On the Mechanism of Action of Phytohemagglutinin in Cellular Immunity (36071)

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Phytohemagglutinin (PHA) possesses significant immunosuppressive activity in various types of cellular immunity: (a) graft rejection (1–7); (b) delayed hypersensitivity (7, 8); and (c) graft-versus-host disease (9, 10). There is increasing interest in the mechanism of PHA in these phenomena. Since PHA has well-known erythroagglutinating, leukoagglutinating, mitogenic and immunogenic properties, attempts are being made to determine which of these factors (or others as yet unknown) is responsible for its action. In this study, the effect of the mitogenic and immunogenic properties of PHA has been studied in graft rejection and delayed hypersensitivity.

Materials and Methods. Materials used. A partially purified PHA, PHA-P, was obtained from Difco Co. and diluted to 19.1 mg/ml of saline (8.7% nitrogen/g of dry wt). Half of the same batch was boiled at 100° for 30 min in a water bath to destroy the mitogenic and erythroagglutinating properties of PHA (11). A more purified preparation of PHA was prepared from *Phaseolus vulgaris* according to the method of Goldberg *et al.* (12). Sugars were obtained from Sigma Chemical Co. and Nutritional Biochemicals Corporation.

Graft rejection. Adult female mice (20–25 g) 1–6 months old were used for grafts. Donor C57BL/6N (H-2^d) skin allografts were applied to C3H/HeN (H-2^k) recipient mice, as described previously (4). This transfer represents a marked histocompatibility difference at the H-2 locus. Recipient mice were given intraperitoneal injections of the agents tested starting 2 days prior to grafting and continuing until rejection of the graft. Graft rejection was determined when 95% of the graft had become dark and inflexible.

Delayed hypersensitivity. Female Hartley guinea pigs (300–700 g) were injected with 0.5 ml of Freund's complete adjuvant containing 10 mg of *Mycobacterium tuberculosis* H37RA (killed)/ml. The dose was divided among four sites including the footpads of both hindlegs and both sides of the neck. Approximately 3 weeks later the guinea pigs were given intraperitoneal injections of the agents tested. Approximately 18 hr later, the fur on the abdomen was shaved and 0.1 ml of BCG (50 μg/ml) was injected intradermally. The areas of erythema, swelling, and induration were measured at 24 and 48 hr later.

Assay for mitogenic activity. Human peripheral lymphocytes were cultured as described by Sample *et al.* (13). Tritiated thymidine (300 mCi/mmole, 7.7 μCi/ml of culture) was added at 48 hr. At 65 hr the cultures were harvested. DNA was precipitated with ice-cold 5% trichloroacetic acid, washed and filtered through Whatman GF/C glass filter papers. Radioactivity was measured in a Packard Tri-Carb liquid scintillation counter (Model 3375).

Results. Properties of the purified PHA mitogenic fraction. In vitro, this fraction called DSF (DNA-stimulating factor), showed a 208-fold purification by incorporation of tritiated thymidine into DNA by human peripheral lymphocytes in culture. The DSF fraction had small amounts of protein and carbohydrate as measured by the method of Lowry *et al.* (14) and by the anthrone method (15). The purified DSF nevertheless, still retained a small amount of erythroagglutinating activity (titer down to 512 from 204,800) and most of its leukoagglutinating

activity.

In vivo, experiments were performed using female albino Swiss Webster mice (18–20 g) of the NIH strain. One 0.3 ml dose of DSF fraction (0.41 mg protein/ml) injected intraperitoneally 3 days prior to testing doubled the number of large lymphocytes in the peripheral blood from 13 to 27%, significantly enlarged the spleen from an average of 0.08 to 0.12 g/spleen, and increased the amount of splenic white pulp and large cells stained heavily with methyl green pyronin for RNA. These biologic effects are more marked than those seen with PHA-P. Single or daily doses of 0.1–0.5 ml of DSF (0.195 to 1.25 mg of protein/ml) injected intraperitoneally beginning 2 days before injection of sheep erythrocytes had, however, no significant effect in lowering antibody production as measured by erythroagglutination tests, whereas PHA-P did (16–18).

Properties of boiled PHA-P. The absence of mitogenic activity of boiled PHA was confirmed by the markedly decreased incorporation of tritiated thymidine into DNA by human peripheral lymphocytes in culture. It became apparent that boiling does not destroy the immunogenic properties of PHA, since immunization of 3 rabbits with boiled PHA-P produced antibodies against the antigen in all rabbits as measured by the Ouchterlony technique; however, boiling apparently does change the antigenic properties, since (a) all animals immunized with boiled PHA-P cross-react with PHA-P antigen (Fig. 1A), whereas only one of 3 animals immunized with PHA-P cross-reacts with boiled PHA-P antigen (Fig. 1B); and (b) the Ouchterlony pattern of serum from animals immunized against boiled PHA-P is slightly different from that obtained with the serum of animals immunized with PHA-P (Fig. 1A). These results show that there was no change in immunogenicity and only minor changes in antigenicity of boiled PHA-P.

Graft rejection. To test the immunogenic mechanism of action of PHA, recipient C3H/HeN mice were given intraperitoneal injections of 0.1 ml of PHA-P or boiled PHA-P starting 2 days prior to grafting and continuing until rejection of the graft. Table I (Expt. A) shows that PHA-P prolonged the average survival time of skin allografts from 13.0 to 16.5 days ($p<.001$), whereas the same dose of boiled PHA-P increased survival only slightly from 13.0 to 14.0 days

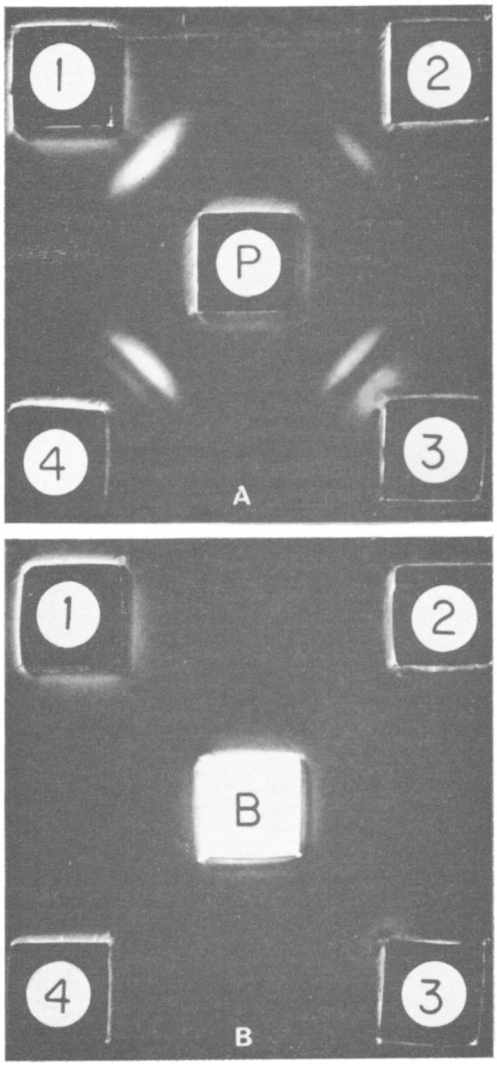

FIG. 1. Ouchterlony plate demonstration of antibody response by immunization of rabbits with PHA-P: (A) well P contains PHA-P; (B) well B contains boiled PHA-P. Well 1 contains normal rabbit serum before immunization with PHA-P; 2 contains normal rabbit serum before immunization with boiled PHA-P; 3 contains rabbit serum 38 days after first injection of boiled PHA-P; 4 contains rabbit serum 38 days after first injection of PHA-P. (B) There is a faint precipitin band near well 4 that does not show clearly in the photograph.

TABLE I. The Effect of PHA and Sugars on Graft Rejection.[a]

Expt.	Host treatment	Dosage (ml)	Conc (mg/ml)	No. of mice	Graft survival time (days) Av	±SD	p value
A	0.85% NaCl	0.1		23	13.0	1.46	
	PHA-P	0.1	50[b]	23	16.5	2.35	$<.001$
	Boiled PHA-P	0.1	50[b]	24	14.0	2.42	$>.05; <.1$
B	Phosphate buffer[c]	0.4		5	12.6	1.67	
	Dilute DSF	0.4	0.04[b]	5	13.2	1.30	$>.5$
C	Phosphate buffer[c]	1.0		14	14.0	2.54	
	Conc DSF	1.0	0.29-0.47[b]	15	14.6	2.06	$>.5$
D	0.85% NaCl	1.0		9	12.1	1.36	
	N-Acetyl-D-galactosamine + PHA-P	1.0	124 50[b]	10	14.9	1.29	$<.001$
	PHA-P	0.1	50[b]	10	14.9	2.33	$<.001$
E	0.85% NaCl	1.0		10	12.8	1.32	
	N-Acetyl-D-galactosamine	1.0	124-200	10	13.3	3.34	$>.6$
F	0.85% NaCl	1.0		8	13.0	1.51	
	D(+)-Galactose	1.0	100	9	13.4	1.94	$>.6$

[a] All agents were given intraperitoneally starting 2 days prior to grafting and continuing until graft rejection.
[b] Protein.
[c] pH 7.5; 5×10^{-3} *M*.

TABLE II. The Effect of PHA and Sugars on Delayed Hypersensitivity.[a]

Agent	Dosage (ml)	Conc (mg/ml)	Delayed hypersensitivity reaction at 24 hr		
			No. of skin tests	Pos (%)	Neg (%)
PHA-P	0.4–0.7	50[b]	16	19	81
Boiled PHA-P	0.4–0.7	50[b]	5	100	0
Conc DSF	1.0–2.0	0.29[b]	3	100	0
0.85% NaCl or phosphate buffer[c]	0.4–2.0		18	100	0
N-Acetyl-D-galactosamine	1.0–2.0	124	4	100	0
N-Acetyl-D-galactosamine + PHA-P	1.0 0.4	124 50[b]	4	75	25
D-Galactose	1.0–2.0	100–124	6	100	0
D-Galactose + PHA-P	1.0 0.4	124 50[b]	2	100	0
Glucose	1.0	124	2	100	0
Glucose + PHA-P	1.0 0.4	124 50[b]	4	0	100
N-Acetyl glucosamine	1.0–2.0	100	2	100	0

[a] All agents were given intraperitoneally 18 hr before skin test with 5 μg of BCG. A positive reaction is defined as an area of erythema greater than 1 cm in diameter with a moderate amount of induration present.

[b] Protein.

[c] pH 7.5; 5×10^{-3} *M*.

($p > .05$). These results suggest that prolongation of skin graft survival by PHA is not due to its immunogenic properties alone.

To test the mitogenic mechanism of action of PHA, the DSF fraction was injected intraperitoneally starting 2 days prior to grafting and continuing until graft rejection. Table I (Expts. B and C) shows that no prolongation of graft survival time was noted with either dilute or concentrated DSF. These latter results illustrate that purified PHA (DSF) behaved differently from Difco PHA-P *in vivo* and suggest that the mitogenic property of PHA is not solely responsible for the prolongation of graft survival, at least in the dosages tested.

Sugars, such as *N*-acetyl-D-galactosamine, and a soluble glycoprotein containing galactose as a determinant inhibit agglutination of erythrocytes and leukocytes, as well as the mitogenicity of PHA (19–21). Since it is believed that specific sugars in the cell receptor site may be responsible for attachment of PHA, large doses of certain sugars were administered intraperitoneally to mice in order to determine: (a) whether the prolongation of graft survival by PHA could be reversed; and (b) whether sugars themselves could compete with sugars on cell surfaces, such as lymphocytes, and influence graft rejection. Table I shows that *N*-acetyl-D-galactosamine in the dose administered did not prevent the prolongation of graft survival by PHA (Expt. D) and that neither *N*-acetyl-D-galactosamine nor D-galactose had any significant effect on graft rejection (Expts. E and F). These results suggest that: (a) the sugars had no effect, or were given in inadequate doses; or (b) larger molecules (*i.e.*, glycoproteins) may be necessary to compete with PHA or cells.

Delayed hypersensitivity. The immunogenic and mitogenic mechanisms of action of PHA were also tested in this form of cellular immunity. Table II shows that PHA-P decreased the delayed hypersensitivity reaction and confirms related findings by others (7, 8). Boiled PHA-P and concentrated DSF had

no effect on delayed hypersensitivity. These results suggest that the mitogenic and immunogenic properties of PHA are not responsible for its effect on delayed hypersensitivity. It is noteworthy that *N*-acetyl-D-galactosamine and D-galactose were able to block the effect of PHA-P on delayed hypersensitivity, while glucose did not. None of the sugars tested were able to decrease delayed hypersensitivity by themselves.

Discussion. Our experiments demonstrate that PHA-P does not appear to act via its immunogenic or mitogenic properties in graft rejection or delayed hypersensitivity. In graft-vs-host disease, on the other hand, Hunter *et al.* (10) reported that PHA-P suppressed this form of cellular immunity by antigenic competition. The explanation for the different mechanism of action of PHA in these types of cellular immunity is unclear at the present time.

The finding that *N*-acetyl-D-galactosamine and D-galactose were able to block the effect of PHA-P on delayed hypersensitivity is the first evidence reported of such an action of simple sugars *in vivo* in cellular immunity. It was also noted that the dosage of PHA-P given to guinea pigs made them sick. When the above sugars were given at the same time as PHA-P, the animals appeared less sick. The competitive inhibition by these sugars in this reaction suggest that they may comprise the binding site of the PHA molecule *in vivo*.

Further studies are in progress testing various fractions obtained during the purification of PHA in an attempt to delineate which fraction contains the factor responsible for the effect of PHA on cellular immunity.

Summary. To study the mechanism of action of phytohemagglutinin in cellular immunity, preparations of phytohemagglutinin were made to separate its mitogenic, erythroagglutinating, and immunogenic properties. Boiled phytohemagglutinin (PHA-P, Difco), which had lost its mitogenic and erythroagglutinating activities, was used to test the immunogenic properties of phytohemagglutinin. A purified mitogenic fraction of phytohemagglutinin was isolated chemically which stimulated the lymphoid system *in vivo*. Untreated PHA-P was able to prolong graft rejection and block the delayed hypersensitivity reaction. Neither boiled PHA-P nor the mitogenic fraction of phytohemagglutinin was able to mimic the actions of the untreated compound. The mechanism of action, therefore, does not appear to rest in either of these properties alone. In the delayed hypersensitivity reaction, intraperitoneal injection of *N*-acetyl-D-galactosamine and D-galactose was able to block partially the effect of phytohemagglutinin on the reaction. These sugars, however, were not similarly effective in blocking the action of phytohemagglutinin on graft rejection.

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