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THE EFFECT OF PHYTOHAEMAGGLUTININ ON THE IMMUNE RESPONSE *IN VIVO*

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SUMMARY

Phytohaemagglutinin (PHA) was found to suppress both the primary and secondary immune response to mammalian and avian erythrocytes in rats and mice. Effective suppression of humoral antibody production occurred when PHA was administered prior to or a few hours after immunization. PHA also induced the appearance of naturally occurring antibody in low titre in unimmunized animals. Variable results were obtained in a study of suppression of humoral antibody production to bacterial antigens. No effect of PHA could be detected on the development of experimental thyroiditis in guinea-pigs.

INTRODUCTION

PHA is known to initiate transformation *in vitro* of lymphocytes into large proliferating basophilic cells (Nowell, 1960). Since the lymphocyte plays an important part in antibody production (Gowans & McGregor, 1963) it seems likely that if PHA is capable of transforming lymphocytes *in vivo*, the immune response might be altered in some observable fashion by pretreating animals with PHA prior to immunization. This report extends initial observations reported earlier (Jennings, 1966) and describes the effect of PHA on both humoral antibody production and experimental thyroiditis in guinea-pigs.

MATERIALS AND METHODS

STUDIES ON HUMORAL ANTIBODY PRODUCTION

Animals

Animals used in this study were inbred Wistar rats and inbred strains of mice; C3H and B10.D2 (new).

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PHA

PHA was obtained from Burroughs Wellcome (England), freeze dried in vials. Each vial contained approximately 50 mg of extract and was reconstituted with 5 ml of distilled water. This will be referred to in the text as a standard preparation of PHA.

Treatment of animals with PHA

In order to test the effect of PHA on antibody production to the erythrocyte antigens in rats and mice, 0·4 ml of a standard 'Wellcome' vial of PHA was injected intraperitoneally at three 24-hr intervals. The last injection was given 24 hr prior to immunization. This procedure of PHA treatment was identical for both the primary and secondary immune response. In additional experiments with rats, 1 ml PHA was injected at variable times after immunization with chicken erythrocytes. In the case of mice immunized with the bacterial antigens, four 24-hourly injections of PHA were given prior to immunization.

Immunization

Erythrocytes. Rabbit, sheep, guinea-pig and chicken erythrocytes were made up as a 30% suspension in saline and inoculated into the intraperitoneal cavity of mice in 0·4 ml volumes. In the case of rats, chicken erythrocytes only were used and inoculated into the peritoneal cavity as a 1% suspension in 1 ml volumes. In a study of the secondary immune response mice were immunized as above with chicken erythrocytes 6–8 weeks after the primary immunization.

Bacterial antigens. Mice were immunized with a variety of bacterial antigens (Burroughs Wellcome) via the intraperitoneal route in the following volumes:

(a) Agglutinable suspension of *Proteus* OX 2 (15× concentrated) 0·2 ml (diluted 1:2).

(b) Agglutinable suspension of *Salmonella london* O (15× concentrated) 0·2 ml (diluted 1:2).

(c) Agglutinable suspension of *Salmonella typhi* H 0·2 ml (undiluted).

Assay of antisera

Mice were usually bled 6 days after primary and secondary immunization; rats were usually bled 7 days after immunization. For the assay of antibodies to *Salmonella london* O and *Proteus* OX 2, doubling dilutions of mouse serum were made up with saline in M.R.C. Perspex haemagglutinating trays in volumes of 0·1 ml. To each well was added 0·1 ml of undiluted bacterial suspension. The trays were then incubated at 37°C for 1 hr, and the end-point of antibody activity was taken as the highest dilution given macroscopically observable agglutination. For the assay antibodies to *Salmonella typhi* H similar titrations were set up, except that 0·2 ml of the bacterial suspension was used for optimum results. In the case of the erythrocyte antibodies, doubling dilutions of either rat or mouse sera were made up exactly as above. To each well 0·1 ml of 2% erythrocytes were added and finally 0·1 ml of fresh guinea-pig serum was added to provide excess complement. Complement and serum controls were set up to detect possible non-specific lytic activity of these reagents. The end-point of antibody activity was taken as the highest dilution giving at least 50% lysis of the added erythrocytes.

STUDIES ON EXPERIMENTAL THYROIDITIS IN GUINEA-PIGS

Animals

Animals used in this study were young adult Hartley strain guinea-pigs.

Immunization

Guinea-pigs were immunized with one injection of homologous thyroid extract in complete Freund's adjuvant as described by Lerner, McMaster & Exum (1964).

Histological examination of thyroids

The guinea-pigs were killed by an overdose of pentobarbitone sodium BP. The thyroid gland was removed, still attached to the trachea, and fixed together with blocks of liver, heart, spleen, kidney, lung, adrenal and intestine in 10% neutral formalin. After fixation each lobe of the thyroid was cut transversely into three blocks which were examined separately. Paraffin sections (5 μ) of the tissues were prepared and stained with Harris's haematoxylin and eosin.

All sections were scored for the presence of thyroiditis as follows:

− = No inflammatory cells present or at the most one or two small foci of lymphocytes.

+ = The presence of inflammatory foci composed of lymphocytes and present in every section examined. The size and shape of acini adjacent to these foci appeared somewhat irregular.

++ = Sections containing numerous inflammatory foci with the adjacent foci fusing to form areas of diffuse infiltration.

Treatment of animals with PHA

Guinea-pigs were injected with 1 ml PHA daily for 30 days. On alternate days the intradermal and intraperitoneal routes were used. On the 5th day of PHA treatment the guinea-pigs were immunized as above. Forty days after treatment with PHA had commenced the guinea-pigs were killed and the thyroids processed as above.

STATISTICAL EVALUATION OF RESULTS

The antibody response was expressed as the negative logarithm to the base 2 of the titre. Means, standard deviations and the significance of differences between control and experimental groups were calculated using the Student's *t*-test (Fisher, 1950).

To test the significance of the incidence of naturally-occurring antibody in control and experimental groups the Fourfold Significance Test was used (Documenta Geigy, 1966).

RESULTS

The effect of PHA on the primary immune response to erythrocytes and bacterial antigens

Table 1 clearly shows that PHA effectively depresses the formation of haemolytic antibodies to chicken erythrocytes in rats and mice. In preliminary experiments with mice it was found that one intraperitoneal injection of 0·4 ml of the standard PHA preparation

TABLE 1. The effect of PHA on the immune response to chicken erythrocytes

Treatment of animals prior to immunization	Animals used	No. of animals	The haemolysin response $-\log_2$ titre			Standard error of mean	Difference between means of experimental and control groups	Significance of difference of means
			Range	Mean	Standard deviation			
Primary immune response								
3 × 24-hourly injections PHA	C3H mice	23	0–4	0·61	±1·13	0·236	6·39	$P<0·001$ (n = 44, t = 19·2)
3 × 24-hourly injections of saline	C3H mice	21	5–8	7·00	±1·07	0·233		
3 × 24-hourly injections PHA	B.10 D.2 (new) mice	20	0–5	1·05	±1·66	0·371	6·52	$P<0·001$ (n = 41, t = 16·2)
3 × 24-hourly injections of saline	B.10 D.2 (new) mice	21	6–9	7·57	±0·73	0·159		
3 × 24-hourly injections PHA	Inbred Wistar rats	20	4–5	5·56	±0·87	0·193	3·26	$P<0·001$ (n = 43, t = 11·2)
3 × 24-hourly injections of saline	Inbred Wistar rats	23	7–10	8·82	±1·05	0·219		
Secondary immune response								
3 × 24-hourly injections PHA	C3H mice	24	1–5	3·17	±1·57	0·321	5·33	$P<0·001$ (n = 47, t = 12·97)
3 × 24-hourly injections of saline	C3H mice	24	7–10	8·50	±1·26	0·257		

The haemolysin response is represented by the negative logarithm to the base 2 of the titre.

TABLE 2. Effect of PHA on primary immune response to bacterial antigens

Antigen used for immunization	Treatment of animals prior to immunization	Animals used	No. of animals	The agglutination response $-\log_2$ titre			Standard error of mean	Difference between means of experimental and control groups	Significance of difference of means
				Range	Mean	Standard deviation			
Salmonella london 'O'	4×24-hourly injections PHA	C3H mice	43	2–8	5·63	±1·51	0·230		
	4×24-hourly injections of saline	C3H mice	44	4–10	6·68	±1·74	0·262	1·05	$P<0·005$ ($n = 76$, $t = 3·01$)
Proteus OX 2	4×24-hourly injections PHA	C3H mice	24	1–8	4·75	±2·07	0·422		
	4×24-hourly injections of saline	C3H mice	24	1–9	5·42	±2·10	0·429	0·67	Not significant ($n = 47$, $t = 1·11$)
Salmonella typhi H.	4×24-hourly injections PHA	C3H mice	12	2–8	4·25	±1·79	0·515		
	4×24-hourly injections of saline	C3H mice	12	6–10	8·25	±1·36	0·393	4·00	$P<0·001$ ($n = 23$, $t = 6·17$)

resulted in inhibition of antibody formation in about 50% of these experiments; with three 24-hourly injections all experiments showed inhibition of haemolytic antibody formation. In experiments with rats, 1 ml of PHA administered up to 3 hr after immunization with chicken erythrocytes depressed the formation of haemolytic antibody; PHA administered 5 and 12 hr after immunization did not affect the formation of antibody. Additional experiments have indicated that as far as haemolytic antibodies are concerned, the immunosuppressive action of PHA is permanent. Titration of antibody at 15, 20 and 25 days after immunization showed that control mice immunized without PHA treatment exhibited a gradual decline in antibody titre with PHA treated mice not showing any signs of delayed appearance of antibody. Similar inhibition of antibody formation was shown with sheep, guinea-pig and rabbit erythrocytes.

In initial studies with bacterial antigens the impression was gained that PHA was only inducing a slight depression of antibody production, and hence four 24-hourly injections of PHA were given. Table 2 shows that the formation of antibodies to the flagellar antigens of *Salmonella typhi* was markedly suppressed, whereas antibody production to somatic antigens was minimally suppressed in the case of *Salmonella london*, and not suppressed at all in the case of *Proteus* OX 2. Initial experiments have shown that inhibition of antibody formation to the flagellar antigen of *Salmonella typhi* is readily demonstrated in rats.

TABLE 3. Comparison of the incidence of naturally-occuring haemolytic antibody in PHA treated rats and normal rats

Animals	Anti-chicken erythrocyte antibody	
	Negative at 1:2 serum dilution	Positive at 1:2 serum dilution or higher
Rats treated with PHA	6	10
Normal rats	20	3

The higher incidence of antibody in PHA treated rats is statistically significant at the 5% level using the four-fold significance test.

The effect of PHA on the secondary immune response to chicken erythrocytes

Table 1 shows that treatment of mice with PHA also depresses the secondary immune response to chicken erythrocytes. Preliminary experiments with bacterial antigens have suggested that, as with studies on the primary immune response, inhibition is readily demonstrated with the flagellar antigens of *Salmonella typhi* but less readily with the somatic antigens of *Proteus* OX 2 and *Salmonella london.*

The effect of PHA on unimmunized mice and rats

Table 3 shows that the incidence of naturally occurring haemolytic antibodies was significantly higher in a population of rats treated with three 24-hourly injections of PHA than in a comparable control population treated with saline injections. Titres of antibody did not appear to be higher in the PHA group.

The effect of PHA on experimental thyroiditis in guinea-pigs

Table 4 shows that PHA does not affect the development of thyroiditis. Similarly, the degree of hyperplasia was virtually identical in both PHA treated and saline treated guinea-pigs.

TABLE 4. The effect of PHA on experimental thyroiditis in guinea-pigs

Treatment of immunized guinea-pigs	Degree of thyroiditis*		
	−	+	++
PHA group	2	2	4
Saline group	2	4	4

* See 'Materials and methods' for notation of thyroiditis.

DISCUSSION

One of the most interesting points to arise from this study was the marked contrast between the effect of PHA on antibody production to erythrocytes and flagellar antigens of *Salmonella typhi* on the one hand, and the somatic antigens of *Salmonella london* and *Proteus* OX 2 on the other. It would seem that the physical and chemical properties of antigens play an important part in the expression of the immunosuppressive action of PHA.

Another interesting point is the inability of PHA to inhibit the development of thyroiditis in guinea-pigs. There is a good evidence (Lerner *et al.*, 1964; Felix-Davies & Waksman, 1961) to indicate that experimental thyroiditis is probably initiated by 'cell-bound' antibodies in contrast to humoral antibodies. Perhaps, therefore, the cellular events involved in the production of cell-bound antibody are distinct from those involved in the production of humoral antibody with the latter processes sensitive and the former processes insensitive to the inhibitory effect of PHA. On the other hand, PHA is itself antigenic (Astaldi *et al.*, 1966; Marshall & Melman, 1966) and it is possible, in view of the extensive series of PHA injections that antibody formation to PHA might have neutralized its immunosuppressive properties *in vivo*.

Any attempt to explain the immunosuppressive properties of any drug is impeded by the fact that the cellular events associated with antibody production have not been precisely defined. In earlier studies with erythrocytes it seemed highly likely that the action of PHA might be simply explained by its ability to agglutinate the injected erythrocytes with subsequent impairment of the immune response. So far experiments have shown that the peritoneal fluid of mice treated with PHA does not agglutinate erythrocytes *in vitro*. The response of mice treated with erythrocytes agglutinated with PHA *in vitro* was compared with the response obtained from abnormal erythrocyte inoculum; the results were difficult to interpret. Generally, agglutination of the erythrocyte inoculum with PHA does not appear to effect the subsequent formation of antibody, but occasionally a very slight depression in antibody production was noted. Currently a different approach is being used

entailing the assay of the activity of PHA preparations with very low agglutinating ability and comparing their immunosuppressive properties with standard PHA preparations. At present, therefore, the role of *in vivo* haemagglutination in the depression of haemolytic antibody formation is still not clear. However, with the demonstration of the inhibition of antibody production to bacterial antigens it would appear that even if PHA depressed haemolytic antibody production by agglutination of the injected erythrocytes, other properties of PHA must be of importance in relation to its immunosuppressive activity.

Another explanation is based on the twin assumptions that the lymphocyte is involved in antibody production, for which there is good evidence (Gowans & McGregor, 1963) and furthermore that PHA can transform lymphocytes to blast-like cells *in vivo* as *in vitro*. It follows from this hypothesis that if lymphocytes are transformed into blasts they might presumably be unable to participate in the formation of antibodies. If an additional assumption is made to the effect that 'committed' lymphocytes are also transformed into blast cells by PHA, with concomitant production of antibody, the higher incidence of antibody in unimmunized animals treated with PHA is readily explained. A process possibly akin to this has been demonstrated *in vitro* (Forbes, 1965). Lymphocytes sensitized with known antigens produced increased amounts of corresponding antibody when incubated with PHA compared to normal cultures. Again, addition of PHA to cultures of lymph node explants taken from animals immunized with bovine serum albumin, induced formation of the corresponding antibody (Halliday & Garvey, 1965).

The third possibility is that the action of PHA does not depend on mitogenic or haemagglutinating properties but on other unknown factors related merely to the presence of proteinaceous material in the peritoneal cavity. In support of this possibility is the demonstration in this laboratory of the immunosuppressive action of a variety of serum proteins and adjuvants in a manner very similar to PHA.

The mechanism of immunosuppressive activity of these materials is at present being explored.

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