MOUSE THYMUS-INDEPENDENT AND THYMUS-DERIVED LYMPHOID CELLS

I. Immunofluorescent and Functional Studies*

By J.-P. LAMELIN, B. LISOWSKA-BERNSTEIN, A. MATTER, J. E. RYSER, and P. VASSALLI

(*From the Department of Pathology and Institute of Histology, University of Geneva, Geneva, Switzerland*)

Lymphoid cells differ in the constitution of their surface membranes as demonstrated by study of their surface antigens. Aside from the surface immunoglobulin (sIg),[1] which has been demonstrated on a proportion of lymphoid cells (1, 2), some antigenic determinants of the cell membranes appear to be specifically related to the pathway of differentiation followed by the cells which bear them. These "differentiation antigens" (3) can thus be used to recognize, among lymphocytes, those which are derived from the thymus, or T lymphocytes, and those which are thymus independent, or B lymphocytes. In the mouse, alloantisera raised against the θ antigen have been widely used for the recognition of T lymphocytes (4). Heteroantisera can also be obtained by immunization of other species with mouse thymocytes (5) or brain (6); after appropriate absorption on mouse tissues, in vivo (5) or in vitro (6), these antisera recognize antigenic determinants which appear to be present only on T lymphocytes and which have been called "mouse-specific lymphocyte antigens," MSLA (5), brain-associated θ antigen, BAθ (6). It is also possible to obtain, by immunization with mouse lymphoid cells depleted in T cells, heteroantisera which after absorption by mouse thymocytes appear to recognize antigenic determinants present on B but not on T lymphocytes, and called for this reason "mouse-specific bone marrow-derived lymphocyte antigen(s)," MBLA (7).

In the present study, antisera directed against MSLA, BAθ, and MBLA (aMSLA, aBAθ, and aMBLA) have been used to label by immunofluorescence

---

[1] *Abbreviations used in this paper:* ABC, antigen-binding cells; aMSLA, aBAθ, and aMBLA, antisera directed against MSLA, etc.; BAθ, brain-associated theta antigen; B cells, thymus-independent lymphocytes; BSA, bovine serum albumin; BSS, balanced salt solution; C, complement; CFA, complete Freund's adjuvant; Fl, fluorescein; Fl-SaRIg, fluoresceinated sheep anti-rabbit immunoglobulin; GaMIg, goat anti-mouse Ig; GPIg, guinea pig Ig: IF, immunofluorescence; iIg, intracellular Ig; MBLA, mouse-specific bone marrow-derived lymphocyte antigen(s); MEM, minimal essential medium; MSH, *Maia squinado* hemocyanin; MSLA, mouse-specific lymphocyte antigen(s); NSS, normal sheep serum; PFC, plaque-forming cells; RaMIg, rabbit anti-mouse Ig; RFC, rosette-forming cells; Rho, tetramethylrhodamine; SE, sheep erythrocytes; sIg, surface immunoglobulin; T cells, thymus-derived lymphocytes; TdR-^{3}H, tritiated thymidine.

the membrane of mouse lymphoid cells, in an attempt to specifically identify
T and B cells. By simultaneous use of two fluorochromes, it has been possible
to show that aMSLA and aMBLA do indeed label two different populations of
lymphoid cells. Combination of immunofluorescent and radioautographic tech-
niques have thus allowed the study of the capacity of B and T cells to bear sIg
and to bind antigen, their "life-span," and their mitotic response after anti-
genic stimulation. The types of Ig chains present on the surface of lympho-
cytes and the relationships between Ig-bearing and Ig-containing cells have
also been explored, as well as how antisera against Ig chain and differentiation
antigen influence the antigenic structure of the cell surfaces ("antigenic
modulation"). A companion paper describes the ultrastructural features of B,
T, and surface Ig-bearing cells (8).

Materials and Methods

Animals.—Mice from CBA/J, CBA/Ca, BALB/c, AKR, C57/BL, and DBA/2 strains
were obtained from C.S.E.A.L.–Centre National de la Recherche Scientifique, Orléans-la-
Source, France, and from the Jackson Laboratory, Bar Harbor, Maine. Nude mice were
kindly donated by Dr. I. Leskovitz (Institute for Immunology, Basel, Switzerland). Swiss
mice, guinea pigs, rabbits, sheep, and goats were obtained from local breeders.

Antigens.—Bacteriophages T4 were grown and purified on sucrose gradient according to
Dickson et al. (9). *Maia squinado* hemocyanin (MSH) was the generous gift of Dr. B. Askonas.
Bovine serum albumin (BSA) (Miles Laboratories Inc., Elkhart, Ind.) was heat aggregated
according to Gell and Benacerraf (10). DBA/2 mastocytoma cells P-815 (kindly donated by
Dr. T. Brunner and Dr. J. C. Cerottini) were grown in ascitic form and washed three times
before use. *Bordetella pertussis* ("vaccine pertussis" Berna) was obtained from the Institut
sérothérapique et vaccinal, Bern, Switzerland. Wild-type *Escherichia coli* heat killed for 1 hr
at 58°C was kindly donated by Dr. J. C. Piguet (Institute of Microbiology, Geneva, Switz-
erland).

Antisera

Antisera against B and T Cells.—

(a) *Rabbit anti-MBLA (anti-B):* Two different sources of B cells were used for immuniza-
tion: (*i*) spleen and lymph node cells from thymectomized, X-irradiated (850 R) CBA/J
mice reconstituted with 5×10^6 bone marrow cells and repeatedly immunized with *B. per-
tussis*; (*ii*) spleen and mesenteric lymph node cells of normal CBA/J mice treated with AKR
anti-θ-C3H serum and rabbit complement (C) freed of dead cells (as judged by trypan blue
test) by a brief osmotic shock (10 sec suspension in distilled water) and freed of erythrocytes
by incubation in ammonium chloride (0.75% in 0.02 M tris[hydroxymethyl]aminomethane
[Tris], pH 7.4).

Rabbits were injected intramuscularly with B cells (100×10^6) emulsified with complete
Freund's adjuvant (CFA), boosted once in CFA and once in saline at 2-wk intervals, and bled
to death 1 wk after the last injection. All sera were heat inactivated for 30 min at 56°C and
kept at −20°C.

The following sequential absorptions (packed absorbent/serum, $\frac{1}{10}$ in volume, 30 min at
0°C) were performed according to Raff et al. (7): (*i*) freshly homogenized mouse liver (once),
(*ii*) mouse red blood cells, and (*iii*) mouse thymocytes (repeatedly, 5–10 times) until the un-
diluted serum was no longer cytotoxic for and did not fix on thymic cells (as judged by in-
direct immunofluorescent assay). Ig fractions were prepared and absorbed according to
Niederhuber (11) until they did not react with thymocytes as mentioned above; Ig fractions

gave usually stronger staining with immunofluorescence. Antisera prepared with both sources of B cells had very comparable cytotoxic activity after proper absorptions. They did not react in agar diffusion against various concentrations of normal mouse serum, and their cytotoxicity was not modified in the presence of mouse serum.

(b) *Rabbit and guinea pig anti-MSLA:* CBA thymic cells (100×10^6) were emulsified with CFA and rabbits and guinea pigs immunized as described for B cells. The heat-inactivated sera were repeatedly absorbed (packed absorbent/serum, 1/1 in volume) with mouse liver homogenates; livers minced with scissors were homogenized with a Dounce homogenizer and large clumps eliminated by sedimentation. The material sedimenting after 5 min at 900 g was washed twice in PO₄-buffered saline and used for absorptions. The sera were tested for cytotoxicity on bone marrow cell suspensions treated with NH₄Cl; depending upon the residual cytotoxicity, the sera were further absorbed with liver homogenates or given a final absorption with bone marrow cells (1:10 in volume). The absorbed sera had no cytotoxicity for bone marrow cells, and similar cytotoxic titers for thymocytes of various strains, including CBA and AKR mice. The cytotoxicity was not modified after absorption with CBA brain.

(c) *Rabbit aBAθ:* Prepared by immunization with CBA/J brain according to Golub (6) and absorbed as described above. The cytotoxic activity of the absorbed serum was identical on CBA and AKR thymocytes and could be absorbed with CBA brain (absorption with AKR brain was not tried).

(d) *Mouse anti-θ-C3H:* Prepared by injecting AKR mice with CBA thymocytes according to Cerottini et al. (12).

Antisera against Mouse Ig Chains.—Rabbit and goat anti-mouse Ig (RaMIg and GaMIg) were obtained by repeated injections with mouse gamma globulin purchased from Pentex Biochemical, Kankakee, Ill., or prepared from normal mouse serum by Pevikon zone electrophoresis followed by gel filtration on Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden). These antisera recognized all types of Ig chains (see below). Antisera directed against γ- (i.e. recognizing γ1, γ2a, and γ2b chains), μ-, α- and, κ-chains were prepared in rabbits using as immunizing antigens: (a) For γ-chains a mixture of mouse 7S Ig and MOPC-70 protein (a γ1-κ IgG), prepared as described above, from which the γ-chains were obtained by mild reduction and Sephadex G-200 filtration in 1 M propionic acid (13). (b) For μ-chains the MOPC-104 E protein (a μ-κ IgM) prepared by sucrose gradient (14) or Sepharose 6 B gel filtration, followed by 3% polyacrylamide gel electrophoresis in glycine-HCl buffer, pH 7. (c) For α-chains the MOPC-315 protein (a α-λ IgA) prepared by immunoadsorption on dinitrophenyl bovine gamma globulin coupled to Sepharose (15), followed by elution with glycine-HCl buffer, pH 2.8, or propionic acid 0.1 M. (d) For κ-chains the chains isolated from urine of mice bearing the plasmocytoma MOPC-41, as described by Potter (16); these chains were electrophoretically pure. The antisera were rendered specific by passage on solid immunoadsorbents made with the following proteins coupled to Sepharose: MOPC-104, polyclonal IgM obtained (as described for MOPC-104) from the serum of BALB/c mice injected with *Trypanosoma brucei* (strain Eatro 1125), a gift from Dr. H. Bazin; MOPC-41 κ-chains and RPC-20 λ-chains (obtained from urine according to Potter); MOPC-315; MOPC-603 (a α-κ IgA) prepared as described by Potter (16). After the appropriate absorptions, the strict specificity of each of these antisera for μ-, γ-, α-, and κ-chains, respectively, was verified by (i) immunodiffusion, (ii) passive hemagglutination using sheep erythrocytes (SE) coupled with diaminobenzidine to the various proteins mentioned above, and (iii) immunofluorescence of cell smears of the various myeloma tumors mentioned above. All the rabbit antisera represented a pool from several animals, immunized weekly for at least several months.

Other Antisera.—Prepared by repeated immunizations of a sheep with rabbit Ig (Pentex Biochemical) and of a goat with phage T4. Goat anti-guinea pig Ig (GPIg) was obtained from Miles-Yeda Ltd., Rehovoth, Israel.

Cytotoxicity Tests.—Complement-dependent cytotoxicity was determined by either ^{51}Cr

release assay (17) or trypan blue dye exclusion test (18) using agarose-absorbed rabbit serum (19) as a source of complement and selecting among the absorbed rabbit sera those which were nontoxic at $\frac{1}{6}$ final concentration on 10^6/ml CBA/J thymocytes after 30 min at 37°C (background values $\simeq$5% dead cells). Cytotoxic index (percentage of ^{51}Cr release or dead cells) was calculated as in reference 6.

Preparation of Cells.—Peripheral and mesenteric lymph nodes were minced with scissors, pressed through wire mesh No. 100, and washed three times in Hanks' balanced salt solution (BSS). Spleen and thymus were teased in Hanks' BSS. Large clumps were sedimented for 5 min and the supernatant was filtered through nylon stocking. Spleen erythrocytes were lysed with ammonium chloride (0.75% in 0.02 M Tris, pH 7.4) and the cells washed three times in Hanks' BSS. In experiments specifically involving analysis of thymic cells, mice were injected intraperitoneally 1 hr before sacrifice with India ink for better identification and removal of the smallest thymus-adherent lymph nodes (20).

Immunofluorescent Assays

Conjugation of Antisera.—Immunoglobulin fractions of antisera, prepared by diethyl-aminoethyl (DEAE) chromatography, were conjugated to fluorescein (Fl) or tetramethyl-rhodamine (RhO) isothiocyanate (Biochemical Labs, Baltimore, Md.) according to Cebra and Goldstein (21) and purified by gel filtration on Sephadex G-25 followed by DEAE chromatography (eluting buffer PO_4 0.25 M, pH 7.5, for Fl conjugates; PO_4 0.01 M, pH 7.5, with increasing NaCl concentrations for Rho conjugates). Fl conjugates had an Fl/protein molar ratio ranging from 2 to 4; Rho conjugates had an OD 280/515 ratio ranging from 1.5 to 2.0. Some of these conjugates were further absorbed on mouse liver powder.

Staining of Cells.—Cell staining was performed in most experiments by an indirect technique. Unconjugated antibody (10–50 μl) was added to 0.1 ml of Hanks' BSS containing 2–3 $\times$ 10^6 cells and 5% BSA (Difco Labs., Inc., Detroit, Mich., 30% BSA stock solution). After 30 min incubation at 0°C followed by three washings in Hanks' BSS, the cells were resuspended in 0.1 ml of Hanks' BSS 5% BSA with 10 μl of the conjugated antiserum incubated for 20 min at 0°C, washed twice in Hanks' BSS, and kept at 0°C until examined in suspension; aliquots were smeared or cytocentrifuged (Shandon Scientific Company, Ltd., London), fixed with ethanol, and kept for further examination, intracellular Ig staining, and/or pictures. When double staining (Fl vs. Rho) was used, the unlabeled antibodies were mixed in the first incubation and the fluorochrome conjugates in the second. Controls were performed by replacement of the unlabeled specific antisera by normal Ig of the same species.

Microscopy.—The preparations were examined with a Zeiss photomicroscope II (Carl Zeiss, New York) equipped with a Philips CS-200 W/4 mercury lamp (Philips Electronic Instruments, Mount Vernon, N. Y.), a Zeiss II Fl condenser for epi-illumination, and a Plan apochromat 40 x/1.0 (oil immersion) objective.

The following combinations of filters were used: (a) for visualization of both fluorescein and rhodamine, *excitation*: KP 490 nm Balzers (Balzers AG, Balzers, Liechtenstein) plus Bg38 2.5 mm Schott (Jenaer Glaswerk Schott und Gen., Mainz, West Germany), *barrier*: Og4 3 mm Schott; (b) for selective visualization of fluorescein, *excitation*: KP 490 nm Balzers plus Bg38 2.5 mm Schott, *barrier*: Monochro Interference Filter, Filtraflex B40 524 nm Balzers; (c) for selective visualization of rhodamine, *excitation*: 2 $\times$ KP 555 nm Balzers plus Bg38 2.5 mm Schott plus Og1c 3 mm, *barrier*: Og3 3 mm Schott.

These last two combinations of filters allow the detection of only one of the fluorochromes with absolute selectivity. Use of these specific filters enhance the brightness of rhodamine, but somewhat decrease that of fluorescein. Experiments evaluating percentage of labeled cells were performed by counting fluorescent cells in the field, then switching to bright-field illumination to count the total cell number.

Analysis of Antigen-Binding Cells (ABC) Using Radioautography and/or Immunofluores-

cence.—MSH and BSA (10 μg) were coupled to 5 mCi of carrier-free ^{125}I (Radiochemical Center, Amersham, England) by the chloramine-T method (22). After removal of the unbound iodine by gel filtration on Sephadex G-25, the labeled MSH and BSA had a specific radioactivity of 40 and 85 μCi/μg, respectively. Detection of MSH- and BSA-binding cells was done according to the technique developed by Naor and Sulitzeanu (23) and Byrt and Ada (24). In brief, lymphoid cells were suspended (10^7/ml) in Hanks' BSS containing 4% normal mouse serum and 1.5 × 10^{-2} M sodium azide. 2-ml aliquots were transferred to Leighton tubes, and the radioactive antigen (100–150 ng) was added and held at 0°C for 60 min with occasional shaking. The cell suspension was then spun down, resuspended in 0.2 ml of plain Hanks', loaded at the top of a normal sheep serum (NSS) gradient (50–75–100%), and spun for 10 min at 900 g. After two additional washings, each cell sample was stained with the appropriate antiserum, using the indirect sandwich technique with fluoresceinated sheep anti-rabbit Ig (Fl-SaRIg) as described above. After final suspension in NSS, cells were smeared on glass slides, fixed in ethanol for 5 min, and dried. The slides were dipped in Ilford K5 nuclear emulsion (Ilford Ltd., Ilford, Essex, England) (gel/water, 1/1) and developed one to several weeks later. For detection of phage T4-binding cells, 100 μg (protein) of T4 were mixed with 3–5 × 10^6 lymphoid cells, incubated at 0°C for 30 min, and washed three times. Subsequent fluorochrome double staining for the detection of T4 and the various cell surface antigens was performed as described above.

Alterations of Cell Surface Antigens.—

(*a*) *Modulation:* 5 × 10^6 spleen cells were suspended in 0.1 ml Hanks' BSS (2–5% BSA) with ⅒ RaMIg serum, aκ or aμ (2–4 mg/ml), aMBLA, aMSLA (final concentration ¼), or C57 anti-CBA antiserum (final concentration ⅙). Cytochalasin B (I.C.I., Mereside Alderley Park, Macclesfield, Cheshire, England) 0.1% in dimethyl sulfoxide was added at a concentration of 10–20 μg/ml medium. Surface antigens present after incubation were detected in the cold by immunofluorescence, either directly with Fl-SaRIg and Fl-RaMIg, or indirectly by aMBLA, aκ, or C57 anti-CBA serum followed by Fl-SaRIg or Fl-RaMIg. Cultures after removal of modulating antibodies were performed in minimal essential medium (MEM) (Grand Island Biological Company, Grand Island, N. Y.) with 10% fetal calf serum and antibiotics.

(*b*) *Enzymatic treatment:* Cell suspensions (20 × 10^6/ml) were incubated at 37°C for 1–2 hr in Hanks' BSS in the presence of either 1–10 mg/ml Pronase (B grade, Calbiochem, Los Angeles, Calif.) with 0.1–2 mg/ml DNase (Worthington Biochemical Corp., Freehold, N. J.), or 50 units/ml *Vibrio cholerae* neuraminidase (Behringwerke A. G., Marburg-Lahn, West Germany). Detection of surface antigens and culture conditions were the same as above.

RESULTS

Detection of B and T Cells by Specific Antisera.—Cytotoxic curves (Fig. 1) show that, after proper absorptions, the rabbit aMSLA and aBAθ sera killed about the same percentage of cells in lymph nodes and spleen as did AKR anti-θ-C3H serum; cytotoxicity of guinea pig aMSLA (not shown) was very similar. aMBLA, as expected, killed more cells in the spleen than in the lymph nodes (Fig. 1). All these antisera had low cytotoxic titers, but no attempts were made in the present experiment to produce stronger antisera by more prolonged immunization, since this could lead to increasing difficulties of absorption. By immunofluorescence, the number of cells stained by a mixture of aMSLA (or aBAθ) and of aMBLA was roughly equal to the sum of the cells stained by each antiserum alone (Table I), strongly suggesting that these sera were directed against different types of lymphocytes and that there was no overlap

in their specificity. Direct evidence for the strict specificity of anti-T and anti-B sera was obtained by incubating lymphoid cells with a mixture of guinea pig aMSLA and rabbit aMBLA, followed by Fl-aGPIg and Rho-aRIg anti-bodies; use of specific filters showed that the labeled cells bore only one fluoro-chrome (Fig. 2), demonstrating that aMSLA and aMBLA were fixed on different populations of cells.

Several points deserve emphasis concerning the detection of B and T cells by immunofluorescence: (a) The percentage of cells stained in the fluorescent
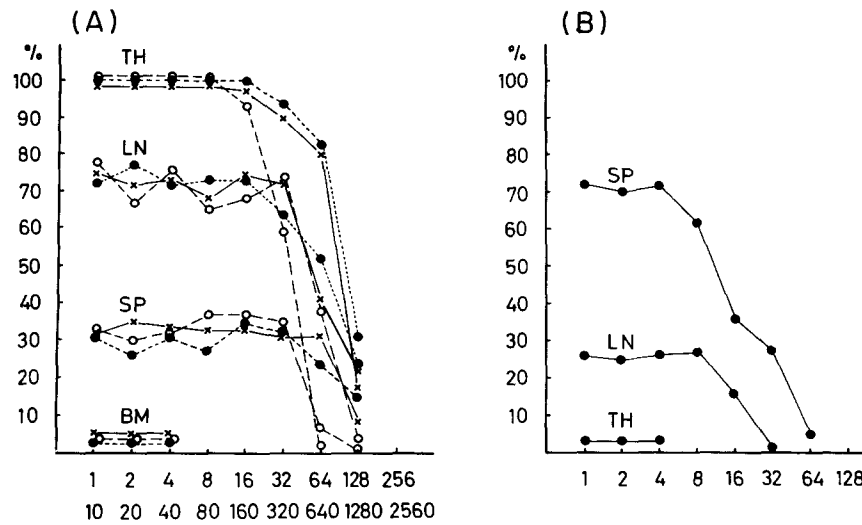

FIG. 1. Cytotoxicity tests with aMSLA, aBAθ, aθ-C3H, and aMBLA. Ordinate: cytotoxic index; abscissa: reciprocal of antisera dilution (for aθ-C3H on the lower line on the left). (A) Results obtained with aMSLA (x—x), aBAθ (O- -O) and aθ-C3H (●- -●) on thymus (TH), lymph node (LN), spleen (SP), and bone marrow (BM) cells. (B) Results obtained with aMBLA.

assay was always inferior to that of the cells killed in the cytotoxic test. Addition of aMSLA (or aBAθ) and aMBLA produced fluorescent staining of between 75 and 90% of the cells, although the mixture usually produced more than 95% of cells stained by trypan blue in the cytotoxic assay. Although, in the spleen, some unstained cells could be accounted for by granulocytes (see below), dead cells probably also accounted for part of the unstained cells, since the percentages of fluorescent cells were always lower in suspensions containing higher percentages of cells stained with trypan blue and since, after removal of dead cells by use of Ficoll-Hypaque gradients (Pharmacia Fine Chemicals, Inc. and Winthrop Laboratories, New York, respectively) (25), the percentage of fluorescent cells stained by anti-T plus anti-B sera reached almost 100. (b) aMSLA and aBAθ sera always gave very comparable

results, and there was no indication in the present experiments of selective fixation on different subpopulations of T cells. (*c*) Fluorescence appears more sensitive than the cytotoxic assay for the detection of surface antigens, since when incubation was performed with concentrations of cells too high to give

TABLE I

*Percentage of Cells Stained by Immunofluorescence with Various Antisera**

Antiserum	Lymph node‡	Spleen‡	Spleen nude§	Spleen Tx‖	Thymus	Antiserum	Spleen¶
aMBLA 1/4	37 (25–35)	51 (50–60)	40	70	1–3	aμ	27 (20–30)
aMSLA 1/4	52 (50–60)	28 (20–35)	1	6	90–95	aγ	19 (15–35)
aMIg (1 mg Ig/ml)	27 (15–30)	35 (35–50)	29	55	1	aμ + aγ	42
aMBLA + aMIg	39	50	—	—	—	aα	3 (2–6)
aMSLA + aMIg	77	63	—	—	—	aκ	46
aMSLA + aMBLA	86	76	—	—	—		

* Percentages established on 300–500 cells (see Materials and Methods). Results from columns presented on the left concern experiments comparing the proportions of B-, T-, and sIg-bearing cells. Results from the column on the right concern an experiment comparing the proportions of spleen cells bearing different types of Ig chains.

‡ Values on the left refer to percentages observed in a given experiment comparing results obtained by antisera alone and in combination. Values between parentheses represent the ranges observed on a large series of experiments with CBA/J and BALB/c mice. Lymph nodes used were either peripheral alone or peripheral plus mesenteric from unimmunized mice. Spleen cell suspensions contained between 5–10% granulocytes as judged by phase-contrast microscopy and staining for peroxidase-containing granules.

§ Spleen cell suspensions containing about 50% dead cells as judged by trypan blue dye exclusion; two experiments gave very close results.

‖ CBA/ca thymectomized (Tx) at 8 wk, irradiated with 850 R, injected with 5 × 10⁶ syngeneic bone marrow cells (treated with AKR anti-θ-C3H + C) and subsequently with 0.5 ml of rabbit antithymocyte serum and killed several weeks later.

¶ Values on the left are percentages observed in a given experiment comparing results obtained with anti-heavy chain antibodies, alone or in combination, to percentage of cells stained with anti κ-chains. Values between parentheses represent the ranges observed in a total of 10 experiments using CBA/J and Swiss mice. Anti-chain antibodies were used at concentrations of 3–10 mg Ig/ml; the percentages were not affected by varying the concentration, except for slight variations in the result obtained with anti-α, which were more difficult to read.

reliable cytotoxic indexes (above 10⁷/ml) the percentage of fluorescent cells remained unchanged. (*d*) aMBLA did not react with granulocytes (identified by phase-contrast microscopy in spleen cell suspension or peritoneal inflammatory exudate) but did react with mononuclear macrophages (identified in peritoneal exudate by India ink ingestion). (*e*) The relative proportion of B and
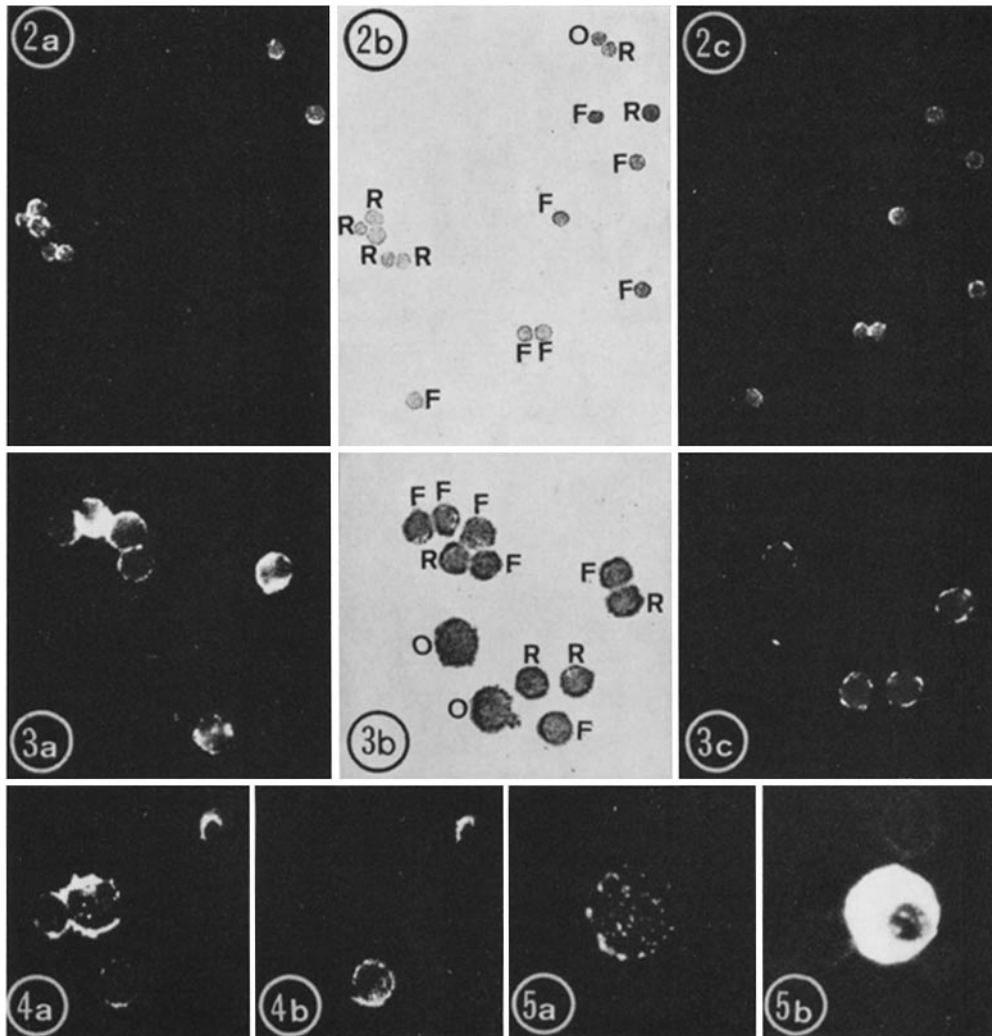
Fig. 2. Smear of spleen cells labeled in suspension with guinea pig aMSLA plus Fl-goat-aGPIg and rabbit aMBLA plus Rho-SaRIg. (2 a) MBLA+ cells revealed by Rho. (2 b) Same area in bright-field illumination: F, Fl-labeled cells; R, Rho-labeled cells, O, unstained cell. (2 c) MSLA+ cells in same field revealed by Fl. One cell (upper right corner on 2 b) is unstained.

Fig. 3. Smear of spleen cells labeled in suspension with aMSLA plus Rho-SaRIg and Fl-GaMIg. (3 a) sIg+ cells revealed by Fl. (3 b) Same area in bright-field illumination: F, Fl-labeled cells; R, Rho-labeled cells; O, unstained cells. (3 c) MSLA+ cells in same field revealed by Rho.

Fig. 4. Smears of spleen cells labelled for MBLA with Rho (4 a) and for sIg with Fl (4 b). Only one of three B cells seen in the center of 4 a is sIg+. (4 b) Spot on the upper right corner is probably a distorted cell bearing the two fluorochromes.

Fig. 5. Plasma cell bearing some sIg: sIg detected with Rho (5 a); iIg detected with Fl (5 b). Cytocentrifugation. The amount of sIg is much lower than usually observed on B cells (compare with Fig. 7 a).

991

T cells can vary markedly, especially in lymph nodes, depending upon the state of immunization.[2] (*f*) There was no condition where a strictly pure population of B or T cells, uncontaminated by the other cell line, was observed. Thymus cell suspensions always contained a small percentage of B, even after careful dissection of adhering small lymph nodes (20) (Table I). Thymectomized; irradiated mice injected with antithymocyte serum still contained some T lymphocytes (Table I). Even the spleens of nude mice have a very few cells labeled by aMSLA (Table I) and indeed some cells characteristic of T cells have been observed by electron microscopy (8).

Which Kinds of Cells Bear Surface Immunoglobulin?—Results presented on Table I show that: (*a*) The percentage of cells stained by a mixture of aMSLA and RaMIg was additive compared with the results obtained with each of these antisera alone, while the percentage of cells stained by a mixture of aMBLA and RaMIg was identical with that obtained with aMBLA alone. (*b*) The number of cells stained by RaMIg was always inferior to that of cells stained by aMBLA; this discrepancy persisted after removal of glass-adhering cells. These results suggest that sIg are present in detectable amounts only on B cells, but that all B cells do not bear sIg. More direct evidence for this conclusion was obtained by double immunofluorescence staining (Figs. 3 and 4): sIg was never found on T cells (Fig. 3) and was always restricted to B cells, although not all B cells bore sIg (Fig. 4), about 20% having no detectable sIg.

It has been suggested that plasma cells do not have sIg (2). In mice immunized with SE it appears that IgM plaque-forming cells (PFC) but not IgG PFC can be destroyed by anti-κ serum plus C (26). Part of the Ig-secreting cells could therefore be sIg— by immunofluorescence and account for some of the B, sIg— cells. To explore this possibility, cell suspensions were incubated with aMBLA or RaMIg to reveal surface antigens and were subsequently smeared and stained for detection of intracellular Ig (iIg). The iIg+ cells were found to be MBLA+ and usually to bear some sIg, but in definitely smaller amounts than other sIg+ B cells (Fig. 5), and some of them had no detectable sIg at all. The same pattern of decreased or absent sIg (or sκ) was found with cells containing γ-chains (stained with specific Fl-anti-γ), suggesting that IgG-containing cells are not markedly different, with respect to sIg, from IgM-containing cells.

In conclusion, the following phenotypes can be recognized among lymphoid cells: MSLA (or BAθ)+, sIg—, iIg— (T cells); MBLA+, sIg+, iIg— (most of the B cells); MBLA+, sIg$\pm$ or —, iIg+ (Ig-containing, and therefore probably secreting, B cells); in addition, since this last category does not seem to account quantitatively for all of the B, sIg— cells, there must be some MBLA+, sIg—, iIg— cells.

Which Kind of Heavy Chains Are Found in sIg+ Cells?—Spleen cell suspensions obtained from mice, either normal or 6 days after immunization with SE were incubated with anti-κ, anti-μ, anti-γ (recognizing γ1 and γ2a and γ2b chains), or anti-α chain sera, followed by indirect staining with Fl-SaRIg (Table I). Percentages of positive cells were easy to establish for all chains except γ, because of a greater unevenness in the degree of cell staining (perhaps due to an unequal strength of the antibodies towards the various subclasses of γ-chains). In two instances the percentage of γ+ cells appeared to be especially high, and the added percentages of μ+ and γ+ cells seemed significantly higher than that observed with cells stained by anti-μ and anti-γ antibodies added simultaneously. Since evidence has been presented that rosette-forming cells (RFC) present early in the immune response to SE might bear more than one class of heavy chains (27), attempts were made to detect by direct staining whether some cells bear both μ- and γ-chains. Unfortunately, although it was possible to prepare a Rho-anti-μ antibody giving good surface staining, none of the Fl-anti-γ antibody prepared gave clear surface staining with the filter system selective for fluorescein (although, as mentioned above, they gave good intracellular staining of plasma cells). It was therefore not possible to explore this point further.

It has been reported that some cells bearing μ-chains on their membranes contain intracellular γ-chains, suggesting a shift from μ-chain to γ-chain synthesis (28). Spleen cells obtained 6 days after immunization with SE were incubated with Rho-anti-μ antibody, smeared, and stained for intracellular γ-chains with Fl-anti-γ antibody. Of 100 cells containing γ-chains, none was found to be sμ+. However, when cells from the same spleens were smeared and stained for intracellular heavy chains with a mixture of these two antisera, 3 out of 200 cells containing heavy chains were found to have both μ- and γ-chains (Fig. 6).

B or T Nature of the Antigen-Binding Cells.—The use of immunofluorescence with double fluorochrome or the combination of fluorescence with radioautography allows the simultaneous detection of ABC and of their B, T, or sIg+ nature. Suspensions of spleen cells from mice immunized with the bacteriophage T4 were incubated with T4 and subsequently treated for the simultaneous detection of T4-bearing cells (detected by Fl-goat anti-T4) and of sIg, MSLA, or MBLA (detected by Rho-SaRIg) (Table II). All of several hundreds of ABC observed were either MBLA+, MSLA−, or sIg+. In the case of sIg, its pattern of distribution on the cell surfaces flattened by cytocentrifugation was strikingly similar to that of the T4 itself (Fig. 7), suggesting that T4 was indeed bound to the cell surface by the sIg themselves. When cells were preincubated with RaMIg before exposure to T4, thus "blocking" the sIg, no cell-binding T4 could be detected. Cytophilic antibodies did not appear to play any significant role in the ABC detected, since (a) removal of glass-adherent cells, presumably macrophages, did not decrease the number of ABC

and (b) only very low percentages of T4-binding cells were observed when spleen cells of normal mice were preincubated in the serum of the T4-immunized mice.

Since T4 could be a "thymus-independent" antigen, other groups of mice were immunized with antigens claimed to be "thymus-dependent", MSH (29) and BSA (30), BSA used for immunization was denatured by heat aggregation,
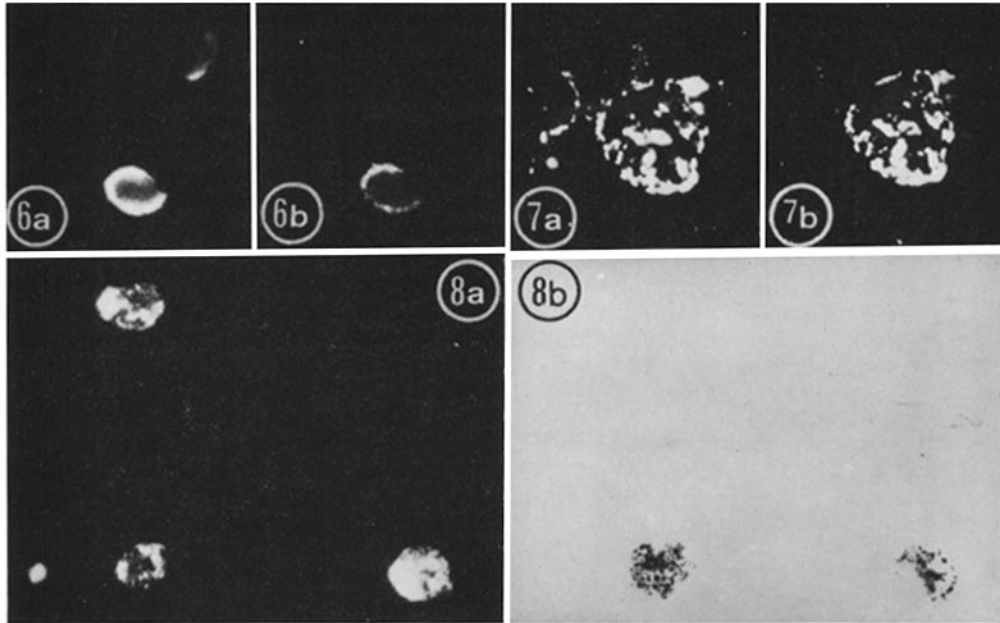

Fig. 6. Plasma cell containing μ-chains detected by Rho (6 a) and γ-chains detected by Fl (6 b). Cytological characteristics of the doubly stained cells were checked by phase-contrast microscopy.

Fig. 7. Spleen cell bearing both sIg (revealed by Rho-RaMIg; 7 a) and T4 phage (revealed by Fl-goat anti-T4; 7 b). The distributions of T4 and sIg on the cell membrane (as revealed by flattening due to cytocentrifugation) are strikingly similar.

Fig. 8. "Short-lived" B lymphocytes. Immunofluorescence (8 a) with radioautography (8 b) of the same field, allowing the detection of TdR-^{3}H grains in cells stained for surface antigens.

since it appears that denaturation favors the induction of delayed-type hypersensitivity (10, 31), which is attributed to the action of T cells. ABC were detected by incubation with highly radioactive antigen (23, 24) followed by labeling of sIg and T cells by fluorescent reagents. On more than 800 ABC detected on radioautographs and analyzed by immunofluorescence for either MSLA or sIg (Table II) only two appeared to be faintly positive for MSLA and one had no detectable sIg; all the others were MSLA— or sIg+. In one experiment, blocking the sIg by incubation with RaMIg before exposure to labeled

MSH suppressed all cell fixation of antigen. However, if T cells have a lower affinity for the antigen than B, sIg+ cells, the amount of antigen bound by T cells could be low and therefore not clearly detectable on radioautographs after 1 wk's exposure. Consequently, radioautographs were exposed for 9–10 wk; the

TABLE II

Nature of Antigen-Binding Cells

Anti-gen	Strain	Immunization	Cells		Technique*	ABC per 1000	Fluorescence of ABC‡ for								
			Col-lected at	Source			Ig			T			B		
							+	±	−	+	±	−	+	±	−
			day												
Phage T4	BALB/c	100 µg i.v.	5–7	Spleen	IF (double fluorescence)	10§	264	0	0	0	0	300	271	0	0
BSA	CBA/J	25 µg of heat-aggregated BSA/footpad in CFA	12	Lymph nodes	RA (1 wk exposure) + IF	12‖	129	0	1	0	0	100	N.D.		
					RA (10 wk exposure) + IF¶	11	98	0	2	0	0	10	N.D.		
MSH	CBA/ca	100 µg alum precipitated + 10⁹ B. pertussis i.p.	7	Spleen	RA (1 wk exposure) + IF	38‖	262	3	0	2**	8	248	N.D.		
					RA (9 wk exposure) + IF	37	96	2	2	N.D.			N.D.		
			21	Spleen	RA + IF (double fluorescence)	8§	50	0	0	0	0	50	N.D.		

* Cells bearing Ig, MSLA, and MBSA were detected by the specific rabbit antisera followed by Fl- or Rho-SaRIg, or simultaneously with Rho-RaMIg and GP aMSLA/Fl-goat aGPIg. Cells binding T4 were detected by Fl-goat anti-T4. IF = immunofluorescence; RA = radioautography.

‡ ± means equivocal.

§ In these experiments, incubation with RaMIg prior exposure to antigen was tried and found to entirely prevent antigen binding.

‖ Cells were considered labeled when they had at least 10 grains. Because of the very low background, ABC could be distinguished without difficulty, even after several weeks' exposure.

¶ Only cells with 10–20 grains were examined for fluorescence.

** Faint labeling.

percentage of labeled cells remained essentially the same (Table II), and no appearance of a second population of more lightly labeled cells could be detected. The immunofluorescent analysis was restricted to the most lightly labeled cells (Table II) and revealed again that on 200 such cells, only four had no detectable sIg (and even these negative cells could have resulted from some fading of the fluorochrome, since technical conditions for immunofluorescence were not as good as on smears examined within a few days after staining).

The RFC appearing after immunization with SE are another example of ABC observed with a thymus-dependent antigen. When spleen cells of mice were tested for RFC 4 and 6 days after immunization with SE, it was found (Table III) that the pretreatment of spleen cells with aMBLA plus C practically abolished all RFC, while aMSLA or aBAθ plus C were without effect; aMBLA had also some inhibitory activity in the absence of C. Since pretreatment with RaMIg without C completely inhibited RFC (not shown); this type of approach too failed to demonstrate significant antigen binding by cells other than B, sIg+ lymphocytes.

B or T Nature of the Short-Lived Lymphocytes.—Repeated injections of thymi-

TABLE III

Inhibition of RFC by aMSLA, aBAθ, and aMBLA

	No. of RFC* per		
	5 × 10⁴‡	3.2 × 10⁴	3.4 × 10⁴
	Exp. 1; day + 4	Exp. 2; day + 4	Exp. 2; day + 6
Control (C)	98, 98	140	612
aMBLA + C	2	6§	3
aMSLA + C	96	149	N.D.
aBAθ + C	96	164	609

* C57/BL mice were immunized intraperitoneally with 0.4 ml of a 1% SE suspension, and killed 4 and 6 days after. To spleen cells incubated for 30 min at 37°C with antiserum or control serum + rabbit C (final concentration 10⁶ cells/ml), an equal volume of 2% SE suspension was added. The mixture was spun down (7 min 700 rpm) and gently resuspended before counting in an hematocytometer (32).

‡ Nucleated spleen cells actually counted.

§ With aMBLA in the absence of C, 65 RFC were counted, indicating an inhibition of about 50%, which could have resulted from steric hindrance by the antibodies fixed on the B cells. This observation was confirmed in other experiments.

dine-³H (TdR-³H; tritiated thymidine) at short intervals during a limited period of time allows the detection of a population of rapidly dividing, or "short-lived" lymphocytes (33). By combining radioautography and immunofluorescence (Fig. 8), it is possible to determine whether these short-lived cells belong mostly to B or T populations. Table IV shows that there were more short-lived cells among B lymphocytes, and more in the spleen than in the lymph nodes. The short-lived populations of spleen and lymph nodes appeared to be different. Since the ratio of T to B cells was about 2:1 in the lymph nodes and 1:2 in the spleen, it can be seen that, while in the lymph node short-lived lymphocytes were about equally divided between B and T cells, in the spleen the short-lived population contained two to three times moie B cells than T cells.

Nature of the Rapidly Dividing Cells Observed during Primary Immune Response.—In the days after injection of antigen in the footpads, there is an outburst of cell divisions in the draining lymph nodes. To study the nature of these

rapidly dividing cells, lymph node cells were obtained 2, 4, and 7 days after immunization with various antigens; the cells in DNA synthesis were labeled by in vitro incubation with TdR-^{3}H, and subsequently stained for identification of T and B cells. The results of Fig. 9 show that the early proliferating cells are in majority T cells, but that later in the immune response B cells proliferation is predominent. With *E. coli* the results were less clear-cut and the proliferative response of the lymph nodes different since, at day 7, the nodes were smaller and contained much less TdR-^{3}H-labeled cells than at day 4.

Alterations of Surface antigens by Modulation and Enzymatic Treatment.—The disappearance of cell surface antigens after incubation of cells at 37°C (but not at 0°C) in the presence of antibodies to these antigens has been called antigenic

TABLE IV

Percentage of "Short-Lived" Cells* among B and T Spleen and Lymph Node Cells

		Exp. 1	Exp. 2
Lymph nodes	T cells‡	5%	4.5%
	B cells‡	15.5	12.0
Spleen	T cells‡	11.0	9.0
	B cells‡	20.5	16.0

* Nonimmunized CBA/J mice were injected intraperitoneally with 2 μCi/g of TdR-^{3}H (Radiochemical Center) every 4 hr during 36 hr and killed 3 hr after the last injection. Lymph nodes (peripheral and mesenteric) and spleen of five mice were pooled in each experiment.

‡ T and B cells were detected by immunofluorescent staining with aMSLA and aMBLA, respectively, followed by Fl-SaRIg, smeared, processed for radioautography as described in Materials and Methods, and developed 7 days later. Fluorescent cells were examined under ordinary light for the presence of grains (Fig. 8); the percentages were established on the analysis of 200 cells.

modulation (3). Recently, modulation of sIg on mouse lymphocytes has been described by Takahashi et al. (26) using cytotoxicity and by Taylor et al. (34) using immunofluorescence. In the present experiments, disappearance and/or reappearance of modulating antibodies and modulated antigens was followed by immunofluorescence (Table V). After 60 min at 37°C of incubation with RaMIg, there was a very strong modulation, both the rabbit Ig and the mouse Ig having practically disappeared from the cell surface. The Fab fraction of the same RaMIg did not modulate at all, (and gave, when Fl-labeled, smooth ring staining); however, when incubation with Fab-RaMIg was followed by incubation with Fl-SaRIg at 37°C, the fluorescence was seen as caps and not rings, indicating that some degree of modulation had taken place (34). Modulation was partially inhibited by cytochalasin B. Anti-κ was found to modulate the associated heavy chain and anti-μ to modulate the associated κ-chain, since after incubation with anti-κ, no chains were detectable on cell surfaces, and after incubation with anti-μ, the percentage of $\kappa+$-cells decreased by a number

roughly equivalent to that of $\mu+$-cells. An interesting finding was that RaMIg modulated MBLA (but not CBA alloantigens) and that aMBLA modulates sIg, although the effect was not as marked as the modulation of sIg by RaMIg.
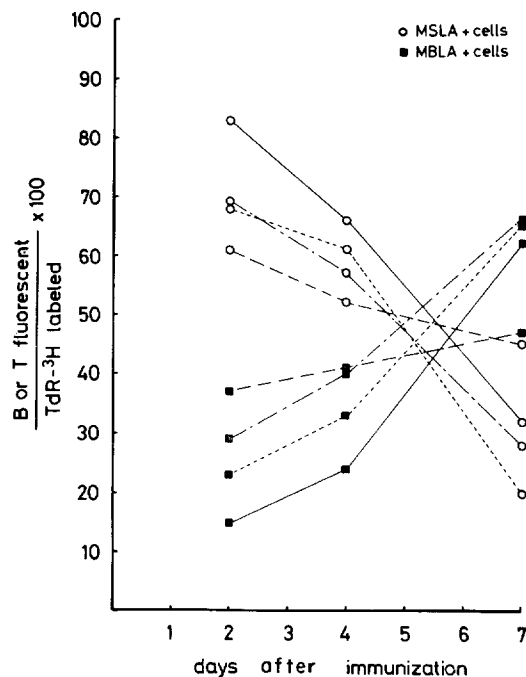

Fig. 9. B or T nature of rapidly dividing cells observed during primary immune responses. Groups of CBA/J mice were injected with the following doses of antigen per footpad: 0.5 × 10^9 B. pertussis (-·-·---); 5 × 10^6 DBA/2 mastocytoma cells (——); 1.5 × 10^8 SE (.........); and 0.5 × 10^9 heat-killed E. coli (-------). Lymphoid cell suspensions of the draining lymph nodes were incubated for 1 hr in MRM containing 10% decomplemented normal sheep serum and 2.5 μCi/ml of TdR-^{3}H. Washings, fluorescein labeling for B or T cells, radioautography (Ilford L4 Nuclear emulsion, gel:water 1:3, 3 days' exposure) were as described in Materials and Methods for detection of ABC. The percentage of total cells which were labeled with TdR-^{3}H varied between 1.2% (day 2) to about 4% (day 7), except for E. coli where there was a decrease in lymph node size and in percentage of TdR-^{3}H-labeled cells (1%) on day 7 compared with day 4. 200 TdR-^{3}H-labeled cells were analyzed for each antigen, 100 for the preparation detecting MBLA and 100 for MSLA (analyses of larger number of cells were found not to modify the results). The ordinate indicates the per cent of TdR-^{3}H-labeled cells bearing MBLA (■) or MSLA (○); it can be seen that for each antigen at a given date, these numbers are reasonably additive.

In contrast, no modulation was observed on spleen and thymus cells after incubation with C57 anti-CBA alloantigen, aMSLA, and aBAθ.

When the reappearance of surface antigens was studied after removal of the modulating agent, washing, and incubation in fresh culture medium, a marked

discrepancy was found between MBLA, which reappears within a few hours, and sIg, which may still be considerably decreased after 24 hr, (Table V). Failure of sIg to reappear rapidly after modulation did not seem to result from an inability of the cells to synthesize detectable amounts of sIg within 24 hr. When

TABLE V

Modulation and Reappearance of sIg MBLA on CBA/J Spleen Cells

Cells incubated with	Cells stained for*	% of cells positive after				
		60 min at 0°C	60 min at 37°C	Further culture without AB		
				2 hr	12 hr	24 hr
RaMIg	Rabbit Ig	40	2			
	Mouse sIg	—	1			
	MBLA	52	27			
	CBA *H-2K*	91	92			
" with cytochalasin B	Rabbit Ig	36	20			
Rabbit aκ	Rabbit Ig	35	1			
	Mouse sIg	—	0			
Rabbit aμ	Rabbit Ig	18	1.5			
	Mouse sκ	42	27			
aMBLA	Rabbit Ig	55	28			
	Mouse sIg	—	15			
" with cytochalasin B	Rabbit Ig	N.D.	55			
RaMIg for 60 min at 37°C followed by cell washing and culture at 37°C	Mouse sIg	N.D.	1	2	6‡	8‡
	MBLA	N.D.	16	N.D.	N.D.	34
aMBLA for 60 min at 37°C followed by cell washing and culture at 37°C	MBLA	N.D.	25˙	33	45	N.D.

Modulation was apparent not only from the decrease in percentage of stained cells, but also from the weaker staining of cells considered as labeled. Between two and eight experiments were performed in each case, and count performed on 300–500 cells.

* See Materials and Methods for detection.

‡ Much weaker staining than on normal cells.

spleen and lymph node cell suspensions were treated with Pronase (Calbiochem; see Materials and Methods), sIg, MBLA, and MSLA could be almost entirely removed as judged by a considerable reduction in, or absence of, immunofluorescence staining. When cells were examined after 4 and 18 hr of culture after Pronase treatment, the three antigens had reappeared, more cells being stained

and the staining more intense after 18 hr than after 4 hr. Disappearance and reappearance of MSLA and BAθ was also observed after Pronase treatment of thymocytes.

It has been reported that neuraminidase treatment of lymphoid cells renders some surface antigens more available (35, 36), perhaps because they are partially "masked" by sialic acid residues. The possibility was therefore explored that neuraminidase might unmask MBLA on T lymphocytes or thymocytes, or T antigens on B cells. After neuraminidase treatment of spleen and thymus cells (see Materials and Methods) no change in the proportions of cells bearing these different surface antigens (sIg included) was observed.

Finally, the effect of inhibition of protein synthesis on the amount of sIg detected by immunofluorescence was explored on spleen cells incubated up to 8 hr in the presence of 1 mg/ml of cycloheximide, a potent inhibitor of protein synthesis. No significant difference with control culture was observed, suggesting that the turnover of sIg is not very fast. After 8 hr, increasing number of dead cells in the culture precluded valid interpretation.

DISCUSSION

B and T cells and Surface Immunoglobulin.—The present experiments show that it is possible to produce rabbit or guinea pig antisera to mouse thymocytes (5) and to mouse brain (6) which, after in vitro absorptions show no cytotoxicity for bone marrow cells even in the presence of rabbit C (which provides a more efficient lytic system than guinea pig C, at least for rabbit and mouse antibodies [37]). Similarly, aMBLA antisera can be absorbed until they show no cytotoxic activity for thymocytes under the same conditions. For these antisera it was felt necessary to adopt especially strict criteria for specificity in cytotoxic tests, since they were not prepared for specific killing of cells but for a more sensitive technique: cell identification by fixation of specific antibodies detected by indirect immunofluorescence. The multiple absorptions resulted in sera with weak cytotoxic titers, but whose fixation on different categories of cells could be simultaneously visualized by immunofluorescence using two fluorochromes, thus offering direct proof of their specificity. It must be pointed out, however, that aMBLA was not strictly specific for the thymus-independent lymphoid cell line, since it recognized macrophages (but no care had been taken to eliminate macrophages among the cells used for immunization).

The association of these antisera and of anti-mouse Ig in experiments using double immunofluorescent staining provided direct confirmation that, in the mouse, the sIg detectable by immunofluorescence is present on B cells (38), sIg was never detected on MSLA+ cells, even in immunized animals where stimulated T cells were certainly present. However, sIg cannot be considered as a strict marker of B cell line, since a number of plasma cells (see below) and of other unidentified MBLA+ cells (present even after removal of glass-adhering cells and thus probably not macrophages) were sIg—.

In agreement with some previous findings using other methods (26, 39), the sIg on mouse spleen cells were found to belong to several classes. Attempts to explore directly by double fluorochrome labeling whether some cells bear two types of heavy chains simultaneously (27, 40) were unsuccessful for technical reasons. The possibility that some cells could bear sμ-chains and contain γ-chains (28) was explored on spleen cells obtained 6 days after immunization with SE, i.e. at a time where both IgG and IgM PFC are present: no such cell was observed among 100 iγ+ cells analyzed. However, when immunoglobulin-containing cells of she spleens were examined for the simultaneous presence of intracellular γ- and μ-chains, 3 out of 200 were found to be unequivocally doubly labeled (Fig. 6), a frequency which is close to the percentage of PFC found in recent studies to be double producers of IgM and IgG (41, 42). This low frequency suggests either that most IgG plasma cells do not arise from IgM plasma cells switching from μ- to γ-chains or that such a switch must take place very rapidly.

As for the relationship between Ig-bearing and Ig-secreting cells, it was found that most cells containing Ig had much less sIg than other sIg+ MBLA+ cells, or had none at all. Since it has been observed that early in the secondary response to BSA a large percentage of the igG PFC could bind the antigen and that this percentage decreased rapidly during the following days (43), it appears that the maturation of plasma cells is accompanied by a progressive loss of sIg.

Antigen-Binding Cells.—ABC observed after immunization with four different antigens were practically all MBLA+, sIg+, MSLA−, or BAθ− cells. AKR ani-θ-C3H plus C has been observed to destroy a number of RFC (32) and of MSH-binding cells (44). However, it has been observed that some mouse anti-θ-C3H sera do not have strict specificity for T lymphocytes and may contain anti-allotype antibodies (45), autoantibodies (46), and antibodies to unidentified alloantigens on B cells (37). Even though some of these antibodies may require rabbit C to kill cells other than T cells (37), their fixation on the surface of B cells may interfere with antigen binding, even when guinea pig C only is used in the assay (it has been found in the present experiments that aMBLA can inhibit a number of RFC in the absence of C). In fact some mouse anti-θ sera fail to inhibit RFC (37, 47),[3] most notably those anti-θ sera prepared in θ congeneic lines by Takahashi et al. (26), who also found, in agreement with the present results, that RFC are MSLA−.

It seems possible therefore to draw the following conclusions concerning the antigen-binding capabilities of B and T cells: (a) The antigen fixed in detectable amounts on cells of immunized mice are bound by the sIg of B cells, since not only ABC are sIg+ cells, but the antigen is fixed on the sites of sIg locali-

---

[3] The AKR aθ-C3H serum used in the present experiments did not kill more cells than aMSLA in the presence of rabbit C and was found not to inhibit RFC.

zation (Fig. 7), and blocking the sIg by anti-Ig gives complete inhibition of binding. This appears to hold true for thymus-dependent as well as for thymus-independent antigens (48), and for ABC in nonimmunized animals as well (49), since ABC to various antigens can be inhibited by prior incubation with anti-Ig (50) and were found in identical percentages among spleen cells of normal and of nude mice (51), the latter being practically devoid of T cells. (b) T cells with specific antigen receptors either are in very small numbers, or more probably have smaller numbers of receptors or receptors with much lower affinity than B cells. If T cells had few receptors but of high affinity, as postulated by Taylor and Iverson (52), it seems unlikely that these cells would have escaped detection on radioautographs exposed for 10 wk. The inability of T cells to bind significant amounts of antigen is in agreement with the failure to observe RFC with the immunizing antigen in bursectomized chicken (53) and in guinea pigs (54) presenting pure delayed hypersensitivity, as well as with the failure of "educated" thymocytes to form rosettes (47). This would also fit with indirect experimental evidence suggesting that cell-mediated immunity requires only very low affinity between the cell-bound receptors of T cells and the antigen (31). In this context it is difficult to understand how the incubation of lymphoid cells with highly radioactive antigen (55, 56) could easily and selectively kill T cells specific for this antigen, since no significant amount of radioactive antigen would be expected to be durably bound to T cells. The possibility should be entertained that some of the killed cells considered to be of T nature were in fact B cells, and that in "hapten-carrier" systems some B cells too might provide some "helper" effect.

Rapidly Dividing Cells.—The study on the nature of short-lived cells in nonimmunized mice, although indicating that there were more short-lived cells among B lymphocytes, did not, however, support a simplified concept equating T cells to long-lived and B cells to short-lived lymphocytes. It revealed a difference in the lymphocyte populations of spleen and lymph nodes which goes beyond a difference in proportion between B and T cells, since it appears that, while in the lymph node the short-lived lymphocytes are about equally divided between B and T cells, in the spleen the short-lived population contains two to three times more B cells than T cells.

Until now, studies of the T or B nature of the cells dividing after immunization have employed the karyotypic analysis of the lymphoid cells of chimeras bearing a T₆ T₆ thymus graft (57). The combination of in vitro incubation of cells with TdR-³H followed by radioautography and of immunofluorescence with aMSLA and aMBLA allows exploration of immune responses, in mice, under more physiological conditions. The present experiments confirm the observations of Davies et al. (57) showing an early wave of T cell divisions followed by a predominance of B cell divisions; it is interesting to point out that rapidly dividing B cells were also very numerous 1 wk after immunization with alloantigenic. The results observed after immunization with *E. coli* were less

clear-cut. Since the immune response to *E. coli* is thymus independent (58), a majority of B cells was expected among the labeled cells. Since *E. coli* contains a variety of antigens, a number of them could be thymus dependent and not play a detectable role in the humoral antibody response; another possibility would be that a thymus-independent antigen, even though not requiring the presence of T cells to initiate a good immune response, is still capable of stimulating their proliferation.

Alteration of Surface Antigens.—The present observations on the disappearance of sIg after incubation of cells at 37°C in the presence of RaMIg confirm and extend those of Takahashi et al. (26), and Taylor et al. (34). The modulating effect appears stronger than in the experiments of Taylor et al. since there was disappearance of practically all detectable rabbit Ig and mouse Ig from the cell surface; this resulted perhaps from the use of hyperimmune antisera. With anti-chain antisera, the disappearance of one type of chain (H or L) under the influence of its specific antiserum was accompanied by the disappearance of its associated chain (L or H), presumably because at the cell surface they are already assembled with covalent bonds in intact Ig molecules. However, sIg are not the only membrane components subject to the modulating effect of antibodies, since the MBLA was also found to be modulated, although less markedly, under the influence of aMBLA. Furthermore, RaMIg led to partial modulation of MBLA, and aMBLA to partial modulation of sIg. The alterations of the cell surface thus included the disappearance of surface antigens not related to the antiserum used and not having any known covalent bond to the antigen fixing the antiserum. These changes in the cell membrane, which are associated with an intense pinocytotic activity leading to the "internalization" of the modulating antibody (8, 34) and probably of the antigens as well, do not affect the whole cell surface, however, since *H-2* histocompatibility antigens appeared unmodified. Furthermore, although they disappeared together, at least in part, sIg and MBLA did not reappear simultaneously. MBLA reappeared within a few hours while sIg was still markedly decreased 24 hr later. This could have resulted from a lesser degree of modulation of MBLA than of sIg, but probably not from a slower speed of synthesis or of transport to the membranes for sIg. Indeed, when both MBLA and sIg were almost completely removed from the cell surface by proteolytic treatment, both were found to reappear within a few hours.

SUMMARY

The simultaneous use on mouse lymphoid suspensions of heterologous antisera directed against thymus-derived (T) cell mouse-specific lymphocyte antigen and brain-associated theta antigen (MSLA and BAθ) or thymus-independent (B) cell mouse-specific bone marrow-derived lymphocyte antigen (MBLA) surface antigens allowed direct proof of the different specificity of these antisera by double immunofluorescence (IF) staining with selective visualization of fluo-

rochromes. These antisera and antisera against mouse Ig and its different types of chains were then used with technique of either double IF staining or IF combined with radioautography, allowing the following conclusions:

(a) Surface Ig (sIg) was found exclusively on B cells and never on T cells, but not all B cells had sIg. Cells containing detectable amounts of Ig were MBLA+, but had less sIg than other B cells or none at all. There was evidence for the existence of a significant number of MBLA+ lymphocytes, neither bearing nor containing detectable Ig.

(b) μ-Chains were the most frequent but not the only heavy chains found on spleen cells; however, it could not be decided with the technique used, if a single cell can bear more than one type of heavy chain. No cell containing γ-chains was found to bear surface μ-chains, although a very few cells containing both μ- and γ-chains were observed.

(c) The antigen-binding cells detected after immunization with bacteriophage T4, bovine serum albumin, *Maia squinado* hemocyanin, and sheep erythrocytes were analyzed for MSLA, MBLA or sIg using double IF, a combination of IF and radioautography, or inhibition of "rosette" formation. Practically all the antigen-binding cells detected were MSLA−, MBLA+, sIg+.

(d) More B cells than T cells were found among short-lived lymphoid cells labeled by repeated in vivo injections of tritiated thymidine, but the results did not support a simplified concept equating T cells to long-lived and B cells to short-lived lymphocytes.

(e) Cells dividing rapidly in the lymph nodes draining the sites of immunization with various antigens were predominantly T cells 2 days after immunization and in majority B cells a few days later.

(f) Incubation of lymphoid cells at 37°C with rabbit anti-mouse Ig or anti-κ chains led to complete disappearance of sIg and to decrease of MBLA ("antigenic modulation"). In the same conditions, anti-MBLA gave partial modulation of MBLA and of sIg; MBLA, however, reappeared much faster than sIg. No modulation of T cell surface antigens by the appropriate antisera was observed. Cell treatment with Pronase could remove MBLA, sIg, MSLA, and BAθ, which reappeared within a few hours. Neuraminidase treatment was without detectable effect on these antigens.

REFERENCES

1. Raff, M. C., M. Sternberg, and R. B. Taylor. 1970. Immunoglobulin determinants on the surface of mouse lymphoid cells. *Nature (Lond.).* **225**:553.
2. Pernis, B., L. Forni, and L. Amante. 1970. Immunoglobulin spots on the surface of rabbit lymphocytes. *J. Exp. Med.* **132**:1001.

3. Boyse, E. A., and L. J. Old. 1969. Some aspects of normal and abnormal cell surface genetics. *Annu. Rev. Genet.* **3**:269.

4. Raff, M. C. 1971. Surface antigenic markers for distinguishing T and B lymphocytes in mice. *Transplant. Rev.* **6**:52.

5. Shigeno, N., U. Hämmerling, C. Arpels, E. A. Boyse, and L. J. Old. 1968. Preparation of lymphocyte-specific antibody from anti-lymphocyte serum. *Lancet.* **2**:320.

6. Golub, E. S. 1971. Brain-associated θ antigen: reactivity of rabbit anti-mouse brain with mouse lymphoid cells. *Cell. Immunol.* **2**:353.

7. Raff, M. C., S. Nase, and N. A. Mitchison. 1971. Mouse specific bone marrow-derived lymphocyte antigen as a marker for thymus-independent lymphocytes. *Nature (Lond.).* **230**:50.

8. Matter, A., B. Lisowska-Bernstein, J. E. Ryser, J.-P. Lamelin, and P. Vassalli. 1972. Mouse thymus-independent and thymus-derived lymphoid cells. II. Ultrastructural studies. *J. Exp. Med.* **136**:1008.

9. Dickson, R. C., S. L. Barnes, and F. A. Eiserling. 1970. Structural proteins of bacteriophage T4. *J. Mol. Biol.* **53**:461.

10. Gell, P. G., and B. Benacerraf. 1959. Studies on hypersensitivity. II. Delayed hypersensitivity to denatured proteins in guinea pigs. *Immunology.* **2**:64.

11. Niederhuber, J. E. 1971. An improved method for preparing anti-B lymphocyte serum. *Nat. New Biol.* **233**:86.

12. Cerottini, J. C., A. A. Nordin, and K. T. Brunner. 1970. Specific in vitro cytotoxicity of thymus-derived lymphocytes sensitized to alloantigens. *Nature (Lond.).* **228**:1308.

13. Fleischman, J. B., R. Pain, and R. R. Porter. 1961. Reduction of γ-globulins. *Arch. Biochem. Biophys. Suppl.* **1**:174.

14. Parkhouse, R. M., and B. A. Askonas. 1969. Immunoglobulin M biosynthesis. Intracellular accumulation of 7 s subunits. *Biochem. J.* **115**:163.

15. Axen, R., J. Porath, and S. Ernback. 1967. Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides. *Nature (Lond.).* **214**:1302.

16. Potter, M. 1967. The plasma cell tumors and myeloma proteins of mice. *Methods Cancer Res.* **2**:105.

17. Wigzell, H. 1965. Quantitative titrations of mouse H-2 antibodies using Cr^{51}-labeled target cells. *Transplantation.* **3**:423.

18. Boyse, E. A., L. H. Old, and I. Chouroulinkov. 1964. Cytotoxic test for demonstration of mouse antibody. *Methods Med. Res.* **10**:39.

19. Cohen, A., and M. Schlesinger. 1970. Absorption of guinea pig serum with agar. A method for elimination of its cytotoxicity for murine thymus cells. *Transplantation.* **10**:130.

20. Leckband, E., and E. A. Boyse. 1971. Immunocompetent cells among mouse thymocytes: a minor population. *Science (Wash. D.C.).* **172**:1258.

21. Cebra, J. J., and G. Goldstein. 1965. Chromatographic purification of tetramethyl-rhodamine-immune globulin conjugates and their use in the cellular localization of rabbit γ-globulin polypeptide chains. *J. Immunol.* **95**:230.

22. McConahey, P. J., and F. J. Dixon. 1966. A method of trace iodination of proteins for immunologic studies. *Int. Arch. Allergy Appl. Immunol.* **29**:185.

23. Naor, D., and D. Sulitzeanu. 1967. Binding of radioiodinated bovine serum albumin to mouse spleen cells. *Nature (Lond.).* **214**:687.

24. Byrt, P., and G. L. Ada. 1969. An in vitro reaction between labeled flagellin or haemocyanin and lymphocyte-like cells from normal animals. *Immunology.* **17**:503.

25. Perper, R. J., T. W. Zee, and M. M. Mickelson. 1968. Purification of lymphocytes and platelets by gradient centrifugation. *J. Lab. Clin. Med.* **72**:842.

26. Takahashi, T., L. J. Old, K. R. McIntire, and E. A. Boyse. 1971. Immunoglobulin and other surface antigens of cells of the immune system. *J. Exp. Med.* **134**:815.

27. Greaves, M. F. 1971. The expression of immunoglobulin determinants on the surface of antigen-binding lymphoid cells in mice. I. An analysis of light and heavy chain restriction on individual cells. *Eur. J. Immunol.* **1**:186.

28. Pernis, B., M. Ferrarini, L. Forni, and L. Amante. 1971. Immunoglobulins on lymphocyte membranes. *In* Progress in Immunology. B. Amos, editor. Academic Press, Inc., New York. 95.

29. Leuchars, E. 1971. The spectrum of thymus dependency. *In* The Role of Lymphocytes and Macrophages in the Immunological Response. D. C. Dumonde, editor. Springer-Verlag New York, Inc., New York. 1.

30. Taylor, R. B. 1969. Cellular cooperation in the antibody response of mice to two serum albumins: specific function of thymus cells. *Transplant. Rev.* **1**:114.

31. Parish, C. R. 1971. Immune response to chemically modified flagellin. II. Evidence for a fundamental relationship between humoral and cell-mediated immunity. *J. Exp. Med.* **134**:21.

32. Greaves, M. F., and E. Möller. 1970. Studies on antigen-binding cells. I. The origin of reactive cells. *Cell. Immunol.* **1**:372.

33. Everett, N. B., R. W. Caffrey, and W. O. Rieke. 1964. Recirculation of lymphocytes. *Ann. N.Y. Acad. Sci.* **113**:887.

34. Taylor, R. B., W. P. H. Duffus, M. C. Raff, and S. de Petris. 1971. Redistribution and pinocytosis of lymphocyte surface immunoglobulin molecules induced by anti-immunoglobulin antibody. *Nat. New Biol.* **233**:225.

35. Simmons, R. L., A. Rios, and P. K. Ray. 1971. Immunogenicity and antigenicity of lymphoid cells treated with neuraminidase. *Nat. New Biol.* **231**:179.

36. Schlesinger, M., and B. D. Amos. 1971. Effect of neuraminidase on serological properties of murine lymphoid cells. *Transplant. Proc.* **3**:895.

37. Greaves, M. F., and M. C. Raff. 1971. Specificity of anti-θ sera in cytotoxicity and functional tests on T lymphocytes. *Nat. New Biol.* **233**:239.

38. Unanue, E. R., H. M. Grey, E. Rabellino, P. Campbell, and J. Schmidtke. 1971. Immunoglobulins on the surface of lymphocytes. *J. Exp. Med.* **133**:1188.

39. Jones, G., G. Torrigiani, and I. M. Roitt. 1971. Immunoglobulin determinants on mouse lymphocytes. *J. Immunol.* **106**:1425.

40. Bankhurst, A. D., and N. L. Warner. 1971. Surface immunoglobulins on mouse lymphoid cells. *J. Immunol.* **107**:368.

41. Nossal, G. J. V., N. L. Warner, and H. Lewis. 1971. Incidence of cells simultaneously secreting IgM and IgG antibody to sheep erythrocytes. *Cell. Immunol.* **2**:41.

42. Cosenza, H., and A. A. Nordin. 1970. Immunoglobulin classes of antibody-form-

ing cells in mice. III. Immunoglobulin antibody restriction of plaque-forming cells demonstrated by the double immunofluorescent technique. *J. Immunol.* **104:**976.

43. McConnell, I. 1971. Antigen receptors on the surface of antibody-secreting cells. *Nat. New Biol.* **233:**177.

44. Humphrey, J. G., G. Roelants, and N. Willcox. 1971. Specific lethal radioactive antigens. *In* Cell Interactions and Receptor Antibodies in Immune Responses. O. Mäkelä, A. Cross, and T. U. Kosunen, editors. Academic Press, Inc., New York. 123.

45. Baird, S., J. Santa, and I. Weissman. 1971. Anti-theta antisera may contain anti-allotype contamination. *Nat. New Biol.* **232:**56.

46. Boyse, E. A., E. Bressler, C. Iritani, and M. Lardis. 1970. Cytotoxic γM auto-antibody in mouse alloantisera. *Transplantation.* **9:**339.

47. Hunter, P., A. Munro, and I. McConnell. 1972. Properties of educated T cells for rosette formation and cooperation with B cells. *Nat. New Biol.* **236:**52.

48. Davie, J. M., A. S. Rosenthal, and W. E. Paul. 1971. Receptors on immunocompetent cells. III. Specificity and nature of receptors on dinitrophenylated guinea pig albumin-^{125}I-binding cells of immunized guinea pigs. *J. Exp. Med.* **134:**517.

49. Davie, J. M., and W. E. Paul. 1971. Receptors on immunocompetent cells. II. Specificity and nature of receptors on dinitrophenylated guinea pig albumin-^{125}I-binding lymphocytes of normal guinea pigs. *J. Exp. Med.* **134:**495.

50. Warner, N. L., P. Byrt, and G. L. Ada. 1970. Blocking of the lymphocyte antigen receptor site with anti-immunoglobulin sera in vitro. *Nature (Lond.).* **226:**942.

51. Dwyer, J. M., S. Mason, N. L. Warner, and I. R. Mackay. 1971. Antigen binding lymphocytes in congenitally athymic (nude) mice. *Nat. New Biol.* **234:**252.

52. Taylor, R. B., and G. M. Iverson. 1971. Hapten competition and the nature of cell-cooperation in the antibody response. *Proc. R. Soc. London B. Biol. Sci.* **176:**393.

53. Kincade, P. W., A. R. Lawton, and M. D. Cooper. 1971. Restriction of surface immunoglobulin determinants to lymphocytes of the plasma cell line. *J. Immunol.* **106:**1421.

54. Roberts, C. I., M. W. Brandriss, and J. H. Vaughan. 1971. Failure of immuno-cytoadherence to demonstrate delayed hypersensitivity. *J. Immunol.* **106:**1056.

55. Basten, A., J. F. A. P. Miller, N. L. Warner, and J. Pye. 1971. Specific inactivation of thymus-derived (T) and non-thymus-derived (B) lymphocytes by ^{125}I-labeled antigen. *Nat. New Biol.* **231:**104.

56. Roelants, G. E., and B. A. Askonas. 1971. Cell cooperation in antibody induction. The susceptibility of helper cells to specific lethal radioactive antigen. *Eur. J. Immunol.* **1:**151.

57. Davies, A. J. S., R. L. Carter, E. Leuchars, V. Wallis, and P. C. Koller. 1969. The morphology of immune reactions in normal, thymectomized and reconstituted mice. I. The response to sheep erythrocytes. *Immunology.* **16:**57.

58. Möller, G., and G. Michael. 1971. Frequency of antigen-sensitive cells to thymus-independent antigens. *Cell. Immunol.* **2:**309.