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PHYTOMITOGEN RESPONSES OF PERIPHERAL BLOOD LYMPHOCYTES IN YOUNG AND OLDER SUBJECTS

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SUMMARY

Quantitation of the response of peripheral blood lymphocytes to the phytomitogens phytohaemagglutinin-M, concanavalin A, and pokeweed mitogen was compared in thirteen young subjects and thirteen older subjects. Both dose of phytomitogen and duration of lymphocyte culture were evaluated. Younger individuals had higher responses to all three phytomitogens after 3 days of culture, whereas older individuals had higher responses after 5 days of culture. The concentration of phytomitogens required to produce maximal stimulation in both groups varied with culture duration.

INTRODUCTION

The *in vitro* response of peripheral blood lymphocytes to the phytomitogens, phytohaemagglutinin (PHA), concanavalin A (Con-A) and pokeweed mitogen (PWM) is widely used to evaluate cell-mediated immunity. Many factors influence this response including cell concentration, types of cells in culture, serum supplement, pH, atmospheric conditions and batch of mitogens (Hughes & Caspary, 1970; Hinz & Chickosky, 1972). The concentration of the mitogen and the duration of culture are important factors in determining the lymphocyte response, especially in distinguishing the response of patients with immune deficiency diseases from normals (Fitzgerald, 1972; Douglas, Kamin & Fudenberg, 1969; Oppenheim, Blaese & Waldman, 1970).

The purpose of the present study was to establish a dose and time response curve in young healthy persons and to compare this to an older healthy group. Today, the study of the immune system in older subjects is of considerable importance but good control data is not available.

MATERIALS AND METHODS

Mitogens

Phytohaemagglutinin-M (PHA-M) (Lot number 585787) was obtained from Difco

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Laboratories, Detroit, Michigan. Five millilitres of sterile PBS was added to the vial containing 50 mg of dry powder and further dilutions were made at 1:20, 1:10 and 1:5 with PBS. These rehydrated concentrations equalled 50 μg, 100 μg, 200 μg, and 1000 μg per 0·1 ml respectively.

Concanavalin A (Con-A) (Lot number 575615) was obtained from Difco Laboratories. Con-A was rehydrated in sterile distilled water to give the following concentrations: 50, 100, 200 and 1000 μg per 0·1 ml.

Pokeweed mitogen (PWM) (Lot number R8248A) was obtained from Grand Island Biological Company (GIBCO), Grand Island, New York. PWM was rehydrated in 5·0 ml of sterile PBS and further dilutions were made at 1:20, 1:10 and 1:5.

0·1 ml of the appropriate mitogen at various concentrations was added to each culture. A new vial of mitogen, but of the same lot, was used each time.

Cell preparation, culture medium and assay system

One hundred millilitres of venous blood was drawn aseptically into a syringe containing preservative-free heparin (heparin sodium, Upjohn Company, Kalamazoo, Michigan). One millilitre of sterile 5% dextran (mol. wt 250,000; Pharmacia, Uppsala, Sweden) was added to the syringe and mixed well. Erythrocytes were sedimented in the inverted syringe for 45–60 min at 37°C, and the leucocyte-rich plasma was expressed into two sterile 50-ml tubes containing an equal volume of MEM with Earle's salts and gentamicin (10 μg/ml) (Microbiological Associates, Bethesda, Maryland) (MEM-G). This mixture was centrifuged at 500 *g* and the resulting cell pellet washed twice more with MEM-G. The cells were then diluted with an appropriate amount of MEM-G which contained 20% foetal calf serum and L-glutamine 0·2 mM/100 ml (Microbiological Associates, Incorporated, Bethesda, Maryland) to yield a final concentration of 500,000 mononuclear cells/ml. Four-millilitre aliquots of the cell suspension were placed into sterile disposable glass culture tubes containing the proper concentration of the three mitogens. The tubes were covered with Morton caps and placed upright in a incubator at 37°C in a moist atmosphere of 95% air and 5% CO_2. Following the proper incubation period (3, 5 and 7 days for mitogens and controls), the cells were pulsed for 2 hr with 4 μCi of [^{3}H]thymidine (6·0 Ci/mM) (Schwarz/Mann, Orangeburg, New York). After the 2-hr pulse, the cultures were placed in ice, centrifuged, and the cell buttons mixed with 2 ml of ice-cold 5% trichloroacetic acid (TCA). The resulting precipitate was washed twice with 1 ml of ice-cold 5% TCA and then washed once with 2 ml of ice-cold methanol, after which it was digested with 0·5 ml hydroxide of Hyamine (10–X) (Packard Instrument Company, Downers Grove, Illinois). The digested material was heated at 70°C for 15 min and then transferred to glass counting vials and 19·5 ml of scintillation fluid [0·3% 2, 5-diphenyloxazole (PPO) and 0·01% 1,4-bis [2-(5-phenyloxazolyl)] benzene (POPOP) in toluene was added to each vial. The vials were counted in a liquid scintillation counter (Amersham/Searle Corporation, Arlington Heights, Illinois) with quenching determined by use of an external standard.

Individuals studied

Twenty-six individuals were divided into two groups according to age: Group I, thirteen young individuals, ages 19–37 with a mean of 27·4 years; Group II, thirteen older individuals, ages 50–83 with a mean of 60·3 years. Both groups were comprised of eight females and five

males. There were seven black individuals in the young group and six in the older group. No individual was receiving any medication at the time of study.

Analysis of results

The response values for the incorporation of [^{3}H]thymidine into DNA was expressed as a ratio of (cpm mitogen cultures − background)/(cpm control cultures − background). Mitogen and control cultures were done in triplicate in most cases, but occasionally in duplicate because of a low cell count. For each concentration of mitogen, the data obtained at 3, 5 and 7 days were expressed as a mean value. Statistical analysis was performed using Student's *t*-test.

RESULTS

Unstimulated cultures

Spontaneous transformation of lymphocytes occurred in unstimulated cultures on days 3, 5 and 7. The counts per minute (cpm) in the young age group were 591, 2090 and 7313 respectively. In the older, the corresponding cpm were 576, 1925 and 8951. Thus, no significant difference was observed in the two groups regarding the degree of spontaneous transformation.

PHA-M

Table 1 and Fig. 1 show the lymphocyte response to PHA-M. At 3 days of culture, the mean response of the young group was significantly higher than the mean response of the

TABLE 1. Lymphocyte response to PHA-M in young and older subjects* (mean ratio ± s.d.)

Culture duration (days)	Subjects	PHA-M (μg)			
		50	100	200	1000
3	Young group	63·6 ± 32·2	90·2 ± 51·9	115·9 ± 44·9	94·9 ± 51·9
	Older group	20·6 ± 14·1†	28·4 ± 19·8†	48·8 ± 31·9†	35·7 ± 23·9
5	Young group	5·2 ± 3·7	9·7 ± 9·2	10·7 ± 5·5	16·8 ± 24·8
	Older group	9·5 ± 6·8	13·6 ± 8·3	27·9 ± 14·3†	30·8 ± 18·2
7	Young group	N.D.	N.D.	3·1 ± 5·4	3·8 ± 3·2
	Older group			3·8 ± 4·1	6·1 ± 4·3

* Expressed as ratio of (cpm mitogen culture − background)/(cpm control culture − background).
† $P < 0{\cdot}005$.
N.D. = not determined.

older group ($P < 0{\cdot}005$), and this was true for the four concentrations of PHA-M used in this study. The peak response in both groups was observed with PHA-M concentration of 200 μg rather than the 1000 μg (undiluted PHA-M) recommended for lymphocyte culture by the manufacturer, Difco.

In 5-day cultures, the lymphocyte responses to PHA-M decreased significantly in both groups. As seen in Fig. 1, the older age group showed a higher response at all concentrations,

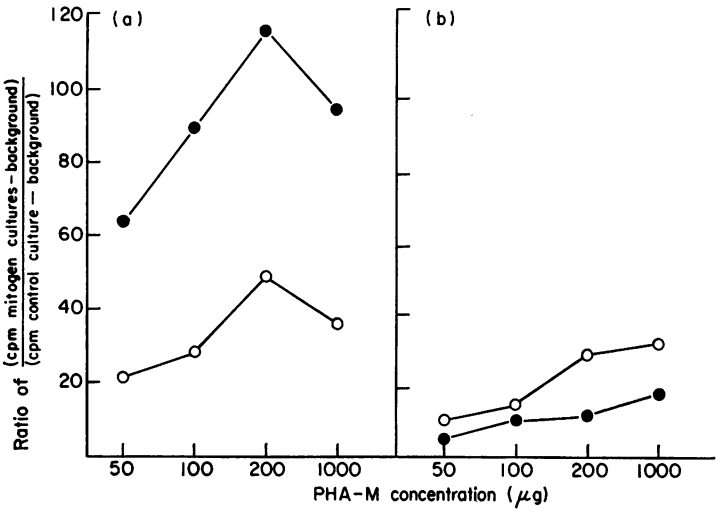

FIG. 1. Lymphocyte response to PHA-M in young (●) and older (○) subjects. (a) Culture duration 3 days. (b) Culture duration 5 days.

but this was statistically significant only at a PHA-M concentration of 200 μg ($P<0{\cdot}005$). The peak response at 5 days was seen at a concentration of 1000 μg in both groups.

In 7-day cultures, data were available only at PHA-M concentration of 1000 μg and 200 μg. The lymphocyte response in 7-day cultures in both groups decreased further compared to 3-and 5-day cultures but did not reach the base line. There was no significant difference between the two groups.

Con-A

Table 2 and Fig. 2 show the lymphocyte responses to Con-A. At 3 days of culture, the maximum response occurred with a 200 μg concentration of Con-A in both groups. The lowest response was seen with a Con-A concentration of 1000 μg. The younger group

TABLE 2. Lymphocyte response to Con-A in young and older subjects (mean ratio ± s.d.)

Culture duration (days)	Subjects	Con-A (μg)			
		50	100	200	1000
3	Young group	65·68 ± 37·86	120·36 ± 53·35	160·69 ± 72·04	20·11 ± 11·99
	Older group	45·34 ± 31·32	43·74 ± 35·95*	68·33 ± 64·92*	17·61 ± 24·92
5	Young group	9·98 ± 7·86	16·46 ± 8·00	22·81 ± 18·50	4·49 ± 4·67
	Older group	23·57 ± 19·74	24·46 ± 15·46	39·86 ± 26·16	2·85 ± 2·58
7	Young group	N.D.	N.D.	1·21 ± 1·40	0·70 ± 0·88
	Older group			3·94 ± 4·90	0·67 ± 0·66

* $P<0{\cdot}005$.
N.D. = not determined.

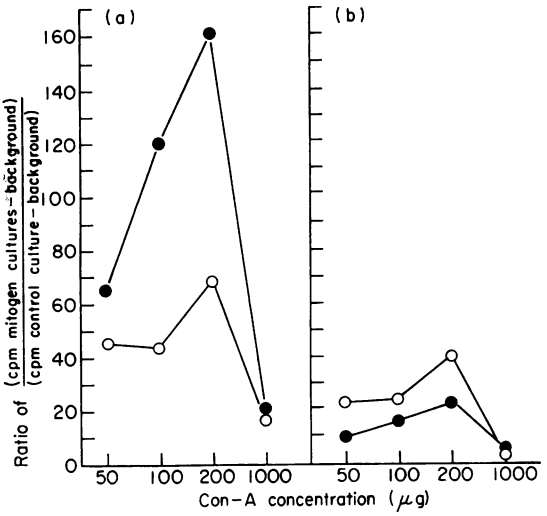

FIG. 2. Lymphocyte response to Con-A in young (●) and older (○) subjects. (a) Culture duration 3 days. (b) Culture duration 5 days.

showed a statistically significant ($P<0{\cdot}005$) higher response compared to the older group at Con-A concentrations of 200 and 100 μg.

In 5-day cultures, no significant difference was detected between the two groups and by 7 days, only minimal stimulation was detectable.

PWM

Table 3 and Fig. 3 show PWM responses. After 3 days of culture in the younger group, the response was lower in magnitude compared to PHA-M and Con-A and the maximum

TABLE 3. Lymphocyte response to PWM in young and older subjects (mean ratio ± s.d.)

Culture duration (days)	Subjects	PWM dilution			
		Undiluted	1:5	1:10	1:20
3	Young group	51·74 ± 20·71	55·47 ± 22·68	72·54 ± 65·79	37·82 ± 17·56
	Older group	26·90 ± 16·65*	34·02 ± 19·65*	21·80 ± 14·64*	19·24 ± 14·81*
5	Young group	13·08 ± 7·47	24·80 ± 14·10	19·39 ± 12·82	15·75 ± 16·41
	Older group	32·60 ± 19·54*	39·00 ± 27·53	44·15 ± 30·52*	33·37 ± 15·99*
7	Young group	N.D.	N.D.	1·92 ± 1·23	2·93 ± 2·11
	Older group			5·65 ± 5·84	7·62 ± 7·98

*$P<0{\cdot}05$.
N.D. = not determined.

response occurred at a dilution of 1:10. The older age group showed significantly lower responses at 3 days for all concentrations of PWM used ($P<0{\cdot}05$). The peak response in the older group occurred at the dilution of 1:5.

By 5 days, the older age group demonstrated a significantly higher response at 1:20, 1:10 and undiluted PWM ($P<0{\cdot}05$) as compared to the younger group.

By 7 days, the lymphocyte response decreased in both groups but was still higher in the older group though not particularly significant.

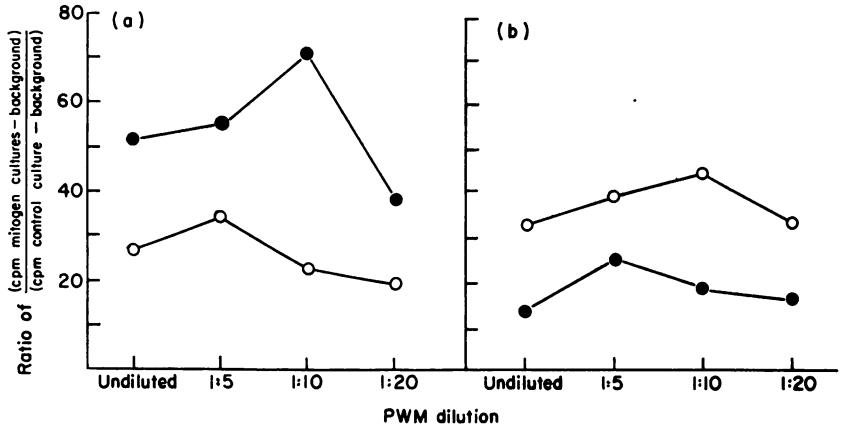

FIG. 3. Lymphocyte response to PWM in young (●) and older (○) subjects. (a) Culture duration 3 days. (b) Culture duration 5 days.

DISCUSSION

The present study clearly illustrates the importance of varying the concentrations of mitogens and culture duration in eliciting optimal lymphocyte transformation in normal individuals. Age is another important variable in the evaluation of lymphocyte response to mitogens. In 3-day cultures, it is clear that the proliferative response to mitogens is depressed in persons over 50 years of age; but when cultures are prolonged to 5 days, the older age group showed a better response than the younger group. This situation is similar to the response of hypogammaglobulinaemic patients to PHA described by Douglas *et al.* (1969).

The concentration of mitogen that produces a peak response varies from one mitogen to another and is influenced by duration of cultures and the age of the person under study. The optimum concentration for PHA in 3-day cultures in both groups was 200 μg or 1:5 dilution of the recommended concentration. However, the optimum concentration in 5-day cultures was 1000 μg or undiluted PHA. The optimum Con-A concentration was also 200 μg of 1:5 dilution for both 3- and 5-day cultures. For PWM, the optimum concentration varied with culture duration with the age of the subjects. In 3-day cultures, the optimal PWM concentration for younger persons was 1:10, whereas in the older group it was 1:5. In 5-day cultures, the reverse occurred, the optimal PWM concentration being 1:5 for the young and 1:10 for the older group.

Fitzgerald (1971, 1972), and Hosking, Fitzgerald & Simons (1971) described a dose–response curve for PHA in normal subjects. The two critical PHA concentrations were 20 and 200 μg. Patients with immunodeficiency states fell below the normal dose–response curve and sometimes could be detected only at a PHA concentration of 20 μg. We used

four concentrations of PHA, 50, 100, 200 and 1000 μg, and our data indicate that older persons show a depressed PHA response at all these concentrations.

It must be pointed out that the three mitogens used were crude commercial preparations and that information relative to the chemical structure of these mitogens is limited. The lymphocyte response varies if different mitogen batches are used. Therefore, one lot of each mitogen was used throughout this study.

Pre-existing delayed hypersensitivity is intact in the older age group (Waldorf, Willkens and Decker, 1968; Grossman *et al.*, 1973); however, DNCB sensitization which measures induction of delayed type hypersensitivity to a new antigen is relatively suppressed (Waldorf *et al.*, 1968; Gross, 1965). Peripheral blood lymphocytes in older subjects have a depressed PHA response (Westring *et al.*, 1964; Pisciotta *et al.*, 1967). However, these studies used morphological methods to detect blastic transformation, the culture duration was of 3 days, and PHA was used in only one concentration.

The depressed lymphocyte response to mitogens in our older subjects at 3 days could be due to a defect in the lymphocytes or to the presence of a serum inhibitor or both. The possibility of a serum inhibitor was not investigated in the present study.

The main value of the present study is to emphasize the need for age-matched controls in lymphocyte cultures. Dose and time response curves should also be performed to clearly establish if a suppressed lymphocyte response to mitogens is present in disease states. For example, in the present study with PHA-M in 5-day cultures, there was a significant difference between the young and older groups only at a concentration of 200 μg. In addition, in 3-day cultures with Con-A, at concentrations of 100 μg and 200 μg, a significantly higher response was observed in the younger group compared to the older group. If only one concentration of mitogen was used, this difference could have been missed.

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