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Two Distinct Populations of Peripheral Lymphocytes in Mice Distinguishable by Immunofluorescence

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Summary. Immunofluorescent staining has been used to detect antigenic determinants on the surface of living lymphoid cells of mice. It has been possible to distinguish two distinct populations of peripheral lymphocytes: one population carries the theta isoantigen and is thymus-dependent; the other has naturally-occurring immunoglobulin determinants on the cell surface and appears to be thymus-independent. The relative proportions of these two populations of cells is characteristic for each type of lymphoid tissue and these proportions have been determined for thymus, lymph node, spleen, and thoracic duct cells.

INTRODUCTION

In view of the increasing evidence for the heterogeneity of lymphocytes (Davies, 1969) and the importance of this heterogeneity to the immune response (Mitchell and Miller, 1968), there is a pressing need for physiological markers to distinguish one type of lymphocyte from another. It has previously been suggested that the theta isoantigen (θ) of mice may be a useful marker for thymus-derived lymphocytes* (Raff, 1969). This was based on the findings that there is a distinct subpopulation of peripheral lymphocytes which carry θ on their surface and that this population is markedly reduced in mice treated with anti-lymphocytic serum (Raff, 1969; Schlesinger and Yron, 1969a), thymectomized mice (Schlesinger and Yron, 1969b; Raff and Wortis, 1970), and mice with congenital absence of the thymus (Raff and Wortis, 1970).

Theta is found mainly in lymphoid tissues and brain and is controlled by a single locus with two alleles: θ AKR, which is found in AKR and RF mice, and θ C3H, which is found in most other strains of mice tested (Reif and Allen, 1964). Thus far θ has been detected mainly by cytotoxic testing. In view of the potential usefulness of this antigen as a marker of thymus-derived lymphocytes it seemed worth while to investigate other methods of detecting θ-bearing cells.

In experiments reported here, immunofluorescence has been used to demonstrate θ on the surface of living lymphocytes and to determine the proportions of θ-bearing cells in the various lymphoid tissues of mice. The effect of ALS and thymectomy on this population was also investigated. Since immunoglobulin (Ig) can be detected on the surface of a proportion of peripheral murine lymphocytes by immunofluorescence (Raff, Sternberg and Taylor, 1970) it has been possible to study the effect of ALS and thymectomy on this population of surface-Ig-bearing cells as well as on θ-bearing cells. It will be shown that these two populations of lymphocytes are distinct and vary inversely in ALS-treated and thymectomized mice.

* The term *thymus-derived lymphocytes* is used throughout this paper as synonymous with the terms *thymus-processed* and *thymus-dependent* lymphocytes.

MATERIALS AND METHODS

Animals

Male and female CBA, BALB/c and (CBA × C57BL/6)F_1 mice, aged 3–8 months, were used except where otherwise stated. In one experiment, germ-free C3H/Jax mice, originally obtained from Pollards Wood Research Station, Institute of Cancer Research, were used. These mice were raised and maintained in sterile isolaters in the germ-free animal house at the National Institute for Medical Research. Full aerobic and anaerobic cultures (Hobson and Mann, 1961) of homogenates of entire intestines of several of these mice were sterile.

A group of CBA mice was thymectomized at 4 weeks of age, irradiated at 10 weeks of age (900 r of whole body X-irradiation at 124 kV and a dose of 37·5 r/min at a distance of 32 cm) and reconstituted with syngeneic adult bone marrow cells given intravenously on the day of irradiation (one donor for one recipient). These mice are hereafter referred to as thymectomized mice. Another group was irradiated and reconstituted as above, but was not thymectomized. The mice were killed 8 weeks after irradiation.

A group of BALB/c mice was treated with antilymphocytic serum (ALS) prepared by the method of Levey and Medawar (1966) beginning on the day of birth and given twice weekly until they were killed at 7–8 weeks of age. Each dose was given subcutaneously and consisted of 0·05 ml during the 1st week, 0·1 ml in the 2nd week, 0·15 and 0·2 ml in the 3rd week and 0·25 ml from then onwards.

Antisera

Anti-θ. Anti-θ C3H (anti-θ) was prepared by immunizing AKR mice with CBA thymocytes (Reif and Allen, 1966). Although these two strains share the same H2 specificities, they differ at the LyA locus (Boyse, Miyazawa, Aoki and Old, 1968) as well as at the θ locus, and theoretically some anti-LyA_1 activity might be expected. This would be important only when CBA (CBA × C57BL/6)F_1 or C3H/Jax cells were used, but not with BALB/c cells which carry the LyA_2 allele. However, the cytotoxic activity of the antiserum was completely absorbed by adult CBA brain, which would not have occurred if significant anti-LyA_1 was present (Boyse *et al.*, 1968). The anti-θ was heat inactivated at 56° for 30 minutes.

Rabbit anti-mouse-immunoglobulin. Rabbit anti-mouse-immunoglobulin (anti-MIg) was prepared by immunizing New Zealand rabbits with mouse immunoglobulin (MIg) prepared by fractionation on DEAE-cellulose (Sober and Peterson, 1958). 0·5 mg of MIg was injected subcutaneously at multiple sites in Freund's complete adjuvant and the rabbits were boosted in the same manner at 1 week and with 0·5 mg given intravenously at 4 weeks. The animals were bled 10 days after the last boost.

Fluorescent antibodies. Anti-θ and rabbit anti-MIg sera were fractionated on DEAE-cellulose (Sober and Peterson, 1958) after precipitation with 40 per cent saturated ammonium sulphate. The fractions which were not absorbed to the DEAE in 0·02 M phosphate buffer at pH 7·5 were conjugated with fluorescein isothiocyanate (FITC) as previously described (Clark and Shepard, 1963; Raff *et al.*, 1970). Normal rabbit serum (NRS) was fractionated on DEAE-cellulose and conjugated with FITC for use as a control conjugate (Fl-NRG).

On immunoelectrophoresis with normal mouse serum the fluorescent rabbit anti-MIg (Fl-anti-MIg) reacted strongly with Ig determinants and weakly with what appeared to

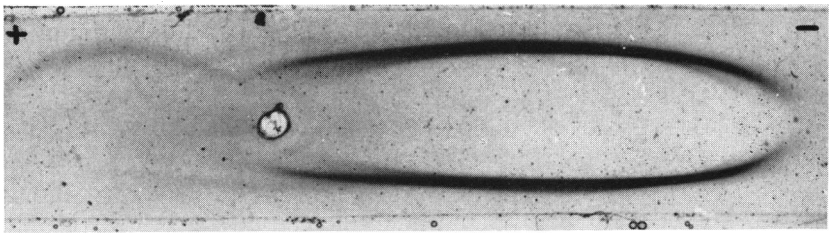

FIG. 1. Immunoelectrophoretic patterns of normal mouse serum with rabbit–anti-mouse immunoglobulin conjugated with fluorescein (Fl–anti-MIg). Normal AKR serum was placed in the well and electrophoresed in 1·2 per cent agar in 0·05 M barbitone buffer, pH 8·2 at 5 V/cm for 90 minutes. Unpurified Fl–anti-MIg (10 mg/ml) was placed in the upper trough and purified Fl–anti-MIg was placed in the lower trough. After 12 hours the slide was washed and stained with 1 per cent amido black.

be transferrin (Fig. 1). In order to make the antiserum more specific, an aliquot of the Fl-anti-MIg was purified by affinity chromatography (Axén, Porath and Ernback, 1967; Wofsy and Burr, 1969), using a column of Sepharose (Pharmacia, Uppsala) to which a purified preparation (Sober and Peterson, 1958) of 5563 γG_{2a} mouse myeloma protein (Askonas, 1961) had been covalently linked (Axén *et al.*, 1967). This myeloma protein preparation showed a single line on immunoelectrophoresis when reacted with an antibody made against normal mouse serum. The Fl–anti-MIg was eluted from the column with 1 M propionic acid, dialysed against phosphate buffered saline, pH 7·3 (PBS) and concentrated by vacuum dialysis. This purified Fl–anti-MIg reacted only with Ig determinants in immunoelectrophoresis with normal mouse serum (Fig. 1). Since the purified and unpurified anti-MIg conjugates gave identical results, the experiments performed with the unpurified conjugate are included in this report (Tables 3 and 6). The properties of both conjugates are outlined in Table 1. The conjugates were absorbed with acetone-dried liver powder (50 mg/ml of conjugate) for 30 minutes at room temperature prior to use.

TABLE 1

CHARACTERISTICS OF THE RABBIT ANTI-MOUSE IMMUNOGLOBULIN FLUORESCENT CONJUGATES*

	FITC concentration (corrected) (μg/ml)	Protein concentration (mg/ml)	F/P ratio
(1) Unpurified conjugate	43·8	7	2·6
(2) Purified conjugate	5·1	1·38	1·48

* Determined as outlined by Holborow and Johnson (1967).

Immunofluorescent staining

Indirect immunofluorescent staining was carried out on living lymphoid cells (Möller, 1961). Cell suspensions were prepared from thymus, lymph nodes (mesenteric, inguinal, brachial, axillary, deep and superficial cervical) and spleen by teasing the tissues with two fine needles into veronal buffered saline containing calcium and magnesium with 5 per cent heat-inactivated foetal calf serum (VBS). Thoracic duct lymphocytes were kindly supplied by Dr J. L. Boak (Boak and Woodruff, 1965). All cell suspensions were passed through a short column of glass wool, and spleen and thoracic duct cells were treated

with tris-buffered NH_4Cl to lyse the RBC (Boyle, 1968). After washing three times in VBS the cell suspensions were adjusted to 20×10^6 cells/ml and 0·025 ml of each cell suspension was mixed with 0·025 ml of neat anti-θ, normal AKR serum (NMS) or VBS in 0·4×4 cm disposable precipitin tubes and incubated for 30 minutes at 37°. The cells were then washed three times in 1 ml of VBS, resuspended in 0·025 ml of neat Fl–anti-MIg and incubated for 15 minutes at room temperature. After washing twice in 1 ml of VBS, the cells were resuspended in 0·05 ml of VBS, and a drop was placed on a glass slide and covered with a coverslip which was then sealed with paraffin. For direct immunofluorescence testing, the cells were incubated with neat fluorescein conjugated anti-θ (Fl–anti-θ) directly, washed twice in VBS and then put onto slides as described. The slides were examined within one hour of preparation, with a Reichert ultraviolet microscope, using a dark field condenser, a BG12 primary filter and an Ilford 107 barrier filter. The light source was an Osram HBO-200 mercury arc lamp. 200–400 cells were examined at a magnification of 504× and scored as outlined below. Photomicrographs were taken using a microscope previously described (Young, 1961), with blue-violet light (400–500 N.M.) from a 250 W high pressure mercury lamp, a primary filter combination consisting of Corning glass 5850, a Wratten 2B (ultraviolet absorber) and a Scholt BG 38 (heat absorber), and an Ilford 105 barrier filter. High speed 35 mm Ektachrome daylight film was used with Leitz Mikas camera equipment.

Cell morphology

Cytocentrifuge preparations (Doré and Balfour, 1965) were made of cell suspensions prepared as described above from the lymphoid tissues of the various kinds of mice used in the experiments reported here. The cells were fixed in absolute methanol, stained with Giemsa stain and 100–200 cells were counted and classified.

RESULTS

DIRECT IMMUNOFLUORESCENCE

When CBA, BALB/c or $(CBA \times C57BL/6)F_1$ thymocytes were treated with Fl–anti-θ, 90–95 per cent of the thymocytes were seen to have faint but definite fluorescent rings around their periphery. This fluorescent staining was completely inhibited when the cells were first incubated with unconjugated anti-θ. No fluorescence was observed when lymphocytes from spleen, lymph nodes or thoracic duct were treated with Fl–anti-θ. Because direct immunofluorescence produced only faint staining of thymocytes and no detectable staining of peripheral lymphocytes, all further experiments were done with indirect immunofluorescence.

INDIRECT IMMUNOFLUORESCENCE

Types of staining

Four types of cells could be distinguished in indirect immunofluorescence tests:

(a) *Negative cells* (Fig. 2a) showed no fluorescence, but their outlines were blue and easily visible.

(b) *Ring cells* (Fig. 2b) showed ring-like fluorescent staining around the periphery of the cells (Möller, 1961); the rings were often discontinuous and at times incomplete. Cells

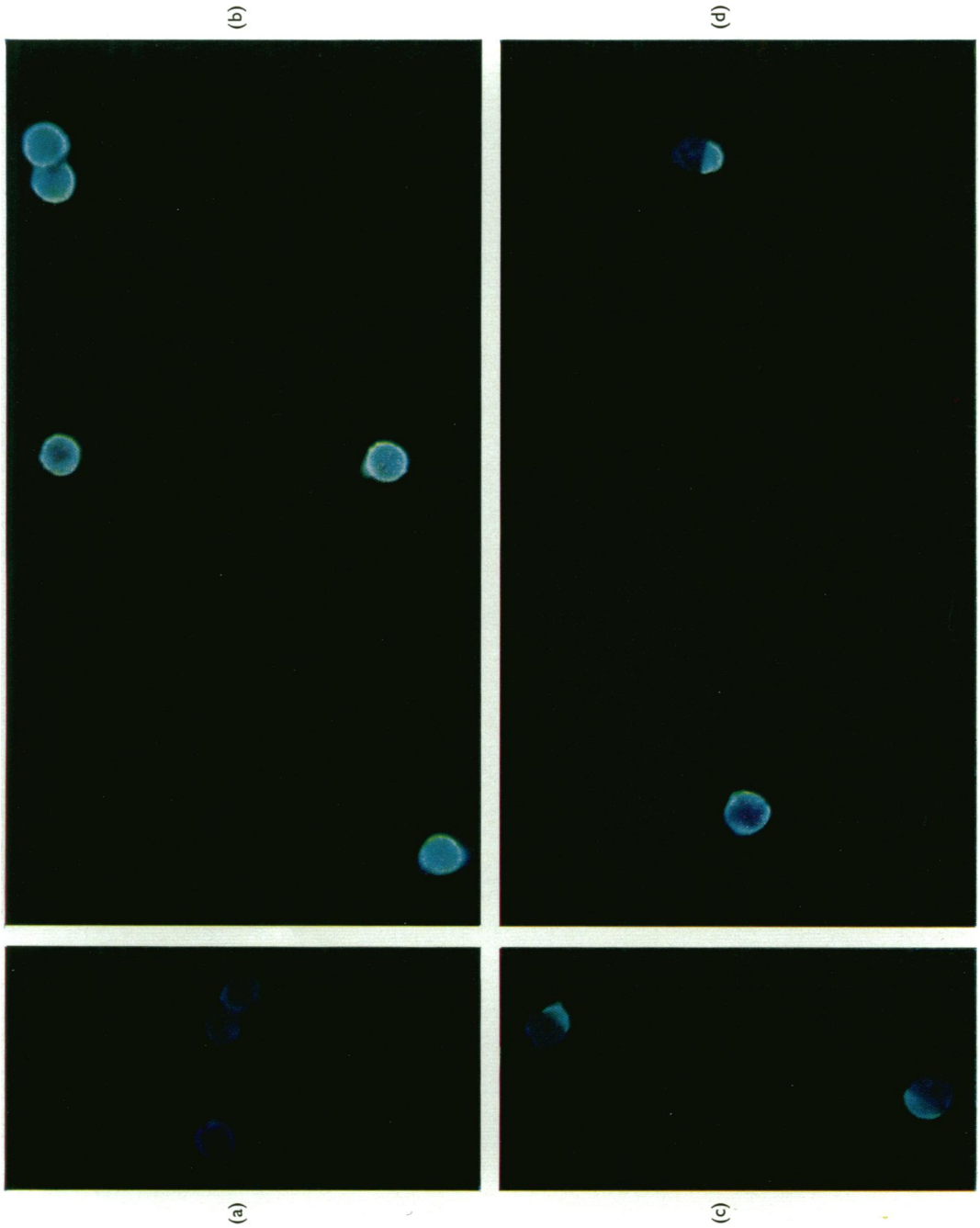

FIG. 2. Photomicrographs of indirect immunofluorescence of living BALB/cells in suspension. (a) Thymocytes treated with veronal buffered saline with 5 per cent foetal calf serum (VBS) and then fluoresceinated rabbit antimouse immunoglobulin (Fl–anti-MIg) showing no fluorescence. (b) Thymocytes treated with anti-θ and then Fl–anti-MIg showing ring fluorescence. (c) Spleen cells treated with VBS and then Fl–anti-MIg showing fluorescent caps. (d) Spleen cells treated with anti-θ and then Fl–anti-MIg showing both ring cells and cap cells. Purified Fl–anti-MIg was used in all cases.

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showing patchy speckling on their surface were sometimes seen and were counted as ring cells.

(c) *'Capped' cells* (Raff *et al.*, 1970; Fig. 2c) showed highly characteristic fluorescent 'caps' occupying one pole of the cell, often overlying a pseudopod-like bulge, and taking up between one fifth to one-half of the cell surface. Cells with caps were readily distinguishable from other types of fluorescent cells and never showed fluorescence on the rest of the cell surface.

(d) *Dead cells* showed diffuse staining of cytoplasm and nucleus (Moller, 1961) and were not scored.

For the most part, only cells treated with anti-θ showed ring staining and these were considered to be θ-bearing cells. Thymocytes tended to show complete and confluent rings while peripheral lymphocytes showed mainly non-confluent and often incomplete rings. Cells with fluorescent caps were seen only with peripheral lymphocytes and their numbers were the same for cells treated with anti-θ, NMS and VBS. These cells have been shown to have naturally-occurring immunoglobulin determinants on their surface (Raff *et al.*, 1970). Not all cells showing pseudopod-like excrescences from their surfaces were fluorescent, and even some thymocytes treated with NMS or VBS showed exactly similar appearing structures—but they were never fluorescent.

Fluorescence control experiments

In all experiments, cells treated with NMS as well as cells treated with anti-θ were studied with Fl–anti-MIg, and in most experiments cells incubated with VBS alone were also tested. In addition, four types of control experiments were done:

(a) Fl–NRG was used in place of Fl–anti-MIg in tests with normal CBA thymus, lymph node and spleen cells which were treated with anti-θ, NMS or VBS. In no instance was surface fluorescence seen, although dead cells were diffusely fluorescent. Even when the cells were pretreated with unfluoresceinated rabbit anti-MIg, Fl–NRG failed to stain the cells.

(b) Thymus, lymph node and spleen cells which were treated with anti-θ, NMS or VBS were incubated with non-fluoresceinated rabbit anti-MIg prior to treatment with Fl–anti-MIg. All types of surface fluorescence were markedly inhibited.

(c) AKR thymus, lymph node and spleen lymphocytes were tested by indirect immunofluorescence as described above. The thymocytes showed no fluorescence, and the peripheral lymphocytes showed mainly cap fluorescence and the number of staining cells was the same in suspensions treated with anti-θ, NMS and VBS.

(d) Ring and cap fluorescence were found to occur when the 15 minute incubation of the cells with Fl–anti-MIg was carried out at 4° instead of at room temperature, excluding the possibility that pinocytosis played a role in the reaction.

Normal mice

With each type of lymphoid tissue and mouse strain used in these experiments the anti-θ and Fl–anti-MIgs were tested in various dilutions to be certain that the percentage of cells staining reached a plateau at high concentrations of the antisera. Although the percentage of fluorescent cells was the same when anti-θ was used neat, or diluted 1 : 2, or 1 : 4, the fluorescence was brighter and thus easier to read when anti-θ was used neat. The same was true when the Fl–anti-MIgs were used neat or diluted 1 : 2 or 1 : 4. Thus all antisera were used neat in all experiments.

TABLE 2

RESULTS OF INDIRECT IMMUNOFLUORESCENCE WITH CBA LYMPHOID CELLS

Experiment No.	Cells sensitized with	Fluorescent staining reaction					
		Lymph nodes (per cent)			Spleen (per cent)		
		Rings	Caps	Negative	Rings	Caps	Negative
1	Anti-θ	80	8	12	49	27	24
	NMS	2	8	90	3	29	68
	VBS	1	10	89	5	30	65
2	Anti-θ	80	11	9	55	20	25
	NMS	1	9	90	5	23	72
3	Anti-θ	81	14	5	52	32	16
	NMS	2	10	88	5	29	66

TABLE 3

RESULTS OF INDIRECT IMMUNOFLUORESCENCE WITH BALB/c LYMPHOID CELLS*

Experiment No.	Cells sensitized with	Fluorescent staining reaction					
		Lymph nodes (per cent)			Spleen (per cent)		
		Rings	Caps	Negative	Rings	Caps	Negative
1	Anti-θ	77	18	5			
	VBS	2	21	79			
2	Anti-θ	74	16	10	36	32	32
	NMS	1	12	87	3	23	74
	VBS	2	18	80	4	20	76
3	Anti-θ	76	12	12	35	35	30
	NMS	0	11	89	5	30	65
	VBS	1	10	89	3	27	70
4	Anti-θ	82	14	4			
5	Anti-θ	78	18	4			
	VBS	2	16	82			

* Unpurified Fl–anti-MIg was used in these experiments.

The percentage of stained cells in normal CBA, BALB/c (CBA × C57Bl/6)F_1 and germ-free C3H/Jax mice are outlined in Tables 2–5. The results with thymocytes are not recorded, as in all experiments 95–100 per cent of thymocytes sensitized with anti-θ showed ring fluorescence (Fig. 2a), and no staining was seen when they were sensitized with NMS or VBS; cells with fluorescent caps were rarely seen. When lymph node cells were tested, 75–85 per cent showed ring fluorescence when sensitized with anti-θ, while 8–22 per cent showed cap fluorescence irrespective of whether they were incubated with anti-θ, NMS or VBS. With spleen cells, 35–55 per cent stained as ring cells with anti-θ and 20–37 per cent as capped cells with anti-θ, NMS or VBS. The percentage of stained cells was similar for the various strains, including the germ-free C3H/Jax mice, although BALB/c peripheral lymphocytes contained relatively fewer ring cells, particularly those from spleen. In the one experiment with thoracic duct lymphocytes, 90 per cent showed ring fluorescence, and 4–6 per cent cap fluorescence.

ALS-treated mice

The results of indirect immunofluorescence with ALS-treated and age-matched untreated control BALB/c mice are shown in Table 6. Thymocytes stained similarly in

TABLE 4

RESULTS OF INDIRECT IMMUNOFLUORESCENCE WITH (CBA × C57BL/6)F_1 LYMPHOID CELLS

Experiment No.	Cells sensitized with	Fluorescent staining reaction								
		Lymph nodes (per cent)			Spleen (per cent)			Thoracic duct lymphocytes (per cent)		
		Rings	Caps	Negative	Rings	Caps	Negative	Rings	Caps	Negative
1	Anti-θ	83	12	5	53	23	24	90	6	4
	NMS	2	15	83	2	26	72	4	5	91
	VBS	0	14	86	0	20	80	2	4	94
2	Anti-θ	75	20	5	45	35	20			
	NMS	3	22	75	7	37	56			
	VBS	4	20	76	10	36	54			

TABLE 5

RESULTS OF INDIRECT IMMUNOFLUORESCENCE WITH GERM-FREE C3H/Jax LYMPHOID CELLS

Cells sensitized with	Fluorescent staining reaction					
	Lymph nodes (per cent)			Spleen (per cent)		
	Rings	Caps	Negative	Rings	Caps	Negative
Anti-θ	84	14	2	42	25	33
NMS	1	13	86	2	30	68
VBS	1	16	83	3	31	66

both groups but there were marked differences in the lymph node and spleen lymphocytes of the two groups. In ALS-treated mice, there was a striking decrease in the percentage of ring cells and a striking increase in the percentage of cells showing cap fluorescence.

Thymectomized mice

The results of indirect immunofluorescence with CBA mice thymectomized as adults, lethally irradiated and reconstituted with syngeneic bone marrow, mice irradiated and reconstituted without thymectomy, and normal mice are outlined in Table 7. Thymectomized mice showed a marked decrease in ring cells and an increase in capped cells when compared to non-thymectomized mice. There was a relatively high percentage of ring cells seen with cells from irradiated animals when treated with NMS. This may have been related to cell injury occurring while harvesting the cells from the fibrotic lymph nodes and spleens of these mice, causing increased stickiness of the cell surface. If one subtracts the percentage of rings seen with NMS from the percentage of rings seen with anti-θ the difference between thymectomized and non-thymectomized mice is even more impressive.

CELL MORPHOLOGY

The cell suspensions of all the tissues examined contained 90 per cent or more lymphocytes except for the spleen suspensions from thymectomized mice, where only 85 per cent of the cells were judged to be lymphocytes. The differential counts from typical experiments are given in Table 8.

DISCUSSION

The use of the Möller technique of immunofluorescence (Möller, 1961) to detect antigens on the surface of living cells has several advantages. One can determine the percentage of cells in a heterogeneous cell suspension which carry a specific antigen, and non-specific staining, which is a major problem when studying non-viable tissues, is virtually non-existent (Möller, 1961).

In this study, direct immunofluorescence, using fluoresceinated anti-θ, proved to be of no value in detecting θ-bearing peripheral lymphocytes. Although thymocytes showed faint ring staining with this method, peripheral lymphocytes did not show any detectable fluorescence. This is consistent with the elegant demonstration using the hybrid antibody technique and electron microscopy that thymocytes have much more θ on their surface than peripheral lymphocytes (Aoki, Igard, Hammerling, de Herven and Old, 1968).

TABLE 6

RESULTS OF INDIRECT IMMUNOFLUORESCENCE WITH ALS-TREATED AND UNTREATED BALB/C MICE*

Experiment No.	Treatment of mice	Cells sensitized with	Fluorescent staining reaction								
			Thymocytes (per cent)			Lymph nodes (per cent)			Spleen (per cent)		
			Rings	Caps	Negative	Rings	Caps	Negative	Rings	Caps	Negative
1	(a) None (normal mice)	Anti-θ	95	0	5	75	15	10	43	30	27
		NMS	0	0	100	3	14	83	3	23	74
		VBS	0	0	100	3	17	80	2	20	78
	(b) ALS	Anti-θ	95	1	4	17	53	30	9	49	42
		NMS	0	0	100	3	41	56	2	49	49
		VBS	0	0	100	4	40	56	4	42	54
2	(a) None (normal mice)	Anti-θ				76	9	15	35	33	32
		NMS				0	7	93	2	30	68
		VBS				0	8	92	3	28	69
	(b) ALS	Anti-θ				12	56	32	11	44	45
		NMS				3	48	49	4	38	58
		VBS				2	42	56	2	36	62

*Unpurified Fl–anti-MIg was used in these experiments.

TABLE 7

RESULTS OF INDIRECT IMMUNOFLUORESENCE WITH THYMECTOMIZED AND NON-THYMECTOMIZED CBA MICE

Experiment No.	Treatment of mice	Cells sensitized with	Fluorescent staining reaction					
			Lymph nodes (per cent)			Spleen (per cent)		
			Rings	Caps	Negative	Rings	Caps	Negative
1	(a) None (normal mice)	Anti-θ	80	11	9	55	20	25
		NMS	1	9	90	5	23	72
	(b) 900 r+BM*	Anti-θ	78	12	10	48	27	25
		NMS	8	18	74	15	23	62
	(c) Tx+900 r+BM*	Anti-θ	38	42	20	36	35	29
		NMS	13	46	41	30	42	28
2	(a) None (normal mice)	Anti-θ	81	14	5	52	32	16
		NMS	2	10	88	5	29	66
	(b) 900 r+BM*	Anti-θ	75	16	9	52	30	18
		NMS	4	12	84	12	23	65
	(c) Tx+900 r+BM*	Anti-θ	19	66	15	18	45	37
		NMS	4	55	41	9	62	29

* BM = reconstituted with syngeneic bone marrow cells.

TABLE 8

DIFFERENTIAL CELL COUNTS OF THYMUS, LYMPH NODES, SPLEEN AND THORACIC DUCT CELL SUSPENSIONS*

Mice	Tissue	Cell type (per cent)					
		Lymphocytes	Lymphoblasts	Plasma cells or plasmablasts	Polymorphonuclear leucocytes	Macrophages	Monocytes
(1) Normal CBA	Thymus	100	0	0	0	0	0
	Lymph node	96	1	1	0	1	1
	Spleen	92	1	1	4	1	1
(2) CBA+900 r+BM†	Lymph node	94	1	1	2	2	0
	Spleen	91	1	1	5	1	1
(3) CBA+thymectomy+ 900 r+BM†	Lymph node	93	2	1	0	2	2
	Spleen	85	3	2	8	1	1
(4) Normal BALB/c	Thymus	100	0	0	0	0	0
	Lymph node	94	1	2	1	2	0
	Spleen	91	1	2	4	1	1
(5) BALB/c, ALS-treated	Thymus	100	0	0	0	0	0
	Lymph node	90	2	2	4	2	0
	Spleen	91	3	1	5	0	0

* A relatively large number of smudge cells were seen in all preparations, particularly in irradiated mice, and these cells were not counted.
† BM = reconstituted with syngeneic bone marrow cells.

Indirect immunofluorescence, using fluorescein conjugated rabbit anti-mouse immunoglobulin (Fl–anti-MIg) produced bright ring fluorescence of θ-bearing cells but introduced the added difficulty of dealing with a population of peripheral lymphocytes that stained directly with the Fl–anti-MIg, without the presence of a middle layer of anti-θ. Fortunately, these cells with naturally-occurring immunoglobulin determinants on their surface (Raff *et al.*, 1970) stained in a very characteristic cap-like fashion and appeared to make up a population of cells that was distinct from θ-bearing cells. For when a cell had a fluorescent cap on one pole, the remainder of its surface was never fluorescent, even if the cells had been sensitized with anti-θ. Also, the percentage of capped cells seen in anti-θ, NMS or VBS treated suspensions was almost always the same. Both of these findings suggest that Ig-bearing cells do not take up anti-θ. The fact that the distribution of θ-bearing and Ig-bearing cells are inversely related and their proportions varied inversely in ALS-treated and thymectomized mice further indicates that these two cell populations are distinct.

The finding that a small number of VBS-treated peripheral lymphocytes showed ring fluorescence may mean that some Ig-bearing cells have Ig determinants more uniformly distributed on their surface, rather than in the characteristic polar fashion. However, the polar concentration of the fluorescence and its tendency to overlie a pseudopod-like bulge of the cell surface, seen in the great majority of these cells, may well be significant. It may be that this structure is in fact the lymphocyte uropod, by means of which lymphocytes have been shown to interact with debris and other cells in culture (McFarland, Heilman and Moorhead, 1966). The advantages of having antibody concentrated on the surface of such an appendage are obvious.

Using cytotoxic testing, it has previously been shown that the number of θ-bearing cells in the peripheral lymphoid tissues of ALS-treated (Raff, 1969; Schlesinger and Yron, 1969a), thymectomized (Schlesinger and Yron, 1969b; Raff and Wortis, 1970), and congenitally athymic (Raff and Wortis, 1970) mice is markedly reduced, suggesting that θ-bearing lymphocytes are thymus-derived. The results obtained here with immunofluorescence in ALS and thymectomized mice reaffirm those findings and lend support to the idea that θ can be used as a marker of thymus-derived lymphocytes.

The finding that the percentage of surface Ig-bearing cells in lymph nodes and spleen of ALS-treated and thymectomized mice was increased suggests that most, if not all of these cells are thymus-independent (marrow-derived) lymphocytes. This is consistent with the reports that peripheral lymphocyte transformation induced by anti-Ig is depressed in bursectomized chickens (Alm and Peterson, 1969; Ivanyi, Marvanova and Skamene, 1969). The lack of Ig determinants detectable by immunofluorescence on the surface of thymocytes is in accord with the report that chicken thymocytes do not transform

TABLE 9

APPROXIMATE ESTIMATES OF THE PERCENTAGES OF θ-BEARING AND SURFACE IMMUNOGLOBULIN-BEARING CELLS IN THE LYMPHOID TISSUES OF MICE

Tissue	θ-bearing cells by cytotoxic testing* (per cent)	θ-bearing cells by immunofluorescence (per cent)	Surface-Ig-bearing cells by immunofluorescence (per cent)
(1) Thymus	97–100	95–100	0
(2) Lymph nodes	65–85	75–85	10–20
(3) Spleen	35–50	30–50	20–35
(4) Thoracic duct	80–85	85–90	5

* Summarized results of experiments reported in Rolf and Wortis, 1970.

in the presence of anti-Ig (Iványi, Skamene and Kurisu, 1970). The finding that most, if not all, thymus-derived peripheral lymphocytes do not have surface Ig determinants detectable by immunofluorescence is disquieting, in view of the likelihood that these cells have antigen-specific receptors on their surface and the indirect evidence that these receptors are Ig molecules or fragments thereof (Greaves, Torrigiani and Roitt, 1969). If Ig determinants are present on these cells they must be relatively few, more diffusely distributed, and/or relatively inaccessible compared to those on marrow-derived lymphocytes.

The proportions of θ-bearing and surface Ig-bearing cells appear to be characteristic for each type of lymphoid tissue, although there is clearly some variation between individual mice and between strains. It is noteworthy that these proportions were very similar in germ-free and normal mice. The percentages of θ-bearing cells obtained by immunofluorescence agree well with those obtained by cytotoxic testing (Raff and Wortis, 1970; Table 9). It should be emphasized that the method of preparing the cell suspensions (e.g. teasing, glass wool, tris buffered NH_4Cl treatment of spleen and thoracic duct lymphocytes) must influence these percentages and to this extent these figures may differ from the *in vivo* proportions of these cell types.

It is clear from these immunofluorescence studies that 5–10 per cent of lymph node cells, and 15–30 per cent of spleen nucleated cells appear to be neither θ-bearing nor surface Ig-bearing, and that this percentage is increased in thymectomized and ALS-treated mice. Some of these cells are likely macrophages, polymorphonuclear leucocytes, monocytes, and perhaps even plasma cells, but some must certainly be lymphocytes, at least by morphological criteria (Table 8).

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