

IN VITRO RESPONSE OF FOETAL LYMPHOCYTES TO PHA, AND A FACTOR PLASMA WHICH SUPPRESSES THE PHA RESPONSE OF ADULT LYMPHOCYTES

J. AYOUB AND S. KASAKURA

Division of Hematology, Department of Medicine, Royal Victoria Hospital, and McGill University Clinic, Montreal

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SUMMARY

The *in vitro* response of cord blood lymphocytes from full-term newborn infants and premature infants to phytohaemagglutinin (PHA) stimulation was investigated. The effect of foetal plasma on adult cellular reactivity also was studied. It was shown that foetal lymphocytes suspended in either foetal or adult plasma reacted significantly less than adult cells in adult plasma. Adult cells suspended in foetal plasma showed a significant suppression of reactivity as compared with adult cells in adult plasma. It seems that foetal plasma contains a factor which suppresses the response of adult lymphocytes to PHA stimulation. At the present, the nature of this inhibitory factor is unknown. However, our demonstration of the relationship between the level of α_1 -fetoglycoprotein (fetuin) and inhibitory activity in premature plasma would suggest that fetuin in foetal plasma might be responsible for its inhibitory activity.

INTRODUCTION

It has been shown that the mammalian foetus, far from being immunologically inert, is capable of a wide range of immunologic activity (Billingham & Silvers, 1963). In the human foetus, immunoglobulin synthesis starts at about the 20th week of gestation as proven by immunofluorescent studies (Van Furth, Schuit & Hijmans, 1965). One of the ways to assess the cellular immunologic status of the foetus is to test the *in vitro* response of foetal lymphocytes to various mitogens. The literature contains several conflicting reports on the response of cord blood lymphocytes to phytohaemagglutinin (PHA) (Lindahl-Kiessling & Böök, 1964; Pentycross, 1969; Jones, 1969). In the present study we compared the reactivity of foetal lymphocytes stimulated by PHA to that of adult cells. We also studied the effect of foetal plasma on the reactivity of foetal and adult cells.

Correspondence: Dr S. Kasakura, Division of Hematology, Royal Victoria Hospital, Montreal 112, Canada.

MATERIALS AND METHODS

Preparation of leucocyte suspensions

Cord blood from full-term newborn infants and prematures (body weight less than 2500 g) was collected under aseptic conditions at the end of the second stage of labour into heparinized plastic culture tubes (Falcon, Los Angeles, California). Normal healthy adults were used as controls. The plastic tubes were centrifuged for 10 min at 500 g. The plasma, buffy coat, and upper portion of the red cells were transferred to another tube. The second tube was kept at 37°C for 60 min to allow the red cells to settle. It was then centrifuged for 5 min at 25 g. The supernatant plasma containing the leucocytes was removed to another tube with a Pasteur pipette.

Preparation of cultures

The leucocyte suspensions were centrifuged for 10 min at 500 g. The cells were resuspended in frozen stored adult plasma, or in frozen stored foetal plasma, using an amount required to adjust the leucocyte count to approximately 7500/mm³. Cultures were prepared as described by Bain & Lowenstein (1964). The plasma, containing the leucocytes, was diluted with medium 199 (Microbiological Associates, Bethesda, Maryland) so that the calculated cell count was about 1500/mm³. The final plasma concentration was 20%, cell-free plasma being added if necessary. 4 ml of the cell suspensions with or without PHA were incubated in 17 × 100 mm disposable plastic culture tubes at 37°C. The usual incubation time was 3 days, but 5-day cultures also were studied in one series of experiments. Cell cultures were set up in triplicate.

Phytohaemagglutinin (PHA) preparation

PHA-M (Difco Laboratories, Michigan) was used as the stimulant. The contents of one vial were dissolved in 5 ml of medium 199. This constituted the undiluted PHA and was stored at -20°C. At the time of an experiment, undiluted PHA was thawed and diluted five times in medium 199. This 1:5 dilution was used in most experiments. 0.25 ml of diluted PHA was added to each culture or 0.25 ml of medium 199 to unstimulated controls. In one series of experiments, various concentrations of PHA were used. Undiluted PHA was diluted 2.5, 5, 10, 20, 40 and 80 times in medium 199, and 0.25 ml was added per culture.

Assessment of the response of lymphocytes to PHA

The degree of transformation induced by PHA was quantitated by estimating [³H]thymidine uptake as described previously (Kasakura & Lowenstein, 1965). At the end of the incubation period, [³H]thymidine (specific activity 2.0 Ci/mM, New England Nuclear Co., Boston) was added to culture tubes to give a concentration of 1 μCi/ml. After 2 hr at 37°C, the cells were washed three times with cold physiological saline. 0.5 ml of hydroxide of Hyamine (Packard Instruments, Illinois) was added to each tube to digest the cells. The tubes were kept in the dark at room temperature for 2 days, and then their contents were transferred to glass counting vials with two washings of absolute ethyl alcohol, 0.3 ml each. The vials were heated for 1½ hr at 75°C. 15 ml of 0.3% PPO (2,5-diphenyloxazole) and 0.01% POPOP (1,4-bis-2-(5-phenyloxazolyl)-benzene) in toluene were added, and the vials were counted in a Liquid Scintillation Spectrophotometer (Packard Instruments). Quenching

was determined by using an automatic external standard and was nearly identical for the samples in each single experiment. Thymidine uptake was expressed as counts/min/culture.

RESULTS

Fig. 1 shows a comparison of the response of premature infant, full-term newborn infant, and adult lymphocytes to PHA stimulation (1:5 dilution). In the experiments shown in Fig. 1(a), the total leucocyte counts in the final cell suspensions from each of the premature, full-term newborns and adult donors were approximately equal. Reactivity was significantly

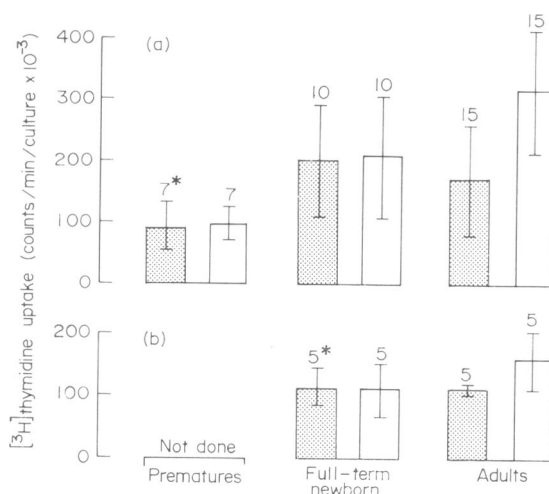


FIG. 1. Comparison of the response of premature infant (<2500 g), full-term newborn infant, and adult leucocytes to PHA stimulation. The mean and SD of thymidine incorporation are depicted. Open bars represent reactivity of cells suspended in adult plasma; bars with black dots represent reactivity of cells in foetal plasma. In Fig. 1(a) the total leucocyte count of premature, full-term newborn infant and adult leucocyte suspensions was identical in each experiment. In Fig. 1(b) the absolute lymphocyte count was identical in each experiment.

* Represents the number of experiments in each group.

higher in adult cells suspended in adult plasma as compared with foetal cells suspended in either foetal or adult plasma ($P < 0.01$ both). Adult cells suspended in foetal plasma showed a significant suppression of their reactivity as compared with adult cells suspended in adult plasma ($P < 0.001$). The responses of foetal cells suspended in foetal plasma were not significantly different from those of foetal cells in adult plasma. Premature lymphocytes suspended in either foetal or adult plasma showed diminished reactivity as compared with full-term foetal cells ($P < 0.005$). In the experiments shown in Fig. 1(b), the lymphocyte counts in the final cell suspensions from each of the adult and foetal cell suspensions were adjusted to be approximately equal (approximately 750 lymphocytes/mm³ in each culture). As a result of this adjustment, the total leucocyte counts in adult cell suspensions usually were twice as high as those of foetal cell suspensions. The results shown in Fig. 1(b) were similar to those shown in Fig. 1(a).

Fig. 2 shows the effect of incubation time on the *in vitro* uptake of [^3H]thymidine by adult and foetal leucocytes stimulated by PHA. All the cell suspensions tested, including adult and foetal cells suspended in either adult or foetal plasma, showed a peak response after 3 days' incubation and a diminished reactivity thereafter. The higher reactivity of adult cells over foetal cells, as well as the suppressive effect of foetal plasma on adult cells, was consistently observed after both the 3- and 5-day incubations.

Fig. 3 shows the effect of different mixtures of adult and foetal plasma on the reactivity of adult lymphocytes stimulated by PHA. The following ratios of adult to foetal plasma

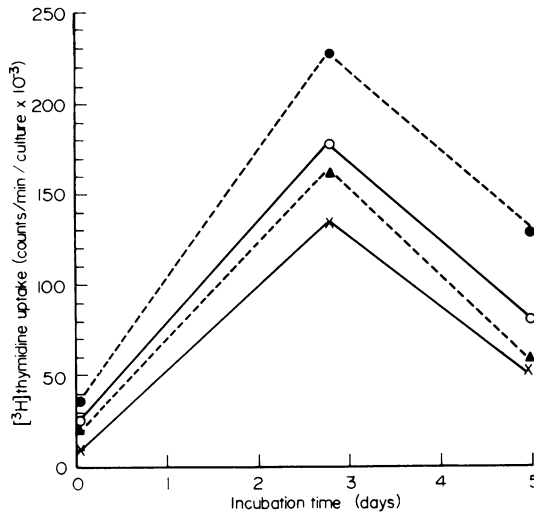


FIG. 2. Effect of incubation time on the *in vitro* incorporation of tritiated thymidine by cord blood lymphocytes, suspended in foetal and adult plasma consecutively, and stimulated by PHA. This figure is based on one sample of adult and one sample of foetal cells. Five similar experiments using different experiments were done. Each of those experiments showed the similar result. Adult controls were set up simultaneously. Mean values of triplicate determinations, given as counts/min, are depicted. ---○---, adult cells in adult plasma; —○—, adult cells in foetal plasma; —▲—, foetal cells in adult plasma; —×—, foetal cells in foetal plasma.

were used for five aliquots of adult cells from a given donor: 4:0, 3:1, 2:2, 1:3 and 0:4. Reactivity (^3H]thymidine incorporation) of leucocytes suspended in a given mixture of adult and foetal plasma was expressed as a percentage of the cellular reactivity in adult plasma (4:0 ratio). As shown in Fig. 3, increasing the ratio of foetal plasma diminished the cellular reactivity.

Table I shows the effects of varying the concentration of PHA on the reactivity of adult lymphocytes suspended in either adult or foetal plasma. The optimal dilution of PHA required to give maximum thymidine uptake by adult cells suspended in adult plasma varied from 1:5 to 1:20, while the optimal dilution of PHA required to give maximum stimulation to adult cells in foetal plasma varied from 1:5 to 1:40. The maximum reactivity of adult cells suspended in adult plasma was consistently higher than that of identical cells suspended in foetal plasma.

TABLE 1. *In vitro* incorporation of tritiated thymidine by adult lymphocytes incubated with varying concentrations of PHA for 3 days

Concentration of PHA-M	Uptake of tritiated thymidine ($\times 10^{-3}$)* Experiment No.									
	I		II		III		IV		V	
	Fet. pl.†	Ad. pl.‡	Fet. pl.	Ad. pl.	Fet. pl.	Ad. pl.	Fet. pl.	Ad. pl.	Fet. pl.	Ad. pl.
1:2.5	44	155	N.D.	281	N.D.	328	75	123	126	211
1:5	59§	170	266	319	273	439§	85	115	166§	215§
1:10	33	173	328	356§	319§	430	114	119	112	156
1:20	52	208§	348§	352	243	380	73	215§	81	81
1:40	47	181	273	198	86	143	123§	52	59	19
1:80	N.D.	N.D.	103	N.D.	32	N.D.	42	N.D.	19	N.D.

* Mean value of triplicate determinations, given as counts/min.

† Cells were suspended in foetal plasma.

‡ Cells were suspended in adult plasma.

§ Optimal dilution of PHA-M required to give maximum thymidine uptake.
N.D. Not Done.

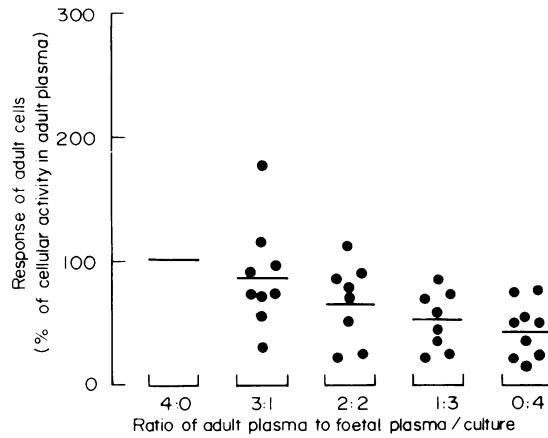


FIG. 3. Effect of different mixtures of foetal and adult plasma on the reactivity of adult lymphocytes stimulated by PHA. Reactivity ($[^3\text{H}]$ thymidine incorporation) of leucocytes suspended in a given mixture of adult and foetal plasma was expressed as % of cellular reactivity in adult plasma. Each point represents the reactivity of adult cells in a given mixture. Mean % value in each mixture is depicted.

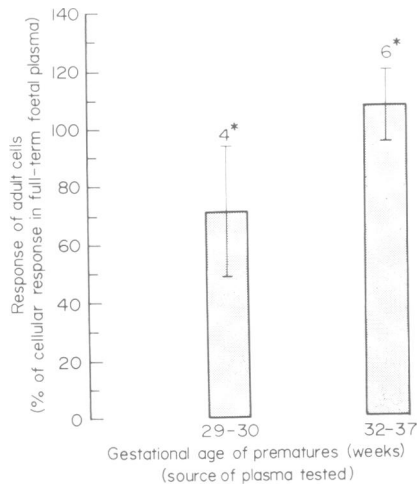


FIG. 4. Effect of premature plasma on the reactivity of adult lymphocytes stimulated by PHA. Leucocytes from a given donor were divided into two aliquots: one was suspended in full-term foetal plasma, and the other in premature plasma. Reactivity ($[^3\text{H}]$ thymidine incorporation) of cells suspended in premature plasma was expressed as % of cellular reactivity in full-term foetal plasma. The left bar represents the mean reactivity of adult cells suspended in premature plasma (the 29th-30th weeks of gestation); the right bar represents the mean reactivity of adult cells in premature plasma (the 32nd-37th weeks of gestation). Vertical lines in each bar represent SD.

* Represents the number of experiments in each group.

Fig. 4 shows the effect of premature plasma on the reactivity of adult lymphocytes to PHA stimulation. Adult cells from a given donor were divided into two aliquots: one was suspended in full-term foetal plasma (as the control), and the other in premature plasma of a given gestational age. These cell suspensions were incubated with PHA for 3 days and their reactivity compared. Reactivity ($[^3\text{H}]$ thymidine incorporation) of adult cells in a given premature plasma was expressed as a percentage of cellular reactivity in pooled frozen stored full-term foetal plasma. Attempts were made to see whether there was any relationship between the suppressive activity of premature plasma and the plasma concentration of α_1 -fetoglycoprotein. Premature plasma was divided into two groups: Group I—plasma obtained from premature infants born during the 29th–30th weeks of gestation; Group II—plasma from premature infants born during the 32nd–37th weeks of gestation. It has been shown by Gitlin & Boesman (1966) that the serum concentration of α_1 -fetoprotein (fetuin) is maximum at the 20th week of gestation and remains on a plateau level up to the 30th week. After the 32nd week of gestation, the amount of fetuin decreases sharply to reach its lowest level just before birth. As shown in Fig. 4, premature plasma belonging to Group I suppressed the reactivity of adult cells much more than did premature plasma belonging to Group II. Premature plasma of the latter group suppressed the reactivity of adult cells to a similar degree to full-term foetal plasma.

DISCUSSION

The literature contains several conflicting reports on the response of cord blood lymphocytes to phytohaemagglutinin (PHA). Some workers found that they were more responsive to PHA than adult lymphocytes (Lindahl-Kiessling & Böök, 1964). Others showed that the PHA response of foetal lymphocytes paralleled that of adult cells (Pentycross, 1969). Recently it was reported by Jones (1969) that the response of cord blood lymphocytes to PHA was lower than adult controls. Our present study confirms this latter report. We have found that foetal lymphocytes suspended in either foetal or adult plasma react to PHA stimulation less than adult cells in adult plasma. There is no adequate explanation for these conflicting reports.

Our present study also has shown that foetal plasma has an inhibitory effect on the response of adult cells to PHA stimulation (Figs 1 and 3). Two possibilities may be considered to explain this inhibitory activity. One possibility is that lymphocytotoxic antibodies originating from the mother might be responsible for this inhibitory activity. It has been shown by Leventhal *et al.* (1970) that lymphocytotoxic sera obtained from multiparous women have a suppressive activity on the *in vitro* response of adult lymphocytes to various blastogenic stimuli. Maternal IgG and lesser amounts of IgM are transferred passively to the foetus (Gusdon, 1969). However, inhibition of the PHA response by antibodies is unlikely since our study included twelve samples of cord blood from newborn infants of primiparous women. These plasmas showed a similar degree of inhibition to those obtained from newborn infants of multiparous women. It has been shown that primipara do not usually have lymphocytotoxic antibodies (Payne & Rolfs, 1958). The other possibility is that α_1 -fetoglycoprotein (fetuin) in foetal plasma may be responsible for its inhibitory activity. It has been shown that α_1 -glycoproteins inhibit the response of lymphocytes to PHA and specific mitogens (Cooperband *et al.*, 1968). More recent studies have demonstrated that fetuin competitively binds PHA (Mendelsohn, Skinner & Kornfeld, 1970). At the present

time we are unable to ascertain whether fetuin is an inhibitory factor in foetal plasma. However, our demonstration of the relationship between the level of fetuin and inhibitory activity in premature plasma (Fig. 4) favours such a possibility.

Contrary to adult lymphocytes, the PHA response of foetal cells in foetal plasma was not significantly different from that of foetal cells in adult plasma. When foetal leucocytes were resuspended in adult plasma, traces of an inhibitory factor might still have existed on their surface. It also is possible that this factor caused irreversible suppression *in vivo*, and the reactivity of the foetal cells did not recover even when they were resuspended in adult plasma.

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