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XVII. THE MIGRATION OF ANTIGEN-BINDING, BONE-MARROW-DERIVED AND THYMUS-DERIVED SPLEEN CELLS IN MICE*

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Summary. The purpose of this investigation was to compare and contrast the migratory behaviour of three sub-classes of spleen lymphocytes in the mouse: cells capable of adsorbing a specific labelled antigen on to their surfaces ('antigen-binding' cells); bone-marrow-derived ('B') cells; and thymus-derived ('T') cells.

Antigen-binding spleen cells were labelled *in vitro* with ^{125}I-polymerized flagellin from *Salmonella adelaide* and injected intravenously into syngeneic recipients. Their distribution within the spleen was studied by radioautography for 24 hours after injection. Nearly all the antigen-binding cells localized and accumulated in and around the germinal centres of the white pulp.

Bone-marrow-derived B-spleen cells which were labelled *in vitro* with ^{3}H-uridine were found to localize in and around germinal centres. The proportion of B cells found in these regions was lower than the proportion of antigen-binding cells and there was not a progressive aggregation of B cells over 48 hours.

Thymus-derived T-spleen cells also labelled *in vitro* with ^{3}H-uridine were located predominantly in the established thymus-dependent periarteriolar regions of the white pulp.

The significance of the localization of antigen-binding cells in the germinal centre regions of spleen is discussed in relation to the B and T components of the spleen lymphocyte population. The conclusion was reached that B lymphocytes activated by contact with antigen lodged preferentially in germinal centre areas.

INTRODUCTION

Since the demonstration of antigen binding *in vitro* by mononuclear lymphocyte-like cells (Naor and Sulitzeanu, 1967; Byrt and Ada, 1969) the immunological significance of this phenomenon has been under investigation. High doses of radioactivity on the surface of antigen-binding cells can selectively abolish an adoptive antibody response, apparently by killing the labelled antigen-binding cells (Ada and Byrt, 1969). The occurrence of antigen binding by lymphocytes after the introduction of antigen into an intact animal has been demonstrated (Mitchell and Abbot, 1971) with evidence suggestive of antigen movement within the spleen occurring by virtue of its association with mobile lymphocytes, an interpretation supported by earlier studies of the movement of large quantities of

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antigen (Nossal, Austin, Pye and Mitchell, 1966) and the histological studies of Pettersen, Borgen and Graupner (1967). Recently Brown, de Jesus, Holborow and Harris (1970) reported that antigen appeared to move on lymphocytes into the germinal centre regions of the spleen.

We have studied by radioautography the distribution of cells labelled *in vitro* with ^{125}I-polymerized flagellin and subsequently injected into normal syngeneic mice. As a basis for comparison of the movement of antigen-binding cells with that of the whole population, normal spleen cells were labelled with ^{3}H-uridine *in vitro* and their distribution examined after injection. Further dissection of the migratory pattern of the whole population of spleen cells was provided by studying the distribution of ^{3}H-uridine labelled thymus-derived (T) or bone-marrow-derived (B) cells (Miller, Basten, Sprent and Cheers, 1971) whose antigen-binding capacities differ from each other, the B cells showing much heavier labelling with a given dose of antigen (Dwyer and Mackay, 1971).

MATERIALS AND METHODS

Animals

Adult (8–16 weeks) CBA mice of both sexes, inbred at the Hall Institute, were used as normal spleen cell donors.

Donors which were thymectomized within 24 hours of birth showed no evidence, visually or microscopically, of thymus remnants.

Donors of 'educated' T cells (Miller *et al.*, 1971) were adult CBA × C57B1 F_1 mice given 750 rads X-irradiation and injected intravenously with 100×10^6 thymus cells from adult CBA donors. The spleens from these animals were used 4 days later as the source of T cells.

Donors of bone-marrow-derived cells, B cells (Miller *et al.*, 1971) were thymectomized at 4 weeks of age, X-irradiated with 750 rads at 7 weeks and injected with 5×10^6 syngeneic bone marrow cells. Their spleens were removed 5 weeks later as a source of B cells.

Recipients were normal CBA mice; mice immunized with 10 μg polymerized flagellin (POL) 4 weeks before use (POL-immunized recipients); or mice immunized with 0·5 ml 20 per cent sheep red blood cells (SRBC) 6 days before use (stimulated recipients) to stimulate the production of germinal centres in the spleen.

Antigen and isotopes

Polymerized flagellin (POL) was prepared from *Salmonella adelaide* by the method of Ada, Nossal, Pye and Abbot (1964).

POL was labelled with ^{125}I by chloramine-T oxidation (Byrt and Ada, 1969). Carrier-free ^{125}I was purchased from the Radiochemical Centre, Amersham, U.K., and mixed (6·6 mCi in 60 μl) with POL (60 μg in 30 μl) and chloramine-T (final concentration 1 mg/ml) added. After 3 minutes, potassium metabisulphite (final concentration 24 mg/ml) was added, followed by KI (M/10, 20 μl) and 20 μl foetal calf serum (FCS). The labelled protein was then separated from iodide in Sephadex (Pharmacia) G-100 which had been pretreated with FCS. The labelled POL contained 60–90 μCi ^{125}I/10 μg.

Media

In all experiments except No. 5, cell manipulations *in vitro* were made in Eagle's medium buffered with Tris (Commonwealth Serum Laboratories, Melbourne) containing FCS (10 per cent), penicillin and streptomycin.

In experiment 5 involving dead cell removal by the bovine serum albumin (BSA) gradient method of Shortman, Williams and Adams (1972), all cell manipulations were made in Eagle's medium buffered with Hepes (Calbiochem) and containing FCS, penicillin and streptomycin.

Density gradients

FCS gradients were prepared by layering 3 ml each 100, 75 and 50 per cent FCS in medium in 15 ml capacity tubes. Cells suspended in medium were centrifuged through the gradient at 300 ***g*** for 8 minutes at 4°C.

BSA gradients were prepared by the method to be reported by Shortman *et al.* (1972). Cells suspended in 5 ml BSA at mouse tonicity (30 per cent BSA in Hepes-buffered Eagle's medium, density 1·093) were layered on to 2 ml BSA (density 1·093) followed by 1 ml 50 per cent BSA and 1 ml medium. After mixing the interfaces, the tube was centrifuged at 3000 ***g***, 10 minutes at 4° to deposit the dense dead cells. Living cells remained in suspension and were resuspended in three times their volume of medium containing 10 per cent FCS, centrifuged at 300 ***g***, 10 minutes, 4°, and resuspended for use.

Preparation of labelled cells

The spleens from five mice (killed by cervical dislocation) constituted each pool. They were diced on a stainless steel sieve into 5 ml medium and allowed to stand 15 minutes over 3 ml FCS. The medium layer was then centrifuged (300 ***g***, 8 minutes, 4°) through 7 ml FCS.

(a) *Antigen-binding cells.* After resuspension (300×10^6/3 ml) the cells were mixed with labelled antigen (15 μg ^{125}I-POL) and held in ice for 30 minutes. The preparation was passed through two FCS gradients to remove excess labelled antigen. The cells were resuspended for injection ($100–200 \times 10^6$/ml) and a sample smeared on gelatin-coated slides for radioautography. The inoculum contained approximately 1 ng POL/100×10^6 cells.

(b) *^{3}H-uridine labelled cells.* In experiment 5 cells were resuspended in BSA for dead cell removal by the BSA gradient technique. For all experiments the cells were resuspended in 3–8 ml medium ($20–100 \times 10^6$/ml), in stoppered 25 ml flasks. ^{3}H-uridine (Amersham TRK178 Batch 44, 30·7 Ci/mM) was added (20 μCi/ml) and incubated with the cells at 37° for 1 hour with slow rocking. Excess label was removed by centrifugation through an FCS gradient before the cells were resuspended for injection as required. A sample was taken for radioautography. Thymus cells used in experiment 5 were prepared from six CBS mice and labelled with ^{3}H-uridine by the same method as spleen cells.

Viability estimations

Viability was estimated by eosin dye exclusion using 0·2 per cent eosin in 15 per cent FCS saline (Black and Berenbaum, 1964). Good correlation was obtained between the number of cells found to be viable by this method and the number found to be labelled with ^{3}H-uridine. Inocula were adjusted for viability which was between 80 and 99 per cent in all cases.

Injections and tissue preparation

Labelled cells were injected slowly into the tail vein in a volume of 0·3–0·5 ml. At intervals from 10 minues to 2 days later mice were killed by cervical dislocation and their spleens removed. After fixation in 10 per cent neutral buffered formalin the tissue was

embedded in paraffin and prepared as sections on gelatin coated slides for radioautography. The slides were dipped in Eastman Kodak NTB-2 emulsion at 42°, dried vertically and stored with anhydrous calcium sulphate (Drierite) for exposure times varying from 4 days to 16 weeks. After development in Kodak D 19b and fixation in Amfix (May and Baker) they were stained with methyl green–pyronin.

Definition of spleen areas

The terminology used in describing labelled cell location in the spleen requires definition. Fig. 1 shows diagrammatically the areas recognized as different categories in making

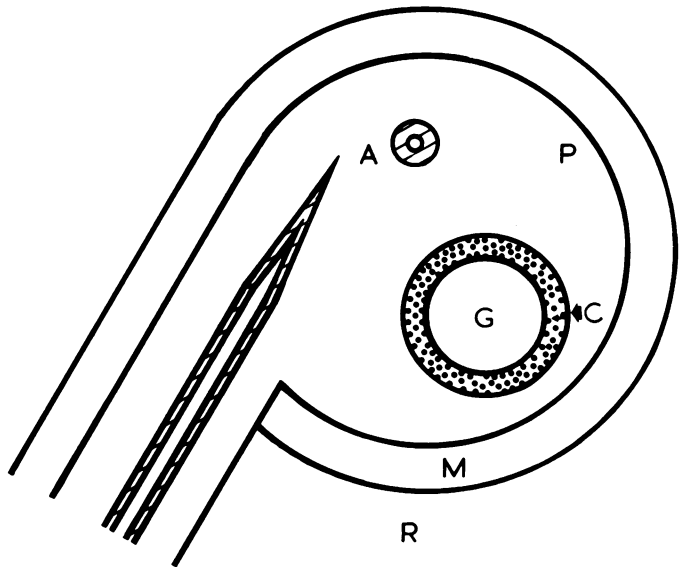

FIG. 1. Schematic diagram of a germinal-centre-containing field of spleen representative of the area scored for labelled cells. A, periarteriolar lymphocyte sheath, within five cell diameters of a sectioned vessel; P, peripheral white pulp; G, germinal centre containing at least two tingible body macrophages and pyroninophilic lymphocytes; C, cuff of lymphocytes within three cell diameters of G; M, marginal zone; R, red pulp.

counts over tissue sections. Thus the red pulp (R) included regions of lymphocytes and plasma cells lying along fibrous septae; marginal zone (M) was the erythrocyte-containing outer border of the white pulp; the diffuse white pulp had a clearly periarteriolar component (A) within five cells of the arteriole, together with a peripheral component (P) which included some periarteriolar and potential primary follicle regions; the germinal centres (G) were defined by the presence of at least two tingible body macrophages together with pyroninophilic blast cells, while the cuff region (C) was defined as the lymphocytes within three cells of such a germinal centre.

Quantitation of labelled cells

In all experiments the sections were coded and then scanned systematically with the grains out of focus under × 155-fold magnification. When an area (1 mm diameter) was

found containing all the features of Fig. 1, the grains were brought into focus and the number of cells containing fifteen or more grains was scored. Labelled cells were verified under high magnification.

The variation in the numbers of germinal centres seen in normal recipient mice limited the numbers of fields available for scoring in the early experiments. Later, more uniformity in the number of germinal centre containing fields was produced by the use of 'stimulated' recipients (*vide supra*).

The number of cells found in each compartment is expressed as the percentage of the total labelled cells counted.

When more than 1×10^6 labelled cells had been injected (experiments 4 and 5) sufficient labelled cells were present in one representative field to allow quantitative assessment of the number of such cells in a unit area of each compartment. Radioautographs were projected at approximately 1000-fold magnification and the labelled cells traced on to bond paper together with the areas occupied by white pulp (A, P and C) germinal centre (G), marginal zone (M) and red pulp (R). Ten such fields were traced and the paper cut. The number of labelled cells per unit weight (10 g) was calculated.

RESULTS

1. LOCALIZATION OF ANTIGEN-BINDING SPLEEN CELLS

In experiment 1, normal mouse spleen cells were labelled with ^{125}I-POL and 35×10^6 viable cells were injected intravenously into four recipients which were killed after 1, 4, 8 or 24 hours. Radioautography of the inoculum showed that after an exposure of 1 day approximately 1/4000 cells was heavily labelled. Sections of spleen from the recipient mice were exposed for 4 days to compensate for the decreased efficiency of radioautography with ^{125}I in sections (Ada, Humphrey, Askonas, McDevitt and Nossal, 1966). The results (Table 1) showed that the most avid antigen-binding cells were rarely found after injection.

When radioautographs of the inoculum were exposed for 15 days, the more lightly labelled antigen-binding cells were detected and the number of labelled lymphocytes (>30 grains) was increased to approximately 3/1000. (Furthermore, since no inhibitor of phagocytosis had been present during the labelling with ^{125}I-POL, 10/1000 labelled phagocytic cells were also present in the inoculum.) Radioautographs of the spleen sections of the recipient mice were exposed for 28 days and the coded sections scored for the numbers of labelled cells present in each of the compartments recognized (Fig. 1).

TABLE 1

COUNTS OF ^{125}I-POL-LABELLED CELLS ON RADIOAUTOGRAPHS EXPOSED 4 DAYS

Time after injection (hours)	No. of longitudinal sections scanned	No. of heavily labelled cells (confluent grains)	Total No. of labelled cells (>15 grains)
1	43	2	18
4	23	0	21
8	21	1	14
24	12	—	1

TABLE 2

DISTRIBUTION OF ^{125}I-POL-LABELLED SPLEEN CELLS WITHIN THE SPLEEN AFTER INTRAVENOUS INJECTION

Time after injection	Expt No.	No. labelled cells/No. fields	Labelled cells (>15 grains) in different regions as the percentage of the total in radioautographs exposed 28 days					
					Diffuse white pulp		Germinal centre and cuff	
			Red pulp (R)	Marginal zone (M)	Periarteriolar (A)	Peripheral (P)	(G)	(C)
10 minutes	2	51/31	57	37	0	2	0	4
1 hour	1	77/40	13	40	5	20	3	19
	2	55/40	18	54	8	15	2	3
4 hours	1	152/80	11	19	3	24	12	31
5 hours	2	15/20	10	23	0	27	13	27
8 hours	1	118/160	4	5	3	26	20	42
24 hours	1	65/131	5	1	0	15	65	14
	2	10/40	0	0	10	30	40	20

In experiment 2, normal spleen cells were labelled with ^{125}I-POL and were injected in a dose of 90×10^6 viable cells into normal recipients which were killed after 10 minutes, 1, 5 or 24 hours.

The results of experiments 1 and 2 are summarized in Table 2. Labelled cells in the red pulp were prominent 10 minutes after injection and declined steadily thereafter. They

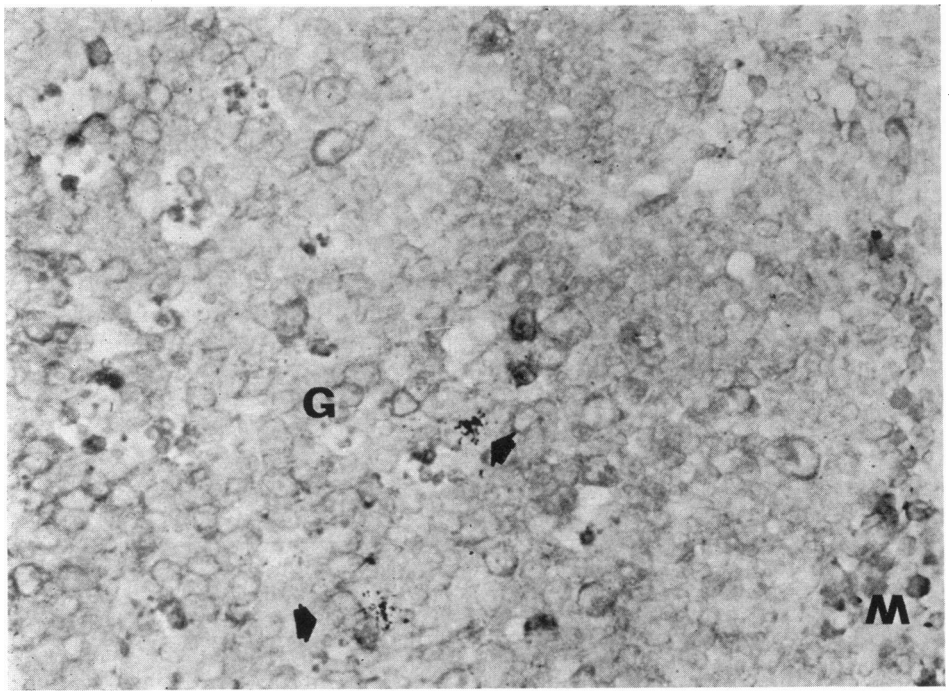

FIG. 2. ^{125}I-labelled antigen-binding cells (arrowed) inside a germinal centre of the spleen 24 hours after injection. G, germinal centre; M, marginal zone. Radioautograph exposed 4 weeks. × 360.

TABLE 3

COMPARISON OF RADIOACTIVITY AND NUMBERS OF LABELLED CELLS AFTER THE INJECTION OF EITHER ^{125}I-POL-LABELLED CELLS OR ^{125}I-POL ALONE

Time after injection	Normal recipients				POL-immunized recipients			
	^{125}I-POL-labelled cells 3000 cps		^{125}I-POL alone 16,000 cps		^{125}I-POL-labelled cells 3000 cps		^{125}I-POL alone 16,000 cps	
	cps/mg spleen	No. labelled cells/No. fields	cps/mg spleen	No. labelled cells/No. fields	cps/mg spleen	No. labelled cells/No. fields	cps/mg spleen	No. labelled cells/No. fields
10 minutes	32	51/31	374	Heavy diffuse label in marginal zone	34	45/20	285	Heavy diffuse label in marginal zone
1 hour	31	55/40	60	Light label throughout marginal zone	32	65/20	37	13/16+ diffuse label
5 hours	9	15/20	12	6/15+diffuse label	9	24/16	11	Light diffuse label in all germinal centres
24 hours	3	6/20	3	Light diffuse label in all germinal centres	4	Light diffuse label in all germinal centres	4	Light diffuse label in all germinal centres

were also seen in the marginal zone for 1 hour after injection, but were virtually absent from this region after 8 and 24 hours. There were no significant numbers of labelled cells close to the arterioles (A); however, the criteria for this category were stringent (within five cells of a sectioned vessel) and some of the thymus-dependent periarteriolar region (Parrot, de Sousa and East, 1966) was included in the peripheral compartment, P. From 1 to 24 hours there was a relatively constant proportion of labelled cells in the peripheral region P. At 24 hours, frequently a labelled cell was seen amongst several pyroninophilic cells (a probable primary lymphoid follicle).

The most interesting feature of the location of antigen-binding cells was the presence of single labelled cells within and around germinal centres (Fig. 2). Such cells were apparent even 4 hours after injection, and was quite evident after 24 hours. Frequently, labelled cells in this area also were pyroninophilic at 8 and 24 hours after injection.

It was possible that the long (28 days) radioautographic exposure may have allowed detection of the hosts' own antigen-binding lymphocytes if free antigen had been released from the injected antigen-binding cells. As a control for this (experiment 2) ^{125}I-POL was prepared; half was used to label normal cells (90×10^6 per inoculum, 3000 cps ^{125}I, approx. 1 ng POL); the remaining antigen was diluted to contain 16,000 cps (4 ng POL) to form a control inoculum containing five times as much ^{125}I-POL as could possibly have come off the POL-labelled cells in the actual experiment. Normal or POL-immunized recipient mice were injected with either labelled cells or labelled antigen and killed after 10 minutes, 1, 5 or 24 hours. The radioactivity of the spleens was assayed before radioautography and the results are presented in Table 3. The use of POL-immunized recipients appeared not to alter significantly the numbers of antigen-binding cells or the amount of antigen alone retained by the spleen over 24 hours. There was, however, a qualitative difference in the location of the injected antigen-binding cells in normal and POL-immunized mice. In the immune animals the labelled cells were predominantly (76 per cent) in the germinal centre and cuff regions at 5 hours after injection and individual cells were no longer apparent at 24 hours. Although some individual antigen-binding cells did appear in the radioautographs after the injection of antigen alone, they were less heavily labelled and occurred less frequently than after the injection of one-fifth the amount of antigen attached to cells. When an equal amount of antigen either alone or attached to cells was injected into normal mice, then, 1 hour after injection, more of that presented on cells was located in the spleen (51 cells/30 fields) than that presented as antigen alone (6·5/30 fields).

2. LOCALIZATION OF NORMAL, T AND B SPLEEN CELLS

To determine what proportion of normal spleen cells migrate to the germinal centre regions of the spleen, ^{3}H-uridine was used to label >95 per cent of a normal spleen cell suspension before injection into normal or stimulated recipients. In experiment 3, normal recipients were injected with 100×10^6 cells labelled with ^{3}H-uridine. Radioautographs of animals killed at 10 minutes, 1, 2, 5 or 24 hours showed qualitatively that the labelled cells were concentrated in the diffuse white pulp but tended to avoid the germinal centre regions.

To provide a comparison with the injection of antigen-binding cells, 100×10^6 unlabelled cells mixed with $0{\cdot}1 \times 10^6$ ^{3}H-uridine labelled cells were injected into normal recipients

TABLE 4

DISTRIBUTION OF ^{3}H-URIDINE-LABELLED SPLEEN CELLS WITHIN THE SPLEEN AFTER INTRAVENOUS INJECTION

Time after injection	Expt. No.	No. labelled cells/No. fields	Labelled cells (> 15 grains) in different regions as the percentage of the total in radioautographs exposed 8–10 weeks					
			Red Pulp (R)	Marginal zone (M)	Diffuse white pulp		Germinal centre and cuff	
					Periarteriolar (A)	Peripheral (P)	(G)	(C)
10 minutes	3	18/60	67	33	0	0	0	0
30 minutes	4	913/20	35	42	3	15	1	3
	5	107/10	22	54	2	18	1	4
1 hour	3	69/40	36	32	6	19	3	4
2 hours	3	69/40	28	18	7	39	1	7
	4	771/10	24	12	18	33	4	9
4 hours	4	924/10	15	6	17	48	2	12
	5	403/10	15	8	15	46	7	10
5 hours	3	60/48	18	25	5	50	0	2
8 hours	5	394/10	13	7	23	42	5	13
24 hours	3	51/40	14	20	8	42	8	8
	4	812/10	8	9	5	59	5	14
	5	244/10	11	9	11	41	7	21

TABLE 5

COMPARISON OF THE DISTRIBUTION OF NORMAL SPLEEN AND THYMUS CELLS WITH THYMUS- AND BONE MARROW-DERIVED SPLEEN CELLS

Time after injection of ^{3}H-uridine-labelled cells	No. labelled cells/No. fields	Type cells injected	Per cent labelled cells (> 15 grains) in different compartments in radioautographs exposed 6 or 8 weeks			
			Marginal zone (M)	Diffuse white pulp		Germinal centre and cuff (G+C)
				Periarteriolar (A)	Peripheral (P)	
30 minutes	145/10	B-spleen	55	1	20	8
	107/10	Normal spleen	54	2	18	4
	135/20	Thymus	37	10	14	4
4 hours	354/10	B-spleen	8	12	31	30
	403/10	Normal spleen	8	15	46	16
	670/10	T-spleen	11	28	43	7
	208/20	Thymus	6	34	51	3
8 hours	288/10	B-spleen	6	11	30	40
	394/10	Normal spleen	7	23	42	18
	638/10	T-spleen	16	20	42	7
	191/20	Thymus	8	25	56	6
24 hours	275/10	B-spleen	12	7	40	33
	244/10	Normal spleen	9	11	41	28
	345/10	T-spleen	22	11	49	5
	74/10	Thymus	0	45	50	5
48 hours	152/10	B-spleen	11	7	40	24
	112/10	Normal spleen	21	14	32	20
	23/20	Thymus	4	39	48	9

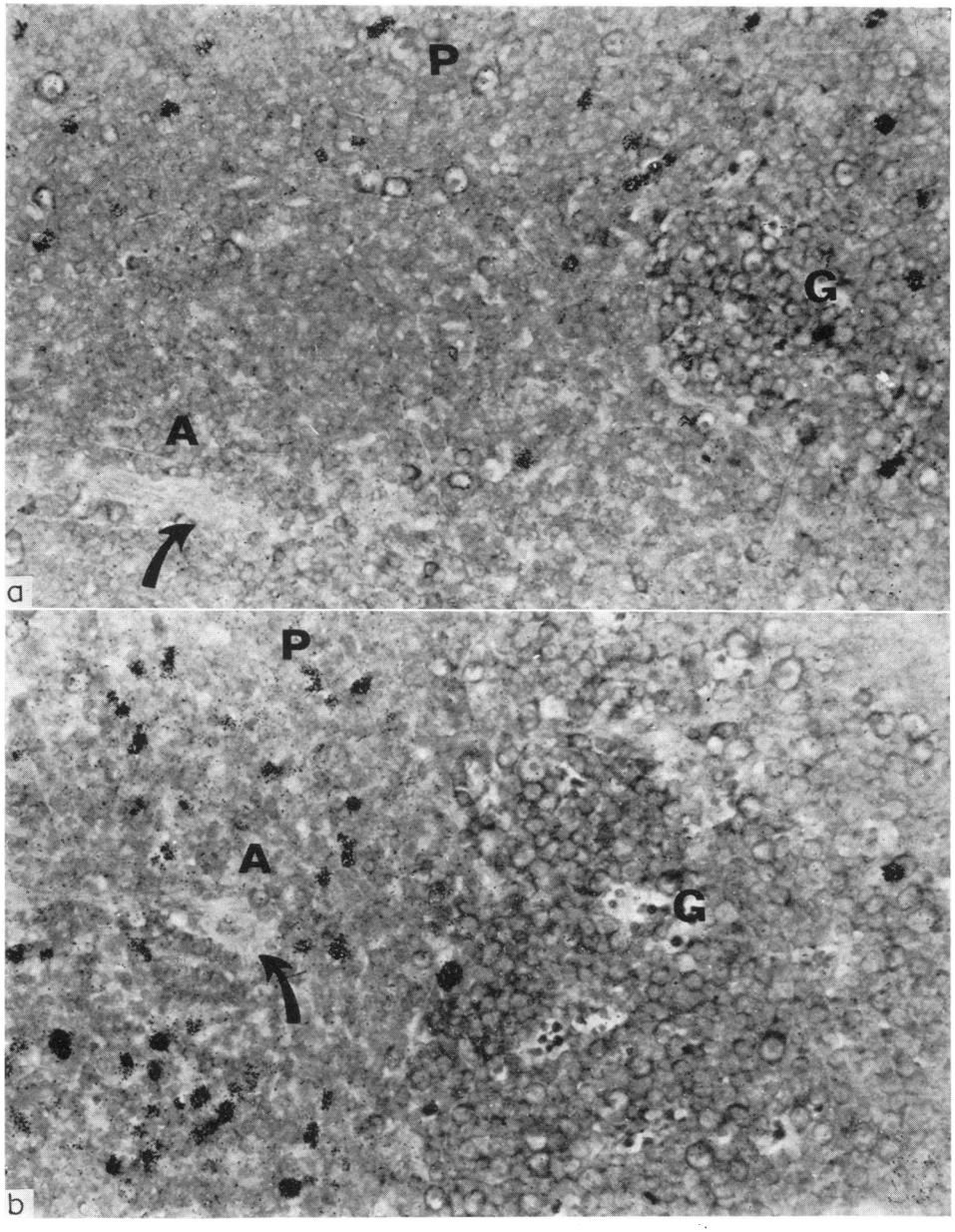

FIG. 3. ^{3}H-uridine labelled cells in spleen white pulp 8 hours after injection. G, germinal centre; A, periarteriolar lymphocyte sheath; P, peripheral white pulp. Arrow, central arteriole. Radioautographs exposed 8 weeks. × 290. (a) Bone marrow derived B spleen cells located in and around a germinal centre. (b) Thymus derived T spleen cells located in the periarteriolar region.

(experiment 3). The numbers of labelled cells in the resultant sections were comparable with those found after the injection of antigen-binding cells. Quantitation of these labelled cells by counting germinal-centre-containing fields (Fig. 1) showed that there was a differ-

ent pattern of migration and localization of ^{3}H-uridine labelled cells from that seen with ^{125}I-labelled antigen-binding cells. In further experiments, 10×10^6 ^{3}H-uridine labelled cells or 5×10^6 cells were injected into stimulated recipients which were killed between 30 minutes and 24 hours later. No significant differences were apparent in the localization of labelled normal cells over the 100-fold range in cell dose, and the results are combined in Table 4. The low proportion of labelled cells found both inside and around germinal centres continued over 24 hours.

To examine subpopulations of normal spleen cells, the distribution of 10×10^6 ^{3}H-uridine labelled cells from either normal or neonatally thymectomized mice was examined. Counting of the labelled cells by microscopy failed to show any significant differences in the localization of these two populations.

A further experiment was carried out to trace the distribution of suspensions of relatively pure thymus-derived spleen cells (T cells), bone-marrow derived spleen cells (B cells), normal spleen cells and thymus cells, using donor mice prepared as described in Materials and Methods and injecting intravenous doses of 5×10^6 viable cells. Recipient animals were killed between $\frac{1}{2}$ and 48 hours after injection and radioautographs of the spleens exposed for 6 weeks (spleen cell inocula, 30 minutes to 24 hours intervals) or 8 weeks (other sections). Table 5 shows the results of differential counting of the radioautographs.

In the marginal zone there were no major departures from the localization pattern seen with normal spleen cells. In the periarteriolar component of the white pulp, the thymus cells were most heavily concentrated, particularly by 24 hours.

T-spleen cells and thymus cells showed different seeding efficiencies in the spleens of the recipient mice, T-cells being some five times more concentrated than thymus cells in the radioautographs (Table 5, col. 2). T-cells and thymus cells were similar, however, in their

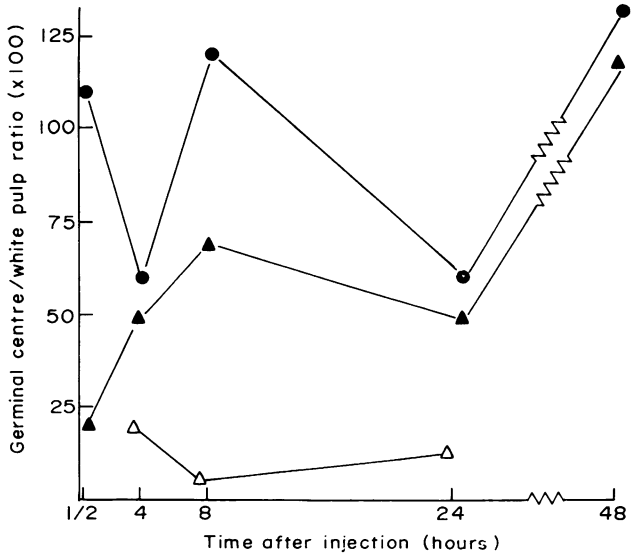

FIG. 4. Germinal centre: remaining white pulp ratio of the number of ^{3}H-uridine labelled cells per unit area (expressed as percentage). ●, B-cells; △, T-cells; ▲, normal cells.

comparative scarcity in and around germinal centres (Table 6). By contrast, B-spleen cells showed a marked tendency to be located in these regions (Table 5). The visual contrast between B and T spleen cells is illustrated in Fig. 3.

Tracing of projected images on to paper allowed a quantitative assessment of the number of labelled cells per unit area (concentration). The concentration of labelled cells in germinal centres was compared with that in the remaining white pulp (Fig. 4). This showed that B-cells were almost equally common in the two regions over 48 hours. T-cells, on the other hand, were only a maximum of one-fifth as concentrated in germinal centres as in the remaining white pulp. Normal spleen cells were intermediate between B-cells and T-cells in their relative germinal centre concentrations, as would be expected since they contain both B and T cells (Miller *et al.*, 1971).

3. COMPARISON OF THE LOCATION OF ANTIGEN-BINDING SPLEEN CELLS WITH NORMAL SPLEEN CELLS OF DIFFERENT ORIGINS

Comparison of Tables 2 and 4 shows that antigen-binding spleen cells were essentially similar to the whole population in their proportional localization over 24 hours in the red pulp, marginal zone and periarteriolar regions of the spleen. The major difference between the two populations was in the progressive proportional increase over 24 hours of labelled antigen-binding cells in the germinal centre regions of the white pulp. Furthermore, when the normal spleen cell population was altered in favour of either thymus-derived or bone-marrow-derived cells, a subpopulation (B-cells) which in normal recipients migrated to the germinal centre regions could be distinguished. Table 6 summarizes the results of all experiments and shows that T-spleen cells behave like thymus cells with very little predilection for germinal centre regions. B-spleen cells, on the other hand, are found in these regions between 4 and 48 hours after injection in a relatively constant proportion. The proportion of antigen-binding cells in germinal centre regions was similar to that of B-cells until 8 hours after injection. By 24 hours, however, there was a substantially greater proportion of antigen-binding cells than B-cells actually in germinal centres.

TABLE 6

COMPARISON OF CELLS FROM DIFFERENT SOURCES IN GERMINAL CENTRE REGION LOCALIZATION

Time after injection	Per cent labelled cells in germinal centre regions (means of all experiments where applicable)									
	Thymus		T-spleen		Normal spleen		B-spleen		Antigen-binding spleen	
	(G)	(C)	(G)	(C)	(G)	(C)	(G)	(C)	(G)	(C)
½–1 hour	0	4	n.d.		2	3	3	5	3	19
4–5 hours	1	2	2	5	3	8	10	20	12	29
8 hours	2	4	2	5	5	13	19	21	20	42
24 hours	0	5	1	4	6	12	12	21	53	17
48 hours	0	9	n.d.		5	15	6	18	n.d.	

DISCUSSION

The phenomenon of antigen retention in lymphoid germinal centres and primary follicles is well established but the function of this prolonged extracellular exhibition of antigen has remained obscure. The most likely explanation seems to be that antigen localization on dendritic follicular cells is an extremely efficient method of presenting antigen to circulating lymphocytes (Nossal and Ada, 1971). Two questions were asked in the experiments reported here. First, do antigen-binding lymphocytes carry antigen to the follicle regions; secondly, is there a sub-population of lymphocytes which normally circulate through follicle and germinal centre regions?

Evidence that individual lymphocytes are responsible for the transport of antigen to germinal centre regions has come from the injection of labelled cells (Brown *et al.*, 1970) and from the injection of antigen (Mitchell and Abbot, 1971). The present experiments show that antigen-binding cells prepared *in vitro* and injected *in vivo* accumulate preferentially in germinal centre regions by migration from the marginal zone.

Although no formal identification of the labelled antigen-binding cells was made by electron microscopy, previous studies indicate that it is only lymphocytes which migrate from the marginal zone to the splenic white pulp in response to antigenic stimulation (Pettersen *et al.*, 1967). The appearance of all the labelled cells from 1 hour after injection suggested that even those cells located in the red pulp and marginal zone were lymphocytes rather than labelled phagocytic cells which had been present in the inoculum.

The validity of studying the distribution of antigen-binding cells labelled *in vitro* has been questioned (Brown *et al.*, 1970). Indeed, when antigen alone is injected, some antigen-binding cells can be found (Mitchell and Abbot, 1971). Such cells, however, are rare and the controls presented here show that even five times as much antigen as was presented on lymphocytes did not introduce significant confusion in the quantitation of the injected labelled cells (Table 3).

An additional problem in this study was the use of relatively high doses of radioactive antigen to label donor lymphocytes. The apparent immunological suicide of heavily labelled cells (Ada and Byrt, 1969) suggests that with this labelling technique the immunologically active cells may not behave normally The conditions of iodination used here were in the range found by Ada and Byrt to produce partial non-reactivity in irradiated recipients. In fact, very few of the most heavily labelled cells were found in the recipients' spleens in these experiments. The labelled cells studied were, therefore, the less avid antigen-binding cells.

The effect of immunization on the localization of antigen is to hasten the appearance of antigen in the germinal centre regions of lymphoid tissue (Nossal *et al.*, 1966; Hanna and Szakal, 1968). Furthermore, in immunized animals, the rate of appearance of marginal zone lymphocytes inside the white pulp is increased (Pettersen *et al.*, 1967). The present work showed that the injection of labelled antigen-binding cells into immunized recipients hastened the progression of these cells from the marginal zone into the germinal centre regions of the white pulp.

When the distribution of antigen-binding cells was compared with that of normal spleen cells, a difference was evident in the proportions of cells located in germinal centre regions, and the experiments with thymus-derived spleen cells and bone-marrow-derived spleen cells showed that T-cells and B-cells differed in their pattern of localization, presumably

because of their different routes of migration. Normal spleen cells showed characteristics consistent with their being a mixture of T and B cells.

It is well established that thymus and thoracic duct cells circulate within the spleen from the marginal zone into the white pulp where they aggregate in the periarteriolar lymphocyte sheath (Goldschneider and McGregor, 1968; Ford, 1969). Normal spleen cells and bone marrow cells, on the other hand, have a wide distribution throughout the white pulp (Parrott, 1967; Austin, 1968; Brahim and Osmond, 1970). The present experiments suggest that B-spleen cells normally circulate through and around germinal centre and primary follicle regions of the spleen. Circulation, rather than seeding and accumulation, is suggested by the fact that the concentration of labelled B cells in germinal centre regions did not increase relative to their concentration in remaining white pulp. Bone-marrow-derived lymphocytes would seem, then, to be the population sought by Ada (1970) to explain a functional role for the prolonged extracellular exhibition of antigen by the dendritic follicular cells.

Although some T-cells were found in germinal centre regions, their normal route of migration through the spleen did not appear to include germinal centre regions. In searching for the anatomical site for the B–T cell interaction discussed by Miller and Mitchell (1968), no evidence was found in this study to suggest the germinal centre as a probable site. Perhaps the most likely area is the spleen marginal zone where large proportions of both T and B cells were found shortly after injection. This region is important as the first site of antigen localization in the spleen (Mitchell and Abbot, 1971); however, if it is the significant area for T–B cell interaction, the time for this interaction must be relatively brief.

Cells which had already bound antigen exhibited behaviour characteristic of B-cells in their predilection for germinal centre regions. Since B-cells are probably the antigen-binding cells detected here (Greaves, 1970) it seems likely that B-cells activated by antigen migrate normally to germinally centre regions and stay there, rather than pass through. No conclusions could be drawn from this work as to whether the antigen carried to germinal centres on lymphocytes is the same antigen seen extracellularly on dendritic follicular cells later in the response.

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