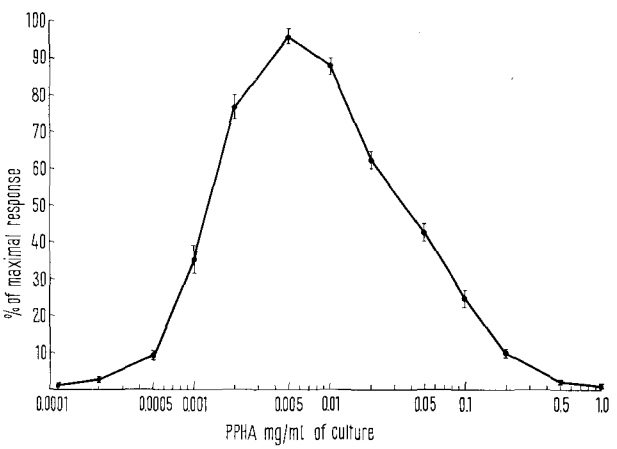
Bio-Assay and Dose-Response of the Mitogenic Activity of the Phytohemagglutinin of *Phaseolus vulgaris*

We have previously reported that the dose-response of normal blood lymphocytes to phytohemagglutinin is a bell shaped curve, exhibiting an optimum dose for maximal response[1,2]. In this report we present in detail this dose-response curve and describe our culture method for assaying the mitogenic potency of various preparations. This assay depends on the quantitation of the uptake of thymidine-2-^{14}C into the DNA which is a more objective and accurate measure of mitogenicity than the determination of mitotic indices.

Materials and methods. Heparinized venus blood (3 IU of heparin/ml of blood) was obtained from normal human healthy subjects showing no sensitivity to penicillin. 20 ml of 6% dextran in 0.14 *M* NaCl (124,000 mol. wt., Sigma Chemical Co., St. Louis, Mo., USA) were added per 100 ml of blood and allowed to sediment at room temperature for 30 min. The supernatant plasma containing the leukocytes was then withdrawn and centrifuged at 625 g for 20 min at 5 °C. The packed leukocytes were washed once with 20.0 ml of tissue culture medium NCTC 109 (Microbiological Associates, Bethesda, Md., USA) and were then suspended in sufficient volume of fresh human serum, to give a count of 5×10^6 leukocytes per millilitre. Cultures were set up in 20 ml screw cap glass vials (Wheaton Glass Co., Millville, N.Y., USA). The caps were modified to permit gas exchange with the atmosphere, by drilling a 0.25 inch hole in their center and inserting a millipore filter, a glass fiber prefilter (Millipore Corp., Bedford, Mass., USA) and a silicone rubber washer between the cap and the vial in that order. 8 ml of NCTC-109 containing 1000 U of potassium penicillin G, protein-phytohemagglutinin (PPHA) purified as previously described[2,3], and 1.0 µc of thymidine-2-^{14}C (New England Nuclear, Corp., Boston, Mass., USA) were added to each vial and mixed with 2.0 ml serum containing 10^7 leukocytes. 4–6 cultures for each PPHA dose were set up and incubated at 37 °C in a 'Dubnoff' water bath shaking gently at 42 strokes per minute. A flow of 2 cubic feet per hour of a 20% O_2, 3% CO_2, 77% N_2 gas mixture was maintained through the instrument. Shaking disperses metabolic products that result in damaging acid conditions, if allowed to accumulate in the cell layer. Gasing also aides in the maintainance of the pH by keeping the CO_2 pressure constant. Aseptic conditions were observed throughout. The cultures were terminated after 48 h by centrifuging the leukocytes at 625 g for 10 min. The packed leukocytes were washed twice with 12.0 ml of 0.14 *M* NaCl and 3 times with 12.0 ml of 1:5 water-ethanol mixture (v/v). They were then dissolved in 1.0 ml of concentrated ammonia by heating in sealed tubes for 1 h at 90 °C. An aliquot was used for DNA determinations by a spectrofluorometric method[4] and the remaining solution was plated on preweighed stainless steel planchets. The dry weight of the cells was determined by reweighing the planchets following drying under an IR-lamp. The incorporated thymidine-2-^{14}C was counted with a low background counter (Beckman Instruments Co., Fullerton, California, USA). The counting times were calculated with the aid of the previously described nomogram[5], to assure a 99% confidence that the tolerable fractional error is not greater than 0.05.

Results and discussion. The data were expressed in 3 different ways: (a) as the total net counts per minute (CPM) of ^{14}C incorporated into the cells of each culture; (b) as CPM/mg of dry weight and (c) as CPM/mg of DNA. Plotting the incorporated amount of thymidine-2-^{14}C against the dose of PPHA resulted in superimposible bell shaped curves regardless of the method used to express the data. This is because the total CPM was found to be linearly related to the total dry weight ($r = 0.89$) and to the total DNA ($r = 0.98$) per culture. Replicate cultures showed good agreement in their ^{14}C uptake, being usually within $\pm$ 10% of the mean. Much greater variation was encountered between corresponding cultures of different experiments, probably because of variations in the number of lymphocytes in each culture and of differences in the human sera that were used. To facilitate comparison of data from different assays, we normalized the results by expressing them as the per cent of maximal response observed in each assay. The normalized data from 23 assays of PPHA are listed in the Table and are plotted semilogarithmically against the dose of PPHA on the accompanying Figure. The reproducibility of this assay method is evident. No effect is discernible below 0.0002 mg of PPHA/ml of culture. The response increases at higher doses reaching a maximum at 0.005 mg and decreases thereafter to control values at 1.0 mg of PPHA/ml of culture. These data clearly demonstrate that there is an optimum dose of PPHA for maximal response of the lymphocytes and that at high doses the PPHA is toxic. In contrast, MACKINNEY[6] and BOYLSTON et al.[7] did not observe this toxic effect using an impure commercial preparation (Difco PHA-P),



Dose-response curve showing the response of normal human blood lymphocytes to various doses of phytohemagglutinin. The vertical bars indicate the standard error of the mean.

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Response of normal human blood lymphocytes to various doses of phytohemagglutinin
mg of PPHA/ml of culture

Assay Number	0.0000 (controls)	0.0001	0.0002	0.0005	0.001	0.002	0.005	0.01	0.02	0.05	0.1	0.2	0.5	1.0
Percentage of maximal response (incorporation of Tdr-2-^{14}C into DNA)														
1	0.3[a]	0.4	2.8	9.2	36.6	80.5	83.1	100	74.1	60.1	33.1	7.8	2.6	0.5
2	0.9			1.2	5.6	39.4	58.7	100	46.8	26.9	30.2	20.4	6.2	1.2
3	2.0			2.0	17.6	66.6	100	78.4	53.9	44.5	25.1	20.7	5.9	1.2
4	3.6	2.5	3.2	14.3	48.9	74.8	100	94.2	57.7	35.9	23.0	5.4	1.8	0.4
5	0.2				44.8	93.2	100	82.1	43.2	30.1	11.1	9.3	0.2	0.1
6	0.2	0.2	0.4	8.1	27.4	71.6	91.4	100	75.4	66.8	52.6	10.9	0.7	0.1
7	2.2	2.8	3.2	13.6		53.1	100	56.5	50.9	32.6	14.5	8.1	1.2	0.6
8	1.4	1.1			12.6		100	95.2		51.7	30.4		0.8	
9	4.3	5.2	11.2	18.6	52.2	88.6	100	87.0	58.6	44.8	30.6	8.9	2.9	0.9
10	0.2	0.3	1.1	5.8	29.9	68.1	100	94.5	73.8	47.8	28.8	10.7	2.1	0.3
11	1.4	1.5	1.4	5.2	34.2	98.8	100	67.7	56.7	58.0	32.6	11.3	2.7	0.5
12	1.5	1.3	1.8	13.0	44.3	84.1	85.1	100	58.9	35.7	21.5	5.1	0.8	0.2
13	0.3	0.3	0.5	5.2	32.0	75.5	86.3	100	64.2	53.7	16.0	6.8	0.5	0.1
14	0.4	0.9	1.2	13.8	68.8	99.6	100	98.8	84.0	52.2	51.7	19.0	6.2	1.2
15	1.1	1.3	3.8	17.6	54.5	91.7	100	91.7	64.5	40.1	17.7	6.4	2.7	1.4
16	0.4	1.8	9.0	16.0	31.8	61.6	100	76.9	57.1	32.1	18.9	13.2	6.6	5.6
17				21.6	63.9	94.8	100	98.7	74.4	53.6	30.5	12.7	0.3	0.0
18	0.5	0.6	0.7	3.0	21.7	74.9	100	91.5	71.3	44.8	11.0	2.6	0.3	0.1
19	0.4	0.6	0.5	1.3	9.9	65.9	100	69.2	58.8	33.5	11.8	1.7	0.2	0.1
20	0.5	0.3	0.5	3.6	21.0	58.7	100	88.4	69.0	42.9	24.9	13.1	0.9	0.2
21	1.4			7.9	13.7	68.8	100	96.0	81.1	31.7	20.7			
22	0.1			7.4	59.6	84.6	100	71.0	44.9	33.4	23.5			
23	1.7	0.6	1.1	6.8	43.2	94.4	100	82.1	52.7	29.1	10.6	3.7	1.6	1.2
Mean	1.1	1.3	2.7	9.3	35.2	76.8	95.9	87.8	62.4	42.7	24.8	9.9	2.3	0.8
S.E.	0.2	0.3	0.8	1.3	3.9	3.4	2.0	2.6	2.5	2.3	2.4	1.2	0.5	0.3

[a] Each value in this table represents the mean of 4–6 replicate cultures.

probably because they did not use sufficiently high doses. Our assays of Difco PHA-P show an optimum dose at 0.02 mg/ml of culture and no response at 5.0 mg/ml indicating that it is toxic. We have similarly found that Difco PHA-M and Burroughs Wellcome PHA are toxic at high doses, which is also suggested by data reported by others[8,9]. We have found that the optimum doses of all commercial preparations are higher than that of PPHA and therefore are not as pure as PPHA. The toxicity at high doses is a property of the phytohemagglutinin molecule, but since PPHA has several different types of subunits, it is not clear whether it is due to the mitogenic subunit or to a separate toxic subunit[2]. This toxic effect is of utmost importance and should be borne in mind when the mitogenicity of various preparations is compared. If we begin with an impure preparation containing only 1% PPHA and purify it to the point that the product is 100% PPHA and then assay both at a dose of 0.5/ml of culture, we will obtain maximal mitogenicity with the impure but no response with the purified. On the other hand, the hemagglutination titer of the purified preparation will be 100 times higher than that of the impure. If we are unaware of the toxic effect of phytohemagglutinin, we might reach the erroneous conclusion that the purified preparation is hemagglutinating but not mitogenic and that we have separated the 2 activities. However, a complete dose-response assay would have clearly demonstrated maximum mitogenicity of the purified preparation at 0.005 mg/ml and no mitogenicity of the impure preparation at this level. Our conclusion would thus be that a 100 fold purification, but no separation of the 2 activities was achieved. It is apparent that correct evaluation of the results of mitogenic assays requires knowledge of the optimum dose of the assayed preparation. The reproducibility of the method described here makes possible the assay of the mitogenic activity of various preparations of phytohemagglutinin with a high degree of accuracy[10].

Zusammenfassung. Es wird eine reproduzierbare, präzise Methode zur Untersuchung mitogenetischer Eigenschaften von Phytohämagglutininpräparaten beschrieben, die Methode beruht auf dem Einbau von radioaktiven Thymidin in DNS.

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