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ACTIVATION OF MURINE THYMOCYTES IN VIVO.

PART II: STUDY OF BLASTOGENESIS, THE SYNTHESIS OF MACROMOLECULES AND THE CYTOTOXIC RESPONSE AFTER STIMULATION WITH PHYTOHEMAGGLUTININ

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ABSTRACT

To determine whether the polyclonal immunomodulator, Phytohemagglutinin (PHA), stimulates murine lymphocytes in vivo *and causes cytotoxic differentiation, we studied blastogenesis and the concomitant synthesis of macromolecules (DNA, RNA and protein) by lymphocytes at different hours after injection of PHA in mice.* ^{51}Cr *release assay was also performed by using allogeneic cells as target to determine whether lymphocytes after stimulation* in vivo *by this activator undergo cytotoxic differentiation. Out of the five doses of Phytohemagglutinin (2.5 μg, 5 μg, 10 μg, 20 μg and 50 μg per mouse) a marginally higher peak of blastogenesis was observed with 20 μg dose at 48hr. No significant peak was observed in synthesis of macromolecules or with cytotoxicity.*

It has been previously shown that thymocytes develop responsiveness to different mitogens such as Phytohemagglutinin (PHA) and Concanavalin A (Con A) at different stages of ontogeny starting from the embryonic state (1-3). Most experiments for activating T cells with polyclonal agents like PHA and Con A have, however, been done *in vitro* (4-9). Recently, it has been shown that Con A can stimulate murine T cells *in vivo* (10-12). Accordingly, this study was designed to determine whether PHA stimulates murine lymphocytes *in vivo* by examining different parameters including blastogenesis, an indicator of activation of lymphocytes, synthesis of macromolecules and efficacy of cytotoxic killer cells.

MATERIALS AND METHODS

Animals: Mice of eight to twelve weeks age were used in all experiments. Breeding nuclei of C57BL and DBA/2 mice were obtained from Tata Cancer Research Institute, Bombay and maintained in our animal colony with pellet food of Hindustan Lever Ltd. and water *ad-libitum*.

Mitogen: Phytohemagglutinin M (PHA-M) was obtained from Difco Laboratories (Detroit, Michigan, USA) and the total amount of a vial (50mg) was dissolved in 5 ml sterilized distilled water. The stock solution of PHA-M was stored at -20°C. Five different doses of PHA-M, 2.5, 5, 10, 20 and 50μg in 0.1 ml, were used intravenously in the experiments.

Isotopes: Radioactively labelled compounds were obtained from the Bhabha Atomic Research Centre, Trombay, Bombay. ^{3}H-thymidine (sp. act. 15.8 Ci/mM),

^{3}H-uracil (sp. act. 38 Ci/mM) and ^{3}H-leucine (sp. act. 68 Ci/mM) were used for measuring the kinetics of DNA, RNA and protein synthesis respectively by the transforming lymphocytes activated with this mitogen. The target cells for cytotoxicity test were labelled with $Na_2{}^{51}CrO_4$ (sp. act. 0.68 to 1.93 Ci/mg).

Serum: Goat *(Capra bengalensis)* blood was collected aseptically from the jugular vein and sometimes at the time of sacrifice of the animal. Collected blood was allowed to stand at room temperature (25-30°C) for 45 min. After clotting of the blood, the serum was collected by centrifugation and aliquots of serum was preserved at -20°C until use (13).

Cell suspension: Spleen, mesenteric lymph nodes, other lymph nodes (cervical, axillary and inguinal lymph nodes pooled together) and peripheral blood were collected from mice aseptically. Cells from secondary lymphoid organs were dissociated in phosphate buffered saline (PBS) with the help of stainless steel wire mesh and by passing through a syringe fitted with 27 gauge needle. Peripheral blood was collected in 3.13% sodium citrate solution. Cell suspensions were layered on Ficoll and Hypaque solution (Sigma Co., USA Product No. F8628) and spun down at 3000 RPM for 15 minutes for the separation of lymphocytes from RBCs and debris. The lymphocytes were collected from the interface and washed three times with PBS.

Measure of blastogenesis: After binding with the mitogen or antigen, metabolic activities of small lymphocytes were augmented and they gradually became transformed into bigger cells termed blast cells. Thus blastogenesis is considered as an indicator of activation of lymphocytes, and the percentage of blast cells in a lymphocyte population stimulated with mitogen or antigen represented a measure of the degree of activation of lymphocytes. Blast cells were counted in a hemocytometer in the presence of trypan blue under the microscope fitted with a special occulometer. Cells with diameters greater than approximately 7 μm were scored as medium sized, whereas cells with diameter greater than 10-11 μm were scored as large. Fifteen mice were used per point in each experiment which was repeated three times. 300-400 cells were counted in three different fields to determine the percentage of blast cells. The proportion of transformed or "blast" cells was derived from the sum of viable medium plus large lymphocytes divided by the total viable lymphocytes counted (14). The percentage count of blast cells was corrected by subtracting the percentage of medium and large lymphoid cells in respective lymphoid organ of normal control mice; the later index usually varied from three to six percent.

Measure of DNA synthesis: C57BL mice were injected with different doses of PHA (10, 20 and 50 μg/mouse). Mice were sacrificed at different hours (24, 48, 72 and 96). Cell suspension from spleen and mesenteric lymph nodes were obtained separately as outlined above and suspended in Minimum Essential Medium (MEM). Cell number was adjusted as $4x10^6$ cells/ml and triplicates of 10^6 cells in 250 μl were transferred by an eppendorf in small glass culture tubes. ^{3}H-thymidine at the dose of 2 μCi was added to each culture tube for measuring the synthesis of DNA by lymphocytes stimulated *in vivo*, and the tubes were then incubated in a humidified atmosphere of 7.5% CO_2 in air at 37°C for eight hours. At the end of incubation 3 ml cold PBS was added to each of the tubes removed by suction. 1 ml cold 10% trichloracetic acid (TCA) in distilled water was added to each tube and all tubes were refrigerated overnight. The TCA precipitate of each tube was then filtered on a small filter disc (Whatmann filter No. 3) under suction and washed with 10 ml of 10% TCA. The filter paper discs were dried and kept in standard scintillation vials for counting by liquid scintillation counter (Packard, USA). A 5 ml scintillation cocktail was prepared by adding 6 gm PPO, 0.05 gm POPOP in one liter toluene.

Measure of RNA synthesis: Cell suspensions from spleen and lymph node were made at different hours of the experiment following the procedure outlined earlier. 250 μl of cell suspension (10^6 cells) in triplicate were incubated with 2 μCi of ^{3}H-uracil for labelling the total RNA, synthesized by the

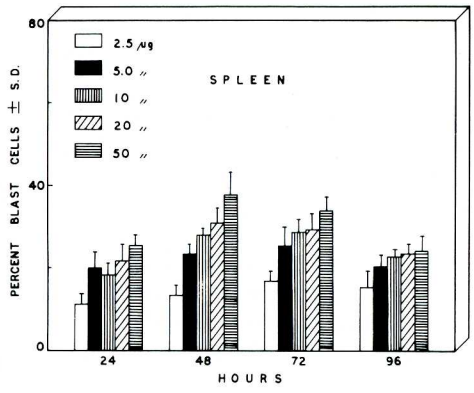

Fig. 1: Blastogenesis of the lymphocytes from spleen at different hours with in vivo *treatment of different doses of PHA.*

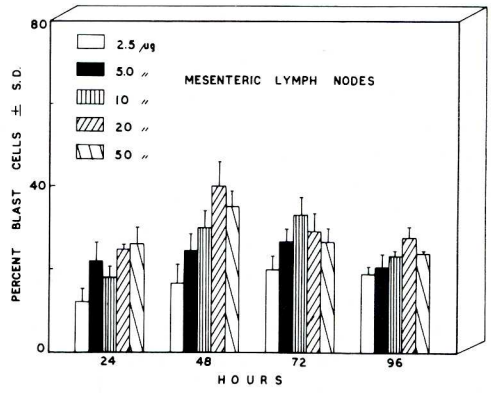

Fig. 2: Blastogenesis of lymphocytes from mesenteric lymph node at different hours with in vivo *treatment of different doses of PHA.*

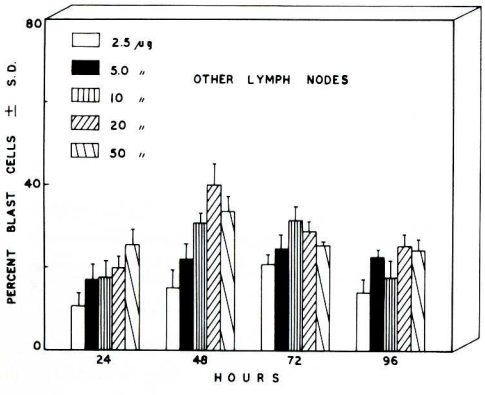

Fig. 3: Percentage of blast obtained at different hours in other lymph nodes with treatment of different doses of PHA.

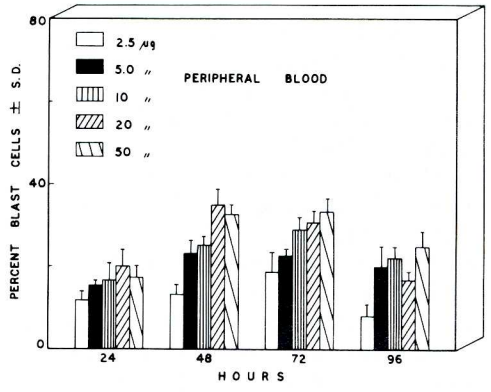

Fig. 4: Percentage of blast obtained at different hours in the lymphocytes of peripheral blood with treatment of different doses of PHA.

activated cells. Period of incubation, termination and further treatment of the cultures up to scintillation counting were same as with measuring DNA synthesis.

Measure of protein synthesis: A dose of 2 μCi of ^{3}H-leucine was used for 10^{6} cells in an aliquot of 250 μl in a tube for labelling the proteins, synthesized by the cells activated *in vivo*. Triplicate cultures for each type of cells were maintained for eight hours as described earlier. After termination of incubation the cultures were washed two times and the cells were precipitated on filter paper discs and processed for final scintillation counting.

Cytotoxicity assay: Cytotoxic ability of the lymphoid cells activated *in vivo* with PHA was determined by using ^{51}Cr release assay (14). Activated lymphocytes from spleen and lymph node of C57BL mice injected with this mitogen constituted the effector cells.

Con A induced blast cells from spleen and lymph nodes of DBA/2 mice were taken as target cells as per method of Melief and co-workers (15). To raise the target cells, DBA/2 mice were injected with 50μg Con A per animal iv. Mice were sacrificed after 48 hr of injection; spleen and lymph nodes were aseptically removed and dissociated using stainless steel grids. Following the method as outlined above the cells were

suspended in MEM supplemented with 10% heart inactivated goat serum, penicillin-streptomycin (50U/ml) and nystatin (50U/ml). Cell number was adjusted to 10^7 cells/ml. An amount of fluid containing 200 μCi of $Na_2{}^{51}CrO_4$ was added in 1 ml of cell suspension containing 10^7 cells and incubated for one and half hours in a humidified atmosphere of 7.5% carbon dioxide in air at 37°C. The tubes containing the cells were shaken three times during incubation for facilitating radioactive labelling. After incubation the cells were washed three times with PBS and the number of cells was adjusted to $4x10^4$ cells/ml. These cells labelled with radioactive chromium were used as target cells for cytotoxicity assay.

The effector cells were obtained from mice injected with 20 μg of PHA. Mice were sacrificed at 24, 48, 72 and 96 hours after injection of the mitogen and the cells were dissociated and suspended in MEM as described earlier. Cell number was adjusted to 10^6 cells/ml.

To 1 ml suspension of effector cells, ^{51}Cr-labelled target cells in 250μl were added at different ratios (100:1, 50:1 and 10:1) and the mixture incubated for six hours. Triplicate tubes for each ratio were maintained. Effector cells were not added in the control cultures for spontaneous release of the isotope from labelled target cells. Instead 1 ml MEM was added in each tube and the tubes were also incubated for six hours. For maximum release of the radioactivity from the target cells 1 ml of distilled water was added instead of medium in the tubes containing labelled target cells only. After incubation, culture tubes were centrifuged except the tubes for maximum release of the radioactivity. One ml supernatant was collected from each tube and the amount of ^{51}Cr released into the supernatant was assessed by using a gamma ray spectrometer (Model No. GR523A, ECIL, India). The percentage of cytotoxicity or ^{51}Cr released by the effector cells was calculated as follows:

$$\frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximum release} - \text{Spontaneous release}} \times 100$$

RESULTS

Blastogenesis: The dose effect of PHA was not well demarcated as with Con A (10) and there was no notable peak in the response with any dose of PHA *(Figs. 1-4)*. Five different doses were used: of these 20 μg PHA per mouse seemed to augment a marginally higher peak at 48 hr. There was little difference in the kinetics and level of blastogenic response of the lymphoid cells from different sources. The blast cells obtained with higher doses of PHA did not become vacuolated at later hours (72 and 96 hrs) as after treatment with Con A (10).

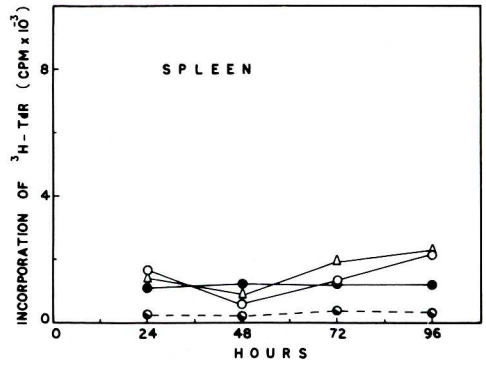

Fig. 5: Incorporation of ^{3}H-TdR by the lymphocytes from spleen at different hours after in vivo stimulation with different doses of PHA. Dose of PHA per animal: o—o 10 μg, ●—● 20 μg, △—△ 50 μg, *and* o—o *control, without PHA treatment. (Same symbols have been used in Figure 6.)*

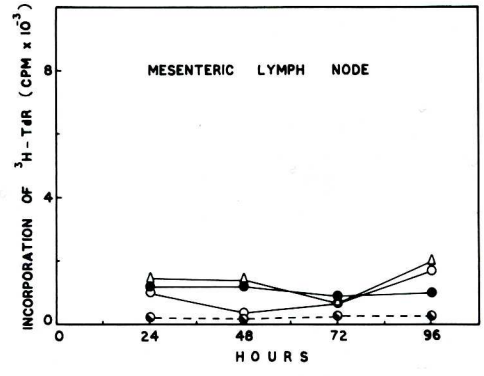

Fig. 6: Incorporation of ^{3}H-TdR by lymphocytes of mesenteric lymph node at different hours after in vivo *stimulation with different doses of PHA.*

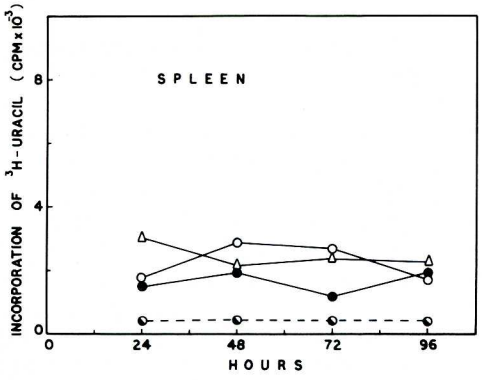

Fig. 7: Incorporation of ^{3}H-Uracil by the splenic lymphocytes at different hours after in vivo *stimulation with different doses of PHA. Dose of PHA per animal: o—o 10 μg, ●—● 20 μg, ▵—▵ 50 μg, and o—o control, without PHA treatment. (Same symbols have been used in Figure 8.)*

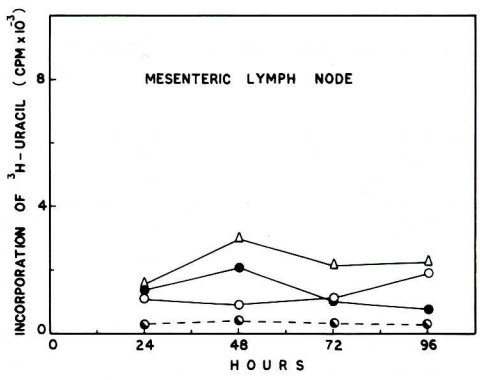

Fig. 8: Incorporation of ^{3}H-Uracil by lymphocytes of mesenteric lymph node at different hours after in vivo *stimulation with different doses of PHA.*

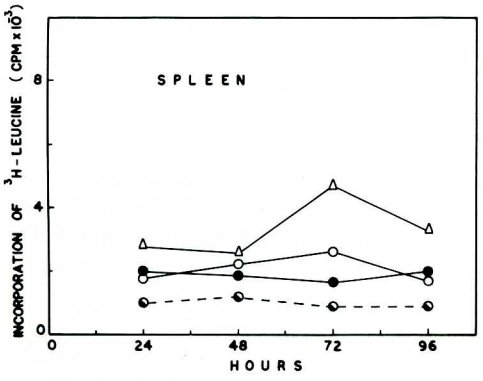

Fig. 9: Incorporation of ^{3}H-Leucine by splenic lymphocytes at different hours after in vivo *stimulation with different doses of PHA. Dose of PHA per animal: o—o 10 μg, ●—● 20 μg, ▵—▵ 50 μg, and o—o control, without PHA treatment. (Same symbols have been used in Figure 10.)*

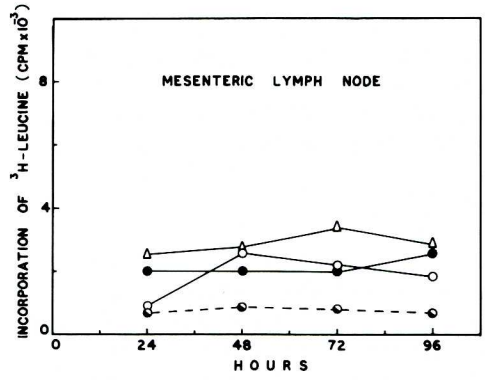

Fig. 10: Incorporation of ^{3}H-Leucine by lymphocytes from mesenteric lymph node at different hours after in vivo *stimulation with different doses of PHA.*

Synthesis of macromolecules: There was minimal incorporation of radioactive precursor into DNA of the lymphocytes with different doses of PHA. This was true with lymphocytes from both spleen and mesenteric lymph node *(Fig. 5,6)*. None of the three doses induced a significant peak of incorporation of ^{3}H-TdR.

Level of incorporation of radioactive uracil by the lymphocytes from spleen and mesenteric lymph node did not attain a significant level at any hour of treatment *(Fig. 7,8)*. This was true with each dose of PHA (10, 20 and 50 μg per mouse).

Similar to kinetics of blastogenesis and synthesis of DNA and RNA, synthesis of protein was also not notably stimulated by PHA *(Fig. 9,10)*. Each dose of PHA induced protein synthesis in spleen and lymph node cells to a similar extent i.e. slightly higher than background. Incorporation of ^{3}H-leucine was slightly higher in spleen cells at 72 hours after administration of 50 μg PHA *(Fig. 9)* but not significantly.

Cytotoxic ability of the lymphocytes stimulated in vivo *with PHA:* The dose of 20 μg PHA per mouse was used to generate the effector cells for this experiment as this dose was marginally better for inducing blast transformation. PHA generated effector cells

Table 1.
Cytotoxic response ($\bar{x}$ ± SD) of the lymphocytes activated with 20 μg PHA *in vivo* against allogeneic target cells.

Experiment No:	Target: Effector	Percentage of cytotoxicity 24 hr	48 hr	72 hr	96 hr
1.	1:100	8.45 ± 2.22	8.40 ± 2.19	21.76 ± 2.65	12.81 ± 2.00
	1:50	16.54 ± 4.04	5.70 ± 1.39	9.57 ± 5.66	17.03 ± 7.25
	1:10	12.31 ± 3.93	7.21 ± 2.11	15.29 ± 4.44	14.42 ± 4.06
2.	1:100	17.31 ± 2.84	17.57 ± 5.18	17.57 ± 4.70	16.18 ± 4.43
	1:50	11.12 ± 3.90	13.40 ± 4.67	10.99 ± 3.96	8.21 ± 2.00
	1:10	12.13 ± 2.66	15.92 ± 3.62	11.75 ± 1.76	17.31 ± 4.73

In control experiment the experimental and spontaneous release were the same.

at any concentration did not mount a significant level of cytotoxicity against allogeneic target cells as seen in *Table 1*. This was true with cells from mice treated with PHA for different length of time.

DISCUSSION

The chief objective of this study was to determine whether lymphocytes were stimulated *in vivo* with PHA and whether stimulated cells undergo wide spectrum differentiation including cytotoxic cells This issue was assessed from several aspects. Measure of the rate of blastogenesis was used to gauge the optimal concentration and time for effective transformation of quiescent lymphocytes into blasts. The process of blast transformation represents the culmination of several biochemical events and affects almost every metabolic pathway (16-21). Synthesis of macromolecules like DNA, RNA and protein accompany blastogenesis and accordingly were also taken into account.

In vivo stimulation with PHA (the dose wise effect) was not well demarcated as with Con A (10) and there was no significant peak in lymphocyte response at any dose. Thus, PHA was a poor stimulator for murine T cells *in vivo*. Similar results for T lymphocytes were obtained by Stobo and his co-workers (22) using an *in vitro* system. There was no vacuolation in the blasts induced with PHA as observed in the blast cells with high doses of Con A (10). Vacuolated cells may signify hyperactivity and physiological exhaustion of the blast cells.

Besides blastogenesis, synthesis of DNA, RNA and protein were also tested for assessing activation of lymphocytes *in vivo*. Rate of synthesis of these substances by lymphocytes of the spleen and mesenteric lymph node were examined as the pattern of blastogenesis of the lymphoid cells from different sources treated with different doses of PHA were found comparable. Thus the experiments with spleen and lymph node cells were considered representative.

No dose of PHA induced significant synthesis of DNA in spleen or lymph node cells. Although there was a feeble blastogenic response by lymphocytes *in vivo*, on the basis of earlier work (22) and our observation, it is concluded that PHA is a poor stimulator for murine lymphocytes whether *in vivo* or *in vitro*. PHA is recognized as a better polyclonal stimulator for human lymphocytes (4,23) and detailed kinetics and site of DNA synthesis augmented by PHA stimulation in human lymphocytes have been elucidated (24-26).

The rate of synthesis of RNA and protein in lymphocytes activated with PHA were studied at 24-hour intervals up to 96 hours. The main purpose was to measure the rate of the synthesis after treatment with PHA and the findings with blastogenesis.

It is likely that the process of blast transformation is more closely related to the

synthesis of RNA and protein than that of DNA. This relationship is expected because large blast cells presumably synthesize greater numbers of building blocks for differentiation.

Finally, PHA was also a poor stimulator for generating cytotoxic cells. These results with PHA suggest that considerable blastogenesis is a prerequisite for differentiation of cytotoxic T-cells, a conclusion suggested by others under different circumstances (2,22).

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