THE PRESENCE OF IgA ON THE SURFACE OF RAT THORACIC DUCT LYMPHOCYTES WHICH CONTAIN INTERNAL IgA

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Thoracic duct lymph contains a minority population of cells consisting of large lymphocytes, all of which can be labeled with a short course of tritiated thymidine in vivo (1) and which do not recirculate (2). Many of these large lymphocytes migrate from the blood into the lamina propria of the small intestine where they become plasma cells (2, 17). The idea that large lymphocytes may be an important source of the IgA-secreting cells in the small intestine is supported by the observation that some of the large cells in rat thoracic duct lymph contain large amounts of internal IgA, but not IgM or IgG_2 (3, 4). By indirect methods these cells were shown to carry surface Ig but its class was not determined (3).

In the present study the IgA-containing cells in rat thoracic duct lymph have been examined by immunofluorescence, and by means of a double-labeling technique it has been shown that the cells with internal IgA also carry large amounts of surface IgA.

Materials and Methods

Methods not described below are in references 3, 5, and 6. For estimation of protein concentrations, standard $E^{280\ nm}_{1\ cm}$ values of 13.5 and 15.0 were used for a 1% solution of IgG and $F(ab')_2$ respectively (7). The purity of Ig was estimated by densitometry on disc gels from polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS PAGE)[1] stained with coomassie blue and quantitated with a Gilford gel-scanner model 2410-S.

Rats. In bred male or female AO, DA, $(PVG/c \times DA)F_1$, and PVG/c rats aged 8–12 wk were used (8) either directly from the specific pathogen-free (SPF) unit, or after housing from weaning in a conventional animal house. Adult thymectomized, irradiated, bone-marrow-restored rats (B rats) were prepared by the method of Howard (9).

Rat IgG_2, IgM, and IgA. Rat IgG_2 purified as previously described (5) was 95% pure, as was IgM IR202 myeloma kindly provided by Dr. Hervé Bazin, Université Catholique de Louvain. Dimeric rat

[1] *Abbreviations used in this paper:* Anti-IgA, anti-Fab, anti-IgG_2, and anti-IgM: purified rabbit $F(ab')_2$ antirat IgA, Fab, IgG_2, and IgM respectively; Antiallotype, purified PVG/c anti-DA Ig-1a allotype antibody; B rats, adult thymectomized, irradiated, bone marrow-restored rats; SDS PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulphate; SPF, specific pathogen-free; TDL, thoracic duct lymphocytes.

IgA was purified from rat lymph which has a much higher concentration of IgA than serum (3, 10). The purification involved the following steps: ultrafiltration; centrifugation to remove lipid; $(NH_4)_2SO_4$ precipitation; DEAE cellulose chromatography; gel filtration on Sephadex-G200 and Sepharose-6B; and Pevikon block electrophoresis. The behavior of the lymph IgA throughout was as described for serum IgA by Bazin et al. (11). The final IgA preparation was 94% pure, gave one line in immunoelectrophoresis with rabbit antirat serum protein, and gave no reaction with anti-IgG_2 or anti-IgM in double-diffusion precipitin analysis.

Purified Anti-Rat Ig Antibodies Labeled with ^{125}I and Fluorescein. Rabbit $(Fab')_2$ antirat Fab and antirat IgG_2 antibodies, and PVG/c anti-DA L chain allotype (Ig-1a) antibody were as previously described (5, 6). Rabbit antirat IgA antiserum was raised against rat IgA from colostrum, rendered monospecific by passage through rat IgG_2-Sepharose 4B beads (3), and purified by absorption to and elution from lymph IgA-Sepharose 4B beads. Fluorescein isothiocyanate (Nordic) was coupled to the purified IgG fraction of the anti-IgA (12), while for cell-surface labeling $F(ab')_2$ anti-IgA was iodinated at 5 μCi/μg. $F(ab')_2$ rabbit antirat IgM was prepared as before (5) excepting that IgM IR202 was used as the immunoabsorbent. All purified antibodies consisted of >90% $F(ab')_2$ or IgG, and the [^{125}I]anti-IgA and [^{125}I]anti-IgM were 56% and 50% active respectively in precipitin assays.

Specificity of ^{125}I-Labeled Antirat IgA Antibody. Labeled antibody was preincubated with various amounts of purified rat Ig fractions, centrifuged, and then tested for binding on rat TDL. The results are shown in Fig. 1, where it can be seen that the binding of the [^{125}I]anti-IgA was specific, being 100% inhibited by a twofold ratio of IgA to antibody and unaffected by a 10-fold excess of IgM or IgG_2. The specificity of the other antibodies was established in the same way (5).

Cell Preparation and Labeling with ^{125}I and Fluorescein-Conjugated Antibodies. TDL from lymph collected at 6°C in the 6-16 hr period after cannulation were used. Binding assays with [^{125}I]anti-Ig were carried out as before (5, 6). Unless otherwise stated 5×10^6 TDL were incubated with 100 μl of anti-Ig at 25 μg/ml. Fluorescein-conjugated anti-IgA at 46 μg/ml was used to stain smears of fresh TDL or cells labeled with ^{125}I antibodies. Smears were fixed in 95% ethanol at 0°C for 1 h, stained for 1 h at 20°C and washed for 2 h. Before autoradiography they were again fixed in 95% ethanol.

Fluorescence Microscopy and Autoradiography. The fluorescent and autoradiographic labels were visualized simultaneously on a Zeiss Universal fluorescence microscope (Carl Zeiss, Oberkochon,

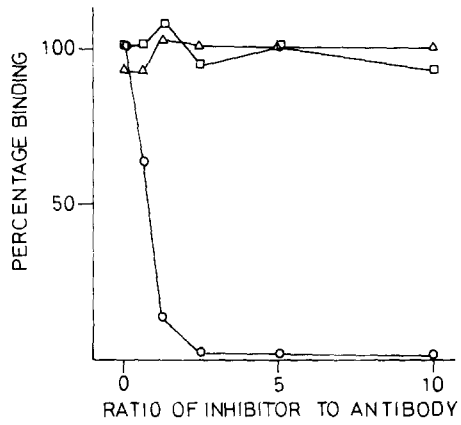

FIG. 1. Specificity of the Binding of [^{125}I]anti-IgA to rat TDL. [^{125}I]anti-IgA antibody was incubated for 30 min at 0°C with rat IgG_2 (□), or IgA (○), or IgM (△) at various mass ratios of inhibitor: antibody as shown on the abscissa. After centrifugation at 10,000 *g* for 30 min, 50 μl in duplicate at each point was added to 5×10^6 TDL and incubated at 0°C for 60 min, and binding measured as cpm ^{125}I bound. The final concentration of antibody was in all cases 3.3 μg/ml. The background binding as measured by cpm of [^{125}I]normal rabbit $F(ab')_2$ bound (<10% of [^{125}I]anti-IgA binding) was subtracted and the results expressed as percentage of control binding.

Wuerttenberg, West Germany): fluorescence was excited by epi-illumination with an HBO 200W/4 mercury vapour lamp; autoradiograph grains appeared red when illuminated by transmitted light from a tungsten iodide lamp employing a dark ground condenser and a KP500 interference filter.

Ilford G5 and K2 nuclear emulsions were used for autoradiography. The autoradiographs were exposed for 1-2 days, and the grain yields were 0.23 and 0.13 per gamma ray for G5 and K2 respectively. Given a spec act of 5 μCi/μg for the [^{125}I]antibodies, 1 grain after 2 days exposure represented 820 molecules bound for G5 and 1,450 molecules for K2 emulsion. The photometric grain-counting method (13) was employed for quantitation of the autoradiography, using a Vickers M74 photometer system rather than the Leitz MPV system (E. Leitz, West Germany) used before (5, 6).

Results

IgA-Containing Cells in Rat Thoracic Duct Lymph. Cells with internal IgA were identified by staining with fluorescein-conjugated antirat IgA antibody. The fluorescent cells in TDL were almost exclusively large lymphocytes; less than 10% had the dimensions of small lymphocytes and less than 1% could be identified morphologically as plasma cells. The most brilliantly fluorescent cells were among the medium-sized lymphocytes. Table I shows that under a variety of conditions about half the large lymphocytes in thoracic duct lymph contained IgA. Rats maintained in a conventional "dirty" animal house had a higher proportion of IgA-containing cells in lymph than SPF rats. Large lymphocytes with internal IgA were also present in lymph from adult thymectomized rats and from rats which lack T cells (B rats).

TABLE I
Proportion of Cells with Internal IgA in Rat Thoracic Duct Lymph

Rat		Period of lymph collection from thoracic duct (h after cannulation)	TDL		
Strain	Status		% Large lymphocytes	% Fluorescent large lymphocytes	% All cells fluorescent ¶
PVG/c	Conventional*	18–24	9.1	60.6	5.7
PVG/c	Conventional	6–16	7.9	52.4	4.3
PVG/c	SPF‡	18–24	3.2	61.8	2.1
PVG/c	SPF	6–16	2.0	39.1	0.9
AO	SPF	60–69	4.3	53.8	2.4
PVG/c	Adult Tx§	17–20	9.4	41.6	4.0
PVG/c	B‖	18–24	12.3	55.0	6.9
PVG/c	B	18–24	17.1	57.0	10.0

* SPF rats maintained from weaning in a conventional animal house.

‡ Rats cannulated on day after delivery from SPF area.

§ Thymectomy at 8 wk of age. Thoracic duct cannulated 5 wk later.

‖ B rats: thymectomy at 8 wk of age. 1,100 rads of radiation and 10^7 bone marrow cells 2 wk later. Thoracic duct cannulated 8 wk later still (9).

¶ Independent count of % of all cells which were fluorescent shows that the great majority, but not all fluorescent cells, are large lymphocytes.

Surface Labeling of TDL with [^{125}I]Anti-Ig Antibodies. Rat TDL were incubated with various concentrations of ^{125}I-labeled antibodies, and the number of molecules bound was measured. In Fig. 2 the binding of [^{125}I]anti-IgA, anti-Fab, and anti-IgM is compared using a single pool of cells. In all cases saturation of binding was approached at concentrations greater than 10 μg/ml. The amount of binding of [^{125}I]anti-Fab was similar in different experiments as was that of [^{125}I]anti-IgM. In contrast, the binding of [^{125}I]anti-IgA varied considerably. This can be seen from Fig. 2 which shows the binding at saturation of [^{125}I]anti-IgA, anti-Fab, and anti-IgM in a number of experiments. In two experiments with TDL from rats housed in an SPF unit lower binding of anti-IgA was observed than in four experiments where rats from a conventional animal house were used. This difference did not occur with anti-Fab and anti-IgM binding (Fig. 2).

Distribution of Surface-Bound [^{125}I]Anti-IgA Determined by Autoradiogra-

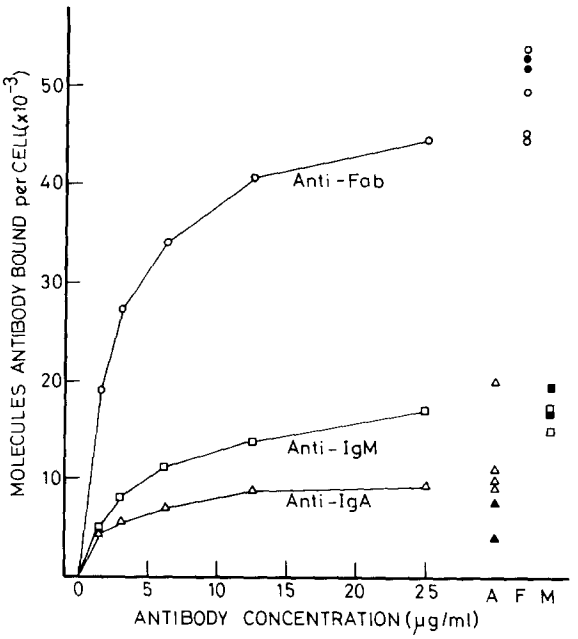

FIG. 2. Binding of [^{125}I]anti-Fab, anti-IgA, and anti-IgM to rat TDL. 2.5 × 10^6 rat TDL plus 5 × 10^6 SRBC (to improve pelleting) were incubated with 50 μl of [^{125}I]antibody (5 μCi/μg) at the concentrations shown on the abscissa. ^{125}I cpm-bound were measured and from this the molecules bound per cell were calculated. Background binding has been subtracted for all points, the controls being incubation of [^{125}I]antibody with 7.5 × 10^6 SRBC or 2.5 × 10^6 TDL plus 5 × 10^6 SRBC with [^{125}I]antibody and specific Ig inhibitor at a ratio of 5:1. The background was linear with increasing antibody and equivalent to 3,000 molecules antibody per cell with [^{125}I]antibody at 25 μg/ml. [^{125}I]antibodies: (○) anti-Fab; (△) anti-IgA; (□) anti-IgM.

The points to the right above A, F, and M show molecules per cell bound after incubation with [^{125}I]anti-IgA, -anti-Fab, or -anti-IgM at 25 μg/ml in a number of experiments. Open and closed symbols are for TDL from animals housed under conventional and SPF conditions respectively.

phy. The percentage of TDL which surface labeled with [^{125}I]anti-IgA was determined by autoradiography. In the lymph from two SPF PVG/c rats 3% and 5% of the cells bound more than 20,000 molecules of anti-IgA compared with 38% and 48% binding anti-Fab. With conventional PVG/c rats more cells were labeled with anti-IgA and the figures for three rats were 15%, 8%, and 9% compared with 38%, 39%, and 40% of cells heavily labeled with anti-Fab antibody.

The distribution of labeling with [^{125}I]anti-IgA is shown in Fig. 3 for TDL from conventional rats. Three categories of cells were identified: unlabeled, lightly labeled (mainly small), and heavily labeled (many large; see Fig. 4). The distribution of cells binding [^{125}I]anti-Fab was also measured, and heavily labeled cells bound 20,000-200,000 molecules of antibody (not shown). Thus, cells heavily labeled with anti-IgA bound as much antibody as most rat B cells bind anti-Fab. In contrast, cells labeled less heavily with anti-IgM antibody and few bound greater than 100,000 molecules. This confirmed the previous observation that many small lymphocytes form TDL-bound anti-IgM at a lower level than anti-Fab (5). However it is still unclear whether this is due to poor availability of IgM determinants or to the presence of an additional unidentified class of surface Ig.

Size Distribution of Cells Surface Labeled with [^{125}I]Anti-IgA. TDL from PVG/c rats were surface labeled with [^{125}I]anti-IgA or [^{125}I]anti-Fab and the diameters of labeled cells (>30,000 molecules of antibody bound) and adjacent unlabeled cells were measured (Fig. 4). It can be seen that the great majority of lymphocytes labeling with [^{125}I]anti-Fab are small cells, presumably B small lymphocytes (6). In contrast, a substantial proportion of the cells surface labeling

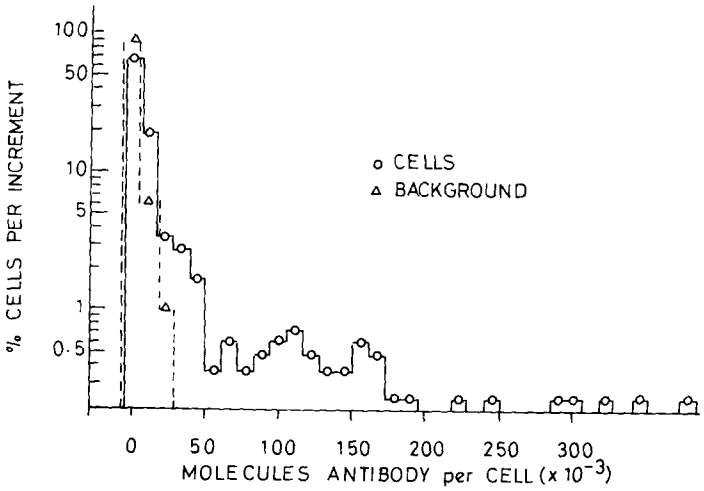

FIG. 3. Distribution of binding of [^{125}I]anti-IgA to the surface of rat TDL. TDL from normal PVG/c rats were labeled with [^{125}I]anti-IgA antibody and processed for autoradiography with Ilford K2 emulsion. Autoradiographs were exposed for 2 days and cell-associated grains measured on 820 cells by the photometric method. (O) shows the distribution of cells into the categories of labeling shown on the abscissa and (Δ) the distribution of 100 background areas read adjacent to the cells. Readings on autoradiographs of cells incubated with antibody plus specific inhibitor were lower than the background areas shown.

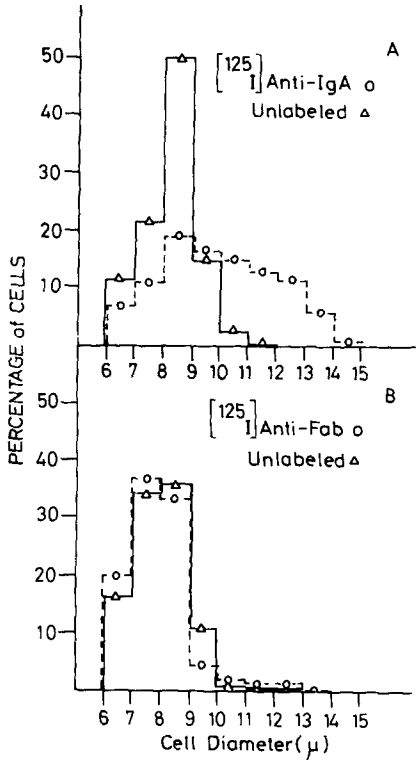

FIG. 4. Size of cells from rat thoracic lymph which surface label with [^{125}I]anti-IgA and [^{125}I]anti-Fab. TDL from normal PVG/c rats were labeled with [^{125}I]anti-IgA and [^{125}I]anti-Fab antibody, processed for autoradiography with Ilford G5 emulsion, and exposed for 1 day. Cells with > 18 grains (30,000 molecules antibody) were identified and their diameters measured with a graticule. Along with each labeled cell, the diameter of the immediately adjacent unlabeled cell was also measured. (A) shows the distribution of 200 labeled (O) and 200 unlabeled (Δ) cells after incubation with [^{125}I]anti-IgA and (B) the same for cells labeled with [^{125}I]anti-Fab.

with [^{125}I]anti-IgA are large lymphocytes although some small lymphocytes are labeled as well. The great majority of the small lymphocytes carrying surface IgA do not contain internal IgA (Table I).

Surface Labeling with [^{125}I]Anti-Ig Antibodies of TDL Containing Internal IgA. TDL were surface labeled with saturating amounts of [^{125}I]antiallotype, -anti-IgA, -anti-IgG$_2$, -anti-Fab, and -anti-IgM. Smears were then fixed, stained with fluorescein-conjugated anti-IgA, processed for autoradiography and examined after exposure for 2 days. The identification of cells containing internal IgA by fluorescence and of cells with surface labeling by autoradiography was performed simultaneously as described in Methods and is illustrated in Fig. 5.

Table II shows that almost all IgA-containing cells were heavily labeled with [^{125}I]anti-Fab antibody. In DA rats this labeling was matched by that with [^{125}I]antiallotype antibody but in (DA × PVG/c)F$_1$ animals 36% of the cells bound less than 15,000 molecules of antiallotype antibody which recognizes the L chain of the Ig-1a (DA) but not that of Ig-1b (PVG/c) (6). The comparison

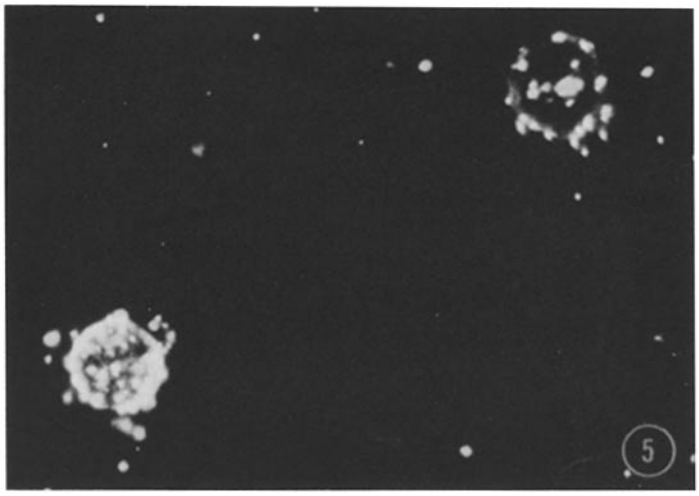

FIG. 5. Two lymphocytes from rat thoracic duct lymph showing surface labeling with [^{125}I]anti-IgA by autoradiography. The preparation had been stained with fluorescein-conjugated anti-IgA and one cell (bottom left), but not the other, also contains internal IgA. Immunofluorescence and autoradiograph viewed simultaneously as described in Methods and in Table III. For photography, the fluorescent cytoplasm was in focus leaving the autoradiographic grains out of focus (× 1,000).

TABLE II

Allelic Exclusion of Surface Ig on TDL with Internal IgA

Rat strain	TDL surface labeled with:	No. TDL with internal IgA in each grain count range:				% TDL with internal IgA with grain counts above or below background	
		0–10	11–20	21–50	>50	<11	>11
DA	[^{125}I]Antiallotype	0	5	14	38	0	100
	[^{125}I]Antiallotype + IgG	98	2	0	0		
	[^{125}I]Anti-Fab	2	1	28	76	1.9	98.1
	[^{125}I]Anti-Fab + IgG	100	0	0	0		
(DA × PVG/c)F_1	[^{125}I]Antiallotype	36	7	0	57	36	64
	[^{125}I]Anti-Fab	0	0	11	96	0	100

TDL from DA and (DA × PVG/c)F_1 rats were incubated with 100 μl of [^{125}I]antirat Ig (5 μCi/μg) antibodies at 25 μg/ml. For DA TDL, surface labeling was performed with and without inhibitor Ig to determine background labeling. The preparations were then stained with fluorescein-conjugated anti-IgA, fixed, dipped in Ilford K2 emulsion and the autoradiographs exposed for 2 days. The table records surface labeling only over those lymphocytes which also contain internal IgA. 10 grains in the autoradiographs represent about 15,000 molecules of [^{125}I]anti-Ig bound.

between the surface labeling of cells from parental and F_1 TDL with respect to antiallotype and anti-Fab antibodies is summarized in the final column in Table II. The results indicate that the surface IgA on cells which contained internal IgA showed the phenomenon of allelic exclusion.

The results of a second experiment (Table III) show that the majority of

TABLE III

Identification of Immunoglobulin on Surface of TDL from Conventional and SPF Rats

PVG/c rats	TDL surface labeled with:	No. cells with internal IgA in each grain count range:				No. cells showing surface labeling but no internal IgA	Total cells scored
		0–10	11–20	21–50	>50		
Conventional	[^{125}I]Anti-Fab	16	32	48	15	NR	111
	[^{125}I]Anti-Fab + IgG	124	0	0	0	NR	124
	[^{125}I]Anti-IgG	131	0	0	0	NR	131
	[^{125}I]Anti-IgG + IgG	120	0	0	0	NR	120
	[^{125}I]Anti-IgM	107	3	0	1	NR	111
	[^{125}I]Anti-IgM + IgM	133	0	0	0	NR	133
	[^{125}I]Anti-IgA*	0	9	42	29	20	100
	[^{125}I]Anti-IgA + IgA*	100	0	0	0	0	100
SPF	[^{125}I]Anti-IgA*	1	11	27	16	15	70
	[^{125}I]Anti-IgA + IgA*	70	0	0	0	0	70
	[^{125}I]Anti-IgM	33	0	0	0	NR	33

Surface Fab, IgG, and IgM recorded only for TDL which also contain internal IgA. Surface IgA recorded for all TDL.

TDL from PVG/c rats were surface labeled with [^{125}I]anti-Ig antibodies with and without inhibitor Ig and then stained for internal IgA as in Table II.

NR, not recorded. Surface labeling only scored on fluorescent cells.

* All cells with surface labeling scored. 10 grains in autoradiographs represent about 15,000 molecules of [^{125}I]anti-Ig bound.

but make the additional point that they carry no surface IgG_2 and very little IgM. It can also be seen that in both conventional and SPF rats at least 20% of the TDL which carry large amounts of surface IgA lack internal IgA. This latter class consists almost exclusively of small lymphocytes (Fig. 4).

Discussion

The finding of IgA-containing cells among the large lymphocytes in the rat IgA-containing cells bound more than 30,000 molecules of [^{125}I]anti-IgA per cell thoracic duct lymph, which was first established by the assay of IgA in detergent extracts of lymphocytes separated by velocity sedimentation (3), has been

confirmed by labeling ethanol-fixed smears with fluorescein-conjugated anti-IgA antibody (4). In the present study it has been shown that about 5% of all the cells in thoracic duct lymph from conventional rats contain IgA while lymph from SPE rats contains about half this number, presumably due to the smaller antigenic load in the gut. The great majority of IgA-containing cells in thoracic duct lymph were large lymphocytes; less than 10% had the dimensions of small lymphocytes and only a very rare cell could be identified morphologically as a plasma cell. These results in the rat are in agreement with the observation originally made in mice that TDL synthesize IgA (14).

In all the animals which were studied about half the large lymphocytes in thoracic duct lymph contained IgA. This was true of lymph from both conventional and SPF rats and also of rats which had been thymectomized in adult life and of adult rats which had been thymectomized, irradiated, and bone marrow restored (B rats). These results suggest that the production of lymph-borne IgA-containing cells is not highly T-cell-dependent. The nature of the large lymphocytes which lack internal IgA is not known, but they are not likely to contain IgM or IgG_2 (3); in normal rats a proportion of them are presumably T cells.

Studies with [^{125}I]anti-Ig antibodies showed clearly that IgA but not IgG_2 or IgM could be found on the surface of the IgA-containing TDL. The bulk of this IgA on cells containing internal IgA was not passively acquired since it was shown to exhibit the phenomenon of allelic exclusion. These results are at variance with the claims of Uhr and Vitetta (15) who found IgM but no IgA on the surface of mouse TDL.

While virtually all cells with internal IgA also carried surface IgA the converse was not true. At least 20% of the TDL which bound large amounts of [^{125}I]anti-IgA contained no internal IgA and these were mainly smaller lymphocytes. Another category of lymphocytes bound smaller amounts of [^{125}I]anti-IgA (less than 30,000 molecules per cell) and most of these also had no internal IgA. The significance of these cells without internal IgA is not clear, but those with large amounts of surface IgA may be precursor cells which will later synthesize IgA. On the other hand, both the lightly and heavily labeled cells which lack internal IgA may be carrying passively acquired Ig. As these are a small minority of Ig-bearing cells this possibility would not be excluded by the previous observation that most Ig-bearing cells in TDL show allelic exclusion (6), nor by the demonstration in the present experiment of allelic exclusion by cells which also contain internal IgA.

The large lymphocytes which enter the blood from thoracic duct lymph migrate selectively into the lamina propria of the small intestine (2, 16, 17). These cells are derived from intestinal lymphoid tissue because Mann and Higgins (18) showed that the output of cells from the abdominal thoracic duct of the rat can be accounted for by the output from the intestinal ducts alone, and also because IgA-containing large lymphocytes have been identified in intestinal lymph (4). There seems little doubt that migrating large lymphocytes provide a major source of IgA-synthesizing cells in the gut because substantial numbers of large lymphocytes which had been labeled in vitro with [^{3}H]thymidine were found, after transfusion, as labeled IgA-containing plasma cells in the lamina

propria of the small intestine (Gowans, to be published). It also seems very likely from the experiments of Craig and Cebra (19) that the precursors of the lymph borne cells reside in Peyer's patches where engagement with antigen also presumably occurs.

Although this scheme accommodates most of the facts a number of points remain unsettled. For example, the signal which determines the migration of large lymphocytes from the blood into the lamina propria has not been identified. It has been shown that antigen in the gut is not a necessary requirement because large lymphocytes migrate into grafts of embryonic intestine (20). Possibly, the signal for migration may be provided by free secretory piece, known to be produced by the epithelial cells of the small intestine (21), which may interact with the surface IgA on large lymphocytes. These considerations do not rule out the possibility that antigen may provide an additional stimulus for the development of IgA-synthesizing cells in the gut. Thus, lymphocytes which had not synthesized IgA but which had migrated into the gut might require a second contact with antigen to drive them to IgA synthesis. Such a mechanism might explain the way in which segmental immunization of the gut favors the local production specific antibody (22).

Summary

The presence of lymphocytes with internal IgA among cells from rat thoracic duct lymph was confirmed by labeling ethanol-fixed smears with fluorescein-conjugated antirat IgA antibody. The number of cells detected was greater in animals kept in a conventional animal house compared with those maintained under specific pathogen-free conditions. Thoracic duct lymph from B rats and adult thymectomized rats also contained cells with internal IgA. The surface Ig of the IgA-containing cells was studied using a double-labeling technique with [^{125}I]anti-Ig to detect surface Ig, and fluorescein-conjugated anti-IgA to identify cells with internal IgA. Cells containing IgA had surface IgA in large amounts, but very little IgM and no surface IgG_2. The surface IgA was not acquired passively.

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