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SELECTIVE DEPLETION OF LYMPHOID TISSUE BY CYCLOPHOSPHAMIDE

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SUMMARY

Selective depletion of lymphocytes from the lymph follicles and cortico-medullary junction in lymph nodes and equivalent non thymus dependent areas of the spleen can be produced by cyclophosphamide (CY) (300 mg/kg) in the mouse and guinea-pig. Despite three such injections on alternate days, thymus dependent areas still contained lymphocytes. Total depletion of lymphocytes from lymph nodes and spleen was produced by combining neonatal thymectomy in the mouse or ALS treatment in the guinea-pig with CY. CY produced depletion of lymphocytes in the cortex of the thymus before the medulla. Maximal depletion occurred at 3 days and in surviving animals repopulation was evident by 7 days at the cortico-medullary junction only. Lymph follicles were found in lymph nodes of neonatally thymectomized CY treated mice following repopulation with bone marrow. These findings suggest that the lymphocytes of the lymph follicles are derived from a population of rapidly dividing cells, part of which at least can be found in the bone marrow.

INTRODUCTION

The selective depletion of lymphoid tissue by neonatal thymectomy has been described by Parrott, de Sousa & East (1966). A similar effect is found following treatment with anti-lymphocyte serum (Turk & Willoughby, 1967). The depletion of lymphocytes from the paracortical areas of lymph nodes and the area of the white pulp of the spleen round the central arterioles is associated with the depletion of the mobile pool of long-lived small lymphocytes, considered to be influenced by the thymus in late foetal or early neonatal life (Denman, Denman & Embling, 1968; Martin & Miller, 1968). Proliferation of lymphocytes in the paracortical area of lymph nodes occurs during the development of a cell-mediated immune response (Oort & Turk, 1965) whereas the germinal centre formation in lymph follicles and proliferation of plasma cell precursors at the cortico-medullary

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junction is a feature of humoral antibody production. Thus lymphoid tissue would appear to contain two compartments of lymphocytes, that associated with cell-mediated immunity and that associated with humoral antibody production. The areas of lymphoid tissue associated with cell-mediated immunity are depleted of lymphocytes selectively by techniques designed to reduce the mobile pool of long-lived small lymphocytes. Cyclophosphamide (CY) was selected for investigation as a compound which would reduce the pool of short-lived small lymphocytes, in view of its strong mitostatic activity. It was therefore possible that this compound might deplete those areas of lymphoid tissue populated by short-lived as opposed to long-lived lymphocytes and which are not under the influence of the thymus in neonatal life. As it is suggested that these cells are continuously replaced from the bone marrow (Davies, 1969), it was also considered of interest to examine the pattern of repopulation of lymphoid tissue by bone marrow-derived cells in animals whose total lymphocyte pool had been depleted by treatment with both thymectomy and cyclophosphamide.

MATERIALS AND METHODS

Animals

Syngeneic C_3H/Bi mice or outbred Hartley strain guinea-pigs were bred in the department and used throughout. The mice were fed on pelleted diet FFG(M) and the guinea-pigs on pelleted diet RGP (E. Dixon & Sons, Ware, Herts.). The guinea-pig diet was supplemented liberally with cabbage and hay. A proportion of the mice were thymectomized at birth (Parrott *et al.*, 1966) and allowed to grow up in a quiet dark room. In all experiments care was taken to have at least five survivors in each group, where a histological assessment was obtained.

Preparation of anti-lymphocyte serum (ALS)

ALS was prepared in rabbits against guinea-pig lymph node and thymus cells, absorbed with guinea-pig erythrocytes (Turk, Willoughby & Stevens, 1968) and sterilized by membrane filtration or ultracentrifugation. Each batch of ALS was tested for potency by its ability to deplete paracortical areas of lymph nodes of small lymphocytes following a 6-day course of 1 ml of ALS intraperitoneally.

Treatment with cyclophosphamide

Cyclophosphamide, 'Endoxana', was kindly given by Ward Blenkinsop and Co. Ltd., Wembley, Middlesex. It was injected dissolved in 0·15 M NaCl in a dose of 300 or 400 mg/kg intraperitoneally. The 300 mg/kg dose was injected singly or repeated three times with intervals of 48 hr. Animals were killed 3 days after the last injection of CY when the peripheral lymphocyte count was at its lowest (Fig. 1). Mice given one injection of 300 mg/kg were also killed 7 days after injection when their peripheral lymphocyte count was returning to normal. Mice thymectomized neonatally 6 weeks previously were also treated with one dose of CY at 300 mg/kg and killed 3 days after the injection.

The action of CY on guinea-pigs treated with ALS was examined by injecting 300 mg/kg on the seventh, fifth and third days before they were killed. At the same time the animals also received daily injections of 1 ml ALS intraperitoneally for a 6-day period.

The survival of mice and guinea-pigs treated with the various schedules is shown in Table 1.

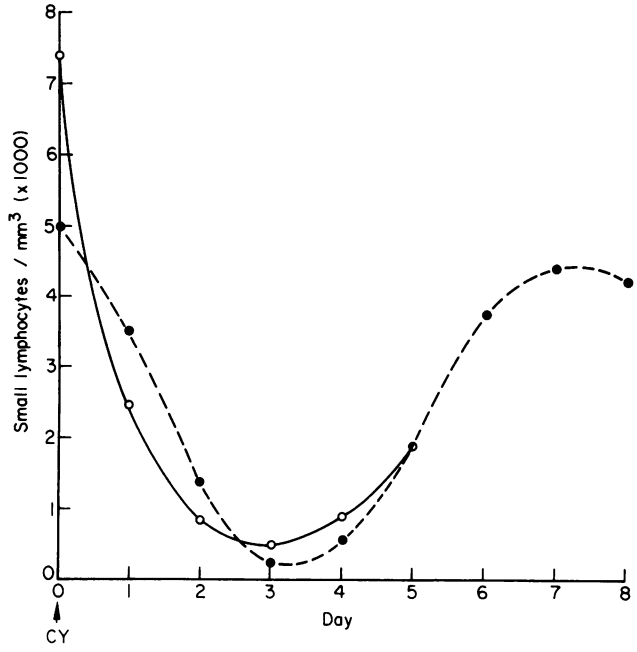


FIG. 1. The effect of one injection of CY (300 mg/kg) on the circulating small lymphocyte count in the mouse (●) and guinea-pig (o).

TABLE 1. Survival of mice and guinea-pigs treated with cyclophosphamide with or without neonatal thymectomy or ALS treatment and the effect of repopulation with bone marrow

Species	Dose of CY (mg/kg)	Other treament	7-Day survival
Mouse	1 × 300	—	15/19
	3 × 300	—	3/15
	1 × 300	TX	3/21
	1 × 300	TX + BM	9/10
	1 × 400	—	2/17
	1 × 400	BM	5/5
Guinea-pig	1 × 300	—	5/5
	3 × 300	—	8/8
	3 × 300	ALS	4/7

TX = neonatally thymectomized mice; BM = mice transfused with 5×10^6 bone marrow cells 9 hr after injection of CY; ALS = 6 day treatment with 1 ml anti-lymphocyte serum.

Transfusion of syngeneic bone marrow in mice

Bone marrow was flushed from donor femurs with tissue culture medium 199 (Burroughs Wellcome), pH 7·4, containing penicillin and streptomycin. Syngeneic recipient mice were injected intravenously with 5×10^6 nucleated cells from donors of the same sex. Bone marrow cells were injected 9 hr after one dose of cyclophosphamide. Thymectomized animals similarly treated with CY were injected with bone marrow cells 9 hr after the injection. A group of thymectomized animals which had not been treated with cyclophosphamide was also injected with bone marrow cells. Mice were killed 1 week after the injection of bone marrow and their lymphoid tissues taken for histological examination.

Preparation of tissues for histology

Cervical, axillary and mesenteric lymph nodes, thymus and spleen were removed by autopsy. The tissues were fixed in Carnoy's solution, sectioned at 5 μ and stained with haematoxylin eosin and pyronin methyl green.

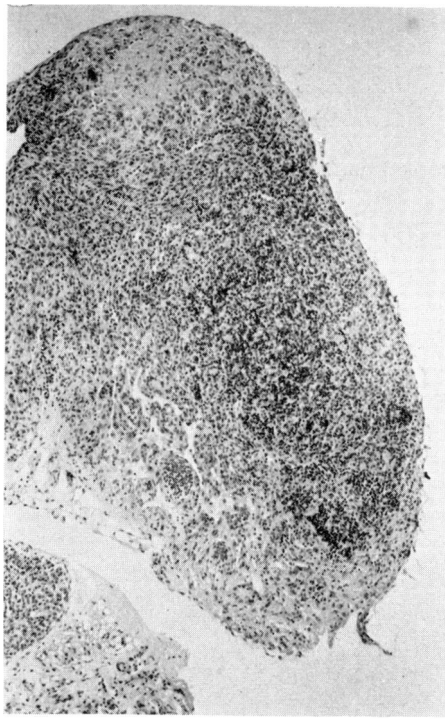
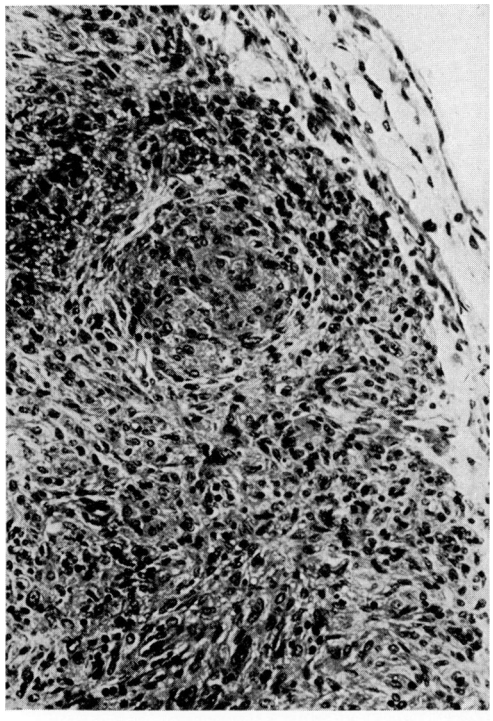


FIG. 2

FIG. 3

FIG. 2. Cervical lymph node from mouse treated with 3 × CY (300 mg/kg) showing lymphocytes surviving in paracortical area, 3 days after last injection of CY. Haematoxylin–eosin. × 132.

FIG 3. Mesenteric lymph node from guinea-pig treated with 3 × CY (300 mg/kg) showing lymphocytes surviving in paracortical area, 3 days after last injection of CY. Haematoxylin–eosin. × 132.



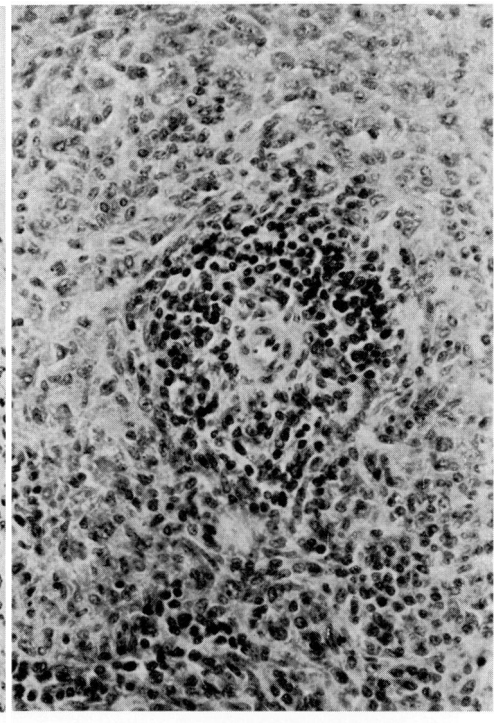


FIG. 4 FIG. 5

FIG. 4. High-power view of depleted lymph follicle from lymph node of guinea-pig treated with 3 × CY (300 mg/kg), the last injection being 3 days previously, showing whorl-like appearance of reticulum cell background. Haematoxylin–eosin. × 384.

FIG. 5. Spleen of mouse treated with 1 × CY (300 mg/kg) 3 days previously, showing lymphocytes remaining round central arteriole. Haematoxylin–eosin. × 528.

RESULTS

Effect of cyclophosphamide alone

Lymph nodes from mice treated with one or three doses of CY showed a similar pattern of selective depletion when examined 3 days after the last dose of CY (Fig. 2). The depletion was mainly limited to the lymphocytes of the lymph follicles, germinal centres and those at the cortico-medullary junction. The lymphocytes round the post-capillary venules, in the paracortical areas were never completely depleted in the same way even after three doses of 300 mg/kg or one dose of 400 mg/kg CY, although these doses were usually fatal. Although the concentration of these cells was less than in lymph nodes from untreated animals there was always a significant number of these cells present, especially round the post-capillary venule. The areas of lymphocyte depletion were well demarcated and the background of reticulum cells remained undisturbed.

A similar picture was seen in the lymph nodes of guinea-pigs treated in the same way (Fig. 3). Lymphocytes in the paracortical areas were also less depleted than those in other parts of the node. Depletion was most marked at the cortico-medullary junction, although

mature plasma cells in the medullary cords were unaffected. Germinal centres and lymph follicles were always depleted. In guinea-pigs the small lymphocytes of the marginal cuff round the germinal centres were not completely depleted by the one dose of CY, although they were usually depleted completely by three doses. As in the mouse, the areas of lymphocyte depletion were identified by the presence of spindle-shaped reticulum cells which form the background structure of the node. The positions in the node which under normal conditions are the sites of lymph follicles or germinal centres could be identified by whorl-like structures of reticulum cells lying under the cortex of the node (Fig. 4). The cells at the centre of the whorls had less of a spindle shape and a more diffuse cytoplasm than those in the periphery.

Lymphocyte depletion was also considerable in the spleen. However, in both mice and guinea-pigs there were always considerable numbers of small lymphocytes left round the central arteriole in the white pulp (Fig. 5) even following three injections of CY (Fig. 6.) As in the lymph nodes, areas of the spleen depleted of lymphocytes showed up the background structure of reticulum cells which would appear to form the basic structure of the organ.

The thymus in the mouse showed depletion of lymphocytes from the cortex only,

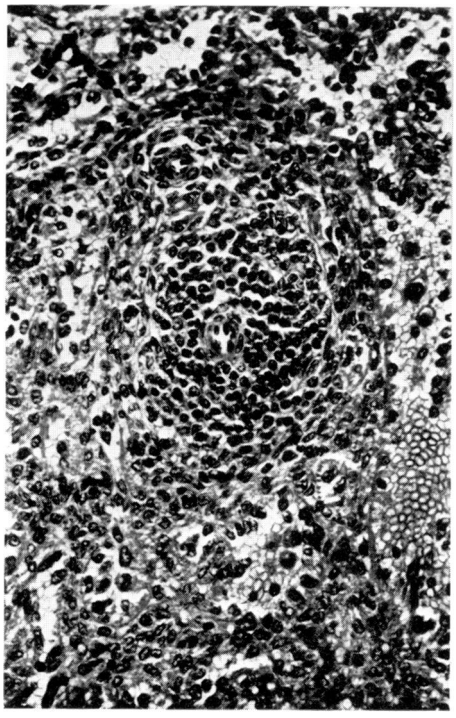


FIG. 6

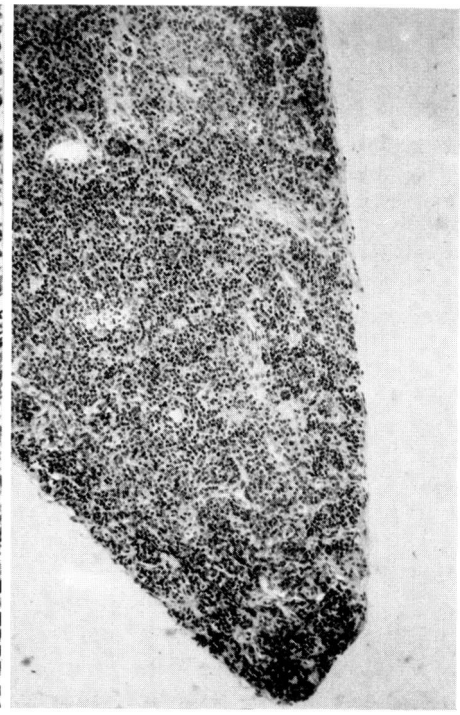


FIG. 7

FIG. 6. Spleen of guinea-pig treated with 3 × CY (300 mg/kg) showing lymphocytes remaining round central arteriole, 3 days after last injection of CY. Haematoxylin–eosin. × 528.

FIG. 7. Thymus of mouse treated with 1 × CY (300 mg/kg) 3 days previously, showing depletion of cortex. Haematoxylin–eosin. × 240.

following one injection of cyclophosphamide (Fig. 7). However, following three doses with intervals of 48 hr there was progressive depletion of lymphocytes from the medulla, although some lymphocytes still remained round dilated capillaries. The rest of the thymus consisted of reticulum cells only. In the guinea-pig one dose of CY did not deplete the cortex of the thymus as in the mouse. Cortical depletion was only found following three doses of cyclophosphamide (Fig. 8) although the degree of depletion of the medulla with this dose was not quite so great as it was in the mouse.

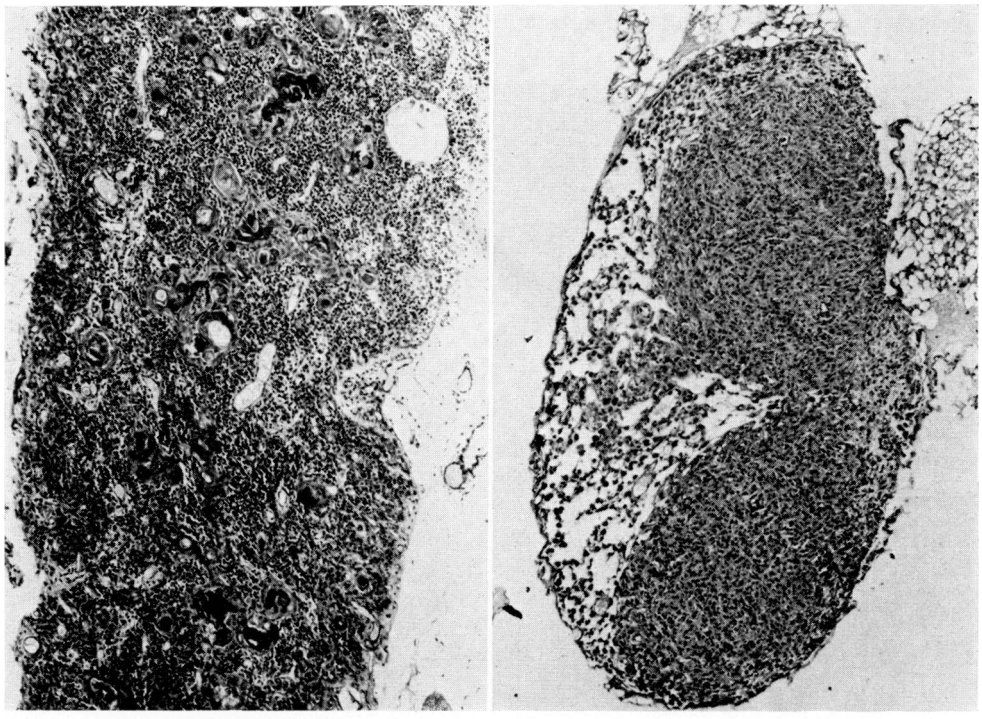


FIG. 8 FIG. 9

FIG. 8. Thymus of guinea-pig treated with 3 × CY (300 mg/kg) showing depletion of cortex, 3 days after last injection of CY. Haematoxylin–eosin. × 132.

FIG. 9. Cervical lymph node of neonatally thymectomized mouse treated with 1 × CY (300 mg/kg) 3 days previously, showing complete depletion of lymphocytes from all areas. Haematoxylin–eosin. × 132.

Initial information on the mode of reconstitution of depleted areas of lymphoid tissue was obtained by examination of thymus, spleen and lymph nodes from mice killed 7 days after the injection of one dose of 300 mg/kg CY. This was at a time when the peripheral leucocyte count had returned to normal. In the lymph nodes previously depleted areas in the medullary cords, cortico-medullary junction and in the cortex were the site of large pyroninophilic cells, many of which were in mitosis. These cells were also seen scattered throughout the paracortical areas. There was also marked infiltration of the medullary cords with polymorphonuclear leucocytes, probably in response to infection. No regular arrangement of lymph follicles and germinal centres could be seen in the cortex.

In the spleen there was also evidence of considerable lymphocyte proliferation in the white pulp, where large pyroninophilic cells could be seen, many of which were in mitosis. There was considerable extra-medullary haemapoiesis in the red pulp which was also infiltrated with polymorphonuclear leucocytes. In the thymus the cortex was completely replaced by proliferating large pyroninophilic cells, many of which were in mitosis.

Effect of combined neonatal thymectomy and cyclophosphamide in the mouse, or combined treatment with ALS and cyclophosphamide in the guinea-pig

The selective depletion of lymphoid tissue following neonatal thymectomy or treatment

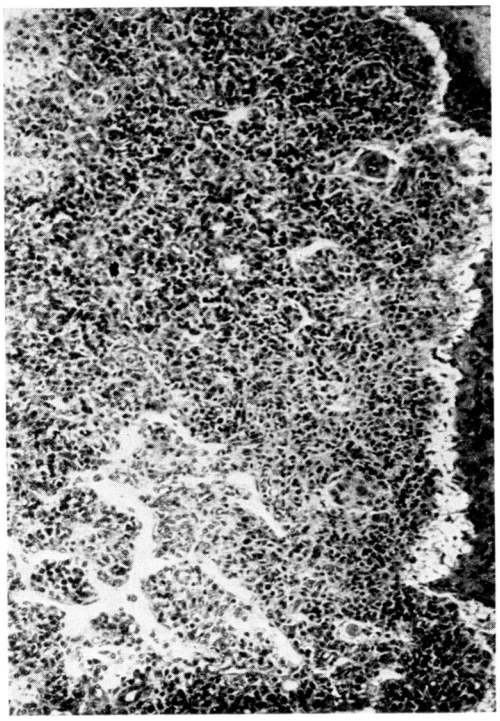


FIG. 10

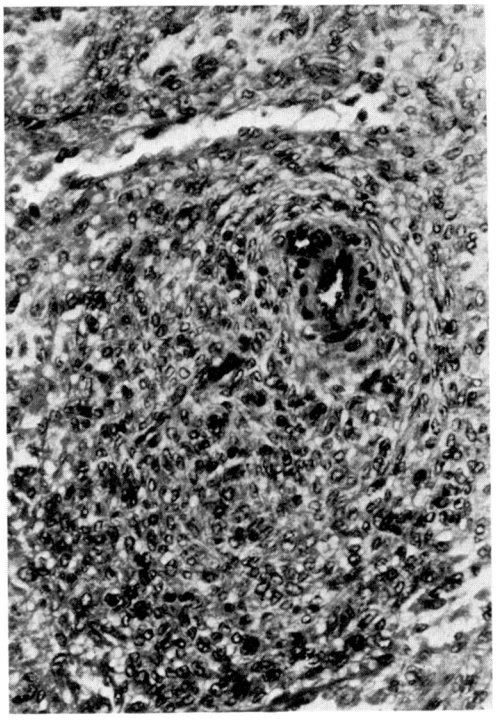


FIG. 11

FIG. 10. Mesenteric lymph node of guinea-pig treated with 3 × CY (300 mg/kg) and ALS, showing complete depletion of lymphocytes from all areas 3 days after last injection of CY. Haematoxylin–eosin. × 240.

FIG. 11. Spleen of neonatally thymectomized mouse treated with 1 × CY (300 mg/kg) 3 days previously, showing complete lymphocyte depletion. Haematoxylin–eosin. × 528.

with ALS has been described previously (Parrott *et al.*, 1966; Turk & Willoughby, 1967). The effect of CY on mice which had been thymectomized neonatally, or in combination with ALS in guinea-pigs, was to deplete those areas of lymphoid tissue which had not been depleted by the thymectomy or ALS. Thus the result of combined treatment in both species was to cause complete depletion of lymphocytes from all areas of lymph nodes and spleen, maximal 3 days after the last dose of CY (Figs 9–12). There was, however,

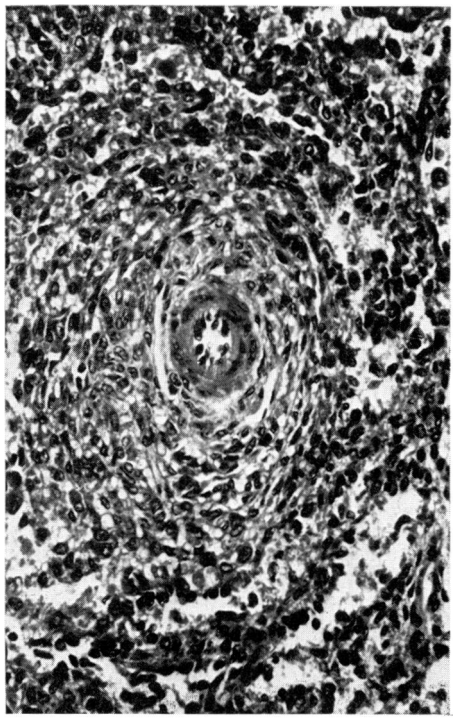
no depletion of mature plasma cells from the medullary cords. Thus those lymphocytes in the lymph nodes and spleen which were not eliminated by one injection of CY were mainly those which were controlled by the thymus in neonatal life and were depleted by treatment with ALS in the adult.

Treatment of thymectomized mice with one dose of 300 mg/kg CY produces an 85% mortality within 7 days. The lymphoid tissue from three mice which survived this regimen for 7 days was examined to see what pattern of repopulation could be achieved. There was marked proliferation of large pyroninophilic cells at the cortico-medullary junction and in the medullary cords which were beginning to show lymphocyte repopulation. Large pyroninophilic cells could also be found scattered throughout the paracortical areas and the subcapsular region of the node. Many of these cells were in mitosis. No regular lymph follicle or germinal centre formation could be seen in any of the lymph nodes examined. In the spleen there was marked mitotic activity and many large pyroninophilic cells throughout the red pulp and much of the white pulp, sparing however the area immediately round the central arteriole.

Effect of transfusion of syngeneic bone marrow cells

Two groups of mice were treated contemporaneously with 5×10^6 nucleated sex matched syngeneic bone marrow cells: (i) neonatally thymectomized + CY 300 mg/kg; (ii) neonatally thymectomized alone. The bone marrow cells were transfused 9 hr after the injection of CY and all the animals were killed 7 days later. The effect of the transfusion of bone marrow cells into thymectomized + CY treated mice was to drop the 7 day mortality of this treatment from 85% to 10%. Examination of the lymph nodes of these mice still showed depletion of paracortical areas. There was an increase in large pyroninophilic cells in mitosis at the cortico-medullary junction. However, the most striking finding in these animals was the presence of lymph follicles under the capsule of the nodes consisting of small lymphocytes (Fig. 13), as these were consistently absent from animals treated with CY without bone marrow replacement. The red pulp of the spleens was the site of intense mitotic activity. Apart from the area round the central arterioles which still remained depleted of cells of the lymphoid series, the spleen was well populated with lymphocytes and also contained scattered areas of large pyroninophilic cells, many of which were in mitosis. Transfusion of bone marrow cells into neonatally thymectomized animals not treated with CY had no effect on the pattern of depletion of thymus dependent areas of lymph nodes and spleen and no increased mitotic activity was seen in any areas.

Lymphoid tissue was also examined from mice treated 7 days previously with 400 mg/kg cyclophosphamide followed 9 hr later by a transfusion of 5×10^6 bone marrow cells. 88% of mice treated at the same time with 400 mg/kg CY alone died within 7 days, mostly within the first 48 hr. After transfusion with bone marrow cells, animals treated in a similar manner survived for at least 7 days. Despite bone marrow transfusion there was less sign of proliferation of lymphocytes in the lymph nodes, spleen or thymus than in animals treated with 300 mg/kg CY which were repopulating their own lymphoid tissues 7 days after CY. The thymus showed marked cortical depletion, although there were signs of lymphocyte repopulation in the medulla, but no large pyroninophilic cells nor mitoses could be seen in this tissue. Large pyroninophilic cells occasionally in mitosis could be seen in the paracortical areas and at the cortico-medullary junction in a proportion of the lymph nodes examined. The medullary cords, especially of the mesenteric lymph nodes,



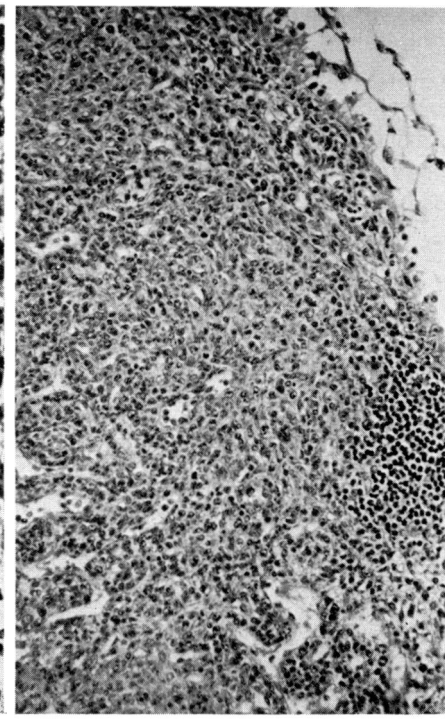


FIG. 12 FIG. 13

FIG. 12. Spleen of guinea-pig treated with 3 × CY (300 mg/kg) and ALS, showing complete lymphocyte depletion 3 days after last injection of CY. Haematoxylin–eosin. × 528.

FIG. 13. Lymph node from neonatally thymectomized mouse 7 days after treatment with 1 × CY (300 mg/kg) followed 9 hr later by 5×10^6 sex matched syngeneic bone marrow cells, showing repopulated lymph follicle. Haematoxylin–eosin. × 240.

contained large numbers of polymorphonuclear leucocytes which were probably part of the response to infection. The spleen showed mainly extra-medullary erythropoiesis. No evidence of lymph follicle formation in the lymph nodes could be found in this group of animals.

DISCUSSION

The differential effect of CY on lymphoid tissues is similar to that described for X-irradiation by Keuning and his colleagues (Keuning *et al.*, 1963; Keuning & Bos, 1967). In these studies sublethal X-irradiation in the rabbit was found to cause depletion of lymph follicles and germinal centres of the spleen without affecting the lymphocytes of the periarteriolar sheath. In the present study in mice and guinea-pigs a sublethal dose of CY (300 mg/kg) or of three such doses, which was mainly lethal, was found to have a similar differential effect on lymphoid tissue. In the lymph nodes there was a marked depletion of lymphocytes in the lymph follicles, germinal centres and at the cortico-medullary junction, with far less effect on the lymphocytes of the paracortical or 'thymus dependent' areas. In the spleen there was a similar sparing of lymphocytes immediately

round the central arteriole, although there was a complete loss of those cells from other areas. In mice treated with one injection of 300 mg/kg CY at 3 days the thymus cortex was depleted and at 7 days when repopulation of other tissues is beginning it was found to be the site of a mass of proliferating large pyroninophilic cells. Depletion of the thymus cortex with one dose of 300 mg/kg CY was not associated with depletion of lymphocytes in the medulla, which only occurred in mice given three doses of 300 mg/kg. Thus the lymphocytes of the medulla appear to need repeated exposure to CY to be depleted whereas one exposure is sufficient to eliminate cortical lymphocytes. This is consistent with the observation in the rat that cortical lymphocytes in the thymus are four times more radio-sensitive than those in the medulla (Trowell, 1961). It is also in agreement with the observation (Sainte-Marie & Leblond, 1964) that most of the cell division in the thymus occurs in the cortex and the suggestion that most of the small lymphocytes in the medulla do not originate locally but migrate there from the cortex.

Despite the effect of CY on depletion of the thymus cells, the lymphocytes of the paracortical areas of the lymph nodes or the thymus dependent areas of the spleen are not completely depleted even after three exposures to 300 mg/kg CY over a period of 1 week. Lymphoid tissue may, however, be depleted completely of small lymphocytes by combining CY treatment with neonatal thymectomy in the mouse or ALS treatment in the guinea-pig. This lack of sensitivity of 'thymus-dependent' lymphocytes to CY could be related to their being 'long-lived' rather than 'short-lived' lymphocytes. The effect of CY on non-thymus-dependent lymphocytes could indicate that these cells are derived from cells which are dividing regularly either *in situ* in the lymphoid organs or in distant sites such as the bone morrow. As these cells are depleted both in the peripheral blood and from the peripheral lymphoid tissues by 3 days, it is likely that if they are destroyed by CY during mitosis, their precursors undergo at least one cell division during this time. Since the peripheral lymphocyte count drops to 3% over this period, it would also appear that 97% of circulating lymphocytes in the peripheral blood consist of cells derived from CY-sensitive precursors and that non CY-dependent lymphocytes form a relatively small proportion of circulating cells at any particular time both in the mouse and in the guinea-pig.

Repopulation of the cortico-medullary junction of lymph nodes is well under way 7 days after the last dose of CY. However, there are still no lymph follicles to be seen in the lymph nodes of these animals at this time. If sex matched syngeneic bone marrow is transfused to neonatally thymectomized mice 9 hr after the injection of CY, 7 days later lymph follicles may be seen in the cortex of the lymph nodes. Bone marrow transfusion to these mice increases their survival potential considerably. This would indicate that the direct toxic effect on these cells does not extend beyond 9 hr after the injection of CY. Moreover, the replacement of bone marrow after CY treatment provides cells which can act as precursors of small lymphocytes in the lymph follicles so that by 7 days after treatment these structures are reformed. In addition repopulation of cells at the cortico-medullary junction appears further developed in mice which have received bone marrow than in those which have not, as there is far less mitotic activity at this site in bone marrow replaced animals than in those which are repopulating themselves following CY treatment. At the same time in neonatally thymectomized CY treated, bone marrow replaced mice there is no evidence of repopulation of the 'thymus-dependent' areas of lymph nodes or spleen. This would suggest that the transfused bone marrow contains cells necessary for the

continuous repopulation of lymph follicles and the cortico-medullary junction without containing precursors of cells which would repopulate the thymus-dependent areas. As lymph follicles are eliminated from lymph nodes in mice as early as 3 days after treatment with CY it would appear that they are being regularly replaced from a rapidly dividing pool of cells. Although bone marrow cells have not been traced directly to these areas, the presence of lymph follicles in bone marrow transfused CY treated thymectomized animals could be taken as presumptive evidence that lymph follicle lymphocytes are being continuously replaced from the bone marrow.

Finally, Petrov *et al.*, (1971) have suggested that cyclophosphamide can have a direct toxic as well as the conventionally accepted mitostatic action on lymphocytes involved in a graft versus host reaction. It would, however, appear that in the conditions of the present experiments the action of CY is mainly mitostatic, in view of the selective sparing of lymphocytes in the paracortical areas of the lymph nodes. Moreover, in as yet unpublished experiments, such lymphocytes can be shown to proliferate normally as part of a cell-mediated immune response to the application of a chemical sensitizing agent to the skin, 3 days after an injection of 300 mg/kg CY.

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