HEMOPOIETIC SPLEEN COLONY STUDIES

IV. Phytohemagglutinin and Hemopoietic Regeneration*

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Plates 61 and 62

(Received for publication 5 June 1967)

Phytohemagglutinin (PHA), an extract from the kidney bean *Phaseolus vulgaris* in which the active principle is a protein molecule with a high affinity for polysaccharides, has a marked mitogenic effect upon peripheral lymphocytes in vitro (1, 2). Most studies of this phenomenon have concerned themselves with the action of PHA upon immune competent lymphocytes. It has been shown that such cells exposed to PHA undergo blastoid transformation, enter mitosis, begin synthesizing protein, and in some cases manifest an anamnestic response (3–5). Recently, however, several investigations have considered the effects of PHA on the marrow lymphocyte and on hemopoiesis. Humble (6) postulated that PHA might result in marrow stimulation in cases of aplastic anemia by causing proliferation and/or transformation of the marrow lymphocyte, which is believed by some workers to represent the stem cell (7, 8). He administered PHA to six patients with symptomatic aplastic anemia and reported evidence of marrow stimulation in all cases. Other workers have reported results suggesting that PHA provides significant but temporary stimulation of hemopoiesis in cases of drug-induced marrow failure, but that it is ineffective in cases of idiopathic aplastic anemia (9–11). In an experiment involving in vitro culture of the aplastic anemic patient's peripheral leukocytes with PHA, followed by injection of the stimulated cells directly back into the patient's marrow spaces, Astaldi et al. (12) found only equivocal indications of hemopoietic stimulation.

Papac (13) working with PHA in vivo in small rodents, looked for radioprotective and myeloproliferative effects after lethal doses of irradiation, but found no conclusive evidence for such effects. We have previously reported that lymph nodes but not thymus of PHA-treated mice undergo a slight increase in their low content of spleen colony-forming units (14). This paper seeks to explore further the action of PHA upon hemopoietic precursor cells.

* This investigation was supported by Public Health Service Grants CA 03367, T1 CA 05021, and K6 CA 14219 from the National Cancer Institute.

Materials and Methods

The animals used and methods for administering X-irradiation, producing endogenous colonies, collecting and injecting bone marrow cells, harvesting and counting spleen colonies, and identifying the types of colonies, have been described in previous publications (15, 16).

Retransplantation of colonies was performed by dissecting out individual colonies from the fresh spleen with sharp pointed scissors and small pointed forceps. The colony was then cut in half; one piece was placed directly into 0.2 ml of Eagle's minimum essential medium (MEM) without serum, and the other was imprinted lightly, cut surface down, three times on a clean glass slide and then placed into the MEM. The cells were expressed from the colony halves by gentle pressure from a smooth-tipped glass rod, and the bits of stroma remaining were removed. In some cases cell counts were performed on the suspensions at this point, in the manner described previously (15), to determine the number of viable nucleated cells. The cell suspension was diluted to 0.5 ml with MEM, taken up in a tuberculin syringe, and injected into a single recipient mouse via a lateral tail vein. The imprints were air dried, fixed for 3 min in absolute methanol, and stained by the May-Grünwald-Giemsa technique (17).

Phytohemagglutinin (PHA) was generally administered by the intraperitoneal route. Rigas and Osgood (18) reported that the purified protein fraction of PHA retained all of the mitogenic activity possessed by the mucoprotein substance and was 50 times more potent. For this reason the more refined PHA-P[1] was used rather than the mucoprotein PHA-M[2]. The dose used was 20 μg per g of body weight of the protein PHA dissolved in isotonic saline and administered in a volume of 0.5 ml. This dose was comparable to the amounts used by MacKinney (19) for the study of human lymphocytes in vitro, but is below the levels found toxic in vivo by Norins and Marshall (20). Mekori et al. (21) reported using 0.3 ml of PHA-M per mouse intravenously, which when prepared in the customary fashion is approximately equal in potency to 10 μg of PHA-P per g of body weight.

RESULTS

Effect of PHA Treatment of the Bone Marrow Donor on the Number and Types of Spleen Colonies Formed.—Two groups of mice were exposed to 850 R whole-body X-irradiation. One group was injected with femoral marrow cells (4×10^4, 8×10^4, or 12×10^4 viable nucleated isogeneic cells) from a pool of three normal donors and the other group was injected with the same number of pooled marrow cells from three donors that had received daily injections of PHA for 3 days prior to sacrifice. All recipient mice were sacrificed 8 days after marrow injection, the spleens removed and the colonies counted. There was a consistent but slight decrease (not quite statistically significant) in the total number of colonies found. The proportions of the various cell types of colonies were not significantly changed (Table I).

Effect of PHA Treatment of the Marrow Recipient on Developing Spleen Colonies.—Two groups of mice were exposed to 850 R whole-body X-irradiation and injected with 4×10^4 cell aliquots of the same marrow suspension from a single isogeneic donor. One group was not treated further, while the other received daily intraperitoneal injections of PHA. All mice were sacrificed 8 days after marrow injection, the spleens were weighed, and sections were made for colony

[1] Difco Bacto-Phytohemagglutinin P, Code 3110, Difco Laboratories, Detroit, Mich.

[2] Difco Bacto-Phytohemagglutinin M, Code 0528.

counting and typing. A third group of animals was irradiated but not injected, to determine the postirradiation weight of spleens without colonies. In the PHA-treated mice there were approximately the same total number of colonies,

TABLE I

Number and Type of 8-Day Spleen Colonies in 850 R-Irradiated Mice Receiving Marrow from Normal or from PHA-Treated Donors

Experiment	Marrow donor pretreatment	Cell dose	No. of mice	Per cent of colony types					Mean total colonies per spleen*
				Ery.	Neutr.	Meg.	Undiff.	Mixed	
1	None	4×10^4	16	55	15	8	4	18	5.8 ± 0.64
	PHA	"	12	59	22	4	7	8	3.6 ± 0.53
2	None	8×10^4	18	53	19	8	6	14	7.9 ± 0.66
	PHA	"	18	49	24	10	4	13	5.8 ± 0.80
3	None	12×10^4	10	57	14	10	4	15	15.0 ± 1.29
	PHA	"	12	54	17	7	5	17	11.8 ± 1.02

In this and other tables, Ery., erythroid; Neutr., neutrophilic; Meg., megakaryocytic.
* ± SE
$0.1 > p > 0.05$ between groups in any experiment.

TABLE II

Number, Type, and Weight of 8-Day Spleen Colonies in Irradiated Recipients of Normal Marrow, Given Daily Injections of PHA until Sacrifice

Group	Cell dose	No. of mice	Mean total colonies per spleen*	Per cent of colony types					Mean spleen weight*	Mean weight all colonies	Mean weight individual colonies
				Ery.	Neutr.	Meg.	Undiff.	Mixed			
									mg	*mg*	*mg*
850 R	—	12	—	—	—	—	—	—	19.7 ± 1.44	—	—
850 R and B.M.‡	4×10^4	18	6.9 ± 0.7	55	16	9	5	15	35.9 ± 2.53	16.2	2.3
850 R and B.M. and PHA	"	18	6.8 ± 0.8	33	5	20	1	41	26.3 ± 1.20	6.6	0.97

* ± SE
‡ B.M., bone marrow cells.

but there was a significantly greater proportion of mixed colonies, more megakaryocytes, and a reduction of the proportion of erythroid, of neutrophilic, and of undifferentiated colonies (Table II). Histologically, the appearance of the developing colonies was quite similar to that of colonies in untreated hosts except

in two regards. In addition to the normal spectrum of cell types usually found in spleen colonies there was an unusually high proportion of large, undifferentiated cells, many of which had a blastoid appearance (Fig. 1). These cells resembled in general appearance the transformed cells found in cultures of peripheral lymphocytes cultured with PHA. The other unusual feature was the finding of hemopoietic cells, nearly always of mixed types, in a peculiar, sharply delineated distribution (Fig. 2). The appearance suggests that sinusoidal passages are packed with proliferating or emigrating hemopoietic cells. It may be significant that the bone marrow sinusoids are normally rich in small lymphocytes,

TABLE III

Mean Cell Counts of the Five Largest 8-Day Colonies of Each Spleen in Irradiated, Marrow-Injected Mice Given Daily Injections of PHA until Sacrifice

Group	No. of mice	Per cent of colony types						Mean total colonies (gross)*	Grand mean No. of cells for five largest colonies
		Erythroid		Granuloid		Mixed			
		No. of colonies	Mean No. of cells	No. of colonies	Mean No. of cells	No. of colonies	Mean No. of cells		
850 R and B.M.‡	12	44	6.8×10^5	4	3.2×10^5	12	17.1×10^5	9.3 ± 2.4	8.6×10^5
850 R and B.M. and PHA	9	19	4.3×10^5	3	2.9×10^5	23	5.6×10^5	8.7 ± 1.1	4.9×10^5

* ± SE

‡ B.M., bone marrow cells.

i.e., the "lymphocyte loading" of Yoffey (7). In general the colonies were smaller (Table II). The size of the colonies was determined in two ways:

Mean colony weight was estimated by determining the mean weight of spleens without colonies and subtracting this from the mean weight of spleens containing colonies. The difference was then divided by the mean number of colonies per spleen to arrive at the mean single colony weight. The colonies developing under the influence of PHA weighed on the average only half as much as normal colonies (Table II).

Mean cell number per colony: In this experiment the same treatment groups were used as for the experiment above, but the colonies were counted grossly in the fresh spleen, and the five largest colonies dissected out, imprinted for identification, and suspended for cell counts. Again there was virtually no difference in the total number of colonies and there were relatively more mixed colonies (Table III). No megakaryocytic or undifferentiated colonies were found, but we have shown that none are to be expected by this method for they

are too small to be grossly visible and dissectable (15). The cell counts verified the impression that the colonies developing under the influence of PHA were smaller. This was particularly true of the mixed colony type. Either PHA reduced the rate of growth of spleen colonies, or, perhaps more likely, increased the rate of emigration of cells out of the colonies and into the sinusoids.

Effect of PHA on the Colony-Forming Unit (CFU) Content of Spleen Colonies.—Two groups of animals were irradiated with 850 R. Each animal was injected with a suspension of all the cells expressed from a single colony, one group receiving normal 10-day colonies while the other group received colonies which developed under the influence of PHA for 10 days as in the experiments above. 10 days after injection the recipients were sacrificed and the spleens prepared

TABLE IV

Mean CFU Content of, and Cell Types of 10-Day Colonies Formed by Retransplantation of 10-Day Colonies Arising in PHA-Treated and Untreated Hosts

Source of transfused cells	No. of colonies	Mean No. of secondary colonies per injected colony*	Per cent of 10-day secondary colony types				
			Ery.	Gran.	Meg.	Undiff.	Mixed
10-day normal colonies	25	12.8 ± 3.1	51	20	8	5	16
10-day colonies arising in PHA-treated hosts	22	4.5 ± 1.3‡	56	17	10	3	14

In this table, Gran., granuloid.

* ± SE

‡ $p < 0.02$.

for histological study. The results are shown in Table IV. The PHA-treated colonies had significantly fewer CFU's, but the spectrum of colonies formed upon retransplantation was almost identical with that of normal colonies.

Effect of PHA on Endogenous Colonies.—Since PHA tended to reduce the number of CFU's in the marrow, it was of interest to study the effect of this agent on the formation and development of endogenous colonies.

Two groups of animals were irradiated with 770 R, and one group was given daily injections of PHA until sacrifice. The spleens were harvested 9 days postirradiation and prepared for histological study. The results are shown in Table V. At this dose of irradiation PHA significantly increased the number of endogenous colonies formed, but there were too few colonies in these experiments to determine whether there was any effect of PHA on the colony types. However, pooled data from these and several other experiments involving PHA and endogenous colonies revealed considerable differences in the proportions of the various colony types found. In particular, as in the case of exogenous marrow colonies, there was an increase in mixed colonies at the expense of the

other types (Table VI). When similar experiments were done giving PHA injections for 3 days *prior* to irradiation, no significant effect on the number of endogenous colonies was noted. It was concluded that PHA administered after irradiation increased the number of endogenous colonies and increased the proportion of mixed endogenous colonies.

TABLE V

Number and Type of Endogenous Colonies Formed in Mice Receiving 770 R and Daily Injections of PHA

Experiment	Group	No. of mice	Mean total colonies per spleen*	No. of colonies of each type				
				Ery.	Neutr.	Meg.	Undiff.	Mixed
1	770 R only	12	0.09 ± 0.009	1	—	—	—	—
	770 R and PHA	10	3.3 ± 0.87‡	10	3	1	—	19
2	770 R only	12	0.17 ± 0.02	1	—	—	—	1
	770 R and PHA	12	3.5 ± 0.93‡	9	5	3	—	24

* ± SE

‡ $p < 0.01$.

TABLE VI

*Distribution of the Various Cell Types of Endogenous Colonies in Irradiated Untreated and Irradiated PHA-Treated Mice**

Treatment	Range of irradiation dose	Range of colony age	No. of mice	No. of colonies	Per cent of endogenous colony types				
					Ery.	Neutr.	Meg.	Undiff.	Mixed
	R	*days*							
Irradiation only	500–700	8–10	87	367	58	12	3.5	8	18.5
Irradiation and PHA	770–850	8–10	51	89‡	23	8	2	2	65

* Pooled data from several experiments.

‡ The smaller number of colonies per spleen of PHA-treated animals is the result of higher dose of irradiation. At comparable irradiation doses, PHA-treated animals had more colonies per spleen (see Table V).

Effect of PHA on Survival after Whole-Body Irradiation.—The increased number of endogenous spleen colonies found after PHA treatment raised the possibility of more rapid hematologic recovery after irradiation and subsequent manifestation as improved survival. Paired groups of animals were irradiated at 600, 700, 800, and 900 R. The mice in one group of each pair were given daily injections of PHA for 21 days postirradiation. The PHA-treated mice had a significantly improved survival at 600 and 700 R, as determined by the Kolmogorov-Smirnov test (22) for the difference between two cumulative percentages

(Text-Fig. 1). At higher doses of irradiation no protective effect was noted. Similar experiments were performed using pretreatment of the mice with injections of PHA on the 3 days prior to irradiation, but no significant difference in survival was noted at any of the four irradiation dose levels.

DISCUSSION

The results indicate that PHA exerts significant effects upon hematopoietic tissues in vivo. These effects are apparently multiple and complex, and not simple of interpretation. Apparently opposing effects were obtained in the endogenous irradiation recovery experiments as compared to the bone marrow transplantation experiments. In the former, PHA administered after irradiation (but not before irradiation) stimulated endogenous hemopoietic spleen colony formation, and enhanced postirradiation recovery and survival. In the transplanted marrow systems, PHA treatment of the donor, if anything, reduced the number of CFU, whereas PHA treatment of the recipient resulted in smaller more differentiated colonies. Whether any of these effects are related to or secondary to previously reported effects of PHA is not clear. The previously reported biological effects of PHA include the following:

Hemagglutinating activity: this leaves the leukocytes free in suspension; first used for a rapid and efficient separation of leukocytes from blood by Li and Osgood (23).

Mitogenic activity in vitro: peripheral leukocytes (later shown to be lymphocytes) incubated in vitro with PHA were shown to transform into blasts and begin dividing (1); PHA provided mitotic stimulation to all of the lines of tissue culture cells to which it was added (24).

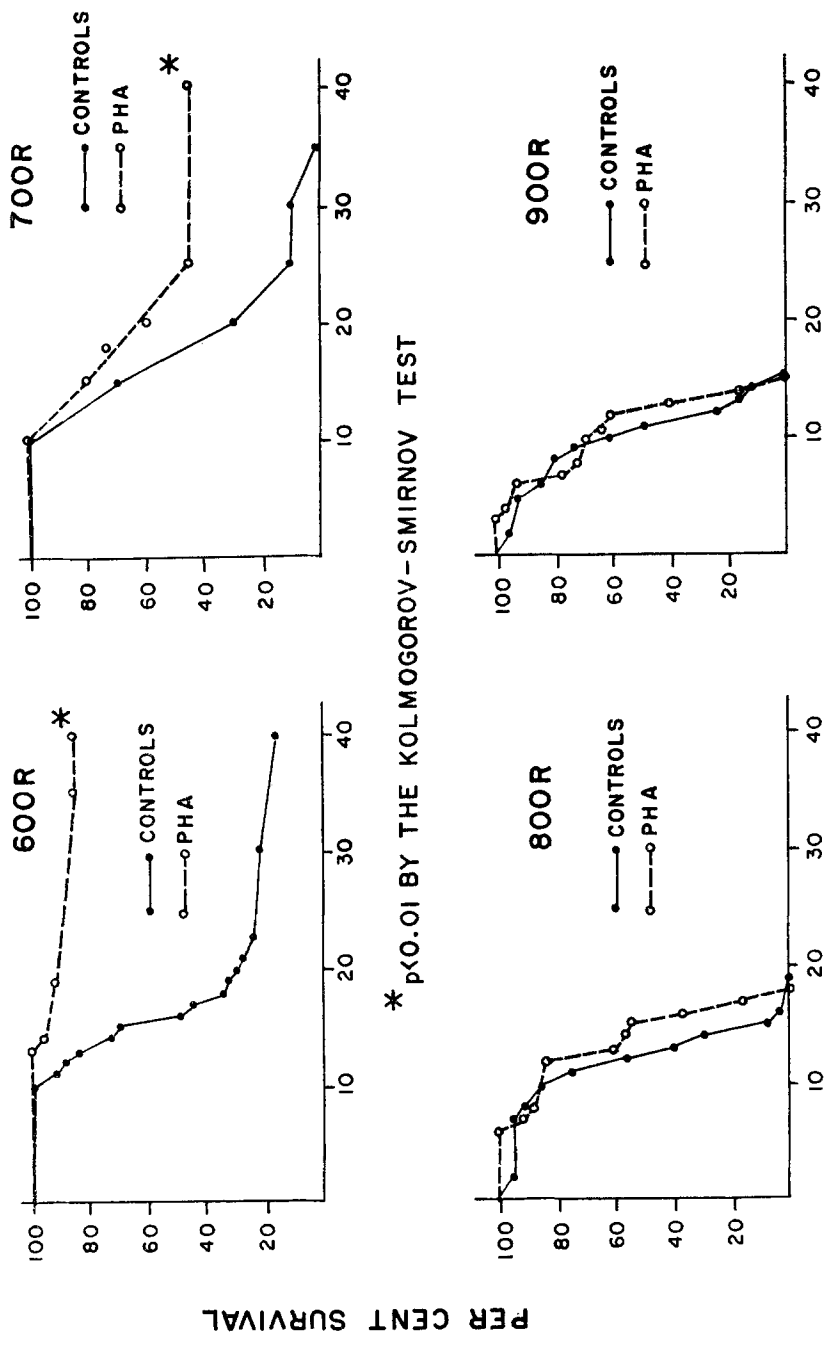
Leukoagglutinating activity: Hirschhorn et al. (4) showed that PHA caused aggregation of lymphocytes prior to transformation and mitosis.

Stimulation of an immune response: lymphocytes after transformation by PHA have been shown to produce γ-globulin (4) and react with a specific anamnestic response (5).

Radioprotective activity: Schrek and Stefani (25) demonstrated that stimulation by PHA conferred a remarkable radioresistance on lymphocytes in vitro, but Papac (13) could not obtain a significantly improved survival of irradiated mice when PHA was administered in one dose immediately after irradiation.

Myeloproliferative activity: Humble (6) found evidence of marrow stimulation in all of six aplastic anemia patients given PHA, and Israel et al. (26) found that PHA increased the hematologic tolerance of patients to massive cancer chemotherapy. However, Papac (13) could find no significant effect of PHA on the cell count or morphology of rodent marrow after irradiation.

Toxicity: several workers (13, 20, 27) have investigated the toxicity of PHA in mice. Respiratory distress, paresis, and sudden death are most commonly reported, especially when PHA was administered intravenously. Arthus reac-



TEXT-FIG. 1. Postirradiation survival of PHA-treated and untreated mice after 600, 700, 800, and 900 R whole-body irradiation. The PHA-treated animals show a significantly improved survival at the lower two irradiation doses, but not at the higher doses. It is interesting to note that endogenous colonies are formed at the lower irradiation doses, but none have been noted after 800 or 900 R in this laboratory. If PHA exerts its in vivo radioprotective effect by causing augmented proliferation of endogenous CFU, the fact that there are no remaining CFU at the higher irradiation doses might explain its lack of effect at those doses.

tions and anaphylaxis were found with repeated injections of PHA in guinea pigs.

An important consideration is whether several of the above are manifestations of the same effect. The hemagglutinating effect, although once believed inseparable from the mitogenic effect (28), has been selectively removed both by drastic chemical procedures (29) and by repeated absorption by red cells (30), leaving only the mitogenic activity. However, many of the other effects of PHA may be explained on the basis of one or the other of these two separable properties. Hirschhorn et al. (4) concluded that leukoagglutination, transformation to blasts, mitosis, and γ-globulin synthesis were different stages in a single response of lymphocytes to PHA.

The toxicity of PHA might well be explained by intravascular hemagglutination and possibly pulmonary emboli. In mice receiving doses of PHA over long periods anaphylaxis might be considered as a mode of toxicity; but in the experience of this laboratory adverse reactions have occurred only acutely, after the first intravenous dose of PHA, and a phenomenon more immediate than allergy must be postulated.

For the purposes at hand, the most interesting effect is that of transformation of cells in vitro. Most work along these lines has been done with peripheral lymphocytes, which are the only cells in peripheral blood that respond to PHA. This phenomenon might be due either to the possibility that PHA has a specific effect on lymphocytes or to the fact that of all the cells in the blood, only the lymphocytes are capable of mitosis. PHA might be a nonspecific stimulant for mitosis. Recent studies have shown PHA to be a stimulant for growth in several lines of cells in tissue culture (24).

At least one action of PHA on the cell membrane is known: agglutination of like cells. As Hirschhorn has suggested, this in itself is necessary and sufficient to trigger new metabolic activities in some cell types. That cell-to-cell contact has some role in regulating cell division is apparent not only from in vitro studies with lymphocytes but also from the well-known tissue culture phenomenon of "contact inhibition."

The rationale for Humble's use of PHA in aplastic anemia (6) rested on the possibility that the PHA transformation of lymphocytes, which are usually present in that disease, might include transformation to stem cells which could repopulate the marrow. The results of work along this line, although not providing unequivocal evidence, are quite suggestive of some kind of myeloproliferative effect which cannot be explained on the basis of hemagglutination or lymphocyte transformation to immune-active cells. Astaldi et al. (12) found signs of increased marrow cellularity and function when they exposed a patient's lymphocytes to PHA in vitro and then reinjected them into the marrow cavity, suggesting that PHA can transform lymphoid cells into myeloid precursors.

The increased number of endogenous colonies after a given dose of irradiation,

the formation of spleen colonies by lymph node cells, and the improvement in postirradiation survival found with injections of PHA or lymphoid cells from PHA-treated donors can all be explained by the hypothesis that PHA may transform a precursor cell (which may appear morphologically as a lymphocyte) into a functionally myeloid CFU. On the other hand, the decreased CFU content of the bone marrow and increased CFU content of the lymphoid organs of PHA-treated mice might in fact represent PHA-induced migration of CFU from marrow to lymphoid organs, and possibly elsewhere. Indeed Micklem (31) has reported an increase in the spleen colony forming units circulating in the blood of mice 72 hr after intravenous injection of 0.3 ml of PHA, and a small increase in the CFU content in the lymph nodes.

The disparity in the results between testing the effects of PHA in the endogenous system and in the transplantation system presents a problem in interpretation. Porteous and Lajtha (32) have cautioned against comparison of hemopoietic regeneration studies in the two systems, pointing out that the transplantation system, compared to endogenous repopulation, is nonphysiologic, involves the trauma of manipulation, and often fails to transplant the stem cells which are manifest in the endogenous system. A consideration of their criticisms has led us to speculate that perhaps the stem cell or CFU exists in two forms–a small lymphocytoid form which is transplantable, and a large, undifferentiated hemohistioblast which (perhaps because of its size, fragility and/or attachment to the reticulum) is not amenable to transplantation in a cell suspension given intravenously. In order to reach the spleen or bone marrow, intravenously transplanted stem cells must first traverse the pulmonary vascular network. If the effect of PHA upon the CFU is indeed to stimulate proliferation involving a blastoid transformation (in a fashion analogous to its effect upon lymphocytes in vitro), the observation of blastoid cells in the PHA-influenced spleen colonies and the results of these experiments are understandable, i.e., PHA-transformed stem cells may not be detectable by transplantation but readily detectable in an endogenous system (increase in endogenous colonies or improved survival postirradiation).

Further experiments in this area should include studies on the in vitro effects of PHA on bone marrow and on the CFU content of lymphocytes from various sources transformed in vitro by PHA.

SUMMARY

The effects of phytohemagglutinin (PHA) were studied in irradiated mice to see if a definite myeloproliferative effect could be demonstrated in vivo. The data obtained suggested the following conclusions.

PHA treatment of the bone marrow donor only, causes a consistent but

slight reduction in transplantable spleen colony-forming unit (CFU) content of the bone marrow 24 hr after the last PHA injection, but no change was found in the proportion of the various colony types.

PHA treatment of the irradiated recipient of normal bone marrow causes no change in the number of spleen colonies. However, 8-day colonies are only about half normal size, are much more likely to be of mixed cell types, contain many large undifferentiated blastoid cells, but fewer transplantable CFU. The spleen sinusoids are packed with hemopoietic cells.

Spleen colonies developing in hosts receiving daily injections of PHA show, in addition to the usual spectrum of cell types, a high proportion of unusual blastoid cells resembling the PHA transformed peripheral lymphocytes seen in vitro. The function of these cells is not known, but they may represent augmented proliferation and/or transformation of stem cells.

PHA administered after irradiation significantly increased the number of endogenous spleen colonies, and, at certain doses of irradiation, improved post-irradiation survival.

PHA administered before irradiation had no effect on the number of endogenous spleen colonies formed, or on postirradiation survival.

On the basis of these and other data, possible modes of action of PHA are discussed.

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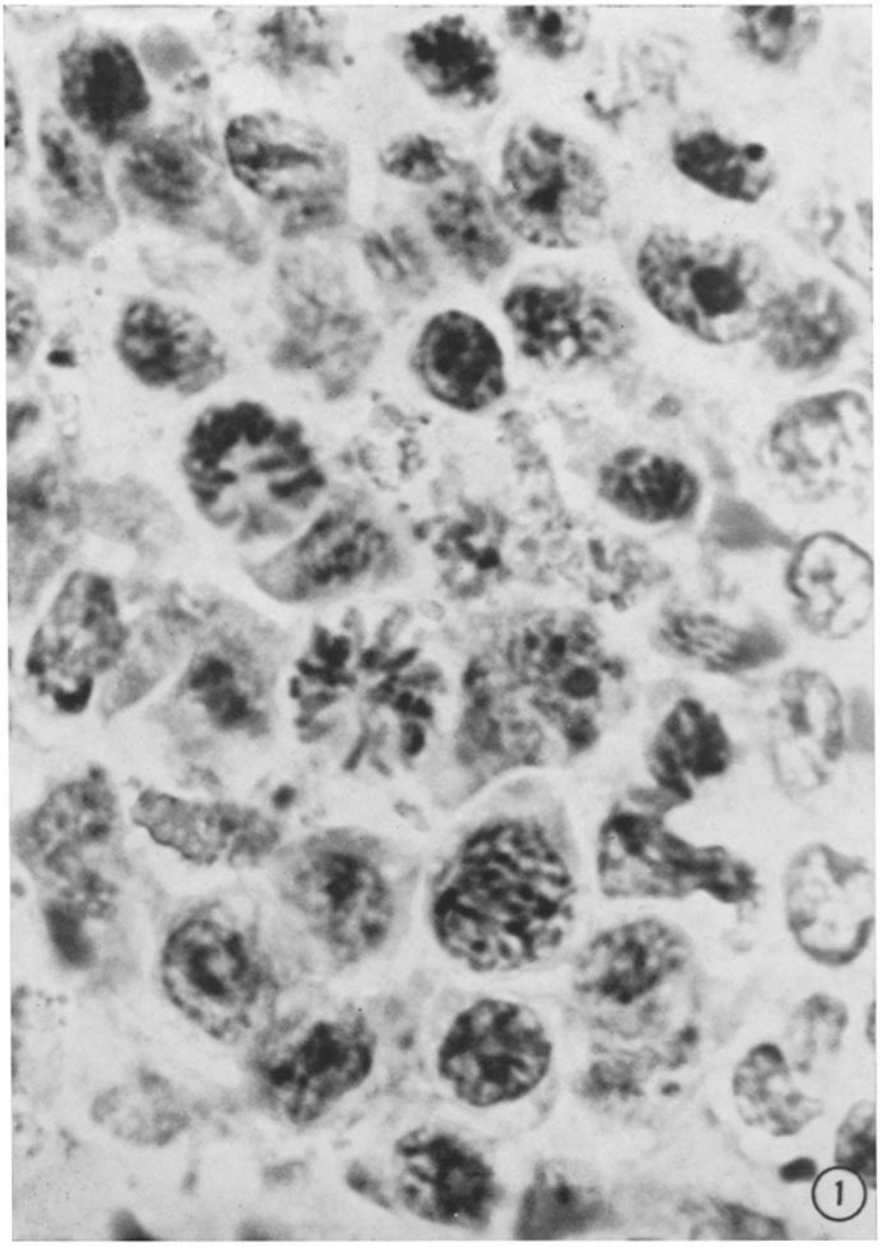
EXPLANATION OF PLATES

PLATE 61

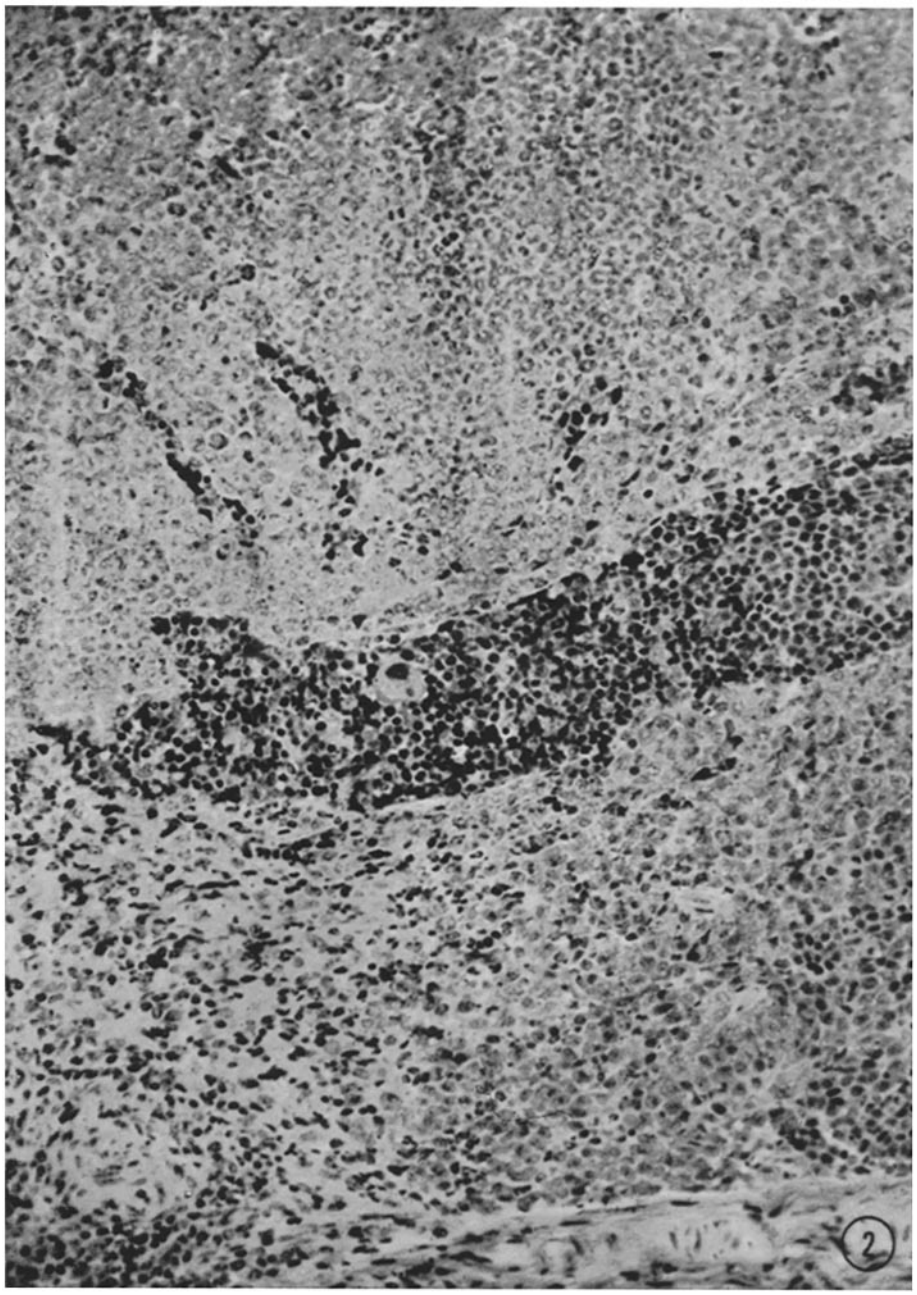
FIG. 1. Large, undifferentiated, blastoid cells appearing in a spleen colony in a $(C_{57} \times Af)F_1$ mouse which received 850 R whole-body irradiation, 8×10^4 viable isogeneic marrow cells, and daily injections of 0.4 mg PHA-P until sacrifice 10 days postirradiation. Such cells are much more common in colonies arising in PHA-treated hosts, and they resemble in many ways the PHA-transformed lymphocytes seen in tissue culture. Hematoxylin and eosin. × 1400.



(Curry and Trentin: Hemopoietic spleen colony studies. IV)

PLATE 62

FIG. 2. Intrasinusoidal mixed hemopoietic cells occurring in the spleen of a (C_{57} × Af)F_1 mouse which had received 850 R whole-body irradiation, 8 × 10^4 isogeneic marrow cells and daily injections of PHA, until sacrifice 10 days postirradiation. Neither the homogeneous mixture of different cell types nor the peculiar intraluminal "packing" of hemopoiesis have been noted in the spleens of colony-bearing hosts not treated with PHA. In the marrow, however, lymphocytes in unusually high concentrations are characteristically found within the sinusoids. Hematoxylin and eosin. × 306.



(Curry and Trentin: Hemopoietic spleen colony studies. IV)