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Response of Sheep Lymphocytes to PHA: Quantitation by Nuclear Volume Measurement and Cell Counts (40764)[1]

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Nowell first described the transformation and proliferation of human peripheral blood lymphocytes in response to phytohemagglutinin (PHA) in culture (1). Since then the *in vitro* growth of lymphocytes in response to various mitogens has become a valuable investigative tool for evaluation of lymphocyte function. Although the PHA-induced lymphocyte blast transformation has been studied in most laboratory animals (2–6), very little information is available on PHA response of sheep lymphocytes (7, 8). We are currently employing this species in studies related to inhalation toxicology.

The mitogenic response is commonly quantitated by determination of the amount of tritiated thymidine ([^{3}H]TdR) incorporated by cells in culture. We report here the observations on sheep lymphocyte blastogenesis in culture as determined by [^{3}H]TdR incorporation and by the serial changes in the nuclear volumes and cell numbers. It is known that both the DNA content and nuclear volume increase before the cell divides (9).

Materials and methods. (i) Animals. Peripheral blood lymphocytes (PBL) from 18 adult Dorset sheep[2] of 3–4 years of age were studied. The animals were clinically normal, free of intestinal parasites, and were fed regular chow.

(ii) Blood samples. Blood was drawn from jugular vein in a syringe containing 10 units of preservative free heparin/milliliter of blood.

(iii) Isolation of lymphocytes. Modification of Boyum's method (10) was used to concentrate lymphocytes from blood. Briefly, 30 ml of heparinized sheep blood was layered over 10 ml of Ficoll–sodium metrizoate (Lymphoprep obtained from Nygaard, Oslo, Norway) in a 50-ml centrifuge tube and centrifuged at 400*g* at 4°C for 40 min. The opalescent "ring" which formed at the junction of Lymphoprep and plasma was aspirated into another tube, washed twice with phosphate-buffered saline (PBS) and suspended in McCoy's 5A modified medium supplemental with 200 m*M*/liter of L-glutamine, 100 units of penicillin and 100 μg of streptomycin per milliliter of medium and 10% heat-inactivated homologous sheep serum. Cell concentration was adjusted to 10^6/ml. Smears made from the cell suspension contained 91–93% lymphocytes, 0–2% granulocytes, and 5–9% monocytes.

(iv) Mitogen. One hundred milligrams of PHA (Difco Laboratories, Detroit, Mich.) was dissolved in 5 ml of sterile distilled water and stored at 4°C until use.

(v) Lymphocyte cultures. Lymphocytes were cultured at 39°C in humidified 5% CO_2 atmosphere in a microculture plate (0.2 ml of cell suspension/well). Two sets of lymphocyte cultures were initiated; one contained 100 μg PHA/ml; the other set was kept under identical conditions but did not contain PHA. The optimal dose of PHA for sheep lymphocyte cultures was previously determined in a pilot experiment (not reported here). Triplicate cultures from each set were grown and counted for each point on the day of initiation and on the first, second, third, fourth, seventh, and ninth day thereafter.

(vi) Determination of cell counts and nuclear volumes. The Coulter Channelyzer Model H_4 System (Coulter Electronics,

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Hialeah, Fla.) was used to determine cell counts and nuclear volumes. The method has been described in detail (11). For cell counts, the cells in the cultures were first dispersed by pipetting and 20 μl of cell suspension was transferred to a cuvette and diluted with 19.98 ml of Isoton-II (Coulter Electronics). For nuclear volume determination, the cytoplasm of cells was lysed by "Zap-Isoton" (Coulter Electronics) and the "stripped" nuclei were fixed in the Fixative Counting Solution (Coulter Electronics).

(vii) Determination of the number of transforming cells by cell counts and nuclear volume measurements. The volume-frequency curve of nuclei of lymphocytes is typically bimodal (Fig. 1). The point of intersection of the two curves (point *X* in Fig. 1) is determined on the day of initiation of culture and remains a constant reference point for that culture. Nuclei larger than the vertical plane at point *X* were considered "transformed." By determining the cell count and the fraction of nuclei "transformed," the actual number of proliferating cells can be calculated (10).

(viii) Determination of proliferative response by uptake of [^{3}H]TdR by lymphocytes. Four hours before harvesting 1 μCi of [^{3}H]TdR (specific activity 1.9 Ci/mmol, Schwarz/Mann, Orangeburg, N.Y.) was added per well. At the termination of cultures the cells were trapped on a glass-fiber filter during washing in Tetertek Cell Harvester (Flow Laboratory, Rockville, Md.) and the cell-associated radioactivity was determined in a liquid scintillation counter.

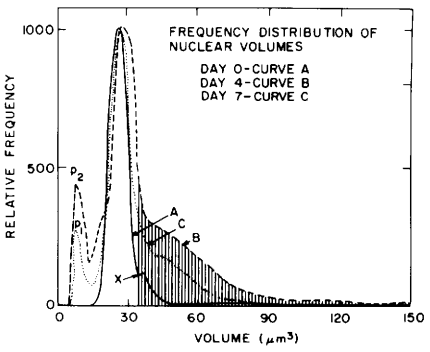

FIG. 1. Volume-frequency distribution curve of sheep lymphocytes. The volume-frequency distribution curves of unstimulated lymphocyte on the day initiation of culture (Day 0) and of PHA-stimulated lymphocyte cultures on Day 4 and Day 7 are represented by Curves A, B, and C, respectively. Smaller peaks p_1 and p_2 indicate the cellular element of 10–15 μm^3 and probably represent cellular debris of dead or dying cells. Nuclei larger than the vertical plane of point *X* were considered "transformed." See under Materials and methods for method of determining point *X*.

Results. Response to PHA of PBL from 18 adult sheep were studied, 14 with nuclear volume and cell count analyses and 4 with [^{3}H]TdR uptake. Ninety-seven to one hundred percent of the cells that were put into the cultures were viable as assessed by the trypan blue exclusion test. In the cultures with PHA, there was a decline in the number of cells on Days 1, 2, and 3 post-initiation. The number of cells then increased steadily from Day 3 onward, reaching maximum on Day 7 of the culture (Table 1). The reduction in the cell count occurred concomitant with the increase in the mean nuclear volume of cells in PHA containing cultures (Fig. 2). The mean nuclear volume of PHA stimulated cells was increased already on Day 1 and reached a maximum after 48 hr of cultures. From Day 2 to Day 4, between 40 and 45% of cells in PHA-stimulated culture were undergoing blast transformation (Table 1). In the cultures not containing PHA a steady decline in the cell numbers with time was observed. This decline in cell numbers was accompanied by an initial reduction in the mean nuclear volume up to the third day followed by a slight increment in the mean nuclear volume and cell count (Fig. 2).

Assessment of proliferation of lymphocytes with and without PHA by using [^{3}H]TdR incorporation is shown in Fig. 3. Proliferation of cells was measured by the ratio of [^{3}H]TdR incorporated by PBL in cultures containing PHA to [^{3}H]TdR incorporated by PBL in cultures not containing PHA on the same day. The ratio (Proliferation Index) increased from Day 1 onward reaching a peak on Day 4.

Discussion. The commonly used method for assessment of proliferation of lymphocytes in culture by [^{3}H]TdR incorporation merely reflects the DNA synthesis phase of the proliferating lymphocytes (13). Addi-

TABLE 1. GROWTH OF SHEEP LYMPHOCYTES IN CULTURE

No. of cultures = 14	Cells in culture/ml × 10^{-5}							Percentage of transforming cells in culture[a]					
	Day							Day					
	0	1	2	3	4	7	9	1	2	3	4	7	9
Mean ± 1 SD	10.66	9.06	7.89	6.46	5.61	4.66	4.05	13.91 (1.46)[b]	14.26 (1.137)	15.32 (1.02)	16.07 (0.93)	18.69 (1.28)	23.15 (1.13)
Cultures without PHA	1.14	1.06	1.38	1.45	1.62	3.74	3.32	2.19 (0.26)	1.78 (0.28)	3.90 (0.44)	3.59 (0.42)	8.76 (1.56)	7.55 (1.18)
Mean ± 1 SD	10.66	6.12	6.95	9.97	12.60	17.50	17.43	22.81 (1.44)	40.15 (2.92)	42.49 (4.67)	44.94 (5.79)	36.00 (6.28)	35.62 (6.12)
Cultures with PHA	1.14	2.07	2.52	3.14	4.97	8.08	7.70	7.30 (0.81)	10.48 (1.57)	7.79 (2.06)	6.83 (2.80)	4.87 (2.91)	4.46 (2.70)

[a] Percentage of transforming cells = (Area under the curve of transforming cells/Total area under the curve) × 100. For details of "Area under the curve," refer to Fig. 1 and Materials and methods.

[b] Number of transforming cells in culture per milliliter × 10^5 is shown within the brackets.

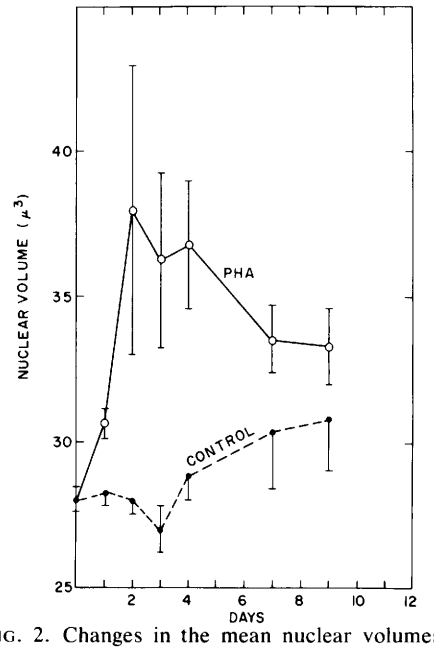

FIG. 2. Changes in the mean nuclear volumes of lymphocytes with time in PHA-stimulated and PHA-unstimulated (control) cultures. Bars in the figure represent (±) ISD of the mean value.

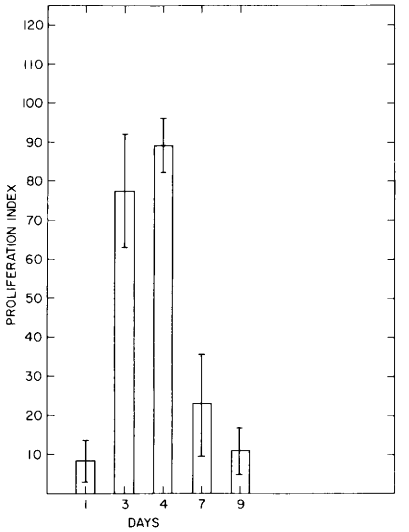

FIG. 3. The ratio of tritiated thymidine ([^{3}H]TdR) incorporated by PHA-stimulated lymphocytes to the [^{3}H]TdR incorporated by lymphocytes in culture not containing PHA on the same day represents Proliferation Index (PI).

tional information of cellular kinetic parameters can be obtained by determining the cell count and the fraction of cells in blast transformation (11). This later method which directly measures cell proliferation in culture shows that the maximal transformation phase of cultured sheep cells in PHA begins early after initiation of culture and reaches a plateau beginning on Day 2. The cell count however continues to increase up to the seventh day of culture. Isotope incorporation indicated peak DNA synthesis phase on Day 4 with a decline in [^{3}H]TdR incorporation on Day 7 and further reduction on Day 9. These data are similar to the response of human lymphocytes to PHA with the difference that the peak incorporation of [^{3}H]TdR in man occurs on Day 3 of PHA-stimulated culture (13). Significance of this difference in peak isotope incorporation between sheep and man is not clear, but may be related to a difference in cell-cycling time or to the "responsiveness' of sheep lymphocyte to PHA. Response of lymphocytes to PHA is known to vary with different species. Knight *et al.* observed that the blast transformation with PHA occurred earlier in lymphocytes from monkeys and rabbits than in the lymphocytes from mice and rats (2). Viability in *in vitro* culture was also found to vary with species studied. In mouse, hamster, and rat PHA-stimulated cultures, less than 20% of the cells (5, 17, and 19%, respectively) were viable at 48 hr in culture (2). Our data indicate that after an average reduction of 42.5% in cell counts during the first 24 hr in the PHA-stimulated culture, there is a steady average increment of 13.56, 43.45, 26.63, and 38.8% on 2, 3, 4, and 7 days, respectively, over the preceding cell count in the culture. This steady increase in the cell count is the net result of the difference in the number of newly formed cells and the number of cells dying in the culture. It is evident, therefore, that at least up to the seventh day in the PHA-stimulated cultures, a significant number of sheep lymphocytes must be viable and proliferating. It is interesting to note that in the sheep lymphocyte cultures not containing PHA, there was only 15% reduction in cell count in the first 24 hr, as compared to 42.5% in PHA-stimulated cultures. This greater re-

duction in the cell count in the first 24 hr in PHA-stimulated cultures remains unexplained and may be due to cytotoxic effects of PHA on certain lymphocyte subpopulations. Stewart *et al.* observed 40–60% reduction in cell count in the first 24 hr in PHA-stimulated cultures of normal human peripheral blood lymphocytes (12).

Our studies indicate that sheep PBL respond well to mitogenic stimulation of PHA and by measuring cell counts and nuclear volumes, potentially more useful quantitative cell kinetics data are obtained than by the [^{3}H]TdR incorporation method in assessing the lymphocyte proliferation.

Summary. Phytohemagglutinin response of peripheral blood lymphocytes (PBL) of sheep was studied. Assessment of proliferative response was performed by determination of nuclear volumes and cell counts in cultures from 14 sheep and by incorporation of tritiated thymidine in cultures in four additional sheep. PBL of sheep were found to transform and proliferate with PHA similarly to human peripheral blood lymphocytes with minor differences. Quantitation of the proliferative response by determining the cell count and nuclear volumes provided more information on cell kinetics in culture than the commonly used isotope-labeled thymidine incorporation method.

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