ACTION OF PHYTOHEMAGGLUTININ IN RABBITS

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ABSTRACT

Phytohemagglutinin (PHA) has a well established mitogenic and blastogenic action when incubated in vitro with lymphoid cells. The action of this substance was studied in vivo and the histological changes induced by PHA in various organs of the rabbit were compared to those induced by a common antigen (human gamma globulin). It is suggested from the data obtained that PHA does not act as a common antigen. In the PHA treated animals the most striking changes were noted in the spleen. There were marked effects on the white pulp but the most significant histologic features were observed in the red pulp and consisted of intense proliferation of cells of the myeloid-erythroid series. This finding suggests that PHA may have a role in the treatment of diseased states of the bone marrow and lymphoid tissues.

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CHARLES K. NASPITZ, M.D.

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science.

Department of Experimental Medicine,
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TABLE OF CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Phytohemagglutinin (PHA), an extract derived from beans of the genus Phaseolus, has been used since the beginning of this century as a means of agglutinating red blood cells. However, the mitogenic and blastogenic properties of PHA were discovered only as recently as 1960 by Hungerford et al (6) who observed that leukocytes cultured in vitro with PHA for three days were transformed to large primitive cells capable of division. In the ensuing years, these in vitro effects of PHA have been many times substantiated with human lymphocytes and those of rabbits, guinea pigs, mice and rats (24, 115). Recently PHA has been utilized therapeutically in the treatment of apparently intractable idiopathic and drug-induced aplastic anemias, with contradictory results (209-223). However, the basis for its therapeutic use has not yet been experimentally validated in animals, nor has it been demonstrated that the blastogenic and mitogenic properties of PHA can be reproduced in vivo. This lack of detailed histopathologic studies of the effects of PHA in vivo, coupled with the potential therapeutic use of PHA, prompted the present systematic histologic study of the in vivo effects following the administration of PHA.

CHAPTER 2

HISTORICAL REVIEW

2.1 INTRODUCTION

Phytohemagglutinin (PHA) is a saline extract derived from beans of the genus Phaseolus. Landsteiner (1), in the early 1900's discovered the hemagglutinating properties of PHA and subsequently did much work on this aspect of PHA. In 1916, Dorset and Henley (2) used PHA to remove red cells in the preparation of anti-serum against hog cholera. Goddard and Mendel (3), in 1929, described a method for the preparation of PHA, injected it into rabbits and mice and found that their material was not toxic. After these earlier reports, no further work was done with PHA until 1949 when Li and Osgood (4) used PHA in order to obtain suspensions of leukocytes free of red blood cells. Osgood and Krippaehne (5) took advantage of the hemagglutinating property of PHA to remove red cells from a suspension of leukocytes used in tissue culture procedures. It was not until 1959 that Hungerford et al (6) described the mitogenic and blastogenic properties of PHA. They found that this substance induced transformation of small lymphocytes in human peripheral blood into large primitive cells. These blast cells were capable of undergoing division. As a large number of cells in mitosis could be obtained by incubating peripheral leukocytes with PHA, this discovery was of great importance in the field of cytogenetics, providing a simple and reproducible method for chromosome analysis. The findings of Hungerford et al (6) were confirmed a year later by Nowell (7) and by Carstairs (8) in 1962 and by Marshall and Roberts (9) in 1963.

2.2. IN VITRO PROPERTIES OF PHA

ERYTHROAGGLUTINATING PROPERTY - PHA agglutinates rabbit, dog, cat, chicken, duck, mouse, rat, sheep, horse, pig, frog, guinea-pig, and, to a limited extent, beef erythrocytes (10). As discussed below, this erythroagglutinating property can be removed by repeated absorption with red blood cells. Perera and Frumin (11) showed that hemagglutination by the extract of the fava bean was inhibited by 5 per cent d-glucose or maltose, but not by 5 per cent d-galactose or lactose. Borberg et al (12) found that N-acetyl-D-galactosamine inhibited hemagglutination induced by PHA.

BLASTOGENIC AND MITOGENIC PROPERTIES - Peripheral white blood cells, thoracic duct cells, thymus cells, lymph node cells and spleen cells obtained from humans and different animals species (13) when incubated in vitro with PHA undergo a similar and characteristic sequence of events resulting in blast cell transformation of a majority of the incubated cells by 72 hours of culture (39)..

LEUKOAGGLUTINATING PROPERTY - PHA has the property of agglutinating white blood cells in vitro. The addition of PHA to cultures of leukocytes in vitro 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 (12) showed that N-acetyl-D-galactosamine inhibits leukoagglutination by PHA without affecting the blastogenic and mitogenic activities of PHA. The blastogenic and mitogenic properties of PHA are the most important ones for 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. These properties will be discussed in detail below.

2.3 PREPARATION AND PURIFICATION OF PHA

Substances with in vitro hemagglutinating activity have been obtained from a variety of plants. The hemagglutinin obtained from the castor bean (Riccinus communis) was used by Erlich (14) in 1897, in his first in vitro studies of the reactions between antigen and antibody. This castor bean-derived hemagglutinin was studied chemically by Osborne, Mendel and Harris (15) who attempted to separate the various active constituents. They found the hemagglutinating activity associated with the "albumin" constituent. Goddard and Mendel (3) prepared a phytohemagglutinin preparation in dry form from navy beans (Phaseolus communis). The active substance was a protein, with the characteristics of an albumin. They reported that chemical changes in the protein which led to denaturation or hydrolytic cleavage were accompanied by a lessened hemagglutinative potency. Wienhaus (16) in 1909 and Souza (17,18) in 1944 obtained partially purified proteins from red kidney beans (Phaseolus vulgaris) with hemagglutinating activity and speculated that this protein was an albumin. Rigas and Osgood (19) in 1955 described a method for the purification of the hemagglutinin obtained from kidney beans. 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 per cent nitrogen, 50.4 per cent total reducing substances estimated as glucose, was heat-labile and very soluble in distilled water. The protein behaved as a homogenous 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 per cent and its total reducing substances 3.4 per cent. 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 the protein form was a non-toxic, powerful hemagglutinin of human erythrocytes and those of the horse, pig, dog, cat, rabbit, chicken and frog.

The findings of Hungerford et al (6) and Nowell (7) that PHA obtained by the method of Rigas and Osgood (19) has the remarkable property of inducing blastogenesis and mitosis when incubated with human peripheral leukocytes stimulated further attempts to separate the various active constituents of PHA and to better characterize their chemical structure. In 1962, Punnett et al (20) reported that they were able to separate two active constituents obtained from the cotyledons of Phaseolus vulgaris, one capable of agglutinating erythrocytes and the other capable of inducing blastogenesis in tissue culture.

In 1963, Punnett and Punnett (21), after extensive fractionation, isolated a blastogenic factor of macromolecular dimensions, free of hemagglutinating activity. This preparation was, however, impure. The activity was stable to heat (50°-70°C for 5 minutes), stable to 0.1 N hydrochloric acid at 0°C for several weeks, but not at 18°C for 24 hours and the activity of the macromolecule was destroyed on treatment with metaperiodate. From their findings, they suggested that the blastogenic and hemagglutinating factors are different molecules or separable portions of one macromolecule. Genest (22) fractionated PHA by ethanol precipitation and observed that the hemagglutinating and mitogenic activities were in the fraction precipitated by 30 per cent ethanol. The agglutinating activity could be removed by Seitz filtration.

Barkhan and Ballas (23), in 1963, working with PHA prepared from red kidney beans, showed that the agglutinating activity of PHA could be removed by absorption with red blood cells without a concomitant loss of mitogenic activity, suggesting that the mitogenic and agglutinating activities are possessed by different entities.

However, when they attempted to separate the mitogenic and agglutinating principles by starch block electrophoresis, they could only recover the hemagglutinating activity. Presumably, the mitogenic activity was destroyed during the fractionation procedure. Removal of the erythrocyte agglutinating activity of PHA was also achieved by Robbins (24). He incubated PHA with packed red cells at 4° C for 25 minutes, centrifuged the cell suspension and repeated the absorption a second time. He found that the supernatant solution did not possess erythrocyte agglutinating activity but had retained essentially all of the blastogenic and mitogenic activity of the original preparation.

Hastings et al (25) observed that when leukocytes were incubated with PHA, mitoses occurred in white blood cell clumps suggesting that this leukoagglutination could be partially responsible for the mitotic activity. Kolodny and Hirschhorn (26) found that absorption of PHA with red cells removed only the erythrocyte-agglutinating property without affecting the leukoagglutinating and mitogenic activities of PHA. However, when PHA was absorbed with white blood cells all three activities were removed. The authors concluded that there was an apparent relationship between the mitotic and white blood cell clumping activities.

The complexity of the crude preparation of PHA was shown by Spitz (27) who observed eight lines of precipitation on immunoelectrophoretic analysis of PHA. Borjeson et al (28), in 1964, purified a mitosis-stimulating factor from the phytohemagglutinin of red kidney beans which was of protein or polypeptide nature and contained six per cent carbohydrate. Maximal numbers of mitoses were induced in cultures of growing lymphocytes with only 0.2 ug/ml of culture medium.

The hemagglutinating property was still present but could be removed by absorption with red blood cells without any detectable loss of the mitosis-stimulating activity. Rigas and Johnson (29), in 1964, obtained a phytohemagglutinin in the protein form which was homogeneous by ultracentrifugation, continuous-flow curtain electrophoresis at pH 8.6, starch gel electrophoresis at pH 8.0, polyacrylamide disc electrophoresis and chromatography on Sephadex G-200 and on DEAE cellulose. The molecular weight of this material was of the order of 128,000 and it possessed both agglutinating and mitogenic activities. An important finding was that the results of the mitogenicity assays of fractions obtained by chromatography suggested the existence of more than one mitogenic substance. Tunis (30), in 1964, isolated an additional cytoagglutinin from kidney bean extracts which was inactive against human red blood cells, but capable of agglutinating nucleated cells.

It has been found by several investigators (31,32,33) that PHA has the ability to precipitate with human serum and sera of a number of animal species. Holland and Holland (33) showed that the hemagglutinating and precipitating activity of PHA did not appear to be associated with the lymphocyte-stimulating factor in that loss of precipitating ability and marked decrease in hemagglutinating titre were not associated with a change in the ability of the PHA to stimulate blood lymphocytes cultured in vitro. Surrey et al (34) isolated several highly purified leukocyte growth factors from red kidney beans. However, all the blastogenic fractions possessed hemagglutinating activity. One minor protein component exhibited strong leukoagglutinating, but no blastogenic or hemagglutinating activity. Rigas et al (35) in an very comprehensive study, isolated a protein phytohemagglutinin of Phaseolus vulgaris (PPHA)

which was apparently homogeneous and had a molecular weight of approximately 128,000. The PPHA molecule was of unusual complexity as it dissociated slowly into eight subunits in 8.0 M urea at neutral pH. It was found to have both hemagglutinating and mitogenic activities, each located on a different subunit.

Rivera and Mueller (36) presented evidence for the existence of at least three separate and distinct activities of PHA which affect lymphocytes: a leukoagglutinin, a factor inducing RNA synthesis and a factor inducing DNA replication (mitogenic factor). Takahashi et al (37), in 1966, purified a phytohemagglutinin of the wax bean (Phaseolus vulgaris) and found that the proteins isolated had physico-chemical properties different from those described for the phytohemagglutinin isolated from the red kidney bean. However, they did not assay their preparation for mitogenic activity. Weber et al (38), in 1967, attempted a chromatographic fractionation of a commercially available preparation of PHA, PHA-P (Difco Laboratories, Detroit, Michigan, U.S.A.) and obtained three peaks: the first peak completely lacked lymphocyte stimulating and agglutinating activities, the second peak possessed strong leukoagglutinating and lymphocyte-stimulating, but no erythroagglutinating activity and the third peak was strongly erythroagglutinating and also possessed leukoagglutinating activity. The latter fraction stimulated lymphocytes to undergo blastogenesis but to a lesser degree than the second peak. These experiments demonstrated that PHA contains at least two different lymphocyte-stimulating substances, which differ from each other in their chromatographic behaviour and their ability to agglutinate human blood cells.

2.4 MORPHOLOGIC CHANGES INDUCED BY PHA IN VITRO

2.4.1. LIGHT MICROSCOPY STUDIES - The small lymphocyte, the predominant cell at the beginning of culture, contains a large nucleus with somewhat condensed chromatin and only a small ring of basophilic cytoplasm. Within 24 hours of culture in the presence of PHA a type of lymphocyte is seen which is larger in size and whose nucleus in more leptochromatic and might even contain a nucleolus. The cytoplasm of this cell is more extensive and more basophilic as compared to the small lymphocyte. Between 48 and 72 hours blast cells appear. These are large cells with deeply basophilic cytoplasm which often contains a clear perinuclear zone which might represent the Golgi apparatus. The nucleus is large with a chromatin pattern which is usually finely stippled although small condensations may also occur and one or more nucleoli are invariably found in these cells (39). The cytoplasm of these blast cells is pyroninophilic (9,40) and shows granules (41) or clear vacuoles (42). These large cells go on to divide and numerous mitotic figures are seen between 48 and 72 hours of culture. The products of their division are not clear at present. There is evidence, on purely morphological grounds from the anaphase figures of the dividing blasts, that indicates that the daughter cell may have the same morphology as the original parent cell (43).

2.4.2 ELECTRON MICROSCOPY STUDIES - Marshall and Roberts (9), in 1962, studied the electron microscopic morphology of the blast cell obtained from human peripheral small lymphocytes after in vitro stimulation with PHA. They found no evidence for an extensive endoplasmic reticulum such as is found in plasma cells.

The authors suggested that the granules and vacuoles seen by light microscopy in the cytoplasm of the blast cells could probably be interpreted in electronmicrographs as being ribosomes and lipids, respectively. The authors noted the similarity of their electron microscopic studies with these reported by Binet and Mathe (44) who studied the blast cell seen in lymph nodes during a graft-versus-host response. Tanaka et al (45), in 1963, described the ultrastructure of the blast cell seen after 72 hours in culture in the presence of PHA. They noted that nucleoli were always prominent with the cytoplasm containing abundant ribosomes and mitochondria. Endoplasmic reticulum was frequently present in the form of small vesicles and the Golgi apparatus was usually well developed. Inman and Cooper (46), in 1963, incubated human peripheral blood and thoracic duct lymphocytes with PHA for three days. Electronmicrographs of the blast cells showed abundant mitochondria. The greatly increased cytoplasmic content of ribosomes indicated active protein synthesis and the highly developed Golgi apparatus was interpreted as reflecting activity associated with cell division. Elves et al (47), in 1964, in similar studies noted an endoplasmic reticulum in the form of rough-surfaced vesicles and sacs and a well developed Golgi apparatus with smooth-surfaced vesicles. They claimed that the most prominent feature of the PHA-induced blast cell was the presence of large electron-opaque osmiophilic bodies, membrane bounded, which seemed to bear some relationship to the endoplasmic reticulum or Golgi vesicles. They also noted that the PHA-induced blast cell is morphologically indistinguishable from cells described by others in lymphoid tissues of animals during host-versus-graft and graft-versus-host reactions.

Inman and Cooper (48), in 1965, studied the relation of ultrastructure to DNA synthesis in the human lymphocytes stimulated with PHA in vitro. The lymphocytes were obtained from peripheral blood and thoracic duct lymph. They noted that cells synthesizing DNA possessed more nucleoli, mitochrondria and elaborate Golgi zones when compared to blast cells not synthesizing DNA. The cells which were synthesizing DNA presented with trace amounts of rough endoplasmic reticulum, increased micropinocytosis and vesicles probably containing glycogen. In some cultures, cells were observed which contained osmiophilic bodies bounded by a membrane in the cytoplasm. These bodies described previously by these authors (48) are probably similar to those described by Elves et al (47). Chapman (49) noted that although endoplasmic reticulum can be detected in most blastoid cells, it is limited in extent. The endoplasmic reticulum showed a paucity of attached ribosomes in contradistinction to the dense packing of ribosomes on the membranes of the endoplasmic reticulum in normal protein-synthesizing and secreting cells. The reason for this restricted development of the endoplasmic reticulum in the PHA stimulated cell is obscure. This particular point was emphasized by the work of Michalowsky et al (50). Lymphocytes from patients immunized with small-pox vaccine were incubated in vitro with this antigen and the blast cells obtained at the termination of culture were compared to the blast cells obtained in response to PHA. Under the electron microscope, the most striking difference was that the rough-surfaced endoplasmic reticulum was much more developed in the antigen stimulated cells.

2.5 METABOLIC CHANGES INDUCED BY PHA IN VITRO

2.5.1 EFFECT ON NUCLEIC ACID METABOLISM - After adding PHA to the lymphocyte cultures, several metabolic alterations occur in the cells undergoing blastogenesis. The dynamics of the lymphocyte response to PHA is well established. Through the use of radioactively-labelled precursers, the synthesis of protein, DNA and RNA has been studied by several investigators in PHA stimulated lymphocyte cultures. McIntyre and Ebaugh (51) showed that RNA synthesis begins within the first 24 hours of exposure to PHA. Cooper and Rubin (52) showed that the earliest change in cultures of lymphocytes with PHA was a decrease in total RNA, detectable after 30 minutes of exposure to PHA. After 1 hour, accelerated synthesis of RNA, shown by incorporation of tritiated uridine was detected, and a progressive increase in the rate of synthesis continued for 24 hours. The uptake and radioautographic localization of H^3-5-uridine, a specific precurser of RNA, were reported by Hayhoe and Quaglino (53), by Winter and Yoffey (54) and by Torelli et al (55). Kay (56) showed that six hours after the addition of PHA a more sustained uptake of H^3-uridine became apparent and the rate of incorporation continued to increase until 48 hours. This was associated with a marked increase in the labelling of ribosomal and transfer RNA. Cooper and Rubin (57) showed that when lymphocytes were stimulated to enlarge and divide by treatment with PHA, most of the rapidly synthesized RNA was non-ribosomal. Mueller and Mahieu (58) have shown that RNA production increased exponentially as soon as PHA was added to the cells and established the exact time-curve of the period of RNA synthesis. The analysis of the newly synthesized RNA by centrifugation in sucrose gradients revealed that the synthesis of all classes of RNA was stimulated by PHA.

DNA synthesis begins 24-36 hours after the addition of PHA. This has been demonstrated by the incorporation of tritiated thymidine (59-62) and P^{32} (51,63) into newly formed DNA. Yoffey et al (64) described in detail, by means of radioautography, the morphological alterations andnuclear DNA labelling with H^3-thymidine, in lymphocytes cultured with PHA.

2.5.2 EFFECT ON PROTEIN SYNTHESIS

Protein synthesis in human lymphocyte cultures incubated with PHA has been described by Bach and Hirschhorn (65), by Elves et al (66) and by Parenti et al (67). Mueller and Mahieu (58) found a sequence of RNA synthesis followed by protein synthesis and DNA synthesis. Sell et al (68) found that after in vitro stimulation of rabbit peripheral lymphocytes with PHA, the sequence of events was: protein synthesis, RNA synthesis, histologic blast transformation, DNA synthesis and mitosis, with the complete sequence requiring 48 hours. Kay (56), studying the effects of PHA on human lymphocyte cultures showed that PHA caused an increase in protein synthesis as determined by the incorporation of C^{14}-leucine. This increase followed the early stimulation of H^3-uridine incorporation, but preceeded the stimulation of ribosomal RNA synthesis. Van Furth et al (69), using the technique of immunofluorescence, reported that normal human peripheral lymphocytes are capable of synthesizing IgG, IgA and IgM when cultured in the absence of any stimulating agent. It is not surprising, therefore, that Elves et al (66), using the fluorescent antibody technique, observed that PHA stimulated human lymphocytes could be stained with fluorescein-conjugated anti-human globulin. Bach and Hirschhorn (65) demonstrated protein production in lymphocytes incubated with PHA by adding C^{14}-labelled amino acids to the cultures and measuring the radioactive C^{14}-incorporation into protein. They found that in the presence of PHA, five million lymphocytes produced 10 ug of protein at the end of 24 hours of culture.

When the medium of these cultures was analysed by immunoelectrophoresis and radioautography, precipitation bands were noted in the γ_1A, γ_2 and γ-globulin regions. By immunofluorescence studies they found that virtually all the cells stimulated by PHA fluoresced with fluorescein-conjugated anti-human 7 S γ-globulin. Some cells showed cytoplasmic fluorescence and others only nuclear fluorescence. They also showed that lymphocytes taken from an individual with multiple myeloma when stimulated with PHA produced the specific myeloma protein in vitro (65). Forbes and Henderson (70), using the same technique as that used by Bach and Hirschhorn (65) also observed immunoglobulin synthesis by human lymphocytes cultured with PHA in vitro. However, neither Pass et al (71) nor Balfour et al (72) were able to detect immunoglobulin synthesis by humal peripheral lymphocytes either in the presence or absence of PHA in vitro. Coulson and Chalmers (73) incubated human peripheral lymphocytes in a serum-free medium and observed that PHA was able to induce blastogenesis in over 50 per cent of the surviving cells. By the two-stage fluorescent-antibody technique they were unable to detect any IgG in the transformed cells.

In cultures of lymphocytes obtained from patients with rheumatoid arthritis and stimulated by PHA, Bartfeld and Juliar (74) found that the cells contained rheumatoid factor demonstrable by the fluorescent antibody technique.

Forbes (75) cultures lymphocytes from one patient with Hashimoto's disease in the presence of PHA and human thyroglobulin. Both of these materials were able to induce the lymphocytes to synthesize specific precipitating antibody and non-specific globulins.

2.5.3 EFFECT ON ENZYME SYNTHESIS -

- Various cytochemical studies have been carried out in lymphocyte cultures stimulated with PHA. Hirschhern et al (76) showed that before mitosis (48 to 72 hours), a sharp increase in acid phosphatase activity occurred in cells stimulated with phytohemagglutinin. Histochemical examination of these cells demonstrated that innumerable granules containing acid phosphatase developed in the cytoplasm before mitosis. It would appear, they state, that the PHA stimulus induces the formation of lysosome-like structures before mitosis. Quagline et al (77) and Gough and Elves (78,79) observed an increase in the content of lactic dehydrogenase in the transformed cells. Gough (80) showed that PHA-transformed cells are negative for lipids, for alkaline phosphatase, for non-specific esterase and for phosphorylase. He found a moderate number of granules positive for 5-nucleotidase. There was some tendency for this enzyme to be localized in cytoplasmic vacuoles in the PHA transformed cells.

2.5.4. EFFECT ON CARBOHYDRATE METABOLISM -

- Quagline and Cowling (81), Quagline, Hayoe and Flemans (77) and Gough and Elves (78,79) 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, suggesting that the cells obtain some of their energy for transformation from glycolysis. This interpretation is supported by the findings of Cooper et al (59), who found a high rate of lactate production in PHA stimulated lymphocytes, which indicated a high rate of glycolysis, and of Pachman (82), who observed an increase in glucose

and oxygen consumption and lactic acid production during 24 hours of culture of equine peripheral lymphocytes with PHA. Hrachovic (83) showed that the addition of PHA to cultures of rat peripheral lymphocytes had a stimulatory effect on the rate of glucose-U-C^{14} oxidation.

MacHaffie and Wang (84) showed that glucose metabolism in intact lymphocytes was altered in the presence of PHA. The operation of the pentose phosphate pathway and to some extent the pentose cycle pathway in lymphocytes was found to be significantly enhanced.

2.6 CELL LOCALIZATION OF PHA

Michalowsky et al (85) studied the cellular localization of the mitogenic principle of PHA in leukocyte cultures. PHA-P (Difco) was labelled with fluorescein isothiocyanate and incubated with human peripheral leukocytes. During the early period of the culture (4 to 9 hours) a distinct cellular localization of fluorescent material was found confined to the nuclei of the majority of the lymphocytes but not in the granulocytes. This localization of fluorescein-conjugated PHA did not change during the entire three day culture. The authors suggest that the labelled mitogenic factor enters the cell and resides inside the nucleus. On the other hand, Razavi (86), also using fluorescein-conjugated PHA, observed fluorescence in white cells of all types during the initial period of culture. Subsequently, the fluorescence was localized to the cytoplasm of the blast cell and was not associated with nuclear structures or the cytoplasmic membrane. Conard (87) obtained tritium-labelled PHA by growing red kidney bean plants (Phaseolus vulgaris) in nutrient solution containing tritiated water. This PHA was added to cultures of normal human blood leukocytes and the results were evaluated by radioautography. Most of the leukocytes were labelled after 4 - 6 hours of culture. At first, the label appeared to be mainly in the cytoplasm but after 2 - 3 days labelling was predominant in large blast cells and mainly localized to the nuclei.

Rieke (88) labelled PHA-P (Difco) with I^{125} and added it to cultures of rat thoracic duct lymphocytes. By radioautography he found that one hour after exposure radioactivity was localized to cell membranes and to a lesser extend within the cytoplasm of the lymphocytes.

As blast cells developed, radioactivity was commonly noted on nuclear membranes and in apparent Golgi zones. Greenland and Oppenheim (89) studied the localization of mercury-labelled PHA (Burroughs Wellcome) in lymphocytes by electron microscopy. Electron-dense spots were seen in greatest numbers in the cytoplasm of lymphocytes but were also, though more rarely, present in the nucleus. This localization of PHA in the lymphocytes was observed in cells from specimens examined as early as one hour after the addition of the PHA and its localization did not alter up to 72 hours. In all of the studies referred to above, it must be emphasized that the PHA preparations used for the localization studies were highly heterogeneous in terms of biologically-active and inert constituents. It is therefore impossible to state catagorically, as several of the authors did, that the localization of label is synonymous with localization of the blastogenic factor since any or all of the constituents of the PHA preparation could have been labelled.

2.7 BLASTOGENIC AND MITOGENIC ACTION OF PHA ON LYMPHOID CELLS FROM NORMAL HUMANS IN VITRO

2.7.1. PERIPHERAL WHITE CELLS - The effects of PHA on cultures of human peripheral leukocytes are well established (7,9,39,42,63,64,90-99). The morphological, metabolic and cytochemical behaviour of the cells in the presence of PHA were discussed above. They are also well described in the excellent reviews by Robbins (24) and by Bach and Hirschhorn (41).

There is good evidence from the reports of Marshall and Roberts (9), MacKinney et al (61), Hastings et al (25) and Tanaka et al (45) that the proliferating blast cells in cultures of human peripheral blood originate from small lymphocytes. Robbins (24) and Lindahl-Kiessling and Book (100) reported a normal response to PHA in cultures of peripheral lymphocytes obtained from foetuses and newborns. Pisciotta et al (101) analyzed the PHA response in peripheral lymphocytes obtained from healthy individuals ranging in age from 1 to 98 years. The ability of the lymphocytes to undergo blast transformation in response to PHA stimulation appeared to reach a maximum at puberty and to decline uniformly with age thereafter. The replacement of autologous with homologous plasma did not alter the blastogenic response of the lymphocytes. These data suggested that the diminished in vitro blastogenic response with age may be attributed to a cellular, rather than a humoral,factor.

2.7.2 THORACIC DUCT CELLS - Cooper (102), Cooper et al (40) and Lindahl-Kiessling and Werner (103) obtained appreciable cell transformation by incubating human thoracic duct cells with PHA in vitro. Thoracic duct lymph usually contains only lymphocytes with the small variety constituting approximately 90 per cent of the cell population.

2.7.3. LYMPH NODE CELLS - Rubio and Zajicek (104) reported that 70 - 90 per cent of the cells aspirated from human lymph nodes and cultured in vitro with PHA transformed to blast cells after three days of incubation. Winkenstein and Craddock (105) cultured human lymph node cells obtained during surgery with PHA and observed a normal response, similar to that manifested by peripheral blood lymphocytes. Similar results were reported by McIntyre and Segel (106).

2.7.4 THYMUS CELLS - Bain and Gould (107), Lischner and Punnett (108) and Wilson (109) obtained successful cultures of cells from human thymus in the presence of PHA. The thymus was obtained from foetuses (20-40 weeks of gestation) and from infants up to one year of age. McIntyre and Segel (106) and Stresselli (110), however, failed to obtain transformation of human thymus cells cultured in vitro with PHA.

In 1965, Claman (111) cultured human thymus cells in vitro with and without PHA. During the initial few hours of culture in the absence of PHA, 5 - 7 per cent of the cells took up tritiated thymidine. This ability to spontaneously incorporate tritiated thymidine waned with time and by 48 - 72 hours of culture the cell population was composed almost solely of small cells. In contrast, suspensions of cells exposed to PHA showed increasing percentages of large cells after longer periods of incubation and by 72 hours approximately 40 per cent of the cells were large blastoid cells which had incorporated tritiated thymidine. The data suggested the existence of two populations of cells in the human thymus, one capable of synthesizing DNA spontaneously for a short period of time and the other a "resting" population of small cells many of which can be transformed into large blast cells in the presence of PHA. Experiments of a similar nature were carried out by Winkenstein and Craddock (105) who stated that the labelling index of the PHA-stimulated thymus cultures was less than the spontaneous rate of proliferation of thymocytes during the initial period of culture.

These observations suggested that the normal human thymus contains at least two populations of lymphoid cells: a major component which shows autonomous and unsustained proliferative activity and does not respond to PHA and a second minor cellular component which transforms and proliferates in response to PHA.

2.7.5 TONSILLAR CELLS - Oettgen et al (112) studied the in vitro behaviour of cultured human tonsillar lymphocytes. In comparison with peripheral blood lymphocytes, these cells showed a higher degree of transformation to large cells and mitosis in control cultures in the absence of PHA. When cultured with PHA, the percentage of transformed cells was high, similar to that observed in cultures of peripheral blood lymphocytes. On the other hand, Goldfarb and Ullal (113) did not obtain blast cell transformation upon incubating human tonsillar lymphocytes with PHA.

2.7.6 SPLEEN CELLS - Bain and Gauld (107) obtained spleen cells from foetuses and infants. Satisfactory responses were obtained when these cells were stimulated with PHA in vitro. Similar results were observed by Cenen and Erkman (114).

2.8 BLASTOGENIC AND MITOGENIC ACTION OF PHA ON LYMPHOID CELLS OF NORMAL ANIMALS IN VITRO

2.8.1 PERIPHERAL WHITE CELLS

RATS - Knight et al (115) reported that rat peripheral lymphocytes survived rather poorly in cell culture in vitro. Only 10 to 20 per cent of the rat lymphocytes remaining after three days were transformed with PHA. Marshall and Roberts (120) and Williams and Ray (121) were unable to demonstrate transformation in in vitro cultures of rat peripheral blood in the presence of PHA. Elred and Schrek (116), however, found that rat peripheral lymphocytes responded better to PHA when incubated in the presence of rabbit serum. Metcalf (117) showed that rat peripheral lymphocytes did not respond to PHA when cultured with rat plasma, but were able to transform well in cultures containing rat or human serum. These findings were confirmed by Rieke and Schwartz (118). Johnson et al (119) found that diffusion chambers placed in the ventral abdominal wall of rats provided an optimal in vivo environment. Under these conditions they obtained successful blastogenic responses to PHA with rat peripheral lymphocytes.

Metcalf and Osmond (122) reported stimulation of rat blood cells by PHA in vitro and by radioautographic analysis concluded that the small lymphocyte was the cell stimulated to grow and replicate DNA.

MICE - Knight et al (115) reported a poor survival with only occasional transformed cells in cultures of mouse lymphocytes in the presence of PHA. Neither Elves (39), Marshall and Roberts (120) nor Willard et al (123) were successful in obtaining survival of mouse lymphocytes in culture.

However, using diffusion chambers placed into isologous hosts, Willard et al (123) observed some transformation under the influence of PHA. Macario (124) obtained a good proportion of blast cells when he cultured mouse peripheral lymphocytes in the presence of PHA, with maximum transformation occurring at 24 - 48 hours of culture.

GUINEA PIGS - Knight et al (115) reported that guinea pig lymphocytes could be stimulated to transform into blast cells by PHA in vitro. Blasts were present at 24 - 48 hours but less frequently at 72 hours. Similar results were obtained by Sabesin (125) who observed that the addition of PHA to cultures of guinea pig lymphocytes induced a blastogenic response in 75 per cent of the cells by 72 hours of culture. Aspegren and Rorsman (126) and Nichols and Levan (127) were also able to obtain blast transformation of guinea pig lymphocytes cultured with PHA. On the other hand, Marshall and Roberts (120) could not demonstrate such transformation with guinea pig peripheral lymphocytes.

RABBITS - Knight et al (115) observed a marked stimulation of rabbit peripheral lymphocytes incubated in vitro with PHA. Similar results were reported by Sabesin (125), Nichols and Levan (127), Fikrig et al (128) and Sell et al (68). Williams and Ray (121) and Marshall and Roberts (120), however, did not observe blast transformation in rabbit lymphocytes after in vitro stimulation with PHA.

MONKEYS - Knight et al (115) reported that blood lymphocytes from rhesus monkeys responded markedly to in vitro stimulation by PHA to a degree comparable to that obtained with human lymphocytes.

HAMSTER - Knight et al (115) were unable to obtain blast transformation by incubating hamster peripheral lymphocytes with PHA in vitro.

2.8.2 THORACIC DUCT CELLS

RATS - Rieke and Schwartz (118), Rieke (129) and Metcalf (117) were able to demonstrate blast formation in cultures of rat lymphocytes derived from the thoracic duct lymph in response to PHA. However, blastogenesis was completely inhibited if the serum was replaced by rat plasma in the culture medium.

RABBITS - Fikrig et al (128) showed that rabbit lymphocytes obtained from the mesenteric lymph undergo transformation when cultured in vitro in the presence of PHA.

2.8.3 LYMPH NODE CELLS

RATS - Metcalf (117) incubated rat lymph node cells with PHA in the presence of either rat serum or human plasma. A large number of blast cells was obtained at 72 hours of culture. These results were confirmed by Metcalf and Osmond (122) who incubated their cells with PHA and tritiated thymidine and subsequently examined the transformed cells by radioautography.

RABBITS - Foft and Ramere (130) observed blast cell transformation in cultures of lymphocytes obtained from rabbit lymph nodes after stimulation with PHA in vitro.

2.8.4 SPLEEN CELLS

RATS - Elrod and Schrek (116), Metcalf (117) and Metcalf and Osmond (122) demonstrated the production of blast cells in cultures of rat splenic lymphocytes in the presence of PHA.

RABBITS - Harris and Littleton (131) and Schrek and Batra (132) demonstrated that rabbit splenic lymphocytes are capable of undergoing blastogenesis in the presence of PHA in vitro.

CHICKENS - Weber (133) cultured spleen cells from 4 - 10 week old white Leghorn chickens in the presence of PHA and observed a marked blastogenic response after 48 hours.

2.8.5 THYMUS CELLS

RATS - Schwartz and Rieke (134) showed that small lymphocytes obtained from rat thymus and cultured in vitro with PHA were transformed into enlarged cells. A progressive increase in responsiveness was detected as the age of the donor animal increased from five hours to 32 days. Thymic cells from newborn animals failed to respond to PHA. Metcalf (117) and Metcalf and Osmond (122) also found a good response of rat thymic lymphocytes to PHA in vitro.

PIGS - Weber (135) studied the in vitro response to PHA of lymphocytes obtained from pig thymus by the incorporation of tritiated thymidine into the DNA of the transformed cells. Within the first hour of culture 6 - 18 per cent of the lymphocytes were synthesizing DNA. During the following 24 hours a sharp decrease in the number of DNA synthesizing cells was observed. This was followed by a definite peak in DNA synthesis and mitotic response in a minority of the cells between 48 - 54 hours. With organ culture techniques, Weber (136) showed that the lymphocytes capable of responding to PHA are located predominantly in the medulla of the thymus.

RABBITS - Schrek and Batra (132) reported that PHA caused slight to moderate enlargement of a few rabbit thymic lymphocytes into cells which were presumably lymphoblastoid.

CHICKENS - Weber (133) cultured thymus cells from 4 - 10 week old chickens with PHA and observed many blast cells by 48 hours of culture.

2.8.6 APPENDIX CELLS

RABBITS - Lymphocytes obtained from rabbit appendix did not transform to blast cells when cultured with PHA in vitro (132).

2.8.7 BURSA OF FABRICIUS CELLS

CHICKENS - Weber (133) was unable to observe any blast cells in cultures of Bursa cells in the presence of PHA.

2.9 BLASTOGENIC AND MITOGENIC ACTION ON HUMAN LYMPHOCYTES IN VITRO :DISEASED STATES

2.9.1 HYPOGAMMAGLOBULINEMIA AND AGAMMAGLOBULINEMIA -- Elves et al (137) observed a decrease in the degree of transformation to blast cells when lymphocytes from a child with congenital agammaglobulinemia were cultured with PHA in vitro. Lymphocytes of patients with chronic lymphatic leukemia associated with hypogammaglobulinemia also presented with a depressed in vitro response to PHA but those from leukemic patients with normal γ-globulin levels exhibited a normal response. In their report, Elves et al (137) suggested a correlation between the decrease in the concentration of the serum gamma globulin and the impaired in vitro response of the lymphocytes to PHA. Their results and hypothesis were however not confirmed by several other investigators (see below). Furthermore, the failure of cord-blood lymphocytes to respond to PHA in vitro as observed by Elves et al (137) was not confirmed by Robbins (24) and Lindahl-Kiessling (100) who observed a normal response.

Robbins (24) showed that leukocyte cultures from patients with hypogammaglobulinemia responded normally to PHA, i.e., 70 per cent of the cells were blast cells after three days of incubation. Rudenberg and Hirschhorn (138) also reported that peripheral leukocytes obtained from agammaglobulinemic patients incubated in vitro with PHA or streptolysin S showed a degree of blast transformation and mitosis comparable to normals. Especially pertinent was their failure to detect gamma globulin in the transformed agammaglobulinemic lymphocytes. They also stated that 10 - 15 per cent of the transformed agammaglobulinemic cells morphologically resembled plasma cells. Based on these findings, they suggested that the absence of plasma cells in agammaglobulinemia is not in itself responsible for the failure of antibody production but is rather the morphologic concomitant of the primary defect (failure of antibody production on exposure to antigenic stimulus).

Termey et al (140) found a decreased synthesis of RNA and DNA in PHA-stimulated agammaglobulinemic lymphocytes. As the presence of an inhibitor in the patient's sera could not be demonstrated, they suggested that the basic defect in agammaglobulinemia is cellular rather than humoral. Cline and Fudenberg (139) cultured lymphocytes from normal and agammaglobulinemic individuals in the presence of PHA and also observed a markedly decreased synthesis of RNA in the agammaglobulinemic, as compared to normal lymphocytes. From their data, they suggested that, in primary agammaglobulinemia, the defect involves an abnormality of quantitative RNA synthesis or RNA stability in the circulating lymphocyte.

2.9.2 LEUKEMIAS - Newell (141) observed that peripheral lymphocytes obtained from patients with chronic lymphatic leukemia (CLL) incubated with PHA in vitro for three days exhibited a marked decrease in the number of blast cells as compared to normal controls. Similar findings were reported by Schrek and Rabinowitz (142), Quaglino and Cowling (81), Bernard et al (143) and Robbins (24). The latter investigator, Oppenheim et al (144), Trubowitz et al (145), Elves (146) and Elves et al (147) all attributed the failure of CLL lymphocytes to undergo transformation in vitro in the presence of PHA to a defect in the cell since they could find no evidence for a plasma inhibitor. Only Astaldi et al (148) reported that the impaired response to PHA of CLL cells is due to a plasma factor. Quaglino et al (149) suggested that the defect in the CLL cell is a failure to synthesize glycogen which is necessary to provide the energy requirements for blast transformation. The correlation between impaired in vitro response to PHA and immunological abnormalities in patients with CLL was suggested by Oppenheim et al (144) and Elves (146). Winter et al (150) cultured lymphocytes of seven CLL patients, who were being treated with either irradiation and/or prednisone, with PHA in vitro and observed that very few of these cells transformed into blasts. They suggested that the failure of these cells to transform in response to PHA

might be the result of cell damage caused by treatment of the patient rather than attributable to an intrinsic defect in the leukemic cell.

However, Sharman et al (151) and Elves et al (147) observed a similar in vitro response to PHA by lymphocytes of CLL patients whether of not they were undergoing immunosuppressive therapy. Robbins (24) suggested that there exist two types of lymphocytes in the CLL blood: The leukemic lymphocytes which do not respond to PHA comprising more than 97 per cent of the total lymphocytes and the normal lymphocytes which react to PHA in vitro comprising less than three per cent of the total lymphocyte population. The relationship between a low lymphocyte response to PHA and a high lymphocyte count in CLL was stressed by Elves et al (147), Bernard et al (143) and Oppenheim et al (144). The dual population of lymphocytes in CLL was reinforced by the finding of Kagan and Johnson (152) who reported that lymphocytes from patients with CLL who had been treated with total body irradiation gave a normal response to PHA in vitro, although their response to PHA was greatly impaired prior to treatment. These patients also presented with improvement in their clinical and hematological pictures subsequent to irradiation treatment. Elves et al (137) showed that CLL patients with normal circulating γ-globulin had a normal in vitro response to PHA. However, the same authors (147) with another series of CLL patients found no correlation between the degree of transformation and the level of γ-globulin in the plasma. This is in agreement with the results of Oppenheim et al (144) and do not support the finding of Schrek (153) that hypogammaglobulinemia in the leukemic patient is associated with poor capacity of the blood lymphocytes to transform under the stimulus of PHA.

Lymphocytes obtained from patients with chronic granulocytic leukemia (154), acute lymphocytic leukemia (155,156), acute granulocytic leukemia (156) and chronic myeloid leukemia (147) manifested a normal blastogenic response when incubated in vitro with PHA.

2.9.3 HODGKIN'S DISEASE - Hersh and Oppenheim (157) noted that lymphocytes from patients with Hodgkin's disease have a depressed in vitro response to PHA. Clinical evaluation of the patients suggested that this impairment was related to the severity of the disease and to in vivo anergy. They also found that manipulations of lymphocyte concentration, ratio of lymphocytes to neutrophils and plasma source did not impart to Hodgkin's lymphocytes the capacity to respond in a normal fashion. Similar results with lymphocytes from patients with Hodgkin's disease were reported by Hirschhorn et al (158), Aisenberg (159), Holm et al (160) and Trubowitz et al (145). However, a normal response to PHA of lymphocytes from Hodgkin's patients was reported by Elves et al (147).

2.9.4 SARCOIDOSIS - Hirschhorn et al (158) noted that cultured peripheral blood lymphocytes from patients with sarcoidosis showed a diminished blastogenic response to PHA in vitro. They related their finding with the depressed immunological features of sarcoidosis. Bucley et al (161) also found a depressed in vitro response to PHA in lymphocytes from patients with sarcoidosis but they noted that this impairment was transitory and paralleled the clinical severity of the disease. Lymphocytes from patients during remission had a normal response to PHA as compared to markedly diminished response when harvested from the patients during periods of exacerbation.

2.9.5 ATAXIA TELANGIECTASIA - The impairment of the in vitro response to PHA of lymphocytes from patients with ataxia telangiectasia was reported by Leikin et al (162), Oppenheim et al (163), Naspitz et al (164) and Hayakawa and Kobayashi (165). Schuler et al (166) found a normal blastogenic response to PHA in vitro with lymphocytes obtained from one patient with ataxia telangiectasia. Oppenheim et al (163) claimed that ataxic cells responded more vigorously to PHA in vitro when incubated in the presence of homologous rather than autologous plasma.

This finding was not supported by the work of Naspitz et al (164) who showed that ataxic cells were equally depressed in their response to PHA in the presence of autologous or homologous plasma. Similar findings were reported by Leikin et al (162),. Naspitz et al (164) suggested that a relationship exists between the impaired delayed hypersensitivity manifested by the patients with ataxia talangiectasia and the failure of their lymphocytes to respond to PHA in vitro.

2.9.6 LIVER DISEASE - Mella and Lang (167) noted that there was an inhibition of mitosis when leukocytes from patients with acute infectious hepatitis were cultured in vitro with PHA. Tobias et al (168) found that peripheral lymphocytes of patients with liver disease manifested a normal degree of blast transformation when incubated in vitro with PHA. Similar results were reported by Heenig and Possnereva (169) with lymphocytes of cirrhotic patients. Winters et al (170) and Holt et al (171) observed a variable response when blood lymphocytes from patients with idiopathic steatorrhea and cirrhosis were cultured in autologous plasma with PHA. Failure to transform into blast cells and to synthesize DNA was complete in some cases. Washed cells from the patients, however, did transform well in response to PHA when incubated in the presence of homologous normal plasma, suggesting the existence of a PHA inhibitor in the plasma of the patients.

2.9.7 BURKITT'S TUMOUR - Pulvertaft (172) in a study of Burkitt's tumour in patients in Africa noted the similar proliferation and morphology of the tumour cells and PHA treated normal lymphocytes in tissue culture in vitro. He suggested that the high incidence of this tumour in that region of Africa may be related to the local tradition of feeding infants with bean pap.

2.9.8 CONGENITAL RUBELLA - Olsen et al (173) presented data which showed that peripheral lymphocytes obtained from babies with the congenital rubella syndrome did not undergo in vitro transformation to blast type cells in the presence of PHA. They suggested the presence of the virus in the embryonic lymphoid system as a possible explanation for this in vitro depressed response.

2.9.9 MYASTHENIA GRAVIS - Housley and Oppenheim (174) studied the in vitro behaviour of leukocytes obtained from patients with myasthenia gravis. They added PHA, various antigens and skeletal muscle and thymus preparations to their cultures and measured the response to these stimuli. The lymphocytes of all the patients thymectomized and non-thymectomized, had a normal blastogenic response to PHA and no response was obtained with any of the antigens, muscle or thymus.

2.9.10 INFECTIOUS MONONUCLEOSIS - Pearmain and Lycette (175) incubated leukocytes from patients with mononucleosis for ten hours without addition of PHA. Numerous mitotic figures were observed, whereas none were apparent in smears prepared before incubation. Rubin (176) also observed that lymphocytes of infectious mononucleosis patients cultured in vitro without PHA proliferated rapidly and synthesized RNA at 10 times the rate of normal, unstimulated lymphocytes. One hour exposure of these lymphocytes to PHA resulted in marked suppression of 4 to 30 S RNA synthesis but only a slight depression of total RNA synthesis, which is in sharp contrast to the stimulation of all RNA synthesis in normal lymphocytes by PHA. Rubin (176) concluded that although it is not clear why PHA treatment induced an inhibition of RNA synthesis in infectious mononucleosis lymphocytes there was a basic difference in the RNA metabolism between these cells and normal lymphocytes.

Inman and Cooper (48) noted the similar untrastructural organization between cultured cells from normal individuals in the presence of PHA and cultured cells from patients with infectious mononucleosis in the absence of PHA.

2.10 BLASTOGENIC AND MITOGENIC ACTION OF PHA ON HUMAN LYMPHOCYTES IN VITRO: OTHER HUMAN CONDITIONS

2.10.1 POSTOPERATIVE STATES - Riddle and Berenbaum (177) noted that the percentage of lymphocytes in the peripheral blood undergoing transformation in response to PHA in vitro was much decreased post-operatively in five patients undergoing a variety of surgical procedures. They suggested that the depression in the PHA response was the result of operative trauma and may have been mediated by substances released from damaged tissues or by steroids released by the adrenals in response to stress.

2.10.2 PREGNANCY - Cemings (178) reported that lymphocytes obtained from women during the third trimester of pregnancy and after delivery exhibited a normal response to PHA in vitro. On the basis of this finding, Cemings suggested that one can not attribute the sustaining of the fetus, which is, to all intents and purposes, an intrauterine graft of foreign tissue, to a major intrinsic defect in the lymphocyte.

2.10.3 RENAL HOMOTRANSPLANT RECIPIENTS - Rubin et al (179) studied patients who had received kidney homografts and were being treated with immunosuppressive drugs. When suppression was effective, the lymphocytes from these patients manifested a poor response to PHA in vitro. In one of the patients there was an increase in the number of blast cells in PHA stimulated cultures several days before the appearance of clinical evidence of rejection. Raising the dosage of immunosuppressive drugs decreased the response in vitro and eliminated signs of rejection. The authors suggested that the in vitro response to PHA may be of value in detecting the onset of escape from immunosuppression. However, Joseph (180) found normal responses to PHA in cultures of lymphocytes from renal hemotransplant patients whether or not they were experiencing a rejection episode. All patients were receiving prednisone and imuran.

2.11 ACTION OF PHA IN ANIMALS IN VIVO

2.11.1 TOXICITY OF PHA

Goddard and Mendel (3), in 1929, injected their own preparation of phytohemagglutinin into rabbits (8mg per kg body weight) and mice (600mg per kg body weight) and observed that it was not toxic in either of these animal species. Elves reported that PHA was non-toxic in C3H mice when doses as high as 500 mg/kg were injected intraperitoneally (39). No histopathologic studies were carried out in the above studies. However, Norins and Marshall (181) found that the intracardiac injection of 100-300 mg PHA (Wellcome) killed all of twelve mice, three rats and eight guinea pigs injected. Lower doses of PHA in these animals caused a illness which was marked by muscular weakness, some degree of peralysis, slumped and hunched posture and inactivity. Papac (182) injected 0.5 ml of PHA-M intravenously in C57BL mice and found it to be lethal for 10 of 24 animals. Naspitz et al (183) found that one bottle of PHA-P (80mg) injected intravenously in rabbits was lethal for 25 - 40 per cent of the animals. However, when this amount of PHA was injected intravenously in four divided doses, at 15 minute intervals, less than 10 per cent of the rabbits died.

Schrek and Stefani (184) noted that the intradermal injection of PHA into guinea pigs induced an erythema in 24 hours whereas rats failed to react to the intradermal injection of PHA. Lycette and Pearmain (185) injected four guinea pigs intradermally with 0.05 ml of PHA and noted a small zone of erythema which developed within three hours and persisted for up to 18 hours.

2.11.2 HISTOLOGIC CHANGES FOLLOWING PHA ADMINISTRATION

Elves et al (186) injected PHA-M intraperitoneally and subcutaneously into rats at weekly intervals for four weeks at which time the animals were sacrificed and the spleens examined histologically.

No abnormal changes were noted in the tissues examined nor was there any alteration in the white cell counts. Gamble (187) studied the effects of intravenously administered PHA-M on the mouse spleen. He observed an increase in spleen weight which reached a maximum on day three following the PHA administration. The number of nucleated cells in the spleen increased as well following the PHA injection, reached a maximum on day four and decreased rapidly over the next two days. The number of cells in mitosis paralleled the curves obtained for nucleated cells. The white pulp of the spleen contained numerous blast cells on day three and four but by day five there were many areas of degenerating cells. This study, as well as that of Elves et al (186), was however limited to changes in the spleen only.

Golub and Weigle (188) injected PHA-M intravenously into mice to determine whether it can facilitate the induction of immunological tolerance to inocuous protein antigens. They observed that PHA had no effect on the induction of a state of immunological unresponsiveness but it did provoke an intense proliferation in spleen cells which was maximum at day three. Sarkany and Caron (189) and Sarkany (190) injected PHA intradermally into guinea pigs followed by an injection of desacetylmethylcolchicine. By histological analysis they showed that PHA had a mitogenic effect on epithelial cells of guinea pig skin in vivo which resembled more closely the picture seen in an allergic rather than an irritant reaction.

2.11.3 EFFECT OF PHA ON ANTIBODY FORMATION - Several studies have been carried out with respect to the role of PHA in the immune response. Singhal et al (191) observed an enhanced formation of circulating antibody in the rabbit following PHA administration.

Rabbits were injected with three antigens simultaneously, BSA (bovine serum albumin), BGG (bovine gamma globulin) and sheep erythrocytes, and were given a single intravenous injection of PHA-M (approximately 80 mg) either seven days or three days before, simultaneously with, or three days or seven days after, the injection of the antigens. The rabbits responded with an enhanced and accelerated primary antibody response to all three antigens when PHA was injected three days prior to or simultaneously with the antigens. The immune response in the other PHA injected rabbits did not deviate from that observed in the non-PHA-injected animals. Gamble (192) carried out a similar experiment with mice rather than rabbits and with different antigens - HGG (human gamma globulin) and rat erythrocytes. He found that the prior injection of PHA facilitated an enhanced immune response to the rat erythrocyte but a depression in the response to HGG. Elves (193) injected PHA (Burroughs) intraperitoneally into Wister rats on three successive days followed by the injection of chicken erythrocytes. A marked suppression in the circulating titer of chicken red cell hemagglutinin was observed in these rats relative to controls.

Using the hemolytic plaque technique of Jerne (347), Spreafico and Lerner (194) reported that the intraperitoneal administration of PHA-P simultaneously with, or prior to, the injection of sheep erythrocytes into mice induced a marked depression of both the primary and secondary immune responses. On the other hand, PHA given after the antigen led to no modification of the immune response. Knight et al (195) reported that the injection of PHA (Burroughs) at the same time or 24 hours prior to the injection of sheep red blood cells into mice induced a marked suppression of antibody production. However, PHA administered after the antigen enhanced the antibody response and this effect was most consistently obtained when PHA was injected two days after the red cells.

All injections were administered via the intraperitoneal route. Elves (196) studied several immunological reactions in PHA-treated mice and rats. When PHA was injected prior to the antigen there was a depression of antibody production to chicken erythrocytes, Salmonella and Proteus. PHA injected after the antigen had little effect on the subsequent antibody titer. However, Elves observed that the route of PHA administration relative to the administration of the antigen was critical. Only when both were given by the intraperitoneal rouge was there any significant impairment of the immune response suggesting that PHA in the peritoneal cavity was capable of obstructing the systemic absorption of the antigen. In the same study, using mice and rats, Elves (196) found that the intraperitoneal injection of PHA into the recipient of a skin homograft did not alter the rejection time. He also noted that the injection of spleen parental cells into the young adult hybrids produced fatal graft-versus-host reactions, but when the doner's spleen cells were first treated with PHA in vitro they were unable to induce a graft-versus-host reaction in the recipient mice.

Mekori et al (197), Feldman and Mekori (198) and Micklem (199) studied the immunological capabilities of intrasplenic clones obtained by the injection of lymph node cells from PHA treated mice into irradiated recipients mice. Micklem noted extensive hepatic necrosis in more than half of the recipient mice.

Markley et al (200) injected PHA-P intraperitoneally into rabbits each of which had an allograft placed on one ear and an autograft on the other ear. PHA-P increased the survival time of these skin allografts from 6.9 days to 15.2 days.

2.11.4 RELATION TO IMMUNOSUPPRESSANT THERAPY

Phytohemagglutinin has been used in combined immunosuppressive therapy in dogs with renal homotransplants. Calne et al (201) reported that PHA administered systemically into dogs which received renal homotransplants demonstrated some immunosuppressive activity when given alone and potentiated the immunosuppressive action of azathioprine (imuran) when these two agents were given together. PHA produced no toxic effect of its own on the marrow. Richter and Mandl (202) noted that PHA is capable of neutralizing the lethal effects of 6-Mercaptopurine (6-MP) administration. Fifty to seventy per cent of the rabbits injected daily subcutaneously with 6-MP (10mg/kg/body weight/day) died within the first twenty days of the experiment whereas only one of eleven rabbits injected daily with PHA (1/4 bottle PHA-M) and 6-MP died.

2.11.5 PROTECTION TO IRRADIATION

The value of PHA in protection from irradiation in vivo has also been investigated. Robinson (203) and Stefani (204) observed a protective effect to irradiation in both the rat and mouse when PHA was administered prior to whole body irradiation. Papac (182) did not observe this radio-protective effect of PHA in mice and rats. However, she injected the PHA after irradiating the animals.

2.11.6 EFFECT ON TUMOUR SURVIVAL

Robinson (205) investigated the effect of PHA administration on tumour cell survival in mice. He injected ascites tumour cells intraperitoneally into mice which were given PHA, in a single injection, at various times prior to and subsequent to the tumour cell injection. Seven days later the peritoneal cavity was washed and the volume of packed cells obtained was determined. The lowest number of tumour cells was obtained when PHA was injected 72 hours before tumour transplantation. PHA had no protective effect when administered 120 hours before tumour transplantation, suggesting that the action of PHA is a transient one. Rubio and Unsgaard (206) injected Erlich's ascites cells into PHA-treated CBA mice. Tumour "takes" occurred in 95 percent of the PHA-treated animals and in 90 per cent of the control animals. They concluded that PHA did not affect the rejection of allogeneic tumour cells.

2.12 ACTION OF PHA IN HUMANS IN VIVO

2.12.1 SKIN REACTIONS FOLLOWING INTRADERMAL ADMINISTRATION -

Lycette and Pearmain (185) skin-tested seven volunteers with an intradermal injection of 0.05 ml of PHA-M. A zone of induration about the injection site, surrounded by an area of erythema, appeared within 15 minutes. On the other hand, Schrek and Stefani (184) found that the intradermal injection of 0.001 ml of PHA in 7 individuals produced a delayed reaction with maximum erythema and induration at 24 hours. Caron (207) reported that synthesis of DNA by adult human epidermal skin maintained in organ culture was increased in the presence of PHA. Aire et al (208) skin tested 38 volunteers and 11 patients with chronic lymphatic leukemia (CLL) with PHA (Wellcome). The response in all the individuals tested had the characteristics of delayed type hypersensitivity. Only 7.3 per cent of the peripheral lymphocytes of CLL patients cultured in vitro with PHA transformed to blast cells whereas the corresponding figure for the normal controls was 64.5 per cent. The finding in the same patient of a positive delayed skin test to PHA and a failure of the lymphocytes to respond to PHA in vitro conflicts with the results reported by Schrek and Stefani (184), who concluded that the in vitro reaction of human blood cells to PHA may be interpreted as a delayed hypersensitivity reaction.

2.12.2 PHA AS A CHEMOTHERAPEUTIC AGENT IN APLASTIC ANEMIA -

The parenteral use of PHA in humans has initially been limited to the treatment of aplastic anemia. Humble (209) in 1963, was the first to use PHA intravenously in four cases of aplastic anemia. In 1964, Humble (210) treated two more cases and described the results of PHA treatment in these six cases. Evidence of stimulation of bone marrow function was seen in all cases and two patients recovered with a normal peripheral blood picture .

The malignant disease process present in two cases was not activated by the PHA treatment. The marrow depression in these six patients was secondary to known causes. In 1966, Humble (211) reported that eight of sixteen patients with aplastic anemia, treated with PHA, showed recovery of hemopoietic function. Retief et al (213), on the other hand, reported no beneficial effects in six patients with aplastic anemia treated with seven daily intravenous injections of PHA. Fleming (212) reported similar negative findings with one patient with aplastic anemia. Aksoy et al (214) injected PHA into three patients with aplastic anemia but their results are difficult to evaluate as they administered androgens and corticosteroids simultaneously with the PHA. Hayes and Spurr (215) administered PHA to two patients with idiopathic aplastic anemia and one with myelofibrosis. One patient with aplastic anemia showed increased marrow erythroid activity; the others had no response. Bushor and Von Deschwanden (216) treated one patient with aplastic anemia, probably induced by chloramphenicol, with an infusion of PHA and reported the cure of the patient. Gruenwald et al (217) treated three patients with varying degrees of bone marrow hypoplasia with PHA but the results obtained did not permit definite conclusions. Astaldi et al (218) reported some improvements in a patient with primary aplastic anemia treated by the intra-bone marrow injection of autologous peripheral lymphocytes which had been incubated with PHA in vitro for 48 hours. Fleming et al (219) treated one patient with aplastic anemia by a method similar to that of Astaldi et al (218) without any positive results. Similar negative results using this same treatment were reported by Thiele and Von Wichert (220) with one patient with aplastic anemia.

Mehra et al (221) treated one patient with aplastic anemia secondary to phenylbutazone with intravenous PHA. The treatment was without obvious benefit. Baker and Oliver (223) also treated

one patient with aplastic anemia induced by phenylbutazone. After seven injections of PHA the patient returned to normal hematologic conditions. Gurling and Leonard (222) also reported some improvement in one case of phenylbutazone-induced aplastic anemia.

2.12.3 PROTECTION TO THE EFFECTS OF IMMUNOSUPPRESSANT DRUGS

Recently, Israel et al (224) reported that the intravenous injection of PHA into 27 cancer patients on chemotherapy permitted the administration of a greater dose of antimetabolite drugs without the accompanying leucopenia. This finding supports the results obtained by Richter and Mandl using rabbits (202).

2.12.4 PROTECTION TO IRRADIATION

Stefani and Donnely (225) reported, in a preliminary study, the possible in vivo radioprotective effect of PHA on bone marrow. Four patients with bronchogenic carcinoma received localized irradiation of whom two received PHA by infusion during a five day period commencing prior to the irradiation. They noted an increase in the percentage of lymphocytes in the bone-marrow in the PHA treated patients.

2.13 ANTIGENICITY OF PHA

Marshall and Norins (226) immunized two rabbits with their own preparation of PHA and analyzed the antisera for their antibody content to the various constituents of PHA. By immunoelectrophoresis they detected some precipitation between the α and β globulins of normal serum and the bean extract whereas after immunization there was precipitation also between γ-globulin and the bean extract, the immune serum precipitating with 5 - 7 components of the bean extract. Immune rabbit sera inhibited the blastogenic effect of PHA when added to cultures of human peripheral leukocytes. Similar inhibitory activity was detected in serum from a patient with aplastic anemia who had been treated with PHA. Marshall and Norins (226) also reported that immediate hemorrhagic skin reactions and anaphylactic shock could be produced in guinea pigs previously immunized with the bean extract emulsified in Freund's adjuvant by the subsequent injection of PHA. Spitz (27) injected rabbits with an extract of red kidney beans which he prepared with Freund's adjuvant. He found eight precipitin lines by immunoelectrophoresis. Nordman et al (227) detected at least six antigens in PHA-P (Difco) by immunoelectrophoresis using immune rabbit serum. Byrd et al (228,229) immunized rabbits by the intravenous administration of PHA-M (Difco). The globulin fractions of the immune rabbit sera were isolated and found to have an inhibitory effect on the mitogenic action of PHA in vitro. Inhibition of cell growth as measured by the mitotic rate was found to be directly proportional to the amount of antiserum incubated with PHA-M.

Marshall and Melman (230) studied the antibody response to the mitogenic factor in sheep and man. One sheep was immunized with an intravenous injection of PHA and sera were obtained from four patients treated for aplastic anemia by the intravenous injection of PHA.

The presence of the antibody was demonstrated by its capacity to inhibit the blastogenic action of PHA.

Astaldi et al (231) injected PHA intravenously into three patients with aplastic anemia. Before treatment, the peripheral lymphocytes of all three patients were PHA-responsive. Two to three weeks after the injections of PHA, the lymphocytes from the same patients cultured under the same conditions failed to react to PHA. The plasma obtained from the three patients at this time, when added to cultures of normal homologous human lymphocytes, inhibited their in vitro response to PHA. The authors suggest that the intravenous injection of PHA in man causes the formation of circulating antibodies capable of neutralizing the blastogenic action of PHA. There can therefore be no doubt as to the antigenicity of PHA in both man and animal and of the capacity of antisera to neutralize the specific mitogens in PHA, aside from the reaction of the antisera with other non-biologically active constituents of PHA.

Richter and Naspitz (232) failed to induce tolerance to PHA in rabbits. Newborn rabbits were injected twice with 80 mg of PHA-M at age three and six days without a single mortality. At age 4 - 6 weeks, the rabbits were sacrificed and the lymph node cells from these animals were incubated in vitro with PHA. After three days of incubation a normal blastogenic response was observed with 40 - 70 per cent of the cells having transformed to blasts. The authors concluded that either the concentration of the blastogenic factor was very low in the whole PHA solution used and therefore insufficient quantities of the active principle were injected into the neonatal rabbits or that tolerance could not be induced to the mitogen.

2.14 OTHER IN VITRO STUDIES WITH PHA

2.14.1 INHIBITION OF PHA-INDUCED BLASTOGENESIS BY VARIOUS AGENTS IN VITRO -

2.14.1.1 CHLOROQUINE - Hurvitz and Hirschhorn (233) observed that chloroquine is capable of inhibiting the response of cultured human lymphocytes to PHA. In the low doses used, this effect was seen only if the drug was added early in culture. Addition of chloroquine after four hours or more of culture was ineffective.

2.14.1.2 MYCOPLASMA HOMINIS - Copperman and Morton (234) showed that sufficiently concentrated non-viable Mycoplasma hominis type I (PPLO) suspensions inhibit the mitogenic action of PHA in lymphocyte cultures. The inhibition could be reversed by removing the PPLO as late as 48 hours after inoculation. Mitosis could be initiated with PHA, stopped by PPLO and recommenced without further addition of PHA by changing the medium.

2.14.1.3 HORMONES - Stefani and Oester (235) showed that the blasteid transformation produced by PHA in human blood lymphocytes in vitro was suppressed by prednisolone when the latter was added to the culture medium 24 hours before PHA. Similar results were obtained by Elves and Israel (95). This effect was markedly reduced or lost when the corticosteroid was introduced into the culture after PHA (235). Tormey et al (236) measured the synthesis of RNA and DNA in cultures of peripheral human lymphocytes in response to PHA. Prednisolone - 21 phosphate, in a concentration greater than one gamma exerted a profound inhibition of nucleic acid synthesis when it was added to the culture simultaneous with PHA. However this inhibitory capacity of the steroid was markedly depressed if added to the culture subsequent to PHA. On the other hand, Cowling and Quaglino (237) reported that the addition of hydrocortisone (2-20mg/ml) to cultures of peripheral lymphocytes simultaneous with the addition of PHA did not inhibit the PHA induced blast transformation.

Inhibition occurred only with concentrations of hydrocortisone known to produce lysis of lymphocytes (100 mg/ml).

These findings suggest that PHA may be capable of imparting to the lymphocytes a resistance to the cytocidal activity of steroid.

2.14.1.4 IMMUNOSUPPRESSANT DRUGS - Hersh and Oppenheim (238) studied the in vitro transformation to PHA of lymphocytes obtained from patients with ocular and malignant diseases receiving chemotherapy. Before treatment, the PHA stimulated cultures showed 71 per cent blast cells but during chemotherapy the number of cells which transformed decreased to 1.5 per cent. This inhibition of the in vitro response was attributed to intrinsic damage to the lymphocyte and not to antimetabolites in the plasma. Intensive in vivo therapy for as short a period as three days with parenteral 6-mercaptopurine and methotrexate, with or without prednisolone, completely abolished the capacity of the lymphocyte to undergo in vitro transformation. Substantial recovery of in vitro blastogenesis occurred within three days after the termination of therapy. Non-toxic therapy with methotrexate or 6-mercaptopurine, which did not induce leukopenia, took 2 - 5 weeks to cause maximum suppression of the blastogenic response in vitro. Actinomycin-D added to lymphocyte cultures stimulated with PHA stopped gamma globulin production, after two hours of incubation with this drug (239). Mitomycin-C has also been shown to be capable of inhibiting DNA synthesis in lymphocytes cultured in vitro (240, 241).

2.14.1.5 EPINEPHRINE - Nowell (242) showed that epinephrine inhibited blastogenesis when added to cultures of human peripheral lymphocytes in the presence of PHA and maintained for the entire duration of the culture (3 days). Epinephrine added only during the final 24 hours of culture had no effect on the mitotic activity.

2.14.2 PROTECTION TO THE EFFECTS OF IMMUNOSUPPRESSANT DRUGS BY PHA - Schrek and Stefani (243) and Stefani and Schrek (244) noted that PHA had a protective effect against the deleterious effects of nitrogen mustard on lymphocytes in vitro. The addition of 1ug/ml of nitrogen mustard to a culture tube containing normal cells killed the entire population. In the PHA-treated suspensions, 40 per cent of the cells were alive after treatment with 1ug/ml of nitrogen mustard and 17 per cent after incubation with 10 ug/ml of nitrogen mustard. The cells were protected as long as PHA was added at any time from four days to one hour before nitrogen mustard. When PHA was added 24 hours after the addition of nitrogen mustard some protection was still observed.

2.14.3 PROTECTION TO THE EFFECTS OF IRRADIATION BY PHA - - Schrek and Stefani (243,245) reported that only 10 per cent 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. In the PHA treated lymphocytes, 2400 r had the same cytotoxic effect as 75 r on the untreated cells. Radioprotection of lymphocytes was observed as long as PHA was added at any time from four days before to two days after irradiation. Stefani and Oester (235) showed that the radioresistance induced by PHA in human blood lymphocytes in vitro was suppressed when prednisolone was added to the culture 24 hours prior to PHA.

2.14.4 INDUCTION OF INTERFERON FORMATION BY PHA - - Wheelock (246) showed that PHA induces the formation of an inhibitor of the cytopathologic effects of Sindbis virus in human leukocyte cultures. The physicochemical and biological properties of this virus inhibitor are similar to those of interferon induced by Newcastle disease virus.

2.14.5 CAPACITY TO FACILITATE VIRUS REPLICATION

Edelman and Wheelock (247) reported that the replication of vesicular stomatitis virus in human leukocyte cultures can be enhanced 6 to 180-fold by treating the cultures with PHA prior to virus inoculation. Their data suggest that a substance is produced in PHA-treated leukocyte cultures which is capable, on transfer to fresh leukocytes, of inducing blast cell formation and enhancing virus replication. The ability of PHA to induce replication of virus in human leukocyte cultures was also reported by Nahmias et al (248) with herpes simplex virus and by Duc-Nguyen and Henle (249) with mumps virus.

2.14.6 PHA-INDUCED ENHANCED SURVIVAL OF LYMPHOCYTES IN VITRO

Several attempts to maintain normal lymphocytes in a continuous cell culture have resulted in failure (250,251). Favier et al (91) added PHA to cultured cells on the 11th day of culture and obtained a typical blastogenic response. Foft and Romero (130) observed that the addition of PHA to the medium and changing the medium every four days resulted in a more prolonged survival of the lymphocytes with an average survival time of 11 days. Naspitz and Richter (252) demonstrated that human peripheral lymphocytes could be adequately maintained in long term culture, in a viable state, by the incorporation of a subthreshold concentration of PHA (non-blastogenic dose) into the medium and changing the medium every seven days. These cells, after 42 days of culture, were capable of undergoing blastogenesis and mitosis upon incubation with blastogenic doses of PHA.

2.14.7 PHA-INDUCED CYTOTOXICITY IN VITRO

Lymphocytes from sensitized animals are able to adhere to and aggregate about cells in culture which carry the specific antigen to which the lymphocyte donor had been previously sensitized.

This aggregation causes destruction of cells within 24-48 hours (253,254). In 1964, Holm et al (255) showed that normal lymphocytes from unsensitized animals damaged allogenic cells in tissue culture to which they were aggregated by PHA. Injury of the target cells was not seen in the absence of PHA nor when the PHA-treated lymphocytes were separated from the target-cells by a millipore filter. Similar results were obtained by Bach and Hirschhorn (41) who showed that lymphocytes added to a monolayer of fibroblasts destroyed that monolayer in eight days whereas, in the presence of PHA, lymphocytes destroyed the fibroblasts in four days.

Holm and Perlmann (258), in 1965, reported that PHA-treated allogeneic or xenogeneic lymphocytes, but not syngeneic lymphocytes, were capable of damaging foetal rat kidney cells in culture. Killed lymphocytes had no effect. They suggested that this cytotoxic effect of normal lymphocytes in the presence of PHA reflects an activity exerted by immunologically competent cells.

In a similar study, in 1967, Holm and Perlmann (257) measured the cytotoxic effect of normal human blood lymphocytes on Chang cells (human foetal liver) in tissue culture by the release of Cr^{51} from pre-labelled target cells. In control cultures, 25 per cent of the Cr^{51} was released. When PHA was added to the system, 50 - 60 per cent of the isotope appeared in the medium. Contamination with erythrocytes or granulocytes did not interfere with the cytotoxic action of the lymphocytes.

Holm et al (160) obtained peripheral blood lymphocytes from patients with Hodgkin's disease and chronic lymphatic leukemia and tested their cytotoxicity to Chang cells in the presence of PHA.

The cytotoxicity of the patient's lymphocytes was significantly lower than that of lymphocytes from healthy donors, thus paralleling the impaired blastogenesis obtained when the patient's lymphocytes were incubated in vitro with PHA.

Using the same system, Holm and Perlmann (258) demonstrated that aggregation of the lymphocytes to the target cells was not necessary in order to obtain destruction of the target cells. Human lymphocytes pretreated with a filtrate of staphylococcus aureus or preincubated with allogeneic lymphocytes manifested streng cytotoxic effects. This same effect was obtained when lymphocytes from a tuberculin-positive donor were prestimulated with PPD in vitro. Additive cytotoxic effects were seen when two stimulants were applied at the same time. The mechanism by which in vitro cell injury and death is effectuated by non-specifically stimulated normal human lymphocytes is at present unknown.

2.15 BLASTOGENIC AND MITOGENIC STIMULANTS OTHER THAN PHA

Following the description of the blastogenic and mitogenic activities of PHA in 1960, several investigators observed that other substances have the same properties when cultured in vitro with white blood cells. The mechanism of action of these other mitogenic agents will be discussed below.

2.15.1 SERUM FACTORS

Sabesin (259) cultured small lymphocytes from rabbits and guinea pigs in vitro without PHA and observed blast transformation in 40-70 percent of the peripheral lymphocytes on the fifth day of cultures in the presence of either fetal calf or autologous serum. On the other hand, Johnson and Russell (260) found that fetal calf serum, but not autologous serum, in the culture medium was blastogenic to lymphocytes incubated without PHA. Similar findings were reported by Woodliff and Onesti (261). However, Caron (262) observed no spontaneous blastoid transformation when the cells were cultured for 1 - 7 days in the presence of autologous or fetal calf serum. Pulvertaft and Pulvertaft (263) reported similar results but they noted spontaneous blastogenesis in cultures of lymphocytes obtained from the umbilical cord-vein. They suggested that this latter transformation is probably due to the mixture of maternal and infant lymphocytes. Gill (264) found some degree of spontaneous transformation in cultures of lymphocytes obtained from penicillin-sensitive patients during episodes of allergic drug reations.

2.15.2 ANTIGENS AND ALLERGENS

2.15.2.1 HUMAN CELLS

Specific antigens have the property of stimulating peripheral blood lymphocytes obtained from the specifically-immunized individual to undergo blastogenesis and mitosis.

In contradistinction to PHA which induces the enlargement of 80 per cent or more of the small lymphocytes in three days of culture, specific antigens stimulate the enlargement of 5 to 40 per cent of the cells after 5 - 10 days of incubation. The significance of this transformation, which provides an in vitro method for the diagnosis of hypersensitivity, will be discussed later, together with a discussion on the mechanism of action of PHA and other mitogens.

In 1926, Timofejewsky and Benewolenskaja (265) suggested that it might be possible to differentiate leukocytes derived from immune=and non-immune donors by a study of the reaction of the cells to microbes and toxins in an in vitro system. In 1927, they reported that human lymphocytes and monocytes transformed into polyblasts and epithelioid cells in the presence of tubercle bacilli (266). Pearmain et al (267), Schrek (268), Hirschhorn et al (97), Marshall and Roberts (120) and Cowling et al (269), showed that the addition of tuberculin purified protein derivative (PPD) to cultures of lymphocytes from individuals sensitive to tuberculin induced the appearance of blast cells and mitosis. The in vitro mitogenic action of PPD on lymphocytes of tuberculin-positive individuals was further confirmed by Coulson and Chalmers (270), Hartog et al (271), Gump et al (272), Rauch (273), McFarland and Heilman (274), Caron (275), Heilman and McFarland (276,277), by Vischer (278) and by Mayron and Baram (279). Aspergren and Rorsman (280), in 1964, observed that tuberculin in culture medium stimulated mitosis in leukocytes of 14 of 20 tuberculin-positive patients and of 4 of 10 tuberculin-negative persons. However, they pointed out that the number of mitoses in lymphocytes of tuberculin-negative patients tended to be lower than in those from the tuberculin-positive individuals. Heilman and McFarland (277) and Ricci et al (281) showed that cultures of peripheral lymphocytes obtained from patients with active

tuberculosis were not transformed after addition of PPD, thus confirming the earlier findings of Pearmain et al (267). Heilman and McFarland (277) found that the inhibitory activity was the property of the tuberculosis serum or plasma.

Lycette and Pearmain (185), in 1963, cultured lymphocytes from individuals immunized with poliomyelitis antigen. The addition of 0.2 ml of Sabin vaccine to the cultures produced blast transformation and mitosis in all cases. Elves et al (66,282) in 1963, observed that tetanus toxoid, typhoid-paratyphoid vaccine (TAB), Hemophilus pertussis vaccine, diphtheria toxoid, polievirus vaccine and small-pox vaccine were able to induce blastogenesis when added to cultures of lymphocytes obtained from patients sensitized to these antigens. That same year, Hirschhorn et al (239) reported similar results using diphtheria toxoid, pertussis vaccine and penicillin as antigens. In 1964, Ling and Husband (63) added a number of antigens (TAB, Tetaxus toxoid, PPD, and endotoxin) to cultures of lymphocytes from sensitized patients. No blastogenesis was observed with TAB or with the endotoxins.

In 1965, Cowling and Quagline (237) determined the optimal dilutions for various antigens (old tuberculin, diphtheria toxoid, TAB vaccine and tetanus toxoid) to induce blastogenesis in cultures of human peripheral lymphocytes after seven days of incubation. Mixtures of antigens showed no summation of effect and the transforming activity of PHA was not inhibited by any of the antigens tested.

Lycette and Pearmain (185), in 1963, cultured lymphocytes obtained from five patients with severe grass hay fever.

A polyvalent extract of grass pollens, which gave strongly positive skin tests, was added to the cultures. Blast cells and miteses were observed after 4 - 6 days of incubation although much fewer in number when compared to cultures of PHA stimulated cells. In 1965, Wiener and Brasch (283) reported that the addition of grass-pollen extracts to lymphocytes from sensitized individuals resulted in a significant increase in the number of miteses. This mitogenic effect was inhibited when leukocytes of desensitized patients were used. Zeitz et al (284), in 1966, observed blast transformation in cultures of peripheral lymphocytes obtained from timothy-sensitive individuals when timothy pollen was added to the lymphocyte cultures. The maximum response was obtained at 10 days of incubation. The response was not changed with hyposensitization treatment, thus not supporting the findings of Wiener and Brasch (283). Girard et al (285) were also able to obtain blastogenesis using ragweed, alternaria and penicillin as antigens. The peak response of blast cells was obtained at 5 - 7 days of incubation. On the other hand, Ricci et al (286) were unable to induce blastogenesis in lymphocyte cultures from patients sensitive to Graminacea pollens when this antigen was added to the cultures. Similarly, Mayron and Baram (279) observed only a slight increase in the rate of nucleic acid synthesis when ragweed pollen extract was incubated with lymphocytes of individuals with ragweed allergy.

Allergic reactions to drugs are common but they present a difficult diagnostic problem for there are few satisfactory in vitro tests to detect specific sensitivity. The fact that a large number of antigens possess the capacity to stimulate specific blastogenesis in vitro prompted several investigators to use this technique as an in vitro diagnostic tool for drug hypersensitivity. Hirschhorn et al (239), in 1963, reported that lymphocytes from patients with allergic sensitivity to penicillin, when cultured with this antibiotic showed blastogenesis and mitosis. Holland and Mauer (287), in 1964, reported

drug-induced in vitro stimulation of peripheral lymphocytes in a patient with a sensitivity reaction to diphenylhydantoin. Similar findings were reported by Caron and Sarkany (288) in a patient with sulphonamide sensitivity. Visher (278) studied lymphocytes of patients sensitive to penicillin, phenylbutazone, isoniazid and p-aminosalicylic acid. Using the technique of incorporation of labelled nucleic acid precursors, he was able to demonstrate stimulation of the lymphocytes of only 2 of 13 penicillin-sensitive patients. With the other drugs all cultures were negative. The lymphocytes of all patients responded well to PHA. Girard et al (285) added penicilloyl-polylysine to cultures of lymphocytes from penicillin allergic patients and found that the number of blast cells was greater than in control cultures. Ripps and Fellner (289) studied 17 subjects each of whom gave ahistory of allergic reactions to penicillin. The lymphocytes of only eleven of these individuals manifested a blastogenic in vitro response to penicillin. These patients reacted to the intra-dermal injection of penicillin or penicilloyl-polylysine. Similar results were reported by Ripps et al (290) in one patient sensitive to tetracycline. Gill (264) also obtained blast transformation using penicillin as an antigen. However, he reported that the spontaneous lymphocyte transformation in vitro, in the absence of any stimulant, was greater than with lymphocytes of non-allergic controls. Halpern et al (291), using several drugs including penicillin as stimulants on leukocyte cultures from allergic patients, reported blast transformation after four days of incubation with the lymphocytes of all the 45 patients tested.

However, Woodliff and Onesti (261) could not reproduce these findings in one patient sensitive to penicillin and Sarkany (292) tested 22 different drugs on cultures of lymphocytes from 40 patients sensitive to these drugs and was able to obtain blastogenesis in only seven cultures. Evans (293), in 1967, using penicillyl-polylysine and potassium benzil penicillin G as antigens, found no difference in response between the lymphocytes of control and antibiotic treated patients.

Hashem and Barr (294) obtained blastogenesis in cultures of lymphocytes from 7 of 9 patients with degenerative nervous disease by incubating the lymphocytes with rabies vaccine (rabbit brain) or with a cell-free brain extract prepared from human embryos. This mitogenic activity was induced in cells obtained from only one of six normal controls. Fowler et al (295) incubated cultures of peripheral blood lymphocytes, obtained from patients with multiple sclerosis, with cerebrospinal fluid, the latter serving as the source of antigen (s). They found a significant increase in the percentage of lymphocyte transformation in their patients in comparison to normal controls. Hashem et al (296) studied lymphocytes from children with eczema using extracts of autologous and homologous skin as antigens. The number of blast cells obtained was greater in lymphocyte cultures of the patients as compared to controls. They suggested that this finding supports the concept of autosensitization to skin in this disease. Fjelde and Kopecka (297), in similar studies, showed that autologous skin extracts induce a slight but definite blastogenic response on leukocytes from atopic patients. Pass et al (71) using a much more objective method to detect blast transformation and mitosis i.e the uptake of tritiated thymidine, were unable to reproduce the results reported by both Hashem et al (296) and Fjelde and Kopecka (297).

Patrucco et al (298) cultured lymphocytes from patients with systemic lupus erythematosus (SLE) with DNA. Blast transformation was obtained with the lymphocytes of all of 12 patients with SLE. No relation could be demonstrated between the degree of in vitro response to DNA and the titer of circulating antinuclear antibody.

Hirschhorn et al (158) reported that lymphocytes from patients with sarcoidosis undergo transformation to blast cells in vitro when cultured for eight days in the presence of Kveim antigen. Cowling et al (299) failed to observe stimulation of lymphocytes from patients with sarcoidosis cultured for five days in the presence of Kveim antigen.

2.15.2.2 ANIMAL CELLS

Aspegren and Rorsman (126) sensitized guinea pigs to tuberculin and observed no blast transformation when this antigen was added to cultures of peripheral blood lymphocytes obtained from the sensitized animals. Tuberculin did not inhibit the stimulating effect of PHA on the lymphocytes. On the other hand, in similar studies, Zweiman et al (301,302) obtained opposite results, i.e., blastogenesis and mitosis after addition of PPD to the cultures. Mills (303) immunized guinea pigs with tuberculin, egg albumin and bovine gamma globulin. Only lymphocytes obtained from animals which presented a delayed reaction to these antigens underwent transformation in vitro when cultured in the presence of the specific antigens. Negative results were obtained in cultures of lymphocytes from guinea pigs with circulating antibodies. Similar results were obtained by Oppenheim et al (304) using PPD and guinea pig albumin conjugated to orthanilic-acid as antigens.

Opposite results to those of Mills (303) and Oppenheim et al (304) were reported by Visher et al (305) and by Visher and Stastny (306).

They immunized rabbits with Keyhole limpet hemocyanin and found that cultures of peripheral lymphocytes from these animals, all of whom had demonstrable circulating antibody, proliferated and incorporated tritiated thymidine in the presence of the antigen. Similar results were reported by Dutton and Eady (307) and by Dutton and Bulman (308).

2.15.3 ANTI-LEUKOCYTE SERUM Grasbeck et al (309,310) immunized rabbits by the intravenous injection of a concentrate of human peripheral leukocytes. All the rabbits produced antisera which were mitogenic in cell cultures of human peripheral leukocytes. The antisera had a lytic effect on the cells which was related to the presence of complement. Absorption of the antiserum with leukocytes removed the mitogen. Holt et al (311) also reported that rabbit anti-human leukocyte antisera had a blastogenic effect on human lymphocytes in vitro and that this activity was independent of the source of the cultured human leukocytes.

Knight and Ling (312) reported that two our of nine rabbits immunized with homologous leukocytes produced antisera which caused blast formation and stimulated DNA synthesis in leukocytes of the donor rabbit in vitro. Heterologous antisera obtained by injecting rabbit leukocytes into rats and guinea pigs were cytotoxic to rabbit leukocytes in vitro. These antisera caused leukoagglutination but did not stimulate DNA synthesis and blast formation.

2.15.4 ANTI-PROTEIN SERUM Sell and Gell (313) reported that rabbit lymphocytes may be stimulated in vitro with specific rabbit antiallotype sera to transform into blast cells and to synthesize DNA. This transformation only occurred when the donor cells were obtained from a given gamma globulin allotype (As4) and cultured in the presence of an antiserum prepared against the given allotype (As4).

Heterologous (sheep, goat and guinea pig) anti-rabbit γ-globulin sera also induced significant blast transformation and DNA synthesis in rabbit lymphocytes. In a subsequent report, Gell and Sell (314) stated that this effect could be obtained with specific antisera directed against all six of the well characterized allotypic determinants of rabbit IgG (As1,2,3,4,5 and 6) and with sheep anti-rabbit whole serum (68). Sell and Gell (315) showed that lymphocytes from the peripheral blood of newborn rabbits heterozygous for IgG allotypes As4 and As5, or As5 and As6, obtained at an age when only the maternal allotypic determinants are detectable in the serum, could be stimulated to transform into blast cells when incubated with antiallotype sera directed against the determinants controlled by both the maternal and paternal chromosomes. The in vitro blastogenic response of rabbit lymphocytes incubated with sheep antisera to rabbit IgG subunits, with sheep antisera to rabbit IgA and IGM and with the $F(ab)^2$ and F(ab) fragments of sheep antibody to rabbit IgG was demonstrated by Sell (316-318).

Oppenheim et al (319) immunized monkeys with purified preparations of human immunoglubulins: IgG, IgA, IgM and Bence Jones protein. Each of the antisera incubated with washed human peripheral lymphocytes stimulated DNA synthesis. The significance of these findings will be discussed later.

2.15.5 ALLOGENEIC CELLS - Schrek and Donnelly (320), in 1961, observed a number of large cells with large nuclei and nucleoli in a culture consisting of a mixture of bloods from two patients with hemochromatosis. However, they did not elaborate on this observation. Bain, Vas and Lowenstein (321), in 1964, reported that when leukocytes from two normal, unrelated subjects were mixed together and cultured, some of them transformed into large basophilic cells that could synthesize DNA and undergo mitosis. The blast cell obtained in the mixed leukocyte reaction

was morphologically similar to that obtained following incubation with PHA although it was a less intense reaction and involved changes is a smaller proportion of the lymphocytes. Erythrocytes, plasma or platelets had no effect in the mixed leukocyte reaction. These authors were the first to suggest a relationship of the "mixed leukocyte reaction" to homograft immunity. Hashem and Carr (323) also noted that the mixing of lymphocytes from two unrelated individuals induced the appearance of blast cells and mitoses in vitro with the exception of one pair of identical twins.

The role of the various cell constituents in the mixed leukocyte reaction has been investigated by Jones (322) who observed that the presence of large numbers of polymorphonuclear leukocytes resulted in higher levels of blastoid transformation than when polymorphs were absent.

Kasakura and Lowenstein (325) and Gordon and Maclean (326) reported that the cell-free medium obtained from single or mixed leukocyte cultures was able to induce blastogenesis when added to homologous leukocytes. Kasakura and Lowenstein (327) studied the physical and chemical properties of this blastogenic material and suggested that it may contain transplantation antigens. It has also been demonstrated that blastogenesis can be obtained when extracts of cells from one individual are mixed with intact cells from another individual (328). Hashem (329) noted that ribosomal RNA extracted from peripheral lymphocytes which had been incubated in vitro with the specific antigens to which

the donor had been sensitized was also capable of promoting transformation and mitosis when added to cultures of autologous lymphocytes. In this case, the RNA appeared to be acting as the antigen to stimulate autologous cells to undergo blastogenesis. Hashem and Rosen (330) fractionated cell extracts and added the fractions to cultures of homologous lymphocytes. They found that blastogenesis was induced by the whole-cell extract, cell-wall nuclear fraction and the ribosomal fraction but not by the mitochondrial fraction.

Bain and Lowenstein (324) carried out genetic studies with the mixed leukocyte reaction. By mixing leukocytes in culture from 15 sibling pairs, they noted that the leukocytes of most individuals reacted less strongly with those of their siblings than with those of unrelated subjects. Bach and Hirschhorn (328) observed a correlation between the degree of the blastogenic response in a culture of lymphocytes from two unrelated individuals and the degree of cross-reactivity of grafts from the two individuals placed on a third unrelated recipient. Oppenheim et al (331) and Moynihan et al (332) examined mixed leukocyte cultures of skin graft recipients and a normal donor before, during and after skin transplantation and observed an increase in lymphocyte proliferation and blastogenesis at the time of graft rejection. Silvers et al (333) reported that mixed leukocyte cultures of rats showed blastogenesis only when the donors of the leukocytes differed at the important Ag-B histocompatibility locus.

This difference was associated with the prompt rejection of skin homografts.

In order to determine whether both donor cells in the mixed leukocyte reaction are responding equally, attempts have been made to inactivate the cells of one of the donor pairs. The treatment of the cells of one individual with mitomycin C (240) on with x-irradiation (334) has been shown to result in the loss of their capacity to undergo blastogenesis in mixed leukocyte reactions; however, these cells are able to stimulate homologous untreated cells to undergo blastogenesis. The lymphocyte transformation obtained by mixing lymphocytes from two unrelated individuals has been studied by several other investigators (335-340) and its application as an in vitro histocompatibility test is currently under investigation in a number of laboratories.

2.15.6 ENZYMES — Mazzei et al (341) showed that trypsin, chymotrypsin and papain have mitogenic action on peripheral lymphocytes from normal persons in vitro.

2.15.7 STREPTOLYSIN - S — Hirschhorn et al (342) showed that a nonantigenic product of the B-hemolytic streptococcus, streptolysin S (SLS), has the ability of inducing blastogenesis when incubated with normal human peripheral lymphocytes. This effect of SLS was not observed when lymphocytes were obtained from patients with untreated rheumatic fever or from an occasional patient with streptococcal infection.

2.15.8 STAPHYLOCOCCAL FILTRATE — Ling and Husband (63) found that a staphyleccecal filtrate (SF) induced transformation in most of the peripheral lymphocytes from normal human doners. The effect was very similar to that of PHA. The SF was prepared by growing Staphyleccocus aureus in medium 199 without antibiotic for four days and removing the organisms by filtration.

The stimulating material was thermolabile and non-dialysable.

2.15.9 MICROWAVE IRRADIATION - Stodolnik-Baranska (343) reported that microwave irradiation could induce blastogenesis and mitosis in cultures of human peripheral lymphocytes. After incubation for five days, the percentage of transformed lymphocytes was five times as high as that in control cultures.

2.15.10 POKEWEED - Farnes et al (344), in 1964, reported that extracts of the plant Phytolacca americana, pokeweed, induced transformation of human lymphocytes in vitro. Berjesson et al (345) described the biological properties of pokeweed (hemagglutinating, leukoagglutinating and mitogenic properties) and the physicochemical characteristics of the constituents in the pokeweed preparation. Chessin et al (346) showed that, in pokeweed stimulated lymphocytes in vitro, RNA synthesis preceded the onset of DNA synthesis by 24 hours and that DNA synthesis was maximal at 72 hours of incubation.

2.16 MECHANISM OF ACTION OF PHA AND OTHER MITOGENS IN VITRO

The mechanism whereby PHA induces blastogenesis and mitosis on normal lymphocytes in tissue culture is still highly controversial in spite of the large number of studies which have been carried out over the past few years. Some have alluded to an immunologic basis for PHA action and others suggest a non-immunological mechanism.

The evidence in favor of an immunologic basis for PHA action is as follows. The morphology of the blast cells obtained after PHA stimulation is very similar, both by light and electron microscopy, to that of blast cells induced as a consequence of incubation with specific antigens (see chapter 2.4). Furthermore, blast cells induced by PHA are capable of synthesizing gamma-globulin (65) similar to cells stimulated by antigen in vitro (66). The protection to x-irradiation afforded by PHA in vitro may appear to be a unique property not possessed by antigens in general. However, it has recently been noted that old tuberculin is also capable of imparting radioprotection when incubated with lymphocytes obtained from PPD-positive patients but not when incubated with cells of PPD-negative patients (348). The authors observed a relation between old tuberculin concentration, blastoid transformation and radioprotection and postulated that the radioprotective effect of old tuberculin had an immunologic basis.

The fact that it is essentially the small lymphocyte which is transformed by PHA in vitro and that it has been shown conclusively that the small lymphocyte possesses all the attributes required for immunologic competence (349) cannot go unnoticed. This cell is capable of undergoing blast transformation under a variety of antigenic stimuli, both in vivo and in vitro. In vivo, blast transformation has been observed in contact hypersensitivity reactions (350,351),

graft-versus-host reactions (349) and skin homograft rejection reactions (352). The morphology of the blast cell arising in each of these immune reactions has been found to closely resemble that of the PHA-induced blast cell in vitro (349, 364). Furthermore, 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 (144, 164), suggesting that PHA is indeed measuring the immunocompetence of the lymphocytes. Blastogenesis has also been observed when lymphocytes are incubated with specific antigens in vitro (see chapter 2.15). Blastogenesis has also been observed when lymphocytes are cultured in the presence of anti-lymphocyte and anti-immunoglobulin antisera or when they are mixed in cell culture with lymphocytes of an unrelated, but not from a genetically-identical, individual. (see chapter 2.15). The presence of blast cells in all of these reactions strongly suggests that this cell plays an important role in immunological processes.

The evidence for the non-immunologic action of PHA is as follows. In PHA stimulated cultures the percentage of transformed cells is 70-90 per cent after three days of incubation whereas, in antigen stimulated cultures, only 5-30 per cent of the cells are transformed after 7-10 days of culture (41). No transformation has been observed after only three days of culture with specific antigen. Furthermore, the in vitro transformation with PHA does not require the presence of macrophages (39,353), whereas macrophages are necessary for the antigen-induced blastogenic response (322,353,354). Recently, however, it has been shown that blastogenesis was observed when PPD was incubated with a highly purified suspension of small lymphocytes obtained from PPD-positive patients (270). Moreover, it has also been demonstrated that PHA can stimulate lymphocytes, previously immunized to various protein antigens, to secrete greater amounts of the specific antibody than can the specific antigens in vitro (75,355).

The non-immunological mechanism of PHA transformation is further supported by the findings that PHA can stimulate epithelial cells in the skin (207), free-living amoeba (356) and non-lymphoid cell lines (357).

There is as yet insufficient data to suggest the mechanism of action of other potent blastogenic stimulants such as pokeweed, streptolysin-S and staphylococcal filtrate although it would appear to be non-immunologic in nature.

If the specific blast transformation of sensitized lymphocytes after exposure to specific antigens, by its high degree of specificity, is immunological in nature, a summation of effects should be obtained when lymphocytes from an individual sensitized to more than one antigen are incubated in the presence of these antigens. However, summation of action was not observed by Cowling and Quaglino (237) and by Girard et al (285). Ling and Husband (63) suggested that they obtained an apparent summation of effects with two different antigens, but they did not present any data.

An alternative hypothesis as to the mechanism of PHA action was proposed by Hirschhorn et al (239). They suggested that PHA exerted its action by attaching to some immunologically nonspecific surface structrue on lymphocytes. This idea was fostered by the observation that PHA causes agglutination of lymphocytes and that if this property of PHA is neutralized by absorption of PHA with lymphocytes, the blastogenic activity is lost as well. A similar view was expressed by Grasbeck et al (309,310), who demonstrated that rabbit anti-human leukocyte antiserum in vitro agglutinated and transformed a high percentage of the cultured lymphocytes. The probability that PHA attaches to the cell surface was suggested by Vassar and Culling (358) who showed that the electrophoretic mobility of lymphoid cells was changed after treatment with PHA. The studies

on the cell localization of PHA (see chapter 2.6), although not conclusive, suggest that PHA is present within the cell, and is not absorbed onto the surface of the cell.

The above hypothesis is unlikely since leukoagglutination does not occur when in vitro blastogenesis is induced by antigens, staphylococcal filtrate, allogeneic lymphocytes and specific anti-allotype sera. Furthermore, Borjesson et al (359) showed that human peripheral lymphocytes coated with Vi polysaccharide completely suppressed the agglutination of these cells when PHA was added to the culture. Nevertheless, the normal complement of blast cells was observed at the end of the culture.

Another theory was proposed by Allison and Mallucci (360). They observed that the blast cell obtained in PHA stimulated cultures had an increased lysosome content and suggested that PHA could unstabilize lysosomal membranes. This change in permeability could induce release of enzymes and initiate cell division. Hirschhorn and Hirschhorn (361) pointed out that two substances which mimic the blastogenic action of PHA (streptolysin S and staphylococcal filtrate) also produced release of enzymes from lysosomes. Moreover, they showed that chloroquine and prednisolone, substances which stabilize the lysosomal membrane, inhibited the blastogenic and mitotic action of PHA. They suggested that small lymphocytes under normal conditions are "repressed" and that when PHA is added to the lymphocyte culture a "derepression" occurs, associated with controlled release of lysosomal enzymes which break down the existing RNA. This interpretation is in agreement with the findings of Cooper and Rubin (52) who demonstrated that, within 30 minutes of the addition of PHA to the cell culture, there was a rapid breakdown of existing cellular RNA, followed by synthesis of increased amount of new cellular RNA. This newly synthesized RNA could regulate the transition of the lymphocyte from the resting state to active growth. The new RNA was shown by

Cooper and Rubin (57) to be nonribosomal. However, they observed that when lymphocytes enlarged and divided in response to a specific antigen, ribosomal RNA was the principle product of RNA synthesis and therefore, the nonribosomal RNA seen after PHA stimulation would not appear to be obligatory for growing lymphocytes.

Beckman (32) showed that PHA precipitated with certain proteins of cell homogenates and the plasma medium in tissue culture and suggested that the precipitation of a serum protein which acts as an inhibitor of mitosis could be responsible for the cell proliferation observed in PHA treated cultures. However, Holland and Holland (33) showed that the removal of the precipitating activity of PHA did not change the ability of PHA to stimulate blood lymphocytes in vitro.

It has been demonstrated that although PHA must be in contact with the lymphocytes for 5-10 minutes in vitro in order to induce transformation, it is not consumed or destroyed during this incubation (95,362). Killander and Rigler (363) observed that changes in the deoxyribonucleoprotein content of the lymphocyte are already discernible within this interval of time.

In conclusion, although a great deal of work has been carried out with PHA, both in vivo and in vitro, and the morphology and metabolism of the transformed lymphocytes have been well documented, the exact mechanism of action of PHA remains to be determined.

CHAPTER 3

METHODS AND MATERIALS

ANIMALS

Adult, white New-Zealand rabbits 2 - 3 kg in weight were used throughout this study.

PHYTOHEMAGGLUTININ

Phytohemagglutinin-M (PHA-M) and Phytohemagglutinin-P (PHA-P) were obtained from Difco Laboratories, Detroit, Michigan, U.S.A. The lyophilized PHA in each vial (approximately 80 mg) was dissolved in sterile medium before use.

ANTIGENS

Human gamma globulin (HGG) was obtained from Pentex Incorporated, Kankakee, Illinois, U.S.A.

MEDIUM

Medium 199 was obtained from Microbiological Associates, Bethesda, Md., U.S.A.

RADIOACTIVE THYMIDINE

Tritiated thymidine, specific activity 5000 mc/Mm, was obtained from The Radiochemical Center, Amersham, England.

ERYTHROCYTE AND LEUKOCYTE COUNTS

Erythrocyte (rbc) and leukocyte (wbc) counts were performed in a Fisher Autocytometer, obtained from the Fisher Scientific Co., Montreal, Quebec, Canada. The autocytometer was adjusted daily to give readings corresponding to those obtained by simultaneous hemocytometer determinations.

TANNED CELL HEMAGGLUTINATION TECHNIQUE.

The tanned cell hemagglutination method as described by Boyden (300) was modified in the following manner. Peripheral blood from a normal, non-immunized sheep was collected into an equal volume of Alsever's solution* and stored at 4°C. When red blood cells were required, they were separated by centrifugation and washed three times with cold saline, or until the last washing was colorless.

Normal rabbit serum (NRS), which was obtained from a normal, non-immunized rabbit, was decomplemented at 56°C for 30 minutes and diluted 100 fold with phosphate buffered saline (PBS)**. This solution will be referred as diluent.

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* Alsever's solution:

Glucose	2.05 gm
Sodium citrate	0.80 gm
Sodium chloride	0.42 gm
Citric acid	0.005 gm
Final pH	6.1
Final volume	1 liter

** PBS: Phosphate buffered saline -
phosphate buffered saline (phosphate buffer, pH 7.3 and physiological saline solution 1:1)
0.15 M phosphate buffer, pH 7.3 was prepared by mixing 215 ml of 0.15 M $Na_2 PO_4$ with 49 ml of 0.15 M $KH_2 PO_4$.

A 2.5% suspension of sheep red blood cells in PBS was tanned by mixing it with an equal volume of 1:20,000 dilution of tannic acid, freshly prepared in PBS from a 1:100 stock solution of tannic acid. The mixture of red cells and tannic acid was incubated at 37°C for 10 minutes and the tanned red cells thus obtained were washed twice with PBS and resuspended in PBS at a final cell concentration of 2.5 per cent. The tanned cells were sensitized by incubating them for 15 minutes at 37°C with the antigen prepared in 6 ml PBS. The cell suspension was centrifuged and the supernatant was discarded. The cells were washed three times with 6 ml of diluent and the final suspension was prepared in 6 ml of diluent (4 ml diluent per 0.1 ml packed tanned sensitized cells) to give a 2.5 per cent cell suspension. The antisera to be treated were decomplemented and serial two-fold dilutions in diluent were prepared so that the final volume in each tube was 1.0 ml. One tenth ml sensitized cells was added to each tube and the hemagglutination patterns were read after 2-3 hours at room temperature. All determinations were performed on the same day.

HISTOLOGIC PROCEDURES.

The rabbits were sacrificed by the intravenous injection of 300 mg nembutal. Immediately after sacrifice of the rabbits, fragments of liver, appendix, spleen, kidney, popliteal lymph node, bone marrow, thymus and sacullus rotundus were fixed in Lillie's buffered formalin for 4-6 days. The tissues were trimmed, processed through different grades of alcohols and xylol and embedded in parafin. The blocks were sectioned at 6 u with a AO Spencer Microtome. The sections obtained were stained with hematoxylin and eosin and methyl green pyronin.

RADIOAUTOGRAPHY

Duplicate sections were deparafinized and the slides thoroughly dried before the procedure of radioautography was begun in a photographic dark room. A safelight filter (Kodak, Wratten Series 2) was used with a 15 Watt bulb at a distance of 3 feet above the working area. Kodak NTB-2 Nuclear Track Emulsion was heated to 37°C in a water bath and kept at this temperature throughout the procedure. The slides containing the tissue preparations were held vertically by the marked end and dipped into the emulsion. The excess emulsion on the back of the slide was wiped off with a soft cloth. The coated slides maintained at a 70 degree angle were allowed to dry for 2 - 3 hours in complete darkness. During this period, the excess emulsion that drained was absorbed onto gauze. The slides were then stored in light-tight slide boxes containing a dessicating agent (Drierite pellets), wrapped in tissue paper. The boxes were kept at 4°C for 6 - 8 weeks, then processed with Kodak D-19 developer for 3 minutes, followed by passage through a bath of water and acid fixation (Kodak) for 5 minutes. These solutions and the water used afterwards during a 30 minute period of washing were maintained at room temperature. After washing, the slides were stained with hematoxylin and eosin.

CHAPTER 4

EXPERIMENTAL PROCEDURES

EXPERIMENT I

Sixteen adult, white, New Zealand rabbits were each injected, intravenously with a vial of PHA-P, diluted in 10 ml of Medium 199 in four divided doses of 2.5 ml over a period of one hour (see discussion). All the rabbits received 100 uc tritiated thymidine immediately following the last aliquot of PHA, another 100 uc 24 hours after the PHA administration (day 1) and another 100 uc 48 hours after the PHA injection (day 2). Non-tritiated thymidine 250 mg per injection, was administered intravenously on days 4, 6, 8, 10, 12 and 14 to prevent or reduce re-incorporation of radioactive thymidine. Eight non-PHA-injected rabbits served as controls; these were treated in an identical fashion with respect to the radioactive and non-radioactive thymidine injections.

White cell counts of the peripheral blood were performed on all the rabbits prior to the PHA injections (basal determination) and at 2 hours, 1, 2, 3, 4, 5, 7, 10, 14 and 18 days after the PHA injection. Red cell counts were done prior to the PHA injection and at 2 hours, 1, 2, 3, 7, 10 and 18 days. These determinations were carried out using the Autocytometer.

The PHA-injected rabbits were sacrificed in groups of two by the intravenous injection of 300 mg nembutal at 6 hours, 1, 2, 3, 4, 5, 8, and 18 days subsequent to the administration of PHA. The control rabbits were sacrificed in groups of two at 1, 3, 8 and 18 days. Fragments of various organs were placed in fixing solution and subsequently cut and stained as described in Chapter 3. Radioautographs were performed as described above.

EXPERIMENT II

Three groups of 16 adult, white, New Zealand rabbits were used. The babbits in Group I were each injected intravenously with one vial of PHA-P in the same way as described for the first experiment. The rabbits in Group II were each injected intravenously with one vial of PHA-M, dissolved in 2.5 ml of Medium 199. The rabbits in Group III each received intravenously 25 mg of Human Gamma Globulin (HGG). All the rabbits received 400 uc tritiated thymidine intravenously 24 hours before sacrifice. Four non-PHA-injected rabbits served as controls and received the same amount of tritiated thymidine 24 hours before sacrifice. White cell counts of the peripheral blood were performed on all the rabbits prior to the PHA-P, PHA-M and antigen injections and at 2 hours, 1, 2, 4, 5, 7 and 10 days. Red cell counts were performed prior to the PHA-P, PHA-M and antigen injections and at 2 hours and 2 days. All cell counts were carried out using the autocytometer. Smears were prepared from peripheral blood obtained from all the rabbits before injection of PHA-P, PHA-M and antigen and at 2 hours and 2 days after injection. The smears were made on glass slides, air dried and stained with Jenner-Giemsa. Differential white blood cell counts were performed by counting one hundred cells.

Peripheral blood was collected from the antigen (HGG)-injected rabbits prior to antigen injection and at days 5, 7 and 10. The blood was allowed to clot at room temperature and serum was obtained after centrifugation.

The antibody titers of these antisera to HGG were determined by the tanned cell hemagglutination technique.

The three groups of rabbits were sacrificed in groups of two by the intravenous injection of 300 mg of nembutal, at 6 hours, 1, 2, 3, 4, 5, 7 and 10 days. The control rabbits were sacrificed 24 hours after the injection of radioactive thymidine. The processing of the tissues and preparation of radioautographs were performed as described in Chapter 3.

CHAPTER 5

RESULTS

EXPERIMENT I

MORPHOLOGIC STUDIES

The histopathologic changes observed in the sections stained with hematoxylin and eosin (H and E) and methyl green pyronine and in the radioautographs, obtained from the rabbits injected with PHA-P, were readily distinguished from tissues obtained from control animals.

Liver

Within 6 hours of the PHA injection and the initial dose of H^3-thymidine, there was moderate infiltration of blast cells in the subendothelial area of many veins. By 24 hours extensive subendothelial infiltration was noted with moderate numbers of cells of similar morphology in hepatic sinusoids. With methyl green pyronine staining, the cytoplasm of virtually all the infiltrating cells exhibited pyroninophilia of moderate degree. The peak subendothelial infiltration of blast cells was reached by 48 hours (Fig.1). This was noticeably less marked at 72 hours, at which time increased numbers of periductal cells were present, and the proportion of pyroninophilic cells was considerably less than at 24 and 48 hours. The cellular infiltration decreased thereafter and within 5 days after PHA injection the experimental sections resembled controls. Megakaryocytes were prominent in hepatic sinusoids from days 3 to 5 in PHA treated animals (Fig.2). No definite evidence of abnormality was observed within the hepatocytes.

Autoradiographs showed extensive labelling of the infiltrating subendothelial cells (Fig.3). In sections from control animals (Fig.4), only occasional periductal cells (which are normally present in small numbers in portal triads) showed nuclear labelling, while in experimental animals at the peak time of infiltration approximately 50 per cent of the infiltrating cells showed nuclear labelling.

A similar extent of nuclear labelling was evident in blast cells present within sinusoids. Nuclei of megakaryocytes showed no labelling, nor did hepatocytes.

Spleen

In splenic sections obtained from control animals, there was a clear demarcation between the white and red pulp. The white pulp contained periarterial lymphoid sheaths and excentric follicles. These follicles occasionally contained small germinal centers. Two concentric zones were noted around the germinal center: a middle zone, of compact darkly-stained small lymphocytes and an outer mantle of pale-stained large lymphocytes (Fig.4A). The sinusoids in the red pulp contained erythrocytes and variable numbers of normoblasts. The parenchyma between the sinusoids contained a large number of mature granulocytes, a variable population of immature myeloid cells and occasional megakaryocytes.

The most striking changes observed in the PHA-treated rabbits consisted of marked enlargement of the zones of white pulp with loss of germinal centers. The enlargement of the white pulp rapidly reached a peak at 24 to 48 hours (Fig.5) and had largely reverted to normal (Fig.6) by 120 hours. The enlargement appeared to be due to an increase in blast cells and undifferentiated reticular cells, and there was in addition invasion of the red pulp by similar cells with a consequent increase in the ratio of blast cells to small lymphocytes. While the degree of cytoplasmic pyroninophilia prior to 48 hours was difficult to evaluate and no marked difference could be seen between experimental and control animals, at 48 and 72 hours experimental animals showed prominent pyroninophilia of virtually all the transformed and/or infiltrating blast cells. This change was present in both red and white pulp cells but was more readily observed in the single cells or small groups of cells in the red pulp. By day 4, the degree of pyroninophilia was again comparable with that of control animals. A marked decrease in splenic polymorphonuclear leukocytes was evident at 24 and 48 hours.

In addition, there was a prominent and early marked increase in normoblasts in the splenic red pulp, reaching peak levels at 6 and 24 hours and a return to normal levels by 48 hours. In addition to the polymorphonuclear leukocyte depletion during this time, nuclear debris was prominent within follicles of the white pulp. Myeloblasts and myelocytes were not definitely increased, but metamyelocytes were prominent at 24 hours.

At day 8 after the PHA-P injection a moderate hyperplasia of the white pulp was again evident. However, the histological picture was different from that seen on day 2, due to the presence of large germinal centers. Also at day 8, a second and more intense peak of myeloid-erythroid infiltration of the splenic red pulp was noted. The myeloid population was represented by myeloblasts, myelocytes and other more mature forms. Normoblastic islands were prominent in the red pulp. This proliferation declined thereafter with only slight myeloid activity detectable by day 18.

Autoradiographs showed labelling of approximlately 10 per cent of white pulp cells and 5 per cent of red pulp cells in control spleens (Fig.7). In experimental animals approximately 60 per cent of the blast cells and reticular cells in white pulp (Fig.8) and 50 per cent of such cells in red pulp (Fig.9) were labelled at 48 hours.

Lymph Node

Histologic changes in the lymph nodes were difficult to evaluate in PHA treated animals in view of the variability of appearances (with respect to size of germinal centers, encroachment on medullary sinusoids, etc.) of lymph nodes from control animals. It was evident however that, at 24 hours, the PHA treated animals exhibited depletion of small lymphocytes (Fig.10) as compared to the control animals (Fig.11) and increase in the number of blast cells in the interfollicular and subfollicular pulp. By 48 hours, normal follicles began to reappear in the lymph node (Fig.12)

PHA-treated animals showed a moderate increase in the number of pyroninophilic cells at 48 and 72 hours, although the change was not as striking as that seen in the liver and spleen.

This increase in the number of pyroninophilic cells appeared in the interfollicular pulp and was also evident to a lesser degree in the germinal centers.

Autoradiographs showed no increase in labelled cells within recognizable follicles, but at 48 hours nuclear labelling in interfollicular and subfollicular pulp had increased from a control level of 5 per cent of cells (Fig.13) to 25 per cent of cells in experimental animals (Fig.14).

Appendix

The appendix was also difficult to evaluate histologically in view of the variability of the appearance of sections from control animals (Fig.15). Sections taken from animals killed at 12, 24 and 48 hours showed an apparent increase in size of the germinal centers which appeared to be largely the result of edema and the accumulation of foamy macrophages (Fig.16). However similar changes were present in occasional control animals, and the significance of these changes in the 12-48 hour period in PHA treated animals is obscure.

Autoradiographs, however, showed a marked increase in nuclear labelling in the cap of cells between the peripheral follicles and the subepithelial follicular collections of lymphocytes and macrophages (Fig.17). While less than 5 per cent of cells in control animals showed nuclear labelling (Fig.18), approximately 20-25 per cent of cells of experimental animals at 24 and 48 hours were labelled.

Thymus

No definite morphological alterations could be assessed from either H and E or methyl green pyronine staining (Fig.19). An impression was gained that there was depletion of cortical small lymphocytes (Fig.20) and an increase in the number of blast cells from 48 to 120 hours.

Autoradiographs showed no increase in the proportion of labelled cells in PHA treated animals as compared with control animals.

Kidney

Only in the 2 animals killed at 48 hours were any abnormalities noted. Perivenous infiltration by blast cells was focally evident in the renal cortex in both PHA-treated animals killed at 48 hours. No similar change was seen in any of the remaining experimental and control animals. Virtually all the infiltrating blast cells showed pyronin staining of the cytoplasm. Approximately 40 per cent of infiltrating cells were labelled (Fig.21).

Bone Marrow

Material was not available from animals killed at 6 hours after the injection of PHA. At 24 hours the bone marrow showed an increase in the number of primitive cells of the granulocytic series with a markedly reduced proportion of metamyelocytes and an increase in primitive white cells. One animal showed prominent normoblastic hyperplasia at 24 hours; in the other 24 hour animal this change was less striking. There was increased nuclear labelling at 48 hours. Bone marrow from animals killed at 72 hours and subsequently did not differ from controls to any marked degree.

RED CELL AND LEUKOCYTE COUNTS

As can be seen in Table I, the white cell counts in all of the experimental rabbits were markedly depressed within 2 hours following the PHA injection. By 24 hours, the leukocyte count had begun to recover and by 48 hours was invariably back to normal levels. The changes in red cell counts did not occur as rapidly nor were they as marked as the changes in white cell counts (Table II). The maximum drop in the red cell count was attained at 24 hours. However, unlike the white cell counts, the erythrocyte counts remained low for up to a week following PHA administration. These results are in sharp contrast to the white and red cell counts performed in control animals (Tables III and IV, respectively), which did not fluctuate to any great extent over the same period of time.

EXPERIMENT II

PHA-P INJECTED ANIMALS

MORPHOLOGIC STUDIES

The histologic appearance of the tissues observed in the animals injected with PHA-P is comparable to that described in Experiment I. The livers of the control animals showed central and portal veins essentially free of infiltrating cells (Fig. 22), whereas livers of PHA-treated animals manifested perivascular infiltration (Fig. 23). The depletion of small lymphocytes in the lymph nodes was again observed at 24 hours (Fig. 24) as compared to control animals (Fig. 25). Normal follicles began to reappear in the lymph nodes by 48 hours (Fig. 26). Nuclear labelling in interfollicular pulp was more intense in the PHA-P injected animals (Fig. 27) as compared to control animals (Fig. 28). In the spleen, the enlargement of the white pulp reached a peak at 48 hours (Fig.29) and, by 120 hours, the morphology of the spleen had reverted essentially to normal (Fig. 30). Furthermore, the nuclear labelling in the white pulp (Fig. 31) and in the red pulp (Fig. 32) of the experimental animals at 48 hours was much greater than the labelling observed in the white pulp (Fig. 33) and in the red pulp (Fig. 34) of control animals. Again, as in Experiment I, a second peak of myeloid-erythroid infiltration was noted in the splenic red pulp by day 8 (Figs. 35 and 36). At 10 days, the majority of myeloid cells and many normoblasts were labelled (Fig. 37). Hyperplasia of the white pulp, with large germinal centers, was again evident at days 7-8 and was prominent by day 10 (Fig. 38). The changes in the appendix of the PHA-P injected animal (Figs. 39 and 40) as compared to the control animal (Figs. 41 and 42) are similar to those described in Experiment I. A relative increase in the thymic medulla was noted in the experimental animals sacrificed at 24 and 48 hours (Fig. 43) as compared to control animals (Fig. 44).

No perivascular infiltration was noted in the kidney.

Peripheral blood

Radioautographs of peripheral blood smears from control animals showed rare labelled medium size lymphocytes. The smears of the PHA-P injected animals were similar to controls at day 1. However, at day 2, several labelled medium size lymphocytes (Fig. 59) and occasional blast cells with moderate label (Fig. 60) were noted. At day 3, several small (Fig. 61) and medium size (Fig. 62) labelled lymphocytes were observed. Similar results were obtained at day 4 (Fig. 63). At days 5 and 7 there was a gradual decrease of labelled lymphocytes and by day 10 the smears were comparable to normals.

PERIPHERAL CELL COUNTS

As can be seen in table V, a sharp uniform decrease in the number of peripheral white cells was observed 2 hours after the injection of PHA-P. The counts returned to normal levels at 24-36 hours.

A marked depression in the red cell count was noted 2 hours after the injection of PHA-P. There was an almost complete recovery by day 2 (Table VIII).

WHITE BLOOD CELL DIFFERENTIAL AND NORMOBLAST COUNT

Before the injection of PHA-P, the differential counts showed an average of 60-70 per cent lymphocytes and 30-40 per cent segmented cells. Two hours after the administration of PHA-P, a considerable decrease in the number of circulating lymphocytes was observed with a consequent increase in the number of segmented cells. The animals then began to recover and by 48 hours the values were similar to those before injection. Normoblasts, in large numbers, were observed in seven of the rabbits 2 hours after the injection of PHA-P.

At 48 hours, only two of these animals had circulating normoblasts (Table XI).

PHA-M INJECTED ANIMALS

MORPHOLOGIC STUDIES

Liver

The livers of the animals sacrificed at 6 hours, 1, 2 and 3 days showed variable but mild periductal infiltration by lymphocytes (Fig. 45). On day 2, rare plasma cells were seen in the infiltrate. All these changes are, however, within the range of variation observed in the livers from control animals. At day 4, a more extensive portal infiltration, including a few plasma cells, was noted in the experimental livers only. Nuclear labelling was not increased as compared to that in control animals. Liver sections from animals sacrificed at days 5, 7 and 10 were considered to have normal morphology.

Spleen

Splenic sections were normal at 6 hours after injection of PHA-M. At day 1, one experimental animal showed an increase in the white pulp but the red pulp in both animals was normal. On day 2, the spleen of one animal showed a mild increase in white pulp with slightly increased germinal centers and external mantle zones (Fig. 46). The red pulp of both animals was characterized by a large number of myeloblasts (60 per cent with nuclear labelling) and prominent normoblastic islands (Fig. 47). A moderate hyperplasia of the white pulp, without large germinal centers, was observed on day 3. The changes in the red pulp were less marked but prominent as compared to those seen on day 2. On day 4, the spleen of one rabbit was essentially normal while the spleen of the second animal displayed hyperplastic white pulp with large germinal centers. The red pulp in both animals contained mainly mature white cells and normoblastic islands. By day 5, one animal showed a slightly hyperplastic white pulp and the other animal had a markedly hyperplastic white pulp with large germinal centers. The red pulp contained a normal white

cell population and prominent numbers of normoblasts. At day 7, hyperplastic white pulp with large germinal centers and prominent external mantles were noted in both animals (Fig. 48). Nuclear debris in the germinal centers was observed in one animal (Fig. 49). The red pulp contained a large number of myeloblasts and prominent normoblastic islands. Sixty per cent of these cells were labelled (Fig. 50). There was a slight increase in the nuclear labelling in germinal centers (Fig. 51) and in the outer mantle zones (Fig. 52) as compared to control animals. By day 10, marked white pulp hyperplasia with large germinal centers was observed in both animals (Fig. 53). The red pulp contained a moderate number of myeloblasts and normoblasts.

Bone Marrow

There was no change in the percentage of mature white cells observed in sections of bone marrow as compared to controls. A questionable increase in nuclear labelling of erythroid and myeloid cells was observed in the speciman obtained from one rabbit sacrificed on day 2.

Lymph node, thymus, appendix and sacculus rotundus

No abnormal changes were observed in these organs throughout the course of the experiment.

Peripheral blood

The peripheral blood smears of the rabbits injected with PHA-M, with the exception of one animal which showed moderate numbers of labelled lymphocytes at day 5, were comparable to controls.

PERIPHERAL CELL COUNTS

There was a slight decrease, generally, in the number of peripheral white cells 2 hours after injection of PHA-M. By 24 hours the counts were back to normal values (Table VI).

A marked depression in the red cell count was noted 2 hours after the injection of PHA-M. Recovery then set in and was almost complete by day 2 (Table IX).

WHITE BLOOD CELL DIFFERENTIAL AND NORMOBLAST COUNT

The differential white cell count of rabbits injected with PHA-M ran a course similar to that observed in rabbits injected with PHA-P. Circulating normoblasts were noted in 11 out of the 16 rabbits 2 hours after the injection of PHA-M (Table XII).

ANTIGEN INJECTED ANIMALS

MORPHOLOGIC STUDIES

Liver

In all the animals the presence and extent of lymphocytic population in portal triads was comparable with that seen in control animals.

Spleen

No abnormal changes were observed in splenic sections from animals sacrificed at 6 and 24 hours. At 2 and 3 days white pulp hyperplasia was observed with slightly enlarged germinal centers but with the outer mantle definitely widened (Fig. 54). The percentage of labelled cells in the white pulp was the same as that observed in control animals. The red pulp was normal in appearance. At day 4, the white pulp was more hyperplastic with a merging of the germinal centers and middle layers (i.e. no germinal centers). In the red pulp, somewhat greater numbers of myeloblasts and normoblasts were noted, but they were within the range seen in control animals. The radioautographs showed no definite increase in the percentage of labelled cells. At day 5, the white pulp was very hyperplastic (Fig. 55). The outer mantle was widened and germinal centers were present but not in all follicles (Fig. 56). Radioautographs showed an increase in the percentage of labelled cells in germinal centers and in the outer mantles (Fig. 57) as compared to control animals. At day 7, an extensive hyperplasia of the white pulp with prominent germinal centers was noted, but the main increase was observed in the middle and outer mantle layers (Fig. 58). The radioautographs were comparable to those seen at day 5. The red pulp did not differ

from control animals but some nuclear debris was present. At day 10, in one of the animals, the white pulp appeared normal and in the other there was partial regression of the hyperplasia seen on day 7. The red pulp showed a moderate increase in the numbers of myeloblasts and normoblasts. Plasma cells were rarely identified throughout the experiment.

Lymph node, appendix, thymus, kidney, bone marrow and sacculus rotundus

No consistently abnormal changes were observed in any of these organs throughout the experiment.

Peripheral blood

No abnormal changes were observed in the white cell counts throughout the course of the experiment (Table VII)? No significant changes were noted in the red cell counts either with the exception of rabbit No. 97 which exhibited a profound depression of the red cell count 2 hours after the injection of the antigen and had still not recovered by 48 hours.

WHITE BLOOD CELL DIFFERENTIAL AND NORMOBLAST COUNT

No significant abnormal changes were observed (Table XIII).

ANTIBODY TITERS

As can be seen in Table XIV, the animals injected with 25 mg of the antigen (HGG) had no demonstrable circulating antibody up to day 10 of the experiment when titers of 2,560 and 5,120 were recorded.

TABLE I

PERIPHERAL WHITE CELL COUNTS IN RABBITS SUBSEQUENT TO PHA-P ADMINISTRATION (EXP. I)

Rabbit No.	Prior to PHA Inj.	2 hrs.	1 day	2 days	3 days	4 days	5 days	7 days	8 days	10 days	14 days	18 days
1	7,200	2,300	sac.									
2	8,500	1,000	sac.									
3	9,900	2,100	7,400	sac.								
4	10,600	1,300	4,300	sac.								
5	7,200	1,300	4,900	8,650	sac.							
6	8,400	1,300	4,150	9,900	sac.							
7	10,000	1,200	4,350	9,200	9,950	sac.						
8	9,800	950	9,100	12,800	15,000	sac.						
9	8,200	900	3,700	7,050	7,750	8,350	sac.					
10	8,000	1,200	3,750	6,050	6,450	7,700	sac.					
11	9,750	1,500	2,750	6,850	7,400	11,000	7,300	sac.				
12	10,400	950	4,500	14,800	15,200	9,500	8,200	sac.				
13	6,200	1,450	8,550	15,000	5,550	5,900	5,400	6,200	5,600	sac.		
14	10,250	1,300	8,700	13,200	12,150	8,000	9,700	7,000	7,500	sac.		
15	9,350	1,150	7,750	18,700	11,250	14,000	13,100	11,800	9,100	7,000	7,300	7,000
16	9,050	1,200	3,000	8,000	8,300	8,500	9,600	9,300	8,500	5,800	10,400	8,500

Sac. = sacrificed

TABLE II

PERIPHERAL RED CELL COUNTS* IN RABBITS SUBSEQUENT TO PHA-P ADMINISTRATION (Exp. I)

Rabbit No.	Prior to PHA Inj.	2 hrs.	1 day	2 days	3 days	7 days	10 days	18 days
1	2.9	2.1	sac.					
2	4.6	4.9	sac.					
3	4.5	3.7	1.9	sac.				
4	5.3	3.2	2.7	sac.				
5	4.2	4.3	2.0	3.4	sac.			
6	4.8	3.1	2.7	2.2	sac.			
7	4.0	4.0	2.3	2.4	1.9	sac.		
8	3.6	3.2	3.1	2.7	1.6	sac.		
9	4.3	2.5	3.2	2.4	2.3	sac.		
10	4.1	4.2	2.9	3.2	2.9	sac.		
11	4.3	2.8	2.6	3.1	2.0	sac.		
12	4.7	3.5	2.5	3.2	2.3	sac.		
13	4.6	4.0	3.4	3.3	2.3	3.3	sac.	
14	5.3	3.8	3.1	2.7	2.9	3.5	sac.	
15	4.7	3.7	1.7	2.2	2.4	2.7	3.4	3.4
16	3.7	3.2	1.5	1.8	2.1	2.2	3.4	3.8

* = $X10^6$
Sac. = sacrificed

TABLE III

PERIPHERAL WHITE CELL COUNTS IN CONTROL RABBITS INJECTED WITH TRITIATED THYMIDINE ONLY

Rabbit No.	Prior to Injection	2 hrs.	1 day	2 days	3 days	4 days	5 days	7 days	8 days	10 days	14 days	18 days
17	11,550	15,000	16,200	sac.								
18	10,550	11,700	11,400	sac.								
19	12,100	9,600	10,500	11,950	8,450	sac.						
20	7,150	8,050	8,750	10,350	10,650	sac.						
21	7,650	6,950	10,850	11,500	12,050	11,000	5,800	8,000	11,000	sac.		
22	8,750	8,500	9,800	6,500	8,550	12,100	9,150	10,000	10,800	sac.		
23	7,750	6,300	7,800	9,550	6,700	6,000	6,900	8,000	11,000	10,700	8,400	7,300
24	4,050	5,200	6,300	5,250	6,800	9,500	9,900	10,300	11,600	10,500	10,000	7,800

Sac. = sacrificed

TABLE IV

PERIPHERAL RED CELL COUNTS* IN CONTROL RABBITS INJECTED WITH TRITIATED THYMIDINE ONLY

Rabbit Number	Prior to Injection	2 hours	1 day	2 days	3 days	7 days	10 days	18 days
17	4.5	4.0	3.6	sac.				
18	3.8	4.1	3.7	sac.				
19	3.4	3.6	3.2	2.7	3.5	sac.		
20	4.8	4.8	4.0	3.5	3.5	sac.		
21	2.7	2.5	2.1	2.6	2.2	2.7	sac.	
22	3.6	3.3	2.7	3.3	2.7	2.7	sac.	
23	4.1	3.4	3.5	3.3	3.3	4.3	4.7	4.1
24	4.1	3.5	4.0	3.3	3.8	4.2	4.4	4.2

* = $X10^6$
Sac.= sacrificed

TABLE V

PERIPHERAL WHITE CELL COUNTS IN RABBITS SUBSEQUENT TO PHA-P ADMINISTRATION (EXP.II)

Rabbit Number	Before Injection	2 hours	Day 1	Day 2	Day 4	Day 5	Day 7	Day 10
64	8,500	1,200	sac.					
65	14,400	1,500	sac.					
101	11,000	4,100	8,500	sac.				
102	7,500	1,800	6,200	sac.				
86	8,700	4,700	5,700	7,400	sac.			
85	13,500	6,700	12,700	12,300	sac.			
87	6,700	2,850	9,750	11,300	sac.			
88	12,300	2,000	5,570	6,800	sac.			
89	5,500	350	5,000	8,200	10,000	Sac.		
90	7,200	1,700	11,800	10,100	10,700	sac.		
91	8,000	1,000	5,100	9,600	ND	16,000	sac.	
95	7,600	1,400	11,400	11,900	ND	11,000	sac.	
99	9,000	3,900	9,500	12,500	ND	ND	10,500	Sac.
84	5,000	700	4,700	6,300	ND	ND	8,000	sac.
103	7,800	1,300	6,100	10,800	ND	ND	ND	9,000
108	8,300	750	6,000	8,000	ND	ND	ND	10,000

Sac. = sacrificed
ND = not done

TABLE VI

PERIPHERAL WHITE CELL COUNTS IN RABBITS SUBSEQUENT TO PHA-M ADMINISTRATION

Rabbit Number	Before Injection	2 hours	Day 1	Day 2	Day 4	Day 5	Day 7	Day 10
61	9,250	8,000	Sac.					
63	10,150	3,000	sac.					
68	8,700	8,100	8,600	sac.				
69	6,000	5,000	10,300	sac.				
70	11,000	8,000	10,500	12,200	sac			
71	16,000	8,700	11,100	10,700	sac.			
72	8,100	6,000	12,200	9,800	sac.			
73	8,600	7,600	11,100	16,700	sac.			
74	13,000	11,000	12,700	17,000	12,500	sac.		
75	15,000	13,000	20,000	14,600	20,000	sac.		
76	12,200	5,600	11,900	7,300	ND	11,500	sac.	
78	8,000	3,700	8,700	9,900	ND	9,500	sac.	
80	8,400	5,400	8,100	7,800	ND	ND	9,000	sac.
79	10,300	9,700	11,000	9,100	ND	ND	12,000	sac.
83	9,500	7,700	12,900	11,800	ND	ND	ND	10,000
82	7,800	5,000	7,500	7,000	ND	ND	ND	6,000

Sac. = sacrificed
ND = not done

TABLE VII

PERIPHERAL WHITE CELL COUNTS IN RABBITS SUBSEQUENT TO ANTIGEN(HGG) ADMINISTRATION

Rabbit Number	Before Injection	2 hours	Day 1	Day 2	Day 4	Day 5	Day 7	Day 10
67	8,200	8,000	sac.					
68	8,700	7,800	sac.					
62	6,700	8,400	10,600	sac.				
61	12,800	14,000	10,700	sac.				
63	9,500	7,400	8,150	9,400	sac.			
64	6,000	5,400	5,200	7,000	sac.			
66	9,500	7,200	9,500	8,000	sac.			
65	6,400	6,100	6,400	7,300	sac.			
77	9,200	8,500	8,500	7,300	9,500	sac.		
67	7,000	7,700	8,200	8,800	9,000	sac.		
81	6,700	6,000	5,800	6,400	ND	5,400	sac.	
92	8,800	8,600	8,300	8,200	ND	7,700	sac.	
93	8,200	12,000	9,300	8,900	ND	ND	9,000	sac.
97	6,700	9,500	7,600	5,700	ND	ND	6,500	sac.
106	6,900	5,200	5,100	7,200	ND	ND	ND	6,000
107	12,300	13,000	12,500	11,300	ND	ND	ND	12,000

Sac. = sacrificed
ND = not done

TABLE VIII

PERIPHERAL RED CELL COUNTS* IN RABBITS SUBSEQUENT TO PHA-P ADMINISTRATION (EXP.II)

Rabbit Number	Before Injection	2 hours	Day 2
64	2.45	1.7	Sac.
65	2.5	1.5	Sac.
101	3.2	0.7	Sac.
102	3.0	2.1	Sac.
86	6.8	1.5	3.0
85	2.4	1.3	2.2
87	6.5	1.2	2.2
88	6.4	0.9	3.0
89	1.6	0.7	2.6
90	3.8	1.1	3.5
91	2.4	1.1	3.2
95	3.1	2.2	3.1
99	2.0	1.5	3.5
84	2.2	1.9	2.8
103	5.8	2.1	3.7
108	3.4	2.0	3.9

* = $X10^6$
Sac. = sacrificed

TABLE IX

PERIPHERAL RED CELL COUNTS* IN RABBITS SUBSEQUENT TO PHA-M ADMINISTRATION

Rabbit Number	Before Injection	2 hours	Day 2
61	2.7	1.5	sac.
63	3.2	3.0	sac.
68	3.4	1.0	sac.
69	2.9	1.5	sac.
70	2.5	1.0	2.2
71	3.0	2.0	2.8
72	4.6	1.4	2.7
73	7.0	1.5	3.1
74	2.8	2.0	3.1
75	2.5	2.3	3.8
76	6.5	2.4	3.0
78	2.1	1.9	2.5
80	2.8	2.1	2.7
79	6.2	1.3	2.0
83	6.8	1.6	3.1
82	5.3	1.3	2.0

* = $x10^6$
Sac. = sacrificed

TABLE X

PERIPHERAL RED CELL COUNTS* IN RABBITS SUBSEQUENT TO ANTIGEN(HGG) ADMINISTRATION

Rabbit Number	Before Injection	2 hours	Day 2
67	2.6	2.9	Sac.
68	2.6	3.5	Sac.
62	2.8	1.8	Sac.
61	2.4	2.0	Sac.
63	1.8	1.4	1.9
64	1.5	1.8	2.0
66	2.0	1.8	2.9
65	1.2	1.7	3.4
77	2.0	1.8	2.9
67	2.6	2.5	2.5
81	2.6	2.9	2.5
92	2.0	2.0	2.7
93	1.5	1.9	1.6
97	3.5	0.95	1.1
106	4.8	3.8	3.3
107	5.6	5.2	3.9

* = $x10^6$
Sac. = sacrificed

TABLE XI

WHITE BLOOD CELL DIFFERENTIAL AND NORMOBLAST COUNT IN THE PERIPHERAL BLOOD OF RABBITS FOLLOWING PHA-P ADMINISTRATION (Exp. II)

RABBIT NUMBER	CELL COUNT BEFORE INJECTION					CELL COUNT 2 HOURS AFTER INJECTION					CELL COUNT 2 DAYS AFTER INJECTION				
	L	M	S	B	N	L	M	S	B	N	L	M	S	B	N
64*	70	1	29	-	-	60	-	40	-	-	-	-	-	-	-
65*	77	-	23	-	1	55	-	45	-	5	-	-	-	-	-
101*	62	2	35	1	-	28	6	66	-	60	-	-	-	-	-
102*	81	1	16	2	-	40	-	60	-	-	-	-	-	-	-
86	77	1	21	1	-	46	-	54	-	4	77	-	23	-	-
85	78	4	18	-	-	58	7	35	-	-	69	3	28	-	-
87	64	4	32	-	-	50	-	50	-	-	61	-	39	-	-
88	68	4	26	2	-	36	-	64	-	-	72	1	27	-	-
89	70	1	29	-	-	10	-	90	-	-	60	-	40	-	-
90	74	1	23	2	-	40	2	58	-	-	80	1	19	-	-
91	78	2	20	-	-	53	7	40	-	39	55	2	43	-	-
95	68	5	24	3	-	56	2	42	-	8	82	1	17	-	10
99	74	2	24	-	-	64	2	34	-	7	56	3	41	-	-
84	48	4	47	1	1	36	2	62	-	1	43	3	51	3	-
103	79	1	20	-	-	60	3	37	-	3	69	-	31	-	35
108	77	1	19	3	-	43	2	55	-	-	54	1	55	-	-

L = lymphocytes
M = monocytes
S = segmented cells (neutrophils + eosinophils)
B = basophils
N = normoblasts
* = sacrificed at 6 and 24 hours

TABLE XII

WHITE BLOOD CELL DIFFERENTIAL AND NORMOBLAST COUNT IN THE PERIPHERAL BLOOD OF RABBITS FOLLOWING PHA-M ADMINISTRATION

RABBIT NUMBER	CELL COUNT BEFORE INJECTION					CELL COUNT 2 HOURS AFTER INJECTION					CELL COUNT 2 DAYS AFTER INJECTION				
	L	M	S	B	N	L	M	S	B	N	L	M	S	B	N
61*	75	2	20	3	-	36	-	64	-	-	-	-	-	-	-
63*	63	-	37	-	-	51	-	49	-	-	-	-	-	-	-
68*	56	1	43	-	1	26	-	74	-	2	-	-	-	-	-
69*	62	-	37	1	1	50	-	50	-	8	-	-	-	-	-
70	66	-	34	-	-	22	1	77	-	12	62	-	38	-	-
71	87	2	10	1	-	42	-	58	-	10	58	-	42	-	-
72	48	3	49	-	-	36	2	62	-	18	59	1	40	-	-
73	88	-	12	-	1	51	-	49	-	4	60	2	38	-	-
74	82	4	11	3	-	16	-	84	-	1	61	1	38	-	-
75	68	1	30	1	-	9	-	91	-	-	68	1	31	-	-
76	79	1	20	-	-	54	-	46	-	15	76	-	24	-	-
78	85	1	14	-	3	40	-	60	-	21	72	3	25	-	2
80	57	2	41	-	-	40	-	58	2	3	68	-	32	-	-
79	80	-	20	-	-	19	1	80	-	-	70	3	27	-	-
83	64	2	34	-	-	34	-	66	-	10	92	-	8	-	-
82	71	-	29	-	-	35	-	65	-	4	64	4	32	-	-

L = lymphocytes
M = monocytes
S = segmented cells (neutrophils + eosinophils)
B= basophils
N = normoblasts
* = sacrificed at 6 and 24 hours

TABLE XIII

WHITE BLOOD CELL DIFFERENTIAL AND NORMOBLAST COUNT IN THE PERIPHERAL BLOOD OF RABBITS FOLLOWING ANTIGEN(HGG) ADMINISTRATION

RABBIT NUMBER	CELL COUNT BEFORE INJECTION					CELL COUNT 2 HOURS AFTER INJECTION					CELL COUNT 2 DAYS AFTER INJECTION				
	L	M	S	B	N	L	M	S	B	N	L	M	S	B	N
68*	75	-	22	3	-	75	-	25	-	-	-	-	-	-	-
67*	86	2	12	-	-	80	-	20	-	-	-	-	-	-	-
62*	65	1	34	-	-	ND	ND	ND	ND	ND	-	-	-	-	-
61*	66	1	31	2	-	65	3	32	-	-	-	-	-	-	-
63	74	1	25	-	-	60	-	40	-	-	72	4	24	-	-
64	65	-	33	2	-	60	4	36	-	-	58	2	40	-	-
66	44	3	48	5	-	67	3	29	-	-	70	1	29	-	-
65	76	1	23	-	-	52	1	47	-	-	58	2	40	-	-
77	60	-	40	-	2	55	-	45	-	-	78	-	22	-	-
67	64	2	33	1	-	53	1	45	1	3	56	4	40	-	-
81	67	1	29	3	-	57	1	40	2	-	70	-	30	-	-
92	65	2	31	2	-	58	-	42	-	-	65	2	33	-	-
93	71	1	28	-	-	62	-	38	-	-	77	-	23	-	-
97	70	2	22	6	-	65	-	35	-	-	68	-	32	-	-
107	66	4	30	-	-	68	-	32	-	-	55	-	45	-	-
106	78	-	20	2	-	76	-	24	-	-	72	1	27	-	-

L = lymphocytes
M = monocytes
S = segmented cells (neutrophils + eosinophils)
B = basophils
N = normoblasts
ND = not done
* = sacrificed at 6 and 24 hours

TABLE XIV

ANTIBODY RESPONSE IN RABBITS INJECTED WITH ANTIGEN(HGG)

Rabbit Number	Days Following Injection	Reciprocal of Hemagglutination Titer
81	5	0
92	5	0
93	7	0
97	7	0
106	10	2,560
107	10	5,120

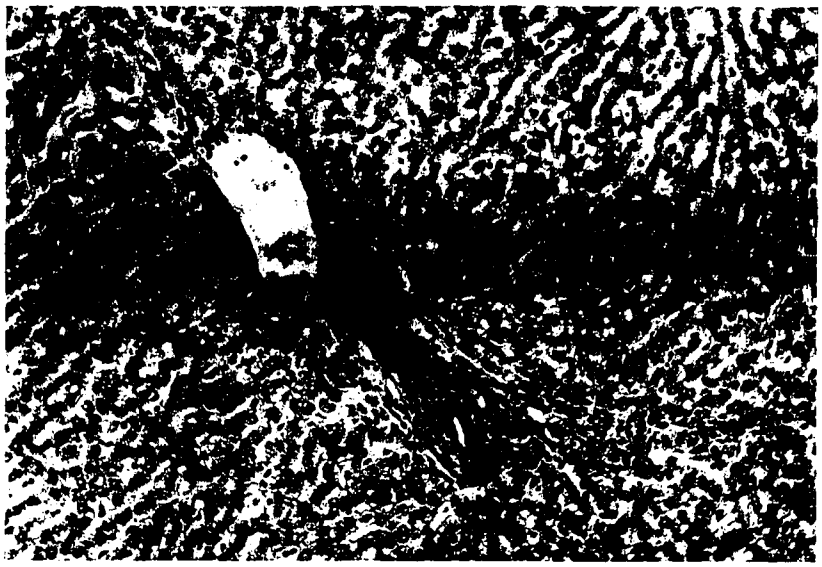

Figure 1 - PHA-P treated animal-2 days-100x
Liver-portal area-subendothelial infiltration of mononuclear cells..

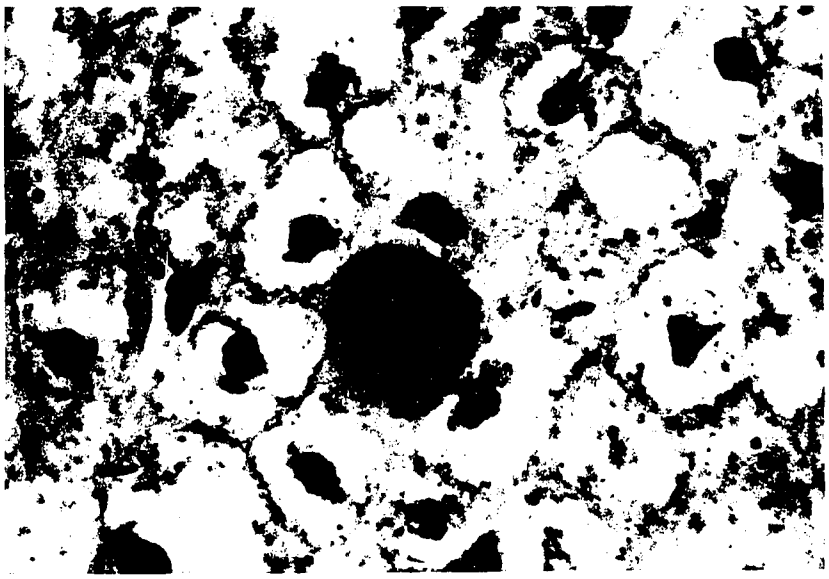

Figure 2 - PHA-P treated animal-4 days-1,000x
Liver-megakaryocyte in hepatic sinusoid

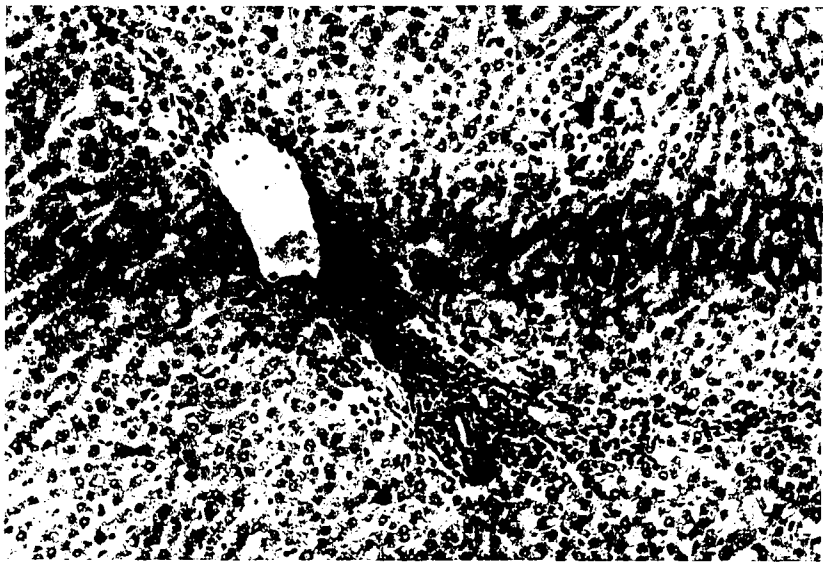

Figure 1 - PHA-P treated animal-2 days-100x
Liver-portal area-subendothelial infiltration of mononuclear cells..

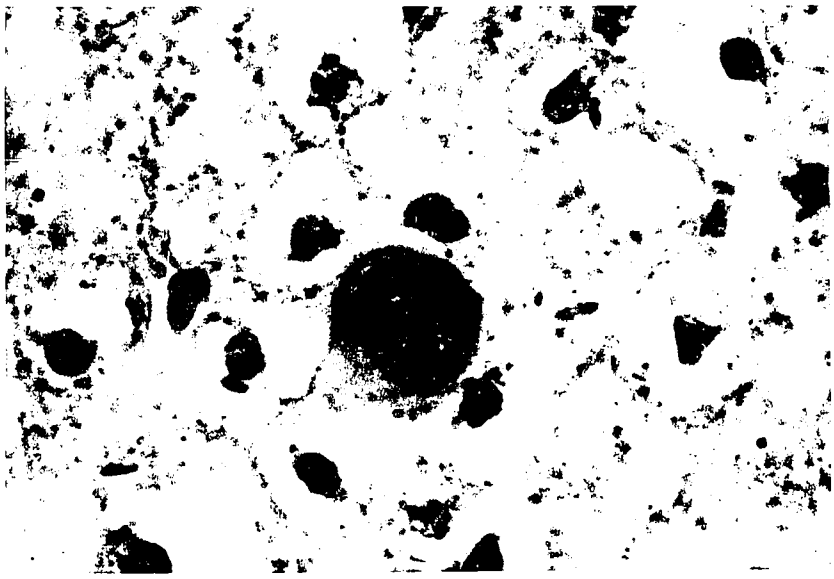

Figure 2 - PHA-P treated animal-4 days-1,000x
Liver-megakaryocyte in hepatic sinusoid

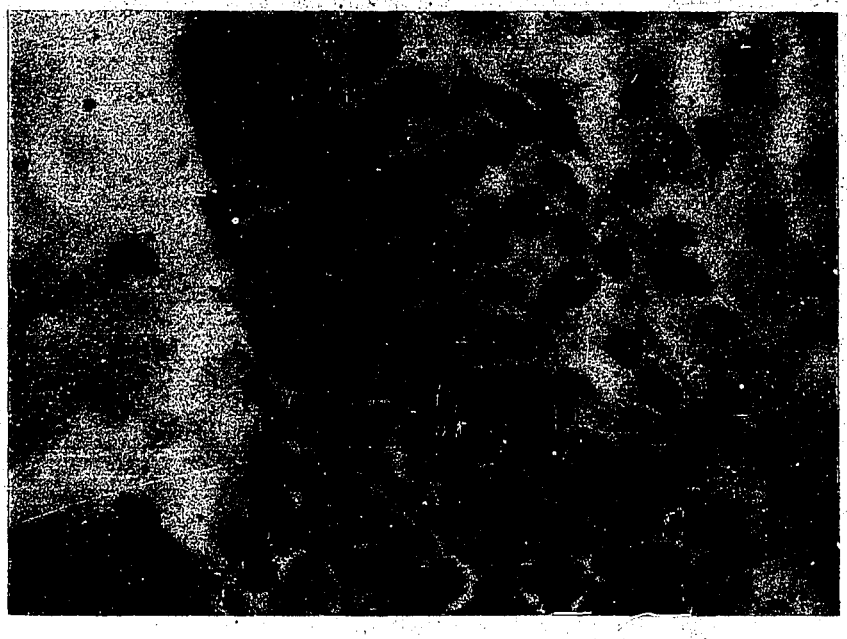

Figure 3 - PHA-P treated animal-2 days-630x
Liver-Radioautograph of cells infiltrating vein in portal area.

Figure 4 - Control animal-100x
Liver-portal area free of infiltrating cells.

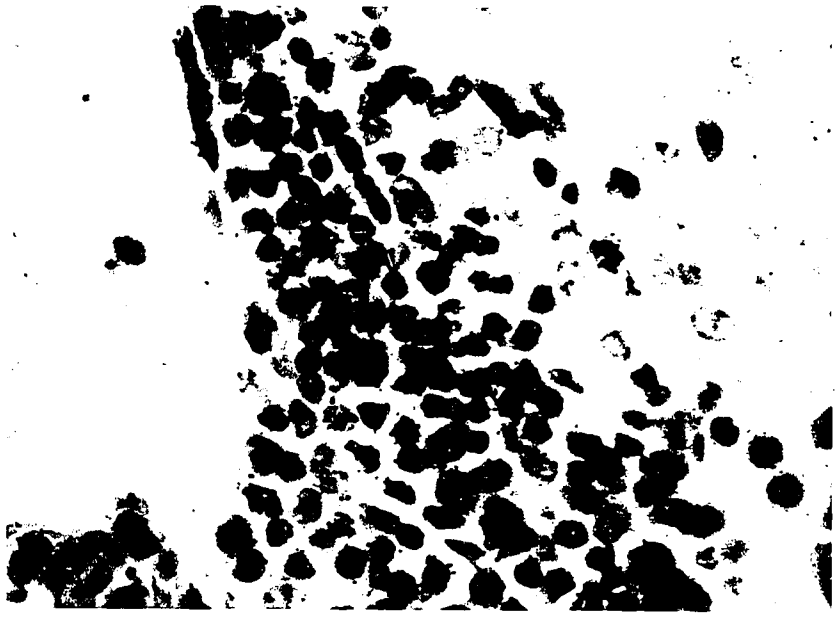

Figure 3 - PHA-P treated animal-2 days-630x
Liver-Radioautograph of cells infiltrating vein in portal area.

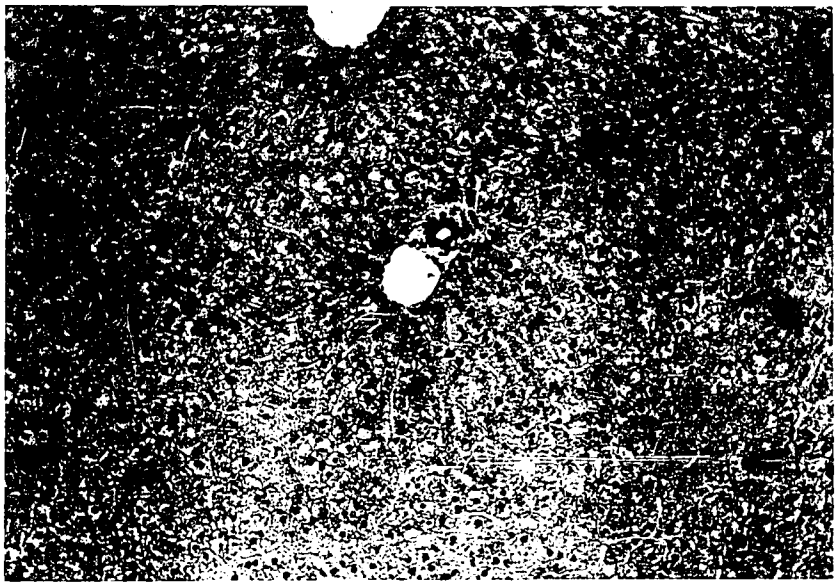

Figure 4 - Control animal-100x
Liver-portal area free of infiltrating cells.

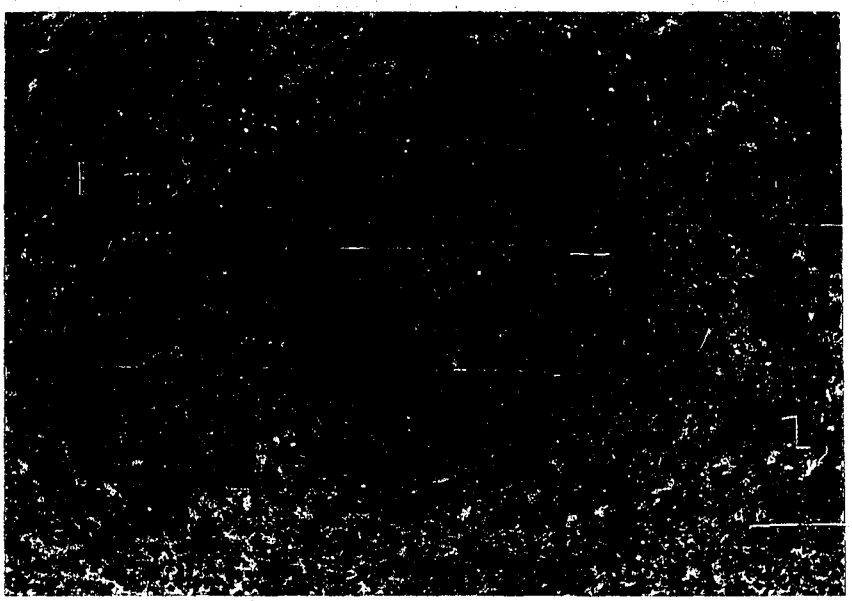

Figure 4A - Control animal-400x
Spleen-white pulp-follicle with a central germinal center, a middle layer of small lymphocytes and an outer mantle of medium and large lymphocytes.

Figure 5 - PHA-P treated animal-2 days-25x
Spleen-increase of white pulp and disappearance of germinal centers.

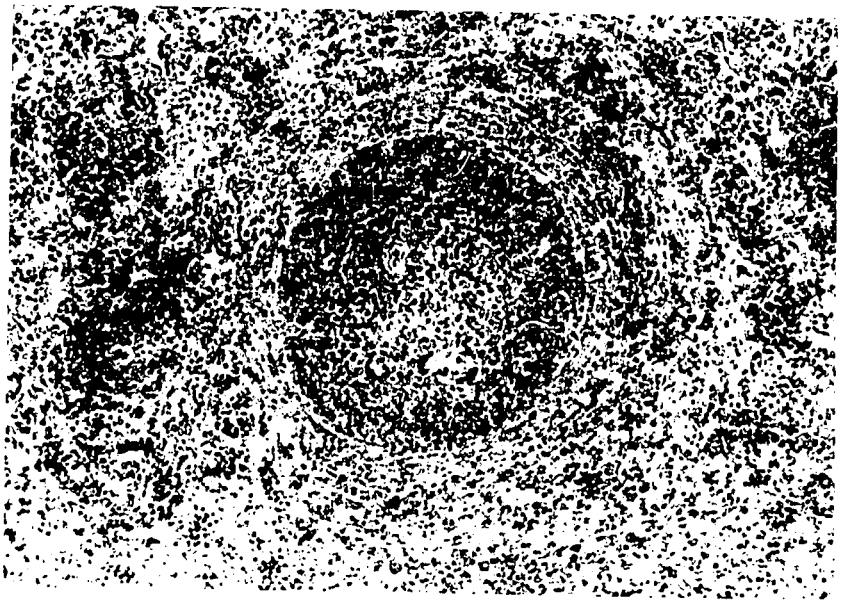

Figure 4A - Control animal-400x
Spleen-white pulp-follicle with a central germinal center, a middle layer of small lymphocytes and an outer mantle of medium and large lymphocytes.

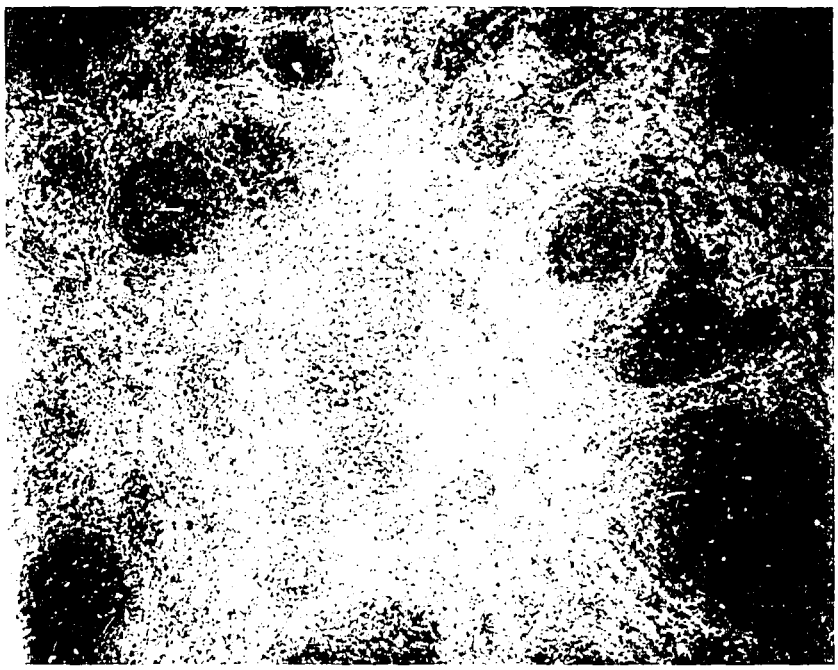

Figure 5 - PHA-P treated animal-2 days-25x
Spleen-increase of white pulp and disappearance of germinal centers.

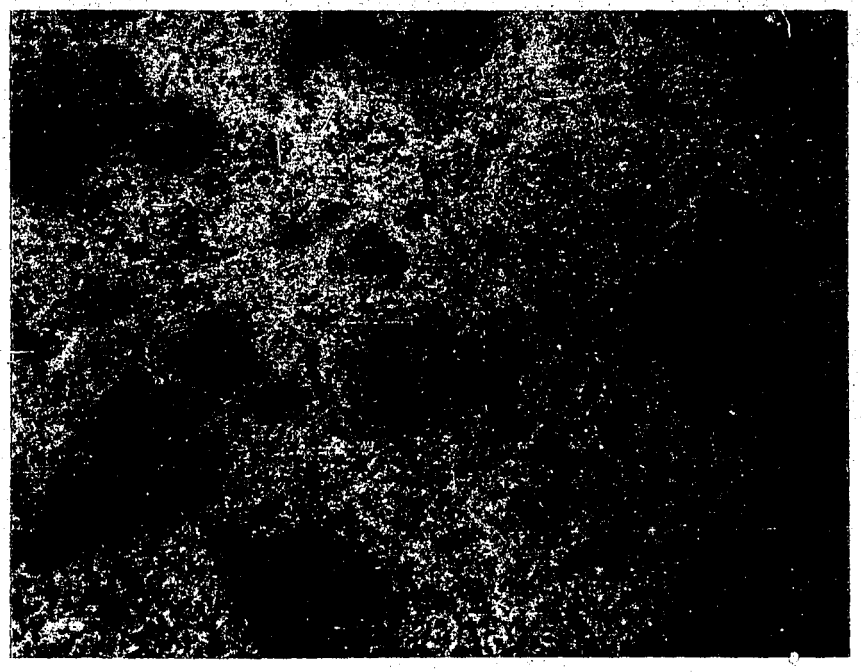

Figure 6 - Control animal-25x
Spleen-normal looking follicles

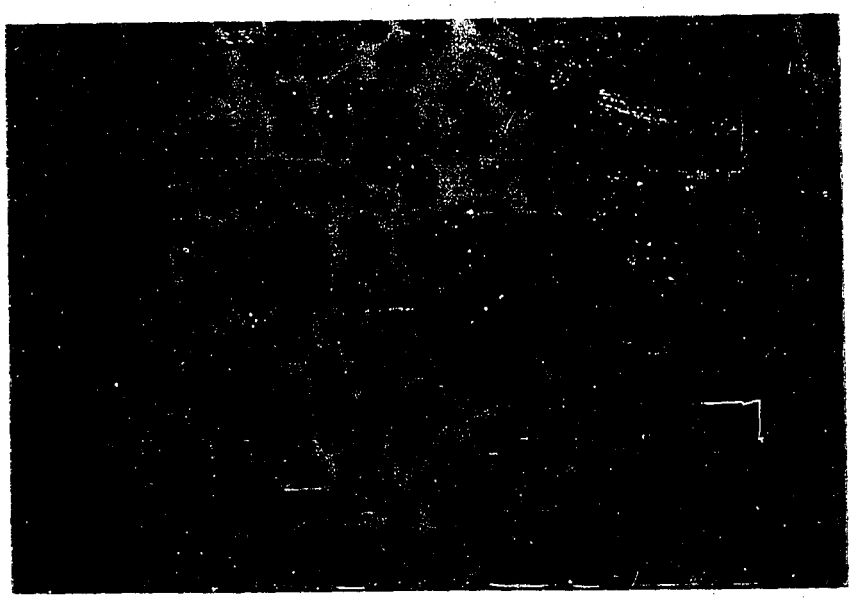

Figure 7 - Control animal-400x
Spleen-Radioautograph

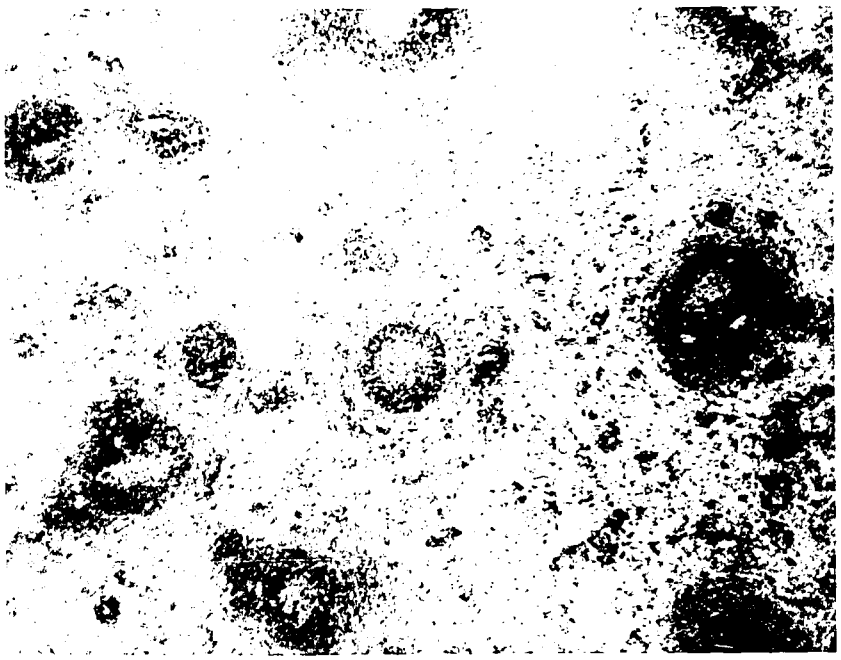

Figure 6 - Control animal-25x
Spleen-normal looking follicles

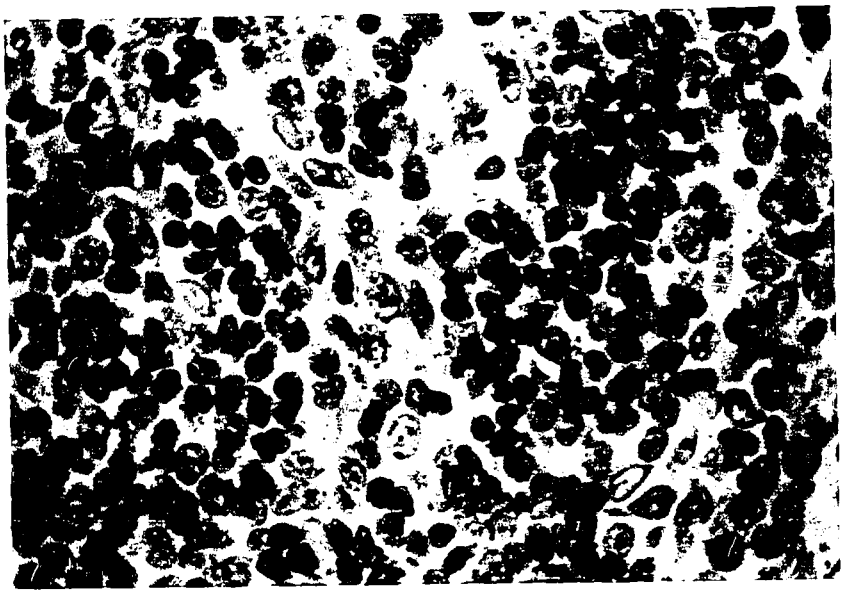

Figure 7 - Control animal-400x
Spleen-Radioautograph

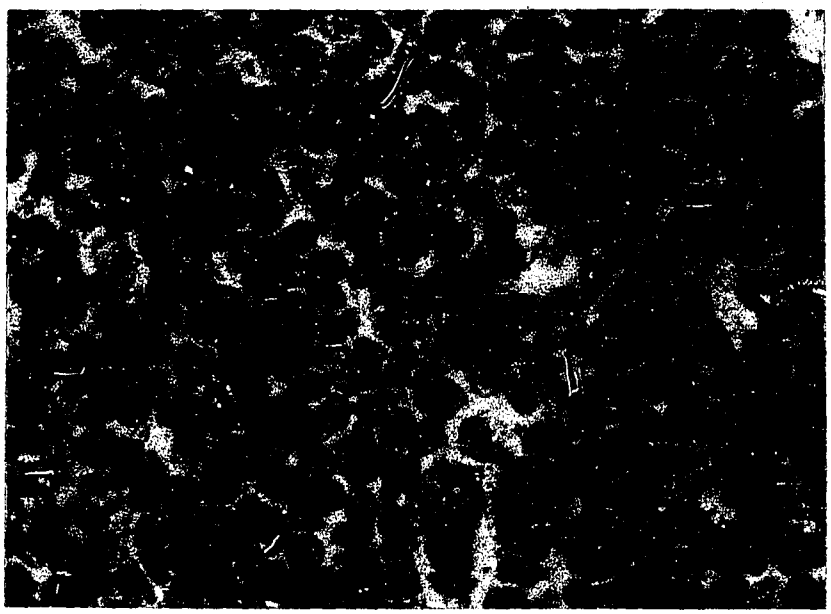

Figure 8 - PHA-P treated animal-2 days-630x
Spleen-Radioautograph of white pulp.

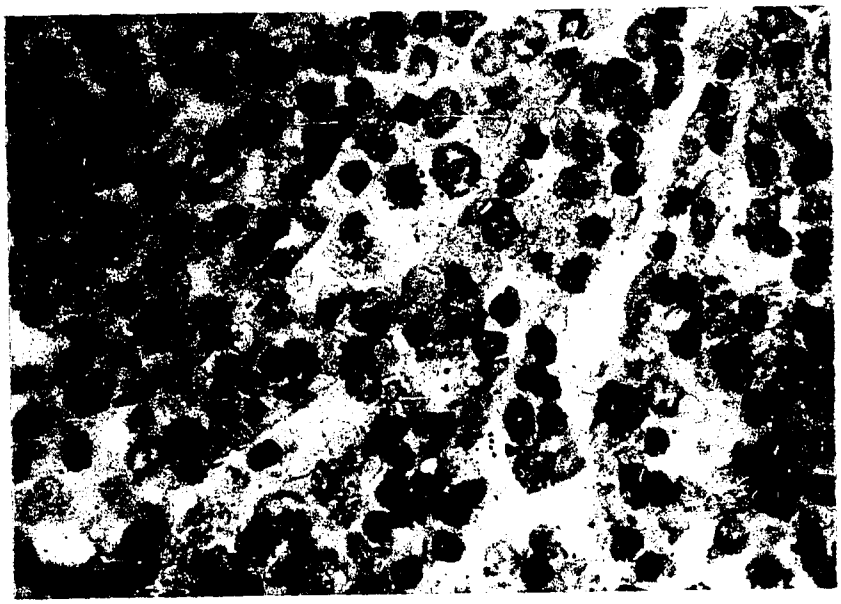

Figure 9 - PHA-P treated animal-2 days-630x
Spleen-Radioautograph of red pulp.

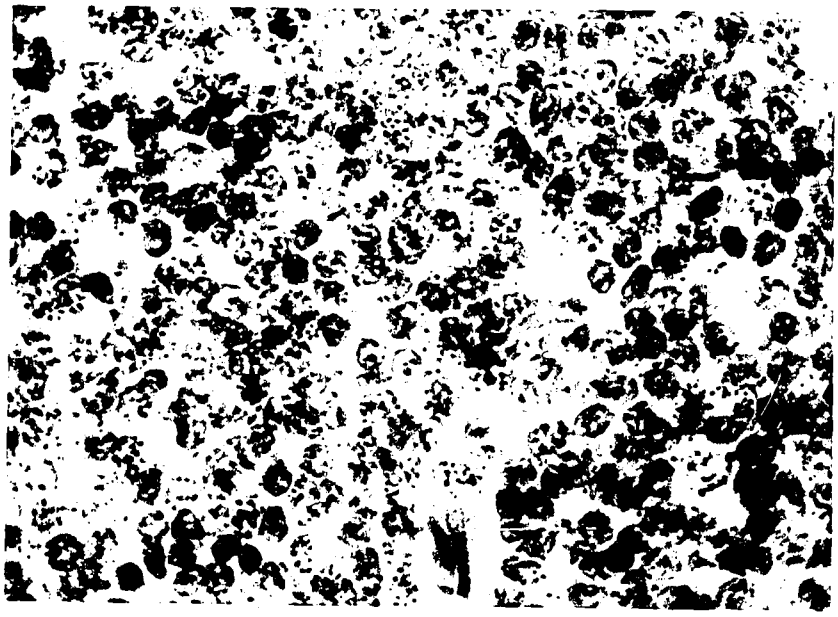

Figure 8 - PHA-P treated animal-2 days-630x
Spleen-Radioautograph of white pulp.

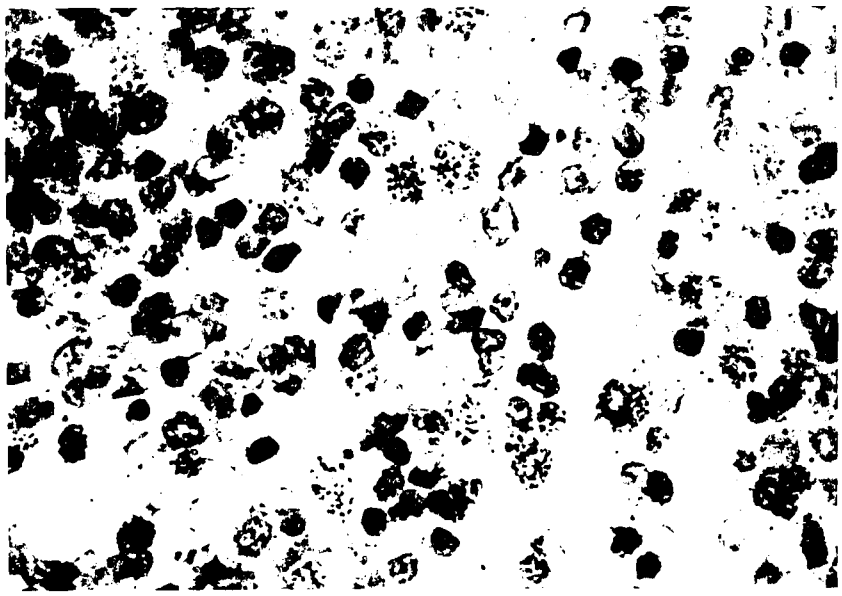

Figure 9 - PHA-P treated animal-2 days-630x
Spleen-Radioautograph of red pulp.

Figure 10 - PHA-P treated animal -1 day-40x
Lymph Node-depletion of small lymphocytes.

Figure 11 - Control animal-40x
Lymph Node.

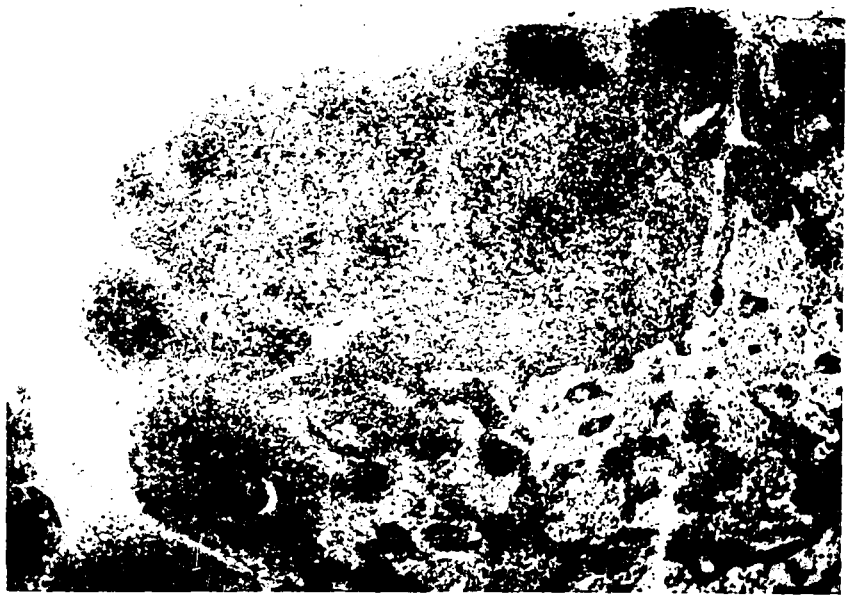

Figure 10 - PHA-P treated animal -1 day-40x
Lymph Node-depletion of small lymphocytes.

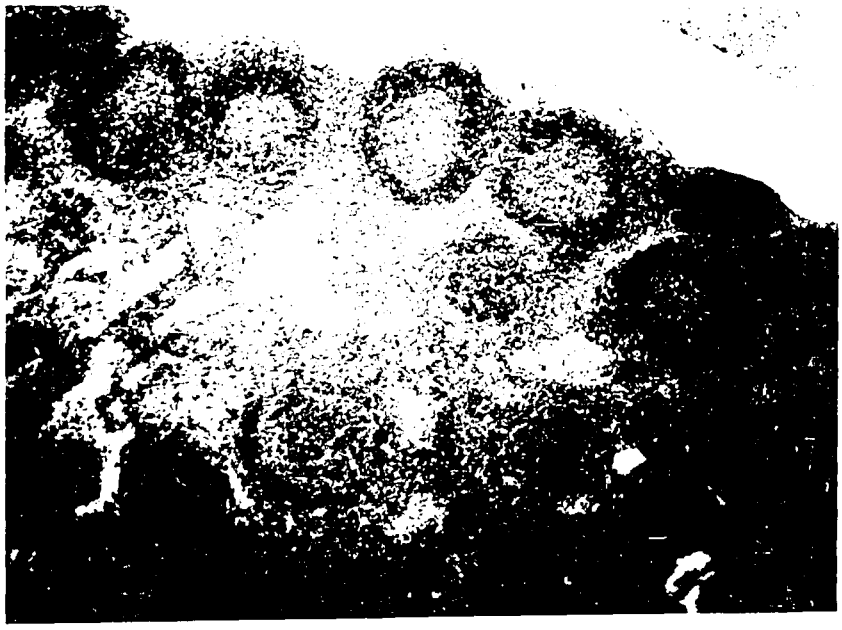

Figure 11 - Control animal-40x
Lymph Node.

Figure 12 - PHA-P treated animal-2 days-40x
Lymph Node-reappearance of normal looking follicles.

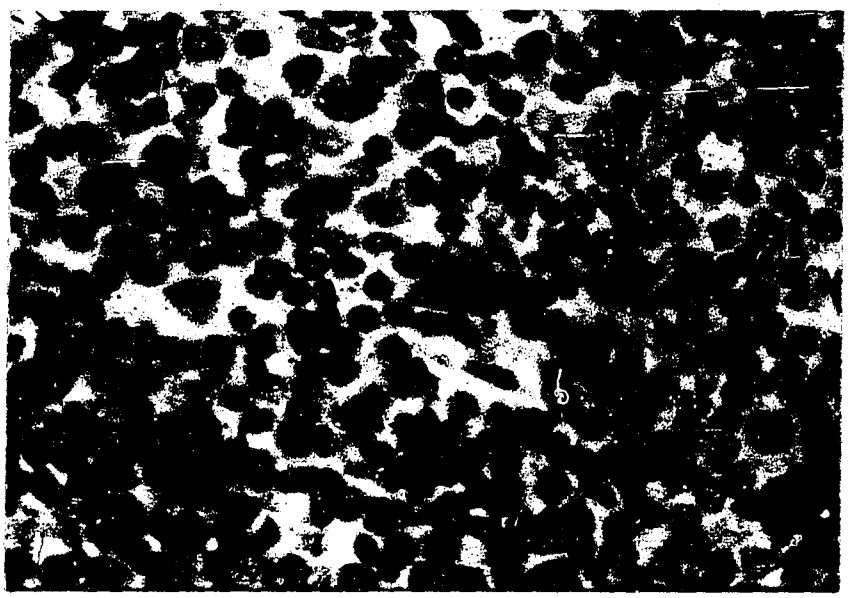

Figure 13 - Control animal-400x
Lymph Node-Radioautograph of interfollicular area.

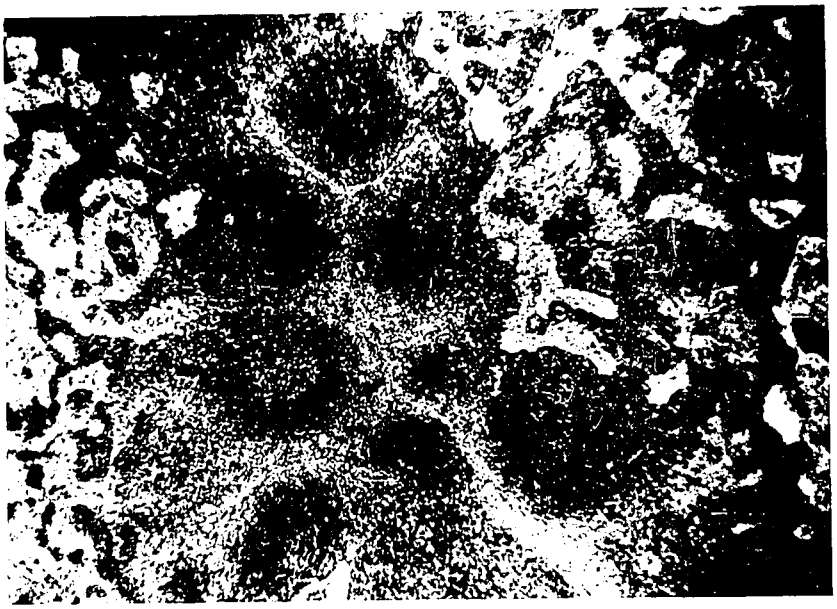

Figure 12 - PHA-P treated animal-2 days-40x
Lymph Node-reappearance of normal looking follicles.

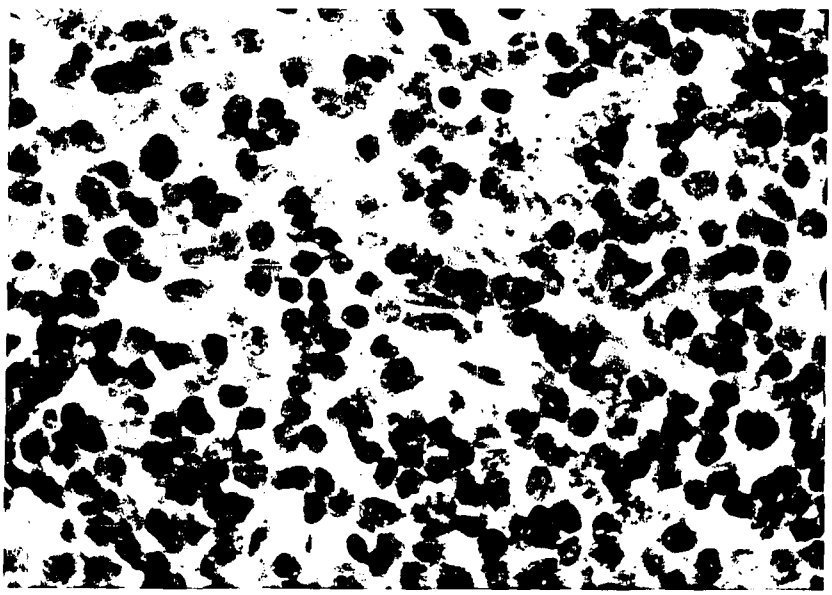

Figure 13 - Control animal-400x
Lymph Node-Radioautograph of interfollicular area.

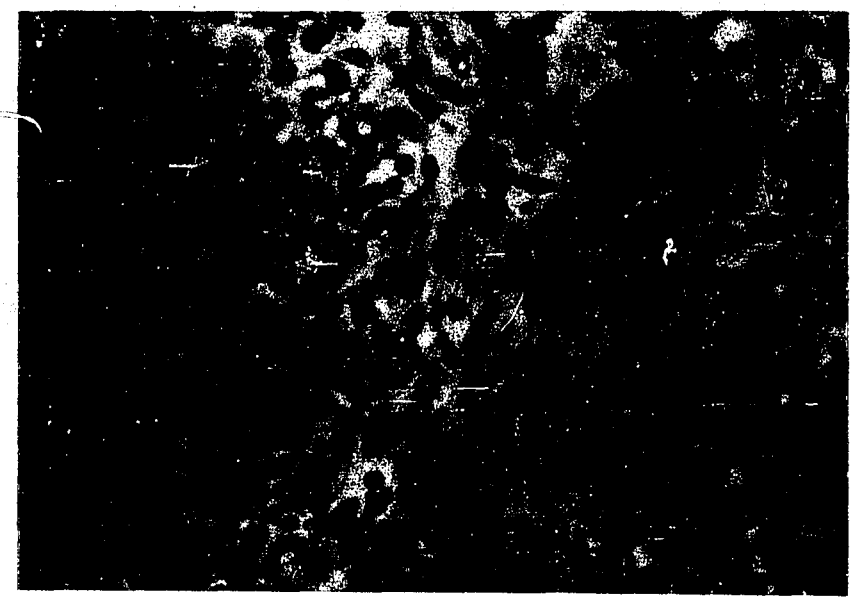

Figure 14 - PHA-P treated animal-2 days-400x
Lymph Node-Radioautograph of interfollicular area.

Figure 15 - Control animal-40x
Appendix

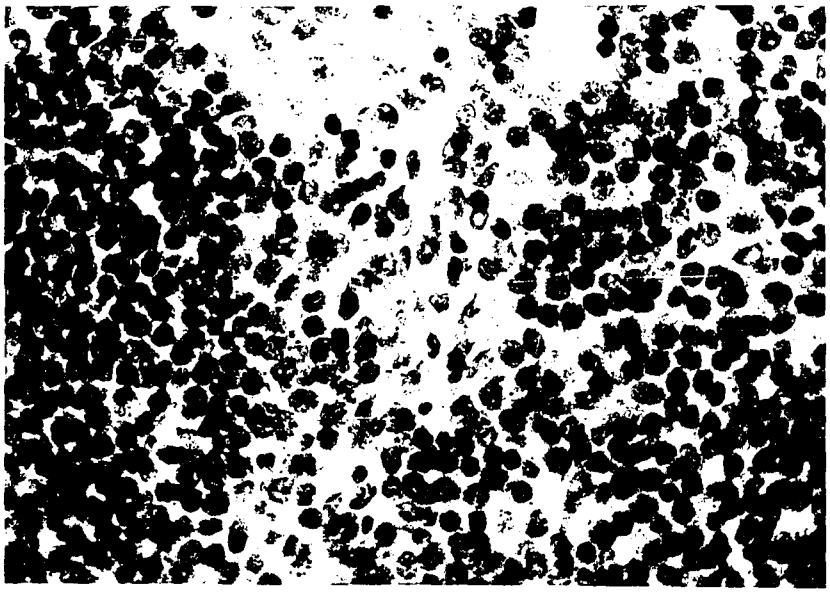

Figure 14 - PHA-P treated animal-2 days-400x
Lymph Node-Radioautograph of interfollicular area.

Figure 15 - Control animal-40x
Appendix

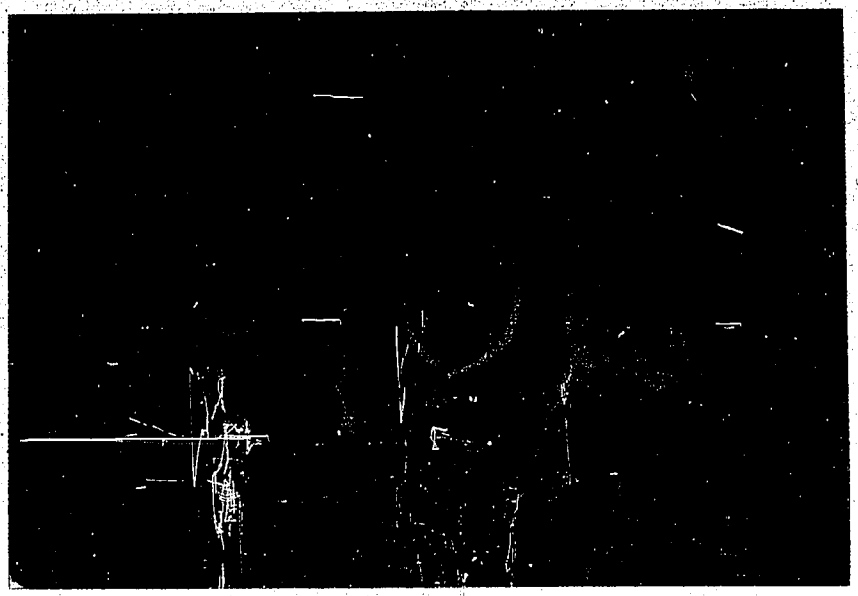

Figure 16 - PHA-P treated animal-2 days-100x
Appendix-edema in follicles.

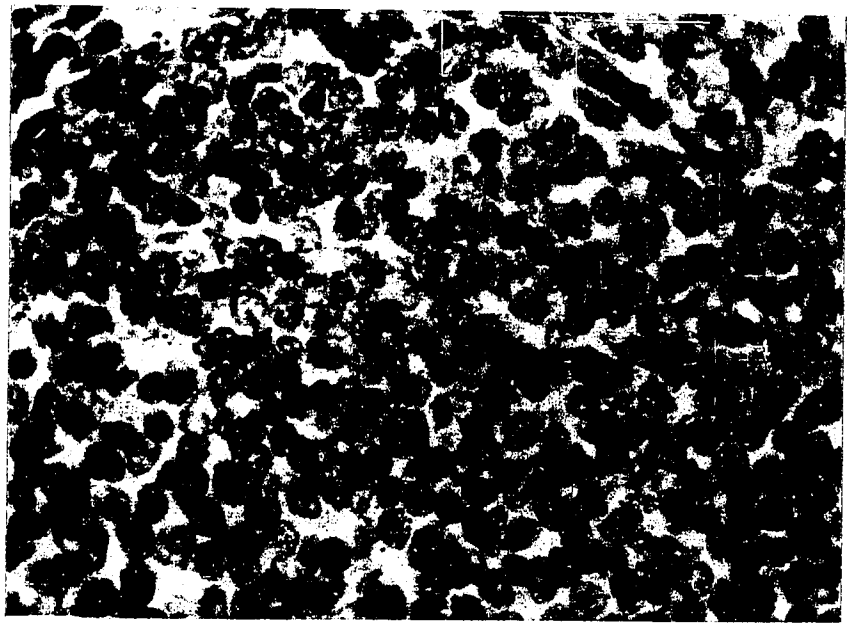

Figure 17 - PHA-P treated animal-2 days-400x
Appendix-Radioautograph of area enclosed by arrows in Figure 16.

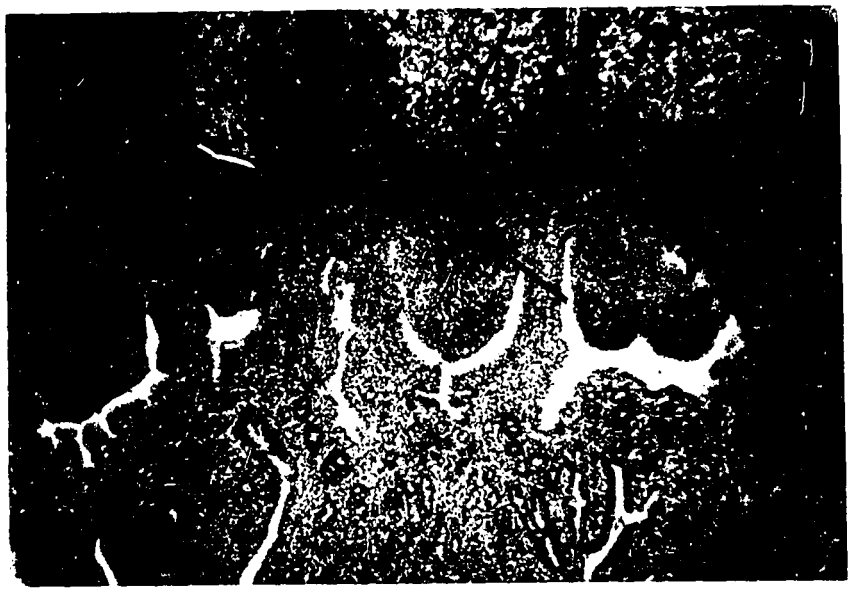

Figure 16 - PHA-P treated animal-2 days-100x
Appendix-edema in follicles.

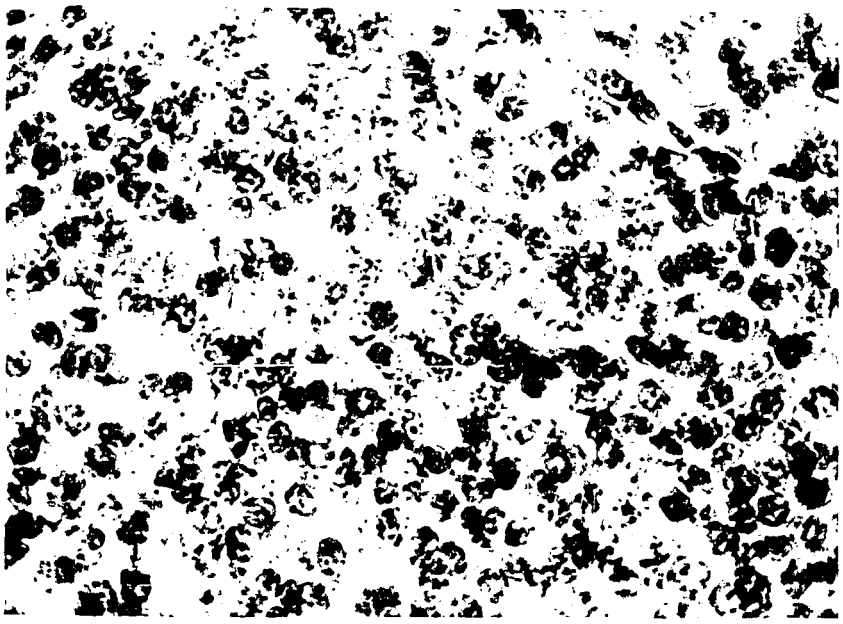

Figure 17 - PHA-P treated animal-2 days-400x
Appendix-Radioautograph of area enclosed by arrows in Figure 16.

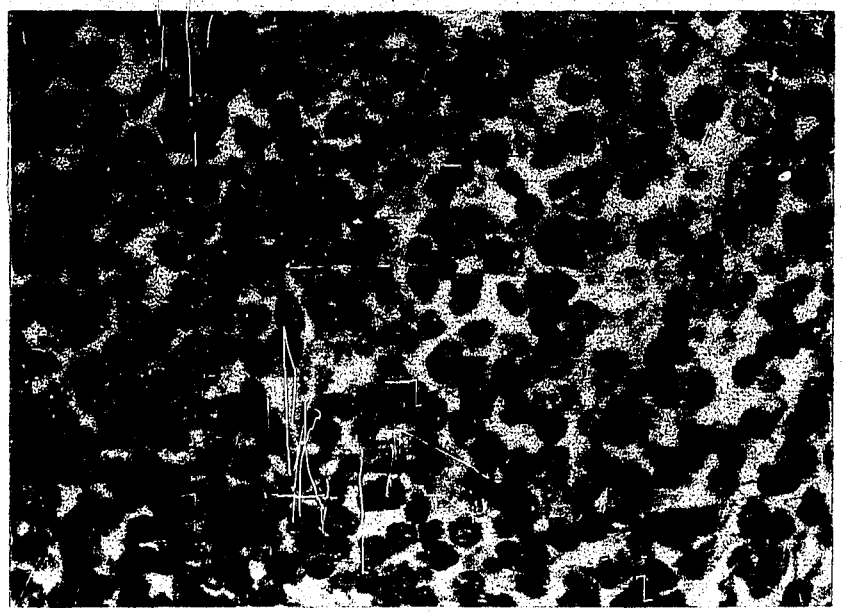

Figure 18 - Control animal-400x
Appendix-Radioautograph of area enclosed by arrows in Figure 15.

Figure 19 - Control animal-25x
Thymus

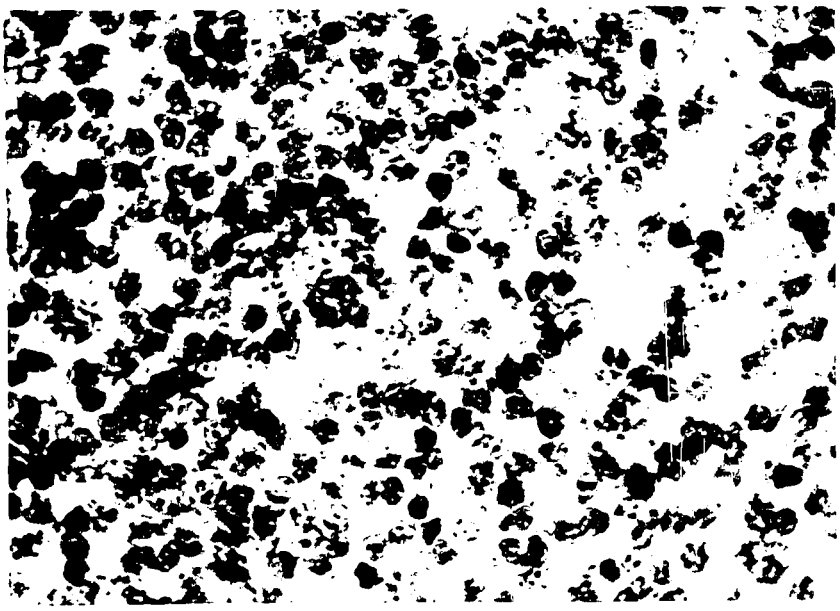

Figure 18 - Control animal-400x
Appendix-Radioautograph of area enclosed by arrows in Figure 15.

Figure 19 - Control animal-25x
Thymus

Figure 20 - PHA-P treated animal-2 days-100x
Thymus-depletion of lymphocytes in the cortex.

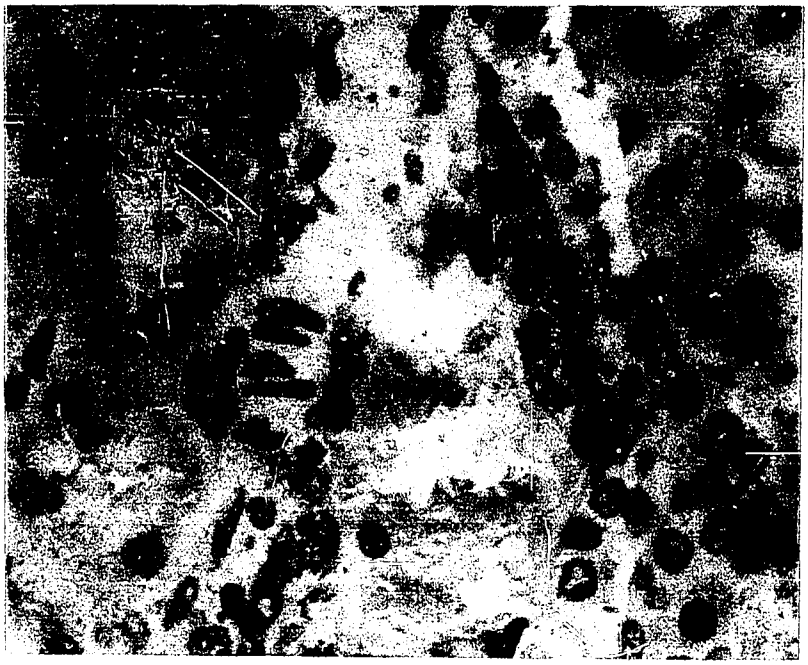

Figure 21 - PHA-P treated animal-2 days-630x
Kidney-Radioautograph-labelled cells infiltrating blood vessel.

Figure 20 - PHA-P treated animal-2 days-100x
Thymus-depletion of lymphocytes in the cortex.

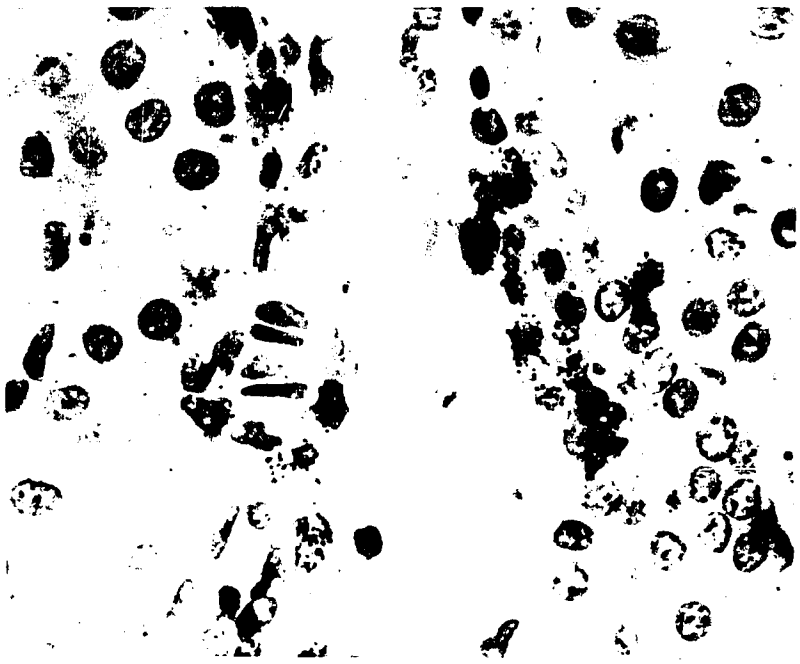

Figure 21 - PHA-P treated animal-2 days-630x
Kidney-Radioautograph-labelled cells infiltrating blood vessel.

Figure 22 - Control animal-40x
Liver

Figure 23 - PHA-P treated animal-2 days-100x
Liver-portal area-infiltration of mononuclear cells.

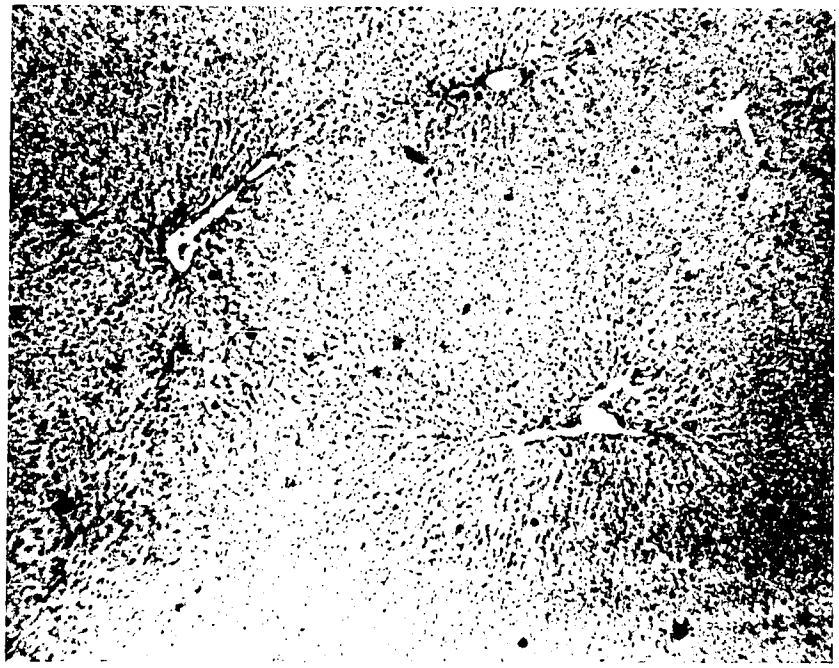

Figure 22 - Control animal-40x
Liver

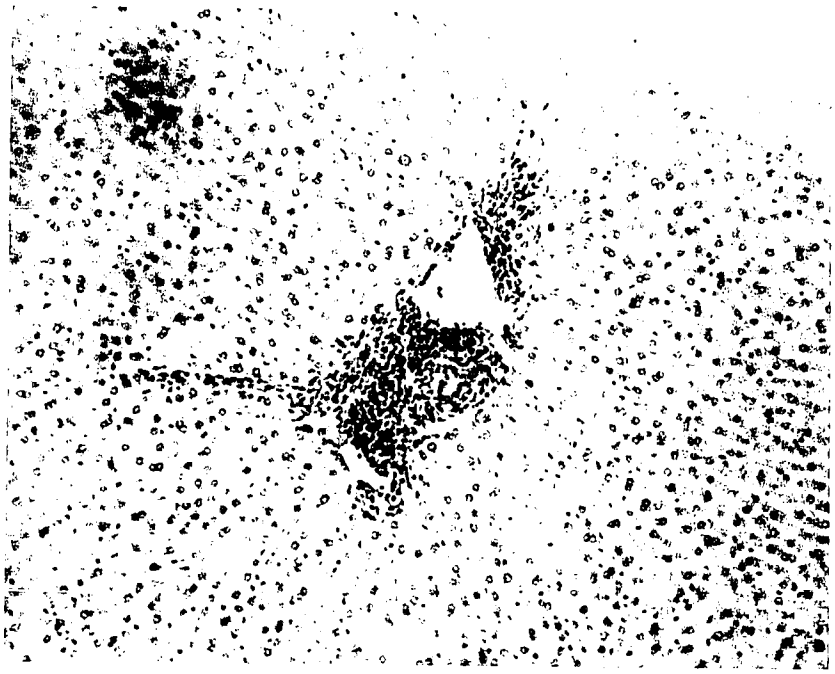

Figure 23 - PHA-P treated animal-2 days-100x
Liver-portal area-infiltration of mononuclear cells.

Figure 24 - PHA-P treated animal-1 day-25x
Lymph Node-depletion of small lymphocytes.

Figure 25 - Control animal-40x
Lymph node.

Figure 24 - PHA-P treated animal-1 day-25x
Lymph Node-depletion of small lymphocytes.

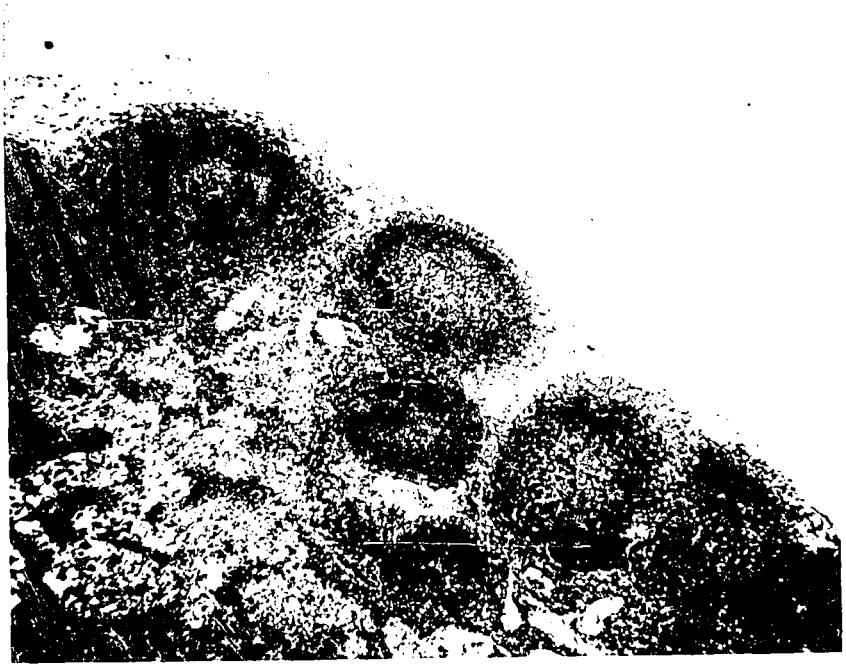

Figure 25 - Control animal-40x
Lymph node.

Figure 26 - PHA-P treated animal-2 days-25x
Lymph Node-reappearance of normal follicles.

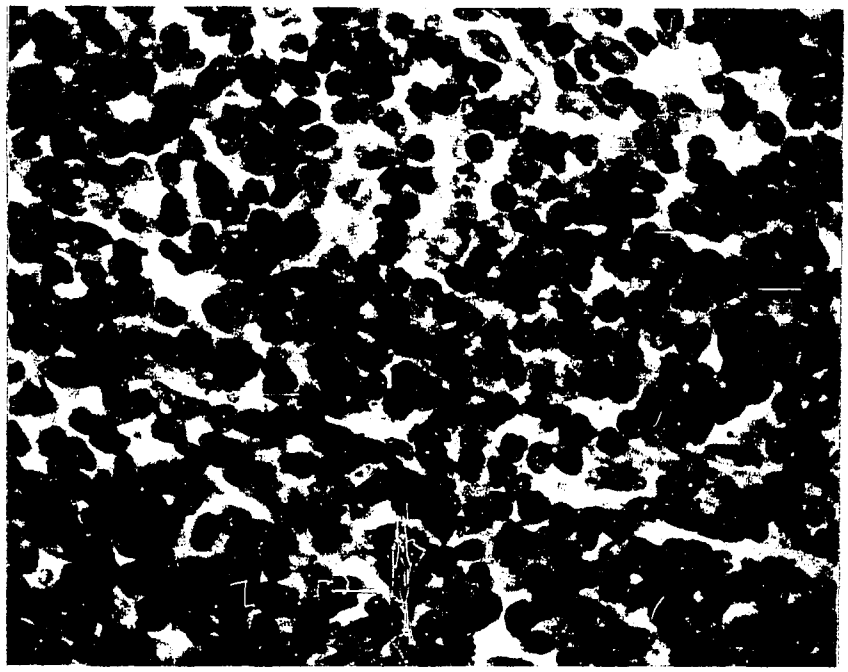

Figure 27 - PHA-P treated animal-2 days-630x
Lymph Node- Radioautograph of interfollicular area.

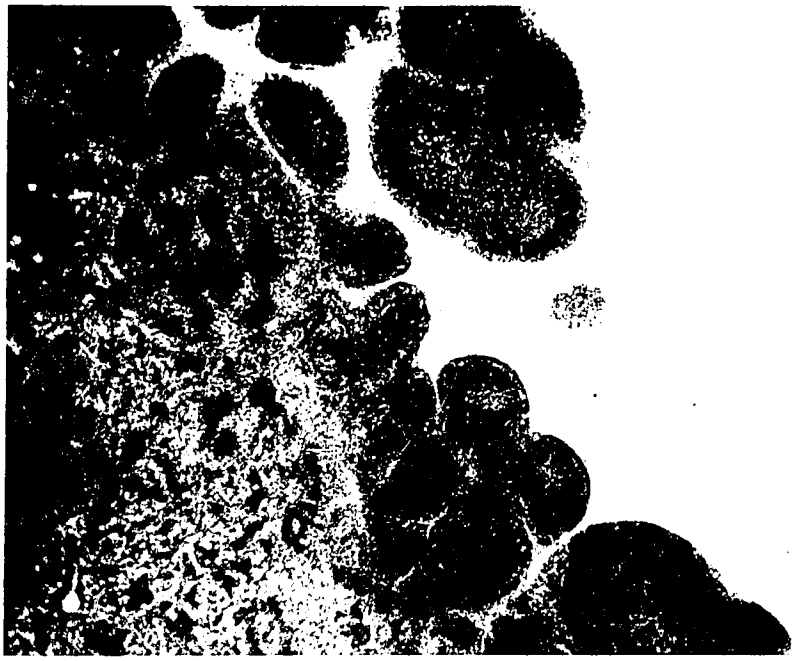

Figure 26 - PHA-P treated animal-2 days-25x
Lymph Node-reappearance of normal follicles.

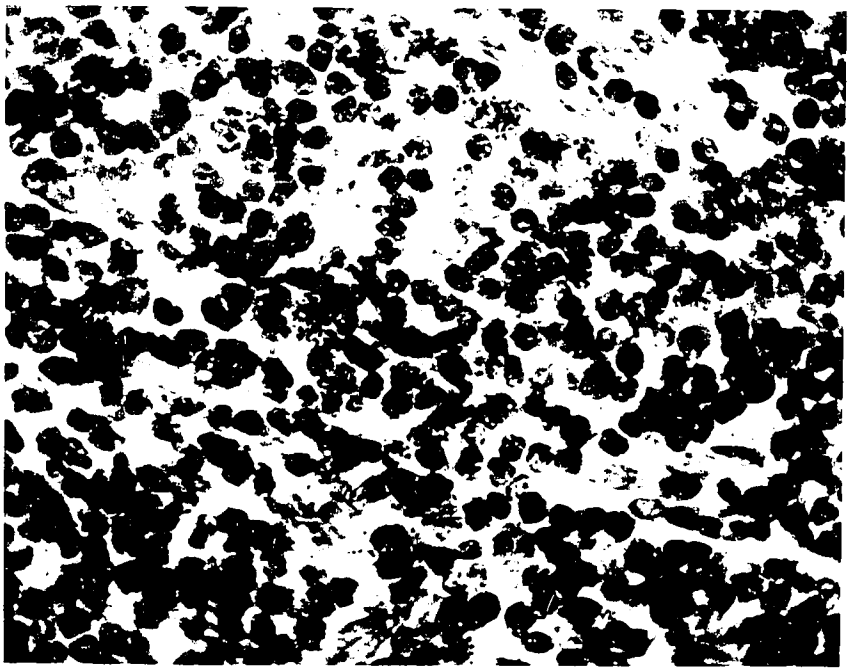

Figure 27 - PHA-P treated animal-2 days-630x
Lymph Node- Radioautograph of interfollicular area.

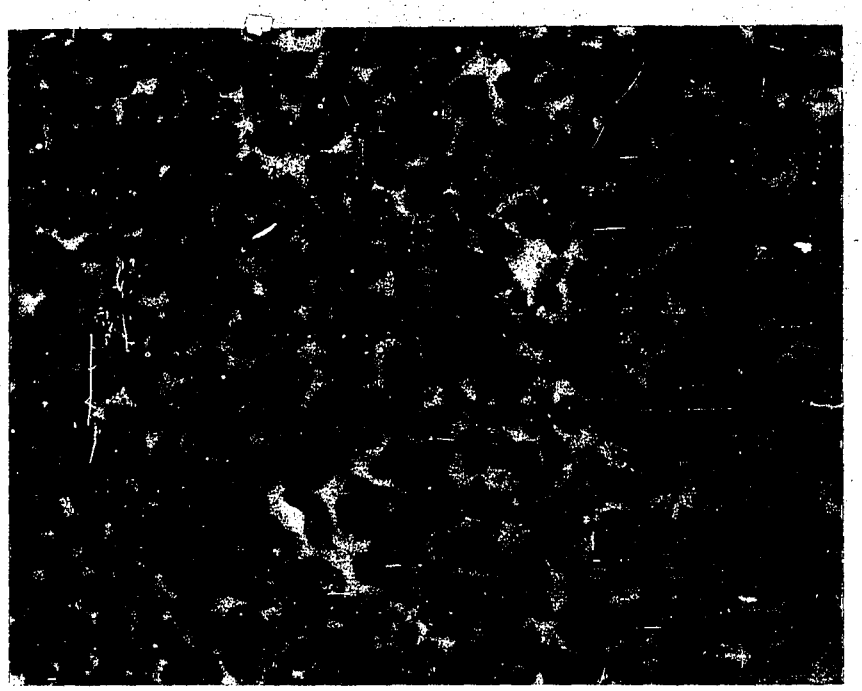

Figure 28 - Control animal-630x
Lymph Node-Radioautograph of interfollicular area.

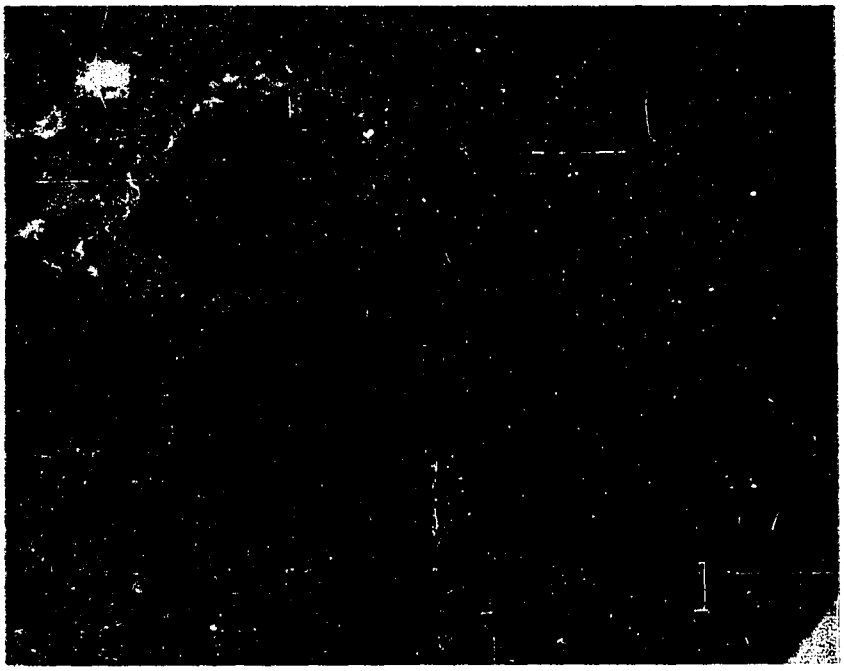

Figure 29 - PHA-P treated animal-2 days-40x
Spleen-enlargement of white pulp.

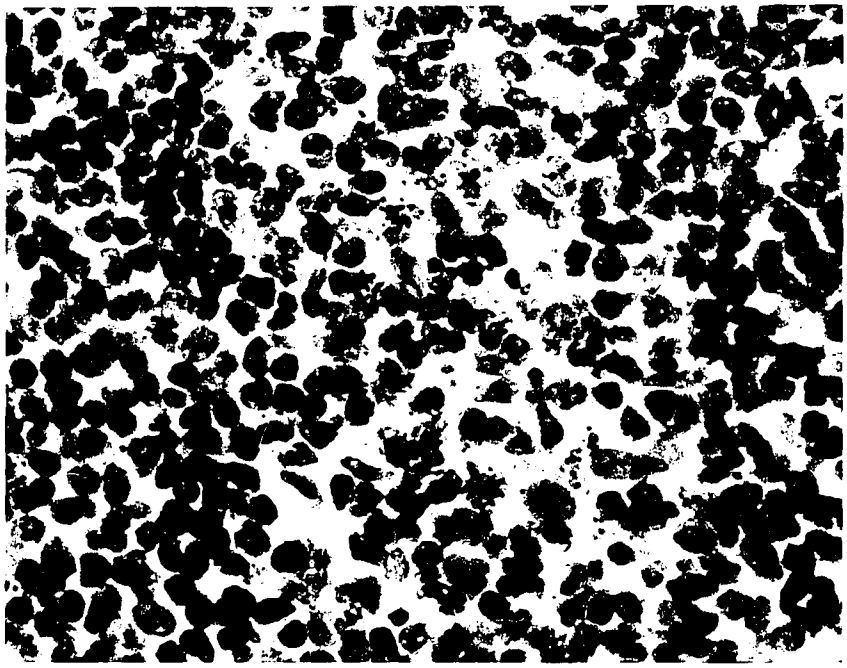

Figure 28 - Control animal-630x
Lymph Node-Radioautograph of interfollicular area.

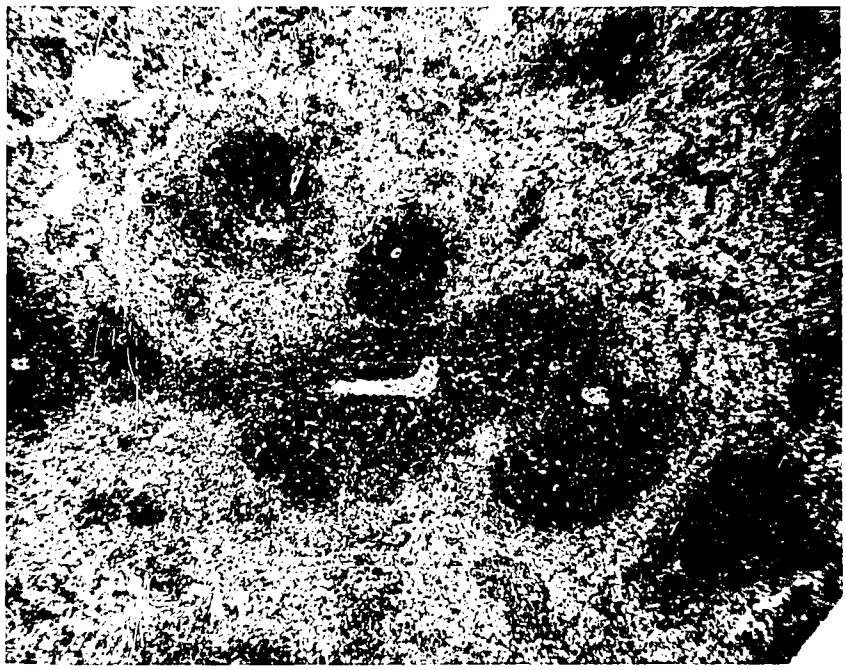

Figure 29 - PHA-P treated animal-2 days-430x
Spleen-enlargement of white pulp.

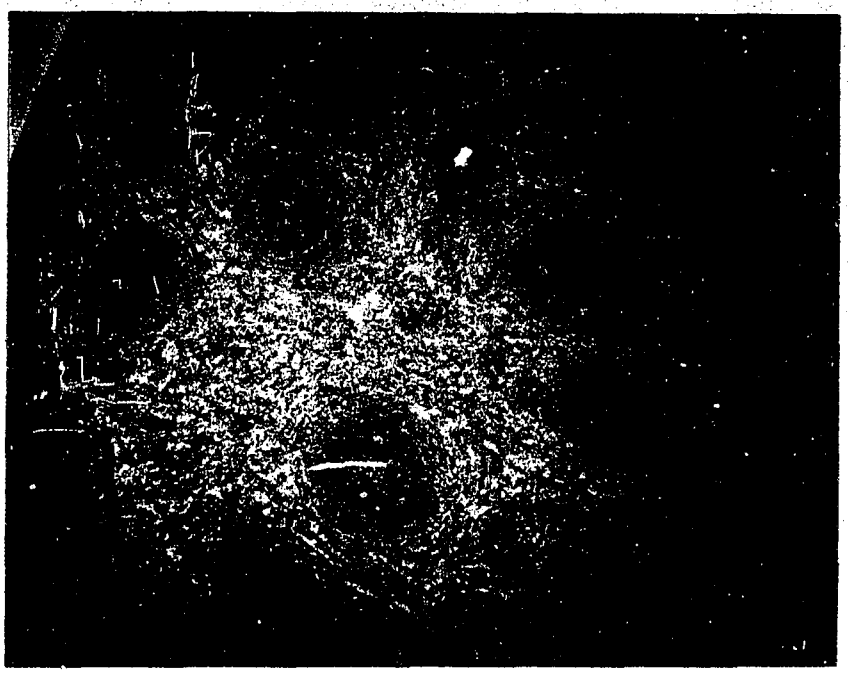

Figure 30 - Control animal-25x
Spleen-normal looking follicles.

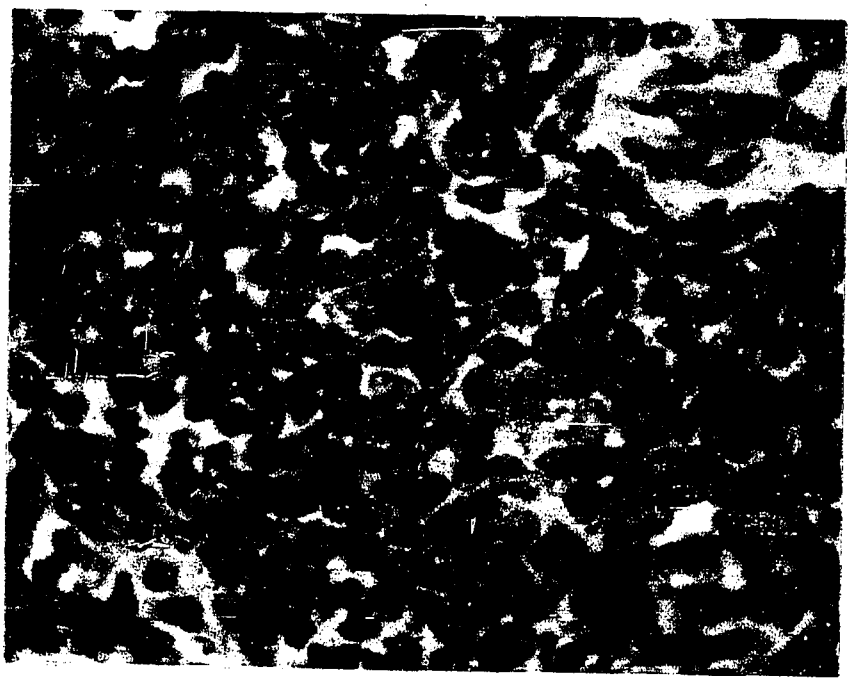

Figure 31 - PHA-P treated animal-2 days-630x
Spleen-Radioautograph of white pulp.

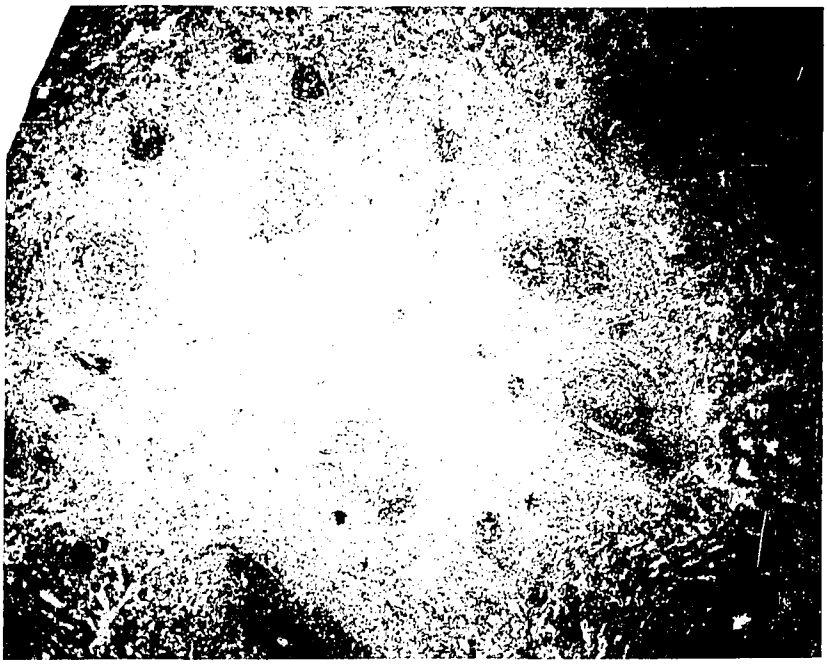

Figure 30 - Control animal-25x
Spleen-normal looking follicles.

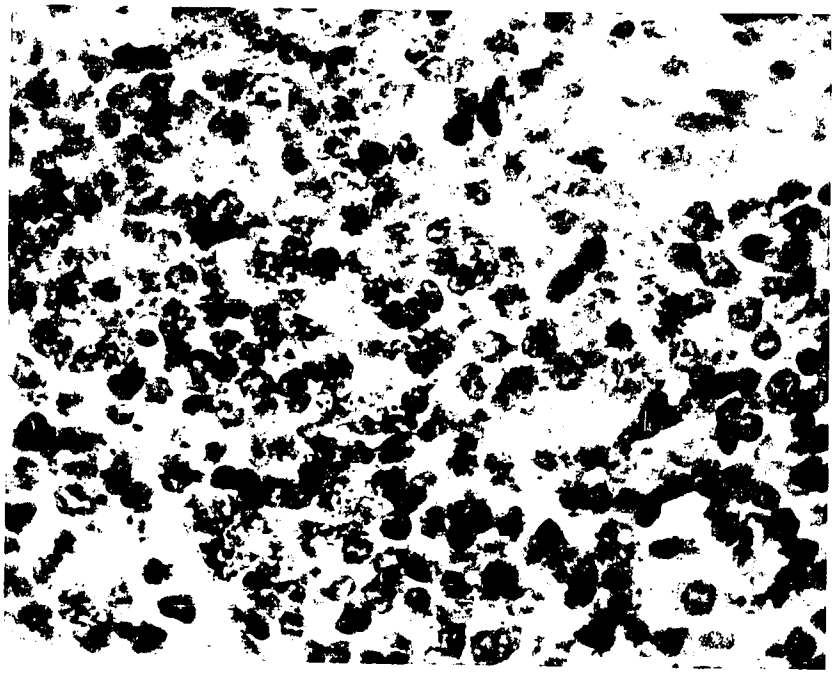

Figure 31 - PHA-P treated animal-2 days-630x
Spleen-Radioautograph of white pulp.

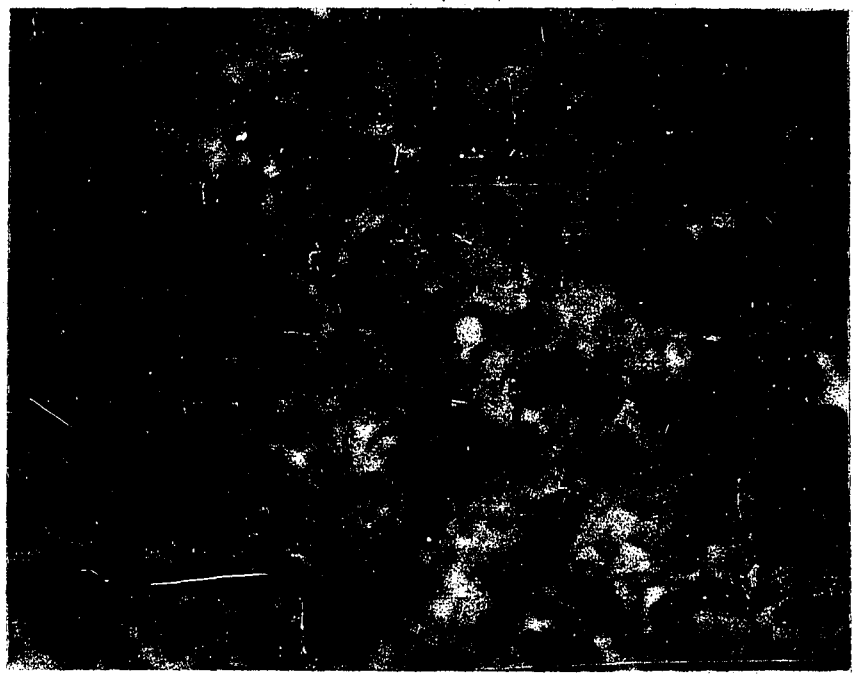

Figure 32 - PHA-P treated animal-2 days-630x
Spleen-Radioautograph of red pulp.

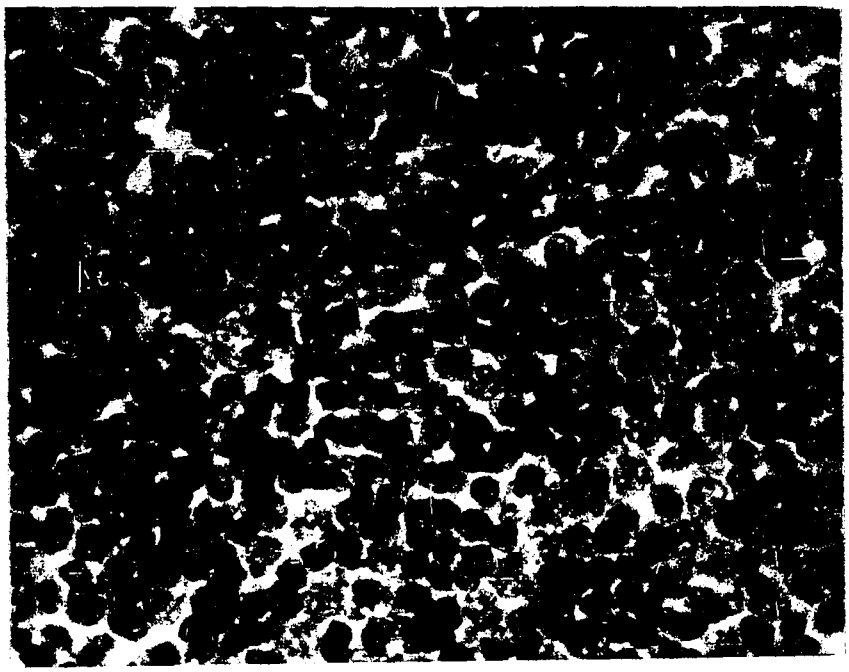

Figure 33 - Control animal-630x
Spleen-Radioautograph of white pulp.

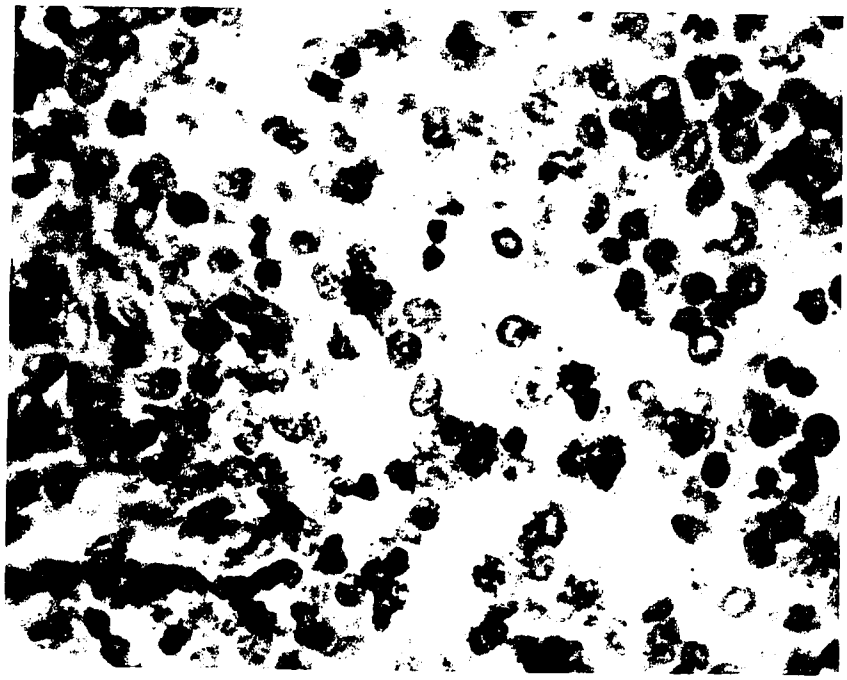

Figure 32 - PHA-P treated animal-2 days-630x
Spleen-Radioautograph of red pulp.

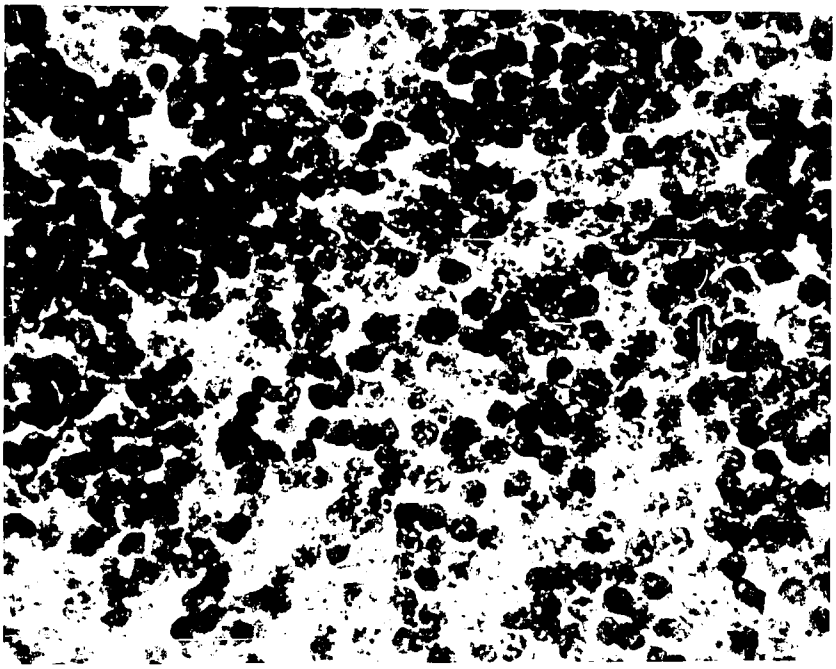

Figure 33 - Control animal-630x
Spleen-Radioautograph of white pulp.

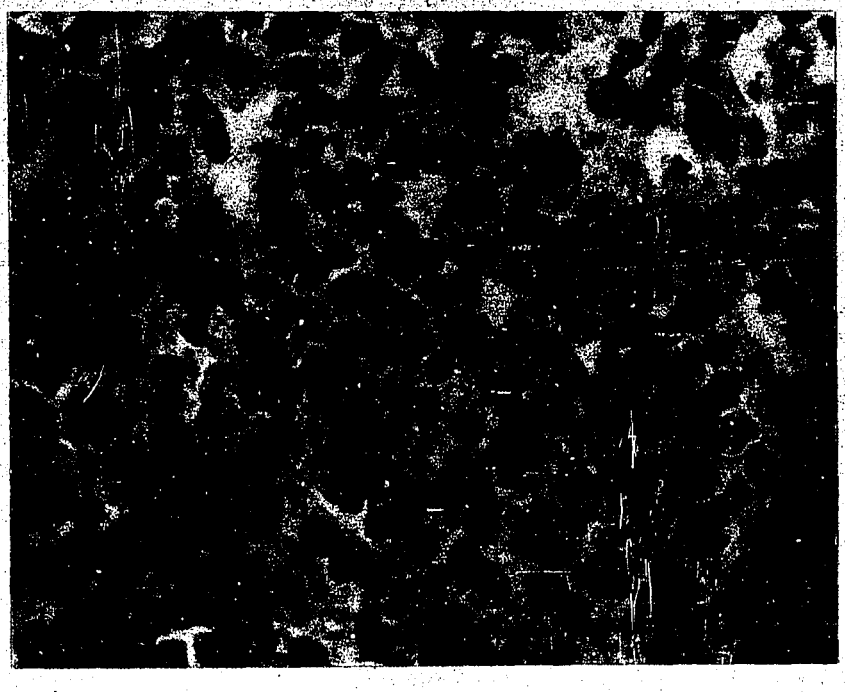

Figure 34 - Control animal-630x
Spleen-Radioautograph of red pulp.

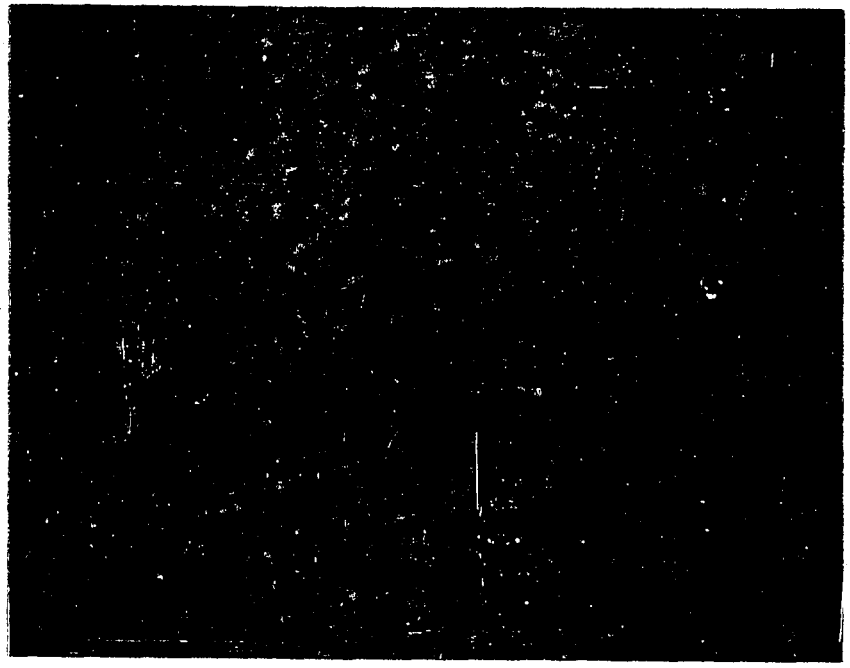

Figure 35 - PHA-P treated animal-8 days-100x
Spleen-normoblastic islands in the red pulp.

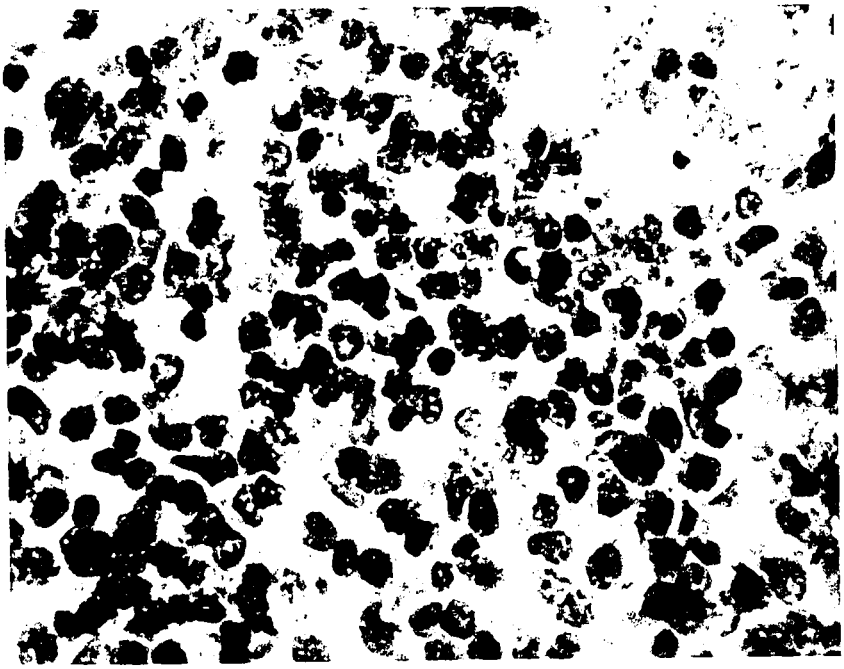

Figure 34 - Control animal-630x
Spleen-Radioautograph of red pulp.

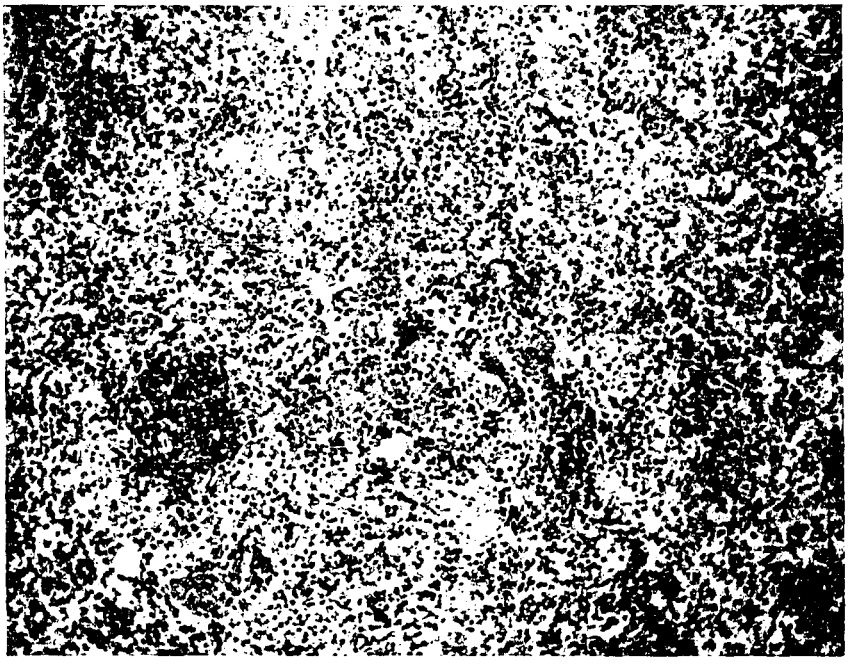

Figure 35 - PHA-P treated animal-8 days-100x
Spleen-normoblastic islands in the red pulp.

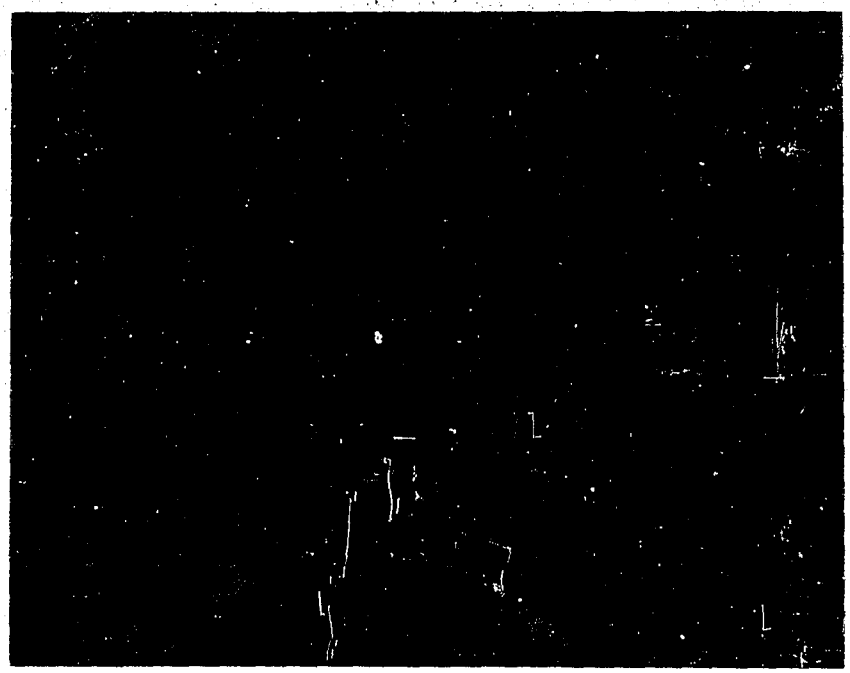

Figure 36 - PHA-P treated animal-8 days-400x
Spleen- myeloid-erythroid infiltration in the red pulp.

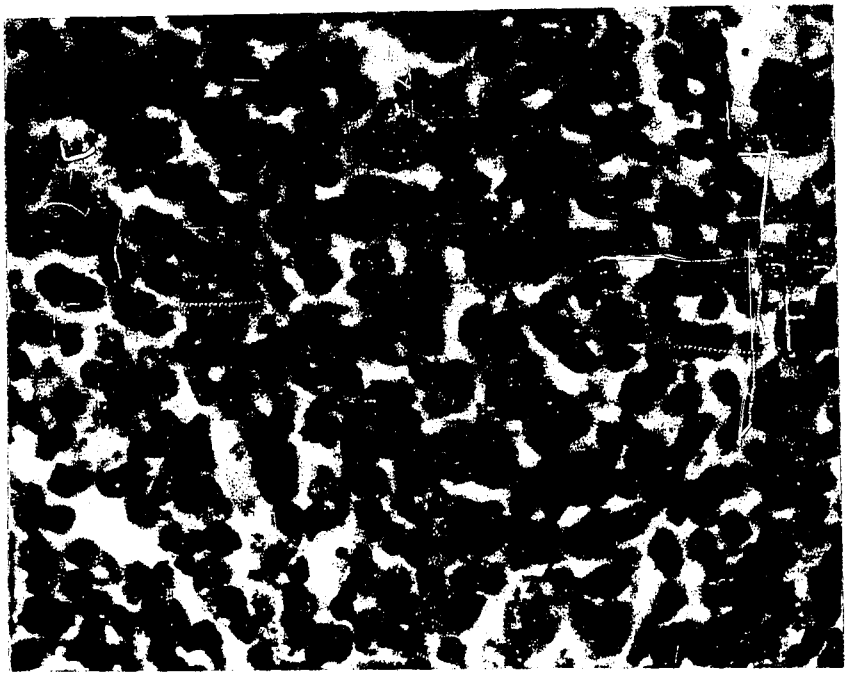

Figure 37 - PHA-P treated animal-10 days-630x
Spleen-Radioautograph of red pulp.

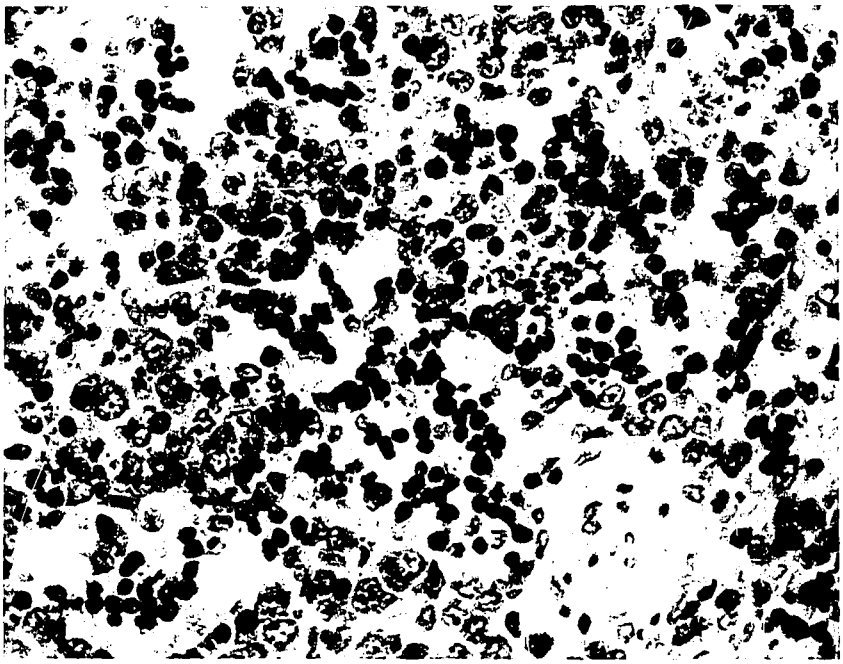

Figure 36 - PHA-P treated animal-8 days-400x
Spleen- myeloid-erythroid infiltration in the red pulp.

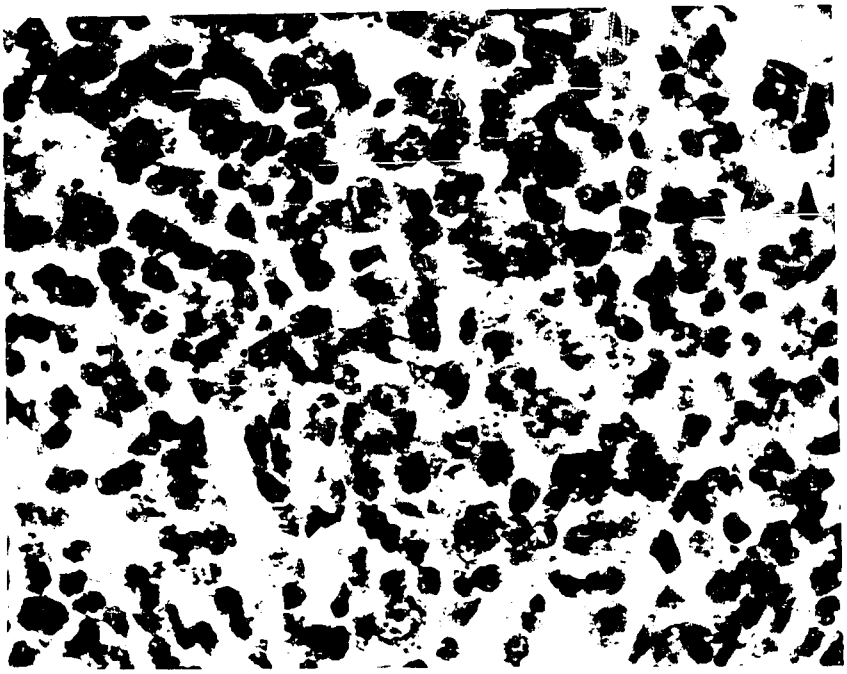

Figure 37 - PHA-P treated animal-10 days-630x
Spleen-Radioautograph of red pulp.

Figure 38 - PHA-P treated animal-10 days-40x
Spleen-hyperplasia of white pulp, large germinal centers.

Figure 39 - PHA-P treated animal-2 days-25x
Appendix-edema in the follicles.

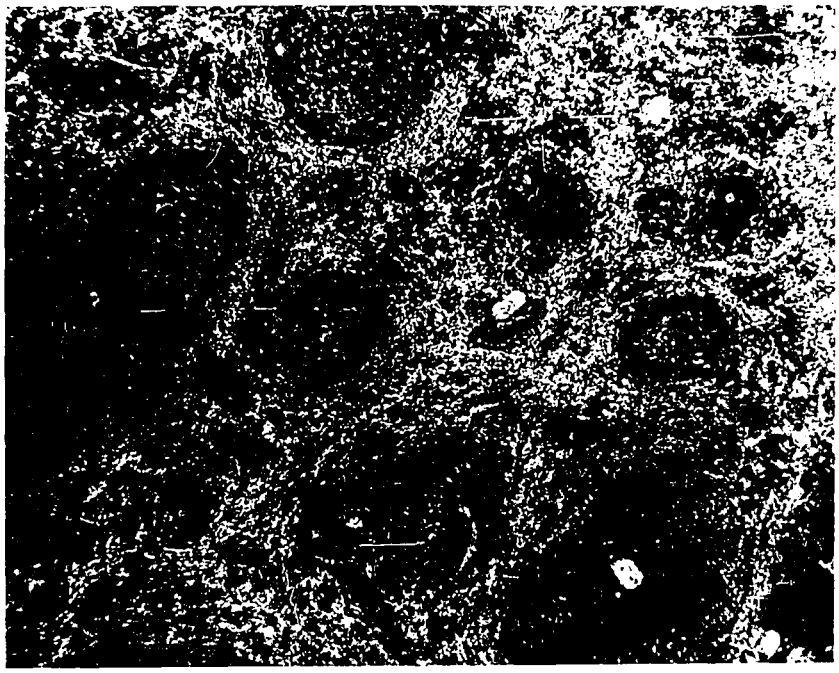

Figure 38 - PHA-P treated animal-10 days-40x
Spleen-hyperplasia of white pulp, large germinal centers.

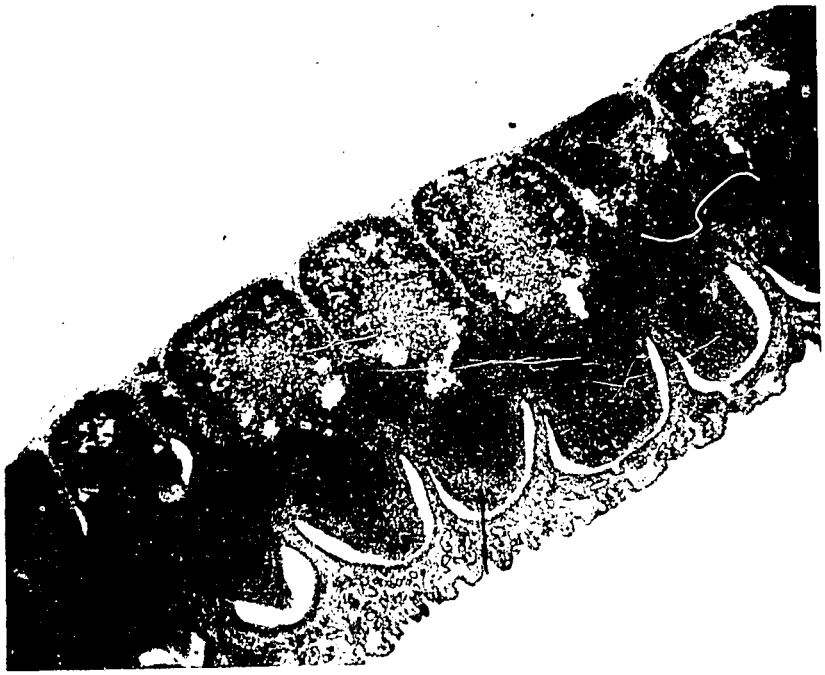

Figure 39 - PHA-P treated animal-2 days-25x
Appendix-edema in the follicles.

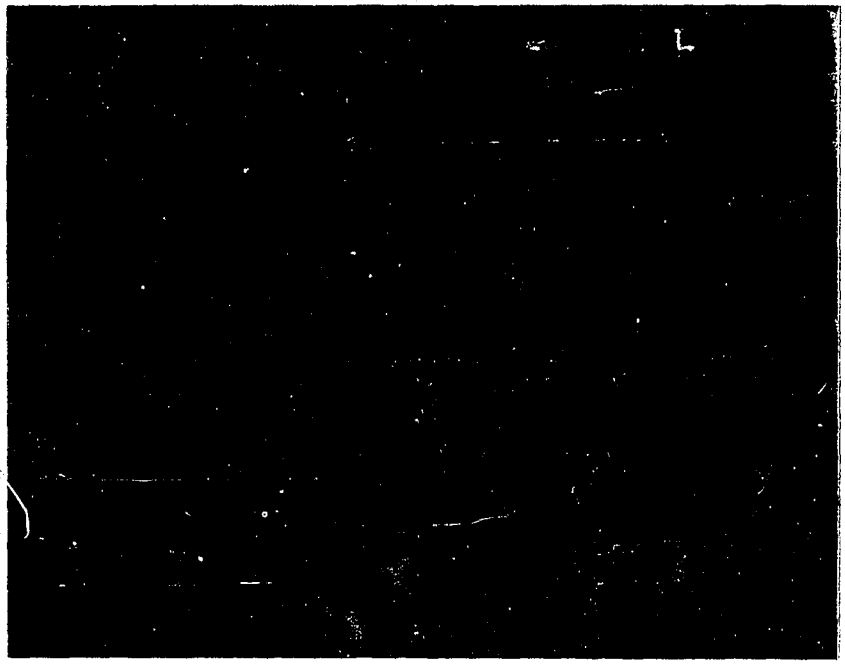

Figure 40 - PHA-P treated animal-2 days-630x
Appendix-Radioautograph of area enclosed by arrows in Figure 39.

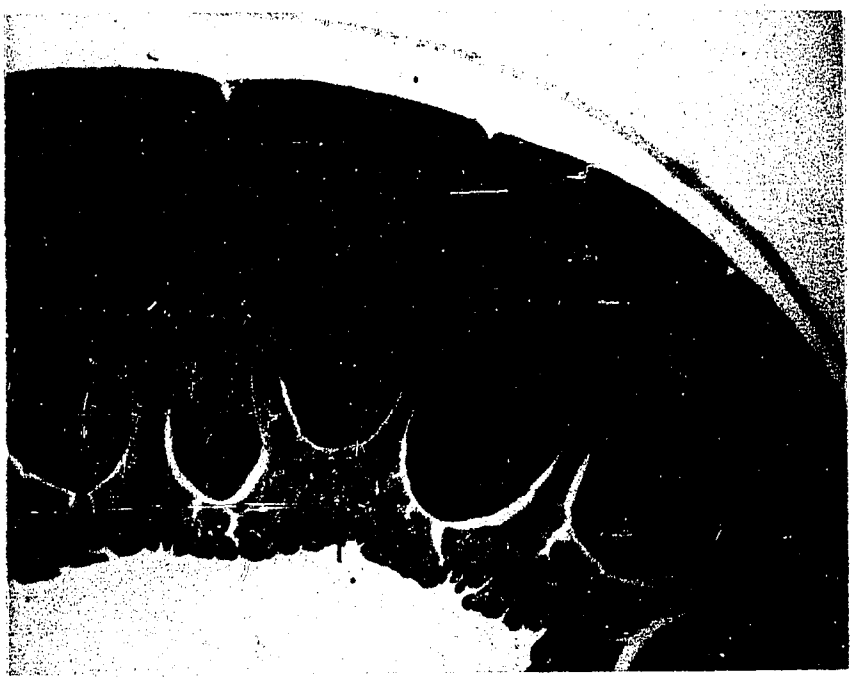

Figure 41 - Control animal-100x
Appendix

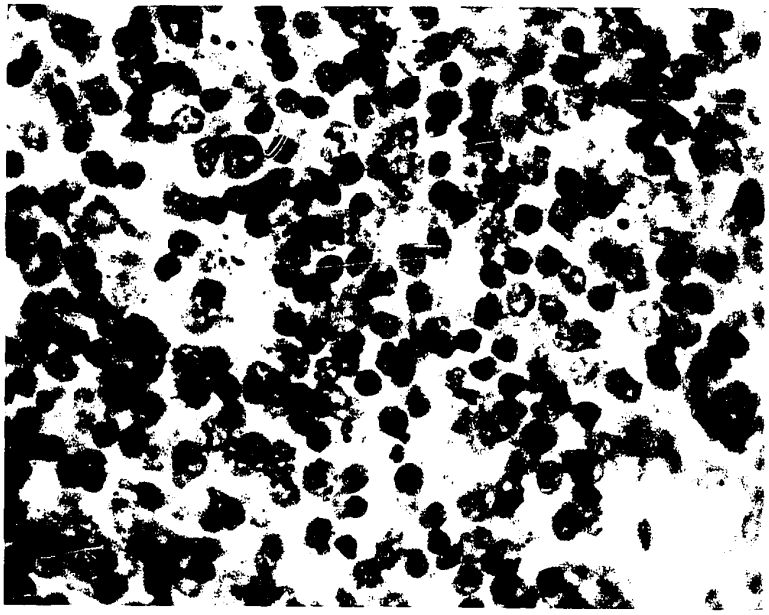

Figure 40 - PHA-P treated animal-2 days-630x
Appendix-Radioautograph of area enclosed by arrows in Figure 39.

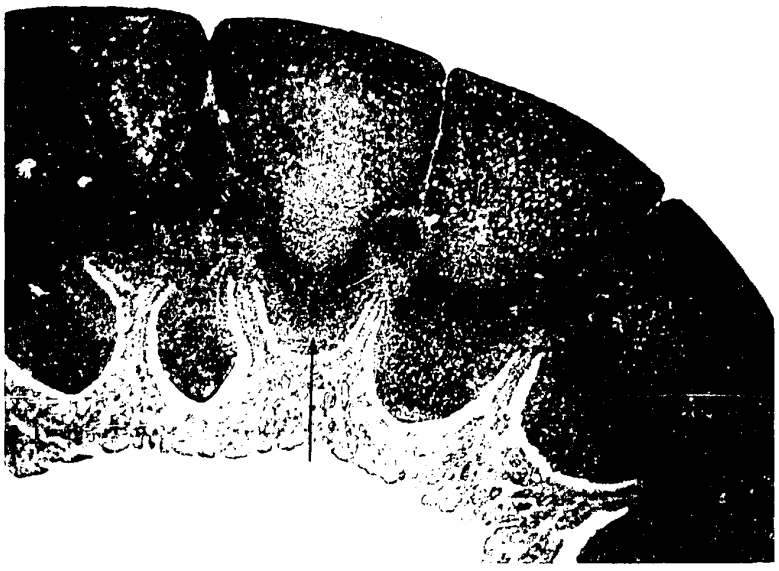

Figure 41 - Control animal-100x
Appendix

Figure 42 - Control animal-630x
Appendix-Radioautograph of area enclosed by arrows in Figure 41.

Figure 43 - PHA-P treated animal-1 day-25x
Thymus-enlargement of medulla

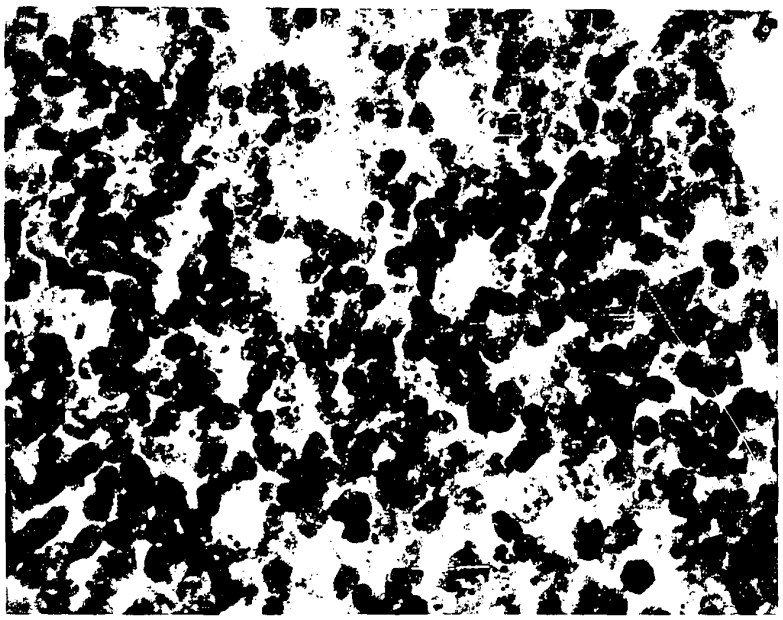

Figure 42 - Control animal-630x
Appendix-Radioautograph of area enclosed by arrows in Figure 41.

Figure 43 - PHA-P treated animal-1 day-25x
Thymus-enlargement of medulla

Figure 44 - Control animal-25x
Thymus

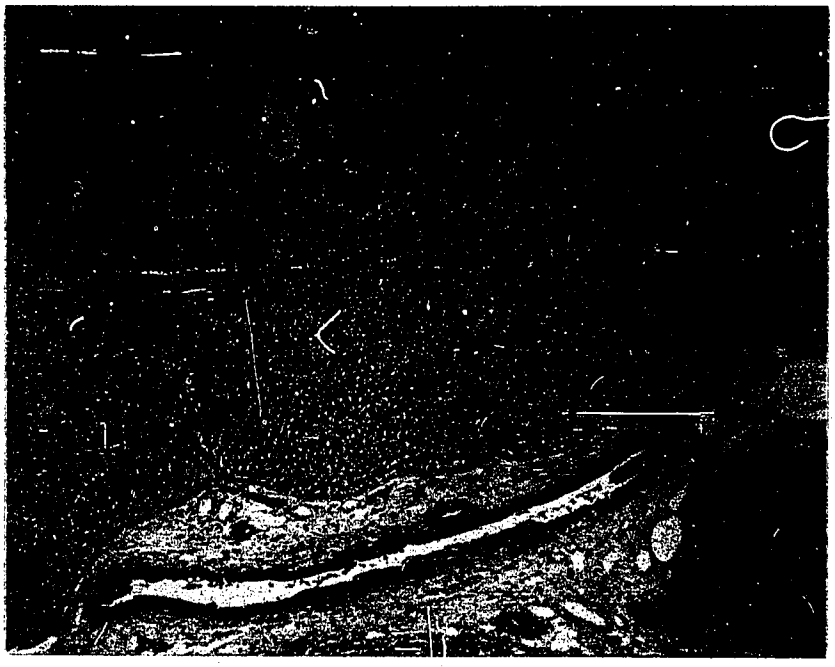

Figure 45 - PHA-M treated animal-2 days-40x
Liver-mild mononuclear infiltration in portal area.

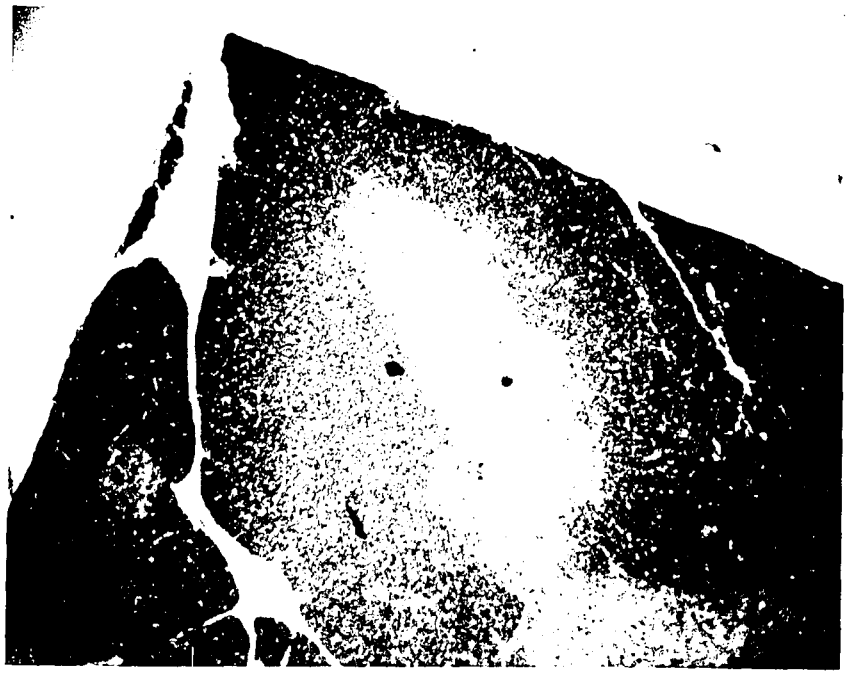

Figure 44 - Control animal-25x
Thymus

Figure 45 - PHA-M treated animal-2 days-40x
Liver-mild mononuclear infiltration in portal area.

Figure 46 - PHA-M treated animal-2 days-40x
Spleen-hyperplasia of white pulp.

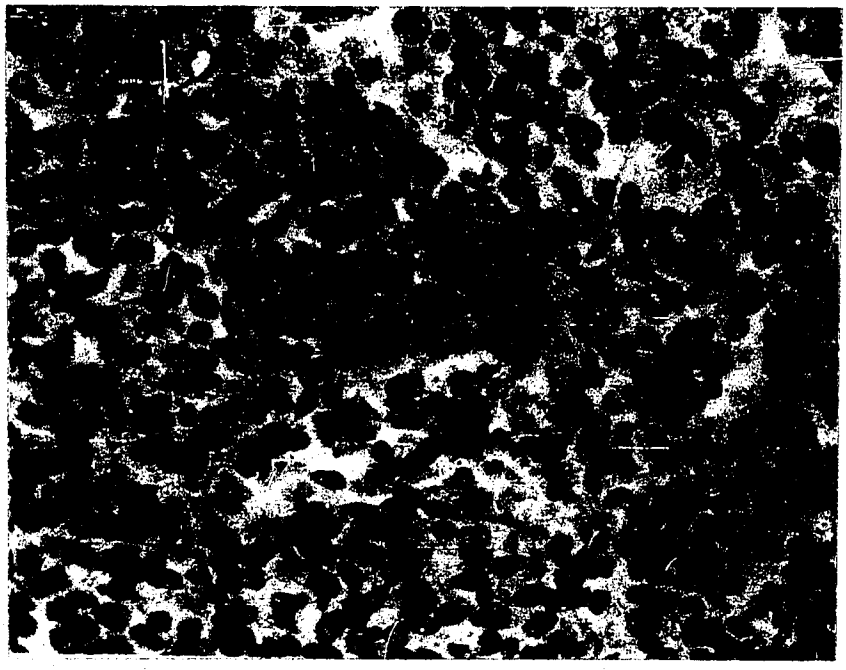

Figure 47 - PHA-M treated animal-2 days-400x
Spleen-Radioautograph of red pulp.

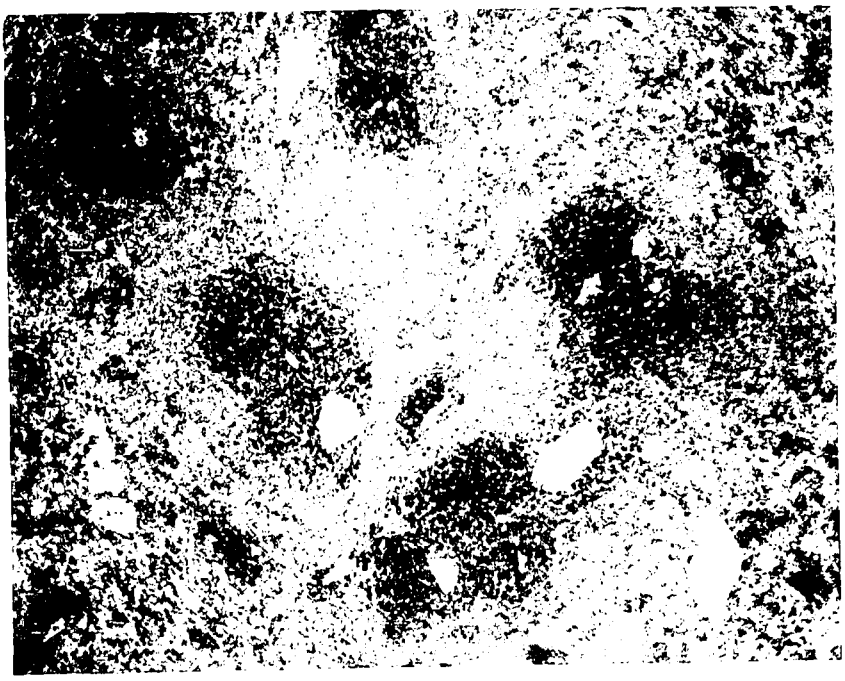

Figure 46 - PHA-M treated animal-2 days-40x
Spleen-hyperplasia of white pulp.

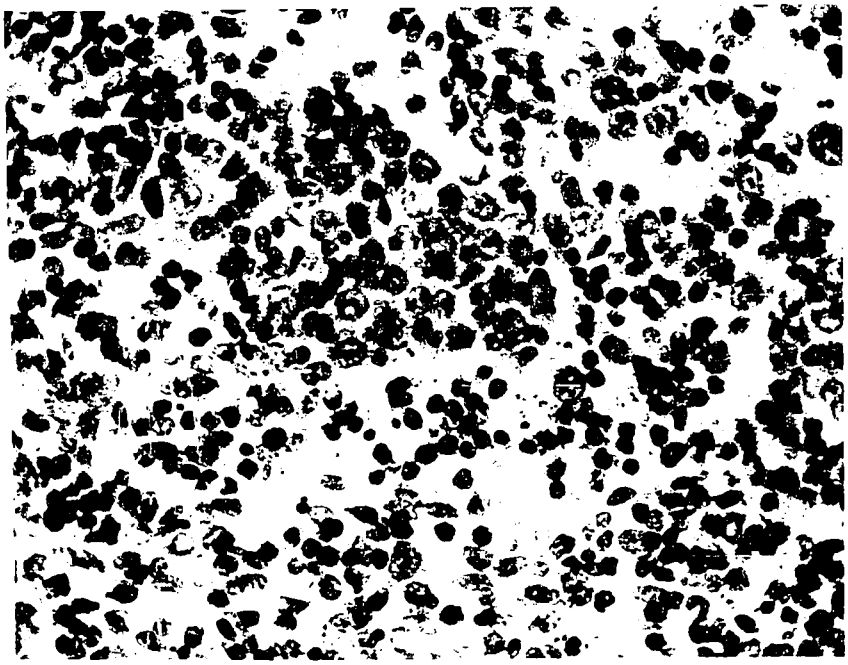

Figure 47 - PHA-M treated animal-2 days-400x
Spleen-Radioautograph of red pulp.

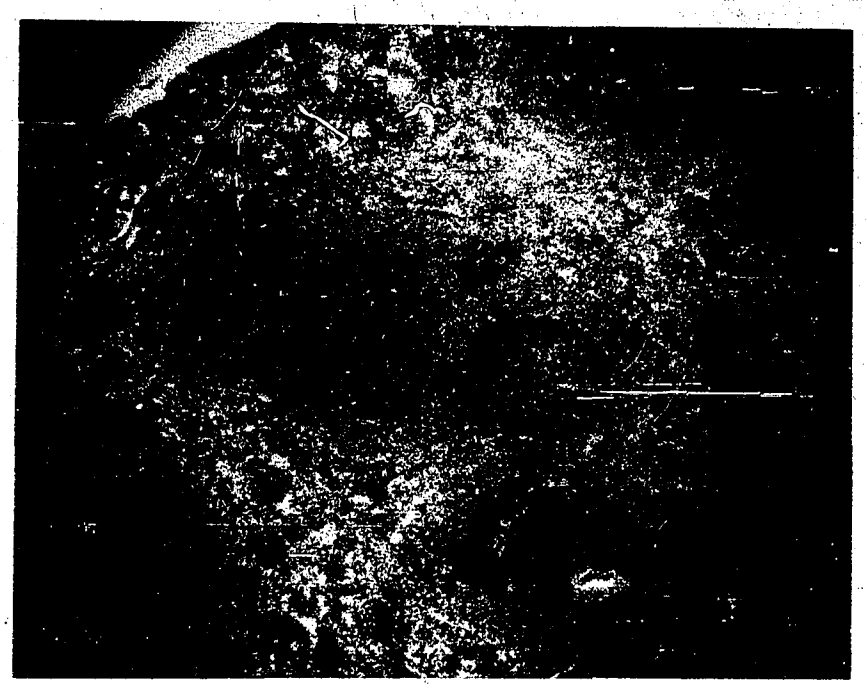

Figure 48 -PHA-M treated animal-7 days-40x
Spleen-hyperplastic white pulp with large germinal centers.

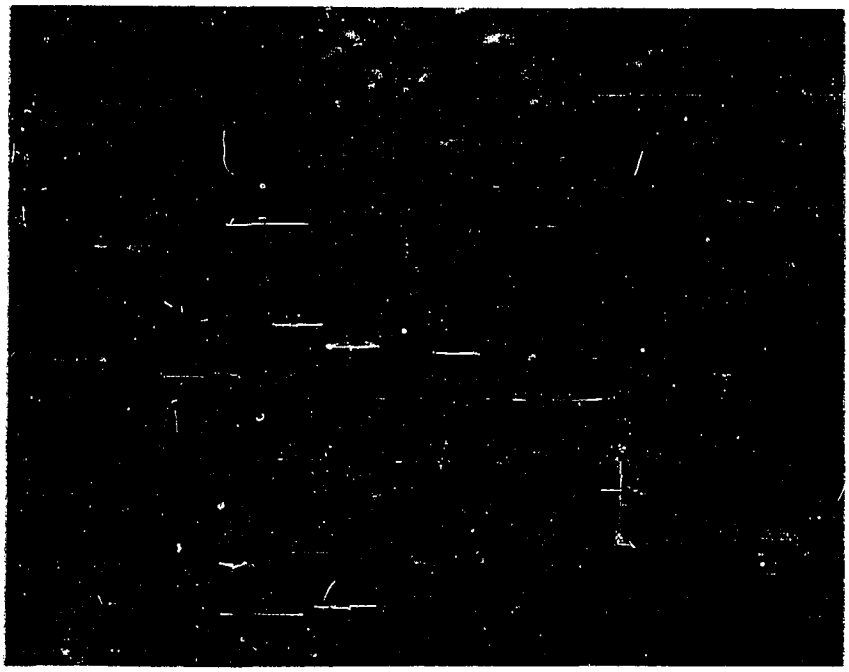

Figure 49 - PHA-M treated animal-7 days-100x
Spleen-follicle with prominent external mantle.

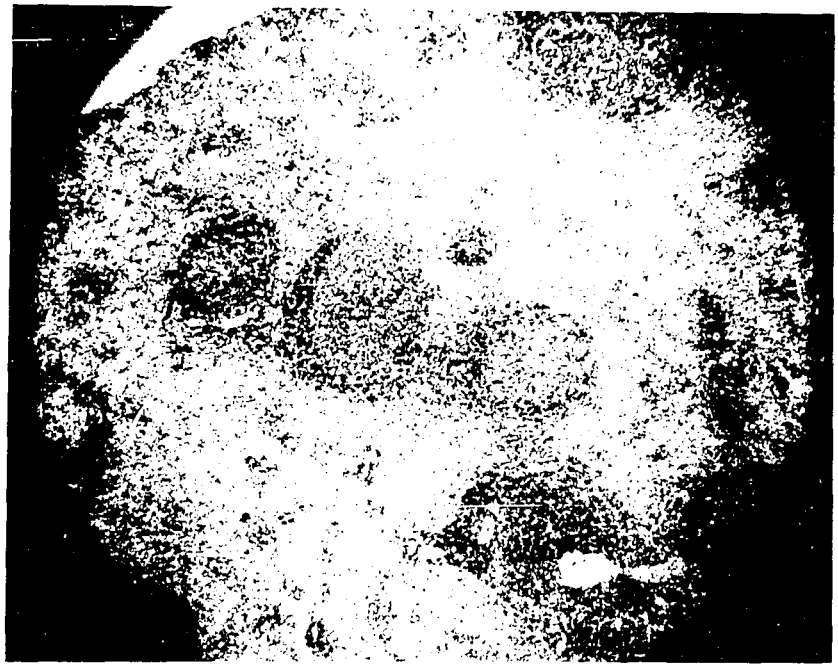

Figure 48 -PHA-M treated animal-7 days-40x
Spleen-hyperplastic white pulp with large germinal centers.

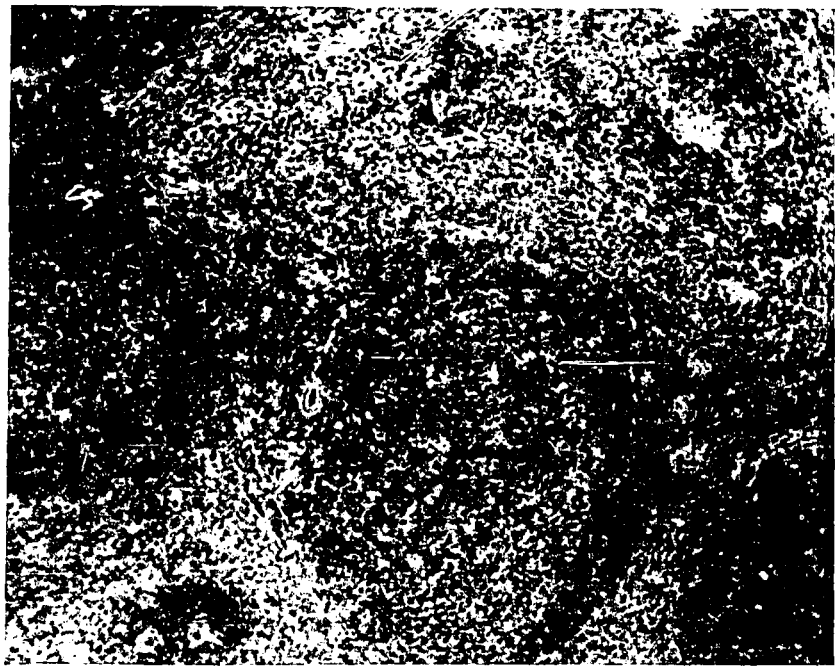

Figure 49 - PHA-M treated animal-7 days-100x
Spleen-follicle with prominent external mantle.

Figure 50 - PHA-M treated animal-7 days-630x
Spleen-Radioautograph of red pulp.

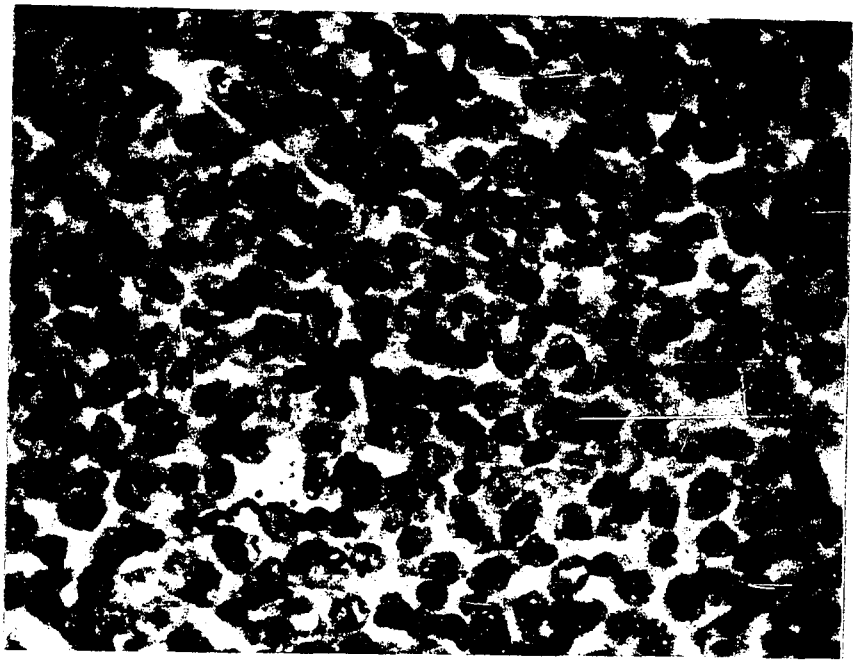

Figure 51 - PHA-M treated animal-7 days-630x
Spleen-Radioautograph of follicle-germinal center.

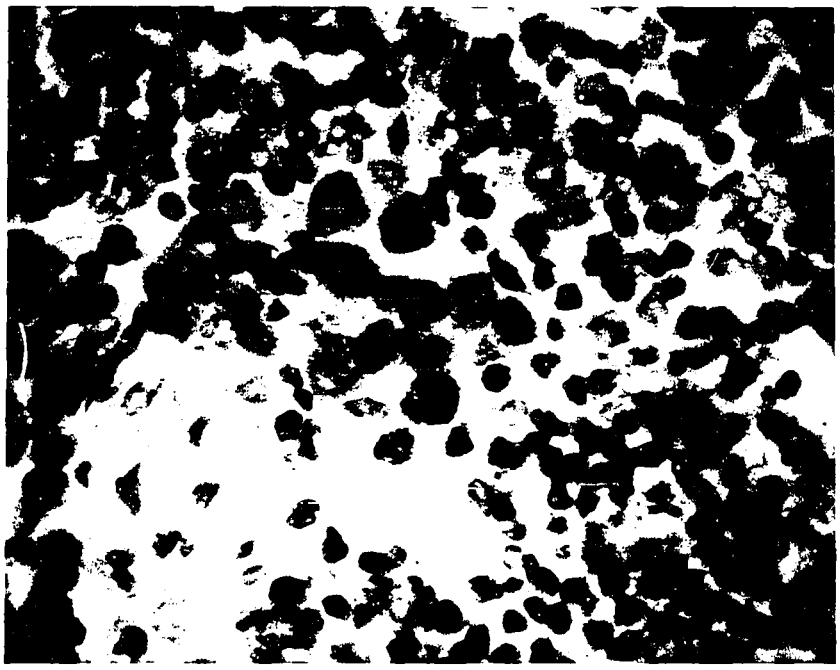

Figure 50 - PHA-M treated animal-7 days-630x
Spleen-Radioautograph of red pulp.

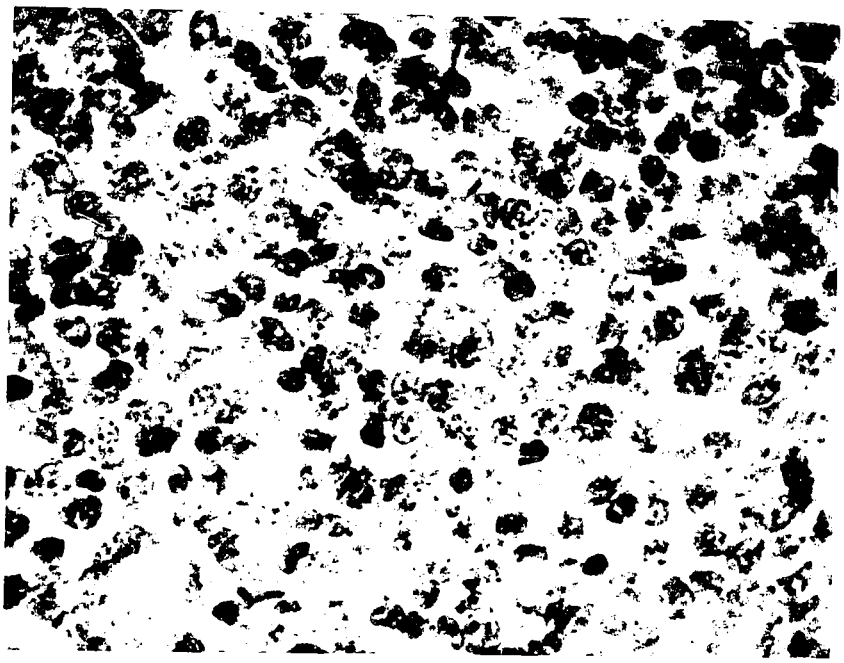

Figure 51 - PHA-M treated animal-7 days-630x
Spleen-Radioautograph of follicle-germinal center.

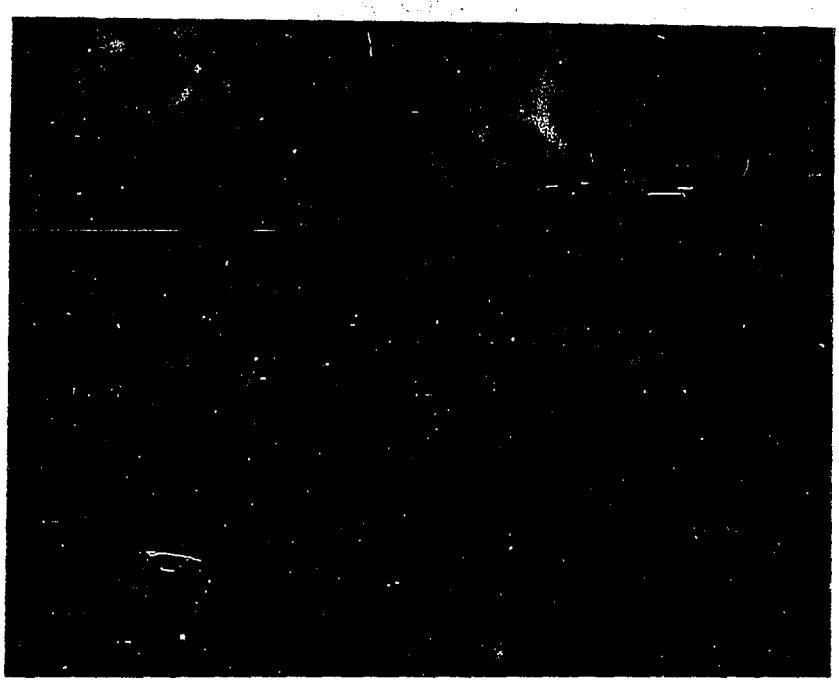

Figure 52 - PHA-M treated animal- 7 days-630x
Spleen-Radioautograph of follicle-external mantle.

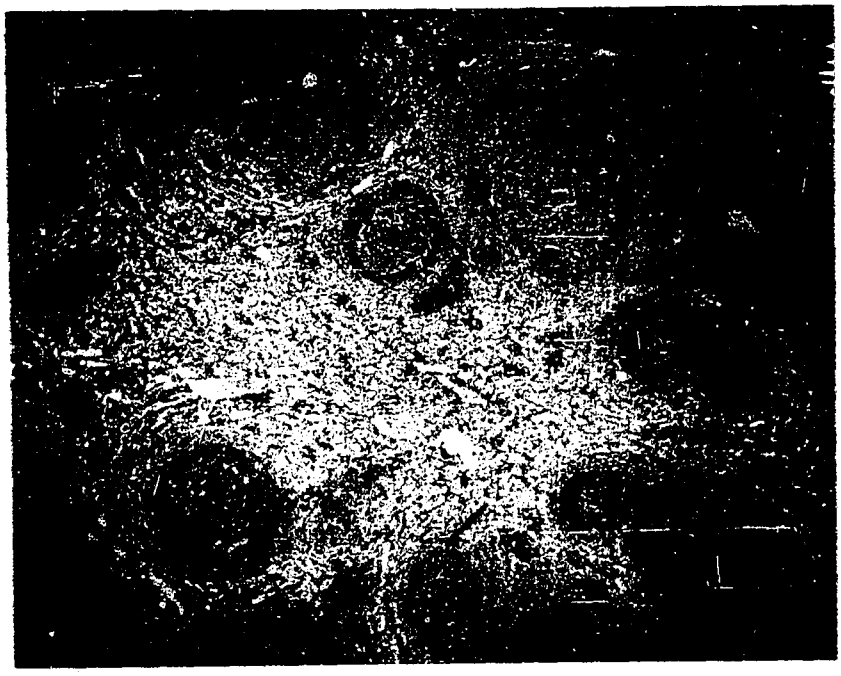

Figure 53 - PHA-M treated animal-10 days-25x
Spleen-hyperplasia of white pulp.

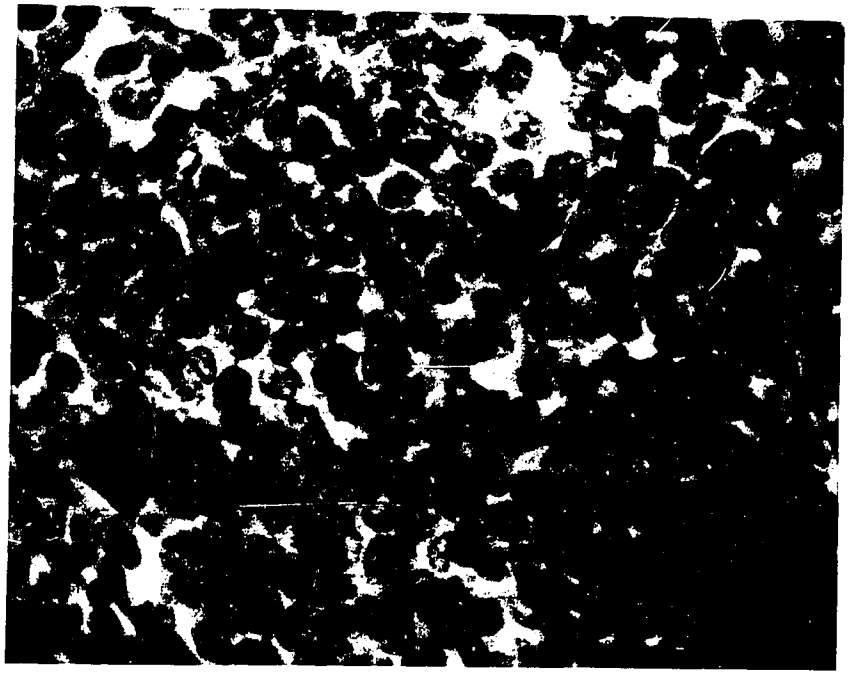

Figure 52 - PHA-M treated animal- 7 days-630x
Spleen-Radioautograph of follicle-external mantle.

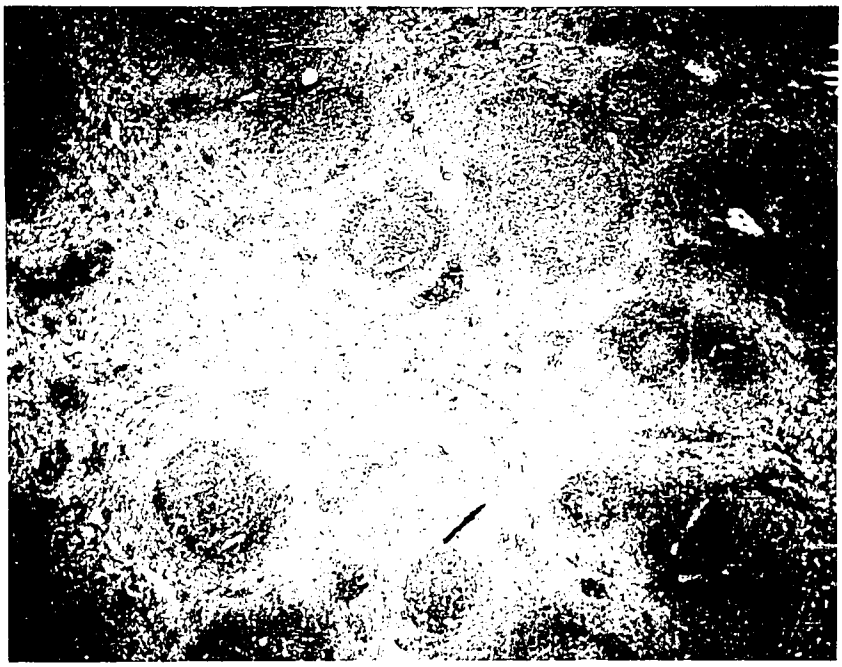

Figure 53 - PHA-M treated animal-10 days-25x
Spleen-hyperplasia of white pulp.

Figure 54 - Antigen treated animal-2 days-40x
Spleen-hyperplastic white pulp - outer mantle enlarged

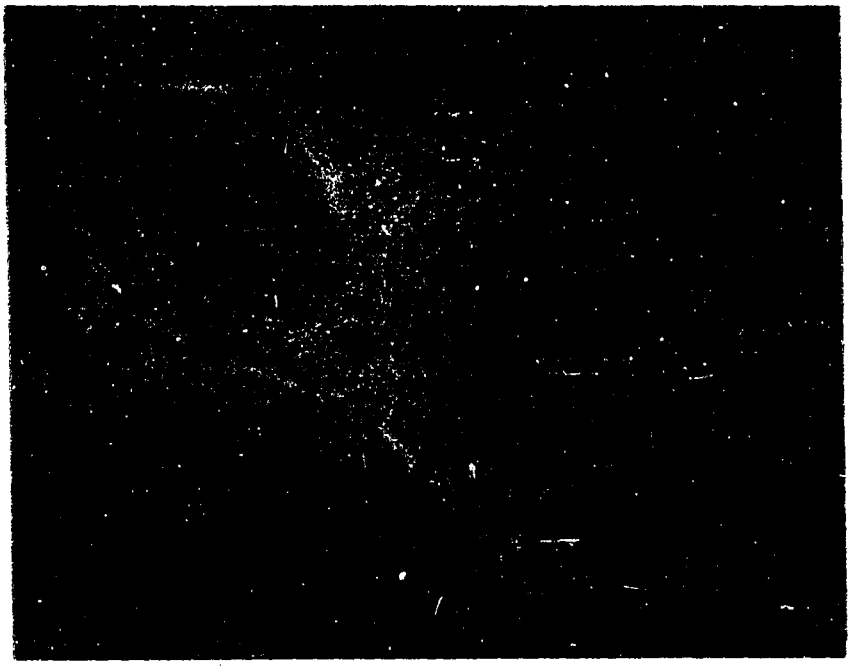

Figure 55 - Antigen treated animal-5 days-40x
Spleen-hyperplastic white pulp.

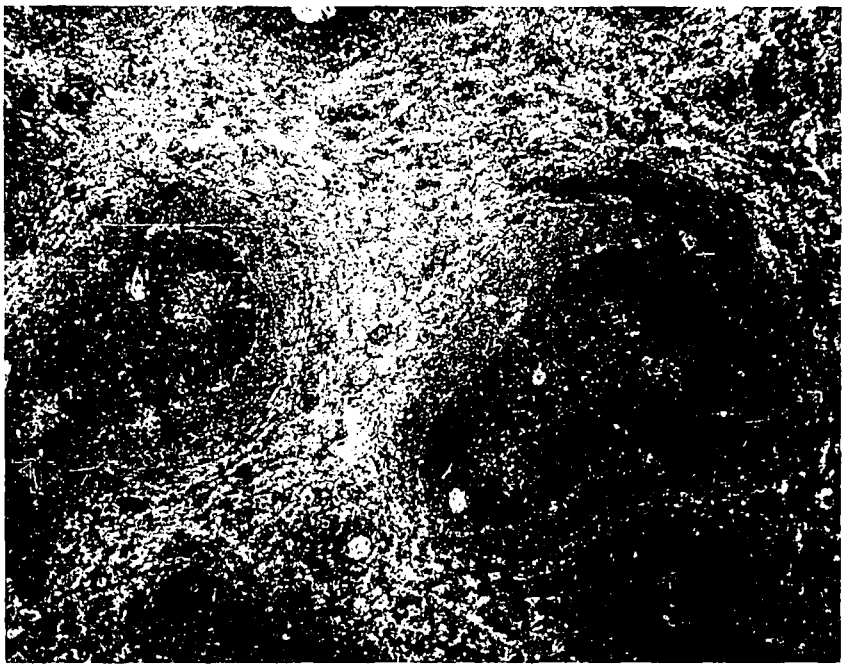

Figure 54 - Antigen treated animal-2 days-40x
Spleen-hyperplastic white pulp - outer mantle enlarged

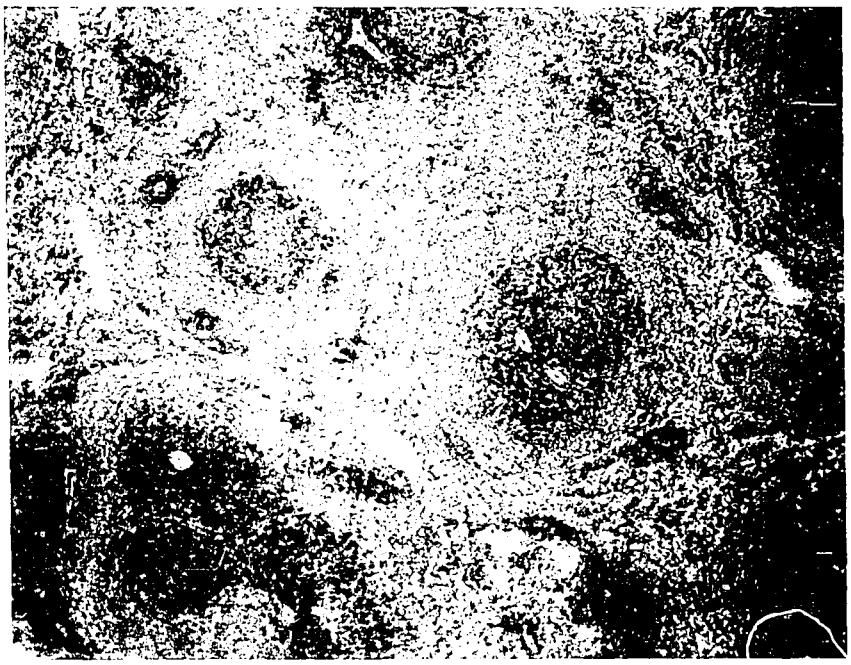

Figure 55 - Antigen treated animal-5 days-40x
Spleen-hyperplastic white pulp.

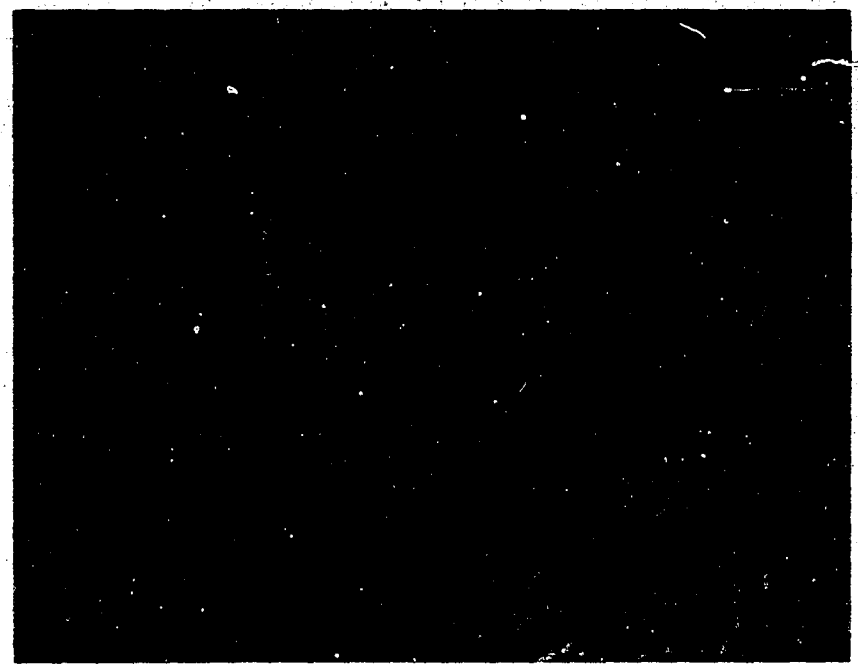

Figure 56 - Antigen treated animal-5 days-100x
Spleen-follicle with enlarged external mantle.

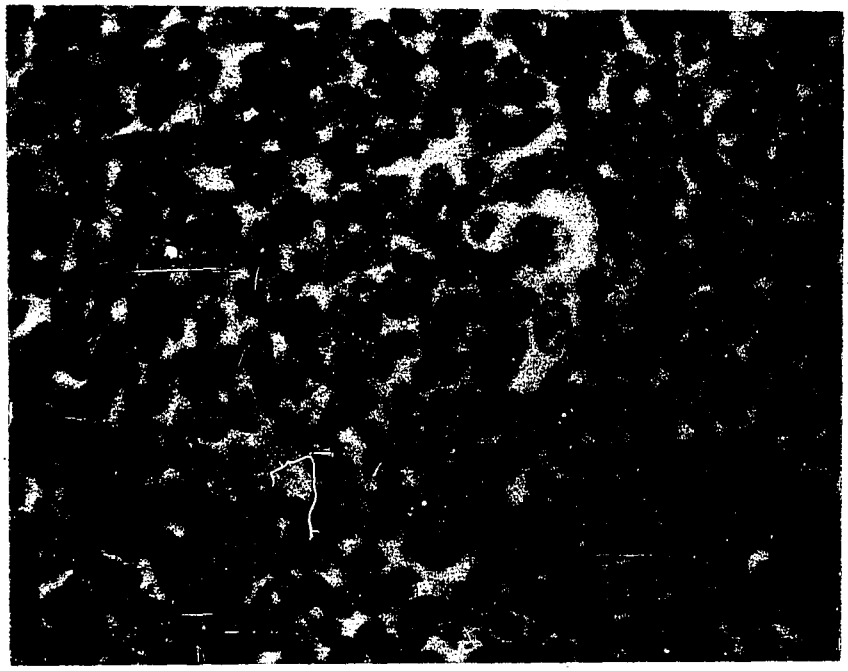

Figure 57 - Antigen treated animal-5 days-630x
Spleen-Radioautograph of white pulp.

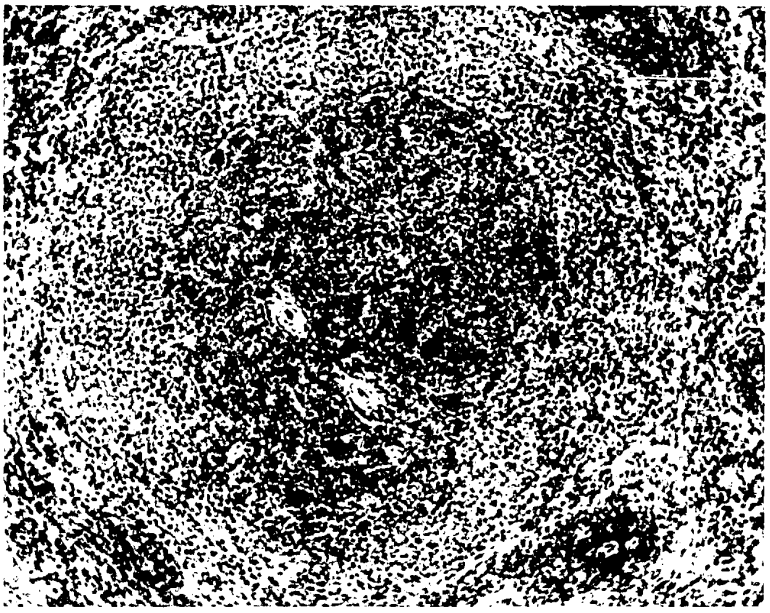

Figure 56 - Antigen treated animal-5 days-100x
Spleen-follicle with enlarged external mantle.

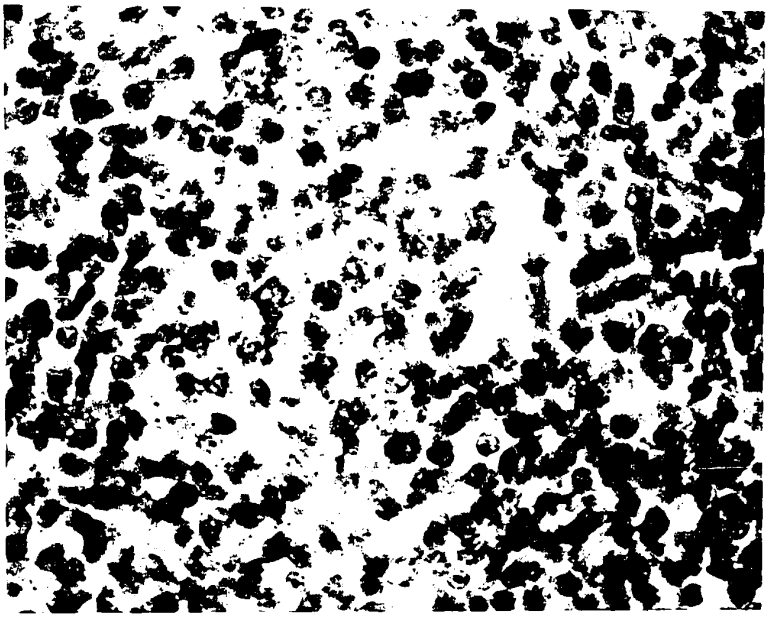

Figure 57 - Antigen treated animal-5 days-650x
Spleen-Radioautograph of white pulp.

Figure 58 - Antigen treated animal-7 days-100x
Spleen-follicle with enlarged middle and external layers.

Figure 59 - PHA-P treated animal-2 days-1,000x
Peripheral blood-Radioautograph-labelled lymphocyte.

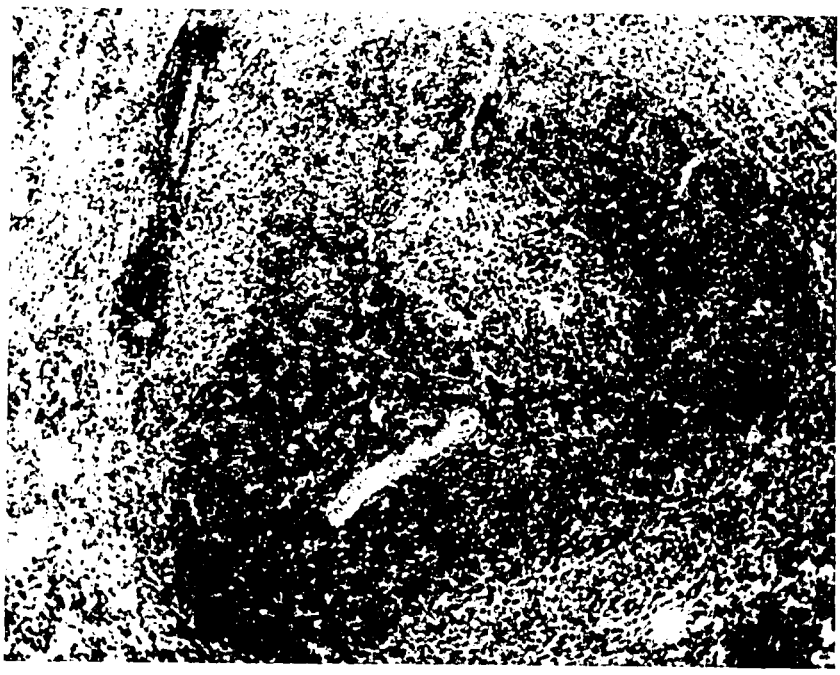

Figure 58 - Antigen treated animal-7 days-100x
Spleen-follicle with enlarged middle and external layers.

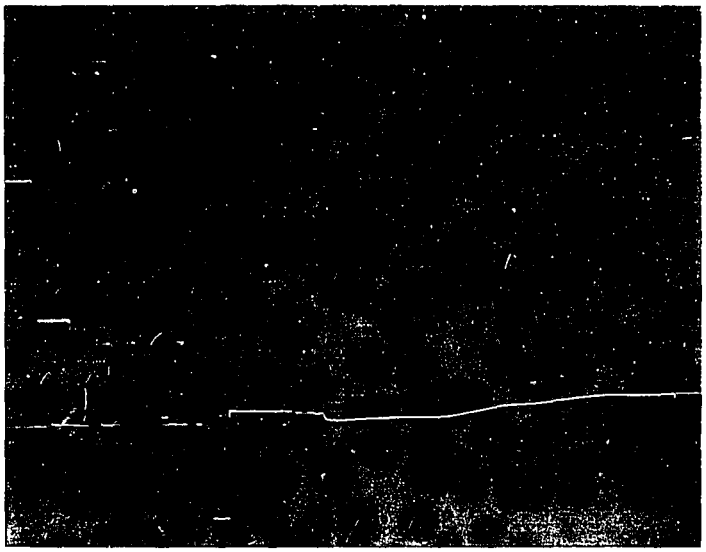

Figure 59 - PHA-P treated animal-2 days-1,000x
Peripheral blood-Radioautograph-labelled lymphocyte.

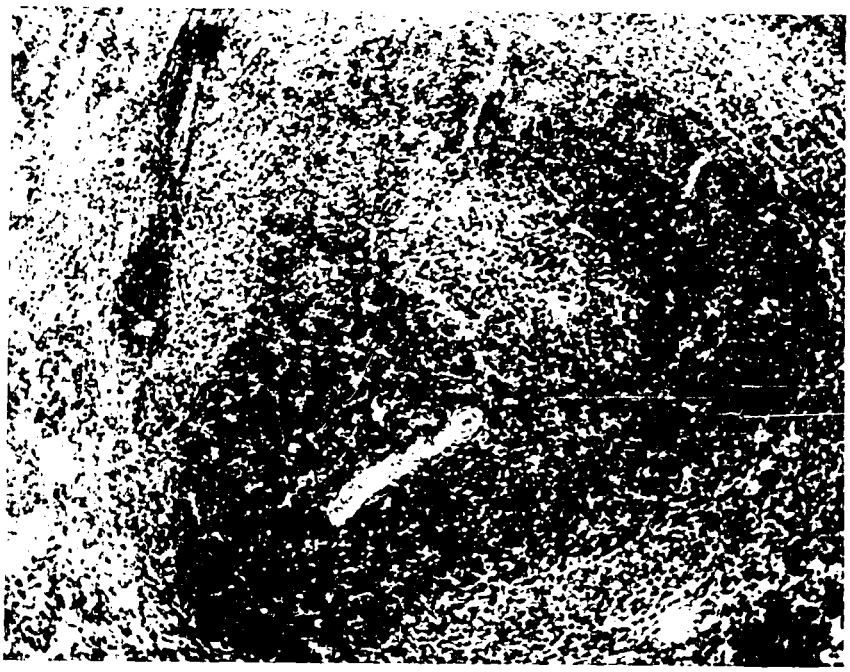

Figure 58 - Antigen treated animal-7 days-100x
Spleen-follicle with enlarged middle and external layers.

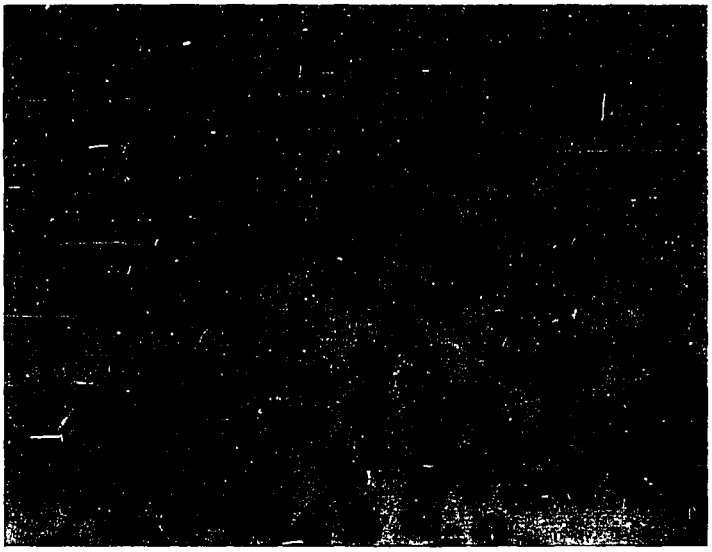

Figure 59 - PHA-P treated animal-2 days-1,000x
Peripheral blood-Radioautograph-labelled lymphocyte.

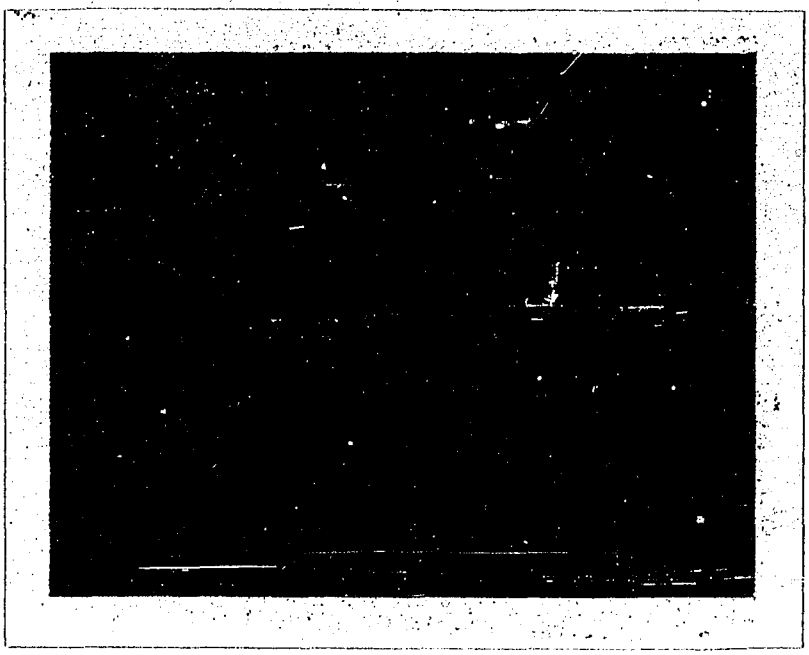

Figure 60 - PHA-P treated animal-2 days-1,000x
Peripheral blood-Radioautograph-moderately labelled blast cell.

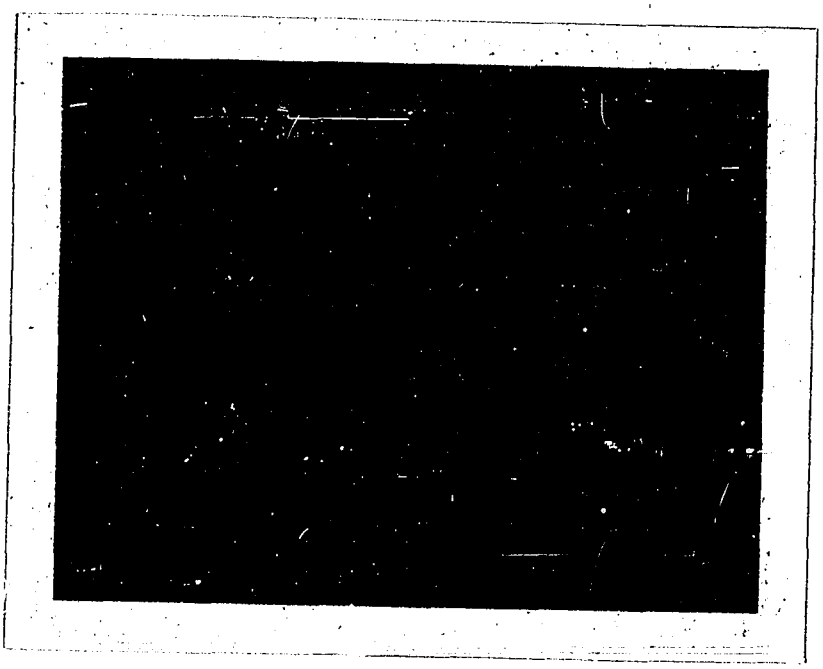

Figure 61 - PHA-P treated animal-3 days-1,000x
Peripheral blood-Radioautograph-labelled small lymphocyte.

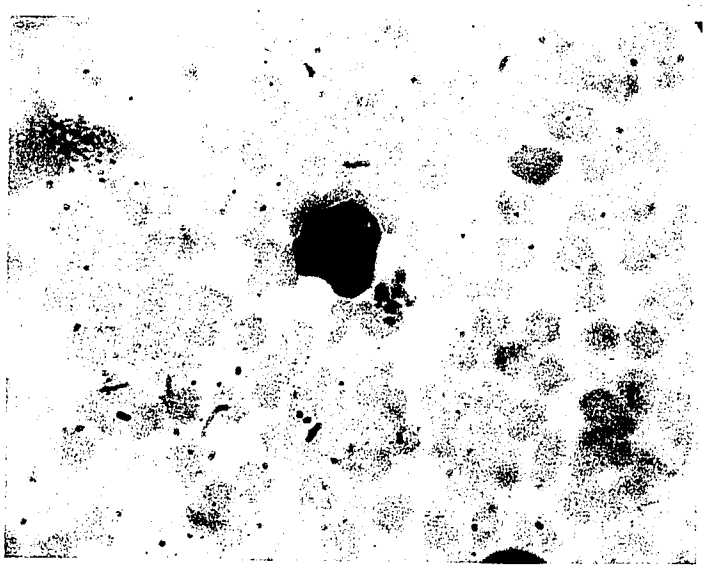

Figure 60 - PHA-P treated animal-2 days-1,000x
Peripheral blood-Radioautograph-moderately labelled blast cell.

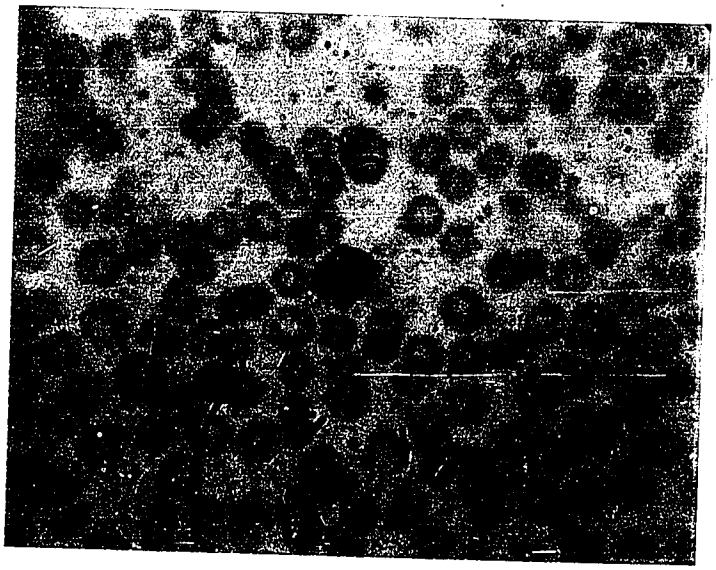

Figure 61 - PHA-P treated animal-3 days-1,000x
Peripheral blood-Radioautograph-labelled small lymphocyte.

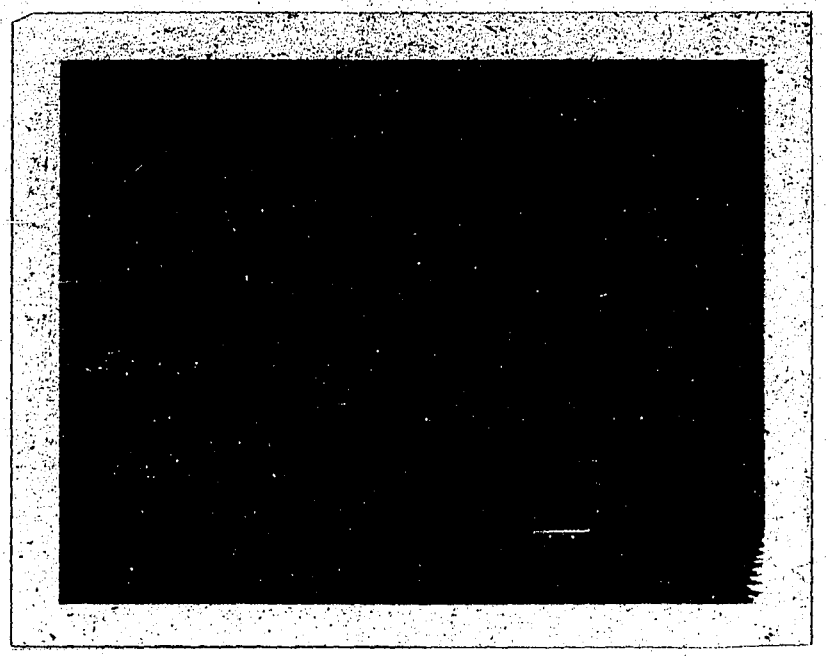

Figure 62 - PHA-P treated animal-3 days-1,000x
Peripheral blood-Radioautograph-labelled medium size lymphocyte.

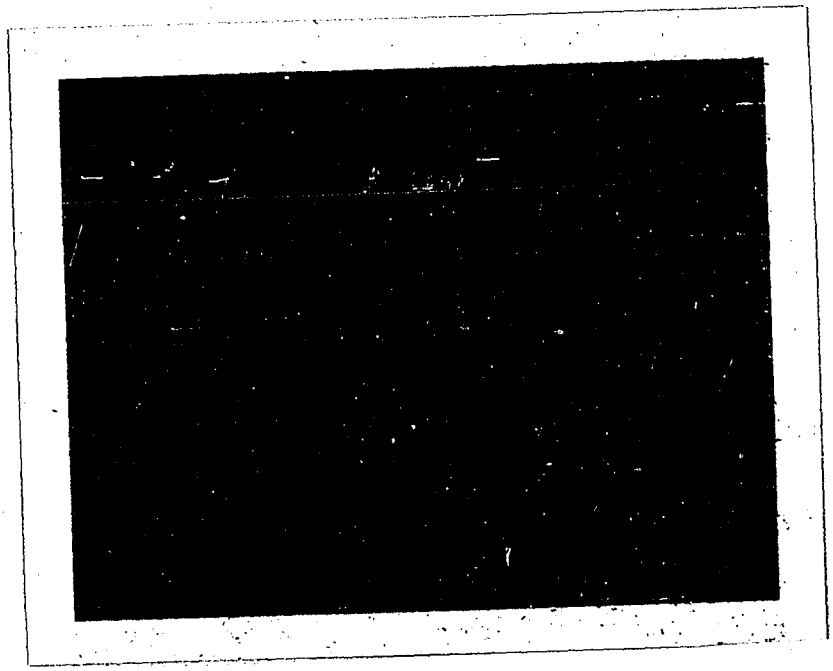

Figure 63 - PHA-P treated animal-4 days-630x
Peripheral blood-Radioautograph-labelled small and medium size lymphocytes.

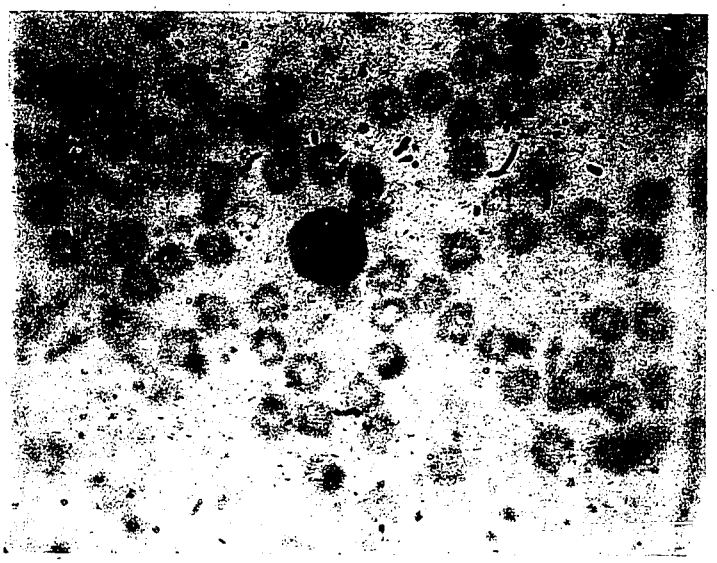

Figure 62 - PHA-P treated animal-3 days-1,000x
Peripheral blood-Radioautograph-labelled medium size lymphocyte.

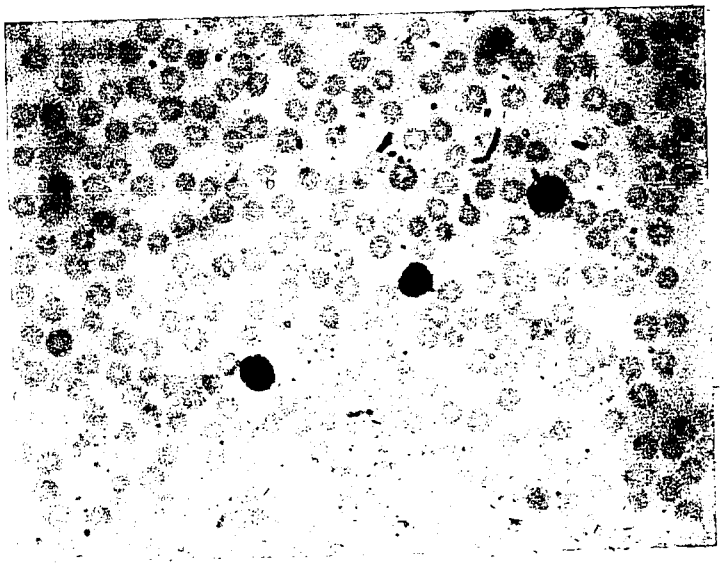

Figure 63 - PHA-P treated animal-4 days-630x
Peripheral blood-Radioautograph-labelled small and medium size lymphocytes.

CHAPTER 6

D I S C U S S I O N

The use of PHA in the treatment of various hematological disorders in a strictly arbitrary fashion (209-223) has accentuated the need for a systematic investigation of the effect of PHA in animals. The study of the histopathological consequences of PHA administration into rabbits was therefore undertaken due to the absence of such an investigation in the current literature. The two commercially available preparation of PHA, PHA-P and PHA-M, were used. As it has been found that PHA-P is 5-50 times more active than PHA-M in terms of in vitro blastogenic activity, depending on the source of the white blood cells (98), it was anticipated that there would be a marked difference in the morphological changes induced by these two PHA preparations in vivo. In fact, a single intravenous injection of 80 mg of PHA-M (one vial) did not have any toxic effect on the rabbits whereas a single intravenous administration of the same amount of PHA-P resulted in the death of 30-40 per cent of the rabbits within two to five minutes after injection, always accompanied by convulsions. Death was probably caused by thrombo-embolic phenomena as a result of intravascular red blood cell agglutination by the PHA-P. It was therefore necessary to divide the contents of each vial of PHA-P into four intravenous injections spaced over a total period of one hour. Such an injection schedule resulted in only infrequent deaths. We refer to this series of injections as a "single" injection, since the time spread for the injections was only one hour.

The hypothesis that the action of PHA in vitro has an immunological basis, i.e., that PHA acts as an antigen, prompted

us to compare the histological changes induced by both preparations of PHA with those subsequent to the administration of human gamma globulin, which has been used many times as an antigen in rabbits (Exp. II). Our initial results with PHA-P (Exp. I) were completely reproducible in the second experiment, thus validating the comparison between the three substances studied. These results demonstrate that a single administration of PHA-P results in marked, immediate changes in various organs with a return to the normal state within 5-6 days with the exception of the spleen. These changes consisted of depletion of small mature lymphocytes in the lymphopoietic organs followed by repopulation of blast cells and infiltration of blast cells in the kidney and in the liver. The intense response in the spleen as compared with the lesser response in other lymphoid tissue (lymph node, appendix) amy possibly be attributed to the fact that PHA-P was administered intravenously rather than regionally.

It has been shown that lymphoid tissues may be divided into central and peripheral tissue (365). A lymphoid organ qualifies as central if its removal prior to a critical period in development compromises the integrity of the remaining lymphoid tissue. The thymus in several animal species and the bursa of Fabricius in the chicken have central function. From these central organs, lymphoid cells migrate to peripheral lymphoid tissues such as the spleen and lymph nodes where they facilitate morphologic and functional maturity (365). In the rabbit it has been shown that the appendix (366,367) and the sacculus rotundus (368) are lymphoid organs with central function and can be compared to the bursa of Fabricius, in morphology and function (369). These consideration are made in order to better understand the effects of PHA on the lymphoid organs in the rabbit.

The absence of appreciable morphologic alteration in the thymus of the PHA-P injected rabbit, coupled with no detectable difference in nuclear labelling when compared to controls, suggests the presence of a blood-thymus barrier or alternatively that the thymic lymphocytes are not as susceptible to the action of PHA in vivo as they are in vitro. The existence of a blood-thymus barrier was suggested by several investigaters (370) and the presence of such a barrier is supported by studies which clearly demonstrate the capacity of thymocytes to undergo blastogenesis in the presence of PHA in vitro, although to a lesser extent than that observed with cells of other lymphoid tissues (105,107-109,111,117,122,134-136). Moreover, the histological changes observed in the appendix with blast transformation of the lymphocytes located between the peripheral follicles and the subepithelial follicular collection of lymphocytes and macrophages implies that the unresponsiveness of the thymus cells to PHA in vivo is not a common denominator of central lymphoid tissue. The changes observed in the sacculus rotundus were inconsistent and a definite abnormal pattern could not be established.

In the lymph nodes, the main blastogenic activity was observed in the interfollicular and subfollicular areas, which correspond to the paracortical area described by Parrott and Sousa (351), Turk (350) and Sousa and Parrott (371). These areas are classified as thymus dependent since they are depleted after neonatal thymectomy (371). In the liver, the infiltrating blast cells appeared to migrate from the circulation to the subendothelial zone and then to the periductal region. The ultimate fate of these cells as to whether they matured to form lymphocytes or differentiated into other mature types of cells could not be determined from this study.

The changes in the spleen subsequent to PHA-P administration were the most striking and involved both the white and red pulp. The initial depletion of the rim of mature small lymphocytes in white pulp follicles was followed by a profound increase in the number of blast cells with hyperplasia of the white pulp and the appearance of groups of blast cells in the red pulp. These changes are interpreted as being due to transformation of small lymphocytes into large blast cells reaching a peak at 48 hours and thus paralleling the in vitro effect of PHA. A commencing repopulation of the periphery of the follicles and the red pulp in the spleen by small lymphocytes was noted by 72 hours and the repopulation appeared completed by 5 days, restoring normal morphology. Thus it appears that the lymphoid cells in the spleen undergo a cycle of changes in response to PHA characterized by initial transformation into blast cells, mitotic activity most intense in the 24-48 hours period as indicated by the incorporation of tritiated thymidine in a high proportion of these cells, followed by maturation to small lymphocytes to repopulate the areas which had been depleted. In fact, at day 4, 50 per cent of the new population of small lymphocytes appearing at the periphery of the follicle were labelled. At day 5, a similar number of small lymphocytes were labelled but the grain count per cell was less than at day 4.

The histological changes observed in the PHA-M injected animals were variable and much less intense than those induced by PHA-P. This was not unexpected, since PHA-P is more potent than PHA-M in terms of blastogenic and mitogenic properties and the dose of PHA-M used may therefore have been only a threshold dose. However, the erythroid-myeloid proliferation in the spleen of the PHA-M treated animals was quantitatively similar to that observed with PHA-P, suggesting that this remarkable extramedullary activity can be induced with smaller doses of PHA-P.

This interpretation of our findings war reinforced by the observation that both PHA-M and PHA-P induced similar changes in the peripheral blood. Both groups of animals had a decrease in the number of peripheral white cells 2 hours after the injection of PHA and the counts were back to normal 24-48 hours later. This transitory decrease in circulating white cells can possibly be attributed to agglutination of the white cells by PHA followed by sequestration. Moreover, the differential cell count showed a significant decrease in the number of mononuclear cells following the administration of PHA. The increase in granulocytes observed at the same period may have resulted from increased granulocytopoiesis stimulated by the rapid destruction of large numbers of mature neutrophils or by a direct stimulatory effect of PHA. In addition, a marked decrease in splenic polymorphonuclear leukocytes was evident at 24 hours, indicating a release of these mature forms into the peripheral blood. By 48 hours, the values observed were similar to those before injection. The initial lymphopenia was probably corrected by the release of newly formed small lymphocytes as indicated by the finding of labelled lymphocytes in the peripheral blood 48 hours after the injection of the PHA-P. The increased erythroid activity in the spleens of the PHA-M and PHA-P treated animals was reflected in the large number of circulating normoblasts observed between 2 and 36 hours after the PHA injection. This was followed by an almost complete recovery of the red cell count at 48 hours suggesting release of fully hemoglobinized non-nucleated red cells into the circulation.

The numerous studies pertaining to histological alterations during the immune response are difficult to compare in view of the different routes of administration, antigens used (particulate or soluble), number of injections and species of animal used. Multiple antigen injections

have often been used to produce a maximum cellular response, but sequential changes are difficult to interpret in such a study. Subcutaneous injection of antigen, especially when given with adjuvant, results in slow absorption on the antigen, thus superimposing cellular reactions of various ages. Furthermore, adjuvants may cause cellular reactions unrelated to antibody formation (372). Fitch and Wissler (372) pointed out that a single intravenous injection of a particulate antigen offers the best conditions to determine the sequential histological changes. However, Ward et al (373) noted that the general physical make-up of the antigen used (particulate or soluble) can be responsible for different histological changes. Most workers used the rat spleen in order to follow the histological sequence of events after the injection of antigen. However, as pointed out by Ward et al (373), the rat spleen is the center of active extramedullary hematopoiesis and consequently qualitative changes attributable solely to a primary antigenic stimulus may be more difficult to discern. In contrast, there is little evidence of extramedullary hematopoiesis in the normal rabbit spleen.

Marshall and White (374) administered either one, two or multiple intravenous injections into rabbits using several particulate and non-particulate antigens. They described the appearance of a large, basophilic cell in the immediate region of the arterioles of the splenic pulp 3 to 4 days after injection of the antigen. This cell was named "activated reticulum cell" and in its later differentiation was presumed to give rise to plasma cells, but only after multiple injections of the antigens. The proliferative changes in the splenic follicles began four days following the intravenous injection of an antigen and, as with the plasma cell reaction, these changes were produced by all antigens employed. However, it was their

impression that the germinal centers reached their greatest development when bacterial antigens were employed. Fagraeus (375), in a classical work with hyperimmunized rabbits, described the formation of plasma cells as the main feature following the injection of an antigen. The precurser cell had a morphology similar to the "activated reticulum cell" described by Marshall and White and was named "transitional cell". Ward et al (373) injected rabbits intravenously with one single dose of bovine gamma globulin. Up to 6 days after injection the histological features of the tissues examined were the same as those of the control animals. After 8 days, histological changes were noted only in the white pulp in the spleen. The splenic follicle showed an enlargement of the germinal center, with a reduction in the size of the mantle layer. These changes declined thereafter and 18 days after administration of the antigen the spleen had a morphology similar to that of control animals. They correlated the appearance of proliferative activity in the germinal center with detectable antibody in the circulation, but could not detect any plasma cells. However, Wissler et al (376) reported that after the intravenous injection of a particulate antigen, the response in the rat spleen was quite consistent and limited to the red pulp. They described that 36 hours after the administration of the antigen, mitotic activity began in the red pulp which reached a peak at days 3-4 while the serum antibody titer reached a peak at day 6. On the other hand, Congdon and Makinodan (377) observed marked cellular alterations in the splenic white pulp of mice given sheep red cells before antibody production could be detected. The most striking observation after primary antigen injection was the loss of germinal centers in the spleen accompanied by extensive proliferation of antibody forming cells throughout the white pulp.

This process lasted about 3 days and was followed by a hyperplastic regeneration of germinal centers. The sequence of histological events suggested that the large, newly-formed cells in the white pulp gave rise to antibody forming cells of the plasmacytic series later seen in the red pulp. Gundersen et al (378) noted changes in the splenic follicle in the rat spleen 2 days after a single intravenous injection of a bacterial antigen but not after the injection of sheep red blood cells. They pointed out that the red pulp is the site of antibody forming cells. Langevoort (379) investigated the histological changes in rabbit spleen after one intravenous injection of horse gamma globulin. He found that plasma cells arose among the lymphocytes of the periarteriolar lymphocyte sheaths of the white pulp and suggested that the sheath lymphocyte was the precursor of the plasma cells. The plasma cell proliferation started at 24 hours after injection of the antigen, was maximal at days 3-4 and by day 7 the periarteriolar lymphocyte sheaths had resumed their normal appearance. Four days after the antigen injection blast cells appeared in the germinal centers, giving rise to numerous medium-sized lymphocytes. The follicles enlarged during the subsequent 5 to 10 days after which the reaction gradually subsided. No relation was found between the follicular activity and antibody formation. The findings of Langevoort (379) are in contradiction to the opinion of other investigators (376,378) that antibody forming cells develop in the red pulp.

A histologic and autoradiographic study by Hanna (380) on the germinal center changes in the mouse spleen revealed a proliferation following a primary sheep red blood cell stimulation. This proliferation was detected in two ways: within intact germinal centers (hyperplasia) and in a dispersed manner with migration. These changes began 2 hours after

the injection of the antigen and an increase in the number of immature lymphocytic cells coincided with germinal center dissociation. These cells migrated into the red pulp where they presumably transformed to plasma cells, the transformation beginning 1-2 days after antigen injection. On the other hand, Craddock et al (381) studied the response of rats to the injection of the same antigen and reported that the development of new germinal centers in the spleen, with prominent phagocytic activity, occurred before the appearance of antibody producing cells but that these cells were not the products of germinal center proliferation.

As can be seen from the above studies, there is no agreement as to the histological changes which occur during the immune response, nor is the antibody forming cell line definitely established. Gowans and MacGregor (349), in their excellent review, pointed out that these conflicting views cannot be reconciled on the basis of existing evidence.

In our experiments, the changes induced by a single intravenous injection of human gamma globulin were confined only to the spleen. The initial changes, observed 2-3 days after antigen administration, consisted of white pulp hyperplasia. The follicle contained a slightly enlarged germinal center but the outer mantle of cells was very enlarged. The dissociation of the germinal center was noted at day four and was characterized by a merging of the germinal center with the middle layer. At day 5, a more marked sequence of events began with a very hyperplastic white pulp - germinal centers were again present but not in all follicles. Mitotic activity was detected in the germinal centers and in the outer mantles in view of the large number of labelled cells in the radioautographs. The most

prominent germinal centers were noted at day 7, and this change was accompanied by a widening of the middle and outer mantle layers. By day 10, the white pulp had almost returned to a morphological state similar to that observed in control animals. No outstanding changes were observed in the red pulp except for some nuclear debris and plasma cells were rarely identified throughout the experiment. Antibody was detected in the circulation of the antigen injected animals only after day 7 when the most striking changes were observed in the splenic white pulp.

Although our results do not closely resemble the histological changes subsequent to antigenic stimulation discussed above, the main purpose of this study was to compare the histological features induced by the injection of PHA and antigen. Evidence both in favor and against the hypothesis that the action of PHA has an immunological basis were discussed in Chapter 2.16. On the other hand, there is no doubt the PHA, when injected into animals, also possesses antigenic properties, in the sense that an antibody (anti-PHA) is formed (see Chapter 2.13). However, from the results presented above, it is clear that the histological changes induced by PHA are quite different from those induced by antigen. PHA induced a marked initial depletion of lymphocytes followed by white pulp hyperplasia at 48 hours and at days 7-8 a moderate hyperplasia of the white pulp was again evident, but different from that seen on day 2, due to the presence of large germinal centers. This histologic picture in the white pulp observed 7-8 days after the injection of PHA is similar to that observed 7 days after the injection of antigen. Furthermore, the red pulp in the PHA-treated rabbit was characterized by myeloid-erythroid activity on days 7-8, a change not seen in the spleen of the

antigen-injected rabbit.

From these data it can be suggested that PHA has at least two active components: one capable of exerting an early and active blastogenic and mitogenic response and the other behaving as an antigen. This is not surprising in view of the complex composition of PHA as discussed in Chapter 2.3.

As was stated previously, the most obvious changes as a result of PHA administration took place in the spleen. The initial depletion of small cells in the white pulp was followed within 24 hours by hyperplasia of the white pulp and the appearance of large numbers of blast cells in both the red and white pulp. Repopulation of the follicular mantles was noted by day 3 and appeared to be complete by day 5, restoring normal morphology. Recent studies in normal animals (see below) suggest that this new population of small lymphocytes was derived by division of the labelled blast cells in the periphery of the follicles, rather than from the blasts in the germinal center. Fliedner et al (382) injected tritiated thymidine into rats and sacrificed them at intervals of time thereafter. They found no evidence that small lymphocytes are produced by division of cells within germinal centers in the rat spleen. Similar findings were reported by Craddock et al (383). Everett and Tyler (384) performed similar studies on the mesenteric lymph nodes of the rat. By radioautographic analysis they showed that the majority of cells in the germinal centers were labelled and had a rapid turnover but no labelled small lymphocytes were detected in germinal centers. Cottier et al (385) presented evidence for the production of the circulating lymphocytes in the spleen independent of germinal center proliferation. Extracorporeal irradiation of the blood of the calf depleted the animal of recirculating

lymphocytes. Such depletion, however, failed to diminish the size of proliferative activity of germinal centers although the surrounding cuffs of small lymphocytes were seriously reduced. Lymphopenia was found to persist for many months despite hypertrophy of germinal centers. However, Koburg, (386) concluded from radioautographs of tonsils of normal rabbits that the small lymphocytes in the follicular mantles probably originated from the adjacent germinal centers.

It has been shown by Everett et al (387) using rats injused with tritiated thymidine, that lymphocytes in the rat have a various life span. There is a short-lived population of lymphocytes mainly located in the thymus and bone marrow and a predominantly long-lived population found in the spleen, lymph nodes and thoracic duct lymph. The lymphoid cells in the germinal centers in the spleen are short-lived (381,382). Fliedner (382) showed that, after a single injection of tritiated thymidine, 30-40 per cent of the cells in the germinal centers in rat spleen were labelled within 1 hour and this fraction remained labelled for about 24-36 hours. However, due to the rapid mitotic activity of many of these cells, the mean grain count over these cells diminished with a half-time of 13.4 hours. Cottier et al (385) (see above) demonstrated that the germinal center cells do not give rise to the long-lived circulating lymphocytes which constitute the majority of the lymphocytes in the peripheral blood. Radiation-induced chromosome aberrations have been used as markers to identify long-lived small lymphocytes capable of surviving for long periods in vivo without dividing. When these cells were stimulated in vitro with PHA, they could be identified by the chromosome alteration. Based on this criterion, Nordman et al (388) estimated that the life span of the circulating human lymphocyte is approximately 550 days. Weber and Nowell (389) cultured lymphocytes from irradiated monkeys in the presence of PHA and observed cells with abnormal chromosomes up to 8 months after irradiation . Such cells were found

in the blood, spleen and lymph nodes. Newell (390), in a similar study, showed that these long-lived lymphocytes divide in vitro in response to tuberculin when obtained from tuberculin-sensitive individuals. Thus it would appear that, in the peripheral blood, the PHA-responsive cell is the long-lived, recirculating, small lymphocyte. However, Metcalf and Osmond (122), working with rats previously injected with tritiated thymidine, cultured cells obtained from the lymph node, spleen, thymus, blood and thoracic duct lymph in the presence of PHA and concluded that both the long-lived and short-lived small lymphocytes respond to PHA.

From our results, it would appear that the regenerating population of small lymphocytes in the spleen after PHA administration had their origin in the small lymphocytes constituting the surrounding cuffs of the germinal centers and can therefore be classified as long-lived lymphocytes. These "cuff" cells were able to respond promptly to the PHA injection, thus confirming the in vitro studies showing the ability of the long-lived lymphocytes to transform to blast cells (388-390). The importance of this observation stems from the fact that the long-lived lymphocyte has an active role in the immune response (387,381,349) and is the carrier of long-term immunological memory (391).

The most important histological findings in the PHA-P injected animals, in relation to the potential use of PHA as a therapeutic agent in human disease, were observed in the red pulp of the spleen. A very intensive myeloid-erythroid activity was detected at 24 hours and a second peak was noted at day 7, reaching the maximum between days 8 and 10. The myeloid population consisted mainly of myeloblasts and myelocytes, cells which are precursors of mature granulocytes. The erythroid

population was represented by large numbers of normoblastic islands. The early increase in normoblasts seen in the red pulp followed rapidly the decrease in the red cell count of the peripheral blood which occurred after the PHA injection. This decrease in circulating erythrocytes is probably the result of hemagglutination and sequestration while the increase in normoblasts could be interpreted as being either a response to the erythrocyte depletion or a direct stimulatory effect of PHA on erythropoietic tissue. The work of Gamble (187) suggests that the latter is the more likely alternative. Although he injected mice with PHA absorbed with mouse red cells, thus removing its erythro-agglutinating property, he nevertheless observed an increase in the number of normoblasts in the spleen.

From our results, it seems that PHA-P has a strong, direct effect in stimulating the appearance of immature forms of the myeloid and erythroid series. The presence of immature cells of the granulocytic series in the spleen may correspond to the proportional increase in primitive myelopoietic cells observed at 24 hours in the bone marrow. It may be suggested that these primitive cells in both the myeloid and erythroid series originate from the small lymphocyte, but such a role for this latter cell is still highly debatable. Some authors of the monophyletic school (392-395) have stated that the lymphocyte is a totipotential cell, giving rise under proper stimulation to any other type of blood cell. This change can be a direct one or the small lymphocyte may be first modified to a large blast cell. On the other hand, the polyphyletic school (392) maintains that there are several different blast cell lines each of them giving rise to a different cell in the blood. Although Wintrobe (392) states that a dogmatic viewpoint on the interrelationships of the blood cells is unjustified at the present time, he gives serious consideration to the modified monophyletic theory of Downey (392) who maintained that the lymphocyte of normal blood does not usually transform

into some other type of cell but the developmental potentialities of the lymphocyte are such that this may occur in an abnormal environment. Such an environment could be provided by the PHA injection into the animal. As discussed above, there was a large number of blast cells derived by transformation of the small lymphocytes in the spleen subsequent to the administration of PHA. These cells may have differentiated into small lymphocytes and into immature forms of the myeloid-erythroid series. This is supported by the finding that the new population of normoblasts and myeloblasts were labelled. The early peak of erythroid-myeloid activity found in our animals could be explained by the prompt response to PHA of a small percentage of cells which was already being activated by other biological mechanisms. The second, more intense and prolonged wave of erythroid-myeloid activity (day 7) was probably the result of blast cell differentiation. Gamble (187) also showed an intense activity in the mouse spleen after the intravenous injection of PHA. He observed a single early peak of normoblast and granulocyte precursor activity. The second and late peak of erythroid-myeloid proliferation was not observed due probably to the termination of the experiment after only 8 days. The failure of Elves et al (186) to obtain significant changes in the rat spleen following intraperitoneal and subcutaneous injections of PHA can be attributed to a difference in the species of animal used and mainly to the different route of administration of the PHA. The sharp increase in immature forms that were found in bone marrow after PHA injection probably supports the concept that lymphocytes in the bone marrow can differentiate into erythroid and myeloid elements (396). It has been suggested that these lymphocytes are not produced in the bone marrow but arrive by migration from other lymphoid organs (397). Labelled lymph node cells (398) and

thymus lymphocytes (399, 400) have been traced to the bone marrow, where they have taken on the morphological characteristics of erythroblasts and myelocytes.

The possibility that the lymphocyte could function as a totipotential hemopoietic stem cell encouraged Humble (209) to use PHA as a therapeutic agent in human aplastic anemia. This disease is characterized by anemia, leukopenia and thrombocytopenia. In the bone marrow, the majority of nucleated cells are lymphocytes and a smear of peripheral blood may contain as many as 70-90 per cent lymphocytes. However, the leukocyte count can be very low and thus absolute lymphocytopenia may be present as well (392). On the assumption that the lymphocytes in patients with aplastic anemia are able to undergo blast transformation in vivo under PHA stimulation, Humble (211) administered 5 intravenous daily injections of PHA into 16 patients with aplastic anemia. After a two day pause, 2 further daily injections were given. There was some recovery of hemopoietic function in eight of the patients. In these patients abnormal large lymphoid cells were seen in the blood 7 days after the first injection. About this time the low white cell count started to rise and granulocytes and reticulocytes tended to increase. Humble (211) also noted that recovery of red cells preceeded that of platelets and he concluded that PHA is of some value in the treatment of aplastic anemia in humans.

Although it is difficult to extrapolate the findings in healthy rabbits after PHA administration to those described in diseased humans after a similar treatment, certain considerations can be made. The demonstration of an increased and previously non-existent extramedullary hematopoiesis in the rabbits injected with PHA suggests that this substance can act

as a potent stimulant, inducing the production of red cells and granulocytes. Occasional circulating megakaryocytes found in our experimental animals could reflect an increased production of these cells. These findings could perhaps explain the red cell, granulocyte and platelet recovery observed by Humble (211) in humans. Also, the circulating blast cell noted by Humble (211) was observed in the peripheral blood of the rabbits injected with PHA. However, the contradictory results observed by other workers (see chapter 2.12.2) following the administration of PHA into patients with aplastic anemia makes clear that further studies are needed. The dose injected in our rabbits (1 vial of PHA) is 5-50 times more potent than the therapeutic dose used by the above authors. Furthermore, experimental studies with multiple injections of PHA are needed. Nevertheless, the report of Israel et al (224) that the intravenous injection of PHA into 27 cancer patients permitted the administration of a greater dose of antimetabolite drugs without the accompanying leukopenia reinforces the potential use of PHA as a valuable therapeutic agent in certain human diseased states of the bone marrow and lymphoid tissue, provided a proper dosage and injection schedule can be worked out.

CHAPTER 7

S U M M A R Y

Two preparations of commercially-available phytohemagglutinin (PHA), PHA-M and PHA-P, were compared with a commonly used antigen, human gamma globulin (HGG) with respect to their effects in the rabbit. Each preparation was injected once intravenously and the rabbits were then sacrificed at predetermined intervals of time. Each rabbit was injected with 400 microcuries of tritiated thymidine 24 hours prior to sacrifice, specimens of various organs (lymph node, spleen, thymus, liver, kidney, bone marrow, sacculus rotundus and appendix) were fixed in buffered formalin and sections were stained directly with hematoxylin and eosin. Other sections were deparafinized, coated with a photographic emulsion, stored in the dark at 4°C for 6-8 weeks and developed and stained with hematoxylin and eosin. Examination of stained sections and radioautographs revealed no permanent changes in the different organs investigated. The only marked changes occurred in the spleens of the PHA-P injected rabbits which consisted of depletion of small cells during the first 24 hours after PHA administration followed by hyperplasia of the white pulp and the appearance of a large number of labelled blast cells in both the red and white pulp. The spleen attained normal morphology by day 5 but by days 7-8 germinal center hyperplasia and myeloid-erythroid activity were observed. By day 18, all the organs were considered to have returned to normal morphology. The histologic changes induced by PHA-M were similar but of much lower intensity. The changes induced by antigen were markedly different from those induced by PHA. The only abnormal feature was hyperplasia of the white pulp with enlarged germinal centers by days 5-7 with a return to almost normal morphology by day 10. The transitory nature of the changes described in the blood forming and lymphoid organs, together with the

absence of degenerative changes generally, suggest that PHA is essentially non-toxic and that the changes observed are in fact entirely due to a specific stimulating activity of PHA on the myeloid and lymphoid tissues. These findings would appear to justify the use of PHA in certain diseases of the bone marrow and lymphoid tissues such as idiopathic aplastic anemia and drug-induced leukopenias, provided a proper dosage and injection schedule can be arrived at. Much more work is obviously required to better elucidate the mechanism of action of PHA and its role as a chemotherapeutic agent.

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