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Activation of murine thymocytes *in vivo*. Part I. Study of blastogenesis and DNA synthesis after stimulation with Concanavalin A

TAPAS K. CHAUDHURI AND ASHIM K. CHAKRAVARTY*
Immunology and Cell Biology Laboratory, Centre for Life Sciences, University of North Bengal, P.O. North Bengal University, Darjeeling 734430, India.

Abstract

The present study establishes that a polyclonal stimulator like Concanavalin A can stimulate the murine lymphocytes *in vivo* in different lymphoid organs and peripheral blood. The process of stimulation has been investigated by studying blastogenesis and DNA synthesis of lymphocytes after injecting this substance in mice. Different doses of this substance cause differential response of blastogenesis and DNA synthesis by the lymphocytes. The implications of the findings and the necessity of the study have been discussed.

Key words: *In vivo* activation, thymocytes, Concanavalin A—blastogenesis and DNA synthesis.

1. Introduction

It has been shown earlier that some other substances than specific antigen can stimulate the T cells *in vitro* to perform cell mediated immunological functions[1–7]. The present study has been designed to determine whether a plant lectin like Concanavalin A (Con A) can stimulate the murine lymphocytes (T cells) *in vivo*. The process of stimulation has been investigated by studying blastogenesis and DNA synthesis of the lymphocytes after injecting this substance in mice.

2. Materials and methods

2.1. *Animals*

Inbred C57BL mice were obtained from Cancer Research Institute, Bombay, and maintained in our laboratory with mice-feed and water *ad-libitum*. Eight to twelve weeks old mice were used for all the experiments.

* For correspondence.

2.2. *Blastogenesis*

C57BL mice were injected with five different doses of Con A ranging from 5 μg to 100 μg per animal intravenously (iv). The rate of blast transformation of lymphocytes in different organs like spleen, mesenteric lymph node, other lymph nodes (cervical, axilary and inguinal lymph nodes pooled together) and peripheral blood was recorded at 24 hr interval up to 96 hr. Cell suspensions from different lymphoid organs and the buffy coat of the sedimented perpheral blood in sodium citrate solution were layered on Ficoll and Hypaque solution (Sigma Co., U.S.A., Product No. F8628) and spun down at 3000 r.p.m. for 15 minutes for separation of lymphocytes from RBCs, debris, etc.

The percentage of blasts was counted according to the method described earlier[1]. Briefly, the proportion of transformed or ' blast 'cells was determined from the sum of viable medium plus large lymphocytes divided by the total viable lymphocytes, counted by hemocytometer in presence of trypan blue. The percentage count of blasts was corrected by substracting the percentage of medium and large lymphoid cells in respetive lymphoid organ of normal control mice ; the latter index usually varied from 3 to 6 per cent.

The experiments were repeated several times with different doses except with 100 μg of Con A, with which only two experiments were performed. Only the mean values are presented in the figures.

2.3. *DNA synthesis*

Synthesis of DNA was measured by the rate of incorporation of ^{3}H-thymidine into DNA as described earlier[1].

Three different doses of Con A, 10, 20 and 50 μg were used for *iv* injection and assays were made at 24 hr intervals up to 96 hr. Cells from spleen and mesenteric lymph node of experimental and control animals were collected by using tissue grinder and suspended in Minimum Essential Medium. Cell numbers were adjusted at 4×10^6 cells/ml. Minimum Essential Medium was supplemented with 10% goat serum, nystatin (50 U/ml) and penicillin-streptomycin (50 U/ml). Because of its easy availability goat serum was used instead of fetal calf serum and we observed that the viability and blastoid transformation of murine lymphocytes in the medium containing goat serum were similar as in the medium with fetal calf serum (unpublished observation). Triplicates of 250 μl cell suspension containing 10^6 cells were taken into glass culture tubes. 2μCi ^{3}H-thymidine (sp. Act. 15·8 Ci/mM, Bhabha Atomic Research Centre, Bombay) was used per tube and cells were incubated for 8 hr at 37° C in a humidified atmosphere of 7·5% CO_2 in air. The cultures were terminated by washing with cold phosphate buffered saline and precipitated with cold 10% tricholoacetic acid (TCA). The TCA precipitates were then filtered on small filter papers (Whatman filter

paper no. 3). Each residue was washed with 10 ml of 10% TCA and filter papers were dried and counted in omnifluor-toluene for total radioactivity.

3. Results

3.1. *Blastogenesis*

The dose-wise effects of Con A on blastogenesis of cell population from spleen, mesenteric lymph node, other lymph nodes and peripheral blood are shown in figs. 1a, 2a, 3 and 4 respectively. The peak of blastogenesis with the cells from all the sources irrespective of the dose of Con A, was effectively reached by 48 hr. The peak was delayed 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 are almost at the same level in all the cases. In other three higher doses, the height of the responses correspond to the amount of Con A injected per animal. With 50 μg and 100 μg Con A per animal, percentages of blastogenesis of cells from mesenteric lymph node are higher at initial and subsequent stages.

The blast cells become more vacuolated or exhausted by 72 to 96 hr and increase in the percentage of vacuolated cells corresponds with the higher doses of Con A. The number of dead cells were found to be more in the mesenteric lymph nodes.

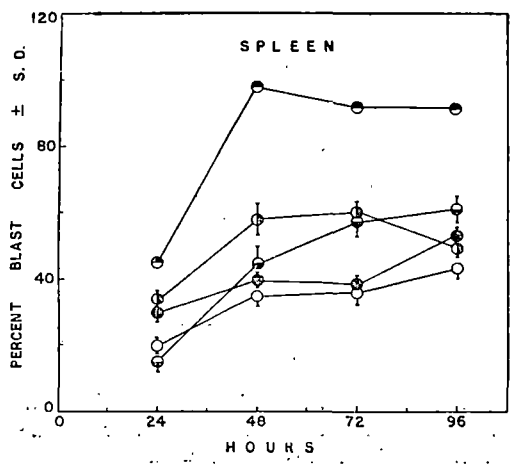

FIG. 1*a*. Kinetics of blastogenesis of lymphocytes from spleen with *in vivo* treatment of different doses of Con A. Dose of Con A per animal: O—O 5 μg; ⊕—⊕ 10 μg; ⊖—⊖ 20 μg; ⊕—● 50 μg and ⊖—⊖ 100 μg. (Same symbols for the different doses of Con A have been used in other figures).

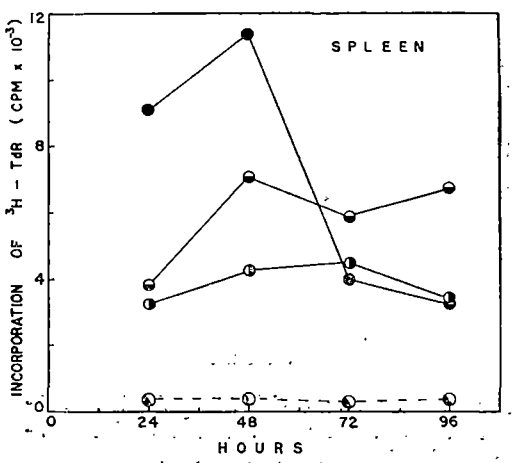

FIG. 1*b*. Pattern of incorporation of ^{3}H-TdR by splenic lymphocytes at different hours after *in vivo* stimulation with different doses of Con A (⊙—⊙ control, without Con A treatment. Same symbols have been used in other figures.).

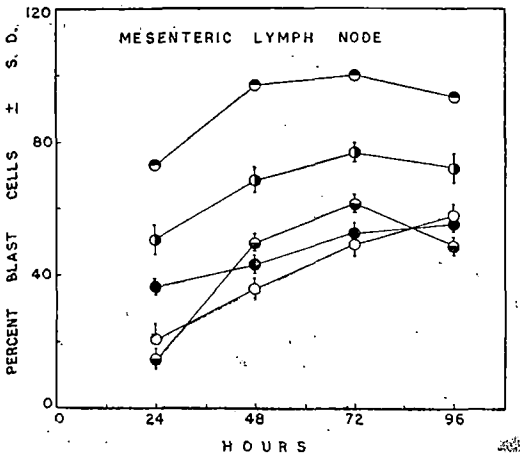

FIG 2*a*. Kinetics of blastogenesis of lymphocytes of mesenteric lymph node with *in vivo* treatment of different doses of Con A.

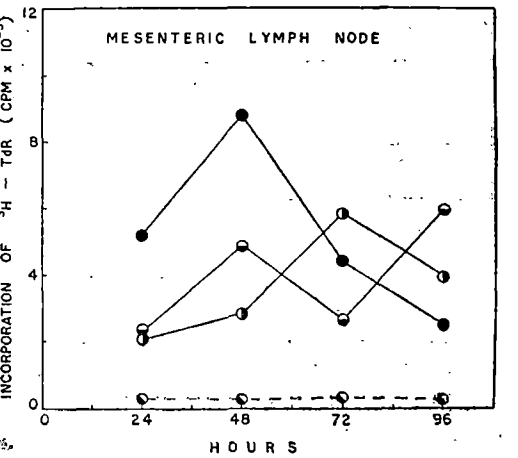

FIG. 2*b*. Pattern of incorporation of 3_H–TdR by lymphocyte of mesenteric lymph node at different hours after *in vivo* stimulation with different doses of Con A.

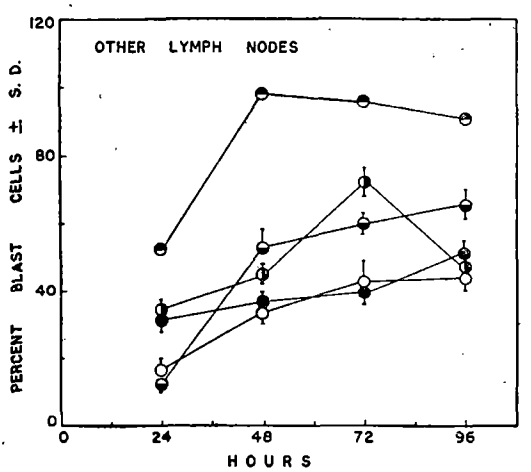

FIG. 3. Percentages of blast obtained at different hours in other lymph nodes with treatment of different doses of Con A.

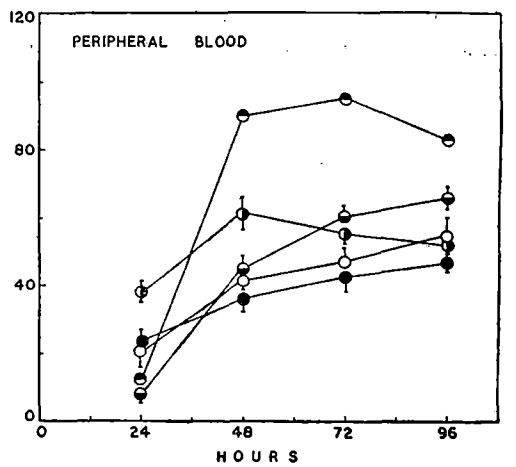

FIG. 4. Percentages of blast obtained at different hours in peripheral blood with treatment of different doses of Con A.

3.2. *DNA synthesis*

Patterns of DNA synthesis by the spleen cells with treatment of three different doses of Con A have been presented in fig. 1b. With all three different doses maximum level of incorporation of radioactive thymidine occurred at 48 hr. 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 synthesis of DNA is higher with lower dose of Con A.

DNA synthesis by cells of mesenteric lymph node follow the pattern as in the case of spleen cells with a few variations (Fig. 2b). Similar to the spleen cells, the rate of synthesis of DNA with 10 μg Con A is higher than that with other doses. But there is not much difference in the height of incorporation of ^{3}H-thymidine (^{3}H-TdR) with the treatment of 20 μg and 50 μg Con A. With the latter dose, the peak of synthesis was at 72 hr. Possibly there are two peaks at 48 hr and at 96 hr with the treatment of 20 μg Con A. Similar tendency has also been observed with the spleen cells (Fig. 1b).

4. Discussion

Our findings indicate that substances like a plant lectin, Con A can stimulate the lymphoid system of mice *in vivo* and cause blastoid differentiation of the lymphocytes. The nature of blast cells and kinetics of differentiation *in vivo* simulate these of *in vitro* treatment of lymphocytes with Con A[6]. In both *in vivo* and *in vitro* set-ups the peak of the responses was around 48 hr. This is interesting for *in vivo* set-up as the substance passes through different systems before coming into contact with the lymphoid cells in secondary lymphoid organs whereas in *in vitro* system the substance gets direct contact with the cells from the very beginning. The *in vivo* blastogenesis with Con A indicates that this substance does not get totally neutralized, digested or excreted before exerting its stimulatory effects to the lymphoid cells. This fact will help one to use this substance *in vivo* for certain specific purposes, especially to stimulate the lymphoid system against some specific antigenic moieties.

Although this substance is generally considered as polyclonal, we and others[1–7] have shown that the lymphoid cells, stimulated with Con A can differentiate into cytotoxic killer cells and can cause lysis of specific target cells. This lysis has deen shown not to depend on the bridging of effector and target cells by tetravalent Con A molecules as the cytotoxic reaction was made in the absence of Con A and in the presence of its competitor α-D-methyl mannoside[1, 8].

It is interesting to note that pretty close to hundred per cent of blats can be obtained from different organs and peripheral blood with 100 μg Con A treatment. As 30–35% T cells are present in spleen and 65–70% of them in lymph nodes and the rest of the lymphocytes are B cells and Con A is supposed to stimulate only the T cells, it is not possible to obtain 100% blastogenesis index without participation of B cells. It is possible that the T cells stimulated with Con A *in vivo* could release certain kind of factors or lymphokines which might cause blastogenic transformation of B cells[9]. There would have been another possibility of making 100% blasts by T cells only if the B cells are already dead. In favour of this possibility we observed (unpublished observation) high percentage of dead cells, more than 50% with 100 μg Con A treatment

(percentage of blasts was counted in reference to viable lymphocytes only as outlined in Materials and methods). We are pursuing certain experiments with neonatally thymectomized mice to resolve these possibilities.

The rate of blastogenesis corresponds to the dose of Con A and was not correlated to the rate of DNA synthesis. Rate of synthesis of DNA was higher with lower dose of Con A (Fig. 1b). As the kinetics of blastogenesis with lymphocytes from different sources after treatment of 5 μg and 10 μg Con A are almost at the same plane and at lower level, it seems that these doses are sub-optimal for blastogenesis. But the 10 μg dose of Con A cause maximum synthesis of DNA by the lymphocytes. Thus it seems that the dose-wise blastogenic response of the lymphocytes might not correlate with their synthesis of DNA.

The higher dose, especially 50 μg Con A, caused low level of ^{3}H-TdR incorporation in our study. This could be as this dose is being supra-optimal for induction of DNA synthesis as similar observation was made by Steen and Lindmo[10] in *in vitro* studies.

Further investigation about cytolytic property of blast cells generated *in vivo* with a polyclonal stimulator like Con A might help to select an effective dose of the substance for generating effector cells for *in vivo* immune responses against different types of antigen.

Acknowledgement

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ACTIVATION OF MURINE LYMPHOCYTES *IN VIVO*: BLASTOGENESIS AND DNA SYNTHESIS AFTER STIMULATION WITH BACTERIAL ADJUVANT

T. K. CHAUDHURI AND A. K. CHAKRAVARTY
Centre for Life Sciences, University of North Bengal, Darjeeling 734 430, India.

ABSTRACT

Freund's complete adjuvant containing killed mycobacteria has been used in this study to stimulate the lymphoid cells *in vivo*. The degree of stimulation with three different doses of this substance was measured in terms of blastogenesis and DNA synthesis by the lymphoid cells in spleen, lymph nodes and peripheral blood. The implication of the findings has been discussed.

INTRODUCTION

IT has been shown by several workers that certain substances other than specific antigen can stimulate the T cells *in vitro* to provide helper factor in antibody response[1,2] and to perform cell mediated immunological functions[3-9]. We have shown that a plant lectin like Concanavalin A (Con A) can stimulate the murine lymphocytes (T cells) *in vivo*[10]. In this investigation, complete Freund's adjuvant known as general stimulator for reticuloendothelial system, has been used for stimulation of lymphocytes *in vivo* in mice. The process of stimulation of lymphoid cells in spleen, lymph nodes and peripheral blood has been investigated by studying blastogenic transformation of the cells; DNA synthesis in the course of blastogenesis has also been measured in the case of spleen and lymph node cells.

MATERAL AND METHODS

Animals

Inbred C57BL mice, obtained from Cancer Research Institute, Bombay and reared in our Centre with mice-feed from Hindusthan Lever Limited., Bombay and water *adlibitum* were used for the experiments. Eight to twleve week old mice were used for all the experiments.

Measure for Blastogenesis

Freund's complete adjuvant (Difco, U.S.A.) containing killed mycobacteria was injected intraperitoneally with three different doses, 0.1, 0.3 and 0.5 ml per animal. The rate of blast transformation of lymphocytes in different organs like spleen, mesenteric lymph node, other lymph nodes (cervical, axillary and inquinal lymph nodes pooled together) and peripheral blood was recorded at 24 hr intervals upto 96 hr. Cell suspensions from different lymphoid organs and the buffy coat of the sedimented peripheral blood in sodium citrate solution were layered on Histopaque (Sigma Co., U.S.A. Product No. F 8628) and spun down at 3000 RPM for 15 minutes for separation of lymphocytes from RBCs, debris, etc.

The percentage of blasts was counted according to the method described earlier[3]. Briefly, the proportion of transformed or 'blast' cells was determined from the sum of viable medium plus large lymphocytes divided by the total viable lymphocytes, counted by hemocytometer in presence of trypan blue. The percentage count of blasts was corrected by subtracting the percentage of medium and large lymphoid cells in respective lymphoid organ of normal control mice; the latter index usually varied from 3 to 6%.

Measure for DNA synthesis

DNA synthesis was measured by the rate of incorporation of ^{3}H-Thymidine into DNA as described earlier[3]. Cells from spleen and mesenteric lymph node of experimental and control animals were collected by using tissue grinder and suspended in minimum essential medium. Cell numbers were adjusted at 4×10^6 cells/ml. Minimum essential medium was supplemented with 10% goat serum, nystatin (50 μ/ml) and penicillin-streptomycin (50 μ/ml). Goat serum was used instead of fetal calf serum as it was easily available. It was observed that viability and blastoid transformation of murine lymphocytes in the medium containing goat serum were similar as in the medium with fetal calf serum (unpublished observation). Triplicates of 250 μl cell suspension containing 10^6 cells were taken into glass culture tubes, 2 μ Ci ^{3}H-thymidine (sp. Act. 15.8 Ci/mM, Bhabha Atomic Research Centre) was used per tube and the cells were incubated for 8 hr at 37° C in a humidified atmosphere of 7.5% CO_2 in air. The cultures were terminated by washing with cold phosphate buffered saline and precipitated with cold 10% trichloroacetic acid (TCA). The TCA precipitates were then filtered on small filter paper (Whatman filter paper no. 3) Each residue was washed with 10 ml of 10% TCA and filter papers were dried and counted in omniflour-toluene for total radioactivity.

TABLE 1

Percentage of blast cells from different Lymphoid organs and Peripheral blood after injecting different doses of Freund's complete Adjuvant in Mice

Adjuvant Dose per animal	0.1 ml				0.3 ml				0.5 ml			
Hours	24	48	72	96	24	48	72	96	24	48	72	96
Spleen	37.5 ±1.4	43.8 ±2.7	50.0 ±3.1	45.4 ±3.0	46.8 ±1.6	51.2 ±1.9	54.6 ±3.0	40.6 ±2.6	38.4 ±2.3	47.3 ±1.8	50.3 ±1.7	44.9 ±0.9
Mesenteric lymph node	40.3 ±2.4	47.1 ±2.0	50.9 ±2.4	44.0 ±1.1	44.4 ±1.6	54.7 ±1.5	57.7 ±1.4	41.2 ±4.7	44.1 ±1.8	53.2 ±1.8	51.6 ±1.4	48.3 ±1.8
Other lymph nodes	35.7 ±1.8	44.9 ±2.0	51.6 ±0.6	46.2 ±2.6	40.6 ±5.7	49.2 ±8.4	52.5 ±0.9	46.2 ±1.8	46.0 ±1.0	51.8 ±1.1	47.1 ±1.8	42.5 ±2.2
Peripheral Blood	38.9 ±1.9	48.7 ±1.8	55.0 ±1.7	50.9 ±2.3	43.6 ±0.6	51.2 ±0.4	51.5 ±0.6	44.1 ±1.6	39.9 ±1.7	46.0 ±1.0	47.4 ±1.4	45.7 ±2.0

Control Experiment—Incomplete Adjuvant 0.3 ml injected per animal:
Percentages of blasts in different organs at different hours varies from 4% to 7%.

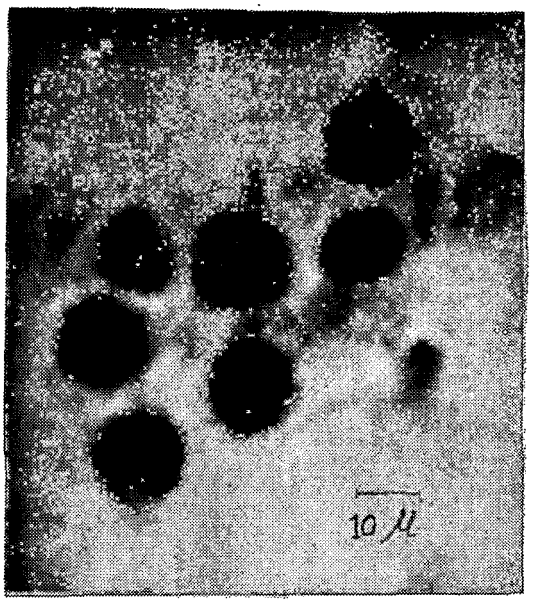

Figure 1. Photomicrograph of the blast cells at 48 hours. Cells were in suspension and stained with Delafield's haematoxylin.

Results and Discussion

Blastogenesis

A good number of lymphoctyes transform into blasts from 72 hr onward with intraperitoneal injection of the complete adjuvant. The blastogenesis peak is reached between 48 and 72 hr in lymphocytes (figure 1) from all the sources with all three doses of Freund's complete adjuvant (table 1). The differences between initial and maximal responses is marginal. The blast cells induced with adjuvant are not too much vacuolated or exhausted by 72 to 96 hr as in case of Con A induced blasts[10].

DNA synthesis

DNA Synthesis by the cells of spleen and mesenteric lymph node have been presented in figures 2 and 3 respectively. In both cases, the dose-dependent patterns of DNA synthesis are similar. Treatment with 0.3 ml adjuvant caused the maximum incorporation of radioactive precursors in DNA. It seems that there could be two cycles of DNA synthesis in course of 96 hr, the first peak might be around 24 hr. This is more evident with the dose of 0.3 ml adjuvant per animal.

In the present investigation, it was observed that complete adjuvant containing killed *Mycobacterium* can stimulate the lymphoid system of mice *in vivo* and cause blastoid differentiation of the lymphocytes. It

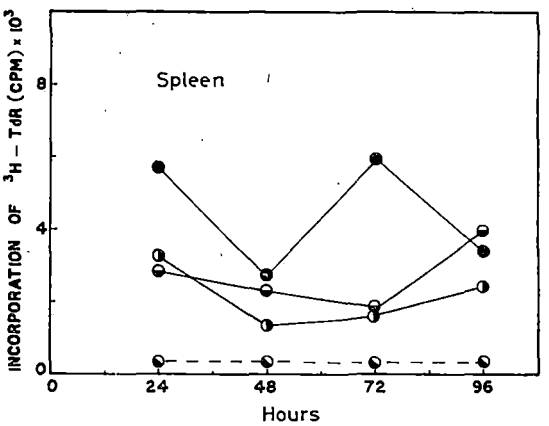

Figure 2. Pattern of incorporation of ^{3}H-TdR by splenic lymphocytes at different hours after *in vivo* stimulation with different doses of Freund's complete adjuvant.

Doses of adjuvant per animal: ◑—◑ 0.1 ml; ●—● 0.3 ml and ◒—◒ 0.5 ml.

Control, without adjuvant: ◔—◔ (same symbols for the different doses of adjuvant and control have been used in other figures).

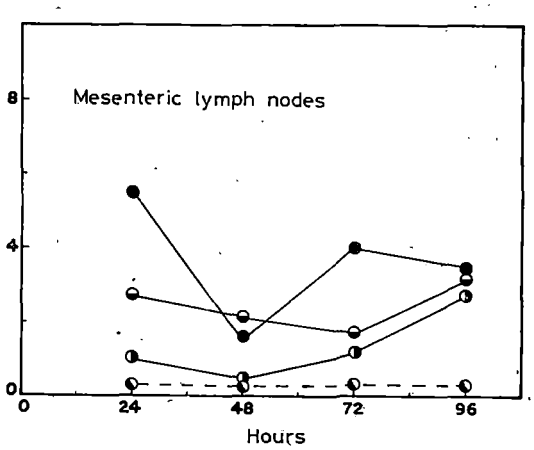

Figure 3. Pattern of incorporation of ^{3}H-TdR by lymphocytes of mesenteric lymph node at different hours after *in vivo* stimulation with different doses of Freund's complete adjuvant.

was known that complete Freund's adjuvant potentiate a given antigen when injected along with the antigen[11,12]. It has also long been recognized that certain microorganism or bacterial extracts increase the host's resistance to a variety of unrelated bacterial or viral infections by stimulating reticulo-endothelial system (RES) and also stimulate immunity against

tumours[13-18]. Different parameters for stimulation of RES like increment in the weight of spleen and liver, phagocytic activity of the RE cells, rate of antibody synthesis, resistance to viral infection with introduction of *Corynebacterium parvaum* in mice have been studied earlier[19,20]. The present investigation provides two parameters, blastogenesis and DNA synthesis to measure the stimulation of lymphoid cells with adjuvant at cellular level.

Furthermore, this study will possibly help to initiate a programme to test the immunologic response of the blast cells activated by the Freund's complete adjuvant containing killed bacteria. It has been shown that although Con A is a polyclonal stimulator, Con A induced blasts can mount cytotoxic response against allogeneic targets including tumour cells[5]. It remains to see whether blasts, induced by Freund's complete adjuvant can have cytolytic property.

This knowledge will possibly help to explain the causative mechanism for host's resistance to unrelated bacterial and viral infections or immunity to tumours[13-18] with injection of microorganisms or bacterial extracts.

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Short Communication

CORRELATION OF BLASTOGENESIS AND DNA SYNTHESIS BY THE MURINE LYMPHOCYTES DURING IN VIVO ACTIVATION WITH CONCANAVALIN A

ASHIM K. CHAKRAVARTY and TAPAS K. CHAUDHURI

Immunology and Cell Biology Laboratory, Centre for Life Sciences, University of North Bengal, Siliguri, Dist. Darjeeling, Pin 734430, India

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Immunology and Cell Biology Laboratory, Centre for Life Sciences, University of North Bengal, Siliguri, Dist. Darjeeling, Pin 734430, India

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SUMMARY: Blastogenesis and DNA synthesis of murine T cells were studied after in vivo stimulation with Concanavalin A; blastogenic response does not dose-wise correlate with the rate of DNA synthesis although these two responses are often considered synonymous.

It has been shown earlier that Concanavalin A (Con A), a plant lectin, can activate murine T cells in vitro to perform cell mediated immune response (Chakravarty and Clark, 1977; Heininger et al., 1976; Bevan, Langman and Cohn, 1976). We have also shown that Con A can stimulate the lymphocytes in vivo when injected intravenously (Chaudhuri and Chakravarty, 1982). For the measure of activation of the lymphocytes, the index of blastogenesis and DNA synthesis by the activated cells are usually taken into consideration. In the course of our in vivo studies, we observed that the rate of blastogenesis sometimes does not correlate with the kinetics of DNA synthesis. This will be discussed here.

Inbred C57BL mice of 8–12 weeks of age were used throughout the study. Three different doses of Con A (Sigma Co., St. Louis, MO, type IV), 10, 20 and 50 μg per animal were injected intravenously; assays for blast transformation and DNA synthesis of activated lymphocytes were made at 24-hr intervals up to 96 hr. Spleen and mesenteric lymphnode of Con A-injected mice and normal control mice were collected and cell suspensions were made separately; lymphocytes were separated form RBCs and debris by using Ficoll and Hypaque (Sigma Co., St. Louis, MO) gradient as outlined elsewhere (Chaudhuri and Chakravarty, 1982).

The proportion of transformed or 'blast' cells was estimated from the sum of viable medium and large lymphocytes divided by the total viable lymphocytes, counted with a hemocytometer in the presence of trypan blue by the method described earlier (Chakravarty and Clark, 1977).

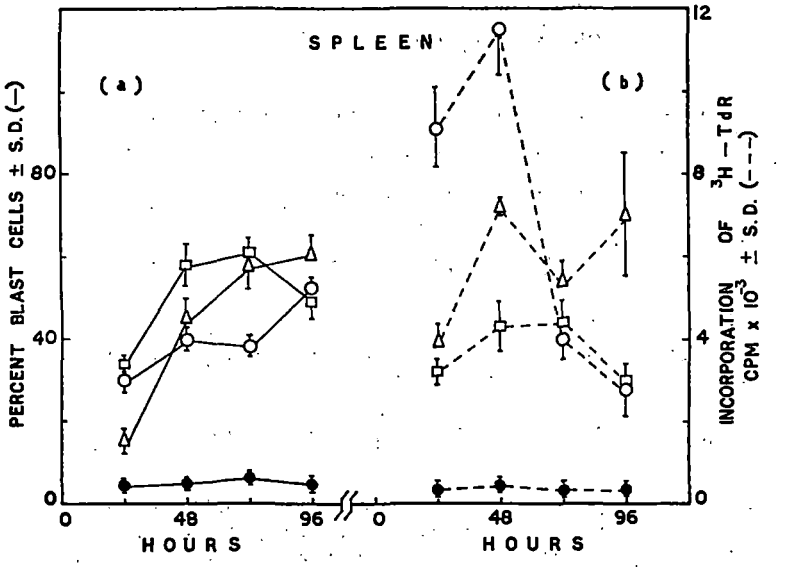

Fig. 1. Rates of (a) blastogenesis and (b) DNA synthesis of lymphocytes from the spleen of the mouse after in vivo stimulation with 10 μg (○), 20 μg (△) and 50 μg (□) of Con A per animal. Control, without Con A treatment (●). Three to four mice were used per point.

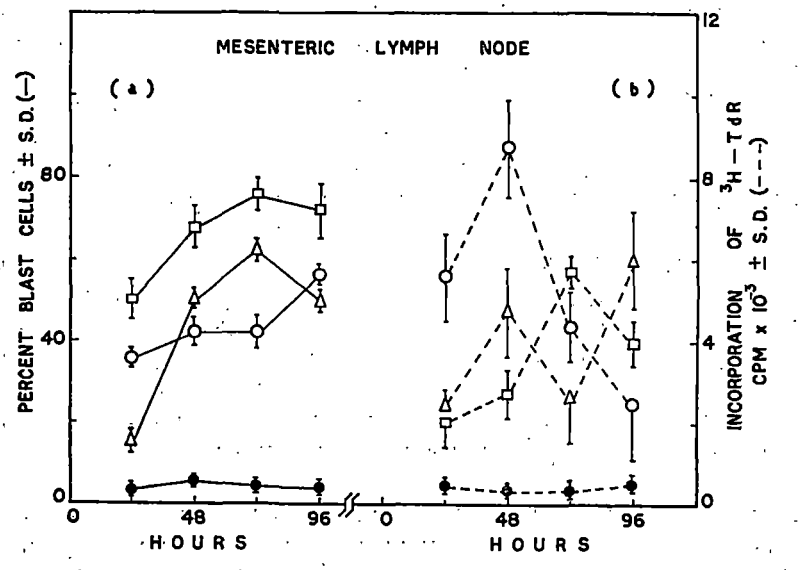

Fig. 2. Rates of (a) blastogenesis and (b) DNA synthesis of lymphocytes from the mesenteric lymph node of the mouse after in vivo stimulation with different doses of Con A (symbols are same as in Fig. 1). Three to four mice were used per point.

For measuring DNA synthesis, triplicates of 250 μl of a cell suspension containing 10^6 cells were taken into glass culture tubes. ^{3}H-Thymidine (sp.act. 15.8 Ci/mM) of 2 μCi was used per tube and cells were incubated for 8 hr at 37 C in a humidified atmosphere of 7.5% CO_2 in air. The cultures were terminated by washing with cold phosphate buffered saline and precipitated with cold 10% trichloroacetic acid (TCA). The TCA precipitates were then filtered on small filter paper. Each residue was washed with 10 ml of 10% TCA and the filter papers were dried and counted in omnifluor-toluene for total radioactivity.

Data of one representative experiment are presented in the figures. Each experiment was repeated three times and they showed similar trends.

The peak of blast transformation with the dose of 50 μg Con A was effectively reached between 48 to 72 hr with lymphocytes from both spleen and mesenteric lymph node. The peak response was somewhat slower with the lower dose of Con A. The height of the response curves for blastogenesis corresponded to the amount of Con A injected per animal (Fig. 1a and 2a). On the other hand, the rate of DNA synthesis by the lymphocytes was at the highest level with 10 μg of Con A, higher than those with the other two higher doses of Con A and the peak response was at 48 hr. The highest dose of 50 μg of Con A per animal caused the maximum level of blastogenesis but a low level of ^{3}H-TdR incorporation. This was true in case of lymphocytes from the spleen as well as from the mesenteric lymph node (Fig. 1b and 2b). Our observations indicate that the in vivo blastogenic response of the lymphocytes did not dose-wise correlate with their rate of DNA synthesis.

Blastogenesis and DNA synthesis are often considered synonymous for indexing blast transformation, but this may not always hold true as we found in this study. We also observed earlier that blocking DNA synthesis with hydroxyurea has essentially no effect on Con A-mediated in vitro blastogenesis (Chakravarty and Clark, 1977); similar observations were made by Nedrud, Touton and Clark (1975) in the course of MLC reaction and blocking of DNA synthesis.

Blastogenesis is microscopically visual reflection of a myriad of complex biochemical events following stimulation with a mitogen like Con A or antigen. These biochemical events include such early membrane-related phenomena as increased permeability to divalent cations, activation of adenyl cyclase and consequent increase in intracellular cAMP. Then syntheses of protein, RNA and DNA occur; it is the synthesis of DNA that eventually results in cell division. Thus it seems that a certain dose of a mitogen like 50 μg of Con A per animal might stimulate the earlier events of blastogenesis to proceed faster and at a higher level but for some reasons DNA synthesis is not been augmented to the same level. This could be the physiological state of the cells after initial reactions, which does not allow DNA synthesis to proceed at faster rate. We have observed that with increase in the dose of Con A and the corresponding higher rate of blastogenesis, the blast cells became more vacuolated and exhausted which were more pronounced by 72 to 96 hr; these could be the signs of dying cells. Steen and Lindmo (1979) have also shown that when human lymphocytes were

stimulated with a hyper-optimal dose of 100 μg Con A/ml, the cellular and nuclear volume increased but there was inhibition of incorporation of radioactive thymidine.

Acknowledgement

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STUDY OF MURINE LYMPHOID CELLS *IN SITU* AFTER STIMULATION WITH CONCANAVALIN A

TAPAS K. CHAUDHURI and ASHIM K. CHAKRAVARTY

Immunology and Cell Biology Laboratory, Centre for Life Sciences, University of North Bengal, Siliguri, Dist. Darjeeling, Pin: 734430, India

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TAPAS K. CHAUDHURI and ASHIM K. CHAKRAVARTY

Immunology and Cell Biology Laboratory, Centre for Life Sciences, University of North Bengal, Siliguri, Dist. Darjeeling, Pin: 734430, India

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SUMMARY: The effects of Concanavalin A (Con A) on the lymphocytes *in situ* were investigated here by studying the blastogenesis and organization of lymphoid cells in the histological preparations of the spleen and the mesenteric lymph node and the cell death at different hours after iv administration of different doses (10, 20 and 50 μg/animal) of the substance. The gradual transformation of the lymphocytes into blasts was observed in the histological preparations of the spleen and the mesenteric lymph node. The rate of the change became higher with higher dose of Con A. It seems that the lymph node was affected more with in vivo Con A treatment than the spleen on account of higher percentage of blast transformation at early hour and lower density of the cells due to cell migration or cell death. Purpose of this study has been discussed.

INTRODUCTION

It has been shown earlier that Concanavalin A (Con A), a plant lectin, can induce the murine T lymphocytes in vitro to transform into blasts and become cytotoxic to nonself target cells (Clark, 1975; Bonavida, 1977; Chakravarty and Clark, 1977). We have also shown that Con A can activate the lymphocytes in vivo and that kinetics of blastogenesis and DNA synthesis of the in vivo stimulated cells are parallel to those of in vitro activated cells (Chaudhuri and Chakravarty, 1981). Thus, it seemed that Con A, a polyclonal activator, could be used for in vivo stimulation of the lymphoid cells for certain specific purposes like mounting cytotoxic response to the target cells with unknown antigenic character, such as malignant cells. Before contemplating the in vivo use of Con A, it is possibly obligatory to know the effect of Con A on the lymphocytes *in situ*. The present study attempted to resolve this point by precisely analyzing the histological sections of the spleen and the mesenteric lymph node at different hours after iv administration of different doses of Con A.

MATERIALS AND METHODS

Animals: Inbred C57BL mice were obtained from Tata Cancer Research Institute, Bombay and maintained in our laboratory with mouse feed and water *ad libitum*. Eight to 12 weeks old mice were used for all the experiments.

Histological preparation: Different doses of Con A (Sigma Co., USA, type IV), 10, 20 and 50 μg per animal, were injected intravenously. The mice were sacrificed at different hours like 24, 48, 72 and 96 hr. Spleen and mesenteric lymph nodes were fixed in Bouins fixative, dehydrated in a graded alcohol series, cleared in xylene, and embedded in paraffin. Five- to 6-μm thick sections were stained with Delafield's hematoxylin and eosin and viewed under a binocular microscope, and photographed when necessary. The subcapsular cortical regions of the histological sections of the spleen and the lymph node were mostly considered for photomicrographs as the majority of the white pulps or congregations of the lymphocytes were located at this site of these organs.

Cell viability test: Different doses of Con A (10, 20 and 50 μg)-injected mice were sacrificed at different hours (24, 48, 72 and 96 hr) and the spleen and the mesenteric lymph nodes were collected. The cells were dissociated from the respective tissue through stainless steel grids. The resultant suspension in phosphate buffered saline (PBS) was repeatedly passaged through a 27-gauge needle mounted on a syringe to obtain a uniform cell suspension. The spleen cell suspension was centrifuged and 0.85% ammonium chloride was added for removing RBCs. The cells were then washed twice with PBS and resuspended in Minimum Essential Medium (MEM). The mesenteric lymph node cells suspended in PBS were centrifuged and resuspended in MEM. The percentage of viable cells was counted with a hemocytometer in the presence of trypan blue. To know the possible percentage of dead cells in the leukocyte population not subjected to mechanical stress for cell separation, peripheral blood was collected in a sodium citrate solution and after removal of RBCs with 0.85% NH_4Cl was washed twice with PBS. The percentage of viable cells in this cell population was determined as indicated earlier.

Results

Within 24 hr, the cells in the spleen of Con A-treated animals looked loosely packed in comparison with those in the normal spleen (Fig. 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 dense appearance than the normal lymphoid cells.

The kinetics of blastogenesis with different doses of Con A has been measured earlier and it was noted that the rate of blastogenesis increased with the increment of the dose of Con A (Chaudhuri and Chakravarty, 1981). This fact was also visualized with the histological preparations of the spleen of the mice injected with 10-μg, 20-μg, or 50-μg Con A per animal. By 48 hr, with the treatment with 10-μg Con A, the small cells seemed to be transforming into the blasts and with 20-μg Con A, the cells were sizewise in heterogenous state—blasts and small lymphocytes were found; but with a 50-μg dose of Con A, most of the cells transformed into blasts giving a picture of uniform-sized cells (Fig. 2).

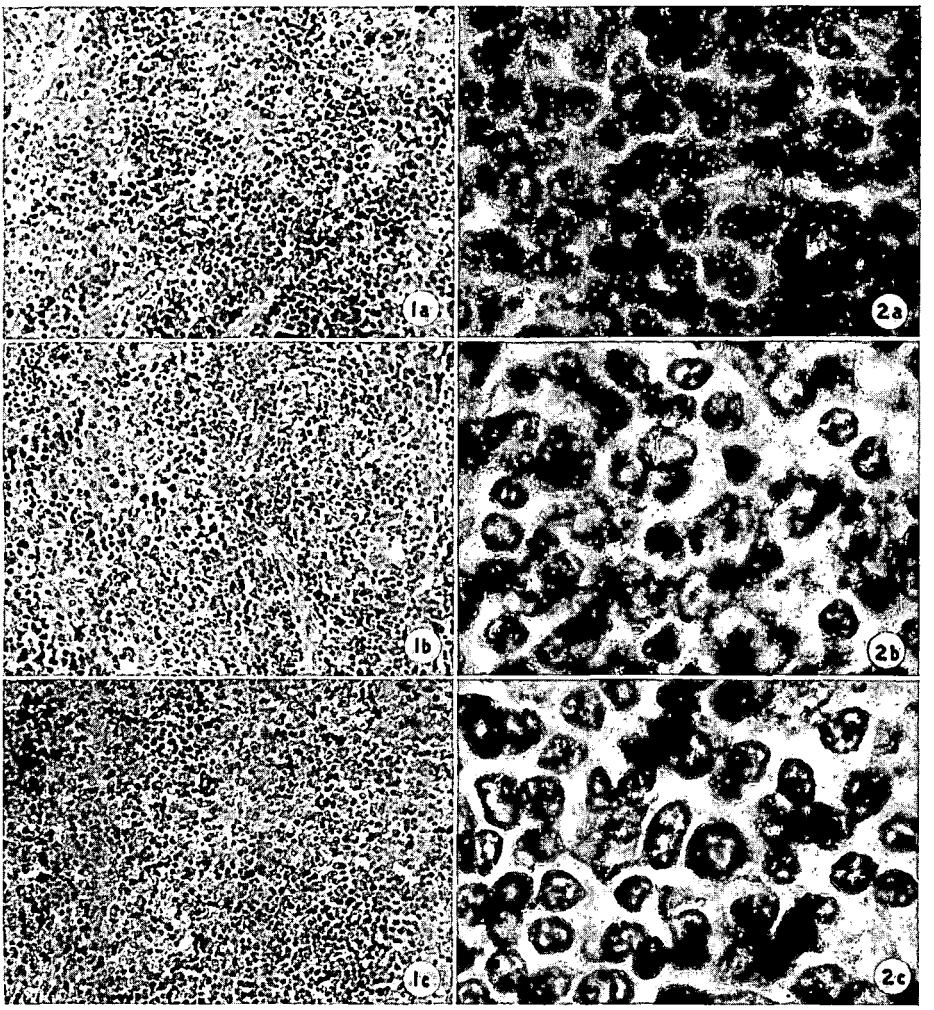

Fig. 1. Photomicrographs showing the organization of lymphoid cells in (a) normal spleen (b) 10-μg Con A-treated spleen (c) 20-μg Con A-treated spleen at 24 hr. Note the loosely packed nature of the spleen of treated animals. $\times$500

Fig. 2. Photomicrographs of the histological sections of the spleen from the animals treated with (a) 10-μg Con A, (b) 20-μg Con A, and (c) 50-μg Con A per animal at 48 hr showing the increased rate of blast transformation with increment of the dose of Con A. $\times$3000

Fig. 3. Photomicrographs of the histological sections of the mesenteric lymph node from the animals treated with (a) 10-μg Con A, (b) 20-μg Con A, and (c) 50-μg Con A per animal showing the differentiation of the cells into blast with all three doses of Con A by 24 hr. $\times$3000

Fig. 4. Photomicrographs of the histological sections of the spleen from the animals treated 96 hr before with (a) 10-μg Con A, (b) 50-μg Con A showing the lower cell density by 50-μg Con A treatment. $\times$1500.

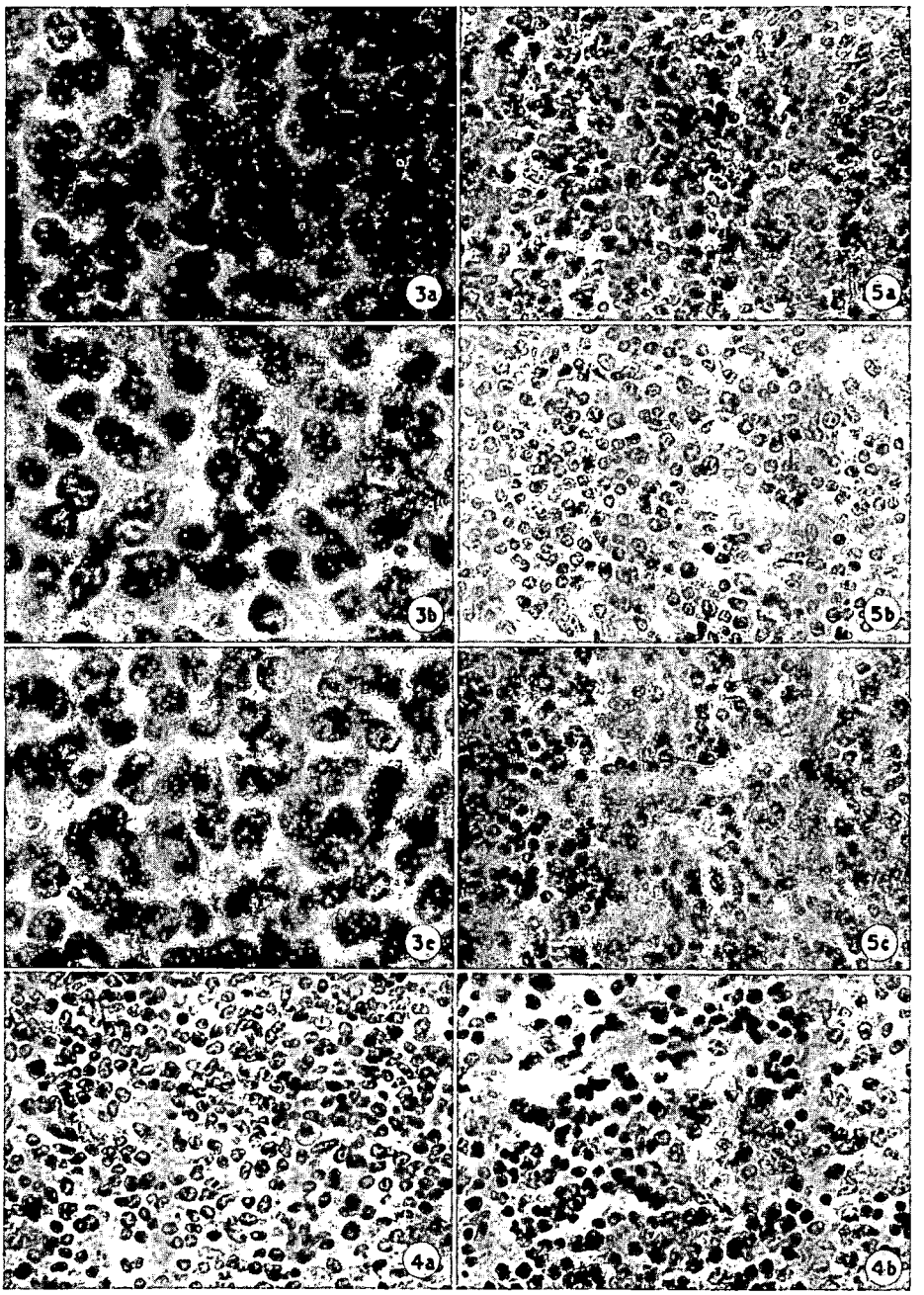

Fig. 5. Photomicrographs of the histological sections of the mesenteric lymph nodes from the animals treated with (a) 10-μg Con A, (b) 20-μg Con A, and (c) 50-μg Con A per animal at 24 hr showing the lower cell density by 50-μg Con A treatment. $\times$1500

The sensitivity of lymph node cells to Con A may be higher than that of the spleen cells. In comparison with the spleen, a higher percentage of blasts in the mesenteric lymph node with in vivo treatment with Con A has also been recorded within 24 hr (Chaudhuri and Chakravarty, 1981) and by 48 hr most of the cells in the histological sections differentiated into blasts with all three doses of Con A (Fig. 3, compare with Fig. 2). We also observed in this study that at 96 hr, the cell density in the spleen of the animal treated with 50-μg Con A was lower than that of the animal treated with 10-μg Con A (Fig. 4). Such picture was seen from 72 hr onward, whereas the cell density in the lymph node was notably low from 24 hr onward (Fig. 5). Such lower density of the cells in lymphoid organs could be for two different reasons. The cells transformed at a higher rate with a higher dose of Con A migrated out of the spleen and the lymph node into the circulation or some of the cells died off with a higher dose of Con A. Although existence of blast cells in the blood vessels of the spleen and the lymphatic spaces of the lymph node were observed and the percentage of blasts in peripheral circulation was significantly high (Chaudhuri and Chakravarty, 1981), further experiments can only prove the first possibility.

Regarding the question of cell death by Con-A treatment, we counted the dead cells in the suspension of lymphoid cells from the spleen, the lymph node and the peripheral blood. A considerably high percentage of dead cells was observed in the suspension of the spleen and the lymph node (Fig. 6); the index was slightly higher with the mesenteric lymph node cells of the animals treated with 50-μg Con A. However, more cell death with a higher rate of blastogenesis in the spleen and the mesenteric lymph node could be due to delicate condition of the cell membrane of the blasts and their susceptibility to the stress of mechanical separation of the cells from the spleen and the lymph nodes. This

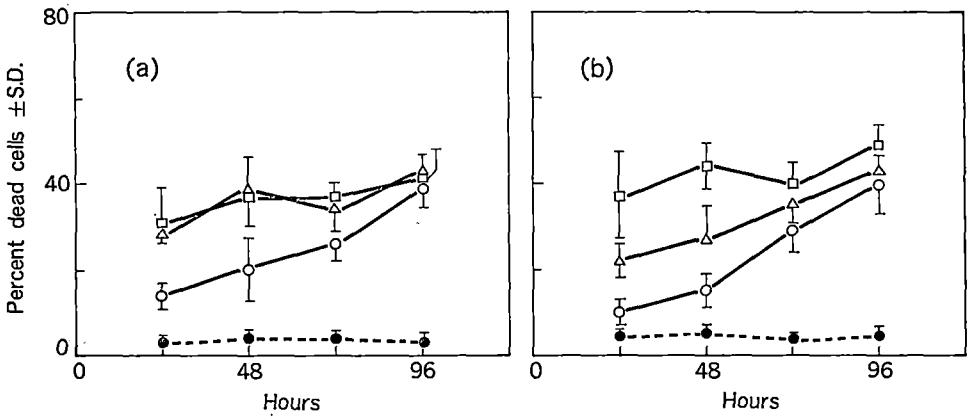

Fig. 6. Percentage of dead cells obtained at different hours (a) in the spleen and (b) in the mesenteric lymph node by in vivo treatment with different doses of Con A. Dose of Con A per animal: ○——○ 10 μg, △——△ 20 μg and □——□ 50 μg; ●——● control, without Con A treatment. (The same symbols for the different doses of Con A were used in Fig. 7).

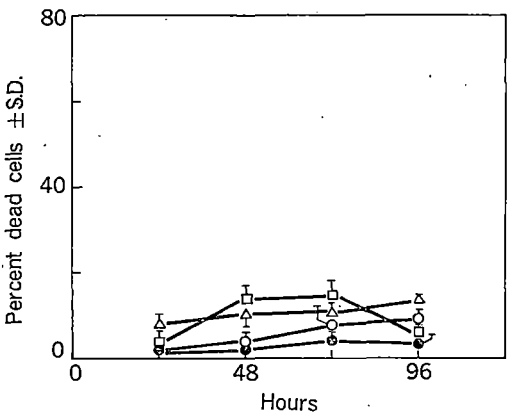

Fig. 7. Percentage of dead cells at different hours in peripheral blood after treatment with different doses of Con A.

seems more plausible when we take into account the lower percentage of dead cells in the leukocyte cell population of the peripheral blood which was collected without any mechanical impact of the tissue grinder or of passage through the syringe. The percentage of dead cells in peripheral blood samples was pretty close to the background count (Fig. 7).

Discussion

It is apparent that the lymph node was affected more with in vivo Con A treatment than the spleen on account of a higher percentage of blast transformation at early hour and a lower density of the cells due to cell migration or cell death. This could be because 65 to 70% of T cells are present in the lymphnode and only 30 to 35% of lymphoid cells are T cells in the spleen; Con A does affect mostly the T cells. Another factor might also be coupled with this. The density of Con A receptors is comparatively higher on the cells of lymph nodes than that on splenocytes, but no such evidence has yet been available. Thus, it seems that in vivo administration of such a T cell mitogenic substance as Con A does not cause adverse effects to the lymphocytes *in situ*.

Elsewhere we and others indicated that in vitro Con A-stimulated T cells can perform as cytotoxic cells against the target with non-identical H-2 antigen (Bevan, Langman and Cohn, 1976; Heininger et al., 1976; Chakravarty and Clark, 1977). In preliminary cytotoxicity experiments we observed that the lymphocytes treated in vivo with Con A were capable of mounting cytotoxic reactions. Thus it will be an interesting proposition to activate the T cells in vivo with a dose of such a polyclonal agent as Con A having less adverse effect on the cells and lymphoid organs so that the polyclonally activated cells will differentiate into cytotoxic cells to mount lytic reaction to the target cells bearing nonself antigen. Some experiments in this direction are already under

way in our laboratory utilizing chemically induced fibrosarcoma as the target.

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