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Local Immune Response to Repeated Topical Antigen Application in the Simian Labial Mucosa

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Minor salivary glands of the oral mucosa in healthy monkeys (*Macaca fascicularis* and *Macaca mulatta*) contain organized structural units suitable for recognizing and processing antigens. A previous study of *M. fascicularis* monkeys provided experimental evidence of retrograde access of oral antigens deep into the minor salivary glands. The present study aimed at exploring the possible immune response of simian oral mucosa to repeated topical application of a chemically defined antigenic solution at the labial and gut mucosa. Ten female *M. fascicularis* animals were challenged topically at the lower lip mucosa at weekly intervals for a variable period of 4 to 8 weeks with a solution consisting of horseradish peroxidase, ferritin, and special India ink. Transmission electron microscopic examination of immunohistochemically treated sections of the labial glands revealed the presence of plasma cells containing specific anti-horseradish peroxidase antibody. These cells resided in the interacinar regions. Enteric and gut priming with the same antigen in four other monkeys, bypassing the oral mucosa, failed to reveal the presence of horseradish peroxidase-positive plasma cells in the labial mucosa of any of the four animals, although in one animal such cells could be identified in a mesenteric lymph node. This is suggestive of the existence, at least in primates, of a local immune response of the oral mucosa independent of systemic involvement.

Based on the concept of secretory immunoglobulin A (s-IgA)-mediated local immunity and on evidence currently available, Mestecky et al. (47) proposed the existence of two pathways of stimulation for a secretory humoral immune response: (i) an explicitly local and topical antigen application or (ii) induction through sensitization of gut- or bronchial-associated lymphoid tissues. With respect to the oral mucosa, it has been postulated that secretory ducts of the minor salivary glands (MSG) "may well be continuously stimulated by natural retrograde flow of oral antigen" (23). This view was also expressed by Brandtzaeg (15) and Crawford et al. (22). This postulate originally followed from physiological data showing that MSG secretions contain a much higher concentration of s-IgA than does parotid or mixed saliva, and it has also been supported by both earlier and recent structural findings. For example, numerous authors have noted the existence of organized lymphoid tissue with germinal centers associated with MSG secretory ducts at various oral mucosal sites in nonhuman primates and in humans (2, 6, 17, 25, 34, 35, 42, 44, 49, 51, 59, 60, 61, 63). In fact, stereological data have suggested that in the simian oral mucosa MSG-related, organized lymphoid tissue occupies a volume of 1 to 5% (35, 60).

Recently, by means of tissue reconstruction methods, MSG duct-related, organized lymphoid tissue, termed "duct/follicle assemblies," was described three dimensionally (61). That study demonstrated that the germinal center of such assemblies faces or embraces particular blind, pouchlike duct formations branching out from a pelvis-like basin where small interlobular ducts fuse. The wall of these blind pouches appears to serve for small lymphocytes and blast-forming T-cells contacting intraductal material such as bacteria (61). Indeed, these organized structures resembling a tonsillar microcrypt can be reached by retrograde passage through secretory ducts by any oral antigen (53).

Based on these experimental findings, we have asked whether oral antigens, being repeatedly presented topically, would not only gain retrograde access to but also trigger a local immune response of the local organized lymphoid tissues, independent of systemic involvement. The present study is an attempt to answer this question.

TABLE 1. Animals (all females) and specimen blocks used for transmission electron microscopy[a]

Animal	Species	Wt (kg)	Challenge site	Tissue	No. of blocks[b] incubated with:	
					HRP	B
1	*M. fascicularis*	2.2	OM	LL	9 (+)	13 (−)
2	*M. fascicularis*	1.8	OM	LL	20 (−)	NA
3	*M. fascicularis*	3.3	OM	LL	11 (+?)	10 (−)
4	*M. fascicularis*	2.5	OM	LL	17 (−)	NA
5	*M. fascicularis*	2.8	OM	LL	19 (−)	NA
6	*M. fascicularis*	3.8	OM	LL	25 (−)	NA
7	*M. fascicularis*	2.8	OM	LL	55 (+)	18 (−)
8	*M. fascicularis*	3.2	OM	LL	40 (−)	NA
9	*M. fascicularis*	3.4	OM	LL	29 (−)	NA
10	*M. fascicularis*	3.8	OM	LL	43 (+?)	12 (−)
11	*M. mulatta*	6.7	GM	LL	13 (−)	NA
				MLN	11 (−)	NA
12	*M. mulatta*	10.3	GM	LL	27 (−)	NA
				MLN	—	—
13	*M. fascicularis*	3.4	GM	LL	17 (−)	NA
				MLN	4 (+)	5 (−)
14	*M. fascicularis*	3.4	GM	LL	15 (−)	NA
				MLN	7 (−)	NA

[a] Abbreviations: B, buffer; LL, lower lip; OM, oral mucosa; GM, gut mucosa; NA, not applicable; —, not obtained.

[b] +, Plasma cells positive for anti-HRP antibodies in one or more Epon blocks; −, plasma cells negative for anti-HRP antibodies in all Epon blocks; +?, plasma cells moderately positive for anti-HRP antibodies in one or more Epon blocks.

MATERIALS AND METHODS

Animals. A total of 14 female monkeys (12 *Macaca fascicularis* and 2 *Macaca mulatta*) of various ages were utilized for this study. The *M. fascicularis* animals weighed 3.0 ± 0.6 kg (range, 1.8 to 3.8 kg), whereas the *M. mulatta* animals weighed 6.7 and 10.3 kg, respectively (Table 1).

Antigenic tracer. The antigenic tracer consisted of a mixture of horseradish peroxidase (HRP) (type VI; molecular weight, ca. 40,000; Sigma Chemical Co.), ferritin (Fluka; molecular weight, 650,000), and special India ink (Pelikan, C 11/1431a; Günther Wagner). It was freshly prepared by dissolving 10,000 U of HRP in 2.5 ml of aqueous ferritin solution (Fe, 10%) and extending the volume to 5 ml with the India ink (53).

Oral mucosa challenge. At weekly intervals, the antigenic tracer was applied topically on the lower lip mucosa of 10 *M. fascicularis* animals for a period of 4 to 8 weeks. Care was taken to prevent the tracer from spreading to posterior regions of the mouth by means of cotton rolls inserted into the lateral vestibular folds and the cheek pouches. These methods have been used previously to achieve retrograde antigen passage (53).

Gut challenge. Four female monkeys (two *M. fascicularis* and two *M. mulatta*) were challenged at weekly intervals for a period of 4 to 8 weeks. The animals were restrained under general anaesthesia (5% pentobarbital sodium solution intraperitoneally) and held in lateral recumbency. A rubber stomach catheter (Rüsch, size 12) was gently passed through the mouth and pharynx to the stomach. The location of the catheter was controlled by aspirating a small quantity of the stomach content into a syringe attached to the outer end of the catheter. A 5-ml portion of the antigenic material was administered, bypassing the oral cavity. Subsequently, 5 ml of distilled water was syringed into the catheter to flush the remaining material into the stomach. Afterwards, the catheter was removed, taking care to avoid having its tip come into contact with the oral mucosa.

Tissue processing. After the appropriate number of antigenic challenges, the animals were perfused through the heart with modified grades of Karnovsky fixative (33), i.e., (i) 200 ml of 6% Macrodex (Pharmacia Fine Chemicals; 270 mOsm) to wash out the blood, (ii) 1,000 ml of 0.4% *para*-formaldehyde–1.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4, 430 mOsm), and (iii) 1,000 ml of 0.5% *para*-formaldehyde–2.5% glutaraldehyde in the same phosphate buffer (pH 7.4, 580 mOsm) (52). Thereafter, the lips and a few randomly selected mesenteric lymph nodes (MLN) were carefully removed, stored for 3 days in 5% glutaraldehyde buffered in 0.02 M sodium cacodylate (pH 7.4, 520 mOsm), and washed for several hours in 0.185 M sodium cacodylate buffer (pH 7.4, 360 mOsm).

The lower lips were divided sagittally into 3- to 4-mm-thick blocks with a sharp razor blade. The skin, subcutaneous tissue, and part of the orbicularis oris muscle were removed. Subsequently, two types of sections were prepared from both lip blocks and MLN: (i) tissue slices about 0.5 mm thick prepared by hand with the aid of a dissection microscope and a razor blade and (ii) cryosections about 50 μm thick prepared with a microtome cryostate (Minatome; International Equipment Co., Div. of Damon Corp.) with Tissue-Tek II O.C.T. Compound (Lab-Tek Products, Div. Miles Laboratories Inc.) to bind the tissue

to the specimen holder. The cryosections were suspended individually in 0.185 M sodium cacodylate buffer and screened through a dissection microscope to select samples suitable for further processing, with the sample sections being distributed over the whole right-to-left range of the lower lip.

Immunoperoxidase reaction. In a series of preliminary trials to develop an appropriate methodology, attempts were made to reduce the nonspecific endogenous peroxidase activity (65–67). The results were not encouraging, and the procedures used enhanced the ultrastructural damage to the tissue. Therefore, all attempts to suppress the nonspecific peroxidase activity were discontinued.

The immunocytochemical reactions were carried out as described by Avrameas and Leduc (4). The procedures used can be summarized as follows: (i) incubation of the tissue sections with homologous antigen, i.e., 1 mg of HRP in 1 ml of 0.185 M sodium cacodylate buffer, for 1 h at room temperature (this step allows the HRP to combine with the specific homologous antibody in the tissue); (ii) washing twice (10 min each) in sodium cacodylate buffer to remove the unbound antigen; (iii) fixation with 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer for 15 min; (iv) washing three times (10 min each) in 0.185 M sodium cacodylate buffer to remove the unbound fixative; and (v) incubation for 30 min at room temperature in the substrate medium to reveal peroxidase activity (28). The substrate medium was freshly prepared by dissolving 10 mg of 3,3'-diaminobenzidine tetrahydrochloride (Fluka) in 20 ml of 0.5 M Tris-hydrochloride buffer (pH 7.6). This solution was filtered, adjusted to pH 7.2, and supplemented with 0.2 ml of 0.1% H_2O_2.

Sections incubated in 0.185 M sodium cacodylate buffer instead of the HRP solution and processed further as described above provided the chemical controls. However, transmission electron microscopic examinations of the buffer-treated control sections were carried out only for those animals for which one or more HRP-treated sections (Epon blocks [Table 1]) revealed the presence of anti-HRP antibody-containing plasma cells. Other possible controls, such as use of nonimmunized animals that did not receive the antigen either topically or by gastric intubation, were not carried out because there was absolutely no chance for this simian population to have come in contact with the antigen used. Furthermore, the number of animals available was very much restricted.

Sections and microscopy. MLN and lip portions not used for immunoperoxidase studies were Epon embedded (43), and semithin sections (about 1 to 2 μm thick) were prepared, using a Reichert OM-U2 ultramicrotome and glass knives. These sections were stained with a mixture of methylene blue and Azure II (62).

All peroxidase-treated and control sections were rinsed with distilled water, postfixed for 1 h in 1.33% OsO_4 buffered in 0.067 M *sym*-collidine (pH 7.4, 360 mOsm) (5), dehydrated in ethanol, and embedded on the flat surface of empty Epon blocks (43). Ultrathin sections were prepared, using diamond knives (Diatome WR) and an LKB-III Ultratome. Since the penetration of HRP into the sections is limited to a few micrometers, care was taken to save the first, most superficial sections, which were then examined without further contrast in a Philips 201 transmission electron microscope.

RESULTS

Repeated topical application of a soluble antigen, HRP, to the simian labial mucosa resulted in the appearance of plasma cells containing specific homologous antibody in the interacinar connective tissue of MSG. Enteric and gut priming with the same antigen in four other animals failed to reveal an anamnestic response in the labial mucosa.

Topical oral mucosa challenge. Of the 10 animals used for topical oral mucosal challenge, 4 revealed the presence of anti-HRP antibody-containing plasma cells in the MSG. Two of these animals (Table 1, animals 1 and 7) showed plasma cells with intense peroxidase activity located in the endoplasmic reticulum cisternae (ER), whereas the other two animals (Table 1, animals 3 and 10) showed plasma cells with less intense peroxidase activity.

In the labial mucosa, MSG reside superficially, and their secretory ducts are rather short (Fig. 1). Topical application of the opaque tracer demonstrated the accessibility of central acinar regions via the duct system (Fig. 1, lower inset). Plasma cells occurred individually or in clusters in the periacinar tissue of MSG (Fig. 1, upper inset). However, in response-positive animals, not all of these cells revealed peroxidase activity. Because retrograde antigen passage occurred randomly, i.e., being restricted to single ducts, and because morphological demonstration of the existence of positively responding plasma cells was the primary aim of this study, the number of such cells, i.e., the proportion of responding versus nonresponding cells, was impossible to assess. Those cells showing a highly electron-dense ER (Fig. 2a, inset) were considered to be positive for anti-HRP antibodies. In addition, nonspecific peroxidase or pseudoperoxidase activities of endogenous origin were expressed in the acinar cells of MSG, all mitochondria (Fig. 2a, inset), and erythrocytes. However, it was possible to differentiate the nonspecific from the specific reaction by comparison with control sections incubated in buffer instead of in HRP solution. In these sections, the interacinar plasma cells did not show ER peroxidase activity (Fig. 2b, inset), whereas the nonspecific reactions of endogenous origin were retained in the acinar cells and mitochondria (Fig. 2b).

Gut challenge. None of the four gut-challenged animals (Table 1) revealed plasma cells with ER peroxidase activity in the labial mucosa (Fig. 3a, lower inset). Nonspecific reactions of endogenous origin could be seen in the acinar cells of the MSG and in erythrocytes (Fig. 3a, upper inset).

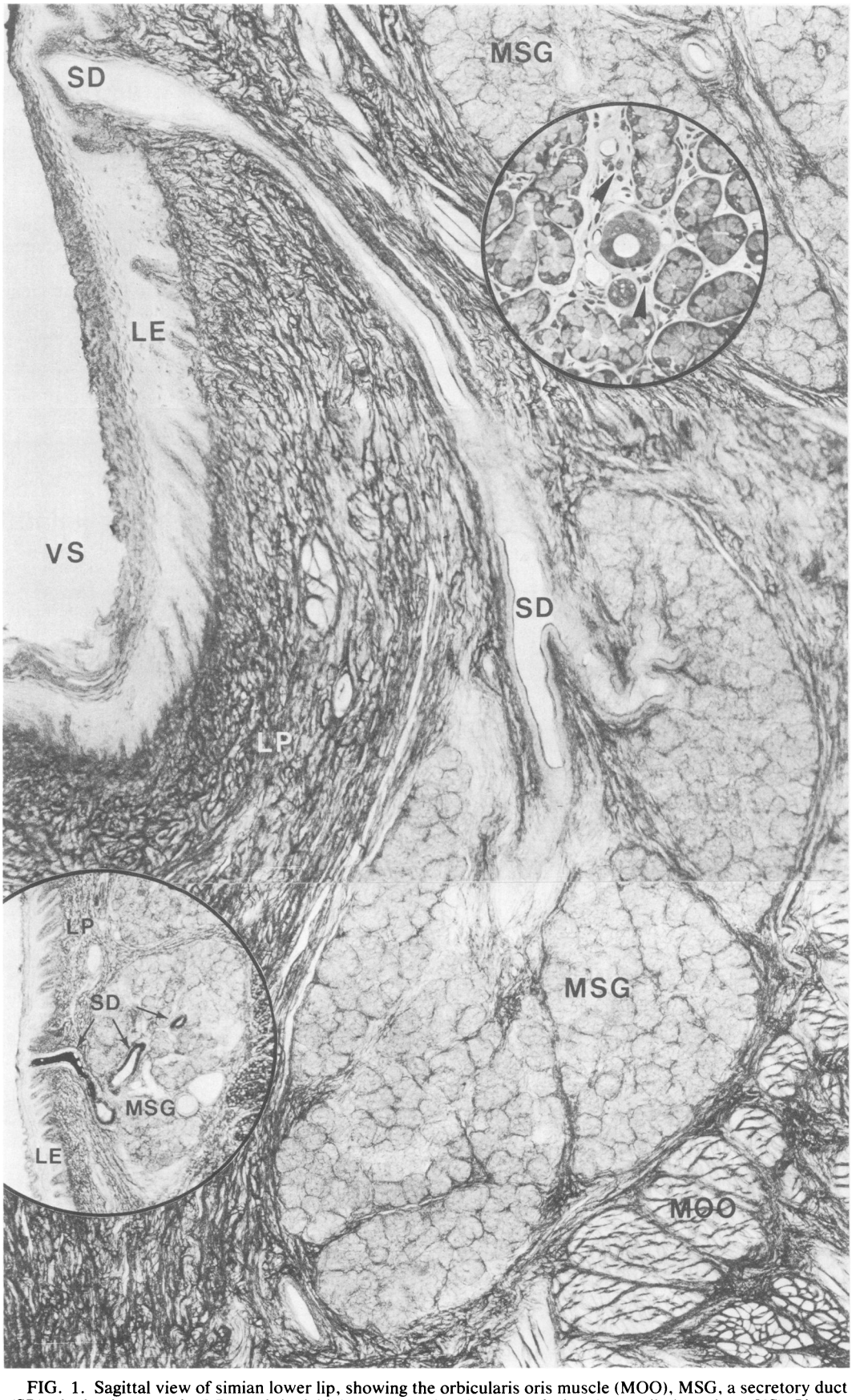

FIG. 1. Sagittal view of simian lower lip, showing the orbicularis oris muscle (MOO), MSG, a secretory duct (SD), the lamina propria (LP), and the labial mucosal epithelium (LE) facing the vestibular sulcus (VS). Plasma cells reside in the interacinar tissue (upper inset, arrow heads). An opaque tracer gained retrograde access deep into the MSG via the secretory duct (lower inset, nonstained cryosection). Magnifications: main figure, ×60, upper inset, ×150, lower inset, ×25.

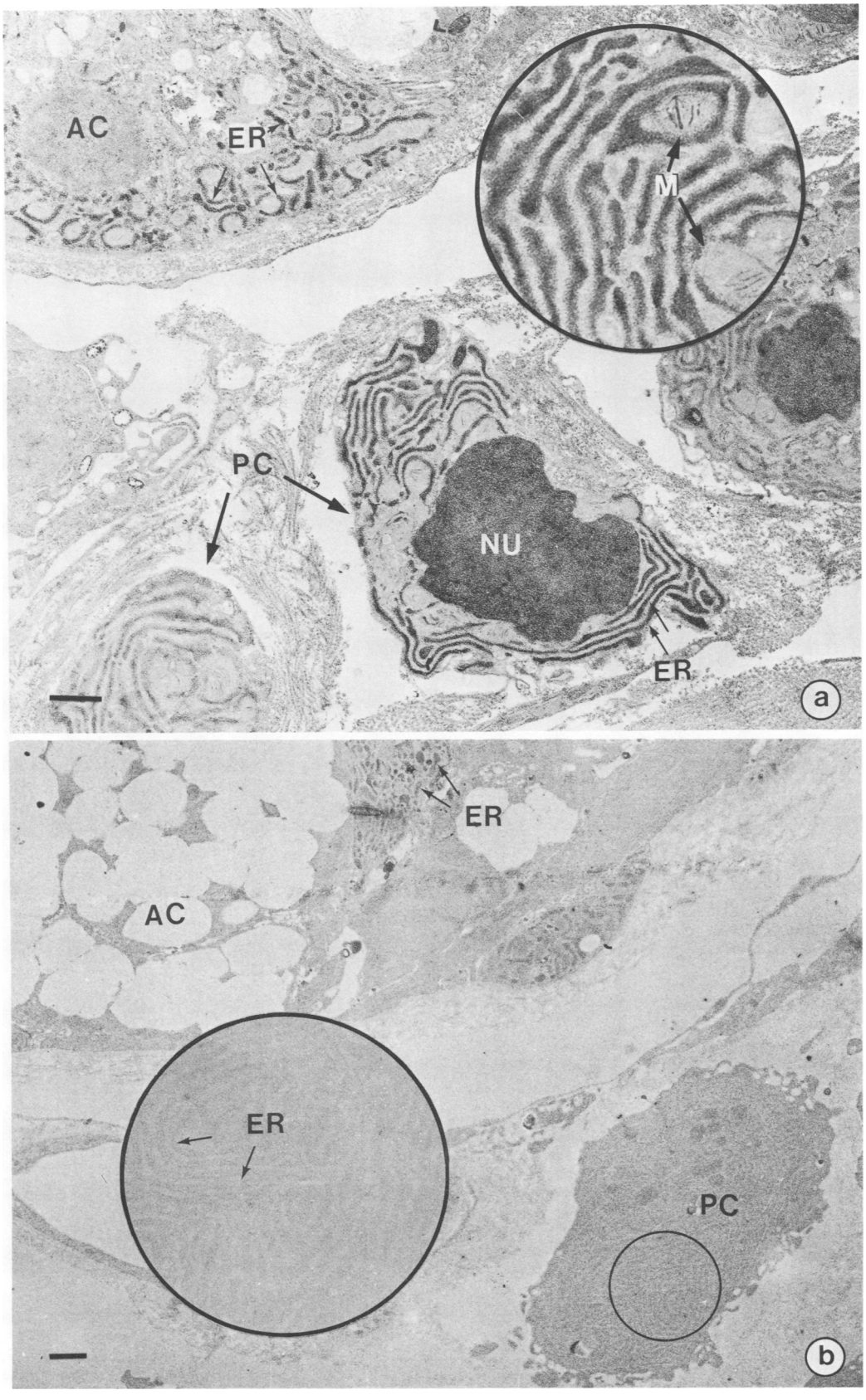

FIG. 2. Local immune response in the simian labial mucosa, showing interacinar plasma cells (PC) of the MSG. (a) Specific anti-HRP antibodies in the ER and nonspecific endogenous reactions in the ER of acinar cells (AC) and mitochondria (M). NU, Nucleus. Magnification, ×8,200 (inset, ×15,300). Bar, 1 μm. (b) Control half of the same section not incubated with HRP. Note the absence of specific peroxidase activity in the ER of plasma cells. Nonspecific endogenous reaction in the acinar cells is retained. Magnification, ×5,800 (inset, ×13,400). Bar, 1 μm.

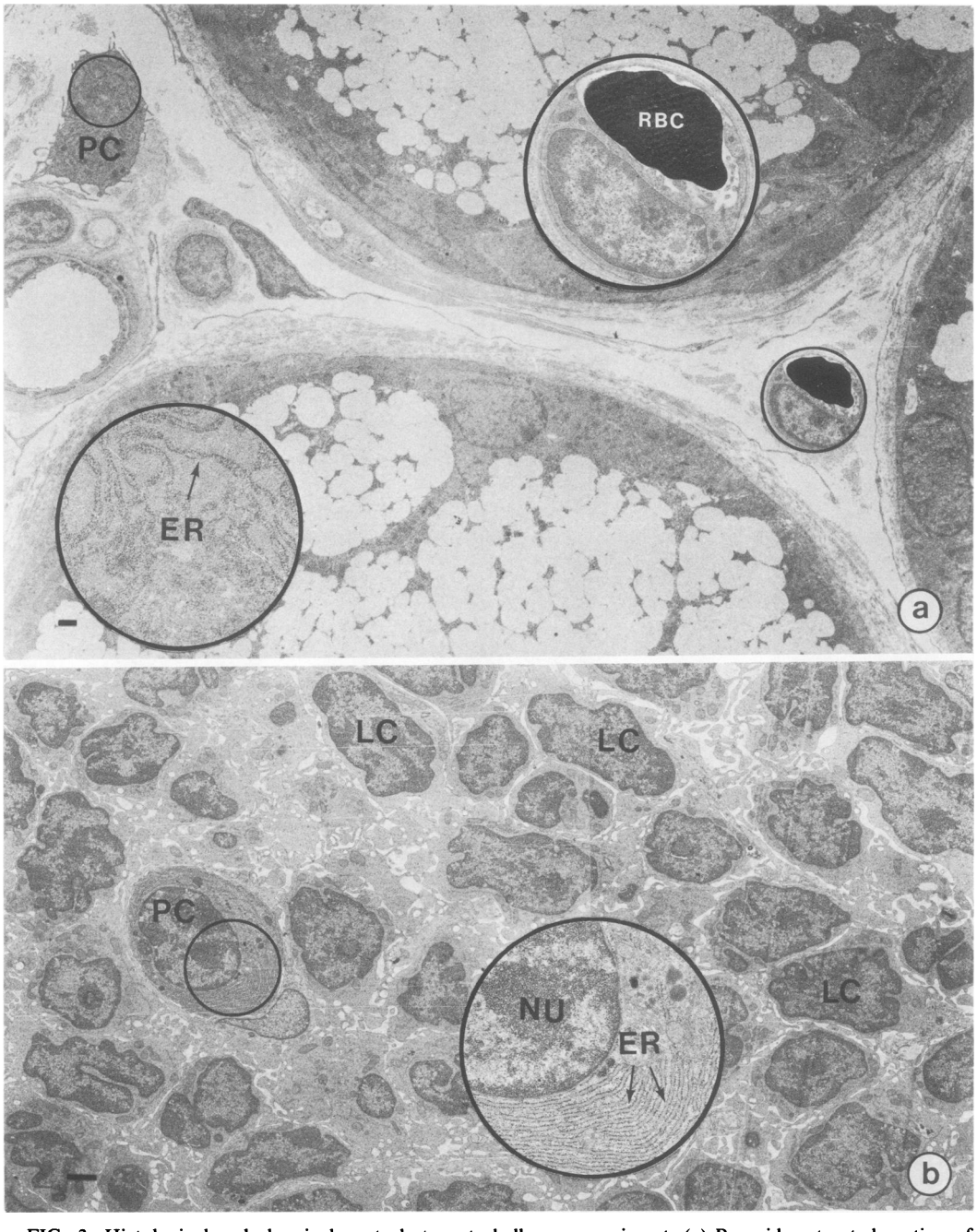

FIG. 3. Histological and chemical controls to gut challenge experiment. (a) Peroxidase-treated section of labial mucosa in a gut-challenged animal. Note the absence of specific reaction in the ER of interacinar plasma cells (PC). Nonspecific endogenous reaction is observed in acinar cells and erythrocytes (RBC). Magnifications: main figure, ×2,600; upper inset, ×5,700; lower inset, ×13,400. Bar, 1 μm. (b) Buffer-treated section of MLN, showing a mature plasma cell among numerous lymphocytes (LC). The plasma cell lacks specific reaction in the ER. NU, Nucleus. Magnification, ×3,400 (inset, ×7,800). Bar, 1 μm.

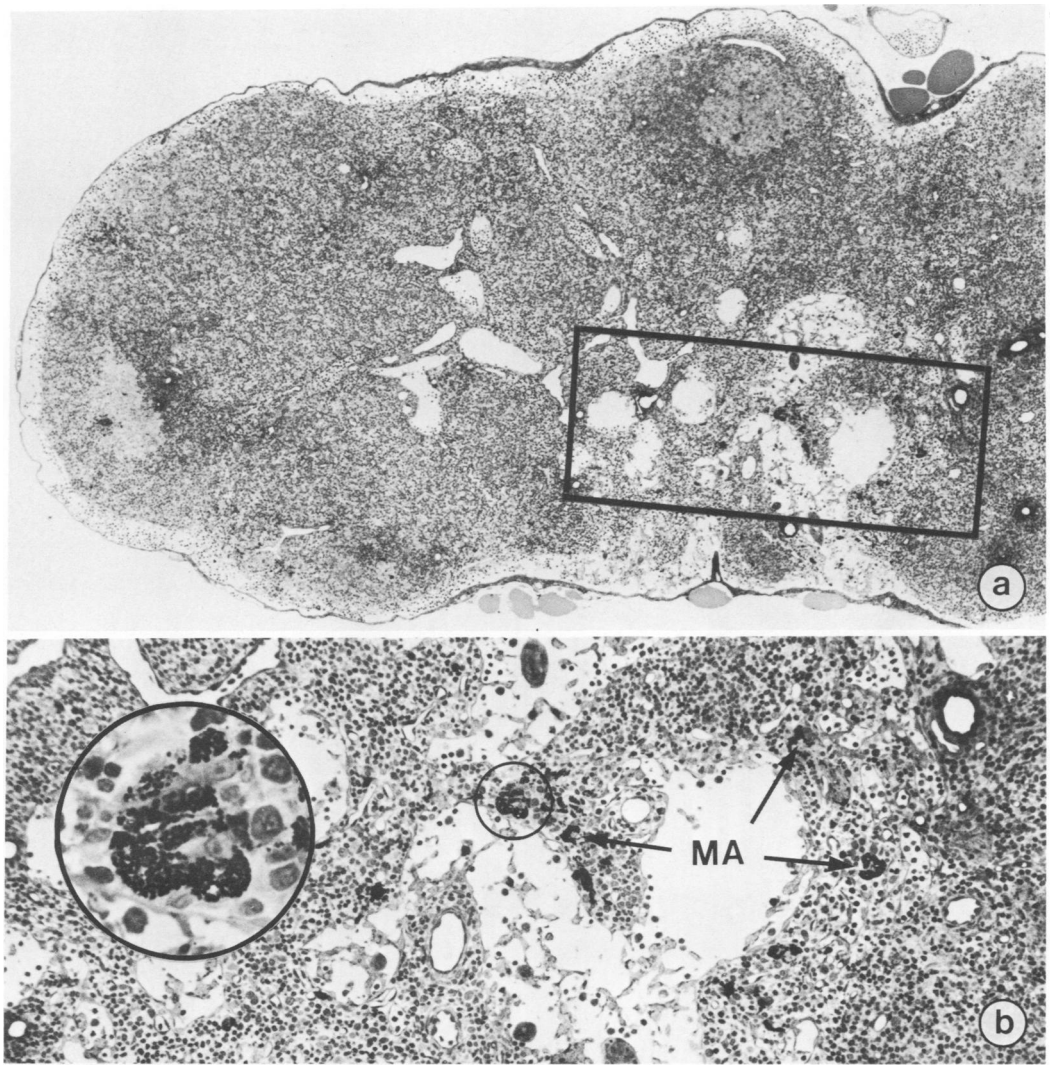

FIG. 4. (a) Cross section of MLN of a gut-challenged animal. Magnification, ×60. (b) Area outlined in (a), showing aggregations of macrophages (MA) containing black material resembling the India ink fraction of the tracer (inset). Magnification, ×150 (inset, ×600).

MLN could be examined in three animals only. Due to heavy adiposity in the abdominal region, MLN could not be retrieved in the fourth animal (Table 1, animal 12). Of the three monkeys examined, one (Table 1, animal 13) revealed the presence of plasmablasts with ER peroxidase activity in a randomly selected MLN. This particular lymph node, when split into halves, showed minute black spots located in the hilus area. Semithin sections revealed concentrations of macrophages containing black material resembling the India ink fraction of the antigenic material (Fig. 4). Noncontrasted, ultrathin sections of HRP-incubated lymph node portions exhibited intense peroxidase activity in the nuclear envelope, ER, and Golgi area of many plasmablasts (Fig. 5). The latter included highly electron-dense circular, cytoplasmic bodies (Fig. 5a, inset). In control sections incubated in buffer, neither plasmablasts nor mature plasma cells showed specific peroxidase activity (Fig. 3b, inset).

DISCUSSION

Considerable attention is being paid to the mucosal defense mechanisms so as to immunize against certain microbial diseases through topical vaccination. An important precondition for

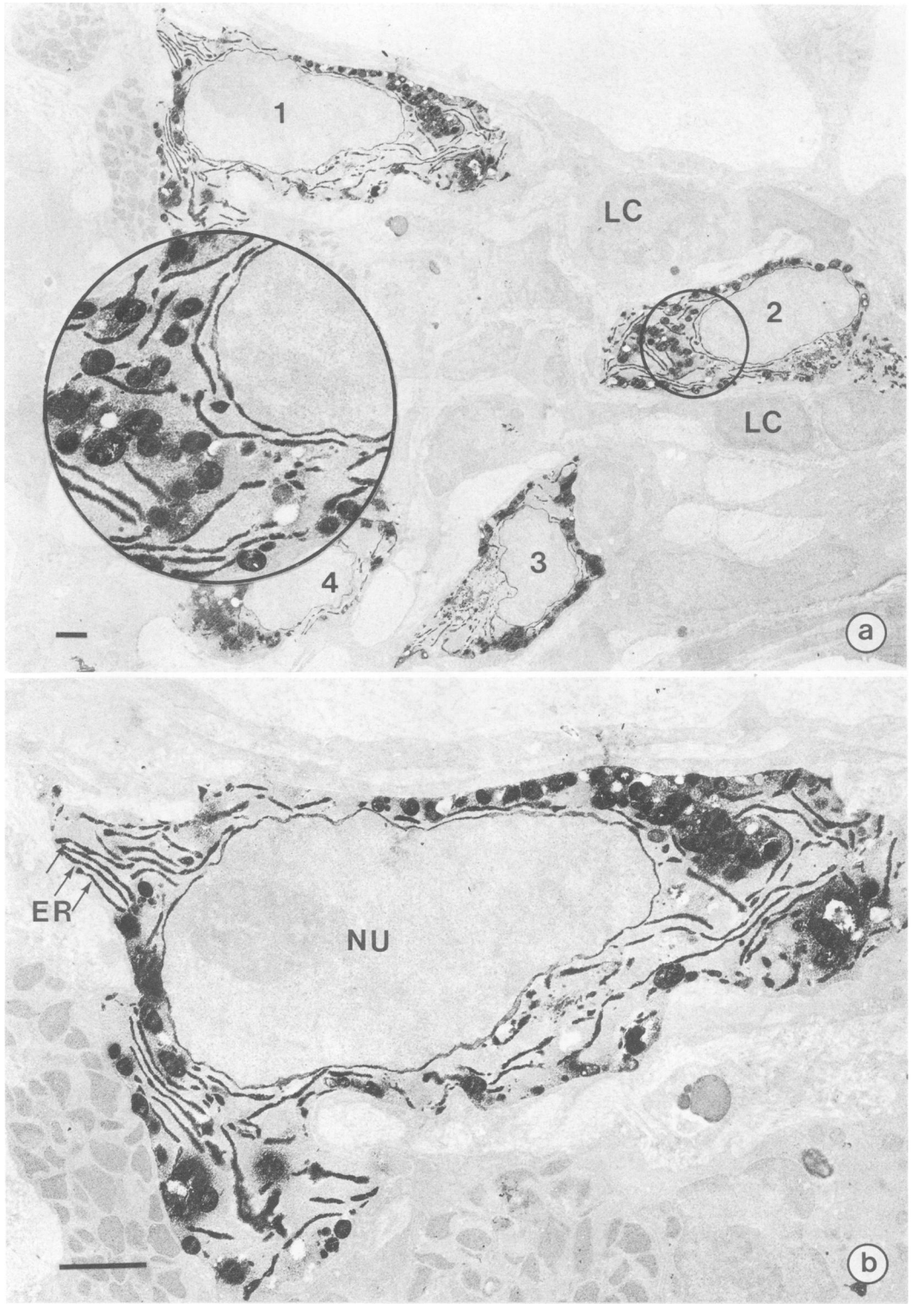

FIG. 5. Immune response in MLN of a gut-challenged animal. Many plasmablasts (no. 1–4) (a) with intense peroxidase activity in the ER (b) and circular cytoplasmic bodies (inset) (a) can be seen. Plasmablast 1 in (a) is shown in (b) at a higher magnification. LC, Lymphocytes; NU, nucleus. Bars, 1 μm. Magnifications: (a), ×4,000 (inset, ×13,400); (b), ×13,400.

this would be the ability of the mucosal tissue to have an immunological memory response (8). A significant feature of the immunological defense at mucosal surfaces is the presence of s-IgA (14, 36, 69) in secretions that bathe the mucous membranes. Depending on the mucosal site, this special class of antibody may be produced locally (e.g., salivary and lacrimal glands, bronchial mucosa [68]), by active transportation and processing of serum IgA (e.g., hepatocytes [24, 41, 56]), or by a combination of both (30). Saliva contains specific antibodies against a variety of microorganisms that are indigenous to the oral cavity (16, 38, 39, 64), and their titer can hardly be changed by parenteral immunization (13). Topical application of a streptococcal vaccine consisting of Formalin-killed cells to the labial mucosa of three humans resulted in a local immune response of MSG in one individual, whereas the other two did not respond (37). However, the route of this local sensitization is still enigmatic.

Currently there are two opposing views regarding the site of antigen recognition and stimulation for the oral mucosal immune response, i.e., common versus local mucosal immunity (31, 47). In the "universal" (9) or the so-called "common mucosal" immune system (7, 10, 11, 45, 46), the antigenic stimulation takes place in either the gut- or the bronchial-associated lymphoid tissue. The committed lymphocytes migrate through the thoracic duct and blood circulation to distant glandular and mucosal sites, where B-cells differentiate further into s-IgA-producing plasma cells. There are two types of evidence in support of this concept. (i) Lymphocyte traffic and homing studies have shown that MLN- and thoracic duct-derived blast cells have a predeliction for gut mucosa (27, 29, 31) and that thoracic duct-derived blasts can selectively lodge in the bowel of isogenic, allogenic, and even xenogenic recipients (50). Other authors, using the adoptive transfer system, were able to show that MLN-derived blasts migrate to distant mucosal and glandular sites, including the mammary and salivary glands (32, 57, 72). Lymphocytes derived from bronchial-associated lymphoid tissue have been shown to repopulate in bowels and lungs of lethally irradiated rabbits (58). Conversely, cells derived from gut associated lymphoid tissue repopulate the bronchial mucosa (8). (ii) Stimulation of one mucosal site can cause a specific immunoglobulin response in remote glandular sites (1, 3, 12, 19, 26). More specifically, antigens derived from *Streptococcus mutans* induce a remote salivary immune response when given by gastric intubation to gnotobiotic rats (48).

On the other hand, a local response to local antigenic stimuli has been shown to occur in female genital mucosa (20, 55) and lower-gut mucosa (54). Stimulation through the conjunctival sac of one eye leads to the appearance of specific antibodies in tears of the immunized but not the contralateral eye (18). Intraductal administration of *S. mutans* results in the synthesis of specific s-IgA by the parotid gland (23). Obviously, these observations cannot be explained in terms of a common mucosal immune system. It is in this context that we interpret the present data, which corroborate and extend the evidence in support of the existence of local mucosal immunity. However, for technical reasons, determination of the class of antibody in HRP-positive plasma cells could not be done.

The reproducibility of this local response, however, was found to be rather low. There may be several reasons for this. Unlike bronchial- or gut-associated lymphoid tissue, the volume density of the mouth-associated lymphoid tissue (40) is low in simian oral mucosa (35, 60), and this volume tends to decline with increasing age (35). At least one-half of the monkeys involved in the present study were known to be more than 4 years old. The organized structures capable of antigen recognition and processing are located rather deep in the MSG (61). To reach them, the antigen has to traverse a tortuous course through the duct system. This is possible (53) but may not be probable in all animals under experimentation. We are conscious of the possibility of at least some portion of the antigenic material, applied to the labial mucosa, passing down the gastrointestinal tract where it might act as an antigenic stimulant in accordance with the concept of common mucosal immunity. The failure of the labial mucosa to respond immunologically to the gut-priming experiments tends to eliminate this possibility. However, the host response observed in the MLN is explainable in view of the ability of the adult mammalian gut to absorb nutritionally insignificant but immunologically sufficient quantities of macromolecules (70, 71) or even materials of particulate nature such as asbestos or mineral fibers (21).

In conclusion, topical application of a soluble antigen to the simian labial mucosa resulted in a specific and structurally detectable immune response in the MSG, whereas enteric and gut priming failed to evoke any response in the oral mucosa. This is suggestive of the existence, at least in primates, of a local immune response independent of systemic involvement.

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