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PRESENCE OF INOSINE MONOPHOSPHATE DURING CELL MEDIATED IMMUNITY IN GUINEA PIGS

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PRESENCE OF INOSINE MONOPHOSPHATE DURING CELL MEDIATED IMMUNITY IN GUINEA PIGS

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Mary Ann Valentine

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and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy with a major in Microbiology.

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SIGNED Mary C. Dale

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ABSTRACT

The presence of the purine nucleotide inosine monophosphate (IMP) was studied in direct relationship to the development and expression of cell mediated immunity in guinea pigs using DNCB or Histoplasma capsulatum as sensitizing antigens. The IMP content of T-cell enriched lymphocytic lysates was measured by isocratic high pressure liquid chromatography (HPLC). Intracellular IMP levels of cells from homologously skin tested sensitized animals were significantly increased one day after skin testing when compared to the concentrations found in these cells during the period following sensitization. Concurrent with these observations were the findings that the absolute lymphocyte counts and histoplasmin stimulated in vitro blastogenic responses increased following sensitization while the PHA-induced proliferative response decreased slightly.

One day after skin testing, when IMP levels had increased, there was a slight decrease in lymphocyte numbers and a marked decrease in the PHA response. Cells collected at this time and cultured in vitro with histoplasmin responded with increased levels of protein production and increased IMP levels. These data suggest (1) the proliferative response of cells from sensitized animals appears to be associated with lower levels of intracellular IMP, and (2) sensitized cells stimulated in vivo with antigen appear to have characteristically higher IMP concentrations.

INTRODUCTION

Studies of lymphoid cells have included the observation of in vivo events such as dermal reactions and anaphylaxis, identification of lymphocytic populations and their interactions, and, most recently, the investigation of intracellular events involved in lymphocyte function. General mitogenic and specific antigenic stimulation of responsive cells cause the initiation of intracellular events. When activated, cells can respond by proliferating, differentiating, or by generating products without proliferation. Immunoglobulin is the major product of bone marrow derived lymphocytes (B-cells) once the cells have undergone proliferation and differentiation into plasma cells. Lymphokines are produced when thymus-derived lymphocytes (T-cells) interact with mitogens and specific antigens (1). An improved understanding of the intracellular activities of these cells has resulted from studying the changes which occur in DNA, protein and purine synthetic mechanisms following stimulation.

The importance of purine metabolism in the immune response of T-cells was first evident from research concerned with immunosuppression. Among the earliest reports were those involved with the purine anti-metabolites - the thioprines - reviewed by Bach (2) and Berenbaum (3). In 1967 Elion showed that the hypoxanthine analogue, azathioprine, affected RNA and protein production well before the inhibition of DNA occurred (4). Later work established that T-cells were especially sensitive to these agents (5-7). Concentrations too low to cause any effect on

antibody production were still effective in causing the suppression of delayed type hypersensitivity (DTH) in rodents (8). The idea developed that purine metabolites were important not only for cellular proliferation, but also for the differentiation and function of T-cells (9, 10).

Aspects of this concept were supported by experiments with isoprinosine (an alkylated form of the purine nucleoside inosine). Hadden and colleagues (11) reported enhanced phytohemagglutinin (PHA) induced lymphocyte proliferation in partially purified human peripheral blood lymphocytes (PBL) following exposure to Isoprinosine. In 1977, Renoux and Renoux (12) described the induction of a T-cell surface marker following relatively small concentrations of this same compound. Additional work with Isoprinosine supported the idea that the substance apparently could potentiate T-cell differentiation (13). Since the compound was not immunogenic, but caused an increase in the numbers of actively rosetting T-cells, it was proposed that the drug acted on T-cells and caused them to differentiate into active populations (14).

The in vitro activity of human lymphocytes exposed to graded concentration of inosine or inosine monophosphate (IMP) has also been investigated. At low concentrations, both IMP and inosine have been found to increase the responsiveness of lymphocytes to PHA (15-17). When cells were cultured with the glutamine analogue, azaserine, to inhibit de novo purine production, neihter IMP nor inosine could prevent the inhibition of DNA synthesis. While it could be argued that a glutamine analogue could affect more than de novo purine synthesis, it was apparent that normally stimulating concentrations of inosine or IMP could not

compensate for the inhibitory effect azaserine produced in lymphocytes. The potentiating effect of the two purines was only evident in normally metabolizing cells.

Yet another line of evidence associating purine metabolites with levels of immunocompetence came from studies attempting to identify the active component(s) of transfer factor preparations. The preparations, first described by Jeter, Tremain and Seebohm using guinea pig cells (18) and by Lawrence using human cells (19), appeared to passively transfer delayed hypersensitivity from immune individuals to naive recipients. While hypoxanthine was reported as the major component of the active fractions of these dialysates (20), other studies suggested that the active component may consist of a peptide portion linked to one or two inosine monosphosphate moieties (21, 22). Whereas these models are yet unproven, the inclusion of a purine base in an immunomodulatory substance is not inconsistent with other lines of evidence mentioned above.

Immunologists and biochemists received impetus to pursue research on purines when reports in 1972 showed a clear association between immunodeficiency disease and the genetic deficit of a purine salvage enzyme, adenosine deaminase (ADA) (23, 24). Within three years another salvage enzyme, purine nucleoside phosphorylase (PNP), was reported to be abnormal in an immunocompetent patient (25). These reports supplied impressive evidence that absence of the normal activity of these enzymes was a direct cause for immune dysfunction. Patients lacking these enzymatic activites presented with severe impairment of T-cell function;

both B- and T-cell activities could be affected in ADA deficient patients (26).

The action of these enzymes involves the sequential reutilization of purine bases as shown in Figure 1. ADA converts adenosine to inosine in an irreversible reaction and PNP can then convert inosine to hypoxanthine for use by the salvage enzyme hypoxanthine-guanine phosphoribosyl tranferase (HGPRT). Purine biosynthesis occurs along two major pathways: (1) the synthesis of nucleotides from reutilized purine bases and nucleosides and (2) the de novo synthesis resulting in formation of IMP from low molecular weight precursors (27). Studies measuring enzyme activities have shown that the major purine pathway of resting lymphocytes is the salvage pathway (28-34). The needs of these cells, primarily energetic and not synthetic, can be met by reutilizing the bases taken into the cells.

Human lymphocytes stimulated by the T-cell mitogen PHA respond with a shift after stimulation from the salvage pathways to increased de novo synthesis of purines (29-31, 33, 35, 36). This is concommitant with an increased uptake of purine bases into the cell (33). The eventual utilization of these bases is altered during cellular proliferation. When the activity of enzymes at the IMP branchpoint was assessed, a shift in production from predominantly adenine nucleotides to approximately equal production of both adenine and guanine nucleotides was reported.(35,37). It was suggested that nucleotide synthesis from IMP was regulated differently in cells producing IMP from de novo pathways than in those deriving their IMP solely by salvage. Control at the branchpoint would be

quite sensitive to intracellular IMP concentrations (35). This concept was supported by other work. When stimulating levels of adenosine were given to cells along with the ADA inhibitor, coformycin, a decrease in PHA stimulation resulted. This suggested the hypothesis that a 'delicately regulated' supply of purine nucleotides may control proliferation (36).

Actual measurements of total intracellular IMP in normal lymphocytes have rarely been reported. One report estimated IMP concentrations to be about 30 picomoles per 10^6 cells (38). Whereas many studies have reported the relative uptake of various radiolabelled purine metabolites (31-34, 39), the quantification of purine metabolism has been done using leukemic cell lines and mitogen stimulated peripheral blood lymphocytes. To our knowledge, assessment of purine metabolism in antigen stimulated lymphocytes has not been reported. The work of Lewis, Cramer and Reed (22) did suggest an increase in the levels of IMP in blood cells after transfer factor was administered to canines, but quantification was not attempted.

In total, studies to date have shown that (1) the administration of purines in vivo and in vitro alter lymphocyte responses and (2) subjects with purine imbalances due to enzyme deficiencies lack normal cell mediated immunity. The importance of IMP to normal immune function was emphasized by these studies. There is, however, no data available to (1) evaluate the changes in IMP concentrations following antigenic stimulation and (2) correlate any potential changes of intracellular IMP levels to the induced states of CMI.

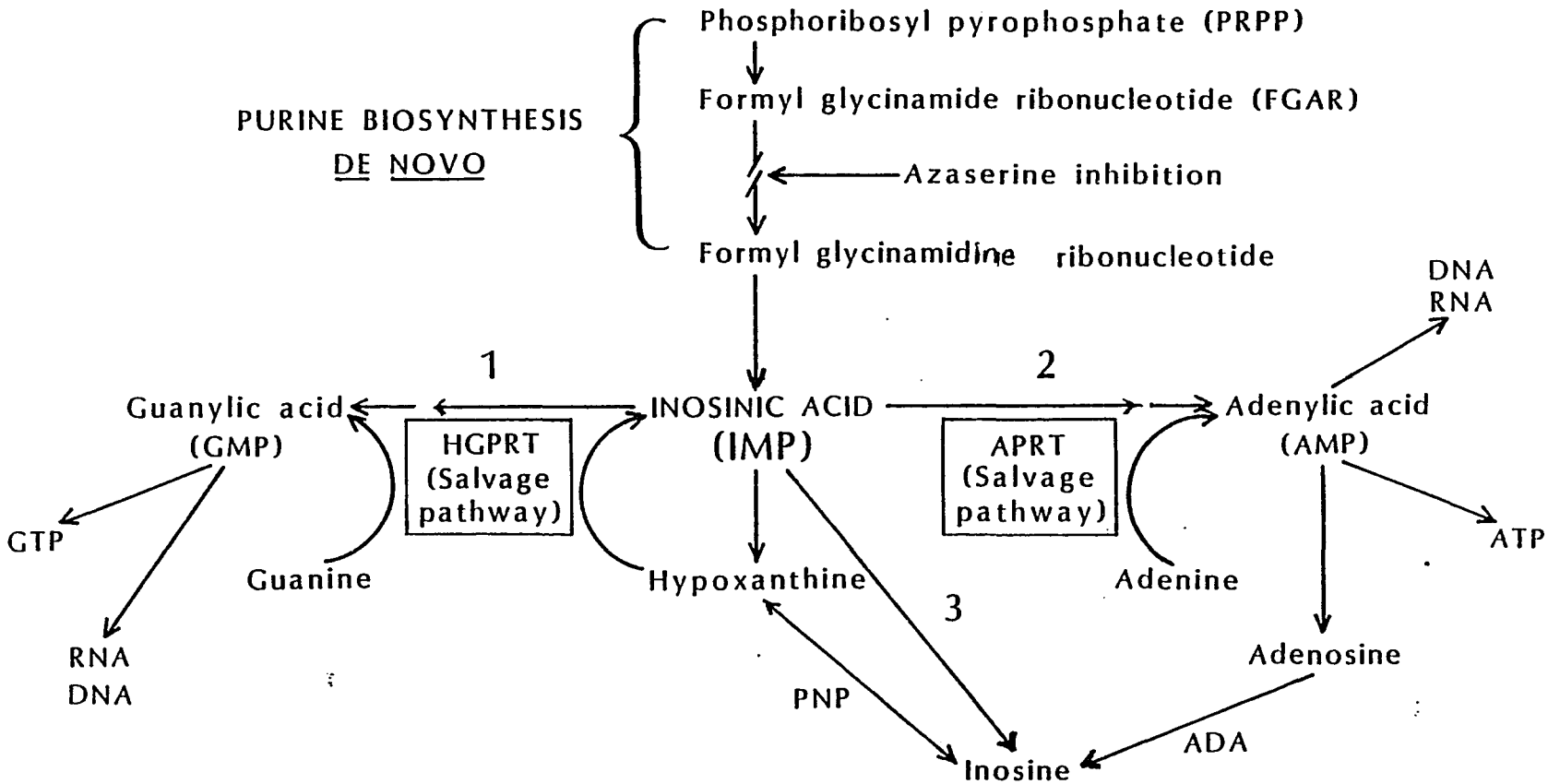

Figure 1. A simplified diagram of the purine biosynthetic pathways 1 = Inosine monophosphate dehydrogenase, 2 = adenylo-succinate synthetase, 3 = 5'nucleotidase. HGPRT = hypoxanthine-guanine phosphoribosyl transferase, ADA = adenosine deaminase, PNP = purine nucleoside phosphorylase.

CMI against many antigens can be evaluated. Of the methods available, skin test procedures can show the in vivo presence of immunity and in vitro activity can be asssessed using several techniques, including the lymphocyte transformation assay (40-43). The cells involved in the in vivo correlate of CMI, skin testing, have been identified as the macrophage or neutrophile and the T_{DH} cell (44). During antigen stimulation at the skin test site, the T_{DH} lymphocyte is believed to be called from the lymph node to the site of stimulus and to direct the dermal response. Lymphocyte transformation, an in vitro correlate of CMI, is reported to be a measure of prior sensitization of the cellular immune system to specific antigens (42). It is based on the premise that lymphocytes from previously sensitized animals will, on subsequent antigenic challenge in vitro, synthesize macromolecules, enlarge, and undergo blast transformation. The uptake of tritiated thymidine into actively synthesized DNA reflects prior sensitivity. Recent work has identified the cells involved in these activites as the macrophage, a population of T helper cells and possibly other lymphocytes as well (45).

Statement of Problem

Several diverse lines of work have suggested that intracellular levels of IMP in sensitized lymphocytes might have critical importance for the proper expression of function by T-cells. Two basic approaches were chosen to determine if a reproducible change in IMP levels would occur during the course of sensitization and subsequent stimulation with homologous antigen. These approaches were (1) to measure IMP

in T-enriched lymphocytic populations obtained from guinea pigs during the course of sensitization against dinitrochlorobenzene (DNCB)and Histoplasma capsulatum and (2) to measure IMP from lymphocytes stimulated in vitro with specific antigen (histoplasmin) or mitogen.

The aims of this work are: (1) to determine if intracellular IMP in T-cell enriched populations changes during the course of sensitization with antigens, (2) to determine if IMP levels in T-cell enriched populations change following in vivo skin test procedures, (3) to determine if alterations in the levels of IMP can be related to active CMI as measured by in vivo and in vitro correlates, and (4) to compare the IMP levels in sensitized and nonsensitized cells to the synthetic activities of the cells cultured in vitro with specific antigen.

MATERIAL AND METHODS

Animals

Out-bred Hartley guinea pigs from a departmental colony were used. Each group consisted of equal numbers of male and female animals whenever possible. The beginning body weights for all animals were between 400 and 1000 grams. Animals were maintained on Arizona Feed's guinea pig chow, water *ad libitum* amd daily supplemental cabbage. Animals were placed in individual cages at the beginning of an experiment and kept undisturbed for at least one week to permit them to adjust to their new environment before the experiment commenced.

Sensitization Methods

Animals were sensitized to 2,4 dinitro-chloro benzene (DCNB) according to the method of Jeter, Tremain and Seebohm (15). This was accomplished by daily painting for six days using five drops of a 2% alcoholic solution of DNCB on the clipped nucheal area of the animals.

The second antigen system consisted of *Histoplasma capsulatum* Scritchfield strain, kindly supplied by Dr. Howard J. Larsh. Subcutaneous injections of formalin killed yeast phase cells were made into multiple sites on the nucheal area. Each animal was sensitized on day one with 25 mg of antigen in 0.3 ml emulsed with equal parts of Freund's incomplete adjuvant. A second sensitizing dose was given on day ten.

Methods for Skin Testing

Delayed type hypersensitivity reactions against DCNB were assayed as follows. Five or ten days after the last painting, animals were skin tested with 0.1% or with both 0.1% and 0.5% DCNB in olive oil. (Purepac, Elizabeth, N.J.) on clipped flank areas. Heterologous reactions were assessed using citraconic anhydride at the same concentration as the homologous antigen, i.e. 0.1% and 0.5% and 0.5% antigen in olive oil. Skin tests were checked at six hours and scored at 18 and 48 hours after application. Reactions were scored as follows: plus/minus, patchy erythema; 1 plus, homogeneous erythema; 2 plus, homogeneous erythema and edema; 3 plus,marked homogeneous erythema and edema; 4 plus, marked homegeneous erythema, and central blanching.

DTH reactions against histoplasmin was assayed as follows. Skin tests were made 20 days after the last sensitizing done, using 0.1 ml given intradermally of a 1:10 dilution of histoplasmin (Parke, Davis and Co., Detroit, Michigan) Reaction sites were observed at six and 24 hours. Millimeters of erythematous indurated area and presence of central blanching or necrosis were noted. Heterologous antigen reactions in histoplasma sensitized animals were checked using five units of human Old Tuberculin (OT, Eli Lilley and Co., Indianapolis, Ind.), administered as 0.1 ml intradermally.

In all experiments representative samples of skin test sites were taken for histological confirmation generously done by Dr. Raymond Reed, professor of Veterinary Science, Department of Veterinary Science, University of Arizona. Tissues were fixed in phosphate buffered formalin,

embedded in paraffin, and sectioned perpendicular to the skin surface through the center of the reaction sites. At least four sections cut at five micron thickness were read per site. Reactions were read for the presence of lymphocytes. Neutrophiles, eosinophiles and basophiles were noted when found.

Preparation of T-enriched Lymphocyte Suspensions

Blood was collected by cardiac puncture without anesthetic in most experiments in which multiple blood samples were needed from an individual animal. Ether was used when anesthetic was required. Four to five ml of blood was collected, defibrinated on glass beads and diluted 1:2 in minimal essential medium (MEM, Grand Island Biological Co., Grand Island, N.Y.) with 5% heat -inactivated fetal calf serum (FCS, heat inactivated for 30 minutes at 56C: Grand Island Biological Co., Grand Island, N.Y.). The dilutions were overlaid onto a Ficoll-hypaque gradient (46) (Ficoll, Pharmacia Fine Chemicals, Piscataway, N.Y.; Hypaque, 50% Winthrop Laboratories, N.Y., N.Y.) (Appendix A). Gradients were centrifuged at 400x g for 20 minutes at 20 C and the cells at the interface were collected, washed in 5% FCS-MEM and centrifuged at 400 x g for 10 minutes. Washed cells were processed to make T-enriched lymphocytic suspensions using two methods. Each will be described separately.

The first method for T-cell enrichment employed nylon wool columns to remove adherent cells and permit non-adherent T-cells to

be separated (47). Pelleted mononuclear cells were resuspended in a minimum of 5% FCS-MEM and layered onto a pre-warmed nylon wool column (3 denier, Fenwall lab, Deerfield, Ill.). After incubating the loaded columns for 45 minutes at 37 C, non-adherent cells were eluted using 5% FCS-MEM and adherent cells were collected separately by mechanically agitating the cells off the wool. Samples thus collected were washed one time in plain MEM, pelleted by centrifugation and resuspended in about 0.2 m. of MEM.

The concentration and purity of cells from each sample were determined as follows: (1) 0.04 ml of the cellular suspension were enumerated by Coulter Counter determinations and (2) a small portion was also removed and the cells permitted to rosette with rabbit erythrocytes (48). The remainder of the sample was frozen for IMP assay, stored at -60 C until all samples were collected, Approximately 85-90% of the eluted non-adherent cells rosetted with the rabbit erythrocytes and an average of 10-20% adherent cells formed rosettes.

In some experiments, several cell populations were collected from each animal. In these cases, the animals were etherized, exsanguinated by cardiac puncture and the blood collections processed as described above. The animals were killed either by creating an air embolism in the vascular system or by cervical dislocation and then spleens, draining axial, brachial and cervical lymph nodes were removed. Spleens and pooled lymph nodes were processed separately into single-cell suspensions by teasing each organ apart and forcing the cells through stainless steel screens (20 gauge, 60 mesh). The cellular suspensions were washed one time in 5% FCS-MEM,

resuspended in the same medium and layered onto Ficoll-hypaque gradients. Gradients were centrifuged and the cells carried through the procedure described above for peripheral blood cells.

The second method for T-cell enrichment employed complement coated erythrocytes (EAC cells) (50). Human group A cells were collected into Alsever's solution and kept refrigerated for two to seven days before use as EAC cells. Erythrocytes were washed three times in cell balanced salt solution (CBSS, Appendix A) and resuspended to a volume of 10% (v/v) with CBSS. The washed cells were incubated with an equal volume of a 1:10 dilution of 2-mercaptoethanol treated human 0 serum which had been dialyzed and heat inactivated (56 C, 30 minutes). After incubation for 30 minutes at 37 C, the cells were washed two times in CBSS and brought to a 10% (v/v) suspension with CBSS. An equal volume of a 1:10 dilution of normal guinea pig serum was added to the cells and incubated for one hour at 37 C in 5% CO2, mixing frequently. After incubation, the cells were washed two times with CBSS and resuspended to a 5% volume (v/v) with CBSS containing 5% heat inactivated FCS for use in EAC rosetting.

EAC lymphocyte separations were performed by mixing equal volumes of EAC suspension with mononuclear cells collected from Ficoll-hypaque gradients at an approximate concentration of 5×10^7 cells per ml. These mixtures were incubated for 20 minutes at 37 C in 5% CO2. After incubation, the mixtures were centrifuged over Ficoll-hypaque for 20 minutes at 2000 x g and all cells above the pellet were collected and washed two times with CBSS. The washed cells were resuspended in about 0.2 ml of CBSS and

stored for IMP assay. Purity of the separation was checked by two methods: (1) E-rosetting (48) and (2) indirect fluorescence using fluorescent-tagged anti-rabbit serum after incubation with rabbit anti-guinea pig T-cell serum raised in the department. By the first method, the T-enriched populations were found to be 91-95% T-cells a value confirmed by the second method.

Cell Preparation for IMP Assay

Stored samples of T-enriched suspension were processed for high pressure liquid chromatography (HPLC) by lysing the cells with ten freeze-thaw cycles in dry-ice-acetone baths alternating with 37 C water baths. Lysates were then precipitated in 60% acetone (v/v) and centrifuged for 45 minutes at 2000 x g at room temperature. The supernatant fluid was removed, brought to 90% acetone (v/v) and the resultant precipitate was centrifuged as described above. Supernatant fluid was removed by careful suction and the second precipitates were allowed to air dry overnight at room temperature (49). All samples were reconstituted to 100 microliters with 1% acetic acid less than one hour before chromatographic assay.

Reverse Phase High Pressure Liquid Chromatography

Isocratic, reverse phase high pressure liquid chromatography (HPLC) as described by Burger et al. (51) was performed to measure IMP. This was done using a Spectra Physics chromatograph (model 3500 B), a chromatronix type HP valve and a 10 microliter sampling loop. The

solvent system was 1% acetic acid which was passed through a 25 cm column bed packed with octadecyl silane (ODS) resin (Partisil PXS 10/25, ODS-2, Whatman Co., Clifton, N.J.). Flow rate was set at 2 ml per minute and pressures averaged between 1800 and 2100 psi. Absorbence was read at a set wavelength of 254 nm. Quanitation was accomplished by comparison of retention time (Rf) and peak height to aqueous standards run under identical conditions. When sample size permitted, the material corresponding to the Rf for IMP was collected, treated with 2.5 Units of alkaline phosphatase (Sigma Chemical Co, St. Louis, Mo.) at pH 8.5 for 30 minutes at 37 C (51). Samples were then rechromatographed, specifically noting the absence of peaks having the Rf of IMP and the appearance of peaks co-chromatographing with inosine. IMP values were calculated from a standard curve. These values were then expressed on a per cell basis using the formula:

$$\text{picograms IMP/cell} = \frac{\text{IMP, pg/ml}}{V \times C}$$

where V represents the volume of lysate before acetone precipitation in milliliters, and C is the number of cells per milliter of sample before lysing. Cell samples were counted from duplicate dilutions with an inherent counting error of 4.6% (52). Variation between replicate samples as determined on HPLC averaged approximately five percent. This results in a total expected variance of 20 to 25% by this method.

Transformation Assays

The responsiveness of peripheral blood lymphocytes (PBL) to

mitogens and specific antigens was assayed using two methods: (1) the incorporation of tritiated thymidine (3H-TdR) into DNA and (2) the incorporation of tritiated leucine (3H-Leu) into protein. Each method will be presented separately.

Culture Medium. Tritiated thymidine uptake was determined in cells cultured in RPMI 1630 (Gibco, Grand Island, N.Y.). The medium contained 10 mM HEPES (N-2-hydroxyethyl piperazine-N-2-ethanesulfonic acid) buffer (Appendix A), 50 micrograms per ml of gentamycin sulfate (Appendix A) (Schering Corp., Kenilworth, N.J.), 2mM/ml of 1-glutamine (Sigma Chemical Corp, St. Louis, Mo.) and sodium heparin at a concentration of 10 U/ml (Organon, Inc., West Orange, N.J.). This medium was adjusted to a pH of 7.3 with sterile NaOH and kept refrigerated between uses.

Tritiated leucine uptake was assayed in cells cultured in Eagle's MEM with Earle's salts and without leucine and glutamine (GIBCO, Grand Island, N.Y.). The medium was supplemented with 1-glutamine, gentamycin and heparin as stated above, and buffered using sodium bicarbonate at a final concentration of 0.024 M (56). The medium was supplemented with sterile normal guinea pig serum (inactivated at 56 C for 30 minutes) at a final concentration of 15%. The pH of this medium was adjusted to 7.3.

Mitogen Bacto-phytohemagglutinin (PHA-P) (Difco,Detroit, Michigan) was prepared as a 10 mg per ml stock solution in unsupplemented 1630 medium. The stock solution was passed through a 0.45 mu filter (Millipore Corp. Bedford, Mass), and stored at -20 C in one ml portions.Stock PHA-P was

diluted on the day of use with appropriate culture medium to 10 micrograms per ml for thymidine uptake or to 500 micrograms per ml for leucine uptake assays. All unused thawed portions were discarded.

Histoplasmin. Commercial histoplasmin (Parke, Davis and Co., Detroit, Mich.) was dialysed overnight against 200 volumes of saline at 4 C. The preparation was removed from the tubing, lyophillized and reconstituted to the original volume in 1630-supplemented medium and then filter sterilized through a 0.45 filter. The preparation was refrigerated until use.

Tritiated thymidine uptake assay. A modified whole blood assay for ^{3}H-TdR uptake was utilized based on the method of Han and Pauly (57). Blood was diluted in sterile supplemented culture medium. All work was done in a laminar flow hood (Model EV, 425, Envirico, Albuquerque, N.M.). Pilot studies were performed to establish the optimal harvest time and concentration of mitogen and histoplasmin for the assay of guinea pig whole blood (Appendix B). The best harvest times were found on day two for mitogen stimulated cultures and day five for antigen stimulated cultures. One tenth ml of 1:10 dilutions of blood were placed in triplicate wells of flat bottomed microtiter plates (Microtest II, Falcon, Oxnard, Ca.). Triplicate control wells received 0.1 ml of medium and triplicate mitogen wells received 0.1 ml of PHA-P resulting in a final PHA-P concentration of 1 microgram per well. Histoplasmin plates were set up using triplicate control wells, triplicate PHA-P wells and triplicate wells containing either a 1:10 or a 1:40 dilution of histoplasmin. The histoplasmin assay was made at a 1:10 and a 1:20

dilution of blood.

Plates were covered and placed in a humidified 5% CO_2 air atmosphere to incubate at 37 C for the days prescribed (Shell Lab incubator, Sheldon Manufacturers, Portland, Ore.). Cultures received a one microCurie pulse in 0.025 ml of tritiated thymidine (Amersham/ Searle Corp., Arlington Heights, Ill: specific activity 5 Curies/mM). After 24 hours, the cultures were harvested on glass fiber strips (Whatman, Inc., Clifton, N.Y.) using a mini-MASH harvester (M.A. Bioproducts, Walkersville, Md.) and 4% acetic acid washes. The strips were dried for two hours in a 55 C oven, the discs transferred to scintillation vials and eight ml of liquid scintillation cocktail (0.1 g POPOP [1,4 bis phenyloxazolyl benzene and 5 g. PPO (2.5 diphenyl-2-methoxy-ethanol) in one liter of toluene) (58) was added. Radioactivity of the vials was counted in a Packard Tri Carb Liquid Scintillation Spectrophotometer (Model 332, Packard Instrument Co., La Grange, Ill.) for one minute with single channel settings at 95% gain and a window setting of 50-1000.

Tritiated leucine uptake assay. Pilot studies were performed based on several methods (59-61) to determine optimal culture conditions for a ^{3}H-Leu uptake assay (Appendix B). Blood was defibrinated using four millimeter glass beads and the mononuclear cells were separated from the blood after centrifuging over Ficoll-hypaque, as described earlier. The cells were washed three times and adjusted to 2 x 10^7 cells per milliliter in leucine-free MEM, supplemented as described. Three 1.8 ml

volumes of the cell suspensions from each animal were then placed into sterile 14 x 100 millimeter plastic tubes (Falcon, Oxnard, Ca.). The first tube received two-tenths ml of culture medium, the second tube received two-tenths of PHA-P dilution, and the third tube received two-tenths of a 1:40 dilution of dialyzed histoplasmin. Tubes were loosely capped, incubated in a humidified 5% CO_2 atmosphere at 37 C for 18 hours and then pulsed for six hours with 2 microCuries of ^{3}H-Leu (Amersham/Searle, Corp., Arlington Heights, Ill.; specific gravity 60 Ci/mM. At the end of the pulse period duplicate two-tenths ml volumes were removed from each tube to a microtiter plate and harvested onto glass fiber strips as described above. Samples were dried and the amount of radioactivity was determined as in the ^{3}H-TdR assay. The remaining samples were rosetted with EAC cells and the T-enriched populations processed for IMP assay.

Statistical Analysis

Data from all experiments were subjected to statistical analysis to establish significant changes and relationships. Various statistical analyses were employed, including analysis of variance (ANOVA), multivariate discriminant analysis, and bivariate and multiple regression analyses. Where appropriate or necessary, computer programs were used. Factorial analysis of variance was done using a Cyber 175 computer (Control Data Corp., Minneapolis, Minn.) with a program adapted from Sokol and Rohlf (62), generously supplied by L.M. Kelley, Ph.D., Department of Microbiology, University of Arizona, Tucson, Arizona.

Discriminant analyses and multiple regression analyses were run using programs identified as Discrim and Regran from Veldman (63).

The values for IMP and absolute lymphocyte counts were used as recorded. Stimulation indices of ^{3}H-TdR uptake assays were calculated by subtracting the $\log_{10}$ geometric mean of unstimulated control cultures from the $\log_{10}$ geometric mean of respective cultures exposed to either PHA-P or histoplasmin stimulants. When arithmetic stimulation indices were of interest, these were calculated by averaging the counts per minute from all sets of triplicate cultures. Averages were then used to determine the response ratios according to the following formula:

$$\text{Stimulation indes} = \frac{\text{average cpm of stimulated culture}}{\text{average cpm of control culture}}$$

where: cpm = counts per minute

Data for the last reported experiment (Table III) were handled identically to that for ^{3}H-TdR uptake assay, converting the cpm of ^{3}H-Leu into logs as described above to establish means and stimulation indices. A summary of all statistical analyses can be found in Appendix C.

RESULTS

IMP Levels in Non-Adherent Lymphocytes from Sensitized Guinea Pigs

The object of these experiments was to examine the levels of IMP during the course of sensitization and subsequent skin testing. Quantification of IMP in T-cell enriched populations of non-adherent lymphocytes was accomplished after the selective removal of adherent cell populations on nylon wool columns. Both adherent and non-adherent cell populations were collected and assayed. The adherent cells, however, required considerable perturbation to remove them from the nylon wool, a process which may have altered their physiological condition and, for this reason, the data for adherent cells will not be presented.

In the experiment presented in Figure 2, animals were sensitized with histoplasma on days one and ten and skin tested on day 30 with either histoplasmin, OT or saline. An analysis of variance (ANOVA) was done to compare the effects of time, treatment and source of lymphoid cells. Statistical comparison of these variables showed there was no difference in the change of IMP among the cell sources - peripheral blood lymphocytes (PBL), nodes and spleen - and for this reason the data were pooled to best show the effects of time and treatment. Data reflect an apparent decrease in IMP concentrations following the initial sensitization with histoplasma followed by a three-fold increase in IMP between day ten and day thirty-one in the animals skin tested with histoplasmin on day thirty ($p<0.001$). Animals treated with OT or saline on day thirty-one did

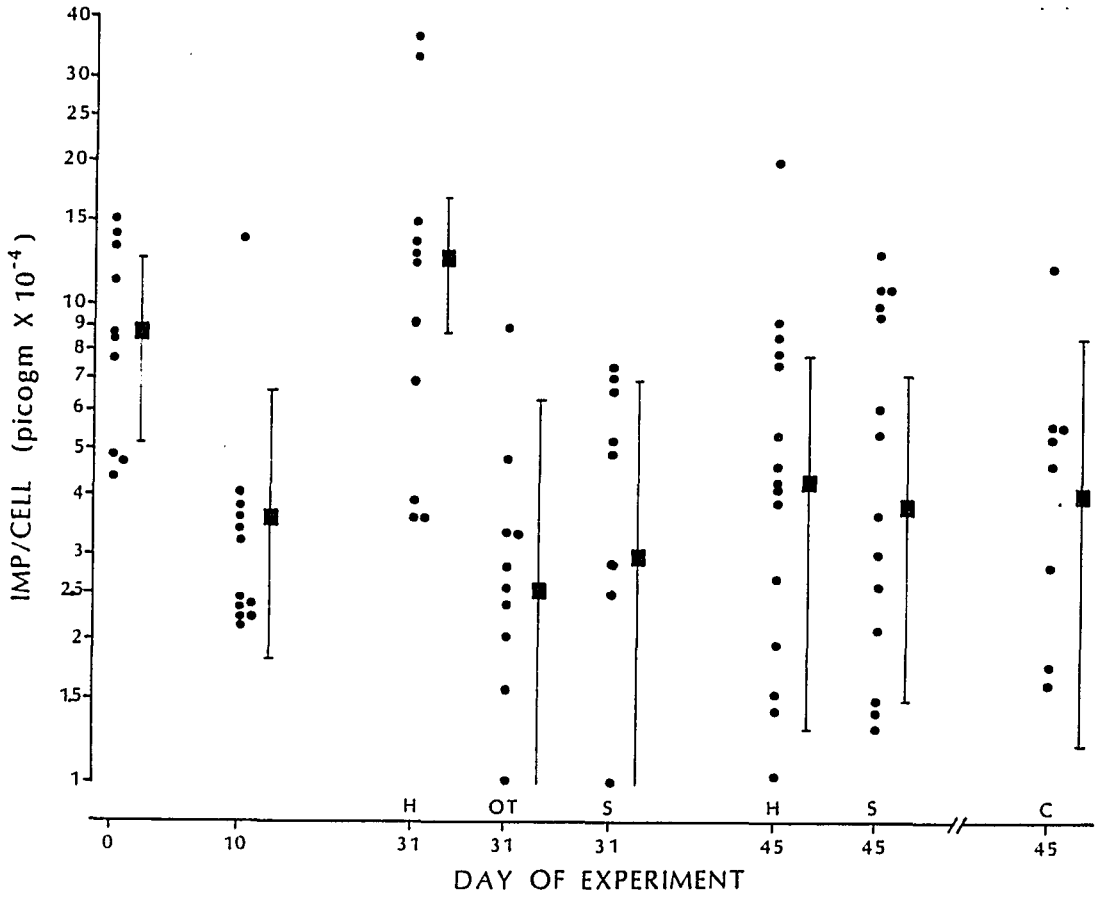

Figure 2. Intracellular IMP from non-adherent lymphocytic lysates collected from histoplasma sensitized guinea pigs. Sensitizing doses of histoplasma were given on days one and and ten and skin testing was done on day 30. Animals were skin tested using 1:10 histoplasmin (H), 5 Unites of old tuberculin (OT) or saline (S). One group of control animals (C) were sham treated with saline on the same schedule as sensitized animals. Each point is the IMP value for one animal. The mean is shown (■) with the standard error at 95% confidence level.

not show this same increase. Measurement for either sensitized or non-sensitized animals on day forty-five, two weeks after skin testing, were essentially the same. Skin test results indicated the presence of active DTH in all animals, as the readings in every case were consistently above 12 mm of indurated erythema.

Since the different lymphoid sources responded with similar changes in intracellular IMP during the course of sensitization, an experiment was designed to follow the response of PBL of individual animals as a function of time. Sensitization with two antigens was done to examine the possibility that the involvment of more cells in a response might enhance the opportunity to detect changes in IMP concentrations during sensitization. Two sets of animals were sensitized to histoplasma and DNCB and subsequently skin tested with both antigens on day 21 (Figure 3). Group I was skin tested with both antigens on day 28. Group II was skin tested with DCNB on day twenty-eight and with histoplasmin on day thirty-five. Data from all sensitized animals were grouped together until day thirty.

Inspection of Figure 3 shows a similar pattern of IMP levels for both groups of animals during sensitization and skin test. This trend was confirmed by an analysis of variance. While statistical analysis could show no significant change in IMP levels as a function of time, each experiment appeared to show a similar pattern of IMP levels during sensitization and skin testing. IMP concentrations were essentially the same in both experiments on day zero. Two weeks after the first skin testing, however, differences in multiple skin tested animals as

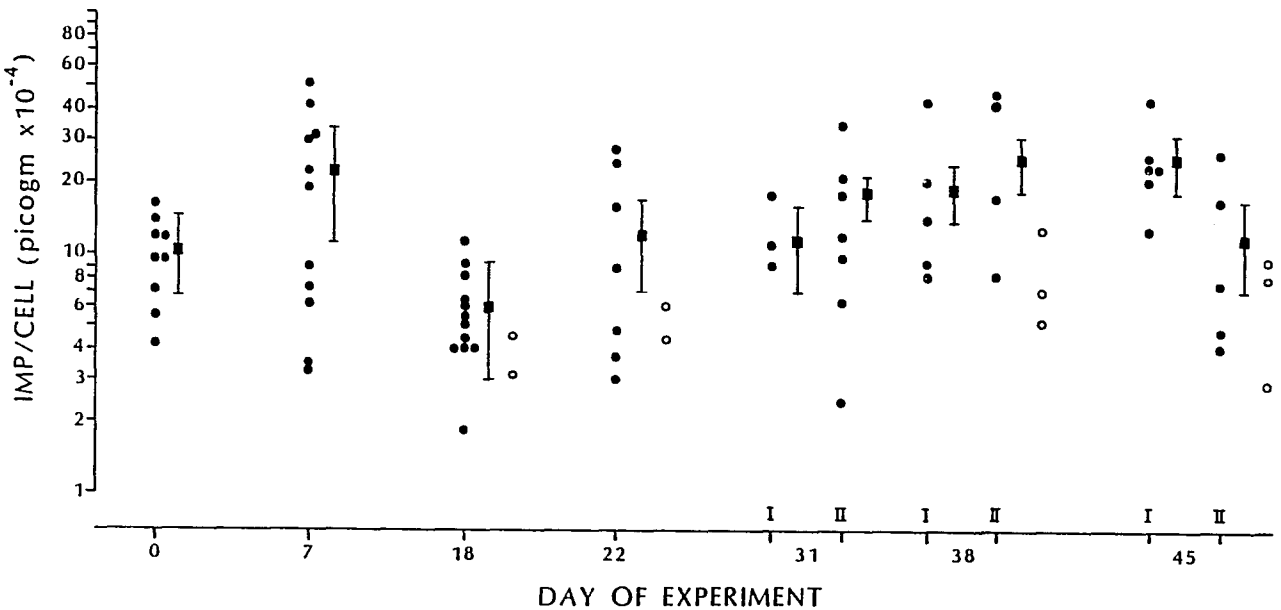

Figure 3. Intracellular IMP from non-adherent blood lymphocytic lysates collected from guinea pigs sensitized to both DNCB and histoplasma. Test animals (●) were sensitized with both antigens and skin tested with 0.1% DNCB and 1:10 histoplasmin on day 21. Group 1 was skin tested with both antigens again on day 29. Group II was skin tested with DNCB on day 29 and with histoplasmin on day 35. Three unsensitized controls (○) were skin tested with both antigens on days 21 and 29. Group averages (■) appear with standard error bars at 95% confidence level.

compared to animals tested one time became apparent. Animals from the second experiment which were dually sensitized and received more than one skin test appeared to maintain higher intracellular IMP than those responding once to a single sensitizing antigen. On day forty-five of the first experiment in which animals were sensitized to histoplasma alone, mean levels of IMP were 4.6± 3.7 and 3.9± 4.5 x 10^{-4} picograms per cell. At this same time, animals responding to more than one skin test had mean levels of 12.5± 4.3 and 25.7 ± 12 x 10^{-4} picograms per cell. These levels were maintained during subsequent skin tests. The measurement of skin test reactivity to histoplasmin and DCNB in these animals is presented in Table I. Animals in group I demonstrated acceptable response to histoplasmin on first skin testing and three of the five subjects maintained the vigor of their original readings. There was considerably less response to DCNB in these animals. Four of the five animals had readings of 1+ or less with only one animal responding with a 3+ reaction. This pattern was essentially repeated on the second DCNB skin test one week later. The histoplasmin response in Group II animals varied and second testing gave mixed readings relative to the first reactions. DCNB response of Group II animals was not especially strong, giving low readings for six of the seven animals on both testings. No direct correlation between IMP concentration and skin test results were possible.

Table I

Histoplasmin and DCNB skin test readings from dually sensitized guinea pigs

group[a]	day of skin test	antigen[b]	Dermal reaction of individual animals						
1	21	H	14x21	17x20	18x21	15x19	16x19		
		D	1+	3+	±	±	±		
	28	H	15x15	3+	±	7x7	10x11		
		D	±	3+	±	±	+		
11	21	H	19x23	11x14	7x10	15x20	16x20	19x24	11x11
		D	3+	1+	±	±	-	-	3+
	28	D	2+	1+	±	+	-	-	2+
	35	H	11x13	14x18	17x17	12x15	17x19	10x12	12x12

a Animals were given sensitizing doses of histoplasma on days one and ten and painted with DCNB on days seven through twelve. Group I was then skin tested with both antigens on days 21 and 28. Group II was skin tested with both antigens on day 21, with DCNB alone on day 28 and with histoplasmin on day 35.

b Reactions for histoplasmin (H) are recorded in millimeters of indurated erythema. Readings for DCNB (D) are graded as described in materials and methods.

IMP Measurements in T-cell enriched Populations and Lymphocyte Transformation Assays of Cells from Sensitized Guinea Pigs

The previous experiments did not provide any obvious correlation between intracellular IMP concentrations in sensitized cells and skin test results. As the introduction of skin test antigen was considered further stimulus by itself, an in vitro correlate was utilized as a non-obtrusive measurement of sensitization. Responses to the polyclonal mitogen PHA-P and the specific sensitizing antigen (histoplasmin) were determined by 3H-TdR uptake using PBL collections taken concurrently with those for IMP assay. This permitted a measure of cells responsive to histoplasmin without directly affecting the in vivo activities. Mononuclear cell suspensions were enriched for T-cells by rosetting the B-cells and monocytes. EAC rosetting was used in preference to nylon wool columns as the rosetting technique provided improved cell yield and purity.

Cells capable of responding to histoplasmin were noted by day sixteen as seen in Figure 4. At no time throughout the experiment did an unsensitized animal respond with greater than a 0.2 log stimulation ratio to histoplasmin, their overall log response being less than 0. In contrast, by day nine two animals in the sensitized groups began to recognize histoplasmin in vitro and by day sixteen, five of the eighteen animals had log response indices of greater than 0.2. Sensitized animals had a wide range of response by day thirty. At that time five subjects still appeared to lack cellular populations that could recognize

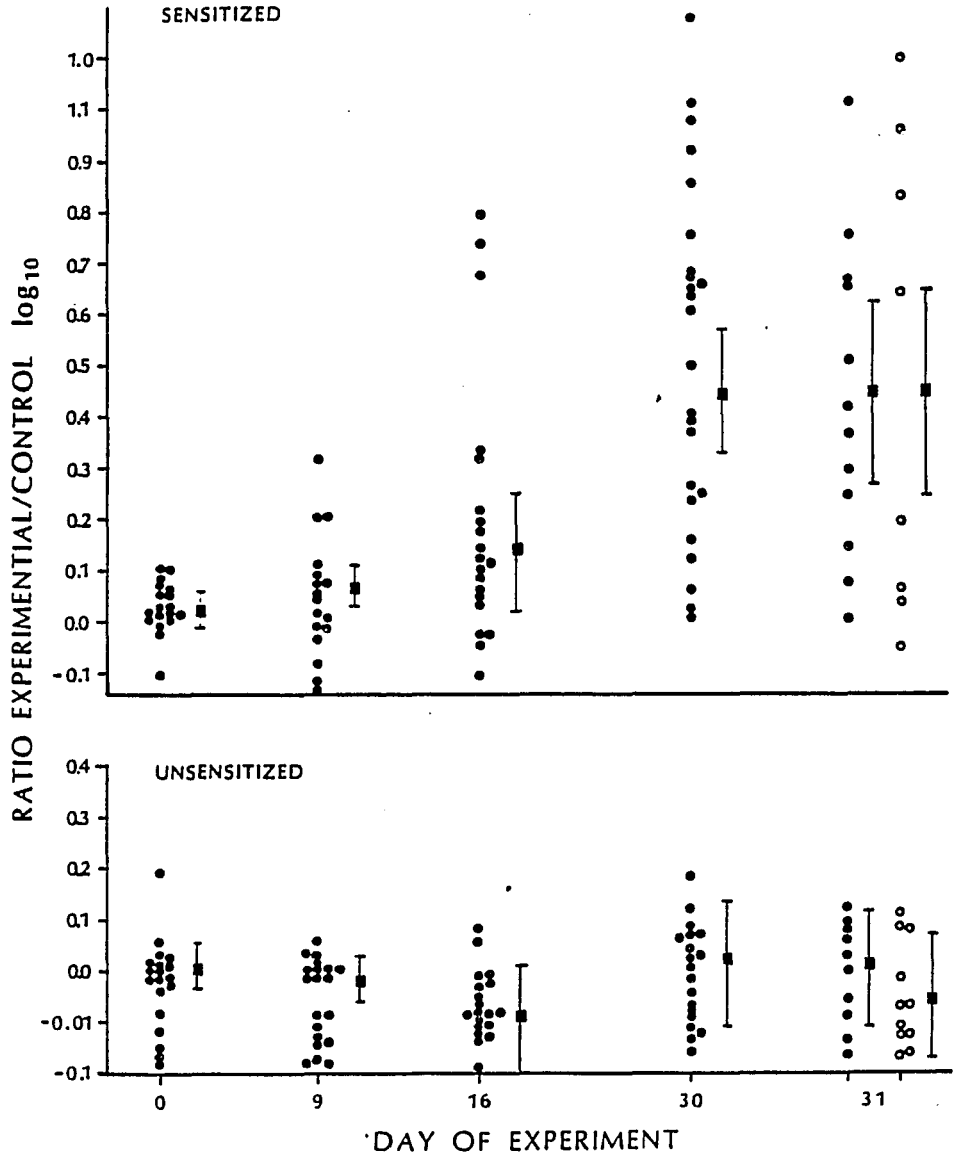

Figure 4. Histoplasmin stimulated tritiated thymidine uptake from guinea pig lymphocytes during histoplasma sensitization. Each point is the $\log_{10}$ of the stimulation index of a single animals's 1:10 blood dilution using 1:40 dilutions of histoplasmin. Geometric means (■) are shown with error bars at 95% confidence level. Groups on day 31 were skin tested with either histoplasmin (●) or saline (○).

and respond to histoplasmin. Other animals gave good response to the sensitizing antigen, resulting in a geometric mean value of 0.44 ± 0.05. (Figure 4).

The effect of prior skin testing on in vitro stimulation by histoplasmin was examined by splitting the sensitized groups of animals on day thirty into one set that received histoplasmin and one that received saline skin tests. When ^{3}H-TdR uptake was again assessed 24 hours after skin test, no significant difference between the two sets of sensitized animals could be found. The geometric mean of the histoplasmin tested group was 0.46 ± 0.18 while the mean for saline skin tested animals was 0.465 ± 0.1. There was no measurable difference in unsensitized animals skin tested with histoplasmin or saline.

In contrast to the histoplasmin response, the 3H-TdR uptake following the polyclonal T-cell mitogen PHA-P was found to exhibit a much different pattern during sensitization and skin testing (Figure 5). During the entire time course of the experiment, unsensitized groups of animals maintained an essentially unchanged response to PHA-P. The response of sensitized animals showed significant differences ($p<0.05$) between day sixteen and day thirty-one. The geometric means for log stimulation indices of sensitized animals dropped from 1.2004 ± 0.007 on day zero to 1.0578 ± 0.07 on day sixteen. By day thirty, the PHA-P responses returned to original levels and were much the same as in unsensitized animals.

One day later, twenty-four hours after skin testing procedures, the PHA-P response in saline tested sensitized animals was apparently

Table II Mean values for lymphocyte counts, IMP and tritiated thymidine uptake assays.

group[b]	day	Absolute lymphocytes/ml ($\times 10^6$)	IMP/cell[c]	Arithmetic Stimulation indices PHA-P	Histoplasmin
H/H	0	2.28 ± 0.4	5.34 ± 4.8	17.2	1.15
	9	2.85 ± 0.4	4.7 ± 4.4	14.6	1.0
	16	4.55 ± 0.6	3.9 ± 2.4	12.5	1.55
	30	4.2 ± 0.5	4.2 ± 4.0	18.2	3.6
	31	2.15 ± 0.6	14.4 ± 6.0	7.9	3.9
H/S	0	2.73 ± 0.4	9.6 ± 5.4	15.4	1.0
	9	3.25 ± 0.4	3.7 ± 4.9	12.6	1.0
	16	4.35 ± 0.6	2.6 ± 2.7	11.1	2.6
	30	3.15 ± 0.5	8.7 ± 4.5	16.2	5.1
	31	3.0 ± 0.6	8.5 ± 6.8	13.0	6.0
S/H	0	2.45 ± 0.4	9.6 ± 5.1	15.9	1.0
	9	2.2 ± 0.4	12.5 ± 4.6	13.5	1.0
	16	2.9 ± 0.6	8.6 ± 2.6	14.3	1.0
	30	2.75 ± 0.5	10.8 ± 4.2	15.9	1.0
	31	2.9 ± 0.6	13.1 ± 6.4	12.6	1.0
S/S	0	2.65 ± 0.4	10.6 ± 4.8	15.4	1.0
	9	2.3 ± 0.4	9.3 ± 4.4	13.4	1.0
	16	3.05 ± 0.6	5.7 ± 2.4	15.4	1.0
	30	2.3 ± 0.5	9.8 ± 4.0	14.2	1.0
	31	2.35 ± 0.6	8.4 ± 6.0	13.0	1.0

a Means represent the average of eight to ten subjects for each day, plus or minus the standard error at 95% confidence. Sensitization and skin testing are as described in the legend of Figure 5.

b Groups are coded: H/H = sensitized and histoplasmin skin tested, H/S = sensitized and saline skin tested, S/H = unsensitized and histoplasmin skin tested, and S/S = unsensitized and saline skin tested.

c IMP is given as picograms per cell x 10^{-4}.

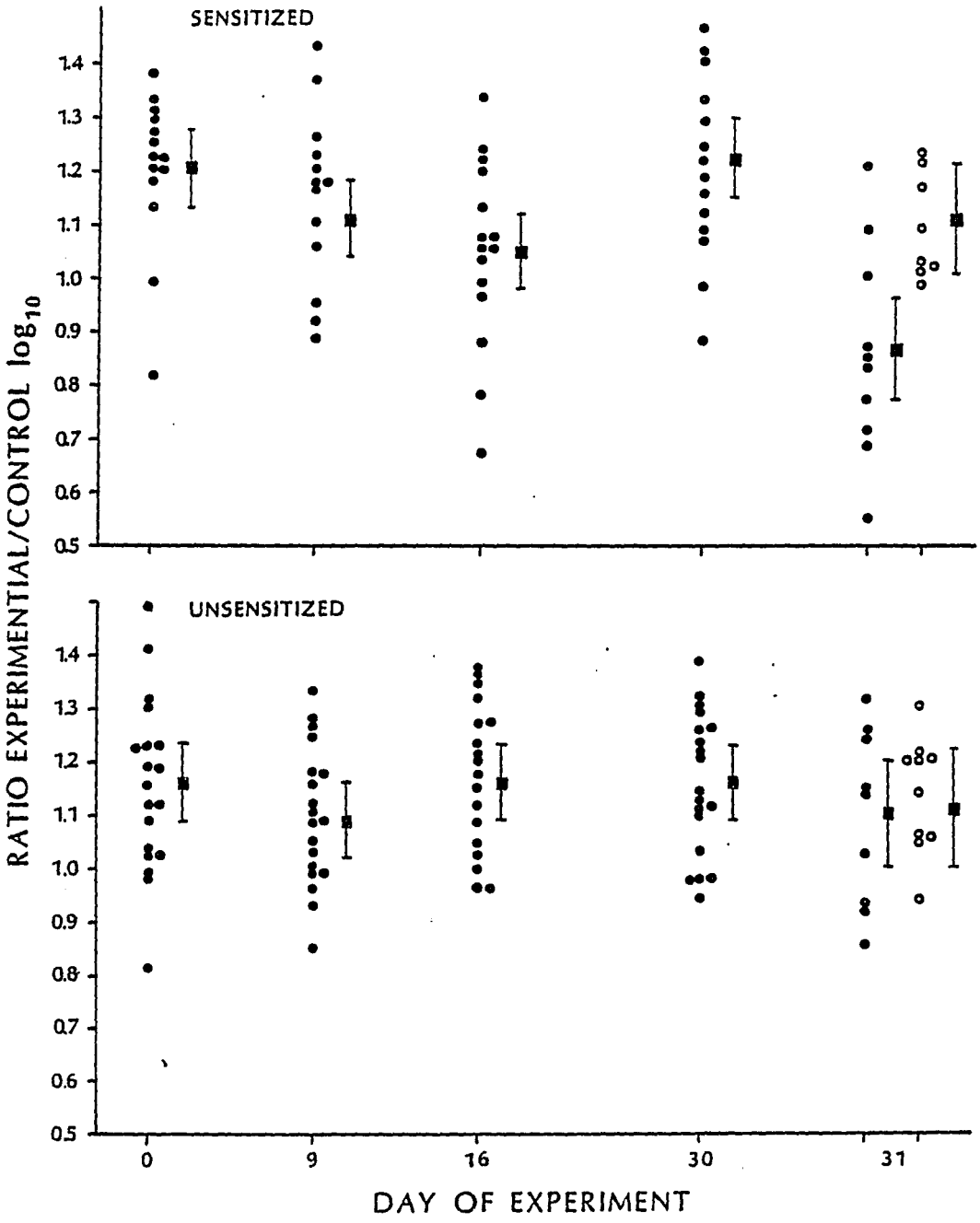

Figure 5. Phytohemagglutinin stimulated tritiated thymidine uptake from guinea pig lymphocytes during histoplasma sensitization. Each point is the $\log_{10}$ stimulation index of a single animal on the given day. Geometric group means (■) are shown with error bars at 95% confidence level.
tested with either histoplasmin (●) or with saline (○).

unchanged. In marked contrast, ^{3}H-TdR uptake in cells from sensitized histoplasmin skin tested animals decreased. The geometric mean on day thirty for this group was 1.22 ± 0.01. One day after histoplasmin skin testing the mean dropped to 0.866 ± 0.01. This decided reduction in mitogen response was evident in no other group. The reduction of ^{3}H-TdR uptake in PBL stimulated with PHA-P was concurrent with the maintained ^{3}H-TdR uptake in cells following histoplasmin stimulation as noted in Figure 4.

At each time that PBL were collected from each animal, absolute lymphocyte counts were obtained. As shown in Table II, unsensitized animals demonstrated no significant change in lymphocyte numbers during the course of the experiment. The absolute lymphocyte counts of sensitized groups increased significantly ($p<0.05$) on days nine, sixteen and thirty. The increase in the number of lymphocytes per milliliter of blood appeared to be concurrent with the decrease in responsiveness to the T-cell mitogen PHA-P and the increased responsiveness of cells towards histoplasmin. None of these trends were noted in unsensitized subjects.

Only homologously skin tested animals which had been sensitized showed a significant change in the absolute lymphocyte count between days thirty and thirty-one. These animals experienced a change from 4.2×10^{6} cells per milliliter (±0.5) on day thirty to 2.15×10^{6} ± 0.6 on day thirty-one. Since the lymphocyte count was determined using Wright's stained blood films of peripheral blood, it was possible to

note any morphological evidence of anemia during the experiment. While a slight polychromasia was evident in several animals on day sixteen, it was not noted on day thirty and thirty-one. An essentially normal blood picture was common to all four groups, including the homologously skin tested sensitized subjects.

Figure 6 presents the variation in intracellular IMP of T-cell enriched populaions of lymphocytes as a function of time and treatment. Control goups of animals maintained an apparently unchanged level of IMP during the entire experiment. Significant differences were noted, however, in the two sensitized groups. Pooled averages of IMP on days nine and sixteen were significantly lower($p<0.05$) in sensitized animals. This was concurrent with the increase in absolute lymphocytes for these sensitized groups.

Comparison of IMP levels between days sixteen and thirty-one in sensitized animals receiving homologous skin tests showed the same increase suggested in a previous experiment. In the three experiments, the levels of intracellular IMP in homologously skin tested animals on day thirty-one did not differ significantly from the values found in these animals at the beginning of the experiment. What can be noted is the significant difference in IMP levels just after sensitization as compared to the levels after homologous skin testing. The real difference of IMP content of these two populations of cells appeared only in animals which were sensitized and then skin tested with the antigen to which they had been exposed. Analysis of the cell populations on day thirty-one showed increased IMP, decreased absolute lymphocyte counts, decreased

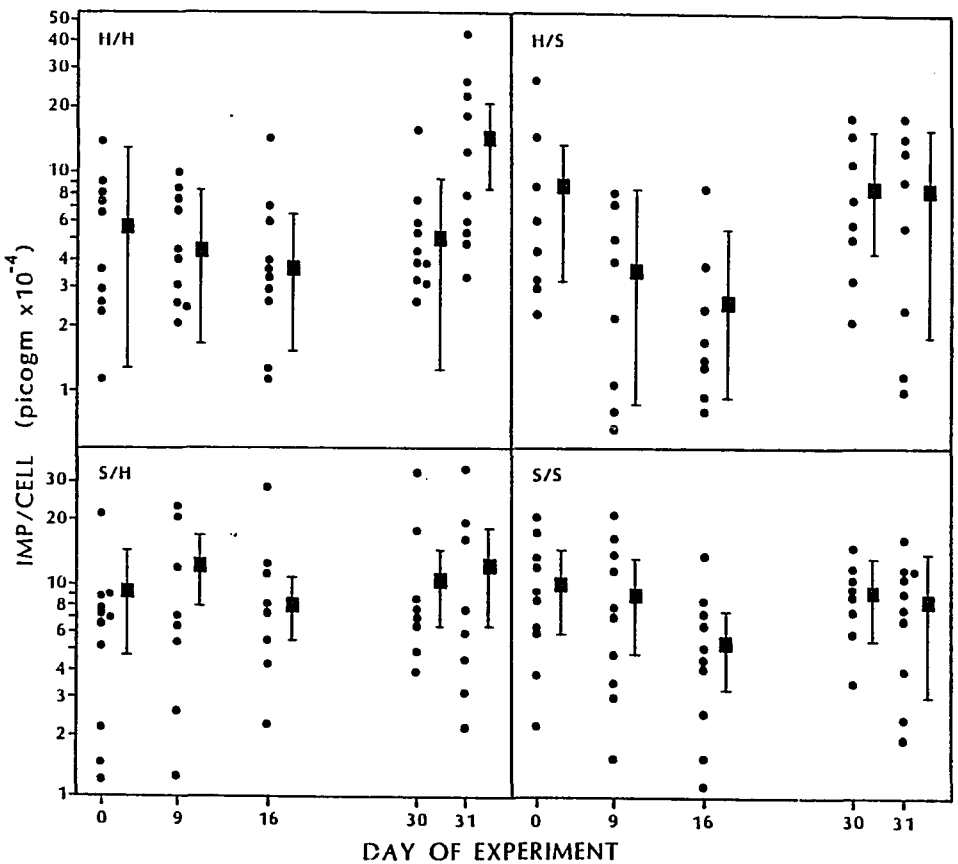

Figure 6. Intracellular IMP during sensitization with histoplasma. T-cell enriched populations were prepared by EAC depletion, lysed and acetone precipitated. Sensitizing doses of yeast phase histoplasma were given on days one and ten. Skin testing was done on day 30. Each point represents one determination done on one animal. Group means (■) are shown with error bars at the 95% confidence level determined by ANOVA. Groups are coded: H/H=sensitized and histoplasmin skin tested, H/S=sensitized and saline skin tested., S/H=unsensitized and saline skin tested.

PHA-P response and an unchanged histoplasmin stimulated ^{3}H-TdR uptake. All four concurrent events were shown to have statistical significance.

Data from this experiment were subjected to further analysis to confirm the trends in responsiveness discussed above. Figure 7 depicts IMP and PHA responses as a function of time for all four groups. The mean of each day is plotted as the center of the sphere and the perimeter describes the 95% confidence limit for all observations on that day. Overlapping spheres share common response. It can be clearly noted that the only real change in the relations between PHA-P stimulation of ^{3}H-TdR uptake and IMP concentrations occurs on day thirty-one in sensitized animals that had been skin tested with the homologous antigen.

All data from this experiment were also examined by inter-correlation analysis to help clarify the relationship between events during the experiment. Factors that were compared included the effect of sensitization, the vigor of skin test response, IMP levels on each day, absolute lymphocyte counts on each day, and 3H-TdR uptake following both PHA and histoplasmin stimulation. Table III presents the positive and negative correlations at the 99% confidence level. Sensitization was shown to affect the numbers of circulating lymphocytes on days nine, sixteen and thirty and to have a positive correlation with histoplasmin stimulation in vitro on days nine, sixteen, thirty and thirty-one. A significant negative relationship was found between sensitization and intracellular IMP on days nine and sixteen. The drop in PHA response was strongly related to the vigor of the skin test response ($r = -.6464$).

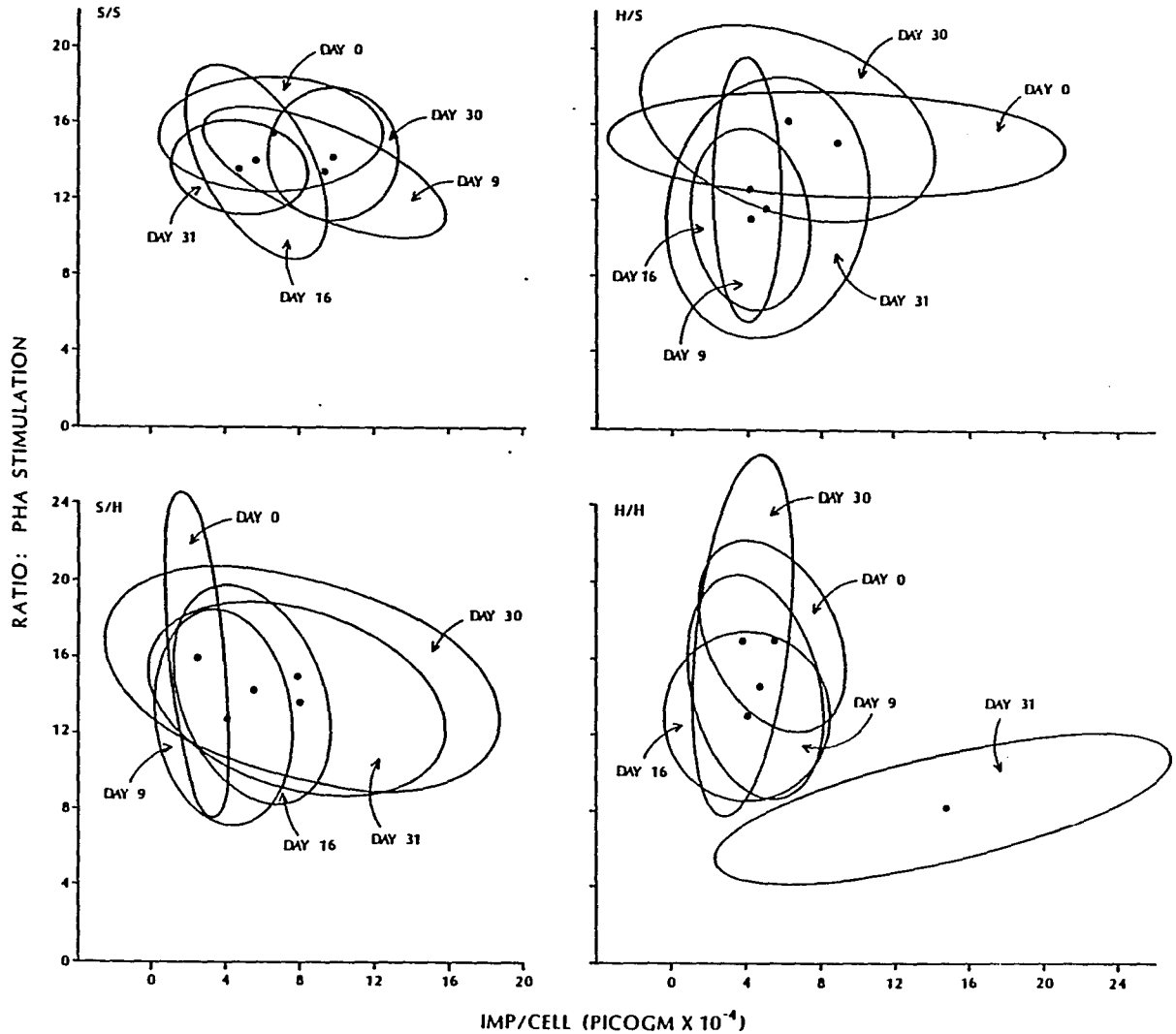

Figure 7. Bivariate analysis of phytohemagglutinin stimulation ratios and IMP concentrations of guinea pig lymphocytes collected during sensitization.

Skin test intensity was also correlated with the ability of histoplasmin to stimulate ^{3}H-TdR uptake in vitro on days thirty and thirty-one.

The profile of variables that occurred on days sixteen and thirty-one showed the interrelationships of events on those two days to be quite different. The increase in absolute lymphocytes that correlated with sensitization on day sixteen had a positive relation to the vigor of the newly noted in vitro histoplasmin responses. At this time, the higher the number of lymphocytes in circulation, the lower the IMP levels in T-cell enriched populations were found to be. No direct relation to lymphocyte number or histoplasmin in vitro response and PHA stimulation was noted. By day thirty-one a strong negative relation was found between PHA responses and the three variables of dermal reactivity,lymphocyte counts, and histoplasmin in vitro responses. It was at this time that the intracellular IMP levels in T-cell enriched populations of homologously skin tested animals were significantly higher than that found on day sixteen in that group.

Intracellular IMP Measurement in in vitro Stimulated Cells

The previous experiments have examined the changes in IMP levels as a function of time following stimulation in vivo. The in vitro correlate of CMI used to establish the presence of antigen responsive cells measures the proliferative abilities of responsive cells. It was noted that the in vivo antigen stimulation of sensitized lymphocytes resulted in a decrease in total T-cell DNA synthesis in mitogen

Table III. Intercorrelation of Variables during Histoplasmin Sensitization

variable [a]	positive	negative
sensitization	skin test	IMP (9,16)[b]
	absolute lymphocytes (9,16,30)	
	histoplasmin-TdR (9,16,30,31)	
dermal reactivity	sensitivization	PHA- TdR(31)
	absolute lymphocytes (30)	
	histoplasmin-TdR(30,31)	
absolute lymphocytes		
day 0	histoplasmin-TdR(9)	-
day 9	sensitization	PHA(31)
day 16	sensitization	IMP(16)
	histoplasmin-TdR(9,16,30,31)	
day 30	sensitization	-
	dermal response	
	histoplasmin-TdR(16,30,31)	
PHA-P stimulation of ^{3}H-TdR uptake		
day 16	-	histoplasmin-TdR(31)
day 31	-	dermal reactivity
	-	histoplasmin-TdR(31)
histoplasmin stimulation of ^{3}H-TdR uptake		
day 9	sensitization	IMP(0)
	absolute lymphocytes(16)	
day 16	sensitization	-
day 30	sensitization	-
	absolute lymphocytes(16,30)	
day 31	sensitization	PHA(16,31)
	dermal reactivity	
	absolute lymphocytes(16,30)	
intracellular IMP concentration		
day 0	-	histoplasmin-TdR(9,16)
day 9	sensitization	-
day 16	-	sensitization
		absolute lymphocytes(16)

a All variables intercorrelated either positively or negatively with an r value greater than 0.418 ($p<0.01$)

b Numbers in parenthesis represent the experimental day for the variable.

stimulated cultures of PBI. Since decreased DNA synthesis coincided with increased IMP levels, and since PBL are not normally blastoid in circulation, a further experiment was designed to investigate the protein activity in stimulated and non-stimulated cells. PBL were collected from animals of the previous experiment on day thirty-one and cultured in vitro either (1) by themselves, (2) with PHA-P, or (3) with histoplasmin. After 24 hours of culture, both protein synthesis and IMP levels were measured. Data for this experiment appear in Table IV.

Comparison of the ^{3}H-Leu uptake between the four groups of animals showed the highest stimulation ratios in histoplasmin stimulated cultures of cells taken from sensitized animals. Log response ratios of greater than 0.17 (arithmetic ratios of 1.5) were set as the level above which significant stimulation occurred (64). By this criteria, only sensitized animals responded with increased protein synthesis following in vitro stimulation with histoplasmin. In the sensitized animals, highest stimulation was noted in homologously skin tested subjects and averaged 0.397. All PHA stimulated cultures had response ratios above 0.17.

Intercorrelation analysis was performed on these data to examine any significant relationships between the amount of protein synthesis as measured by ^{3}H-Leu uptake and IMP levels (Table V). This analysis established four significant correlations. First, the ability of histoplasmin to stimulate a change in IMP levels over control cultures was directly correlated to the ability of PHA to stimulate IMP changes in

Table IV

IMP levels and tritiated leucine uptake following in vitro stimulation

groups[b]	IMP control	IMP PHA	IMP histoplasmin	$\log_{10}$ stimulation ratio for[c] PHA	$\log_{10}$ stimulation ratio for[c] histoplasmin
H/H	7.4	8.3	9.9	0.3277	0.5833
	10.0	15.0	23.0	0.5810	0.6358
	1.67	21.0	3.4	0.2308	0.0411
	1.87	22.0	48.0	0.4495	0.3063
	12.0	9.3	16.0	0.2319	0.4165
H/S	10.7	34.0	22.0	0.3622	0.6440
	3.8	5.9	3.7	0.2040	0.8812
	8.1	10.1	7.4	0.3224	0.5185
	3.5	5.4	3.0	0.3008	0.1148
	15.0	24.0	11.7	0.2027	0.1453
S/H	3.4	3.9	7.3	0.4289	0.1165
	1.7	1.6	2.4	0.2550	-.0052
	3.0	13.1	8.9	0.3560	0.0813
	2.9	18.0	4.4	0.6706	0.0415
	9.2	10.4	7.1	0.4641	0.0809
S/S	0.7	2.6	1.0	0.5375	0.1324
	24.0	20.0	17.0	0.3984	0.0435
	1.2	16.0	1.7	0.7189	-.0029
	16.0	12.0	9.3	0.2408	0.0806
	17.0	25.0	8.1	0.4622	0.0397

a Ficoll-hypaque separated PBL were incubated in 2 ml volumes at 2×10^7 cells per milliliter and stimulated with 50μg per ml of PHA-P or a 1:40 dilution of histoplasmin. After 18 hours, 2 Ci of ^{3}H-Leu was added and the cultures were incubated another six hours and harvested.

b Groups of five animals were identified as:H/H=sensitized and histoplasmin skin tested, H/S = sensitized and saline skin tested, S/H = unsensitized and histoplasmin skin tested, S/S = unsensitized and saline skin tested. Skin testing had been done 24 hours before initiating culture.

c Each value represents the geometric mean of triplicate counts per minute of control cultures subtracted from the geometric mean of stimulated cultures.

these cells ($p<0.05$) Second, a strong positive relationship ($p<0.01$) between sensitization and protein synthesis following histoplasmin exposure was found. Third, sensitization showed a negative relationship to the ^{3}H-Leu uptake in PHA stimulated cultures. And, fourth, sensitization demonstrated a positive correlation to intracellular IMP from histoplasmin stimulated cultures. When cells from sensitized animals were exposed in vitro to antigen, both protein synthesis and IMP concentrations increased.

Table V

Intecorrelation coefficients for in vitro stimulated cultures

	sensitization	skin test	Δ IMP PHA	Δ IMP Histo	^{3}H-leucine assay PHA	^{3}H-leucine assay Histo
variable:[b]						
sensitization	1.0000	0.000	0.2191	0.4472	-.4449	0.6584
skin test	-	1.000	0.0020	0.3596	0.0828	0.1150
IMP-PHA	-	-	1.000	0.4962	0.3019	0.0814
IMP-Histo	-	-	-	1.000	0.1352	0.4097
^{3}H-Leu/PHA	-	-	-	-	1.000	-.0518
^{3}H-Leu/Histo	-	-	-	-	-	1.000

a Pearson's product moment correlation coefficient significant at :

$r1/_5$ = 0.4444 at 0.05
0.561 at 0.01

b Variables used in comparison were sensitization, effect of skin testing, the change from control in IMP levels after PHA (IMP-PHA), the change from control in IMP levels after histoplasmin (IMP-histo), protein synthesis after PHA (^{3}H-Leu/PHA) and after histoplasmin (^{3}H-Leu/histo) stimulation.

DISCUSSION

When antigen is presented to elicit cell mediated immunity (CMI) it may be taken up by local phagocytic cells and carried to the draining regional lymph nodes for presentation to precursor cells. (44). Precursor cells must then undergo two processes - differentiation and proliferation. Approximately four days after exposure to contact sensitizing agents, the nodes become hyperplastic and maximal numbers of blastoid cells develop (65). At this time, the host is unresponsive to the skin test antigen(66). Simultaneous with this period of proliferation, several events concerned with cell responses are known to occur, and, in our experiments, these events have been related to levels of IMP.

During the proliferative period, a slight but significant decrease in tritiated thymidine uptake following PHA stimulation of PBL was noted. At this same time, the number of circulating lymphocytes increased, the first antigen sensitive lymphocytes were detected in the circulation by stimulation assay, and the average IMP concentration in T-cell enriched populations was slightly decreased. A slight decrease in the levels of IMP was noted in all the experiments but was never shown to result in levels significantly different from those observed at the beginning of the experiments. In the later experiments, however, intercorrelation analysis did show this change to correlate significantly both with the process of sensitization and with the increase in circulating lymphocytes that occurred at that time.

The slight decrease in average IMP levels is subject to several possible interpretations. Decreased IMP may be associated with a general decrease in all of the cells measured, or, alternatively, with an increase in a proportion of cells having lower intracellular IMP. Several observations favor the second interpretation.
At the cellular level of the immune response, increasing numbers of lymphocytes were found in the circulation at the same time as the first antigen responsive cells capable of proliferative responses following histoplasmin exposure in vitro were detected. These observations may imply the appearance of new populations of lymphocytes entering into the circulation and that these cells would have lower than average IMP levels. The fact that this slight decrease in IMP was noted in all the cell sources examined - blood, nodes and spleen - suggests that during sensitization a small population of lymphocytes with lower intracellular levels of IMP may be introduced into the general circulation. The idea receives support from the strong negative correlation between IMP concentrations and the number of lymphocytes in circulation found on day sixteen.

Antigen sensitive cells appearing in peripheral circulation on day sixteen were capable of in vitro proliferation following exposure to the antigen. If, as discussed above, these newly responsive cells were a population with lower IMP levels, it may follow that antigen stimulated cells capable of in vitro proliferation may have characteristically lower IMP concentrations. Germane to this idea is the report that growth rates

as reflected in increasing numbers of proliferating liver cells have been found to correlate directly with the activity of the enzyme IMP dehydrogenase (67,68), the enzyme responsible for conversion of IMP into xanthine monophosphate on the pathway to guanosine monophosphate (GMP).

The incipient proliferative response of cells to antigen stimulation in vitro occurred at a time when mitogen-induced proliferation was slightly decreased. These events were concurrent with increased numbers of circulating lymphocytes and since the sensitizing antigen should stimulate T-cell responses (69,70), the decrease in PHA-induced blastogenesis is not likely to be due to an actual decrease in the number of circulating T-cells. This transient anergy to PHA is in accord with previous reports. Sensitization with a variety of both live and killed agents has been found to induce a temporary decrease in mitogen induced in vitro proliferation(71-77). Tewari et al. (78) has recently reported suppression of the blastogenic response to both T-cell mitogens concanavalin A (con-A) and PHA for a period of two weeks past immunization with live yeast cells of Histoplasma capsulatum in mice. The increased antigen-induced and decreased mitogen-induced proliferative responses they noted were entirely consistent with our observations.

The profile of events changed significantly one day after skin testing. Intercorrelation analysis showed a strong positive correlation between (1) in vitro proliferation to histoplasmin, and (2) the vigor of the skin test in sensitized subjects. In marked contrast, the stronger the skin test and in vitro histoplasmin responses, the less the proliferation response to PHA. IMP measurements at this time showed a significantly

increased concentration in the T-cell enriched population as compared to day sixteen. The two striking changes following the skin tests are the decrease in PHA response and the increase in IMP levels.

Previous studies have also shown that peripheral lymphocytes obtained after homologous skin testing of sensitized subjects have reduced response in vitro to PHA (79,80). Both human histoplasmosis patients and guinea pigs sensitized to heat killed M. tuberculosis showed transient anergy to PHA following skin testing. The decreased mitogenic response may be attributed to: (1) a decrease in mitogen responsive cells either due to dilution by unresponsive cells or by sequestration of non-responsive cells, (2) induction of non-specific suppressor T-cells, (3) alteration of the activities of macrophages, or (4) alteration of general T-cell populations to make them less responsive to mitogens. The actual cell types and the cell cooperations involved in mitogen- and antigen-induced proliferation have not been determined satisfactorily (81-83). While none of the above possiblities explaining the decrease in mitogen-induced proliferation need be mutually exclusive, the last mentioned possibility is in accord with several observations (84).

It is difficult to clearly interpret the meaning of the increase in IMP concentrations following skin testing in relation to the proliferative response. However, a study was done to correlate IMP changes with a second macromolecular activity - protein synthesis. This study was designed to relate the amounts of protein and IMP synthesized non-sensitized, and sensitized cells following in vitro stimulation with

PHA or antigen. This study showed increased protein synthesis and IMP levels following histoplasmin stimulation of sensitized cells in vitro.

Previous studies in the literature have not satisfactorily defined the roles of IMP in the immune response. However, some available studies indicate:

1) Greater quantities of bases enter into lymphocytes following mitogen stimulation (33).
2) Administration of inosine caused increased RNA content in brain cells.(85).
3) Incorporation of deoxy inosinetriphosphate RNA in vitro increased the initiation but not translation of message (86).

These observations coupled with reports showing that lymphokine production in vitro is associated with a non-dividing cell (81, 87-89) give support to the close association of IMP increases in antigen stimulated cells producing protein. It has been suggested that protein synthesis in activated lymphocytes is more likely a molecular event closer to in vivo activation than is DNA synthesis (60,90).

Based on these observations and the events summarized in Figure 8, the following is suggested. During sensitization the lymphocyte population increases in number and in the presence of antigen sensitive cells in circulation becomes apparent. Analysis of these cells shows a decrease in PHA responsiveness and decreased IMP concentrations. Immediately after skin testing, the cells remaining in circulation show a slight decrease in

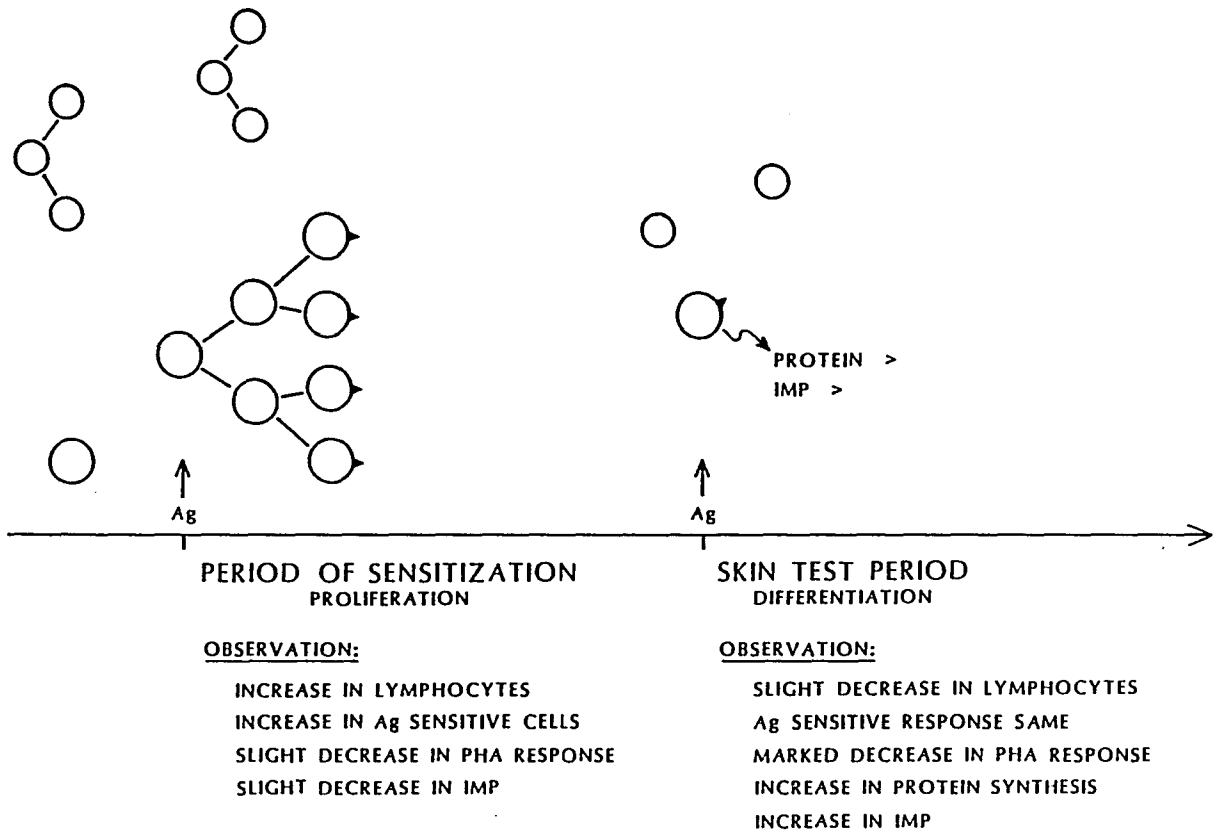

Figure 8. Summary of observations during sensitization and skin testing.

number, the same proliferative response to antigen and a marked decrease in PHA response. These cells, when cultured with antigen, have increased protein synthesis and IMP concentrations. These observations may support the idea that there exists a regulatory mechanism in circulating antigen sensitive lymphocytes which function to block the usage of IMP for DNA synthesis, thus permitting the resultant increased intracellular IMP to be used for protein synthesis in effector cells.

APPENDIX A

PREPARATION OF REAGENTS

Ficoll-Hypaque : Ficoll 400 is supplied as a powder from Pharmacia Fine Chemicals. Sodium Hypaque, brand of diatrizoate sodium, is supplied as a sterile 50% solution from Winthrop Labs. A solution with a final specific gravity of 1.076 is prepared as follows:

Ficoll 400	9 grams
Double distilled water	115 ml.
Hypaque	30 ml.

Ficoll is dissolved in the water and then Hypaque is added. The solution is autoclaved in loosely capped bottles and, after cooling, the specific gravity checked by refractometer.

Cell Balanced Salt Solution: Cell balanced salt solution was prepared as follows:

Dextrose	1000 grams
KH_2PO_4	0.060 grams
$Na_2HPO_4.7H_2O$	0.358 grams
$CaCl_2.2H_2O$	0.186 grams
KCl	0.400 grams
$MgCl_2.6H_2$)	0.200 grams
$MgSO_4.7H_2$)	0.200 grams
NaCl	8.00 grams
Phenol Red(0.05%)	2 ml
H_2O (distilled)	1000 ml

The solution was passed through a 0.45 n Millipore filter and stored at 4 C.

HEPES Buffer HEPES (N-2-hydroyethylpiperazine-N'-2-Ethanesulfonic acid) is supplied in powder form by Grand Island Biological Company and was prepared in a 100 x concentration as follows:

NaCl	0.800	grams
KCl	0.040	grams
Na_2HPO_4	0.010	grams
Dextrose	0.100	grams
HEPES	23.800	grams
H_2O (distilled)	100	ml

HEPES buffer was passed through a 0.45 Millipore filter and stored at -20 C until use.

Gentamycin Sulfate: Gentamycin sulfate was obtained from Schering Corporation. The antibiotic was prepared as a 100x stock solution of 5mg per ml in saline using the correction factor supplied by the manufacturer. Gentamycin was passed through a 0.045 u Millipore filter and stored at -20 C until use.

APPENDIX B

PILOT STUDIES

Phytemagglutinin Stimulation of Tridiated Thymidine Uptake: Parameters for harvest time, blood dilution and mitogen concentration were optimized in repeated pilot studies. Animals were bled by cardiac puncture and the blood was immediately diluted in sterile culture medium to concentrations of 1:10, 1:20, 1:30 and 1:40. Triplicate wells of a microtiter plate received 0.1 ml of the blood dilutions followed by 0.1 ml of PHA-P resulting in a final concentration per well of 1, 2.5, 5 or 10 micrograms. Three identical plates were set up for pulsing at 24, 48 and 72 hours. After a 24 hour pulse, the plates were harvested and counted as described in materials and methods. Highest stimulation ratios were found using a blood dilution of 1:10, a PHA-P concentration of one microgram per well and harvesting at 48 hours. Typical results appear in Figure B-1 below.

Histoplasmin Stimulation of Tridiated Thymidine Uptake: Harvest time, blood dilutions and Histoplasmin concentrations were optimized by the same basic procedures used for PHA optimization. Blood was diluted to concentrations of 1:10, 1:20, and 1:40. Histoplasmin was diluted to concentrations of 1:10 and 1:40 and plates were harvested on days five, six and seven. Highest stimulation ratios were found on day five. Optimal blood dilutions was either 1:10 or 1:20, optimal histoplasmin dilution was either 1:10 or 1:40 and both parameters appeared to vary with individual animals. For this reason, both blood and histoplasmin concentrations were used in experiments. Figure B-2 below shows the results for two sensitized animals:

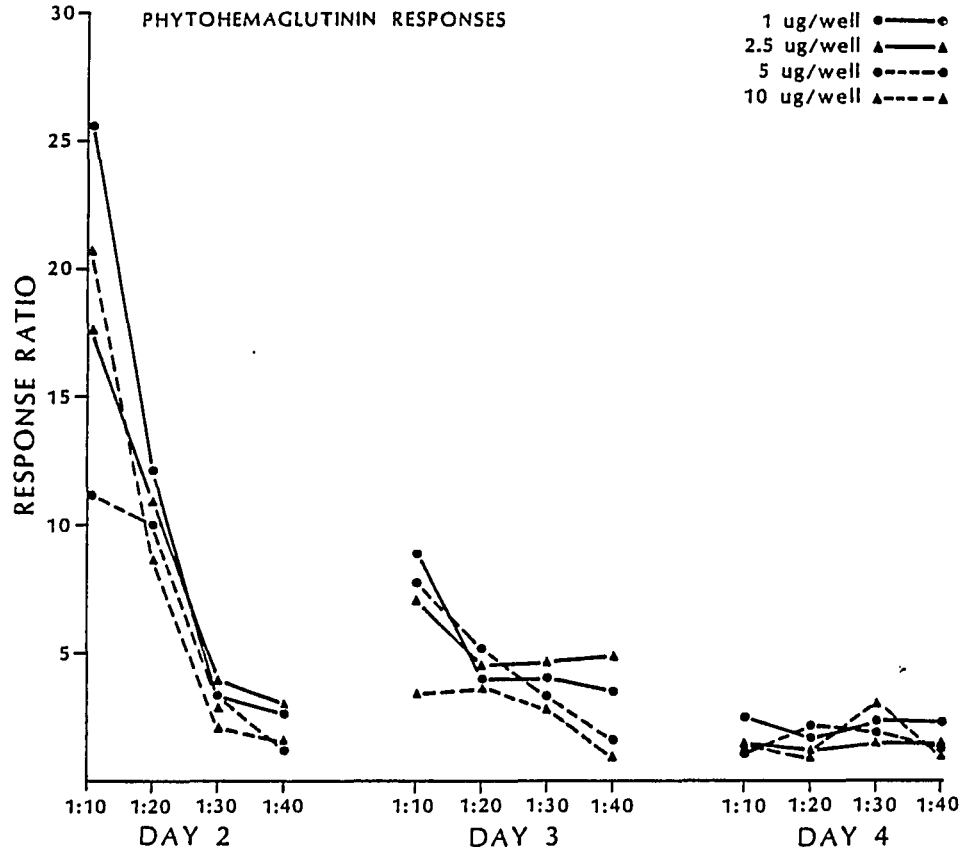

Figure B-1 Pilot study results for a whole blood blastogenesis assay using phytohemagglutinin as stimulant. Blood dilutions of 1:10, 1:20, 1:30 and 1:40 were done on each day using four PHA concentrations. Arithmetic response ratios are shown.

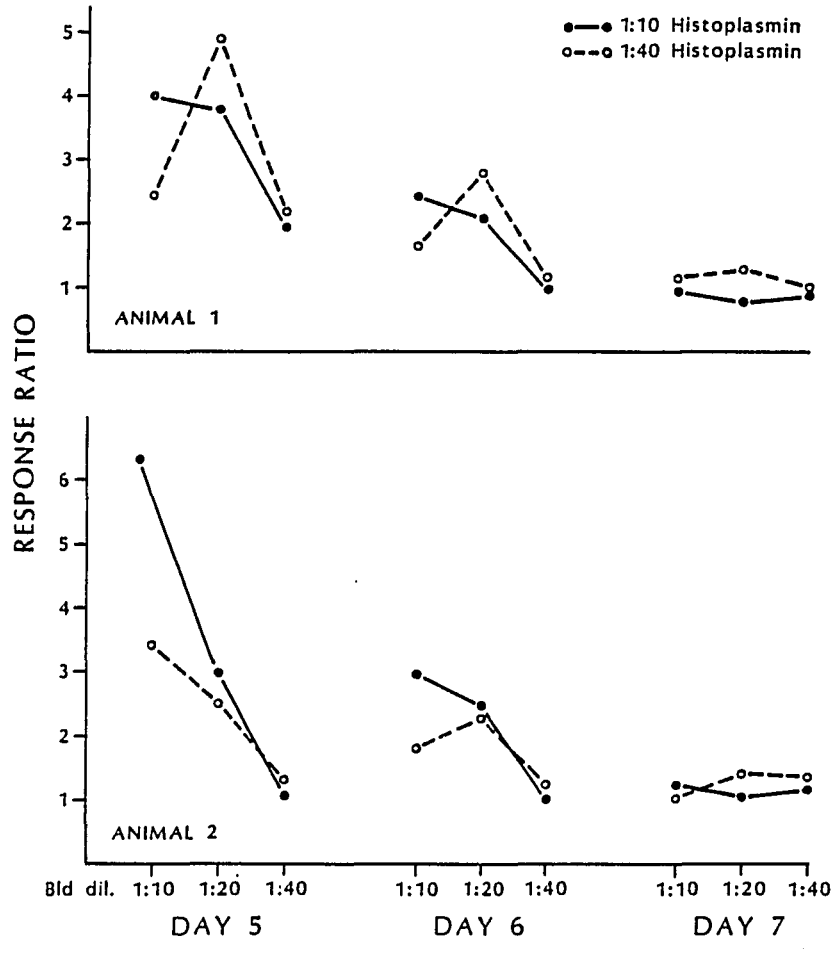

Figure B-2 Pilot study results for a whole blood blastogenesis assay using 1:10 and 1:40 dilutions of histoplasmin. Blood was diluted to 1:10, 1:20, or 1:40 on each day. Arithmetic response ratios are shown.

Tridiated Leucine Uptake Pilot: Parameters for a macro culture technique were optimized in regards to cell number, PHA-P and histoplasmin concentrations. Mononuclear blood cells were sterilely collected from a Ficoll-hypaque gradient and adjusted to concentrations of 2×10^7 or 2×10^6 per ml. Three 1.8 ml volumes of these cellular suspensions then received either 0.2 ml of medium for control cultures, 0.2 ml of PHA-P dilutions ,or 0.2 ml of histoplasmin dilutions. PHA-P was diluted to result in final concentrations of 10, 50 or 100 micrograms per ml and histoplasmin was diluted at 1:10, 1:20 or 1:40. These were incubated and harvested as described in material and methods. Optimal conditions were found using 2×10^7 cells, a PHA concentration of 50 micrograms per ml and a histoplasmin concentration of 1:40. Results appear in the following tables:

	PHA-P concentration, ug/ml			
cell concentration:	0	10	50	100
2×10^6	403	475	954	799
	753	909	1591	1452
2×10^7	819	795	2357	1947
	1011	1486	4740	2119
	926	1033	5185	4553
		Histoplasmin dilution		
	con	1:10	1:20	1:40
2×10^6	580	614	812	1392
	455	493	606	683
2×10^7	959	1061	1288	2965
	1131	1203	1381	2735

APPENDIX C

SUMMARY OF STATISTICAL TABLES

The following tables refer to Corresponding figure numbers in the text.

source of variation	degrees of freedom	sum of squares	mean square	F ratio	p value
cell source	2	74.4598	37.2299		ns
time+treatment	7	1299.4392	185.6341	4.56	<0.001
source x timextrtm.	14	638.9730	45.6255	1.12	ns
Error	73	2971.2133	40.7015		

Table 2. Intracellular IMP from non-adherent lymphocyte lysates collected from histoplasma sensitized guinea pigs.

groups	1	76.4598	76.6812	ns	
times	5	2217.3105	443.4621	2.34	0.055
groups x times	5	2026.2176	405.2435	2.14	ns
Error	48	9080.1666	189.1700		

Table 3 Intracellular IMP from non-adherent blood lymphocytic lysates collected from guinea pigs sensitized to both DNCB and histoplasma.

Appendix C-- Continued

Univariate F-tests

day	Error mean square	F ratio	p value
C	0.0040	1.6731*	0.2017
9	0.0083	9.6262*	0.0040
16	0.0601	9.1606*	0.0048
30	0.0710	24.0010*	0.0001
31	0.0776	9.3911**	0.0003

* = F_{35}^{1}

** = F_{33}^{3}

Table 4 Histoplasmin stimulate tritiated thymidine uptake from guinea pigs lymphocytes during histoplasmin sensitization.

Univariate F-tests

day	error mean square	F ratio	p value
0	0.0204	0.6426*	0.5662
9	0.0239	0.0934*	0.7595
16	0.0234	4.5426*	0.0379
30	0.0237	1.1109*	0.2995
31	0.2249	7.1379**	0.0011

Table 5 Phytohemagglutinin Stimulated Tritiated Thymidine Uptake from Guinea Pig Lymphocyte During Histoplasma Sensitization

Appendix C -- Continued

Univariate F-tests*

day	error mean square	F tests	p value
0	54.9170	1.6990*	0.1983
9	44.4715	9.0132*	0.0051
16	14.4614	7.7684*	0.0084
30	38.2357	2.7919*	0.1000
31	91.3547	0.1468**	0.7054

* = $_{35}^{1}E$ ** = $_{33}^{3}E$

Table 6 Intracellular IMP during Sensitization with Histoplasma.

Univariate F-tests

IMP values:

0	56.6757	0.8536	0.5227
9	45.4806	0.3464	0.0302
16	14.1613	0.3583	0.0242
30	38.8586	1.3986	0.2598
31	89.3598	0.9771	0.2598

Absolute lymphocyte counts

0	0.7410	0.0027	0.9588
9	0.6792	9.6029	0.0041
16	1.4644	13.6802	0.0010
30	1.2780	9.7114	0.0039
31	1.9834	0.0046	0.0039

Table II Mean values for lymphocyte counts, IMP and Tritiated Thymidine Uptake Assays.

Summary of ANOVA of H-TdR uptake of cells stimulated with saline or histoplasmin.*

Source of variation	degrees of freedom	sum of squares	error mean square	F ratio	p value
Blood dilution	1	50.6477	-	-	-
Histoplasmin dilution	1	0.1096	0.1096	2.58	ns
Sensitization	1	8.8217	8.8217	207.95	<0.001
skin test	1	0.0093	0.0093	1.0	ns
days	4	6.0154	1.5038	35.45	<0.001
Blood dil. x s.t.	1	0.1671	0.1671	3.94	<0.005
Sens x skin test	1	0.2924	0.2924	6.89	<0.01
Sens.x day	4	5.9525	0.4881	35.08	<0.001
Error	648	27.4894	0.0424		

S.D. = 0.0206

* All other interactions were insignificant and omitted for table simplification.

Table 111. Significant Intercorrelation of coefficients of Variables During Histoplasm Sensitization.

Listing of Pearson's product moment correlation coefficients. Significance $r1/_{35}$ = 0.325 at 0.05, 0.418 at 0.01

variable	correlate	r values on day 9	16	30	31
sensitization					
	skin testing	-.617	-	-	-
	IMP-	-.450	-.426	-	-
	Abs. lymphs-	-.460	-.53	0.466	0.675
	histo-TdR	0.429	o.465	0.646	-.405
	PHA-TdR	-	-.339	-	
skin testing					
	PHA-TdR	-	-	-	.415
skin test reactivity					
	sensitization	0.617	-	-	-
	abs, lymphs	-	0.372	0.528	-
	histo TdR	-	-	0.326	0.456
	PHA-TdR	-	-	-	-.6464
IMP					
day 0	histo-TdR	-.443	-.402	-	-
day 9	sensitization	0.452	-	-	-
	abs. lymphs	-.387	-.384	-	-
	histo-TdR	-	-	-.395	-
day 16	sensitization	-.426	-	-	-
	abs. lymphs	-	-.421	-	-
	histo-TdR	-.327	-.348	-.326	-
day 31	PHA-TdR	0.338	0.378	-	-
Absolute lymphocyte counts:					
day 0	histo TdR	0.433	0.386	-	-
day 9	sensitization	0.464	-	-	-
	IMP	-.387	-	-	-
	PHA-TdR	-	-	-	-.646
day 16	sensitization	0.530	-	-	-
	skin test react.	-	0.372	-	-
	IMP	-.384	-.421	-	-
	histo TdR	0.444	.593	0.468	0.426
day 30	sensitization	0.466	-	-	-

Appendix C --Continued

variable	correlate	9	16	30	31
	skin testing	0.466	-	-	-
	skin test react	0.528	-	-	-
	histo TdR	-	0.483	0.422	0.419
PHA stimulation of ^{3}H-TdR uptake:					
day 0	-				
day 9	IMP	-	-	-	0.338
	histo TdR	0.327	-	-	-
day 16	sensitization	-.338			
	IMP	-	-	-	0.378
	histo TdR	-	-	-	-.445
day 30	histo TdR	o.327	-	-	-
day 31	sensitization	0.405			
	skin testing	-.415			
	skin test react	-.646			
	histo TdR	-	-	-	-.440
histoplasmin stimulation of ^{3}H-TdR uptake					
day 0	skin test react.	0.370			
	histo-TdR	-	-	0.334	-
day 9	sensitization	0.428			
	IMP	-	-	-.327	-
	abs. lymphs	-	0.444	-	-
	PHA TdR	-	-	0.327	-
day 16	sensitization	-0.465			
	IMP	-.402	-.367	-.348	-
	abs. lymphs	0.341	0.593	0.483	-
day 30	sensitization	0.645			
	skin test react	0.326			
	abs. lymphs	0.346	0.468	0.422	-.318
day 31	sensitization	0.675			
	skin test react	0.465			
	abs. lymphs	-	0.426	0.419	-
	PHA TdR	-	-.445	-	-.440

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