REVISTA ESPAÑOLA DE FISIOLOGIA, 33, 181-186. 1977

Studies on T Cells in Newborns. — Higher Reactivity of Umbilical Cord Blood Lymphocytes to PHA as Measured by Whole Blood Microtechnique

R. Ruiz-Requena, F. Garrido, A. Martín-Andrés and C. Osorio

Departamento de Fisiología y Bioquímica
Universidad de Granada (Spain)

(Received on November 3, 1976)

R. RUIZ-REQUENA, F. GARRIDO, A. MARTIN-ANDRES and C. OSORIO. *Studies on T Cells in Newborns. — Higher Reactivity of Umbilical Cord Blood Lymphocytes to PHA as Measured by Whole Blood Microtechnique.* Rev. esp. Fisiol. 33, 181-186. 1977.

The capacity of human foetal lymphocytes to respond to PHA and to form E-rosettes have been compared with data from adult individuals. For this purpose a microculture system that uses whole blood and avoids the problems of lymphocyte separation, has been developed.

Foetal lymphocytes reached optimal stimulation with lower dosis of PHA (31.2 μg/ml) as compared with adult cells (125-252 μg/ml). However their quantitative response (measured by ^{14}C-thymidine uptake) was equal in both groups. In addition, peripheral T cells (E-rosetting cells) reached values of 36.47 ± 9 % in newborn and 49.6 ± 10 % in normal adult controls. These results are discussed as to the status and development of cellular inmunity in human foetus.

The capacity of human foetal lymphocytes to respond to mitogens has been demonstrated by several authors (1-3). Nevertheless it is not clear whether these lymphocytes are more susceptible than normal adult lymphocytes to phytohaemaglutinin. For example AYOUB and KASAKURA (3) found the PHA of foetal lymphocytes to be lower than the adult lymphocyte response whereas LEIKEN *et al.* (14) have shown the foetal response to be equal to that of adult cells and CAMPBELL *et al.* (5) have shown that the response of cord blood lymphocytes was higher than that of control adult lymphocites. The comparison was established only when a single or two different doses of phytohaemaglutinin was used to stimulate the lymphocytes. More significant data however can be obtained by the use of dose response curves (10). Thus, since the maximal response for different lymphocyte populations may occur at different doses of PHA, comparisons should be made at the optimal concentration of mitogen for a given test cell (15, 16).

In the present study we have examined the dose response of human cord blood and adult lymphocytes to PHA and compared the results with the number of

E-rossetting lymphocytes in both groups. For this purpose a microculture system using whole blood has been developed which proved to be reliable and reproducible. This method avoided the problems of lymphocyte separation and was also suitable for screening a large number of samples with relatively small volumes of blood.

Materials and Methods

Collection of blood samples. 10 ml of cord blood was collected at birth from 36 donors and similarly 20 ml of venous blood from adult controls (28 adults 20 to 30 years old). All samples were cultured within 17 hours of collection, usually within 6 hours.

Preparation of cultures. A whole blood microtechnique similar to that described by KISSLING and SPECK (17) was used in setting up the cultures. Tubes (0.8 × 1.5 cm) were set up in triplicate, each containing 1 ml of Eagle's medium [MEM] supplemented with 10 % of foetal calf serum and cloxacillin and ampicillin (100 μg per ml), to which 0.1 ml of the blood sample was added. PHA (Wellcome Grade Reagent, HA 15) was diluted in PBS (phosphate buffer saline) to give a final concentration of 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, 500, 1.000 μg/ml. 20 μl of different concentrations of PHA solutions were added in triplicate to tubes with either foetal or adult blood. Both were also cultured in triplicate without PHA. The cultures were gassed in a vacuum dessicator (9) to give a final atmosphere of 10 % oxygen, 4 % CO_2 and 86 % N_2 and incubated at 36° C.

Assessment of lymphocytes response to PHA. DNA synthesis, as an index of blastogenesis, was measured by incorporation of ^{14}C-thymidine. The doses of isotope used ranged between 0.02 μCi/ml to 0.6 μCi/ml and were added to the cultures in 20 μl. The response as a function of time, was measured by ^{14}C-thymidine incorporation after 24, 48, 72, 96, 120, 144 and 168 hours. In the standard assay 0.32 μCi/ml was used. At the end of each culture the cells were washed four times and resuspended in 0.5 ml of Eagle's medium; 0.2 ml aliquots were transferred into round discs (Whatman 3 MM), in duplicate and dried. The radioactivity was measured according to a standard technique (9) in a liquid scintillation counter.

Rossette formation by T lymphocytes. The percentage of peripheral mononuclear cells binding sheep red blood cells (SRBC) was determined in cord and adult blood. Lymphocytes were isolated by density gradient centrifugation using Ficoll-Hypaque, according to the method of Böyum (4). The cells were washed in MEM, counted using phase contrast microscopy and the concentration adjusted to 6 × 16^6/ml.

0.5 ml of the lymphocyte suspension was mixed with an equal volume of sheep red blood cells (0.5 %). This mixture was incubated at 37° C for 5 min and centrifuged at 400 g for another 5 min. Finally the pellet was kept at 4° C for 1 hour, gently resuspended and the rosetting cells counted. Lymphocytes binding 3 or more SRBC were considered to have formed E-rosettes. All cells were counted using phase contrast microscopy.

Results

Figure 1 shows the time course of adult lymphocytes transformation during seven consecutive days with two different concentrations of PHA (125 μg/ml, 250 μg/ml) added to the whole blood. It is obvious that for both PHA doses the maximal blast transformation is reached at aproximately 72 hours.

The optimal ^{14}C-thymidine concentration was determined by assessing increasing doses of isotope. Compared with the

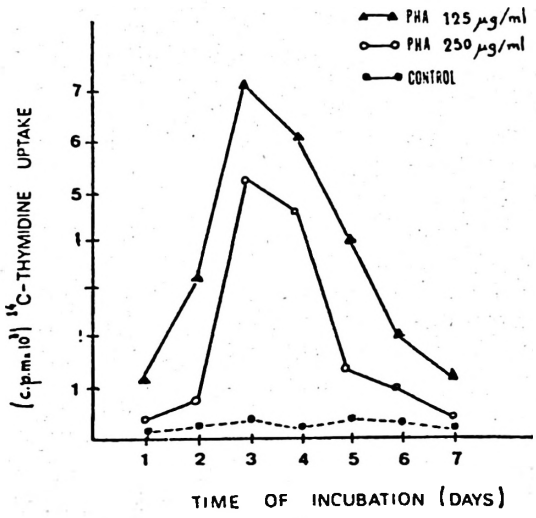

Fig. 1. *Time course of lymphocyte response to PHA as assesed by ^{14}C-Thymidine uptake using the whole blood microtechnique.* Cultures of peripheral blood from adults individuals were stimulated with 125 µg of PHA during seven consecutive days. At the end of each period the lymphocyte transformation was quantified by DNA synthesis. Control cultures without mitogen were also performed. Optimal ^{14}C-Thymidine uptake was reached at 72-96 hours.

control value without PHA, amounts of ^{14}C-thymidine below 0.08 µCi were insufficient to detect any response. Above this value however, incorporation rose rapidly until a plateau was reached at doses close to 0.32 µCi/ml. For the standard test system it was therefore selected this concentration (fig. 2).

The proportion of T cells in a population of lymphocytes separated from whole blood as measured by their rosettting capacity with SRBC in both newborns and other individuals are shown in figure 3. The difference between the mean of the two groups was statistically significant (newborns 36.47 ± 9 % and adults 49.64 ± 10.6 % respectively $P < 0.001$).

Cord blood lymphocytes are able to respond better to lower doses of PHA than lymphocytes from adult individuals (the optimal concentration range in the first group between 15.6 and 31.2 µg/ml, in the second group between 125 and 250 µg/ml). Nevertheless their quantitative response (cpm of ^{14}C-thymidine) reached approximately the same value in both groups (fig. 4).

Discussion

The immunological status in the early and late phases of human foetal developement is poorly understood. It is known that human embryos are able to responde as early as 20-22 weeks to PHA (7, 8, 11) to generate positive MLR and citotoxic T lymphocytes *in vitro* (13) as

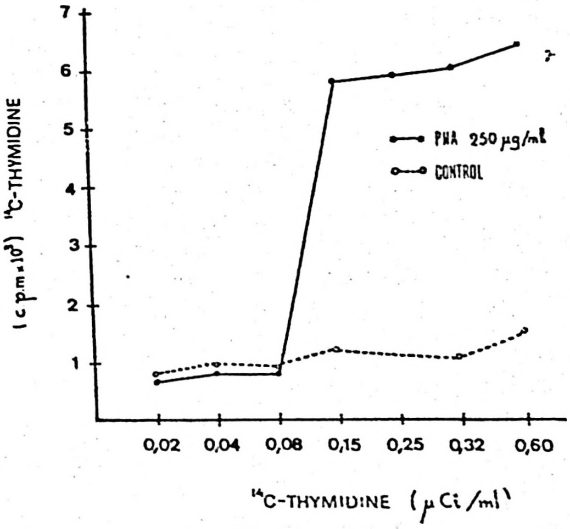

Fig. 2. *Estimation of µCi of radioactive nucleoside required to measure mitogenic stimulus on lymphocytes using PHA.* Different amounts of isotope were used in 72 hour cultures with 250 µg of phytohaemaglutinin in the microassay system. 20 µl of ^{14}C-Thymidine at different concentrations were incubated for further 16 hours. Control cultures without mitogen received also the same amount of labelled molecules. The uptake was recorded starting at 0.16 µCi, with a plateau following increasing amount of isotope. For the standard culture system, 0.32 µCi was selected.

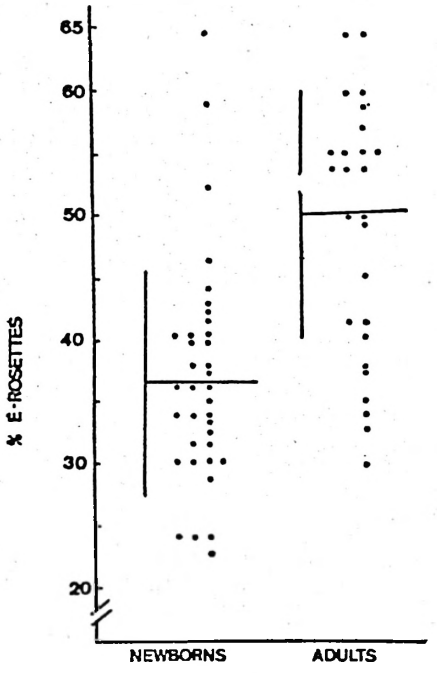

Fig. 3. *Percentage of peripheral T cells detected by their E-rosseting capacity in newborn and adult individuals.*
Partially purified mononuclear cells were used for measuring the capacity to bind sheep red blood cells in both groups. The mean in healthy control was 49.64 ± 10.63 and in foetal blood 36.47 ± 9.14.

well as having the capacity for inducing GVH reaction (12).

The experimental work have been concentrated on comparing the T cell numbers and T cells response to PHA in the human foetus at delivery, with those figures obtained from adult individuals (20 to 30 years) (18). For this purpose a whole blood microculture with isotope quantitation has been developed. Having examined the parameters of the response as shown in figures 1 and 2 the standard test system was adopted.

It was found that the number of circulating T cells in cord blood is lower than that of adult individuals. This is in agreement with AIUTI *et al.* (1). Apparently the newborn would have the capacity for increasing the number of T cells when a thymus factor acts on lymphocyte membranes suggesting the presence of precursor T cells that are not able to bind SRBC. This would explain the low figures obtained compared with adult individuals.

Surprisingly the response to a thymus dependent mitogen (PHA) by these T cells reached optimal conditions at concentrations smaller than that for adult lymphocytes (fig. 4). This suggest a higher susceptibility of these cells to PHA and is in accordance with the results of WEBER *et al.* (19). Nevertheless the proliferative capacity seems to be the same in both groups and there is no evidence for a lower response of foetal blood to mitogens compared to other types of lymphocytes as previously reported (3).

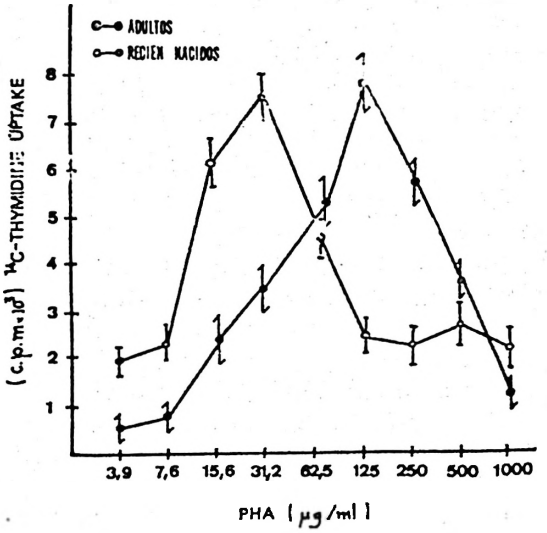

Fig. 4. *Profile of blast transformation by adult and newborn lymphocytes using a wide range of PHA concentrations.*
The capacity to produce blast cells by adult and newborn lymphocytes under PHA stimulus has been compared. For this purpose we used a wide range of PHA concentrations (from 3.9 µg/ml to 1000 µg/ml). In adult lymphocytes the optimal response was reached at 125 µg/ml while in newborns it was at 15.6 µg/ml.

The present data support the contention that human foetal lymphocytes: *a*) Are able to respond to PHA quantitatively similar to adult lymphocytes. *b*) Reached maximal response to lower dosis of mitogen and *c*) Their E-rossetting capacity is impaired compared to adult cells. Finally it could be favoured the existence of immature precursor T cells normally present on human foetus at delivery.

Resumen

Se compara la capacidad de linfocitos de fetos humanos y linfocitos de personas adultas para responder a PHA y formar rosetas directas (E-rosetas). Con este fin se ha puesto a punto un sistema de microcultivo utilizando sangre total que evita la purificación previa de los linfocitos.

Los linfocitos fetales alcanzaron una estimulación óptima con dosis más bajas de PHA (31,2 μg/ml) en comparación con células de adultos (125-252 μg/ml). Sin embargo, la respuesta fue cuantitativamente igual en ambos grupos. Las cifras de linfocitos T (formadores de E-rosetas) en recién nacidos fueron 36,47 $\pm$ 9 %, mientras en adultos alcanzaron 49,6 $\pm$ 10 %.

Se discuten estos resultados en relación con el desarrollo de la inmunidad celular en el feto humano.

References

1. AIUTI, F., SCHIRRMACHER, P., AMMIRATI and FIORILLI, M.: *Clin. Exp. Immunol.*, 20, 499-503, 1975.
2. ALEMÁN, P.: Tesis Doctoral, Granada (Spain), 1976.
3. AYOUB, J. and KASAKURA, S.: *Clin. Exp. Immunol.*, 8, 427-434, 1971.
4. BÖYUM, A.: *Scand. H. Clin. Lab. Invest.*, 21, Supplement 97, 1968.
5. CAMPBELL, A. C., WALLER, C., WOOD, J., AYNSLEY-GREEN. A. and YU, V.: *Clin. Exp. Immunol.*, 18, 789-796, 1974.
6. CARR, C. C., STITES, D. P. and FUDENBERG, H. H.: *Cell Immunology*, 5, 21-29, 1972.
7. CLAMAN, H. N.: *Proc. Soc. exp. Biol. Med.*, 121, 236-243, 1966.
8. CLAMAN, H. N. and BRUNSTETTER, F. H.: *Lab. Invest.*, 18, 757-761, 1968.
9. FESTENSTEIN, H.: *Lancet*, 27, 182-186, 1968.
10. GARRIDO, F., GARCÍA-PUCHE, J. L., OSORIO, C.: *Sangre*, 20, 255-260, 1975.
11. HAIWARD, A. R. and SOOTHILL, S. F.: *Ciba Foundation Symposium*, 5, 261-267, 1972.
12. HOFMAN, F. and GLABERSON, A.: *Europ. J. Immunol.*, 3, 479-482, 1973.
13. KAY, H. E. M., DOE, J. and HOCKLEY, A.: *Immunology*, 18, 393-396, 1970.
14. LEIKEN, S., MOCHIR-FATEMI, F. and PARK, K.: *J. Pediat.*, 72, 510-571, 1968.
15. LINDALCH-KIESLING, K. and BOOK, J. A.: *Lancet*, 2, 591-597, 1964.
16. LISHNER, H. W. and PUNNET, H. H.: *Lancet*, 2, 540-543, 1966.
17. KISSLIN, M. and SPECK, B.: *Lancet*, 19, 451-452, 1973.
18. RUIZ-REQUENA, R.: Tesis Doctoral, Granada (Spain), 1976.
19. WEBER, T. H., SANTENSON, B., VALDEMAR, T. and SKOOG, M. L.: *Scand. J. Haemat.*, 11, 177-183, 1973.