CELL SURFACE IMMUNOGLOBULIN*

VII. Synthesis, Shedding, and Secretion of Immunoglobulin by Lymphoid Cells of Germ-Free Mice

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Previous studies of enzymatically iodinated splenocytes from normal BALB/c mice indicate that cell surface Ig is primarily 8S IgM (1) which is rapidly released on a fragment of plasma membrane (shedding) during incubation of iodinated cells (2). In contrast, labeling of the same cell population with [³H]tyrosine results in secretion of radioactive IgG, 19S IgM, and 8S IgM all unassociated with plasma membrane (2). To explain these findings, we suggested (3) that Ig might be transported to the cell surface in post Golgi vesicles where Ig destined to have a surface phase would be attached to the inner surface of a Golgi vesicle and Ig to be secreted would be "free" in the vesicle. We also suggested that the exteriorization of cell surface and secreted Ig might be independently regulated.

In order to study further these pathways and to obtain information about the kinetics of the exteriorization of Ig, it was desirable to perform studies in a system in which only one class of Ig was involved. Our preliminary studies with germ-free mice indicated that their splenocytes synthesize only IgM and would be a suitable cell system. The results with this system indicate that shedding of cell surface IgM and secretion of IgM appear to involve two different pathways that are independently regulated. Small lymphocytes shed Ig; plasma cells secrete Ig. The results also indicate that splenocytes from germ-free mice synthesize an excessive proportion of IgM. It is suggested that germ-free mice lack a population of stimulated T cells capable of inducing the "switch" from IgM to IgG synthesis and also of suppressing IgM synthesis.

Materials and Methods

Preparation of Iodinated Lymphoid Cells.—Male germ-free mice of 4–6 wk of age were purchased from A. R. Schmidt Division, Sprague Dawley, Madison, Wis. (C3H) or from

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Charles River, Mass. (CD-1). Nonaxenic animals of the same strain and age were purchased from Jackson Laboratories, Bar Harbor, Maine (C3H) or from Charles River (CD-1). Splenic lymphocytes were prepared and iodinated as previously described (4). Other cell populations that were employed include thoracic duct lymphocytes (5) obtained from the first 24 h of drainage or lymph node cells from pooled axillary, cervical, and inguinal nodes.

Cell Culture.—1–2 × 10^8 iodinated or unlabeled cells were suspended at a concentration of 1 × 10^7 cells/ml in Eagle's minimal essential medium (MEM) containing nonessential amino acids (Grand Island Biological Co., New York) and 10% fetal calf serum. In experiments in which cells were labeled with [^{3}H]tyrosine, the medium lacked both tyrosine and fetal calf serum for the first 60 min of labeling. The cells were labeled with 20 μCi/ml of [^{3}H]tyrosine [3, 5-^{3}H]-L-tyrosine (50 Ci/mmol, New England Nuclear, Boston, Mass.). The cells were then pelleted and resuspended in MEM and 10% fetal calf serum for further incubation. Viability was determined by trypan blue exclusion.

After the incubation period, the cell suspension was centrifuged at 1,000 g for 10 min, and the lysates and secretions prepared and processed as previously described (1). The cells were lysed into phosphate-buffered solution (PBS)[1] containing 0.5% Nonidet P40, (NP40, Shell Chemical Corp., New York) containing 0.5 M iodoacetamide (recrystallized). In all experiments NP40 (sometimes containing 0.5% iodoacetamide) was added to the secretions to a final concentration of 0.5% before dialysis.

Isolation of Radioactive Ig.—Dialyzed lysates and secretions were concentrated by pervaporation and centrifuged for 10 min at 10,000 g. Small aliquots were precipitated in trichloracetic acid (TCA), and the remainder immunoprecipitated using rabbit antiserum directed to mouse μ, γ, κ, and λ chains or to bacteriophage-$\phi\chi$ (control) (2). The resultant complexes were precipitated with goat antirabbit Ig. Washed precipitates were dissolved and electrophoresed within 24 h on sodium dodecyl sulfate (SDS) agarose-2.5% acrylamide gels or were reduced and alkylated and electrophoresed on 5% acrylamide gels (1). 19S human IgM, and mouse IgG (or μ, γ, and L chains obtained after reduction and alkylation of these proteins) were used as markers on companion gels that were electrophoresed simultaneously. All gels were fractionated and radioactivity determined (1).

Electronmicroscopy.—Suspensions of spleen cells from axenic and control mice were pelleted, fixed for 15 min with 1% glutaraldehyde (6) in PBS, postfixed for 20 min with 1% osmium tetroxide (7) in PBS, dehydrated in a series of alcohols and propylene oxide, and embedded in Epon resin (8). Sections of specimens were cut perpendicular to the plane of the pellet in the center of the pellet with a diamond knife on a Porter-Blum microtome and stained with uranyl acetate, and lead citrate (9) and examined with a JEOL JEM-100B electron microscope. All cells in randomly selected sections were photographed and classified according to morphological types. Cell profiles which did not include a cross section of a nucleus were not classified.

RESULTS

Immunoglobulin Synthesis and Secretion in Splenocytes.—The amount of protein and Ig synthesized and secreted by splenocytes from axenic and control mice was studied. Thus, cells were labeled for 60 min with [^{3}H]tyrosine and then incubated in complete medium for an additional 120 min.

When axenic mice were employed, an average of 7% of the total acid precipitable radioactivity from the lysate and 68% from the medium was specifically immunoprecipitated as Ig (Table I). The average ratio of radioactivity

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[1] *Abbreviations used in this paper:* MEM, minimal essential medium; NP40, Nonidet P40; PBS, phosphate-buffered solution; SDS, sodium dodecyl sulfate; TCA, trichloracetic acid.

TABLE I

Immunglobulin Synthesis, Secretion, and Shedding by Mouse Splenocytes

Isotope*	Splenocytes	Acid precipitable radioactivity/10⁸ cells‡	cpm specific precipitate/ cpm control precipitate§		Total protein that was Ig‖		Ig released into incubation medium in 2 h
			Lysate	Medium	Lysate	Medium	
		$cpm \times 10^6$			%		%
[³H]tyrosine	Axenic (+)	2.2 ± 1.1	17.3 ± 2.2	35.9 ± 5.3	7.3 ± 1.8	68 ± 3	45 ± 4
	Nonaxenic (++)	1.7 ± 1.0	5.4 ± 0.9	13.4 ± 2.7	2.0 ± 1.1	73 ± 4	48 ± 6
¹²⁵I	Axenic (++)	4.9 ± 1.8	13.1 ± 2.1	4.6 ± 1.6	10.5 ± 1.6	8 ± 2	22 ± 4
	Nonaxenic (++)	3.5 ± 1.7	4.0 ± 0.7	2.8 ± 0.6	5.1 ± 0.5	8 ± 3	29 ± 6

Results of experiments using C3H are shown. Several experiments were performed in CD-1 mice with similar results.

(+) Eight experiments.

(++) Three experiments.

* 1-2 × 10⁸ cells were labeled for 60 min with [³H]tyrosine or were radioiodinated. Cells were washed and incubated for an additional 120 min.

‡ Acid precipitable radioactivity in the cell suspension was determined after labeling and before incubation. Figures in this table are averages followed by the range.

§ Ig in cells and medium determined after 2 h incubation.

‖ 100 (cpm specific precipitate − cpm control precipitate)
   ————————————————————————————————
   acid precipitable radioactivity.

in specific to control precipitates was 17 from the lysates and 36 from the medium. When specific precipitates were dissolved and electrophoresed on SDS gels, the majority of the intracellular Ig was recovered as 8S IgM; approximately 10-25 % of the radioactivity was recovered as 19S IgM and 5-10% as free chains (Fig. 1). Similar results were obtained when the cells were lysed into NP₄₀ containing 0.5 M iodoacetamide, suggesting that the molecular forms of IgM recovered were not due to self-assembly or disulfide exchange during cell lysis. In addition, when ¹²⁵I-labeled mouse 19S IgM (isolated from MOPC 104E ascites fluid) was admixed with unlabeled cell cultures or NP₄₀ lysates of unlabeled cells and precipitated with anti-Ig, 95 % could be recovered in its 19S form. This observation suggests that degradation of 19S IgM was not contributing substantially to the recovery of 8S IgM and "free" μ- and L-chains from the ³H-labeled lysates. No IgG was detected either by specific precipitation with monospecific anti-γ or as γ-chain by electrophoresis of reduced and alkylated precipitates (formed with antimouse μ, γ, λ, K) on SDS-acrylamide gels.

In contrast to the lysates, greater than 90% of the immunoprecipitable radioactivity in the secretions was recovered as 19S IgM with small amounts of free chains. No IgG was detected in the secretions (Fig. 2). As with the lysates, these findings were not changed by the addition of iodoacetamide to secretions. These results suggest that the small numbers of 19S IgM molecules in the lysate are those destined for immediate secretion and that assembly of IgM monomer to polymer is an intracellular event.
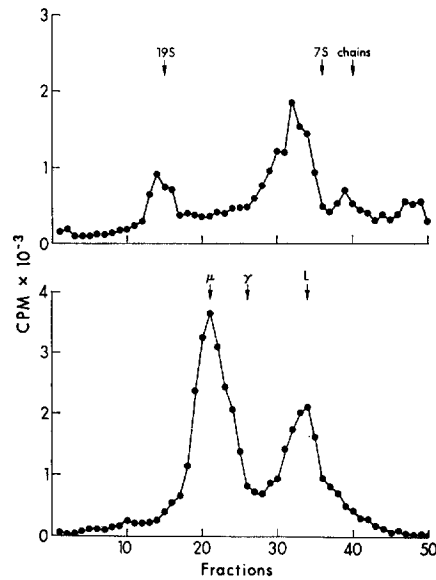
FIG. 1. [³H]tyrosine-labeled Ig synthesized by splenocytes from axenic mice. Dissolved immunoprecipitates were electrophoresed on SDS-agarose 2.5% acrylamide gels (above) or were reduced and alkylated and electrophoresed on SDS-5% acrylamide gels (below) with appropriate markers.

When splenic lymphocytes from unimmunized, nonaxenic mice of the same age were labeled in a similar manner, it was found that an average of 2% of the acid precipitable radioactivity from the lysates and 73% from the medium could be specifically immunoprecipitated. In contrast to the situation with germ-free mice, however, the ratios of specific to control precipitates for the lysates and medium were much less, an average of 5 and 13 respectively. The most likely explanation is that splenocytes from nonaxenic mice synthesize proteins which bind to immune complexes; synthesis of such proteins is reduced in lymphoid cells from axenic mice. The anti-Ig containing immunoprecipitates from both lysates (Fig. 3) and secretions (Fig. 4) contained considerable amounts of IgG in addition to 8S and 19S IgM.

These experiments indicate that in splenocytes from axenic mice a higher proportion of their protein synthesis is devoted to Ig compared to splenocytes from control mice. Also, splenocytes from axenic mice do not synthesize detectable IgG.

Ig Synthesis and Secretion in Nonsplenic Lymphocytes.—The preceding experiments indicated absence of IgG synthesis in splenocytes from axenic mice. It was possible that IgG-synthesizing cells had migrated from the spleen to lymph nodes and/or to the pool of circulating lymphocytes. To test these possibilities, similar incorporation experiments to the above were performed
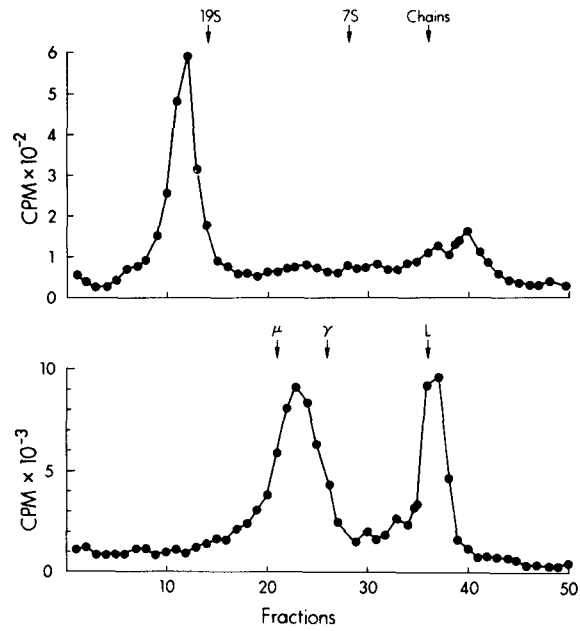
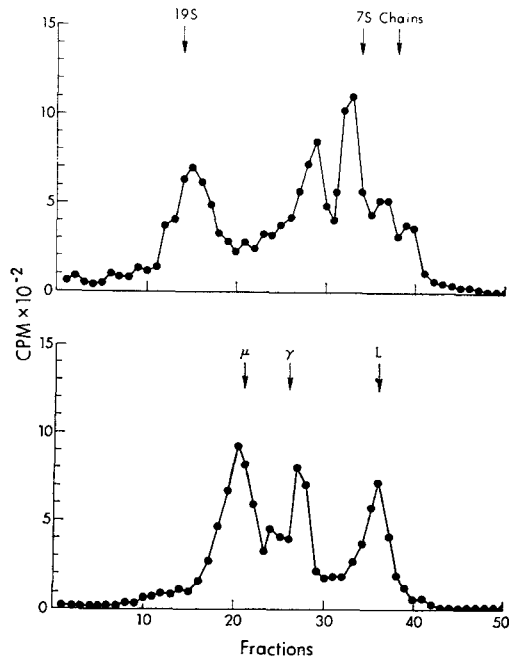Fig. 2. [³H]tyrosine-labeled Ig secreted by splenocytes from axenic mice. See Fig. 1.

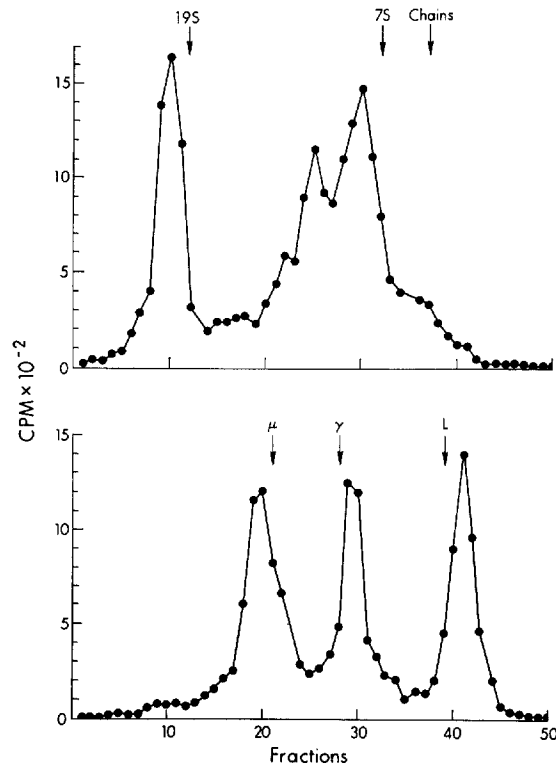Fig. 3. [³H]tyrosine-labeled Ig synthesized by splenocytes from control mice. See Fig. 1.

Fᴏɪ. 4. [³H]tyrosine-labeled Ig secreted by splenocytes from control mice. See Fig. 1.

with lymph node cells and thoracic duct lymphocytes from axenic C3H mice. Immunoprecipitates were analyzed after reduction and alkylation by acrylamide gel electrophoresis in SDS. Thoracic duct cells were of particular interest because studies in normal BALB/c mice had indicated that such cells synthesize and secrete almost exclusively IgA.[2]

The results were analogous to those shown in Figs. 1 and 2. These experiments indicate that only IgM is synthesized by lymphoid cells from axenic mice.

Cell Surface Ig in Splenocytes.—Enzymatic iodination was used to characterize the class and molecular form of Ig on the surface of splenocytes from axenic mice. When cells from C3H axenic mice were radioiodinated, lysed, and the Ig specifically precipitated from the lysate, an average of 10% of the total labeled cell surface protein was recovered as Ig and the average ratio of radio-

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[2] Uhr, J. W., and E. S. Vitetta. 1974. Cell surface Immunoglobulin. VIII. Synthesis, secretion, and cell surface expression of immunoglobulin in murine thoracic duct lymphocytes. *J. Exp. Med.* **139**: 1013.
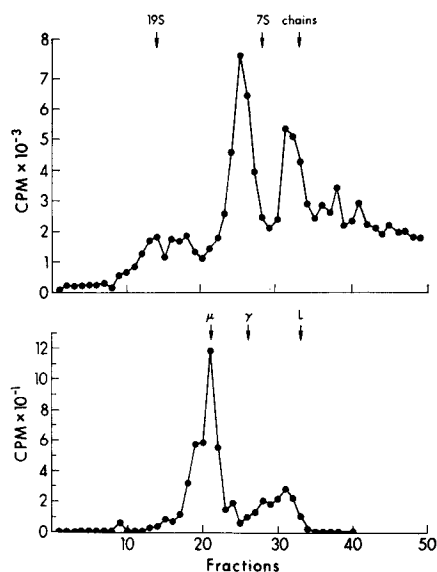
FIG. 5. Radioiodinated cell surface Ig from splenic lymphocytes of axenic mice. See Fig. 1

activity in specific to control precipitates was 13 (Table I). Comparable values for control mice were 5 and 4 respectively. As seen in Fig. 5, the majority of the cell surface Ig on splenocytes from axenic mice appeared to be 8S IgM accompanied by a substantial amount of radioactivity in free chains and small amounts in molecules of mol wt greater than 2×10^5 daltons. No Ig peaks were seen when control precipitates were electrophoresed. However, a small peak corresponding to 30–40,000 daltons was usually observed. When the 8S IgM monomer was eluted from the agarose gel, concentrated by pervaporation, reduced and alkylated, and electrophoresed on a 5% acrylamide gel, μ, and L chains were obtained with a ratio of radioactivity in μ/L of 3–4. This ratio is similar to that obtained from surface IgM in nonaxenic mice of other strains (1).

When radioiodinated cells from germ free mice were cultured for 3–6 h, the cell surface IgM was released into the medium, as previously observed using lymphocytes from nonaxenic, unimmunized mice (2). As seen in Table I, 8% of the acid precipitable radioactivity recovered from the secretions was Ig. Analysis of this Ig by acrylamide or agarose-acrylamide gel electrophoresis revealed the same molecular forms as those present on the cell surface indicating that the released cell surface Ig did not polymerize. Unlike the Ig recovered from the secretions of [3H]tyrosine-labeled cells, there was no increase in the proportion of Ig to total protein in the secretions of the 125I-labeled cells relative to that associated with the cells.

Kinetics of Release of Cell Surface and Total Ig.—In two experiments, separate aliquots of lymphocytes from C3H germ-free mice were either prelabeled

for 60 min with [³H]tyrosine or were radioiodinated. In two additional experiments, the same aliquot of cells was labeled with both [³H]tyrosine and ¹²⁵I. In all four experiments, the cells were then washed in MEM containing 10% fetal calf serum and incubated for 6 h at 37°C. At intervals during the incubation, aliquots of cells were removed and the lysates and secretions prepared as previously described. In all experiments cell viability remained above 90%.

Fig. 6 shows the results of a representative experiment in which "double-labeling" was used. As can be seen, the [³H]tyrosine-labeled Ig was lost from the cells more rapidly than the ¹²⁵I-labeled cell surface Ig. The half-lives of total and cell surface Ig based on the initial slopes were 2–3 and 5–6 h respectively. Similar values were obtained in the single label experiments. The half-life of release of the cell surface Ig is similar to that obtained from experiments with nonaxenic mice of other strains (1). These experiments suggest that shedding of 8S IgM and secretion of 19S IgM are independent pathways.
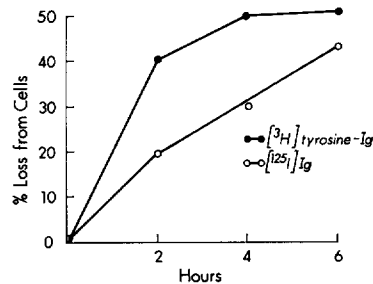

FIG. 6. Kinetics of release of radioiodinated cell surface Ig and [³H]tyrosine-labeled Ig from splenocytes of axenic mice during in vitro incubation.

Ultrastructural Studies of Splenocytes.—The preceding findings indicate that splenocytes from axenic mice synthesize a higher proportion of Ig to total protein than splenocytes from control mice. This was unexpected in the light of several reports that germ-free mice have a paucity of plasma cells (10–12).

To study the morphological basis of the above biosynthetic activities, suspensions of splenocytes from germ-free and control C3H and CD-1 mice were examined by electron microscopy. The results are summarized in Table II and Fig. 7. The majority of cells in both germ-free and control cell suspensions were small and medium-sized lymphocytes. The cells were characterized by scanty cytoplasm and spherical nuclei with marginal and occasionally central heterochromatin (Fig. 7). However, significant differences in the proportion of plasma cells and lymphoblasts were observed between the germ-free and control populations. Thus, in the cells from germ-free mice the proportion of plasma cells was three-to fivefold greater and the proportion of lymphoblasts markedly reduced compared to cells from control mice. The plasma cells which were characterized by a small, dense, round, often eccentric nucleus and a large

TABLE II

Electronmicroscopic Analysis of Splenocytes of Axenic and Control Mice

Cell Type	Percent distribution			
	C3H		CD-1	
	Axenic*	Control*	Axenic*	Control*
	%		%	
Plasma cell	24	8	18	4
Lymphoblast	1	12	2	14
Lymphocyte	74	79	78	82
Other	2	1	2	1

* Number of cells examined for the four categories of mice listed in the above headings were 123, 155, 115, and 114 respectively.

cytoplasmic area containing numerous lamellae of rough endoplasmic reticulum appeared similar in the populations studied (Fig. 7). Lymphoblasts which were identified as large cells which exhibit an irregular cytoplasmic outline with a large irregularly shaped nucleus, and a finely granular nucleoplasm also appeared similar in all cell suspensions studied.

These morphological findings together with the biosynthetic results suggest that the small amount of antigenic stimulation in germ-free mice is sufficient to cause a marked differentiation of lymphocytes to IgM-secreting plasma cells.

DISCUSSION

The major findings to emerge from these studies are: (a) Cultured lymphoid cells from axenic mice synthesize only one major class of Ig, IgM; control mice also synthesized IgG; (b) the proportion of IgM relative to synthesis of total protein is approximately threefold higher in axenic mice; (c) the proportion of plasma cells is also approximately threefold higher and the proportion of lymphoblasts 10-fold lower in the spleens of germ-free mice; (d) the kinetics of shedding and secretion of IgM and the molecular forms of the molecules released indicate that there are two different pathways for releasing Ig from lymphoid cells; and (e) lymphoid cells from axenic mice synthesize and secrete less non-Ig proteins which coprecipitate with immune complexes than lymphocytes from control animals.

The finding that lymphoid cells of axenic mice synthesize IgM only is unexpected. There are numerous reports claiming that germ-free animals, human fetuses, and chick embryos synthesize IgG (13–19, footnote 3). Some of these reports rely on demonstration of IgG on lymphocytes by immunofluorescence. The interpretation of such evidence is complicated by the presence of Fc

[3] Lawton, A. R., J. W. Davie, R. S. Asofsky, and M. D. Cooper, unpublished results.
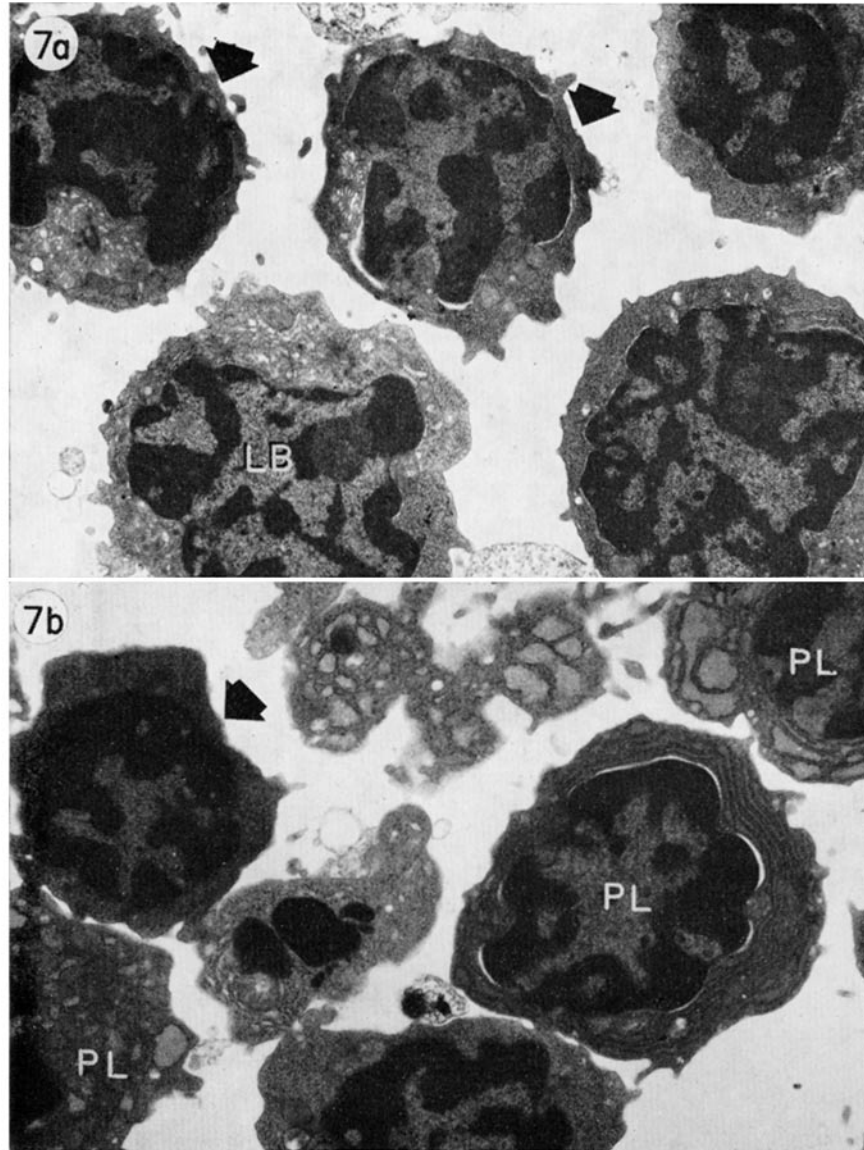
Fig. 7. Electronmicrographs of splenocytes from control (a) and axenic (b) C3H mice. (a) Arrows indicate lymphocytes which are the predominant cell type in both control and axenic spleen cell populations. A higher proportion of splenocytes from control mice were lymphoblasts (LB). (b) A higher proportion of plasma cells (PL) were present in splenocytes of axenic compared to control mice. × 13,300.

receptors on both B (20–22) and T cells (23, 24, footnote 4) which would allow binding of maternal Ig. It should also be stressed that the rate of Ig synthesis in lymphocytes is very slow compared to plasma cells and that our biosynthetic studies might not detect synthesis of cell surface IgG and IgA in lymphocytes. The above reports, however, include studies of incorporation of isotopic amino acids into IgG in human fetal tissue (13, 14), into IgG and IgA by germ-free newborn piglets (16, 17), and into IgG by bursal cells in chick embryos (19). It is possible that mammalian and avian fetuses could be exposed to maternal antigens as well as to pathogenic microorganisms. Regardless, it should be emphasized that the approach for detection of synthesis of IgG in our experiments is a simple one: the experimental results depend upon labeling cells with radioactive amino acids, immunoprecipitation of cell lysates with multispecific antimouse Ig, and analysis of solubilized precipitates by SDS-acrylamide gel electrophoresis. We considered the possibility that cells synthesizing detectable amounts of IgG were present in other organs besides the spleen. This possibility was rendered unlikely by the absence of such synthesis in lymphocytes from pooled lymph nodes and thoracic duct lymph. The results with thoracic duct lymphoid cells are particularly striking because in nonaxenic animals such cells synthesize almost exclusively IgA.[2] In germ-free mice, however, thoracic duct lymphoid cells do not synthesize IgA but synthesize and secrete IgM.

Another unexpected finding was the demonstration by electron microscopy of a markedly increased proportion of plasma cells and a paucity of lympho-blasts in the spleens of germ-free mice. These observations are consistent with the conclusion that 19S IgM was secreted mainly or exclusively by such plasma cells rather than small lymphocytes since plasma cells can be over 100-fold more active in Ig synthesis per cell than lymphocytes (25, footnote 5). This interpretation correlates well with the kinetic studies of shedding and secretion. Shedding occurs with a slower half-life (5–6 h) than secretion (2–3 h); moreover, shed IgM is monomeric (8S) and secreted IgM is polymeric (19S). These findings suggest two different pathways of release. The simplest explanation is that plasma cells secrete 19S IgM as mentioned above and that small lymphocytes shed 8S IgM. It is also noteworthy that 8S IgM was secreted by splenocytes from control mice but was not secreted by axenic splenocytes. Perhaps stimulated B lymphocytes (lymphoblasts) are responsible for secreting 8S IgM.

The unusually large number of plasma cells in the spleens of germ-free mice and the paucity of lymphoblasts could be related phenomena. These spleens may lack a sizeable population of stimulated T cells that would have induced the switch from IgM to IgG (or IgA) and that would have also suppressed

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[4] Hudson, L., J. Sprent, and J. F. A. P. Miller, unpublished observations.

[5] Vitetta, E. S., and J. W. Uhr, unpublished results.

the IgM response. Thus, the overresponse of IgM synthesis and secretion in these mice may be due to the absence of an effective population of suppressor T cells (26, 27). An additional possibility is that antibody-induced inhibition of synthesis of IgM antibody (28) is less effective with IgM than IgG antibody.

The above observations are consistent with the model of differentiation depicted in Fig. 8. The assumption underlying the model is that germ-free mice have received minute amounts of antigen. Thus, major features of the model are: (a) antigen dependence of all differentiation steps in contrast to the model of Cooper et al. (29) (They hypothesize an antigen-independent differentiation of stem cells to multiple clones of B lymphocytes with sequential expression
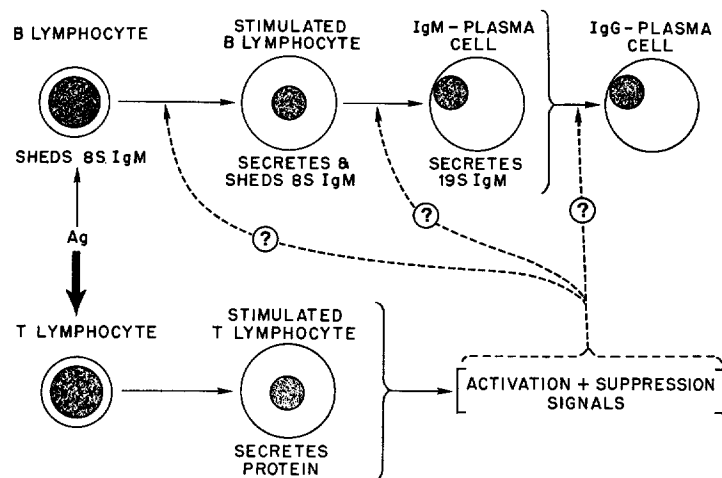

Fig. 8. Model of differentiation of small lymphocytes to plasma cells emphasizing biosynthetic and cell surface changes and dependence of differentiation on antigen and stimulated T cells.

of IgM and IgG [or IgA]); (b) a lower threshold of antigenic stimulation for differentiation to IgM-secreting plasma cells than for either IgG or IgA-secreting plasma cells or a population of stimulated T lymphocytes; (c) mediation of the antigen dependent "switch" via a population of stimulated T lymphocytes; and (d) change from a cell that synthesizes primarily cell surface Ig and that can shed in vitro (small lymphocyte) to a cell that exports Ig primarily without a cell surface phase (plasma cell). The above model is consistent with previous studies of the shift from IgM to IgG (30–33) and with current concepts of the cooperative role of T cells in the differentiation of B cells (see 34). The possibility that T helper cells have a higher threshold of stimulation than B cells could be related to the nature of the immunogen. Thus, germ-free mice may be immunized predominantly by bacterial antigens in their food such as lipopolysaccharides which are potent mitogens for B cells (35).

The absence of stimulated lymphocytes may explain an additional observation of interest. Splenocytes and lymphocytes from axenic mice synthesize less non-Ig protein that coprecipitates with antigen-antibody complexes than comparable cell populations from normal mice. These findings suggest that there are a number of proteins synthesized and secreted by lymphoid cells whose synthesis depends ultimately on antigenic stimulation. At least one of these proteins with a mol wt of 30–40,000 daltons appears to bind selectively to antigen-antibody complexes (25). It will be of interest to determine whether these proteins are of B- or T-cell origin and their role in cell cooperation.

SUMMARY

Lymphoid cells from the spleen, lymph nodes, and thoracic duct of axenic and control mice were incubated with [^{3}H]tyrosine and synthesis and secretion of protein and Ig studied. It was found that only IgM was synthesized by cells from axenic mice whereas cells from control mice also synthesized IgG. Splenocytes from both axenic and control mice had 8S IgM on their surface. Radiolabeled splenocytes from axenic mice were incubated to determine the kinetics of release of ^{125}I-labeled cell surface IgM and [^{3}H]tyrosine-labeled IgM. Cell surface IgM was shed as 8S with an initial half-life of release of 5–8 h whereas [^{3}H]tyrosine-labeled Ig was secreted as 19S with an initial half-life of 2–3 h. These findings suggest that two independent pathways are involved. It is suggested that small lymphocytes shed 8S IgM and plasma cells secrete 19S IgM.

It was observed that lymphoid cells from axenic mice synthesize a higher proportion of IgM relative to total protein. Electron microscopic examination of splenocytes from such mice revealed a markedly higher proportion of plasma cells and a paucity of lymphoblasts compared to controls. It was suggested, therefore, that axenic mice lack a population of stimulated T cells which can induce a switch from IgM to IgG synthesis and which is capable of suppressing IgM synthesis. Lymphoid cells from axenic mice synthesize and secrete less protein that coprecipitates with antigen-antibody complexes.

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