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Comparison of cellular and humoral immune responses to rabies and Sindbis virus in mice

by

Howard Thomas Hill

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
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INTRODUCTION

The humoral response to agents producing infectious disease has for years been measured serologically. The researcher, veterinarian and physician have used these serological results to determine the resistance or protection developed by the vaccinated or infected host. Relatively new discoveries such as the identification of thymus and bursa derived lymphocytes and the function of these cells have demonstrated that the humoral response is only part of the immune mechanism. In addition, the observation has been made that human patients with immunological deficiency diseases were still capable of responding to certain antigens. The responses observed were determined by whether the immunological deficiency affected the cells responsible for humoral or cell-mediated immunity. It has been generally accepted that antibody plays a major role in preventing reinfection by a pathogenic organism. However, antibody may not be produced early enough or in sufficient amounts in a primary infection to bring about recovery from the infection. Other mechanisms such as interferon and cell-mediated immunity may play far more important roles than antibody in altering the course of a primary infection. Since immunized animals possess both types of immunity and both are closely interrelated, it is difficult to
evaluate the role of cell-mediated versus antibody-mediated immunity.

Although rabies is one of the oldest diseases known to man, researchers have yet to establish the entire pathogenesis of this disease. Cell-mediated immunity could possibly be involved in some immunopathogenic phenomena which are responsible for the final outcome of the disease. Even more important is the realization that as with resistance to many diseases, in rabies prophylaxis or postexposure treatment, cell-mediated immunity may be equally as important as antibody-mediated immunity. There have been only two published reports on the cell-mediated aspects of rabies. These reports are conflicting in their results. Turner (1973) concluded from his work that there was no cell-mediated immunity in rabies-immune mice. Wiktor et al. (1974) on the other hand recently reported that they could demonstrate the presence of cell-mediated immunity in rabbits vaccinated against rabies.

The World Health Organization (WHO) Expert Committee on Rabies has recognized the need for a better understanding of cell-mediated immunity to rabies virus. They have suggested that it may be crucial for the protection of the exposed individual. In its sixth report (1973) this committee specified that one area of research that should be pursued is the investigation of the mechanism of *in vivo* protection after
exposure to rabies with reference to the role of cell-mediated immunity.

This study was designed to determine if in fact cell-mediated immunity is involved in rabies infections and if so, how the cell-mediated response compares temporally to the humoral response. No published reports were present at the initiation of this study which demonstrated the presence of cell-mediated immunity in rabies vaccinated or infected animals. Therefore it was necessary to establish the standards and examine the kinetics of the reaction in the in vitro microculture test before proceeding with the specific experiments that would identify whether or not sensitized lymphocytes could be stimulated by rabies virus in vitro.

As the laboratory in which this author was working was not previously involved in lymphocyte stimulation studies, it was necessary to establish expertise using the in vitro microculture lymphocyte stimulation test. Sindbis virus, which has recently been demonstrated to transform sensitized lymphocytes (Griffin and Johnson, 1973) provided a viral model which was used to develop the microculture lymphocyte stimulation test.
LITERATURE REVIEW

Cell Mediated Immunity

The defense mechanisms that protect animals from the onslaught of infectious agents that continually confront them are numerous and intimately interrelated. In order for scientists to study these defense mechanisms logically it has been necessary to divide this problem into components and study them individually. The immune system is but one aspect of nature’s defense mechanism which protects the animal kingdom from infectious diseases.

The immune system itself is a dichotomous system. Prior to 1942 the knowledge of immunity consisted primarily of what we knew about antibodies and their biological functions. In 1942 Landsteiner and Chase discovered that reactivity could be transferred to unsensitized hosts by lymphoid cells but not by serum. Since the work of Landsteiner and Chase, volumes of information have been accumulated concerning the immune system. It is now widely recognized that the system is dichotomous, consisting of cellular and humoral responses each of which is mediated by different types of lymphocytes.

Glick et al. in 1956 described the role of the bursa of Fabricius, a hindgut lymphoid organ in the chicken, in the development of humoral immunity in the chicken. Warner et
al. (1962) were the first to describe a functional dissociation of the chicken immune system based on differences in thymic and bursal influence. This work was confirmed repeatedly using the chicken as a model by Good et al. (1966), and Cooper et al. (1966). This discovery of a functional dissociation of the immune system arose from the fact that there are two different types of lymphocytes in the chicken. One type is a thymus-derived or thymus-dependent lymphocyte (T cell) which is responsible for the reactions of cell mediated immunity (CMI) and the other type being an independent or bursa-dependent lymphocyte (B cell) responsible for humoral immunity.

A similar situation involving T and B cells exists in mammals (Miller, 1961, 1962; Good et al., 1962). However the mammalian analog for the bursa of Fabricius has not been demonstrated and it appears that a single corresponding central lymphoid organ will not be found (McCluskey and Leber, 1974).

Classically T cells have been considered the mediators of cellular immunity having a primary role in delayed hypersensitivity while B cells were the cells responsible for antibody production (McCluskey and Leber, 1974). Recent research in the field of cellular and humoral immunity has indicated that the functions of T and B cells are not as precisely defined into cellular and humoral functions as
was once believed. In the case of B cells, recent findings by Yoshida et al. (1973) indicated that B cells can produce and release an effector substance (to be discussed later). The particular effector substance described by these workers is migration inhibition factor (MIF) which is one of the lymphokines. In the past the elaboration of these lymphokines has been correlated with a state of delayed hypersensitivity, mediated by T cells (Sonozaki and Cohen, 1972). It was concluded by Yoshida et al. (1973) that, although activation of lymphocytes for MIF production by specific antigen was a function of T cells, B cells were also activated by mitogenic agents, such as purified protein derivative (PPD), which act nonspecifically.

Likewise, T cells have more than the mediation of cellular immunity as their biological function. Claman et al. (1966) recognized that thymus dependent cells had an enhancing effect on the ability of marrow cells to produce antibody. These workers concluded that one cell population contains cells capable of making antibody ("effector cells"), but only in the presence of cells from the other population ("auxiliary cells"). The exact mechanism by which T and B cells interact during the induction of the antibody response is not fully understood. However, it is known that B cells will not produce antibodies to haptenic determinants on an immunogen unless other determinants on the immunogenic
molecule are recognized by T cells (Miller and Mitchell, 1970; Segal et al., 1972). Segal et al. (1971) reported that this bicellular cooperation was necessary in both the primary and secondary response to dinitrophenyl (DNP). The T cell is referred to as a "helper" cell (Yoshida and Cohen, 1974) in the process of antigen recognition. This "helper" cell is not directly involved in the production of antibody, but the switching on of the antibody synthesis depends on the cooperation between T and B cells (Segal et al., 1971).

Another function of T cells which in the past had been excluded from their classical role as mediators of cellular immunity was their cytotoxic or killer activity. Koprowski and Fernandes (1962) reported that immune lymphocytes would aggregate around and destroy target cells. This cytotoxic reaction can however be induced by phytohemagglutinin using nonimmune lymphocytes (Holln et al., 1964). Speel et al. (1968) have made use of the cytotoxic function of immune lymphocytes to study cellular immunity to mumps virus. These workers evaluated their results using specific immune lymphocytes as killer cells against epithelial cell cultures infected with a persistent noncytocidal mumps virus, as a manifestation of the efferent arc of a homograft response to a viral infection.
Mechanisms of Lymphocyte Reactions

Lymphocytes play a key role in tissue destruction by participating in delayed hypersensitivity, homograph and graft-vs.-host reactions. This cytotoxic action of sensitized lymphocytes is either mediated through the elaboration of soluble factors known as "lymphokines" (Dumonde et al., 1969) or by their direct attack on target cells as described previously. The lymphokines released from sensitized lymphocytes when stimulated include migration inhibition factor (MIF), lymphototoxic factor, skin reactive factor, macrophage chemotactic factor, mitogenic or blastogenic factor, cloning inhibitory factor, proliferation inhibiting factor, inhibitor of DNA synthesis, transfer factor and interferon (Bloom, 1971a; Yoshida and Cohen, 1974).

Although it is widely assumed that many of the reactions of cell-mediated immunity are dependent on the activity of one or more of these lymphokines, there is little evidence to date that any of these lymphokines, which are the putative mediators of such T-cell-dependent reactions, are in fact produced by T cells. Two notable exceptions have been reported by Sonozaki and Cohen (1972) and Yoshida et al. (1973). As mentioned previously this latter group was able to show that MIF was specifically produced by T cells but that it could also be produced by B cells when
stimulated with PPD which is a B cell mitogen. Sonozaki and Cohen (1972) attacked the problem directly by proving that the macrophage disappearance reaction (MDR) which is an in vitro correlate of delayed hypersensitivity was mediated by a lymphokine that was produced by T cells. This work made use of the fact that guinea pig lymphocytes are divided into two populations on the basis of whether or not they possess receptors for the third component of complement on their surface. Bianco et al. (1970) reported that guinea pig lymphocytes that had the complement-receptors were B cells while the noncomplement-receptor lymphocytes were T cells. Using this separation technique, Sonozaki and Cohen (1972) were able to confirm that the lymphokine, a soluble product that is the mediator of the MDR, was produced by the T cells.

The important information that is lacking concerning the lymphokines is what role these products, that have been so extensively studied in vitro, play in vivo. It is generally accepted that some of the lymphokines do participate in reactions in vivo and that similar substances, induced by nonimmunologic means, may play a corresponding role (Yoshida and Cohen, 1974).

Migration inhibition factor (MIF) and lymphotoxin are two of the most widely studied of the lymphokines. Some of the other lymphokines will only be mentioned briefly.
Migration inhibition factor was involved in the first model for studying delayed-type hypersensitivity. Rich and Lewis (1932) made the observation that the migration of cells from spleen or lymph node explants obtained from tuberculous rabbits or guinea pigs were markedly inhibited when tuberculin was added to the tissue culture medium. This reaction is now recognized as being mediated by MIF. Unfortunately very little interest was stimulated by this observation until George and Vaughn (1962) developed the capillary tube migration test which provided an accurate and relatively simple way to study delayed hypersensitivity reactions involving MIF in an *in vitro* system. MIF was first thought to be an immunoglobulin due to its solubility and immunological functions (Bloom, 1971b). However, the elution of MIF on Sephadex G-100 (Yoshida and Reisfeld, 1970) indicated that fractions with MIF activity had molecular weights of 12,000 and 67,000 daltons. More recently the molecular weight of MIF has been reported to be 23,000 daltons (Rocklin *et al.*, 1972). Rocklin (1974) recently reported that leukocyte inhibitor factor (LIF), like MIF, was elaborated by sensitized lymphocytes following stimulation by specific antigen. This factor has a molecular weight of 69,000 daltons and this may correspond to the high molecular weight (67,000 daltons) MIF molecule reported by Yoshida and Reisfeld (1970). Rocklin (1974) reported that LIF is
distinctly different from MIF. LIF selectively inhibited the migration of polymorphonuclear leukocytes, but did not affect the migration of guinea pig or human monocytes. The molecular weight of MIF is smaller than any known immunoglobulin indicating that MIF is not such a molecule. Bloom (1970) summarizing much of the work on the chemical analysis of MIF concluded that this molecule is most likely a glycoprotein.

The relation of MIF's effect on macrophages in vitro to observed activation in vivo is a subject as yet unsettled. However it was reported that supernatant fluids from tuberculin-stimulated mouse lymphocytes had the capacity to confer resistance to infection by virulent tubercle bacilli onto normal macrophages (Patterson and Youmans, 1970). The soluble factor was not specifically identified as MIF but regardless, this report represented the first direct in vitro assay of acquired cellular resistance to tuberculosis, a disease widely known for its cell-mediated response. Of equal interest were the findings of David (1971) that MIF-treated macrophages had increased phagocytic capacity, were metabolically more active and exhibited increased membrane activity. All three characteristics also describe macrophages at the site of a delayed hypersensitivity reaction in vivo.

It is clear from a report by Dumonde (1967) that MIF
does react with macrophages, the evidence being that the migration inhibitory activity could be removed from active supernatant fluids by absorption with macrophages. The immunochemical specificity of antigen-induced inhibition of peritoneal exudate cell migration was studied using chemically defined antigens such as the alpha, dinitrophenyl-L-lysine series (David and Schlossman, 1968). These studies indicated that this response was very specific which suggests the presence of a highly specific binding site functioning as the cellular receptor for antigen on the sensitized lymphoid cell or on some "processing" cell (David and Schlossman, 1968).

The in vitro reaction involving MIF is a two step reaction (Bloom and Bennett, 1966). Lymphocytes from a sensitized donor are the immunologically active cells which when interacting in vitro with the specific antigen releases a soluble material, MIF, which is capable of inhibiting the migration of normal macrophages. The macrophages merely act as indicator cells for MIF. The in vitro test measures only some of the initial steps that occur in in vivo reactions of cellular hypersensitivity (Bloom, 1971a). This test does not take into account the release of lysosomal enzymes from macrophages and the subsequent reactions that follow. Also the MIF test does not assess the affect of other mediators, blood clotting factors, or local tissue conditions such as
the condition of blood vessels (Bloom, 1971a).

Another important and widely studied lymphokine is lymphotoxin (LT) or as it is sometimes referred to, cyto-toxin. This product of sensitized lymphocytes was first described by Ruddle and Waksman (1968), using antigen sensitive cells. These workers reported that cell-free supernatant fluids, obtained from cultures in which direct lymphocyte-target cell cytotoxicity had been induced by PPD stimulation of tuberculin-sensitive lymphocytes, had a slight, but discernible cytotoxic or growth inhibitory activity on monolayers of rat fibroblast. Granger and Williams (1968) using phytohemagglutinin (PHA) stimulation of unsensitized lymphocytes were subsequently able to demonstrate the presence of LT by the ability of the supernatant fluid to diminish the incorporation of labeled amino acids into protein by mouse L cells, the target cells. Other criteria such as vital staining and morphological criteria have also been used to detect cytotoxicity (Granger and Kolb, 1968). Granger and Williams (1971) reported that LT binding to target cells does not suppress macromolecular biosynthesis and induces cell cytolysis probably by degrading the cell plasma membrane.

Bloom (1971b) suggested that LT like MIF is not an immunoglobulin based on the facts that LT acts nonspecifically on a variety of target cells and its production can be
induced by nonspecific mitogens. Granger (1969) reported that elution studies from Sephadex G-100 columns indicated a molecular weight of 80,000 daltons for human LT. Kolb and Granger (1970) reported that mouse LT had a slightly higher molecular weight in the range 90,000 to 150,000 daltons.

The effects of LT on target cells have been somewhat elucidated by morphological studies in which the cells were found to be rounded up, vaculated and detached from glass (Bloom, 1971b). Bloom (1971b) has also reported that unlike direct lymphocyte toxicity, LT cytotoxicity requires 24 to 48 hours for maximal effect. The susceptibility of target cells is quite variable with mouse L cells being the most sensitive (Williams and Granger, 1969). Less than 1 µg of purified LT can be detected using mouse L cells as the target cells (Granger, 1969).

Work by McCluskey et al. (1963) involving cells at the sites of delayed hypersensitivity reactions indicated that the majority of the cells are rapidly dividing nonsensitive mononuclear cells, and only a few of these cells are specifically sensitized. The obvious hypothesis forthcoming was that sensitive lymphocytes interacting with antigen could produce a chemoactive factor (CF) that would attract macrophages to the site. Ward et al. (1970) demonstrated the presence of CF in cultures of antigen-stimulated lymphocytes.
These workers demonstrated the production of CF to be immunologically specific. On Sephadex G-100 columns, CF activity was found in fractions where MIF peak activity resided, however CF activity was separated from MIF activity by gel electrophoresis (Remold and David, 1974).

Blastogenic or mitogenic factor was reported simultaneously by Kasakura and Lowenstein (1965) and Gordon and McLean (1965). These two research teams found that supernatant fluids from mixed leukocyte cultures contained a substance that was mitogenic for unrelated lymphocytes. This finding has particular significance when trying to explain the kinetics of lymphocyte reactions. Remold and David (1974) concluded that it is possible that mitogenic factor or a similar substance can "turn on" or "recruit" nonsensitive cells thus providing a mechanism for expanding a cellular reaction and enlarging the production of other mediators.

Transfer factor (TF), a molecule composed of polypeptides and polynucleotides with a molecular weight of less than 10,000 daltons (Lawrence, 1974), is another factor released from lymphocytes that plays an important role in the transfer of delayed hypersensitivity (DH) in humans. This active moiety that was extracted from leukocytes had the same capacity as viable cells in the transfer of DH and cellular immunity, thus the term "transfer factor" (Lawrence,
1954). Warwick et al. (1960) and Lawrence (1969b) found that multiple antigenic specificities of DH could be transferred with TF and the transferred DH and its intensity was concordant with, and dictated by, the particular specificities expressed by the donor.

The understanding of the mechanism by which TF can transfer DH was greatly enhanced by the finding of Lawrence (1955) that one could serially transfer DH. Extracts of blood leukocytes from a sensitive donor were used to transfer streptococcal DH to a primary recipient. Three days post-transfer an extract of lysed leukocytes was prepared from this primary recipient and passed to a secondary recipient. Lawrence (1955) was also able to transfer tuberculin DH in a similar serial fashion. These experiments by Lawrence weaken proposed theories of a "superantigen" or an undiscovered type of immunoglobulin as the mediator of this transfer of DH. The minute quantities of material (TF) initially employed for transfer and the dilutional effects of serial transfer would eliminate such possible mechanisms.

Although the exact mechanism of transfer is not completely understood, Lawrence (1969a, 1974) reported that TF, in addition to conferring DH and cell-mediated immunity to diseased immunologically deficient recipients, causes a new population of antigen-responsive lymphocytes to appear in the recipient circulation. The responsiveness of the recipient's lymphocytes can
be shown by their transformation and proliferation and/or elaboration of such lymphokines as MIF and LT when exposed to specific antigens to which the donor is sensitive.

Presently the studies involving TF are primarily centered around immunotherapy (Lawrence, 1974). Experimental and clinical use of TF in restoration of cellular immunity in patients with congenital and acquired cellular immunodeficiency disease, tumors and intracellular infections have been quite rewarding. Although clinical studies using TF to treat viral diseases are fewer, some favorable results do exist. Patients with disseminated vaccina infections have been successfully treated using viable leukocytes (Hathaway et al., 1965; Kempe, 1960). Lawrence (1974) reported that Moulias and his coworkers in France successfully treated patients suffering from measles "giant cell" pneumonia and subacute sclerosing panencephalitis, a sequela to measles, with measles-positive transfer factor. These same workers successfully treated a newborn child suffering from congenital herpes virus infection with transfer factor.

The ultimate understanding of the mechanism of cellular immunity may revolve around our present knowledge or theoretical knowledge of the actions of the mediators of CMI and their interaction with TF.

Lawrence (1974) summarizes the present state of our knowledge by explaining that TF appears to confer or uncover
specific antigen-receptor sites on the recipient's circu-
culating lymphocytes which on contact with specific antigen
proliferate to form new clones of cells with particular
specificities. These cells produce the soluble mediators,
lymphokines, that probably magnify the response by recruit-
ment of other cells.

Lymphocyte Transformation

The discovery by Nowell in 1960 that phytohemagglutinin
(PHA), an extract from the kidney bean, Phaseolus vulgaris,
could stimulate peripheral blood leukocytes to enlarge and
divide, and the discovery by Hastings et al. (1961) and
Carstairs (1961) that the stimulated cells were lymphocytes
provided a cell culture system to study many aspects of im-
munology, cell biology and genetics.

Lymphocyte transformation has been defined as the mor-
phological enlargement of small lymphocytes to large lympho-
blasts in vitro (Robbins, 1964). The resultant transformed
cell, a lymphoblast, has been described as a large cell hav-
ing a basophilic cytoplasm and one or more nucleoli (Oppen-
heim, 1968). In concert with this definition morphological
criteria were first used to determine the degree of trans-
formation in stimulated lymphocyte cultures. The first and
simplest to be used was the evaluation of the percentage of
lymphoblasts on coverslip preparations of cultured cells
stained with May-Grunwald Giesma stain (Valentine, 1971).
One of the most reproducible and quantitative techniques for
the measurement of lymphocyte response to a stimulus is an
assay of the total radioactive thymidine uptake by a culture.
This quantitative measure of tritiated thymidine as an indica­
tor of total DNA synthesis by lymphocytes is a sensitive indica­
tor of their transformation index (Oppenheim, et al., 1965).

Lymphocytes can be stimulated with a variety of ma­
terials. Oppenheim (1968) has summarized the stimulants
and divided them into 4 classes. Nonspecific stimulants
comprise the first class and are probably the most widely
studied. Phytohemagglutinin is the prototype of this group
of very active stimulants. Other stimulants in this class
that have been widely studied include pokeweed mitogen (PWM)
from Phytolacca americana (Farnes et al., 1964) and concanaval­
in A (Con A) (Douglas et al., 1969). Other less notable
stimulants listed in Oppenheim's first class include
staphylococcal filtrate (Ling et al., 1965) and streptolysin
(Hirschhorn et al., 1964).

Different responses to PHA, Con A and PWM have been ob­
served between B and T cells in the mouse. Douglas (1972)
reported that mouse B cells were more responsive to PWM where­
as mouse T cells were more responsive to PHA and Con A. B
cells have been activated by PHA bound to Sepharose (Greaves
and Bauminger, 1972), however Janossy and Greaves (1971) reported that the responsiveness to PHA is predominantly, if not uniquely, a property of T lymphocytes. None of these nonspecific stimulants of lymphocytes need prior sensitization of cell donors to be effective (Oppenheim, 1968).

Oppenheim's second class of stimulants are tissue antigens of which homologous lymphocytes (Bain et al., 1964), homologous macrophages (Marshall et al., 1966), calf and other sera (Johnson and Russell, 1965) are the most important. Products of leukocytes are also used as stimulants but the live cells are usually much more effective (Oppenheim, 1968). This group of stimulants does not fit the description of specific or nonspecific stimulants. For example, the cocultivation of lymphocytes from genetically unrelated individuals will cross stimulate, resulting in a mixed lymphocyte response (MLR) (Hirschhorn and Hirschhorn, 1974).

The MLR does not require presensitization (Wilson et al., 1967), but it does require the recognition of genetic differences between the two populations of lymphocytes giving genetic specificity to the reaction.

Specific antisera stimulators make up the third class of mitogens. Antisera prepared against the lymphocytes themselves or against serum proteins are also very vigorous in vitro stimulators (Grasbeck et al., 1963). Rabbit antirabbit immunoglobulin allotypes and sheep antirabbit
immunoglobulin (Sell and Gell, 1965) have been demonstrated as antisera type stimulants.

Oppenheim (1968) has termed the fourth class of mitogens specific antigen stimulants because they require previous lymphocyte sensitization of the donor by a given mitogen. This is the largest class of stimulants and will continue to grow even larger as more antigens are tested as to their specific ability to transform sensitized lymphocytes. Some of the more widely studied stimulants in this class are ragweed pollen (Lycette and Pearmain, 1963), purified protein derivative (PPD) (Marshall and Roberts, 1963), tetanus toxoid, typhoid-paratyphoid vaccine, diphtheria toxoid (Elves et al., 1963), *Haemophilus influenza* (Alford, 1973), *Neisseria gonorrhoeae*, *Neisseria catarrhalis* (Esquenazi and Streitfeld, 1973), *Mycoplasma pneumonia* (Fernald, 1972), *Mycoplasma mycoides var. mycoides* (Roberts et al., 1973), Australia antigen (Laiwah et al., 1973), vaccinia virus (Elves et al., 1963), Epstein-Barr virus (Gerber and Lucas, 1972), herpes simplex (Rosenberg et al., 1972), mumps virus (Smith et al., 1972), Sindbis virus (Griffin and Johnson, 1973), and rabies virus (Wiktor et al., 1974).

The mechanism involving the activation of lymphocytes and their subsequent transformation by specific antigen is presently unsettled but is an area of active research. The
need for macrophages and their interaction in lymphocyte cultures that are specifically stimulated has been demonstrated repeatedly (Hersh and Harris, 1968; Seeger and Oppenheim, 1970; Waldron et al., 1973). Waldron et al. (1973) using highly purified lymphocytes obtained from the lymph nodes of guinea pigs immunized with complete Freund's adjuvant, demonstrated no response upon stimulation with PPD, but a good response with PHA. When macrophages were added to these immune lymphocyte cultures there was a marked enhancement of the PPD-induced lymphoproliferative response. These workers concluded that macrophages play an obligatory role in the presentation of antigen to immunospecific T cells but apparently have no function in the lymphoproliferative response to nonspecific mitogens such as PHA.

The actual manner in which T cells recognize antigens is unclear. B lymphocytes have detectable membrane immunoglobulin receptors that are capable of binding the antigen for which the cell is specific (Davie and Paul, 1971; Davie et al., 1971). However T cells probably rely on some means other than immunoglobulins as receptors (Rosenthal and Shevach, 1973). Some workers have observed immunoglobulin on the membrane of T lymphocytes (Marchalonis et al., 1972; Hammering and Rajewsky, 1971) whereas others have failed to detect these immunoglobulins in significant
quantities (Vitetta et al., 1972; Perkins et al., 1972).

Cohen et al. (1973) and Waldron et al. (1974) have demonstrated that the obligatory role of the macrophage, in sensitized lymphocyte cultures stimulated with specific antigen, is one of binding and presenting the antigen to the as yet unidentified receptor on the lymphocyte. Cohen et al. (1973) have concluded that there are at least two mechanisms of antigen binding to the macrophage surface. One involves sites on the macrophage of low avidity which are effective only at relatively high antigen concentrations and which appear not to be immunoglobulins. The other sites involve cytophilic antibody which in hapten-carrier systems is hapten-specific and of high avidity. The latter system is the primary means by which small amounts of antigen are concentrated by immune macrophages in quantities sufficient to stimulate lymphocytes (Cohen et al., 1973). Waldron et al. (1974) have demonstrated that after binding of the antigen to immune macrophages, there is a metabolic dependent sequestration of bound antigen by the macrophages. The binding and the sequestration of the bound antigen in or on the macrophage requires approximately 1 hour (Waldron et al., 1974).

Lipsky and Rosenthal (1973) demonstrated that the interaction between the macrophage and lymphocyte must involve physical interaction in order to manifest an antigen-
dependent immune response in vitro. This demand for actual physical contact between these two cells is the purpose for using rounded bottom wells or tubes for in vitro cultivation of immune lymphocytes in order to provide close proximity of reactant cells (Rosenthal; Lipsky and Rosenthal, 1973).

Less than 2 percent of the lymphocytes, upon stimulation with antigen in vitro, respond to a given antigen by transformation into blast-like cells (Valentine, 1971). These transformed lymphocytes undergo a clonal proliferation to yield the much larger number of transformed cells seen when cells are examined on the 4th and 5th day (Marshall et al., 1969). Because this proliferation serves to magnify the initial response of a small number of cells to antigen, a determination on day 4 of the incorporation of radio-labelled thymidine into a DNA preparation has served as a convenient measurement of the initial immunological reaction (Valentine, 1971). In comparison, the receptor to nonspecific mitogens such as PHA and Con A are much more universal (Hirschhorn and Hirschhorn, 1974) and therefore many more lymphocytes are initially stimulated by these mitogens. Jones (1973) reported that PHA induced 11 to 26 percent and

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Con A induced 29 to 46 percent of the initial population of lymphocytes to proliferate.

DNA synthesis begins by 24 hours after PHA stimulation and peaks between 48 and 72 hours, however stimulation of sensitized lymphocytes with specific antigens cause a maximum response between the 3rd and 4th day (Hirschhorn and Hirschhorn, 1974). Marshall et al. (1969) reported that antigen stimulated cultures have a generation time of 8 to 14 hours by the 3rd day of cultivation. Under the usual conditions of culture, the lymphocytes will continue to divide up to 5 times before dying (Hirschhorn and Hirschhorn, 1974).

Adler et al. (1970) have reported on the media and cultural conditions required for optimal in vitro stimulation of mouse lymphoid cells. These workers demonstrated that RPMI 1640 medium supplemented with 5.0 percent fresh, heat inactivated, human serum provided the best medium for mouse lymphocytes. Adler et al. (1970) reported that human serum varies considerably in its capacity to support lymphocyte stimulation and recommended that serum from a single donor be used for each complete experiment.

These workers also reported that inactivation of serum at 56 C for 30 minutes was required to negate the cytotoxic effect of human serum upon mouse lymphocytes. Storage of human serum at 4 C or 20 C for any significant period of time
resulted in a medium which gave undependable and variable degrees of stimulation (Adler et al., 1970). A protein-free medium supplemented with either 0.5 percent isologous or homologous serum has also been used for mouse lymphocyte cultures (Peck and Click, 1973).

Cellular and Humoral Immune Responses to Rabies and Sindbis Virus

Griffin and Johnson (1973) have demonstrated the ability of Sindbis virus to elicit both a strong cellular and humoral response in mice. These workers reported that sensitization of the mice with live virus was necessary for the primary response but not for the secondary response. The Sindbis virus antigen used for in vitro stimulation was more effective when given live than when ultraviolet light (UV) inactivated virus was used. The UV inactivation resulted in a 15-20-fold reduction of the response (Griffin and Johnson, 1973). Rosenberg et al. (1972), on the other hand, have demonstrated that infectious herpes simplex virus (HSV) markedly depressed the ability of viral antigens to stimulate lymphocytes, thus requiring the use of inactivated HSV antigen to stimulate in vitro lymphocyte cultures. These authors theorize that viruses that replicate in macrophages and/or lymphocytes might depress the ability of these cells to respond in vitro. The obligatory role of both the macrophage and lymphocyte in
the lymphocyte response to specific antigen has been discussed previously.

Griffin and Johnson (1973) demonstrated that Sindbis virus sensitized lymphocytes were present in draining lymph nodes after footpad inoculation with Sindbis virus by day 3-4 and the in vitro stimulation response of these lymphocytes was maximal on day 6. The cells in these draining lymph nodes showed a rapid decline in specific incorporation of tritiated thymidine (\(^{3}\text{H}-\text{Tdr}\)) in response to virus antigen and reached control levels by 16 days postinoculation. These workers also demonstrated a secondary response in the draining lymph node cells resembling the primary response in magnitude but slightly accelerated in its occurrence.

The humoral response to Sindbis virus was also maximal on day 6, dropping slightly by day 8. The secondary humoral response was greater in magnitude than the primary response (Griffin and Johnson, 1973). Like the cellular response, there was little or no humoral response when the primary sensitizing antigen was inactivated virus. And again like the cellular response, the secondary humoral response could be elicited with inactivated virus following a primary sensitization with live Sindbis virus.

Bell et al. (1966), attempting to differentiate between animals previously vaccinated against rabies from those recovering from a rabies infection made use of a phenomenon
involving rabies virus called cerebro-neutralization. This is a phenomenon first described by Kubes and Gallis (1944) whereby brain tissue from rabies infected, vaccinated or recovered animals neutralize rabies virus. Bell et al. (1966) described cerebro-neutralizations as an autosterilizing property which is possibly responsible for abortive rabies infection.

Although these workers did not specifically define the mechanism of this phenomenon, they did suggest that cell-mediated immunity, circulating antibody or antibody produced locally in the brain may be responsible for cerebro-neutralization. The active component of cerebro-neutralization has been referred to as either rabies neutralizing substance (RNS) (Gough et al., 1974) or rabies inhibitory substance (RIS) (Wilsnack and Parker, 1966). Gough et al. (1974) demonstrated that RNS is circulating antibody that has probably crossed the blood-brain barrier. These workers suggested that the increased permeability of the blood-brain barrier allowing antibody to cross might be a consequence of lesions produced by the encephalitis that occurs with rabies infection. This work by Gough et al. (1974) significantly weakens the possibility that cell-mediated immunity is involved in cerebro-neutralization.

Turner (1973) in an attempt to determine the mechanisms of resistance provided by rabies vaccines in mice was unable
to demonstrate cell-mediated immunity to rabies virus. This worker passively transferred spleen cells or serum from immune mice and then challenged the recipients as a means to determine cell-mediated or humoral immune responses. Turner (1973) was able to demonstrate that both vaccinia immune serum and spleen cells significantly protected suckling mice against lethal infection with vaccinia virus. However, rabies immune serum conferred protection against rabies while transferred rabies immune spleen cells provided no protection against rabies.

At the initiation of the research, reported in this dissertation, no one had demonstrated either *in vivo* or *in vitro* cell-mediated immunity to rabies virus although the possibility of such immunity had been alluded to. The World Expert Committee on Rabies (1973) suggested that the time elapsing between the processing of rabies antigen derived from vaccine and the stimulation of cell-mediated immunity might be crucial for the protection of a rabies exposed individual.

Recently Wiktor *et al.* (1974) demonstrated *in vitro* cell-mediated immunity to rabies virus using spleen lymphocytes from rabbits immunized with live or inactivated rabies virus. These workers reported that they could detect no difference when either live or inactivated viruses were used for immunization of rabbits or for stimulation of spleen
lymphocytes. Wiktor et al. (1974) were able to demonstrate a maximum stimulation ratio 8 days after immunization with rabies virus. The response was still present at 175 days after inoculation but it was relatively low at that time. The lymphoproliferative response could be obtained with a minimum of $10^{5.5}$ pfu per 0.2 ml of virus. These workers also demonstrated lymphocyte cultures obtained from rabbits immunized with the ERA strain of rabies were stimulated equally with the same virus or with high egg passage (HEP) or challenge virus strain (CVS) rabies virus. Wiktor and his coworkers also demonstrated that purified virions, virus glycoprotein preparations and "soluble antigen" were capable of stimulating sensitized spleen lymphocytes. Nucleocapsids had no stimulatory effect on blast transformation.

Wiktor et al. (1974) summarized their findings by stating, "Whereas antirabies antibodies play an important part in protecting animals against rabies, the role of the immune cells in either protection of the animal or human or as a component of some possible immunopathogenic phenomenon is still unclear."

Without sounding apologetic, this author would like it understood that this literature review in no way is intended to be a complete review of cell-mediated immunity. Volumes have been written on this subject and current
research in this area is voluminous. As research progresses, more questions arise. But with the answers to these questions we increase our basic understanding of one of Mother Nature's greatest feats, the immune system. The author has attempted to review some of the most recent literature as well as give a general understanding of cell mediated immunity as it will apply to the following research.
MATERIALS AND METHODS

Cell Cultures

Porcine kidney_{2A} cells

The pig kidney_{2A} (PK_{2A}) cell line was used for the tissue-culture propagation of the ERA strain of rabies virus, for serum-virus-neutralization tests (SVN) to determine anti-rabies antibodies in mouse serum and for titration of the ERA strain of rabies virus originating from suckling mouse brains and tissue-culture fluids. The PK_{2A} cells were propagated at 37°C in 250 ml plastic tissue-culture flasks^1 or in glass roller bottles. The growth medium consisted of minimum essential medium^3 (MEM) (Eagle) with Earle's salts and L-glutamine plus 5 percent fetal calf serum and 0.22 percent sodium bicarbonate. The medium was adjusted with CO_{2} to approximately pH 7.2 before use. Monolayers of cells were trypsinized with Trypsin-EDTA^4 (1X), diluted 1 to 4 in complete MEM and dispensed into plastic tissue-culture flasks.

^1 Falcon Plastics, Los Angeles, California.

^2 Bellco Glass Inc., Vineland, New Jersey.

^3 Grand Island Biological Co., Grand Island, New York.

^4 Difco Laboratories, Detroit, Michigan.
plates for virus titration or SVN tests, or into flasks or roller bottles for ERA rabies virus propagation.

**Chicken embryo fibroblast cell cultures**

Chicken embryo fibroblast (CEF) cell cultures were used for the propagation and titration of Sindbis virus and for SVN tests to determine mouse anti-Sindbis antibody titers. A modification of the method described by Cunningham (1955) was used to prepare the CEF cell cultures. Ten-day-old embryos were removed from the shell and washed in warm Hanks' balanced salt solution (BSS). The head, legs, wings, and viscera were removed and discarded. The remaining embryonic tissue was washed twice in warm Hanks' BSS and then forced through a 10 ml plastic syringe into a fluted-side Erlenmeyer flask containing a Teflon-covered stirring bar and 0.25 percent trypsin in Hanks' BSS, pH 8.0-8.4. Twenty ml of trypsin solution was used for each embryo. The tissues were trypsinized for 30 minutes at room temperature and then poured through 2 layers of sterile cheese cloth into 50 ml conical screw-capped tubes. The cell suspension was centrifuged for 5 minutes at 1500 revolutions per minute (RPM). The supernatant was discarded, the pellet was resuspended in Hanks' BSS and centrifuged as

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1 Linbro Chemical Co., New Haven, Connecticut.
before. This washing process was repeated twice and the pellet was then resuspended, at the rate of 1 ml of packed cells per 200 ml of Medium 199\(^1\) with Hanks' salts and L-glutamine supplemented with 10 percent fetal calf serum and 0.35 percent sodium bicarbonate. The CEF cell suspension was dispensed into 60 x 15 mm plastic tissue culture dishes,\(^2\) in 5.0 ml volumes, for Sindbis virus titration and SVN tests as stated previously. The CEF cell cultures were grown in 250 ml plastic tissue culture flasks\(^2\) for Sindbis virus propagation.

**BHK-21/13S cells**

The baby-hamster-kidney 13S cloned cell line (BHK-21/13S) was used for the propagation of the high egg passage (HEP), Flury strain, rabies virus. The BHK-21/13S cells were propagated at 37 C in glass roller bottles. The growth medium consisted of BHK-21 medium with L-glutamine\(^3\) supplemented with 10 percent tryptose phosphate broth,\(^3\) 10 percent fetal calf serum and 0.22 percent sodium bicarbonate. The pH in each roller bottle was adjusted with CO\(_2\) to approximately 7.2 before incubation.

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\(^1\)Grand Island Biological Co., Grand Island, New York.

\(^2\)Falcon Plastics, Los Angeles, California.

\(^3\)Grand Island Biological Co., Grand Island, New York.
Viruses

Propagation of the ERA strain of rabies virus in PK\textsubscript{2A} cells

The ERA strain\textsuperscript{1} of rabies virus was received at the 177 passage level on PK\textsubscript{2A} cells. This virus was propagated to the 179th passage, concentrated and purified as described later, and used as the ERA rabies virus lymphocyte stimulating antigen.

Three-day-old PK\textsubscript{2A} cell monolayers grown in glass roller bottles were rinsed with warm Dulbecco's phosphate buffered saline (PBS) before inoculating. The virus was absorbed at 37°C for 90 minutes after which time the inoculum was removed and 100 ml of MEM supplemented with 3 percent fetal calf serum was added. The tissue-culture fluids were harvested after incubation at 37°C for 96 hours. These fluids were centrifuged at 1000 g for 10 minutes to remove the cellular debris and the supernatant fluid was stored at -70°C until the purification procedure.

\textsuperscript{1}Obtained from Dr. A. Strating, Animal and Plant Health Inspection Service, Ames, Iowa.
Propagation of HEP (Flury strain) rabies virus in BHK-21/13S cells

High egg passage (Flury strain)\(^1\) rabies virus was propagated on confluent 3-day-old monolayers of BHK-21/13S cells grown in glass roller bottles. The monolayers were washed once with warm Dulbecco's PBS before inoculating each roller bottle with this virus. The virus was absorbed at 37 °C for 90 minutes after which time the inoculum was removed and 100 ml of maintenance medium was added. The maintenance medium was similar to the growth medium used for the propagation of the BHK-21/13S cells except that it contained 0.4 percent bovine serum albumin instead of fetal calf serum. The HEP rabies virus infected cells were incubated at 34 °C for 96 hours before harvesting. The tissue-culture fluids were centrifuged at 1000 g for 10 minutes to remove the cellular debris and the supernatant fluid was stored at -70 °C until the purification procedure.

Suckling mouse adapted rabies virus, ERA strain

The 178th passage of the ERA strain of rabies on PK\(_{2A}\) cells was adapted to suckling mouse brain serially passing the virus in 2-day-old suckling mice. The first passage mice received \(10^{4.6}\) plaque-forming units (PFU) of the ERA virus.

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\(^1\) Obtained from Dr. R. E. Dierks, Veterinary Research Institute, Iowa State University, Ames, Iowa.
strain of rabies virus. Brains were harvested when the mice showed clinical signs of infection. The brains were ground in a mortar with a pestle with the aid of a carborundum abrasive. A 20 percent suspension was made in cold Dulbecco's PBS containing 100 units of penicillin and 100 μg of streptomycin per ml. This suspension was centrifuged at 12,000 g for 15 minutes and a 1 to 100 dilution of the supernatant was used as the inoculum for the subsequent passages. The supernatant from the 4th suckling mouse brain passage was used to sensitize mice to the ERA strain of rabies virus.

Propagation of Sindbis virus in CEF cells

Strain AR3391 of Sindbis virus was received at an undetermined passage level in CEF cells. Twenty-four hour CEF monolayers in 250 ml plastic tissue-culture flasks were rinsed with warm Dulbecco's PBS before being inoculated with Sindbis virus. The virus was absorbed at room temperature for 90 minutes, aspirated off and Medium 199 supplemented with 2 percent fetal calf serum was added. Tissue-culture fluids were collected after incubating at 37 C for 48 hours. The fluids were centrifuged at 1000 g for 10 minutes to remove the cell debris and stored at -70 C until

1 Obtained from Dr. D. E. Griffin, Department of Neurology, The Johns Hopkins University School of Medicine, Baltimore, Maryland.
used to sensitize mice to Sindbis virus. Tissue-culture fluids from uninfected CEF monolayers were prepared in the same manner and used to inoculate control mice.

Virus Purification

Both ERA and HEP rabies virus suspensions were purified before being used for antigen stimulation of sensitized lymphocytes. The purification technique is similar to that reported by Obijeski et al. (1974) with minor modifications due to different laboratory equipment available. Tissue-culture fluids containing either ERA or HEP rabies virus were harvested from cell cultures or thawed from -70 C storage and centrifuged at 4 C for 30 minutes at 8000 x g to remove the cellular debris. The supernatant fluids were mixed with sodium chloride (NaCl) polyethylene glycol (PEG) at the ratio of 23 gm NaCl and 70 gm PEG per liter. This mixture was stored at 4 C for 3 hours after which the precipitate was collected by centrifuging at 10,000 g for 30 minutes at 4 C. The precipitate was drained and resuspended in 30 ml TSE buffer (0.01 M-tris, pH 7.4, 0.15 M-NaCl and 0.002 M-EDTA) and homogenized with a Tenbroeck tissue grinder. The suspension was clarified by centrifuging at 5000 g for 10 minutes. The resulting supernatant fluid was layered over 5 ml of 30 percent (w/v) sucrose in TSE buffer and centrifuged for 3 hours at 25,000 RPM in a Spinco SW 37
rotor. The pellets were suspended in 6 ml TSE buffer, homogenized, clarified by centrifugation, then layered on a 14 ml combination density:viscosity gradient of potassium tartrate and glycerol in TE buffer (0.01 M-tris, pH 7.4 and 0.002 M-EDTA). Virus preparations were centrifuged to equilibrium at 25,000 RPM for 18 hours at 4 C in a Spinco SW 27 rotor and the virus band was removed with a pipette and dialyzed for 3 hours at 4 C against 4 liters of TE buffer. After a second similar centrifugation, the virus band was collected and dialyzed overnight at 4 C against PBS (pH 7.7). Tissue culture fluids from uninfected PK 2A were prepared in the same manner and used as a control stimulant.

Using this procedure one obtains approximately 100-fold reduction in volume and a corresponding 100-fold increase in virus titer. Purity was ascertained by the lymphocyte stimulation response to control cultures.

**Nutrient Agar-Overlay Medium**

A double concentration of MEM with Earle's salts supplemented with 6 percent fetal calf serum and 0.12 percent sodium bicarbonate was added to an equal volume of 1.8 percent "Lonagar" #2. Just prior to using this medium 10,000 units of penicillin and 10,000 µg of streptomycin were

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1Oxoid marketed by Colob Laboratories, Inc., Chicago Heights, Illinois.
added to each 100 ml. The final concentration of each component was 0.9 percent agar, 3 percent fetal calf serum and 0.05 percent sodium bicarbonate.

Neutral-Red Agar-Overlay Medium

A neutral-red agar-overlay medium was added to the tissue culture plates used for Sindbis SVN tests and Sindbis virus titrations to aid in the discernibility of plaques. This medium contained 1 percent "lonagar" #2 and 10 mg of neutral red per 100 ml of medium.

Carboxymethyl Cellulose-Minimum Essential Medium Overlay

A semisolid overlay medium was used to overlay rabies virus, ERA strain, infected monolayers of PK2A cells used in SVN and virus titration tests. This overlay medium was prepared by mixing equal volumes of double strength MEM (Eagle's) and 1.5 percent sterile carboxymethyl cellulose\(^1\) (CMC). This medium was supplemented with 3 percent fetal calf serum, 0.12 percent sodium bicarbonate, 100 units of penicillin and 100 μg of streptomycin per ml.

\(^1\)Hercules Inc., Wilmington, Delaware.
Serum-Virus-Neutralization Tests

**Serum-virus-neutralization test for rabies antibodies**

Mouse antirabies antibody titers were determined using a method described by Strating et al. (1975) with slight modifications. Mouse serum was inactivated at 56°C for 30 minutes and serial 2-fold dilutions were made using cold Dulbecco's PBS as the diluent. To these serum dilutions, an equal amount of previously titered ERA virus suspension was added that would produce approximately 75 PFU per plate. The serum-virus mixture was incubated at room temperature for 60 minutes before being inoculated onto confluent monolayers of PK2A cells. The tissue-culture medium was aspirated from the plate and each plate was rinsed with warm Dulbecco's PBS. One-half ml of each dilution of the serum-virus suspension was then inoculated into each of two 35 x 10 mm wells of a plastic tissue-culture plate, containing the 3-day-old monolayers of PK2A cells. The unneutralized virus was allowed to absorb at room temperature for 90 minutes after which time the inoculum was removed. The inoculated monolayers were rinsed once with incomplete MEM and then overlaid with CMC-MEM overlay medium. The tissue-culture plates were again incubated at 37°C in 5 percent CO2 for 4 to 5 days depending on when plaques could be seen by the naked eye. Care was taken to avoid disturbing the plates during this
later incubation period as this overlay medium is a semi-solid and plaque formation could be distorted if the plates were jarred.

When plaques were visible, the overlay was aspirated off and the monolayers of cells were rinsed twice with warm PBS pH 7.6. To better delineate plaques the monolayers were then stained by adding 2 ml of 0.05 percent neutral-red in PBS pH 7.6 per well. The plates were incubated at 37 C for 2 hours, after which time the excess stain was removed and the plaques counted and recorded. The antibody titers were expressed as the reciprocals of the serum dilutions which produced 50 percent reduction in the number of viral plaques when compared with the number of plaques in the control plates.

**Serum-virus-neutralization test for Sindbis antibodies**

The mouse serum containing anti-Sindbis antibodies was inactivated at 56 C for 30 minutes and serial 2-fold dilutions were made as described for rabies SVN tests. Sindbis virus, previously titrated, was diluted so that the final concentration would produce approximately 75 PFU per well. Equal amounts of virus and serum were mixed and incubated at room temperature for 60 minutes. One-half ml of each dilution of the serum-virus mixture was then inoculated into each of two 60 x 15 mm plastic tissue-culture plates.
containing 24 hour confluent monolayers of CEF cells which had previously been rinsed with warm Dulbecco's PBS.

The virus was allowed to absorb for 90 minutes at room temperature before being aspirated. Each plate was then overlayed with 5 ml of nutrient agar-overlay medium. The plates were incubated at 37 C in a 5 percent CO₂ atmosphere for 48 hours. To aid in the differentiation of plaques, 2 ml of a neutral-red agar-overlay was added to each plate. The plates were incubated in the dark at room temperature for an additional 6 hours before the plaques were counted. The antibody titers were expressed as the reciprocal of the serum dilutions which produced 50 percent reduction in the number of viral plaques when compared with the number of plaques in the control plates.

Virus Titrations

Ten-fold serial dilutions of the virus to be titrated were made in cold Dulbecco's PBS. One-half ml of each dilution of ERA rabies virus was inoculated into each of two 35 x 10 mm wells of a plastic tissue-culture plate containing a 3-day-old confluent monolayer of PK₂A cells. The virus was absorbed, inoculum removed, CMC-MEM overlay medium added, plates incubated and monolayers stained as described for the rabies SVN test. The plaques were counted and the titers of the virus were expressed in PFU per ml of virus
suspension.

One-half ml of each dilution of Sindbis virus was inoculated into two 60 x 10 mm plastic tissue-culture plates containing a 24 hour confluent monolayer of CEF cells. The adsorption, addition of the nutrient agar-overlay medium, incubation and staining was as described for the Sindbis SVN tests. The plaques were counted and the titers of the virus were expressed in PFU per ml of virus suspension.

Experimental Animals

All mice used in the following experiment were BALB/c inbred mice. Breeding stock was purchased commercially and the 6-to-8-week-old mice used for lymphocyte stimulation studies were raised at the Veterinary Medical Research Institute.

Preparation of Lymphocyte Culture Additive

Phytohemagglutinin

Phytohemagglutinin-P² (PHA) was reconstituted with RPMI-1640 medium³ to give 10 μg per 10 μl, the quantity

1 Obtained from Carworth Division of Becton, Dickinson and Co., New City, New York.
2 Difco Laboratories, Detroit, Michigan.
3 Grand Island Biological Co., Grand Island, New York.
used per ml of cells in all experiments unless stated otherwise in the text.

**Sindbis virus lymphocyte stimulating antigen**

Sindbis virus antigen used for lymphocyte transformation was prepared as a 20 percent suspension of suckling mouse brain from 4-day-old BALB/c mice that were infected intracerebally with Sindbis virus approximately 40 hours prior to harvest. Brains from infected mice were removed, ground in a mortar with a pestle with the aid of an abrasive and diluted up to a 20 percent suspension with Dulbecco's PBS. This virus suspension was clarified by centrifugation at 12,000 g for 15 minutes to eliminate the toxicity reported by Griffin and Johnson (1973). A 10 μl volume of this preparation contained 10^{7.4} PFU Sindbis virus. Normal suckling mouse brain (SMB) was prepared in an identical manner as a control stimulant. Both preparations were stored at -70 C in 0.2 ml aliquots until used, at which time they were thawed and added to the lymphocyte cultures.

**Rabies virus lymphocyte stimulating antigen**

Rabies virus, strains HEP and ERA, which had been propagated in tissue-culture, purified and stored at -70 C were thawed just prior to being added to the lymphocyte cultures. The ERA strain contained 10^{6.0} PFU per 10 μl and the HEP strain contained 10^{7.1} PFU per 10 μl.
Rabies virus (HEP) ribonucleoprotein

Ribonucleoprotein (RNP) was isolated from BHK-21 cells infected with the HEP strain of rabies virus. The procedure used for the isolation and purification of the RNP has been described previously (Schneider et al. 1973). This RNP preparation was used as a stimulant in order to partially characterize that portion of the virus which is responsible for the in vitro stimulation of rabies virus sensitized lymphocytes.

Mouse Inoculation—Sensitization

In order to sensitize mice to rabies or Sindbis virus, the animals were inoculated with $10^{5.5}$ PFU of the 4th SMB passage rabies virus, ERA strain, or $10^{4.3}$ PFU of the tissue-culture propagated Sindbis virus. Mice were anesthetized with ether and 0.025 ml of virus was inoculated subcutaneously into each foot pad. Control mice for rabies experiments were inoculated with an equal amount of normal suckling mouse brain diluted to contain the same amount of brain tissue as in the virus suspension. Control mice for Sindbis virus sensitization were inoculated with a quantity of CEF tissue-culture fluid equal to that contained in the Sindbis virus suspension.
Click's Mouse Lymphocyte Medium

Click's (CM-10) EHAA medium was used to culture mouse lymphocytes (Katz-Heber et al., 1973). This medium was supplemented with 0.5 percent homologous mouse serum. Experiments were conducted comparing results using this medium with results using RPMI-1640. Mouse serum used to supplement this medium was prepared as described by Peck and Click (1973). Mouse blood collected by cardiac puncture was allowed to clot at room temperature. The clot was loosened from the sides of the tube and then kept at 4°C for one-half hour. The blood was centrifuged for 10 minutes at 400 g and the serum was aspirated being careful to exclude red blood cells. The serum was immediately used in the Click's medium.

Preparation of Mouse Lymphocyte Cultures

Mouse lymphocyte cultures were prepared using a modification of the technique described by Adler et al. (1970) and Griffin and Johnson (1973). Mice were anesthetized with ether and exsanguinated by intracardiac puncture. The serum was saved for serological tests. Lymph nodes that drain the foot pad area (axillary, brachial and popliteal) were aseptically removed and washed in RPMI-1640 medium. Lymph node cells

1Altick Associated, 4201 Odana Road, Madison, Wisconsin.
were obtained by teasing the nodes apart in RPMI-1640 medium with two 27 gauge hypodermic needles attached to 3cc syringes. The cell suspension was further dispersed by gently aspirating and expelling the suspension through the 27 gauge needles. This process was repeated 3 times. For each study, pools of draining lymph node cells from 3 to 4 mice were used. The cell suspensions were washed 2 times by centrifuging at 100 g for 10 minutes and resuspending in incomplete RPMI-1640 medium. After the second centrifugation, the cell pellet was resuspended in RPMI-1640 (pH 7.1) supplemented with 5 percent fresh heat-inactivated human serum, 1 mM HEPES buffer, 2 mM L-glutamine, 100 units penicillin and 100 μg streptomycin per ml. The human serum for each experiment was from the same donor. All complete medium prepared as described above was used the same day. Cell concentrations were determined manually using commercial blood diluters\(^1\) and a hemocytometer. Using this method of preparation these lymph node cell suspensions usually consisted of 95 percent or higher mononuclear cells of which approximately 90 percent were viable as determined by the trypan blue exclusion technique. All cell suspensions were diluted to give \(1 \times 10^6\) leukocytes per ml unless stated otherwise.

\(^{1}\)Unipette, Becton, Dickinson and Co., Rutherford, New Jersey.
Antigens or PHA were added at the rate of 10 μl per culture and were incorporated into the cultures at the initiation of the incubation period.

Triplicate cultures, each containing 0.2 ml of the lymphocyte cell suspension, were prepared for each of the test antigens, PHA and control cultures. Lymphocyte cultures were incubated in U-bottom plastic microtiter plates at 37 C in a 5 percent CO₂ humidified atmosphere for 78 hours. At the end of this incubation, 1 μ Ci of tritiated thymidine (³H-Tdr) with a specific activity of 6.7 Ci/mM, in a volume of 0.05 ml, was added to each culture. These radioactively pulsed cultures were incubated for an additional 18 hours making a total of 96 hours that the lymphocytes were in culture.

Termination of Lymphocyte Cultures for DNA Analysis

At the termination of the 96 hours of incubation the DNA from the cultured lymphocytes was harvested using a semi-automatic multiple-sample cell harvester (Hartman et al.,

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¹Cook Engineering Co., Alexandria, Virginia

²New England Nuclear Corp., Boston, Massachusetts.

³Otto Hiller Co., Box 1294, Madison, Wisconsin.
Lymphocytes were aspirated onto glass fiber filters\textsuperscript{1} previously soaked with a 4 mg/ml solution of thymidine, and rinsed with PBS saline pH 7.6. The cultures were then precipitated with cold 10 percent trichloroacetic acid (TCA) followed by a washing with absolute ethanol. The filters containing the TCA precipitated material were removed from the harvester and placed in glass scintillation counting vials and dried at room temperature overnight. Ten ml of scintillation fluid (5 gm 2.5-Diphenyloxazole/liter of toluene) was added to each counting vial. The $^{3}$H-TdR incorporated into TCA-precipitable material was counted in a Beckman model LS-230 liquid scintillation counter.\textsuperscript{2}

\textsuperscript{1}Catalogue #934 AH, Reeve Angel Co., Clifton, New Jersey.

\textsuperscript{2}Model LS-230, Beckman Instruments Inc., Scientific Instrument Division, Fullerton, California.
RESULTS

Kinetics of the Microtiter In Vitro Mouse Lymphocyte Stimulation Test

In order to determine that all lymphocyte preparations were capable of being transformed in vitro, each preparation of lymphocytes was stimulated with PHA. The optimum concentration of PHA and optimum concentration of cells per ml of culture fluid were determined (Table 1, Figure 1). As the concentration of cells increased the incorporation of $^3$H-Tdr in unstimulated control cultures increased causing a proportionately lower stimulation ratio (PHA stimulated culture divided by unstimulated culture). High concentrations of PHA are toxic to the cultures and each lot of PHA has a different activity. Therefore, once the optimum PHA concentration was determined for a particular lot of PHA, enough of that particular lot was diluted and frozen in small aliquots for all subsequent experiments. The optimum cell concentration using 0.2 ml of cells per culture was $1.0 \times 10^6$ cells per ml (Table 1) and the optimum PHA concentration for such culture was 3 $\mu$g per ml (Table 2, Figure 2).
Table 1. Counts per minute of incorporated $^3$H-thymidine and indices of stimulation (S/R) of varying concentration of mouse lymph node lymphocytes stimulated with varying amounts of PHA

<table>
<thead>
<tr>
<th>PHA Concentration ($\mu$g/ml)</th>
<th>$1 \times 10^6$ cells</th>
<th>$2 \times 10^6$ cells</th>
<th>$4 \times 10^6$ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ave cpm $^a$</td>
<td>Range cpm</td>
<td>S/R $^b$</td>
</tr>
<tr>
<td>0</td>
<td>820</td>
<td>(703-905)</td>
<td>1,002</td>
</tr>
<tr>
<td>2</td>
<td>11,015</td>
<td>(6,209-16,762)</td>
<td>13.4</td>
</tr>
<tr>
<td>10</td>
<td>69,560</td>
<td>(60,436-77,280)</td>
<td>84.8</td>
</tr>
<tr>
<td>40</td>
<td>29,934</td>
<td>(25,406-34,022)</td>
<td>36.5</td>
</tr>
</tbody>
</table>

$^a$ cpm = counts per minute.

$^b$ counts per minute of the stimulated culture / average counts per minute of the control culture = stimulation ratio.
Figure 1. PHA dose-response curve with mouse lymph node lymphocyte cultures of differing cell concentrations
Table 2. Dose response of mouse lymph node lymphocytes (1 x 10⁶ cells/culture) stimulated with PHA

<table>
<thead>
<tr>
<th>PHA Concentration μg/ml</th>
<th>cpm\textsuperscript{a} of Incorporated $^3\text{H}$-Tdr</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>877</td>
</tr>
<tr>
<td>1.5</td>
<td>1,369</td>
</tr>
<tr>
<td>3.0</td>
<td>38,115</td>
</tr>
<tr>
<td>7.5</td>
<td>31,176</td>
</tr>
<tr>
<td>15.0</td>
<td>28,810</td>
</tr>
<tr>
<td>150.0</td>
<td>6,915</td>
</tr>
</tbody>
</table>

\textsuperscript{a}cpm = counts per minute.
Figure 2. PHA dose-response curve using \(1 \times 10^6\) mouse lymph node lymphocytes per culture
Stimulation of Sensitized Lymphocytes by Sindbis Virus

The ability of Sindbis virus to stimulate specifically sensitized mouse lymphocytes from draining lymph nodes is illustrated in Table 3. Sindbis virus antigen, propagated in SMB, and SMB antigen were incubated with lymphocytes from Sindbis inoculated mice. Sindbis virus antigen was also incubated with lymphocytes from control mice inoculated with CEF tissue culture. The stimulation of specifically sensitized lymphocytes by Sindbis virus, the lack of stimulation of these cells by SMB antigen and the fact that nonsensitized cells were not stimulated by Sindbis virus antigen indicated that this transformation reaction was specific.

The peak incorporation of $^3$H-Tdr into cultured sensitized lymphocytes by Sindbis virus antigen was determined to be 10 days. The temporal response of this reaction was determined over a 30 day period (Figure 3). Sensitized lymphocytes were possibly present in the draining lymph nodes of inoculated mice by day 2 and were obviously present by day 3. After the peak incorporation of $^3$H-Tdr into specifically sensitized lymphocytes in response to the Sindbis virus antigen, there was a rapid decline in responsiveness of these cells to control or nearly control levels by day 20.

By comparison (Figure 3) the humoral response, as indicated by titers of circulating neutralizing antibodies, in mice inoculated subcutaneously with Sindbis virus appeared
Table 3. Stimulation in vitro of mouse lymph node lymphocytes by Sindbis antigen after in vivo sensitization with Sindbis virus

<table>
<thead>
<tr>
<th>Days After Sindbis Inoculation</th>
<th>Lymphocytes From Sindbis Sensitized Mice</th>
<th>Lymphocytes From Control Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control cpm(^a)</td>
<td>SMB Antigen cpm</td>
</tr>
<tr>
<td>1</td>
<td>471</td>
<td>543</td>
</tr>
<tr>
<td>2</td>
<td>362</td>
<td>693</td>
</tr>
<tr>
<td>3</td>
<td>341</td>
<td>434</td>
</tr>
<tr>
<td>4</td>
<td>182</td>
<td>1,216</td>
</tr>
<tr>
<td>6</td>
<td>843</td>
<td>1,990</td>
</tr>
<tr>
<td>8</td>
<td>659</td>
<td>2,268</td>
</tr>
<tr>
<td>10</td>
<td>432</td>
<td>556</td>
</tr>
<tr>
<td>12</td>
<td>384</td>
<td>624</td>
</tr>
<tr>
<td>14</td>
<td>607</td>
<td>521</td>
</tr>
<tr>
<td>16</td>
<td>1,180</td>
<td>114</td>
</tr>
<tr>
<td>18</td>
<td>442</td>
<td>585</td>
</tr>
<tr>
<td>20</td>
<td>546</td>
<td>534</td>
</tr>
<tr>
<td>30</td>
<td>489</td>
<td>543</td>
</tr>
<tr>
<td>Average</td>
<td>480</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) cpm = counts per minute.

\(^b\) counts per minute of the stimulated culture - average counts per minute of the control culture = stimulation ratio.

\(^c\) Contaminated culture.
Figure 3. Temporal development of Sindbis virus responsive cells from draining lymph nodes and of neutralizing antibody, after subcutaneous inoculation of Sindbis virus
slightly slower than did the cellular response. The neutralizing antibodies reached a peak at 8 days, slightly earlier than the peak cellular response. The neutralizing antibody titers declined from their peak of 1:6,400 to 1:3,200, remaining constant at 1:3,200 to the end of the experiment.

Stimulation ratios in this study were determined by dividing the CPM of the stimulated cultures by the average CPM of the control nonstimulated cultures. The average CPM of the control cultures was used in order to avoid the extreme variations in the stimulation ratio caused by small differences in the control.

Kinetics of Stimulation of Sensitized Lymphocytes by Rabies Antigen

Lymphocytes from draining lymph nodes of mice inoculated subcutaneously with the ERA strain of rabies virus were cultured in RPMI-1640 medium supplemented with 5.0 percent fresh human serum and in Click's EHAA medium, supplemented with 0.5 percent mouse serum. This experiment was designed to determine if a medium that did not require human serum, which is difficult to obtain, could support lymphocytes during transformation. The results of this experiment (Table 4) indicated that Click's EHAA medium failed to adequately support lymphocytes cultured in the microculture system. Stimulation
Table 4. Comparison of the responsiveness of rabies (ERA) sensitized mouse lymph node lymphocytes to rabies (ERA) stimulation when cultured in Click's EHAA medium or RPMI-1640 medium

<table>
<thead>
<tr>
<th>Days After ERA Inoculation</th>
<th>Control cpm(^a)</th>
<th>Rabies (ERA) Antigen cpm</th>
<th>S/R(^b)</th>
<th>PHA cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Click's EHAA Medium(^c)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>779</td>
<td>2,888</td>
<td>3.7</td>
<td>4,803</td>
</tr>
<tr>
<td>8</td>
<td>1,035</td>
<td>668</td>
<td>0.6</td>
<td>17,242</td>
</tr>
<tr>
<td></td>
<td>RPMI-1640 Medium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1,577</td>
<td>27,134</td>
<td>17.2</td>
<td>34,426</td>
</tr>
<tr>
<td>8</td>
<td>884</td>
<td>23,576</td>
<td>26.7</td>
<td>37,073</td>
</tr>
</tbody>
</table>

\(^a\) cpm = counts per minute.

\(^b\) Counts per minute of the stimulated culture / counts per minute of the control culture = stimulation ratio.

\(^c\) Supplemented with 0.5 percent homologous mouse serum.

Ratios of rabies stimulated lymphocytes were markedly lower than those cultured in RPMI-1640 medium. In addition, the results using Click's EHAA medium were quite variable.

Lymph node lymphocytes harvested from mice inoculated subcutaneously 6 days previously with the ERA strain of rabies virus or SMB were cultured in RPMI-1640 supplemented...
with 5.0 percent fresh human serum. Rabies (ERA) antigen or PHA was incubated with SMB sensitized lymphocytes and rabies (ERA) antigen, PK2a tissue culture fluid, or PHA was incubated with rabies sensitized lymphocytes. Lymphocyte cultures were harvested daily after an 18 hour pulse with 1 μci of $^3$H-Tdr per culture. Results of this experiment demonstrated (Table 5) that the maximum response to the non-specific mitogen, PHA, occurred before the specific response of the sensitized lymphocytes to the rabies antigen. A slight response to PHA was detected at 24 hours reaching a peak response at 3 days (Figure 4). By contrast rabies sensitized lymphocytes that were stimulated *in vitro* with rabies (ERA) antigen developed no significant uptake of $^3$H-Tdr until the third day in culture. The maximum response of specifically stimulated cultures occurred on day 4 (Figure 4). The stimulatory response of these cultures was still present at day 6.

**Effect of Concentration of Virus on the Stimulation of Sensitized Lymphocytes**

Lymphocytes from draining lymph nodes harvested from mice 7 days after subcutaneous inoculation with rabies virus were stimulated by various concentrations of inactivated rabies virus antigen. As illustrated in Table 6, stimulation was directly related to concentration of the antigen.
Table 5. Counts per minute of incorporated $^3$H-thymidine and indices of stimulation (S/R) during a six-day-culture period of lymphocytes\textsuperscript{a} from SMB and rabies (ERA) sensitized mice stimulated with rabies antigen or PHA

<table>
<thead>
<tr>
<th>Days in Culture</th>
<th>SMB Sensitized Lymphocytes</th>
<th>Rabies (ERA) Sensitized Lymphocytes</th>
<th>Rabies (ERA) Sensitized Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Antigen</td>
<td>PHA</td>
</tr>
<tr>
<td></td>
<td>cpm\textsuperscript{b}</td>
<td>cpm</td>
<td>S/R\textsuperscript{c}</td>
</tr>
<tr>
<td>1</td>
<td>1,173</td>
<td>1,094</td>
<td>0.9</td>
</tr>
<tr>
<td>2</td>
<td>941</td>
<td>816</td>
<td>0.9</td>
</tr>
<tr>
<td>3</td>
<td>1,004</td>
<td>1,035</td>
<td>1.0</td>
</tr>
<tr>
<td>4</td>
<td>958</td>
<td>1,208</td>
<td>1.3</td>
</tr>
<tr>
<td>5</td>
<td>711</td>
<td>936</td>
<td>1.3</td>
</tr>
<tr>
<td>6</td>
<td>782</td>
<td>883</td>
<td>1.1</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Sensitized lymphocytes were obtained from mice 6 days after subcutaneous inoculation with rabies (ERA) virus.

\textsuperscript{b}cpm = counts per minute.

\textsuperscript{c}counts per minute of the stimulated culture \over counts per minute of the control culture = stimulation ratio.
Figure 4. Time-course of rabies (ERA) sensitized mouse lymph node lymphocytes stimulated with SMB, rabies (ERA), PHA and no stimulant as the control.
• «CONTROL • • TISSUE CULTURE FLUID STIMULATED • RABIES (ERA) STIMULATED • PHA

$3^\text{H}$-THYMIDINE INCORPORATED (CPM)

DAYS IN CULTURE

1 2 3 4 5 6
Table 6. Stimulation of sensitized lymphocytes\(^a\) by different concentrations of rabies (ERA) viral antigen

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>PFU/culture</th>
<th>cpm(^b)</th>
<th>S/R(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabies (ERA)</td>
<td>10^6.0</td>
<td>48,943</td>
<td>51.6</td>
</tr>
<tr>
<td>Rabies (ERA)</td>
<td>10^5.5</td>
<td>22,604</td>
<td>23.8</td>
</tr>
<tr>
<td>Rabies (ERA)</td>
<td>10^5.0</td>
<td>18,774</td>
<td>19.8</td>
</tr>
<tr>
<td>Rabies (ERA)</td>
<td>10^4.0</td>
<td>6,148</td>
<td>6.5</td>
</tr>
<tr>
<td>Rabies (ERA)</td>
<td>10^3.0</td>
<td>1,506</td>
<td>1.6</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>948</td>
<td>1.0</td>
</tr>
<tr>
<td>PHA</td>
<td></td>
<td>38,115</td>
<td>40.2</td>
</tr>
</tbody>
</table>

\(^a\)Sensitized lymphocytes were obtained from mice 7 days after subcutaneous inoculation with rabies (ERA) virus.

\(^b\)cpm = counts per minute.

\(^c\)counts per minute of the stimulated culture = stimulation ratio.
Sensitized lymphocytes were stimulated with as little as $10^{4.0}$ PFU per culture. Lesser concentrations of virus did not stimulate sensitized lymphocytes.

**Stimulation of Sensitized Lymphocytes by Rabies Virus**

The ability of rabies virus to stimulate specifically sensitized mouse lymphocytes from draining lymph nodes is illustrated in Table 7. Ultraviolet light inactivated rabies (ERA) virus which had been propagated in PK$_{2A}$ cells prior to inactivation and tissue culture fluid from uninfected PK$_{2A}$ cells were incubated with lymphocytes from mice inoculated with rabies virus. Control lymphocytes from SMB inoculated mice were incubated in the presence of inactivated rabies antigen.

These results demonstrated that the transformation and incorporation of $^3$H-Tdr of rabies sensitized lymphocytes stimulated with rabies (ERA) antigen is specific. Control cultures from SMB inoculated mice did not respond to rabies (ERA) antigen and rabies sensitized lymphocytes did not respond to the control antigen, PK$_{2A}$ tissue culture fluid. However rabies sensitized lymphocytes responded dramatically to rabies antigen in vitro.
Table 7. Stimulation in vitro of mouse lymph node lymphocytes by rabies (ERA) antigen after in vivo sensitization with rabies (ERA) virus

<table>
<thead>
<tr>
<th>Days After ERA Inoculation</th>
<th>Lymphocytes From Rabies (ERA) Sensitized Mice</th>
<th>Lymphocytes From Control Mice (SMB)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control cpm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Tissue Culture Fluid cpm</td>
</tr>
<tr>
<td>2</td>
<td>552</td>
<td>1,466</td>
</tr>
<tr>
<td>3</td>
<td>561</td>
<td>997</td>
</tr>
<tr>
<td>4</td>
<td>359</td>
<td>580</td>
</tr>
<tr>
<td>6</td>
<td>1,577</td>
<td>367</td>
</tr>
<tr>
<td>8</td>
<td>884</td>
<td>802</td>
</tr>
<tr>
<td>10</td>
<td>1,191</td>
<td>967</td>
</tr>
<tr>
<td>12</td>
<td>982</td>
<td>559</td>
</tr>
<tr>
<td>14</td>
<td>1,445</td>
<td>982</td>
</tr>
<tr>
<td>16</td>
<td>888</td>
<td>771</td>
</tr>
<tr>
<td>20</td>
<td>243</td>
<td>998</td>
</tr>
<tr>
<td>30</td>
<td>871</td>
<td>881</td>
</tr>
<tr>
<td>Average</td>
<td>868</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> cpm = counts per minute.

<sup>b</sup> Counts per minute of the stimulated culture / average counts per minute of the control culture = stimulation ratio.
Temporal Development of the Primary and Secondary Cellular and Humoral Responses of Sensitized Lymphocytes to Rabies Virus

Lymphocytes harvested from draining lymph nodes of mice inoculated with rabies (ERA) virus were stimulated when cultured in vitro with rabies ERA antigen as early as 3 days. Maximum stimulation as indicated by incorporation of $^3$H-Tdr occurred at 6 days (Table 7, Figure 5). This response declined rapidly and returned to control levels by the day 20. Stimulation ratios in these temporal experiments were determined by dividing the CPM of specifically stimulated cultures by the average CPM of the unstimulated cultures.

The secondary response of lymph node lymphocytes was studied following the subcutaneous inoculation of rabies virus into mice 30 days after the primary sensitization with rabies virus. When these lymphocytes were cultured with UV inactivated rabies (ERA) virus a secondary response was observed (Table 8, Figure 5). This secondary response was similar in magnitude to the primary response but it occurred earlier. The peak incorporation of $^3$H-Tdr in the lymphocytes occurred on day 2 and declined rapidly. By day 12 the stimulation ratios of the lymphocyte cultures had returned to control levels.

By comparison the humoral response to rabies virus in mice occurred later than the cellular response (Figure 5).
Table 8. Comparison of the responsiveness of mouse lymph node lymphocytes stimulated with rabies (ERA) antigen after a primary sensitization with live rabies (ERA) and a secondary sensitization 30 days later with live or UV inactivated rabies (ERA) virus

<table>
<thead>
<tr>
<th>Days After Second ERA Inoculation</th>
<th>Stimulated With Live Rabies (ERA) Antigen</th>
<th>Stimulated With UV Inactivated Rabies (ERA) Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control cpm</td>
<td>Tissue Culture cpm</td>
</tr>
<tr>
<td>2</td>
<td>2,110</td>
<td>1,958</td>
</tr>
<tr>
<td>3</td>
<td>1,181</td>
<td>1,245</td>
</tr>
<tr>
<td>4</td>
<td>2,320</td>
<td>2,843</td>
</tr>
<tr>
<td>6</td>
<td>1,174</td>
<td>2,076</td>
</tr>
<tr>
<td>8</td>
<td>1,061</td>
<td>1,054</td>
</tr>
<tr>
<td>10</td>
<td>661</td>
<td>1,095</td>
</tr>
<tr>
<td>12</td>
<td>819</td>
<td>994</td>
</tr>
<tr>
<td>16</td>
<td>405</td>
<td>355</td>
</tr>
<tr>
<td>25</td>
<td>931</td>
<td>684</td>
</tr>
<tr>
<td>Average</td>
<td>1,185</td>
<td></td>
</tr>
</tbody>
</table>

a cpm = counts per minute.

b \( \frac{\text{counts per minute of the stimulated culture}}{\text{average counts per minute of the control culture}} = \text{stimulation ratio.} \)
Figure 5. Temporal development of rabies virus responsive cells from draining lymph nodes and of neutralizing antibody after a primary and secondary subcutaneous inoculation of rabies (ERA) virus.
The cellular response was present 3 days after inoculation whereas the humoral response was not present until 6 days after inoculation. The in vitro cellular response was transient, lasting approximately 16 days while neutralizing antibodies remained at high levels.

Both cellular and humoral secondary responses occurred more rapidly than primary responses. The secondary cellular response was approximately equal in magnitude to the primary response while the secondary humoral response was greater than the primary humoral response.

Live Versus UV Inactivated Rabies Virus in the Primary and Secondary Cellular Response

A comparison of the responsiveness of lymph node lymphocytes to a primary rabies sensitization with live versus UV inactivated rabies virus demonstrated that live rabies virus was much more effective than inactivated rabies virus in sensitizing mouse lymphocytes (Table 9). Mice inoculated with inactivated rabies virus produced a maximum stimulation ratio of 8.7 on day 3. This peak response quickly declined to control levels. By comparison the primary response of lymphocytes to rabies antigen in vitro after inoculation with live (ERA) rabies was much greater reaching a maximum stimulation ratio of 35.5 on day 6. Live rabies virus was also a much better stimulator of the primary humoral response. The
Table 9. Comparison of the responsiveness of mouse lymph node lymphocytes sensitized in vivo with either UV inactivated or live rabies (ERA) virus and stimulated in vitro with rabies (ERA)

<table>
<thead>
<tr>
<th>Days After ERA Inoculation</th>
<th>Mouse Lymphocytes Sensitized With UV Inactivated Rabies (ERA)</th>
<th>Mouse Lymphocytes Sensitized With Live Rabies (ERA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control cpm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Rabies (ERA) Antigen cpm</td>
</tr>
<tr>
<td>2</td>
<td>948</td>
<td>690</td>
</tr>
<tr>
<td>3</td>
<td>947</td>
<td>6,396</td>
</tr>
<tr>
<td>4</td>
<td>292</td>
<td>2,896</td>
</tr>
<tr>
<td>6</td>
<td>326</td>
<td>1,446</td>
</tr>
<tr>
<td>8</td>
<td>558</td>
<td>2,283</td>
</tr>
<tr>
<td>10</td>
<td>967</td>
<td>1,268</td>
</tr>
<tr>
<td>14</td>
<td>923</td>
<td>1,379</td>
</tr>
<tr>
<td>18</td>
<td>988</td>
<td>959</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>731</strong></td>
<td><strong>959</strong></td>
</tr>
</tbody>
</table>

<sup>a</sup> cpm = counts per minute.

<sup>b</sup> Counts per minute of the stimulated cultures divided by average counts per minute of the control cultures = stimulation ratio.
inoculation of live rabies virus stimulated the primary antibody response to a titer of 2,560 at day 30 whereas after UV inactivated virus a titer of only 160 was demonstrated at day 16 (Table 10).

The stimulation \textit{in vitro} of lymphocytes after a secondary sensitization with either live or UV inactivated rabies virus was not markedly different (Table 8). In both cases mice had been inoculated 30 days prior to the secondary stimulation with live rabies (ERA) virus. The only major difference noted between the live and UV inactivated virus as a secondary stimulator occurred on the second day after inoculation. Lymphocytes from mice that had received the inactivated virus were stimulated to a greater extent. On all other days the stimulation ratios were comparable. By day 12 stimulation ratios of both groups had declined to control levels. The secondary humoral responses after inoculation with live or UV inactivated rabies virus were also quite comparable (Table 10).

Comparison of the Stimulation of Rabies (ERA) Sensitized Lymphocytes by the HEP and ERA Strains of Rabies Virus

Lymphocytes harvested from draining lymph nodes of mice 6 days after subcutaneous inoculation with the ERA strain of rabies virus were stimulated with both the HEP and ERA strain of rabies \textit{in vitro} (Table 11). Lymphocyte cultures
Table 10. Rabies antibody titers in mice after primary and secondary sensitization with live or UV inactivated rabies (ERA) virus

<table>
<thead>
<tr>
<th>Days After ERA Inoculation</th>
<th>Primary Humoral Response</th>
<th>Secondary Humoral Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Live-Rabies (ERA) Virus</td>
<td>UV Inactivated Rabies (ERA) Virus</td>
</tr>
<tr>
<td>2</td>
<td>&lt;20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;20</td>
</tr>
<tr>
<td>3</td>
<td>&lt;20</td>
<td>&lt;20</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>80</td>
<td>40</td>
</tr>
<tr>
<td>8</td>
<td>1,280</td>
<td>40</td>
</tr>
<tr>
<td>10</td>
<td>1,280</td>
<td>80</td>
</tr>
<tr>
<td>12</td>
<td>2,560</td>
<td>80</td>
</tr>
<tr>
<td>14</td>
<td>2,560</td>
<td>160</td>
</tr>
<tr>
<td>16</td>
<td>2,560</td>
<td>160</td>
</tr>
<tr>
<td>20</td>
<td>2,560</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>25</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>30</td>
<td>2,560</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> Reciprocal of the serum dilution.

<sup>b</sup> ND = not done.
stimulated by the homologous virus did have slightly higher stimulation ratios than did cultures stimulated by the heterologous virus.

Stimulation of Sensitized Lymphocytes by Live Virus as Compared to Inactivated Virus

Both live and inactivated rabies virus cultured with rabies sensitized lymphocytes caused lymphocyte stimulation. No appreciable difference between the live and inactivated rabies virus antigens was demonstrated (Table 11).

Stimulation of Sensitized Lymphocytes by Rabies Ribonucleoprotein

Rabies virus RNP failed to cause any significant stimulation of rabies sensitized lymphocytes (Table 11). Final concentrations of 0.1 mg and 0.01 mg of RNP had no effect on rabies sensitized lymphocytes.
Table 11. Stimulation of rabies (ERA) sensitized lymphocytes when cultured in the presence of live or UV inactivated HEP or ERA strain of rabies or with HEP ribonucleoprotein (RNP)

<table>
<thead>
<tr>
<th>Stimulating Antigen</th>
<th>$^3$H-Thymidine Uptake $^a$</th>
<th>S/R $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERA live</td>
<td>24,627</td>
<td>14.7</td>
</tr>
<tr>
<td>ERA UV</td>
<td>26,429</td>
<td>15.8</td>
</tr>
<tr>
<td>HEP live</td>
<td>21,012</td>
<td>12.6</td>
</tr>
<tr>
<td>HEP UV</td>
<td>23,155</td>
<td>13.8</td>
</tr>
<tr>
<td>RNP (1.0 mg/ml)</td>
<td>2,162</td>
<td>1.3</td>
</tr>
<tr>
<td>PHA</td>
<td>38,905</td>
<td>23.2</td>
</tr>
<tr>
<td>None</td>
<td>1,674</td>
<td>1.0</td>
</tr>
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</table>

$^a$cpm = counts per minute.

$^b$Counts per minute of the stimulated culture = stimulation ratio.
The role of the cellular versus humoral immune response in viral diseases and the interrelationship between these two responses is a subject of great concern. In the case of rabies a great deal of research has been conducted on humoral immunity aspects of this disease but little work has been done on cellular aspects. Unsatisfactory laboratory methods as well as a poor understanding of the dichotomy of the immune system held back advancements in cell-mediated immunity. The discovery by Nowell in 1960 that PHA stimulated peripheral blood leukocytes to transform into blast cells was very important as this helped pave the way for in vitro cell-mediated studies. The tremendous interest during the last decade concerning the role of the lymphocyte in immunological diseases and oncogenesis has also contributed to our understanding of the immune system especially in regard to cellular aspects. Techniques of harvesting and purifying lymphocytes, new lymphocyte culture methods and media as well as new equipment such as the semiautomatic multiple-sample processor (Hartzman et al., 1972) have made it possible to study cell-mediated immunity in vitro.

Rosenberg et al. (1972) using herpes simplex virus and vaccinia virus have reported quantitative information on the preparation and nature of the viral antigens, the time of
appearance and magnitude of the response and the specificity of the viral stimulated lymphocyte response. Using a similar technique but in microculture this author was able to demonstrate that rabies virus will stimulate sensitized lymphocytes in vitro. This lymphocyte stimulation has been accepted as an in vitro corollary of delayed hypersensitivity or cell-mediated immunity (Valentine, 1971). It is therefore postulated that in vivo cell-mediated immunity is an important consideration in rabies.

The two reports in the literature dealing with the presence or absence of cell-mediated immunity in rabies inoculated animals are conflicting. Turner (1973) concluded from his experiments that lymphocytes in rabies-immune mice do not contribute to protection whereas Wiktor et al. (1974) demonstrated that rabies virus antigen stimulated rabies sensitized lymphocytes. The experimental methods used by these workers differed considerably. Turner (1973) failed to demonstrate cell-mediated immunity to rabies virus by transferring lymphocytes from immune mice to nonimmune mice, challenging the mice with virulent rabies virus and looking for protection. Wiktor et al. (1974) successfully demonstrated in vitro cell-mediated immunity using lymphocytes from rabies vaccinated rabbits and stimulating these lymphocytes with rabies viral antigen.

The different methods used by these workers probably
did not account for the different results obtained. Turner's negative findings may have resulted from the fact that he did not obtain his lymphocytes to transfer until 21 days after immunization of the mice. Results from this present study (Figure 5) demonstrated that the in vitro stimulation of sensitized lymphocytes by rabies virus disappeared by day 20. Therefore it is probable that Turner (1973) would have demonstrated cell-mediated immunity to rabies virus if he had harvested the lymphocytes earlier. Turner's technique was adequate to demonstrate cell-mediated immunity to vaccinia virus proving that his procedure was capable of demonstrating this type of immune response.

Wiktor et al. (1974) demonstrated that the stimulatory effect of sensitized lymphocytes by rabies virus antigen was not present until 8 days after intravenous inoculation. These workers also demonstrated that this response was maximal at 8 days and that the lymphocyte response, although low, was still present 175 days after inoculation.

Results from the present study differ from those of Wiktor and his coworkers in that lymphocytes stimulated with rabies virus were present by day 3, maximal on day 6 and back to control levels by day 20. Different results may be attributed to the route of inoculation used to sensitized animals, kind of animals used, potency of inoculum and the conditions under which the lymphocytes were cultured. The
dilution of the inoculum caused by the intravenous route of inoculation used by Wiktor and his coworkers may account for the greater lag period between inoculation and lymphocyte stimulation that these workers demonstrated. The extended period during which these workers could demonstrate the lymphocyte stimulation response is unexplainable.

Rosenberg and Notkins (1974) recently reported on the temporal lymphocyte stimulation response using herpes simplex virus (HSV). Results obtained by these authors using HSV and those reported here (Table 7, Figure 5) using rabies virus are quite similar. Herpes simplex virus sensitized lymphocytes were stimulated as early as 3 days after subcutaneous inoculation of rabbits. The lymphocyte stimulation ratio reached a maximum of 10.7 seven days after inoculation and was near control levels by day 20. Although rabies virus caused a greater stimulation ratio, the occurrence of the response, its peak and its disappearance were approximately the same as seen with HSV. Stimulation ratios are affected by control cultures which may be higher in the macroculture technique, thus causing the relatively low stimulation ratio reported by Rosenberg and Notkins.

When comparing live versus UV inactivated rabies virus as a sensitizer of lymphocytes one finds that inactivated virus is inferior to live preparation (Table 9). Likewise the primary humoral response was much lower with inactivated
rabies virus (Table 10). Rosenberg and Notkins (1974), using inactivated HSV, also found less cellular and humoral response than when infectious HSV was used. Wiktor et al. (1974) however demonstrated that lymphocytes from rabbits inoculated with beta propiolactone-inactivated rabies virus were stimulated quite significantly when exposed to rabies virus in vitro.

One would suspect that live viruses would produce greater sensitization of lymphocytes than killed preparations based on the potential of live virus to replicate eventually providing a greater mass of antigen to which the lymphocytes would be exposed. The discrepancy between this present report of a poor cellular response obtained when using inactivated rabies virus and the report by Wiktor et al. (1974) may be accounted for by a difference in antigenic potency. The inactivated vaccine used by Wiktor et al. had a potency in mice of 40 times that of the reference NIH vaccine Lot 178. This value indicated a relatively high antigenic mass.

The secondary cellular and humoral responses were comparable when using live or UV inactivated rabies virus to inoculate mice (Tables 8, 10). The magnitude of the secondary response in draining lymph node cells resembled the primary response, while neutralizing antibody titers increased above the primary humoral response (Figure 5). The time course of
the secondary responses was similar to the primary response. Both the cellular and the humoral responses were accelerated (Tables 8 and 10, Figure 5). Peak lymphocyte stimulation was seen 2 days after the second inoculation and a rise in neutralizing antibody titers was also seen at this time.

The observation that inactivated rabies virus would cause a good secondary cellular response but a poor primary response is again possibly a matter of antigenic mass. Since a large amount of antigen is required to sensitize the lymphocytes in the primary response, one obtains best results with a replicating live virus. However, once sensitized it takes less antigenic mass to reactivate these lymphocytes. Griffin and Johnson (1973) described similar results using live and inactivated Sindbis virus to sensitized mice for a primary and secondary cellular response.

The secondary humoral response was also present when mice were inoculated with inactivated rabies virus. These findings may also be explained on the basis of antigenic mass available in the inoculum and the fact that presensitized lymphocytes require less antigen stimulus to be reactivated than they do to be sensitized initially.

The time course of the primary and secondary humoral responses demonstrated the existence of the classical anamnestic response one would expect. An interesting observation that possibly one may not expect is that the secondary
cellular response also appeared as an "anamnestic response," at least in its time of appearance.

The stimulation of rabies sensitized lymphocytes appeared to be quite specific. Lymphocytes from control mice inoculated with SMB (suckling mice were used to propagate the sensitizing rabies virus) did not respond in vitro to PK<sub>2A</sub> tissue culture fluid (stimulating rabies virus antigen was propagated in PK<sub>2A</sub> cells) (Table 7).

The use of dilutions of rabies virus antigen used to stimulate sensitized lymphocytes demonstrated that the lymphocyte stimulation reaction is dose dependent (Table 6). A minimum of 10<sup>4.0</sup> PFU per lymphocyte culture was required for any stimulation. Maximum stimulation was obtained using the most concentrated viral suspension as the stimulating antigen.

In this present study and the study by Wiktor et al. (1974), the live and inactivated rabies virus antigens were equally as effective in stimulating the DNA-synthesis of cultured rabies virus-sensitized lymphocytes. Griffin and Johnson (1973) reported that live Sindbis virus antigen was more effective than inactivated virus in stimulating Sindbis virus-sensitized lymphocytes. Rosenberg et al. (1972) however, reported that UV inactivated HSV antigen was more effective than live virus in stimulating HSV-sensitized lymphocytes. These differences may in part be due to the fact
that some viruses such as Sindbis do not replicate in mononuclear cells (Johnson, 1965) whereas HSV does replicate in these cells (Nahmias et al., 1964). Viruses that replicate in mononuclear cells destroy these cells and a poor stimulatory response results.

Lymphocytes from mice inoculated with the ERA strain of rabies virus could be stimulated with both the HEP and ERA strains of rabies. The homologous strain caused a slightly greater stimulation than the heterologous strain. One would have to carefully quantitate the amount of virus in each virus-stimulating preparation before any absolute conclusion could be drawn from such data. Incomplete virus particles would also have to be accounted for in the preparation as they may also have stimulatory properties.

Ribonucleoprotein derived from HEP infected BHK cells did not stimulate rabies sensitized lymphocytes (Table 11). This observation is in agreement with the results obtained by Wiktor et al. (1974). One would expect that RNP would be a poor stimulator as this is an internal component of the virus particle and not readily processed or presented to the lymphocyte during the sensitization process.

The kinetics of any lymphocyte stimulation test must be established for each laboratory, as slight variations in technique will alter the response dramatically. Results of these studies can only be used as guidelines from one
laboratory to another. Kinetic studies of rabies lymphocyte stimulation indicated that the maximal response was present at 4 days of incubation (Table 5, Figure 4). These cultures were pulsed with 1 μCi of $^{3}$H-Tdr 18 hours before termination of the experiments. Using this schedule one is able to conduct the pulsing and harvesting of cultures within a normal 8 hour working day, a fact that is important when many different tests are being conducted simultaneously. The PHA response is maximal at 3 days (Figure 4), but it is still adequate at 4 days to indicate whether or not the cultured lymphocytes were capable of being transformed.

Cell concentration is an important variable in lymphocyte stimulation studies. One attempts to use as many cells as possible in order to increase the number of sensitized cells in a culture. The limiting factor is the available nutrition. Using the microculture system to culture mouse lymph node cells, 1 million cells per ml is the optimum cell concentration based on the response to PHA and the need to conserve the limited supply of cells (Table 1, Figure 1). The optimum PHA concentration using 1 million cells per ml was 3 μg per ml (Table 2, Figure 2). This level of PHA will vary depending on the activity of a particular lot of this mitogen.

An attempt was made to find a medium that could be used for lymphocyte cultures that did not require fresh human
serum. Studies comparing Click's EHAA medium supplemented with 0.5 percent fresh mouse serum with RPMI-1640 medium demonstrated that the latter medium was far superior to Click's EHAA medium (Table 4).

Sindbis virus was used as a model in this study. The primary cellular and humoral responses of Sindbis inoculated mice were as expected and as reported previously by Griffin and Johnson (1973) (Table 3, Figure 3).

This study is only a part of what will undoubtedly follow concerning cellular immunity or the interaction of cellular and humoral immunity in rabies infections. A problem such as the enhancing or inhibiting effect of specific antibody on sensitizing virus is one that should be considered in the future. As suggested by Rosenberg et al. (1972), if the virus is complexed with specific antibody before stimulating sensitized cells this would suggest that the host has evolved a highly effective means of protecting the cells of the immune system from destruction by infectious virus, while at the same time allowing viral antigens to stimulate the immune system. Rosenberg et al. (1972) has also suggested that the ability of virus-antibody complexes to stimulate sensitized lymphocytes might prove to be a useful tool for detection of circulating noninfectious virus-antibody complexes in chronic viral infections.

Cell-mediated immunity to rabies or other viruses may
act to protect the host by several possible mechanisms. For example, virus infected cells may be sought out and destroyed by sensitized lymphocytes similar to the destruction seen in a graft rejection. Furthermore, stimulated lymphocytes may protect by release of one or more of the lymphokines which act to destroy viruses or virus infected cells.

In conclusion this study has demonstrated that rabies sensitized lymphocytes can be stimulated in vitro with rabies antigen, a response that has been widely accepted as a corollary of cell-mediated immunity. This study has also demonstrated the use of a microculture method for this test. The advantages of this method over standard tube lymphocyte culture methods include both a savings in time of preparing and harvesting lymphocytes as well as a savings in expense of supplies. Furthermore this study has added to our basic knowledge of both the primary and secondary humoral and cellular response to rabies virus as well as the relationship of these two responses to one another.

A quotation from John R. David (1971) summarizes the point we are at in our understanding of cell-mediated immunity to viral disease,

The sum of our new information, as is so often the case when new areas of investigation are explored, makes us realize more clearly than before how little we know and how much is still to be
done. It is readily apparent that significant progress in defining cellular reactions may be important in maintaining man's health and in ameliorating some of his most devastating diseases. Undoubtedly, this alone would suffice to keep us struggling with the problem. But there is also the luckily incurable scientific urge to know precisely that which is unknown, as the mountaineer climbs the mountain because it is there. And the cells are there, with most of the secrets still to be uncovered (p. 410).
SUMMARY

In vitro lymphocyte stimulation and virus-neutralization tests were employed to study the relationship between cellular and humoral responses to rabies virus in mice. Specifically sensitized lymphocytes harvested from mouse lymph nodes were present as early as 3 days after inoculation. The peak cellular response was present by day 6. Neutralizing antibody appeared later reaching a peak at 12 days. The ability of sensitized lymphocytes to respond to viral antigen was transient. This cellular response disappeared by day 20 but the neutralizing antibody levels remained high. Reinfection 30 days after the primary infection resulted in a sharp increase in the responsiveness of lymphocytes to viral antigens as well as a sharp rise in neutralizing antibody levels. The lymphocyte cultures were propagated in microculture plates, 0.2 ml per culture, in RPMI medium. Stimulation of lymphocytes was determined by the incorporation of $^3$H thymidine into the DNA. This reaction was demonstrated to be quite specific and dose dependent. Ultraviolet light inactivated rabies virus was as equally effective as live virus in stimulating specifically sensitized lymphocytes. However, inactivated rabies virus was ineffective in comparison to live virus when used as a primary sensitizing agent. The secondary response of lymphocytes was initiated equally well by inactivated and live rabies virus.
BIBLIOGRAPHY


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