THE UPTAKE OF TRITIUM-LABELLED THYMIDINE BY LYMPHOID TISSUE

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In 1885 Flemming, in one of the first studies of mitotic division in mammals, described in lymphoid tissue a nodular formation consisting of a clear central portion surrounded by a dark rim. The central portion he described as a germinal centre, because of the numerous mitoses which it contained. The daughter cells after division would be pushed—or migrate—outwards to form the peripheral darker zone of small lymphocytes which then entered the lymph sinuses, and were carried in the lymph stream to the blood. Flemming described germ centres in lymph nodes (ox and rabbit) Peyer's patches (rabbit) and lingual tonsils (man). The light central portions of the nodules had been noted previously by Brücke (1854), His (1862—he termed them 'Vacuolen'), and Frey (1874), but their significance had not been appreciated.

Flemming's view of the germ centre held the field for many years. (For a review of the earlier literature see Latta, 1922.) Latta (1921, 1922) seems to have been one of the first to challenge it seriously. He thought it was a region of degeneration—more especially in the spleen—consequent upon impairment of the blood supply to the central portion of large nodules. Hellman (1939, 1943) termed the germinal centres 'reaction' centres, regarding them as areas of reaction to noxious substances reaching them via the blood stream.

Maximow (1927) came to the conclusion that the germinal centres were structures which showed cyclic changes, and this view was substantiated and amplified by the later work of Conway (1937). When cell division was active, numerous mitoses could be seen. During the time when activity was at a low ebb there was no sign of cell formation, and it was the appearances at this stage which suggested degenerative changes. The position has been complicated by the fact that in some animals, e.g. cat (Röhlich, 1928), monkey (Taliaferro & Cannon, 1936) and rat (Kindred, 1938) the germinal centre may consist of two distinct zones, an inner active portion next to the medulla, and an outer inactive portion near the capsule.

In recent years still other views have been put forward concerning the structure and function of the germinal centre. Thus Trowell (1957) and Hamilton (1957) have suggested that there is considerable re-utilization of lymphocytes, ingested by the macrophages which are often a conspicuous feature of the germinal centres. These macrophages may be quite large, and their pale cytoplasm contains dark-staining bodies, noted by Flemming (1885) and termed by him 'tingible Körper'.

Yet another interpretation is that advanced by Ortega & Mellors (1957), who thought, following some earlier observations of Leduc, Coons & Connolly (1955) that the germinal centres were glandular in function and produced gamma globulin.

The various views which have been put forward concerning the germinal centres fall essentially into two main categories. According to one group of observers, the germinal centre is a region of active cell proliferation, whereas according to the second view it is not. The evidence for cell multiplication rested initially upon the observation of mitoses. More recently it has been provided by the uptake of radio-active precursors into newly synthesized DNA (e.g. Andreasen & Ottesen, 1945; Yoffey, Hanks & Kelly, 1958; Walker & Leblond, 1958; Cronkite, Bond, Fliedner & Rubini, 1959; and many others). The site of such uptake can be identified in radioautographs of sections. In the present work we have endeavoured to study the extent of cell multiplication by the use of tritium-labelled thymidine.

A discussion of the significance of labelling after the administration of tritiated thymidine, and a report on the appearance of labelled cells in thoracic duct lymph and blood will be found elsewhere (Everett, Reinhardt & Yoffey, 1960). Briefly, it may here be stated that tritiated thymidine is incorporated rapidly and specifically into DNA which is being synthesized prior to mitosis. Cells which take up thymidine are therefore growing cells which will subsequently divide.

MATERIAL AND METHODS

The work has been performed on 15 male guinea-pigs, of a mixed strain, weighing approximately 400 g. Observations have already been published on the appearance of labelled cells in the thoracic duct lymph of these animals (Everett, Reinhardt & Yoffey, 1960).

Tritiated thymidine (Schwarz), with specific activity of either 0·36 or 1·9 c./m-mole was given intraperitoneally or intravenously in doses of 1 μc./g. body weight and at varying intervals afterwards under sodium pentobarbital anaesthesia (4 mg./100 g. body weight) the thoracic duct lymph was collected for 1–3 hr. and the animal then killed. Samples of thymus, spleen, mesenteric lymph nodes and Peyer's patches were fixed in 10% formol saline or Bouin's fluid, and after paraffin embedding were cut at 6μ. In addition, suspensions were made of the cells of the thymus and mesenteric lymph nodes by cutting these tissues into small pieces and teasing in homologous serum. Smears were made of these suspensions and fixed in absolute methyl alcohol for 4 min. The tissue sections and the tissue smears were stained in haemalum for 2 hr. and in 1% eosin in 70% alcohol for 5 min. Subsequent to the transfer through 95% and 100% absolute alcohol the slides were dipped in a 1% solution of nitrocellulose in ether alcohol, dried in air for 30 min., and dipped a second time. They were then dried overnight at 37° C. Coated radioautographs were then prepared as described previously (Everett, Reinhardt & Yoffey, 1960) using NTB-3 emulsion (Eastman Kodak). The slides were exposed for 2–5 weeks and developed as previously described (*loc. cit.*). The radioautographs of the tissue smears, after washing, were stained once more using a 0·5% Giemsa solution in phosphate buffer of pH 6·4 at 5° C. for 12–15 hr.*

* Radioautographs are now prepared of tissue smears without the coating of nitrocellulose and without staining with haemalum and eosin. These preparations, after development, can be stained in a few minutes with conventional blood stains (Everett, Rieke, Reinhardt & Yoffey, *Ciba Symposium on Haemopoiesis*, 1960).

RESULTS

Sections. Labelling is most intense before any of the labelled cells have begun to divide. The best autographs are therefore those obtained within the first few hours after giving thymidine, before mitosis causes dilution of the label.

Pl. 1, fig. 1, shows the subcapsular sinus and adjacent cortical tissue of a mesenteric lymph node 4 hr. after the administration of thymidine. The most heavily labelled cells, one of which is in the sinus, are large lymphocytes. Some labelled medium lymphocytes may also be seen. The appearances suggest a steady migration of large lymphocytes into the sinus. Pl. 1, fig. 2, from the same gland, shows a germinal centre in which most of the cells are labelled. Pl. 2, fig. 3 on the other hand, shows a nodule in which considerably fewer cells are labelled—though those which are labelling do so intensely—and the germinal centre is much less active than that depicted in Pl. 1, fig. 2. Germ centres in different parts of the gland may show great variations in their proliferative activity.

Pl. 2, fig. 4, is from a Peyer's patch of the same animal, and shows a fair number of labelled cells. The lymphoid tissue of Peyer's patches is usually the site of proliferation which may be a good deal more active than shown in Pl. 2, fig. 4. Pl. 2; fig. 5, is from the spleen, and shows active proliferation, with many large lymphocytes labelling heavily.

Table 1. *Number and size distribution of labelled cells in mesenteric lymph node of guinea-pig at varying times after administration of tritiated thymidine*

No. of animal	Hr. after thymidine	No. of cells counted	Labelled cells, % of total	Size distribution of labelled cells				
				Large	Medium	Small	Damaged*	Total
100	0·5	2018	4·0	11	55	0	14	80
101	1	4122	3·8	28	83	7	38	156
94	3	4029	3·8	27	98	17	10	152
88	4·75	5132	3·0	20	105	17	12	154
89	5	4080	5·7	20	131	50	30	231
103	7	6189	4·4	32	148	53	42	275
106†	7	4037	6·8	14	103	53	103	273
104	12	6164	6·6	0	97	289	21	407
90	14·5	5130	5·5	11	111	134	27	283
105†	17·5	5091	3·7	24	88	38	36	186
92	27	3054	6·7	7	46	116	35	204
95	52·5	4100	11·7	1	62	401	17	480
91	78	4043	9·0	5	84	243	30	362
93	14·3	4036	15·3	0	59	540	20	619
96	190·5	4057	8·9	0	31	326	3	360

* Some of the damaged cells could be identified as reticulum cells, others as lymphocytes in different stages of disintegration. But in most cases the degree of damage was such as to make accurate identification impossible.

† High background.

Cell suspensions. Table 1 shows the distribution of labelled cells in the lymph node suspensions, and Table 2 in suspensions of thymic cells. The problem of cell size in smears is always a very difficult one, since it depends upon a number of factors, including the nature of the medium in which the cells are suspended and the speed with which the smears are made. The slower the smear the greater the cell spread, and the apparent size of the cells. Absolute measurements are not therefore

of much value in smears, and in practice the relative size—i.e. the size in relation to other lymphocytes or to red blood cells—has proved the most useful guide.

The figures for size distribution in Tables 1 and 2 can only be regarded as an approximation in the middle part of the range. There is no doubt about the really large lymphocytes, or the small. But it is often difficult to decide whether a cell should be classified as a medium lymphocyte which is a little larger than usual, or a large lymphocyte which is rather smaller. The cytoplasm of the larger lymphocytes is generally more basophilic, and in doubtful cases this may be a useful additional criterion.

Table 2. *Number and size distribution of labelled cells in suspensions of thymus of guinea-pig at varying times after administration of tritiated thymidine*

No. of animal	Hr. after thymidine	No. of cells counted	Labelled cells, % of total	Size distribution of labelled cells				
				Large	Medium	Small	Damaged*	Total
100	0·5	500	12·6	14	18	2	29	63
101	1	500	9·6	2	5	1	40	48
94	3	900	10·5	4	24	2	65	95
88	4·75	1300	7·8	8	36	5	53	102
89	5	500	4·0	1	7	3	9	20
103	7	1100	14·2	13	50	29	64	156
106†	7	600	3·2	1	2	2	14	19
104	12	500	1·6	0	0	3	5	8
90	14·5	1000	10·6	12	21	36	37	106
105	17·5	500	2·0	0	0	8	2	10
92	27	1000	8·9	2	20	24	43	89
95	52·5	1000	11·7	1	12	46	58	117
91	78	1100	17·6	11	22	139	22	194
93	143	400	9·7	0	8	20	11	39
96	190·5	300	29·3	0	0	64	24	88

* Damaged; accurate indentification impossible.
† High background.

Subject to considerations such as these, the general trend seems clear. In the early stages there are hardly any labelled small lymphocytes, the bulk of the labelling cells being large and medium, whereas in the latter stages the number of labelled small lymphocytes gradually increases, while that of the large and medium cells steadily diminishes. This is in accord with the trend previously found in the cells of thoracic duct lymph (Everett, Reinhardt & Yoffey, 1960), and would appear to indicate that the cell content of the lymph reflects the changes in the parent lymphoid tissue. This orderly sequence of changes in size distribution of lymphocytes seems to fit in best with the view that as far as the lymphoid tissues are concerned, the small lymphocytes are the end stage of a series of cell divisions (cf. Sainte-Marie & Leblond, 1958).

The changes in intensity of labelling seem to fit in with this view. Though the actual grain counts have not been performed, the appearances strongly suggest that the grain count of the labelled cells steadily diminishes with the passage of time. This was equally the case both in lymph node and thymus, though it was our impression that there was a more rapid dilution of label in the thymus. In Expt. 96 (190·5 hr.) many of the labelled small lymphocytes had only one or two grains. There was some doubt at first as to whether one could in fact count these cells as

labelled or not, but in this instance there was fortunately a very clear background, which simplified the decision.

A further point of interest concerns the large number of damaged cells. Whilst many of these were undoubtedly reticulum cells, large numbers were also lymphocytes, and one could readily observe a graded series of damaged cells, ranging from those which could be clearly identified as lymphocytes which had been only slightly damaged, to cells which had been damaged so severely that if seen in isolation they would not have been recognizable. It seems apparent that many of these damaged cells, the 'smudge' or 'basket' cells thought so often to be effete and dying, are on the contrary cells which are actively growing (cf. Cronkite *et al.* 1959). It may possibly be the case that all growing cells, at certain phases of the mitotic cycle, are readily damaged in the process of making a smear.

DISCUSSION

The significance of the germinal centre

From appearances such as those seen in Pl. 1, fig. 2, it would appear that the germinal centre can be an area of active proliferation. Even when the centre is less active, as in Pl. 2, fig. 3, there still seems to be an appreciable amount of cell proliferation. The variation in activity of the different germinal centres seems to be brought out with especial clarity in the thymidine autographs, and would appear to accord best with the view that they are the seat of cyclic changes.

The evidence of DNA synthesis does not altogether rule out some degree of re-utilization, as suggested by Trowell (1957) and Hamilton (1957). However, if re-utilization does occur, it quite obviously does not dispense with the need for active formation of new DNA on a considerable scale. Similarly, the occurrence of energetic DNA synthesis does not rule out the possibility of some degree of re-circulation, as suggested by Yoffey & Drinker (1939). But the fact that new formation of DNA can be so marked as indicated in Pl. 1, fig. 2, seems to argue against anything like a major re-circulation of lymphocytes, as suggested by Sjövall (1936) and Gowans (1959). It is difficult to reconcile the evidence of thymidine uptake with the view of Latta (1922) that the germinal centre is primarily an area of degeneration, or that it is a reaction centre to noxious substances (Hellman, 1939), unless reaction implies active cellular proliferation presumably of cells other than lymphocytes. As far as Latta's (1922) view is concerned, it is perfectly true that in large germinal centres the central portion may show no mitotic activity, but in such cases it will usually be found that the peripheral part of the germ centre contains numerous dividing cells.

Lymphocyte formation in the spleen

The extent to which lymphocyte formation may occur in the spleen has always been a matter of doubt. Morris (1914) compared the white cell content of blood in the splenic artery with that in the vein, and found the count in the splenic vein to be appreciably above that in the artery. Pearce, Krumbhaar & Frazier (1918) were unable to demonstrate a clearcut difference, and concluded that '...detailed comparison of the arterial and venous blood of the spleen offers no evidence to indicate by the methods used that the spleen has an important role in blood formation'.

More recently, Fichtelius (1958) has suggested that the spleen may be constantly taking up lymphocytes from the blood stream. Appearances such as those in Pl. 2, fig. 5, do not rule out such a possibility, but they make it seem most unlikely. It seems improbable that a spleen which itself produces large numbers of lymphocytes would be in need of many lymphocytes from elsewhere. Furthermore, if the protective effect of spleen shielding (Jacobson, Marks, Gaston, Robson & Zirkle, 1949) is cellular in origin, this too would imply an active production of cells by the spleen.

SUMMARY

In thirteen healthy male guinea-pigs the uptake of tritiated thymidine has been investigated in lymph nodes, thymus, spleen and Peyer's patches. Radioautographs have been prepared both in cell suspensions, and in sections. The germinal centres frequently show extensive DNA synthesis, and this is interpreted to support the view that they are centres of cell proliferation. Lymphocytopoiesis is also active in Peyer's patches and in the spleen. In the lymphoid tissues small lymphocytes are formed by the repeated division of large cells.

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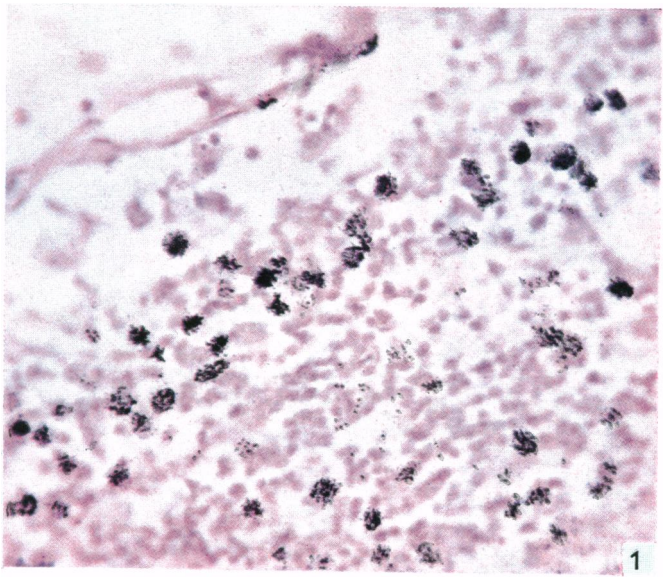

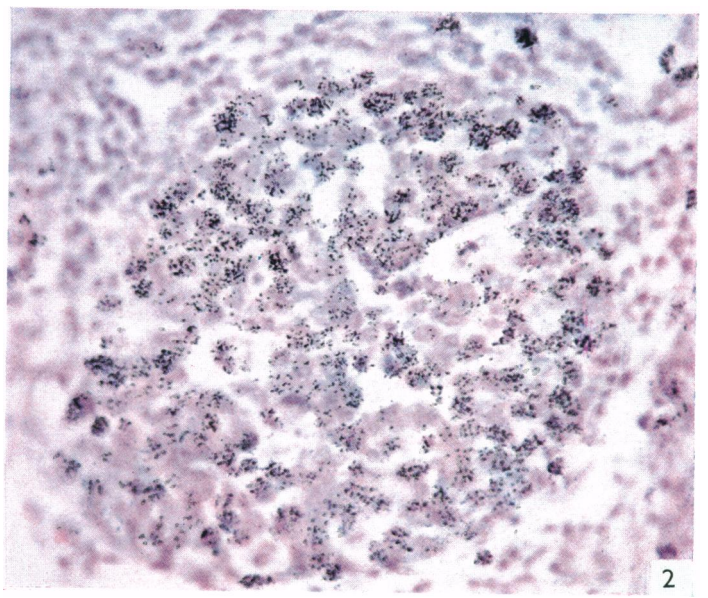

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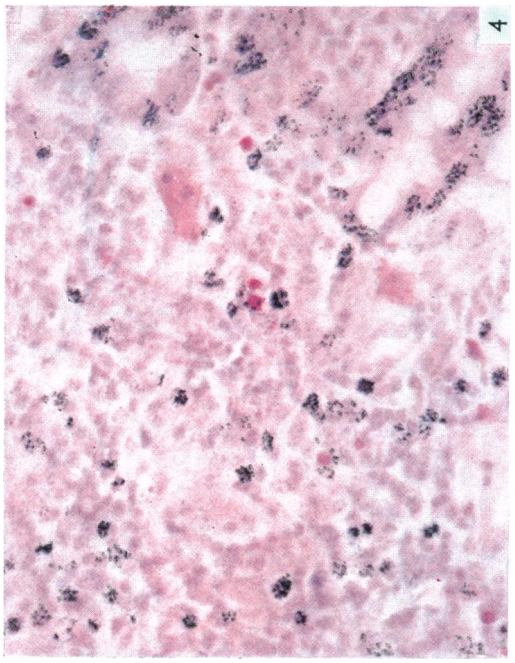

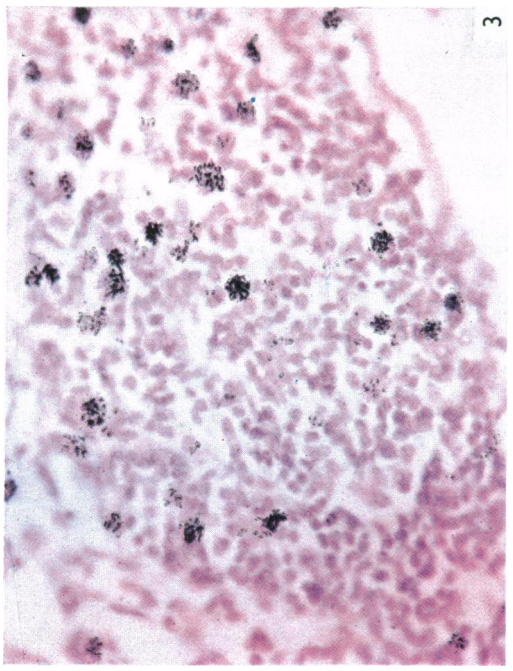

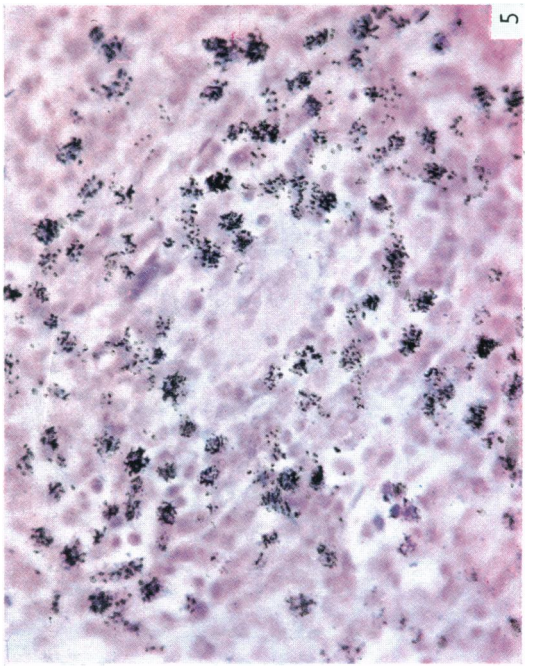

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Mesenteric lymph node, 4 hr. after tritiated thymidine. The section shows the periphery of a lymphoid nodule and the adjacent subcapsular region. A heavily labelled large lymphocyte is lying free in the subcapsular sinus. A considerable number of labelled large lymphocytes are lying in the lymphoid tissue close to the sinus. (×552.)

Fig. 2. Mesenteric lymph node of guinea-pig 4 hr. after tritiated thymidine. A young germ centre with many labelling cells. (×552.)

PLATE 2

Fig. 3. Mesenteric lymph node of guinea-pig. A lymphoid nodule with dense labelling of only a few cells. Compare with fig. 2. (×552.)

Fig. 4. Peyer's patch of guinea-pig, 4 hr. after tritiated thymidine. Note the heavy labelling of scattered cells in the patch, as also of the adjoining mucosal cells. (×368.)

Fig. 5. Spleen of guinea-pig 4 hr. after tritiated thymidine A perivascular lymphoid sheath showing many heavily labelled medium and large lymphocytes. (×552.)