ANTIBODIES OF THE IgA TYPE IN INTESTINAL PLASMA CELLS OF GERMFREE MICE AFTER ORAL OR PARENTERAL IMMUNIZATION WITH FERRITIN*

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The potential significance of the intestinal mucosa as an antibody-forming tissue has repeatedly been discussed (1–7) since the classical report of Davies (8), "An investigation into the serological properties of dysentery stools." Although there is no lack of compelling indirect evidence for the synthesis of "coproantibodies" (1, 2) by the intestinal lymphoid tissue in response to local antigenic stimulation, direct proof of such activity in the plasma cells of the lamina propria of the gut still remains to be furnished. A renewal of interest in this field has resulted from the discovery by Tomasi and Zigelbaum (9) of IgA as the major immunoglobulin in human external secretions, as well as from the demonstration that plasma cells of the IgA class far outnumber all other types of immunoglobulin-synthesizing cells in the lamina propria (10, 11).

The present investigation was aimed at demonstrating the participation of the intestinal plasma cells of the mouse in the antibody response to a soluble antigen, horse spleen ferritin, administered by the oral route. Advantage was taken of the availability of the sequential immunofluorescence technique (12, 13) to assign individual antibody-producing cells to four different immunoglobulin classes, IgG_1, IgG_2, IgM, and IgA. The choice of germfree, rather than conventional, animals was motivated by the hope that this would facilitate the immunohistological analyses of the lymphoid tissues which, in germfree animals, contain only limited numbers of plasma cells.

Materials and Methods

Animals.—The experiments were carried out on 3–6 month old germfree mice of the C_3H strain. The sterility of the environment was maintained throughout the experimental period.

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Antigen.—Horse spleen ferritin (Sigma Chemical Co., St. Louis, Mo.) was sterilized by filtration through a Millipore membrane.

Immunization Procedures.—The mice were grouped in separate cages according to the schedule given in Table I. Two groups received a single injection of 0.5 mg ferritin (0.25 ml)

TABLE I

Immunization Procedures

Group	No. of mice	Animals	Route of ferritin administration	Immunization schedule	Interval between last stimulation and study
			Single parenteral stimulation		
1	5	1–5	Subcutaneous	Day 0: 0.5 mg ferritin + incomplete Freund's adjuvant	8–12 days
2	6	6–11	Intraperitoneal	Day 0: 0.5 mg ferritin + incomplete Freund's adjuvant	8–12 days
			Repeated parenteral stimulation		
3	4	12–15	Subcutaneous	Day 0: 0.5 mg ferritin + incomplete Freund's adjuvant Day 10: 1 mg ferritin without adjuvant Day 20: 1 mg ferritin without adjuvant	10 days
4	4	16–19	Intraperitoneal	Day 0: 0.5 mg ferritin + incomplete Freund's adjuvant Day 10: 1 mg ferritin without adjuvant Day 20: 1 mg ferritin without adjuvant	10 days
			Enteric stimulation		
5	4	20–23	Oral	1 mg ferritin/ml of drinking water for 30 days	12 hr
			Controls		
6	1	24	Subcutaneous	Day 0: incomplete Freund's adjuvant	10 days
7	1	25	Intraperitoneal	Day 0: incomplete Freund's adjuvant	10 days
8	2	26		No stimulation	

mixed with an equal volume of Freund's incomplete adjuvant (Difco Laboratories, Inc., Detroit, Mich.). In two other groups this stimulation was repeated twice, without adjuvant, at 10 day intervals. One group of four mice was given ferritin in the drinking water for 30 days. The last three groups served as controls (Table I).

Antisera.—Antisera directed against the heavy polypeptide chains of the four major immunoglobulin classes of the mouse, IgG_1, IgG_2, IgA, and IgM, were prepared in rabbits as described in a previous publication (14). The specificity of these reagents, after appropriate

absorptions, was found to be satisfactory, except for the anti-IgG_2 antiserum, which retained a weak capacity to react with light chains.

The antiserum against horse spleen ferritin was obtained from a rabbit immunized with purified antigen.

Immunohistochemical Methods.—

Fluorescent antisera: The IgG fractions of the antisera were isolated by precipitation with half-saturated ammonium sulfate followed by elution from DEAE-cellulose by means of a 0.01 M phosphate buffer, pH 7.5. The preparations were brought to pH 9.2 after concentration to a protein content of 10–20 mg/ml, and conjugated with fluorescein isothiocyanate at a ratio of 1 mg of fluorochrome/100 mg of protein. The fluorescent proteins were freed of unreacted fluorescein by passage through a Sephadex G-25 column.

Tissue specimens: After removal from the isolator, the animals were bled by cardiac puncture. Their spleens, mesenteric, axillary, and inguinal lymph nodes, as well as samples of the duodenum, ileum, and colon, were snap-frozen with Dry Ice and processed to 3–5 μ thick sections in a Bright-Pearse cryostat. In order to reduce sampling errors, sections were obtained from at least three different parts of each specimen.

Identification of cells containing antibodies against ferritin: Tissue sections were successively incubated with a 0.01% solution of ferritin and, after washing with buffered saline, with the fluoresceinated rabbit antiserum against ferritin. Control sections included one slide incubated with fluorescent rabbit antiferritin only, and one slide exposed first to ferritin and then, after washing, to fluorescent rabbit antiferritin absorbed with an excess of ferritin.

Allocation of antibody-containing cells to the different classes of mouse immunoglobulins: The technique of sequential immunofluorescent staining, originally proposed by Pernis (12), was applied as described in detail in a previous publication (13).

Briefly, microscopic fields showing a number of antiferritin-containing plasma cells were first photographed and then overexposed to a strong beam of ultraviolet light for 10–20 min, until all cytoplasmic fluorescence had faded away. A control photograph was then taken, using the same exposure time as used for the initial photograph. The cover slips were gently removed, and the sections, after washing with buffered saline, were incubated with fluorescent antiserum directed against one of the mouse immunoglobulin heavy chains. The microscopic fields already photographed were localized and photographed again. The sections were then incubated with an antiserum directed against another class of mouse immunoglobulins and photographed once more, and this procedure was repeated for the remaining two antisera against mouse heavy chains. For any given field, the comparison between the successive photographs made it possible to identify the classes of immunoglobulins to which belonged the antibody activity in any individual cell.

In order to facilitate the analysis of the pictures, the sequence of anti-Ig antisera was usually opened with the reagent which would contribute the smallest number of fluorescent cells to the field. For this reason, the following sequence was often chosen for lymph nodes and spleens: ferritin-antiferritin; control; anti-IgA; anti-IgM; anti-IgG_1; anti-IgG_2. For intestinal tissue this sequence was changed to: ferritin-antiferritin; control; anti-IgM; anti-IgG_1; anti-IgG_2; anti-IgA.

Cell counts: In some of the tissue specimens, several microscopic fields were photographed at a standard magnification (25 × objective), and counts of fluorescent cells were made on the enlarged prints. These counts included, for each field, the total number of antiferritin-containing cells, as well as their allocation to different immunoglobulin classes.

Quantitative Determination of Antibody Activity in the Four Classes of Serum Immunoglobulins.—The procedure employed for this purpose has been fully described elsewhere (15). Briefly, this technique is based on the differential determination of each immunoglobulin

class before and after removal of the specific antiferritin antibodies which it contains. This elimination of antiferritin—including nonprecipitating antibodies and antibodies of low affinity—is achieved by first combining all available antiferritin with an excess of antigen, followed by the precipitation of antigen-antibody complexes by means of an excess of rabbit antiserum against ferritin.

Quantitations of IgG_1, IgG_2, IgA, and IgM, before and after removal of the antiferritin antibody, were carried out by means of the method of single radial immunodiffusion, using a pool of normal C_3H sera as a standard.

RESULTS

Immunohistochemical Studies.—

Localization of plasma cells containing antiferritin antibodies: The plasma cells containing antibodies against ferritin stood out very clearly on the dark background, owing to their bright cytoplasmic fluorescence (Figs. 1–6, FRT). In

TABLE II

Identification in the Spleen of Cells Containing Antibodies Against Horse Spleen Ferritin and of Macrophages Containing the Antigen

Cells	Ferritin + rabbit antiferritin	Rabbit antiferritin	Rabbit antiferritin absorbed with ferritin	Rabbit antiferritin absorbed with mouse spleen
Antibody-containing	+*	−	−	−
Antigen-containing	+	+	−	+

* + = reaction; − = no reaction.

peripheral and mesenteric lymph nodes they were fairly evenly distributed in the spaces between the follicles. In the mucosa of the small intestine, most of the fluorescent cells were concentrated at the bases of the villi. There was no preferential accumulation of antiferritin-containing cells near the Peyer patches. The fluorescent cells tended to be somewhat more numerous in the colon than in the small intestine.

No fluorescent cells were found in sections of nodes or mucosa incubated with fluoresceinated rabbit antiferritin alone, or in the sections exposed to antigen and absorbed antibody.

The identification of antiferritin-producing cells in the spleen raised a special problem owing to the presence, in this tissue, of a great number of cells containing the antigen. The latter cells, presumably macrophages, were found mostly at the rims of the lymphoid follicles. They were readily distinguished from antibody-containing cells because their positive reaction to fluoresceinated rabbit antiferritin was also observed in slides which had not been incubated with the antigen. Appropriate controls (Table II) indicated that this reaction was specifically due to horse spleen ferritin. Genuine antiferritin-containing cells occurred scattered throughout the red pulp and, unlike the antigen-

containing macrophages, were not found in close contact with the lymphoid follicles.

Immunoglobulin classes of the antiferritin-containing plasma cells (Table III): In eight of the immunized animals the immunoglobulin classes of the antiferritin-containing plasma cells were determined by the method of sequential fluorescent staining. Some difficulty was encountered with the proper allocation

TABLE III

Immunoglobulin Classes of the Plasma Cells Containing Antibodies Against Ferritin

Group	Animals	Tissue	No. of fields studied	Total antiferritin cells	Average antiferritin cells per field	Immunoglobulin classes of antiferritin cells			
						IgG ($G_1 + G_2$)	IgA	IgM	Not clearly identified
2. Single intraperitoneal stimulation	11	Spleen	2	9	4.5			6	3
		Peripheral lymph nodes	1	3	3			3	
		Mesenteric lymph nodes	1	1	1			1	
		Intestine	1	2	2		2		
3. Repeated subcutaneous stimulation	13, 15	Spleen	1	19	19	6	6		7
		Mesenteric lymph nodes	3	64	21	28	15	1	20
		Intestine	2	4	2		4		
4. Repeated intraperitoneal stimulation	16, 18, 19	Spleen	2	64	32	28			36
		Peripheral lymph nodes	3	200	67	92	1	2	105
		Mesenteric lymph nodes	2	200	100	150	15	1	34
		Intestine	7	94	13.5		73		21
5. Enteric stimulation	20, 22	Mesenteric lymph nodes	3	5	2		4		1
		Intestine	6	66	11		58		8

of individual cells to the IgG_1 and IgG_2 classes, owing to the rather weak fluorescence produced by the corresponding antisera. For practical purposes, therefore, these classes were merged together into a single immunoglobulin class, called IgG in Table III. In addition, a number of cells could not be classified as to the type of immunoglobulin which they contained.

Immunoglobulin Levels and Antibody Activity in the Serum.—The results of this analysis are set out in Table IV. The concentrations of the different immunoglobulins are expressed as percentages of the corresponding values ob-

TABLE IV

Antiferritin Antibodies in Serum and Lymphoid Tissues

Animal	Serum								Antibody-containing cells in lymphoid tissues§			
	Concentrations*				Antibody activity‡				Spleen	Peripheral lymph nodes	Mesenteric lymph nodes	Intestine
	IgG_1	IgG_2	IgA	IgM	IgG_1	IgG_2	IgA	IgM				
						Group 1—Single subcutaneous stimulation						
1	4.5	4.5	4	123.5	–	–	–	–				
2	5.5	5.5	4	121	–	–	–	–				
3	18.5	14	9	160	–	–	–	–	–	–	–	–
4	4.5	8.5	8.5	172.5	–	–	–	–	+	+	–	–
5	4.5	6	7	153	–	–	–	–	+	+	+	–
						Group 2—Single intraperitoneal stimulation						
6	7.5	10.5	4	200	–	–	–	–	–	–	+	–
7	6.5	9.5	4	268	–	–	–	–	+	–	+	–
8	6.5	11.5	9	380	–	–	–	–	+	–	+	–
9	15.5	16.5	10	275	–	–	–	–	–	–	+	–
10	22	18	8.5	254	27.3	19.4	–	–	–	+	+	–
11	25	21	8.8	265	20	14.1	11.4	–	+ (+ in M)	+++ (++ in M)	+ (+ in M)	+ (+ in A)
						Group 3—Repeated subcutaneous stimulation						
12	29	19.5	9.3	169	44.8	33.3	–	–	+	+	–	–
13	54	29.5	9	160	33.3	21.7	–	–	++ (+ in G) (+ in A)	+++	+ (+ in G) (+ in A)	++ (++ in A)
14	16.5	7	9.2	182.5	25	–	–	11	+	+	–	–
15	62	36.5	9.2	210	24.2	13.7	–	–	+	+++	++ (++ in G) (+ in A)	+
						Group 4—Repeated intraperitoneal stimulation						
16	160	53	8.7	205	18.8	13.2	24.1	–	+++	+++	++++ (+++ in G) (+ in A)	+
17	34	16.5	7	200	58.8	27.3	–	3.8	++	++	++	++
18	93	38	7.8	191	18.3	31.6	–	–	+++	+++	+++	++ (++ in A)
19	58	27	8.2	260	17.2	11.1	–	20.2	+++ (++ in G)	++++ (+++ in G) (+ in M) (+ in A)	+++ (+++ in G) (++ in A)	++ (++ in A)
						Group 5—Enteric stimulation						
20	5.5	11.5	9.2	124	–	–	32.2	–	+	–	+ (+ in A)	++++ (++++ in A)
21	7.5	15	7.5	130	–	–		–	–	–	–	–
22	15.5	8	8.8	157	–	–	11.4	–	–	–	+ (+ in A)	+++ (+++ in A)
23	9.5	14	8.5	123	–	–		–	+	+	+	++
						Groups 6–8—Nonimmunized animals						
24	<2	7	<2	95	–	–	–	–	–	–	–	–
25	<2	11	<2	130	–	–	–	–	–	–	–	–
26	7.5	22	<2	120	–	–	–	–	–	–	–	–

* Percentages of values found in a pool of serum from conventional adult C3H mice.

‡ Percentages of the immunoglobulin found to combine with ferritin.

§ – = no antibody activity or antibody-containing cells detected; + = 1–5 cells/tissue section; ++ – 5–20 cells/tissue section; +++ = 20–100 cells/tissue section; ++++ = more than 100 cells/tissue section.

served in a pool of sera from conventional adult C_3H mice. Values indicated for antibody activities represent the percentage of total immunoglobulin in each class which was found to combine with ferritin.

Types of Antibody Response in the Different Groups (Tables III and IV).—

Group 1—Single subcutaneous stimulation: In these animals no antibody activity was detected in the serum, and the immunoglobulin levels were only slightly higher than those of nonimmunized, germfree mice.

Of the three mice studied, two (Nos. 4 and 5) showed a small number of antibody-containing cells (less than 10/section) in their lymph nodes and spleens, whereas no response was observed in the intestinal mucosa. The third animal (No. 3) apparently failed to respond.

Group 2—Single intraperitoneal stimulation: In two mice (Nos. 10 and 11) weak antibody activity due to IgG_1 and IgG_2 was detected in the serum. This was reflected by a rise of the IgG_1 and IgG_2 concentrations. In all animals of this group the IgM concentrations in the serum were also found to be moderately increased.

All animals had developed a small number of antibody-containing plasma cells, most of which were located in the mesenteric lymph nodes (Fig. 1, FRT). In the single mouse (No. 11) which showed a fairly good response, occasional antiferritin-containing cells were also present in the intestine (Fig. 2, FRT). In the last-mentioned tissue, such cells were all of the IgA class (Fig. 2, arrows in b_3), whereas the few plasma cells containing antiferritin antibodies in the spleen and lymph nodes from the same animal (No. 11) were of the IgM type (Fig. 1, FRT; M; arrow in c_2). The presence of numerous IgM cells devoid of antibody activity against ferritin (Fig. 1, M, in a_1, a_4, b_2, and c_4) could account for the high level of IgM in the serum and for the failure to detect any antibody activity in this immunoglobulin class in spite of the histological evidence. None of the antiferritin cells could be clearly allocated to the IgG classes.

Group 3—Repeated subcutaneous stimulation: In the sera from all mice of this group, antibodies against ferritin were present mainly in the IgG_1 and IgG_2 classes. Moderate increases in the concentrations of these immunoglobulins were noted in two animals (Nos. 13 and 15).

Antibody-containing cells were found in the four mice and were relatively abundant in the peripheral lymph nodes (mainly the axillary nodes), less so in the spleen and mesenteric nodes. Intestinal plasma cells containing antiferritin were found in the two animals which showed the highest antibody response. These mice (Nos. 13 and 15) were investigated in detail. In the spleen and lymph nodes, slightly less than half of the plasma cells containing antibodies against ferritin belonged to the IgG classes, whereas one-fourth were of the IgA class and virtually none of the IgM class. In contrast, all the antibody-containing cells in the intestine were of the IgA class.

Group 4—Repeated intraperitoneal stimulation: This was the group showing

the highest antibody response. In the serum, the antiferritin activity was due to IgG_1 and IgG_2 in all four animals and also, less constantly, to IgA (No. 16) or IgM (Nos. 17 and 19). Increases in the levels of both IgG_1 and IgG_2 were noted in all animals of this group.

Dense populations of antiferritin-containing plasma cells were found in the spleen as well as in the mesenteric and peripheral lymph nodes (Fig. 3, FRT). Moderate numbers of such cells were also present in the intestinal mucosa in all animals (Fig. 4, FRT). In the three mice (Nos. 16, 18, and 19) investigated, the majority of the antiferritin cells in the lymph nodes and spleen were of the IgG type. In these sites less than 5% of the plasma cells containing antibody against ferritin were of the IgA class, and such cells were relatively more numerous in the mesenteric lymph nodes than in the spleen or peripheral lymph nodes. The IgM class was hardly represented at all. Among the wealth of antiferritin-containing cells (Fig. 3, FRT) in a section from an axillary lymph node, none was of the IgA type (Fig. 3, A) and only one could be allocated to the IgM class (Fig. 3, AM; arrow in b_2). The overwhelming majority of the antibody-containing cells obviously belonged to the IgG_1 class (Fig. 3, AMG_1), and only few could be classified as IgG_2 (Fig. 3, AMG_1G_2 ; arrow in a_3). Several IgM cells (Fig. 3, AM) were found not to react with ferritin. In the intestinal mucosa, all the plasma cells containing antiferritin antibodies were of the IgA class (Fig. 4). There were also many other IgA cells which did not react with ferritin (Fig. 4, MG_1G_2A, in a_1 , b_1 , d_3 , and d_4). The single plasma cell of the field shown in Fig. 4, M (b_1), which contained IgM, failed to react with ferritin.

Group 5—Enteric stimulation: In the serum, antibody activity was found in two mice (Nos. 20 and 22) and was present exclusively in the IgA class. In the four animals of this group, the immunoglobulin levels were only sightly higher than those of nonimmunized, germfree mice.

In three mice, antibody-containing cells were present in the small and large intestine (Fig. 5, FRT) and were particularly numerous in the two animals (Nos. 20 and 22) in which serum antibodies against ferritin had been detected. In the same two mice, altogether only five antiferritin plasma cells were observed in six sections of the mesenteric lymph nodes. Four of these cells proved to be of the IgA class, whereas none could be typed as either IgG_1 or IgG_2 (Fig. 6). All the antiferritin cells in the intestine were of the IgA type (Fig. 5).

Groups 6–8—Nonimmunized animals: The immunoglobulin levels of these mice were in the range usually found in germfree mice. No antibody against ferritin could be detected in either the serum or the lymphoid tissues.

DISCUSSION

Immunoglobulin Levels in the Serum.—In the three animals which had not been immunized with ferritin, the immunoglobulin concentrations in the serum were within the range typical for germfree mice of the stock employed in these

studies.[1] These levels were increased in all animals that received ferritin, but in many cases the major part of the increment was not represented by specific antibody (Table IV). Similar findings have been made by Asofsky et al. (16) with regard to antibody and "nonantibody" IgM in germfree mice immunized with ferritin. Barth et al. (17), however, who used conventional adult mice, reported the absence of any significant rise in the immunoglobulin levels after a single intraperitoneal injection of ferritin or hemocyanin. In the present study, as in that of Asofsky et al. (16), the demonstration of small increases in immunoglobulin concentrations after immunization was presumably facilitated by the use of germfree animals having very low concentrations of immunoglobulins in their serum.

Response of Extraintestinal Lymphoid Tissues to Parenteral Immunization.— In general, the quantitative and qualitative data on the serum antibody response were in good agreement with the immunohistochemical findings. The latter seemed to be a more sensitive index of antibody formation than the former, as was particularly evident in animals 4–9, which received only minimal stimulation.

Single parenteral stimulation: Although a comparison between our data and those from the literature must be made with due regard to differences in experimental conditions, which are known to influence the antibody response (18), several of the findings reported here are in line with previous observations. The data of Sell (19) indicate that germfree mice, after a single intraperitoneal injection of bovine γ-globulin, respond slowly and progressively within the first month. It may be, therefore, that the apparent absence of circulating antibodies in animals 1–9, after a single parenteral stimulation, was due to the short interval between the injection and the study.

As could be expected from a primary response, the majority of the antiferritin-containing plasma cells, in lymph nodes and spleen, were of the IgM type. The failure to detect antibodies of this class in the serum was not surprising, because the cells which produced them were quite scarce and represented only a minor proportion of the total population of IgM-synthesizing plasma cells. A serum antibody response of the IgM type has, however, been observed by Adler (20), Bazin (21), and Asofsky et al. (16) in mice that received a single injection of ovalbumin or ferritin.

In two mice (Nos. 10 and 11) antibody in the serum was found mainly in IgG_1 and IgG_2, in agreement with reports from other investigators (18, 20, 21). Still, in these two animals, none of the antiferritin-containing cells from the spleen and lymph nodes appeared to belong to any of the IgG classes. It may be, of course, that antiferritin IgG cells were confined to unexplored sites of the

[1] Unpublished observations.

lymphoid system, such as the bone marrow or the peritoneal granulomas caused by the adjuvant (22).

Repeated parenteral stimulation: In these groups, vast numbers of antibody-containing cells were found in the lymph nodes adjacent to the sites of injection, i.e. the mesenteric nodes after intraperitoneal immunization and the axillary nodes after subcutaneous injection.

In both the plasma cells and the serum, the antibodies against ferritin were confined mainly to IgG_1 and IgG_2 (especially the former), although the two other classes had some share in the response. The predominance of IgG_1 and IgG_2 antibodies in the serum of mice repeatedly immunized with a variety of soluble antigens has also been reported by Merryman and Benacerraf (23), as well as by Bazin (21).

Role of the Intestinal Mucosa in the Antibody Response.—The main purpose of the present investigation was to obtain direct proof of the synthesis of specific antibody by intestinal plasma cells following antigenic stimulation by the oral route. In addition, it was felt desirable to evaluate the participation of the same tissue in the response to parenteral immunization.

It is well known that the intestinal mucosa is a very active lymphoid tissue and that it is a preferential site of synthesis of IgA (10, 11, 24–26). Much indirect evidence suggests that these mucosal IgA-producing plasma cells are part of a local defense mechanism of the body. Their occurrence in the lamina propria of the gut is dependent upon the presence of antigenic material in the intestinal lumen. They are extremely reduced in numbers as long as the gut remains sterile, as in the fetus (27) and germfree adult (25). After birth, or upon exposure of a germfree adult mouse to a normal septic environment, the intestinal mucosa becomes populated by IgA-producing plasma cells in a matter of a few weeks.[2] Similarly, IgA, which is known to be the predominant immunoglobulin in normal and pathological (28, 29) human intestinal secretions, as well as in washings of the intestinal contents of normal dogs (30) and mice, is absent from intestinal secretions of germfree mice (31). Although direct evidence for a local origin of intestinal secretory IgA is lacking, there are several indirect arguments in favor of such a view (27, 31). "Coproantibodies," i.e., antibodies found in intestinal secretions and directed against antigenic material from the gut, have repeatedly been demonstrated to be of the IgA class (5–7, 32).

Antibodies present in intestinal secretions have generally been considered to originate in the intestinal mucosa rather than in the blood, mainly on the basis of the lack of chronological relationship between peak antibody titers in serum and feces (1–3, 8, 33, 34) and because of the higher fecal antibody titers observed after oral as compared to parenteral vaccination (4, 35–37). Also, it has been reported by Felsenfeld et al. (5) that the toxin-neutralizing capacity of

[2] Manuscript in preparation.

IgA isolated from the serum, saliva, and gastric fluid of vervet monkeys with experimental cholera was distinctly lower than that of IgA from jejunal contents.

To our knowledge, however, we are still short of direct arguments pointing to the mucosal plasma cells of the gut as sites of synthesis of coproantibodies. Recent investigations on the immunological activity of the gut have been concerned less with the plasma cells of the lamina propria than with adjacent lymphoid structures such as Peyer's patches and mesenteric lymph nodes. Cooper et al. (38) inoculated *Salmonella* and *Shigella* antigens into intestinal blind loops of rats, and found that plaque-forming cells appeared first in the spleen and subsequently in Peyer's patches. This was interpreted as evidence against a significant share of the gut in the immunological response against antigens of enteric origin. On the other hand, Felsenfeld et al. (5) have shown that in vervet monkeys orally immunized with *Vibrio cholerae*, antibody-producing cells were more numerous in the mesenteric lymph nodes than in the spleen. Using the method of passive cutaneous anaphylaxis, Levanon et al. (39) obtained evidence that suspensions of intestinal cells from guinea pigs orally immunized with heat-killed *Salmonella typhimurium* were able to sensitize nonimmunized animals to the antigen.

The results presented here indicate that in germfree mice immunized with ferritin the intestinal plasma cells participated in the synthesis of specific antibody, whatever the route of administration of antigen. After parenteral stimulation, the share of the gut in the antibody response was modest in comparison to that of extraintestinal lymphoid tissues. After oral immunization, however, the intestine became the major site of antibody production. It is of interest that the antiferritin antibodies found in the intestinal plasma cells were always exclusively of the IgA class, irrespective of the route and frequency of administration of the antigen, as well as of the immunoglobulin classes of antibodies synthesized elsewhere in the body. This observation may be paralleled to the finding that the plasma cells acquired by the gut of the germfree mouse, upon exposure to a septic environment, almost exclusively belong to the IgA class.[2] These findings are somewhat at variance with those of Crandall et al. (24), who reported an increase in numbers of intestinal plasma cells of the IgM type after experimental infection of rabbits with *Trichinella spiralis*. It must be noted, however, that in rabbits the predominance of IgA-type plasma cells in the gut is less pronounced than in the mouse.

The selective commitment of the intestinal mucosa to the production of antibodies of the IgA type requires some comment. It is possible that the plasma cells involved in this response are descended from a population of mucosal lymphoid cells predestined to synthesize IgA, rather than other types of immunoglobulins, upon antigenic stimulation. Alternatively, the mucosal lymphoid cells would not possess any such predifferentiation, but antigens would be

processed by the intestinal mucosa in such a way as to become selectively effective in eliciting the formation of antibodies of the IgA type. The local antigen-processing cells postulated by the latter hypothesis might be either the macrophages from the lamina propria or the epithelial cells. One would, of course, have to assume that antigens brought in from the circulation undergo the same treatment as that applied to antigens resorbed from the intestinal lumen, since in both cases the antibody response is of the IgA type (Table III).

It may be relevant to mention the intimate relationships which have been described between the epithelium of the gut and small lymphocytes, in this connection called "theliolymphocytes" (40, 41). The role of the epithelium as a foster tissue for theliolymphocytes, suggested by Fichtelius et al. (40, 41), might be conceived to extend to the predifferentiation of these lymphoid cells toward the production of IgA-type antibodies, according to the first hypothesis outlined above. Alternatively, if a special mechanism of antigen processing were to be retained, the epithelium might be cast in the role of the antigen-processing tissue, and its physical contact with theliolymphocytes viewed as reflecting the transfer of antigenic material from the former to the latter.

In the experiments described here, the antibody response to orally administered ferritin, though predominantly localized in the intestine, was found also to involve extraintestinal lymphoid tissues, particularly the mesenteric lymph nodes. This was not unexpected, since it is known that soluble antigens from the intestinal lumen may gain access to the circulation (42). Such an explanation does not, however, account for the striking finding that after oral immunization virtually all the antiferritin-containing cells from extraintestinal sites belonged to the IgA class, whereas they were primarily of the IgG_1 and IgG_2 types when the same antigen was given parenterally. If, as suggested in the second hypothesis outlined above, the gut has the ability to process antigen in a special way, one might conceive that antigens modified in such manner, after entering the circulation, would induce the same selective IgA response in extraintestinal sites as is observed in the intestine. In this event, all circulating antibody to soluble antigens administered by the oral route ought to be of the IgA class. This was so in the experiments reported here (Table IV, group 5). However, in their studies on circulating antimilk antibodies in infants, Coombs et al. (43) noted that antibodies of the IgG type nearly always predominated over those of the IgA class. Similarly, in rabbits immunized with bovine serum albumin, Rothberg et al. (44) found no difference in antibody class distribution whether the antigen was given orally or parenterally; in both cases, IgA-type antibodies were demonstrated only in those antisera which had the highest antibody titers.

The alternative view would be that the IgA-type antiferritin cells found in extraintestinal sites actually originated in the gut. Having received their antigenic stimulation in the intestinal mucosa, they would, after emigration to the

lymph nodes and spleen, synthesize the same IgA types of antibodies they would have produced if they had never left the lamina propria. The particular abundance of antiferritin IgA cells of the mesenteric lymph nodes, as compared to the other lymph nodes, after oral immunization, is equally compatible with both views, since the mesenteric nodes are the first relay on the efferent lymph route by which antigens as well as lymphoid cells from the gut enter the blood. It is possible that those cells from the thoracic duct lymph of the mouse, which were shown by Mandel and Asofsky (26) to synthesize IgA in vitro, were emigrants from the intestinal mucosa, on their way to the blood.

SUMMARY

In adult germfree C_3H mice immunized with horse spleen ferritin, either subcutaneously or intraperitoneally, plasma cells containing specific antibodies were found in lymph nodes and spleen and, in smaller numbers, also in the lamina propria of the intestine. In extraintestinal sites, these antiferritin-containing plasma cells were mainly of the IgM class after a single stimulation, and of the IgG_1 class after repeated stimulation. In the intestine, all the antiferritin-containing cells appeared to be of the IgA class. Circulating antibodies, after repeated stimulation, were for the major part IgG_1 and IgG_2.

In germfree mice given ferritin in their drinking water, antiferritin-containing cells were abundant in the intestinal mucosa, much less numerous in the mesenteric lymph nodes, and extremely scarce in other lymphoid tissues. All these cells, whatever their location, appeared to belong exclusively to the IgA class. Similarly, all the circulating antibody in these animals was found to be IgA.

These findings illustrate the role of the gut as a site of antibody synthesis, as well as its selective commitment to the production of antibodies of the IgA class.

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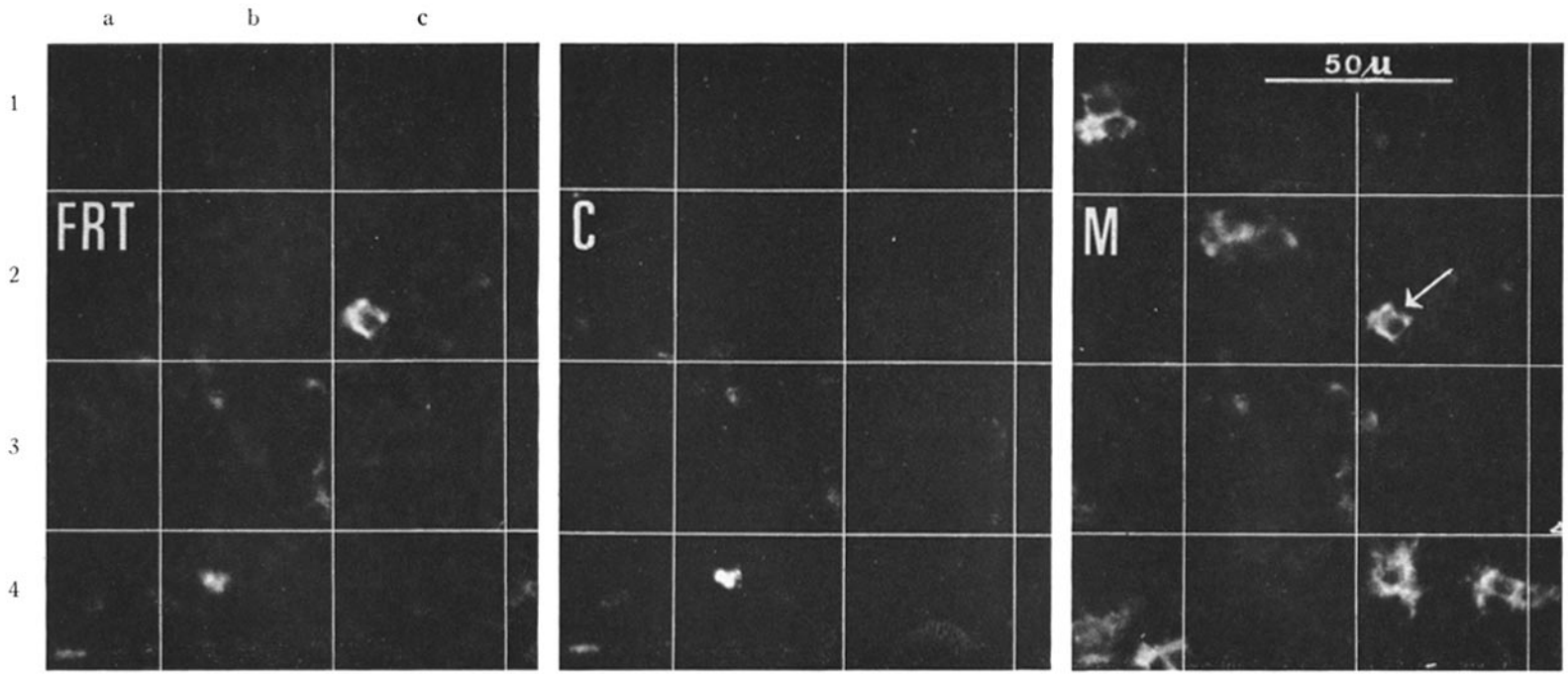

FIG. 1. Section of a mesenteric lymph node from a mouse given a single intraperitoneal stimulation with ferritin. The tissue was exposed to the reagents in the following sequence: ferritin-antiferritin (FRT); control photograph after irradiation with ultra-violet light (C); anti-IgM (M). The single antiferritin plasma cell (FRT, in c_2) belonged to the IgM class (M; arrow in c_2), whereas the majority of the plasma cells containing this immunoglobulin (M, in a_1, a_4, b_2, and c_4) failed to react with ferritin.

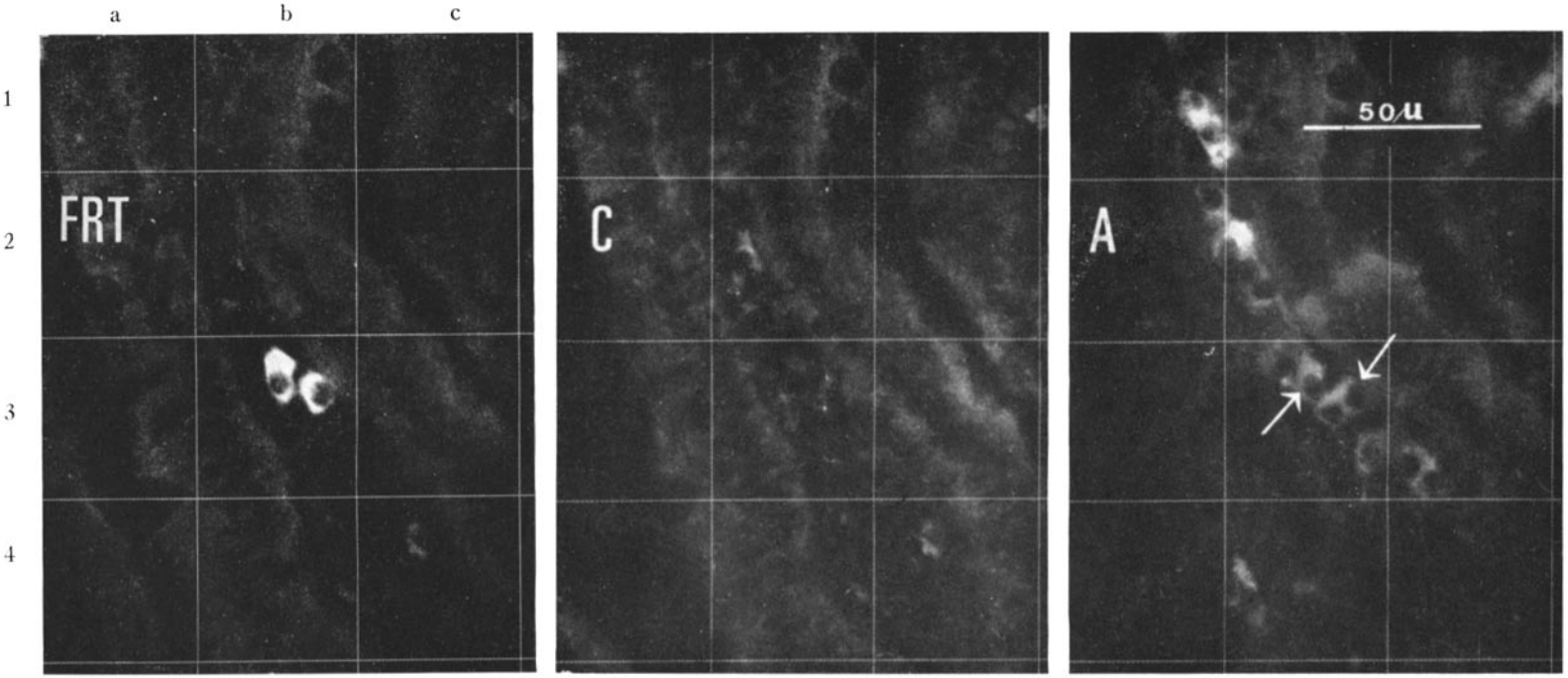

FIG. 2. This axial section through an intestinal villus from a mouse given a single intraperitoneal injection of ferritin was studied in the following sequence: ferritin-antiferritin (FRT); extinction of the fluorescence upon irradiation (C); anti-IgA (A). The two plasma cells containing antibodies against ferritin (FRT, in b_3) obviously belonged to the IgA class (A; arrows in b_3).

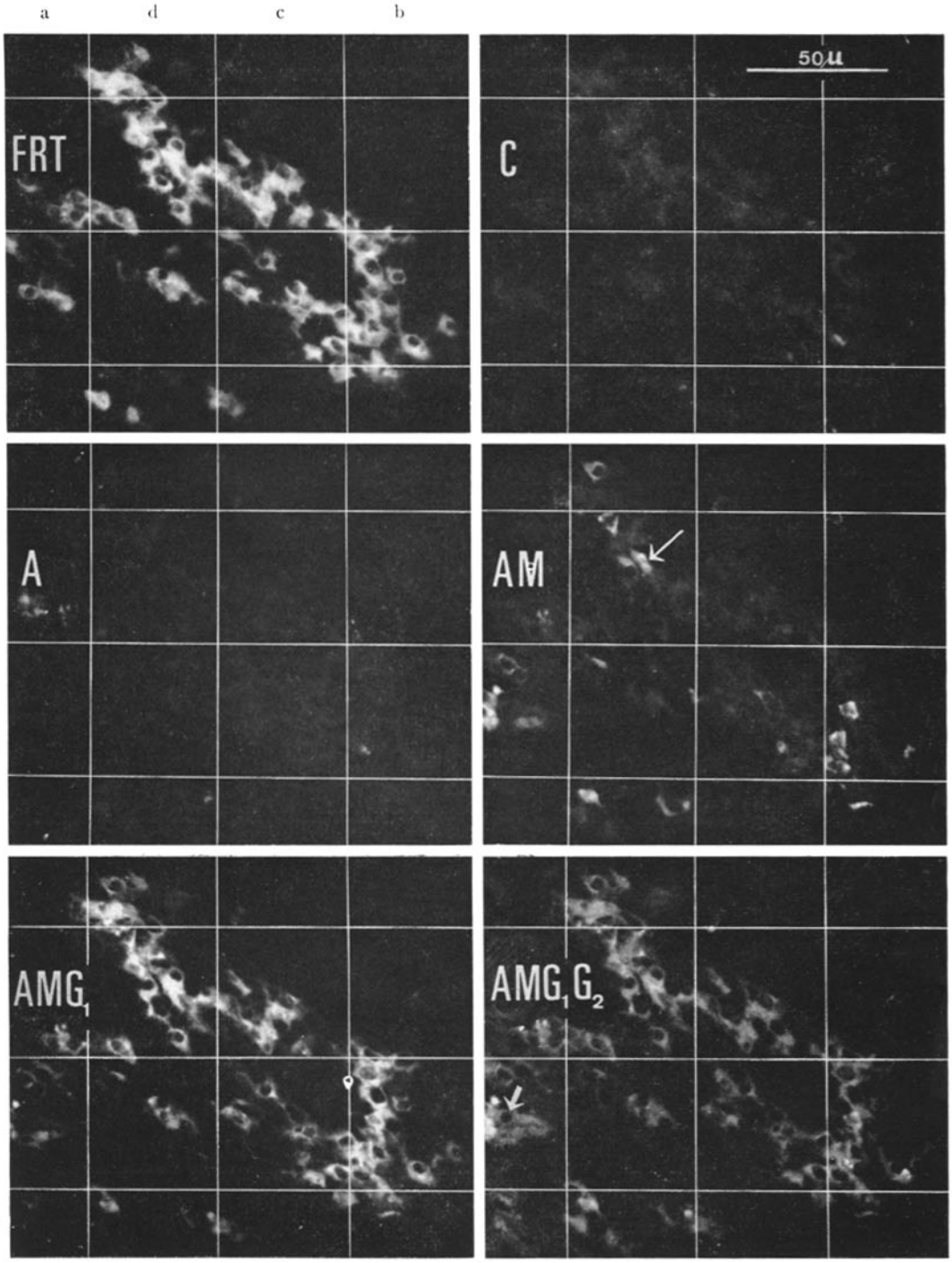

FIG. 3. Section of an axillary lymph node from a mouse repeatedly immunized by the intraperitoneal route. Sequence: ferritin-antiferritin (FRT); exposure to ultraviolet light (C); anti-IgA (A); anti-IgM (AM); anti-IgG_1 (AMG_1); anti-IgG_2 (AMG_1G_2). None of the numerous plasma cells containing antiferritin antibodies (FRT) reacted with the antiserum against IgA (A). Only one could be classified in the IgM class (AM; arrow in b_2); the vast majority belonged to the IgG_1 class (AMG_1), and one was found to be an IgG_2 plasma cell (AMG_1G_2; arrow in a_3).

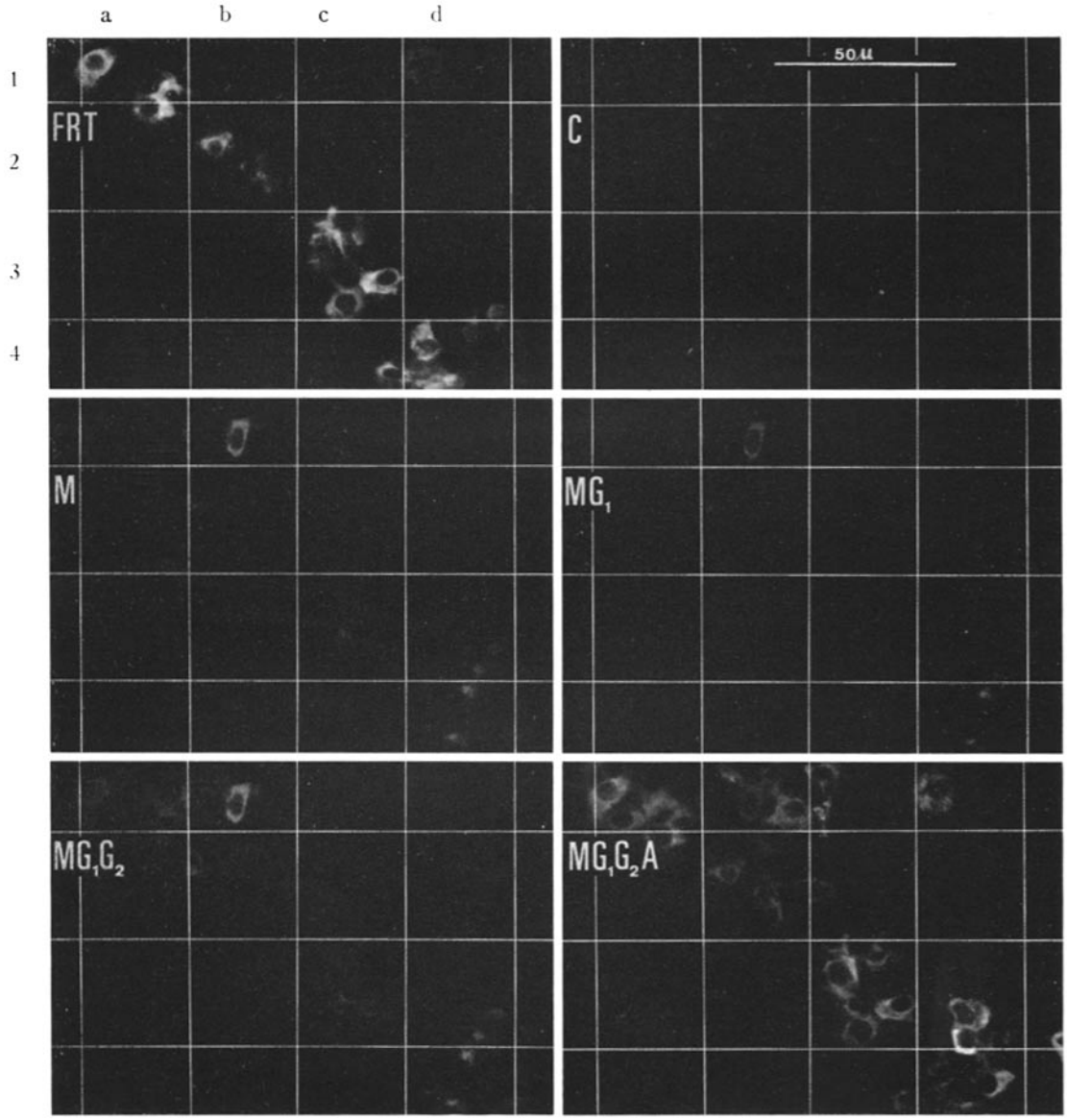

FIG. 4. Lamina propria of the duodenum from a mouse repeatedly immunized with ferritin by the intraperitoneal route. Sequence: ferritin-antiferritin (FRT); control after exposure to ultraviolet light (C); anti-IgM (M); anti-IgG_1 (MG_1); anti-IgG_2 (MG_1G_2); anti-IgA (MG_1G_2A). The single IgM plasma cell (M, in b_1) present in the preparation did not react with ferritin. The last incubation with the anti-IgA antiserum revealed all the antiferritin plasma cells present in the field, as well as a few other IgA cells (MG_1G_2A, in a_1, b_1, d_3, and d_4) of unknown specificities.

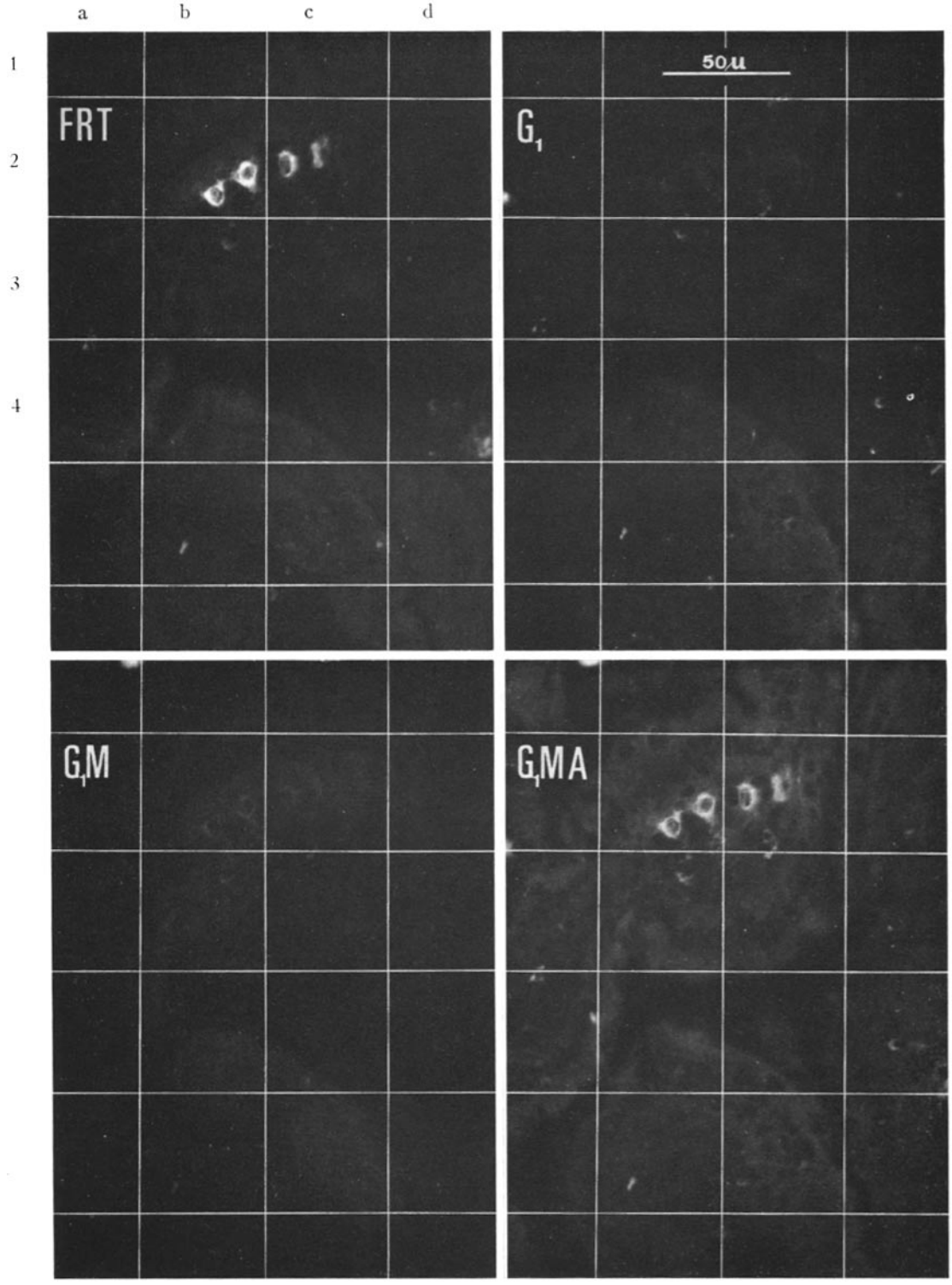

FIG. 5. Duodenum from a mouse given ferritin by the oral route. Sequence: ferritin-antiferritin (FRT); irradiation (not shown); anti-IgG$_1$ (G$_1$); anti-IgM (G$_1$M); anti-IgA (G$_1$MA). Only this last incubation revealed the four antiferritin plasma cells (FRT and G$_1$MA, in b_2 and c_2) present in the field.

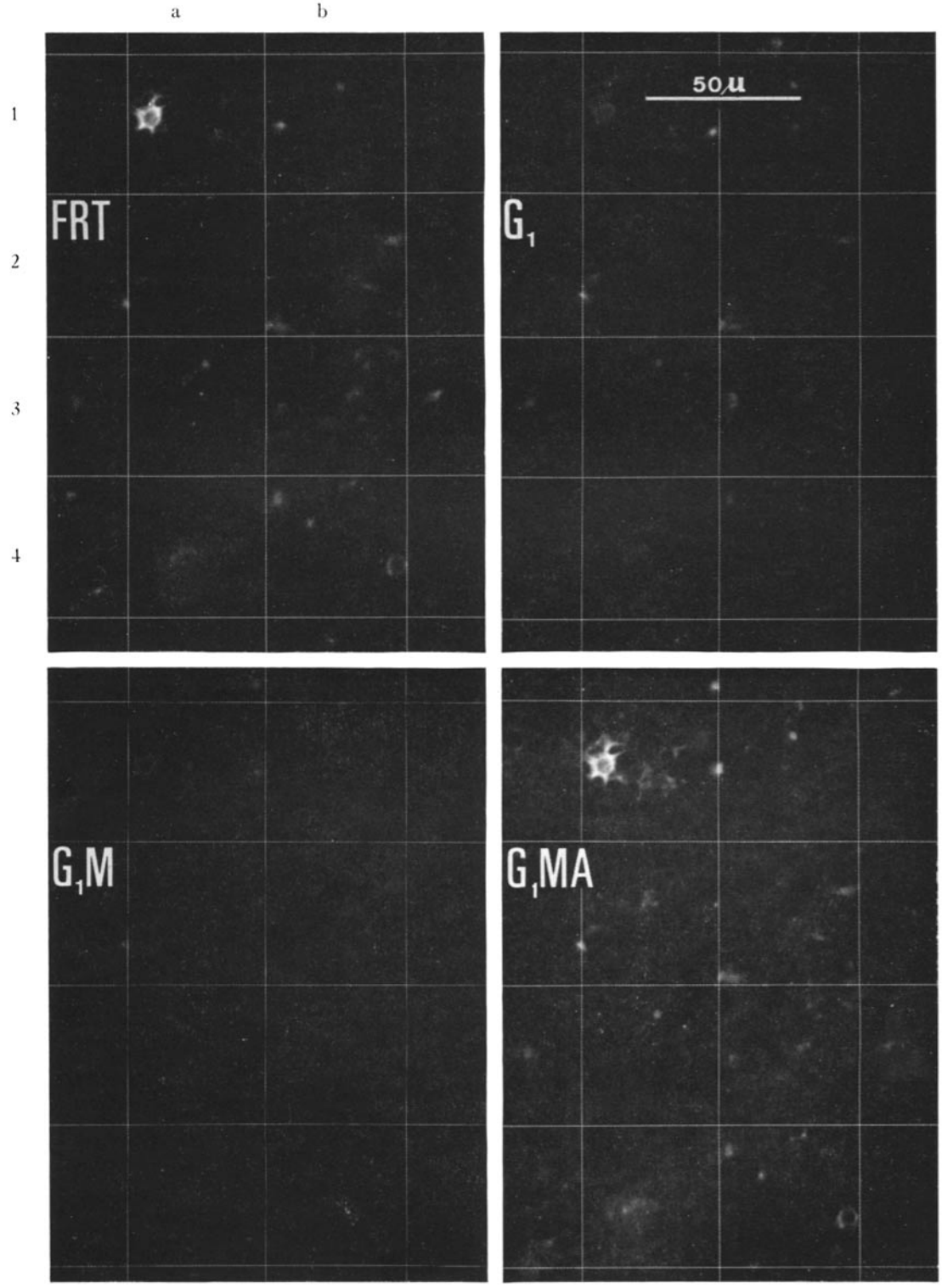

FIG. 6. Section through a mesenteric lymph node from a mouse given ferritin by the oral route. Sequence: ferritin-antiferritin (FRT); irradiation (not shown); anti-IgG$_1$ (G$_1$); anti-IgM (G$_1$M); anti-IgA (G$_1$MA). The single plasma cell containing anti-ferritin antibody (FRT, in a$_1$) failed to react with anti-IgG$_1$ and anti-IgM (G$_1$ and G$_1$M, in a$_1$), but was revealed by the antiserum against IgA (G$_1$MA, in a$_1$).