4-1-1973

Lymphocyte-Induced Polymorphonuclear Leukocyte Stimulation

Christine Kull (Walsh)

LYMPHOCYTE-INDUCED POLYMORPHONUCLEAR LEUKOCYTE STIMULATION

CHRISTINE ANN KULL

1973

Permission for photocopying or microfilming of "Lymphocyte-Induced Polymorphonuclear Leukocyte Stimulation"
(TITLE OF THESIS)

for the purpose of individual scholarly consultation or reference is hereby granted by the author. This permission is not to be interpreted as affecting publication of this work or otherwise placing it in the public domain, and the author reserves all rights of ownership guaranteed under common law protection of unpublished manuscripts.

Christine Kell
Signature of Author

5/12/73
Date

LYMPHOCYTE-INDUCED POLYMORPHONUCLEAR LEUKOCYTE STIMULATION

CHRISTINE ANN KULL

A Thesis Presented to the
Faculty of the Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

Department of Internal Medicine

April 1, 1973

Dedicated

to

MY PARENTS

ACKNOWLEDGMENT

The authoress wishes to express her thankfulness to her advisor, Dr. Stuart Finch, for his encouragement and useful suggestions, to Drs. Elisha Atkins and Phyllis Bodel for their spontaneous consultations, and to the personnel in Dr. Finch's laboratory for their help and tolerance.

TABLE OF CONTENTS

INTRODUCTION

The participation of different cell types in reactions of cellular hypersensitivity is well established (1). Delayed hypersensitivity, autoimmunity, homograft rejection, certain allergic manifestations, immunologic responses to cancer cells, and defense against intracellular bacteria, viruses, fungi, and parasites are included among the immunologic events in which these cells take part. The cells involved are mainly lymphocytes, which become sensitized to antigens through contact, and macrophages, which use their phagocytic property to attack, destroy, and sequester autologous or foreign substances. With the recognition that more than one cell type was connected with these events, it became necessary to establish the means of communication between them. It is becoming clear that cell-free factors produced by the sensitized lymphocytes subsequent to stimulation by specific antigen can involve macrophages as well as other cell types (2). It is, therefore, of interest to determine whether lymphocytes can have an effect on polymorphonuclear leukocytes. This present study investigates the communication between these cell types by using lysozyme production and glucose-1-^{14}C oxidation as indicators of polymorphonuclear leukocyte stimulation.

In studying the various factors already described, experimenters have generally started with lymphocytes derived

from an individual having cellular hypersensitivity to a particular antigen (3). The lymphocytes were stimulated *in vitro* by incubating the cells with that antigen for a given time, usually 24 hours. The production of the various factors was usually highly specific in that lymphocytes produced them *in vitro* only against the antigen with which sensitization of the lymphocytes was accomplished *in vivo*. Old tuberculin has been commonly used as the antigen and was chosen as such for this study. However, it has been recently noted that certain mitogenic agents have the ability to stimulate lymphocytes nonspecifically with the production of active factors (4). Phytohemagglutinin is such an agent and was also used in this investigation.

In relating the results of this study, it is the purpose of this thesis:

1. to report that human sensitized lymphocytes exposed to tuberculin have a stimulatory effect on polymorphonuclear leukocytes as indicated by the latter's increased glucose-1-^{14}C oxidation and lysozyme production;
2. to establish time factors involved in this event;
3. to report similar results using lymphocytes nonspecifically stimulated by phytohemagglutinin;
4. to suggest the mechanism by which this phenomenon occurs.

CHAPTER I

REVIEW OF LITERATURE

A review was made of aspects of the following four areas related to the work presented in this thesis: phytohemagglutinin, tubercle bacillus antigen, lysozyme, and glucose metabolism through the hexose monophosphate shunt.

Phytohemagglutinin

Phytohemagglutinin (PHA) is a saline extract derived from beans of the genus Phaseolus. Goddard and Mendel (5), in 1929, described a method for the preparation of PHA from navy beans (Phaseolus communis). The active substance was a protein with the characteristics of albumin. In 1909, Wienhaus (6) partially purified proteins from red kidney beans (Phaseolus vulgaris) and speculated that this protein was an albumin. In 1955, Osgood and Rigas (7) completed the purification. The final products were a mucoprotein (MPHA) and a protein (PPHA). The mucoprotein behaved as a homogeneous substance by electrophoretic analysis over the pH range 5.8 to 8.6. Below pH 5.8, it dissociated into a protein phytohemagglutinin and an inactive polysaccharide. This mucoprotein contained 6.5% nitrogen, 50.4% total reducing substances estimated as glucose, and was heat-labile and very soluble in distilled water. The protein behaved as a homogeneous substance by electrophoresis over the pH

range 2.0 to 8.0 and its isoelectric point was 6.5. The nitrogen content of this protein was 14.6%, and the content of its total reducing substances was 3.4%. It was a heat-labile euglobulin, insoluble in distilled water, but very soluble in saline or buffer solutions. This phytohemagglutinin, in either the mucoprotein or in the protein form, was a non-toxic, powerful hemagglutinin of human, horse, pig, dog, cat, rabbit, chicken, and frog erythrocytes.

The hemagglutinating properties of PHA were discovered by Landsteiner (8) in the early 1900's. This erythro-agglutinating property can be removed by repeated absoprtion with red blood cells. Perera and Frumin (9) demonstrated that hemagglutination by the extract of the fava bean was inhibited by 5% d-glucose or maltose but not by 5% d-galactose or lactose. Borberg et al (10) found that N-acetyl-D-galactosamine inhibited hemagglutination induced by PHA.

It was not until 1959 that Hungerford et al (11) described the mitogenic and blastogenic properties of PHA. They found that this material induced transformation of small lymphocytes in human peripheral blood into large primitive cells, which were capable of undergoing division. The discovery that a large number of cells in mitosis could be obtained by incubating peripheral leukocytes with PHA was of great importance in the field of cytogenetics since it provided a simple and reproducible method for chromosome analysis. These findings stimulated further attempts to separate the various active constituents of PHA. Barkhan and Ballas (12)

showed that the agglutinating activity of PHA could be removed by absorption with red blood cells without a concomitant loss of mitogenic activity, which suggests that the mitogenic and agglutinating activities are possessed by different entities. The presence of at least two distinct mitogens in PHA was suggested by Richter and Naspitz (13) who observed a difference in reactivity of human peripheral lymphocytes to the various PHA preparations tested.

PHA also has the property of agglutinating white blood cells in vitro. The addition of PHA to cultures of leukocytes results in many clumps of cells by 72 hours of culture. This leukoagglutinating property can be removed by absorption with white blood cells. Borberg et al (14) demonstrated that N-acetyl-D-galactosamine inhibited leukoagglutination by PHA without affecting the blastogenic and mitogenic activities of PHA. The blastogenic and mitogenic properties of PHA are the most important ones because they provide a method for the in vitro study of cellular enlargement and division, and demonstrate that the small lymphocyte is not a "dead end" cell.

A discussion of the effect of PHA on the carbohydrate metabolism of lymphocytes is appropriate. Quaglino and Cowling (15) and Gough and Elves (16) showed by the PAS-reaction that there is a marked accumulation of glycogen in the small lymphocyte and intermediate cells after 24 hours of culture with PHA. The content of this substance decreased in the later stages of transformation, and only rarely were

blast cells found which contained blocks of PAS positive material. This suggests that the cells obtain some of their energy for transformation from glycolysis. This interpretation is supported by the findings of Cooper *et al* (17) who found a high rate of lactate production in PHA stimulated lymphocytes which indicated a high rate of glycolysis. In addition, Pachman (18) observed an increase in glucose and oxygen consumption and lactic acid production during 24 hours of culture of equine peripheral lymphocytes with PHA. (See Chapter 4 for discussion of role of hexose monophosphate shunt in lymphocytes.)

The metabolism of PHA in *in vitro* leukocyte cultures with respect to the fate of the mitogen during the three day blastogenic response was investigated by Naspitz and Richter (19). They demonstrated that incubation of the leukocytes with PHA for only 5 minutes followed by incubation for 72 hours in medium devoid of PHA was sufficient to initiate mitosis and blastogenesis. However, the minimal incubation time with PHA required to induce maximum blastogenesis was 6 hours. It was also demonstrated that the mitogen is bound to cells in culture and the findings suggest that this cell-bound mitogen is not degraded during the three day incubation period.

There is still much debate about the mechanism whereby PHA induces blastogenesis and mitosis on normal lymphocytes in tissue culture despite a large number of studies in this area. Immunological and nonimmunological bases for PHA

action have been proposed.

Evidence in favor of an immunologic basis of PHA action is as follows. The morphology of the blast cells obtained after PHA stimulation is very similar, both by light (20, 21, 22, 23) and by electon microscopy (24, 25, 26, 27), to that of blast cells induced as a consequence of incubation with specific antigens. Moreover, blast cells induced by PHA are capable of synthesizing gamma-globulin (28, 29, 30, 31), similar to cells stimulated by antigen *in vitro*. In several human diseased states, a good correlation between impaired delayed hypersensitivity *in vivo* and depressed *in vitro* transformation of lymphocytes with PHA was found (32, 33), which suggests that PHA is measuring the immunocompetence of the lymphocytes.

The evidence for the non-immunologic action of PHA is as follows. In PHA stimulated cultures, the percentage of transformed cells is 70-90% after three days of incubation, whereas in antigen stimulated cultures, only 5-30% of the cells are transformed after 7-10 days of culture (34). No transformation has been observed after only 3 days of culture with specific antigen. Furthermore, the *in vitro* transformation with PHA does not require the presence of macrophages (35, 36), whereas they are necessary for the antigen-induced blastogenic response (37, 38, 39). In addition to lymphocytes, PHA can stimulate epithelial cells in the skin (40), free-living amoeba (41), and non-lymphoid lines (42).

Tubercle bacillus antigen

Koch observed in 1890 that viable tubercle bacilli incubated subcutaneously into guinea pigs evoke a much more intense inflammatory reaction in previously infected than in uninfected animals. Since a similar result was obtained with killed tubercle bacilli, he was led to examine bacterial extracts and found that filtrates of cultures of Mycobacterium tuberculosis were also effective. Injected in non-toxic amounts, they elicited after many hours an inflammatory reaction in tuberculous but not in normal animals. After concentration by boiling and removal of bacterial debris, the culture filtrate was called tuberculin.(43).

Old tuberculin (OT) today is prepared by autoclaving or boiling a culture of tubercle bacilli, concentrating it 10-fold on a steam bath, filtering off the debris, and adding glycerol as a preservative. In this impure product, the active constituent is a protein remarkable for its heat stability. After being autoclaved, it remains soluble and retains specific determinants of the protein in the infecting bacilli. Stock solutions retain full potency for years when stored at 5°C.(44).

A slightly more refined tuberculin, called "purified protein derivative (PPD), is now in use. It is prepared by growing M. tuberculosis in a simple synthetic medium, autoclaving, removing debris by filtration, concentrating the filtrate by ultrafiltration, and precipitating several times with 50% saturated ammonium sulfate. The product is a mix-

ture of small proteins (average M.W. 10,000), together with traces of nucleic acid and polysaccharides (45).

The lipids and waxes in the tubercle bacillus were felt by Raffel and Forney (46) to constitute the adjuvant. According to Raffel et al (47), it is bacause of the presence of such adjuvants that tuberculous guinea pigs develop a delayed type as well as the immediate type of hypersensitivity following sensitization with egg white, whereas nontuberculous guinea pigs, similarly injected with egg white, develop only the anaphylactic type of reaction. This "immediate" reaction is accompanied by a whealing type of skin reaction to egg white. As demonstrated by Dienes (48), if the guinea pig receives antigen like egg white in combination with paraffin oil and killed acid-fast bacilli (complete adjuvant), delayed hypersensitivity develops and the skin reaction to egg white is of the delayed type. The method of administration of antigen bears an important role. The delayed tuberculin response is obtained when the antigen-adjuvant mixture is given intramuscularly, intraperitoneally, or intradermally but not intravenously (49).

Lymphocytes play an important role in mediating the delayed tuberculin response. Chase (50) demonstrated that tuberculin hypersensitivity cannot be transferred from a sensitized guinea pig to a normal guinea pig skin by means of the host's serum, but that it is possible to transfer tuberculin sensitivity with cells from hypersensitive guinea pigs to normal guinea pigs. Lymphocytes are involved in this

type of transfer. Sensitivity may be transferred with cells obtained from the lymph nodes or spleen of the sensitized donor (51). The cellular passive transfer of tuberculin sensitivity can be demonstrated when labeled cells are used. A number of these cells appear at the local site of the PPD reaction, although according to some, they are in the minority since the host also supplies similar cells (52). This type of transferred delayed sensitivity in man lasts for one or two years. The degree of sensitivity thus transferred is determined by the ssnsitivity of the donor and the amount of cells transferred.(53).

The nature of the factor which is transferred by cells is not completely known. The transfer factor which resides in the lymphocyte is not to be found, at least in man (54), in the DNA or RNA in the cells. The evidence suggests that the transferred factor in not antigen, because sensitization develops too quickly for it to be due to antigenic stimulation, nor is it antibody for no antibody is detectable in cell extracts (55). Jeter et al (56) have analyzed leukocyte extracts from guinea pigs hypersensitive to tuberculin. They suggest that this factor is most likely a dialyzable non-protein substance, that has a molecular weight of about 10,000 and resists RNAase treatment. It may be lyophilized without undergoing any change. Additional transfer of tuberculin sensitivity can be made with cells obtained from recipients previously sensitized by cell transfer (57). However, such secondary transfer is usually not very strong. The

addition in vitro of tuberculin to leukocytes obtained from a PPD positive donor yields the transfer factor in the supernate (58). This factor may be separated from viable human cells and still be active on transfer.

As stated previously (see Phytohemagglutinin), in 1960, PHA was shown to stimulate both mitoses and formation of blast-like cells in cultures of normal human lymphocytes. Soon after this observation was made, it was found that lymphocytes could also be stimulated to divide by antigens to which the individual was sensitive. Pearmain et al (59) substituted tuberculin for PHA in peripheral blood leukocyte culture from persons sensitized to tuberculin, and observed a marked phase of mitosis. Schrek (60) found that PHA and PPD produced similar effects on human lymphocytes. Both reagents stimulated mitotic activity and the formation of large blast-like cells. Coulson and Chalmers (61) also demonstrated blastogenesis when PPD was incubated with a highly purified suspension of small lymphocytes obtained from PPD positive patients.

Schrek and Stefani (62) reported that only 10% of the cells in cultures of human peripheral lymphocytes survived six days after 75 r irradiation. On the other hand, PHA-treated suspensions irradiated with 75 to 2400 r survived. This protection to x-irradiation afforded by PHA in vitro appeared at first to be a unique property not possessed by antigens in general. However, it was later noted by Stefani (63) that old tuberculin is also capable of imparting radio-

protection when incubated with lymphocytes obtained from PPD positive patients but not when incubated with cells of PPD negative patients. He observed a relation between old tuberculin concentration, blastoid transformation, and radioprotection, and postulated that the radioprotective effect of old tuberculin had an immunologic basis.

Rich and Lewis (64) were the first to describe that cells from spleens and lymph nodes of tuberculin sensitive animals showed poor migration in the presince of tuberculin. Later, George and Vaughan (65) studied the migration of guinea pig peritoneal exudate cells out of capillary tubes and showed that the inhibition of migration by tuberculin or egg albumin paralleled delayed skin reactivity to these antigens. Using this capillary tube technique, David et al (66) showed that as few as 2.5% of peritoneal cells from sensitive guinea pigs mixed with normal cells caused inhibition of the whole population and that the sensitive cells must be viable. Inhibition does not occur if the protein synthesis of the cells is blocked by puromycin (67). Peritoneal exudate consists mainly of macrophages with a minority of lymphocytes and polymorphonuclear leukocytes. Bloom and Bennett (68) removed the lymphocytes by tissue culture methods and showed that the remaining cells were no longer inhibited by antigen, but that the re-addition of even a small amount (0.6%) of peritoneal lymphocytes from sensitive animals resulted in migration inhibition. It was found that sensitive lymphocytes interacting with specific antigen produce a sol-

uble, nondialyzable material that mediates the inhibition of macrophage migration (69, 70). Kostiala (71) found that sensitivity to tuberculoprotein in guinea pigs sensitized with killed tubercle bacilli in adjuvant lasts at least one year as determined by peritoneal cell migration inhibition.

Lysozyme

Lysozyme was first described in 1922 by Sir Alexander Fleming (72). He also discovered a gram positive coccus, Micrococcus lysodeikticus, which was extremely sensitive to enzymatic lysis, and used it to develop an assay for lysozyme. Since then, many substances (73, 74, 75) capable of lysing Micrococcus lysodeikticus have been included under the name "lysozyme". In general, a lysozyme is a low molecular weight (about 15,000) basic protein which is stable at acid pH but unstable at alkaline pH. It can resist boiling in glacial acetic acid for several minutes. Lysis of Micrococcus lysodeikticus must yield the appropriate degradation products which can be detected by reagents for reducing sugars and N-acetyl-hexosamines which are derived from glucosamine and muramic acid. Highly purified lysozyme was first prepared by Alderton (76) and Fevold (77) who adsorbed lysozyme on Bentonite, washed it with buffered phosphate and pyridine solutions to remove other proteins, and removed it from the Bentonite in acid pyridine solution. Osserman and Lawler (78) applied this technique to lysozyme of human origin.

The relationship between leukocytes and lysozyme was

also first observed by Fleming (79) when he noted that infected urine and pus from abscesses contained large amounts of lysozyme. Flanagan and Lionetti (80) were the first to present quantitative data on the distribution of lyaozyme in plasma and the formed elements of the blood. They found no significant lysozyme activity in red blood cells or platelets. Although saline washings of leukocytes had very little enzymatic activity, several times as much enzyme was released when these cells were frozen and thawed. However, no activity was found in lymphocytes alone. Finch et al (81) found no strong relationship between human serum lysozyme and total white blood count, but did observe a significant correlation between lysozyme and granulocyte count. No conclusive relationship existed between the fifty highest and the fifty lowest absolute monocyte counts and the serum lysozyme. Males had slightly higher serum lysozyme than females and a gradual increase in serum lysozyme was observed with increasing age.

Cohn and Hirsch (82) used ultracentrifugation to separate and analyze the subcellular elements of rabbit polymorphonuclear leukocytes. They found that 50% of lysozyme was found in the formed cytoplasmic granules and that the other 50% was unbound and free in the cytoplasm. By preparing an antibody to rat kidney lysozyme and tagging it with a fluorescent label, Glynn and Parkman (83, 84) observed that the label was about equally distributed between granules and cytoplasm in rat polymorphonuclear leukocyte granules. When

heat-killed E. coli were added to these granulocytes, the granules were observed to coalesce into large bright-staining areas. Parkman (85) labelled the bacteria and by double fluorescent studies concluded that the lysozyme was surrounding the bacteria within cytoplasmic vacuoles.

Work has been done to correlate lysozyme with leukocyte turnover. Finch and associates (86, 87, 88) studied serum lysozyme in various hematologic disorders. They found elevated levels in acute monocytic leukemia, acute myelomonocytic leukemia, and acute and chronic granulocytic leukemias. However, there were usually lower than average values in acute and chronic lymphocytic leukemias. Elevated lysozyme levels were also found in polycythemia vera, myeloid metaplasia, and granulocytosis (infection). Below normal values were found in agranulocytosis and aplastic and hemolytic anemias. The conclusion was made that a valid positive relationship existed between serum lysozyme and granulocyte turnover rate.

Different investigators have used various methods for altering intracellular and serum lysozyme in the experimental situation. Myrvik (89) demonstrated that the amount of lysozyme present in alveolar macrophages of rabbits could be greatly increased by causing granulomatous lung disease induced by BCG innoculation. Cohn and Weiner (90, 91) found increases in all hydrolytic lysosomal enzymes studied following BCG stimulation. Carson and Donnenberg (92) hypo-

thesized that lysozyme might work synergistically with other agents in tuberculous infection. However, although they observed increased lysozyme levels in macrophages of animals treated with heat-killed Mycobacterium tuberculosis, they could not demonstrate changes in protease or lipase content.

It has been found that various substances injected into experimental animals will disrupt leukocytes with consequent release of lysozyme. Kerby (93, 94) measured increases in lysozyme as a function of leukocyte injury by various bacterial derivatives. Ribble (95, 96) followed increases in plasma lysozyme activity after injecting rabbits with typhoid vaccine. He found that lysozyme activity increased 200% within two hours after injection, during which time the rabbit became leukopenic. The increase in serum lysozyme was directly proportionate to the degree of leukopenia. However, if the animals had been pretreated with nitrogen mustard, there was no rise in serum lysozyme after typhoid vaccine was administered. Similarly, there was no elevation if the animals were made tolerant to the vaccine.

Changes in serum lysozyme, properidin, and white blood counts after treatment of rats with x-irradiation, zymosan and endotoxin were studied by Hook et al (97). After 600 to 800 r of whole body x-irradiation, serum lysozyme fell to 20% of normal values. Serum lysozyme levels fell rapidly and remained low for 24 hours after the intraperitoneal injection of zymosan. With endotoxin, there was

a slight initial rise in serum lysozyme, followed by a fall to subnormal levels for the next 48 hours. It was concluded that lysozyme was a good index of cellular injury to white blood cells caused by irradiation and endotoxin.

A guinea pig antiserum to rabbit leukocyte lysosomes was prepared by Quie and Hirsch (98). When this was added to intact granulocytes, approximately 80% of the enzymes, including lysozyme, which were experimentally obtainable by other methods, were released.

Glucose metabolism through the hexose monophosphate shunt

The discovery by Warburg (99) in 1931 of the oxidation of glucose-6-P to 6-phosphogluconate established the existence of a new reaction in glucose metabolism which differed from the Embden-Meyerhof (EM) pathway. In the following decade, the studies of Warburg, Lipmann, Dickens, Dische and others demonstrated that phospho-gluconate is decarboxylated to CO_2 and a pentose phosphate, which is then reconverted to a hexose phosphate. The detailed mechanism of this "hexose monophosphate shunt" (HMS) was not elucidated until much later, between 1950 and 1955, by Cohen, Racker, Horecker and their coworkers (100).

The HMS encompasses a series of reactions that include a number of novel transformations and compounds as well as several of the intermediates and enzymes encountered in glycolysis. It is in effect a multicyclic process whereby 3 molecules of glucose-6-phosphate give rise to 3 molecules of CO_2 and 3 five-carbon residues. The latter are rearranged

to regenerate 2 molecules of glucose-6-phosphate and 1 molecule of glyceraldehyde-3-phosphate. Since two molecules of glyceraldehyde-3-phosphate can regenerate a molecule of glucose-6-phosphate by reactions which are essentially a reversal of glycolysis, the pathway can account for the complete oxidation of glucose independent of the citric acid cycle. As in the E-M glycolysis pathway, oxidation is achieved by dehydrogenation. However, in the case of the shunt pathway, NADP and not NAD is used as a hydrogen acceptor. It is, therefore, the important generator of NADPH necessary for synthesis of fatty acids and the functioning of various hydroxylases. It also serves as a source of D-ribose as well as of 4-carbon and 7-carbon sugars. Commencing with glucose-6-phosphate, there is no further requirement for ATP. The enzymes of the shunt pathway are found in the extramitochondrial soluble portion of the cell (101).

It is clear that the HMS is markedly different from the E-M pathway of glycolysis. Oxidation occurs in the first reactions, and CO_2, which is not produced at all in the E-M pathway itself, is a characteristic product. This fact has been utilized in experiments designed to evaluate the relative proportions of glucose metabolized by the E-M pathway compared with the shunt pathway. In 1953, Bloom et al (102, 103) initiated the first use of specifically ^{14}C-labeled glucose to measure the relative amount of glucose metabolized by the HMS and the E-M pathway. They measured

the yield of ^{14}C in CO_2 produced from glucose-1-^{14}C and glucose-6-^{14}C by tissue slices prepared from different organs in rats. In the glycolytic pathway, carbons 1 and 6 of glucose are both converted to the methyl carbon of pyruvic acid and are, therefore, metabolized in the same manner. In the HMS, carbons 1 and 6 of glucose are treated differently, carbon 1 being removed early by decarboxylation with the formation of labeled CO_2. With liver, Bloom et al observed that there was a much greater yield of $^{14}CO_2$ from glucose-1-^{14}C than from glucose-6-^{14}C, and they concluded that in liver the pentose pathway accounted for at least 60% of the CO_2 formed from glucose. This finding aroused much interest because up to that time it had been considered that the majority of carbohydrate metabolism occurred via the E-M pathway and Krebs cycle. Shortly thereafter, investigators in the laboratories of Weinhouse, Chaikoff, Cheldelin and Wang published similar studies and the use of the yield of ^{14}C in fatty acids and other products was soon introduced as a means of measurement (104, 105, 106, 107).

In mammalian striated muscle, there appears to be no glucose oxidation via the HMS, and catabolism proceeds entirely via glycolysis and the citric acid cycle. However, the shunt is active in adipose tissue, adrenal cortex, thyroid, testis, lactating mammary gland, and leukocytes (see Discussion for activity of HMS in leukocytes). The special advantages that accrue to the organism by virtue of the HMS, other than pentose formation for nucleotide synthesis, appear to derive

from the fact that no additional ATP is required, there is no dependence upon the availability of the 4-carbon dicarboxylic acids of the citric acid cycle, and, probably most significant, this pathway employs NADP as the exclusive electron acceptor. It is not clear whether ATP can be efficiently formed by mitochondrial oxidation of NADPH produced in the cytoplasm, but to the extent that this does occur, it would permit generation of 3 moles of ATP per TPNH formed or 36 moles for the total combustion of glucose. This value compares favorably with the yield of ATP from glycolysis and the citric acid cycle. However, it appears likely that most of the NADPH generated by the HMS is employed as a reducing agent, particularly in the synthesis of fatty acids and steroids. This is consonant with the distribution of the enzymes concerned with these processes. Liver, lactating mammary gland, testis, and adrenal cortex are active sites of fatty acid and/or steroid synthesis, whereas these processes are not prominent in the metabolism of striated muscle tissue in which the HMS is not known to occur. In those cells in which HMS oxidation is possible, it appears that the rate-limiting factor is the availability of NADP, thus indicating a coupling of glucose oxidation with the requirements of NADPH-utilizing reactions (108).

CHAPTER II

MATERIALS AND METHODS

1. General

Erlenmeyer flasks and syringes were siliconized when total leukocytes were being obtained, but not when only lymphocytes were desired.

2. Subjects

All experiments were performed on minimally heparinized fresh human venous blood (4 U.S.P. units of heparin[1] per ml of whole blood) obtained from healthy adult volunteers. Subjects were divided into P.P.D. positive or negative groups by presence or absence of induration measuring 10 mm or over at the site of the intracutaneous injection of 0.1 ml of Intermediate Strength, 5 U.S. units (TU), Tuberculin Purified Protein Derivative[2](109,110).Certain individuals were retested with Second Strength, 250 U.S. units (TU) after a negative reaction, and were discounted from either group after a positive reaction, since only strongly positive subjects were desired.

---

[1]Sodium Heparin, 1,000 U.S.P. units per ml, from intestinal mucosa, Upjohn Company.

[2]Connaught Medical Research Laboratories, Willowdale, Ontario, Canada.

3. Old Tuberculin

Old tuberculin (OT) obtained from the Massachusetts Department of Public Health (Lots 50 and 50c)[3] contained 2,000 mg OT per ml and was bottled in pyrogen-free glassware (111, 112). It was diluted with sterile normal saline to 1:20, giving a concentration of 100 mg per ml.

4. Tuberculin Purified Protein Derivative

Second Strength Tuberculin Purified Protein Derivative containing 250 U.S. units (TU) per 0.1 ml or 0.05 mg PPD per ml (as described in Section 2) was used undiluted.

5. Phytohemagglutinin

Bacto-Phytohemagglutinin-P (PHA-P) (5 ml per vial) was obtained from Difco Laboratories, Detroit, Michigan, and was diluted a further 20 times (113) before use.

6. Modified Krebs-Ringer phosphate buffer (KRP)[4]

KRP buffer pH 7.4 was modified to contain one-fifth of the suggested concentrations of $CaCl_2$ and $MgSO_4$ (to prevent precipitation) (114). Four units of heparin were added per ml of buffer.

---

[3]Supplied through the courtesy of Dr. Elisha Atkins, Yale University School of Medicine, New Haven, Connecticut.

[4]Supplied through the courtesy of Dr. Phyllis Bodel, Yale University School of Medicine, New Haven, Connecticut.

7. Heparinized buffered tissue culture medium[5]

Minimum Essential Medium (Eagle)[6] with $NaHCO_3$ (3.5 ml of 5.6% sterile sodium bicarbonate solution for each 100 ml of medium) was stored at 4°C. Four units of heparin were added per ml of medium. Before use, CO_2 was run through the medium for 3 minutes to obtain a pH of approximately 7.4. During experimentation, flasks containing medium were tightly sealed with parafilm.

8. Leukocyte Suspension

Total leukocyte counts from each 50 ml or 100 ml blood sample were done in a "Coulter counter" and 100 leukocytes were differentiated on Wright's stained blood smears made on cover glass. White blood counts (WBC) ranged from 6,000 to 10,000 cells per cu mm with lymphocytes usually making up 25-35 per cent of the total.

Two methods were used to remove red blood cells from whole blood:

a. Sedimentation

Red blood cells were settled by allowing the heparinized whole blood to stand 90 minutes at room temperature. The supernate was then pipetted off using a standard Pasteur pipette.

---

[5]Supplied through the courtesy of Dr. Phyllis Bodel, Yale University School of Medicine, New Haven, Connecticut.

[6]Flow Laboratories, Inc., Rockville, Maryland.

b. Centrifugation

The heparinized blood was placed in centrifuge tubes and spun at (40 X g) for 10 minutes at 4°C. The supernate was then pipetted off as in method a.

WBC and differential were done on the supernate resulting from either method and the supernate was then spun at (600 X g) for 15 minutes. The plasma was removed and respun at (1,000 X g) for 20 minutes. The cell-free supernate was saved for resuspension of the cell button in various experiments (see Experimental Procedures).

9. Separation of lymphocytes by nylon column (115)

The nylon columns[7] were made by preparing Dupont nylon and putting 0.7 g of it into a standard Pasteur pipette. The preparation of the nylon consisted of washing it by weighing 250 mg of nylon in 4 liters of water with 40 ml of Dupinol. It was then rinsed in tap water and heated from 65° to 70° for 30 minutes. It was washed in tap water overnight and rewashed with distilled water 7 times and then air-dried. The dried, washed nylon was then combed with a nylon comb and brushed to remove any lumps or snags. The column held approximately 1.75 ml.

The column was wetted with heparinized buffered tissue

---

[7]Obtained through the courtesy of Dr. Fred Kantor, Yale University School of Medicine.

culture medium (see section 7) from a 3 cc syringe, noting the volume used. The cell button obtained from 100 ml of whole blood was resuspended in 0.2 ml of cell-free autologous plasm (see section 8) and 1.3 ml of heparanized buffered tissue culture medium. This concentrated leukocyte suspension was then slowly injected into the column and the replaced wetting medium was discarded. The column was then allowed to stand 25 minutes at room temperature, after which 2.0 ml of medium were slowly injected into the column while catching the lymphocytes suspended in approximately 0.2 ml of plasm and 1.8 ml of medium. The final differential usually indicated greater than 90% lymphocytes. The average yield of this procedure was 7.5×10^7 lymphocytes from a starting sample of 100 ml of blood.

10. $^{14}CO_2$ assay for measuring glucose-1-^{14}C oxidation

Glucose-1-^{14}C oxidation was measured by assaying $^{14}CO_2$ production according to a modification of the technique described by De Chatelet, Cooper and McCall (116).

Whole blood, leukocyte suspensions or controls to be assayed were prepared in triplicate siliconized 25 ml Erlenmeyer flasks to a total volume of either 4.0 or 6.5 ml as described below (see Experimental Procedures). Included in the total was either 0.1 or 0.2 ml glucose-1-^{14}C[8] (specific

[8]New England Nuclear Corporation, Boston, Massachusetts

activity: 54.2 mCi per mmole. The isotope was dissolved in normal saline to give a concentration of 2 mcCi per ml and stored frozen. Each flask was stoppered and fitted with plastic well-sticks[9] into which 0.2 ml of hydroxide of hyamine was introduced with a syringe and needle. The flasks were then incubated in a shaking water bath at 37° C for a specified length of time (see Experimental Procedures). At the end of this period, 1.0 ml of 5 N H_2SO_4 was added by syringe and needle to the contents of the flask to stop the reaction and release $^{14}CO_2$. Gentle agitation at 37° C was then resumed for an additional 30 minutes in order to trap $^{14}CO_2$ in the hyamine. Radioassay of the $^{14}CO_2$ in hyamine was done in a liquid scintillation counter using Bray's solution as a counting fluid. Counting was done for 10 min and the values obtained were divided by 10 to give counts per min. Results were expressed as the average of the counts of the three flasks.

11. Lysozyme assay

Plasma lysozyme activity was measured by the turbidimetric method of Smolelin and Hartnell (117) with the modification of Litwack (118). The lysozyme activity of an aliquot of frozen serum from a single normal donor along with that of a known concentration of hen-egg white lysozyme were

---

[9]Kontes Glass Company, Fireland, New Jersey.

used to standardize and control each series of unknown determinations. Values were expressed as micrograms (mcg) of egg-white lysozyme activity per ml of plasma.

12. Experimental Procedures

Five groups of experiments were performed. They are each given a Roman numeral which they will retain through other sections for easier cross-referencing.

I In order to compare the glucose-1-^{14}C oxidation of isolated leukocytes from PPD positive subjects and that of leukocytes from PPD negative subjects after incubation with OT for 2 hours at 37° C, triplicate 25 ml Erlenmeyer flasks were prepared according to Table 1.

The cell buttons obtained from 50 ml of whole blood from a PPD positive and a PPD negative subject were resuspended to 4 X 10^7 leukocytes per 0.5 ml of autologous plasma (see section 8). Glucose-1-^{14}C oxidation was determined by $^{14}CO_2$ assay (see section 10). Four positive and four negative subjects were used.

Table 1 on following page.

Table 1

Protocol for Glucose-1-^{14}C Oxidation Study
in Comparison Between
Leukocytes from PPD Positive and Negative Subjects
After Incubation with OT for 2 Hours

Components	PPD Positive Subject	PPD Negative Subject	Control
Number of Leukocytes	4 X 10^7	4 X 10^7	---
Plasma	0.5 ml	0.5 ml	---
Old Tuberculin (1:20; 100 mg/ml)	0.5 ml	0.5 ml	0.5 ml
Heparinized KRP buffer	2.9 ml	2.9 ml	3.4 ml
Glucose-1-^{14}C (0.2 mcCi/0.1 ml)	0.1 ml	0.1 ml	0.1 ml
Total	4.0 ml	4.0 ml	4.0 ml

II In order to measure glucose-1-^{14}C oxidation of whole blood from 4 PPD positive and 4 PPD negative subjects after incubation for 2 hours with OT, PPD, or saline, triplicate 25 ml Erlenmeyer flasks, again containing a total volume of 4.0 ml, were prepared.

Twelve flasks, each containing 0.2 ml of glucose-1-^{14}C and 3.3 ml of whole blood from a PPD positive subject and a duplicate group of flasks with blood

from a PPD negative subject were allowed to incubate as previously described (see Section 10) for 1 hour. At the end of this period, 1.0 ml of 5 N H_2SO_4 was injected into a set of 3 flasks from each group in order to stop the reaction. The remaining sets of flasks from each group received 0.5 ml of OT (1:20 or 100 mg per ml), 0.5 ml of PPD (1:1 or 0.05 mg per ml), or 0.5 ml of N saline and were allowed to incubate for an additional 2 hours. At this time, the remaining reactions were stopped and $^{14}CO_2$ was assayed.

III Lysozyme levels were used as an indicator of leukocyte stimulation after simultaneous incubation of whole blood from a PPD positive and a PPD negative subject with OT for 1, 3, 5, 8, and 24 hours.

Five ml of whole blood from a PPD positive subject and 0.5 ml of OT (1:20 or 100 mg per ml) were added to each of fifteen 25 ml Erlenmeyer flasks. A duplicate group of flasks containing blood from a PPD negative subject was simultaneously tested. Triplicate sets from each group were allowed to incubate in a shaking water bath at 37° C for 1, 3, 5, 8, and 24 hours. Triplicate baseline flasks containing 5 ml of whole blood and 0.5 ml of N saline were prepared for each group. At the end of each

period, the contents of the flasks were centrifuged at (1,000 X g) for 20 min, and the supernatant cell-free plasma was assayed for lysozyme (see section 11). Assays were also performed on the baseline plasmas. Six different subjects were used.

TV $^{14}CO_2$ assay was done on whole blood from PPD positive subjects after incubation for 2 hours with lymphocytes stimulated with OT, with unstimulated lymphocytes, or with OT and medium without lymphocytes. The experiment consistes of two parts.

STEP 1

The cell button obtained by centrifugation (see section 8) of 100 ml of whole blood from a PPD positive subject was resuspended in 0.2 ml of cell-free autologous plasma and 1.3 ml of heparinized buffered tissue culture medium for separation of lymphocytes by nylon column (see Section 9). The resulting suspension of lymphocytes in 0.2 ml of plasma and 1.8 ml of medium was added to 4.8 ml of plasma and 13.2 ml of medium. Ten ml of this final suspension were placed in flask "A" to which 0.5 ml of OT (1:20 or 100 mg per ml) was added. The remaining 10 ml were placed in flask "B" to which 0.5 ml of N saline was added. Flask "C" was prepared by adding 0.5 ml of OT to 2.5 ml of plasma and 7.5 ml of medium (without lymphocytes). After running CO_2

through them for 3 min and sealing them tightly with parafilm (see section 7), the 3 flasks were incubated in a shaking water bath at 37°C for 24 hours.

Step 2

At the end of this period, 50 ml of blood were obtained from the same donor as in Step 1 and triplicate 25 ml Erlenmeyer flasks were prepared according to Table 2 for $^{14}CO_2$ assay after 2 hours of incubation (see section 10). Three positive subjects were used.

Table 2

Protocol for Glucose-1-^{14}C Oxidation Study of Whole Blood after Incubation for 2 Hours with OT-stimulated and Unstimulated Lymphocytes and Control

Components	Set 1	Set 2	Set 3
Whole blood from same donor as in step 1	3.3 ml	3.3 ml	3.3 ml
Flask "A"	3.0 ml	------	------
Flask "B"	------	3.0 ml	------
Flask "C"	------	------	3.0 ml
Glucose-1-^{14}C in normal saline (0.2 mcCi/0.1 ml)	0.2 ml	0.2 ml	0.2 ml
Total	6.5 ml	6.5 ml	6.5 ml

V $^{14}CO_2$ assay was again performed on whole blood from PPD positive subjects. However, this time, the 2 hour incubation was carried out with lymphocytes stimulated with PHA, with unstimulated lymphocytes, or with PHA and medium without lymphocytes.

A procedure identical to that in IV was used except that 1.0 ml of phytohemagglutinin was added to flasks "A" and "C" instead of 0.5 ml of OT, and 1.0 ml instead of 0.5 ml of N saline was added to flask "B". Four positive subjects were used.

CHAPTER III

RESULTS

The design and results of experiments are summarized in tabular form. In each case, results are expressed as the average of triplicate flasks. In experiment I, independent t-test analysis was done. In experiments II, III, IV, and V, where the subject served as his own control, the data were processed by paired t-test analysis.

Experiment I

A comparison was made between the glucose-1-^{14}C oxidation of leukocytes from PPD positive subjects and that of leukocytes from PPD negative subjects after incubation with OT for 2 hours. Table 3 indicates the results of 4 separate experiments. Because the leukocyte concentration was adjusted in each case to 4×10^7 cells per ml, the PPD positive group could be compared directly to the PPD negative group. The average counts per min were 21,695 $\pm$ 1,140 (mean $\pm$ S.E.M.) for the PPD positive group and 20,961 $\pm$ 8,900 for the PPD negative group. No significant difference could be found between the two groups (p greater than 0.5).

Table 3

Comparison between Glucose-1-^{14}C Oxidation of Leukocytes from PPD Positive and Negative Subjects after Incubation with OT for 2 Hours

	(A) PPD Positive (Counts/min)	(B) PPD Negative (Counts/min)	(C) Control (Counts/min)
	22,871	19,393	188
	19,980	22,425	190
	19,592	22,574	109
	24,337	19,450	188
Mean ± S.E.M.	21,695 ± 1,140	20,961 ± 8,900	

Comparison of (A) and (B) $t = 0.5062$
$p > 0.5$

Experiment II

Measurements were made of glucose-1-^{14}C oxidation of whole blood from PPD positive and PPD negative subjects after incubation for 2 hours with OT, PPD or saline. The results of 4 tests are indicated in Table 4. The number of counts in the 2 hours of incubation with antigens or saline was determined by subtracting the number of counts in the reaction without antigens or saline stopped at one hour from the counts obtained in the reactions stopped at three hours, the first hour being without and the next two hours being with antigens or saline (see Experimental Procedures II). Since whole blood was used, the number of leukocytes naturally varied in each sample and average counts per min would be meaningless. Each person, therefore, served as his own control and analysis of data was done by paired t-test.

Table 4

Glucose-1-^{14}C Oxidation of Whole Blood

from PPD Positive and PPD Negative Subjects

after Incubation for 2 Hours with OT, PPD or Saline

PPD Positive Group

Subject	(A) Saline (Counts/min)	(B) PPD (Counts/min)	(C) OT (Counts/min)
1	2,228	2,111	2,196
2	4,126	4,559	5,210
3	2,358	2,517	2,891
4	3,023	3,190	3,245

Comparison of (A) and (B) $t = 1.8327$, $p > 0.1$

Comparison of (A) and (C) $t = 0.1015$, $p > 0.3$

PPD Negative Group

	(D) Saline (Counts/min)	(E) PPD (Counts/min)	(F) OT (Counts/min)
1	4,482	3,693	3,249
2	2,676	1,984	3,334
3	2,881	3,532	3,541
4	4,479	3,768	3,927

Comparison of (D) and (E) $t = 1.1211$, $p > 0.3$

Comparison of (D) and (F) $t = 0.2231$, $p > 0.8$

No significant difference could be found in the PPD positive group between the flasks with OT and those with saline ($p>0.1$), nor between those with PPD and those with saline ($p>0.3$). The same was true for the PPD negative group, where no significant difference existed between the flasks with OT and those with saline ($p>0.3$), nor between the flasks with PPD and those with saline ($p>0.8$).

Experiment III

Lysozyme production was used as an indicator of leukocyte stimulation after simultaneous incubation of whole blood from a PPD positive and a PPD negative subject with OT for 1, 3, 5, 8 and 24 hours. Results are given in Table 5. Again subjects served as their own controls by comparing results at different lengths of incubation with baseline values. The per cent increase of lysozyme production was only determined for results with statistical significance by dividing the incubation value in excess of the baseline value and multiplying by 100. No significant difference was found in the PPD negative group at any incubation time: $p>0.9$ at 1 hour, $p>0.4$ at 3 hours, $p>0.6$ at 5 hours, $p>0.2$ at 8 hours, $p>0.1$ at 24 hours. However, in the positive group, statistical significance was not seen at 1 hour ($p>0.1$), but was observed starting at 3 hours where per cent increase was $1 \pm 0.9\%$ (mean $\pm$ S.E.M.) ($p<0.05$). Per cent increase was $10 \pm 4\%$ ($p<0.02$) at 5 hours, $19 \pm 5\%$ ($p<0.02$) at 8 hours, and $112 \pm 32\%$ ($p<0.02$) at 24 hours.

TABLE 5

Lysozyme Production of Whole Blood

From PPD Positive and Negative Subjects

After Incubation with OT

POSITIVE GROUP

Subject	Base line	1 h *	3 h *	% Incr.	5 h *	% Incr.	8 h *	% Incr.	24 h *	% Incr.
1	15.5	15.7	16.0	3	16.2	5	17.0	10	24.0	61
2	3.8	4.3	4.3	1	4.5	18	4.8	26	10.4	174
3	6.5	6.3	6.5	0	7.0	8	7.8	20	16.8	100
Mean±S.E.M.			1±.9		10±4		19±5		112±32	
Comparison with Baseline		t=2.907 p>0.1	t=5.875 p<0.05		t=8.873 p<0.02		t=8.814 p<0.02		t=7.788 p<0.02	

NEGATIVE GROUP

Subject	Base line	1 h *	3 h *	5 h *	8 h *	24 h *
1	8.2	8.5	8.8	9.3	9.5	10.0
2	6.8	6.8	7.3	7.6	7.8	7.5
3	11.8	11.5	12.0	12.3	11.8	12.5
Comparison with Baseline		t=0 p>0.9	t=1.057 p>0.4	t=.460 p>0.6	t=1.924 p>0.2	t=2.483 p>0.1

* mcg/ml

Figures 1, 2, and 3 depict the graphs of the results of the three individual experiments. In figure 1, there was clearly a rise in the curve between 8 and 24 hours for the PPD positive subject, while the curve of the PPD negative subject remained fairly flat. In Figure 2, although the baseline value for the PPD positive subject was lower than that of the negative subject,

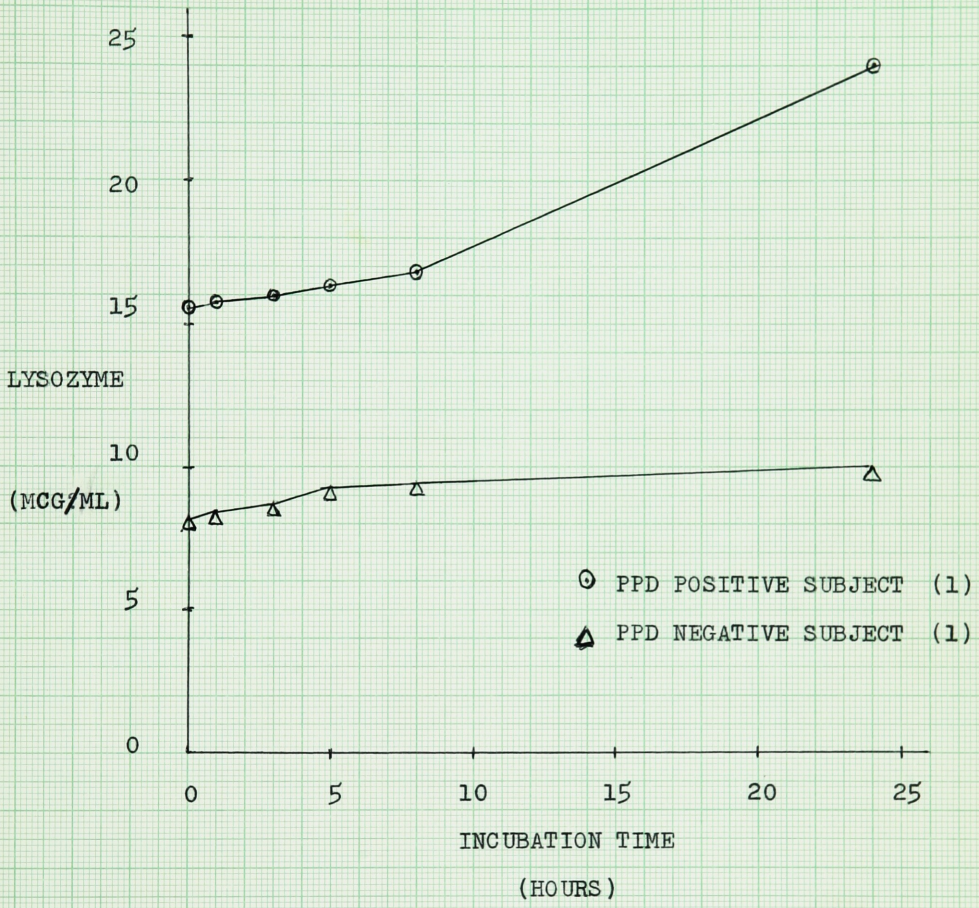

Figure 1: Comparison of lysozyme production of whole blood from a PPD positive and a PPD negative subject after incubation with OT.

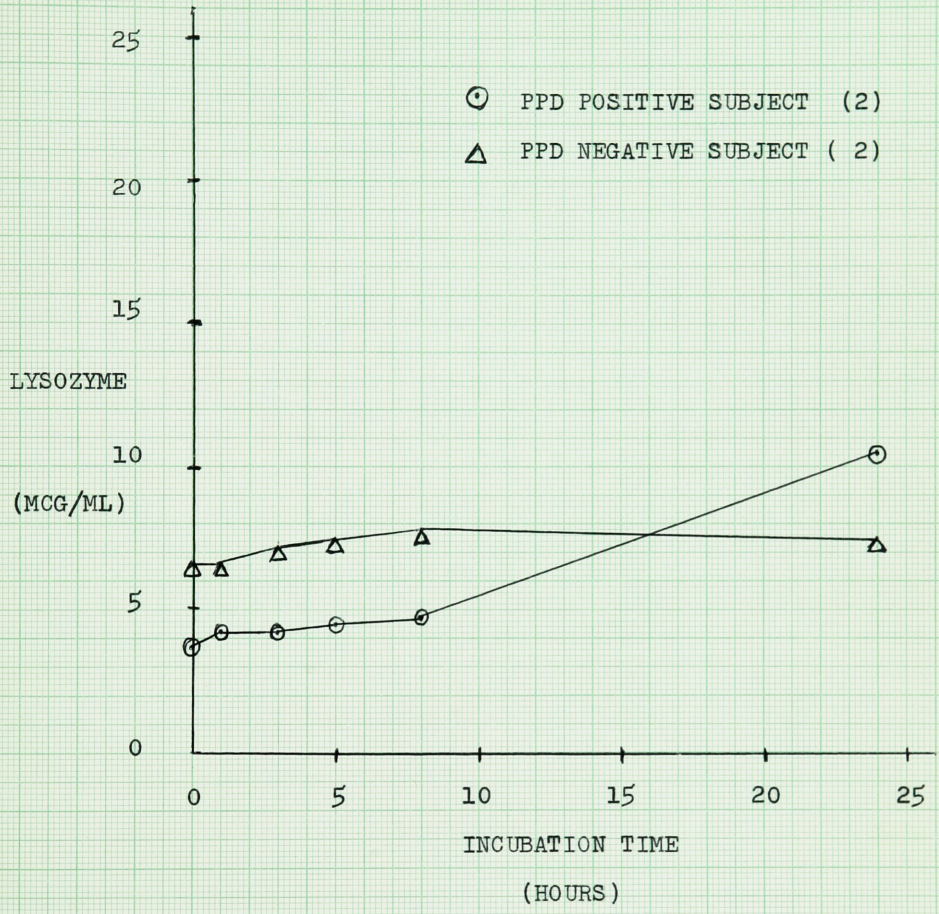

Figure 2: Comparison of lysozyme production of whole blood from a PPD positive and a PPD negative subject after incubation with OT.

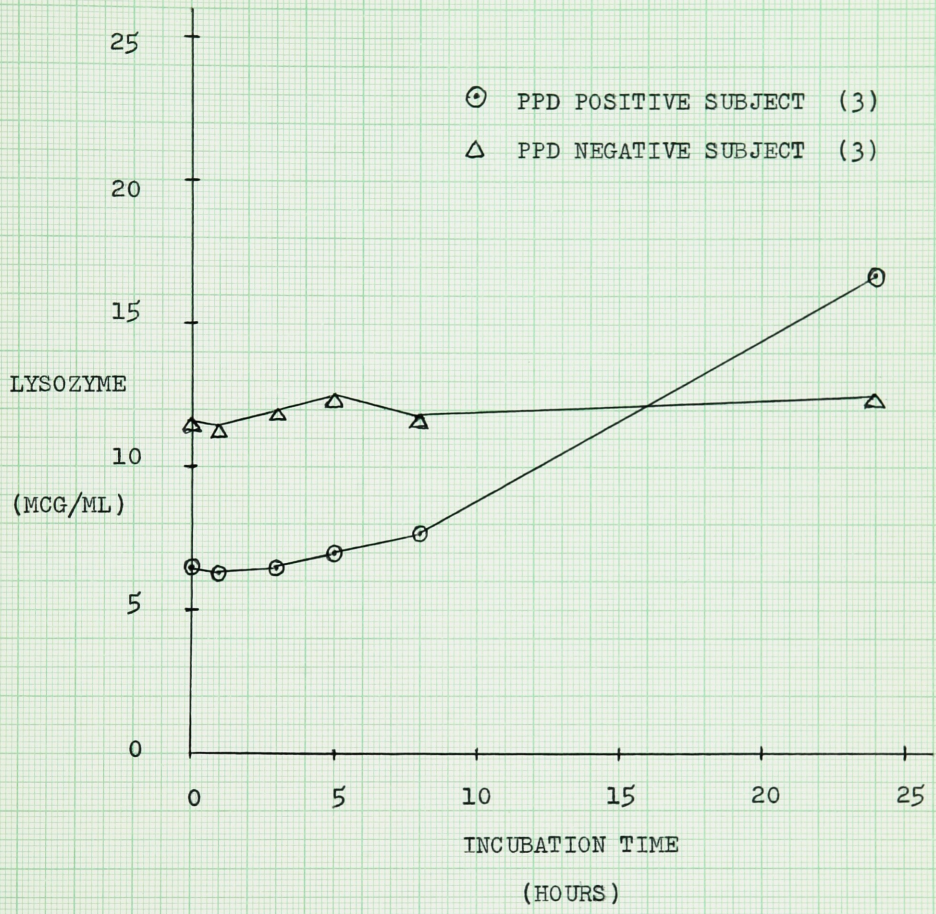

Figure 3: Comparison of lysozyme production of whole blood from a PPD positive and a PPD negative subject after incubation with OT.

by the end of 24 hours the amount of lysozyme was greater in the PPD positive sample. This again occurred in the experiment depicted in Figure 3.

Experiment IV

$^{14}CO_2$ assay was done on whole blood from PPD positive subjects after incubation for 2 hours with lymphocytes stimulated with OT, with unstimulated lymphocytes, or with OT and medium without lymphocytes. The results of these tests are included in Table 6.

Table 6

Glucose-1-^{14}C Oxidation of Whole Blood after Incubation for 2 Hours with OT-stimulated and Unstimulated Lymphocytes and Control

Subject	(A) Stimulated (Counts/min)	(B) Unstim. (Counts/min)	% Incr.	(C) Control (Counts/min)	% Incr.
1	10,528	2,029	419	1,501	601
2	16,280	6,293	159	5,572	192
3	13,179	2,211	489	2,372	462
Mean ± S.E.M.			356 ± 101		418 ± 121

Comparison between (A) and (B) $t = 13.712$, $p < 0.01$

Comparison between (A) and (C) $t = 17.553$, $p < 0.01$

Comparison between (B) and (C) $t = 1.349$, $p > 0.1$

The per cent increases of $^{14}CO_2$ production were obtained in each case by dividing the stimulated value in excess of the unstimulated value and in excess of the control value by the unstimulated value and the control value respectively, and

multiplying by 100. No significant difference was found between the unstimulated values and the control values ($p>0.1$). However, the blood incubated with stimulated lymphocytes showed an increase of 356 ± 101% ($p<0.01$) in glucose-1-^{14}C oxidation above the blood incubated with unstimulated lymphocytes. Similarly, there was an increase of 418 ± 121% ($p<0.01$) above the control values.

Experiment V

$^{14}CO_2$ assay was again done on whole blood from PPD positive subjects. However, in this case the 2-hour incubation was carried out with lymphocytes stimulated with PHA, with unstimulated lymphocytes, or with PHA and medium without lymphocytes. Results of 3 tests are given in Table 7.

Table 7

Glucose-1-^{14}C Oxidation of Whole Blood after Incubation for 2 Hours with PHA-stimulated and Unstimulated Lymphocytes and Control

Subject	(A) Stimulated (Counts/min)	(B) Unstim. (Counts/min)	% Incr.	(C) Control (Counts/min)	% Incr.
1	15,562	2,658	485	2,750	466
2	16,880	3,689	358	3,033	457
3	16,158	3,158	412	3,295	390
Mean ± S.E.M.			418 ± 36		438 ± 24

Comparison between (A) and (B) $t = 71.213$, $p < 0.001$

Comparison between (A) and (C) $t = 38.520$, $p < 0.001$

Comparison between (B) and (C) $t = 0.5378$, $p > 0.5$

The per cent increases were obtained in the same manner as in experiment IV. As in that experiment, no significant difference was observed between the unstimulated values and the control values ($p>0.5$). Blood incubated with stimulated lymphocytes again showed an increase in glucose-1-^{14}C oxidation over blood incubated with unstimulated lymphocytes or with antigen and medium without lymphocytes. There was an increase of $418 \pm 36\%$ ($p<0.001$) above the unstimulated value and an increase of $438 \pm 24\%$ ($p<0.001$ above the control values.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

Glucose oxidation in polymorphonuclear leukocytes has been shown to be stimulated by the act of phagocytosis (119) and by the administration of certain membrane-active agents (120). It was of interest to determine whether this aspect of polymorphonuclear leukocyte metabolism might be affected by lymphocyte-induced stimulation. In experiments IV and V, it can be seen that glucose-1-^{14}C oxidation was dramatically increased in the group incubated with stimulated lymphocytes as compared to the group incubated with unstimulated lymphocytes. The average increase in $^{14}CO_2$ production in the group incubated with specifically stimulated lymphocytes, i.e., with OT, was 356 ± 101% of the group incubated with unstimulated lymphocytes. Similarly, there was an average increase of 418 ± 36% in the group incubated with nonspecifically stimulated lymphocytes, i.e., with PHA, over the group incubated with unstimulated lymphocytes.

However, because, as was stated previously, oxidation of glucose is increased during phagocytosis, it was necessary to determine whether significant amounts of phagocytizable matter might have formed during the 24-hour incubation. It was also important to confirm that OT and PHA did not in themselves produce a stimulatory effect on

$^{14}CO_2$ production in 2 hours of incubation. To test these two possibilities, media and antigen were incubated for 24 hours and then added to whole blood for 2 hours before $^{14}CO_2$ assay. These control flasks exhibited the same unstimulated rate of glucose-1C oxidation as the flasks incubated with unstimulated lymphocytes.

The fact that little, if any, stimulation of glucose utilization is produced by the antigen itself in the first two hours of incubation of whole blood was confirmed in experiment II. No significant difference in glucose utilization was found in either the PPD positive or the PPD negative groups between blood incubated with OT or PPD and blood incubated with saline. Similarly, in experiment I, no significant difference in glucose utilization was found between a fixed number of leukocytes from PPD positive subjects and those from PPD negative subjects after incubation with OT for 2 hours.

Further studies concerning the time factor involved in activating the lymphocyte before it can exert its effect on polymorphonuclear leukocytes were carried out in experiment III. In the PPD positive group, no significant increase in lysozyme production over baseline values could be seen after incubation with OT for 1 hour. However, after 3 hours, there was a slight but significant increase ($1.0 \pm 0.9\%$) which became larger after 5 hours ($10 \pm 4\%$), after 8 hours ($19 \pm 5\%$), and markedly after 24 hours ($112 \pm 32\%$). The

fact that this increase was not due to the effect of the antigen on the polymorphonuclear leukocytes themselves can be seen in the lack of increase in the PPD negative group. Two classes of materials have previously been mentioned which stimulate leukocyte oxidation. These are phagocytizable particles (121), and certain membrane-active substances which may stimulate pinocytosis (122), such as endotoxin, deoxycholate, digitonin (123), and dilute anti-macrophage serum (124, 125). However, these materials act very quickly upon being introduced into the leukocyte culture. Therefore, the activation of lymphocytes mentioned above appears to act by a different mechanism, one involving prolonged intracellular processes. However, once these processes are underway, the lymphocytes exert their action on polymorphonuclear leukocytes quickly as evidenced by the rapid (2 hour) increase in glucose utilization in experiments IV and V. This latent period is interesting, especially in light of the fact that a period of 2 to 3 days was sometimes found by Mackaness (126) to be required for the development of enhanced nonspecific bactericidal capacity in mouse peritoneal macrophages. For example, macrophage bactericidal capacity was enhanced only 2 to 3 days after normal mice received Bacille Calmette-Guérin (BCG) plus lymphocytes from BCG-immunized donors.

The question might be raised as to how much is contributed by the added stimulated lymphocytes themselves to the increase in glucose utilization. As described in the

Review of Literature, specifically ^{14}C-labeled glucose is used in the estimation of HMS activity. In 1956, Kits (127) reported that in splenic and thymic lymphocyte preparations, the HMS is operative as evidenced by experimental findings using ^{14}C specifically labeled glucose as substrates. However, Beck (128) has estimated, on the basis of findings in enzyme studies with white blood cell homogenates, that approximately 3% of the glucose utilized was by way of the HMS. MacHaffie and Wang (129) reported that radiorespirometric experiments with ^{14}C specifically labeled glucose as substrate demonstrated that glucose is utilized by intact lymphocytes predominantly by way of the glycolytic pathway, leading to the formation of lactate and pyruvate. Their results also revealed that the HMS is playing a rather minor role in being responsible for the utilization of not greater than 2% of the catabolized glucose. More recently, it was shown by MacHaffie and Wang (130) that the glucose metabolism in intact lymphocytes was found to be altered in the presence of phytohemagglutinin. The operation of the HMS and, to some extent the pentose cycle pathway in lymphocytes was found to be enhanced. This finding was interpreted as reflecting an additional need of biosynthetic intermediates and NADPH by the lymphocytes to accommodate mitotic activity induced by phytohemagglutinin. However, the activity of the HMS was still small in comparison to the activity of the glycolytic pathway.

It is, therefore, assumed that the dramatically increased

glucose-1-^{14}C oxidation in experiments IV and V must be due to stimulation of some other element(s) in the whole blood. Erythrocyte and platelet metabolism has not been shown to be enhanced by stimulated lymphocytes, OT or PHA (131). In the red blood cell, both the E-M pathway and the HMS are active (132). However, Murphy (133) calculated the $^{14}CO_2$ production in erythrocytes in experiments with ^{14}C-labeled glucose and showed that the portion of glucose transformed in the HMS was only 5-10% of the total utilized glucose. Similarly, DeLoecker and Pranherd (134) studied human red cells in phosphate buffer with glucose-1-^{14}C and found that these cells utilized 20.6 microliters of O_2 per ml of cells in 3 hours and 2.05 micromoles of glucose per ml of cells per hour, and produced 4.4 micromoles of lactate. From the CO_2 produced, it was calculated that only 8.5% of the glucose was metabolized via the HMS. The respiratory coefficient for thrombocytes is about equal to unity (135, 136). However, experiments with ^{14}C-glucose have shown that exogenous glucose does not serve as the principal substrate for oxidative metabolism in thrombocytes (137). Glucose oxidation accounted for as little as 6-10% of oxygen consumption. Respiration was largely independent of sugar addition. Monocytes, eosinophils and basophils are present in very small numbers in whole blood in comparison to polymorphonuclear leukocytes, and their contribution to glucose metabolism may be condidered negligible.

Furthermore, another parameter was used to indicate

the stimulatory effect of sensitized lymphocytes, i.e., lysozyme production. As noted in the Review of Literature, a positive relationship exists between serum lysozyme and both granulocyte count and granulocyte turnover. However, lymphocytes alone have little if any lysozyme activity.

Speculation can be made about the function of the increased glucose-1-^{14}C oxidation in polymorphonuclear leukocytes incubated with activated lymphocytes. Although HMS activity cannot be equated with glucose-1-^{14}C oxidation, the shunt does play a major role in carbohydrate metabolism in the polymorphonuclear leukocyte (see Review of Literature). Two biochemical processes can be looked at, both of which utilize reduced nicotinamide adenine dinucleotide phosphate (NADPH), which is produced by the HMS, and regenerate its oxidezed form (NADP), the level of which appears to regulate HMS activity (138). The first process is synthesis of membrane lipids. In phagocytosis, where plasma membrane is expended in the formation of phagosomes and presumably regenerated, increased incorporation of precursors into phospholipids has been demonstrated (139). Similarly, the stimulation of HMS activity in macrophages treated with migration-inhibitory-factor (MIF)-rich fractions may reflect increased turnover or even net synthesis of membrane involved in locomotion (140), spreading and ruffled border activity.

On the other hand, increased glucose-1-^{14}C oxidation might be related to enhanced microbicidal capacity. Reduced

pyridine nucleotides, possibly from the HMS, might be oxidized to produce hydrogen peroxide (141, 142, 143). Gee et al (144) have reported hydrogen peroxide-generating mechanisms, the activation of which increases during phagocytosis, in guinea pig alveolar macrophages, while Paul et al (145) have made similar observations with mouse peritoneal macrophages. In addition, Cotran and Ditt (146) have reported endogenous peroxidase in guinea pig peritoneal macrophages. Klebanoff (147) has clearly implicated hydrogen peroxide with a peroxidase in the bactericidal capacity of polymorphonuclear leukocytes. One can speculate that stimulation of glucose-1-^{14}C oxidation in polymorphonuclear leukocytes incubated with stimulated lymphocytes may reflect activation of a peroxide-forming mechanism which is potentially microbicidal. Moreover, this microbicidal capacity would be enhanced by the increased lysozyme production observed in experiment III.

Work with macrophages has already been done in this area. Simon and Sheagren (148) have observed that when whole peritoneal exudate cells taken from guinea pigs sensitized to PPD or other protein antigens were incubated with the sensitizing antigen for 24 hours, the resulting macrophage monolayers killed Listeria monocytogenes much better than the same cells incubated without antigen. Furthermore, the same workers have found that sensitive lymphocytes can confer this "immunity" on normal macrophages in vitro. Patterson and Youmans (149) have reported that

lymphocytes from M. tuberculosis-immunized mice, when cultured in vitro with the same organism produced supernates which inhibited the growth of this organism in normal macrophages. This raises the question of the possible mechanism by which the postulated lymphocyte stimulation of polymorphonuclear leukocytes occurs.

The capacity of sensitized cells to affect a population of other cells may result from cell to cell contact or indirectly through the release into the medium of a material. Bloom and Bennett (150) have obtained evidence to support the latter possibility. They studied migration using highly purified populations of macrophages and peritoneal lymphocytes from tuberculin-sensitive guinea pigs. They reached two important conclusions: (1) sensitive lymphocytes are necessary for inhibition of macrophage migration; (2) inhibition of migration of normal macrophages can be accomplished by a cell-free supernate of sensitive lymphocytes that have been incubated with specific antigen (PPD) for 24 hours. Furthermore, David (151) has shown that inhibition of protein synthesis prevents the specific inhibition of migration. The material produced is now ordinarily referred to as MIF (migration inhibitory factor). At about the same time, sensitized lymphocytes were shown to be cytotoxic for cells to which the lymphocytes had been specifically sensitized (152, 153). Although many studies have suggested that this cytotoxicity was mediated directly by the lymphocytes, others have indi-

cated that cytotoxicity could be achieved by soluble mediators (154, 155). Some investigators have also found that stimulation of lymphocytes by antigen will cause them to produce a soluble substance which causes non-sensitive lymphocytes to divide (156, 157). The interaction of sensitive lymphocytes with specific antigen has yielded soluble materials which have several other biological activities (158). In addition to MIF, a cytotoxic factor and a blastogenic factor, the following have been described: a factor which causes reddening of the skin and infiltration by mononuclear cells typical of the delayed reaction (159), a factor chemotactic for macrophages (160) and a factor chemotactic for polymorphonuclear leukocytes (161), a factor with interferon activity (162), and factors which inhibit the growth of cultured cells without killing them (163, 164). However, for the most part, what has really been demonstrated is a number of different activities. It is unlikely that each of the activities separately identified is actually a function of a distinct substance. Therefore, it can be speculated that still another substance is produced by activated sensitized lymphocytes which stimulates polymorphonuclear leukocytes as evidenced by increased glucose-1-^{14}C oxidation and production of lysozyme, or that one of the already existing "factors" has this additional effect.

The fact that stimulation of polymorphonuclear leukocytes occurred when they were incubated with lymphocytes ac-

tivated by a specific antigen, i.e., OT, and also with lymphocytes activated by a so-called non-specific agent, i.e., PHA, is noteworthy. An important question asked is whether when a lymphocyte is activated, it is in effect "turned on" for everything it is capable of making regardless of the conditions, or whether there are circumstances when this is selective and only certain activities are released. Recently, it has become evident that lymphocytes can be activated by a nonimmunologic stimulant, such as a mitogen, to produce soluble substances with activities similar to those stimulated by specific antigen. In early *in vitro* studies, Granger and Kolb (165) provided strong indications that destruction of target L cells by *either* immune or PHA-stimulated normal mouse lymphocytes was not the direct result of aggressor-target cell contact, but was instead associated with lymphocyte activation and consequent secretion by lymphocytes of a soluble non-specific cell toxin. Pick *et al* (166) have described mitogen-induced release of MIF and skin reactive factor. The results of this present study indicate that both with specific stimulation with OT and non-specific stimulation with PHA, lymphocytes have produced what appears to be the same stimulatory effect on polymorphonuclear leukocytes.

CHAPTER V

SUMMARY

Reactions of cellular hypersensitivity often involve the participation of different cell types. The communication between lymphocytes and polymorphonuclear leukocytes was investigated. Human sensitized lymphocytes exposed to old tuberculin were found to have a stimulatory effect on polymorphonuclear leukocytes as indicated by the latter's increased glucose-1-^{14}C oxidation and lysozyme production. Similar results were obtained using lymphocytes non-specifically stimulated by phytohemagglutinin. Activation of lymphocytes required a latent period (24 hours) followed by rapid stimulation of polymorphonuclear leukocytes (2 hours). These results suggest that increased glucose-1-^{14}C oxidation and lysozyme production may be related to enhanced microbicidal activity. A possible mechanism by which the postulated lymphocyte stimulation of polymorphonuclear leukocytes occurs is the secretion into the medium by the sensitized lymphocytes of a soluble substance which has biological activity. This activity may be due to a new material or it may be a function of one of the already described lymphocyte "factors".

REFERENCES

1. Lawrence, H. S., and Landy, M.: Mediators of Cellular Immunity. Proceedings of an International Conference, August, Michigan. New York: Academic Press, 1969.

2. Intersociety Symposium on In Vitro Correlates of Delayed Hypersensitivity, 51st Annual Meeting of the Federation of American Societies for Experimental Biology, Chicago. Fed. Proc. 27:3, 1968.

3. World Health Organization: Cell-Mediated Immune Responses. Report of a WHO Study Group. WHO Tech. Rep., Ser. 423, WHO, Geneva, 1969; Int. Arch. Allerg. 36 (No. 6): 7, 1969.

4. David, J.R.: Cellular hypersensitivity and immunity; inhibition of macrophage migration and the lymphocytic mediators. Progr. Allerg. (in press).

5. Goddard, J. R., and Mendel, L. B.: Plant hemagglutinins with special reference to a preparation from the navy bean. J. biol. Chem. 82:447-463 (1929).

6. Wienhauns, O.: Zur Biochemie des Phasins. Biochem. Z. 18:228-260 (1909).

7. Rigas, D. A., and Osgood, E. E.: Purification and properties of the phytohemagglutinin of phaseolus vulgaris. J. biol. Chem. 212:607-615 (1955).

8. Landsteiner, K.: The specificity of serological reactions. C. Ch. Thomas. Springfield, Illinois (1936).

9. Perera, C. B., and Frumin, A. M.: Hemagglutination by fava bean extract inhibited by simple sugars. Science 151:821 (1966).

10. Borberg, H., Woodruff, J., Hirschhorn, R., Gesner, B., Miescher, P., and Silber, R.: Phytohemagglutinin: inhibition of the agglutinating activity by N-Acetyl-D-Galactosamine. Science 154:1019-1020 (1966).

11. Hungerford, D. A., Donnelly, A. J., Nowell, P. C., and Beck, S.: The chromosome constitution of a human phenotypic intersex. Amer. J. hum. Genet. 11:215-235 (1959).

12. Barkhan, P., and Ballas, A.: Phytohemagglutinin: separation of hemagglutinating and mitogenic principles. Nature, Lond.200:141-142 (1963).

13. Richter, M., and Naspitz, C. K.: The in vitro blastogenic response of lymphocytes of ragweed sensitive individuals. J. Allergy, in press.

14. Borberg, H., Woodruff, J., Hirschhorn, R., Gesner, B., Miescher, P., and Silber, R.: Phytohemagglutinin: inhibition of the agglutinating activity by N-Acetyl-D-Galactosamine. Science 154:1019-1020 (1966).

15. Quaglino, D., and Cowling, D. C.: Cytochemical studies on cells from chronic lymphatic leukemia and lymphosarcoma cultured with phytohemagglutinin. Brit. J. Haemat. 10:358-364 (1964).

16. Gough, J., and Elves, M. W.: Studies of lymphocytes and their derivative cells in vitro. I. Biochemical constituents. Acta haemat. 36:344-349 (1966).

17. Cooper, H. L., Barkhan, P., and Hale, A.J.: Mitogenic activity of phytohemagglutinin. Lancet i:210 (1961).

18. Pachman, L. M.: The small circulating lymphocyte--in vitro studies of respiration and glycolysis. Fed. Proc. 26:527 (167).

19. Naspitz, C. K., and Richter, M.: The metabolism of phytohemagglutinin during incubation with human peripheral lymphocytes. Brit. J. Haemat, in press.

20. Elves, M. W.: The effect of phytohemagglutinin on lymphoid cells; in Elves' The Biological Effects of Phytohemagglutinin, pp. 11-26 (The Robert Jones and Agnes Hunt Orthopaedic Hospital Management Committee, Oswestry, England (1966).

21. Cooper, E. H., Barkhan, P., and Hale, A. J.: Observations on the proliferation of human leucocytes cultured with phytohemagglutinin. Brit. J. Haemat. 9:101-111 (1963).

22. Bach, F. H., and Hirschhorn, K.: The in vitro immune response of peripheral blood lymphocytes. Sem. Haemat. 2:68-89 (1965).

23. Elves, M. W.: The Lymphocytes, p. 202 (Year Book Medical Publishers, Chicago, 1967).

24. Marshall, W. H., and Roberts, K. B.: The growth and mitosis of human small lymphocytes after incubation with a phytohemagglutinin. Quart. J. exp. Physiol. 48: 146-155 (1963).

25. Inman, D. R., and Cooper, E. H.: The relation of ultrastructure to DNA sythesis in human leukocytes. I. Atypical lymphocytes in phytohemagglutinin cultures and infectious mononucleosis. Acta Haemat. 33:257-278 (1965).

26. Chapman, J. A.: Electron microscopic studies of the PHA transformed lymphocyte; in Elves' The Biological Effects of Phytohemagglutinin, pp. 67-70 (The Robert Jones and Agnes Hunt Orthopaedic Hospital Management Committee, Oswestry, England 1966).

27. Michalowski, A., Bartosiewicz, W., and Kozumowski, J., Blastic transformation in leukocyte culture. Lancet 1130-1131 (1966).

28. Bach, F., and Hirschhorn, K.: gamma globulin production by human lymphocytes in vitro. Exp. Cell Res. 32: 592-595 (1963).

29. Forbes, I. J., and Henderson, D. W.: Globulin synthesis by human peripheral lymphocytes. In vitro measurements using lymphocytes from normals and patients with disease. Ann. Intern. Med. 65: 69-79 (1966).

30. Ripps, C. S., and Hirschhorn, K.: The production of immonoglobulins by human peripheral blood lymphocytes in vitro. Clin. exp. Immunol. 2:377-398 (1967).

31. Turner, K. J., and Forbes, I. J.: Synthesis of proteins by human leukocytes in vitro. II. Chemical characterization. J. Immunol. 96:926-935 (1966).

32. Naspitz, C. K., Eisen, A. H., and Richter, M.: DNA synthesis in vitro in leukocytes from patients with ataxia telangiectasia. Int. Arch. Allergy, in press.

33. Oppenheim, J. J., Wolstencroft, R. A., and Gell, P. G. H.: Delayed hypersensitivity in the guinea-pig to a protein-hapten conjugate and its relationship to in vitro transformation of lymph node, spleen, thymus, and peripheral blood lymphocytes. Immunology, Lond. 12:89-102 (1967).

34. Bach, F. H., and Hirschhorn, K.: The in vitro immune response of peripheral blood lymphocytes. Sem. Haemat. 2:68-89 (1965).

35. Elves, M. W.: The effect of phytohemagglutinin on lymphoid cells; in Elves' The Biological Effects of Phytohemagglutinin, pp. 11-26 (The Robert Jones and Agnes Hunt Orthopedic Hospital Management Committee, Oswestry, England 1966).

36. Oppenheim, J. J., Hersh, E. M., and Block, J. B.: The response to various stimuli of human peripheral lymphocytes which have been 'purified' by passage through glass bead columns; in Elves' The Biological Effects of Phytohemagglutinin, pp. 183-189 (The Robert Jones and Agnes Hunt Orthopaedic Hospital Management Committee, Oswestry, England 1966).

37. Heilman, D., and McFarland, W.: Cellular interaction during antigen-induced blastogenesis in leukocyte cultures. Fed. Proc. 26:528 (1967).

38. Jones, A. L.: The effect of polymorphonuclear leucocytes on the blastoid transformation of lymphocytes in mixed leucocyte cultures. Transplant. Bull. 4:337-343 (1966).

39. Oppenheim, J. J., Hersh, E. M., and Block, J. B.: The response to various stimuli of human peripheral lymphocytes which have been 'purified' by passage through glass bead columns; in Elves' The Biological Effects of Phytohemagglutinin, pp. 183-189 (The Robert Jones and Agnes Hunt Orthopaedic Hospital Management Committee, Oswestry, England 1966).

40. Caron, G. A.: A tritiated thymidine study of the effect of phytohemagglutinin on adult human skin in organ culture; in Elves' The Biological Effects of Phytohemagglutinin, pp. 151-157 (The Robert Jones and Agnes Hunt Orthopaedic Hospital Management Committee, Oswestry, England 1966).

41. Agrell, I. P. S.: Phytohemagglutinin as a mitotic stimulator on free-living amoebae. Exp. Cell Res. 42:403-408 (1966).

42. Ioachim, H. L.: Effects of phytohemagglutinin in vitro on cells other than lymphocytes. Nature, Lond. 210:919-921 (1966).

43. Eisen, H. N.: Delayed type hypersensitivity reactions. In: Mechanisms of Hypersensitivity. Henry Ford Hospital International Symposium. Boston, Little, Brown, 1958.

44. Clinical Immunology and Allergy, Leo H. Criep, Grune and Stratton, New York, 1969, p. 713.

45. Clinical Immunology and Allergy, Leo H. Criep, Grune and Stratton, New York, 1969, p. 713.

46. Raffel, S., and Forney, J.E.: The role of the "wax" of the tubercle bacillus in establishing delayed hypersensitivity. I: Hypersensitivity to a simple chemical substance, picryl chloride. J. Exper. Med. 88:485, 1948.

47. Raffel, S., Arnaud, L. E., Dukes, C. D., and Huang, J. S.: The role of the "wax" of the tubercle bacillus in establishing delayed hypersensitivity. II: Hypersensitivity to a protein antigen egg albumin. J. Exper. Med. 90:53, 1949.

48. Dienes, L.: The technique of producing tuberculin type of sensitization with egg white in tuberculous guinea pigs. J. Immunol. 17:531, 1929.

49. Baldwin, E. R.: Studies in immunity to tuberculosis. I. Hypersusceptibility or anaphylaxis. J. Clin. Res. 22:189, 1910.

50. Chase, M. W.: The cellular transfer of cutaneous hypersensitivity to tuberculin. Proc. Sci. Exper. Biol. Med. 59:134, 1945.

51. Harris, S., and Harris, T. N.: Transfer of cells from lymph nodes of rabbits following regional injection of antigens. Fed. Proc. 10:409, 1951.

52. Chase, M.: The cellular transfer of cutaneous hypersensitivity. J. Bact. 51:643, 1946.

53. Chase, M. W.: The Allergic State in Bacterial and Mycotic Infections of Man, ed. 2. Dubos, R. J. (Ed.): Philadelphia, Lippincott, 1952, p. 198.

54. Epstein, W. L., and Kligman, W. M.: Transfer of allergic contact delayed sensitivity in man. J. Invest. Dermat. 28:28, 1957.

55. Nyka, W., Schariff, M., and Ehr, W. J.: Delayed Hypersensitivity and its possible relation to antibody formation. In Mechanisms of Hypersentivity. Henry Ford Hospital International Symposium. Boston: Little, Brown, 1958.

56. Jeter, W. S., Lawrence, K. A., and Seebohm, P. M.: Analysis of leucocyte extracts from guinea pigs hypersensitive to tuberculin and 2-4-dinitrochlorobenzene. J. Bact. 74:680, 1957.

57. Chase, M. W.: Immunological reactions mediated through cells. In: Pappenheimer, A. M., Jr. (Ed.): The Nature and Significance of the Antibody Response. New York, Columbia University Press, 1953, p. 156.

58. Chase, M. W.: Models for hypersensitivity studies. In: Cellular and Humoral Aspects of the Hypersensitive States. New York, Paul B. Hoeber, 1959, p. 251.

59. Pearnain, G., Lysette, R. R., and Fitzgerald, P. H.: Tuberculin induced mitosis in peripheral blood leukocytes. Lancet i.637 (1963).

60. Schrek: Cell transformation and mitosis produced in vitro by tuberculin purified protein derivative in human blood cells. Amer. Rev. resp. Dis. 87:734, 1963.

61. Coulson, A. S., and Chalmers, D. G.: Response of human blood lymphocytes to tuberculin PPD in tissue culture. Immunology, Lond. 12:417-428 (1967).

62. Schrek, R., and Stefani, S.: Resistance of phytohemagglutinin-treated lymphocytes to X-rays and nitrogen mustard. Nature, Lond. 200: 482-483 (1963).

63. Stefani, S.: Old tuberculin-induced radioresistance on human lymphocytes in vitro. Brit. J. Haemat. 12:345-350 (1966).

64. The value of allergy in tuberculosis as revealed by tissue culture studies. Bull. Johns Hopkins Hospital. 50:115-135, 1932.

65. George, M., and Vaughan, J. H.: In vitro cell migration as a model for delayed hypersensitivity. Proc. Soc. exp. Biol. Med. 111:514-521, 1962.

66. David, J. R., Lawrence, H. S. and Thomas, L.: Delayed hypersensitivity in vitro. II. Effect of sensitive cells or normal cells in the presence of antigen. J. Immun. 93:274-278, 1964.

67. David, J. R.: Suppression of delayed hypersensitivity in vitro by inhibition of protein synthesis. J. exp. Med: 1125-1134, 1965.

68. Bloom, B. R., and Bennett, B.: Mechanism of a reaction in vitro associated with delayed-type hypersensitivity. Science 153:80-82, 1966.

69. Bloom, B. R., and Bennett, B.: Mechanism of a reaction in vitro associated with delayed-type hypersensitivity. Science 153:80-82, 1966.

70. David, J. R., Lawrence, H. S., and Thomas, L.: Delayed hypersensitivity in vitro. III. The specificity of hapten-protein conjugates in the inhibition of cell migration. J. Imm. 93:277-282, 1964.

71. Kostiala, A.: Delayed Hypersensitivity in the guinea pig immunized with killed tubercle bacilli in adjuvant. II. The effect of Tuberculin Purified Protein Derivative concentration on the inhibition of peritonial cell migration.

72. Fleming, A.: On a remarkable bacteriolytic element found in tissues and secretions, Proc. Roy. Soc. London 93:306, 1922.

73. Fromageot, C., and Jolles, P.: Étude comparée de quelques proteines lysantes appartenant a la famille des lysozymes, Experientia Suppl. II: 181, 1955.

74. Javregui-Adell, J., et al.: The disulfide bridges of hen's egg-white lysozyme. Biochem. Biophys. Acta 107:97, 1965.

75. Jolles, P.: Recent developments in the study of lysozymes. Angew. Chem. (Eng.) 328:28, 1964.

76. Alderton, G., et al.: Isolation of lysozyme from egg white. J. Biol. Chem. 157:43, 1945.

77. Fevold, H., and Alderton, G.: Lysozyme. Biochem. prep. 1:67, 1949.

78. Osserman, E. F., and Lawlor, D. P.: Serum and urinary lysozyme (muramidase) in monocytic and myelomonocytic leukemia. J. Exp. Med. 124:921, 1966.

79. Fleming, A.: On a remarkable bacteriolytic element found in tissues and secretions. Proc. Roy. Soc. London 93:306, 1922.

80. Flanagan, P., Lionette, F.: Lysozyme distribution in blood. Blood 10:497, 1955.

81. Finch, S.C., Lamphere, J.P., and Jablon, S.: The relationship of serum lysozyme to leukocytic and other constitutional factors. Yale J. Biol. Med. 36:350, 1964.

82. Cohn, Z. A., and Hirsch, J. G.: The isolation and properties of the specific cytoplasmic granules of rabbit polymorphonuclear leukocytes. J. Exp. Med. 112:983, 1960.

83. Glynn, A. A., and Parkman, R.: The distribution of cell lysozome. Proceedings of the 3rd International Symposium on Fleming's Lysozyme. Milan, Italy, 1964.

84. Glynn, A. A., and Parkman, R.: Studies with an antibody to rat lysozyme. Immunology 7:724, 1964.

85. Parkman, R.: Lysozyme, a thesis presented to the Yale Medical School, New Haven, Connecticut, 1965.

86. Finch, S. C., Gnabasik, F. J., and Rogoway, W.: Lysozyme and Leukopolesis. Proceedings of the 3rd International Symposium on Fleming's Lysozyme. Milan, Italy, 1964.

87. Finch, S. C., Gnabasik, F. J., and Rogoway, W.: The relationship between serum lysozyme and leukocyte turnover. Med. et Hygiene. 22:972, 1964.

88. Speece, A. J.: Histochemical distribution of lysozyme activity in organs of normal mice and radiation chimeras. J. Histo. 12:384, 1964.

89. Myruik, Q. N., Leake, E. S., Oshima, S.: A study of macrophages and epitheliod-like cells from granulamatous (BOG induced) lungs of rabbits. J. Immun. 89:745, 1962.

90. Cohn, Z. A., and Wiener, E.: The particulate hydrolases of macrophages II Biochemical and morphological response to particulate ingestion. J. Exp. Med. 118:1009, 1963.

91. Cohn, Z. A., and Wiener, E.: The particulate hydroses of macrophages I Comparative enzymology, isolation, and properties. J. Exp. Med. 118:991, 1963.

92. Carson, M. E. and Dannenberg, A. M.: Hydrolytic enzymes of rabbit mononuclear exudate cells. J. Immun. 94:99, 1965.

93. Kerby, G. P.: A method for the detection of leukocyte injury based on a release of a lysozyme-like enzyme. Proc. Soc. Exp. Biol. Med. 81:129, 1952.

94. Kerby, G. P.: Release of enzyme from human leukocytes on damage by bacterial derivatives. Proc. Soc. Exp. Biol. Med. 81:381, 1952.

95. Ribble, J. C., and Bennett, J. L.: Lysozyme release as an indication of in vivo leukocyte injury by endotoxin. Clin. Research 7:41, 1959.

96. Ribble, J. C.: Increase of plasma lysozyme activity following injections of typhoid vaccine. Proc. Soc. Exp. Biol. Med. 107:597, 1961.

97. Hook, W. A., Carey, W. F., and Muschel, L. H.: Alterations in serum lysozyme and properdin titers of mice following X-irradiation or treatment with zymosan or endotoxin. J. Immunol. 84:569, 1960.

98. Quie, P. G., and Hirsch, J. G.: Antiserum to leucocyte lysosomes: its cytotoxic, granulolytic, and hemolytic actions. J. Exp. Med. 120:149, 1964.

99. Warburg, O., and Christian, W.: Biochem. Z. 242, 206 (1931).

100. White, A., Handler, P., and Smith, E.: Principles of Biochemistry. New York: McGraw Hill Book Co., 1968, p. 415.

101. White, A., Handler, P., and Smith, E.: Principles of Biochemistry. New York: McGraw Hill Book Co., 1968, p. 416.

102. Bloom, B., and Stetten, D., Jr.: J. Amer. chem. Soc. 75, 5446 (1953).

103. Weaver, G., and Landau, B. R.: Endocrinology (in press).

104. Wenner, C. E., and Weinhouse, S.: J. biol Chem. 219, 691 (1956).

105. Abraham, S., Hirsch, P. F., and Chaikoff, I. L.: J. biol. Chem. 211, 31 (1954).

106. Cheldelin, V. H.: In: Metabolic Pathways in Microorganisms. New York: John Wiley & Sons, 1961.

107. Cheldelin, V. H., Wang, C. H., and King, T. E.: In: Comparative Biochemistry (M. Florkin and H. S. Mason, eds). Vol. 3. New York: Interscience, 1962.

108. White, A., Handler, P., and Smith, E.: Principles of Biochemistry. New York: McGraw Hill Book Co., 1968, p. 417.

109. Lardi, S.: Preparation, Purification, and Stability of Tuberculin. Appl. Microbiol. 11:408, 1963.

110. U. S. Public Health Service Regulations. Biological Products, Title 42, Part 73. Additional Standards: Tuberculin.

111. Atkins, E., and Heijn, C., Jr.: Studies in tuberculin fever. III. Mechanisms involved in the release of endogenous pyrogen in vitro. J. Exp. Med. 122:209, 1965.

112. Hull, W. J., Francis, L., and Atkins, E.: Studies on tuberculin fever. IV. The passive transfer of reactivity with various tissues of sensitized donor rabbits. J. Exp. Med. 131:484, 1970.

113. Richter, M., and Naspitz, C. K.: The Variation in Response of Human Peripheral Lymphocytes to Phytohemagglutinin in vitro. Int. Arch. Allergy 32:288, 1967.

114. Malavista, S. E., and Bodel, P. T.: The Dissociation by Colchicine of Phagocytosis from Increased Oxygen Consumption in Human Leukocytes. J. of Clinical Investigation 46:787, 1967.

115. Cooper, L.: Ribonucleic Acid Metabolism in Lymphocytes Stimulated by Phytohemagglutinin. The Journal of Biological Chemistry 243:35, 1968.

116. De Chalelet, L. R., Cooper, M. R., and McCall, C. E.: Dissociation by colchicine of the hexose monophosphate shunt activation from the bactericidal activity of the leukocyte. Infect. Immunity 3:66-72, 1971.

117. Smolelin, A. N., and Hartsell, S.E.: Determination of Lysozyme. J. Bact. 58:731-736, 1949.

118. Litwack, G.: Photometric determination of lysozyme activity. Proc. Soc. Exp. Biol. Med. 89:401-403, 1955.

119. Sbarra, A. J., and Karnovsky, M. L.: The biochemical basis of phagocytosis. I. Metabolic changes during the ingestion of particles of polymorphonuclear leukocytes. J. Biol. Chem. 234:1355, 1959.

120. Graham, R. C., Jr., Karnovsky, M. J., Shafer, A. W., Glass, E. A., and Karnovsky, M. L.: Metabolic and morphologic observations on the effect of surface-active agents on leukocytes. J. Cell Biol. 32:629, 1967.

121. Sbarra, A. J., and Karnovsky, M. L.: The biochemical basis of phagocytosis. I. Metabolic changes during the ingestion of particles of polymorphonuclear leukocytes. J. Biol. Chem. 234:1355, 1959.

122. Karnovsky, N. L.: Metabolic basis of phagocytic activity. Physiol. Rev. 42:143, 1962.

123. Graham, R. C., Jr., Karnovsky, M. J., Shafer, A. W., Glass, E. A., and Karnovsky, M. L.: Metabolic and morphologic observations on the effect of surface-active agents on leukocytes. J. Cell Biol. 32:629, 1967.

124. Cohn, Z. A., and Parks, E.: The regulation of pinocytosis in mouse macrophages. II. Factors inducing vesicle formation. J. Exp. Med. 125:213, 1967.

125. Musher, D., Keusch, G., and Weinstein, L.: Morphologic and metabolic effects of antimacrophage serum (A. M. S.). Clin. Res. 18:445, 1970. Abstr.

126. Mackaness, G. B.: The influence of immunologically committed lymphoid cells in macrophage activity in vivo. J. Exp. Med. 129:973, 1969.

127. Kits: The role of the hexose monophosphate shunt in tumors and lymphatic tissue. Cancer Res. 16:70, 1956.

128. Beck, W. S., and Valentine, W. N.: The aerobic carbohydrate metabolism of leukocytes in health and disease. Cancer Res. 12:818, 1952; 13:309, 1953.

129. MacHaffie, R. A., and Wang, C. H.: Carbohydrate metabolism in lymphocyte. J. Amer. Coll. Health Assn. 13:126, 1964.

130. MacHaffie, R. A., and Wang, C. H.: The effect of phytohemagglutinin upon glucose catabolism in lymphocytes.

131. Good, R. A., and Fisher, D. W.: Immunobiology. Stamford, Conn.: Sinauer Associates, Inc., 1971.

132. Holmes, William L.: Blood Cells as a Tissue. New York: Plenum Press, 1970.

133. Murphy, J. R.: J. Lab. Clin. Med., 55:286, 1960.

134. De Loecker, W. C. J., and Pranherd, T. A. J., Clin. Chem. Acta 6, 641, 1961.

135. Campbell, E. W., Small, W. J., Dameshek, W.: J. Lab Clin. Med. 47:835, 1956.

136. Lohr, G. W., Waller, H. D., Gross, R.: Deutsch Med. Wschr, 86:946, 1961.

137. Seitz, Joseph F.: The biochemistry of the cells of blood and bone marrow. Springfield, Illinois: Charles C. Thomas, 1969.

138. Beck, W. S.: Occurrence and control of the phosphoglucomate oxidation pathway in normal and leukemic leukocytes. J. Biol. Chem. 232:271, 1958.

139. Oren, R., Farnham, A. E., Saitor, K., Milofsky, E., and Karnovsky, M. L.: Metabolic patterns in three types of phagocytizing cells. J. Cell. Biol. 17:487, 1963.

140. Fach, F. H., Alter, B. J., Solliday, S., Zoschke, D. C., and Janis, M.: Lymphocyte reactivity in vitro. II. Soluble reconstituting factor permitting response of purified lymphocytes.

141. Iver, G. Y. N., Islam, M. F., and Quastel, J. H.: Biochemical aspects of phagocytosis. Nature, Lond. 192:555, 1961.

142. Paul, B., and Sbarra, A. J.: The role of the phagocyte in host-parasite interactions. XIII. The direct quantative estimation of H_2O_2 in phagocytizing cells. Biochem. biophys. Acta 156:168, 1968.

143. Selvaraj, R. J., and Sbarra, A. J.: Relationship of glycolytic and oxidative metabolism to entry and destruction in phagocytosing cells. Nature, Lond. 211:1972, 1966.

144. Gee, J. B. L., Vassallo, C. L., Bell, P., Kaskin, J., Barford, R. E., and Field, J. B.: Catalase-dependent peroxidative metabolism in the alveolar macrophage during phagocytosis. J. Clin. Invest. 49:1280, 1970.

145. Paul, B., Strauss, R. R., Jacobs, A. A., and Sbarra, A. J.: Function of H_2O_2, myeloperoxidase and hexose monophosphate shunt enzymes in phagozytizing cells from different species. Infec. Immun. 1:338, 1970.

146. Cotran, R. S., and Litt, M.: Ultrastructural localization of horseradish peroxidase and endogenous peroxidase activity in guinea pig peritoneal macrophages. J. Immun. 105:1536, 1970.

147. Klebanoff, S. J.: Iodination of bacteria. A bactericidal mechanism. J. Exp. Med. 126:1063, 1967.

148. Simon, H. B., and Sheagren, J. N.: Cellular immunity in vitro. I. Immunologically mediated enhancement of microphage bactericidal capacity. J. Exp. Med. 133:1377, 1971.

149. Patterson, R. J., and Youmans, G. P.: Demonstration in tissue culture of lymphocyte mediated immunity to tuberculosis. Infec. Immun. 1:600, 1970.

150. Bloom, B. R., and Bennett, B.: Delayed hypersensitivity in vitro. The mechanism of inhibition by antigen of cell migration. Fed. Proc. 25:355, 1966.

151. David, J. R.: Suppression of delayed hypersensitivity in vitro by inhibition of protein synthesis. J. Exp. Med. 122:1125, 1965.

152. Govaerts, A.: Cellular antibodies in kidney homotransplantation. J. Immun. 85:516, 1960.

153. Rosenau, W. and Moon, H. D.: Lysis of homologous cells by sensitized lymphocytes in tissue culture. J. nat. Cancer Inst. 27:471, 1961.

154. Granger, G. A., and Williams, T. W.: Lymphocyte cytotoxicity in vitro. Activation and release of a cytotoxic factor. Nature, Lond. 218:1253, 1968.

155. Ruddle, N. H. and Waksman, B. H.: Cytotoxicity mediated by soluble antigen and lymphocytes in delayed hypersensitivity. III. Analysis of mechanism. J. Exp. Med. 128:1267, 1968.

156. Maini, R. N., Bryceson, A. D. M., Wolstencroft, R. A., and Dumonde, D. C.: Lymphocyte mitogenic factor in man. Nature, Lond. 224:43, 1969.

157. Valentine, F. T., and Lawrence, H. S.: Lymphocyte stimulation. Transfer of cellular hypersensitivity to antigen in vitro. Science 165:1014, 1969.

158. Lawrence, H. S., and Landy, M.: Mediators of cellular immunity (Academic Press, New York, 1969).

159. Bennett, B., and Bloom, B. R.: Reactions in vivo produced by a soluble substance associated with delayed-type hypersensitivity. Proc. nat. Acad. Sci., Wash. 59:756, 1968.

160. Ward, P. A., Remold, H. G., and David, J. R.: Leukotactic factor produced by sensitized lymphocytes. Science 163:1079, 1969.

161. Ward, P. A., Remold, H. G., and David, J. R.: The production by antigen-stimulated lymphocytes of a leukotactic factor distinct from migration inhibitory factor. Cellular Immun. 1:162, 1970.

162. Green, J. A., Cooperband, S. R., and Kibrick, S.: Immune specific induction of interferon production in culture of human blood lymphocytes. Science 164:3886, 1969.

163. Green, J. A., Cooperband, S. R., and Kibrick, S.: Inhibition of target cell production by supernatants from cultures of human peripheral lymphocytes. J. Immun. 105-48, 1970.

164. Lebowitz, A., and Lawrence, H. S.: Target cell destruction by antigen-stimulated human lymphocytes. Fed. Proc. 28:630, 1969. (abstr.)

165. Granger, G. A., and Kolb, W. P.: Lymphocyte in vitro cytoxicity. Mechanisms of immune and non-immune small lymphocyte mediated target L cell destruction. J. of Immun. 101:111-120, 1968.

166. Pick, E., Brostoff, J., Krejci, J., and Turk, J.: Interaction between sensitized lymphocytes and antigen in vitro. II. Mitogen-induced release of skin reactive and macrophage migration inhibitory factors. Cellular Immun. 1:92, 1970.

YALE MEDICAL LIBRARY

Manuscript Theses

Unpublished theses submitted for the Master's and Doctor's degrees and deposited in the Yale Medical Library are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but passages must not be copied without permission of the authors, and without proper credit being given in subsequent written or published work.

This thesis by has been used by the following persons, whose signatures attest their acceptance of the above restrictions.

NAME AND ADDRESS	DATE
Debra D. Moore B225 Cedar Street.	9/20/76
Ernest A. Laudano 308 Spring Rd	9-21-78