Specificity of Inhibition of Lymphocyte Activation *in Vitro* by Antiphytohemagglutinin Antisera (35842)

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(Introduced by S. E. Mergenhagen)

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Human blood lymphocytes cultured *in vitro* can be induced to enlarge, initiate DNA synthesis, and divide by the addition to the culture medium of extracts of the kidney bean *Phaseolus vulgaris* known collectively as phytohemagglutinin (PHA) (1). In the course of attempts to purify this lymphocyte stimulant it has been noted that the crude extracts contain several active fractions (2–4) and that the physicochemical properties of different batches of beans show some variation (4). More recently it has been shown that relatively pure preparations of PHA can be fractionated into at least two components (5, 6). Both these are active in stimulating lymphocyte DNA synthesis, but one, PHA-H, also possesses potent erythroagglutinating activity, while the other, PHA-L, does not. These results, together with observations that different PHA preparations have different effects on lymphocytes (7, 8), suggest that bean extracts might contain a family of lymphocyte stimulants rather than a single mitogen.

A number of other preparations will activate lymphocytes in culture. These include extracts of several other legumes, as well as preparations of a more diverse biological origin (9). Some of these stimulants, such as antilymphocyte serum, pokeweed mitogen (10), and concanavalin (11) are known to have physical and chemical properties different from those of PHA, but their effects on lymphocytes are, in general, similar. It seemed possible that at least some of these stimulants might have a common mechanism of lymphocyte activation mediated by a common functional group.

It is known that neutralizing antisera can be raised against the mitogenic components of PHA (12, 13). We have prepared antisera to four purified PHA fractions, and determined whether any differences between PHA preparations, or similarities between PHA and other lymphocyte stimulants, might be detectable with immunological techniques.

Materials and Methods. Lymphocyte stimulants. The relatively pure preparations of PHA used in these experiments were made available to us by the Wellcome Research Laboratories, Beckenham, Kent, through Drs. J. A. C. Parke and G. Hitchings. These included PHA-L and PHA-H, prepared as described by Allen *et al.* (6), and three preparations which contained both activities, PHA-E118, PHA-E316(Q1), and PHA-Q1. Each of these five preparations caused the greatest stimulation of lymphocyte DNA synthesis when added at concentrations between 1 and 5 μg/ml to lymphocyte cultures containing 10% serum or plasma. While the mitogenic potencies of PHA-H and PHA-L were similar, the erythroagglutinating potency of the PHA-H was at least 32 times that of the PHA-L.

Bactophytohemagglutinin-P and -M were purchased from Difco Laboratories, Detroit, Michigan. The optimum mitogenic concentrations of these preparations were determined to be 20 and 200 μg/ml, respectively, under the conditions of our experiments.

Pokeweed mitogen was obtained from Grand Island Biological Co., Grand Island, New York, and was diluted with Eagle's medium to the recommended concentration. Rabbit-antihuman-thymocyte serum was provided by Dr. T. Francis, National Institute of Dental Research, Bethesda and con-

canavalin A by Dr. M. Leon, St. Luke's Hospital, Cleveland, Ohio. Wax bean hemagglutinin was given by Dr. I. Liener, University of Minnesota, and staphylococcal filtrate and lentil extract by Dr. N. R. Ling, University of Birmingham.

Preparation of antisera to phytohemagglutinin. Antisera were prepared to PHA-E118, PHA-Q1, PHA-H, and PHA-L. At least two antisera were made to each preparation. Rabbits were injected in the foot pad and neck with 0.5–1.0 mg of PHA emulsified with complete Freund's adjuvant. One month later the rabbits were given six weekly intravenous injections of 0.4–1.0 mg of PHA. Antisera were obtained by venipuncture 1 week after the final injection, and were heat inactivated and filtered before use. The presence of precipitating antibodies against PHA was demonstrated by double diffusion in agar.

Preparation of PHA–anti-PHA complexes. A series of complexes of PHA-E118 with anti-PHA-E118 was prepared as follows: fixed amounts of PHA were incubated with doubling dilutions of anti-PHA for 2 hr at 37° and then for at least 48 hr at 4°. The amounts of anti-PHA used ranged from one fourth to four times the amounts needed to neutralize the mitogenic effect of PHA. The precipitated complexes were then separated from the supernatants by centrifugation, washed, and reconstituted in Eagle's medium.

Preparation and incubation of lymphocyte cultures. Human lymphocyte cultures were prepared from heparinized venous blood. The blood was allowed to sediment and the leukocyte-rich plasma was withdrawn. The cells were pelleted, washed with Eagle's medium, and resuspended at 2×10^6/ml in Eagle's medium containing 20% decomplemented calf serum. In many experiments phagocytic cells were removed from the leukocyte-rich plasma by passing it down a column of nylon fiber or cotton wool. After this procedure the lymphocytes were resuspended in Eagle's medium with 10% calf serum at 1×10^6/ml.

Aliquots (1 ml) of cell suspension were cultured in 12 × 75-mm tubes or 1 dram vials at 37°, in an atmosphere of air containing 5% CO_2. At the times indicated, 1 μCi/ml of methyl-^{3}H-thymidine (6 Ci/mmole) was added to the culture medium, and its incorporation into DNA was determined (14) after a further 2 hr. Results are shown as the mean incorporation of duplicate cultures.

Results. All the preparations of anti-PHA were able to inhibit completely the activation of lymphocytes by the corresponding PHA. Table I shows an example in which lymphocytes were incubated with or without PHA-E118 for 68 hr and then with ^{14}C-leucine and ^{3}H-thymidine for a further 2 hr. The PHA-dependent increase in the incorporation of ^{14}C-leucine into protein and ^{3}H-thymidine into DNA was completely prevented by anti-PHA-E118. Normal rabbit serum was not inhibitory. Indeed, it was stimulatory in a number of experiments. None of the anti-PHA preparations significantly affected the rate of protein synthesis by unstimulated lymphocytes, nor did they reduce the rate below unstimulated levels in cultures incubated with PHA. This shows that the anti-PHA was not toxic to lymphocytes, a conclusion further supported by the observations that the small lymphocytes exposed to PHA and anti-PHA remained morphologically intact and excluded 0.4% trypan blue, and that the total DNA content of the cultures did not decrease.

TABLE I. Effect of Anti-PHA and Normal Rabbit Serum on the Incorporation of ^{14}C-Leucine and ^{3}H-Thymidine by Lymphocytes Cultured for 68 hr.

Isotope	Stimulant	None (cpm)	Serum Anti-PHA-E118 (cpm)	Normal rabbit (cpm)
^{14}C-Leucine	None	252	204	222
	PHA-E118	2652	228	3534
^{3}H-Thymidine	None	279	227	227
	PHA-E118	13,840	201	22,884

Titration studies, with doubling dilutions of anti-PHA, such as those shown in Fig. 1,

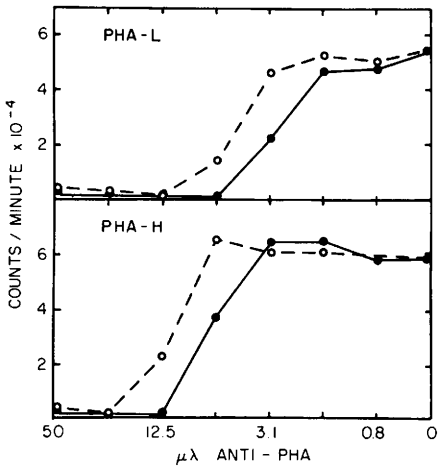

FIG. 1. Titration of 2 μg of PHA-L (upper); or 2 μg of PHA-H (lower) with doubling dilutions of anti-PHA-L (●); and anti-PHA-H (○). Lymphocyte stimulation was assessed as the incorporation of ^{3}H-thymidine into DNA 48 hr after the addition of PHA and anti-PHA.

gave sharp end points. Similar results have been obtained by others (15) showing that the avidity of the antiserum for PHA is much greater than that of the lymphocyte binding sites. Other experiments (14) have shown that anti-PHA can inhibit lymphocyte stimulation even when it is added after the binding of PHA to lymphocytes is complete. This confirms that the anti-PHA has a greater avidity for PHA than the cell receptors.

It seemed possible that PHA extracts might contain a number of different mitogenic components, and that different preparations might contain different stimulants. We therefore determine whether antisera to a relatively pure PHA preparation, PHA-E118, could prevent lymphocyte activation by another relatively pure preparation, PHA-E316, and by two less pure preparations, PHA-P and PHA-M. In the course of the purification of preparations such as PHA-E118 between 80 and 90% of the mitogenic activity present in the crude extract is lost (4) and the mitogenic activities of the pure preparations are much less heterogeneous than those of PHA-M. The specific activity of PHA-E118 is about ten times that of PHA-P and a hundred times that of PHA-M. Nevertheless, Table II shows that anti-PHA-E118 could completely inhibit DNA synthesis by lymphocytes incubated with any of these preparations of PHA. This inhibition could be reversed by excess PHA (shown for PHA-P in Table II). It seems, therefore, that all the mitogenic fractions of these PHA preparations contain antigenic groups in common.

TABLE II. Specificity of Anti-PHA-E118 Against Various Preparations of PHA.

Stimulant	(μg)	Anti-PHA-E118 (cpm[a])	Normal serum (cpm[a])
None		597	513
PHA-E118	2.5	374	24,086
PHA-E316	5	667	15,291
PHA-P	10	376	3161
	50	6992	9558
PHA-E118	2.5	749	53,609
PHA-M	200	827	18,233

[a] ^{3}H-Thymidine was added 66 hr after the PHA.

All the above PHA preparations contain both PHA-H and PHA-L, but we also found that antisera raised against these purified fractions could completely inhibit the mitogenic activity of each other and of PHA preparations containing both activities. As we could not exclude the possibility of minor cross-contamination of PHA-H and PHA-L, we titrated each of these PHA preparations against anti-PHA-H and anti-PHA-L. Figure 1 shows that the anti-PHA-L preparation we used was approximately twice as active as the anti-PHA-H against both PHA-L and PHA-H. This indicates that the cross-inhibition is not due to minor cross-contamination of the two PHA preparations, but to their mitogenic activities being antigenically indistinguishable.

The results shown in Table II and Fig. 1 demonstrate that the mitogenic fractions of all the preparations of PHA that we have tested have an antigenic determinant in common. However, such a determinant need not necessarily be identical with the active site of the PHA. To determine whether anti-PHA inhibited lymphocyte activation merely by precipitation of PHA, we prepared a series of

complexes of PHA with anti-PHA. We then separated the precipitated insoluble antigen–antibody complexes from the soluble complexes in the supernatants, and compared their ability to stimulate lymphocytes. Table III shows that the precipitated complexes

TABLE III. Effect of PHA–Anti-PHA Complexes on Lymphocyte ^{3}H-Thymidine Incorporation.

Stimulant		Precipitates (cpm[a])	Supernatants (cpm[a])
PHA		—	68,251
PHA–anti-PHA complex	1	596	993
	2	922	809
	3	2046	762
	4	19,421	16,696

[a] ^{3}H-Thymidine incorporation was determined following a 4-hr pulse given 4 days after the stimulant.

prepared in PHA excess (complex 4) were as capable of lymphocyte activation as the corresponding supernatants. Complexes prepared at lower PHA:antibody ratios (complexes 1 and 2) were not stimulatory, whether precipitated or not. Indeed, they could neutralize additional PHA. This demonstrates that the neutralization of PHA must involve masking or distortion of the active site, and supports the contention of Simons *et al.* (15) that the antigenic determinant sites of PHA are identical, or closely related, to the active site.

We have investigated the effect of anti-PHA on lymphocyte activation by a number of other nonspecific stimulants. Those we have studied include extracts of three legumes; the hemagglutinins of the wax bean (a variety of *Phaseolus vulgaris*) and the lentil, and the concanavalin A of the jack bean. We have also included the pokeweed mitogen, the active staphylococcal culture filtrate and antithymocyte serum. We tested each stimulant at a number of concentrations, including some which gave suboptimal lymphocyte DNA synthesis, and used a concentration of anti-PHA at least five times the minimum necessary to completely inhibit PHA-induced lymphocyte DNA synthesis.

Experiment 1 of Table IV shows that anti-PHA completely inhibited lymphocyte activation by the wax bean hemagglutinin. Titration showed that this inhibition could be overcome by excess hemagglutinin. The titration curve was very sharp, as noted above for PHA, indicating that the antibody has a high affinity for the hemagglutinin, comparable to that for PHA. The anti-PHA did not affect the activity of any of the other stimulants (Table IV, Expts. 2 and 3) even at suboptimal stimulant concentrations. There was thus no evidence for any cross-reactivity between these agents and PHA.

Discussion. This work shows that antisera of high avidity can be prepared against PHA in rabbits, and that such antisera can prevent lymphocyte activation without cytotoxicity. The study of lymphocyte stimulation by PHA–anti-PHA complexes suggests that these antisera act by masking or distorting the active site of PHA rather than by precipitation.

The demonstration that antisera to relatively pure PHA preparations are capable of complete neutralization of the mitogenic activity of much cruder preparations demonstrates that all the stimulants found in the much more heterogeneous mitogenic fractions of the crude extracts are either identical or very similar. Similarly, the demonstration that the mitogenic activities of the erythroagglutinating PHA-H and the nonerythroagglutinating PHA-L were immunologically indistinguishable shows that both contain the same, or very similar, mitogenic group. Such a group could be either a mitogenic PHA subunit (16) complexed with different nonmitogenic subunits in different preparations, or even a mitogenically active nonprotein hapten attached to different carrier proteins in different preparations (17). There are undoubtedly differences in the effects on lymphocytes of different preparations of PHA (7, 8) but these could be explained by the presence of different carrier groups or contaminating proteins modifying the lymphocyte response.

The mitogenic factor of the wax bean hemagglutinin was shown to be very similar to that of PHA. This was not too surprising, as the wax bean is a variety of *Phaseolus vulgaris* and earlier studies had shown similari-

TABLE IV. Specificity of Anti-PHA Against Other Nonspecific Lymphocyte Stimulants.[a]

Expt.	Stimulant		Anti-PHA (cpm)	Normal serum (cpm)
1.	None		152	158
	PHA-Q1	2 μg	273	28,106
	Wax bean hemagglutinin	20 μg	16,772	14,586
	Wax bean hemagglutinin	10 μg	16,603	21,036
	Wax bean hemagglutinin	5 μg	292	15,198
	Wax bean hemagglutinin	2.5 μg	336	16,059
	Wax bean hemagglutinin	1.2 μg	217	9657
2.	None		161	145
	PHA-Q1	2 μg	249	10,533
	Staphylococcal filtrate	0.2 ml	8276	9724
	Staphylococcal filtrate	0.1 ml	5240	5634
	Staphylococcal filtrate	0.05 ml	2891	2590
	Lentil extract	0.0125 ml	10,793	13,974
	Lentil extract	0.0031 ml	7216	6880
3.	None		129	107
	PHA-E118	2.5 μg	152	20,086
	Pokeweed mitogen	0.05 ml	6130	5123
	Pokeweed mitogen	0.005 ml	4199	3273
	Concanavalin A	50 μg	4519	3407
	Concanavalin A	25 μg	2544	1508
	Antithymocyte serum	0.05 ml	6338	6986
	Antithymocyte serum	0.01 ml	3907	3209

[a] The anti-PHA used was anti-PHA-Q1 in Expts. 1 and 2; and anti-PHA-E118 in Expt. 3. ^{3}H-Thymidine incorporation was determined 48 hr after the addition of stimulant in Expt. 1, and 72 hr after stimulant in Expts. 2 and 3.

ties between this mitogen and PHA-M (18, 19). There was no evidence for the presence of any cross-reactivity between PHA and any of the other lymphocyte stimulants studied. This is consistent with the thesis that the binding groups of these stimulants differ from those of PHA and implies that these stimulants may trigger lymphocyte activation by action at different loci.

Summary. Antisera to various purified preparations of the lymphocyte stimulant PHA have been prepared in rabbits. These antisera neutralized lymphocyte activation by PHA but were not toxic. Using such antisera, we were not able to detect any differences between the mitogenic components of different purified preparations of PHA, including the separated erythroagglutinating and nonerythroagglutinating fractions. Antisera prepared against purified fractions of PHA also inhibited the mitogenic effects of crude PHA extracts. These antisera also prevented lymphocyte activation by the closely related wax bean hemagglutinin but did not inhibit stimulation by concanavalin A, lentil extract, pokeweed mitogen, staphylococcal filtrate, or antithymocyte serum.

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