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Mode of Action of Anti-Lymphocyte Globulin

II. CHANGES IN THE LYMPHOID CELL POPULATION IN RATS TREATED WITH ANTI-LYMPHOCYTE GLOBULIN

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Summary. Lymphopenia in rats which had been made tolerant to normal rabbit immunoglobulin G(IgG) was induced by the administration of rabbit anti-rat immunoglobulin G(IgG). The rats were injected with tritiated thymidine ([^{3}H] thymidine) and the labelling pattern in lymphoid tissues was studied. The thymus weight decreased with continued lymphopenia and this could be explained by the release of small lymphocytes into the circulation. Lymphopoiesis in the thymus and spleen was not inhibited by anti-lymphocyte globulin. Plasmacytosis was noted in the lymph nodes. These findings support the idea that anti-lymphocyte globulin acts mainly on peripheral lymphocytes and suppresses the immune function mediated by these cells.

INTRODUCTION

A companion study has shown that there was little localization of rabbit anti-rat lymphocyte immunoglobulin G(IgG) in the lymphoid tissues of recipient rats. It was concluded that the most obvious effects of the antiglobulin, namely destruction or inactivation of circulating lymphocytes, accounted for most of its immunosuppressive action (Denman and Frenkel, 1968). However, these findings did not exclude the possibility that the formation of lymphocytes and plasma cells in lymphoid tissues was nevertheless inhibited by the administration of anti-lymphocyte globulin. It has been shown, for example, that the immunosuppressive properties of anti-lymphocyte globulin are potentiated by thymectomy (Jeejeebhoy, 1965; Monaco, Wood and Russell, 1966), but it is not clear whether it is the removal of a source of newly formed lymphocytes or of cells secreting a lymphocytopoietic factor (Klein, Goldstein and White, 1965), which is responsible for this potentiation. Similarly, there is uncertainty about the mechanism by which antithymocyte serum induces a specific depletion of thymus-dependent areas of lymph nodes (Turk and Willoughby, 1967). The purpose of this report is to give the results of a detailed study of the proliferative activity in the lymphoid tissues of rats receiving anti-lymphocyte globulin. In order to remove any complicating effects of an immune reaction against the heterologous rabbit globulin, the recipient rats were made tolerant initially to normal rabbit immunoglobulin G(IgG).

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MATERIALS AND METHODS

Experimental animals

Inbred Fischer rats were used in all experiments.

Preparation of rabbit anti-rat immunoglobulin G

Rabbit anti-rat lymphocyte immunoglobulin G (subsequently referred to as ALG) was prepared as previously described (Denman and Frenkel, 1968).

Induction of tolerance to normal rabbit immunoglobulin G

Immunoglobulin G(IgG) was isolated from pooled rabbit serum by DEAE column chromatography (LoSpalluto, Miller, Dorward and Fink, 1962). Newborn rats received intraperitoneal injections of 50 mg of rabbit IgG within 12 hours of birth, then 50 mg daily for 14 days, followed by 50 mg weekly until the 10th week of life. One week after the end of this course, antibody to rabbit IgG could not be detected in the rat sera by capillary precipitation (Boyd, 1956), agar diffusion (Ouchterlony, 1953), or immunoelectrophoresis (Scheidegger, 1955). These rats are subsequently referred to as 'tolerant' rats.

Absolute lymphocyte counts

Absolute lymphocyte counts were determined from haemocytometer counts and differential counts on blood films stained with May–Grünwald–Giemsa.

Labelling with tritiated thymidine ([^3H]thymidine)

Rats were injected by the tail vein with 1 μc/g body weight of [^{3}H]thymidine in 1 ml normal saline (specific activity 6·7 c/mmole; New England Nuclear Corporation).

Autoradiography

Autoradiographs were prepared with NTB-3 Kodak dipping emulsion. Imprints were exposed for a standard 3 weeks and were stained with May–Grünwald–Giemsa. Sections of 3 μ thickness were exposed for 6 weeks and were stained with haematoxylin.

Analysis of autoradiographs

Differentiated cell counts were made on imprints from the thymus, spleen, bone marrow, brachial, aortic and mesenteric lymph nodes. Several preparations from each tissue were examined and a minimum of 4000 cells from each tissue was counted. Thymus lymphocytes were classified as 'large', 'medium' or 'small' by the criteria of Sainte-Marie and Leblond (1965). Larger cells with big nuclei filling the cell and with chromatin of a reticular character were counted as 'reticulum cells' (Frenkel, Sugino, Bishop and Potter, 1963). Although the same classification was adopted for spleen, lymph node and marrow counts, large primitive cells in these sites were simply termed 'blast' cells because the intermingling of cell types in these tissues made their precise differentiation uncertain.

Cells with more than three grains above background were counted as labelled. The mean grain count and the percentage labelling were determined for each category of cell.

Autoradiographs of tissue sections were used to assess the distribution of labelled cells.

Determination of specific activity

The specific activity of the isolated thymus DNA was determined by liquid scintillation counting, using the method of Sugino, Frenkel and Potter (1963). Quantitative counting of [^{3}H]thymidine in other lymphoid tissues was not undertaken because of the admixture of cell types.

Experimental design

(*a*) *Rats given ALG:* twenty 12-week-old tolerant rats were given daily intraperitoneal injections of ALG in quantities that were sufficient to induce a persistent lymphopenia of 1000 lymphocytes/mm^3 or less.

As controls, tolerant rats of the same age received daily injections of 10 mg of normal rabbit IgG.

Lymphopenic and control rats were given single injections of [^{3}H]thymidine between the 8th and 15th days of the experiment and were killed 1, 8, 24, 74 or 100 hours after labelling. The wet weights of the thymus and spleen were noted; tissues for histological examination were fixed in Carnoy's solution, and relevant specimens were obtained for autoradiography and DNA extraction.

(*b*) *Rats receiving endotoxin:* twenty-six rats were injected with *Salmonella* lipopolysaccharide (Difco) 1·5 μg/g body weight in 1 ml normal saline by the tail vein. The MLD 50 for this strain was shown to be 3·0 μg/g body weight in preliminary experiments. [^{3}H]Thymidine was injected between 1 and 5 days after the endotoxin and the rats were killed within 8 hours of labelling.

RESULTS

PERIPHERAL WHITE CELL COUNTS

Persistent lymphopenia was achieved with daily injections of 10–12 mg of ALG per rat; the granulocyte count was not affected (Fig. 1). The peripheral white cell counts of the control rats were not affected by normal rabbit IgG.

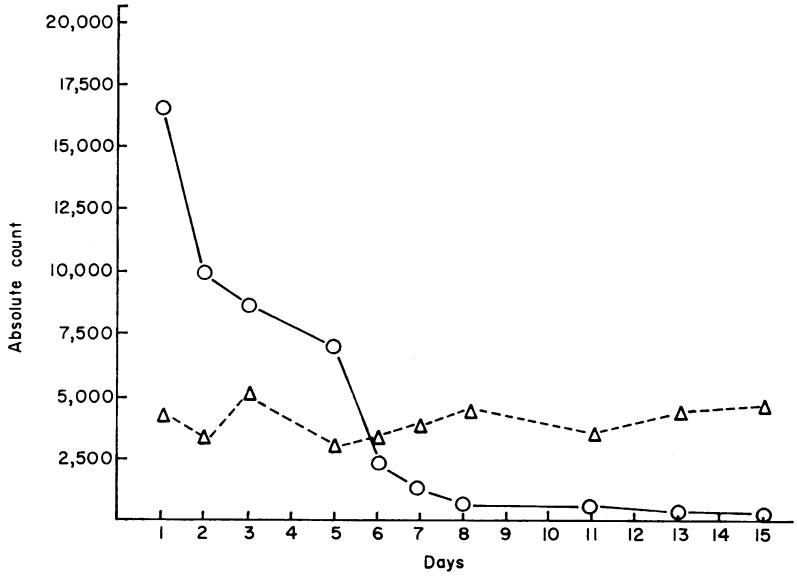

FIG. 1. Peripheral white cell count in rats receiving anti-lymphocyte immunoglobulin G(IgG). Twenty Fischer rats received 8–10 mg rabbit anti-rat lymphocyte immunoglobulin G(IgG) daily. White cell counts are mean values from a minimum of ten rats, performed 24 hours after preceding injection of anti-lymphocyte IgG. ○, Lymphocytes; △, polymorphs.

GENERAL CONDITION

Apart from some slight weight loss, the lymphopenic rats remained healthy throughout the 2 weeks of the experiment.

THYMUS CHANGES

The thymus weight of the lymphopenic rats (mean 180 mg, S.D. 15) decreased as compared with the controls (mean 290, S.D. 25) and histological examination revealed that the weight loss was accompanied by a reduction in cortical thickness.

The percentage of small lymphocytes in thymus imprints fell progressively with continued lymphopenia and this reduction could be correlated with the loss of thymus weight (Fig. 2). The percentage of other cell types rose correspondingly. Since the thymus DNA

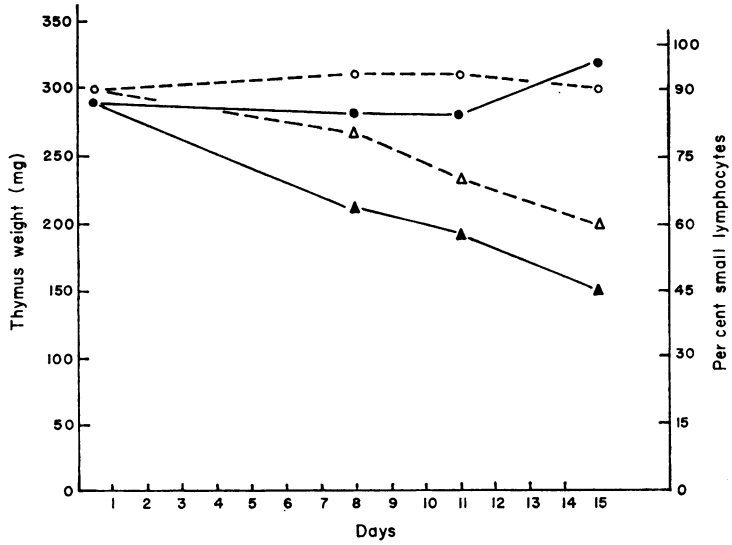

FIG. 2. Relation between thymus weight and small lymphocyte counts in the thymus in rats receiving anti-lymphocyte immunoglobulin G (IgG) and in controls. Thymus weight = wet weight. Per cent small lymphocytes determined by differential counts of thymus imprints. ○, Counts, controls; ●, weights, controls; △, counts treated; ▲, weights, treated.

content per milligram of tissue was comparable in lymphopenic and control rats (25 μg DNA/mg tissue), it is probable that the numbers of cells per milligram of tissue were also similar, and it is unlikely therefore that there was any significant reduction in the numbers of cell types other than small lymphocytes.

The percentage of thymus cells labelled by [^{3}H]thymidine was even higher in the lymphopenic rats than in the controls (Fig. 3), a finding which indicated that DNA synthesis had not been inhibited by the administration of ALG. It is unlikely, moreover, that this agent had interfered with cell division and maturation, since a high percentage of small lymphocytes were labelled 24 hours after the injection of [^{3}H]thymidine. The mean grain count in the reticulum cells and large lymphocytes (Figs. 4 and 5) were similarly higher in the lymphopenic rats than in the controls, and declined exponentially after labelling.

The autoradiographs of thymus sections showed widespread distribution of labelled cells in both lymphopenic and control rats.

The evidence from the autoradiographs that lymphopoeisis was not affected by ALG

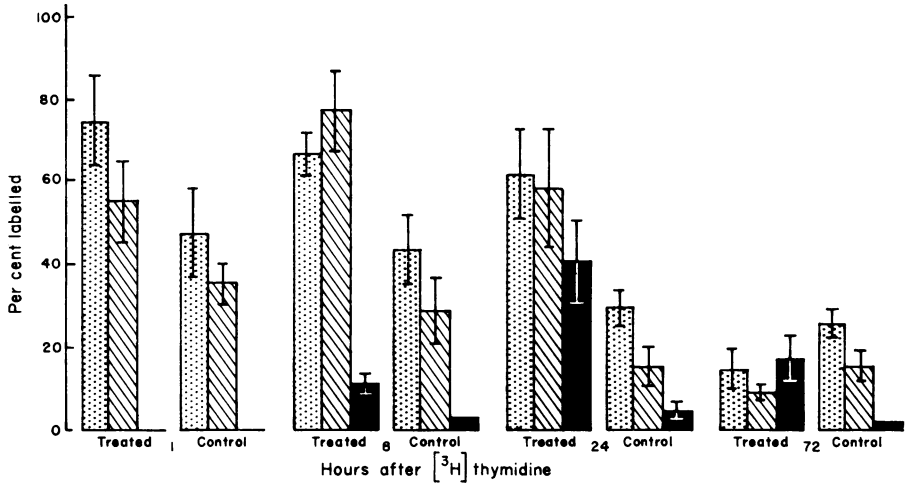

FIG. 3. Percentage of cells labelled by [^{3}H]thymidine in the thymus of rats receiving anti-lymphocyte immunoglobulin G (IgG) and in controls. Figures are mean values of results in four rats ± 1 S.D. Stippled columns, reticulum cells; cross-hatched columns, large and medium lymphocytes; solid columns, small lymphocytes.

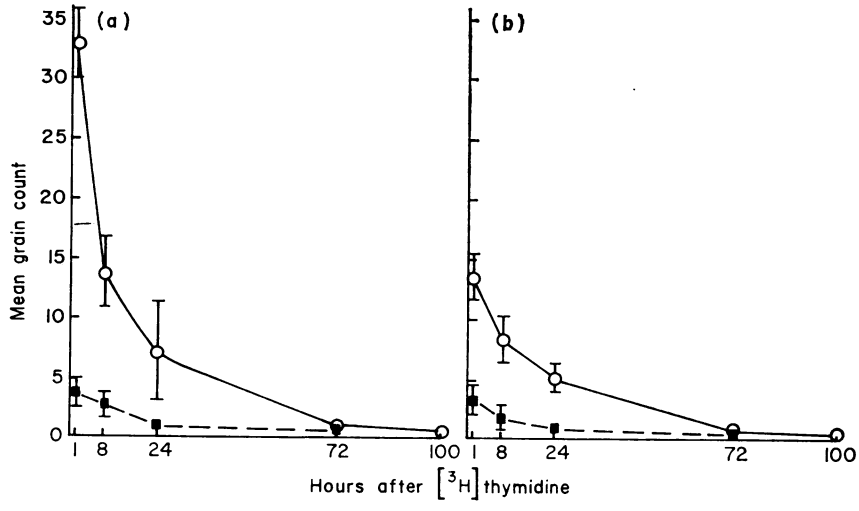

FIG. 4. Mean grain counts of: (a) thymus reticulum cells, and (b) large lymphocytes in rats receiving anti-lymphocyte immunoglobulin G (IgG) and in controls. Figures are mean values of results in four rats ±1 S.D. Mean grain counts calculated as grains per *total* numbers of cell in each category, labelled and unlabelled. ○, Lymphopenic rats; ■, control rats.

was supported by the specific activity of the isolated thymus DNA (Table 1). The specific activity of the DNA extracted from the thymus of lymphopenic rats was in fact higher than that obtained from control thymuses.

TABLE 1

INCORPORATION OF TRITIATED THYMIDINE INTO THE THYMUS DNA OF RATS RECEIVING ANTI-LYMPHOCYTE IMMUNOGLOBULIN G (IgG) AND CONTROLS

	Hours after i.v. [^{3}H]thymidine			
	1	8	24	72
Lymphopenic rats	40·2* (28·6–53·4)	66·8 (27·8–196·6)	126·8 (16·6–234·0)	10·3 (1·4–18·8)
Controls	8·4 (1·0–17·4)	5·9 (1·4–12·0)	1·2 (1·0–1·4)	5·6 (1·1–9·9)

Four animals in each group. Mean values and range are shown for each group.
* Counts/min/μg DNA.

SPLEEN CHANGES

Although spleen weights were not significantly altered by ALG treatment, severe depletion of small lymphocytes from the white pulp was seen histologically. The reduction of small lymphocytes was confirmed by differential counts of splenic imprints in which a large proportion of cells were reticulum cells, ranging from 40·7 to 83·6 per cent compared to 15·3–20·8 per cent in the controls. As in the thymus, a high proportion of these cells were labelled (Table 2), and their mean grain count also declined exponentially in the 100

TABLE 2

PERCENTAGE OF SPLENIC RETICULUM CELLS LABELLED BY [^{3}H]THYMIDINE IN RATS RECEIVING ANTI-LYMPHOCYTE IMMUNOGLOBULIN G (IgG) AND IN CONTROLS

	Hours after [^{3}H]thymidine			
	1	8	24	72
Lymphopenic rats	65·3 (29·9–88·4)	53·2 (45·0–62·0)	37·3 (4·8–46·9)	21·3 (9·5–39·3)
Controls	11·8 (5·7–16·4)	6·8 (5·0–8·5)	8·3 (2·0–20·3)	4·5 (1·0–11·0)

Four rats in each group. Results are mean values and range.

hours following labelling (Fig. 6). Since cells of the myeloid, erythroid and plasma cell series were no more numerous in the spleen of the lymphopenic rats than of the controls, it seemed probable that the labelled reticulum cells were lymphocyte precursor cells. This conclusion was supported by the autoradiographs of tissue sections in which the labelled cells were distributed at the periphery of the white pulp from which lymphocytes had been depleted. A significant proportion of the surviving small lymphocytes (11·5 per cent on average) in the spleens of the lymphopenic rats were labelled 24–100 hours after [^{3}H]thymidine had been injected, another indication that the maturation of labelled stem cells had not been disturbed.

LYMPH NODE CHANGES

The lymph nodes were uniformly enlarged and loss of architecture, lymphocyte depletion and plasmacytosis were evident histologically. Imprints from different lymph nodes

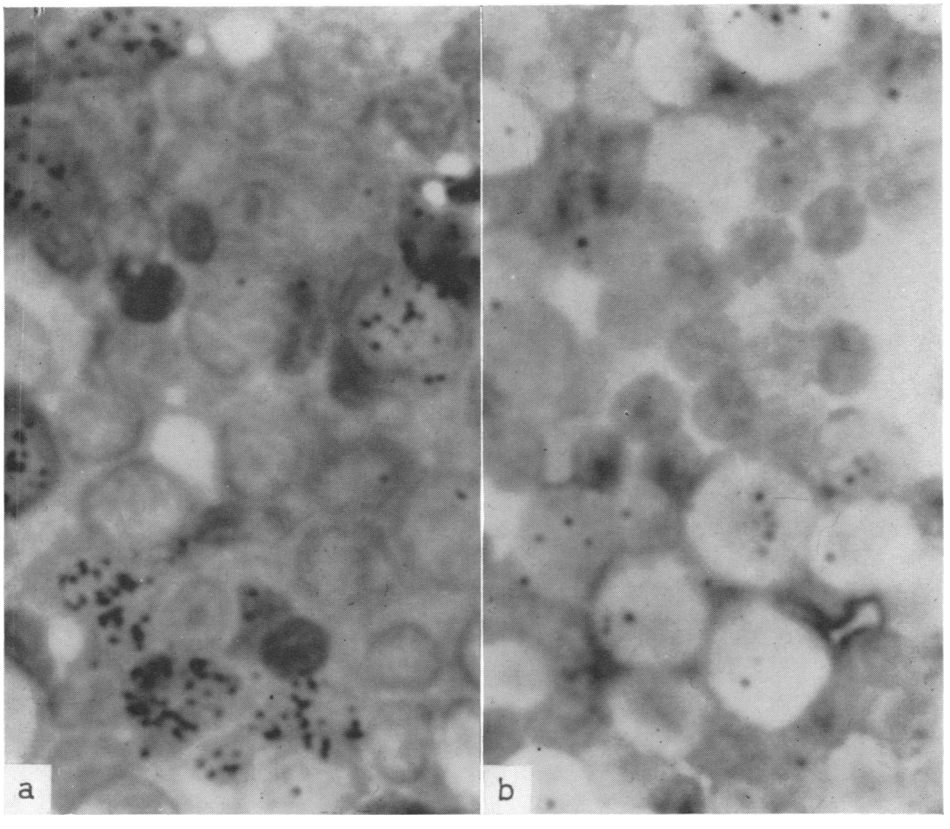

FIG. 5. Figure comparing the intensity of [^{3}H]thymidine labelling in the thymus cells of rats receiving anti-lymphocyte immunoglobulin G (IgG) and in controls. Imprints taken from the thymus of rats killed 1 hour after [^{3}H]thymidine labelling. Note that grains are more frequent over the labelled cells of: (a) the lymphopenic rat, than of (b) the control.

(*Facing p.* 120)

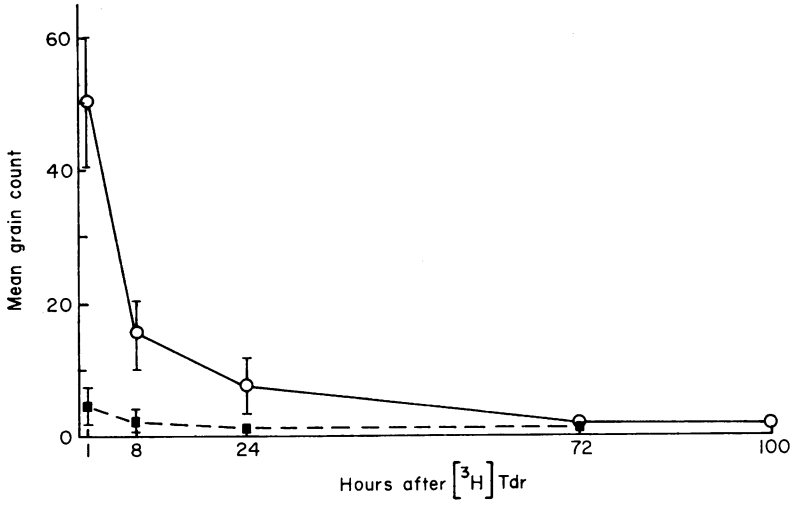

FIG. 6. Mean grain count of splenic reticulum cells in rats receiving anti-lymphocyte immunoglobulin G (IgG) and in controls. Figures are mean values of results in four rats ± 1 S.D. Mean grain count calculated as grains per *total* numbers of cells in each category, labelled and unlabelled. ○, Lymphopenic rats; ■, control rats.

in the same animal showed only minor differences and were therefore analysed collectively. 'Blast' cells and plasma cells comprised a high percentage of the lymph node cells from lymphopenic rats and the percentage of small lymphocytes was correspondingly reduced (Table 3). These 'blast' cells were maximally labelled 1 hour after the injection of [^{3}H] thymidine, whereas the plasma cells only became labelled 24–72 hours after injection

TABLE 3

DIFFERENTIAL CELL COUNTS OF LYMPH NODE IMPRINTS FROM RATS RECEIVING ANTI-LYMPHOCYTE IMMUNOGLOBULIN G (IgG) AND CONTROLS

	'Blast' cells	Plasma cells	Small lymphocytes	Others
Lymphopenic	9·7 (6·1–12·8)	11·0 (1·1–30·0)	46·9 (36·9–59·4)	32·4
Control	1·2 (0·4–1·6)	0·1 (0·01–0·2)	95·0 (94·0–97·2)	3·7

Results given are the mean and range of four rats in each group.

(Table 4). It was concluded that the majority of the 'blast' cells were plasma cell precursors. In contrast with the spleen and thymus, lymph node reticulum cells were not labelled more frequently in lymphopenic rats than in controls, and their grain counts did not fall exponentially after labelling. However, up to 6 per cent of the surviving small lymphocytes in the lymph nodes became labelled 24–100 hours after the injection of [^{3}H]thymidine.

Autoradiographs of lymph node sections showed a fairly uniform distribution of labelled cells through the cortex.

TABLE 4

PERCENTAGE OF LABELLED CELLS IN THE LYMPH NODES OF RATS RECEIVING ANTI-LYMPHOCYTE IMMUNOGLOBULIN G (IgG) AND CONTROLS

	Hours after i.v. [^{3}H]thymidine				
	1	8	24	72	100
Reticulum cells					
ALG	27·2 (20·1–37·2)	36·0 (22·6–41·0)	30·5 (19·1–41·7)	21·5 (9·2–26·5)	15·6 (5·2–29·3)
Control	18·0 (7·5–29·0)	19·3 (11·6–27·0)	22·7 (8·8–31·3)	8·4 (5·8–17·5)	–
'Blast' cells					
ALG	72·9 (63·0–83·0)	73·4 (67·4–80·0)	63·6 (50·5–72·0)	26·7 (5·8–37·0)	20·2 (12·4–32·0)
Control	35·9 (32·8–40·0)	34·2 (28·6–35·7)	35·3 (29·3–45·1)	19·3 (5·0–37·2)	–
Plasma cells					
ALG	0	4·3 (2·5–5·9)	28·6 (14·3–52·5)	26·7 (8·1–37·0)	12·3 (7·2–18·7)
Control	0	1·8 (0–5·5)	1·0 (0–1·3)	0	–

Results are mean values with range. Four rats in each group.

BONE MARROW CHANGES

Forty per cent of the bone marrow cell population of lymphopenic rats were reticulum cells, and of these 26–52 per cent were labelled 1 hour after the intravenous injection of [^{3}H]thymidine. Small lymphocytes constituted 5 per cent of the bone marrow cells of control rats, whereas less than 1 per 1000 nucleated cells from lymphopenic rats were lymphocytes. There was no disturbance of the erythroid and myeloid series.

TABLE 5

CHANGES IN THYMUS WEIGHT, DNA CONTENT AND [^{3}H] THYMIDINE INCORPORATION IN RATS GIVEN i.v. ENDOTOXIN 1·5 μg/g BODY WEIGHT

Days after endotoxin	No. of rats	[^{3}H]thymidine incorporation (counts/min/μg DNA)	DNA content (μg/mg thymus)	Thymus/body weight ratio (mg/g)	Thymus weight (mg)
1	4	3·3	23·4	1·04	132
2	6	4·6	16·6	0·64	94
3	8	3·4	13·4	0·57	85
4	2	4·2	11·3	1·06	137
5	4	14·3	14·1	1·10	134
6	2	4·0	16·4	0·88	125
Controls* (±1 S.D.)	22	6·2±1·4	24·9±4·9	1·80±0·33	257±46

There is a reduction of thymus weight, thymus/body weight ratio and thymus DNA content. [^{3}H] Thymidine incorporation is below controls' levels until the 5th day after endotoxin when there is a rise accompanying thymus regeneration. Results are mean values for each group.

* Rats of the same age, sex and strain.

ENDOTOXIN EXPERIMENT

The reduction in thymus weight following ALG administration superficially resembled the involution which accompanies various forms of stress (Dougherty, 1952). Accordingly, the utilization of [^{3}H]thymidine following administration of endotoxin to rats was compared with that in rats with ALG-induced lymphopenia. After intravenous endotoxin the fall in thymus weight was associated with a reduction in DNA content and [^{3}H] thymidine incorporation (Table 5), in complete contrast to the picture in lymphopenic rats (Table 1). These differences were reflected in the autoradiographs from endotoxin treated animals which showed a reduction in the percentage of labelled cells and their grain counts compared with both ALG treated and control rats.

DISCUSSION

As a general conclusion, it can be stated that the capacity of lymphoid cells to incorporate [^{3}H]thymidine and, by inference, to synthesize DNA, was not suppressed by anti-lymphocyte globulin. However, the labelling patterns in the different lymphoid organs need separate interpretation.

Thymus

Small lymphocytes develop from stem cells in the adult rat thymus by a sequence of division and maturation which has been analysed in detail by Sainte-Marie and Leblond (1965). Although the origin of these stem cells is uncertain, a study of the regenerating thymus after X-irradiation has indicated that, morphologically, they are reticulum cells (Frenkel *et al.*, 1963). The pattern of labelling in the lymphopenic rats did not differ from that seen in the controls and was consistent with a lymphocytopoietic sequence from reticulum cells to small lymphocytes. The reduction in thymus weight was proportionate to the loss of small lymphocytes and could be explained by postulating that these cells were released into the peripheral blood in greater numbers than normal. There is experimental evidence for some migration of thymocytes into the blood stream (Nossal, 1964; Ernstrom, Gyllensten and Larssen, 1965). The loss of small lymphocytes from the thymus in ALG treated animals cannot be attributed to stress involution, since the reduction in thymus weight after endotoxin was accompanied by a considerable inhibition of [^{3}H] thymidine incorporation and of reticulum cell labelling. It is also unlikely that newly formed small lymphocytes were phagocytosed by reticulum cells functioning as macrophages since, if this had been the case, the mean grain count and percentage labelling of these cells would have increased in the 100 hours following [^{3}H]thymidine injection. The continued release of small lymphocytes into the circulation would explain, at least in part, the potentiating effects of thymectomy on the immunosuppression accompanying anti-lymphocyte globulin administration (Jeejeebhoy, 1965; Monaco *et al.*, 1966).

Spleen

For reasons already given it was concluded that the reticulum cells in the spleen were functioning as lymphocyte precursor cells and that here also their maturation was not inhibited by anti-lymphocyte globulin. This conclusion is supported by the findings of Bari (1965) in a study of post-irradiation regeneration in the spleen, in which it was demonstrated by combined electronmicroscopy and autoradiography that the reticulum cell was the lymphocyte precursor cell. In additional control experiments we have found

(Denman, Frenkel and Ziff, unpublished observations) that the labelling pattern in the rat spleen following the injection of endotoxin or acetylphenylhydrazine is quite different from that produced by anti-lymphocyte globulin.

Bone marrow

It seems likely that the reticulum cell hyperplasia in the bone marrow was also associated with lymphocyte regeneration. The importance of myeloid cells as a source of lymphoid cells is well known (Ford, 1966), but the effect of long-term treatment with ALG on thymic cell proliferation is not yet known (Denman, Denman and Holborow, 1966) and is being further investigated.

Lymph nodes

The increased numbers of plasma cells seen in the lymph nodes of the lymphopenic rats were not labelled until 24 hours after the injection of [^{3}H]thymidine and presumably developed from labelled 'blast' cells. This is the pattern of plasma cell labelling normally seen in rat lymph nodes (Nossal, Mitchell and McDonald, 1963). Since the reticulum cells in the lymph nodes were labelled less frequently than those in other sites and their mean grain count did not fall exponentially, it is possible that, unlike similar cells in the bone marrow, thymus and spleen, they were not functioning as lymphocyte precursor cells. Moreover, cells with these labelling characteristics are seen frequently in the lymph nodes of normal rats (Craddock, Nakai, Fukuta and Vanslager, 1964). It is possible that the labelled small lymphocytes in the lymph nodes of lymphopenic rats were derived from plasmablasts (Ford, Gowans and McCullagh, 1966). Since small lymphocytes evidently act as memory cells for immunological events (Gowans and Uhr, 1966), this mechanism provides another explanation for the relative resistance of the secondary antibody response to anti-lymphocyte globulin. The cause of the plasmacytosis in lymph nodes is uncertain; it may have been related to infection or directly to the depletion of small lymphocytes as in homologous disease (Stastny, Stembridge, Vischer and Ziff, 1965) and after neonatal thymectomy (Parrott, De Sousa and East, 1966).

Certain other possibilities need to be considered. It is known that antibody against human buffy coat cells induces a 'blast' transformation of the target leucocytes (Grasbeck, Nordman and De la Chappelle, 1963), and it could be argued that anti-lymphocyte globulin might produce the same phenomenon *in vivo* in rats. However, anti-lymphocyte globulin has limited access to lymphoid tissues (Denman *et al.*, 1968) and 'blast' cells were not seen in the peripheral blood at any time during the experiment. Moreover, the 'blast' conversion of rat lymphocytes by anti-lymphocyte globulin is inhibited by complement (Vischer and Denman, unpublished observations), a phenomenon which has also been reported with antiserum to human lymphocytes (Holt, Ling and Stanworth, 1966).

It is difficult to make a direct comparison between the intensity of labelling in the lymphopenic and control rats. Although [^{3}H]thymidine is rapidly cleared from the peripheral blood (Hughes, 1959) and incorporated into newly formed DNA, there is evidence for the existence of a pool of thymine nucleotides within the thymus (Potter and Nygaard, 1963). If this pool were smaller in lymphopenic rats than in the controls, it would be more enriched by [^{3}H]thymidine in the former. Consequently, the mean grain count of the thymic precursor cells and the specific activity of the isolated DNA could be increased in the lymphopenic rats even in the absence of increased cell proliferation. Accordingly, the differences observed in the present experiments should be treated with

caution. Nevertheless, the intensity of reticulum cell labelling in the thymus, spleen and bone marrow in the rats receiving anti-lymphocyte globulin suggested the possibility that the peripheral lymphopenia had induced a compensatory hyperplasia of lymphocyte stem cells.

Certain conclusions can be reached about the mode of action of anti-lymphocyte globulin. The finding that division and maturation of cell populations in the bone marrow, thymus and spleen were not suppressed by anti-lymphocyte globulin provides additional support for the view that this agent acts primarily on peripheral lymphocytes and on the immune responses mediated by these cells. Plasma cell proliferation in the lymph nodes was also unaffected by anti-lymphocyte globulin, and this finding may explain the relative resistance of the secondary antibody response to this form of immunosuppression.

ACKNOWLEDGMENTS

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