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STUDIES ON THE RELATIONSHIP OF IMMUNE INDUCTION OF INTERFERON AND CELL-MEDIATED IMMUNITY

by

William Charles Wallen

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF MICROBIOLOGY
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
WITH A MAJOR IN MOLECULAR BIOLOGY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1972
I hereby recommend that this dissertation prepared under my direction by William Charles Wallen entitled \textit{STUDIES ON THE RELATIONSHIP OF IMMUNE INDUCTION OF INTERFERON AND CELL-MEDIATED IMMUNITY} be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy.

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ABSTRACT

The purpose of this study was to examine the inter-relation between production of interferon and the blastogenic response by lymphoid cells after incubation with the general mitogens phytohemagglutinin (PHA), concanavalin A (Con A), and pokeweed mitogen (PWM) or with the specific antigen purified protein derivative (PPD). Spleen cells involved in interferon production were examined to determine whether or not they belonged to the same population of thymic-dependent lymphocytes capable of undergoing blastogenic transformation.

Detectable levels of interferon were produced in non-immunized mice in one to three days after an intravenous (i.v.) injection of PHA or Con A. Splenectomy inhibited the capacity of mice to produce serum interferon following intravenous administration of PHA. Serum interferon was detected two to four days following an i.v. injection of PPD only in mice which previously had been immunized with the attenuated Mycobacterium tuberculosis strain, Bacillus Calmette-Guerin (BCG).

PHA, Con A, PWM, and PPD induced the production of an antiviral factor from mouse spleen cell cultures. Many of the physical-chemical properties of this factor resembled those of standard mouse serum interferon. Mouse spleen
cells, that were cultured in the presence of PHA, Con A, PWM, or PPD were examined concomitantly for the production of interferon and blastogenesis. The concentrations of the various mitogens (PHA, Con A, and PWM) and the specific antigen PPD that resulted in production of the highest titers of interferon by mouse spleen cells also induced the maximal blastogenic response. However, the amount of interferon produced by mouse spleen cells in cultures was not directly proportional to the amount of $^3\text{H}-\text{TdR}$ incorporated into TCA-insoluble material after exposure to the various mitogens or PPD. Con A stimulated a greater blastogenic response in spleen cell cultures than PHA; however, PHA induced higher titers of interferon from the same cell cultures.

The cells involved in the production of interferon were studied by separation of spleen cells from normal and BCG-immunized mice into six fractions by centrifugation through discontinuous gradients of bovine serum albumin. The cells capable of producing interferon in response to PHA, Con A, PWM, or PPD were localized exclusively in fraction V; in contrast, the blastogenic response to the mitogens and PPD was found in fractions I through IV and not in fraction V.

Spleen cells fractionated on BSA gradients were treated with anti-theta serum in the presence of complement to remove cytolytically the thymic-dependent lymphocytes
(T-cells) from each fraction. T-cell-depleted fractions of spleen cells were used to determine the role of the thymic-dependent lymphocyte in induction of interferon. The interferon and the blastogenic responses were inhibited in all of the cell fractions treated with anti-theta serum and then exposed to PHA, Con A, or PPD but not PWM.

The PHA-induced interferon response by T-cell-depleted fraction V cells was reconstituted by the addition of a soluble factor derived from PHA stimulated fraction IV cells which had not been treated with anti-thymocyte serum.

These studies demonstrate that induction of interferon from mouse spleen cells by PHA, Con A, or PPD requires the interaction of at least two cell types; a thymic-dependent cell and a thymic-independent cell. The interferon-producing cells were shown to belong to a thymic-independent population of spleen cells; however, the thymic-dependent lymphocyte was shown to play an essential role, through the production of a soluble factor, in the inductive phase of the interferon response. PWM also was found to be able to stimulate the production of interferon but it did not require the presence of thymic-dependent lymphocytes for the induction phase. PWM was able to directly activate the thymic-independent population of spleen cells to obtain the production of interferon.
INTRODUCTION

The initiation of host defense mechanisms which enhance recovery from viral infections is a complex phenomenon. The production of interferon, a potent antiviral protein, and the stimulation of specific humoral and cellular immune mechanisms have been ascribed important roles in the defense against diseases of viral etiology. Numerous attempts have been made to segregate these responses to determine their relative importance. The protective role of each of these responses has been the subject of several recent reviews (Gresser and Lang, 1966; Allison, 1967; Glasgow, 1970; Merigan, 1970; Notkins, Mergenhagen, and Howard, 1970).

Humoral Immunity in Viral Infections

Humoral immunity towards viruses is mediated by circulating antibody, principally belonging to IgG, IgM, and IgA classes of immunoglobulin. The role of humoral immunity is primarily prophylactic by preventing the initiation of an infection and by preventing the dissemination of infectious virus through the circulation to new target organs. Antibody functions by specifically interacting with virus particles and interfering with their adsorption to cells. Viruses are capable of producing new antigenic determinants
on the surface of infected cells (Pasternak, 1969; Allison, 1971) and the interaction of these determinants with antibody plus complement may result in direct cell destruction (Porter, 1971). This provides a mechanism by which antibody may play a direct role in recovery from some viral infections.

Although antibody is highly efficient at inhibiting the rapid spread of virus extracellularly, it is relatively inefficient in inhibiting a number of viruses which spread via cell to cell contact. Although antibody has been shown to be protective in some viral infections, it may not be of primary importance in recovery from many types of viral infections. Therefore, the role of antibody in viral infections, in general, is difficult to assess and its relative importance depends on the specific etiologic agent causing each viral disease.

Interferon in Viral Infections

In addition to antibody, there is another humoral component which is thought to contribute to the host's ability to combat viral infections. This circulating component has been termed interferon and has been the subject of a number of recent reviews (Hilleman, 1968; Wheelock, Larke, and Caroline, 1968; DeClerq and Merigan, 1970; Colby and Morgan, 1971). The antiviral activity of interferon was first demonstrated in 1957 (Isaacs and
Lindenmann, 1957). Interferon, which is produced early in viral infections, is a species specific, potent, and broadly active antiviral protein, effective both in vivo and in vitro (Vilcek, 1969). It has subsequently been found to function at the intracellular level to inhibit the replication of a large number of viruses (Friedman and Sonnabend, 1970). Interferon is a protein with a molecular weight ranging from 18,000 to greater than 100,000 daltons. The differences in molecular weight probably reflect purity of the preparation from which the determination was performed and of the polymeric variation in its sub-unit structure (Merigan, 1967b). The relative importance of interferon in recovery from viral infections, by itself, is difficult to assess because it is now associated to some degree with the immune system.

There are a large number of agents which have been found to induce interferon, including several species of ribonucleic acid (RNA) (Ho, 1966) and deoxyribonucleic acid (DNA) (Ho and Kohler, 1967) viruses, rickettsia (Kazar, 1966), bacteria (Stinebring and Youngner, 1964), chlamydia (Merigen and Hanna, 1966), and protozoa (Rytel and Jones, 1966). Also, microbial extracts, including viral double-stranded RNA (Tytell et al., 1967), bacterial endotoxins (Smith and Wagner, 1967), and exotoxins (Friedman and Cooper, 1967), fungal products such as statalon (Kleinschmidt, Cline, and Murphy, 1964) and helenine
(Lampson et al., 1967), plant lectins such as phytohemagglutinin (Wheelock, 1965) and synthetic polymers such as maleic anhydride: divinyl ether copolymer (Merigan, 1967a), and polyinosinic acid:polycytidilic acid (poly I:C) (Field et al., 1967) have been found to induce interferon.

**Role of Cell-Mediated Immunity in Viral Infections**

Cell mediated immunity is mediated by sensitized thymic-dependent lymphocytes which initiate a cellular response towards foreign antigens. Antigens which stimulate this response are recognized by these cells through a specific recognition mechanism. The cell-directed immune response is dependent upon the thymus; an organ which has been shown to exert a strong influence on the development of immunological competence by the host (Miller, Marshall, and White, 1962; Miller and Osoba, 1967). In many species, the immune responses are dependent on functionally different types of small lymphocytes which have been classified into two groups: thymic-dependent lymphocytes (T-cells) and bone marrow (thymic-independent) lymphocytes (B-cells (Meuwissen, Stutman, and Good, 1969; Roitt et al., 1969). In mice, these cell types can be distinguished by several biological properties. B-cells possess high amounts of immunoglobulin on their surfaces and possess a distinct antigenic determinant, mouse bone marrow-derived lymphocyte antigen (Raff, Nase, and Mitchison, 1971). B-cells are responsible for
initiating the production of humoral antibody (Kreth and Williamson, 1971). T-cells have little immunoglobulin bound to their surfaces and possess a unique theta antigenic determinant, which is shared only with cells belonging to the nervous system (Rief and Allen, 1964; Raff, 1969; Schlesinger and Yron, 1969). B-cells have been shown to lack the theta antigen (Raff, 1971). The T-cells are responsible for directing the cell mediated immune response, though T-cells have been found to have an important function in the humoral immune response to some antigens (Kreth and Williamson, 1971).

Upon contact with an antigen, the sensitized thymic-dependent lymphocytes undergo a number of metabolic alterations which lead to transformation into blast-cells (Mills, 1966). Blastogenesis can be defined as the reversible dedifferentiation of small lymphocytes to larger lymphoblasts. This process eventually leads to mitosis of these cells. Histologically, during the process of blastogenic transformation, the nuclei enlarge and develop nucleoli and the cytoplasm increases in size and content and becomes more basophilic (Ling, 1968).

Antigen-induced transformation of lymphocytes into blast-cells has been shown to correlate with the delayed hypersensitivity response, as detected by a positive tuberculin-type of skin-test following antigenic challenge (Oppenheim, 1968). Many of the cellular events which occur
following activation of sensitized lymphocytes by specific antigen can be simulated by exposure of lymphocytes to a number of plant lectins such as phytohemagglutinin (Nowell, 1960), concanavalin A (Leon and Powell, 1968), or pokeweed mitogen (Farnes et al., 1964; Chessin et al., 1966). Phytohemagglutinin (PHA) is an extract of the red kidney bean Phaseolus vulgaris. Concanavalin A, also a plant lectin, is extracted from the jackbean, Canavalin ensiformis. Pokeweed mitogen is obtained as an extract of the plant, Phytolacca americana. These plant extracts (mitogens) are capable of interacting with the surface of small lymphocytes and stimulating a state of enhanced metabolic activity. Among the biochemical changes which occur during the process of transformation are: alteration of phospholipid content in lymphocyte membranes (Fisher and Mueller, 1968; Lucas, Shohet, and Merler, 1971), acetylation of nuclear histones (Pogo, Allfrey, and Mirsky, 1966) and increased RNA, protein, and DNA synthesis (Sell, Rowe, and Gell, 1965).

Metabolic activity following stimulation of lymphocytes by mitogens or antigens reaches a maximum between two and three days. RNA synthesis has been found to be initiated within thirty minutes following exposure to PHA (McIntyre and Ebaugh, 1962). Initiation of protein synthesis coincides with RNA synthesis (Torelli et al., 1963). DNA synthesis reached its maximum between 72 and 96 hours for human peripheral lymphocytes (Cooper, Barkhan, and Hale, 1963;
Michalowski, 1963) and between 48 and 72 hours for mouse spleen lymphocytes (Adler et al., 1970).

Lymphocyte blast-cell transformation due to antigenic stimulation of sensitized lymphocytes differs from stimulation of lymphocytes by non-specific mitogens. Quantitatively, fewer cells become transformed into blast cells; usually ranging from 5% to 20% following exposure to specific antigens compared to 20% to 90% following exposure to general mitogens. These values are greatly influenced by the origin of cells (species or organ) involved and the particular mitogen or antigen used. The sequence of biochemical changes proceeds more slowly after stimulation by antigen than by general mitogen (Cowling, Quaglino, and Davidson, 1963; Marshall and Roberts, 1963). Differences also are found in the size of the RNA synthesized following stimulation by either antigen or mitogen (Cooper and Rubin, 1965). Along with these biochemical and morphological changes, a number of biologically active proteins are produced as a result of stimulation of lymphocytes by antigens or mitogens. These proteins have been designated "effector molecules" which mediate the inflammatory response of cell-mediated immunity. Some of the factors known to be released following lymphocyte interaction with an antigen or mitogen include: migration inhibition factor (MIF) (Bloom and Bennett, 1966; David, 1968), skin reactive factor (SRF) (Bennett and Bloom, 1968; Pick, Krejci, and Turk, 1969), lymphotoxin (LT) (Ruddle and Waksman, 1968; Granger et al.,
1969), proliferation inhibitory factor (PIF) (Green et al., 1970), blastogenic factor (BF) (Valentine and Lawrence, 1969), chemotactic factor (CF) (Ward, Remold, and David, 1969), transfer factor (TF) (Thor, 1967; Lawrence, 1969), and interferon (IF) (Wheelock, 1965; Glasgow, 1966; Richmond, 1969). Stimulated lymphocytes release many of these factors within a short time after stimulation. These factors may act to amplify the immune response, activate macrophages, suppress intracellular replication of viruses, and eradicate the infection.

The role of cell-mediated immunity in recovery from viral infections is, as yet, incompletely understood. An increasing body of evidence derived from the study of humans with primary immunological deficiency diseases points to a very important, possibly essential role for a thymus-dependent cell-mediated immune response in the recovery from a number of viral infections (Fulginiti et al., 1968). From numerous clinical reports on viral infections in children in immune deficiency states, the general conclusions are that individuals exhibiting humoral deficiencies (e.g., Bruton's Syndrome) but with intact thymic-dependent functions, generally recover from viral infections with a normal course of symptoms. In contrast, individuals with dysfunction or aplasia of the thymus (e.g., DiGeorge's Syndrome) but possess intact humoral immunity, who do not exhibit cell-mediated immunity, experience the most severe consequences
with viral infections (O'Connell, Karzon, and Barron, 1964; Hanson, Johansson, and Vahlquist, 1966; Nahmias et al., 1967). Another group manifesting decreased resistance to viral infections are individuals receiving immunosuppressive agents in the management of rejection of transplanted organs (Craighead, Henshaw, and Carpenter, 1967). Viral infections most frequently associated with diseases in individuals with depressed cell-mediated immunity include Herpes simplex, vaccinia, measles, rubella, varicella-zoster, and cytomegalovirus (Glasgow, 1970; Notkins et al., 1970).

Animals subjected to neonatal thymectomy, treated with anti-lymphocyte serum or immunosuppressive chemicals also have been found to have an enhanced susceptibility to certain viral infections. Hirsch et al. (1968) showed a decreased host resistance in mice to vaccinia and herpes viruses following suppression of cellular immunity by treatment with anti-thymocyte serum. They also found that suppressed mice responded normally with the production of antibody and serum interferon levels comparable to control mice. Conversely, Friedman et al. (1962) suppressed both the delayed hypersensitivity and antibody defense mechanisms of adult guinea pigs with x-irradiation and methotrexate and found that the animals recovered from vaccinia virus infections as rapidly as control, non-suppressed animals. They also noted that the treated animals produced as much serum interferon as control animals. From this evidence, they
(Friedman et al., 1962) concluded that cellular immunity played little or no role in recovery from viral infections but that interferon most likely was instrumental in recovery. In another study (Pincus and Flick, 1963), the ability of rabbits to develop rapid and severe vaccinia dermal reactions was shown to correlate with their ability to demonstrate a delayed hypersensitivity response. The depression of the cell-mediated immune capacity, by treating rabbits with anti-mononuclear-cell serum locally, resulted in a pronounced inability of the rabbits to produce the typical vaccinia lesion (Pincus and Flick, 1963). Flick and Pincus (1963) were able to establish neonatal unresponsiveness to vaccinia virus which resulted in the rabbits inability to demonstrate a delayed hypersensitivity response to vaccinia virus as an adult. The neonatally unresponsive rabbits, as well as the anti-mononuclear-cell serum treated rabbits, frequently developed disseminated vaccinia infections after intradermal administration of the virus. It was concluded from these experiments, that cellular immunity played a central role in recovery from vaccinia virus infection.

Blanden (1970) studied the effect of a depressed cell-mediated immune response upon mice infected with ectromelia virus, the etiological agent for mousepox. He showed that anti-thymocyte serum treatment did not interfere with the production of antibody to ectromelia virus. He also found that the interferon response was slightly
elevated in mice treated with anti-thymocyte serum and infected with ectromelia virus. Anti-thymocyte serum treatment was shown to delay significantly the cell-mediated immune response as measured by a delayed hypersensitivity reaction in the foot of the mice. This response was directed against ectromelia virus antigen which was injected into the foot of mice previously sensitized to the virus. This delay, it was suggested, was responsible for allowing the virus to replicate and spread to target organs resulting in death of the animals. He concluded that cell-mediated immunity probably contributed an "essential acquired recovery mechanism" (Blanden, 1970) to the normal host and that normal interferon and neutralizing antibody responses were insufficient to contain pox virus infections.

It is apparent that extensive conflict exists over the relative importance of each of the host's major protective mechanisms, even after attempts to segregate them have been employed. Analysis of the individual responses is complicated by the overlapping effects of experimental procedures. For example, many of the procedures employ anti-lymphocyte serum to suppress cell-mediated immunity. Anti-lymphocyte serum has been shown to interfere with both cell-mediated immune responses and thymic-dependent antibody production (James, 1970; Levey, 1970). Anti-lymphocyte serum also has been found to have differential effects on the interferon response; this effect being related to the
interferon-inducing agent employed (Glasgow, 1971). Interferon inducers such as poly I:C have also been shown to have multiple effects; such as anti-tumor activity (Gresser, Fontaine, and Coppey, 1967; Levy, Law, and Rabson, 1969), enhancement of graft vs. host (GVH) responses (Cantor, Asofsky, and Levy, 1970), and an increase in both humoral and cellular antibody formation (Turner, Chan, and Chirigos, 1970). It is also important to bear in mind that each of the responses—humoral immunity, cellular immunity, and interferon—are induced differentially by various challenging viruses. Therefore, it is difficult to generalize about the relative importance of each response. Defense against viruses must be considered individually and therapeutic approaches employed which will encourage the most effective combination of responses. It is also very likely that each of these responses are closely related to one another and may act concomittantly to prevent viral infections.

**Relationship of Interferon to Cellular Immunity**

The relationship between interferon production and the cellular immune response has been suggested. However, few studies have been published. Investigations into the cell types involved in the production of interferon have demonstrated that cells belonging to the reticuloendothelial system (RES) are primarily responsible for production of circulating interferon *in vivo* (DeSomer and Billiau, 1966;
Fruitstone et al., 1966; Subrahmamyan and Mims, 1967). The RES consists of leukocytes (including lymphocytes, macrophages, mononuclear cells, and reticulum cells) present in the spleen, lung, and liver, and they have been shown to be effective in producing interferon (Kono and Ho, 1965). Gresser (1961) provided the first evidence that human peripheral leukocytes could produce significant levels of interferon \textit{in vitro} following exposure to Newcastle disease virus (NDV). High titers of interferon were also obtained from human peripheral leukocyte suspensions incubated with NDV and Sendai virus (Falcoff et al., 1966; Wheelock, 1966). Of the different cell types present in leukocyte populations, the lymphocytes have been suggested to be the cells which act as the major source of interferon (Ho, Postic, and Ke, 1968). Cells producing interferon in the host have been shown to be radiosensitive. It was shown that the interferon response to NDV following x-irradiation of mice could be restored by reconstitution with bone-marrow cells (DeMaeyer-Guignard, DeMaeyer, and Jullien, 1969).

The above studies demonstrate that the interferon-synthesizing capacity and the cell-mediated immune response involves morphologically similar cell types. Other studies also have shown that these two defense mechanisms are interrelated. Interferon has been induced in human peripheral leukocyte cultures following stimulation by the general mitogen, PHA (Wheelock, 1965). Peritoneal cells from mice
immunized against chikungunya virus produced two to ten times more interferon than peritoneal cells from unsensitized mice following challenge with this virus. The induction of interferon was immunologically specific because cells sensitized to chikungunya virus failed to produce detectable levels of interferon when challenged with a heterologous virus, encephalomyocarditis virus (Glasgow, 1966). Various non-viral antigens also have been used to induce interferon from sensitized cells. The addition of the specific antigens purified protein derivative (PPD), tetanus toxoid (TT), and diphtheria toxoid (DT) to sensitized human peripheral leukocytes in vitro showed an inverse relationship between the amount of interferon which could be obtained and the length of interval since the individual's last immunization (Green, Cooperband, and Kibrick, 1969). Peritoneal cells from mice sensitized to Mycobacterium tuberculosis (BCG) produced interferon in vitro when cultured in the presence of PPD (Milstone and Waksman, 1970). In addition, PPD has been shown to stimulate increased levels of circulating interferon in mice sensitized to BCG in vivo (Stinebring and Absher, 1970). Mice, after initial exposure to BDG, required 14 to 21 days before they could respond to PPD with the production of interferon. This time period correlates with known temporal requirements for active sensitization of mice to elicit a cell-mediated immune response. Other investigators have confirmed and
extended the concept that immune specific stimulation of lymphocytes can result in the production of interferon (Lackovic and Borecky, 1970; Epstein, Cline, and Merigan, 1971a, 1971b).

These published investigations demonstrate that a relationship may exist between cell-mediated immune responses and the production of interferon. This relationship may have an important bearing on the host's ability to initiate a mobile cellular response capable of delivering sufficient interferon to the site of infection. This capacity would assist the host in controlling the spread of viruses. Thus, it becomes important to investigate the relationship between specific immune mechanisms and the production of interferon.

Statement of the Problem

Interferon production has been suggested to be related to cell-mediated immunity because it can be elicited by stimulation with antigens (Green et al., 1969) and general mitogens (Wheelock, 1965). Prompted by these findings a study was undertaken with the aim of assessing this relationship. The objectives of the present investigations were to: (1) examine temporally the production of interferon in the serum of normal, immune, or splenectomized mice following intravenous administration of general mitogens or a specific antigen; (2) examine the physical-chemical properties of the interferon-like activity obtained
following mitogen stimulation of mouse spleen cells \textit{in vitro}; (3) examine, both temporally and quantitatively, the relationship of lymphocyte blastogenesis and interferon production by spleen cell cultures; (4) examine the types of cells involved in the production of interferon following stimulation by mitogens and an antigen; and (5) examine the role of these cells in interferon production and the mechanisms involved in the induction of interferon by mitogens and antigens.

To accomplish these objectives, serum interferon levels were determined at various times after intravenous injection of PHA or Con A into normal mice and PPD into mice immunized with BCG. Next, some of the physical-chemical properties of the interferon-like factor were examined following induction by PHA from mouse spleen cell cultures. These characteristics then were compared to known properties of standard mouse serum interferon. The relationship between blastogenesis and interferon production was examined in a number of ways. The temporal course of the production of interferon \textit{in vitro} was examined over a 96 hour period. Then, concomittant cultures of spleen cells were examined to compare the degree of blastogenesis with the amount of interferon produced following exposure to various concentrations of the mitogens or PPD.

Bovine serum albumin (BSA) discontinuous density gradient centrifugation was employed to fractionate spleen
cell populations from normal and BCG-immunized mice. This technique was used to determine whether the interferon producing cells belonged to the same or to a different population of spleen cells which undergo blastogenesis. Finally, the role of the various cell types in the production of interferon and the mechanisms involved in the interferon induction process were examined in several ways. Spleen cells from normal or BCG-immunized mice were separated into cell populations of different densities by the BSA gradient technique and treated with anti-theta serum and complement in vitro. This procedure was employed to remove selectively the thymic-dependent lymphocyte population so that the role of the T-cell population in the production of interferon could be assessed. The remaining cells were cultured in the presence of PHA, Con A, PWM, and PPD and the cultures were examined for subsequent interferon production and increase in DNA synthesis. Reconstitution experiments were performed involving the addition of various fractions of spleen cells activated by PHA or their supernatant fluids, to T-cell deficient cultures which by themselves, were unable to produce interferon upon exposure to PHA. The resultant cell-cell or supernatant fluid-cell mixtures were examined then for restoration of the ability of these cultures to produce interferon.
MATERIALS AND METHODS

Ten- to fifteen-week-old CD-1 mice (Charles Rivers, Wilmington, Mass.) were used throughout these experiments. Strain AKR mice (Jackson Laboratories, Bar Harbor, Maine) were used as recipients of C3H (Jackson Labs.) thymocytes for the production of anti-theta serum. The mice were housed in groups of 5 mice per cage and fed an antibiotic-free diet ad libitum. Ten-week-old mice (CD-1) were injected subcutaneously with 300 µg of BCG in complete Freund's adjuvant every two weeks for a six week period. Twenty-six days after the last injection, the sensitized mice were used for experiments involving induction of interferon in vivo and in vitro.

Blastogenesis Inducing Agents

The plant lectins, phytohemagglutinin (PHA-P; Difco, Detroit, Mich.), concanavalin A (Con A; Calbiochem, San Diego, Calif.), and pokeweed mitogen (PWM; Grand Island Biological Co. [GIBCO], Grand Island, N. Y.) were used as non-specific stimulants of lymphocyte blastogenesis. Spleen cells from mice immunized with BCG were cultured with purified protein derivative (PPD; Parke Davis Co., Kalamazoo, Mich.) as a specific antigenic stimulant of lymphocyte blastogenesis.
Interferon Produced In Vivo

CD-1 mice were injected intravenously in the tail vein with 0.1 ml of PHA (1:10 diln. of stock), Con A (100 µg), or PPD (50 µg) diluted in physiologic saline. At appropriate intervals, the mice were bled by cardiac puncture and the blood was allowed to clot for eighteen hours at 4°C. The serum was harvested and adjusted to pH 2.0 with 2N hydrochloric acid and held for 5 days at 4°C. The serum was adjusted to pH 7.0 with 2N sodium hydroxide and heated to 60°C for 60 minutes. The serum was then stored at -70°C until assayed for concentration of interferon.

Splenectomy

Spleens were removed from 10-week-old mice by a lateral incision in the abdominal wall after being anesthetized with an intravenous injection of sodium pentobarbital (6 µg/gram of body weight). The incisions were closed by sutures and the mice were allowed to recover for six weeks prior to use in the experiments. They were maintained on an antibiotic-free diet and did not show any overt signs of infection as a result of the surgery.

Spleen Cell Cultures

Fresh spleens were obtained from mice and the cells were removed by making an incision through the spleen and teasing the cells from them. The cells were placed in
Hank's balanced salt solution with 0.5% dextran (HBSS-D) and separated into single cell suspensions by carefully drawing the cells through a narrow gauge needle (26G). After two washings in HBSS-D, the cell viability was determined using the trypan blue dye exclusion test and the total mononuclear-cell numbers were determined by counting the cells in a Neubauer counting chamber. The spleen cells were resuspended at a cell concentration of 5 x 10^6 mononuclear cells per ml in McCoys-5A medium (Flow Labs Inc., Rockeville, Md.) supplemented with 5% heat-inactivated (56°C for 30 minutes) fresh human serum, penicillin (50 units/ml), and streptomycin (50 μg/ml). The spleen cells were cultured in plastic tubes (Falcon Plastics, Oxnard, Calif.) at 37°C in a humidified 5% CO₂ atmosphere.

**Assay of DNA Synthesis by Lymphocytes**

PHA, Con A, PWM, or PPD were added to each culture of mouse spleen cells. General mitogen-stimulated cultures were incubated for 48 hours; cells stimulated with PPD were cultured for 72 hours. Sixteen hours prior to harvest, 1 μCi of tritiated-thymidine (³H-TdR; New England Nuclear, El Cerrito, Calif., sp. act., 6.7 Ci/mM) was added to duplicate cultures. At harvest, the cells in each culture were resuspended uniformly and 0.1 ml of each culture was applied to duplicate 2.3 cm Whatman (3MM) (W & R Balston, Ltd., England) filter pads following the technique of Mans
and Novelli (1961). The pads were air dried and the cells on the pads were precipitated in 10% TCA and washed once each in ethanol, ethanol:acetone (1:1), and finally washed twice in acetone. The precipitated radioactivity was assayed by liquid scintillation spectrometry. The scintillation fluid consisted of 15.14 g of 2-(5-phenyloxazobyl) (PPO) plus 0.3785 g of 1,4-bis-2-(4ME-5-phenyloxazobyl)-benzene (POPOP) per gallon of toluene. Counting was performed by a Beckman Scintillation Counter with an efficiency between 15 to 25% on the filter pads.

Cytological Assay of Lymphocyte Transformation to Blast Cells

Cell cultures not exposed to $^3$H-TdR were examined for evidence of morphological blast-cell transformation. After appropriate culturing periods, the cultures were centrifuged at 60 x g for 10 minutes to sediment all cells and the culture fluid was removed. The cells were resuspended in 0.25 ml of phosphate buffered saline (pH 7.2) (Cooper, 1967). Each cell suspension was spotted in triplicate on clean glass slides, air dried, and stained with Wright's and Giemsa stains (Gurr, 1962). The percentage of blast-cell transformation per culture was determined by examining 500 stained cells. The morphological characteristics of transformed blast-cells have been previously described (Yoffey et al., 1965).
Assay of Interferon

Mouse L cells (clone 929) were obtained from Dr. C. Gauntt (University of Arizona Medical Center, Tucson). The cells were cultured in a growth medium consisting of Eagle's minimal essential medium (MEM-Gibco, Grand Island, N. Y.) supplemented with 7% calf serum and 1% non-essential amino acids in Blake bottles. For assay of interferon, confluent monolayers of cell cultures were removed by exposure to 0.25% trypsin and were suspended in growth medium. Approximately $1.6 \times 10^6$ cells were seeded to 60 mm plastic petri dishes (Falcon Plastics) and incubated for 16 to 18 hours at 37°C in a 5% CO₂ atmosphere. Cell-free fluids, whether serum or supernatant fluids from spleen cell cultures, were assayed for interferon by diluting the fluids in MEM supplemented with 5% fetal calf serum. Appropriate dilutions were incubated for 18 hours on duplicate L cell monolayer cultures. After removal of the fluids, the monolayer cell cultures were washed twice with warm phosphate-buffered saline and 80 plaque forming units (PFU) of vesicular stomatitis virus (VSV) were added to each cell culture. Adsorption of the virus was allowed to proceed for sixty minutes at 37°C and the inoculum was redistributed every 15 minutes. An overlay medium consisting of 1% Bacto-agar (Difco) and MEM (without phenol red) supplemented with 2% fetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin, 1% non-essential amino acids, 1%
glutamine, and 0.08% protamine sulfate was added to each cell culture. The cells were incubated for 48 hours and the number of plaques were enumerated. The interferon titer was considered the reciprocal of that dilution which produced a 50% reduction in PFU when compared to control cultures.

**Bovine Serum Albumin (BSA) Density Gradient Fractionation of Mouse Spleen Cells**

Spleen cells were obtained as described previously. Portions of the whole cell populations were removed prior to density gradient centrifugation and were kept in culture medium at 4°C during fractionation of the remaining cells. The remaining cells were centrifuged at 200 x g and were suspended in 1.5 ml of a solution of 21% bovine serum albumin (BSA) in phosphate buffered saline. A six layer discontinuous BSA gradient was prepared by modification of a previously described technique (Dicke, Van Hooft, and Van Bekkum, 1968; August et al., 1970; Dicke, 1970). The gradient consisted of 21% to 29% BSA layered in 2% increments with a final layer of 33% BSA. Each BSA layer was prepared from stock 35% BSA in 0.15 M tris (Hydroxymethyl) aminomethane (tris, pH 7.2) at 320 mOsm and diluted to the appropriate concentration with phosphate buffered saline (PBS). Each gradient containing 5 to 8 x 10^8 cells was centrifuged in a 16 x 150 cm siliconized glass tube at 4°C for 30 minutes at 1,000 x g. Following centrifugation, the cells were found to be distributed in layers at the interfaces throughout the gradient.
The cells were harvested from each gradient layer and washed twice in HBSS-D. The cells were counted, viability was determined by the trypan blue dye exclusion test, and each fraction was distributed into culture medium at $5 \times 10^6$ mononuclear cells per ml.

**Production and Assay of Mouse Anti-theta Serum**

Isoantiserum directed against the C3H-theta antigen of mouse lymphocytes was prepared by single, repeated weekly injections of $1 \times 10^7$ viable thymocytes from strain C3H mice into strain AKR recipient mice for a 10 week period (Rief and Allen, 1964). The mice were bled by cardiac puncture eight days after the last immunization. Normal AKR mouse serum was used as control serum. Sera were pooled and stored at $-70^\circ$C. Cytotoxic titer of the anti-theta (C3H) serum was determined by incubating serial two-fold dilutions of the isoantiserum (diluted in McCoys-5A medium) with target cells (thymocytes and spleen cells from different strains of mice) for 30 minutes at $37^\circ$C and then adding fresh guinea pig complement (diluted 1:5 in McCoys-5A medium) for an additional 30 minutes at $37^\circ$C. Cells were washed twice with McCoys-5A medium and their viability was determined by the trypan blue dye exclusion test. The cytotoxic activity directed against C3H, AKR, and CD-1 strain mouse thymocytes and spleen cells is presented in Fig. 1. The AKR anti-theta (C3H) serum was found to be
Fig. 1. Cytotoxicity Assays of the AKR Anti-theta (C3H) Serum against Thymocytes and Spleen Cells of Different Strains of Mice — The percentage of dead cells was determined by trypan blue dye exclusion following incubation of the cells in the presence of anti-theta serum and complement. Control cells were incubated in the presence of homologous non-immune serum and complement and the number of dead cells in this control was subtracted from each group. The target cells used were CD-1 mouse thymocytes (●●●) and splenocytes (○○○), C3H mouse thymocytes (★★★) and splenocytes (☆☆☆), and AKR mouse thymocytes (■■■) and splenocytes (□□□). Each cell type was tested at a concentration of 100 x 10^6 cells per ml.
cytotoxic for 35% of C3H mouse spleen cell lymphocytes and was cytotoxic for greater than 70% of C3H mouse thymocytes. Very little cytotoxic activity was noted against AKR mouse spleen cells or thymocytes. The cytotoxic activity was completely removed by absorbing the antiserum with C3H mouse brain cell homogenate but not with AKR mouse brain cell homogenate or with C3H mouse liver tissue homogenates. The anti-theta (C3H) serum at a dilution of 1:10 resulted in cell death for 37% of spleen cells and 78% for thymocytes from CD-1 mice. Control AKR mouse serum was cytotoxic for less than 5% of CD-1 mouse spleen cells and 8% of CD-1 mouse thymocytes, a level which was comparable to the viability of these populations after treatment with homologous non-immune serum and complement.
RESULTS

Induction of Interferon In Vivo

The in vivo production of serum interferon following mitogenic or antigenic challenge was examined in normal and splenectomized mice. The results of this study are presented in Table 1. No interferon was detected in the serum of normal mice at 6 hours after injection of the PHA (0.1 ml of a 1:10 diln. of stock) or Con A (100 μg). Serum interferon was initially detected at 24 hours after injection of these mitogens. Maximum levels of serum interferon were achieved by 72 hours and the levels were decreasing by 96 hours following administration of the mitogens. Splenectomized mice, injected i.v. with PHA, produced little or no interferon within a 96 hour period; serum interferon was detected at 48 hrs in only one of three mice in this group with a titer of 10 units per ml. Normal or splenectomized control mice, injected i.v. with 100 μg of BSA, failed to produce any interferon at any time over the 96 hour test period.

Intravenous administration of 50 μg of PPD into BDG-immunized mice did not produce any interferon at 6 hours post-injection. Interferon was initially detected within 24 hours of injection of PPD and continued to be produced in increasing amounts up to 96 hours, reaching a titer of 40
Table 1. Temporal Production of Circulating Interferon by Normal Immune and Splenectomized Mice Following Mitogen and Antigen Administration

<table>
<thead>
<tr>
<th>Compound Injected</th>
<th>Splenectomy</th>
<th>Interferon Titers at Times (hours) Post-injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>PHA (1:10)</td>
<td>-</td>
<td>0 (0)</td>
</tr>
<tr>
<td>PHA (1:10)</td>
<td>+</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Con A (100 µg)</td>
<td>-</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Control C</td>
<td>-</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Control D</td>
<td>+</td>
<td>0 (0)</td>
</tr>
<tr>
<td>PPD (50 µg)</td>
<td>-</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Control F</td>
<td>-</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

a. Each compound was injected (i.v.) in the tail vein.

b. Values represent mean serum interferon titers from 3 mice per group expressed in units per ml. Values in parentheses represent the number of mice producing interferon per group.

c. Normal control mice were injected with 100 µg of BSA to determine the effects of injection of protein.

d. Splenectomized control mice were injected with 100 µg of BSA.

e. Mice immunized with BCG were injected with 50 µg of PPD.

f. Normal non-immune mice were injected with 50 µg of PPD.

g. ND = Not done.
units per ml. No interferon was produced by normal non-immune mice challenged with 50 μg of PPD.

These results demonstrate that interferon can be induced in vivo both by the general mitogens, PHA and Con A, and by the specific antigen, PPD. Injection of a non-specific protein (BSA), to which the mice were not immune, did not result in the production of interferon.

Physical-chemical Properties of Interferon Induced by PHA

The physical-chemical characteristics of the antiviral activity stimulated in vitro by exposure of spleen cells to PHA were similar to those of a mouse standard serum interferon supplied by the National Institutes of Health. The antiviral activity induced by PHA was trypsin-sensitive, deoxyribonuclease resistant, and was not sedimentable following centrifugation at 100,000 x g. Similar properties were found for the mouse serum interferon standard (Table 2). A few differences were found between the two interferons. The interferon induced by PHA was found to be partially labile when heated to 60°C for 60 minutes and exposed to pH 2.0 for 5 days at 4°C whereas the mouse serum interferon standard was not labile when treated under the same conditions. PHA-induced interferon had a low level antiviral activity across species barriers as some protection was demonstrated in HeLa monolayers against VSV. The mouse serum interferon standard failed to demonstrate
Table 2. Comparison of Physical-Chemical Properties of PHA Induced Interferon and the NIH Standard Mouse Serum Interferon

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Interferon Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PHA-IF</td>
</tr>
<tr>
<td>Titer (L-cells-VSV)</td>
<td>160</td>
</tr>
<tr>
<td>Trypsin (0.25%-60 min)</td>
<td>0</td>
</tr>
<tr>
<td>DNase</td>
<td>160</td>
</tr>
<tr>
<td>Heat (60°C-60 min)</td>
<td>20</td>
</tr>
<tr>
<td>pH 2.0 (5 days-4°C)</td>
<td>40</td>
</tr>
<tr>
<td>Virus specificity: MM virus</td>
<td>80</td>
</tr>
<tr>
<td>Vaccina Virus</td>
<td>80</td>
</tr>
<tr>
<td>Species specificity: HeLa cells</td>
<td>20</td>
</tr>
<tr>
<td>Sedimentation (100,000 xg)</td>
<td>160</td>
</tr>
</tbody>
</table>
any antiviral activity in HeLa cell monolayers. Both sources of interferon exhibited no virus specificity and had no direct effect on plaque titers when incubated with the challenge virus prior to plaque assay.

Temporal Induction of Interferon by Mitogens and an Antigen

Temporal studies were conducted to determine the kinetics of induction of interferon by PHA, Con A, or PPD in spleen cell cultures in vitro. PHA (0.1 ml of a 1:100 dilution of stock), Con A (10 μg/ml), and PPD (5 μg/ml) were utilized for the temporal study for induction of interferon. Table 3 shows the results of the time course study for the production of interferon in vitro. Interferon induced by PHA or Con A was detected in the cell cultures at 24 hours and maximum titers were obtained at 72 and 48 hours respectively. The interferon induced by PPD was detected at 48 hours and reached maximum levels by 72 hours. Both the mitogen and the antigen-induced interferon titers remained relatively stable throughout duration of the culture period after reaching maximum titers.

Relationship Between Incorporation of Tritiated-Thymidine and Production of Interferon by Cell Cultures In Vitro

Studies were conducted to examine the relationship of incorporation of $^3$H-TdR and production of interferon by mouse spleen cells activated in vitro by mitogens or antigen. After culturing the cells for appropriate time periods in
Table 3. Temporal Elaboration of Interferon by Stimulated Spleen Cell Cultures

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Interferon Titer (units/ml) at Post-inoculation&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6</td>
</tr>
<tr>
<td>PHA (0.1 ml of 1:100)</td>
<td>0</td>
</tr>
<tr>
<td>Con A (10 μg)</td>
<td>0</td>
</tr>
<tr>
<td>PPD (5 μg)</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Interferon titers are expressed in units/ml.

the presence of mitogens or PPD, the cultures were assayed for incorporation of $^3$H-TdR into TCA-insoluble material and supernatant fluids were assayed for interferon content. Various concentrations of each mitogen or antigen were examined for their ability to stimulate DNA synthesis and interferon production. The PHA (Fig. 2a), CON A (Fig. 2b), and PWM (Fig. 2c) dose-response curves demonstrated that maximum levels of interferon were obtained with the same doses of mitogens which stimulated maximum incorporation of $^3$H-TdR. Suboptimal doses of PHA and Con A (one-tenth of the optimal stimulatory concentration) resulted in the loss of greater than 75% of the amount of interferon produced; however, these same concentrations of mitogens resulted in less of a reduction in incorporation of $^3$H-TdR by the cells.
Fig. 2. DNA Synthesis and Interferon Production by Mouse Spleen Cells Following Activation by Various Concentrations of PHA, Con A, PWM, and PPD — Spleen cell cultures were incubated with mitogens or antigen (PPD) for appropriate time periods. Twenty-four hours prior to harvesting the cultures, 1 μCi/ml of $^3\text{H}$-TdR was added to the cultures. All mitogen-treated cultures were harvested at 48 hours and PPD-treated cultures were harvested at 72 hours and assayed for amount of $^3\text{H}$-TdR incorporated into TCA-insoluble material and for interferon. The results for different mitogens or antigen (PPD) are shown in the panels. (A) phytohemagglutinin (PHA-P), (b) Concanavalin A (Con A), (C) pokeweed mitogen (PWM), (D) purified protein derivative (PPD). (●—●, $^3\text{H}$-TdR incorporation in counts per minute [CPM]; o---o, interferon titer in units/ml.) The mean values of six experiments are presented (±1 S.D.). Values for incorporation of $^3\text{H}$-TdR into TCA-insoluble material represents CPM in experimental cell cultures minus CPM in control cell cultures. The control cell cultures (not exposed to mitogens or antigen) had CPM values ranging from 500 to 600 CPM per culture.
Fig. 2. DNA Synthesis and Interferon Production by Mouse Spleen Cells Following Activation by Various Concentrations of PHA, Con A, PWM, and PPD
Various concentrations of PPD were examined for their ability to induce interferon production and stimulate the incorporation of $^3$H-TdR into TCA-insoluble material by spleen cell cultures from BCG-sensitized mice (Fig. 2d). The cell cultures were incubated in the presence of PPD for 72 hours. The amount of DNA synthesized and the amount of interferon elaborated into the supernatant fluid were measured. As was found for the mitogens studied, the concentration of PPD (5 μg) which optimally stimulated DNA synthesis also resulted in production of maximum titers of interferon. In non-sensitized control mice, no enhanced stimulation of DNA synthesis or production of interferon occurred at any concentration of PPD tested, i.e., 0.1 to 25 μg/ml.

A comparison of the optimal dose of each stimulant (PHA, Con A, PWM, and PPD) for DNA synthesis and interferon production was made to correlate the degree of blastogenesis and the amount of interferon produced from cultures of the same spleen cell population (Table 4). PHA induced significantly higher levels of interferon (80 units/ml) than did the other mitogens examined; however, it can be seen that Con A resulted in a greater proportion of the spleen cells undergoing blastogenesis (42% for Con A vs. 32% for PHA). PWM and PPD activated fewer cells to undergo DNA synthesis as measured by a reduced incorporation of $^3$H-TdR and transformation of fewer lymphocytes to blast cells, and yielded
Table 4. Relationship of DNA Synthesis, Blast Cell Transformation, and Interferon Titers Obtained Using Optimal Stimulatory Concentration of Each Mitogen or Antigen

<table>
<thead>
<tr>
<th>Compound</th>
<th>$^3$H-TdR Incorporation (CPM)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>S.I.&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% Blast Cells</th>
<th>Interferon Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA (1:100)</td>
<td>4020 (+320)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>6.1</td>
<td>32</td>
<td>80 (+10)</td>
</tr>
<tr>
<td>Con A (10 µg)</td>
<td>6610 (+800)</td>
<td>10.0</td>
<td>42</td>
<td>35 (+8)</td>
</tr>
<tr>
<td>PWM (1:10)</td>
<td>3040 (+280)</td>
<td>4.6</td>
<td>21</td>
<td>15 (+5)</td>
</tr>
<tr>
<td>Control&lt;sup&gt;c&lt;/sup&gt;</td>
<td>660 (+50)</td>
<td>1.0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>PPD (5 µg)</td>
<td>2710 (+260)</td>
<td>4.9</td>
<td>16</td>
<td>17 (+3)</td>
</tr>
<tr>
<td>Control&lt;sup&gt;d&lt;/sup&gt;</td>
<td>550 (+70)</td>
<td>1.0</td>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>

a. Incorporation of $^3$H-TdR into TCA-isoluble materials is expressed in counts per minute (CPM).

b. S.I. = Stimulation Index which represents the ratio of the CPM in experimental cultures to CPM in control cultures not exposed to mitogen or antigen.

c. Control culture from normal mice only.

d. Control cultures from BCG-sensitized mice only.

e. Mean values of six experiments (± one S.D.).
less interferon than cell cultures that had been exposed to PHA or Con A.

These results indicated that although optimal mitogenic concentrations of each stimulant were required to obtain maximum interferon titers, the degree of induced blastogenesis, as reflected both by the $^3$H-TdR incorporation and the percentage of transformed blast cells within each culture did not correspond to the amount of interferon produced.

**Effect of Anti-theta Serum on DNA Synthesis and Interferon Production by Spleen Cell Cultures**

Mouse AKR anti-theta (C3H) serum directed against the theta antigen present on CD-1 thymic-dependent lymphocytes (T-cells), was used in the presence of complement to selectively destroy the splenic T-cell population. T-cell depleted cell cultures were examined for their ability to respond to mitogens and an antigen (PPD) with the production of interferon. Non-treated, normal AKR mouse serum-treated, and AKR anti-theta (C3H) serum treated CD-1 spleen cells were washed, counted, and seeded at $5 \times 10^6$ mononuclear cells per culture. The cell cultures then were exposed to optimal concentrations of PHA, Con A, PWM, and PPD and the blastogenic response and interferon titers were measured as previously described. The results are presented in Table 5.

Anti-theta serum treatment of spleen cells in the presence of complement resulted in a 70% or greater
Table 5. Production of Interferon and Incorporation of $^3$H-Tdr into TCA-insoluble Material in Cell Cultures Treated with Anti-theta Serum Prior to Exposure to PHA, Con A, PWM, or PPD.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Treatment</th>
<th>$^3$H-Tdr Incorporation (CPM)</th>
<th>S.I. b</th>
<th>Interferon (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA</td>
<td>None</td>
<td>3410</td>
<td>6.4(±1.2)</td>
<td>80(±20)</td>
</tr>
<tr>
<td></td>
<td>NMSC</td>
<td>3220</td>
<td>6.8(±1.1)</td>
<td>60(±20)</td>
</tr>
<tr>
<td></td>
<td>Anti-theta Serum d</td>
<td>1190</td>
<td>2.5(±0.2)</td>
<td>0</td>
</tr>
<tr>
<td>Con A</td>
<td>None</td>
<td>5130</td>
<td>9.5(±1.2)</td>
<td>35(±10)</td>
</tr>
<tr>
<td></td>
<td>NMS</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Anti-theta Serum</td>
<td>2250</td>
<td>4.8(±1.2)</td>
<td>0</td>
</tr>
<tr>
<td>PWM</td>
<td>None</td>
<td>3190</td>
<td>5.9(±0.6)</td>
<td>40(±10)</td>
</tr>
<tr>
<td></td>
<td>NMS</td>
<td>3040</td>
<td>5.8(±1.1)</td>
<td>20(±5)</td>
</tr>
<tr>
<td></td>
<td>Anti-theta Serum</td>
<td>2560</td>
<td>5.4(±0.3)</td>
<td>40(±15)</td>
</tr>
<tr>
<td>PPD f</td>
<td>None</td>
<td>2860</td>
<td>4.3(±0.7)</td>
<td>20(±5)</td>
</tr>
<tr>
<td></td>
<td>NMS</td>
<td>2590</td>
<td>4.2(±1.1)</td>
<td>20(±5)</td>
</tr>
<tr>
<td></td>
<td>Anti-theta Serum</td>
<td>610</td>
<td>1.2(±0.3)</td>
<td>0</td>
</tr>
</tbody>
</table>

a. Compounds were used at their optimum concentrations (see Fig. 2).

b. S.I. = Stimulation Index = the ratio of CPM in experimental cultures:CPM in control cultures.

c. NMS = Normal AKR mouse serum.

d. Anti-theta Serum = AKR anti-theta (C3H) Serum.

e. ND = Not done.

f. Spleen cells were obtained from mice immunized with BCG.
reduction in incorporation of $^3$H-TdR into TCA-insoluble material in cultures exposed to PHA and PPD. DNA synthesis in cell cultures incubated with Con A was reduced by approximately 50% while DNA synthesis in cell cultures incubated with PWM was not significantly affected. The interferon response was inhibited completely by treatment of cells with anti-theta serum prior to exposure to PHA, Con A, PWM, or PPD. In contrast, interferon production in cell cultures exposed to PWM was not affected by prior treatment with anti-theta serum. Spleen cells treated with normal AKR mouse serum in the presence of complement failed to exhibit enhanced DNA synthesis or interferon production in response to any of the mitogens or the antigen (PPD).

These results show that the production of interferon by spleen cells exposed to PHA, Con A, and PPD was inhibited by treatment of the cells with anti-theta serum and indicated the involvement of the T-cell population in the immune induction of interferon. Although the T-cells are important in obtaining interferon in response to T-dependent mitogens, it is evident that they are not absolutely required for interferon production. Another pathway circumventing the T-cell may exist because PWM induced the production of interferon from the spleen cell cultures depleted of T-cells.
Distribution of Mouse Spleen Cells in Discontinuous BSA Density Gradients

Spleen cells were separated into fractions by centrifugation through a discontinuous BSA density gradient. The cell fractions were found to localize at the various interfaces of the gradient. The fractionation of these cells apparently depended on their relative densities. Morphologically, the cell types that were distributed in each fraction throughout the gradient were heterogeneous. The per cent of total cells recovered at each density layer of BSA is presented in Table 6. Fractions III and IV were found to contain the largest proportion of the cells (25% and 27% respectively).

Differential counts were made on 500 cells stained with Wright's and Giemsa stains from each fraction (Table 7). The less dense fractions of the gradients, fractions I and II, contained equal numbers of small and medium-sized lymphocytes, the majority of the blastoid forms, and some cells resembling plasmacytes. The medium-sized lymphocytes were classified as such on the basis of their larger size (10-15 μ), more diffuse chromatin patterns and a greater ratio of cytoplasm to nucleus than small lymphocytes. Morphologically, fractions III and IV contained greater than 95% small lymphocytes. These populations were contaminated with less than 5% large mononuclear cells. Fractions V and VI primarily contained small lymphocytes and large
Table 6. Distribution of Cells Following BSA Discontinuous Density Gradient Fractionation of Mouse Spleen Cells

<table>
<thead>
<tr>
<th>Fractions (%) BSA</th>
<th>% Cells Recovered(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-I (21%)</td>
<td>8.7 (+ 1.1)</td>
</tr>
<tr>
<td>F-II (23%)</td>
<td>16.9 (+ 2.3)</td>
</tr>
<tr>
<td>F-III (25%)</td>
<td>20.6 (+ 3.9)</td>
</tr>
<tr>
<td>F-IV (27%)</td>
<td>25.4 (+ 2.8)</td>
</tr>
<tr>
<td>F-V (29%)</td>
<td>11.6 (+ 1.3)</td>
</tr>
<tr>
<td>F-VI (33%)</td>
<td>16.8 (+ 2.1)</td>
</tr>
</tbody>
</table>

\(^a\) Approximately 800 x 10\(^6\) spleen cells were added to each gradient. Each value is the mean of six gradients (+ S.D.) and represents the per cent of the total recoverable cells present in each layer.
Table 7. Morphological Types of Cells in Each Layer of the BSA Density Gradients Following Fractionation of Mouse Spleen Cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Unfractionated Cells</th>
<th>I (21%)</th>
<th>II (23%)</th>
<th>III (25%)</th>
<th>IV (27%)</th>
<th>V (29%)</th>
<th>VI (33%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large Blast Cells</td>
<td>5.0 (+0.4)\textsuperscript{a}</td>
<td>7.4</td>
<td>4.6</td>
<td>0.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Large Mononuclear Cells</td>
<td>16.4 (+1.1)</td>
<td>17.8</td>
<td>7.7</td>
<td>1.4</td>
<td>2.0</td>
<td>34.0</td>
<td>42.8</td>
</tr>
<tr>
<td>Medium Sized Lymphocytes</td>
<td>15.4 (+1.9)</td>
<td>42.3</td>
<td>40.5</td>
<td>2.6</td>
<td>1.2</td>
<td>6.6</td>
<td>4.6</td>
</tr>
<tr>
<td>Small Lymphocytes</td>
<td>63.0 (+3.5)</td>
<td>42.5</td>
<td>47.2</td>
<td>95.6</td>
<td>96.6</td>
<td>61.2</td>
<td>52.2</td>
</tr>
<tr>
<td>Polymorphonuclear Cells</td>
<td>0.2 (+0.1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
<td>0.2</td>
<td>0.4</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Mean values from four different gradients (± S.D.).
mononuclear cells. The large mononuclear cells were characterized by increased size (25 μ), indented nuclei, and abundant, vacuolated cytoplasm. The viability of the first five fractions after 72 hours of culture was greater than 80% whereas the viability of fraction VI was 60% to 65%.

**DNA Synthesis and Interferon Production by Different Mouse Spleen Cell Fractions Following Incubation with PHA and Con A**

Spleen cells were separated on discontinuous BSA density gradients, and the individual fractions were assayed for their capacity to synthesize DNA and their ability to produce interferon in vitro, in response to exposure to PHA and Con A. The results of six experiments are presented in Figs. 3 and 4.

Cells from each fraction were stimulated with optimum concentrations of PHA and Con A as determined previously (see Fig. 2). The highest level of incorporation of \(^3\)H-TdR into TCA-insoluble material following exposure to PHA occurred in fraction IV. Fraction III showed some stimulatory response while the other fractions exhibited little or no DNA synthesis. Exposure of each cell fraction to Con A resulted in a similar response pattern, although the degree of stimulation was greater in each individual fraction than that of PHA. Maximum incorporation of \(^3\)H-TdR into TCA-insoluble material occurred in fractions III and IV. Fraction IV responded with the highest increment of DNA
Fig. 3. Tritiated-Thymidine Incorporation and Interferon Production Following Exposure to PHA by Mouse Spleen Cell Populations Fractionated on Discontinuous BSA Density Gradients — Each BSA fraction of cells was cultured in the presence of phytohemagglutinin (PHA-P) for 48 hours and then examined for $^3$H-TdR incorporation into TCA-insoluble material and for the amount of interferon elaborated into the supernatant fluid. (●●●, cultures exposed to PHA; ○○○, control cultures.) The mean values of six experiments are presented (+ 1 S.D.).
Fig. 3. Tritiated-Thymidine Incorporation and Interferon Production Following Exposure to PHA by Mouse Spleen Cell Populations Fractionated on Discontinuous BSA Density Gradients
Fig. 4. Tritiated-Thymidine Incorporation and Interferon Production by Fractions of BSA Gradient-Fractionated Mouse Spleen Cells Following Exposure to Concanavalin A — Each fraction of cells was cultured in the presence of concanavalin A (Con A) for 48 hours and then examined for incorporation of \(^{3}\text{H}-\text{TdR}\) into TCA-insoluble material and for the amount of interferon elaborated into the culture fluid. (••••••, cultures exposed to Con A; o--o, control cultures.) The mean values of six experiments are presented (± 1 S.D.).
Fig. 4. Tritiated-Thymidine Incorporation and Interferon Production by Fractions of BSA Gradient-Fractionated Mouse Spleen Cells Following Exposure to Concanavalin A
synthesis after exposure to Con A compared to control cultures; fractions III and II exhibited less, though significant, DNA synthesis.

The interferon-producing cells were found in fraction V after exposure to all mitogens tested and to PPD. Insignificant levels of interferon were produced by cells from the other fractions. PHA was found to induce more interferon in cells of fraction V than did Con A. However, cells from fraction V of both cultures exhibited a relatively low level of DNA synthesis compared to cell fraction IV. In contrast, Con A stimulated more DNA synthesis in cells of fraction IV than did PHA. Therefore, Con A stimulated more cells to synthesize DNA; however, PHA stimulated a higher production of interferon than Con A. This indicates that these two responses, DNA synthesis and interferon production are not directly related.

These results showed that cells responding with enhanced DNA synthesis were localized in fraction IV, yet this subpopulation of cells produced little or no interferon. In contrast, the interferon-producing population of cells was found in fraction V. Thus, the interferon-producing cells belong to a population of cells which were different from cells synthesizing DNA following exposure to PHA or Con A.
DNA Synthesis and Interferon Production by Different Mouse Spleen Cell Fractions Following Incubation with PWM

Treatment of spleen cells with anti-theta serum prior to incubation with PWM had no inhibiting effect on DNA synthesis or interferon production (refer to Table 5). This is in direct contrast to the inhibiting effect that depletion of T-cells had upon the PHA- and Con A-induced responses of DNA synthesis and interferon production. This result suggests that PWM may be activating a different population of spleen cells than the other mitogens. To examine this possibility, spleen cells were separated into different fractions on BSA density gradients. Different fractions of spleen cells were exposed to PWM and the synthesis of DNA and interferon production were measured. The results of six separate fractionation experiments are presented in Fig. 5.

The capacity for synthesis of DNA was found to be localized in cells of fractions I and II. Fraction II showed the highest level of incorporation of $^3$H-TdR into TCA-insoluble material when compared to control cultures. Very little DNA synthesis was detected in cells from fractions III through VI. The PWM-induced interferon response was again found in cells of fraction V. Control cultures from each fraction did not produce any interferon.

These data demonstrate that cells undergoing PWM-induced DNA synthesis belonged to a different population of cells from those cells which produce interferon. PWM
Fig. 5. Tritiated-Thymidine Incorporation and Interferon Production Following Exposure to PWM by Fractions of Mouse Spleen Cells Fractionated on Discontinuous BSA Density Gradients — Each fraction of cells was cultured in the presence of pokeweed mitogen (PWM) for 48 hours and then examined for incorporation of $^3$H-TdR into TCA-insoluble material and for the elaboration of interferon into the culture fluid. ($\bullet\cdots\bullet$, cultures exposed to PWM; $\circ\cdots\circ$, control cultures.) The mean values of six experiments are presented ($\pm 1$ S.D.).
Fig. 5. Tritiated-Thymidine Incorporation and Interferon Production Following Exposure to PWM by Fractions of Mouse Spleen Cells Fractionated on Discontinuous BSA Density Gradients
activated a DNA synthesis response in a cell population of lighter density than those stimulated by PHA or Con A. However, all three mitogens induced production of interferon in the same fraction of cells, i.e., fraction V.

**DNA Synthesis and Interferon Production by Different Spleen Cell Fractions of Cells from BCG-Immunized Mice Challenged with PPD**

Spleen cells from mice immunized with BCG were fractionated into six populations on discontinuous BSA density gradients. Each fraction was challenged with an optimum stimulatory dose of PPD (refer to Fig. 2) to determine the immune-induced DNA synthesis and interferon production responses. The results of four experiments are presented in Fig. 6. Maximum stimulation of incorporation of $^3$H-TdR into TCA-insoluble material by PPD occurred in cells of fraction III and some stimulation also occurred in cells from fraction IV. Apparently, some concentration of antigen-reactive cells occurred in fraction III, because greater stimulation of DNA synthesis was found in this fraction than in the whole unfractionated population. A similar concentration of reactive cells also was noted in gradient fractions of cells exposed to non-specific mitogens, PHA and Con A. Production of interferon in response to PPD occurred maximally in fraction V but no significant incorporation of $^3$H-TdR into TCA-insoluble material was found in that fraction. No fraction of spleen cells from
Fig. 6. Tritiated-Thymidine Incorporation and Interferon Production by Fractions of BSA Gradient-Fractionated Mouse Spleen Cells Sensitized to BCG Following Exposure to PPD -- Each fraction of cells was cultured in the presence of purified protein derivative (PPD) for 72 hours and then examined for incorporation of \(^{3}\text{H}-\text{TdR}\) into TCA-insoluble material and the production of interferon. (••••, cultures exposed to PPD; o---o, control cultures.) The mean values of six experiments are presented (± 1 S.D.).
Fig. 6. Tritiated-Thymidine Incorporation and Interferon Production by Fractions of BSA Gradient-Fractionated Mouse Spleen Cells Sensitized to BCG Following Exposure to PPD
non-immunized mice produced interferon in response to PPD, confirming that this response was immunologically specific. These data demonstrate that cells involved in DNA synthesis in response to PPD and those involved in the production of interferon belong to separate populations of spleen cells.

**Effect of Anti-theta Serum Treatment on BSA Gradient Fractionated Spleen Cell Populations**

Individual fractions of spleen cells were treated with anti-theta serum and complement to remove thymic-dependent lymphocytes (T-cells). Cell numbers were found to be decreased to varying degrees in each fraction following this treatment. The per cent reduction in cells found in each fraction is presented in Table 8.

Cells in fraction III and IV, which are primarily small lymphocytes, were the most sensitive to cytolysis by anti-theta serum and complement. Greater than 50% of the cells residing in both of these fractions were shown to possess the theta antigenic determinant. The other four fractions contained less than 20% of cells sensitive to anti-theta serum.

These results show that the thymic dependent lymphocytes (T-cells) concentrate in fractions III and IV, yet some T-cells (up to 19%) remain in all fractions prior to treatment with anti-theta serum. This difference in density among the lymphocytes may represent dynamic maturational processes or reflect some as yet undetermined
Table 8. Effect of Anti-theta Serum on Recovery of Cells from Different Fractions of Cells from BSA Density Gradients

<table>
<thead>
<tr>
<th>Fractions</th>
<th>% Cells Recovered</th>
<th>% Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before (^a)</td>
<td>After (^b)</td>
</tr>
<tr>
<td>F-I (21%)</td>
<td>9.1 (+0.8)</td>
<td>8.3 (+1.4)</td>
</tr>
<tr>
<td>F-II (23%)</td>
<td>18.2 (+0.5)</td>
<td>15.1 (+2.3)</td>
</tr>
<tr>
<td>F-III (25%)</td>
<td>22.7 (+1.7)</td>
<td>10.0 (+4.9)</td>
</tr>
<tr>
<td>F-IV (27%)</td>
<td>24.1 (+1.3)</td>
<td>10.2 (+3.7)</td>
</tr>
<tr>
<td>F-V (29%)</td>
<td>11.3 (+1.1)</td>
<td>9.1 (+0.9)</td>
</tr>
<tr>
<td>F-VI (33%)</td>
<td>14.6 (+0.9)</td>
<td>12.0 (+1.5)</td>
</tr>
<tr>
<td>Normal Population</td>
<td>100</td>
<td>67.7 (+3.5)</td>
</tr>
</tbody>
</table>

\(^a\) Per cent of the total viable cells present in each fraction prior to treatment with anti-theta serum.

\(^b\) Per cent of total viable cells recovered in each fraction following cytotoxic treatment with anti-theta serum and complement. Values represent means of 5 gradients (+ S.D.).
functional difference which contributes to the density differences.

**Effect of Anti-theta Serum on DNA Synthesis and Interferon Production by BSA Gradient Fractionated Spleen Cells Challenged with PHA, Con A, PWM, and PPD In Vitro**

The previous results (refer to Table 5) suggested a T-cell requirement for DNA synthesis and interferon production by unfractionated mouse spleen cells. Both of these responses to PHA, Con A, and PPD were reduced or eliminated by anti-theta serum treatment. Interestingly, neither DNA synthesis nor interferon production following challenge with pokeweed mitogen was effected by treating the cell fractions with anti-theta serum. Spleen cells were fractionated on discontinuous BSA density gradients and then each fraction of cells was treated with anti-theta serum and complement to remove the T-cell subpopulation of lymphocytes. Each fraction was challenged with optimum concentrations of PHA, Con A, PWM, or PPD to determine the effect of T-cell depletion on DNA synthesis and interferon production. The results of these experiments are presented in Tables 9 and 10. The results of studies involving PHA and Con A were similar and will be discussed first. The treatment with anti-theta serum of fractions of cells separated by centrifugation on BSA density gradients revealed that the DNA synthesis response was severely depressed in fraction IV cells exposed to PHA or Con A (Table 9). The interferon
Table 9. Effect of Anti-theta Serum on the DNA Synthesis Response of Mouse Spleen Cell Fractions Upon Exposure to PHA, Con A, PWM, and PPD

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Treatment</th>
<th>BSA Density</th>
<th>3H-TdR Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gradient Fraction</td>
<td>CPM&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PHA</td>
<td>None&lt;sup&gt;c&lt;/sup&gt;</td>
<td>IV</td>
<td>5030</td>
</tr>
<tr>
<td></td>
<td>Anti-theta Serum</td>
<td>IV</td>
<td>460</td>
</tr>
<tr>
<td>Con A</td>
<td>None</td>
<td>IV</td>
<td>6270</td>
</tr>
<tr>
<td></td>
<td>Anti-theta Serum</td>
<td>IV</td>
<td>1275</td>
</tr>
<tr>
<td>PWM</td>
<td>None</td>
<td>II</td>
<td>3260</td>
</tr>
<tr>
<td></td>
<td>Anti-theta Serum</td>
<td>II</td>
<td>2890</td>
</tr>
<tr>
<td>PPD&lt;sup&gt;d&lt;/sup&gt;</td>
<td>None</td>
<td>III</td>
<td>3870</td>
</tr>
<tr>
<td></td>
<td>Anti-theta Serum</td>
<td>III</td>
<td>520</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values from six gradients are expressed as mean counts per minute (CPM) per 0.1 cc of cell suspension.

<sup>b</sup> S.I. = Stimulation Index = ratio of CPM in experimental cultures to CPM in control culture (± S.D.).

<sup>c</sup> Normal mouse serum plus complement treatment of these fractions were found to have responses comparable to normal cultures in which no treatment occurred.

<sup>d</sup> Spleen cell cultures were obtained from BCG-sensitized mice.
Table 10. Effect of Anti-theta Serum on the Interferon Response by Fraction V Cells Subsequently Incubated with PHA, Con A, PWM, or PPD

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Treatment</th>
<th>BSA Density Gradient Fraction</th>
<th>Interferon (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA</td>
<td>None</td>
<td>V</td>
<td>120 (+20)(^a)</td>
</tr>
<tr>
<td></td>
<td>Anti-theta Serum</td>
<td>V</td>
<td>0</td>
</tr>
<tr>
<td>Con A</td>
<td>None</td>
<td>V</td>
<td>70 (+20)</td>
</tr>
<tr>
<td></td>
<td>Anti-theta Serum</td>
<td>V</td>
<td>5 (+5)</td>
</tr>
<tr>
<td>PWM</td>
<td>None</td>
<td>V</td>
<td>35 (+10)</td>
</tr>
<tr>
<td></td>
<td>Anti-theta Serum</td>
<td>V</td>
<td>40 (+10)</td>
</tr>
<tr>
<td>PPD(^b)</td>
<td>None</td>
<td>V</td>
<td>20 (+0)</td>
</tr>
<tr>
<td></td>
<td>Anti-theta Serum</td>
<td>V</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) Values are expressed as mean of six experiments. The amount of interferon is expressed as the titer obtained from culture fluids (+ S.D.).

\(^b\) Spleen cell cultures were obtained from BCG-immunized mice.
response, which was found only in fraction V cells, was also removed by a prior treatment with anti-theta serum (Table 10). Normal mouse serum did not affect either response upon subsequent challenge with PHA or Con A.

PPD stimulation of DNA synthesis in spleen cells from BCG-sensitized mice occurred principally in fraction III cells. This response was depressed by treating cells of this fraction with anti-theta serum (Table 9). The PPD-induced production of interferon was found only in cells of fraction V and also was eliminated by anti-theta serum treatment of cells prior to exposure to PPD (Table 10).

In contrast, the PWM-induced synthesis of DNA and production of interferon were found not to be sensitive to treatment with anti-theta serum. After elimination of theta-bearing lymphocytes, DNA synthesis remained unchanged in fraction I and II following exposure to PWM (Table 9). The PWM-induction of interferon, normally present in fraction V, also was unaffected by a prior exposure of cells to anti-theta serum. These results demonstrate that while DNA synthesis and interferon production in response to PHA, Con A, and PPD were T-cell dependent, these similar responses when induced by PWM were not T-cell dependent. These findings relate an important role for thymic dependent lymphocytes in the induction phase of interferon production for at least two general mitogens (PHA and Con A) and one antigen (PPD). These results show that one mitogen, PWM, activated
a different subpopulation of spleen cells for production of interferon and this induction occurred via a mechanism which is independent of the T-cell subpopulation of lymphocytes. The PWM-induced interferon response residing in fraction V is, therefore, an expression of a T-cell independent sub-population of spleen cells. These findings suggest that induction of interferon is a complex process which is cell-dependent as well as inducer-dependent.

Reconstitution of the Interferon Response in Anti-theta Serum-treated Spleen Cells by Activated Cells and a Supernatant Fluid Factor from Phytohemagglutinin-Stimulated Cultures

Mitogen-stimulated lymphocytes have been shown to elaborate a factor which is capable of stimulating other cell types to heightened activity, i.e., macrophage activation factor, which increased macrophage bacteriocidal activity (Simon and Sheagren, 1971). Since there is a dissociation between cells incorporating $^3$H-TdR and those producing interferon, it was possible that stimulated lymphocytes also might produce a factor which activated or restored the ability of the interferon producing cells to produce interferon. Depletion of T-cells from fraction V of BSA gradients was found to inhibit the production of interferon in response to stimulation by PHA (refer to Table 10). The ability to reconstitute this response by stimulated cells or the supernatant fluid from cells of the various BSA gradient fractions after exposure to PHA was examined.
Cells from different fractions were cultured in the presence of PHA for 24 hours. After centrifugation at 60 x g for 10 minutes, the supernatant fluid was removed and the cells were washed twice in fresh McCoy's-5A medium. Both the cells (at a ratio of 1:1) and the supernatant fluid from each cell fraction were recombined with cells from fraction V which had been treated previously with anti-theta serum and complement to remove the thymic-dependent lymphocytes. The final cell concentration in each culture was 5 x 10^6 cells per ml. One ml of conditioned medium (24 hr) from non-activated spleen cells was added to the cell to cell recombinant cultures as culture medium. The conditioned medium did not contain interferon. All cultures were incubated for an additional 48 hours prior to harvest. The mixed cell cultures then were examined for their ability to synthesize and produce interferon. The results of these experiments are presented in Table 11.

PHA-activated fraction I cells alone or when added in equal numbers to T-cell-depleted fraction V cells were unable to restore the capacity for production of interferon. Supernatant fluid from fraction I cells also was unable to restore the ability of these cells to produce interferon. Neither of these recombinations resulted in any significant restoration of the DNA synthesis response as measured by incorporation of ^3H-TdR into TCA-insoluble material. PHA-stimulated cells from T-cell-depleted fraction IV cell
Table 11. Reconstitution of the Interferon-production Response of T-cell Depleted Cultures by PHA-activated Spleen Cells or Supernatant Fluid from Different BSA Gradient Fractions of Cells

<table>
<thead>
<tr>
<th>Basic Culture (Fraction)</th>
<th>Anti-theta Serum Treatment</th>
<th>Reconstituting Fraction</th>
<th>Anti-theta Serum Treatment</th>
<th>³H-TdR Incorporation a (CPM)</th>
<th>Interferon b (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>705</td>
<td>100(±27)</td>
</tr>
<tr>
<td>V</td>
<td>+</td>
<td>I(cells) d</td>
<td>-</td>
<td>1205</td>
<td>0</td>
</tr>
<tr>
<td>V</td>
<td>+</td>
<td>IV(cells)</td>
<td>-</td>
<td>5975</td>
<td>60(±13)</td>
</tr>
<tr>
<td>V</td>
<td>+</td>
<td>IV(cells)</td>
<td>+</td>
<td>870</td>
<td>0</td>
</tr>
<tr>
<td>V</td>
<td>+</td>
<td>V(cells)</td>
<td>-</td>
<td>1670</td>
<td>70(±12)</td>
</tr>
<tr>
<td>V</td>
<td>+</td>
<td>V(cells)</td>
<td>+</td>
<td>315</td>
<td>0</td>
</tr>
<tr>
<td>V</td>
<td>+</td>
<td>I(S.F.)</td>
<td>-</td>
<td>320</td>
<td>0</td>
</tr>
<tr>
<td>V</td>
<td>+</td>
<td>IV(S.F.)</td>
<td>-</td>
<td>1010</td>
<td>5(±3)</td>
</tr>
<tr>
<td>V</td>
<td>+</td>
<td>IV(S.F.)</td>
<td>+</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>V</td>
<td>+</td>
<td>V(S.F.)</td>
<td>-</td>
<td>660</td>
<td>7.5(±2)</td>
</tr>
<tr>
<td>V</td>
<td>+</td>
<td>V(S.F.)</td>
<td>+</td>
<td>140</td>
<td>0</td>
</tr>
</tbody>
</table>

a. Mean values are presented from four experiments containing duplicate cultures per sample. Values represent counts per minute (CPM) of tritiated-thymidine incorporated into TCA-insoluble material.

b. Interferon values are presented as mean titers in units per ml.

c. 5 x 10^6 cells from each designated fraction were cultured for 24 hours and then exposed to PHA for 48 hours prior to harvest.

d. 2.5 x 10^6 cells (cell:cell recombinations) or 5 x 10^6 (for supernatant fluid recombinations) from each designated fraction were cultured for 24 hours and then combined with cells or supernatant fluid (S.F.) from the reconstituting fraction and cultured for 48 hours prior to harvest.
cultures alone were unable to produce interferon but when added to T-cell-deficient fraction V cell cultures, were able to partially restore the capacity for interferon production to 60% of normal yield. The supernatant fluid from this cell population was also able to restore the capacity to produce interferon but only to a very low degree. When fraction IV was treated with anti-theta serum prior to exposure to PHA, neither the remaining cells nor the supernatant fluid was able to restore capacity for production of interferon. Cells from fraction V which were not exposed to anti-theta serum were able to restore 70% of the capacity to produce interferon when recombined with anti-theta serum treated fraction V cells. The supernatant fluid from PHA-treated cells in fraction V was also able to restore the capacity to produce interferon, though to less than 10% of the normal response. Removal of the T-cells from the reconstituting cell fraction by anti-theta serum treatment eliminated the restoration of interferon producing capacity of cells in fraction V. Only PHA stimulated cells of fraction IV and to a lesser extent, the supernatant fluid of this fraction of cells, were able to induce a significant incorporation of $^{3}H$-TdR into TCA-insoluble material. Neither cells nor supernatant fluid from either fraction I or V had any significant enhancing effect on the DNA synthesis of cells in fraction V cell cultures depleted of T-cells.
These results demonstrate that PHA stimulated the production of a factor which is capable of inducing the production of interferon from cells normally incapable of producing interferon upon exposure to this mitogen. Fraction IV cells, which by themselves were incapable of producing interferon, upon exposure to PHA and mixing with fraction V cells, which had been depleted of their T-cells, gained the ability to produce interferon. Non-stimulated cells from fraction V which had been treated with anti-theta serum also produced interferon, though only in very low amounts, after being cultured in the presence of PHA-activated supernatant fluids from either fractions IV or V but not from fraction I. These data suggest that, although transfer of PHA-activated cells results in production of more interferon, PHA-activated cells from fractions IV and V produce a soluble factor which is capable of inducing interferon production by non-activated fraction V cells.
DISCUSSION

The intravenous administration of PHA (1:10 dilution of stock), Con A (100 µg), and PPD (50 µg) to mice resulted in the production of circulating interferon within 24 hours. The level of interferon increased to maximum titers at 72 hours. The maximum interferon titer achieved following mitogen induction (27 units in PHA-treated cell cultures at 72 hours) was relatively low and occurred much later than that induced by other non-specific inducers such as poly I:C (250 units at 2 hours) or endotoxin (400 units at 2 hours) (Youngner, 1970). This probably reflects differences in the mechanism by which these materials stimulate the production of interferon and the cell types involved in the response. Mitogens have been reported to stimulate interferon only from leukocyte populations such as human peripheral leukocytes (Wheelock, 1965; Cooper, 1967) or mouse peritoneal leukocytes (Glasgow, 1966) whereas other inducers such as poly I:C are capable of stimulating the production of interferon from a wide variety of cell types maintained in tissue culture such as HeLa cells, L-cells, and RK-13 cells.

Intravenous injection of PPD into mice immunized with BCG also resulted in the production of circulating interferon. This response was shown to be immunologically specific since injection of comparable quantities of PPD
into non-immune mice did not elicit the production of any interferon. The maximal induction of interferon by 50 μg of PPD occurred at a later time than the induction of interferon following administration of mitogens; in addition, interferon continued to be produced through 96 hours. This response corresponded temporally with the time required for induction of cell-mediated immune responses in mice (Blanden, 1970). Non-specific interferon-inducing agents, such as poly I:C or endotoxin, stimulate a relatively early release of interferon into the serum (Stinebring and Youngner, 1964; Youngner and Hallum, 1968), whereas, in this study, the mitogen and antigen-induced interferon responses occurred at later times. Similar data have been presented by Epstein et al. (1971b) using immune human peripheral leukocytes stimulated with PPD; maximum interferon production was found to occur at seven to eight days after exposure to PPD in vitro. Neither general mitogens nor PPD stimulated the production of detectable levels of interferon at six hours after injection. This suggests that the interferon measured in these experiments represents de novo synthesis of interferon and not release of interferon as has been found in leukocytes treated with endotoxin (Youngner and Stinebring, 1965). This delay in production of interferon may be due to the fact that not all cell types can respond to mitogens or antigens and that some complex cell
interaction may be required to obtain the production of interferon by this mechanism.

Splenectomy has been shown to reduce the host capacity to produce serum interferon in response to viral inducers (Fruitstone et al., 1966). The results of this study showed that removal of the spleen also severely reduced the ability of the mice to produce circulating interferon after injection of PHA. The spleen, therefore, plays a major role in the production of serum interferon following the administration of PHA in vivo.

The results from in vitro studies demonstrate that mitogen- and antigen-induced interferon-like activity from mouse spleen cells possess physical-chemical characteristics similar to the mouse serum interferon standard and that they meet criteria for acceptance as interferons (Lockart, 1966). A few minor distinctions were noted. Mitogen-induced interferon was partially heat sensitive at 60°C after 60 minutes and was partially inactivated at pH 2.0 after 5 days, whereas the mouse serum interferon standard was not. Wheelock (1965) has reported the heat sensitivity and pH lability properties of mitogen-induced human peripheral leukocyte interferon and the results were in agreement with the results presented herein. Interferon induced by PHA in the mouse spleen cell cultures was found to have some antiviral activity on HeLa cell monolayers, which are of human origin. This suggests an ability of mitogen-induced
interferon to cross species barriers; however, this capacity was not found with the serum-interferon standard induced by NDV. The ability of different preparations of interferon to cross species barriers has been reported (Desmyter, Rawls, and Melnick, 1968). The significance of these differences in the properties of interferon remain unclarified.

The temporal induction of interferon from spleen cells after exposure to PHA or Con A correlated with the blastogenic response. Maximum blastogenic activity, as measured by the incorporation of $^3$H-TdR into DNA occurred in mouse spleen cells at 48 to 72 hours (Adler et al., 1970) and maximum interferon titers also were obtained during this time period. Maximum antigenic (PPD) stimulation of incorporation of $^3$H-TdR into DNA was somewhat delayed, occurring maximally at 72 to 96 hours; this time was similar to the time of production of maximum titers of interferon. These results suggested that a temporal relationship between DNA synthesis and interferon production exists in mouse spleen cells following exposure to mitogens or specific antigens. A similar temporal relationship between DNA synthesis and interferon production was found to occur for human peripheral leukocytes upon exposure to an antigen (PPD) (Green et al., 1970; Epstein et al., 1971a).

The mitogen and antigen dose-response experiments suggested that there may not be a quantitative correlation
between the degree blastogenesis and the amount of interferon produced. It was found that the concentration of each mitogen or antigen which was required to obtain the highest level of incorporation of \(^3\text{H-TdR}\) also was required to obtain maximum production of interferon. However, when other concentrations of the mitogens or PPD were examined, a correlation between DNA synthesis and interferon production was not evident. A ten-fold excess of PHA or Con A resulted in a greater inhibition of the DNA synthetic response than the interferon-producing capacity of the cells. Doses of one-tenth of the optimum PHA or Con A concentration showed the opposite; i.e., interferon production was more markedly reduced than was synthesis of DNA. The interferon and DNA synthetic responses were reduced equally following exposure to PWM or BPD at both excess and suboptimal levels. The data with PHA or Con A suggest that the response of interferon production has a higher threshold for activation than does stimulation of DNA synthesis. The PWM and PPD dose-response experiments demonstrated a narrower concentration range for stimulation of DNA synthesis and interferon production than PHA or Con A. At an optimal stimulatory dose, Con A stimulated a greater level of incorporation of \(^3\text{H-TdR}\) into DNA and transformed a higher per cent of spleen cells into blast cells than the other mitogens tested, yet Con A did not induce as much interferon as PHA did. Despite stimulation of a lower level of
blastogenesis by PHA than Con A. PHA induced the highest amount of interferon. It was apparent that the elevated DNA synthetic activity of the spleen cells reflected the morphologic blast cell transformation response but neither response accurately reflected the level of the interferon which was produced. These data demonstrate that a direct correlation does not exist between the degree of blastogenesis observed in the spleen cells and the amount of interferon produced by them.

It has been suggested that PHA and Con A stimulate blastogenesis in the T-cell subpopulation of lymphocytes (Greaves and Roitt, 1968; Janossy and Greaves, 1971) while PWM preferentially stimulate bone-marrow dependent lymphocytes (B-cells) (Parkhouse, Janossy, and Greaves, 1972). Both B and T cells are thought to be required for the PPD-induced cell-mediated immune response (Greaves, Torrigiani, and Roitt, 1969; Roitt et al., 1969; Williams and Waksman, 1969). The differences in the types of cells activated by these stimulants may, in part, explain the differences observed in the dose vs. interferon-produced response curves. Each mitogen or antigen should stimulate preferentially only a specific subpopulation of cells and the amount of interferon produced should then reflect the number of each type of cells involved in this response. Spleen cells treated with anti-theta serum to remove the T-cells lost the ability to be stimulated by PHA, Con A, or PPD to initiate synthesis
of DNA and to produce interferon. Yet this same T-cell deficient population of cells retained the capacity to respond to PWM and synthesize increased amounts of DNA and produce interferon. These experiments showed that these mitogens activated different cell types and that the interferon producing cells, stimulated by PWM, belong to a thymic-independent cell population. One hypothesis which could explain these results is that the interferon-producing cells belong to a subpopulation of non-theta cells and require an interaction with another cell type (i.e., T-cells for T-cell requiring mitogens like PHA) to obtain the interferon response. Data presented by DeMaeyer-Guignard et al. (1969) on irradiated mice which had been reconstituted with rat bone marrow cells resulting in rat-mouse chimera's, demonstrated that bone-marrow cells were responsible for the production of interferon since the interferon which was produced possessed rat specificity. This finding corresponds with the above hypothesis that interferon is produced by thymic-independent cells. The separation of spleen cells into six populations by fractionation on a bovine serum albumin density gradient allowed examination of this hypothesis. Separation of heterogeneous populations of cells is frequently based on differences in density of the various cell types. A discontinuous albumin gradient technique for cell separation was initially employed by Harris and Joseph (1966). They examined the morphology of various
fractions of guinea pig bone-marrow cells. They found that blast cells such as myeloblasts, proerythrocytes, and lymphoid blast cells localized in the light density fractions (17 to 22% BSA). Small and medium sized lymphocytes predominated in the middle fractions (24 to 26% BSA), while large normoblasts predominated in the heavier fractions (28 to 29% BSA). Raidt, Mischell, and Dutton (1968) employed a four fraction albumin discontinuous gradient and found that DNA synthesis stimulated by PHA occurred in cells predominantly in a single fraction which banded at a 26% BSA concentration. This demonstrated a functional difference in these cells and showed that non-responding lymphocytes could be separated from responding lymphocytes as far as elevated DNA synthetic responses were concerned.

Although complete morphological separation was not achieved following centrifugation of spleen cells through the BSA density gradient, a functional separation into populations which differed in responses to mitogens and antigens in DNA synthesis and interferon production, was achieved. After centrifugation through the gradient, fractions III and IV were found to contain the largest numbers of cells. Morphologically, this cell population was composed of greater than 95% small lymphocytes. The upper fractions (I and II) contained large numbers of less dense small lymphocytes and, in addition, a number of cells resembling plasmacytes and blast cells. The lower fractions
(IV and V) contained primarily dense small lymphocytes and some large mononuclear cells.

Spleen cells from normal mice which localized in fractions III and IV of the BSA density gradients were stimulated to incorporate $^3$H-TdR after exposure to PHA or Con A. Spleen cells from mice sensitized to BCG responded, upon subsequent exposure to PPD, with synthesis of DNA. These cells were found primarily in fraction III. In contrast, cells activated to synthesize increased levels of DNA after exposure to PWM localized in the less dense portion of the BSA gradients, i.e., in fractions I and II. Relatively little or no activation of cells by PWM occurred in cells in fractions more dense than fraction II. It is, therefore, evident that DNA synthesis following exposure to PWM occurred in cells quite distinct from those which responded to PHA, Con A, or PPD. Despite the heterogenous distribution of cells in the gradients which responded to mitogens with the synthesis of increased levels of DNA, the cells producing interferon were found to be more homogenous in their distribution in the gradient. Cells having the capacity to produce interferon in response to all three mitogens and the specific antigen, PPD, were found only in fraction V. Very little incorporation of $^3$H-TdR was found in this fraction of cells in response to any of the mitogens or PPD. The results demonstrated that the cells which responded to stimulation with the production of interferon
belonged to a separate population of cells which differed functionally from those cells which responded by increased synthesis of DNA.

Anti-theta serum treatment of unfractionated spleen cells abolished the ability of the cells to synthesize DNA or produce interferon following exposure to PHA, Con A, or PPD and yet the cells could be activated by PWM. Exposure of individual fractions to the same agents after treatment with anti-theta serum had the same results. Interferon production in response to PWM in this same fraction remained unaffected. The phrase "production of interferon" does not necessarily imply de novo synthesis but it is meant to reflect the appearance of interferon in the culture fluid.

The results suggest that there is at least a two cell requirement for the induction of interferon from spleen cells in vitro; an immune-recognition cell, a T-cell for T-dependent mitogens such as PHA, and an interferon-producing cell which lacks the theta antigen. Support for this two cell requirement concept is provided by work involving the separation of leukocyte populations by glass adherence techniques (Milstone and Waksman, 1970; Epstein et al., 1971b). In these reports it was shown that glass-adherent mononuclear cells predominantly macrophages and large lymphocytes played an important role in the production of interferon following stimulation of the sensitized cells by an antigen (PPD). Individually, neither the glass
adherent nor the non-adherent population were capable of producing much interferon; yet when combined, they produced elevated levels of interferon. The work reported herein also demonstrated a multiple cell requirement for antigen stimulation of the interferon response. Since there is a T-cell requirement for production of interferon in response to some but not all mitogens, the data would fit the following hypothesis: A thymic-dependent lymphocyte serves as a recognition cell to initiate the interferon response and interacts with a thymic-independent cell through cell to cell contact or by the production of a soluble factor which stimulates that cell to produce interferon. The theta-antigen-bearing recognition cell that is present in fraction V (the interferon-producing fraction) could be derived from one of two sources: (1) the presence of the T-cell may represent incomplete separation of these cells into one fraction and the T-cells present in fraction V represent contamination by T-cells from fraction IV, or (2) a functionally separate T-cell subpopulation which does not respond to stimulation to mitogens by synthesis of DNA but functions only in the induction of interferon, and may or may not be present also in fraction IV. Because of the rather complete separation of cells synthesizing elevated amounts of DNA (fraction III) from cells producing interferon following exposure to PPD, the latter hypothesis is favored. The existence of functionally different subpopulations of T-cells
has been previously suggested (Raff and Cantor, 1971). Two populations of thymic-dependent lymphocytes have been found and are designated T₁ and T₂. Functional differences between these cells remains speculative but it is possible that non-DNA synthesizing T-cells which have a role in antigen recognition may be the cells involved in the interferon response.

Dissociation of the response between $^{3}\text{H}-\text{TdR}$ incorporation and the production of another mediator of cellular immunity in response to mitogenic stimulation has been suggested (Rocklin, Reardon, et al., 1970). It has been shown that certain immune patients have lymphocytes which can respond to Candida albicans by increased synthesis of DNA but do not produce migration inhibition factor (MIF) (Rocklin, Chilgren, et al., 1970). This unilateral response was explained as demonstrating either a dual functional capacity of the same responding cell or a multiple cell requirement for the production of MIF. The results of the present work also showed that cell types which were responsible for increased DNA synthesis differed from cell types involved in the production of interferon. These data support the concept that the mediators of cellular immunity are elaborated by different cell populations from those undergoing an enhanced DNA synthetic response. Dissociation between the DNA synthesis response and the mediator-production response has significant implications for clinical
techniques which attempt to determine the ability of a host to initiate a cellular immune defense against a viral infection. The results presented in this work suggest that a complete cellular response requires a complex interaction of different cell types to obtain the production of at least one of the mediators of cellular immunity and may reflect a similar requirement for the production of other mediator molecules. Methods which measure only one or two of these capabilities do not guarantee that all of the mediators which play an important role in defense against viral infections will be produced. Therefore, it may be necessary to measure several of the important mediators of cellular immunity before conclusions can be made concerning the immunological competence of an individual.

Investigation into the role of the T-cells in the interferon-production response showed that depletion of the theta-antigen bearing cells rendered the remaining spleen cells unable to respond to PHA with the production of interferon. PHA was used to stimulate non-T-cell depleted spleen cell cultures. The resulting activated cells or their supernatant fluid were found to be capable of restoring the ability of T-cell-depleted fraction V cells to produce interferon. Therefore, evidence has been presented which showed that lymphocytes exposed to PHA produced a soluble factor which was capable of inducing interferon from spleen cells that normally were unable to respond to stimulation by
PHA with the production of interferon. Recombination of T-cell-depleted fraction V cells with PHA-stimulated cells restored this response better than activated supernatant fluid. The lower response obtained with supernatant fluid suggested that although a soluble factor is released following lymphocyte stimulation and this factor activated the interferon production response, this material may be very labile, rapidly degraded, or in low concentration. Alternatively, it might indicate that this factor is required in concentrations which are not normally detectable and is best achieved through cell to cell contact. The latter explanation could mean that the factor is either quickly diluted beyond its effective levels when released into the supernatant fluid or it is not produced in sufficiently high quantities to be accurately detected by this technique. Since fraction I cells contained few T-cells and could not restore the interferon response in T-cell depleted fraction V cells, it is suggested that the interferon stimulating factor is associated with activated T-cells. Anti-theta serum removed the ability of both fraction IV and V cells or their supernatant fluid to restore the interferon-production response. These data support the concept that this factor is related to activated T-cells.

These results also showed that although anti-theta serum eliminated the ability of the spleen cells to produce interferon in response to PHA stimulation, the
interferon-producing cells themselves were not removed by this treatment. Supernatant fluid from cells of fraction IV that had been exposed to PHA induced production of interferon in cells of fraction V which normally could not be induced by PHA to produce interferon after being depleted of T-cells. Therefore, the cells producing interferon most probably belong to a subpopulation of spleen cells which lack the theta antigenic marker. Support for this suggestion comes from the fact that PWM also is able to by-pass the T-cell requirement and is able to induce the production of interferon from T-cell depleted spleen cells. The accumulated evidence suggests that the interferon-producing cell itself belongs to a bone-marrow derived subpopulation of lymphocytes.

The data presented in this study suggest that there are at least two subpopulations of T-cells; both recognize mitogens such as PHA or antigens; yet only one of them is capable of undergoing increased DNA synthesis and proliferation. The production of interferon as a result of antigen or general mitogen stimulation can be explained by the following hypotheses:

Mechanism 1: Antigen or mitogen interacts with the blastogenic T-cell, stimulates DNA synthesis, and results in the production of a soluble factor which is capable of stimulating the production of
interferon in the thymic-independent interferon-producing population of cells.

Mechanism 2: Antigens or mitogens interact with a non-blastogenic population of T-cells and indirectly stimulate the production of interferon.

Mechanism 3: Antigens or mitogens interact with a T-cell independent population of cells which results directly or indirectly in the production of interferon.

As regards mechanisms 1 and 2, the non-blastogenic T-cell may be present in both BSA gradient fractions IV and V; therefore, it is possible that this is the cell involved in the release of the soluble factor which stimulated the production of interferon and not the blastogenic cell. This would mean then, that the cell undergoing increased DNA synthesis belongs to a separate T-cell subpopulation which is not involved in the response which results in the production of interferon. The data presented herein are not sufficient to differentiate between these two possibilities.

Evidence for the existence of mechanism 3 was presented in experiments involving the production of interferon following stimulation by PWM. Interferon was produced in T-cell depleted spleen cells and therefore, does not involve a mechanism which requires the T-cell dependent population of cells. Either PWM interacts directly with the
interferon-producing cells or stimulates the production of interferon through the activation of some other thymic-independent cell which does not belong to the interferon-producing population.

In conclusion, these studies showed that interferon can be induced both by a number of mitogens (i.e., PHA, Con A, and PWM) and in a specific manner by PPD. These studies demonstrated that temporally, though not quantitatively, interferon production is associated with the cellular immune response both in vivo and in vitro. This relationship was shown to be complex association of at least two cell types. Although there is a thymic-dependent cell requirement for the inductive phase of the response (except for the PWM-induced response), the interferon producing cells do not belong to the T-cell subpopulation of lymphocytes. A dissociation between the synthesis of DNA and the production of interferon was demonstrated when the cells responding with the production of interferon were found to belong to a different subpopulation of cells from those undergoing an elevated DNA synthetic response and that these populations could be separated from one another by density gradient centrifugation. Finally, PHA was found to stimulate lymphocytes into an active state which allows them, through the release of a soluble factor, to stimulate the production of interferon by non-activated spleen cells, which have been depleted of their T-cells; thereby rendering
them unable to respond to PHA with the production of interferon.
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