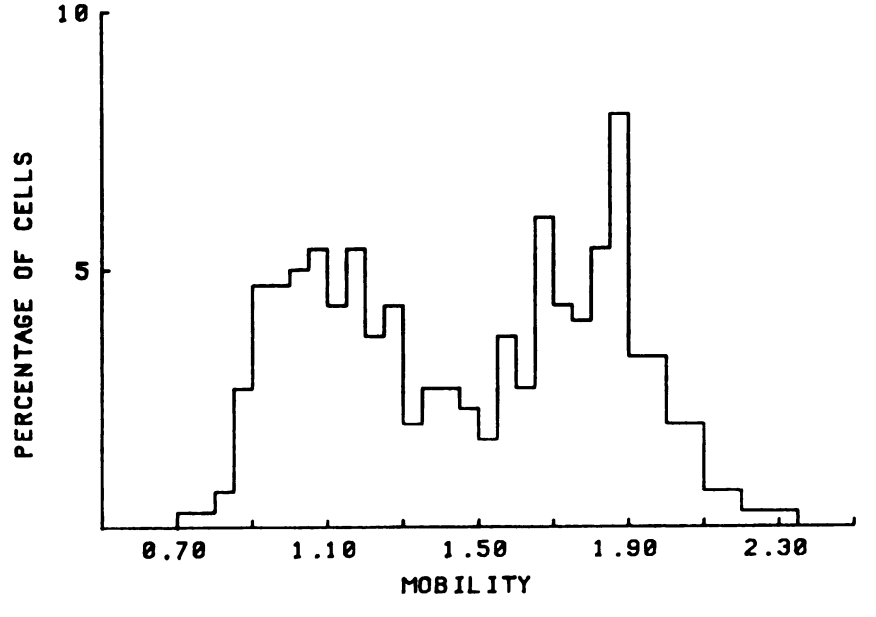

Figure 16. The Mobility Distribution of Spleen Cells from Tumor-resistant Mice 11 days after the Second Injection of the Tumor.

III. The Presence of Surface Nucleic Acids on Spleen Lymphocytes and Their Modulation by Tumor.

Electrophoresis of whole cells is one of the few ways to investigate the surface of cells without destroying the cells in the process. In this section we will discuss the use of electrophoresis to investigate the surface of spleen lymphocytes for the presence of DNA and RNA. Surface DNA and RNA have been related to immune reactions. Here I will show that surface DNA and RNA on the spleen lymphocytes changes with the response of the mouse's body to the growth of a tumor.

A. SDS-PAGE gel electrophoresis of enzymes DNase and RNase

The enzymes used in this study were examined using SDS-PAGE electrophoresis to check on the purity of the enzymes. The DNase from bovine pancreas consists of 4 different DNases which differ from each other by small changes in their composition (49). One component, DNase D, is present only in very small amounts. The other three components are present in the amounts 4:1:1 for DNase A, DNase B and DNase C. The molecular weight of the DNase was reported to be around 31,000 daltons. The results from the SDS-PAGE electrophoresis showed a double band from the DNase sample with both the coomassie blue stain and the silver stain. No other bands were seen in the DNase sample. The DNase band was between the marker bands carbonic anhydrase (molecular

weight of 29,000 daltons) and albumen (molecular weight of 45,000 daltons). The RNase used throughout this series of experiments was from bovine pancreas and is reported to consist of 5 components, 4 active components and one inactive component (50). Its molecular weight was reported by Miles Laboratories to be 13,683 daltons. Only one band was seen in the SDS-PAGE gel for the RNase sample and it was below the lowest marker band (carbonic anhydrase).

B. The presence of DNA on the surface of spleen lymphocytes

One possible origin of surface nucleic acids on the lymphocytes is the binding of nucleic acids released from dead and lysed cells. This breakage would occur due to the handling of cells during isolation. To test this, spleen cells were incubated with cell debris. Approximately 10^8 splenic lymphocytes were lysed by a hand homogenizer. Normal lymphocytes and DNase treated lymphocytes were incubated with the cell debris for 45 minutes on ice. The cells were then washed once in BSS and then prepared normally for electrophoresis. The results are reported in Table 8. The incubation of the cells with the cell debris had no significant effect on the mobility of the cells even at the 0.05 level.

Table 8. The effect of incubating lymphocytes with cell debris upon the mobility of the lymphocytes.

	Control	DNase
Spleen Lymphocytes	1.16 (Ø.25)	1.16 (Ø.24)
Spleen lymphocytes + incubation with cell debris	1.11 (Ø.25)	1.17 (Ø.29)

Note: The cells were treated first with the DNase before being incubated with the cell debris. The standard deviation is in parenthesis.

First normal spleen lymphocytes were examined for the presence of DNA. The presence of DNA on the surface of the spleen lymphocytes was tested by enzymatic digestion with DNase. Statistical testing of the mobility of normal splenic lymphocytes following DNase treatment suggest that there is no detectable surface DNA. The observed level of significance, P value, for the difference between the distributions was greater than Ø.2Ø implying that there was no effect due to the DNase treatment. T lymphocytes were also tested for the presence of DNA)m and they also showed no detectable difference between the normal and DNase treated mobility distributions ($P > Ø.2Ø$). Figures 17 and 18 give the mobility histograms of the normal spleen population and normal T spleen lymphocytes after DNase treatment respectively. The presence of DNA on B cells was not tested separately.

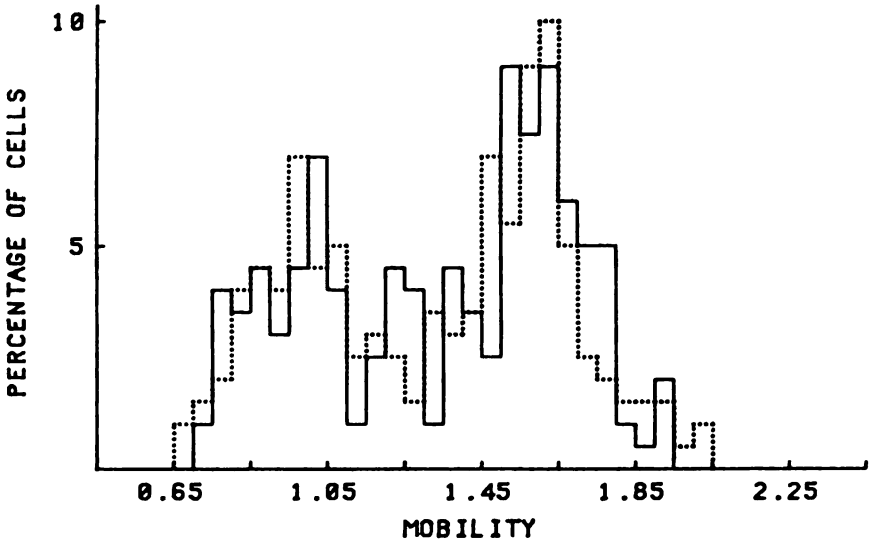

Figure 17. The Effect of DNase on the Mobility of Spleen Lymphocytes. Control is solid line. DNase treated is dotted line.

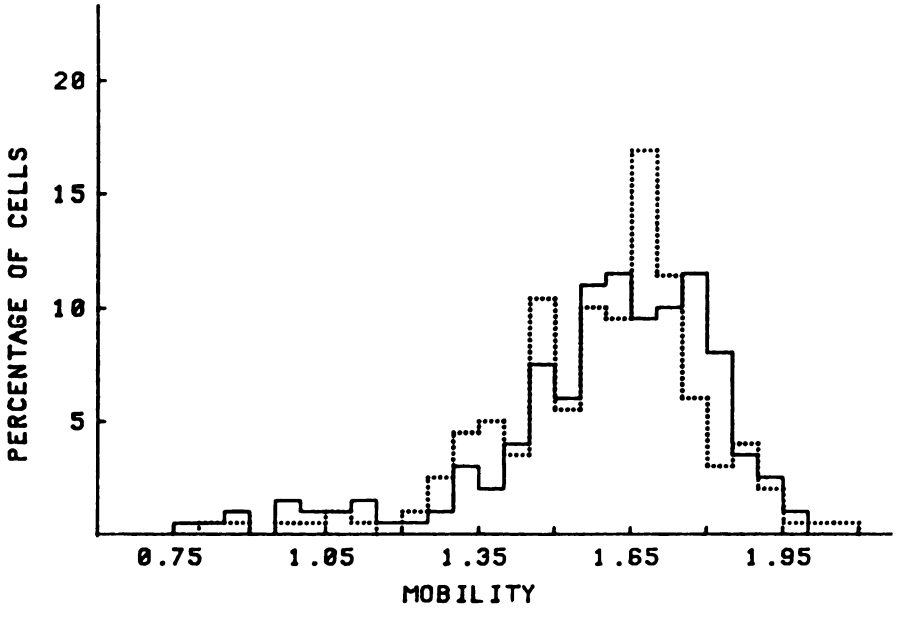

Figure 18. The Effect of DNase on the Mobility of Spleen Lymphocytes. Control is solid line. DNase treated is dotted line.

The ascites tumor S-180J was injected intraperitoneally at 1 x 10^6 cells per mouse on day zero. On following days the mice were sacrificed and their spleens were removed for DNase enzymatic treatment. Several spleen were pooled for each experiment. After enzyme treatment the cells were examined using whole cell microelectrophoresis. Figures 19 and 20 shows the effect of DNase upon the mobility of the spleen cells with time after the inoculation for two separate experiments. Table 9 contains the P values of the effect of the DNase digestions upon the electrophoretic mobility. Examining Table 9, one can see that there is no effect upon the mobility distribution initially. After day 5 in experiment 1 DNase makes a significant difference. In experiment 2, DNase treatment makes a significant difference in the mobility distribution only after day 10.

Table 9. The observed levels of significance for the DNase treatment of spleen cells with time after the inoculation of the tumor.

	Exp. #1		Exp. #2	
DAY	Smirnov	Chi-squared	Smirnov	Chi-squared
2	0.157	>0.250	0.183	>0.250
4			0.071	0.101
5	<0.001	0.004		
6			0.033	0.220
8			0.116	0.240
9	<0.001	0.002		
10			<0.001	<0.001
13	<0.001	<0.001		

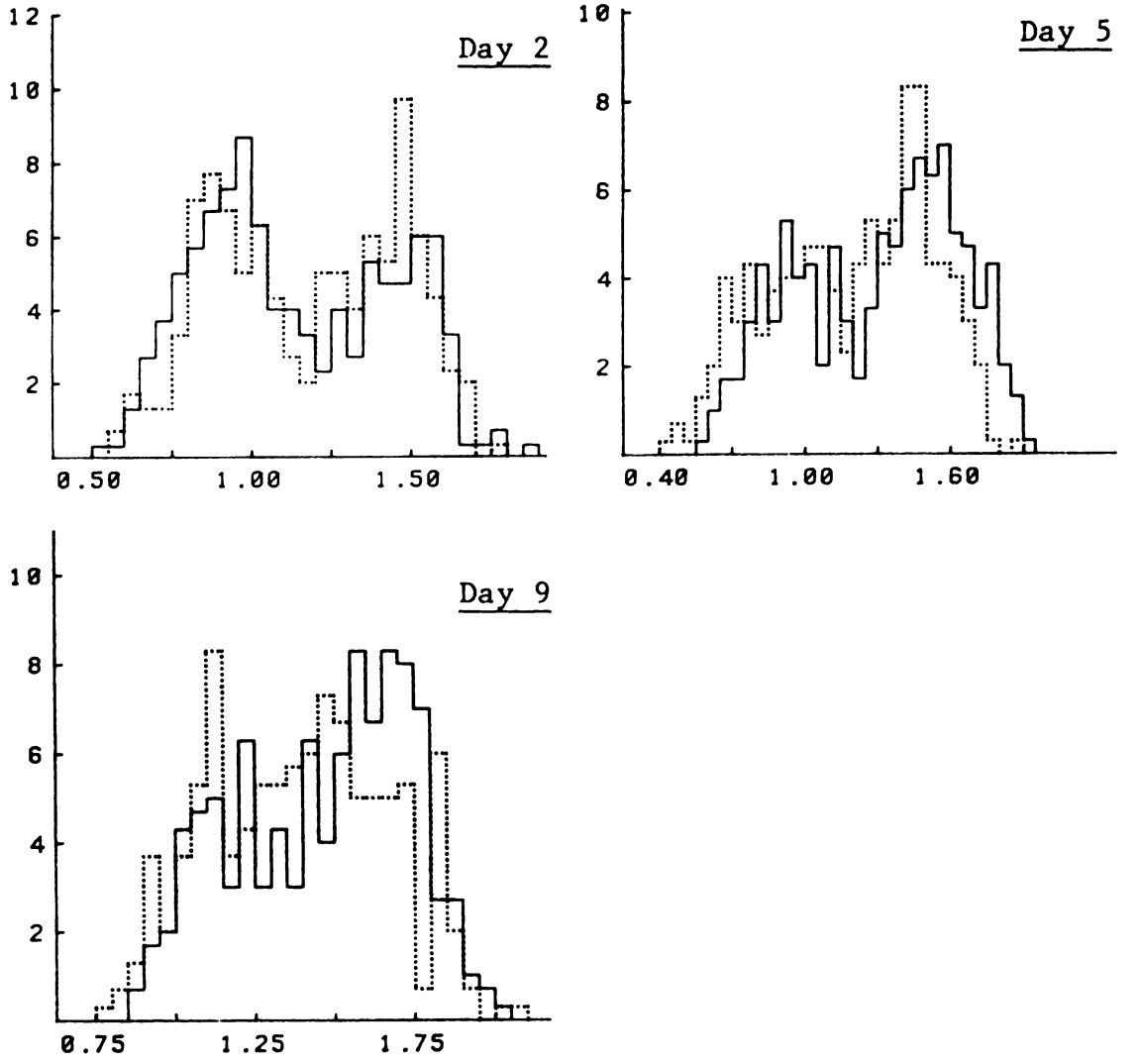

Figure 19. The Effect of DNase (dotted lines) upon the Mobility Distribution of Spleen Cells after the Inoculation of the Mice with S-180J.

Figure 20. The Effect of DNase (dotted line) on the Mobility Distribution of the Spleen Cells after the Inoculation of the Mouse with S-180J.

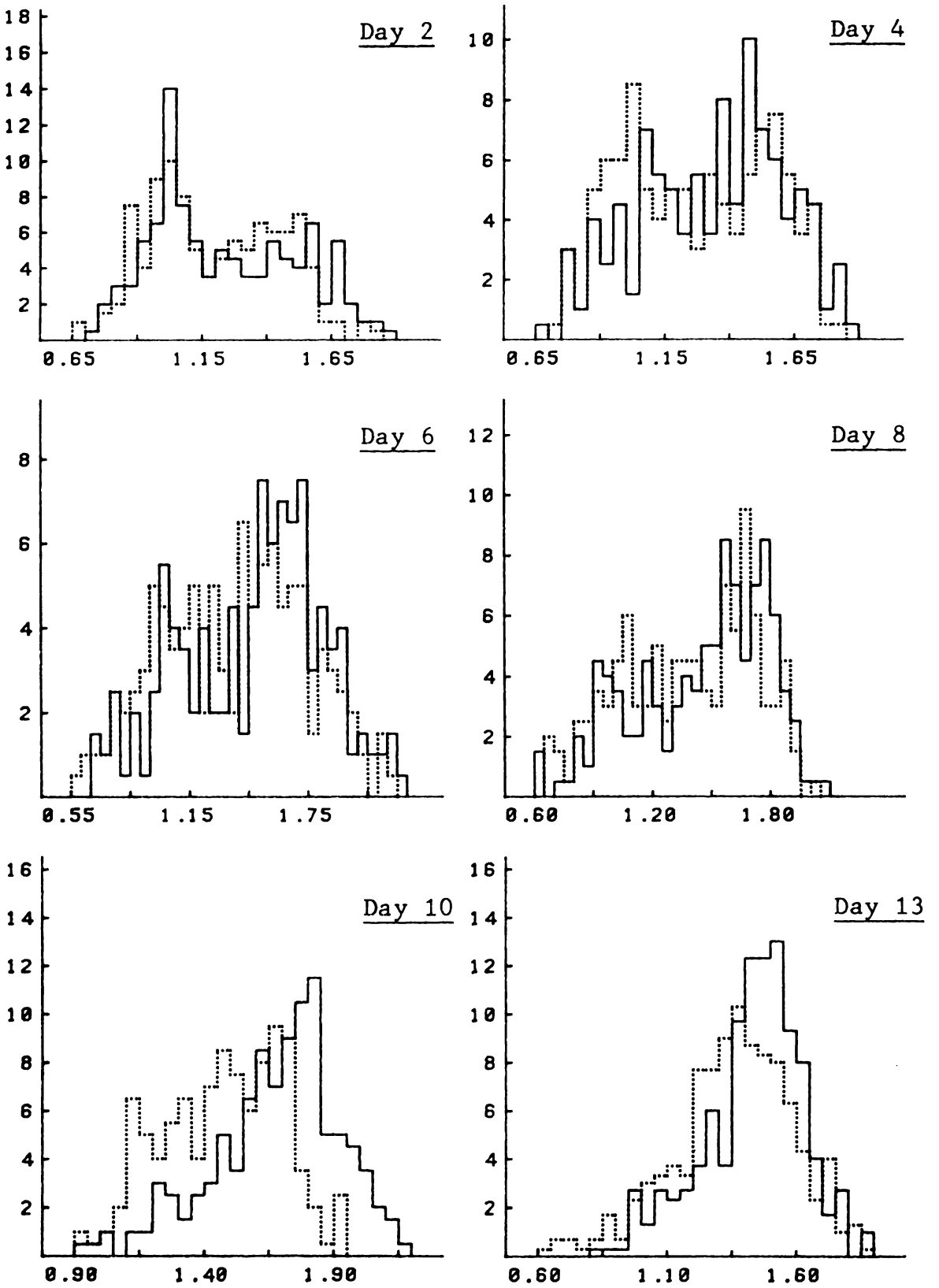
Day 2
18
16
14
12
10
8
6
4
2
0.65
1.15
1.65
Day 4
10
8
6
4
2
0.65
1.15
1.65
Day 6
8
6
4
2
0.55
1.15
1.75
Day 8
12
10
8
6
4
2
0.60
1.20
1.80
Day 10
16
14
12
10
8
6
4
2
0.90
1.40
1.90
Day 13
16
14
12
10
8
6
4
2
0.60
1.10
1.60

Figure 20.

To examine which major type of lymphocytes contain surface DNA, nylon wool columns were used to isolate spleen T lymphocytes. Figure 21 shows the effect of DNA)m on the spleen T lymphocytes from mice inoculated with tumor, and Table 10 lists the P values. From this one can see that there is no induced DNA on the surface of the spleen T lymphocytes. Due to the lack of DNA seen on the T cells, the DNA that appears after day 5 is due to DNA being present on the B lymphocytes and other accessory cells that adhere to the nylon wool. There is no data for the spleen B cells directly, except for one sample 9 days after tumor injection that showed no detectable DNA with P >0.02 (Figure 22).

Table 10. The observed levels of significance values for the DNase enzyme treatment of spleen cells separated into T and B subpopulations.

DAY	CELL Type	Smirnov	Chi-squared
2	T	>0.200	0.212
4	T	>0.200	0.168
6	T	>0.200	0.015
8	T	>0.200	>0.250
9	T	>0.200	>0.250
9	B	>0.200	>0.250

Figure 21. The Effect of DNase (dotted line) on Spleen T Lymphocytes from Mice Inoculated with the Tumor.

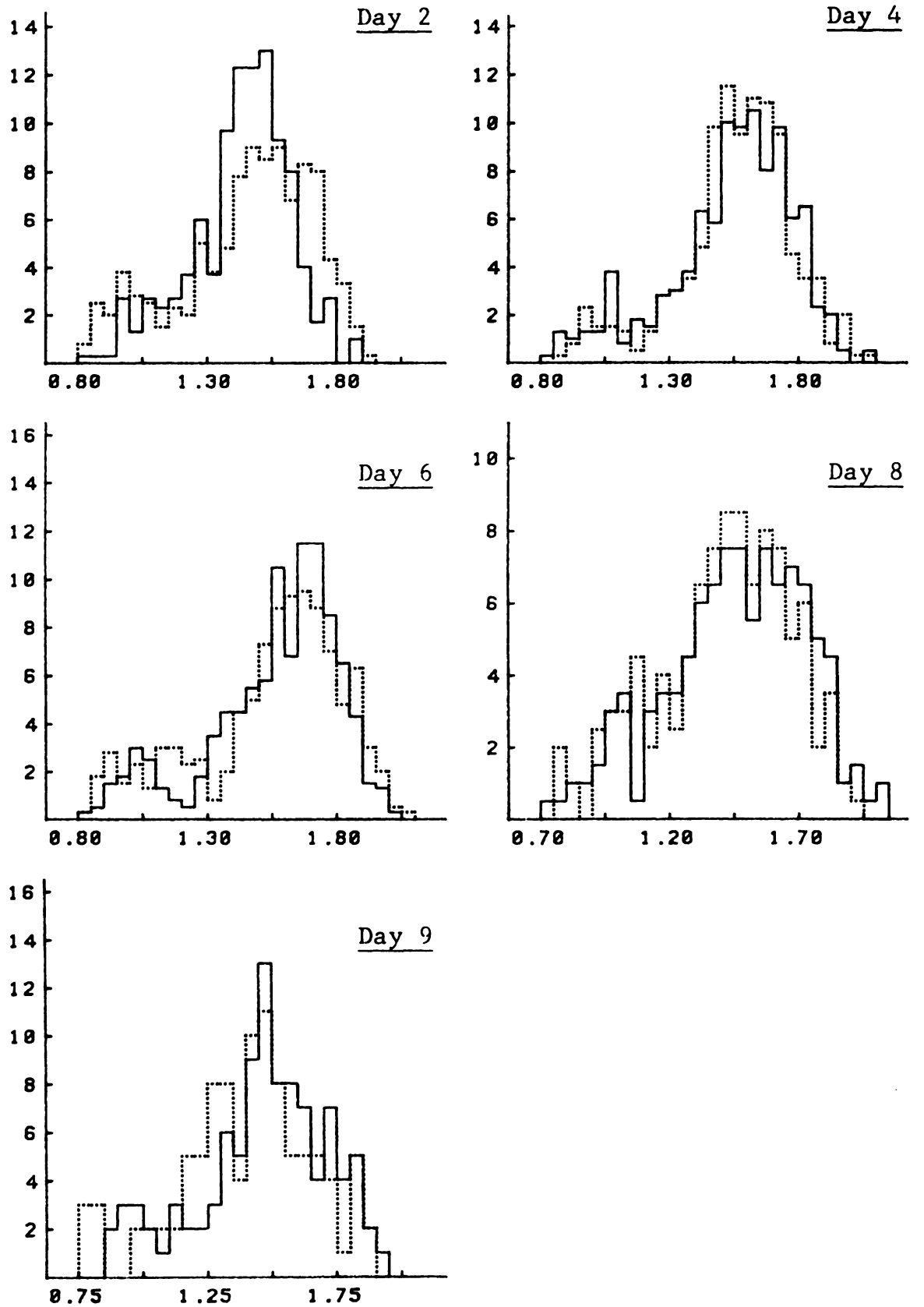
Day 2
14
12
10
8
6
4
2
0.80
1.30
1.80
Day 4
14
12
10
8
6
4
2
0.80
1.30
1.80
Day 6
16
14
12
10
8
6
4
2
0.80
1.30
1.80
Day 8
10
8
6
4
2
0.70
1.20
1.70
Day 9
16
14
12
10
8
6
4
2
0.75
1.25
1.75

Figure 21.

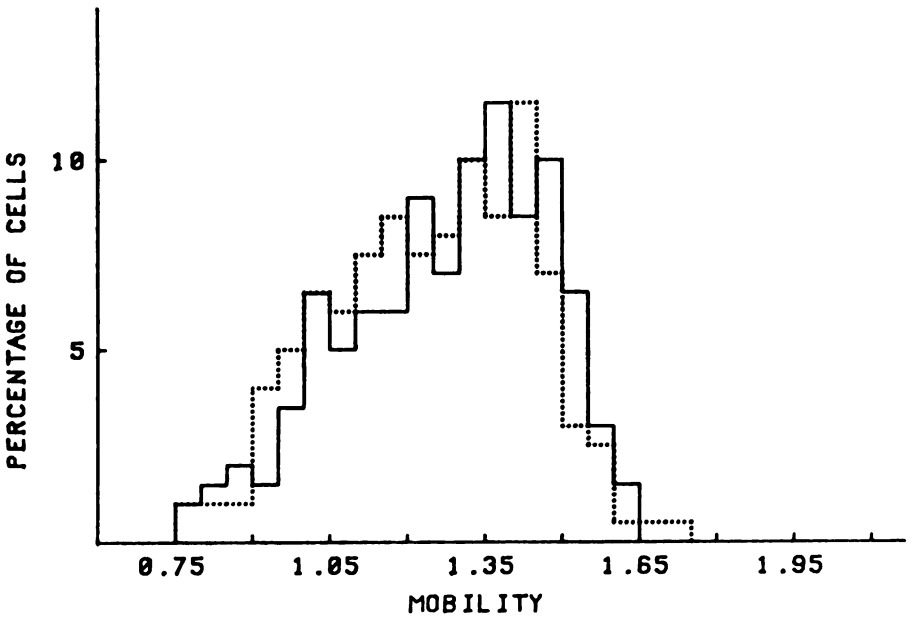

Figure 22. DNase Treatment (dotted line) of B Lymphocytes from Mice 9 days after Tumor Inoculation.

To examine the difference between the primary and secondary immune reaction to the tumor, S180J, mice were inoculated with the tumor and then given cisplatin injections to cure them. Twenty one days later, the tumor was reintroduced into mice that showed no sign of the disease. At various times the spleens of these mice were removed and the cells were examined electrophoretically. The effect of

DNase upon the mobility distributions of these spleen cells is shown in Figure 23. The effect of DNase treatment on the mobility of the spleen cells is shown in Table 11. Before the second inoculation of the tumor, there was no DNA present on the surface of lymphocytes. However, two days after the second inoculation, there was a shift in the mobility of the DNase treated cells to lower values, presumably due to the loss of surface charge resulting from the removal of DNA by DNase treatment. On days 4 to 6, DNase has no effect upon the mobility of the cells. By day 9 though, the DNase treatment lowered the mobility of the spleen cells significantly.

Table 11. The observed levels of significance for the DNase enzyme treatment of spleen cells that isolated from mice that are reinoculated with tumor after being cured of the tumor.

DAY	Smirnov	Chi-squared
-1	Ø.14Ø	Ø.Ø61
2	<Ø.Ø1Ø	<Ø.ØØ1
4	>Ø.2ØØ	>Ø.25Ø
6	>Ø.2ØØ	>Ø.25Ø
9	<Ø.Ø1Ø	<Ø.ØØ1

Figure 23. The Effect of DNase (dotted line) on the Spleen Lymphocytes after a Second Injection of the Tumor.

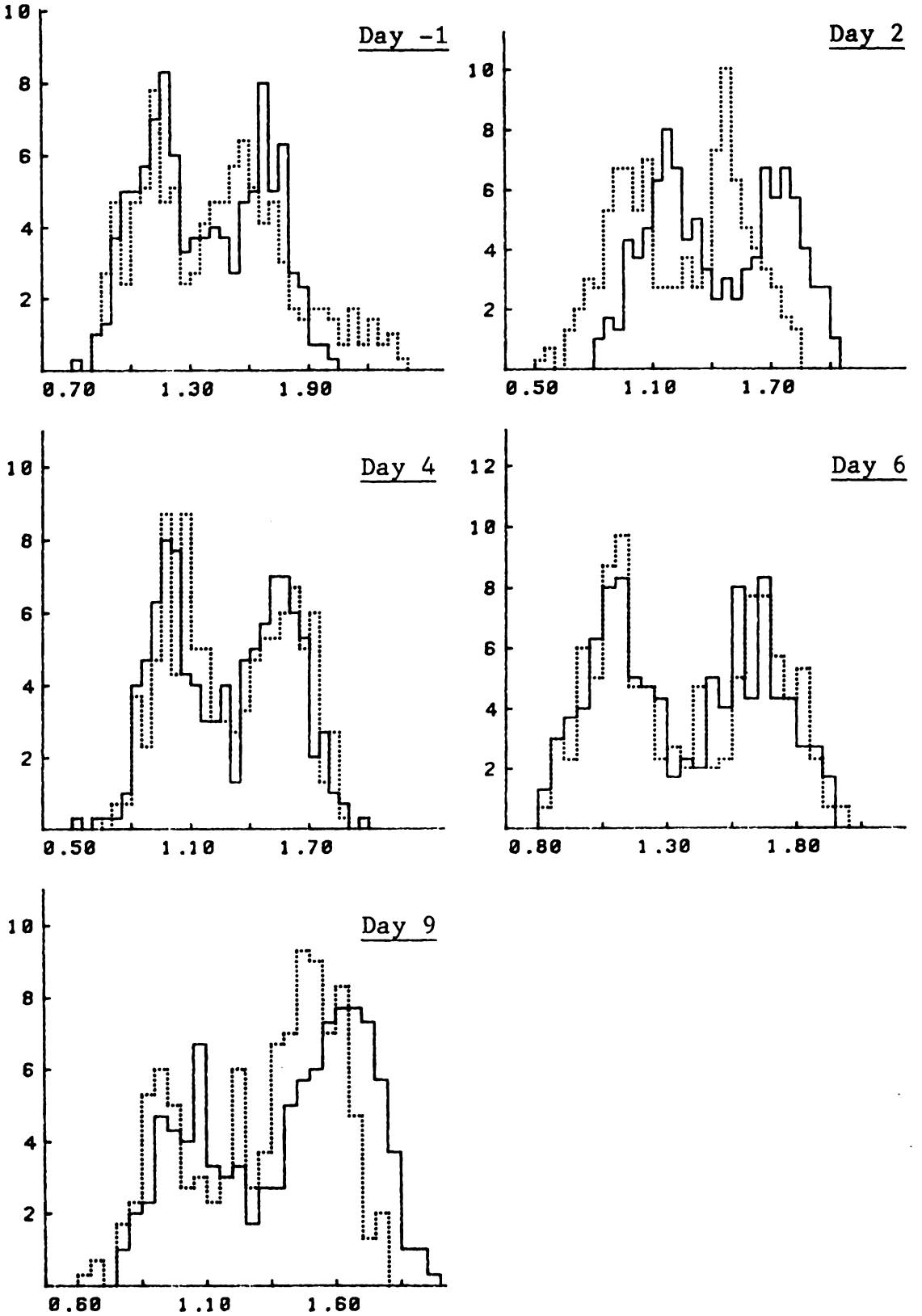
Day -1
10
8
6
4
2
0.70
1.30
1.90
Day 2
10
8
6
4
2
0.50
1.10
1.70
Day 4
10
8
6
4
2
0.50
1.10
1.70
Day 6
12
10
8
6
4
2
0.80
1.30
1.80
Day 9
10
8
6
4
2
0.60
1.10
1.60

Figure 23.

In order to determine which type of lymphocyte contains DNA)m, the spleen cells were separated by nylon wool columns into B and T cells populations.

The presence of DNA on spleen B lymphocytes after a second injection into previously cured mice is illustrated in Table 12 which gives the P values for the effect of DNase treatment on the mobility of the spleen B lymphocytes. Figure 24 shows the mobility distributions of the spleen B lymphocytes from mice after the second injection of the tumor for the experiment 2 in Table 12. In both experiments the mobility of the B lymphocytes show significant differences due to DNase treatment before the second injection of tumor. After the second injection in experiment 1, DNase treatment did not cause a significant change in the mobility of the lymphocytes. In experiment 2, the presence of DNA does not begin to appear until day 8. The mice are beginning to die 12 days after the second injection.

Table 12. The observed levels of significance for the DNase enzyme treatment of spleen B lymphocytes that were isolated from mice that are reinoculated with tumor after being previously cured of that same tumor.

	EXP #1		EXP #2	
DAY	Smirnov	Chi-squared	Smirnov	Chi-squared
-2			<Ø.ØØ1	<Ø.ØØ1
-1	<Ø.ØØ1	<Ø.ØØ1		
2	>Ø.2ØØ	>Ø.25Ø	<Ø.ØØ1	<Ø.ØØ1
5			<Ø.ØØ1	Ø.ØØ5
6	>Ø.2ØØ	>Ø.25Ø		
8			Ø.Ø12	Ø.Ø2Ø
11			>Ø.2ØØ	>Ø.25Ø

Figure 24. The Mobility Distribution of DNase treated (dotted line) and Control (solid line) Spleen B Lymphocytes from Mice Reinoculated with S-180J after being previously cured of the same tumor.

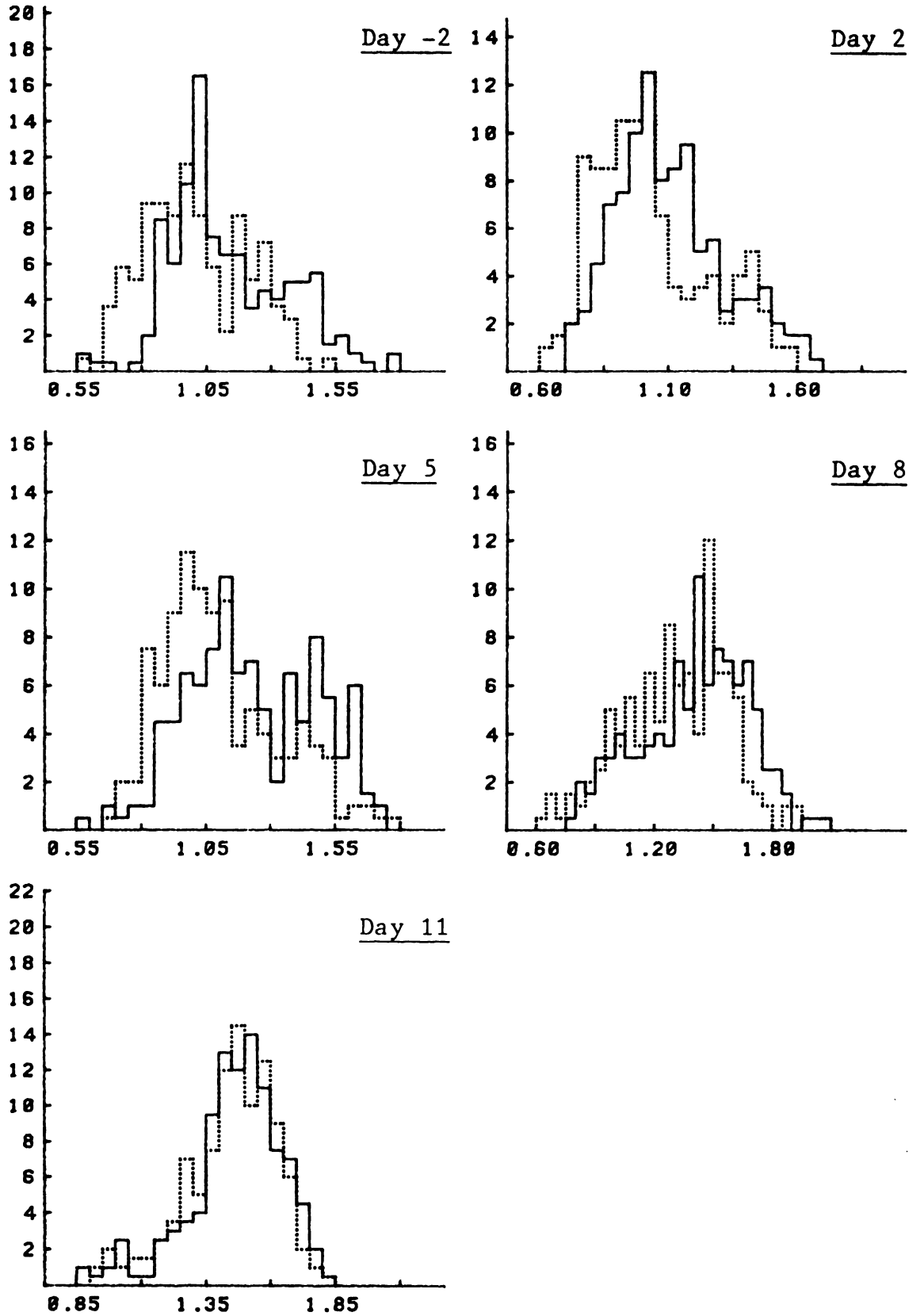
Day -2
Day 2
Day 5
Day 8
Day 11
0.55
1.05
1.55
0.60
1.10
1.60
1.20
1.80
0.85
1.35
1.85

Figure 24.

The presence of DNA upon the spleen T cells with the reinoculated mice is similar to the presence of DNA upon the B lymphocytes. Figure 25 (Experiment 2 in Table 13) gives the mobility distribution showing the effect of DNase upon isolated spleen T lymphocytes. Table 13 lists the P values of the two nonparametric tests analyzing the effect of the enzyme DNase upon the mobility distribution. In experiment 2, DNase treatment of the T cells has an effect until day 11, except for day 2 which is borderline significant at Ø.Ø5 for the Chi-Squared test. In experiment 1, DNase treatment has an initial effect on the mobility before the second injection of the tumor, but day 2 and day 6 after the injection, it has no effect. However by day 9, DNase treatment did change the mobility distribution.

Table 13. The observed levels of significance for the DNase enzyme treatment of spleen T lymphocytes that were isolated from mice that are reinoculated with tumor after being previously cured of that same tumor.

	EXP #1		EXP #2	
DAY	Smirnov	Chi-squared	Smirnov	Chi-squared
-2			<Ø.ØØ1	<Ø.ØØ1
-1	Ø.ØØ3	Ø.Ø1Ø		
2	Ø.Ø24	Ø.Ø34	Ø.Ø12	Ø.Ø86
5			<Ø.ØØ1	Ø.Ø25
6	Ø.194	Ø.222		
8			<Ø.ØØ1	Ø.Ø39
9	<Ø.ØØ1	<Ø.ØØ1		
11			Ø.116	>Ø.25

Figure 25. The Mobility Distribution of DNase treated (dotted line) and Control (solid line) Spleen T Lymphocytes from Mice Reinoculated with S-18ØJ after being previously cured of the same tumor.

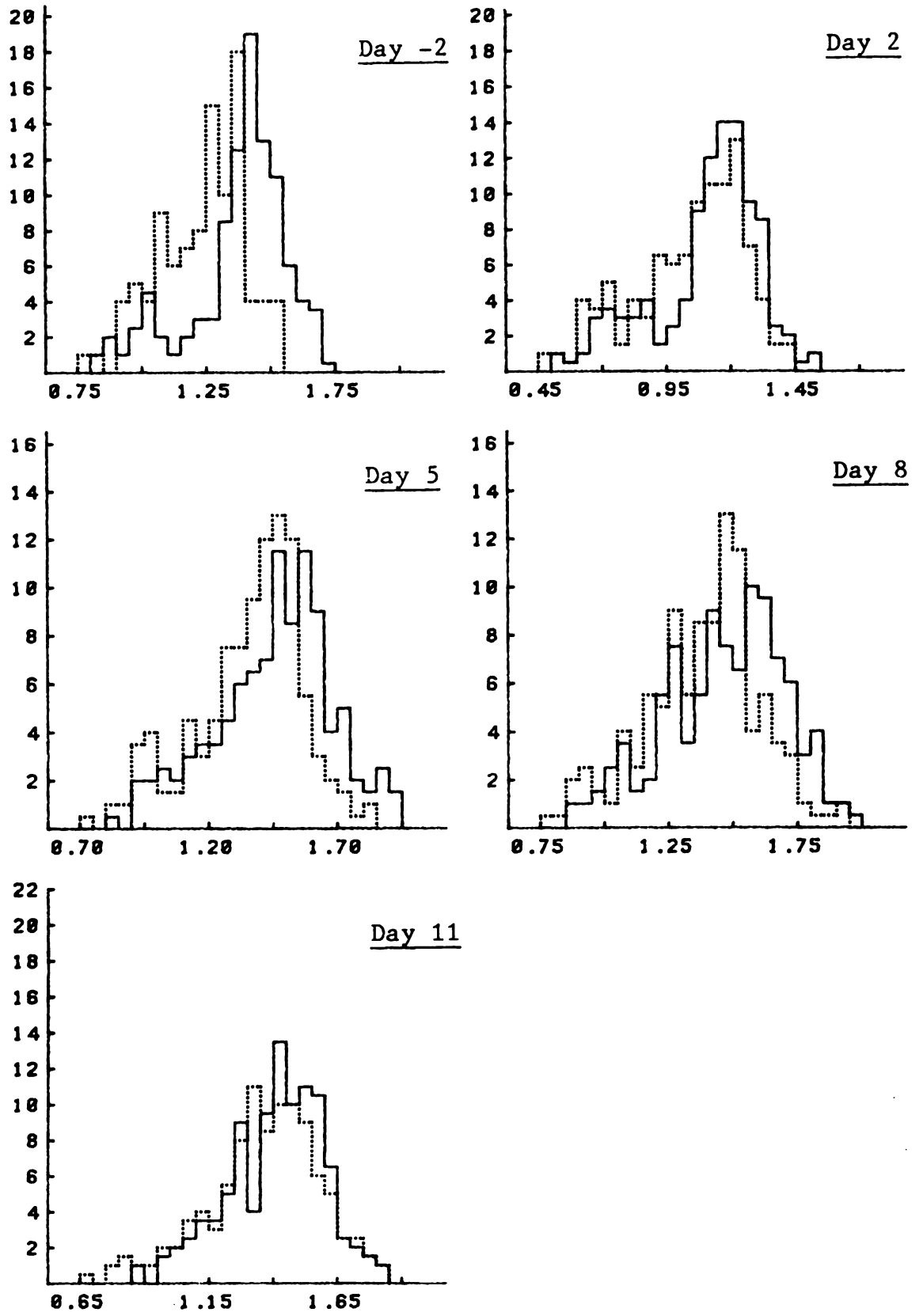
Day -2
Day 2
Day 5
Day 8
Day 11
0.75
1.25
1.75
0.45
0.95
1.45
0.70
1.20
1.70
0.75
1.25
1.75
0.65
1.15
1.65

Figure 25.

Table 14. The presence of DNA)m on spleen lymphocytes which are being exposed to the tumor for a second time.

DAY	Type of Cells: Whole spleen	B lymphocytes	T lymphocytes
-2		+	+
-1	-	+	+
2	+	+	+
4	-		
5		+	+
6	-	-	-
8		+	+
9	+		+
11		-	-

+ indicates the presence of DNA at level of Ø.Ø5
- indicates the absence of DNA at the level of Ø.Ø5

Table 14 summarizes the results which indicate when DNA is expressed on the spleen lymphocytes before and after the second inoculation of the mice with the tumor. The expression of surface DNA appears to occur at the same time in both types of spleen lymphocytes. The discrepancy on day -1 in Table 14 between the whole spleen sample examined for nucleic acids and the separated spleen samples illustrates the increased sensitivity that can be achieved with electrophoresis when examining a less heterogeneous population of cells.

C. The presence of RNA on the surface of spleen lymphocytes

RNA has been shown to be present on the surface of many cells. In this section I will examine the presence of surface RNA on the normal spleen lymphocytes, spleen cells

from mice stimulated with tumor, and from mice that were cured of a tumor and then reinoculated with the same tumor.

One possible origin of surface nucleic acids on the lymphocytes is the binding of nucleic acids released from dead and lysed cells. To test this, spleen cells were incubated with cell debris as previously described. Normal lymphocytes and RNase treated lymphocytes were incubated with the cell debris for 45 minutes on ice. The results are reported in Table 15. The incubation of the cells with the cell debris had no significant effect on the mobility of the cells even at the Ø.Ø5 level.

Table 15. The effect of incubating lymphocytes with cell debris upon the mobility of the lymphocytes.

	Control	RNase
Spleen Lymphocytes	1.16 (Ø.25)	1.15 (Ø.25)
Spleen lymphocytes + incubation with cell debris	1.11 (Ø.25)	1.16 (Ø.27)

Note: The cells were treated first with the RNase before being incubated with the cell debris.

There is no detectable RNA upon normal spleen cells, at least as measured by differences in electrophoretic distributions following RNase treatment ($P > 0.2$). Figure 26 gives both the control and RNase treated normal spleen cell populations.

The S-180J tumor was injected into mice. At various times the spleens were removed and treated with RNase. The resulting mobility distributions, and the changes caused by RNase treatment are seen in Figure 27 (experiment 2 in Table 16). The statistical test P values are in Table 16. The conclusions that can be drawn from the data of the two experiments presented in Table 16 are almost opposite. In experiment 1, RNase treatment causes a significant change in the mobility distributions every day except day 9. In experiment 2, the only time that RNase treatment caused a significant change was day 10.

Table 16. The observed levels of significance for the RNase treatment of spleen cells with time from the inoculation of the tumor on day 0.

	Exp. #1		Exp. #2	
DAY	Smirnov	Chi-squared	Smirnov	Chi-squared
2	<0.001	<0.001	>0.200	>0.250
4			>0.200	0.198
5	<0.001	0.005		
6			>0.200	0.103
8			>0.200	0.117
9	>0.200	0.035		
10			<0.001	<0.001
13	<0.001	<0.001		

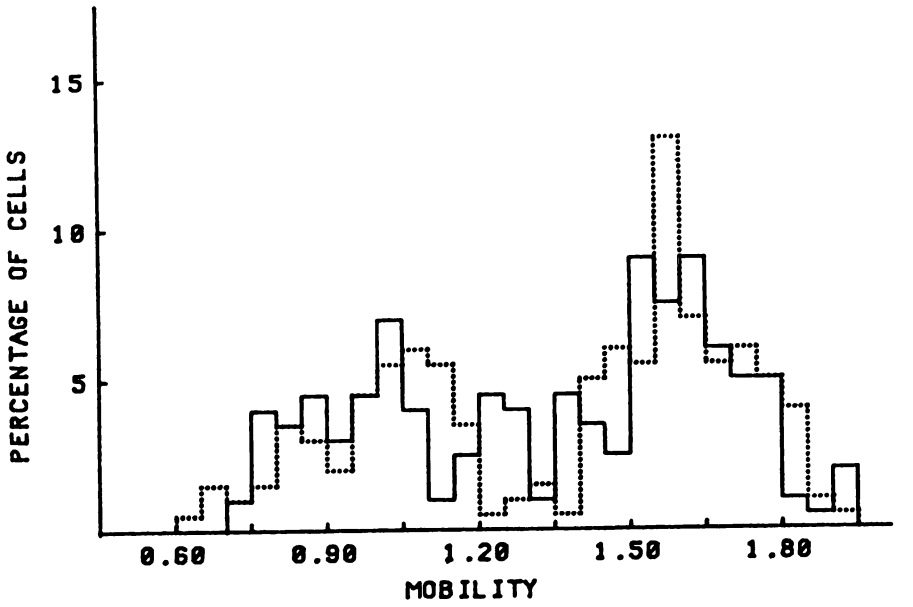

Figure 26: The Mobility Distribution of RNase treated (dotted line) and Control Spleen Lymphocytes.

Figure 27. The Mobility Distribution of RNase Treated (dotted line) and Control (solid line) Spleen Cells from Mice Injected with S-180J.

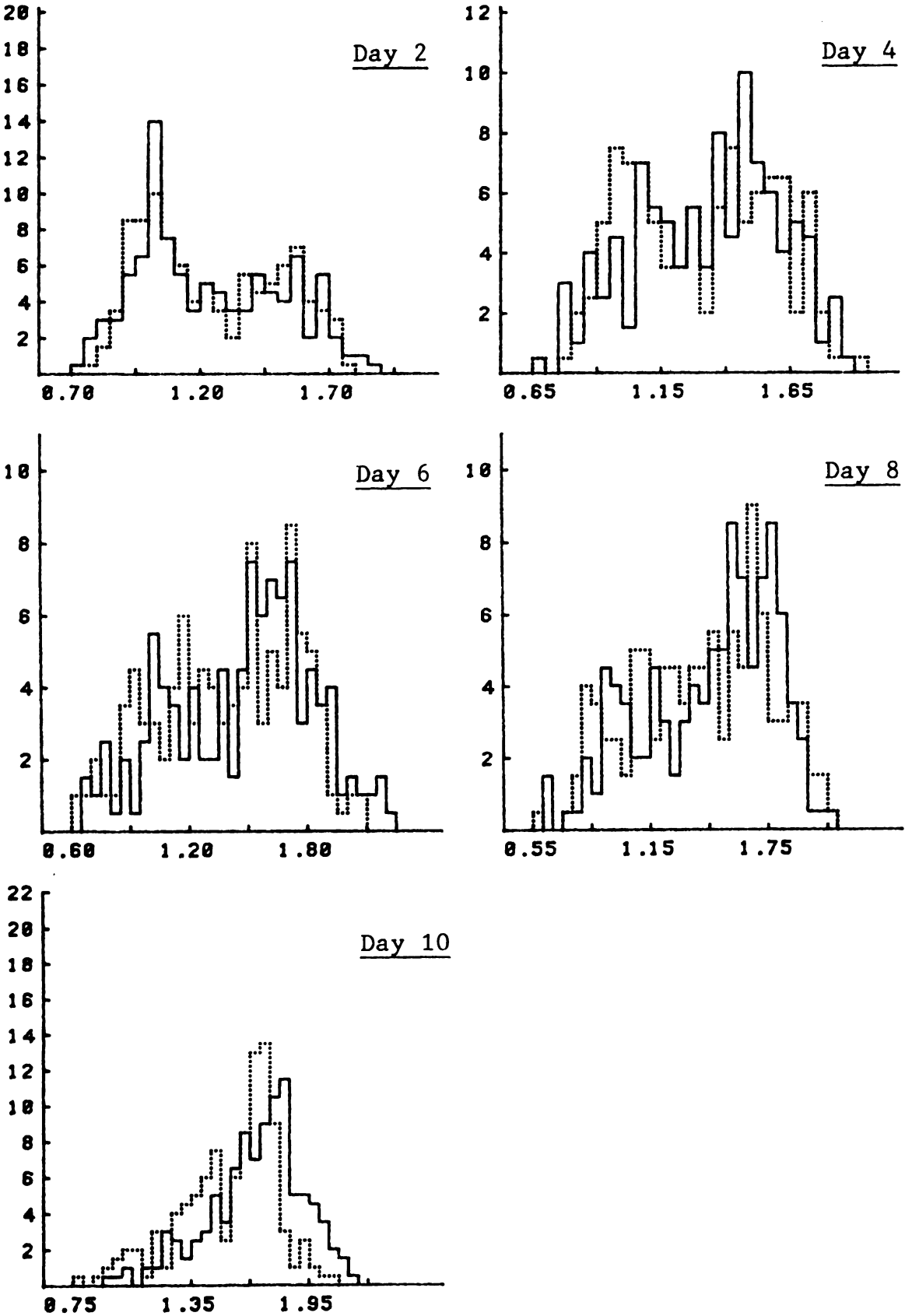
Day 2
20
18
16
14
12
10
8
6
4
2
0.70
1.20
1.70
Day 4
12
10
8
6
4
2
0.65
1.15
1.65
Day 6
10
8
6
4
2
0.60
1.20
1.80
Day 8
10
8
6
4
2
0.55
1.15
1.75
Day 10
22
20
18
16
14
12
10
8
6
4
2
0.75
1.35
1.95

Figure 27.

As with the DNase study, I analyzed the presence of surface RNA following a second injection of the tumor. The effect on the whole spleen population of a second injection of tumor into mice previously cured of the tumor is seen in Figure 28 and the P values arising from the statistical tests are in Table 17. These show that the RNA is expressed before the injection and remains expressed until 4 days after the injection of the tumor. From days 4 to 6 the expression of RNA is gone. By day 9 however it returns. From the graphs it is seen that no subset of the cell population is expressing RNA only.

Table 17. The observed levels of significance for the RNase enzyme treatment of spleen cells that are from mice that are reinoculated with tumor after being cured of the tumor.

DAY	Smirnov	Chi-squared
-1	<Ø.ØØ1	<Ø.ØØ1
2	<Ø.ØØ1	<Ø.ØØ1
4	>Ø.2ØØ	>Ø.25Ø
6	>Ø.2ØØ	>Ø.25Ø
9	<Ø.ØØ1	<Ø.ØØ1

Figure 28. The Effect of RNase (dotted line) upon the Mobility Distribution of Spleen Cells from Mice Reinoculated with S-18ØJ after being Cured of the same tumor.

In order to accentuate any small effects due to RNase digestion that may be hidden when examining the whole spleen, separated T and B lymphocytes populations were examined.

Figure 29 presents the effect of RNase treatment on the mobility distributions of spleen B lymphocytes following a second injection. Table 18 contains the P values of the Smirnov and Chi-squared test comparing the mobility distribution of the control and RNase treated samples. The results drawn from the data in Table 18 again appears contradictory. In experiment 2, RNase treatment has an effect on the mobility distribution every day, except day 8 which is borderline significant at Ø.Ø5 in the Chi-squared test. Experiment 1 showed RNase treatment had an effect only on day -1 and day 9.

The effect of RNase treatment upon splenic T cells is given in Figure 3Ø (experiment 2 Table 19). Table 19 gives the P values of the test statistics. In experiment 1, RNA is expressed only on day 9 at an Ø.Ø1 level of significance while before the injection it is only significant at Ø.Ø5. Experiment 2 only showed significant effect due to RNase treatment before the injection and on day 5 after the injection.

Table 18. The observed levels of significance for the RNase enzyme treatment of spleen B lymphocytes that were isolated from mice that are reinoculated with tumor after being previously cured of that same tumor.

	Exp 1		Exp 2	
DAY	Smirnov	Chi-squared	Smirnov	Chi-squared
-2			<0.001	<0.001
-1	0.005	0.004		
2	>0.200	>0.250	<0.001	<0.001
5			<0.001	<0.001
6	>0.200	>0.250		
8			0.003	0.050
9	<0.001	<0.001		
11			<0.001	0.016

Table 19. The observed levels of significance for the RNase enzyme treatment of spleen T lymphocytes that were isolated from mice that are reinoculated with tumor after being previously cured of that same tumor.

	Exp 1		Exp 2	
DAY	Smirnov	Chi-squared	Smirnov	Chi-squared
-2			<0.001	<0.001
-1	0.017	0.033		
2	>0.200	>0.250	>0.200	>0.25
5			<0.001	0.003
6	>0.200	>0.250		
8			>0.200	>0.25
9	<0.001	<0.001		
11			>0.250	>0.25

Figure 29. The Effect of RNase (dotted line) on the Mobility Distribution of Spleen B Cells (solid line) in a Secondary Response to S-18ØJ.

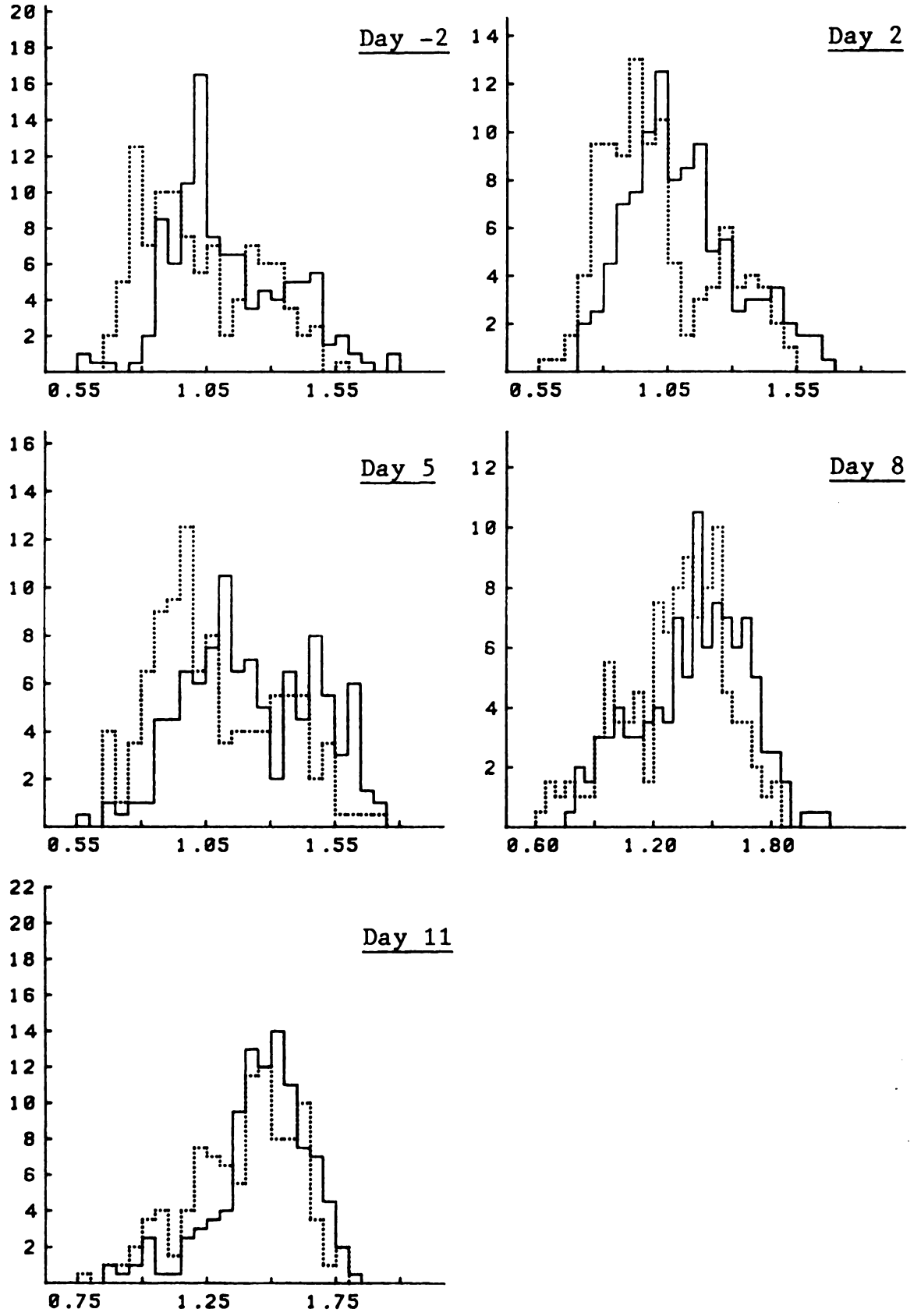

Figure 29.

Figure 30. The Effct of RNase (dotted line) on the Mobility Distribution of Spleen T Cells (solid line) in a Secondary Response to S-180J.

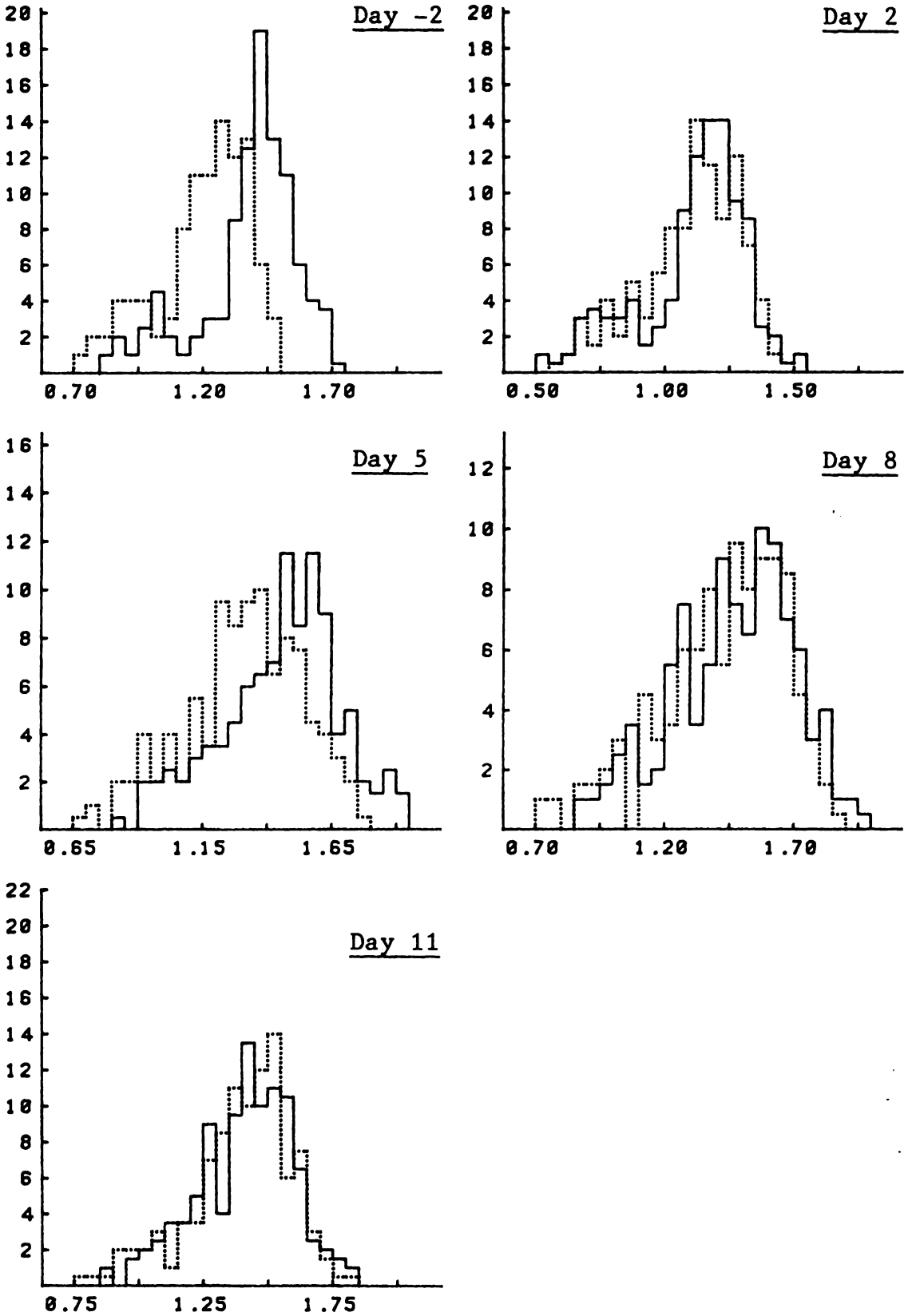
Day -2
20
18
16
14
12
10
8
6
4
2
0.70
1.20
1.70
Day 2
20
18
16
14
12
10
8
6
4
2
0.50
1.00
1.50
Day 5
16
14
12
10
8
6
4
2
0.65
1.15
1.65
Day 8
12
10
8
6
4
2
0.70
1.20
1.70
Day 11
22
20
18
16
14
12
10
8
6
4
2
0.75
1.25
1.75

Figure 30.

The effect of RNase upon the mobility of the spleen cells is summarized in Table 20. The two P values for the Smirnov and Chi-squared test were averaged to give a single P value. Note that the results are presented by experimental run because of the differences between the two sets of data.

Table 20. The presence of RNA on the surface of spleen lymphocytes which are being exposed to the tumor for a second time after the mice had been previously cured.

	Type of Cells				
DAY	Whole spleen	B lymphocytes		T lymphocytes	
		Exp.1	Exp.2	Exp.1	Exp.2
-2			+		+
-1	+	+		+	
2	+	-	+	-	-
4	-				
5			+		+
6	-	-		-	
8			+		-
9	+	+		+	
11			+		-

+, - indicates the presence, absence of RNA at a significance level of 0.05

D. Summary of the presence of membrane associated nucleic acids

A summary of the time dependence of DNA and RNA on spleen lymphocytes is given in Table 21 for the first injection of the tumor and in Table 22 for the second injection. There are some differences, but the majority of the time when DNA is on the surface of the cells, RNA is also on the surface of the cells regardless of the type of lymphocyte. There are major differences between experiments. Also there are some minor differences between the whole spleen cell population and separated T and B lymphocyte subpopulations.

Table 21. The presence of RNA and DNA on the surface of spleen lymphocytes which are being exposed to the tumor for the first time.

	Exp. #1		Exp. #2	
DAY	DNA	RNA	DNA	RNA
2	-	+	-	-
4			-	-
5	+	+		
6			-	-
8			-	-
9	+	-		
10			+	+
13	+	+		

+, - indicates the presence, absence of DNA or RNA at significance level of 0.05

Table 22. The presence of RNA and DNA on the surface of isolated B and T lymphocytes and unseparated splenic lymphocytes which are being exposed to the tumor for a second time after being previously cured.

	Whole spleen		B lymphocytes				T lymphocytes			
DAY	DNA	RNA	DNA		RNA		DNA		RNA	
			#1	#2	#1	#2	#1	#2	#1	#2
-2				+		+		+		+
-1	-	+	+		+		+		+	
2	+	+	-	+	-	+	+	+	-	-
4	-	-								
5				+		+		+		+
6	-	-	-		-		-		-	
8				+		+		+		-
9	+	+			+		+		+	
11				-		+		-		-

+, - indicates the presence, absence of DNA or RNA at significance level of 0.05

E. The presence of nucleic acids on lymphocytes from mice resistant to the tumor after a second injection of the tumor

In each secondary inoculation experiment there were a few mice who proved to be resistant to the tumor (did not died by 14 days or show signs of the tumor externally or internally after 6 days) after the second injection of the tumor. The presence of surface nucleic acids was examined using electrophoresis and enzymatic digestion.

In order to obtain mice that were resistant to the second injection of tumor, survivors were examined for any signs of the tumor while removing the spleen. If no signs of the tumor were present several days after the expected time of death then the mouse was assumed to be free of the

tumor. Figures 31 and 32 show a whole spleen sample treated with DNase and RNase, respectively, 22 days after the second injection of the tumor. Statistical testing (Table 23) shows that there is no detectable RNA, but that DNA is present on these cells. Enzyme treatment of separated spleen lymphocytes from resistant mice 13 days after the second injection of the tumor is shown in Figures 33 to 36. Figures 33 and 34 show the effect of nuclease treatment upon the B lymphocytes. Nuclease treatments showed enough differences to conclude that both DNA and RNA appear on the B lymphocytes. The lack of effects upon T lymphocytes seen in Figures 35 and 36 allows the conclusion that no surface nucleic acids are detected. Close examination of the whole spleen sample (Figures 31 and 32) indicates an effect on the T cells, but this might be explained by difference in the time following the second injection. The whole spleen sample was examined at 11 and 22 days after the second injection whereas the separated samples were examined only 13 days after the second injection.

Table 23. The presence of nucleic acids on the surface of spleen cells from tumor resistant mice.

Day	Whole Spleen		Nylon Wool Separated			
			Adherent		Effluent	
	DNA	RNA	DNA	RNA	DNA	RNA
11	+	+				
13			+	+	-	-
22	-	-				

+, - indicates the presence, absence of DNA or RNA at the significance level of 0.05.

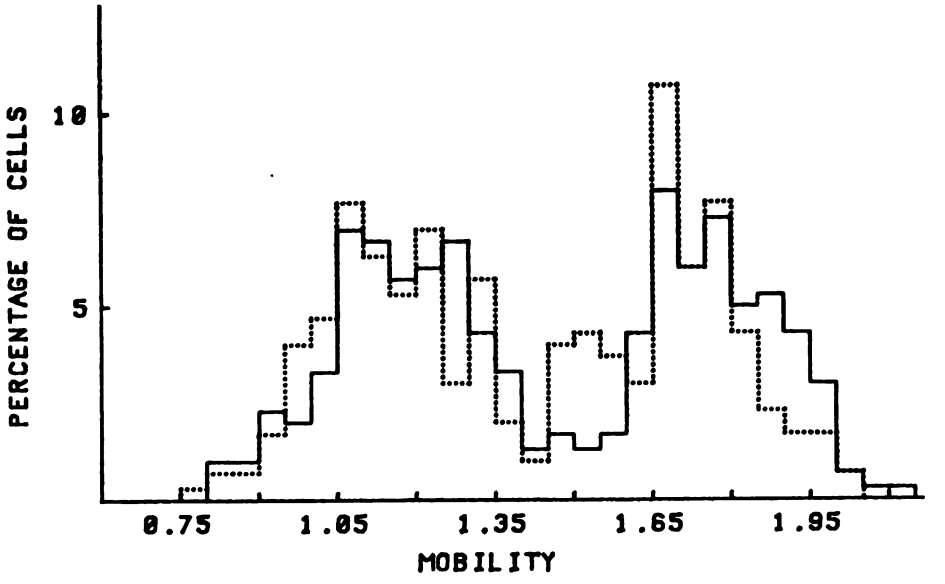

Figure 31. The Effect of DNase (dotted line) on the Mobility Distribution of Spleen Cells (solid line) 22 days after the Second Injection of the Tumor.

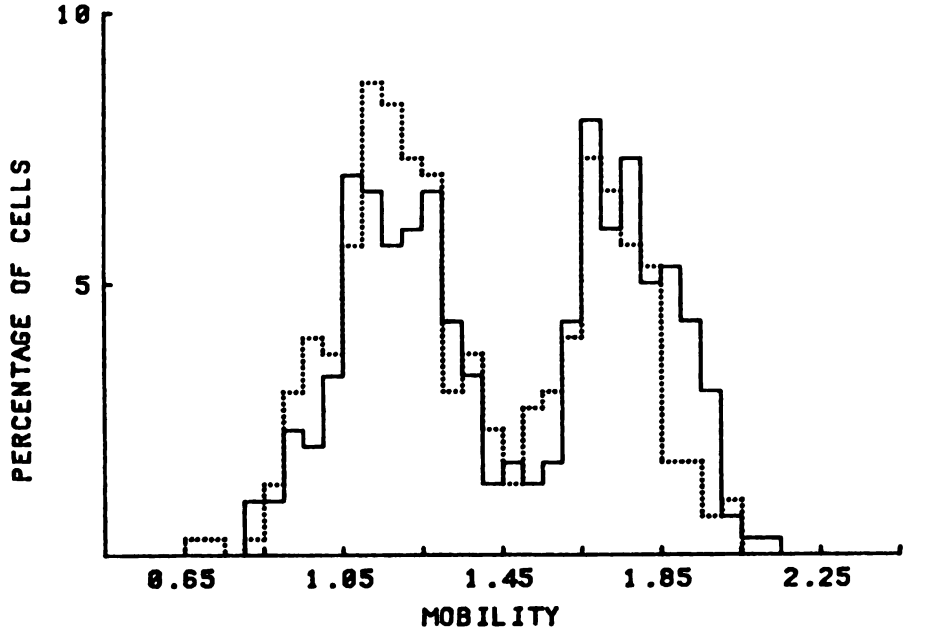

Figure 32. The Effect of RNase (dotted line) on the Mobility Distribution of Spleen Cells (solid line) 22 days after the Second Injection of the Tumor.

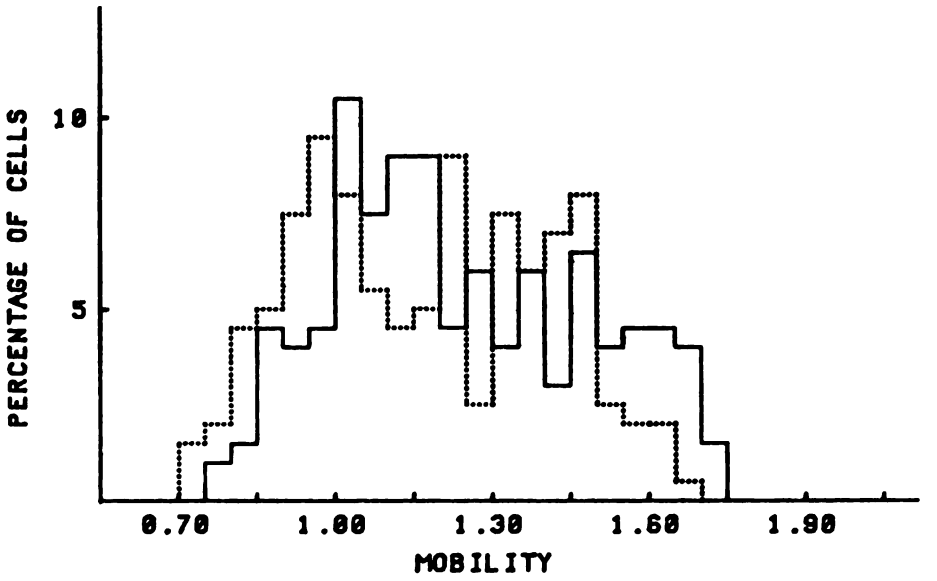

Figure 33. The Effect of DNase (dotted line) on the Mobility Distribution of B Lymphocytes (solid line) 13 days after a Second Injection of S-180J.

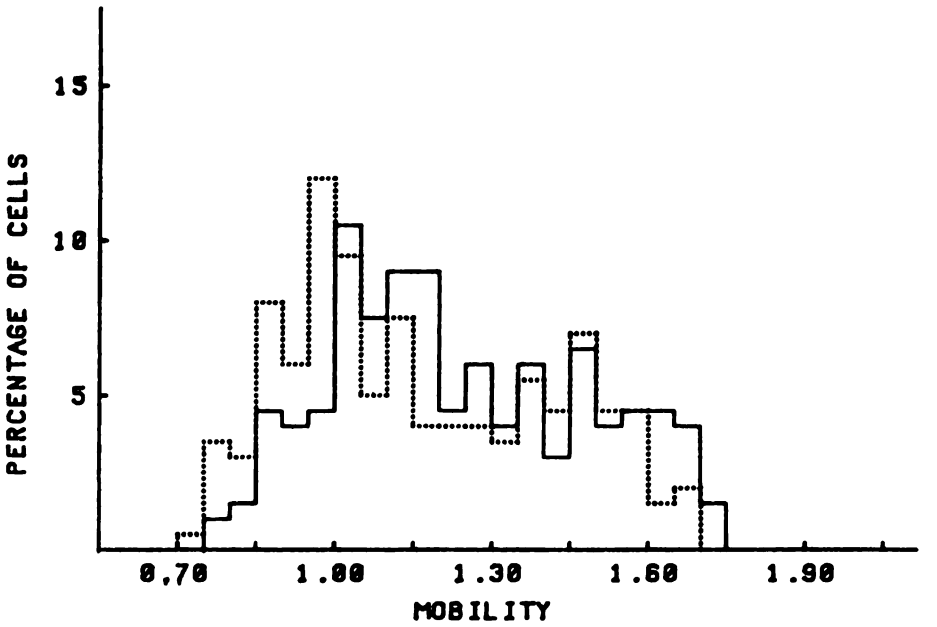

Figure 34. The Effect of RNase (dotted line) on the Mobility Distribution of B Lymphocytes (solid line) 13 days after a Second Injection of S-180J.

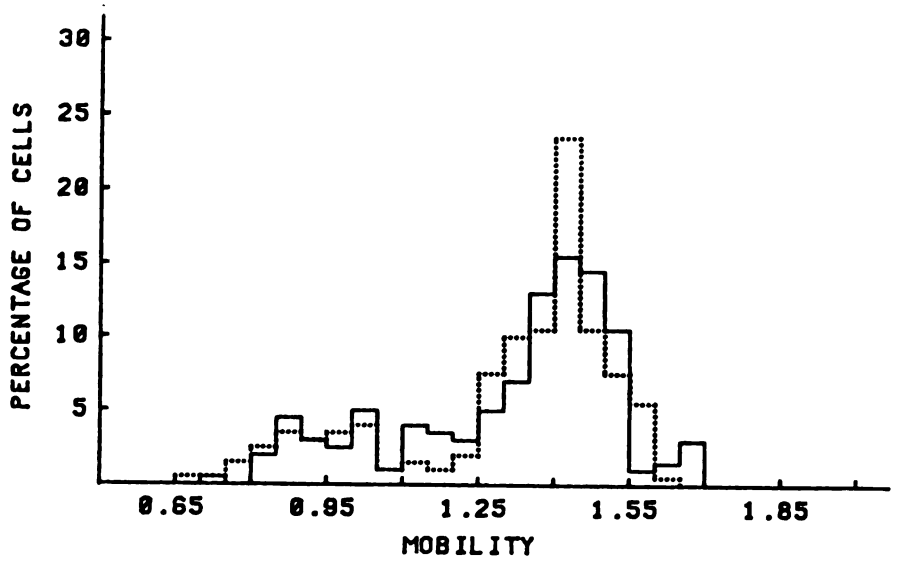

Figure 35. The Effect of DNase (dotted line) on the Mobility Distribution of T Lymphocytes (solid line) 13 days after the Second Injection of S-180J.

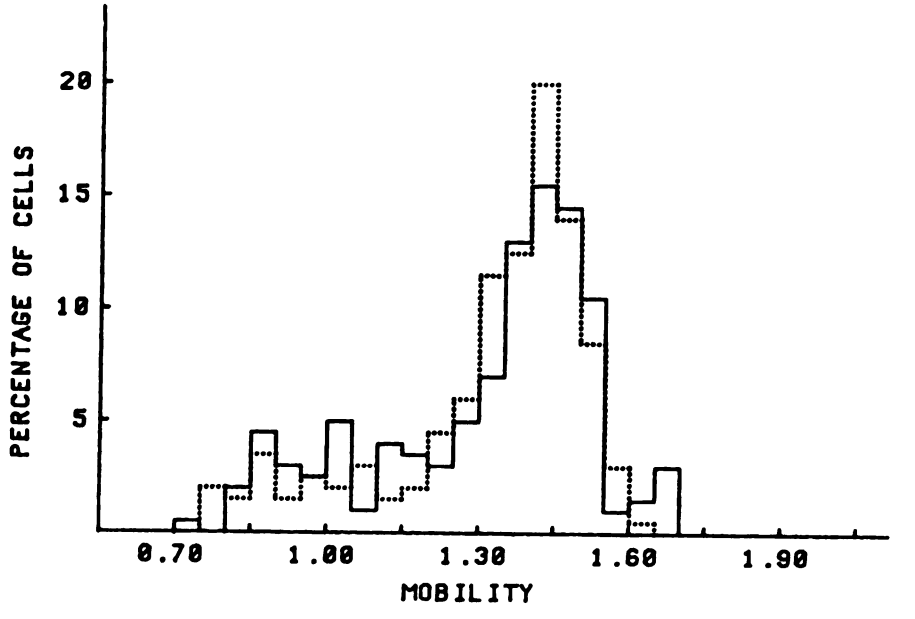

Figure 36. The Effect of RNase (dotted line) on the Mobility Distribution of T Lymphocytes (solid line) 13 days after the Second Injection of S-180J.

IV Summary of Conclusions

1. The microelectrophoretic mobility distribution of normal spleen lymphocytes is a bimodal distribution. The peaks are not normal gaussian distributions.
2. Nylon wool column effluent spleen cells are found predominantly in the high mobility peak of the normal spleen cells.
3. Thy 1.2 surface antigen cells were found mainly in the effluent of the nylon wool column.
4. Spleen cells that were adherent to a nylon wool column were found predominantly in the low mobility peak on the normal spleen cells. A small portion of the nylon wool adherent cells showed a intermediate mobility between the normal spleen cells high mobility peak and the normal spleen cells low mobility peak.
5. Spleen cells with surface IgG were enriched in the nylon wool adherent cells.
6. When the tumor, S180J, is injected into a mice the average mobility of the B lymphocytes (nylon wool adherent cells) increases with time and approaches the mobility range of the T lymphocytes at around 10 days.
7. The effect of a second injection of the tumor into mice previously cured of the tumor upon the electrophoretic mobility distribution is similar to effects the first injection produced.

8. Mice which survived the second injection of the tumor retained the bimodal mobility distribution pattern of the spleen cells. The mobility of the B lymphocytes did not increase.
9. There is no DNA or RNA on the surfaces of normal unstimulated spleen lymphocytes. There was still no surface nucleic acids detected when the cell populations examined were made more homogeneous by isolating lymphocyte subpopulations with nylon wool columns.
10. When the tumor, S180J, was injected into mice, both DNA and RNA appear on the surfaces of the spleen lymphocytes. The time dependence of the nucleic acid detection on the cells did not show a discernible pattern after the injection.
11. When the tumor S180J was reinjected into mice previously cured of the tumor, the lymphocytes of mice that die of the tumor showed surface DNA and RNA.
12. The increase in the mobility of the B lymphocytes after the injection of the tumor was not due to the cells binding nucleic acids from the media or from nucleic acids synthesized in the cells.

DISCUSSION

The research presented was an attempt to elucidate the changes in the surface charge and in the presence of nucleic acids on the surface of splenic lymphocytes with the mouse being subjected to the stress of a tumor. Both DNA and RNA have been implicated in the immune response (1, 51). With the complex interplay of the cells involved in the immune system, it is not surprising that there was also a complex interplay of the nucleic acids on the surface of the spleen lymphocytes as well as changes in the surface charge of the lymphocytes.

The initial hypothesis was that the surface charge of the lymphocytes would decrease with the introduction of the tumor. This would lower the electrostatic repulsion barrier leading to easier cell-cell contact between the cells of the immune system. Appendix IV contains a discussion of the electrostatic forces involved in cellular adhesion. The result of these experiments shows that there is an increase in the surface charge of the nylon wool adherent cells, B lymphocytes, with the introduction of a tumor into mice and that this increase in the surface charge of the B lymphocytes does not occur in the B lymphocytes from mice

that were able to survive the second inoculation of the tumor. The other result arising from these experiments is that nucleic acids can appear on the surface of activated lymphocytes, but were not seen on the surface of spleen lymphocytes from normal mice.

The large increase in the surface charge of the B lymphocytes was not a specific reaction due to a small subpopulation of cells. Ford (52) examined the migration of lymphocytes during both primary and secondary immune responses using lymphocytes labeled with radioactive isotopes injected into non-immune irradiated recipient mouse. He found that in a primary response the percentage of cells responsive to strong transplantation antigens, the number of specific memory cells, was about one percent. The upward shift in the mobility of the B lymphocytes after the introduction of the tumor into the mice is due not to one percent of the cells, but to at least 50% of the cells changing their external surface charge.

Chollet and associates (33) found minor changes in the percentages of human T and B lymphocytes after revaccination with tetanus toxoid. The B cell percentage was measured by electrophoretic mobility and EAC-rosettes (sheep red blood cells coated with non-hemolytic amounts of IgM antibodies and reacted with complement). The T cell percentage was determined by E rosettes, high electrophoretic mobility, and mitogen stimulation. The changes due to the toxoid seen in the percentage of B lymphocytes (low mobility) were signifi-

cant ($P < 0.01$). The percentage of B lymphocytes as determined by electrophoresis decreased from 17% at the injection of the toxoid to a low of 9% on day 3, but, returned to normal on day 8. However when the percentage of B lymphocytes was determined by EAC-rosette formation there was no significant change in the percentage of B lymphocytes after revaccination ($P > 0.05$). The percentage of high mobility T lymphocytes from revaccinated patients increased two-fold. Following the studies presented here, I saw no major changes in the percentage of T lymphocytes except for one value (Table 6, Exp.#2, day 2) whereas the percentage of B lymphocytes showed a rise at day 2, but drops off to a low of 9% by day 11 as determined by electrophoresis. As with Chollet's data, when I examined for B lymphocytes after tumor injection by means other than electrophoresis, I found that B lymphocytes did not disappear, but gradually shifted to higher mobilities after the tumor inoculation. The difference between Chollet's data and mine was that the changes in his low mobility cell percentages were temporary, lasting less than 8 days, whereas the effect of the S-180J tumor was a permanent (until the death of the mouse) reduction in the percentage of low mobility cells.

Normally the higher the surface charge, the higher the repulsion barrier to cell-cell contact. However in the case of cells with the same sign of charge but, of different potentials, an electrostatic attractive force arises. Bierman (53) and Derjahuin (54) described a mechanism by

which electrostatic attraction can occur for surfaces of like charge. Bierman's model for the attractive force was two parallel flat plates of different potentials and same signs separated in a univalent electrolyte solution. The electrical interaction would become attractive when the separation distance of the two plates is less than

$$L = [(DkT)/(8piMe^2)]^{(\emptyset.5)} * \ln(\tanh Z'/\tanh Z)$$

where $Z = eY/4kT$ and $Z' = eY'/4kT$. Y and Y' are the surface potential of the two plates. M is the concentration of the electrolyte in millimolar multiplied by Avogadro's number. D is the dielectric constant of the solvent and e is the electronic charge. Pethica (55) calculated the range of these electrostatic attractive forces and projected that the range would be unlikely to exceed 15 Angstroms. Thus the electrostatic forces would be repulsive at long range, but would become attractive at short range. Curtis (56) discounts this electrostatic attractive force as insignificant, but the repulsion barrier to cells in normal physiological saline is calculated to be 1Ø Angstroms from the surface (57) and true cell-cell contact occurs at 3 to 6 Angstroms. This could lead to a system which would hinder cell-cell contact at long range (greater than 15 Angstroms), but would increase the probability of cells binding when the cells approached close together. The increase in the surface charge of the B cells would hinder the cell-cell adhesion by increasing the electrostatic repulsion barrier, but as the surface charge of the B cells approaches that of the T cells

(after tumor inoculation) the attractive electrostatic forces also increased but only at short range.

The theory of the electric double layer as presented by Gouy (58) and Chapman (59) assumes that the charge is uniformly distributed on the membrane. The alternate theory of discrete charge groups on the membrane is more informative in terms of cell-cell contact. If the charges on the membrane were arranged in a mosaic pattern then the pattern could confer specificity on the binding between cells (55). Binding would consist of aligning the areas of opposite sign, or of allowing small projections at places of low potential to initiate binding. Aggarwal and associates (60) showed that the DNA existed as patches on the plasma membrane of tumorigenic cells. Brown (61) developed a theoretical model allowing for discrete charge on the membrane, instead of the uniform charge model of Gouy and Chapman. Brown's model predicted that the range of any discreteness of the charge was limited to within 25 Angstroms from the charge in an ionic aqueous solution. Beyond 25 Angstroms the uniform model of surface charge accurately described the potentials involved.

There is no actual evidence for mosaic charge distributions on the surface of lymphocytes. Pethica (55) showed electrophoretic evidence that red blood cells lack basic groups on their surface and thus could not exhibit attractive forces due to mosaic distribution of charge. But lymphocytes and platelets do have basic groups near the

membrane surface and could adhere by the mosaic attractive mechanism. Weiss and associates (62) provided evidence that ribonuclease and neuraminidase susceptible sites on the surface of glutaraldehyde-fixed Ehrlich ascites tumor cells were independent and arranged in clusters. If the nucleic acids is arranged on the surface of lymphocytes in patches or clusters (as suggested by Aggarwal and associates), then cell-cell attachment would be further enhanced by mosaic attraction when nucleic acids are expressed on the surface of lymphocytes after stimulation. Any cytoskeleton induced rearrangement of charged surface components would also provide areas of different potential.

This can be further expanded since most cellular adhesion requires calcium ions. Calcium ions appear to be important for the maintenance of close contact binding (less than 3 Angstroms). Weiss and Mayhew (63) showed that RNA on the surface of RPMI# 41 cells and Ehrlich ascites tumor cells are a stronger binder of calcium ions than the carboxyl groups of the peripheral sialic acids as suggested by Pethica (55). This would imply that nucleic acids on the surface of lymphocytes have two mechanisms to increase the attractive force for cellular adhesion and that these two mechanisms can work together. The binding of calcium ions by the nucleic acids and the mosaic pattern of nucleic acids on the surface of the lymphocytes both strengthen the attractive forces at close range (less than 1Ø Angstroms).

The increase in the average mobility of the B lymphocytes after the introduction of the tumor could be caused by either a new population of cells entering the spleen or a morphological change in the cells presently in the spleen, or a combination of both. It is not possible to distinguish which mechanism is responsible using electrophoresis.

Different stages in the maturation of B lymphocytes exhibit different electrophoretic mobilities. Zeiller and associates (64) examined the differentiation of B lymphocytes in the mouse spleen and divided it into two stages. The first stage is defined as the continual regeneration of antigen reactive, virgin B lymphocytes which arise from immature precursor cells. These cells are of intermediate and high electrophoretic mobility. The second stage is the transformation of the virgin B lymphocytes into antibody-forming cells and memory B cells following antigenic stimulation. These cells are of low mobility. Shortman (65) analyzed the differentiation of B lymphocytes and distinguished two stages, nonspecific activation and antigen specific activation. The nonspecific activated B lymphocytes were of medium to high mobility. The antigen specific activated B lymphocytes were of low mobility. Briefly during the maturation process of the B lymphocytes the mobility decreases. Shortman's and Zeiller's results would imply that the increase in the mobility of the B lymphocytes, (from mice inoculated several days earlier with the tumor), could be due to the influx of new antigen reactive virgin B

lymphocytes into the spleen. The mice which survived the tumor showed no change in the mobility of the B lymphocytes and presumably there was no major influx of activated B lymphocytes into the spleen.

The function of the nucleic acids on the surface on the spleen lymphocytes is much debated. I presented some information that suggested the presence of the nucleic acids along with calcium ions would facilitate cell-cell contact. It would explain why nucleic acids were usually found on both T and B cells together if this were a major mechanism for cell-cell binding.

There are interpretations for the role of the nucleic acids on the surface of lymphocytes other than electrostatic roles. Surface DNA has been proposed as a means of transferring information between cells (15, 43). Rogers and associates (22) have shown the excreted DNA from PHA-stimulated lymphocytes is exchanged between cells and that this DNA is actively capped by other lymphocytes.

The data I presented suggests that the lymphocytes only from mice with the S-180J tumor expressed surface nucleic acids. The affect of the tumor on the surface charge of the spleen lymphocytes is a general one because only a few lymphocytes are known to respond specifically to a antigen (66). This implies that the presence of nucleic acids is a general response to the tumor and is not limited to the few lymphocytes which are specifically stimulated for a specific antigen.

What controls the activation of the lymphocytes? When the human immune system was exposed to a non-lethal challenge of tetanus toxoid, the general response lasted 8 days (33). When mice were faced with a lethal tumor the surface charge of the B cells increased and surface nucleic acids were found. When lymphocytes were removed from an animal and cultured, whether they required mitogenic stimulation (19) or not (26, 29) they released DNA into the media. The release of this DNA was related to the concentration of calcium ions present in the medium (22). The external concentration of the excreted DNA was governed by a homeostatic mechanism (11, 15). This excreted DNA was available for accumulation by other lymphocytes (22). Exogenous DNA decreased the response of lymphocytes to mitogens. The suppressor cells from spleens of AKR mice have DNA on their surface (24). It may be that the purpose of surface DNA and excreted DNA is to provide a general immunosuppressive response instead of assisting cell-cell binding.

The function of membrane RNA has also been debated. RNA has a history of being directly involved in the immune response (1). Some of the humoral phenomenon induced by RNA are antibody synthesis, allotype transfer, autoantibody production, transplantation immunity, isograft rejection and homograft protection. Cell-mediated responses include tumor immunity, macrophage induction and lymphokine induction.

Future Experiments

There are two new aspects of lymphocyte surface structure noted in this research. They are that surface nucleic acids appear on tumor exposed lymphocytes and the surface charge of spleen B lymphocytes increases after the inoculation of the S-180J tumor into mice that were not resistant enough to survive the tumor. This raises questions of which subpopulations of lymphocytes are involved in each of these aspects. I answered this question only to the accuracy of the types of lymphocytes which are separated on a nylon wool column. Future research should concentrate on isolating subpopulations of lymphocytes with known immunological activity and markers for examination. Only by testing homogeneous populations of lymphocytes isolated according to the standard immunological methods can we correlate the wealth of published research on lymphocytes with presence of surface nucleic acids and changes in surface charge.

Techniques for isolating homogeneous populations of lymphocytes include selective rosette formation, immunoabsorbent columns and fluorescent cell sorting.

The ability of lymphocytes to bind to sheep red blood cells has been used to isolate both T and B lymphocytes. T lymphocytes will spontaneously form rosettes with sheep red blood cells. B lymphocytes will form rosettes, EAC-rosettes (erythrocyte-antibody-complement rosettes), with sheep red blood cells coated with non-hemolytic amounts of IgM anti-

bodies and reacted with mouse complement (33). The technique of isolating lymphocytes by forming rosettes between lymphocytes and sheep red blood cells is still variable and results vary among different laboratories (67).

B lymphocytes will also adhere to a column of G-200 Sephadex conjugated with anti-mouse $F(ab')_2$ serum. Less than 3% of surface Ig bearing cells will pass through such column (with human lymphocytes) (68). Separation for other characteristics than T or B distinction is possible. Russel and Golub (24) isolated spleen cells with DNA on their surface from AKR mice by treating the spleen cells with rabbit anti-DNA antibody and then passing the spleen cells over a cell affinity chromatography column with anti-rabbit Ig (24).

The development of the immunofluorescence cell sorter has allowed cells to be individually selected for a particular characteristic. It provides both detection and isolation. The problem with this technique as a preparatory step for electrophoresis is the low numbers of cells processed compared with the large numbers of cells needed in routine microelectrophoresis (about 10^7 to 10^8 cells). This problem can be overcome with either capillary electrophoresis or laser doppler electrophoresis.

Immunological markers are convenient for identification of the type of lymphocytes. Thy 1 or theta alloantigen is used to distinguish thymic-derived lymphocytes, T lymphocytes. The antigens, Ly 1.1 and Ly 1.2, are used to dis-

tinguish the helper and effecter T lymphocytes, whereas Ly 2 antigens distinguish the cytotoxic and suppressor T lymphocytes. Russel and Golub (24) used I-J surface antigens to distinguish one suppressor cell population in the spleen of AKR mice. B lymphocytes are usually distinguished by the presence of surface immunoglobulin and can be further identified with the heteroantiserum, MBLA.

By using a immunofluorescence cell sorter, one could select cells expressing a particular antigen. The effect of immune modulators like a tumor or a antigen (tetanus toxoid) on a particular subset of lymphocytes could then be studied. Also if cells could be separated based on surface charge (free flow electrophoresis) the cells could be correlated between surface charge, type of surface antigens, reaction to mitogens, rosette formation, ability to produce antibody, and other immunological tests to determine function and type of lymphocyte. In this manner it might be possible to determine whether the increase in mobility of the B lymphocytes after tumor injection was due to the appearance of new cells in the spleen from other lymphoid organs, changes in the surface properties of the current cells in the spleen (differentiation of the small B lymphocytes into plasma cells), or multiplication of spleen cells.

Russel and Golub (24) found that 18% of the spleen cells in the AKR mouse expressed surface DNA and were suppressor cells. I found that the DNase treatment of the spleen cells in ICR mice did not effect a specific mobility

value only, but was spread across the range of mobilities.

In an attempt to simplify the current system, a stimulus other than a allogeneic tumor should be used. It would also help simplify if the electrophoresis equipment could be scaled down such that one mouse could provide enough cells for both electrophoretic and immunological examination. Chollet and associates (33) examined the effect of revaccination of tetanus toxoid on the mobility of human blood lymphocytes. The reaction to the revaccination upon the mobility of the lymphocytes was minor and temporary. The response of the immune system to an syngeneic tumor system, like the P815 mastocytoma in the DBA/2 mouse would be more representative of allogeneic tumors.

APPENDICES

APPENDIX I

DATA COLLECTION SOFTWARE

This appendix discusses and lists the software program that was used on the ZX-81 computer to collect and organize the electrophoresis data. The program is specific for the ZX-81. It contains hardware specific instructions for the Mindware 100 printer and the VOTEM from Down East Computers. The VOTEM has been modified to extend the initial voltage range of 0 to 1 volt to 0 to 200 volts DC. The program requires an sixteen kilobyte random access memory extension be added to the basic computer.

During the experiment, the software program collects the timing signals and converts them into microelectrophoretic mobilities. After the data collection is finished, the program calculates the mean and standard deviation from the data and arranges the microelectrophoretic mobilities into a histogram. The timings for the cells and the voltages are printed after they are measured to create a paper record minimizing possible data lost due to equipment failure. A calculator can be used to reduce the data from the printed record when an equipment failure occurs.

The program listing below is altered from the original to increase understanding. The Z-80 machine code instructions in the first REM statement is given as hexadecimal numbers with commas separating the numbers. These numbers have to be POKED into the memory locations consecutively beginning at address 16514. The other change from the original program listing is comments in brackets "[]"'s and in lower case characters. Comments are omitted in the working program to conserve memory but are included here to improve understanding of the program. Indentation is added to assist in recognizing blocks of code.

```
PROGRAM EPM

1 REM 21,8B,40,01,00,00,11,00,00,1B,7A,B3,C8,DB,FE,07,30,
      F7,03,1B,7A,B3,C8,DB,FE,07,38,F7,E9
   [ code is hexadecimal, commas not included in program ]
10 REM "EPM"          [ name of the program ]
15 POKE 16417,3       [ sets a specific printing mode in the
                        Mindware 100 printer ]
20 LET VC=0.0092269   [ calibration constant for the VOTEM ]
25 GOTO 2000          [ main program starts at line 2000 ]


     [ subroutine listing ]

100 REM DECIMAL FORMAT
    [ subroutine rounds X to DEC decimal places and
      returns the value in X ]
105 LET XL=INT(ABS X)
110 LET XR=INT((ABS X-XL)*10**DEC +0.5)/10**DEC
115 LET X=SGN X *(XL+SR)
120 RETURN

200 REM A(J,I) TIMING
    [ subroutine counts the difference in registers 16436
      and 16437 and converts it into seconds ]
205 SLOW
     [ ZX-81 has two modes. Fast turns off monitor.  In
       slow the screen is refreshed 60 times a second.]
210 IF INKEY$="" THEN GOTO 210      [ wait for signal V ]
211 IF INKEY$="V" THEN GOTO 215     [ start timing ]
```

```
212 IF INKEY$="Q" THEN LET CELL=J-1 [ end experiment early ]
213 IF INKEY$="Q" THEN GOTO 2040
214 GOTO 210
215 POKE 16437,255                  [ initialize registers ]
220 POKE 16436,255
225 IF INKEY$<>"N" THEN GOTO 255
    [ stop timing on pressing n ]
230 FAST
235 LET X=(65535-PEEK 16436-256*PEEK 16437)/60
     [ conversion formula, converts the number of screen
       refreshes to seconds ]
240 LET A(J,I)=X
245 GOSUB 100               [ round seconds for printing ]
250 RETURN

300 REM A(J,1) RETIMING
  [ subroutine allows retiming of an erroneous data entry ]
305 LET I=1                 [ reset proper index ]
310 GOSUB 200               [ retime cell A(J,1) ]
315 LPRINT J; TAB 4; X
320 RETURN

350 REM A(J,2) RETIMING
  [ subroutine allows retiming of an erroneous data entry ]
355 LET I=2                   [ reset index ]
360 GOSUB 200                 [ retime cell A(J,2) ]
365 LPRINT J; TAB 10; X
370 RETURN

400 REM MEAN AND STAN DEVIATION CALC AND LPRINTING
[ routine calculates the mean and the standard deviation ]
405 LET SUM=0
410 LET SUM2=0
415 FOR J=1 TO CELL
420     LET SUM=SUM + B(J)
425     LET SUM2=SUM2 + B(J)**2
430 NEXT J
435 LET MEAN = SUM/CELL
440 LET SD=SQR(SUM2/CELL-MEAN**2)
445 LET DEC=2
450 LET X=MEAN      [ round and print mean mobility ]
455 GOSUB 100
460 LPRINT
465 LPRINT "MEAN =";X
470 LET X=SD        [ round and print standard deviation ]
475 GOSUB 100
480 LPRINT "STAN DEV=";X
485 RETURN
```

```
500 REM TIMING INSTRUCTIONS
     [ subroutine prints on the monitor the instructions to
       on how to time the cells ]
505 SLOW
510 CLS              [ clear screen and print instructions ]
515 PRINT AT 5,0; "TIMING INSTRUCTIONS"
520 PRINT AT 7,0; "PRESS ""V"" TO START TIMER"
525 PRINT "PRESS ""N"" TO STOP TIMER"
526 PRINT "PRESS ""Q"" TO STOP DATA COLLECTION EARLY"
530 PRINT AT 12,0; "PRESS ""M"" TO ACCEPT PAIR"
535 PRINT "PRESS ""1"" TO RETIME CELL LEFT"
540 PRINT "PRESS ""2"" TO RETIME CELL RIGHT"
545 PRINT
550 PRINT "**** KEYS ACTIVATED ****"
555 PRINT
560 PRINT "SWITCH MONITOR TO CAMERA"
565 RETURN

600 REM MOBILITY CALCULATIONS
  [ routine updates voltage, calculates the mobility
  as timings are available to avoid storing the voltages ]
605 IF INT(J/10)=J/10 THEN LET Y=VC*USR16514
     [ every 10 readings the applied voltage is measured ]
     [ usr16514 is the machine code routine that reads the
     output frequency of the VOTEM ]
     [ vc is number of counts per volt ]
610 LET B(J)=(1/A(J,1) + 1/A(J,2))*RBC*KC*1.758/Y
     [ A(j,i)=timings in seconds ]
     [ rbc= daily red blood cell correction factor ]
     [ B(j)= electrophoretic mobility of cell j ]
     [ kc= cell constant ]
     [ 1.758 = cross sectional area of chamber in cm^2 ]
620 IF INT(J/10) = J/10 THEN LPRINT "VOLTS = ";Y
625 RETURN

700 REM TIMING SUBPROGRAM
     [ subroutine controls the collection of the data ]
705 GOSUB 500                [ print the instructions ]
710 LPRINT "TIMING ARRAY"    [ print heading ]
725 FOR J=1 TO CELL
    [ cell=total number of cells to be measured ]
726     LET DEC=2
        [ round all timings to hundredths of second ]
730     FOR I=1 TO 2
735         GOSUB 200          [ time one cell ]
740         IF I=1 THEN LPRINT J;TAB 4; X;
745         IF I=2 THEN LPRINT TAB 10;X
750     NEXT I
755     IF INKEY$=""  THEN GOTO 755
                      [ wait for instruction ]
756     IF INKEY$="M" THEN GOTO 775
                      [ accept these timings ]
760     IF INKEY$="1" THEN GOSUB 300
                      [ retime the left ]
```

```
765     IF INKEY$="2" THEN GOSUB 350  [ retime the right ]
770     GOTO 755    [ repeat until m is pressed ]
775     GOSUB 600   [ calculate mobility, update voltage ]
780 NEXT J          [ repeat until cell cells are timed ]
785 RETURN

900 REM CELL CONSTANT CALCULATION
   [ routine instructs and calculates the cell constant ]
905 CLS
910 PRINT "FILL THE SYSTEM WITH O.1M KCL AT 25 DEGREES C"
911 PRINT
915 PRINT "TURN ON THE POWER SUPPLY TO 5 MA"
920 PRINT "ENTER THE ELECTRICAL CURRENT IN AMPS!"
921 PRINT
925 INPUT X
930 PRINT X; " AMPS ??"
931 PRINT
935 PRINT "WHEN READY TO COMPUTE CELL CONSTANT ENTER ""R"""
940 INPUT Z$
945 IF Z$<>"R" THEN GOTO 931
950 LET Y=USR 16514     [ obtain voltage ]
951 LET KC=(0.01288*VC*Y)/(X-VC*Y/2.327E5)
954 PRINT
955 PRINT "CELL CONSTANT = "; KC
957 PAUSE 200
960 RETURN

1000 REM CELL CONSTANT ENTRY
     [ subroutine allows for entry of cell constant ]
1005 CLS
1010 PRINT "ENTER KC, CELL CONSTANT "
1015 INPUT KC
1020 PRINT KC, "OK?", "ENTER Y/N"
1025 INPUT Z$
1030 IF CODE Z$=51 THEN GOTO 1010
1035 IF CODE Z$=62 THEN RETURN
1040 GOTO 1020

1100 REM SUBLIST OF PARAMETERS
     [ routine prints list of experimental conditions ]
1100 LPRINT A$          [ date ]
1115 LPRINT B$          [ cell type ]
1120 LPRINT C$          [ experiment code ]
1125 RETURN

1200 REM LISTING EXPERIMENTS CONDITIONS
     [ routine prints a complete list of exp conditions ]
1205 GOSUB 1100
1210 LPRINT "RBC = "; RBC
1215 LPRINT CELL; " CELLS"
1220 LPRINT "KC= ";KC
1230 RETURN
```

```
1300 REM INITIALIZATION
     [ subroutine asks for experiment description ]
1305 CLS          [ clear screen ]
1310 PRINT"DO YOU WISH TO COMPUTE THE CELL CONSTANT?","Y/N"
1315 INPUT Z$
1320 IF CODE Z$=62 THEN GOSUB 900
1325 IF CODE Z$=51 THEN GOSUB 1000
1330 CLS
1335 PRINT AT 2,0; "DATE IS ";
1340 INPUT A$
1345 PRINT A$
1350 PRINT AT 4,0; "CELL TYPE IS ";
1355 INPUT B$
1360 PRINT B$
1365 PRINT AT 6,0; "EXPERIMENT CODE IS ";
1370 INPUT C$
1375 PRINT C$
1395 PAUSE 200
1400 CLS
1405 PRINT "RBC MOBILITY =? "
1410 PRINT "ENTER 1.35 IF THIS IS RBC"
1415 INPUT RBC
1420 LET RBC=1.35/RBC
1425 PRINT "RBC= ";RBC
1430 PRINT "HOW MANY CELLS?"
1435 INPUT CELL
1440 PRINT CELL; "CELLS"
1442 PAUSE 200
1445 RETURN

1500 REM HISTOGRAM CALCULATIONS
 [ routine sorts the mobilities in a frequency histogram ]
1505 LET MAX=0
1510 LET MIN=100
1515 FOR J=1 TO CELL  [ find the min and max mobilities ]
1520     IF MAX<B(J) THEN LET MAX=B(J)
1525     IF MIN>B(J) THEN LET MIN=B(J)
1530 NEXT J
1535 LET HREG=INT(20*MAX) - INT(20*MIN) +1
     [ hreg=number of intervals needed in histogram ]
1540 DIM C(HREG)     [ matrix C holds the frequencies ]
1545 FOR J=1 TO CELL
1550     LET X=INT(20*B(J)) - INT(20*MIN) - 1
1555     LET C(X)= C(X) + 1
1560 NEXT J
1565 RETURN

1600 REM HISTOGRAM LISTING
     [ subroutine print the frequency histogram ]
1605 LPRINT
1610 LPRINT "HISTOGRAM"
1615 GOSUB 1100
1620 LPRINT "MOB MP"; TAB 12; "FREQ"
1625 LET X=INT(MIN*20)/20+0.025
```

```
1630 FOR I=1 TO HREG
1635     LPRINT X+0.05*(I-1); TAB 12; C(I)
1640 NEXT I
1645 RETURN

1700 REM HISTOGRAM GRAPH
     [ subroutine graphs the histogram ]
1705 LPRINT
1710 LPRINT "HISTOGRAPH"
1715 GOSUB 1100
1720 LPRINT "MIN MOB MP="; INT(20*MIN)/20+0.025
1730 FOR J=1 RO HREG
1735     IF C(J)>28 THEN GOSUB 1800
1740     IF C(J)>28 THE GOTO 1780
1745     LET X=INT(C(J)/2)
1750     FOR I=1 TO X
1755         LPRINT "@";
     [ @ should be a solid block graphics character ]
1760     NEXT I
1765     LET X=C(J)-X*2
1770     IF X=1 THEN LPRINT "#"; TAB 14; C(J)
     [ # should be a left half solid block ]
1775     IF X=0 THEN LPRINT TAB 14; C(J)
1780 NEXT J
1790 LPRINT "MAX MOB MP="; INT(20*MAX)/20+0.025
1795 RETURN

1800 REM GRAPHS C(J)>28
1805 FOR I=1 TO 13
1810     LPRINT "@";
1815 NEXT I
1820 LPRINT "*";C(J)
1825 RETURN

1900 REM MOBILITY ARRAY LPRINTING
     [ subroutine prints all of the mobilities ]
1905 LPRINT
1910 LPRINT "MOBILITY ARRAY"
1915 GOSUB 1100
1920 LET DEC=3
1925 FOR I=1 TO CELL STEP 2
1930     FOR J=I TO I+1
1935         IF J=CELL+1 THEN GOTO 1970
1940         LET X=B(J)
1945         GOSUB 100
1950         IF J=I THEN LPRINT J; TAB 4; X;
1955         IF J=I+1 THEN LPRINT TAB 10; X
1960     NEXT J
1965 NEXT I
1970 RETURN

[ end of subroutines ]
```

```
2000 REM MAIN PROGRAM
2005 SLOW
2010 GOSUB 1300          [ initialization ]
2015 GOSUB 1200          [ list experimental parameters ]
2020 DIM A(CELL,2)       [ dimension timing matrix ]
2025 DIM B(CELL)         [ dimension mobility matrix ]
2035 GOSUB 700           [ collect data ]
2044 FAST
2045 GOSUB 400           [ calculate mean and standard
                           deviation ]
2050 GOSUB 1500          [ sort data into histogram ]
2055 GOSUB 1600          [ list frequency histogram ]
2060 GOSUB 1700          [ graph histogram ]
2065 GOSUB 1900          [ list mobilities ]
2070 STOP                [ end of program ]
```

APPENDIX II

NONPARAMETRIC STATISTICS

The distributions encountered in this research are not normal distributions. Because of this, nonparametric testing methods were used exclusively. The two major statistical testing methods that were used are the Smirnov's test and the Chi-squared test. Both tests measure the difference between two distributions, but in different ways. A major reference for nonparametric statistics is the book, Practical Nonparametric Statistics, by W. J. Conover (69).

The Smirnov test, sometimes called the Kolmogorov-Smirnov test, is a measure of the difference between two empirical distributions. It is used to determine whether the distribution functions associated with the data are from identical populations. Other tests such as the median test, the Mann-Whitney test or the parametric t test could be appropriate, but they are only sensitive to differences in two means or medians and may not detect differences of other types, such as differences in variances. In the Smirnov test, the assumptions are:

1. The data consist of random samples,
2. The samples are mutually independent,
3. The measurement scale is ordinal or higher,
4. The random variables of the samples must be continuous for the test to be exact, otherwise test is conservative.

The null hypothesis is that the two unknown distributions are the same. The two-sided alternative hypothesis is that the distributions differ. The alternative hypothesis for the one-sided test is that the distributions shift in relationship to each other in a specific direction, either right or left.

The Chi-squared test is a test for the differences in probability at each class in the populations. In this case it is used to compare mobility distributions to see if they come from an identical population. The data is set up basically as a row X column contingency table. The rows are the different samples while the columns are the frequency of the different mobility classes. The assumptions involved in this test are:

1. Each sample is a random sample,
2. The results of the samples are mutually independent,
3. Each observation, each mobility determination, can be categorized into exactly one of the column categories, mobility classes.

The null hypothesis is that all of the probabilities (expected frequencies of the cells) in the same column, mobility interval, are equal to each other, with the

alternate hypothesis being that they differ in the same column. The Chi-squared test measures the difference between the two distributions and serves as a means to detect significant differences.

The difference between the Smirnov test and the Chi-squared test is that the Smirnov is based upon differences in the two empirical distributions from the data and the Chi-squared test is based upon examining the total differences in the probability at each mobility interval separately. The results of both tests are reported as the observed level of significance. The observed level of significance, the P value, is the smallest level of significance that would result in the rejection of the null hypothesis. In this way the degrees of freedom for the Chi-squared test and the sample size for the Smirnov test do not have to be reported and the reader does not have to look up the quantile level in a table. If the P value is close to zero (a test statistic greater than the Ø.999 quantile), then the alternate hypothesis is accepted and there is sufficient difference between the populations not to be due to chance. If P is close to one (a small test statistic), then the null hypothesis that the two samples are identical is accepted. But since statistical tables rarely give quantile values less than the Ø.75 quantile and interpolation to values less than Ø.75 are given to error, P values greater than Ø.25 (test statistic values smaller than the Ø.75 quantile level) are simply reported as $P \geq Ø.25$.

APPENDIX III

GENERAL PROPERTIES OF LYMPHOCYTES

This Appendix covers the general properties of B and T lymphocytes.

The source of all lymphocytes is the bone marrow. Once a cell is released from the bone marrow it can become a T or B lymphocyte depending on its path. T lymphocytes travel through the thymus which confers upon the cell the ability to differentiate and mature into cells which are competent to perform as helper cells and participate in activities associated with cell-mediated immunity. B lymphocytes do not pass through the thymus and differentiate into antibody producing cells.

T cells are distinguished by surface antigens. Theta, sometimes called Thy 1, antigen is the primary antigen used for distinguishing T cells from B cells. Other surface antigens are the Ly antigens which are used to separate different subpopulations of T cells. Human T lymphocytes will also form rosettes spontaneously with sheep erythrocytes.

B cells are normally distinguished by the high level of surface immunoglobulin. Mouse specific B lymphocyte antigen, MBLA, is a heteroantiserum which is generated by immunizing rabbits with lymph node lymphocytes. MBLA will react with the B cells of the bone marrow and spleen, and the plaque forming cells. B cells have surface receptors for Fc portion of the Ig molecule and complement. Sheep erythrocytes coated with antibody will form rosettes, EA rosettes, with B cells acting on the Fc receptor on the surface of the B cell. The sheep erythrocytes are reacted with anti-erythrocyte antibody and then complement is added to form an antigen-antibody-complement complex on the surface of the erythrocyte which will bind to a complement receptor on the surface of the B cell to form a rosette.

Null cells are lymphoid cells which lack both the theta antigen and surface immunoglobulin.

Mitogens are substances which induce a cell to initiate mitosis. The mitogens, concanavalin A and phytohemagglutinin, are specific for T lymphocytes. Bacterial lipopolysaccharide and lipoprotein are specific mitogens for B lymphocytes.

APPENDIX IV

FORCES INVOLVED IN CELLULAR ADHESION

This appendix will briefly cover the forces involved in cellular adhesion. Two good reviews covering cell-cell interaction are Curtis (56) and Weiss (57).

There are two major regions in cellular adhesion. It is called close adhesion when the cells are nearer than 20 Angstroms. In close adhesion the cells exhibit low surface potential, lacking the energy to separate over the repulsive barrier. The cells are held together by the London forces and short range ionic and chemical bonds. The second type of adhesion occurs at the secondary maximum at 100-200 Angstroms. London forces and electrostatic forces with their longer range are the major forces. Between these two minimums there is a repulsive potential energy barrier. When cells are bound together, less than 5 Angstroms, it requires kinetic energy to surmount the barrier to separate the cells.

The potential energy of interaction is the energy need to place two cells at a specific distance. This potential is illustrated in Figure 36. When the potential energy of interaction is positive the cells require energy to put them

at this distance. As the cells get closer there is considerable repulsion barrier to overcome before the energy becomes attractive, potential energy less than zero.

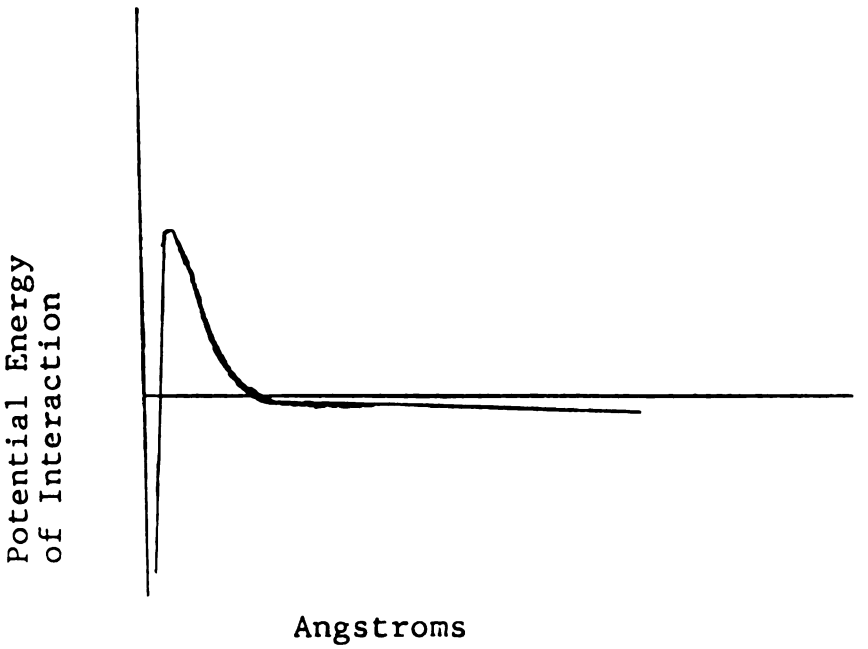

Figure 37. The Total Energy of Interaction for Two Spheres.

The cellular medium modulates adhesion between cells. Alkaline solutions tend to de-adhere cells. Calcium ions are important in the maintenance of adhesion. Calcium being a divalent cation lowers the surface potential as its concentration increases. In general the lower the surface potential the stronger the adhesion due to the reduction in the repulsive electrostatic barrier holding cells apart. Similarly the concentration of monovalent ions does modulate the electric double layer around a cell thus affecting its effective surface potential. The major forces involved in cell adhesion are; 1. the electrostatic potential of the surfaces, 2. the London-van der Waals forces between the surfaces, 3. the deformability of the surfaces and their interfacial viscosity with the bulk medium, and 4. the bulk properties of the medium. The electrostatic force is not always a repulsive one. It arises from the surface membrane charged groups. The zeta potential is a measure of the surface potential measured at the slip plane. The repulsive electrostatic forces are significant when the electric double layers from two cells begin to overlap (around 100 to 200 Angstroms in biological media).

Electrostatic attractive forces arise from surfaces which differ in sign or differ in potential with the same sign. Attractive electrostatic forces between cells occur when the surfaces are a mosaic of positive and negative charges and proper orientation between the two membranes occurs but only at short range, less than 15 Angstroms.

The London-van der Waals forces are believed to be the major attractive forces between surfaces with a range up to 1000 Angstroms. The London dispersion force is due to the fact that electrons oscillate around their position. While the net electric effect is nil, they create a fluctutating dipole which polarizes the surrounding area.

The role of calcium in adhesion is complex. As the concentration of calcium ions (other divalent ions work as well) is increased in the media of the cells, it lowers the surface potential. The surface potential is inversely correlated with the energy to detach cells from a substrate. Others, Steinberg (66) and Rappaport (70), suggest that calcium ions will function as 'bridges' between two surfaces.

The energy of repulsion for two spheres of radius a and with a separation distance of H is :

$$V_r = 0.5*(D*a*Y^2)*\ln(1+e^{-KH})$$

where D is the effective dielectric constant, K is the Debye-Huckel constant, and Y is the surface potential. The attractive energy of the London force for the same system is:

$$V_a = -\left(\frac{A * a}{12 * H}\right)$$

where A is the effective van der Waals constant and a is the radius of curvature of the approaching cell processes. In a study on the flocculation of leucocytes (71), A was found to range from 10^{-14} to 10^{-15} ergs. The resultant energy, V is

the sum of V_a and V_r. Factors that effect both V_r and V_a will only shift the interaction energy. In order to effect the repulsion barrier, any change must alter the ratio of V_a/V_r. Both V_r and V_a are proportional to the radius of curvature, but any changes in the radius of curvature will affect V in total and will not alter the V_a/V_r relationships. But reducing the radius of curvature will reduce the repulsive energy barrier between the primary and secondary minimum. V_r is most easily effected by factors that affect the electric double layer. Since the surface potential is squared in the V_r equation, minor changes in the surface potential will produce major changes in the repulsion energy.

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