STUDIES ON LYMPHOID TISSUE DAMAGE
WITH A TOXIC PHYTOHEMAGGLUTININ AND
ITS SUBFRACTIONS IN MICE

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INTRODUCTION

Since the striking activity which induces blastogenesis in peripheral lymphocytes was demonstrated by Nowell[1], phytohemagglutinin (PHA), an extract of red kidney beans (*Phaseolus vulgaris*), has been the subject of extensive investigations in the fields of hematology, cytology, immunology and clinical use. Although many plant extracts being furnished with hemagglutinating activity are toxic to animals, PHA from red kidney beans has been reported innocuous by Goddard and Mendel[2] and Elves[3], when administered from routes other than intravenous application. However, PHAs prepared in our laboratory from the beans by the methods of Börjeson *et al.*[4] and by Rigas and Osgood[5] with minor modifications, were found to be lethally toxic to mice when administered intraperitoneally. They were also highly active in hemagglutination (HA) and blast-formation (BF) induction. In order to clarify the mode of action and the nature of the toxic components, further purification of PHA by CM-cellulose column chromatography was carried out. The in vitro activities and toxicity against mice, as well as immunochemical properties of subfractions of PHAs were studied. The mode of action of toxic components was analysed by using adrenalectomized mice.

MATERIALS AND METHODS

Preparation of PHAs and their subfractions: According to the method of Börjeson *et al.*[4], 1 kg of red kidney bean flour was extracted in 4 l of phosphate buffered saline (PBS)(0.01 M phosphate and 0.14 M NaCl pH 7.2) at 4°C for 24 hrs. The extract was heated in a water bath at 80°C for 10 min., the inert precipitate occurred was removed by centrifugation and the supernatant was fractionated by successive ethanol precipitation at −5°C. Precipitate obtained at the ethanol concentration 30–65 volume percent was designated as PHA(Bö) in this paper.

Another PHA was prepared essentially as described by Rigas and Osgood[2]. The PBS

extract of kidney bean was fractionated by addition of ammonium sulfate. The precipitate formed at 50 to 70 percent saturation of ammonium sulfate was collected and dissolved in PBS. The preparation was reprecipitated in the same manner. The final precipitate was dissolved in PBS and dialyzed against phosphate buffer (0.01 M pH 7.0). Then the PHA preparation was passed through a column of Amberlite IRC-45 (Nakarai Chem. Co., Kyoto). The eluate was concentrated with polyethylene glycol (Mw. 6,000) and dialyzed against PBS. This preparation was designated PHA(Ba) in this paper. PHA-P and PHA-M (Difco) was obtained commercially from Difco Laboratories (Detroit, Michigan).

PHAs were further subfractionated by chromatography on column of CM-cellulose (0.9 mEq/g, Brown Company, Berlin, N.H.). The columns were eluted stepwise with acetate buffers with different molar density and pH. The elutes with the buffer 0.01 M pH 4.2, 0.03 M pH 5.0, 0.1 M pH 6.0 and 1.0 M pH 6.0 were designated as CM-I, CM-II, CM-III and CM-IV, respectively. All fractions were concentrated with polyethylen glycol and dialysed against PBS in the cold.

Nitrogen counts of PHAs and each fraction were estimated by microkjeldahl method with a slight modification by Koch and McMeekin[6] and approximate protein contents was conventionally estimated using a coefficient protein/nitrogen: 6.25.

Hemagglutination: Hemagglutinating activity of each preparation was assayed by the microtitration method. Human erythrocytes (HRBC) were washed three times with cold PBS and were resuspended in PBS at the concentration 10^8/ml. Equal volume of serially diluted preparation from PHA and HRBC suspension was mixed and incubated for 2–4 hrs at room temperature. Hemagglutinating activity of each preparation was expressed as minimal protein amount required to cause hemagglutination.

Assay for blastogenic activity: Lymph node cells from ICR mice were cultured in a medium RPMI 1640 supplemented with 10% mouse serum plus 50 units of penicillin and 50 μg of strep-tomicin at a concentration of 5×10^6/ml. Three ml of cell suspension was incubated at 37°C in a small Petri dish under 95% air and 5% CO_2 gas.

PHAs and their subfractions were added into the culture dish at 24 hr of cultivation. Three days after stimulation the culture cells were harvested and their smears were made. In order to examine blast formation, the smears were stained with acridine-orange according to the method of Dukes *et al*[7]. The preparations were examined under a fluorescence microscope and blast cells in 1,000 cultured cells were counted.

Toxicity test: All the used were 8-week-old, female ICR/JCL mice weighing around 20 g obtained from Nihon Kurea Co. Ltd. (Osaka ,Japan) After an intraperitoneal injection of test preparation, mortality of the mice was examined at 24 and 48 hrs. The dead mice, if any, and survived ones, after sacrificing, were subjected to routine histological examination at suitable experimental periods appearing in the text.

Adrenalectomy: Each mouse was anesthetized with ether and each adrenal was exposed carefully by dorsal skin incision. After removal of the adrenals, the mice was sutured and fed with 1% sodium chloride solution. Two weeks later adrenalectomized mice were used for toxicity assay.

RESULTS

Chromatographic subfractionation of PHAs: A typical chromatogram of PHA on CM-cellulose column was shown in Fig. 1.   Three major peaks (CM-II, CM-III, and CM-IV) and one minor peak (CM-I) were observed in all chromatograms of PHAs.   However, the size of peak CM-II varied in each sample of PHAs.

Lethal toxic effect of PHA and their subfractions on mice: An intraperitoneal injection of 5 mg of PHA(Ba) caused lethal intoxication to mice within 48 hrs after administration, whereas the same abount PHA-P (Difco) and PHA(Bö) showed no lethal toxicity on Mice (Table 1). One mg of PHA(Ba) caused acute death of mice when it was administered intravenously. Similar toxicity was observed in the case of fraction CM-I from PHA(Ba) when it was injected into mice either intraperitoneally or intravenously (Table 2).   Histopathological examination of these dead mice revealed severe congestion and the hemorrhage in the spleen, lungs, kidneys and the adrenals.   These findings are coincidental with the previous reports in which an intravenous injection of PHA resulted in immediate death of mice[8].

Lymphoid tissue damage with PHAs and their subfractions: Following an intraperitoneal injection of 2 mg of PHA(Ba), marked reduction of the thymus weight was observed at 16 and 32 hrs after administration.   The control animals injected the same dosage of bovine gamma globulin (BGG) as antigen, and the group of mice injected 5 mg prednisolone intraperitoneally showed no remarkable weight loss of thymus (Table 3).

Time course study of histology of the thymus showed karyorrhexis of the thymic cells and pronounced phagocytosis of the retiuclocytes in the cortex as early as 2 hrs after injection.   Then, the unmber of cells of the cortex tended to reduce and the border line between the cortex and
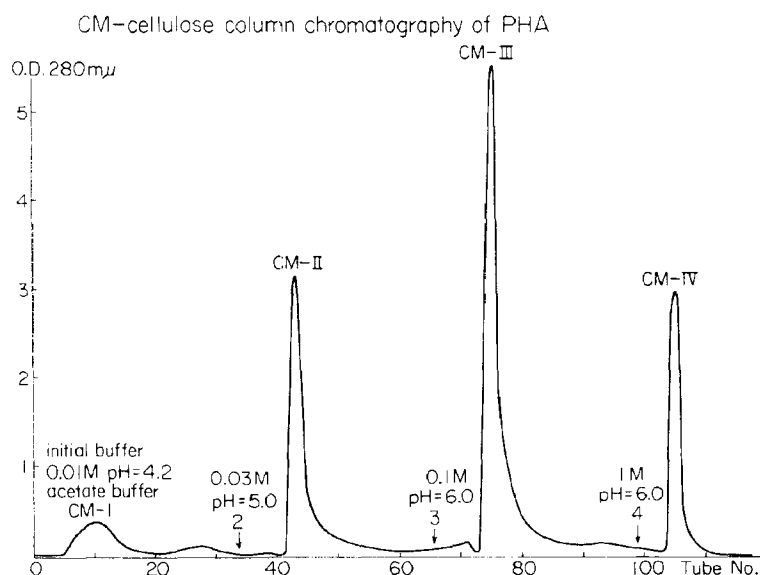

Fig. 1   Cm-cellulose chromatogram of PHA (Ba).

PHA (250 mg) was chromatographed on column (1.5 cm × 25 cm).
Initial buffer: :0.01 M acetate buffer, pH 4.2.   The column was
then eluted stepwisely with 0.03 M, pH 5.0, 0.1 M, pH 6.0 and 1 M,
pH 6.0.   Flow rate, 30 ml/hr with effluent collected in 5 ml fractions.

Table. 1   Toxicity of PHA Preparations

	Dose (mg)	Route	Time (hr)	Mortality	Damage in Thymus	Spleen	Lymph node	Others
PBS		i.p.	24, 48	0/6	—	—	—	—
BGG	5	//	24, 48	0/6	—	—	—	—
PHA-M (Difco)	2.5	//	24, 48	0/6	—	—	—	—
PHA-P (Difco)	5	//	24	0/3	+	+	—	Hemorrhage in adrenal
//	5	//	48	0.3	++	++	--	//
PHA (Bö)	5	//	24	1/3	++	++	—	Hemarrhage in lung
PHA (Ba)	1	//	24	0/4	+	+	+	—
//	2	//	24	0/4	++	+	+	—
//	2	//	48	0/4	+++	++	+	—
//	5	//	24	2/6	++	++	+	Hemorrhage in lung, adrenal
//	5	//	48	3/3	+++	+++	—	//
//	10	//	24	3/4	++	+++	++	Hemorrhage in adrenal
PHA (Ba)	1	i.v.	10 min	4.4	—	—	—	—

Table. 2   Toxicity of subfractions of PHAs

	Dose (mg)	Route	Time (hr)	Mortality	Damage in Thymus	Spleen	Lymph node
PHA (Bö)	5	i.p.	24		++	++	
CM-I (Bö)	2	//	48	0/2	++	+	
CM-II (Bö)	5	//	48	0/2	—	—	
CM-III (Bö)	5	//	48	0/2	+	+	
CM-IV (Bö)	5	//	48	0/2	++	++	
PHA (Ba)	5	i.p.	24		++	+++	+
CM-I (Ba)	2	//	24	0/6	++	++	
//	2	//	48	4/4	+++	++	
CM-II (Ba)	2	//	48	0/2	—	—	--
CM-III (Ba)	2	//	48	0/2	++	+	—
CM-IV (Ba)	2	//	48	0/2	+	+	—
//	5	//	48	0/2	+	+	—
CM-I (Ba)	0.6	i.v.	immed.	3/3	—	—	—

Table. 3　Effects of PHA and BGG on Thymus and Spleen Weights of ICR Mice

Treatment Time (hr)	Thymus weight* BGG	PHA	Spleen weight* BGG	PHA
1	54.3± 7.0	51.6± 9.8	160.3±46.7	176.8±50.0
2	61.3± 9.4	57.3+11.1	148.1±12.8	165.6±35.4
4	46.0±15.7	43.3± 6.2	121.4±38.8	138.1±14.6
8	57.5±10.0	59.8±18.2	167.3±32.3	172.5±40.1
16	56.5±10.7	24.9± 6.2	172.0±37.0	120.0±18.9
32	57.8±13.1	17.0± 5.2	153.6± 9.8	133.6±26.2

* Mean ±S. D. of four mice given 2 mg of PHA or BGG i.p.

meddula tended to become obscure. Thymic cell migration, destruction and phagocytosis by reticulocytes were observed more extensively at 8 and 16 hrs. These results in thinning cortex, at 32 hrs the cortex became scanty in cellularity and remained only network appearance of reticular cells in the most area of it. The histological profile of those thymi, namely reduced number of cells in the cortex and aboundant number in the medulla, may be able to call it as "conversion" in cellularity (Fig. 2~5).

Reduction in the weights of the spleens after the PHA treatment was not so remarkable as that of the thymi (Table 3). Histological examination revealed that the appearance was due to congestion and hemorrhage in the red pulp and perifollicular zone of the white pulp. While, lymphocytic and myelocytic elements were reduced and vanishing. Extramedullary hemopoietic foci in the red pulp were significant and the folliuclar atrophy due to small lymphocyte reduction became apparent after 4 hrs of the dosage. Karyorrhexis and enhanced phagocytosis of reticular cells could be seen in the atrophied follicles some times with occurrence of hemorrhage (Fig. 6~9).

Lymph nodes were also sensitive to PHAs, but not so significant in the case of PHA-P (Difco). After a 2 or 5 mg of PHA(Ba) injection, lymphocyte depression was observed in the lymph nodes histologically. In case of a 10 mg application, marked reduction of lymphoid cells and atrophy of the nodes were recognized.

Effects of PHA(Ba) on the peripheral leukocytes and the red blood cells were tested in the female DDD mice by time course study. As shown in the Fig. 10, intraperitoneal injection of PHA(Ba) caused remarkable leukopenia 2 hrs later, this leukopenia persisted most 12 hrs after injection. The mice received same dosage of BGG as antigen showed remarkable leukocytosis at 2 hrs after administration. The leukopenia induced by PHA administration was not affected significantly by adrenalectomy. Contrally, the red blood cell counts of the mice received PHA were rather higher than that of the mice injected with BGG (Fig. 11)

As for subfractions of PHAs, strong toxicity to lymphoid tissue was observed in CM-I(Ba) and CM-I(Bö). However, CM-I(Bö) did not show the lethal toxicity to the mice at the given dosage. Another fraction with toxicity was CM-IV in the case of PHA(Bö) and CM-III in the case of PHA(Ba). Both CM-II subfractions showed no lymphoid tissue damage at the given dosage in the mice.
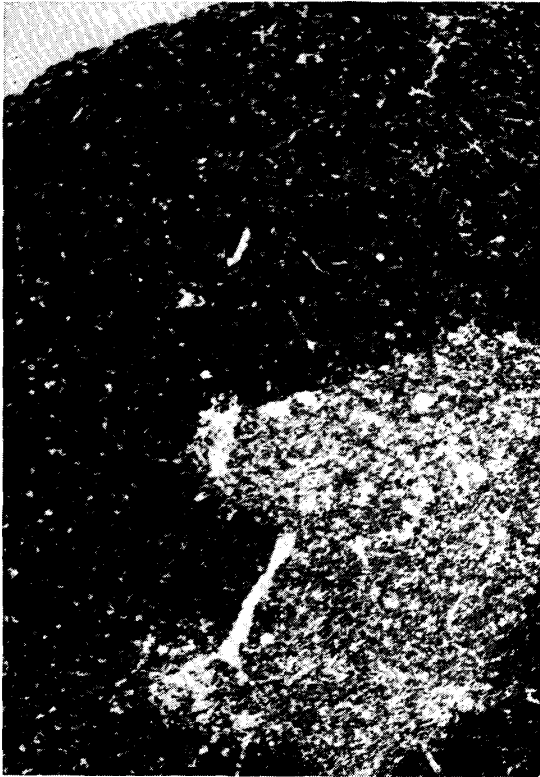
Fig. 2   Normal thymus with abondant small lymphocytes in cortex   (4×10, H. E. stain)
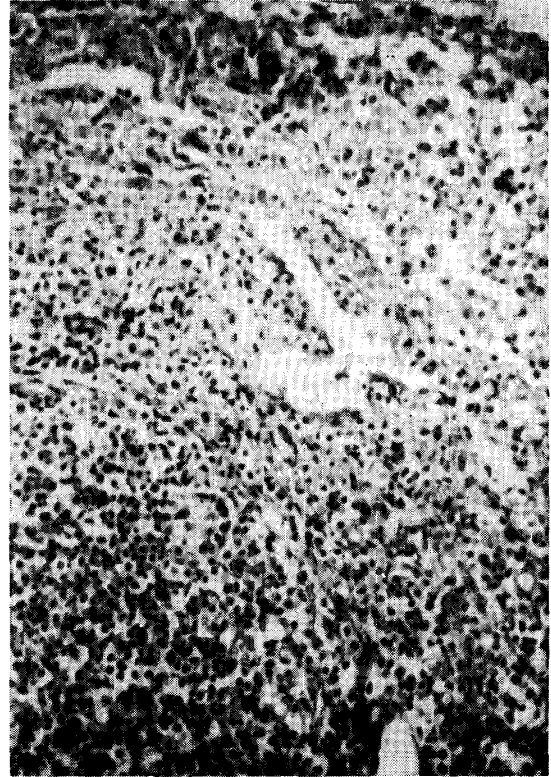

Fig. 3   Thymus 16 hrs after treatment of PHA. Marked reduction of lymphocytes in cortex was seen.   (10×10, H. E. stain.)
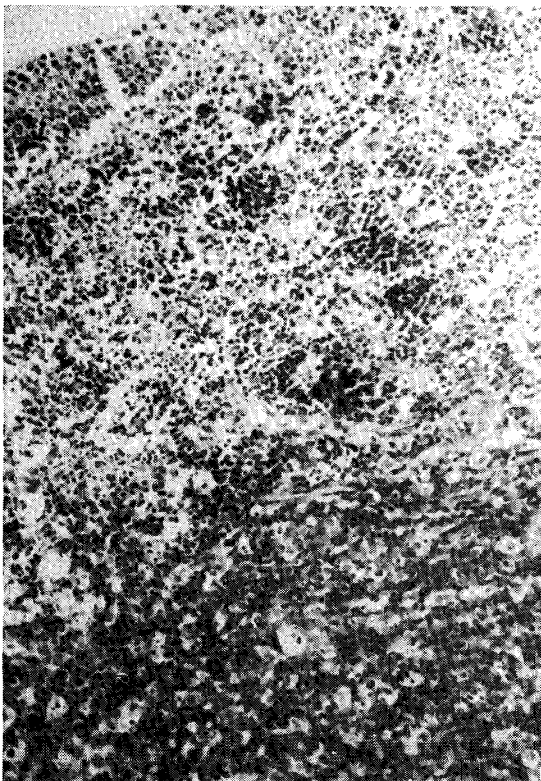

Fig. 4   Thymus 8 hrs after treatment of PHA. "Conversion" of cellularity was dominant and karyorrhexis in the cortex was seen. (10×10, H. E. stain).
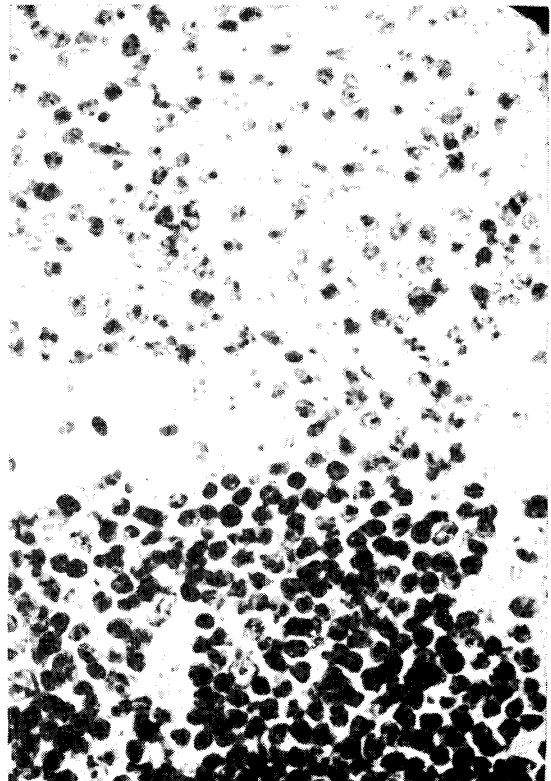

Fig. 5   Higher magnification of Fig. 4 Typical keryorrhexis was seen. (40×10, H. E. stain)

Fig. 6 Normal Spleen with dominant lymphoid
follicle and marginal lymphocytes.
(10×10, H. E. stain)

Fig. 7 Spleen 8 hrs after treatment of PHA.
Marginal lymphocytes were disappeared
and mraked hemorrhage in red pulp was
seen. (10×10, H. E. stain)

Fig. 8 Spleen 4 hrs after treatment of PHA,
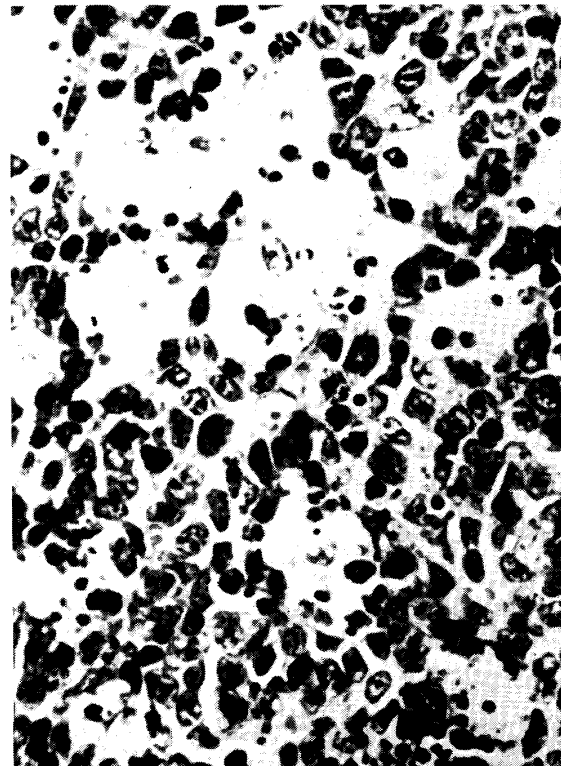Atrophy of lymphoid follicle was dominant.
(10×10, H. E. stain)

Fig. 9 Higher magnification of Fig. 8.
Karyorrhexis in the follicle was seen.
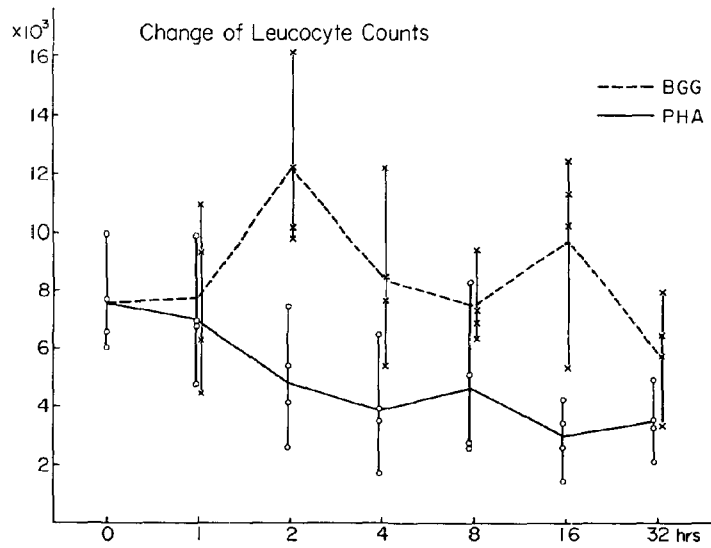(40×10, H. E. stain)

Fig. 10 Changes of leukocyte counts of mice treated with
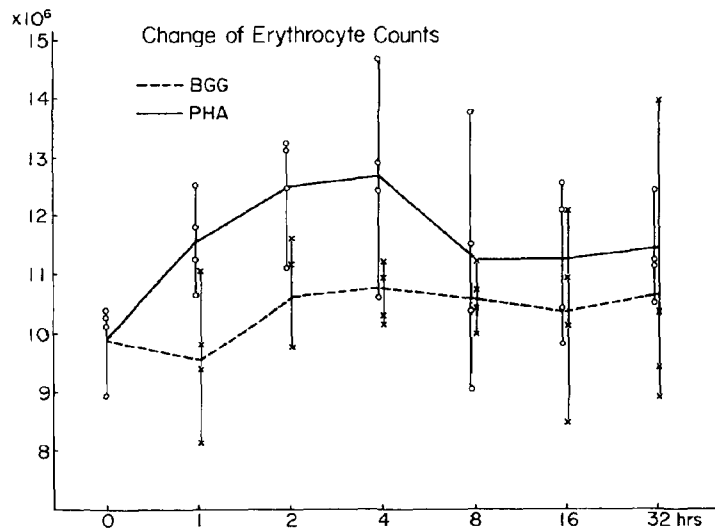2 mg of PHA or BGG.

Fig. 11 Changes of erythrocyte counts of mice treated
with 2 mg of PHA or BGG.

Hemagglutinating activity and blastogenicity: Activities in hemagglutination and blast-formation of PHAs and their chromatographic subfractions are tabulated in Tables 4 and 5, respectively. Fractions CM-IV(Ba) and (Bö) were most active in hemagglutination test and CM-I comes next. Blastogenicity appeared most significantly in fractions CM-III(Bö), CM-IV (Bö), and CM-I(Ba).

Table. 4   Hemagglutinating activity of PHAs and their subfractions

	Activity* μg/ml
PHA-P (Difco)	50
PHA (Bö)	
CM-I (Bö)	10
CM-II (Bö)	1, 000<
CM-III (Bö)	20
CM-IV (Bö)	5
PHA (Ba)	
CM-I (Ba)	5
CM-II (Ba)	1, 000<
CM-III (Ba)	20
CMIV- (Ba)	5

* Activity is expressed as minimal protein concentration to exhibit hemagglutination.

Table. 5   Blastogenic activity of PHAs and their subfractions

		Differential count of lymphocytes		
		Small	Medium and large	Blast
Control	at beginning	94. 9%	4. 1%	1. 0%
	at 72 hrs	96. 3	3. 4	0. 3
PHA (Bö)				
CM-I (Bö)	72 hrs	85. 3	10. 2	4. 5
CM-II (Bö)	//	—	—	—
CM-III (Bö)	//	68. 5	19. 4	12. 2
CM-IV (Bö)	//	62. 7	27. 7	9. 7
Control	at beginning	92. 8	5. 8	1. 4
	at 72 hrs	92. 9	5 9	1. 2
PHA (Ba)				
CM-I (Ba)	72 hrs	71. 9	20. 1	8. 0
CM-II (Ba)	//	82. 3	15. 8	1. 9
CM-III (Ba)	//	72. 4	23. 9	3. 7
CM-IV (Ba)	//	81. 4	16. 3	2. 3
PHA-P (Difco)	//	87. 7	8. 8	3. 5

DISCUSSION

PHAs prepared in this laboratory from red kidney beans is revealed to be toxic to mice. An intraperitoneal application of only 2 mg of the preparation, as a rule, resulted in death of the mice within one or two days of dosage. However, PHA from *Phaseolus vulgairs* has been considered to be innocuous to animals for a long time. In fact, PHA-P (Difco), even when

dosed in mice with 200 mg/kg body weight, seemed to provoke no macroscopic and microscopic damage in the present investigation. Only an observation was reported previously by Honavar, et al.[9] concerning with the toxicity of PHA from red kidney beans. They observed serious weight loss, ending to death eventually, of the mice given this lectin with daily meals. An extract from black beans, Phaseolotoxin A, was described to be toxic by Jaffé and Gaede[10]. The mice which received intraperitoneal injection of this lectin with a dose of 50 to 75 mg/kg body weight were lethal in their experiments. The toxicity of PHAs prepared in this laboratory, therefore, may be similar to that of black kidney beans.

In contrast, it is well known that plant extracts furnished with hemagglutinating activity are toxic to animals and that this property is considered to be due to hemagglutination in blood streams resulting in microembolisms. The immediate death after an intravenous application of PHA preparations in this study may be considered to be such phenomenon caused by hemagglutination.

Histological examination of mice dosed with toxic PHAs reveals striking lymphocyte depression in the thymus and spleen. In the mice under serious intoxication, the lymphoid tissue damage observed in the thymus could be said as "conversion" in cellularity on histological profile as marked destruction and disappearance of lymphoid cells in the cortex. In the spleen, lymphoid cell destruction in the white pulp was also observed in addition to congestion and hemorrhage in the red pulp. These lymphoid tissue damage including leukopenia in the peripheral blood may be considered due to direct toxic action of PHAs on such tissues. Leukopenia was demonstrated by a PHA injection in the adrenalectomized mice and an administration of prednisolone did not cause such a striking lymphoid tissue damage observed in PHA-treated mice. These observations might exclude the possibility that toxicity of PHAs was accomplished by involvement of corticosteroids. On the one hand, PHA is well known to be antigenic to animals[11]. However, those lymphoid tissue damages were not observed in the mice injected with a dose of BGG, a good antigen to this animal.

Nature of active components of PHA has not been consistently discussed as yet. This may be due to multipotent character of PHA. For example, Kolodny and Hirschhorn[12], and Holland and Holland[13] reported that erythroagglutinating activity of PHA is completely absorbable by erythrocytes without remarkable impairment of the other activities. Leukoagglutinating activity of PHA is considered to be intrinsically connected with its leukocyte mitogenic activity by Kolodny and Hirschhorn[12] and Nordman et al[14]. While Rivera and Mueller[15], and Börjeson et al.[16] contend that leukoagglutinating activity is separable further from the mitogenic activity. By a starch block gel electrophoresis, Barkhan and Ballas[17] demonstrated that hemagglutinating activity was detectable in fractions moving to anode where mitogenic activity was seen no longer. Therefore, there is a possibility that these three activities and toxicity of PHA are carried on the different chemical components. In this paper, PHA-P (Difco) and PHAs prepared in our laboratory are further fractionated into four components, namely, CM-I, CM-II, CM-III, and CM-IV by CM-cellulose column chromatography. As summarized in Table 6, subfraction CM-I from these PHAs was demonstrated to be most toxic to mice and this was neigher antigenic nor precipitable with serum proteins (Baba, M. et al. unpublished observation). Clear separation of activities was observed in the

Table. 6 Biological Activity of Subfractionated PHAs

PHAs	HA	BT	LTD
PHA-P (Difco)			
CM-I (Bö)			
CM-II (Bö)	—	ND	—
CM-III (Bö)			
CM-IV (Bö)			
CM-I (Ba)			
CM-II (Ba)	—	—	—
CM-III (Ba)			
CM-IV (Ba)			

HA: Hemagglutinating activity
BT: Blast transforming activity
LTD: Lymphoid tissue damage

fraction of PHA(Bö). In this case, prominent hemagglutinating activity was observed in the fraction CM-IV, and the most strong blastogenic activity was detected in the fraction CM-III. However, in the case of PHA(Ba), prominent blastogenic activity was located in fraction CM-I and CM-III.

Recently, Goldberg et al.[18] suggested that the mitogenic agent in PHA may not be of protein, nucleic acid, nor carbohydrate. The date from our experiments were suggestive the possibility that each activity of PHA, as well as lymphoid tissue toxicity, could be separated into a potent substance. Chromatographic separation and further purification of the potent substances should be expected.

SUMMARY

PHAs prepared from red kidney beans in the laboratory were tested in hemagglutinating, blastogenic and toxic activities and found to be more potent in these activities than PHA-P (Difco). Especially, our PHAs showed prominent toxicity to mice. An intraperitoneal application of 2 mg of PHA(Bö) or PHA(Ba) was enough to kill adult mouse and caused severe damage in lymphoid tissues. The "conversion" of cellularity in the thymus and marked depression of lymphoid elements with intensive hemorrhage were observed histologically as signs of toxicity.

PHAs were further investigated by CM-cellulose column chromatography. Toxic component could be separated from the fraction furnished with hemagglutinating activity or blastogenic activity.

ACKNOWLEDGMENTS

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