RESULTS

BLASTOGENIC TRANSFORMATION OF LYMPHOCYTES IN VIVO WITH MITOGENIC STIMULUS

Antigenic or mitogenic stimulus activates the lymphoid cells by initiating series of biochemical events; then the lymphocytes gradually differentiates into blasts. Instead of measuring the different biochemical activities often the degree of blast transformation of a lymphoid cell population is considered as the measure of activation of lymphocytes. Blasts are counted on the basis of the size of the cell as indicated in the materials and methods. Kinetics of the blastogenesis have been studied after treating the lymphoid cells with all the three agents employed in this study, Con A, PHA and Freund's complete adjuvant.

Blastogenesis with the treatment of Concanavalin A (Con A) :

Blastogenesis in the lymphoid cell population of spleen, mesenteric lymph node, other lymph nodes and peripheral blood at different hours after intravenous injection of Con A have been shown in figures 4 to 7. Peak of blastogenesis with the cells from all the sources, irrespective of the dose of Con A, was effectively reached by 48 hours in most of the cases. The peak was delayed by 24 hours in case of cells from other lymph nodes, treated with 50 µg Con A.

The responses with the dose of 5 µg and 10 µg Con A were almost at the same level in all the cases. With three other higher doses, the height of the responses corresponded to the amount of Con A injected per animal. Percentages of blast cells from mesenteric lymph node were higher at initial and subsequent stages with 50 µg and 100 µg Con A per animal. Thus there was a dose dependent blastogenic response of the murine lymphoid cells in vivo with the treatment of Con A.

The blast cells became more vacuolated or exhausted by 72 to 96 hours with the higher doses of Con A, especially with 100 µg dose.

Blastogenesis with the treatment of Phytohaemagglutinin (PHA) :

The dose-wise effect of PHA was not well demarcated as with Con A and there was no notable peak in the response with any dose of PHA (Fig. 8 to 11). Five different doses of the substance were used, of them 20 µg PHA per animal seemed to augment a marginally higher peak at 48 hours. There was not much difference in the kinetics and level of blastogenic response of the lymphoid cells from different sources. The blast cells obtained with the higher doses of PHA did not become vacuolated at late hours like 72 or 96 hours as in case of the treatment with Con A.

Blastogenesis with the treatment of Freund's complete adjuvant (FCA) :

Percentage of blast cells from different lymphoid organs and peripheral blood after injecting different doses of FCA have been presented in the Table 2. A good number of lymphocytes transform into blasts from 24 hours onward with intraperitoneal injection of the FCA. Peak of blastogenesis was reached between 48 and 72 hours in lymphocytes from all the sources with all three doses of FCA. The difference between initial and maximal response is marginal. Out of the three doses, percentage of blast cells was much higher with the dose of 0.3 ml FCA. The blast cells induced with FCA are not too much vacuolated or exhausted by 72 to 96 hours as in case of Con A induced blasts.

SYNTHESIS OF DNA AFTER IN VIVO STIMULATION OF THE LYMPHOCYTES

Blast transformation of stimulated lymphocytes is usually accompanied by the synthesis of DNA. Thus it became almost imperative to study the rate of synthesis of DNA by the lymphocytes to test the degree of activation of the lymphocyte population in in vivo situation.

DNA synthesis with the treatment of Con A :

Patterns of DNA synthesis by the spleen cells with the treatment of three different doses of Con A have been presented in the figure 12. The dose of 100 µg Con A was not used for studying the synthesis of macromolecules as this dose was found to affect the lymphocytes quite severely in terms of vacuolisation of the lymphoid cells presumably for hyperactivity of them and higher percentage of cell death to be discussed later.

With all three different doses, maximum level of incorporation of radioactive thymidine occurred at 48 hours. However, at different hours, the rate of DNA synthesis with the dose of 50 µg Con A is not appreciably different. Furthermore, the rate of the synthesis of DNA is higher with lower dose of Con A and thus the dose of 10 µg Con A per animal augmented the maximum level of incorporation of ^{3}H-thymidine (^{3}H-TdR).

DNA synthesis by cells of mesenteric lymph node follow the pattern as in the case of spleen cells with a few variations (Fig.13). Similar to the spleen cells, the rate of synthesis of DNA by the cells with 10 µg Con A treatment is higher than that with other doses. There is not much difference in the levels of

incorporation of ^{3}H-TdR with the treatment of 20 µg and 50 µg Con A. With the latter dose, the peak of the synthesis was at 72 hours. Possibly there are two peaks at 48 hours and at 96 hours with the treatment of 20 µg Con A. Similar tendency has also been observed with the spleen cells.

DNA synthesis with the treatment of PHA :

There was not much incorporation of radioactive precursor into DNA of the lymphocytes with the treatment of the different doses of PHA. This was true with the lymphocytes both from spleen and mesenteric lymph node (Fig.14,15). Any of the three doses could not induce any significant peak of incorporation of ^{3}H-TdR. Similarly there was no peak in the blastogenesis induced with PHA.

DNA synthesis with the treatment of FCA :

Synthesis of DNA by the cells of spleen and mesenteric lymph node have been presented in figure 16,& 17. In both the cases, the dose dependent patterns of DNA synthesis were similar. Treatment with 0.3 ml FCA caused the maximum incorporation of radioactive precursors into DNA.

It seemed that there could be two cycles of DNA synthesis in course of 96 hours, the first peak might be around 24 hours and the second one at 72 hours. This was evident with the dose of 0.3 ml FCA per animal with which a significant level of DNA synthesis could be induced.

RNA SYNTHESIS WITH IN VIVO STIMULATION OF THE LYMPHOCYTES

Responsiveness of spleen and lymph node lymphocytes to in vivo stimulation by different mitogens employed in this study was also investigated by measuring the rate of RNA synthesis of the stimulated lymphocytes. This was done in reference to the level of incorporation of ^{3}H-Uracil by the lymphocytes at different hours.

RNA synthesis with the treatment of Con A :

The dose of 50 µg Con A per animal elicited a reasonable response in both spleen and lymph node lymphocytes. The peak of the response was observed at 48 hours (Fig.18,19). There was no significant incorporation of ^{3}H-Uracil by the lymphocytes with the spleen treated with 50 µg Con A, it did seem to be significant (Fig.18).

RNA synthesis with the treatment of PHA :

Level of incorporation of radioactive uracil by the lymphocytes from spleen and mesenteric lymph node did not reach to a significant level at any hour of the treatment (Fig.20,21). This was true with all the three doses of PHA, 10 µg, 20 µg and 50 µg per animal.

RNA synthesis with the treatment of FCA :

Apparently it seemed that there was no particular pattern of synthesis of RNA with the treatment of different doses of FCA. Several experiments have been performed and then seemingly two types of incorporation could be discerned which have been presented in figure 22 & 23 and expressed as the percentage of maximum incorporation. Actual values for the level of incorporation of ^{3}H-Uracil have been presented in Table 3 and 4.

In the first type (Fig.22 & 24), the incprporation of radioactive precursor was high at 24 hours both with spleen and lymph node cells when the animals were injected with 0.3 ml FCA; the dose of 0.5 ml per animal could not elicit a reasonable response.

In the second type, with 0.3 ml FCA the rate of RNA synthesis was not as high at 24 hours as in the first type, but it kept going high with time (Fig.23 & 25). The dose of 0.5 ml caused a reasonable response and it seemed to be biphasic with two peaks at 24 and 72 hours. Both spleen and lymph node lymphocytes showed the similar pattern of synthesis of RNA with these two doses of FCA.

Irrespective of the type, the response with 0.1 ml FCA per animal was found reasonable and the peak was attained at 72 to 96 hours.

PROTEIN SYNTHESIS BY THE LYMPHOCYTES AFTER IN VIVO STIMULATION WITH MITOGENS

Synthesis of protein is an usual phenomenon with transformation of small lymphocytes into bigger sized blast cells after they got activated with mitogenic substances. The rate of synthesis of the protein by the lymphocytes after being stimulated in vivo has been measured here by the rate of incorporation of ^{3}H-leucine.

Protein synthesis with the treatment of Con A :

With the dose of 10 µg Con A per animal the incorporation of ^{3}H-leucine was highest as it was in case of the incorporation of

^{3}H-TdR (Fig.26 & 27). The incorporation of ^{3}H-leucine was maximum at 48 hours in the cells from both spleen and mesenteric lymph node. The dose of 50 µg Con A per animal could elicit a response with a reasonable peak at 48 hours in case of spleen cells only. The dose of 20 µg Con A was not effective in inducing spleen and lymph node cells to incorporate ^{3}H-leucine.

Protein synthesis with the treatment of PHA :

Similar to the kinetics of blastogenesis and synthesis of DNA and RNA, the synthesis of protein was also not caused to a remarkable degree with the stimulation of PHA (Fig.28 & 29). The all three doses of the substance induced the synthesis of protein in spleen and lymph node cells almost to the same extent, slightly higher than that of the background level. The incorporation of ^{3}H-leucine was slightly higher in spleen cells at 72 hours with the treatment of 50 µg PHA (Fig.28) but not to a significant level.

Protein synthesis with the treatment of FCA :

Kinetics of protein synthesis in spleen and lymph node cells were high after *in vivo* stimulation with the dose of 0.1 ml and 0.3 ml FCA per animal (Fig.30 & 31). There were possibly two peaks, one in between 24 and 48 hours and another at 96 hours. With the treatment of 0.5 ml FCA per animal, incorporation of radioactive

leucine was at peak at 24 hours and was almost at background level at other hours.

CYTOTOXIC FUNCTION OF THE LYMPHOCYTES STIMULATED IN VIVO WITH DIFFERENT MITOGENS

Usually antigenic or mitogenic stimulus drives down the T cells through the path of differentiation to killer cells which can cytolyse the allogeneic target cells. To test whether lymphocytes stimulated in vivo with the different mitogenic substances could also undergo similar differentiation, cytotoxic function of these cells was measured in ^{51}Cr release assay. This assay is based upon the finding that radio-active chromium ions ($^{51}Cr_3O_4^-$) deffusing into a cell retained in the cytoplasm for a considerable period of time. This internal ^{51}Cr is released into supernatant fluid following cell membrane damage caused by cell mediated cytotoxic response of the effector lymphocytes.

In this study allogeneic target cells were pre-labelled by incubation with radioactive sodium chromate ($Na_2{}^{51}CrO_4$) for one and half hours. After necessary washes the target cells were incubated with effector cells, and the amount of radioactivity released into the medium was measured after 6 hours of incubation; the

level of radioactivity released in the supernatant indicated the degree of efficacy of activated lymphocytes to lyse target cells.

Cytotoxic ability of the lymphocytes stimulated with Con A :

Percentages of cytotoxicity of the lymphocytes from the mice treated in vivo with Con A for different hours have been presented in Table 5. Activated cells manifested the cytotoxic ability from 24 hours onward. There was no significant change in the percentage of cytotoxicity with the cells treated for different hours with 10 µg and 50 µg Con A per animal. Notable increase in the cytotoxic ability was observed with the cells treated with 20 µg dose for 48 hours.

Specificity of this cytotoxic reaction was tested by incubating the cells with non-activated and Con A activated syngeneic effector cells (Table 6 & 7). Neither of the cell types could mount any effective level of cytotoxicity. Although the percentages of cytotoxicity with activated cells at 1:10 target and effector ratio in experiment 2 (Table 7) seem to be slightly higher than other counts; these higher counts might not have much significance when they are judged in reference to the other concentrations of effector cells within the experiment.

* This has to be better stated. I presume the exp^t is with effector C57Bl animals activated and non activated. Targets obtained from C57Bl animals (activated).

Cytotoxic ability of the lymphocytes stimulated with PHA :

The dose of 20 µg PHA per animal was employed to generate the effector cells for this experiment as this dose was found marginally better for inducing blast transformation. PHA-generated effector cells at any concentration could not mount a significant level of cytotoxicity against allogeneic target cells as it would be revealed from Table 8. This was true with the cells from the animals treated with PHA for different length of time.

Cytotoxic ability of the lymphocytes stimulated with FCA :

Here cytotoxic ability of the lymphocytes activated *in vivo* by treatment of 0.3 ml FCA per animal has been tested as the dose was capable of inducing higher percentage of blasts and higher level of DNA synthesis than the other doses of this substance. Degree of cytotoxicity caused by these cells was higher than that induced by PHA (Table 9). In both the experiments the percentage of cytotoxicity seemed slightly higher with the cells treated for 24 hours.

STUDY OF THE LYMPHOID CELLS IN SITU AFTER STIMULATION WITH DIFFERENT MITROGENS

As Con A and FCA could induce the lymphoid cells in vivo to transform into blasts and higher rate of synthesis of macromolecules, it was possibly obligatory for this investigation to study the condition and organisation of the lymphoid cells in situ after in vivo activation of the cells. It was done by studying the histological preparations of spleen and mesenteric lymph node obtained from the animals treated with different doses of Con A and an effective dose of FCA for different hours. PHA treatment was not included in this part of the study as it was a poor activating agent for mouse lymphocytes in in vivo.

Condition of the lymphoid cells in situ after stimulation with Con A :

Within 24 hours of the treatment lymphoid cells in the spleen of Con A treated animals look loosely packed in comparison to the normal spleen (Plate 1). The loosely packed nature of the spleen of treated animals is possibly the reflection of gradual transformation of lymphocytes into blast cells which are comparatively bigger and with less denser appearance than the normal lymphoid cells.

Kinetics of blastogenesis with different doses of Con A can also be visualized from the histological preparations of the spleen of the mice injected with 10 µg, 20 µg and 50 µg Con A per animal. By 48 hours, with the treatment of 10 µg Con A, the small cells seems to be transforming into the blasts (Plate 2 Fig. 1) and with 20 µg Con A, the cells are sizewise in heterogenous state- blasts and small lymphocytes can be found (Plate 2 Fig. 2); but with 50 µg dose of Con A, most of the cells transform into blasts giving a picture of uniformed sized cells (Plate 2 Fig. 3). At 96 hours, the cell density in the spleen of the animal treated with 50 µg Con A is lesser than that of the animal treated with 10 µg Con A (Plate 3). Lessening in number of cells possibly begin from 72 hours.

Sensitivity of lymph node cells to Con A may be more than that of the spleen cells as by 48 hours most of the cells in the histological sections differentiated into blasts with all three doses of Con A (Plate 4 compare with Plate 2). Density of the cells in lymph node is notably less from 24 hours onward (Plate 5). Where- as lesser cell density could only be visible in spleen at later hours.

It was interesting to note that with the hyperactivities of the lymphoid cells in terms of metabolic rate, increasing in size

and cell division, there was increase in the size of the secondary lymphoid organs like spleen and lymph node(Table 10). Noticeable increment in size of spleen and mesenteric lymph node could be recorded from 48 hours onward with the treatment of all three doses of Con A.

Condition of the lymphoid cells in situ after stimulation with FCA :

Histological sections of spleen from the animal treated with 0.3 ml FCA for 24 hours show that lymphoid cells start to divide and transforming into blasts mostly at the central part of white pulps and in consequence white pulps look less compact. At 48 hours the number of dividing cells are more and the cells in white pulps appear to be very loosely packed and dispersed in all directions, some of them in good number invaded the sub-capsular region of the spleen (Plate 6 Fig. 1,2). At 72 hours the number of blast cells are lesser than at 48 hours, the white pulps seem to be more compact in comparison to the situation at 48 hours (Plate 6 Fig. 3). Organization of the lymphoid cells in the white pulps returns to the almost normal condition by 96 hours when enormous number of dividing or blast cells cannot be detected.

In general it seems that in vivo treatment of FCA affects the lymph node comparatively to a lesser degree than the spleen.

This is a contrast with Con A treatment where lymph node has been affected more than the spleen. By 24 hours dividing cells can be detected in white pulps of the mesenteric lymph node and some of them are on way to blast transformation (Plate 7 Fig. 1). Number of blast cells seem to be at its peak by 48 hours of treatment giving a picture of dispersion of blast cells from loosely packed white pulps at cortical region towards medulla of the lymph node (Plate 7 Fig. 2). At 72 hours the cell number in white pulps are still higher but in more compact organisation indicating possible wane in the process of blastogenesis (Plate 7 Fig. 3). Then gradually the process of hypercellularity and blastogenesis get back to base level as indicated by the organisation of the cells by 96 hours of treatment (Plate 7 Fig. 4). Apparently severe degree of cell death was not observed in the histological preparations of spleen and lymph node from the animals treated with FCA for different hours.

Similar to the Con A treatment, spleen and mesenteric lymph node increased in size with the injection of 0.3 ml FCA (Table 11). Maximum increment in size could be observed at later hours of the treatment.