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Lymphoid Tissue Architecture

EXPERIMENTAL ANALYSIS OF THE ORIGIN AND DISTRIBUTION OF T-CELLS AND B-CELLS

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Summary. The distribution of bone marrow-derived cells, thymus-derived cells and tissue-bound immunoglobulin was examined using indirect immunofluorescence. It was demonstrated that primary follicle small lymphocytes are bone-marrow derived in the absence of the thymus, they lack thymus-associated antigens, and bear high concentrations of surface-associated immunoglobulins. Thymus-antigen positive cells make up the majority of, if not all, diffuse cortex lymphocytes, do not bear concentrations of surface immunoglobulin detectable in this system, are found as rare cells in primary follicles, and are present in germinal centres. A mechanism is proposed for the thymus–bone marrow interaction in the immune response.

INTRODUCTION

Cellular co-operation in the genesis of cellular and humoral immunity has been well documented (Miller and Mitchell, 1968; Mitchell and Miller, 1968; Claman, Chaperon and Triplett, 1966; Cantor and Asofsky, 1970). At least three cell types have been variously implicated in these responses: a thymus-derived, θ-positive [thymus alloantigen (Reif and Allen, 1964)], lymphocytic cell (Mitchell and Miller, 1968; Raff, 1970); a bone marrow-derived thymus-independent lymphocytic cell which is the precursor of high-rate antibody-forming cells (Mitchell and Miller, 1968); and a surface-adherent, presumably phagocytic cell (Mosier and Coppelson, 1968; Pierce, 1969; Fishman, 1961; Ford, Gowans and McCullagh, 1966). The first two are believed to be antigen-specific and clonally predetermined (Burnet, 1959), presumably via cell-surface antigen specific receptors (Byrt and Ada, 1969; Mäkelä, 1970; Wigzell and Mäkelä, 1970; Naor and Sulitzeanu, 1967; Truffa-Bachi and Wofsy, 1970; Paul, 1970), whereas the third cell type has been reported to have either a non-specific (Forbes, 1969) or specific (Fishman, 1961) necessary role in both the afferent and efferent portions of the immune loop. The lymphoid tissue distribution of these cells is only partially defined, and therefore their sites of interaction are unknown.

Only recently has the morphology of lymphoid tissue been clarified in terms of cell population dynamics and migration streams. Gowans and Knight (1964) described in the rat a population of small lymphocytes which recirculates between the blood stream and

lymphatics, passing into lymph nodes through the walls of specialized blood vessels, the postcapillary venules (Marchesi and Gowans, 1964). This population of cells is represented histologically by the diffuse cortex* of the lymph nodes and the periarteriolar white sheath of the spleen; these areas contain the major portion of labelled immigrant thoracic duct lymphocytes and are specifically depleted upon chronic drainage of thoracic duct lymph (Gowans and McGregor, 1965). These have been termed 'thymus-dependent areas', since the same pattern of depletion is seen in animals thymectomized early in life (Metcalf, 1960; Parrott, de Sousa and East, 1966; Waksman, Arnason and Jankovic, 1962), and the migration of cells from the thymus has been shown to be restricted to these areas (Weissman, 1967).

At least two areas other than the diffuse cortex are distinguishable in lymph nodes. The medulla consists of a sessile population rich in plasma cells and macrophages, and is also the presumed emigrant pathway of recirculating lymphocytes and, following local immunization, of basophilic and antibody-forming cells to the efferent lymph (Hall and Morris, 1965). In the outer cortex of lymph nodes (and adjacent to the periarteriolar white sheath of the spleen) lie agglomerations of densely packed small lymphocytes, the primary follicles. The origin of the cells in the primary follicles is not known, although these structures are not depleted in neonatally thymectomized hosts (Weissman, 1970). Experiments suggesting they are bone-marrow derived either lacked cytological markers (Weissman, 1970) or were terminated before the appearance of histologically recognizable follicles (Balner and Dersjant, 1964). A schematic diagram of a normal mouse lymph node is shown in Fig. 1.

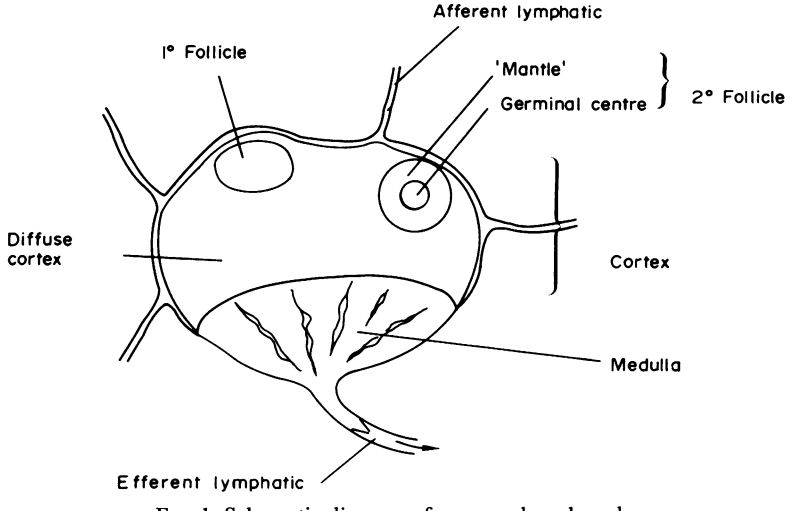


FIG. 1. Schematic diagram of a mouse lymph node.

The purpose of these experiments is to clarify the spatial distribution in lymphoid tissue of thymus-derived lymphocytes and bone-marrow-derived thymus-independent lymphocytes. We here demonstrate that follicular lymphocytes are bone-marrow derived in the absence of the thymus, lack thymus-associated antigens, and bear high concentrations of surface-associated immunoglobulins; that thymus-antigen positive cells make up the majority, if not all, diffuse cortex lymphocytes, are found as rare cells in primary follicles,

* Diffuse cortex = paracortex = parafollicular cortex.

and are present in germinal centres in secondary follicles. A portion of this work has been previously presented (Gutman and Weissman, 1971).

MATERIALS AND METHODS

Mice

Mice used were C57BL/6J (or C57BL/Ka), BALB/cJ, and (C57BL/6J × BALB/cJ)F_1,* all produced by brother–sister mating in this laboratory and maintained on Purina lab chow and water *ad libitum*.

Irradiation

Mice were given a whole-body dose of 900 R (250 Kvp X-rays, 0·5 M Cu + 1·0 mm Al added filtration, half-value layer 1·10 mm Cu, dose rate 80 R/min).

Injections

Irradiated mice were given 3×10^6 newborn liver cells the same day or the day following irradiation. Bicillin, 1000 units injected intraperitoneally, was sometimes administered to prevent the subsequent development of pneumonia.

Cell suspensions

Thymus, lymph node, or spleen was minced with a scalpel and passed through a 200-gauge wire mesh. The cells were washed once and resuspended in cell suspending medium (CSM) consisting of medium 199 + 5 per cent foetal calf serum. Bone marrow was obtained by aspiration from the femur and washed as above.

Cytotoxicity

^{51}Cr-labelled cells were incubated for 1 hour at 37° in serial two-fold dilutions of antiserum plus guinea-pig complement. Cytotoxicity was calculated by the release of ^{51}Cr according to the following formula:

$$100 \times \frac{(\text{Experimental}) - (\text{Complement control})}{(\text{Freeze-thaw}) - (\text{Complement control})}.$$

Plaque-forming cells

Haemolytic plaque-forming cells (PFC) were measured using a modification of the Jerne plaque-forming assay (Mishell and Dutton, 1966). Killing of PFCs was done by a 30-minute incubation of the cells at 37° with one volume of specific antiserum and one volume of guinea-pig complement.

Anti-*H*-2^d serum (BL anti-C) and rabbit anti-BALB thymus serum were prepared as previously described (Gutman and Weissman, 1971). This latter serum, following absorption with 20 per cent v/v of CBF_1 newborn liver cells, was designated R anti-T. Fluoresceinated goat-anti-mouse 7S globulin was obtained as a lyophilized powder from Cappel Laboratories, Inc. (Downington, Pa.). Fluorescein-conjugated sheep anti-rabbit 7S globulin was obtained from the Institut Pasteur, Paris.

Immunofluorescence

Cell surface antigens were detected by indirect immunofluorescence on 6 μm frozen

* Abbreviations used: BL = C57BL/6J; C = BALB/cJ; CBF_1 = (C57BL/6J × BALB/cJ)F_1.

sections, fixed very briefly (<5 seconds) in acetone. Fluorescent staining was carried out as follows: 20 minutes incubation at room temperature with specific antiserum (BL anti-C used 1:2; or R anti-T, used 1:8–1:16), 5 minutes wash in PBS, 20 minutes incubation with the appropriate fluoresceinated anti-globulin serum (goat anti-mouse used 1:2; sheep anti-rabbit used 1:40–1:60), 5 minutes wash in PBS. The sections were then rinsed and mounted in buffered glycerine (10 per cent PBS, pH 8·0) and examined under a Zeiss microscope with an HBO 200 W mercury-vapour lamp, exciter filter BG 3, barrier filter 47. Control serial sections with various combinations of normal rabbit or mouse sera and fluorescent sera were routinely examined for non-specific fluorescence. Acridine orange staining was performed after the method of Bertalanffy and Nagy (1962). Acetone-fixed sections were rehydrated in 70 per cent ethanol and distilled water, dipped in 1 per cent acetic acid followed by distilled water, stained for 3 minutes in 0·01 per cent acridine orange, rinsed 2 minutes in pH 6·0 phosphate buffer, destained for 2 minutes in 0·1 M $CaCl_2$ in distilled water, rinsed in buffer, and mounted in buffer under a coverslip. These slides were examined for fluorescence as with fluorescein. Black and white photographs were taken with a Zeiss 35-mm camera back on Tri-X film, colour slides on High-Speed Ektachrome using ESP-1 processing (ASA = 400).

RESULTS

ORIGIN OF FOLLICULAR LYMPHOCYTES

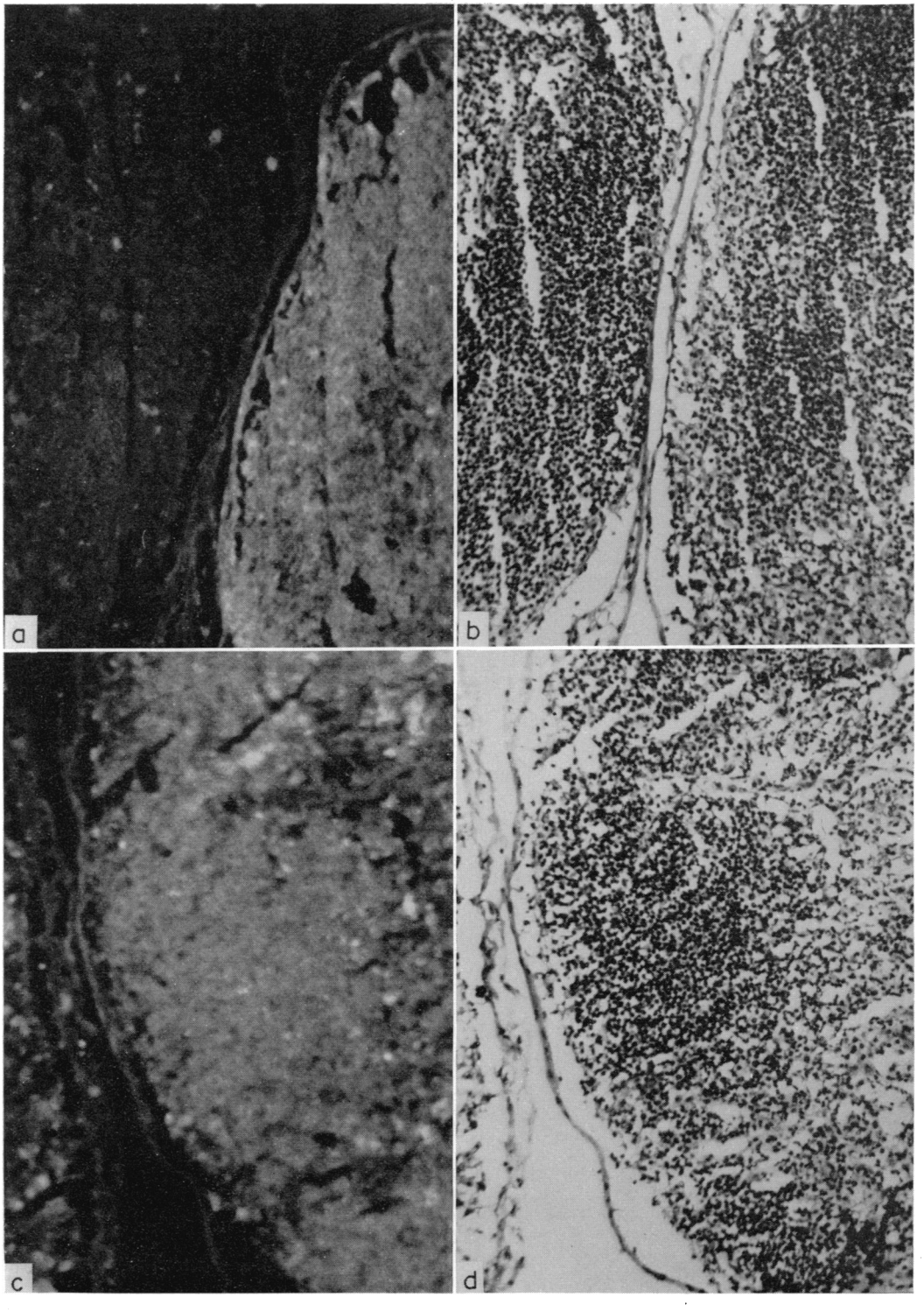
The origin of primary follicle small lymphocytes was investigated in BL (H-2^b) mice which had been thymectomized, irradiated and repopulated with CBF_1 (H-$2^d \times H$-2^b) newborn liver cells. H-2^d cell surface specificities were detected by indirect immunofluorescence, using BL anti-C serum followed by fluoresceinated goat anti-mouse Ig. Fig. 2 shows the specificity of the BL anti-C serum in cytotoxicity; the serum is highly toxic for C cells and not at all for BL cells. The specificity of the immunofluorescence system is illustrated in Fig. 3a; the CBF_1 lymph node is brightly fluorescent, while the BL tissue is dark. (The source and dilution of the fluoresceinated anti-mouse Ig were chosen so as to minimize the fluorescence due to endogenous immunoglobulin in the tissues).

Fig. 3c shows a lymph node from a BL mouse, adult thymectomized, lethally irradiated (900 R) and restored with CBF_1 newborn liver. Nineteen days after restoration, the distribution of H-2^d (present only on the CBF_1 cells) was determined by immunofluorescence. The primary follicle shown in the Figure is brightly fluorescent, demonstrating its origin from the injected newborn liver cells.

DISTRIBUTION OF THYMUS-ANTIGEN POSITIVE (T) CELLS

The localization of cells bearing thymus-specific cell surface antigens was determined by indirect immunofluorescence, using rabbit anti-C thymus absorbed with newborn liver (R anti-T) followed by fluoresceinated sheep anti-rabbit Ig. Fig. 4 shows the specificity of

FIG. 3. Detection of BALB/c cell surface antigens by indirect immunofluorescence (see Materials and Methods). (a) Lymph node from normal C57Bl/Ka (left) or (C57BL/Ka × BALB/c)F_1 (right) showing specific fluorescence of F_1 tissue. (c) Primary follicle in the lymph node of a thymectomized and irradiated C57BL/Ka mouse, reconstituted with (C57BL/Ka × BALB/c)F_1 newborn liver. The peripheral lymph nodes were removed 19 days after reconstitution. Note the fluorescence of the cells in the primary follicle, indicating their origin from the injected newborn liver. (b) and (d) Haematoxylin-eosin stain of sections serial to those on the left. (× 180).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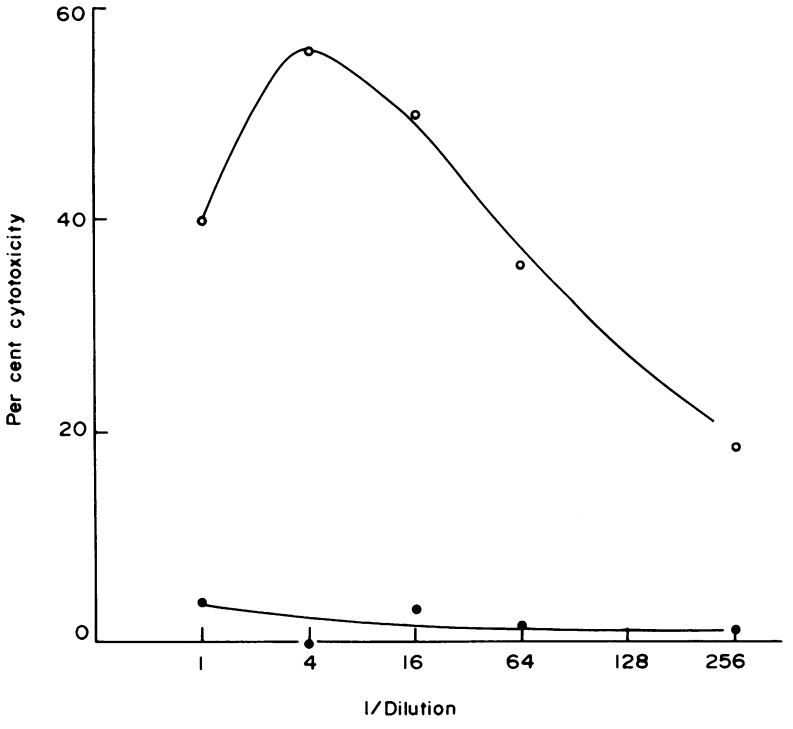


FIG. 2. Cytotoxicity by ^{51}Cr release of C57BL/Ka anti-BALB/c serum tested against thymus cells. (●) C57BL/Ka cells; (○) BALB/c cells.

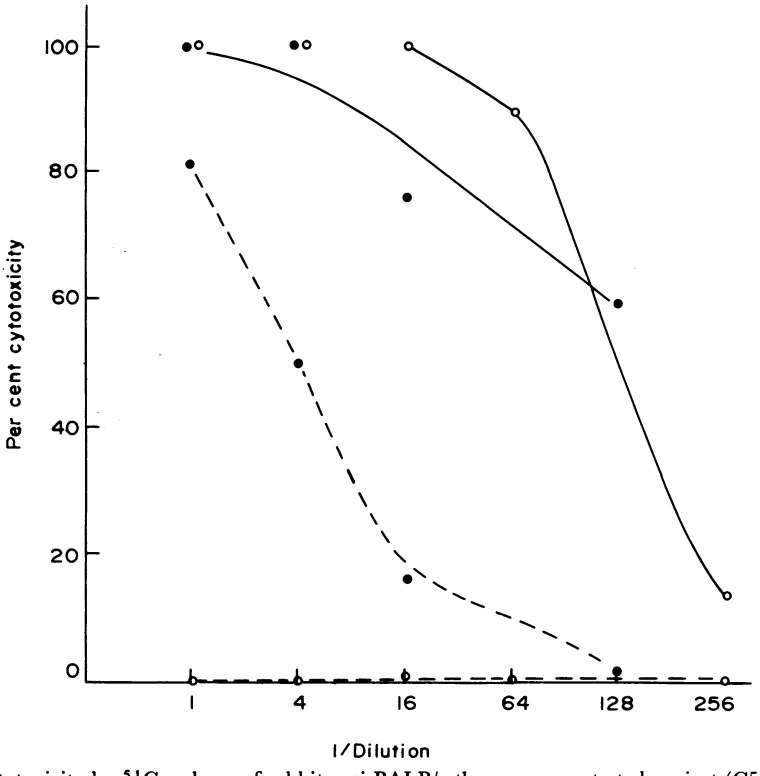


FIG. 4. Cytotoxicity by ^{51}Cr release of rabbit anti-BALB/c thymus serum tested against (C57BL/Ka × BALB/c)F_1 (—) thymus and (- - -) bone marrow (B.M.), (●) before and (○) after absorption with newborn F_1 liver (NBL).

the rabbit anti-C thymus serum in cytotoxicity. After absorption with newborn liver the serum is toxic only to thymus cells. Table 1 shows the toxicity of this serum to PFCs. After absorption, the R anti-T serum shows no toxicity against CBF_1 PFCs.

TABLE 1

KILLING OF HAEMOLYIC PLAQUE-FORMING CELLS[1]

Serum	No. PFCs remaining[2]
Normal Mouse Serum	114±2·0
Anti-*H*-2[d] 1 : 10	13±1·2
R anti-T[3] 1 : 10	113±11·3
R anti-T 1 : 40	114±8·2

[1] $(C57BL/6 \times BALB/c)F_1$ immune spleen cells were pretreated with antiserum and guinea-pig complement at 37° for 1 hour, then tested for haemolytic plaques to sheep red blood cells (see Materials and Methods).

[2] PFCs per aliquot, expressed as mean± standard error.

[3] Rabbit anti-mouse thymus, absorbed with newborn liver.

Fig. 5a shows the pattern of fluorescence exhibited by this serum on normal CBF_1 lymph nodes. The cells in the diffuse cortex are uniformly fluorescent, while those in the primary follicle are dark with the exception of a few scattered fluorescent cells. That this pattern of staining is related to the distribution of specific cell surface antigens and not due to trivial anatomical considerations is indicated by the fluorescence pattern in Fig. 5b.

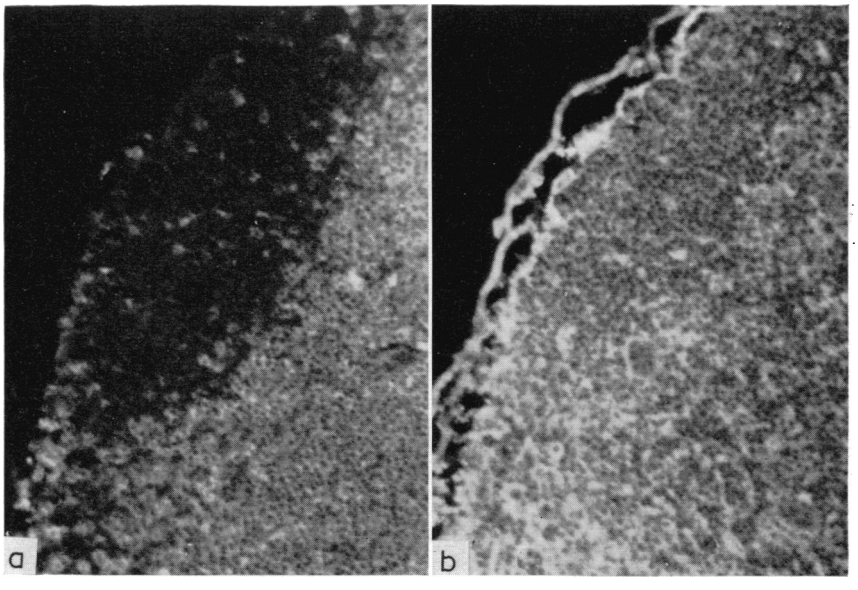


FIG. 5. (a) Indirect immunofluorescence with rabbit anti-mouse thymus, absorbed with newborn liver (R anti-T), shown on normal mouse lymph node. The unstained primary follicle is surrounded by brightly stained diffuse cortex cells. (b) A section serial to (a) but stained with rabbit antimouse lymph node, partially absorbed with thymus. Note the staining of the primary follicle with this serum. (× 130).

This is a section similar to the one in Fig. 5a, but stained with rabbit anti-CBF_1 lymph

node (partially absorbed with thymus). There is uniform fluorescence of the follicular and diffuse cortex (similar to that seen with rabbit anti-peritoneal exudate cells, see below). Fig. 6 shows a higher power view of a primary follicle stained with R anti-T; a few

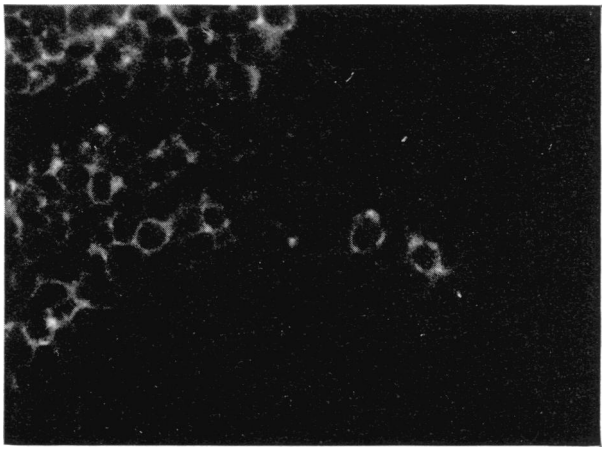


FIG. 6. Higher power view of a normal lymph node stained with R anti-T. Two fluorescent cells are visible in the primary follicle. The edge of the primary follicle abuts on a patch of fluorescent diffuse cortex cells (upper left). (× 650.)

fluorescent cells are seen against a carpet of uniformly dark cells. Fig. 7 shows similar high power views of lymph node diffuse cortex and medulla stained with R anti-T, contrasted with the pattern shown on the same areas by rabbit anti-mouse peritoneal exudate cells. R anti-T shows virtually uniform staining of the diffuse cortex (Fig. 7a), and spotty staining of the medulla (Fig. 7b). The anti-peritoneal cell serum shows the reverse pattern, i.e. spotty staining in the diffuse cortex (Fig. 7c) and extensive staining in the medulla (Fig. 7d). This, again, is considered evidence that the antisera are in fact detecting specific cell surface antigens, and not being distributed on a purely anatomical basis.

Fig. 8a shows a secondary follicle stained with R anti-T; one can see fluorescent cells associated with the germinal centre, surrounded by a non-fluorescent mantle area. The same secondary follicle stained with rabbit anti-mouse peritoneal exudate cells is seen in Fig. 8b; the pattern is different in that the whole germinal centre is stained, indicating again the staining with R anti-T is related to the presence of a specific antigen(s) detected by the R anti-T serum.

Fig. 9 is the pattern of fluorescence shown by R anti-T on the lymph node of an adult thymectomized, lethally irradiated, newborn liver restored BL mouse. In the area normally occupied by densely packed fluorescent lymphocytes, one sees few positive cells on a dark background.

DISTRIBUTION OF IG POSITIVE CELLS

The pattern of staining shown by a polyvalent rabbit anti-mouse Ig serum (L'Age-Stehr and Herzenberg, 1970) is shown in Fig. 10a. One sees specific fluorescence in two areas,

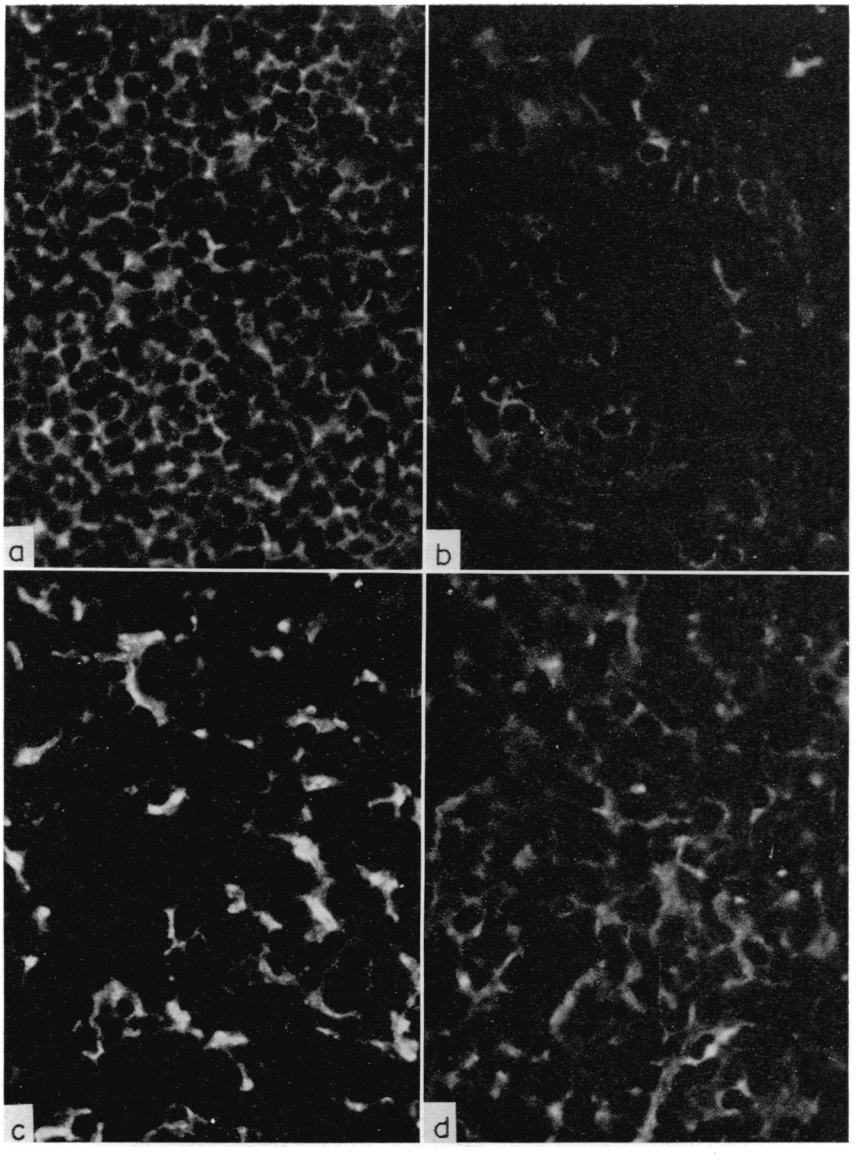


FIG. 7. Indirect immunofluorescence on normal mouse lymph node. Rabbit anti-T stains diffuse cortex (a) uniformly, and medulla (b) spottily. Rabbit anti-mouse peritoneal exudate cells stains the diffuse cortex (c) spottily, and the medulla (d) uniformly. (× 520.)

the medulla and the follicles, both primary and secondary. This fluorescence is completely inhibitable by normal mouse serum as seen in Fig. 10b.

Fig. 11 shows a primary follicle on the left (a–d) and a secondary follicle on the right (e–h), stained with normal mouse serum, acridine orange, R anti-T serum, or R anti-mouse Ig. The patterns of staining are as described in the text above. Note the definition of the germinal centre afforded by acridine orange staining; the blast cells in the germinal centre have RNA-rich cytoplasm, staining red, while the close-packed small lymphocytes

of the 'mantle' and the primary follicle on the left show bright green, nuclear fluorescence.

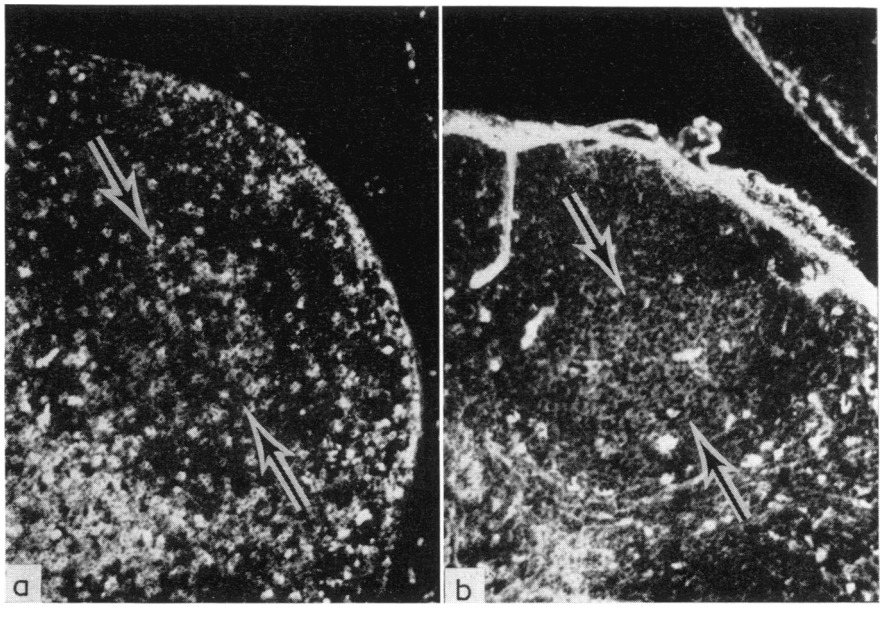


FIG. 8. (a) Rabbit anti-T fluorescence on a normal lymph node. Note the presence of T-positive cells in the germinal centre (between arrows). The brightly staining cells in the lower left are diffuse cortex cells. (b) The same germinal centre, stained with rabbit anti-mouse peritoneal exudate cells. The germinal centre is uniformly stained. (× 520).

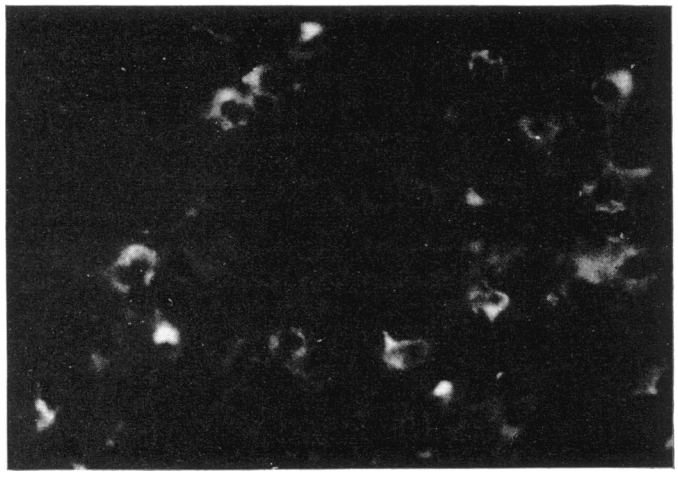


FIG. 9. Rabbit anti-T staining of a lymph node from a thymectomized lethally irradiated newborn liver restored mouse. In the area normally occupied by heavily stained diffuse cortex cells, one sees isolated and rare positive cells. (× 520).

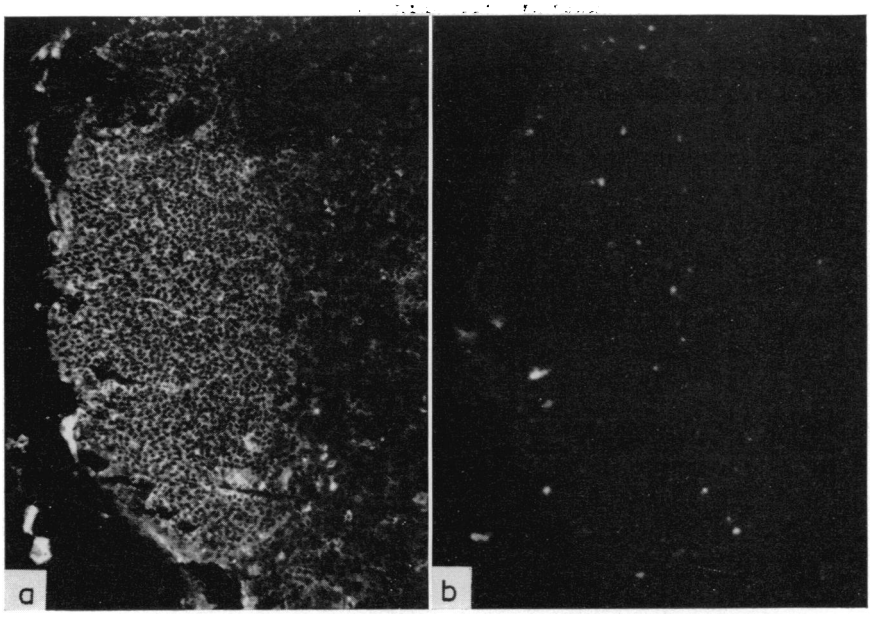


FIG. 10. (a) Immunofluorescent staining of normal mouse lymph node with a polyvalent rabbit anti-mouse immunoglobulin serum. The primary follicle cells stain brightly, while most of the diffuse cortex cells are unstained. (b) The fluorescence shown by the same serum following absorption with normal mouse serum. The fluorescence shown in (a) is totally lost. (× 180.)

DISCUSSION

The experiments presented here and in our previous work (Gutman and Weissman, 1971) establish these points: most follicular lymphocytes are bone-marrow derived in the absence of a thymus, lack thymus-associated cell membrane antigens, and possess appreciable concentrations of surface immunoglobulin. Among these, however, are a small number of cells positive for thymus antigen. Paracortical lymphocytes, on the other hand, possess thymus-associated cell membrane antigens, do not possess high concentrations of surface immunoglobulins, and are rare in thymus-less hosts. At least some germinal centre cells also possess thymus-associated cell membrane antigens, and at least some express cell-associated immunoglobulins. We do not know whether the T-positive cells in germinal centres themselves bear surface immunoglobulin.

The finding of T-antigen positive cells in germinal centres is new, and of considerable potential interest. This complements evidence that bursa-derived lymphocytes may also populate germinal centres (Good, 1970; Durkin, Theis and Thorbecke, 1972). In neonatally thymectomized, irradiated, bone-marrow protected mice, germinal centres are rare, whereas primary follicles may be abundant (Weissman, 1970). It may be that at least under some circumstances T-cells are required for the development of germinal centres. Alternatively, it may be that non-thymus-derived germinal centre cells may express antigens in common (or cross-reacting) with thymus cells; it is known, for example that θ-antigen is expressed in normal brain tissue (Reif and Allen, 1964).

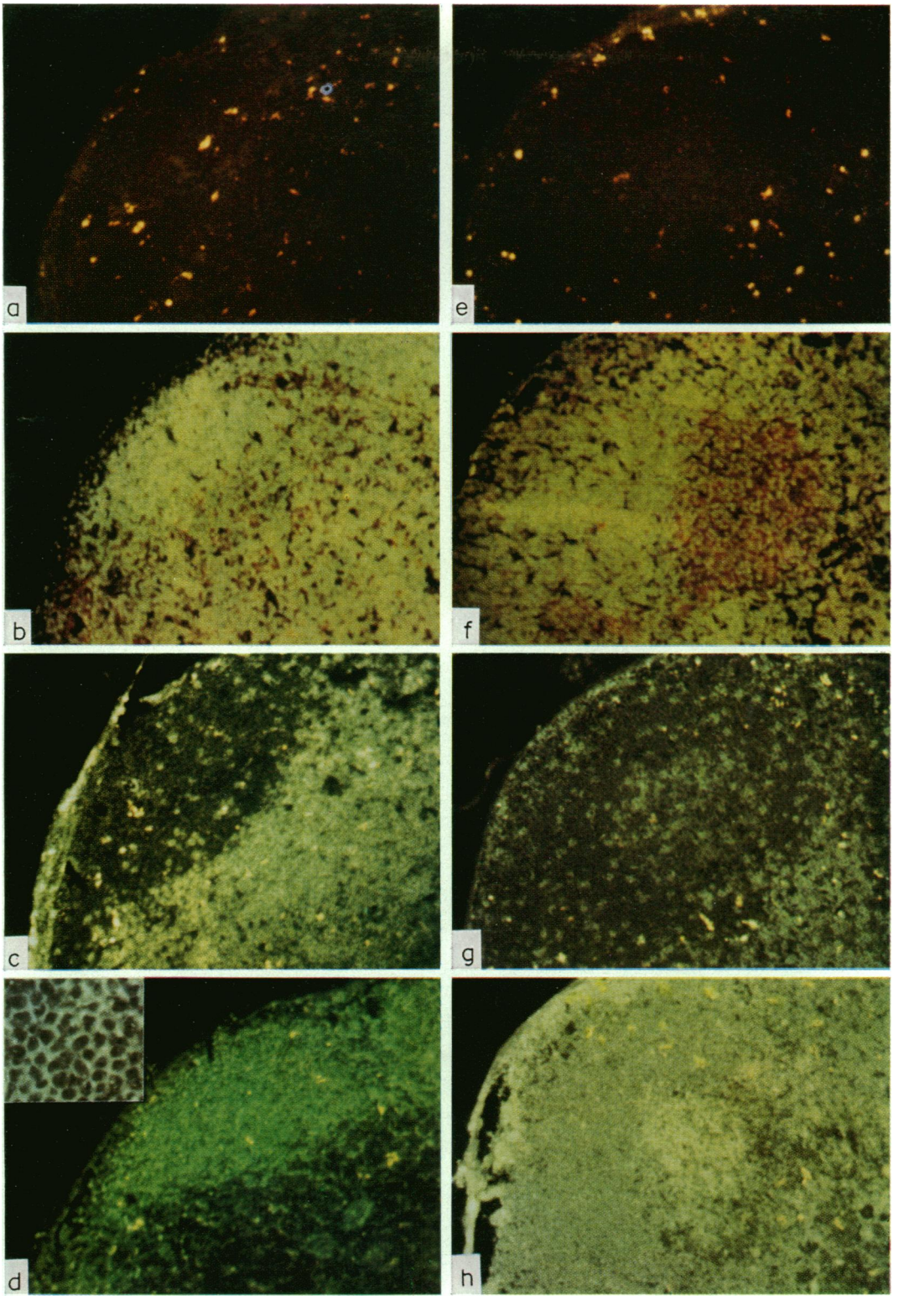
The thymus-antigen positive cells in the paracortex probably constitute a population of cells which include the thymus-derived antigen-reactive cell (T-ARC). The precursor of the antibody-forming cell, however, the bone-marrow or 'bursal-derived' antigen-reactive

cell (B-ARC), has not been morphologically identified or localized to any specific area in lymphoid tissue. We have previously proposed (Gutman and Weissman, 1971) that the follicular lymphocyte is the B-cell, the immediate precursor of antibody-forming cells. If follicular lymphocytes are B-ARCs, then they might be expected to possess antigen-binding receptors on their surface which are immunoglobulin in nature. Our finding of cell-surface associated immunoglobulin on follicular lymphocytes, along with the work of Dukor, Bianco and Nussenzweig (1970), confirms this expectation. In addition, there has recently been described (Howard, 1972; Howard, Hunt and Gowans, 1972) a sub-population of rat thoracic duct lymphocytes which contains B-cells and homes selectively to lymphoid follicles, adding further support to the concept of follicular lymphocytes as B-cells. We do not yet know the immediate origin of follicular lymphocytes; they may arise directly from the bone marrow, or, in analogy to the thymus-derived cell, may undergo differentiation and/or proliferation in some central lymphoid organ ('bursa-equivalent').

The site or mechanism of the T-B interaction have not been identified. If this interaction takes place by direct cell-cell contact in the bloodstream or lymph, one must consider the probability of such an event given the time limitations. It is known that antibody-forming cells are found in significantly higher numbers by 48–72 hours following antigenic stimulation (Jerne, Nordin and Henry, 1963). If T cells and B cells for a specific antigen each have a frequency of 1/5000 (an estimate of the frequency of antigen-binding cells in normal animals [Byrt and Ada 1969; Naor and Sultizeanu 1967]), there is a relatively short time for an exceedingly rare event ($1/5000^2 = 4 \times 10^{-8}$) to occur. This time limitation takes on special significance when one considers that most antibody-forming cells are derived from dividing cells (Nossal and Mäkelä, 1962; Tannenberg and Malaviya, 1968; Dutton and Mishell, 1967; Ellis, Gowans and Howard, 1967), and that both proliferation and differentiation must occur before one sees significant numbers of high-rate antibody-secreting PFCs.

If the T-B interaction takes place within lymphoid tissue, the geographic separation of T and B cells indicated by our experiments places severe restrictions on possible mechanisms. It seems likely that non-random interaction of T and B cells must take place, and that this non-random set of events might be triggered by antigenic stimulation, or directed by the pattern of antigen deposition. Follicular antigen deposition is well-documented, and has been demonstrated for a variety of antigens, both early in an immune response in primary follicles, and later in secondary follicles (for review see Miller, 1971). Such antigen is bound to the dendritic processes of reticular cells, which apparently abut on most, if not all follicular lymphocytes (Nossal *et al.*, 1968). It seems unlikely to us that such a precise relationship between antigen-binding reticular cells and thymus-independent small

FIG. 11. Indirect immunofluorescent staining of normal mouse lymph node, showing a primary follicle on the left (a–d) and a secondary follicle with germinal centre on the right (e–h). (All magnifications × 130.) (a, e) Fluorescent control (normal rabbit serum followed by fluoresceinated sheep anti-rabbit globulin). (b, f) Acridine orange stain; note the definition of the germinal centre in (f), with the RNA-rich cytoplasm of the blast cells staining red. (c, g) Primary follicle (c) stained with rabbit anti-mouse thymus antigen (R anti-T). The follicle is dark, surrounded by brightly staining diffuse cortex. Some positive cells are seen in the follicle. A secondary follicle (g) stained with R anti-T shows some positive cells in the germinal centre (centre of picture) as well as in the surrounding 'mantle' area. The brightly stained cells in the lower right are part of the diffuse cortex. (d, h) Primary follicle (d) stained with rabbit anti-mouse immunoglobulin. The follicle is brightly fluorescent, surrounded by non-staining diffuse cortex. The insert is a higher power view (× 520) of the follicular fluorescence, showing its membrane-associated nature. The secondary follicle (h) shows the same pattern of staining in the 'mantle', surrounding an even more brightly staining germinal centre. No diffuse cortex is visible in this picture.



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lymphocytes is either non-functional or related to immunological tolerance (Ada and Parish, 1968). In fact, the evidence that immunologically tolerant hosts are capable of follicular localization of the tolerogen has been disputed (Balfour and Humphrey, 1967). We propose, therefore, that follicular antigen localization may be a prerequisite for the triggering of specific B-cells. According to this view the rare event of a specific T-cell coming into direct contact with a specific B-cell is avoided, as follicular localization of antigen could bring most, if not all, follicular lymphocytes into contact with antigen within a very short time.

If antigen localized in the follicle is the stimulus for B-cell activation how can one account for the role of the T-cell in the T-B interaction? We suggest that antigen-activated T-cells are responsible for follicular localization of antigen, either directly or indirectly. The *indirect* involvement of the T-cell in follicular localization of antigen might require T-cell activation of some other cell type (presumably a B-cell) which then synthesizes specific antibody cytophilic for reticular cells in the primary follicle. This activation would be expected to be non-random and antigen-dependent. The *direct* involvement of T-cells might be manifested by T-cell synthesis of release in primary follicles or specific antigen-binding receptors, perhaps antibodies, cytophilic for the dendritic processes of reticular cells. Consistent with this interpretation are the recent experiments of Feldmann and Basten (1972), who have demonstrated the *in vitro* release by T-cells of an antigen-specific soluble mediator. If this is true, and the released factor is immunoglobulin, why haven't experiments designed to determine the origin of antibody-forming cells (Miller and Mitchell, 1968; Mitchell and Miller, 1968; Jacobsen, L'Age-Stehr and Herzenberg, 1970) detected T-cell antibodies? One possibility is that the antibodies are of a special type (IgX?) that was not detectable by the techniques used. Another is that T-cells would necessarily synthesize this antibody in appreciable amounts only very early in the course of an immune response; by 4 or 5 days after stimulation, the exponential increase in high-rate antibody-forming B-cells would dilute this pool beyond detection.

The above model has many testable predictions. Follicular localization of thymus-dependent antigens ought to be T-cell dependent, and mediated by cytophilic/opsonic immunoglobulins (or other antigen-specific factors). Follicular lymphocytes should be B-ARCs, the direct precursors of antibody-forming cells. T-cell activation might either lead to entry of T-cells into follicles, or fixation of already migrating T-cells in follicles.

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