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SURFACE CHARGE PROPERTIES OF IMMUNOCYTES

by

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ABSTRACT

The aim of this investigation was to explore the possibility of identifying and isolating the subpopulation of immunocompetent cells of strain A mice, capable of binding to the hapten DNP, in terms of their unique cell surface charge properties. The animals were immunized with $(DNP)_{35}$-BSA and the electrophoretic mobilities (EPM) of their immunocytes, expressed in units of micron/sec/volt/cm (referred to hereafter as units) were measured in a specially designed cell electrophoresis chamber.

The EPM of lymph node cells of control animals peaked bimodally at the values of 0.8750 and 1.4000 units, while those of immunized animals peaked at the values of 0.8176 and 1.3417 units. Upon incubation with various samples of the synthetic mono α-DNP-poly-L-lysine (αDNP-PLL) of differing chain lengths, the cell population of the immunized animals originally possessing the low EPM value of 0.8176 unit exhibited a partially reduced electrophoretic mobility. A partially elevated electrophoretic mobility was observed in the low EPM cell population of the immunized animals when incubated with the ε-succinilated derivatives of various samples of the synthetic αDNP-PLL of differing chain lengths. Incubation of the lymphocytes of both control and immune animals with the poly-trimethyl ammonium derivatives of αDNP-PLL resulted in clumping of the cells, thus making it impossible to study the EPM of the cells interacting with this highly positively charged polymer.

To further characterize the low and high electrophoretic mobility cell populations, normal animals were treated with the immunosuppressive drug cyclophosphamide. The cell population with the low EPM cells was reduced in cell numbers, while the cell population with the high EPM cells remained relatively intact indicating that the low EPM cells were, in all probability, the more rapidly dividing B cells. On the other hand, treatment of cells of normal animals with AKR anti-C_3H θ-antiserum or with neuraminidase resulted in the reverse effect, i.e. the cell population with the high EPM cells was substantially reduced; hence one may suggest that this population contained T cells.

Studies with DNP-Biogel affinity chromatography using the batch method for fractionation of hapten binding cells indicated that the method was not 'specific' enough due to the natural surface stickiness of the cells and the incapability of displacing all the antigen binding cells from the gel by hapten.

The practical use of cell electrophoresis as a tool for immunological investigations was further explored in studies of the Macrophage Electrophoretic Migration Inhibition Factor (MEMIF). Three specific antigenic systems were investigated, namely, the guinea pig MCD tumor system, the A/J mice 1509 tumor system and the synthetic DNP antigen system. In all cases, this factor(s) was released specifically by the

sensitized cells upon incubation with the immunizing antigens.

On the basis of all these data indicating that cells belonging to different classes of lymphocytes possess distinct charges, it is suggested that preparative cell electrophoresis may be developed into a powerful tool for the separation of immunocyte and,also possibly, for immunological diagnoses.

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SCOPE OF THE PRESENT INVESTIGATION

The immune response comprises all the phenomena that result from the interaction of cells of the immune system with antigen. As a consequence of such interactions, cells may become specialized to mediate cellular immune responses (such as delayed hypersensitivity, homograft rejection, or 'graft versus host' (GVH) reaction), or to synthesize and secrete one of several classes of immunoglobulins. Our current understanding of this complex interaction of cells, antibodies and other soluble mediators has been greatly aided by the use of animal models. With *in vitro* culture techniques, information has been provided regarding the contribution of different lymphoid cell types to the immune responses.

Lymphocytes are the central cells for all immune responses and many attempts have, therefore, been made to isolate them. By the use of physical methods, many workers have attempted to separate populations of lymphoid cells into fractions displaying a limited degree of physical heterogeneity in size and charge, on the supposition that homogeneity in physical properties reflects singularity in biological function. For example, the widely used sedimentation velocity method leads to the separation of cells into fractions differing in size and shape and possessing different functions. However, as with the other "non-specific" methods, such as density gradient centrifugation, chromatography on glass beads and nylon column or free flow electrophoresis, these procedures lead at best to the isolation of repertoire of cells enriched with respect to a given type

of cells possessing a distinct immunological function, i.e. hemolysin-forming cells, rosette-forming cells, suppressor cells, null cells, natural killer or cells involved in graft-versus-host reaction. To overcome this problem, specific methods such as affinity chromatography with antigen or antibody chemically coupled to agarose, Sepharose, Sephadex or polyacrylamide gels have been used widely. However, large numbers of cells adhere nonspecifically to the polymeric matrix due to their stickiness and it is difficult to release intact viable cells from the immunosorbents.

The net electrical surface charge of an immunocyte, measured in terms of the corresponding electrophoretic mobility, is the result of charges contributed by the immunoglobulin-like molecules of the antigen binding receptor sites and by the diverse chemical groups constituting the rest of the complex cell membrane. It has been shown in this laboratory (149) that conjugates, consisting of the immunizing hapten coupled to different polyionic carrier macromolecules, can specifically bind to the antigen combining sites of the appropriate immunocytes and thus lead to a marked change in the electrophoretic mobilities of these cells. Hence, it was anticipated that this method may be the basis of the development of an immunologically specific procedure for cell fractionation. Therefore, the objective of this study was to develop a reliable and simple system for cell electrophoresis with a view to establishing surface charge properties of immunocytes and the conditions for altering their electrophoretic mobility.

This study was undertaken with the following specific aims:

(1) to construct a closed, all glass microelectrophoresis chamber for the reproducible measurements of the net electrophoretic mobility (EPM) of immunocytes;

(2) to establish the EPM patterns of lymph node cells of control and immunized mice of strain A/J;

(3) to identify hapten specific binding cells in terms of the alteration of EPM of these immunocytes on interaction with synthetic, polyionic conjugates of haptens (or antigens);

(4) to identify hapten specific binding cells by the immunofluorescence technique;

(5) to isolate hapten specific binding cells by specific immunosorbents;

(6) to identify the θ-positive subpopulation of lymphocytes in terms of the alteration of their electrophoretic pattern on interaction with anti-θ antibodies;

(7) to investigate the effect of neuraminidase on the surface charge properties of lymph node cells;

(8) to establish if the in vivo administration of the immunosuppressive drug Cyclophosphamide could affect the surface charge properties of the lymph node cells;

(9) to establish if measurements of EPM of peritoneal macrophages could be used as diagnostic properties in systems involving cellular immunity to tumor antigens or synthetic antigens.

LITERATURE REVIEW

I. CELLS INVOLVED IN IMMUNE RESPONSES

(a) The Immunologically Competent Cells

The immune response manifests itself in two basic forms, namely humoral and cellular immunity. Both of these manifestations are due to small lymphocytes (70), whose precursors originate from the bone marrow (117). The immunologically competent cells (68) consist of a very heterogenous population of cells with regard to their origin (39), migratory pathways (92, 165), life span (51, 166), their functional dependence on the central lymphoid organs (thymic or bursal dependence) (183), their functional capacities (236-239, 244, 245, 254, 258), their ability to form rosettes with red blood cells (240-242, 253, 255), their surface markers, immunoglobulins, and receptors (243, 246-248, 250-252) and their radiosensitivity (249).

The delayed type of hypersensitivity reaction represents the classical 'cell mediated immunity' (CMI) reaction. Skin graft rejections represent another example of CMI, since this type of immunity is easily transferable by lymphoid cells from immune to normal animals. The transfer of spleen and lymph node cells into hosts, which are genetically unable to reject them (163), results in another manifestation of CMI designated as the 'Graft Versus Host' (GVH) reaction, which is due to the attack of the grafted cells against the host's - this immunological reaction is manifested by splenomegaly, hepatomegaly and wasting disease.

Cells that are capable of releasing antibodies into circulation are called antibody-forming cells. The detection of antibody formation at the single cell level can be achieved

by variations (87) of the hemolytic plaque technique introduced by Jerne and Nordin (89). Cell surface immunoglobulins can be detected by the method of immunocytoadherence (99, 197, 19), microdrop assays (123, 7), immunofluorescence technique (250, 259) and electron microscopy for detection of anitbody complexes (45).

With the technique of immunocytoadherence using sensitized spleen cells and SRBC under mild centrifugation, one can separate out clusters or 'rosettes' of cells comprising one nucleated cell surrounded by a group of SRBC. The rosette forming cells comprise a) large and small lymphocytes, b) plasma cells, c) blast cells, and d) macrophages (167). These can be explained by the fact that plasma cells secrete antibodies, macrophages have passively bound antibodies which lead to their rosette formation, while the lymphocytes have limited quantity of membrane bound antibodies (50). The presence of 'receptors' on the surface of the lymphocytes has been demonstrated by their ability to bind to antigen coated column; thus, the cells emerging from such columns have no capacity to produce antibodies on adoptive transfer, while the cells eluted from the column are endowed with this capacity (188). Similarly the removal of rosette forming cell by mild centrifugation results in loss of this capacity (24).

Mitchison (108) formulated the 'receptor hypothesis' which was based on the assumption that antigen sensitive cells have antibody-like receptors on their surface which interact with the immunogen.

There are effector cells of cellular immunity (257) which react with antigen leading directly or indirectly to tissue damage. These effector cells have been detected by the macrophage migration inhibition test (44, 256); GVH (163); including Simonsen-CAM test (21); adoptive transfer of delayed hypersensitivity or graft rejection and in vitro damage to target cells (119). The precursors of normoblasts and myeloblasts, which are colony-forming units, are responsible for the formation of erythroid and myeloid cells in irradiated spleens (178). It is possible to use chromosome analysis of dividing Kupffer cells in regenerating liver to detect the presence of macrophage precursors (83). The bone marrow-derived precursors of thymus and lymph node cells have been shown to be the true self-perpetuating stem cell pool of the lymphoid system by the parabiosis technique (120).

(b) The Thymus and Central Lymphoid Tissue

The functional capacity of the thymus had not been visualized until Miller (112) showed that neonatal thymectomy in mice prevented the proper development of all cell-mediated and some antibody responses. Neonatal thymectomy resulted in impaired ability to reject grafts, to induce GVH reactions, and to make antibody to some antigens especially proteins, bacteria, and red cells. Miller's finding on the importance of thymus in the immune response was in accord with the clinical studies of Good (67), who associated the human congenital immunological deficiency diseases with the independent deficiency of this subpopulation of cells. This phenomenon was also well demonstrated with the aid of chicken as an experimental model (185).

The population of cells that fail to develop after neonatal thymectomy was termed 'thymus-dependent' lymphocytes (T cells). Their blast transformation can be induced by plant mitogens such as phytohaemagglutinin (PHA) and inactivated by heterologous antilymphocyte sera (ALS) (48). They also have the ability to release mediators upon the interaction with specific antigens or mitogen (9, 20, 36, 43, 44, 48, 94, 97, 136, 138, 184, 206).

The study of the T cells in immune responses was made possible with the discovering of their antigenic markers. The existence of a system of isoantigens (Θ) in mice specific for the thymus-dependent cells was described by Reif and Allen (377), using dye exclusion cytotoxicity tests. This antigen was found to be present in brain and in a subpopulation of lymphocytes in the lymph nodes and spleen of the animals. The antigen was found to be controlled by a single locus with two alleles: Θ-AKR was found in AKR, RF and a few substrains while Θ-C_3H was found in most other inbred strains of mice (225). Anti-Θ antisera can be prepared by injecting C3H mice thymocytes into AKR mice or vice versa. Other alloantigenic systems such as TL (thymus leukemia) Ly, and MSLA (mouse specific lymphocyte antigen)were extensively reviewed by Raff (378).

(c) The Bone Marrow and Hemopoiesis

It has been found that x-irradiation of mice with doses between 800 to 1000 rads results in the death of mice within two

weeks unless the animals are injected with hemopoetic cells so as to restore mainly their granulocytes and platelets. With radiomarkers and chromosome markers, it has been shown that the restorative cells are of donor origin (59). Bone marrow is found to be the best for restoration while spleen and peripheral blood are less effective and lymph node, thymus and thoracic duct lymphocytes are ineffective. Colony forming units in the spleen can be observed with the donor cells in limited numbers (7). Each colony is shown to originate from one cell precursor (13), which is found to be related to the small lymphocytes (41).

It is interesting to note that though bone marrow can repopulate spleen, lymph nodes and the thymus, spleen and lymph node cells can only repopulate the spleen and the lymph nodes, but not the thymus (60). Hence the bone marrow can be considered as the ultimate source of the stem cells which are capable of supplying all the other lymphoid organ through the pluripotential precursors whose developments are dependent on the local humoral environments (195). With the thymectomized animals, there is a reduction of the repopulated lymphocytes, hence one of the lymphocyte-inducing environments is provided by the thymus. There has been effort spent trying to identify the non-thymus derived cells, some of the more successful markers being the mouse-specific B lymphocyte antigen (MBLA) as defined by Raff et al. (145) and the cell surface immunogloblin by Greaves and others (71, 247, 264).

(d) The Macrophages

Macrophages are found to be responsible for the elimination of foreign material from the circulation and its degradation (37,

207). These cells are also important for concentrating the antigen and presenting them to the immunocompetent cells (107). Studies on animals exposed to total body x-irradiation have shown that the injection of macrophages with antigen promotes the recovery of the immune reponse (52, 140). Antibody formation to SRBC can be induced *in vitro* only in the presence of macrophages (53, 61, 121).

There are also reports, which are disregarded by most modern immunologists, to the effect that the reaction of macrophages with antigen yields an "immunogenic RNA" extract which, when added to lymph node cells in culture or upon injection into animals,may elicit antibody formation (6, 54, 137). Both antigen-induced transformation and antibody responses to some antigens *in vitro* require the participation of macrophages (80, 160).

II. CELL COOPERATION IN THE IMMUNE RESPONSE

(a) Effects of the Carrier Portion of the Antigen Molecule on Immunogenicity (the Carrier Effect)

The recognition of the foreigness of the carrier of an antigen is vital for maximal antibody production against the hapten. Homologous hapten-protein conjugates are one-thousand fold more effective in stimulating anti-hapten antibody than conjugates of the hapten with a protein unrelated to the original carrier, even though the new carrier may be highly immunogenic molecules (106). The response to the carrier molecule of the hapten-carrier antigen is genetically controlled, at least for DNP-poly-lysine in the guinea pig system and in the mouse system to polypeptides (10, 266, 267). The introduction of a spacer molecule between hapten and carrier does not impair the immunogenicity of the antigen; hence it is concluded that the hapten and the carrier do not act as one determinant (10). Ir gene product or the Ia molecule dictates, by its ability to combine with an antigen fragment at a specific site on the fragment, the nature of the antigenic determinant that stimulates helper or DTH T cells (268). The specific Ir gene may function to select B cell clones (269). In the guinea pig, the minimal size of the combining site controlled by the PLL Ir gene-Ia molecule is a sequence of three to four amino acids (266, 267).

Using lymphoid cells stimulated *in vitro* with a hapten-carrier conjugate and transferred them into x-irradiated animals for a subsequent challenge with the original or different carrier attached to the hapten, it is obvious that cells responding to the carrier and the hapten are different and that

both types of cells are needed for the anti-hapten antibody response. However, antibody production against a hapten can be enhanced by the presence of 'helper cells' with immunological reactivity to the carrier (110). The helper cells are sensitive to treatment with anti-θ serum; hence it is concluded that they are thymus-derived i.e. T cells (143). They may bind to the carrier through a receptor on their surface, however, the exact nature of which is controversial.

(b) Cell Cooperation in the Antibody Response

It is evident that thymectomy does impair the antibody response especially to thymus dependent antigens (150) with the exception of some T-independent antigen (265). The injection of different types of cells with SRBC into irradiated mice illustrates that a mixture of thymus cells and bone marrow cells is necessary for the production of hemolytic foci and for induction of plaque forming cells (32). Both thymus and bone marrow cells are found to be radiosensitive (34).

Studying the spleen cells with regard to their ability to adhere on plastic surfaces, one can divide them into adherent and nonadherent cells. The presence of both types is necessary to mount an antibody response. The adherent cells are found to be radioresistant, while the nonadherent cells are at least partially radiosensitive (122). From experimental evidence, it has been suggested that the adherent cells are macrophages and are generally referred to as A cells (accessory cells) by Osoba (128).

(c) Cell Cooperation in the Cell Mediated Response

Thymocytes and bone marrow cells are needed to elicit typical cell mediated immunity such as GVH reactions (11). The cytotoxicity to mouse fibroblast by sensitized rat thymocytes was increased when mixed with normal spleen cells (96).

(d) Antigenic Competition and Cell Cooperation

The injection of one antigen may alter the response to another unrelated antigen. The alteration may range from a mere delay in response to a total suppression of the immune response for the unrelated antigen (171).

The phenomenon of antigenic competition is expected to be affected by the physiological state of the animal, the form, dose and route for the injection of the antigen, the genetic characteristics of the animals, the kind of adjuvants employed and the past immunization history of the animals in question. However, generally, the mechanism of this phenomenon is not well understood.

(e) Immunological Memory and Cell Cooperation

The secondary antibody response occurs after repeated injection of the same antigen. It is long lasting and may persist for months. Its lag phase is shorter than for the primary response and the slope for the secondary antibody response is steeper and at the higher level. Antibodies produced in the secondary response usually have exceptionally high affinity, determined by the dose of the antigen used for primary and secondary responses.

The cellular aspect of immunological memory can be well illustrated by Miller's double transfer experiment in which thymus cells were transferred into irradiated mice for a week, then the

spleen cells were mixed with normal bone marrow cells and transferred to a secondary host (117). Response to SRBC can be achieved only if both hosts had received the antigen. Horse RBC used for the immunization of primary host does not give the anti-SRBC response to the second host. This illustrates that specificity and memory rests with the T cell population.

III. THE MECHANISM OF CELL COOPERATION

From the previous chapter, one can visualize that three cell types participate for the elicitation of the immune response, i.e. the thymus-derived cells (T), the bursal equivalent (B) also generally referred to as bone marrow derived cell and the accessory cells (A). The following mechanisms have been proposed in relation to the cooperation of A, B and T cells.

(a) Concentration of Antigens

The basic assumption is that T cells, through their cell surface receptors, termed IgX by Mitchison (109), bind to the carrier portion of the antigen and present the haptenic portion of the antigen to the B cells with appropriate anti-hapten receptors, which are thus stimulated leading to the anti-hapten antibody production. Taylor (175) further suggests that macrophages may serve as helper cells along with the T cell in cell cooperation.

(b) Soluble Factor

Upon the interaction of T cell with specific antigen, a soluble factor(s) may be released with effects on the other cells. Some of these factors have been characterized and tested (91, 57, 208). Purified nonimmunized guinea pig B cells population may be stimulated by PPD or LPS to produce migration inhibition factor (MIF). However after reconstitution with T cell population or with the whole cell population, B cell MIF production is abolished. This observation is the result of yet another cellular factor produced by the T cells with the ability to suppress the B cell activities. This may account for the fact that B cells do not appear to play a role in the reaction of

cell-mediated immunity *in vivo* (256).

(c) Accessory Cells

The accessory cells are most likely macrophages. The macrophage is a phagocytic cell capable of taking up antigens by endocytosis which are partly degraded. The antigen thus modified is capable to elicit the immune response and is known as immunogen (180). It is believed by some investigators that some of the degraded antigens combined with the RNA of the macrophages, which, upon extraction, is capable of transferring immunity (53, 54).

(d) Function of Immunocompetent Cells in Genetic Control of Immune Responsiveness

With the discovery of the specific immune response (Ir) genes in the I region of the major histocompatibility complex (MHC) of mammals (270), their roles in the cell cooperation have been reviewed by many distinguished authors (268, 271, 272, 273, 274, 275). Ir genes are expected to have the capacity to channel the response of the animal toward T helper or T suppressor (276). T helper cells carry the murine cell surface marker Ly 1 antigen while the T suppressor cells carry Ly 23 antigen. Immunogen favors the differentiation of Ly 123 cells to Ly 1 cells, while tolerogen favours the differentiation of Ly 123 cells to Ly 23 cells.

The role of macrophage in the genetic control of immune responsiveness is becoming very important (277) since the demonstration of a new class of alloantigen (Ia molecule) on it as well as on B cells (278). Basically, a function of the Ir gene expressed on B cells and macrophages concerns the

ability of Ia molecules on the cells to form, together with antigen, an immunogenic complex capable of stimulating T cells or being acted upon by specific T cell clones (268). The different mechanism of macrophages in genetic control of immune responsiveness have been proposed by A.S. Rosenthal et al. (273) and E.M. Shevach (271).

(e) Modulation of Immune Responses by Suppressor T Cells

Regulation of the immune response involves specific and non-specific interactions between different immunocytes with their products (279-282). The suppressor cells modulate both humoral and cell-mediated immunity utilizing antigen-specific and nonspecific mechanisms (283). The nonspecific suppressor cells can be activated by mitogens such as Concanavalin-A (Con-A) and phytohemagglutinin (PHA) (284-289) or by simple in vitro cultures (290-295). Specific suppressor cells can be activated by allogeneic cells (290, 296-299), allografts (296, 299, 291, 292, 300) and graft-versus-host reactions (293, 301). The suppressive activities can be assayed by the inhibition of DNA synthesis after the mitogenic stimulation (286, 287, 289) in mixed lymphocyte cultures (MLC) reactions (286, 295, 296, 297, 302), in delayed hypersensitivity reaction(303) and the inhibition of graft-versus-host reaction (304). Suppressor cells can also inhibit the generation of cytotoxic cells to allogeneic cells (285, 295, 301, 305, 315, 316) and tumor cells (297, 302, 306, 307).

The suppressor cells have been shown to be thymus-dependent lymphocytes (285, 287, 292, 297, 302, 304, 305). In the murine system, they belong to T lymphocyte subset with

surface marker Ly 2,3 (297, 237, 308). Macrophages, in some cases, have also suppressive activities (277, 280, 297). Suppressor cells can be cortisone-sensitive (302) or cortisone-resistant (310) and cyclophosphamide sensitive (311, 312). They can be adherent or non-adherent to glass and nylon wool (284, 287, 289, 302, 305, 307). The suppressor cells show a variety of specification in their activities and their ability to produce suppressive factors (286, 288, 313, 314).

It has been a scientific goal for modern immunologists to search for the procedures to induce specific suppressor cells production (276, 283, 317) so as to understand better the mechanism of disease and the immune response phenomenon (318, 319).

IV. SURFACE CHARGE PROPERTIES OF IMMUNOCYTES

(a) Introduction

Electrophoresis is one of the methods of characterizing cells as a function of their surface charge (344, 345). Most of the cell types investigated to date have been shown to have characteristic cell membrane net charges. The net electrical surface charge of an immunocyte, measured in terms of the corresponding electrophoretic mobility, is expected to be the result of charges contributed by the immunoglobulin-like peptides in the antigen binding receptor sites and by the diverse chemical groups constituting the rest of the cell membrane (346, 347). In view of the complex mosaic of antigens to which an animal is constantly exposed in its normal environment and in view of the complex chemical composition of cell membranes, *a priori*, one does not expect mobilities of unfractionated immunocytes of normal animals to differ significantly from those of animals (of the same species) which have been immunized with a given antigen. However, the evidence reviewed below indicates that such differences exist.

(b) Cell Surface Charge Properties of Immunocyte Subpopulations

The chemical moieties contributing to the charge properties of mammalian cells have been widely studied. With the aid of enzymatic degradation procedures, sialic acid is found to contribute most of the negative charge properties of the cells (63, 348, 354).

A large number of investigations have been done on the electrokinetic properties of immunocytes. Thus, for example, the surface charge of lymph node cells from rabbits immunized

with a complex vaccine of Salmonella typhi and paratypi A and B, or Vibrio cholera, is shown to differ from that of normal cells (168). Moreover, the surface charge of immune lymph node cells (168) or of peripheral blood lymphocytes (17) is found to be reduced on treatment with the homologous, specific antigen. The feasibility of altering significantly the net surface charge of lymphoid cells from animals immunized with a hapten-carrier conjugate by reacting them with the specific hapten, coupled to different polyionic carrier macromolecules, has been demonstrated in this laboratory (149).

In a study on small lymphocytes of the rat thoracic duct, Ruhenstroth-Bauer and Christine (151) find that there are two populations, one with slow and the other with fast electrophoretic mobilities. From a comparison of the histograms of electrophoretic mobilities of lymph node cells of normal rats and of rats immunized with four complex vaccines, it has been deduced that the relative concentrations of two subpopulations of these cells changes on immunization and that the new type paralleling the kinetics of antibody formation can be detected (135).

(c) The Electrophoretic Differentiation of T and B Cells

It has been established that the electrophoretic mobility of lymphoid cells of spleens of nude homozygous mice is 1.81 ± 0.01 $\mu m\ s^{-1}V^{-1}cm^{-1}$, (the unit used, i.e. $\mu m\ s^{-1}V^{-1}cm^{-1}$ represents micrometer per second per volt per centimeter)(193). On the other hand, spleen lymphoid cells of heterozygous nude mice provide a trimodal mobility profile, having respectively

mobilities of 0.81; 1.20 and 1.37 $\mu m\ s^{-1}V^{-1}cm^{-1}$. The cells with the lowest mobility are determined as B cell and the other two subpopulations of higher mobility as T cells (346). The low EPM cells show adherence to nylon wool while the high EPM cells can form spontaneous rosette with SRBC (259, 355).

The subpopulation with characteristics of T cells have been investigated by Sabolovic and Dumont (154). The cortisone-resistant PHA responsive cells have higher electrophoretic mobilities, whereas cortisone-sensitive cells are found to have lower electrophoretic mobilities as compared to the mean electrophoretic mobility of the T cells. Four subgroups (T_1, T_2, T_3, T_4) of thymocytes and 2 subgroups (T_4, T_5) of lymphocytes from the peripheral lymphoid organ have been determined by the cell electrophoresis technique (260, 261, 262).

The organ specific distribution of the T and B cells has been investigated with the Zeiss Cytopherometer (190). The percentages of cells in the fast and slow groups corresponded to the percentages of T and B cells in the thymus, lymph nodes and the spleen. Hence T and B cells may be characterized by their different electrophoretic mobilities and it has also been observed that there is a change in the net surface charge during maturation from thymocyte to T-lymphocyte (262). Further work done by Wiig (193) on the T cells depleted and B cells depleted animals confirms that T cells have fast electrophoretic mobilities while B cells have slow electrophoretic mobilities.

(d) Differential Interaction of Anti-Lymphocyte Serum with Sub-Population of Immunocytes

In terms of differences in their EPM, the rat lymph node cells have been divided into Class A and B, corresponding to the T and B cells. Using rat lymph node cells and anti-lymphocyte serum against normal lymphoid cells, ALS(N), and lymphoid cells from animals immunized with Salmonella typhi, ALS(I), it has been shown that ALS(I) can interact with a different class of cells C in addition to the above two classes differentiated by their electrophoretic mobilities. Class C cells are shown to be antibody producing cells that evolved during immunization (133).

Differences are observed in the EPM after the interactions between anti-lymphocyte serum with T and B lymphocytes in both rat (132) and mouse (15). Antilymphocyte serum produced against the spleen cells can decrease the mobilities of lymphocytes and platelets, but not the mobility of erythrocytes.

(e) Free-Flow Electrophoresis for the Elucidation of Immunological Events at the Cellular Level

Free-flow electrophoresis is a refined method for the fractionation of particles or cells according to their net surface charge (344, 345). The apparatus developed originally by Dr. K. Hannig of the Max-Planck-Institut in Munich and now commerically available consists of an electrophoresis chamber with a buffered medium flowing in a constant speed carrying the cells, under a strong electrical field. The fractionated cells can be harvested through the different tubing running at different distance between the electrode at the bottom of the chamber.

Since its development, free flow electrophoresis has been widely used for the fractionation of immunologically competent cells. Two 19S hemolysin-producing cells fractions have been obtained from the lymph nodes of the rat and their immunological functions are confirmed in vitro (198). In accordance with the findings of the micro-electrophoresis method, free-flow electrophoresis leads to the separation of two main subpopulations of lymphocyte. In functional tests, antibody precursor cells are found only in the low mobility region, while the high mobility region contains cells responsible for the GVH reaction (199).

Cells with high and low electrophoretic mobilities are found in the rat thoracic duct with percentages of 75 and 25 respectively. After prolonged cannulation of the thoracic duct, the percentage of cells with high electrophoretic mobility decreases while those with low electrophoretic mobility increases (200). There are differences in mobility patterns of lymphoid cells from different organs, i.e. bone marrow lymphocytes have low electrophoretic mobility, thoracic duct lymphocytes have high mobility, whereas the cells of lymph nodes and spleen have high and low EPM lymphocytes. Natural killer cells have also been enriched by method of free-flow electrophoresis (356).

The relationship of mobility to the sialic acid content on the cell surface has also investigated by digestion with neurominidase (350, 351, 346); the bimodal pattern has found to be reduced to a single peak (124).

As stated above, the cells with high and low electrophoretic mobilities from the mouse lymph nodes have been demonstrated

to be thymus-dependent and thymus-independent cells, respectively. The high mobility cells carry the Θ isoantigen, respond to PHA *in vitro*, and induce GVH reaction in newborn F_1 hybrid mice. Nearly all low mobility spleen cells have complement receptors on their surface. About 70% of the low mobility spleen cells are sensitive to anti-MBLA serum and undergo "cap" formation when incubated with FITC-conjugated anti-Ig. Only low mobility cells respond to *E. coli* lipoplysaccaride (5), indicating the presence of cells with antigen receptor in the low mobility spleen cell.

By use of free-flow electrophoresis it is possible to fractionate various subpopulations of T cells (260-262) and of B cells (203); i.e. the antibody forming and the *in vitro* colony forming cells of the mouse bone marrow (202).

(f) Macrophage Electrophoretic Mobility (MEM) Test for Lymphocyte Sensitization

Field and Caspary (56) have discovered that blood lymphocytes of patients with malignant neoplasms are sensitized to a basic protein derived from the nervous system. Such sensitized lymphocytes, when incubated with the basic protein, release a factor which reduces the electrophoretic mobility of normal guinea pig peritoneal macrophages and modified sheep red blood cells (357, 358). Since then this test has been used to show the presence of sensitized lymphocytes to thyroglobulin, to components of thyroid tissue in patients with Graves' syndrome (55), to purified protein derivative of tuberculin and to Kveim antigen in Sarcoidosis (57). From a variety of human malignant neoplasms, it is possible to prepare by acid

extraction as basic protein (proteolipid) with a similar molecular weight to that of the encephalitogenic protein of brain (16-18,000 daltons). This protein has "antigenic" activities in the MEM test for cancer cells and the activity is related to the presence of tryptophan. An analysis of the most active material so far prepared suggests many important differences from myelin basic protein and from histones (312, 26).

Of 464 patients with cancer, 463 have been correctly diagnosed by measuring their lymphocyte sensitization to encephalitogenic factor or the cancer basic protein in term of EPMs. Leukemia,advanced tuberculosis, asthma, sarcoidosis, systemic lupus erythematosus, and a number of rare conditions may interfere with a correct diagnosis. In multiple sclerosis and all other neurological diseases in which there is an appreciable destruction of nervous parenchyma, sensitization of lymphocytes have been found (214). The usefulness of MEM test in cancer diagnosis has been confirmed by other workers (141). It has been shown that this migration inhibition-factor, which is detected by the slowing of the electrophoretic mobility of the normal guinea pig peritoneal macrophages, is different from the migration inhibition factor which inhibits the migration of normal guinea pig peritoneal macrophages in the absence of an electric field (81).

Macrophage slowing factors production is inhibited either by puromycin or inhibitors of protein synthesis (28). It is also dependent on the dose of antigen and on the number of lymphocytes (27).

(g) Studies related to Cell Electrophoresis

Cell electrophoresis has been employed in various studies. It has been found that myelocytic leukemic cells have high negative mobility (124, 359); sterility in man is related to the surface charge of spermatozoa (111); the electrophoretic mobility of lymphocyte changes in pattern during immune adherence for the intermediate cells (127); polylysine reduces the electrophoretic mobility of BP8 cells whereas lysine HCl neutralized the effect of polylysine and heparin can reverse completely the effects of polyamines or protamine (105); fast syngenic lymph node cells can protect C_3H mice against fatal challenge with BP8 tumor in vivo (176); surface charge/unit area is found to be constant throughout the cell cycle of synchronized L5178-Y mouse lymphoblasts (159); heparin can induce biphasic changes in the electrophoretic mobilities of platelets (78), EDTA induces increase in platelet surface charge associated with the loss of aggregability (360); cell proliferation rather than the level of differentiation is the significant factor determining surface charge of living cells (361); lectin stimulation increase EPM of lymphocytes (362); and the changes of the electrophoretic mobilities of the intermediate cells in immune hemolysis to the attachment of the different components of the complements has been investigated (209).

Each of the above studies represents a distinct problem of considerable interest, supporting thus that cell electrophoresis has a potential of being developed into a powerful tool for research in various areas of cell biology.

EXPERIMENTAL

INTRODUCTION

The experiments performed in this study are reviewed in nine sections. The experimental layout began with the construction of the all glass closed microelectrophoresis chamber that met the biophysical requirements for electrophoretic studies involving cells. Then the experiments proceeded with establishing the electrophoretic mobilities of the normal and immunized mouse lymph node cells. In order to establish the electrophoretic mobility of the hapten (DNP) specific binding cell and the possibility of altering its mobility by interaction with charged molecules incorporating the hapten, the monohaptenic polyionic polymers, αDNP-poly-l-lysines and their derivatives and αDNP, ε-isothiocyanate fluorescein-lysine were synthesized.

The efficiency of DNP-specific biogel affinity column as a method for cell fractionation was investigated with reference to the cell electrophoresis technique. To identify functional (i.e. T and B) subpopulations among lymph node cells, the cells were selectively depleted by treatment in vitro with anti θ serum. Also, to establish if the net surface charge of the immunocytes was contributed mainly by sialic acid, cells were treated with neuraminidase. The effect of the immunosuppressive drug cyclophosphamide was also investigated.

Finally the practical use of the microelectrophoresis as a possible sensitive tool in clinical diagnosis was investigated in terms of the Macrophage Electrophoretic Migration Test (MEM) for Lymphocyte sensitization.

I. THE MICROELECTROPHORESIS CHAMBER

(a) Material

For construction of the glass chamber, pyrex plate (6" x 6" x 1/8") were purchased from Versa Glass Ltd., 89 Quinn Street, Manchester, England. The electrodes were made from "half hard" fine silver strips (0.394" x 0.010" x 1") and fine silver wires (0.04" in diameter) obtained from Johnson Malthey and Mallory Ltd., 110 Industry Street, Toronto 15, Ontario.

Acrylamide (CH_2: $CHCONH_2$), N', N'-methylene-bis acrylamide ((CH_2:$CHCONH)_2CH_2$), and N,N,N',N'-tetramethylethylenediamine (TEMED), $(CH_3)_2NCH_2CH_2N(CH_3)_2$, were purchased from Eastman Kodak Company, Organic Chemicals Division, Rochester, New York. Tris (Hydroxymethyl)Aminomethanol was ordered from Fisher Scientific Company and was of Reagent grade.

(b) Instrument

The microscope used was a standard Carl Zeiss research microscope (Cat. #47-09-34) provided with optical photo changer (Cat.#47-30-10) to a square projection attachments (Cat.#47-30-80) capable of holding a photographic film (4" x 5") inserter (Cat.#47-30-84). The objective was an Achromat UD 40/0.65 effective 25/0.41 (Cat.#46-20-46) with a working distance of 6.6 mm.

(c) Method

The Construction of the Microelectrophoresis Chamber

The parts for the electrophoresis apparatus are represented in Figure 1 and in Plate 1 and the assembled cells in Plate 2. The microelectrophoresis chamber was made in the

Figure 1 The microelectrophoresis chamber and its electrode compartments.

THE MICROELECTROPHORESIS CHAMBER AND ITS ELECTRODE COMPARTMENTS

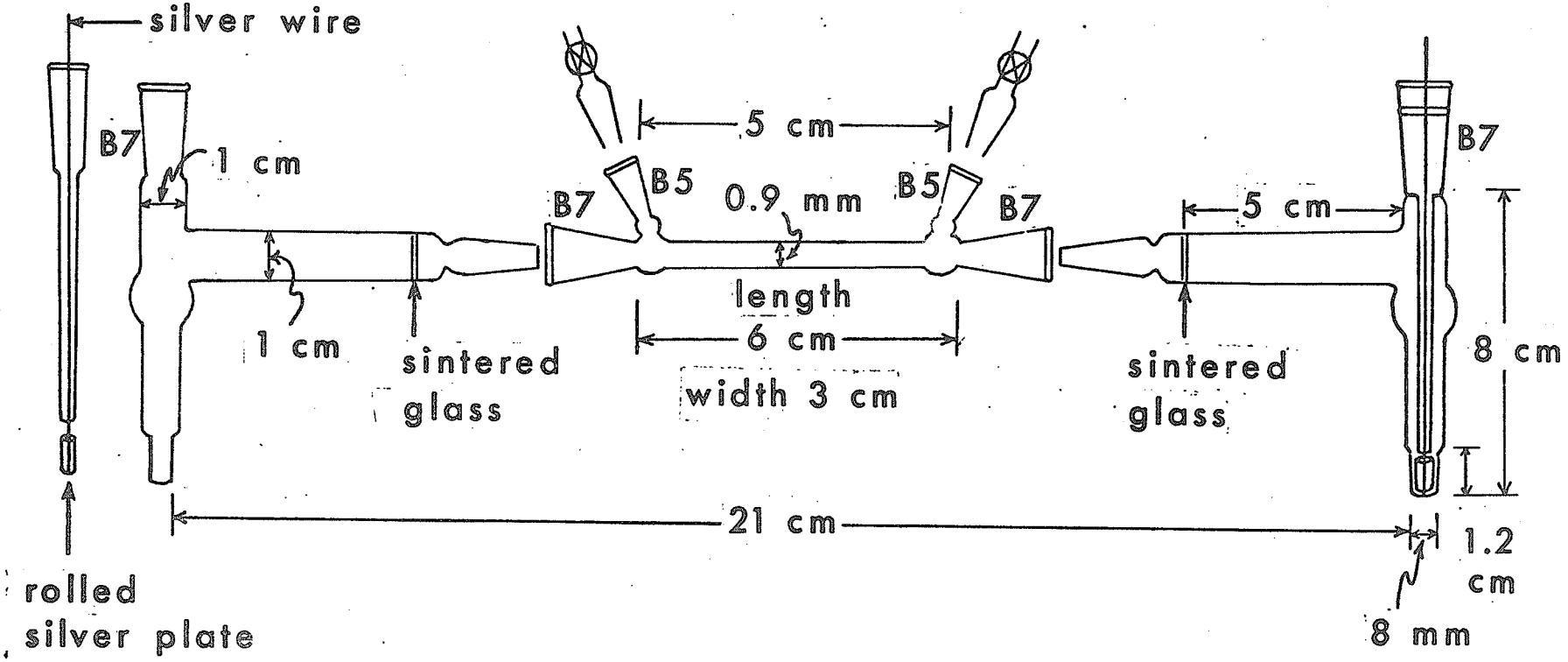


Figure 1

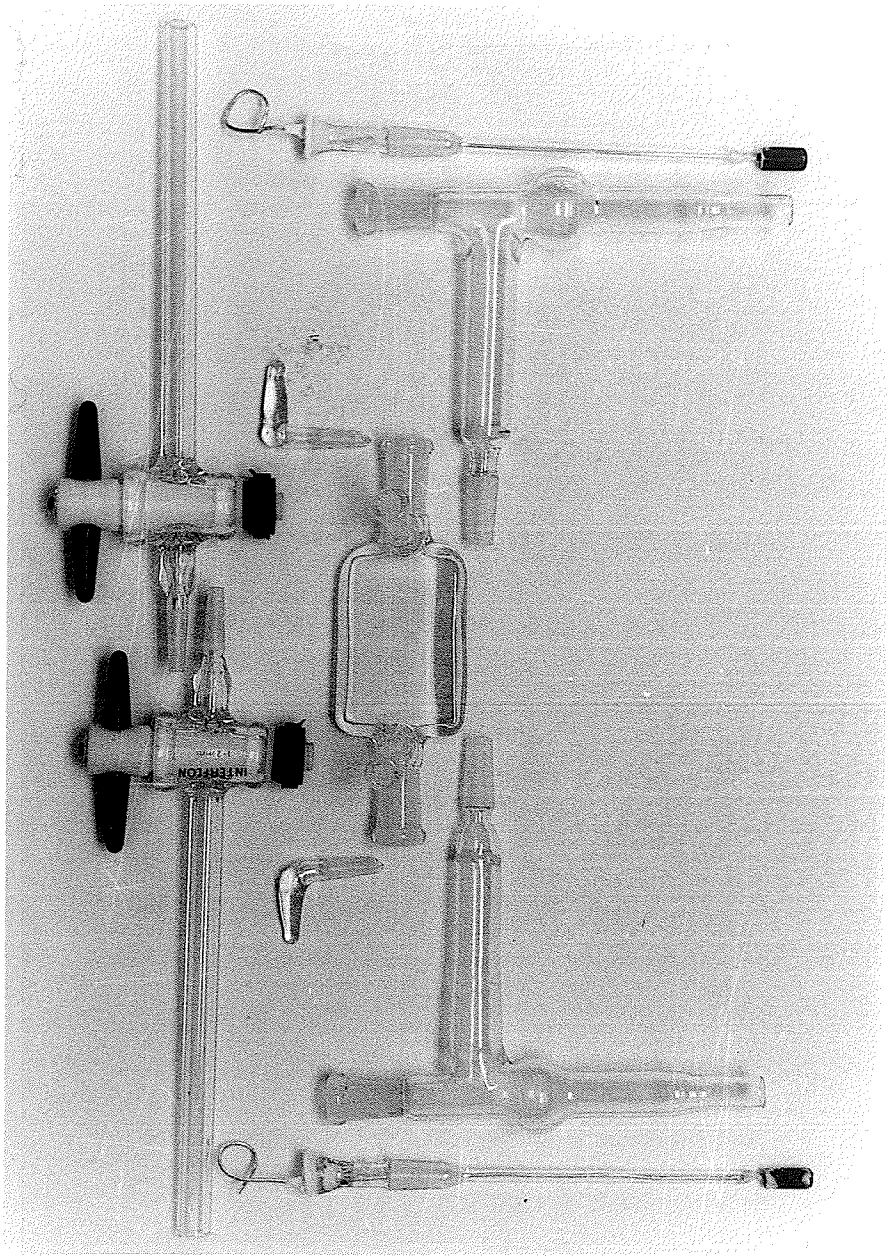


Plate 1: Cell Microelectrophoresis Parts.

glass workshop of the Chemistry Department of the University of Manitoba. After several trial models designed by the author, the final cell chamber was made from the optical free glass plate provided by Versa Glass Ltd.. The parallelepipedal glass chamber (6 cm. x 3 cm. x 0.9 cm.) was joined to a B7 female at each end and to a B5 joint at an angle of 60° to the B7 joint (Figure 1). The capacity of the chamber itself together with the dead space at each end was two ml..

The Construction of the Electrode Compartment

The T-shape electrode compartments were made of one cm. internal diameter tubes (Figure 1), and each was provided with B7 female and male joints for connection to the chamber and the electrode, respectively. A coarse sintered disc was sealed into each electrode compartment to act as a permeable partition between this electrode compartment and the cell chamber. The electrode consisting of electrode silver wire and silver plate was made in the factory of the Birks Jewellery, Winnipeg, Manitoba by "lethering" together, i.e. by hammering without soldering. This electrode system was chosen since it was a reversible silver-silver chloride, non-polarizing electrode and did not promote gas formation. The top of the electrode compartment was filled with the buffer used in the electrophoresis chamber and the bottom with 20% KCl, as normally recommended for the classical free electrophoresis (235). In this system, the reactions involved at the two electrodes are: At the Cathode (i.e. negative electrode, conversion of AgCl to Ag.)

$K^+ + e \longrightarrow K^\cdot$

$AgCl + K^\cdot \longrightarrow Ag^\cdot + KCl$

At the Anode (i.e. positive electrode, reduction of Ag ion to Ag atom)

$Cl^- - e \longrightarrow Cl^\cdot$

$Cl^\cdot + Ag \longrightarrow AgCl$ (Ag is deposited on the electrode in the form of AgCl)

To begin with, the electrodes were anodized in 20% KCl using a platinum wire as the cathode for 15 minutes with an electric current of 5 milliamperes before assembly.

Polymerization of Acrylamide for Gel Formation

The component for preparing the gel were mixed according to the ratio indicated in Table 1. After mixing, the solution was quickly degassed under vacuum and poured into the female joint about the sintered disc in an upright position with the electrode compartme filled with PBS and closed by the electrode B7 joint. The electrode compartment was placed for 20 minutes at a distance of about three inches from a Buchler fluorescent light unit which provided the photochemical energy for initiating the polymerization reaction. Any excess gel above the female joint was removed with a sharp scapel under PBS so as to provide a wet and flat surface. The presence of the gel was necessary to reduce the diffusion of the contaminating ions from the electrode chamber from entering the cell chamber.

Circuit and Determination of Field Strength

The Heatkit regulated power supply, Model PS-4 (Heatkit Co., A subsidary of Daystrom Inc., Benton, Harbor, Michigan) was used. A polarity switch designed to alter the polarity

TABLE I

POLYACRYLAMIDE GEL COMPOSITION

	VOLUME RATIOS	COMPONENTS/100 ML		pH (25°C)
GEL	1	Acrylamide	30 g	
		Bisacrylamide	0.8 g	
		Water to Volume		
	1	Tris (Hydroxymethyl) Aminomethanol	18.15 g	9.1
		1 N HCl	25 ml	
		Temed (N,N,N',N'-tetramethylethylenediamine)	0.24 ml	
		Water to Volume		
	2	Ammonium Persulfate	0.28 g	
		Water to Volume		

of the current without changing the current was made by the Electronic workshop of Faculty of Medicine of the University of Manitoba. The power supply was capable of supplying a constant DC electric current of 5 milliamperes within the experimental time.

The field strength in volts per cm. was obtained from either (a) the applied potential and the distance between the electrodes or (b) Ohm's law using conductance and current data. From the latter relation, it follows that

Field strength = I.R./q

where I = current in ampere

R = specific resistance in ohm

q = cross-sectional area of the cell in cm^2

The conductance was measured with a LKB Conductivity Bridge Type 3216 B. The author found that both a and b method yielded identical values for the field strength because there was absence of polarization. Hence method (a) was adopted. The distance between the electrodes was 35 cm.

Electrophoretic mobilities were expressed as the distance moved in unit time under unit potential gradient and correspondingly the mobility unit was in micron per second per volt per cm., i.e. μm/sec/volt/cm.

The Determination of the Electrophoretic Mobility of Cells

There were lots of biophysical problems associated with cell electrophoresis, however, there was no exact solution for the hydrodynamic equation for the rectangular chamber,

but at a ratio of width to depth of the cell chamber of 20:1, the stationary levels were located at 0.21 and 0.79 of the total depth of the cell chamber (1). The exact depth from the inside top surface and bottom surface of the cell chamber was precisely determined and achieved by adjusting the vernier scale mounted on the arm of the microscope by first focussing on the inside top surface and then the bottom surface of the cell chamber.

The microscope was equipped with the photographic unit as mentioned. A 4" x 5" (10.2 x 12.7 cm.) Kodak Contrast Process Ortho Film with Extra thick base was used with the film holder mounted on the projector. The light inlet was set at "number 10" on the scale. The intensity of the current was 5 milliamperes. The exposure time was 10 seconds. After the film was developed, the velocity of the cells was calculated. The distance travelled by the cells was measured by comparing the length of the tracts on the film with the print of the film of a stage micrometer's scale under the same magnification. Each division on the scale on the film was equivalent to 10 microns in length (Plate number 3). Each photographic film covered a field of about 290 microns x 440 microns at the stationary phase with about 50 to 70 cells.

Temperature

Since it was important to avoid heat generated during the experiments, the experimental time was limited to 10 seconds. Heat elevation during an experiment would induce

viscosity changes in the medium, as well as convection, which would lead to serious errors in the determination of the electrophoretic mobilities of the cells. All the experiments were run at room temperature unless otherwise stated.

Setting up of the Instrument

With the help of a spirit level, the cell holder with the electrophoresis chamber fixed into position was adjusted and levelled by three levelling screws at the bottom of the mounting platform of the microscope. The chamber should be accurately horizontal so as to eliminate the effect of any gravitational components and the optically flat surface should be perpendicular to the objectives. A cell suspension of 10^5 cells/ml. was pipetted into the chamber through the B5 female joint. Grease was not used in any of the joints. Caution was taken so no air bubbles would trap inside the cell chamber.
The inner surface of the upper wall and the lower wall of the cell chamber were observed to have thin layers of cells attaching to them. The stationary phase was pin-pointed with the help of the vernier scale on the arm of the microscope.

Cleaning and Filling

The chamber could be cleaned with either CrO_4/H_2SO_4, CrO_3/HNO_3, alcoholic KOH, pyroneg or 1M NaCl. Usually the cell chamber was cleaned with 1M NaCl, then followed by rinsing with distilled water and siliconized with Siliclad (Clay Adams, Division of Becton, Dickinson and Company, Parsippany, N.Y.07054).

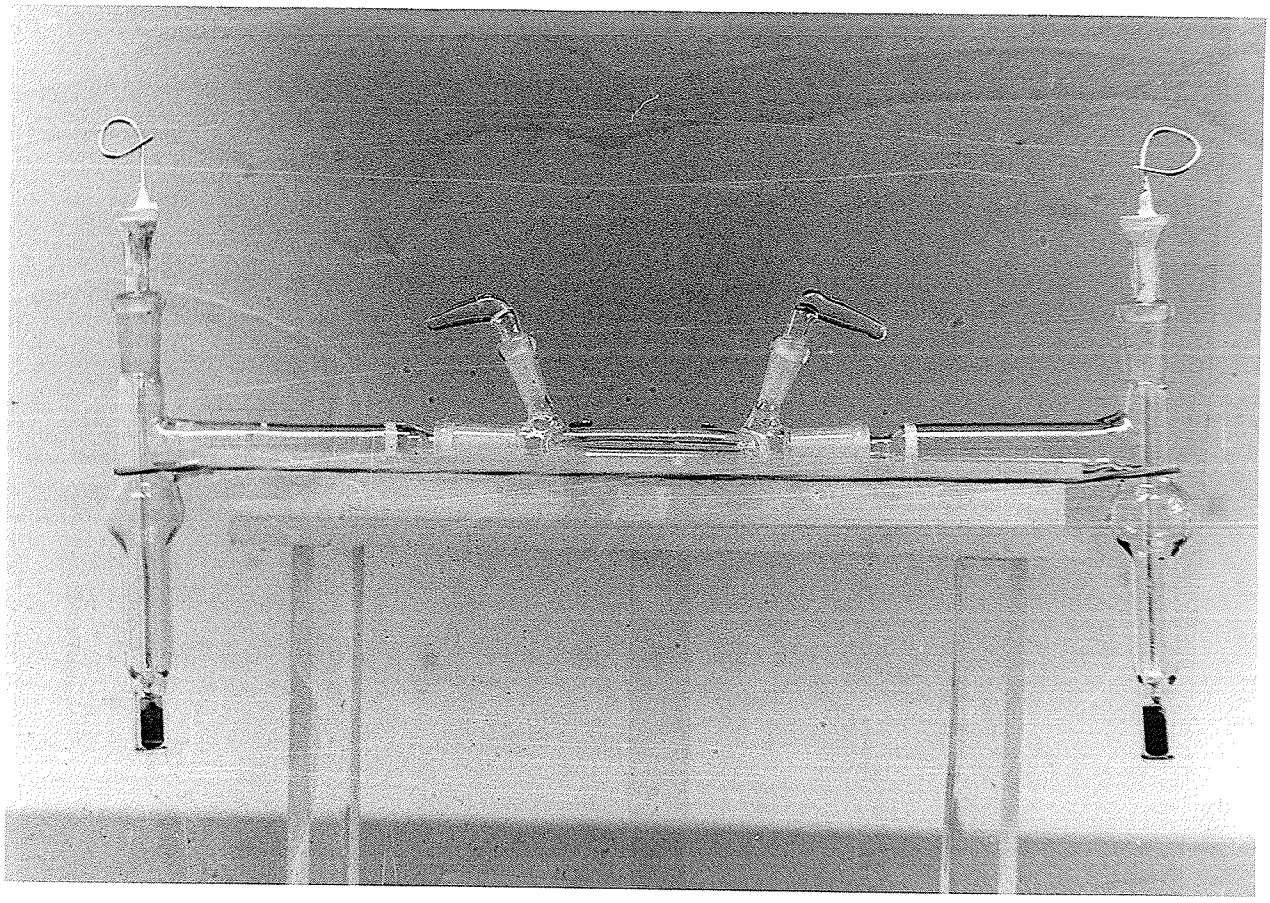


Plate 2: The Microelectrophoresis Chamber

The chamber was then throughly rinsed with double distilled water. Before the cell suspension was put in, the cell chamber was prerinsed with the suspending medium. At the end of the experiment, all the fluid was removed from the chamber with a polythene cannula attached to a water suction pump.

As mentioned earlier no grease was used on the stoppers or sockets.

The water used for the preparation of solution was freshly distilled at least twice from a pyrex glass or quartz still. The distilled water supply from several individual laboratories were checked with no significant changes in the electrophoretic mobilities for the calibration curve using the author's own red blood cells.

Calibration of the Cell Microelectrophoresis Unit

The human red cell had so far gained the widest use as a calibration particles. Each time when an experiment was performed, the cell was calibrated with the red blood cell of the author. The blood was obtained by cutting the middle finger with a sterile disposable blood lancet. Three drops of blood were suspended in 5 ml. of PBS. pH 7.2 and centrifuged down at 1500 rpm for 10 minutes. The cells were washed three times with PBS. Finally the cells were suspended in 0.5 ml. PBS and counted with a haemacytometer, the volume was adjusted to a concentration of 10^5 cell/ml.

(d) Result and Conclusion

The electrophoretic mobility of human red blood cell was found to be 1.28 ± 0.03 microns/sec/volt/cm. which was in

good agreement with the values given in the literature (Table II) for the stationary phase.

The relationship between the distance from the inner surfaces of the cell chamber and the electrophoretic mobility of the red cells was investigated (Figure 2). The profile of the distribution of the electrophoretic mobility of the red cells as a function of the cell chamber depth was in agreement with the hypothetical curve (209) and could be fitted into the theoretical mathematic equation.

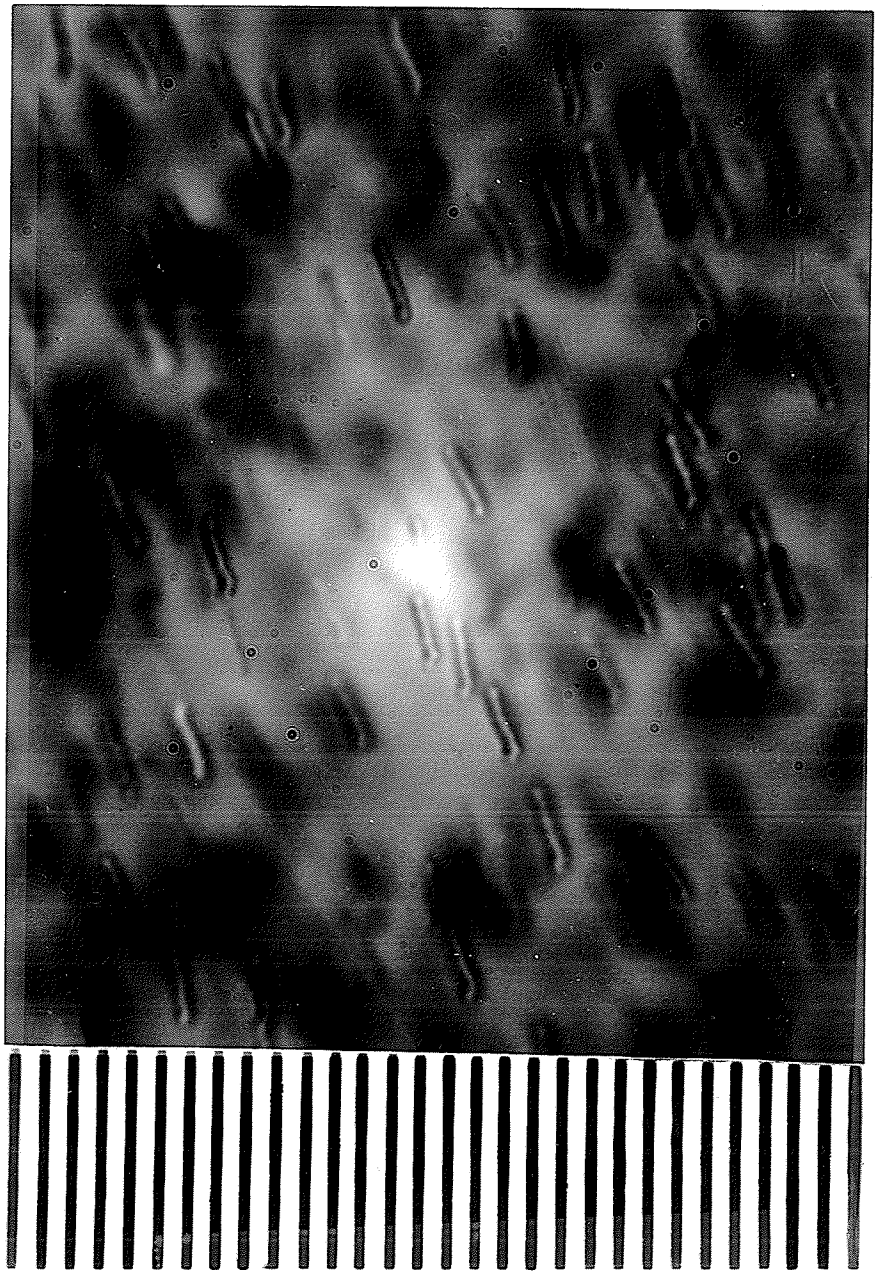


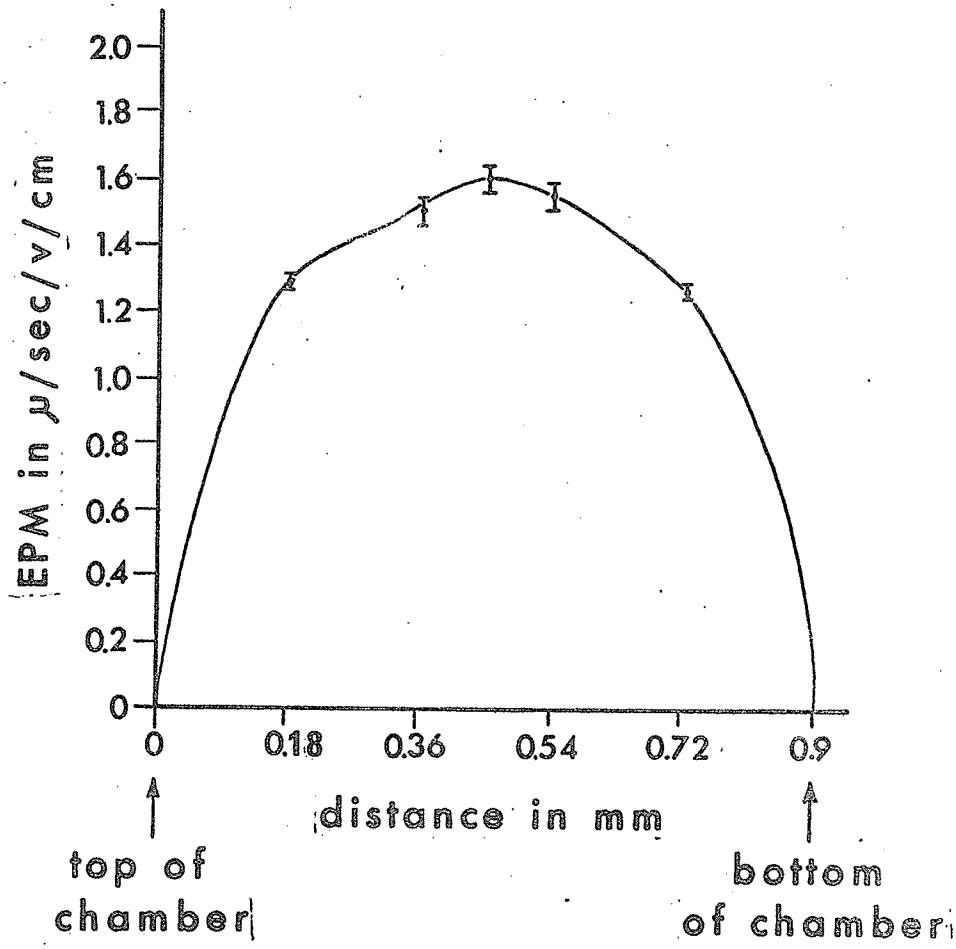
Plate 3: The tracts of Human Red Blood Cells covered under the influence of an electric field strength of 80/35 volt/cm.
Scale, 1 Division = 10 microns.

TABLE II

ELECTROPHORETIC MOBILITY OF HUMAN RED CELLS
AT pH 7.4 and 25°C

REFERENCE	APPARATUS	SUSPENDING MEDIUM	I	MOBILITY μ/sec/v/cm.
Abramson (210)	Rectangular horizontal positive	Phosphate	0.172	1.30
Brody (211)	Rectangular	Phosphate	0.172	1.28
		Chloride	0.145	1.07 ±0.02
Bangham (212)	Cylindrical	Phosphate	0.172	1.28 ±0.03
		Chloride	0.145	1.07 ±0.02
Rhie & Sehon (149)	Rectangular	Phosphate	0.167	1.30
Chollet et al. (259)	Cylindrical	Phosphate Saline	0.145	1.28 ±0.01
Vassar et al. (355)	Cylindrical	Saline	0.145	-1.08 ±0.03
This Study	Rectangular	Phosphate	0.172	1.28 ±0.03

Figure 2 The Electrophoretic Mobility of Human Red Blood Cells versus the distance from the inner surface of the cell chamber in mm. The field strength was 80/35 volt/cm. The current was 5 milliamperes. The time of exposure was 10 seconds.


EPM in μ/sec/v/cm
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
0.18
0.36
0.54
0.72
0.9
distance in mm
top of chamber
bottom of chamber

II. THE ELECTROPHORETIC MOBILITY PATTERNS OF LYMPH NODE CELLS OF CONTROL AND IMMUNIZED A/J MICE

(a) Material

All chemicals used were of reagent grade. Bovine serum albumin (BSA) was purchased from Armour Pharmaceutical Co., Illinois, U.S.A. 2,4-Dinitrobenzenesulfonic acid (DNBS) was purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A., and the compound was recrystallized three times from ethanol before use. Dowex was purchased from Dow Chemical of Canada Ltd., Richmond Hill, Ontario. Potassium carbonate was obtained from Fisher Scientific Company, Toronto, Ontario. Strain A mice (A/J) were ordered from the Jackson Laboratory, Maine, U.S.A.. Freund's complete adjuvant and RPMI 1640 culture medium were obtained from Difco Laboratories, Detroit, Michigan, U.S.A. Penicillin-Streptomycin No.507, and foetal calf serum were purchased from Grant Island Biological Co., Grant Island, N.Y., U.S.A..

(b) Method

The Preparation of DNP_{35}-BSA

To a mixture of 1.0 g BSA in 50 ml. of distilled water containing of 1.0 g of potassium carbonate was added 1.0 g of recrystallized DNBS in small portions. The mixture was stirred at room temperature for 24 hours. The protein conjugate was dialyzed against distilled water (9.4 litres) for two days or deionized by passing through a Dowex (400 mesh), 1 x 8 cm. column (bed volume 5 ml.), previously equilibrated with water.

Lyophilization gave 1.2 g of a yellow product. The extent of conjugation of DNP to BSA was determined spectrophotometrically at $O.D._{280}$ (215). It was then found that an average of 35 groups were coupled to each molecule of BSA.

Immunization Schedule

A/J mice, 6-8 weeks old, were immunized at the four foot pads with 20 μgN of DNP_{35}-BSA in 50% emulsion of Freund's Complete adjuvant in 0.2 ml. PBS. After two weeks, the animals were boosted with the same dose of antigen in FCA subcutaneously at the back. The animals were bled after four days and the peripheral lymph nodes were excised. A weak precipitin line appeared on the Ouchterlony double diffusion method in 1% agar plates between the serum and 500 μgN/ml of DNP_{35}-BSA after incubating overnight at room temperature in a closed humid plastic box. The agar plates were gifts from Dr. S. Fujimoto. It was the intention of the author to sacrifice the animals on this day so that the EPM experiments could be done before the peak of the secondary antibody response. The presence of a large number of plasma cells with possible surface specific immunoglobulins to DNP would affect the interpretation of the EPM data. A group of animals were immunized with 50% of emulsion of FCA in 0.2 ml. PBS as controls, so as to bring out the difference, if any, contributed by the antigen alone to the electrophoretic mobility profiles of the cells. Normal virgin animals were not tested because of the difficulties to obtain peripheral lymph nodes from them. The peripheral lymph nodes were dissected out from the control and immunized animals, and were placed in 5 ml. of culture medium RPMI 1640 containing

0.1% puromycin and streptomycin and 5% foetal calf serum. The cells were teased out from the lymph nodes with two 26 gauges needles in a petri dish. The cells were filtered into a Falcon 30 ml. culture flask through a 200 mesh platinum single cell filter and were incubated at 37°C for 45 minutes each side. The non-adherent cells were pipetted out and transferred into a test tube. The cells were centrifuged at 1,500 rpm for 15 minutes. The cell button was washed with 15 ml. of PBS, pH 7.2. This procedure was repeated twice. The cell concentration was adjusted to 10^5 cells/ml. with PBS. The viability of the cells was determined by the dye exclusion test with 0.2% Trypan Blue in PBS. The electrophoretic mobilities of the cells were determined as described earlier. For each experiment, about 150-300 cells pooled from three animals were determined for their electrophoretic mobilities. The results were computed and illustrated on the histogram with percentage of cells versus the electrophoretic mobilities. Altogether six experiments with 3 animals each were done for the control and test system. The EPM values of the peaks for each of the corresponding experiments were found to be reproducible. Their collective histograms were computed and the mean percentage of cells and their S.D. with their respective electrophoretic mobilities were plotted in the final histogram (Figure 3 and 4). These experiments were done at the same time with Section III and served as the basic EPM profiles for the control and immunized animals before the specific incubation with the antigens and the inhibition tests with the dialysable hapten.

(c) Result and Conclusion

The lymph node cells of both control and immunized animals exhibited a bimodal EPM curve; the control cell sub-

populations had EPM peaked at 0.8750 and 1.4000 micron/sec/v/cm (Figure 3). However, the immune cell subpopulations had EPM peaked at 0.8176 and 1.3417 micron/sec/v/cm (Figure 4).

The results indicated that the electrophoretic mobilities of the cells at the peaks of the immune animal histogram were slower than the corresponding peaks for the control animal cells. They may correlate with the findings for the inverse relationship between the charge of the antibodies by Sela et al. (57) and for the cells by Rhie and Sehon (149) with that of the immunizing antigen. In our case, the immunizing antigen is DNP_{35}-BSA which is negatively charged.

It was observed that the fast electrophoretic mobility cell population was of a higher percentage in the lymph node cells of the control animal while the low electrophoretic mobility cell population comprised a higher proportion of lymph node cells in the immunized animal. Anatomically, one could observe that the size of the lymph node of the immunized animals at the time of excision was about three to four times larger than that of the control animal.

The electrophoretic mobility of our finding for the low electrophoretic mobility cells and that of the high EPM cells of the control animal correlated with those virgin animals of Wiig (190), the values for the lymph node cells of C3H/A/BOM mouse were 0.855 ± 0.113 μm/sec/v/cm. and 1.394 ± 0.113 μm/sec/v/cm. (191)

Figure 3 The EPM Profile for Lymph Node Cells of A/J Mice Immunized with FCA. The field strength was 80/35/v/cm and the current 5 milliamperes.

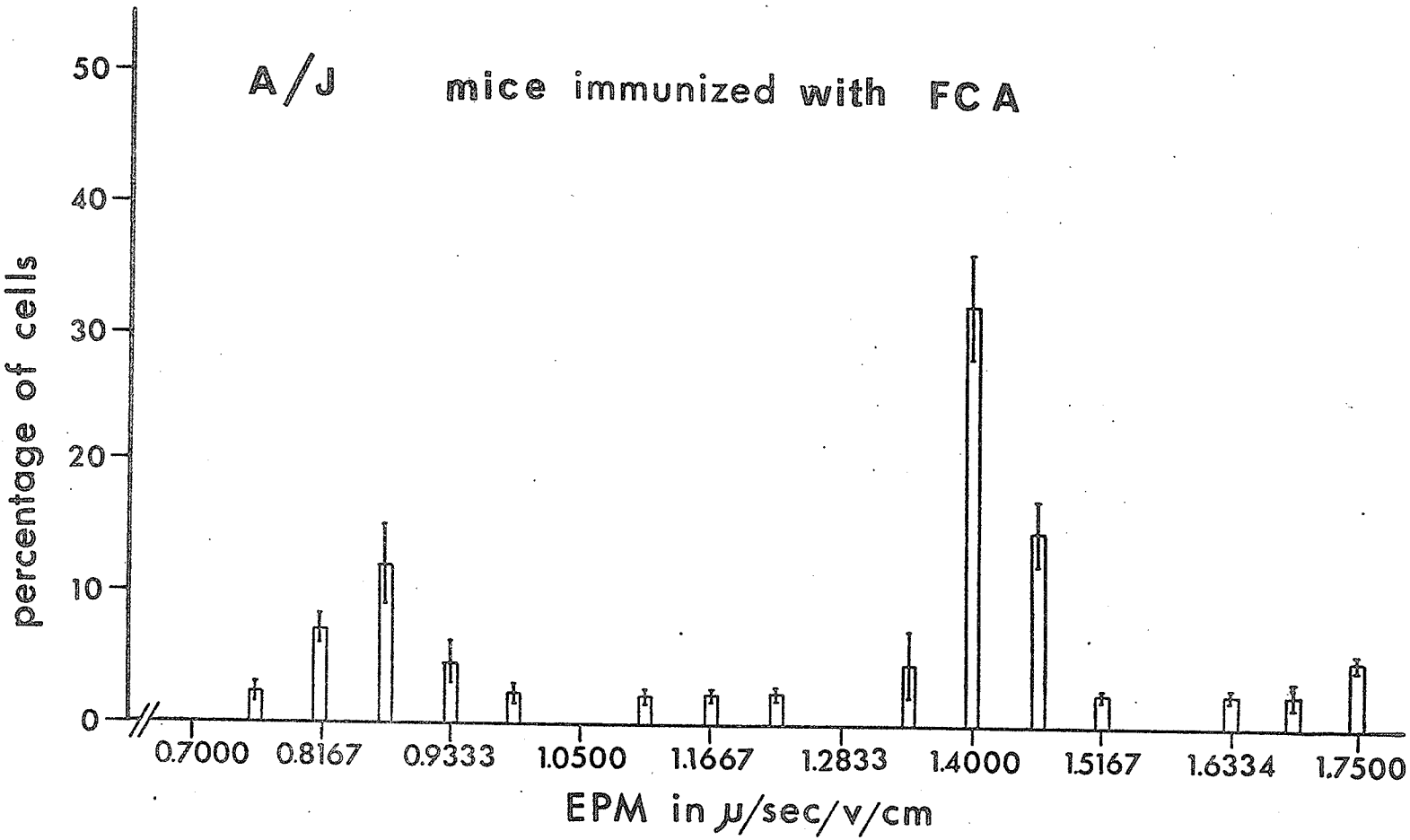
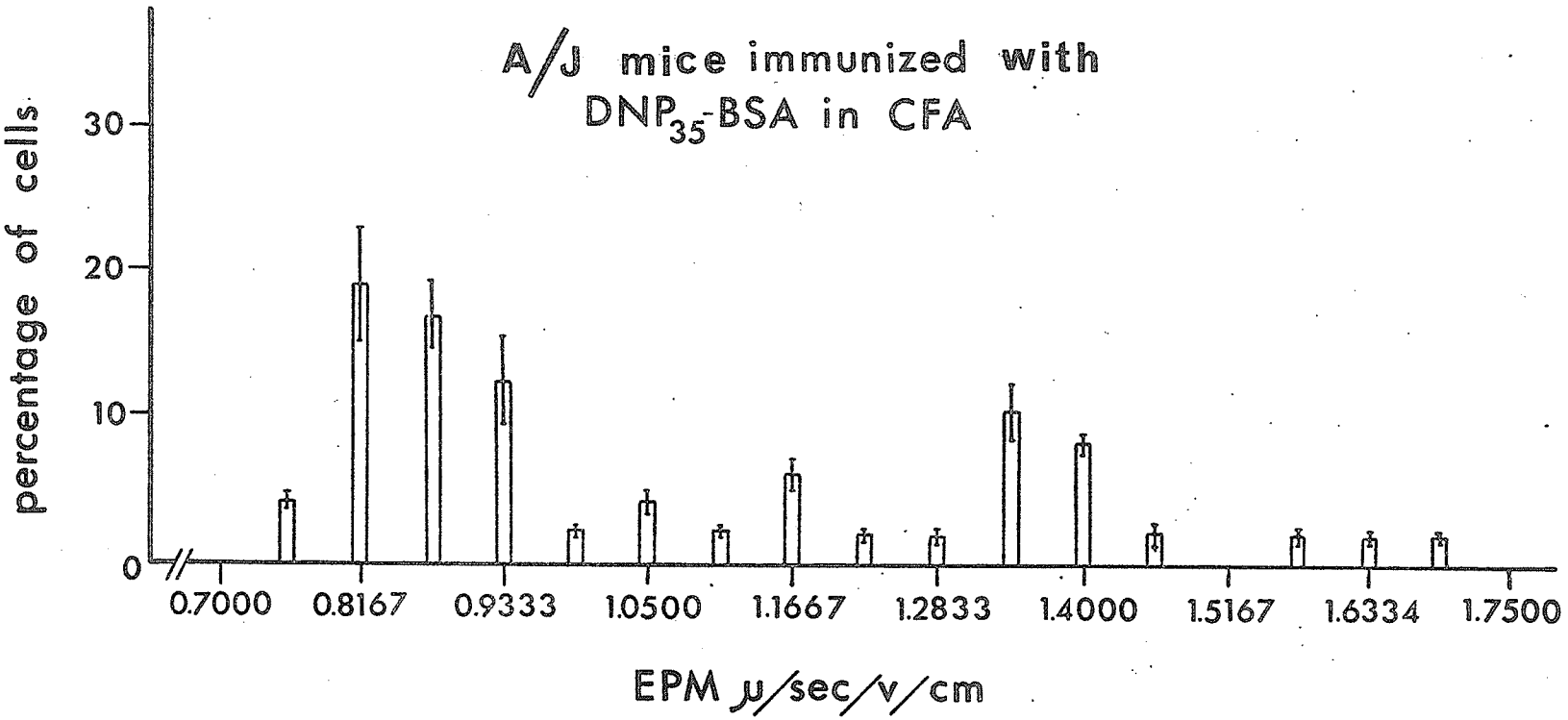

A/J mice immunized with FCA
percentage of cells
50
40
30
20
10
0
0.7000
0.8167
0.9333
1.0500
1.1667
1.2833
1.4000
1.5167
1.6334
1.7500
EPM in μ/sec/v/cm

Figure 4 The EPM Profile for Lymph Node Cells of A/J Mice Immunized with 20 μgN DNP_{35}-BSA in FCA. The field strength was 80/35/v/cm and the applied current 5 milliamperes.


A/J mice immunized with
DNP35-BSA in CFA
percentage of cells
30
20
10
0
0.7000
0.8167
0.9333
1.0500
1.1667
1.2833
1.4000
1.5167
1.6334
1.7500
EPM μ/sec/v/cm

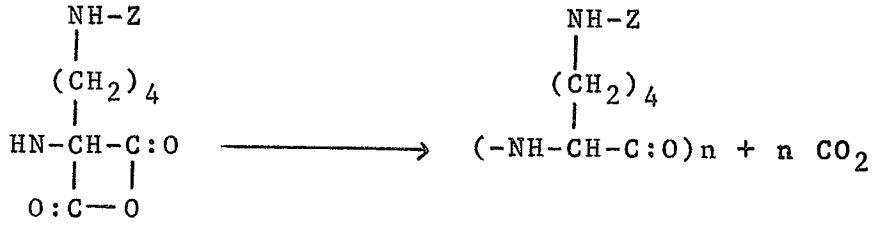
III. THE IDENTIFICATION OF THE HAPTEN SPECIFIC BINDING CELLS BY POLYIONIC CONJUGATES OF HAPTEN

(a) Material and Method

ε,N-αN-carboxy-L-lysine-anhydride (the Leuch's anhydride), ε,N-carbobenzoxy-L-lysine, α,N-ε,CBZ-poly-L-lysine (M.W.15,000) and α,N-ε,CBZ-poly-L-lysine (M.W.150,000) were purchased from Miles Lab., Inc. Elkhart, Indiana. Triethylamine, dioxane, sodium hydride (57% suspension in oil) and succinic anhydride were supplied by Fisher Scientific Co. (Figure 5).

The Synthesis of the Poly-L-lysine Homo-Polymer

The general reaction governing the polymerization with the Leuch's anhydride (216, 217) was as follows:



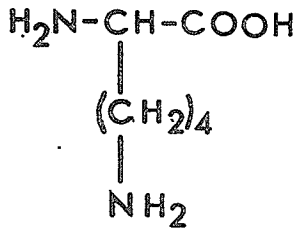
(I) (II)

To a solution of 0.5 g of ε,N-carbobenzoxy α,N-carboxy-L-lysine anhydride (I) in 20 ml. of anhydrous dioxane was added 0.2 ml of triethylamine and the resulting mixture was stirred at room temperature for four days. The polymer was poured into 40 ml of distilled water and the precipitate was collected by centrifuging at 15,000 rpm for 30 minutes. The polymer (II) was dried in vacuo over phosphorus pentoxide.

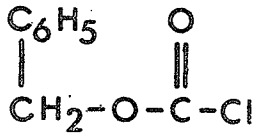
Synthesis of Univalent DNP-ε-N-carbobenzoxy-poly-L-lysine (III)

To 100 mg. ε,N-carbobenzoxy-poly-L-lysine of M.W.15,000 or 150,000 contained in a 50 ml. reaction flask,

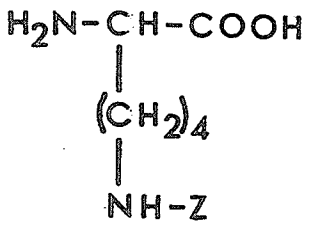
Figure 5 Synthesis reagent.



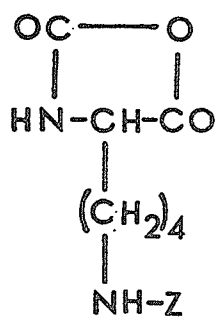
L-lysine



carbobenzoxy(Z) chloride



∈, N-carbobenzoxy-L-lysine



∈, N-carbobenzoxy ∝, N-carboxy-L-lysine-anhydride

Figure 5

equipped with a reflux condenser, was added 10 ml of anhydrous dioxane, and the whole system was kept under nitrogen.

Thereafter, 100 mg of sodium hydride (57% in oil suspension) was added into the reaction flask. The reaction mixture was refluxed for four hours under nitrogen.

Two hundred mg. of 1-fluoro-2,4-dinitro benzene (DNFB) in 5 ml. of dioxane was added dropwise to the reaction flask and the mixture was stirred at room temperature overnight (220, 228). The solution was acidified to pH 3.4 with 0.1N HCl and was filtered. The residue was washed with water and the combined filtrate was dialysed against several changes of 2 liters of distilled water until the dialysate was colourless. The homogenous solution was lyophilized and 110 mg. of a yellow fluffy product was collected (Figure 6).

Cleavage of the Carbobenzoxyl group

The CBZ-homopolymer (100 mg) was dissolved in 5 ml. of anhydrous hydrogen bromide (30-35% in glacial acetic acid). The solution was stirred for one hour at room temperature (221) and the homopolymer was precipitated by addition of 20 ml.of anhydrous ether, the precipitate was collected, washed with ether and dried *in vacuo* over phosphorus pentoxide (step III, Figure 7).

Succinic Acid Monomethyl Ester

A solution of 25 g. of succinic anhydride was reacted with 100 ml. of methanol in a 250 ml. reaction flask for four hours at room temperature, excess methanol was evaporated by using a rotatory evaporator. The white solid was crystallized from benzene. Several recrystallizations from the same solvent gave a pure product, m.p. 54° - 55°C.

Figure 6 Flow chart for synthesis of DNP-PZLL.

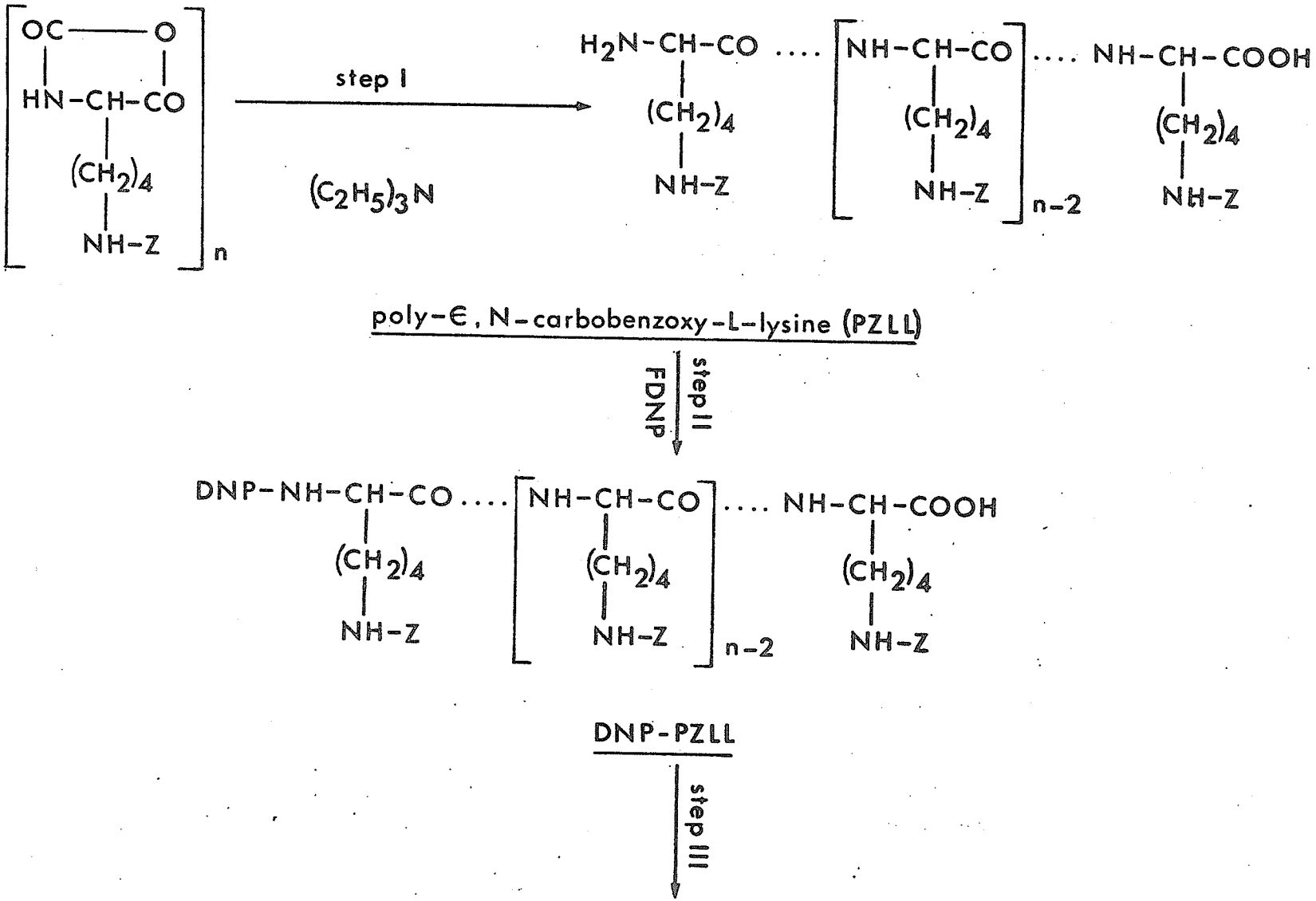

step I
$(C_2H_5)_3N$
poly-ε, N-carbobenzoxy-L-lysine (PZLL)
step II
FDNP
DNP-PZLL
step III

Figure 6

Figure 7 Flow chart for synthesis of DNP-PLL derivatives.

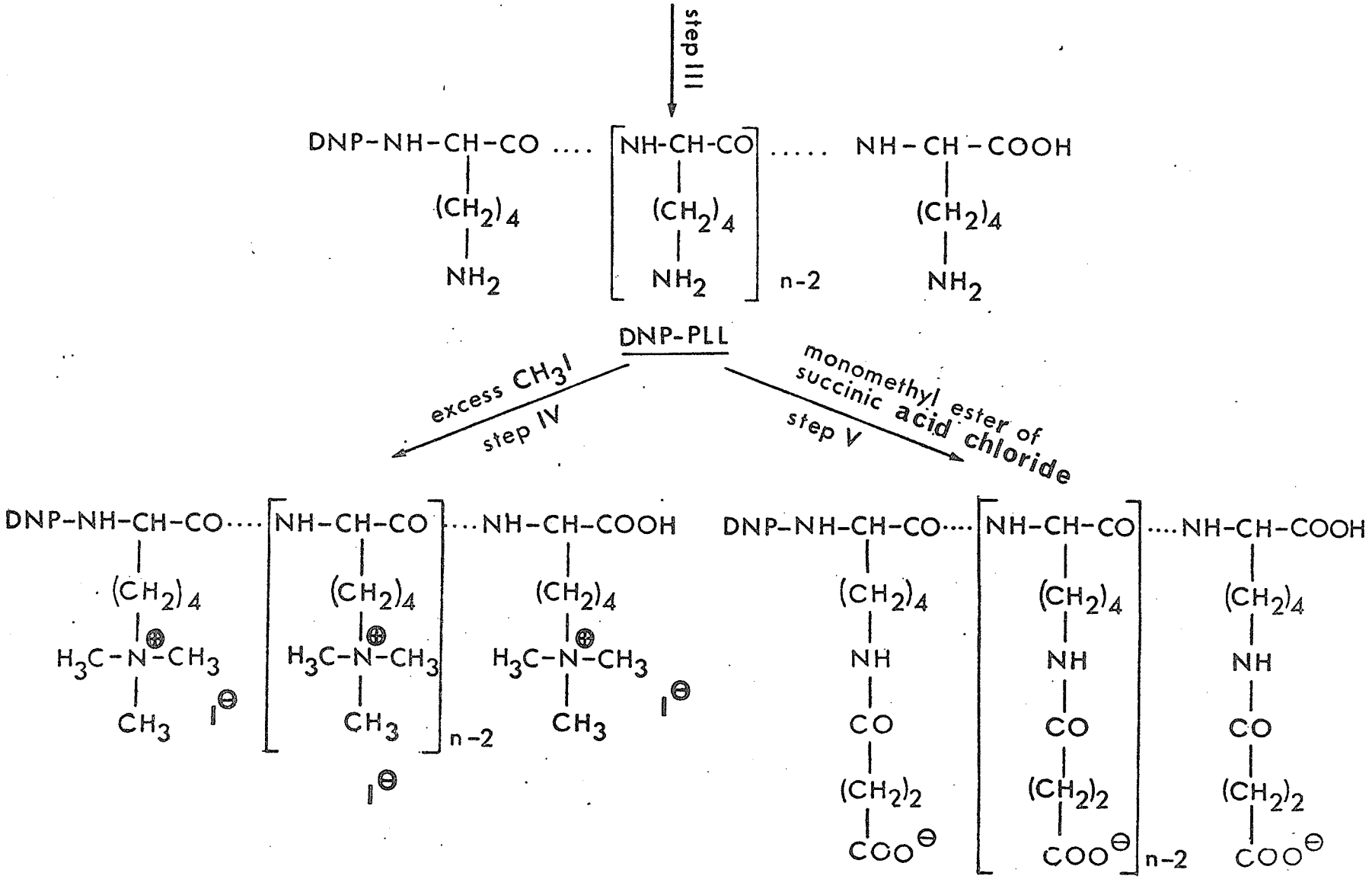

step III
DNP-NH-CH-CO .... [NH-CH-CO ..... NH-CH-COOH
(CH2)4
NH2
n-2
DNP-PLL
excess CH3I
step IV
monomethyl ester of succinic acid chloride
step V
H3C-N-CH3
CH3
I⊖
Poly-trimethyl ammonium derivative of α-DNP-PLL
NH
CO
(CH2)2
COO⊖
polysuccinilated derivative of α-DNP-PLL

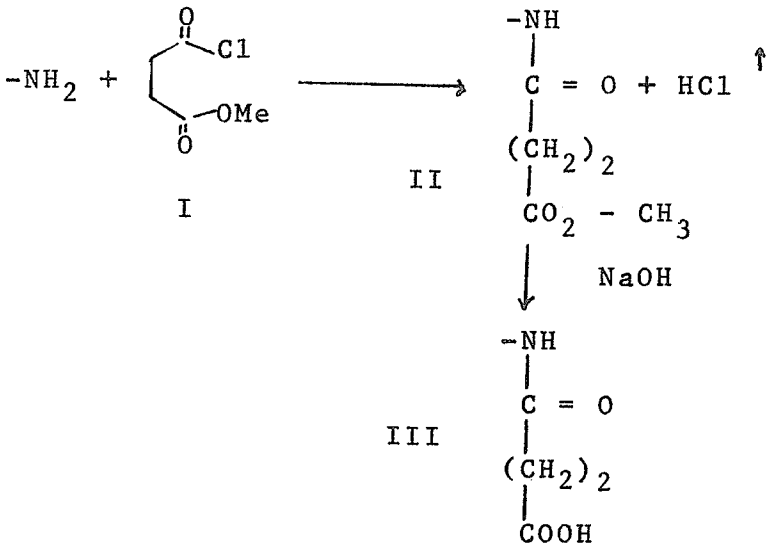
Figure 7

Succinic Acid Chloride Monomethyl Ester

Ten grams of monomethyl ester of succinic acid and 20 ml. of thionyl chloride (Canlab.) was refluxed for three hours. Excess thionyl chloride was removed by distillation and succinic acid chloride monomethyl ester was distilled under reduced pressure at 78°C.

The Synthesis of the Polysuccinilated Derivative of ∝-DNP-PLL (III)

To a solution of 5 mg. of ∝DNP-PLL in 10 ml. of dried dioxane was added 0.3 ml. of (I) and the reaction mixture was stirred at the room temperature for 20 hours. The excess solvent and the reagent were removed by a rotatory evaporator. The compound (II) was redissolved in 10 ml. of dioxane and 5 ml of 1N NaOH solution was added. The solution was acidified to pH 3.5 with 1 N HCl and was filtered. The filtrate was exhaustively dialyzed with water at 4°C and lyophilized to (III).



The percentage of succinic acid coupled to ∝DNP-PLL was determined by the nihydrin test using alanine as the

calibration curve. It was thus shown that 70% of the ε-amino groups had reacted.

Synthesis of Poly-Trimethyl Ammonium Derivative of αDNP-PLL

To a solution of 5 mg. of DNP-PLL in 10 ml of anhydrous dioxane was added 5 mg. of sodium hydride. The solution was stirred for 15 minutes under nitrogen and 0.3 ml. of methyl iodide was added. The reaction was left at room temperature for four hours and then acidified to pH 3.5 with 1N HCl. The solution was filtered and the filtrate was evaporated to dryness by the rotatory evaporator. The product was dissolved in 2 ml. of distilled water and dialysed against distilled water (41) for 24 hours in the cold room at $4^{o}C$ (222).

The Interaction of Immunocytes with DNP-polymer Conjugates

The lymphocytes from the regional lymph nodes of the control and immunized animals were excised and treated as described in the previous chapter. The lymphocytes, after removal of adherent cells, were determined for their viability by the dye exclusion test. The EPM profiles of the control and the immunized animals were determined before each experiment, and the collective computed data was reported in Section II. Approximately a ratio of 10^{8} molecules of the hapten conjugate per cell in 5 ml. (2×10^{6} cells per ml.) of PBS, pH 7.2 were incubated at room temperature for one hour. The cells were washed with 5 ml. of PBS three times. Again the viability of the cells were determined, and then the electrophoretic mobility of the cells was measured.

After the incubation of the hapten conjugate, half of the cells were incubated with αDNP-lysine HCl, at a ratio

larger than 10^8 molecules per cell. The suspension was incubated in a polystyrene tube under the same conditions as before, and the cells were washed three times with PBS. Their electrophoretic mobilities were determined.

(b) Result and Conclusion

The EPM of the Control A/J Lymph Node Cells treated with αDNP-PLL$_{15,000}$, Inhibition Test with αDNP-lysine HCl

The histogram of the electrophoretic mobility of the cells versus the percentage of the cells remained constant, though the peak for the low EPM cells became broader. This might be due to a low percentage of the antigen binding cells specific to the DNP in the control animals. In fact it was reported by Wofsy (194) that the antigen binding cells for the normal animal was around 0.1 in 10^4 cells.

The inhibition test with αDNP-lysine HCl did not change the profile of the histogram (Figure 8).

The EPM of the Immunized A/J Lymph Node Cells treated with α DNP-PLL$_{15,000}$, Inhibition Test with αDNP-lysine HCl

The high EPM cells seemed to remain as the non-treated immune cells, at an EPM of 1.3417 μ/sec/v/cm. The low EPM cells of 0.8176 μ/sec/v/cm shifted to peak at a mobility of 0.2333 μ/sec/v/cm. Half of the low EPM cell population maintained the original mobility of 0.8176 μ/sec/v/cm. In between there was a minor peak of mobility 0.5250 μ/sec/v/cm. On the inhibition test,

Figure 8 Histogram of percentage of control cell against EPM. Cells treated with αDNP-$PLL_{15,000}$. Inhibition Test with αDNP-Lysine HCl. Field Strength 80/35 v/cm. Current applied 5 milliamperes.

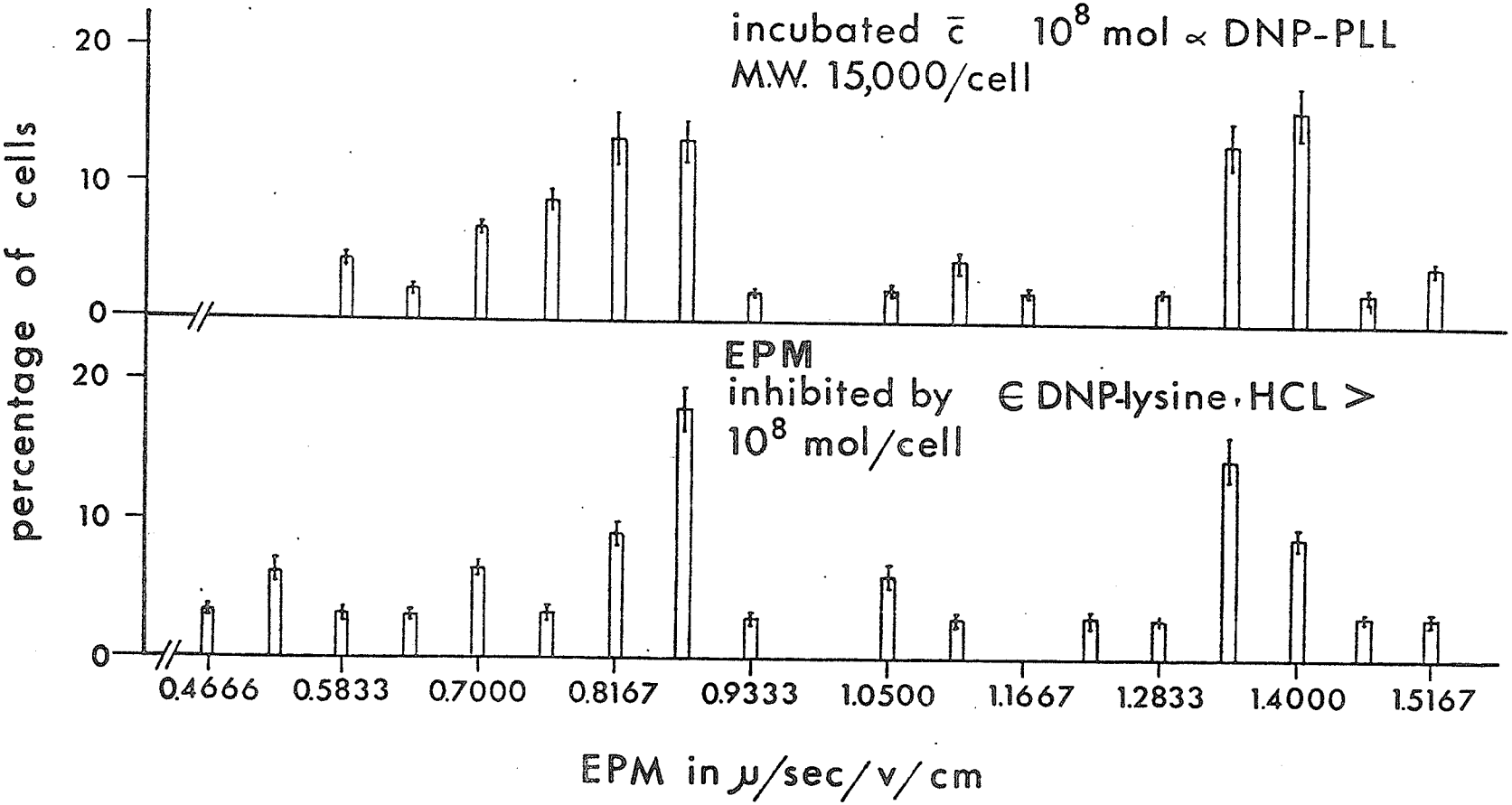

incubated c̄ 10^8 mol ∝ DNP-PLL
M.W. 15,000/cell
EPM
inhibited by ∈ DNP-lysine · HCL >
10^8 mol/cell
percentage of cells
20
10
0
20
10
0
0.4666
0.5833
0.7000
0.8167
0.9333
1.0500
1.1667
1.2833
1.4000
1.5167
EPM in μ/sec/v/cm

Figure 8

a portion of the 0.2333 μ/sec/v/cm cells increased to 0.6416 μ/sec/v/cm. As a conclusion, half of the original low EPM cell, which were antigen binding with different affinity, became part of the cells bound with the DNP-PLL and could not be displaced out by ϰDNP-lysine HCl or it might be possible that the length of the carri of the PLL prevented some of the hapten ϰDNP-lysine HCl to gain access to the cell receptors (Figure 9). The control experiment ruled out non-specific binding of ϰDNP-poly-L-lysine.

The EPM of the Control A/J Lymph Node Cells Treated with αDNP-PLL$_{150,000}$. Inhibition test with αDNP-lysine HCl

As in the previous experiment with the lower molecular weight hapten (αDNP-PLL$_{15,000}$), there was no obvious change in the EPM pattern of the control lymph node cells (Figure 10).

The EPM of the Immunized Lymph Node Cells Treated with αDNP-PLL$_{150,000}$. Inhibition Test with αDNP-lysine HCl

Cells treated with αDNP-PLL$_{150,000}$ caused a portion of the low EPM cells to migrate in different directions. The low EPM cells broke down to peak at 0.2916 and -0.2333 μ/sec/v/cm. The inhibition test shifted the peak to 0.2333 and 0.5833 μ/sec/v/cm. The other high EPM cells remained all through the tests. This showed that a portion of the low EPM cells were antigen binding, and the binding affinity varied (Figure 11) as in αDNP-PLL$_{150,000}$.

Figure 9 Histogram of EPM versus percentage of immunized cells. Animal immunized with 20 μgN DNP_{35}-BSA at the foot pad and boosted after two weeks with the same dose subcutaneously. Cells have been incubated with the hapten αDNP-$PLL_{15,000}$. Inhibition test by α DNP-lysine HCl.

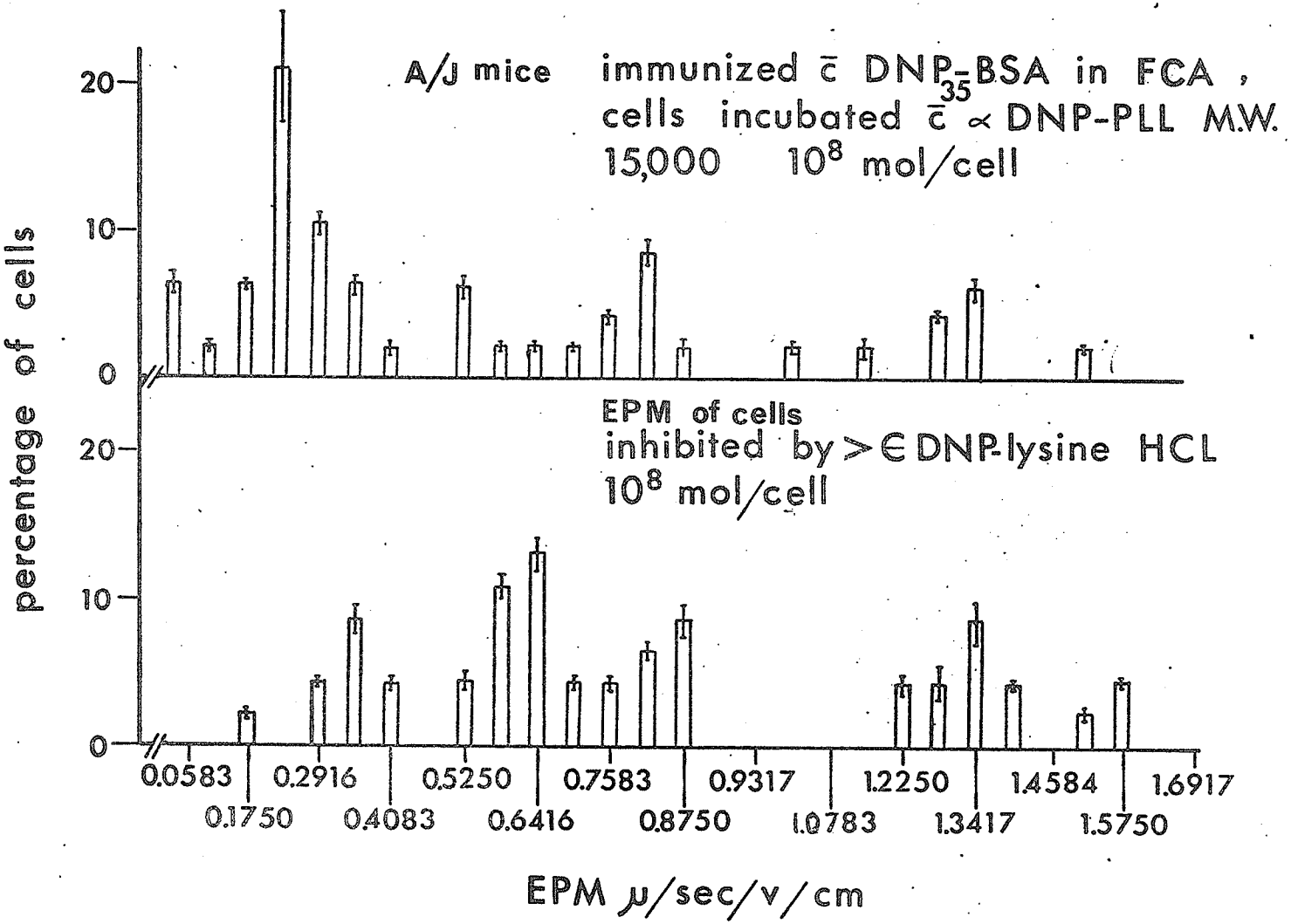

A/J mice immunized c̄ DNP35-BSA in FCA ,
cells incubated c̄ ∝ DNP-PLL M.W.
15,000 10^8 mol/cell
EPM of cells
inhibited by > ∈ DNP-lysine HCL
10^8 mol/cell
percentage of cells
20
10
0
20
10
0
0.0583
0.1750
0.2916
0.4083
0.5250
0.6416
0.7583
0.8750
0.9317
1.0783
1.2250
1.3417
1.4584
1.5750
1.6917
EPM μ/sec/v/cm

Figure 9

Figure 10 Histogram of EPM versus cell percentage. Cells are treated with αDNP-$PLL_{150,000}$. Inhibition test with αDNP-lysine HCl. Field strength 80/35 v/cm. Current applied 5 milliamperes.

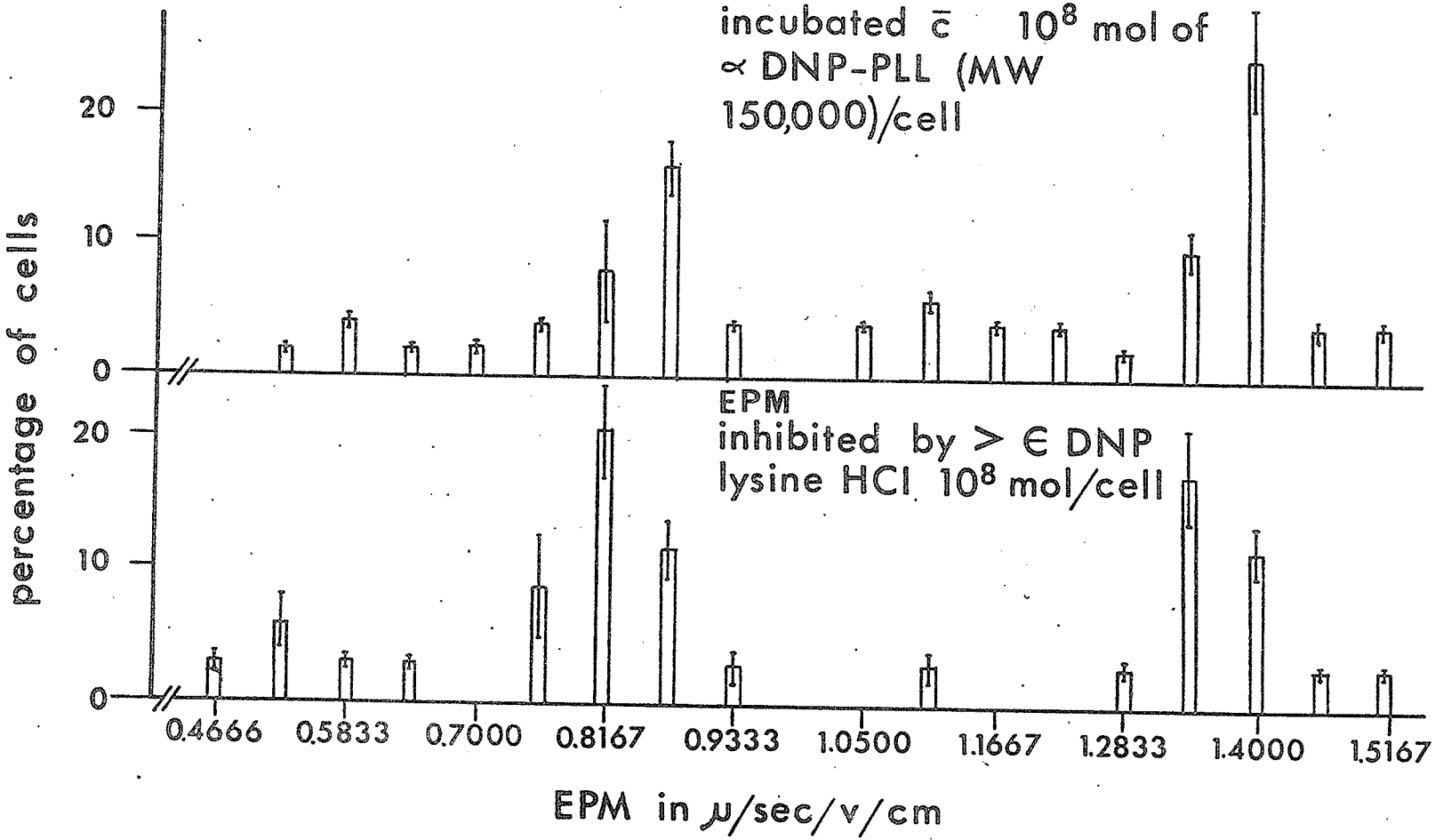

incubated c̄ 10⁸ mol of
∝ DNP-PLL (MW
150,000)/cell
EPM
inhibited by > ∈ DNP
lysine HCl 10⁸ mol/cell
percentage of cells
20
10
0
20
10
0
0.4666
0.5833
0.7000
0.8167
0.9333
1.0500
1.1667
1.2833
1.4000
1.5167
EPM in μ/sec/v/cm

Figure 10

Figure 11 Histogram of EPM versus cell percentage (immunized). Cells are treated with αDNP-$PLL_{150,000}$. Inhibition test with αDNP-lysine HCl. Field strength 80/35 v/cm. Current applied is 5 milliamperes.

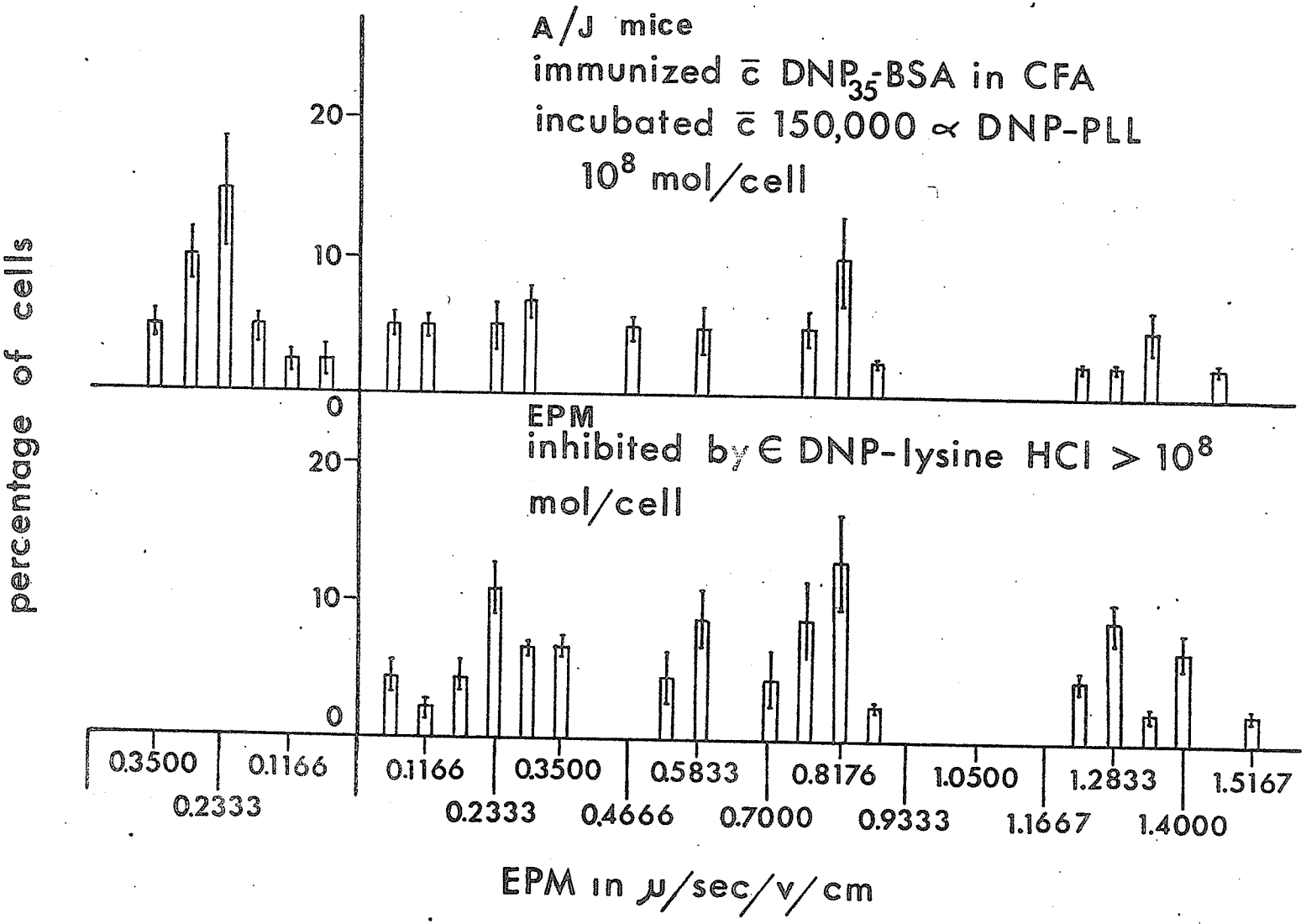

A/J mice
immunized c̄ DNP35-BSA in CFA
incubated c̄ 150,000 ∝ DNP-PLL
10^8 mol/cell
EPM
inhibited by ∈ DNP-lysine HCl > 10^8
mol/cell
percentage of cells
20
10
0
20
10
0
0.3500
0.1166
0.2333
0.1166
0.3500
0.2333
0.4666
0.5833
0.7000
0.8176
0.9333
1.0500
1.1667
1.2833
1.4000
1.5167
EPM in μ/sec/v/cm

Figure 11

The incubation with the antigen was shown to be able to change the electrophoretic mobility of the lymph node cells from the immunized animal for the bacterial antigens (169) and for the synthetic antigen (149).

The EPM of the Control A/J Lymph Node Cells Treated with the ε succinilated derivative of α DNP-PLL$_{15,000}$ Inhibition Test with αDNP-lysine HCl

The incubation of the lymph node cells of the control A/J mice with the highly negative charged antigen did not change the EPM pattern. No change of the pattern was obtained in the inhibition test (Figure 12).

The EPM of the Immunized A/J Lymph Node Cells Treated with the ε succinilated derivative of α DNP-PLL$_{15,000}$, Inhibition Test with αDNP-lysine HCl (Figure 13)

The pattern of the high EPM cells did not change as significantly as the low EPM cells. Part of the low EPM cells' mobility was elevated to about 1.1667 and 1.3400 μ/sec/v/cm. On the inhibition test, the cells with EPM of 1.3400 μ/sec/v/cm could not be depleted while cell with EPM 1.1667 was shifted indicating that there were difference in affinity for the antigen binding cells.

The EPM of the Control A/J Lymph Node Cells Treated with the ε succinilated derivative of αDNP-PLL$_{150,000}$, Inhibition Test with αDNP-lysine HCl

The incubation of the cells with the highly negative charged antigen did not change the EPM pattern of the control

Figure 12 Histogram of the EPM versus cell percentage for the control animal lymph node cells. Cells are treated with ε succinilated derivatives of ∝DNP-$PLL_{15,000}$. Inhibition test by ∝DNP-lysine HCl. Field strength 80/35 v/cm. Current applied 5 milliamperes.

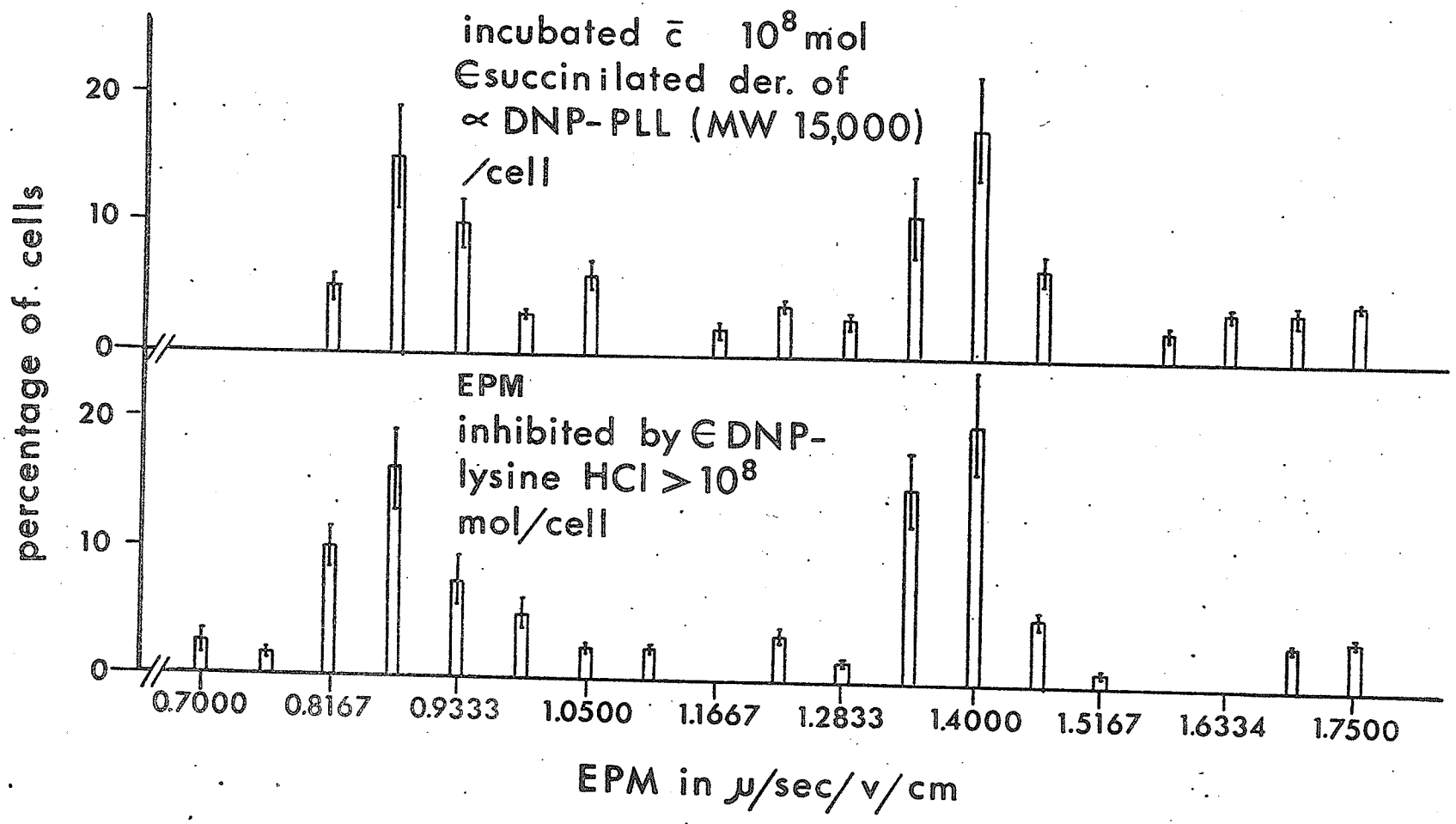

incubated c̄ 10⁸ mol
∈succinilated der. of
∝ DNP-PLL (MW 15,000)
/cell
EPM
inhibited by ∈ DNP-
lysine HCl > 10⁸
mol/cell
percentage of cells
20
10
0
0.7000
0.8167
0.9333
1.0500
1.1667
1.2833
1.4000
1.5167
1.6334
1.7500
EPM in μ/sec/v/cm

Figure 12

Figure 13 Histogram of the EPM of the immunized A/J lymph node cells treated with the ε succinilated derivative of αDNP-$PLL_{15,000}$. Inhibition test with αDNP-lysine HCl. Field strength 80/35 v/cm. Current applied 5 milliamperes.

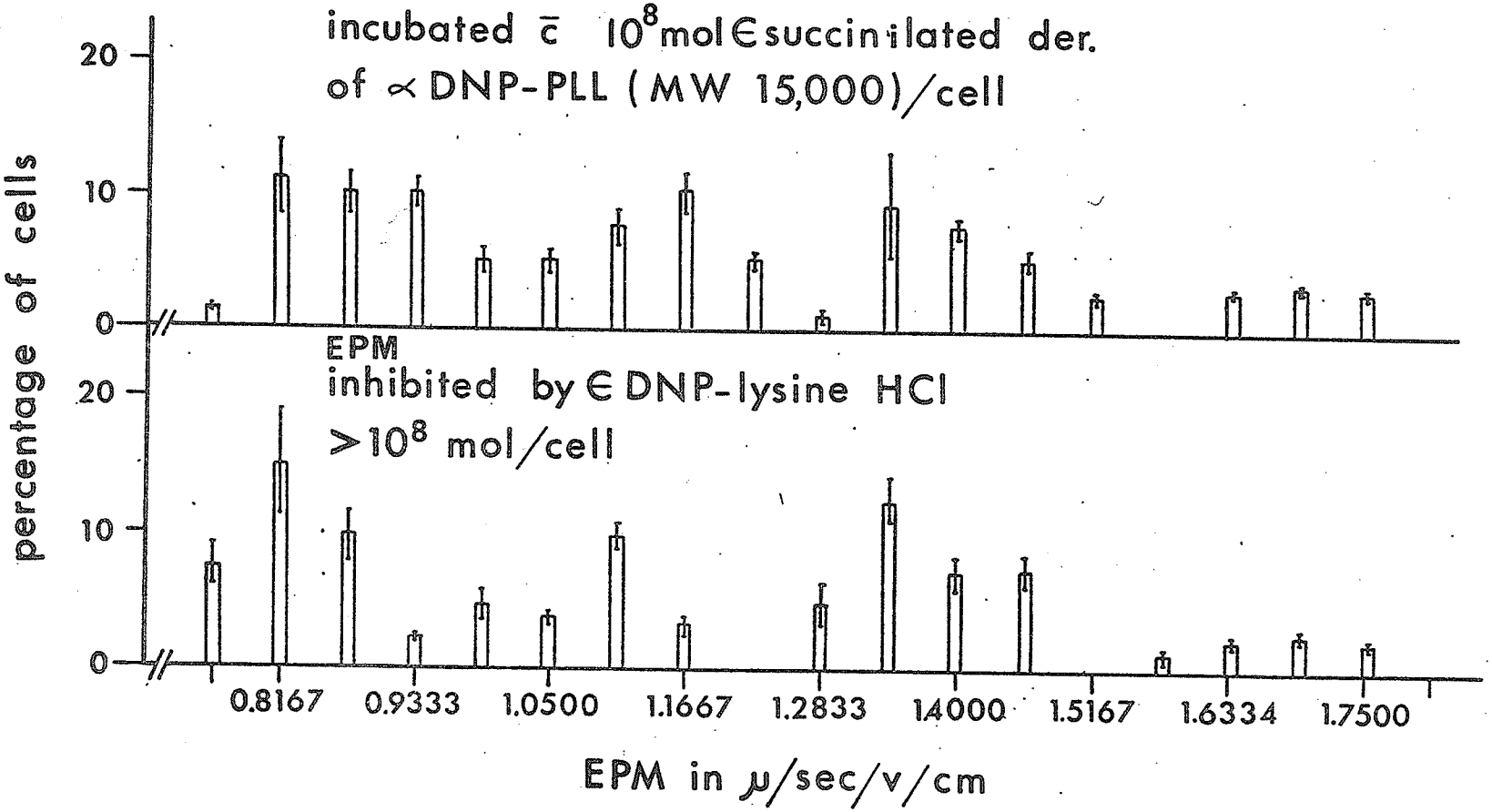

incubated c̄ 10^8 mol ∈ succinilated der.
of ∝ DNP-PLL (MW 15,000)/cell
20
10
0
EPM
inhibited by ∈ DNP-lysine HCl
>10^8 mol/cell
20
10
0
percentage of cells
0.8167
0.9333
1.0500
1.1667
1.2833
1.4000
1.5167
1.6334
1.7500
EPM in μ/sec/v/cm

Figure 13

A/J mice lymph node cells. No change in pattern was observed in the inhibition test (Figure 14).

The EPM of the Immunized A/J Lymph Node Cells Treated with ε Succinilated Derivative of ∝DNP-$PLL_{150,000}$, Inhibition Test with ∝DNP-lysine HCl

The low electrophoretic mobility cells of the lymph nodes of the immunized A/J mice were affected with part of the cell subpopulation shifted to EPM of 1.1667 and 1.7500. On the inhibition test using the hapten ∝DNP-lysine HCl, a small portion of the cells was shifted back to the original mobility. However, a large proportion of the cells remained with a high electrophoretic mobility indicated that the affinity for binding might be very high with these cells (Figure 15).

The selective shift of the portion of the low electrophoretic mobility cells upon incubation with the charge hapten indicated the possibility of fractionation of the specific hapten binding cell with electrophoresis.

The Interaction of the Control and Immunized A/J Lymph Node Cells with the Trimethyl Ammonium Derivative of ∝DNP-PLL

The trimethyl ammonium derivatives of the hapten ∝DNP-$PLL_{15,000}$ was synthesized as described before. This highly positively charged hapten caused cell crumping together upon treatment with either control or immunized cells. The cell clumping could not be redissociated by washing with PBS. Hence it was impossible to observe the EPM of the individual cells.

Figure 14 Histogram of the control A/J lymph node cells. EPM versus percentage of cells. Cells treated with the ε succinilated derivative of αDNP-PLL$_{150,000}$. Inhibition test with α DNP-lysine HCl. Field strength 80/35 v/cm. Current applied 5 milliamperes.

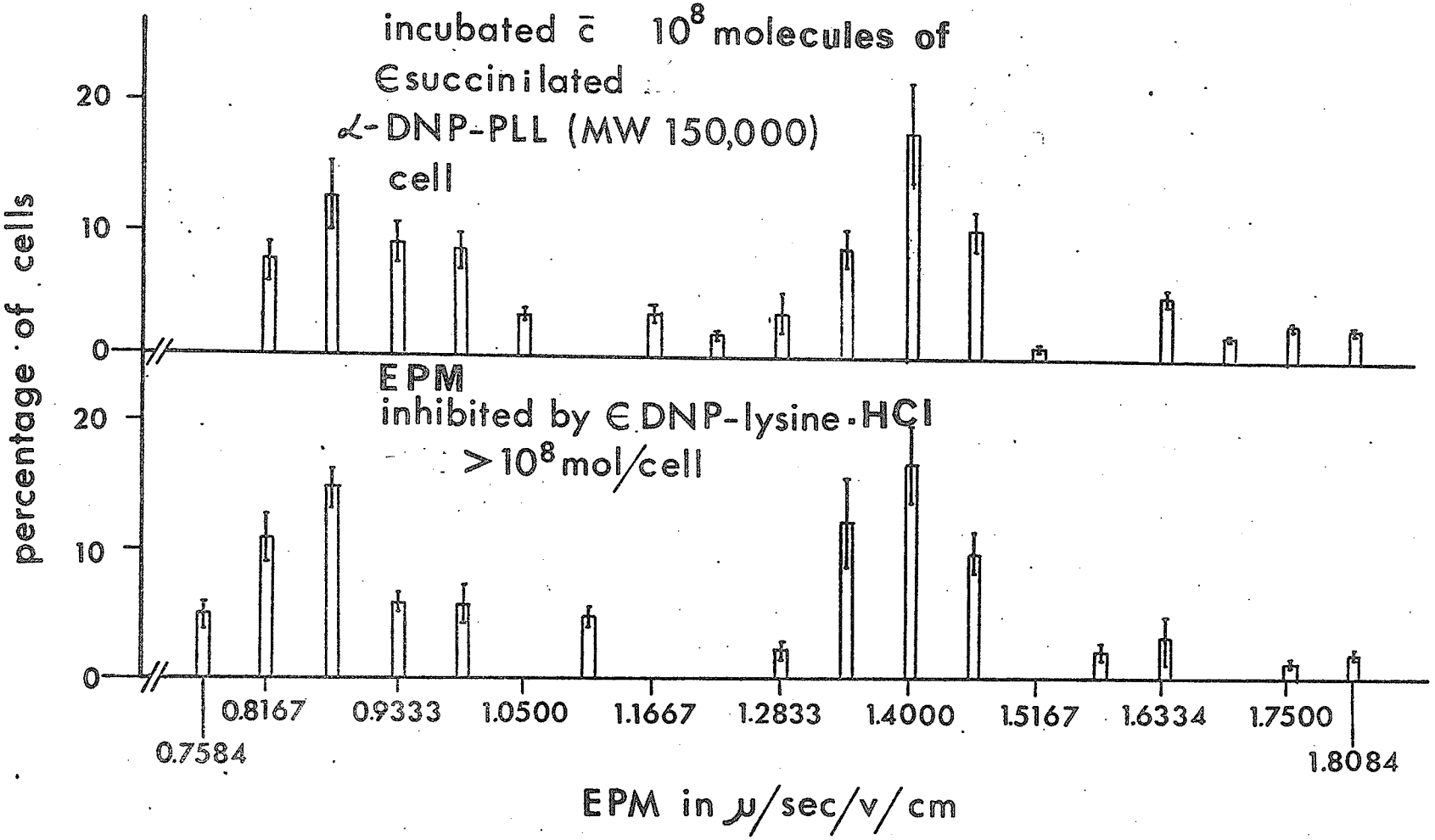

incubated c̄ 10⁸ molecules of
∈succinilated
α-DNP-PLL (MW 150,000)
cell
20
10
0
EPM
inhibited by ∈DNP-lysine·HCl
> 10⁸ mol/cell
20
10
0
percentage of cells
0.7584
0.8167
0.9333
1.0500
1.1667
1.2833
1.4000
1.5167
1.6334
1.7500
1.8084
EPM in μ/sec/v/cm

Figure 14

Figure 15 The EPM of the immunized A/J lymph node cells treated with ε succinilated derivative of ∝ DNP-$PLL_{150,000}$. Histogram EPM versus cell percentage. Field strength 80/35 v/cm. Current applied 5 milliamperes.

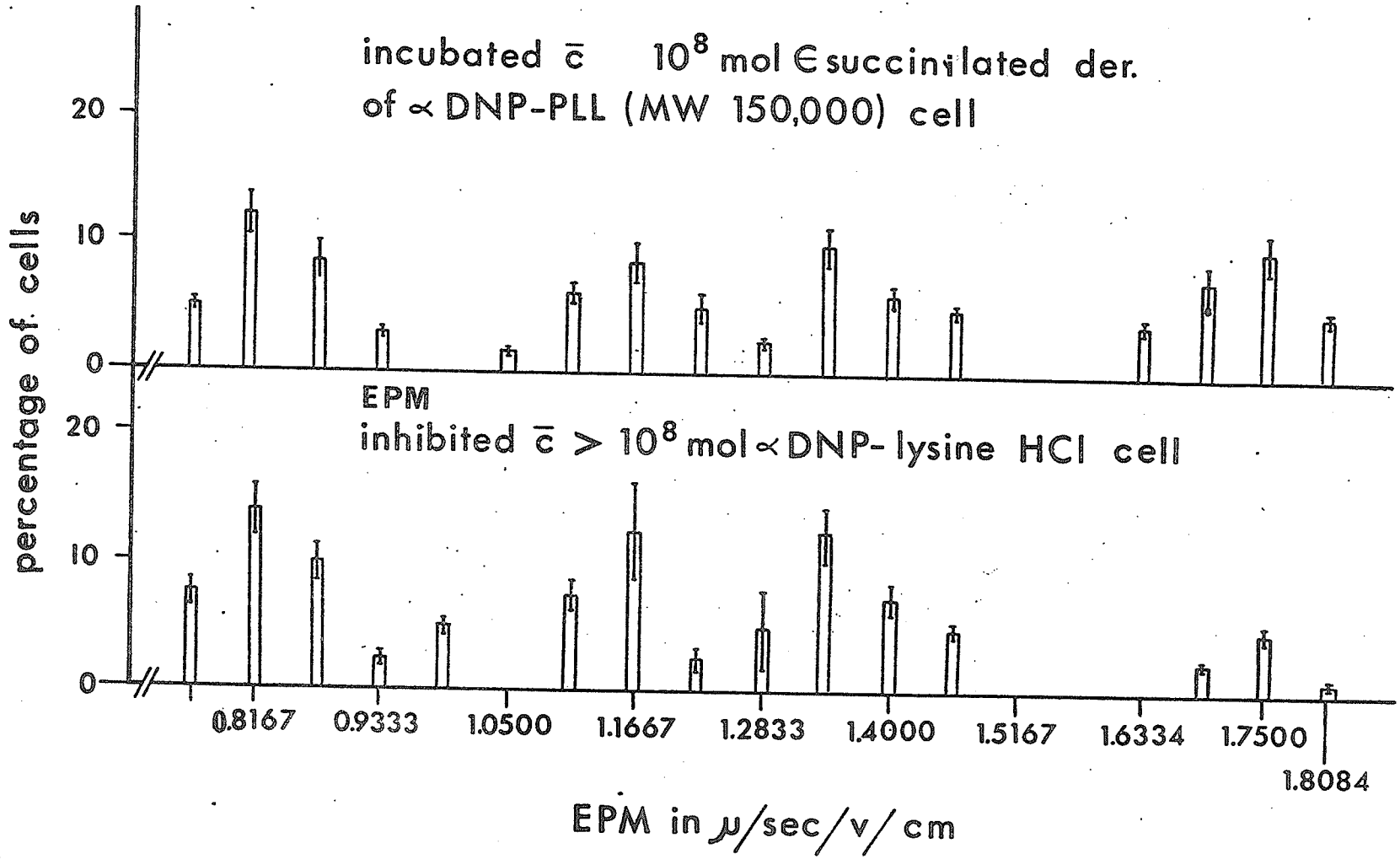

incubated c̄ 10⁸ mol ∈ succinilated der.
of ∝ DNP-PLL (MW 150,000) cell
20
10
0
EPM
inhibited c̄ > 10⁸ mol ∝ DNP- lysine HCl cell
20
10
0
percentage of cells
0.8167
0.9333
1.0500
1.1667
1.2833
1.4000
1.5167
1.6334
1.7500
1.8084
EPM in μ/sec/v/cm

Figure 15

Aggregation of the cells with the positively charged molecules was conceivable since all mammalian cells were found to be negatively charged.

One would conclude with confidence that the low electrophoretic mobility cells contained a population of hapten specific binding cells.

IV. THE IDENTIFICATION OF THE HAPTEN BINDING CELL BY THE IMMUNOFLUORESCENCE TECHNIQUE

(a) Material and Method

The Preparation of α-N-DNP-lysine

The methodsused by Sanger (219) and Sober (220) were adopted. α-N-CBZ-lysine (5 mg.) was allowed to react with 0.3 ml. of 1-fluoro-2,4-ditrobenzene in a mixture of 10 ml. of 0.1 M $NaHCO_3$ and 5 ml of 50% methanol at room temperature over night. The bright yellow solution was extracted with ether, and the aqueous portion was brought to pH 2 with citric acid. This was extracted with EtOAc followed by washing with water, then the sample was dried, and filtered. The filtrate was evaporated by the rotavapor on a water bath under reduced pressure. The only product, α-N-DNP-ε-N-Z-lysine was treated with 5 ml. of 20% HBr in acetic acid at room temperature for one hour. Ether was added and the precipitate was centrifuged at 15,000 rpm for 15 minutes. The precipitate was collected, washed with water, and dried with acetone. The melting point was 240-243°C. A sample of 0.65 mg. was obtained.

The αDNP-lysine synthesized here was used for the following reaction. Identical experiment was carried out by using αDNP-lysine HCl as the starting material.

The Synthesis of αDNP, ε-isothiocyanate fluorescein-lysine

Five ml. of αDNP-lysine containing 1 mg. of peptide/ml. was mixed with 4 ml. of 0.15 M NaCl solution and 1 ml. of 0.5 M,

pH 9.0 Na_2Co_3 - $NHCO_3$ buffer. The solution was cooled to 4^oC with an ice bath. Five mg. of fluorescein isothiocyanate was slowly added, and the reaction was left at 4^oC for 18 hours with gently mixing to avoid foaming. The product was fractionated with G10 Sephadex column with column size 1.0 x 100 cm., bed volume 50 ml., rate of flow was 18 ml/hr. The sample of the 1st peak was collected and stored at -20^oC in small aliquots in plastic tubes or lypholysed.

The Modification of the Cell Electrophoresis Microscope

The power supply for the Carl Zeiss microscope was adapted to project UV light. The UV light passed through a FITC filter. (Cat. #91-00-31). The other photographic conditions remained the same.

The Incubation of the Fluorescein Hapten with the Cells

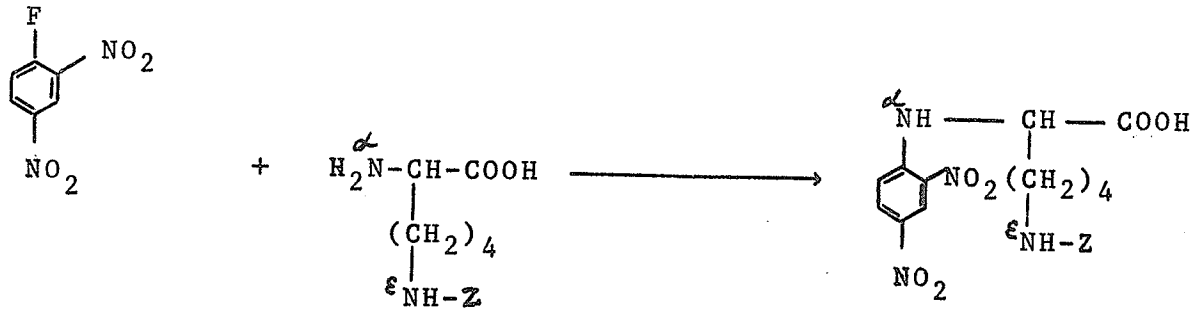
The regional lymphocytes from the control and immunized animals were obtained as described before. The lymphocytes, after the removal of the attaching cells were incubated with the fluorescein antigen, containing 10^8 molecules/cell, in a shaker at room temperature for one hour. Then the cells were washed with PBS three times. The electrophoretic mobility was observed.

(b) Result and Discussion

The antigen binding cells containing the DNP receptor bound to the α DNP- ε-fluorescein isothiocyanate and a 'broader' EPM track was observed on the film. The number of the antigen binding cells in the control animal was negligible. Normally

Figure 16 Synthesis of αDNP- ε-isothiocyanate fluorescein-L-lysine.

Synthesis of α DNP-ε-isothiocyanate fluorescein-L-lysine



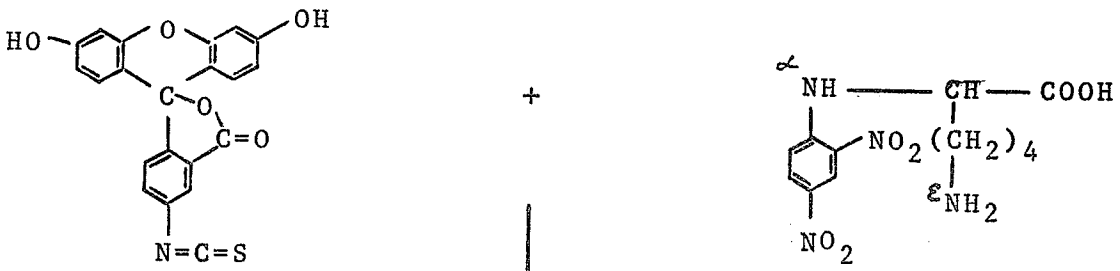
1-fluoro-2,4-dinitrobenzene

ε-CBZ-L-lysine

HBr

Fluorescein Isothiocyanate

α DNP-L-lysine



α DNP-ε-isothiocyanate fluorescein-L-lysine

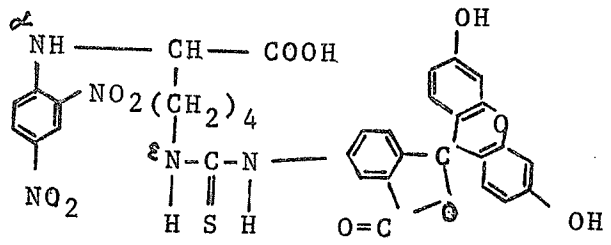


FIGURE 16

only one or two in a series of films were observed. For the immunized animal, up to three cells were detected on a single film. The EPM of the antigen binding cell to DNP was found to be 0.7608 μ/sec/v/cm. The EPM was found to be constant with a slight deviation. The difference in EPM between these binding cell and the subpopulation of the low electrophoretic mobility cells of the previous experiments was contributed by the differences in the charge of the DNP-PLL polymer and the ∝DNP-lysine-ε-isothrocyanate fluorescein compounds which bound to the cell surface.

V. THE IDENTIFICATION OF THE HAPTEN BINDING CELL BY THE HAPTEN SPECIFIC BIOGEL AFFINITY COLUMN

(a) Material and Method

Cell electrophoresis reviewed that nearly half of the low EPM cell population were antigen binding for the immunized animals. However the method of affinity chromatography and the rosette formation with the hapten coupled to the SRBC indicated that the number of antigen binding cells remained around 0.1 to 1 in 10^4 cells (194). Hence affinity chromatography with the hapten chemically bound to the column was used to examine the previous experimental data.

The Preparation of N-(2,4-dinitrophenyl)-6-Polyacrylamide-Hexamine

Biogel P6, 16-20 mesh; P6,400 mesh and P6, 100-200 mesh were ordered from BioRad Co.. It was observed that Biogel P6 400 mesh was the most effective by virtue of its large surface area. Biogel was polyacrylamide gel beads which did not have any surface charges.

Five grams of Biogel P6 400 mesh were allowed to swell in 100 ml. of distilled water containing 5 M of 1,6 hexadiamine solution (50 ml) (Eastman Kodak Co., Rochester, New York). The mixture was warmed at 90°C for 8 hours with stirring.

The beads were washed with several litres of saline, pH 7.2 and with 500 ml. of 0.1 M sodium tetraborate (pH 9.2). Excess solvents were decanted to a level where the beads were

just covered and five grams of recrystallized sodium 2,4, dinitrobenzene sulfonate was added. The reaction was stirred for 16 hours at room temperature. The material was filtered, washed several times with saline until the filtrate gave a negative test for DNP (223).

The broken beads were separated out by the flotation method, and was discarded.

The Interaction of the DNP-Biogel with the Lymph Node Cells

The immunized A/J lymph node cells were obtained as described in the previous chapters. One ml. of the packed gel in suspension was mixed with 10^6 lymphocytes in 4 ml. of phosphate buffer saline pH 7.2. The gel and cell suspension were left at room temperature in a tube for one hour on a rotator. Then the tube was left upright. The cell suspension on the supernatant was pipetted out and termed the "unfractionated cells". The original cell suspension was termed"whole cell"(Figure 17).

The Dissociation of the Antigen Binding Cell with a Monovalent Hapten ∝DNP-lysine HCl

The biogel with cells attaching to it was washed with 15 ml. of PBS and was resuspended in 5 ml. of PBS. Fifty μg of ∝DNP-lysine HCl was added to the solution and the suspension was left on the rotator at room temperature for 1 hour. The tube with the suspension was left upright. When the gel settled down, the supernatant which contained cells termed the'fractionated cells'was pipetted out. The cells were centrifuged down and washed three times with 15 ml. of PBS.

Figure 17 The EPM of cells versus percentage of cells histograms for the whole cell; fractionated and unfractionated cells with BioGel P6 400 mesh. EPM taken in PBS, pH 7.2. Current applied 5 milliamperes. Field strength 80/35 v/cm.

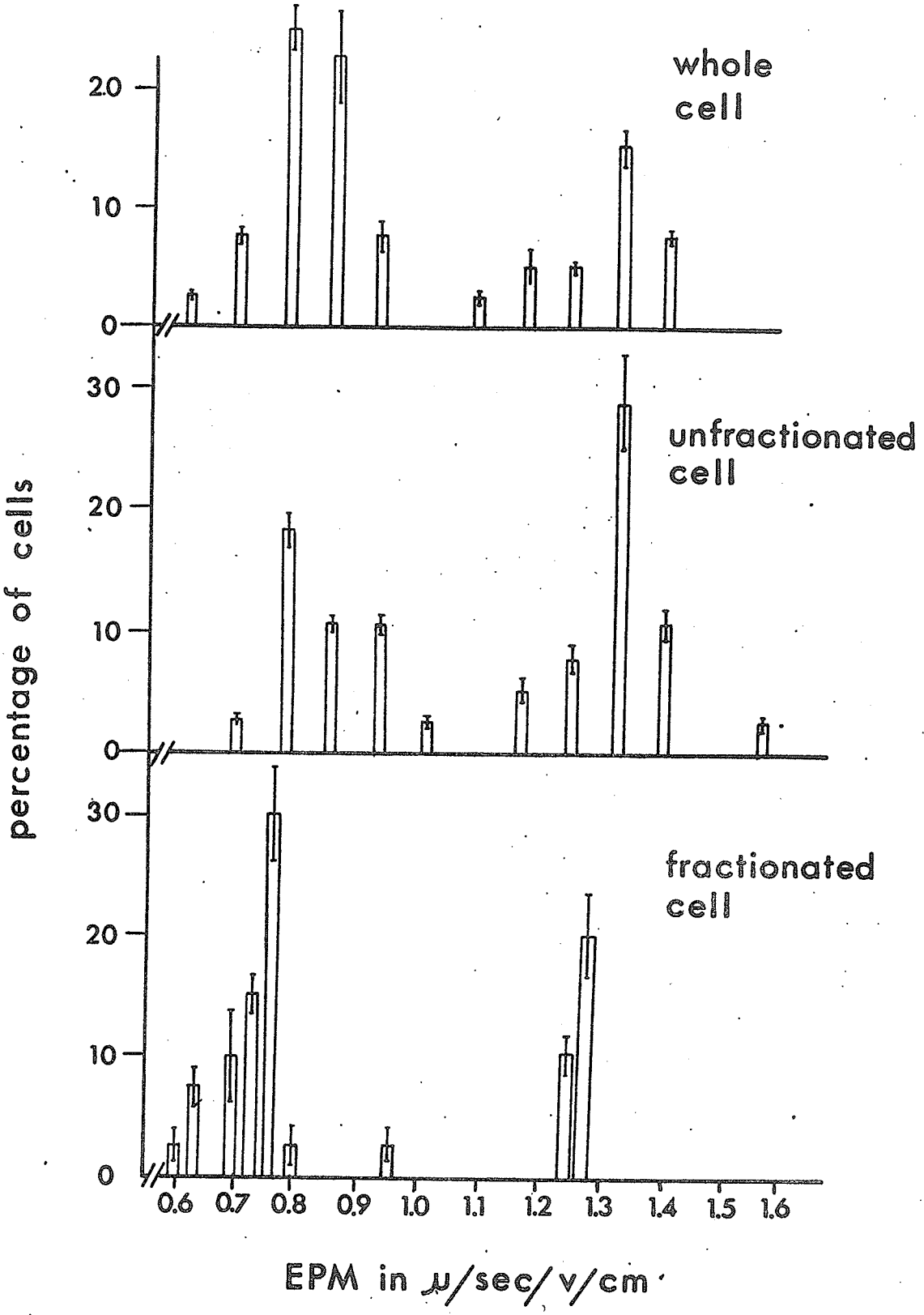

whole cell
unfractionated cell
fractionated cell
percentage of cells
20
10
0
30
20
10
0
30
20
10
0
0.6
0.7
0.8
0.9
1.0
1.1
1.2
1.3
1.4
1.5
1.6
EPM in μ/sec/v/cm

Figure 17

All three kinds of cells were adjusted to have 10^5 cells per ml.. The viability was determined with the dye exclusion test. The viability was found to be higher than 95%. The EPM was observed (Figure 17).

(b) Result and Conclusion

It was found that 9% of cells could be fractionated by the above method. The specific cells fractionated by the column were richer in the low electrophoretic mobility cells; as for the unfractionated cells, the reverse was true. But the contaminating population was still fairly large. The non-specificity of the 'specific' affinity chromatography might be due to the stickness of the cell surface. Hence the non-specific cells would be expected to attach to the beads also. Chollet et al. found that the low EPM cells (19.9% of the whole cell population) could adhere to the nylon wool, and were positive with fluorescein antibody to γ-globulin (259), they were B lymphocytes. The high affinity cells could not be displaced by the αDNP-lysine HCl. Some of the hapten and DNP-lysine used for displacing the specific binding cells from the column might bind to the hapten specific receptor on the cell surface causing difference in their electrophoretic mobilities as compared with the experiments done with the αDNP-PLL polymer. Further studies on the various problems associated with affinity chromatography should be performed.

VI. THE IMMUNOLOGICAL SPECIFICITY OF THE HIGH ELECTROPHORETIC MOBILITY LYMPH NODE CELLS

(a) Material and Method

Preparation of anti Θ Serum

C3H and AKR mice were obtained from Jackson Lab. C3H thymocytes were prepared from young C3H mice by teasing the thymus in 5 ml. of RPMI 1640 culture medium with 5% foetal calf serum in a petri dish. A pool of three animals was used. For immunization, 10^6 C3H thymocytes in 0.5 ml. were injected intravenously through the tail vein to AKR mice weekly. The serum was collected at the end of the seventh week (224, 191).

Using guinea pig complement, the cytotoxic activity of the anti-Θ serum was tested on normal A/J mice lymph node cells using the trypan blue dye exclusion test. It was found that about 62% of the lymph node cells of the normal animals were killed by anti-Θ antiserum (1 ml. of anti-Θ serum to 10^6 lymph node cells with 2 ml. of fresh guinea pig serum for 1 hour at room temperature).

The damaged cells were removed by the method of Boehmer and Shortman (231), using low ionic strength medium on a small glass wool column made by a siliconized pipette. The viability of the cells after passing through the column was more than 95%.

(b) Result and Conclusion

It was found that treatment with the anti-Θ serum of the lymph node cells could selectively deplete the high electrophoretic mobility cells (Figure 18). Hence the high EPM cells

Figure 18 The EPM of the A/J lymph node cells after treated with anti-θ serum (1 ml. of anti-θ serum, 10^6 lymph node cells and 2 ml. of fresh guinea pig complement) were incubated for 1 hour at room temperature.

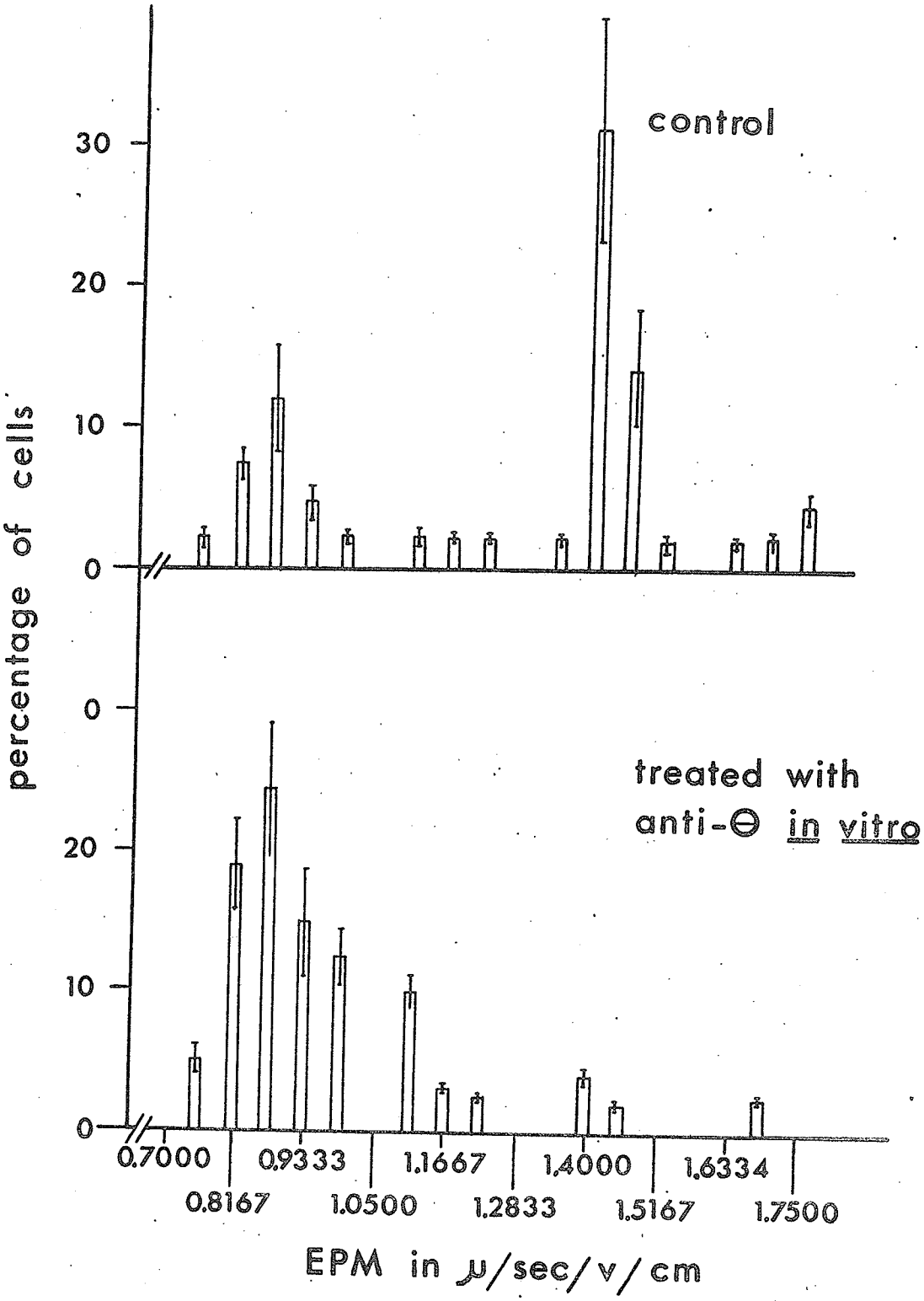

control
30
20
10
0
percentage of cells
0
20
10
0
treated with
anti-Θ in vitro
0.7000
0.8167
0.9333
1.0500
1.1667
1.2833
1.4000
1.5167
1.6334
1.7500
EPM in μ/sec/v/cm

Figure 18

bear the θ antigen and are thymus dependent. In the previous chapter, it was established that the high electrophoretic cells were not hapten specific.

VII. THE EFFECT OF NEURAMINIDASE ON THE CELL SURFACE CHARGE PROPERTIES

Sialic acid had been quantitatively the most important identified anionic group on the surface of cells (129, 124, 225, 347-354). The difference in the number of sialic acid groups exposed on the surface of lymphocytes might be responsible for their differences in electrophoretic mobility. Neuraminidase treatment of control and immune cells would reveal changes in net charges due to sialic acid.

(a) Material and Method

The Treatment of Cells with Neuraminidase

The control and immune cell were treated with *Vibrio cholera* neuraminidase (Behringwerke, Marburg am Lahn, Germany), with 10^5 cells/ml. in solution containing 50 units of enzyme/ml. in 5 ml. of PBS pH 7.2 at $25^{\circ}C$ for 30 minutes.

The cells were then washed with 15 ml. of PBS and their EPM were taken.

(b) Result and Conclusion

Nordling et al. (124) obtained a single EPM peak of lymphocytes treated with neuraminidase instead of the original bimodal pattern. We observed that the low EPM cells appeared to be more resistant to neuraminidase digestion while the high EPM cells were completely reduced in their EPM (Figure 19, 20). This might be due to the presence of the high number of hapten receptors or surface immunoglobulins on the low EPM cells which covered up some of the sialic acid molecules (346). The result indicated that sialic acid was responsible for contributing mainly to the cell EPM.

Figure 19 The changes of the EPM of the control with neuraminidase.

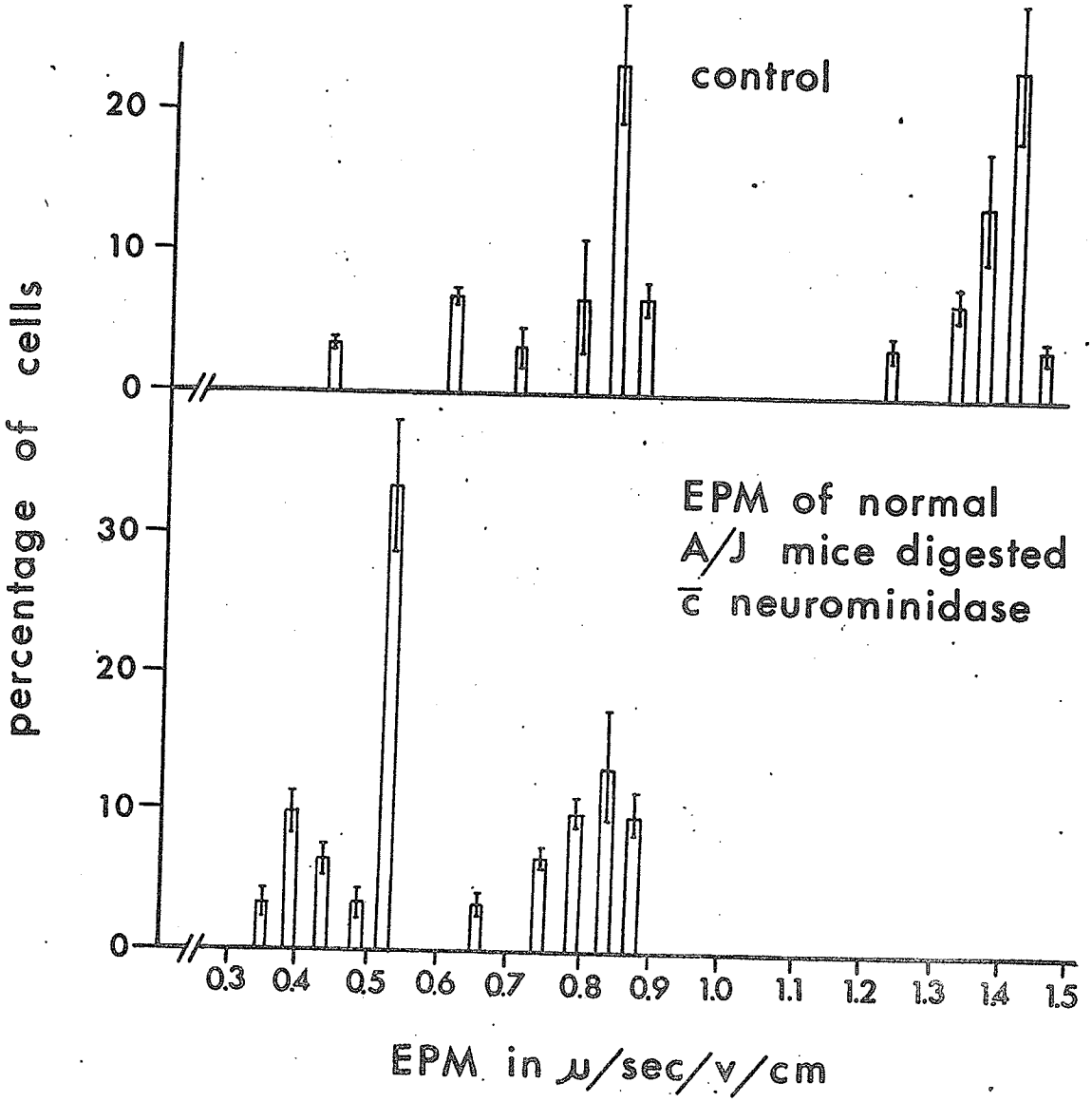

control
EPM of normal
A/J mice digested
C̄ neurominidase
percentage of cells
20
10
0
30
20
10
0
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
1.3
1.4
1.5
EPM in μ/sec/v/cm

Figure 19

Figure 20 The changes in EPM of the immune cell with neuraminidase.

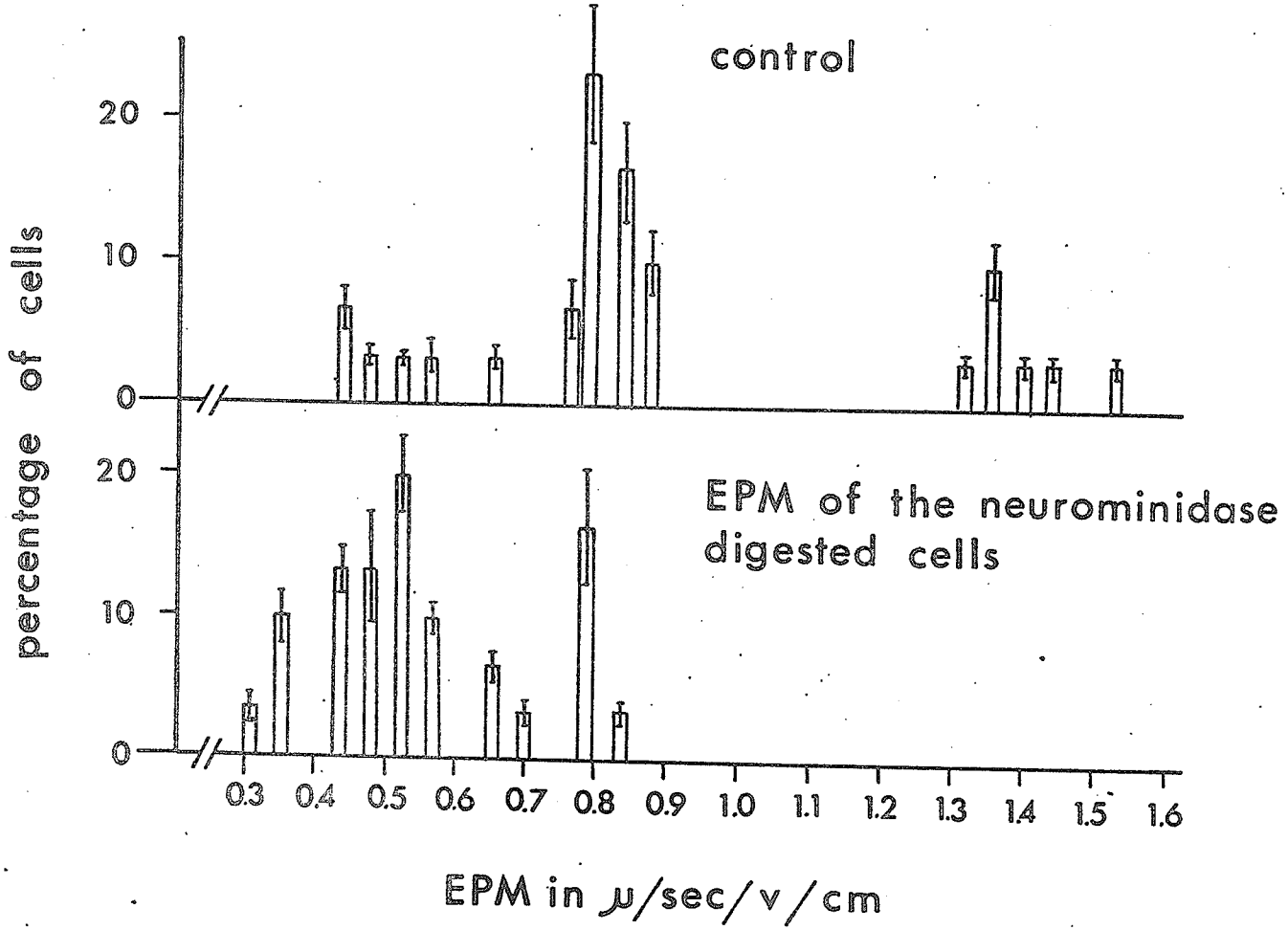

control
EPM of the neurominidase digested cells
percentage of cells
20
10
0
20
10
0
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
1.3
1.4
1.5
1.6
EPM in μ/sec/v/cm

Figure 20

VIII. THE EFFECT OF IMMUNOSUPPRESSIVE DRUG (CYCLOPHOSPHAMIDE TO THE LYMPH NODE CELL DISTRIBUTION)

Turk and Poulter (227) and Wiig (191) had independently reported that cyclophosphamide could depress the immune response by an alkylating reaction. It was important to observe the different capacity of the action of this immunosuppressive drug on the B and T cells.

(a) Material and Method

The Preparation of the Animal

Normal healthy 6-8 weeks A/J mice from Jackson were injected intraperitoneally 7.5 mg of Cyclophosphamide (Pharmacia) in 0.2 ml.. The animals were sacrificed after three days. The EPM of the regional lymph nodes was observed.

(b) Result and Conclusion

Anatomically, atrophy could be observed on the lymph nodes of the treated animals. The population of the fast EPM cells (T cells) remained intact while the low EPM cells (haptenic binding cells) were depleted (Figure 21).

This could be explained by that cyclophosphamide, as an alkylating agent, was capable to kill the more active dividing cells. The B cells were short lived lymphocytes and they turned over much faster than the T cell, since the latter was mostly long lived lymphocytes. The B cell population was expected to be depleted much faster than the T cells (226). Hence in conclusion, the low EPM cells (haptenic binding cells) were B cells.

Treatment of animal with cyclophosphamide might form an alternative method to select the T cells *in vivo*.

Figure 21 The effect of the cyclophosphamide on the lymph node cell population *in vivo*.

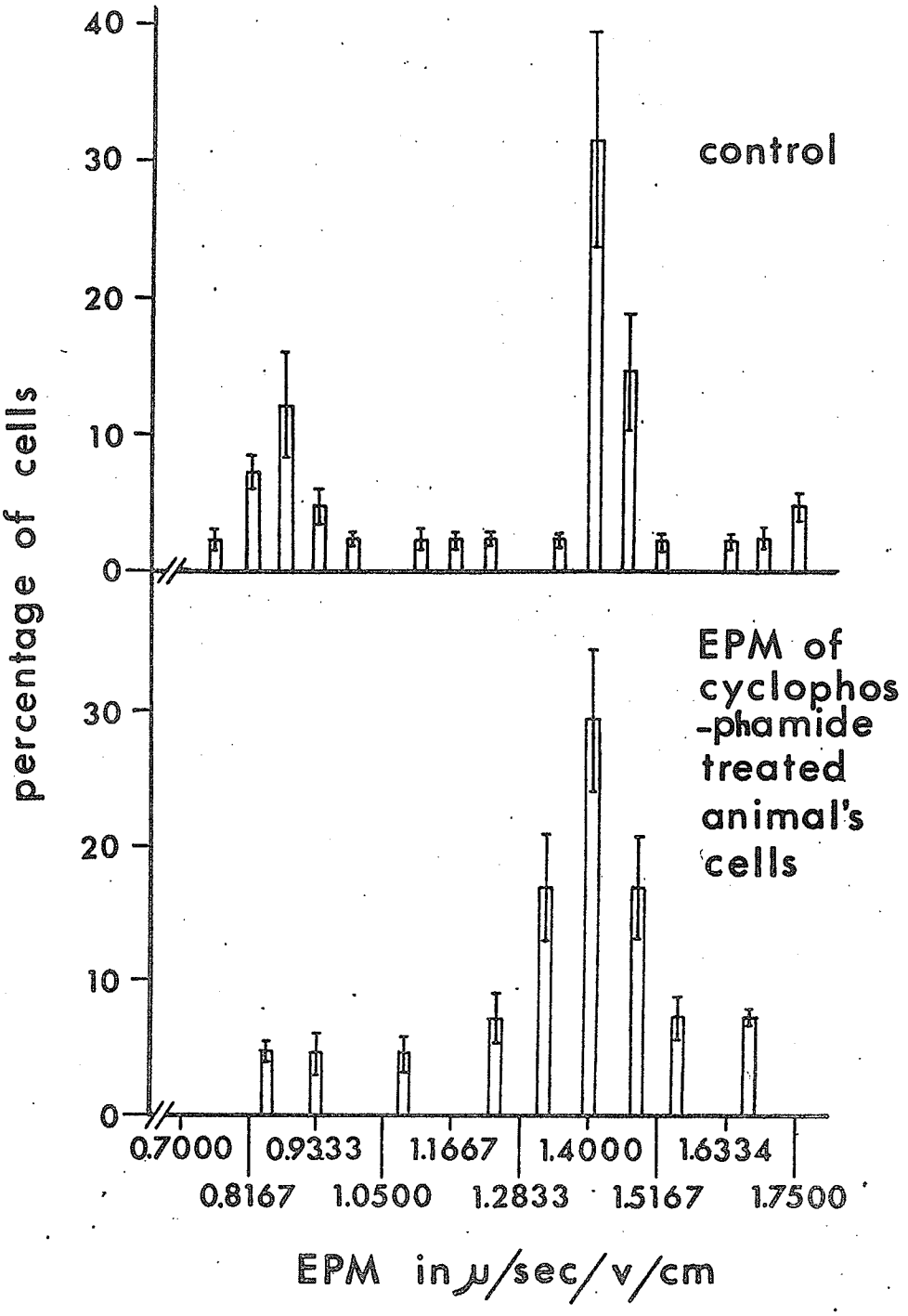

40
30
20
10
0
control
percentage of cells
EPM of cyclophos -phamide treated animal's cells
30
20
10
0
0.7000
0.8167
0.9333
1.0500
1.1667
1.2833
1.4000
1.5167
1.6334
1.7500
EPM in μ/sec/v/cm

Figure 21

IX. MACROPHAGE ELECTROPHORETIC MIGRATION (MEM) TEST FOR LYMPHOCYTE SENSITIZATION

The macrophage slowing factor from the sensitized lymphocyte had been demonstrated by many workers (26, 28-31, 33, 55-57, 141, 214, 215). In this section, lymphocytes from mice or guinea pigs sensitive to specific tumor antigens or to synthetic antigens were found to release a factor(s), which upon exposure to the specific sensitizing antigen, was able to reduce the electrophoretic mobility of normal guinea pig peritoneal macrophages.

(a) Material and Method

The Treatment of Guinea Pig Peritoneal Macrophage

Macrophages were obtained by washing the peritoneal cavity of normal guinea pigs with 10 ml. of minimal Eagle's medium. Before use, the cells were washed three times with 15 ml. of PBS and adjusted to a concentration of 10^6 cells/ml. One ml. of peritoneal macrophages were mixed with 5 ml. of control or test supernatants and incubated for 1 hour at room temperature on a rotator. The macrophages were washed three times with PBS again, were resuspended with PBS and the electrophoretic mobility was determined.

The Macrophage Electrophoretic Migration (MEM) Test

Three immunological systems were studied with the MEM test, i.e. immune reaction of syngeneic (strain 13) guinea pig lymphocytes against a methylcholanthrene induced tumor (MC-D) in vitro (228, 363, 364); the reaction of lymphocytes from A/J mice bearing a methylcholanthrene induced sarcoma (1509A) against tumor cells; and finally the reaction of lymphocytes from A/J mice immunized with bovine serum albumin conjugated with

dinitrophenol (DNP_{35}-BSA) to the immunizing antigen. The results of the MEM test for three immune systems were presented in Table III, IV and V respectively.

The In Vitro System

A strongly antigenic methylcholanthrene-induced sarcoma D (MC-D), transplantable in inbred Sewall Wright strain 13 guinea pig, was studied by I. Berczi et al. with in vitro culture technique (228). The emergence of foci of lymphoblast-cells was observed in the MC-D tumor line, capable of rapidly destroying the tumor cells in the culture. In the first group of experiments this culture system was investigated where tumor cell destruction took place in vitro, by immune syngeneic lymphoblast cells. Cell free supernatants of pure tumor cell cultures without the syngeneic lymphoblast cells were collected and used as control, the test supernatants were collected from cultures where tumor cell destruction took place by immune lymphoblasts. Macrophages (10^6 cells) of normal guinea pigs were exposed to 5 ml. of these control and test supernatants separately for one hour at room temperature on a rotator in a test tube at dilutions given in Table III. There was a progressive decrease (22-260%) in the macrophage electrophoretic mobility with increasing concentration of test supernatants. The macrophages exposed to the concentrated test supernatants (Experiments 10, 11, 12) actually migrated to the opposite direction (cathode) while the macrophages exposed to the control supernatants were heading for the anode.

The In Vivo System

In the second group of experiments (Table IV) 10^6 lymph node cells from normal virgin A/J mice (N) and those bearing

TABLE III

Reaction of syngeneic guinea pig (strain 13) lymphoblasts against a methylcholanthrene - induced sarcoma (MC-D) *in vitro*. Macrophages in control have been exposed to cell free supernatants of MC-D cultures. In the test system supernatants of cultures, with immune lymphoblasts was used. Negative mobility values (experiments 10 - 12) represent a complete change in macrophage charge, so that they migrate towards the cathode instead of the anode.

THE MACROPHAGE ELECTROPHORETIC MIGRATION (MEM) TEST FOR LYMPHOCYTES SENSITIZATION

(a) *In Vitro* System

The MCD Guinea Pig Strain XIII Tumor System

Expt. #	Control (MCD Sup)	Test (MCD + LBC) sup.	dilution	% decrease
1.	0.5615 ± 0.0300	0.4187 ± 0.0100	1/6	25.43
2.	0.5616 ± 0.0010	0.3649 ± 0.0100	1/4	35.00
3.	0.5204 ± 0.0100	0.3661 ± 0.0200	1/4	29.65
4.	0.5600 ± 0.0100	0.3400 ± 0.0200	1/4	22.00
5.	0.5450 ± 0.0100	0.3784 ± 0.0100	1/4	29.13
6.	0.5340 ± 0.0141	0.3511 ± 0.0141	1/4	34.25
7.	0.5810 ± 0.0010	0.3846 ± 0.0223	1/4	33.49
8.	0.5500 ± 0.0331	0.3650 ± 0.0282	1/4	33.63
9.	0.5601 ± 0.0300	0.3550 ± 0.0400	1/4	32.00
10.	0.5318 ± 0.0173	-0.9543 ± 0.0284	1/1	260.00
11.	0.5318 ± 0.0173	-0.7916 ± 0.0363	1/1	248.00
12.	0.5318 ± 0.0173	-0.7951 ± 0.0233	1/1	249.00

TABLE IV

Reaction of lymph node cells from A/J mice bearing a methyl-cholanthrene induced sarcoma (1509A) to 1509A cells (experiments 13 - 15) or to 1509A tissue culture supernatants (experiments 16, 17).

THE MACROPHAGE ELECTROPHORETIC MIGRATION (MEM) TEST FOR LYMPHOCYTES SENSITIZATION

(b) In Vivo System

The A/J 1509A Tumor Cell System

Expt. #	Control (1509A Tumor cell c̄ N cell)	Test (1509A Tumor cell c̄ I cells)	% decrease
13.	0.5617 ± 0.0374	0.3106 ± 0.0244	44.70
14.	0.5714 ± 0.0616	0.4133 ± 0.0346	27.66
15.	0.5614 ± 0.0515	0.4258 ± 0.0458	24.15
Expt. #	Control (1509A sup c̄ N cell)	Test (1509A sup c̄ I cell)	% decrease
16.	0.5550 ± 0.0424	0.3526 ± 0.0264	36.47
17.	0.5620 ± 0.0390	0.4173 ± 0.0700	25.73

sarcoma 1509A (I) (gifts from Dr. S. Fujimoto) were exposed separately in 5 ml. of PBS for 1 hour at room temperature on a rotator to 10^6 1509A sarcoma cells taken from another animal (Experiment 13) or from tissue culture (Experiment 14, 15). In the other experiments (16, 17) the lymph node cells (N and I) were exposed only to 1509A culture supernatants. The cell free supernatants were obtained by centrifugation at 1,500 rpm for 15 minutes. The normal guinea pig peritoneal macrophages were exposed to the supernatants under the conditions as described before. Their EPM were determined after washing. Table IV showed that inhibitions of macrophage electrophoretic mobility were detected ranging from 24 - 45%.

The Artifical System

In the third group of experiments (Table V), artifical antigen, DNP_{35}-BSA was used. A/J mice were immunized with 20 μg N of this antigen emulsified with equal amounts of Freund's complete adjuvant and injected into the foot pads at a total volume of 0.2 ml. The animals were boosted with the same dose of antigen subcutaneously on the back. Regional lymph node cells were excised from immunized animals four days after the second injection. In experiment 18 of Table V, 10^6 normal or immunized lymphocytes were exposed to 0.1 mg. of antigen. In Experiment 19, 10^5 lymphocytes were exposed to the same amount of antigen for one hour in PBS at room temperature on a rotator. Cell free supernatants were obtained by centrifugation as described before. The supernatants then were used for incubation with macrophages. The data of macrophage electrophoretic migration was presented

TABLE V

Reaction of lymph node cells from mice immunized with dinitrophenyl-bovine serum albumin with the specific antigen (DNP_{35}-BSA).

THE MACROPHAGE ELECTROPHORETIC MIGRATION (MEM) TEST FOR LYMPHOCYTE SENSITIZATION

(c) Artificial System

The DNP System on the A/J Mice

Expt. #	Control (DNP_{35}-BSA $\bar{c}$ N Cell)	Test (DNP_{35}-BSA $\bar{c}$ I cell)	% decrease
18.	1.1317 ± 0.0754	0.4660 ± 0.0574	58.82
19.	0.9129 ± 0.0707	0.6951 ± 0.0842	23.85

in Table V. It was evident that higher number of immune lymphocytes gave rise to a higher percentage of inhibition. Peculiarly the electrophoretic mobility of macrophages exposed to control supernatants was much higher than values obtained in the previous experiments. This might be explained by the possibility that some of the DNP_{35}-BSA (a highly negatively charged antigen), might adhere to the surface of macrophages non-specifically, increasing their original negative charge, and resulted in a higher electrophoretic mobility. Other possibilities could not be excluded at this point.

(b) Result and Conclusion

The results indicated that mouse and guinea pig lymphocytes sensitized to tumor or synthetic antigens released a factor which was able to decrease or competely change the negative charge of normal guinea pig macrophages. This factor was produced during lymphoblast mediated killing of tumor cells in vitro (363) as well as by lymphocytes of tumor bearing mice upon exposure to intact tumor cells or even to cell free supernatants of tumor cell cultures with lymphoblasts. This latter experiment indicated that the tumor "antigen" interacting with the sensitized lymphocytes was released at least in the case of 1509A sarcoma during in vitro culture. The fact that this macrophage electrophoretic migration slowing factor obtained with mice immunized with DNP-BSA strongly supported the assumption that this factor was released by the sensitized lymphocytes during the interaction with antigen. We did not know whether this factor was identical to MIF or to the leukocyte adherence inhibition factor (365).

We had no evidence at this moment to know whether the antigen involved in these animals tumor systems was similar to the encephalitogenic protein as in the case of human tumors. However, the great similarity of the behaviour of human and animal lymphocytes from tumor bearing hosts suggested that animal models might be used to obtain more information about the nature of this reaction under more carefully controlled experimental conditions.

GENERAL DISCUSSION

Many procedures had been developed to enrich the specific lymphocyte subpopulations. The erythrocytes could be removed by the combined phagocytosis with the density gradients centrifugation (367, 368). Monocytes and granulocyte (and platelets) could be depleted by passing the leucocytes through a column of glass wool (369), cotton batting (370), nylon fibres (371) or glass beads (372). Part of the B lymphocytes was found to adhere non-specifically on nylon fibre column (330). Human T lymphocytes were shown to have receptors for sheep red blood cells (SRBC) (332, 333) while B lymphocytes were demonstrated to have monkey red blood cell receptors (334) and Fc receptors (335, 336). They formed rosettes when incubated with the red cells alone or with antibody. With centrifugation on a density gradient, the non-rosetted cells were separated out at the interphase, the rosetted cells in the pellet might be harvested by the lysis of the SRBC with a hypertonic solution. However, there were difficulties associated with the competitive displacement of the cells out from the specific hapten immunosorbent by the dialysable hapten (87, 188). Cell electrophoresis was developed to investigate the different surface charge properties of the immunocytes, as a possible specific cell fractionation technique.

The non-polarizing reversible silver-silver chloride electrode gave no gas formation in the electrode compartment so that the net electrophoretic mobility could be measured (Section I). The EPM of red cells was found to be fairly

constant (1) due to the relatively constant charged moieties on the cell surface. The value of EPM with the calibration of the author's red blood cells was 1.28 ± 0.03 micron/sec/volt/cm. at the stationary phase in our cell electrophoresis system which agreed with the literature (Table II). The immunizing antigen used in these studies was DNP_{35}-BSA which was negatively charged. The peaks of the bimodal EPM profile for the lymphocytes from the immunized animals were slower than that of the control one (Figure 3 and 4, Section II), verifying the finding of Rhie and Sehon (149) in the inverse relationship between the immunocytes and that of the immunizing antigen. The low electrophoretic mobility cell subpopulation was higher in percentage than the high electrophoretic mobility cell subpopulation for the immunized animal lymph node cells as compared with that of the control animals. B cells, T cells, mast cells, granulocytes and basophils were shown to possess receptors with various types of Ig specificities (247, 251, 337). B lymphocytes were shown to bind Ag-Ab complex (338, 375) and fluorescein-labelled aggregated Ig (339). Addition of anti-Ig sera to culture of lymphoid cells <u>in vitro</u> could stimulate metabolic changes and cell proliferation (158). With radio-iodinated specific antisera to Ig, the uptake of radioactivities by the cells could be demonstrated by radioautography (125, 373). Quantitative determination of the amount of membrane bound Ig on lymphoid cells had been made by the binding of horseradish peroxidase labeled antibody (374). In EPM study of human lymphocytes, the pretreatment with polyvalent antisera caused a reduction

in the net negative charge of cells (16). Clonal selection concepts held that the interaction of antigen with specific preformed Ab receptors on the cell surface triggered clonal proliferation. The membrane-bound Ig on the lymphocyte surface could have been derived exogenously by the Ig bound to it through the Fc receptor on the lymphocytes surface (12, 335, 336) or had been derived endogenously from the lymphocytes themselves. The low electrophoretic cells might have more receptors for the immunizing antigen than that of the high electrophoretic mobility cells, hence in response to the antigen, they proliferated more than the latter. With quantitative studies in the uptake of ^{125}I-labelled specific antibodies to Ig, it was shown that the B cell populations had about 140 to 440 times more anti-Ig binding sites than T cell populations (125). The low electrophoretic cells could be considered to be B cells while the high electrophoretic cells were T cells (5, 124, 176, 191, 193, 259, 355). In the case of the cell-mediated immunity, the subpopulation of the high electrophoretic mobility cell was larger than that of the low electrophoretic mobility cells (77) since T cell was responsible mostly for the cell-mediated immunity.

The electrophoretic mobility of the immunocytes could be altered with the specific incubation of the immunization antigens (149, 168). Using different synthetic polyionic derivatives of DNP-PLL as the incubating antigen (Section III), half of the low electrophoretic mobility cells of the immunized

animals with DNP_{35}-BSA was altered in their EPM, while the high electrophoretic mobility cell subpopulation remained relatively the same indicating that the low electrophoretic mobility cell population contained cell subpopulations which could bind to the haptenic determinant DNP specifically, possibly through an antibody-like receptor or by the cell bound antibodies on the plasma cells or L cells (248). With other antigen and animal systems, the percentages of antigen binding cells were reported to range from 0.002 to 1 (322). However the sensitivity of different assaying techniques varied a lot. The specificity of the alternation of the EPM of the low electrophoretic mobility cell by the incubation with the antigens in the immunized animals reported in this study was furnished by the failure of the incubating antigens to change the EPM profile of the control animals.

The inhibition test performed with the dialysable hapten DNP-lysine HCl was incomplete, probably due to the difference in the affinity between the antibody-like receptors and the incubating antigens. The incubating antigens, αDNP-ε-polyionic derivatives of lysine, might block the competitive binding by the dialyzable hapten by the virtue of the length and charges of their carrier. During the whole process of the experiment, the cells had been shown to be living by the trypan blue viability test. It had been shown that the physiologically active antigen binding cells could regenerate their receptor (232) within a matter of few hours. Since it might also be possible that some of the receptors of the lymphocytes were

regenerated at the time the dialysable hapten was incubating with the cells.

Using cell electrophoresis and ∝DNP-ε-isothiocyanate fluorescein L-lysine as the incubating hapten, it was possible to identify the hapten binding cells by the broader and lighter tracks on the black and white photo negatives (Section IV). Actually, fluorescein labelled cells had been injected through a glass-nozzle in a very dilute concentration, and excited by a beam of light, the emission was detected by a photomultiplier which would excite an electric field. The cells travelling through the electric field would then be deflected and collected in the tube at the bottom of the apparatus (376).

The isolation of the hapten specific binding cells by DNP-BioGel affinity column was shown to have problems involving the non-specific binding of the cells to the gel by the virtue of their stickiness on the cell surfaces (Section V). Also the cells with high affinity receptor to the hapten would not be able to be eluted out by the free hapten.

The T cells carried θ antigen on their surfaces (143, 144). The selective depletion of the high electrophoretic cells by the anti-θ antisera indicated the presence of T cells in that subpopulation (Section VI).

The density of the exposed sialic acid on the surface of immunocytes determined their electrophoretic mobility (124, 346, 347, 350, 351). The B cell subpopulation was found to have lower electrophoretic mobility than the T cells, probably due

to the fact that the surface of the B cells had more Ig-like receptors to the immunizing antigens (346, 355, 356, 359) and covered up some of the sialic acid. This assumption was further supported by the fact that the B cells were more resistant to neuraminidase digestion than the T cells (Section VII).

It had been shown that B cells were short lived lymphocytes, and they turned over much faster than the T cells (51, 166). Cyclophosphamide, an immunosuppressive drug, was able to kill the dividing cells by an alkylating reaction (226). After the in vivo administration of 7.5 mg. of cyclophosphamide to the mice after 3 days, the low electrophoretic mobility cells were found to be depleted (Section VIII), confirming the finding of Turk and Poulter (226), suggesting the possible selective depletion of B cells by this immunosuppressive drug.

The practical use of cell electrophoresis as a tool in immunological studies, utilizing the cell surface charge properties was investigated (Section IX). Lots of experiments had been done by many workers involving the detection of lymphocytes sensitized to human tumor antigens (26, 28-31, 33, 55-57, 141, 214, 215). Animal models were employed in our experiments; the guinea pig MCD tumor system, the A/J mice 1509A tumor system and the artificial DNP system were investigated. In all the experiments, mediator(s) was released specifically by the sensitized cells upon the incubation with the immunizing antigens. The factor(s) was probably a positively charged protein material which could bind non-specifically to the normal peritoneal macrophages of the guinea

pig. The possibility that this factor was an antigen-antibody complex could not be ruled out (207).

CONCLUSION

With a well constructed microelectrophoresis chamber, the electrophoretic mobility profile of lymphocytes were analysed. The high EPM cells were demonstrated to be T cells by means of the selective depletion by the anti-θ antiserum. The low EPM cells of the lymph node were demonstrated to be B cells by their binding capacity with the charged hapten, by an immunofluorescence technique, the "selective" depletion by the immunosuppressive drug, cyclophosphamide, and by the hapten binding affinity column. The efficiency of cell fractionation by the affinity column had to be questioned owing to the natural stickiness of the cell surface and also because of the difficulties in eluting the bound cells effectively with the hapten.

The ability of the hapten with the different polyionic charged carriers to identify the specific population of the hapten binding cells by the cell electrophoresis technique provided an alternate method for specific cell fractionation, especially with the help of the preparative deflection-continuous free flow electrophoresis apparatus. The main advantages of the method suggested above were that it combined the immunochemical specificity with the mildness of free flow electrophoresis and did not involve the contact of cells with solid surfaces (as would be in the case in the affinity chromatography). However, the possibility of the regeneration

of the receptors and the difficulties of displacing out the incubating antigen would add to the problems.

In principle, this method of the possible fractionation of immunocytes with highly charged conjugates had the capacity to be extended to the fractionation of immunocytes sensitized to the more complex antigens (e.g. transplantation or tumor associated antigens)subjected to being able to attach covalently polyionic molecules to these antigens. But more parameters had to be adjusted to ensure the proper interpretation of the data.

It was also anticipated that this method might prove valuable for the elucidation of the repertoire of cells involved in the delayed type of hypersensitivity and in the cell cooperation. Thus, by using a hapten attached to the polyionic molecules, which differ radically from that of the 'carrier' of the sensitizing antigen, one might be able to isolate, by deflection electrophoresis, the hapten-specific binding cells; similarly one would be able to isolate the cells reacting with the 'carrier' portion of the antigen by attaching the 'carrier' to an appropriately charged polyionic group.

The macrophage electrophoretic mobility inhibition test for lymphocyte sensitization might be developed into an extremely sensitive method. The factor responsible for the inhibition was specifically released by the sensitized lymphocytes during the incubation with the antigen. In order to elucidate the role of this factor in the immune response, the relationship

between it and MIF, or the leukocyte adherence inhibition factor, or other lymphokines will have to be established.

The cell microelectrophoresis method appears therefore to be a useful tool for immunological research and hence additional studies for investigating its potential appears to be justified.

CLAIM OF ORIGINALITY

1. A microelectrophoresis cell was constructed which provided optimal conditions for cell electrophoresis.
2. The EPM profile of the control A/J Mice lymph node cells was found to be bimodal, and peaked at 0.8750 and 1.4000 micron/sec/v/cm. The EPM profile of the Immuned Lymph Node Cells was found to be biomodal and peaked at 0.8176 and 1.3417 micron/sec/v/cm.
3. αDNP-PLL$_{15,000}$ and $_{150,000}$ and their derivatives were synthesized and were found to be able to induce specific changes of the EPM of the low EPM immuned cell population.
4. The hapten binding cells were identified with an immunofluorescence technique.
5. The efficiency of the hapten affinity chromatography method for cell isolation was questioned.
6. The depletion of the high EPM cells with anti-θ antiserum was confirmed.
7. The contribution of sialic acid to the surface charge properties of cells was confirmed. B lymphocytes were found to be more resistent to neuroaminidase digestion than the T lymphocytes.
8. The possibility of the depletion of B cells with cyclophosphamide *in vivo* was confirmed.
9. Macrophage Electrophoretic Migration was demonstrated to be a very sensitive test for lymphocyte sensitization.

LIST OF ABBREVIATIONS

A Cell	Accessory cell.
A/J Mice	Strain A Mice developed by the Jackson Lab., USA.
B lymphocyte	Thymus independent lymphocyte.
C	Control animals immunized with FCA.
CMI	Cell mediated immunity.
DNBS	2,4-Dinitrobenzenesulfonic acid.
DNP_{35}-BSA	35 molecules of dinitrophenol were coupled to one bovine serum albumin molecule.
αDNP-PLL	αdinitrophenol-poly-L-lysine.
DNP	Dinitrophenol.
EPM	Electrophoretic mobility.
FCA	Freund's complete adjuvants.
GVH	Graft versus host reaction.
I	Immunized animal.
MBLA	Mouse specific B lymphocyte antigen.
MCD	Tumor methylcholanthrene induced sarcoma.
MEMIF	Macrophage electrophoretic migration inhibition factor.
N	Normal control animal.
PBS	Phosphate buffer saline.
T lymphocyte	Thymus dependent lymphocyte.
θAg	θAlloantigen of the mouse T lymphocyte.

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