1967

Effects of phytohemagglutinin (PHA) on homograft rejection

Brent Douglas Kehn
University of Nebraska Medical Center

THE EFFECTS OF PHYTOHEMAGGLUTININ (PHA) ON HOMOGRAFT REJECTION

Brent D. Kehn

Submitted in Partial Fulfillment for the Degree of
Doctor of Medicine

February 1, 1967

Omaha, Nebraska

TABLE OF CONTENTS

THE EFFECTS OF PHYTOHEMAGGLUTININ (PHA) ON HOMOGRAFT REJECTION

Advisor: P. G. Rigby, M.D.

Introduction

Phytohemagglutinin (PHA) is a mucoprotein extract of the red kidney bean, Phaseolus vulgaris, which has hemagglutinating and leukoagglutinating properties. The leukoagglutinating fraction remains in solution following agglutination of red blood cells with PHA.[1] This leukoagglutinating property has been shown to promote synthesis of RNA, DNA, and gamma globulin and is known to induce mitosis in a large fraction of lymphocytes in vitro.

Although considerable work has been done with PHA in vitro, relatively little is known about its in vivo effects. It has been used in humans with aplastic anemia, who subsequently showed evidence of increased bone marrow avtivity in six cases.[2] Gamble[3] found that intravenous injection of PHA in mice resulted in an increase in the cellularity, the proportion of immature cells, and the weight of the spleen. These changes were most marked three days following administration.

Calne, et. al.,[4] used combined PHA and azathioprine (IMURAN) therapy in an attempt to prolong renal homotransplant survival times in dogs. Intravenous PHA potentiated the immunosuppressive action of azathioprine and was thought to have some immunosuppressive activity when used alone.

Because of the stimulating effect of PHA on lymphocytes in vitro, it is feasible that there could be a profound effect on the immune system of the organism, if adequate concentrations could be attained in vivo. If generalized dedifferentiation occurred in the lymphoid system of the organism following PHA administration, one might suspect that the ability to respond to antigenic stimulus could be severely compromised. This project was designed to determine whether PHA in vivo has any effect on the ability of a host to reject a skin homograft.

Materials and Methods

Animals - Closed colony White Swiss and inbred C57BL mice obtained from Roswell Park, Buffalo, New York, were used. They were fed autoclaved Purina^{R} chow, tap water in autoclaved dispenser

bottles, and were kept in an air-conditioned environment. Mixed male and female White Swiss and virgin female C57BL mice were used. They were picked two to three months old and weights varied from twenty-seven to forty-four grams.

Phytohemagglutinin Dosage and Administration - Phytohemagglutinin M, lot no. 661601, was obtained from General Biochemicals Company.

PHA was injected in doses of 0.5 ml. into the tail veins of the mice using sterile tuberculin syringes and 27 ga. needles.

The maximum dose tolerated was determined by injecting increasing amounts of PHA into the tail veins. Most of the animals survived 0.5 ml. whereas most animals died with injections of 1.0 ml; therefore 0.5 ml. was used as the basic dosage given in one injection.

Phytohemagglutinin M (General Biochemicals) used in this project is apparently not as toxic to mice as PHA-M (Difco) which has been used extensively by other investigators. Whereas Micklem[5] found a mortality rate of 50% using 0.4ml. PHA (Difco) most of the animals in this project survived the standard dose of 0.5 ml.

PHA (General Biochemicals). However, several of the mice in Group I showed gross hepatic necrosis on autopsy apparently similar to that found in 50% of Micklem's animals.

Skin Grafting and Observation - The host was anesthetised with 0.3 ml. of 6 mgm. % Nembutal^{R} and the fur on the back was removed with an electric razor. Tomasine^{R} aerosol skin antiseptic was then applied and approximately one cm.^{2} of skin was removed from between the scapulae. Donor tail skin of corresponding size from C57BL mice was then applied. The operative site was covered with Neosporin^{R} powder and dressed with sterile filter paper bandages held in place with non-allergic tape (Blenderm^{R}). The dressing was removed four days post-operatively and the skin grafts observed daily thereafter. Rejection was determined by visual inspection of the graft according to the criteria presented by Billingham and Silvers.[6]

Procedure - The mice were divided into four groups (see Table I).

Group I consisted of eight White Swiss male (WSM) and eight White Swiss female (WSF) mice.

Four were immediately sacrificed and body weights and spleen weights were recorded and spleen smears were made. The remaining twelve animals were given 0.5 ml. of PHA and sacrificed on subsequent days in groups of two beginning two days following injection. Body weights, spleen weights, and peripheral WBC counts were recorded and spleen smears were made.

Group II consisted of five WSM and five WSF mice which received 0.5 ml. of PHA and the skin homograft on the same day. Another dose of PHA was given three days later.

Group III consisted of five WSM and five WSF mice which received 0.5 ml. of PHA three days prior to skin graft, on the day of skin graft, and three days following skin graft.

Group IV consisted of four animals treated like those in Group III except that they were injected with saline rather than PHA.

Spleen Examinations - Following necropsy of the animals in Group I the spleen was removed. Fat was trimmed from its capsule and the spleen was placed on a dry filter paper and immediately weighed on a Mettler analytical balance to the

nearest .0001 gram. A small piece of the spleen with a drop of saline was then macerated between two glass slides and a smear made in the same manner as peripheral blood smears. These were stained with Wright's stain and examined for morphological changes. The WBC counts on the animals in Group I were done by standard manual techniques.

Results

Peripheral WBC counts done on the animals in Group I revealed counts ranging from 6,100 cells/mm^3 to 16,850 cells/mm^3. No trend was established over six days following PHA administration. The peripheral WBC count was inconclusive as a criteria of the *in vivo* effect of PHA, perhaps because of many variables in production, release, and sequestration of leukocytes.

Gamble[3] proved earlier that PHA affected spleen weight and cellularity and this was found to be true in these animals. A ratio was made in all cases by dividing spleen weight by the total body weight of the mice. The ratios are shown in Table II. The average spleen weight of the control animals was 0.1144 grams and the average ratio of spleen weight to mouse weight was .00286. The values

obtained from the animals that received PHA are seen to be moderately elevated with very little overlap into the control group, both in splenic weights and in spleen weight-mouse weight ratios.

The accompanying photomicrographs illustrate the differences observed in the spleen smears under high and low power magnification. Photomicrograph #1 is from a mouse which received no PHA and photomicrograph #2 is from a mouse, four days following administration of PHA intravenously. The absence of small, dark staining lymphocytes and the presence of larger, more immature cells is quite evident in the second photomicrograph. High power magnification of normal spleen cells as compared with those stimulated by PHA are shown, respectively, in photomicrographs #3 and #4.

The twenty-four White Swiss mice bearing skin homografts from C57BL mice all rejected their grafts without any significant deviation from the normal rejection time. Neither dosage of PHA nor timing of its administration in relation to application of the skin homograft caused any prolongation or shortening of the rejection time. The length of time that a homograft will remain compatible was

previously determined at this laboratory to be 9 ± 1 days[7]. Rejection times in this project ranged 8-11 days with only one of the grafts becoming incompatible on day 11. Table II summarizes the findings in Groups II, III, and IV.

Discussion

The Role of Lymphocytes in Homograft Rejection - Lymphoid cells are known to play a prominent role in the rejection of homografts. The most important cell of the lymphoid series in this respect appears to be the small lymphocyte[8]. This is apparent in that procedures that prevent the normal development of lymphoid tissue or remove mature lymphocytes from the organism have been shown to compromise that organism's immunological competence. In rats, chronic drainage from a thoracic duct fistula will produce a depletion in small lymphocytes. Prolonged compatibility of homografts was subsequently found in these animals[9]. Also, neonatal thymectomy results in inadequate lymphoid tissue development and a subsequent depressed number of mature lymphocytes. Ability to reject homografts in these animals is impaired and infusion of lymphoid cells has been

shown to restore immune competence.[10]

Even stronger evidence for the involvement of lymphocytes in homograft response is provided by studies of graft versus host reactions. These reactions occur when the lymphoid cells of a compatible graft attack the histocompatibility antigens in the cells of the host.[11] It is reasonable to assume that the reverse process is occurring when a recipient is successfully rejecting its graft.

The mechanism of the actual destruction of the homograft is unclear. Lymphoid cells and other leukocytes physically invade the substance of the graft[7,12] but whether destruction is the result of a direct cytotoxic effect or whether some humoral agent is involved is not known.

The in Vitro Effect of Phytohemagglutinin on Lymphocytes - When lymphocytes of normal blood are cultured in vitro there is minimal evidence of mitogenic activity. The addition of PHA to normal lymphocyte cultures results in transformation of more than 70% of the cells to a blastoid form. The cytoplasm of these blastoid cells stains intensely with the dye, pyronin, indicating the presence of high concentrations of ribonucleic acid.[13] Human

and equine lymphocytes increase their rates of RNA synthesis in a matter of minutes following exposure to PHA. However, quantitative extraction of RNA with perchloric acid reveals that during the first one and one-half hours following exposure to PHA there is a steady decline in the amount of cellular RNA. Furthermore, sedimentation studies reveal that all three major RNA fractions (28S, 16S, and 4S) are involved in this decline. After a lag period of seven to twenty-four hours there is evidence of net synthesis of RNA.[14] It is postulated that PHA initiates degradation of RNA involved in the metabolic processes of the resting state. This interruption of resting-cell activities then permits the onset of growth. In an elaborate experiment by Pogo, Allfrey, and Mirsky[15] histone acetylation of basic chromosomal proteins was demonstrated to precede the increase in RNA synthesis. Thus, it was postulated that this mechanism resulted in gene activation leading to increased synthesis of RNA and other proteins.

If the concentration of PHA remains high enough and the cells are exposed to it long enough, DNA synthesis will occur in most cells.[15] DNA response

to PHA requires hours as opposed to the almost immediate RNA production, and very little DNA synthesis occurs in the first twenty-four hours following the addition of PHA to the lymphocyte culture media. After DNA synthesis has begun, the cells involved assume the blastoid form described above, and mitoses occur. Most susceptible cells show maximal morphological changes by seventy-two hours following incorporation of PHA into the media. Ultramicroscopy reveals that these cells are rich in ribosomes and mitochondria. The endoplasmic reticulum is in the form of small dilated or flattened vesicles or sacs and is often seen proximal to large electron-opaque bodies which are characteristic of PHA-stimulated cells. The Golgi apparatus is well developed and in some cells is quite extensive.[17]

The in Vivo Effects of Phytohemagglutinin - There has been much less research on the in vivo effects of PHA as compared to the volume of published reports concerning the in vitro effects of this substance. It is interesting that some of the earliest recorded subjects to receive PHA were humans. Because of the similarity of the blastoid cells resulting from PHA stimulation to hemocytoblasts

or blood stem-cells, Humble[2] used small intravenous injections of PHA in patients suffering from aplastic anemia. In these patients the anemias had developed as a result of drugs, radiation, or a combination of either of the first two and a malignant tumor. In all of these patients there was evidence of increased bone marrow activity which was attributed to PHA stimulation.

Since that time, investigators working with irradiated rats,[18] and humans with aplastic anemia[19] have had much less enthusiasm about the effectiveness of PHA in stimulating hematopoiesis. It is conceded by these authors that possibly PHA may be more effective in secondary aplastic anemias.

Recently it was demonstrated that injection of lymph node cells obtained from mice pre-treated with PHA into irradiated, syngeneic recipients resulted in macroscopic colonies of lymphoid cells in the recipient's spleen.[20] Normal and hyperimmunized donor cells failed to produce such nodules in the recipients and it was postulated that PHA triggered the <u>in vivo</u> proliferation of the lymphoid cells.

Direct administration of PHA intravenously in mice results in increased splenic weight. Histo-

logically, the splenic parenchyma shows increased cellularity and a greater proportion of immature cells.[3] These criteria were used in this project to insure that the preparation of PHA employed (General Biochemicals) was biologically active.

PHA and Homograft Rejection - Intravenous administration of PHA in divided doses of 1.0 to 1.5 ml. failed to significantly alter first-set homograft rejection times. Animals receiving lesser doses of PHA demonstrated evidence of lymphoid mitogenic activity in their spleens.

The failure of PHA to render these animals immunologically tolerant may be due to a variety of factors. A likely possibility is that an inadequate concentration of PHA or an inadequate period of exposure to PHA was attained for it to exhibit its full biological effect. Toxicity becomes a problem in higher dose levels and is usually manifested by sudden death. Since PHA is a relatively crude extract, further biochemical study may, in the future, yield a pure substance of high mitogenic activity and lower toxicity.

In vitro studies reveal that PHA affects 70-95% of the cells in lymphocyte cultures. The percentage

of lymphoid cells that are affected *in vivo* is unknown. Even if PHA concentration was optimal, there might still exist *in vivo* enough functional, mature lymphocytes to maintain unaltered defenses against foreign antigens.

A less likely possibility exists that, in spite of a profound effect on morphology and kinetics of the lymphocyte, PHA stimulation may not alter its ability to react with foreign histocompatibility antigens.

Presently unknown factors are almost surely involved in the interaction of PHA and the functions of *in vivo* lymphoid cells. Further work should be valuable not only in the field of immunology but also in gaining knowledge concerning basic cellular kinetics.

Summary

Phytohemagglutinin (PHA) is a mucoprotein extract of the red kidney bean, *Phaseolus vulgaris*. Besides hemagglutinating and leukoagglutinating abilities, it causes transformation and mitosis in the majority of cells in lymphocyte cultures. Mitogenic activity *in vivo* is demonstrated by increased cellularity and immaturity of the lymphoid

elements of the spleen in mice.

The purpose of this paper was to determine if PHA, by itself, has an effect on the immunological competence of mice. Skin homografts were applied and various regimens of PHA were employed. No significant alteration of first-set rejection time was found.

A discussion of the role of lymphocytes in homograft rejection and *in vitro* and *in vivo* effects of PHA was presented.

TABLE I

Days After PHA	Sex	Spleen Wt.	Mouse Wt.	Ratio
2	m	.1465	38	.00386
	f	.1453	39	.00373
3	m	.1685	33	.00510
	f	.1395	34	.00410
4	m	.1232	37	.00334*
	f	.1622	35	.00464
5	m	.1689	41	.00412
	f	.1786	34	.00526
6	m	.1265	35	.00362
	f	.2365	35	.00678
9	m	.1142	38	.00301
	f	.1325	31	.00427
Controls	m	.1225	44	.00278
	f	.1351	40	.00338
	m	.1207	39	.00309
	f	.0795	37	.00214

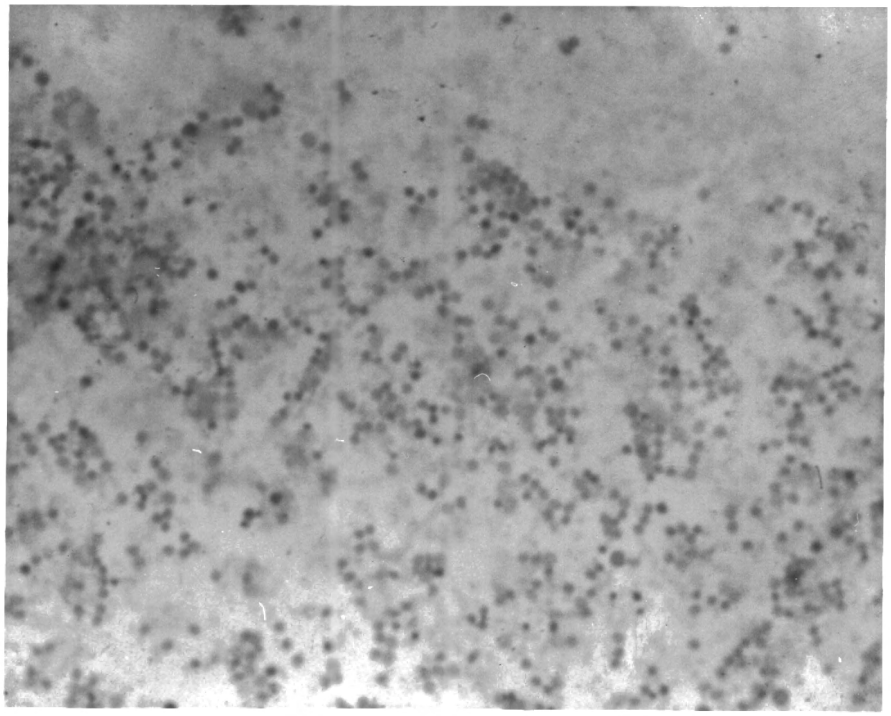
Spleen weights and spleen weight/mouse weight ratios following intravenous PHA administration - The average spleen weight of mice, two to nine days following PHA administration, is 0.1535 grams as compared to the control average of 0.1145 grams. The average ratio of spleen weight/mouse weight in PHA-treated animals is .00433; average control animal ratio is .00286.

* A portion of this animal's spleen was lost during splenectomy.

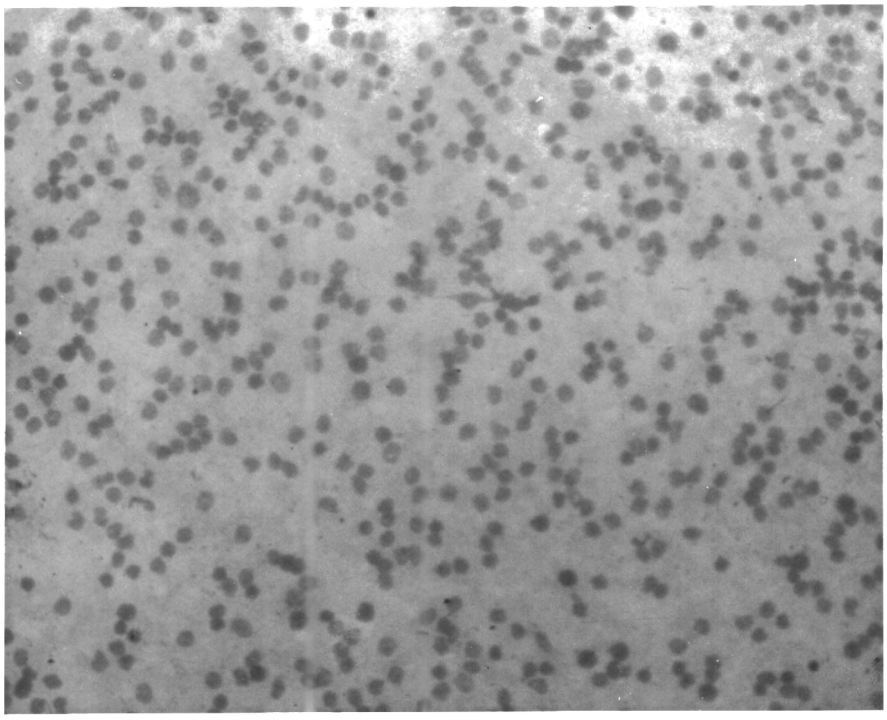
TABLE II

Group	No. of Mice	Days between Homograft and PHA Administration			Rejection Date			
		-3	0	+3	+8	+9	+10	+11
II	7	PHA	PHA & Homograft	PHA	3	2	1	1
III	9	-	"	"	2	3	4	-
IV	4	Saline	Saline & Homograft	Saline	-	2	2	-

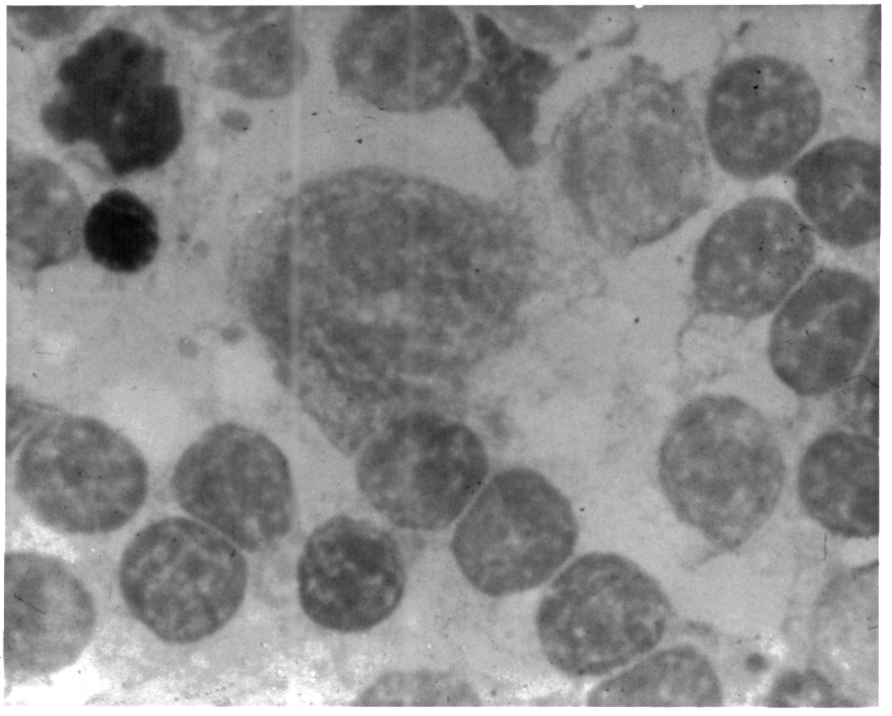
Homograft rejection times in Groups II, III, and IV. No significant difference in homograft rejection is apparent between PHA-treated and saline control animals.



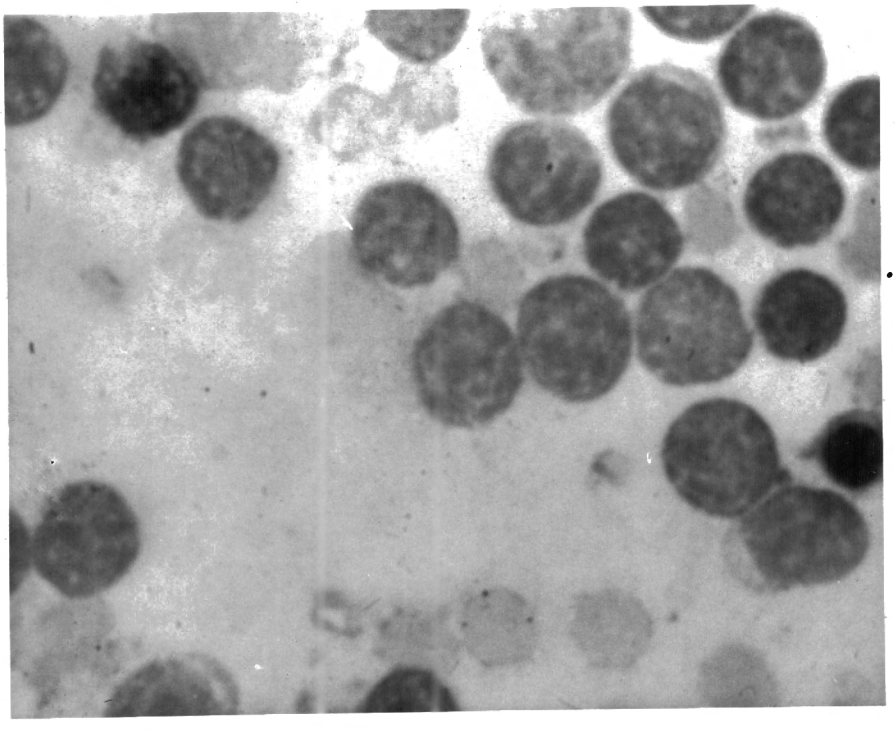
Photomicrograph #1 - Low power magnification of a control spleen smear.



Photomicrograph #2 - Low power magnification of a spleen smear, four days following PHA administration.



Photomicrograph #3 - High power magnification of a control spleen smear.



Photomicrograph #4 - High power magnification of a spleen smear, four days following PHA administration.

BIBLIOGRAPHY

1. Barkhan, P., and Ballas, A., Phytohemagglutinin: Separation of Hemagglutinating and Mitogenic Principles, Nature 200:141 (Oct) 1963.

2. Humble, J. G., The Treatment of Aplastic Anemia with Phytohemagglutinin, Lancet i:1345 (June) 1964.

3. Gamble, C. N., The Effects of Phytohemmaglutinin on Mouse Spleen Cells in Vivo, Blood 28:175 (Aug) 1966.

4. Calne, R. Y., and others, Preliminary Communication: Combined Immunosuppressive Action of Phytohemagglutinin and Azathioprine (Imuran) on Dogs with Renal Homotransplants, Brit. Med. J. 2:154 (July) 1965.

5. Micklem, H. S., The Effect of Phytohemagglutinin on the Spleen Colony Forming Capacity of Mouse Lymph Node and Blood Cells, Transplant. 4:732 (Nov) 1966.

6. Billingham, R. E. and Silvers, W. K., Transplantation of Tissues and Cells, Wistar, Philadelphia, 1961.

7. Dietrich, M. L. and Rigby, P. G., The White Blood Cell Response to Skin Homografts in Mice, Transplant. 4:416 (July) 1966.

8. Gowans, J. L., The Role of Lymphocytes in the Destruction of Homografts, Brit. Med. Bull. 21:106 (May) 1965.

9. McGregor, D. D. and Gowans, J. L., Survival of Homografts of Skin in Rats Depleted of Lymphocytes by Chronic Drainage from the Thoracic Duct, Lancet i:629 (Mar) 1964.

10. Dalmasso, A. P. and others, Reconstitution of Immunologic Capacity in Mice Thymectomized at Birth, J. Exp. Med. 118:1089 (Dec) 1963.

11. Billingham, R. E. and Brent, L., Quantitative Studies on Tissue Transplantation Immunity, Phil. Trans. B. 242:439 (1959).

12. Prendergast, R. A., Cellular Specificity in the Homograft Reaction, J. Exp. Med. 119:377 (Mar) 1964.

13. Marshall, W. H. and Roberts, K. B., The Growth and Mitosis of Human Small Lymphocytes after Incubation with a Phytohemagglutinin, Quart. J. Exp. Physiol. 48:146 (April) 1963.

14. Cooper, H. L. and Rubin, A. D., RNA Metabolism in Lymphocytes Stimulated by Phytohemagglutinin: Initial Responses to Phytohemagglutinin, Blood 25:1014 (June) 1965.

15. Pogo, B., Allfrey, V., and Mirsky, A., RNA Synthesis and Histone Acetylation During the Course of Gene Activation in Lymphocytes, Information Exchange Group No. 7: Nucleic Acids and the Genetic Code (Mar) 1966.

16. Tormey, D. C. and Mueller, G. C., An Assay for Mitogenic Activity of Phytohemagglutinin Preparations, Blood 26:569 (Nov) 1965.

17. Elves, M. W. and others, Electron Microscopy Study of Lymphocytes, Lancet i:306 (Feb) 1964.

18. Papac, R. J., Effect of Phytohemagglutinin on Marrow Regeneration in Rodents, Lancet i:63 (Jan) 1966.

19. Hayes, D. M. and Spurr, C. L., Use of Phytohemagglutinin to Stimulate Hematopoiesis in Humans, Blood 27:78 (Jan) 1966.

20. Mekori, T., Chieco-Bianco,L., and Feldman, M., Production of Clones of Lymphoid Cell Populations, Nature 206:367 (April) 1965.