PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **179**, 522–528 (1985)

Retention of Ingested Latex Particles in Peyer's Patches of Germfree and Conventional Mice[1] (42133)

M. E. LEFEVRE, D. D. JOEL, AND G. SCHIDLOVSKY

Medical Research Center, Brookhaven National Laboratory, Upton, New York 11973

Abstract. Conventional and germfree mice ingested a suspension of 2-μm latex particles in drinking water for a 15-day period. Number and distribution of intestinal Peyer's patches did not differ significantly in the two types of mice. Cleared Peyer's patches were compared with regard to size and particle content. The location of particles within Peyer's patch follicles of germfree mice was similar to that of conventional mice, but the latter had significantly larger follicles and greater accumulations of latex particles. Latex concentration varied with patch location. Proximal patches contained the majority of particles in germfree mice, whereas particles were most abundant in distal patches of conventional mice. The results show that particle uptake into Peyer's patches takes place even in the complete absence of bacteria in the gut.

Previous publications have described the accumulation of 2-μm polymeric microspheres (latex particles) in intestinal Peyer's patches of mice that ingested the particles (1–4). The present report extends this investigation to the germfree mouse to test the possibility that bacterial activity in the intestine plays a role in particle uptake. Several investigators have described the colonization of the Peyer's patch epithelium by bacteria which show little affinity for nearby epithelial cells of the villi (5, 6). It could be postulated that this or similar bacterial colonization might produce occasional cell destruction or denudation, permitting the entry of particles. Alternatively, bacterial metabolites might stimulate phagocytosis by macrophages or mucosal epithelial cells. Accordingly, it might be expected that particle uptake in Peyer's patches of conventional mice would differ from that of germfree mice. The experiments reported here show that the complete absence of bacteria in the gut does not prevent particle accumulation in murine Peyer's patches. However, the abundance and distribution of the particles differ in germfree and conventional mice.

Materials and Methods. *Latex particles.* Commercially obtained polyvinyltoluene latex (PVT; Dow Chemical Co.; mean particle diameter ± SD, 2.02 ± 0.014 μm) was diluted to a concentration of 2.5×10^9 particles/ml with distilled water. The susupensions were autoclaved (30 min at 250°F, 15 lb pressure) in 1-liter Pyrex bottles (American Sterilizer Co.). The sterile suspensions were then shaken manually and sonicated for 30 min in a water-bath sonicator. Coulter sizing of suspensions before and after autoclaving showed negligible aggregation of particles.

Experimental design. Female germfree mice (Charles River Laboratories CD-1[CR]BR), 3–4 weeks old at the time of receipt, were housed in three groups of three to five each in separate isolators under temperature- and light-controlled conditions. After a week, one group (Group A) was conventionalized by seeding their isolator with feces of specific-pathogen-free mice. These mice remained in an isolator and were subject to the same conditions as the germfree mice. After 42 days, a time sufficient for bacterial colonization to take place and intestinal morphology to become normalized (7, 8), Group A and one group of germfree mice (Group B) were deprived of water and given suspensions of latex particles to drink. The sterile latex suspension was transferred into the isolators and poured into glass drinking bot-

[1] The research described in this report involved animals maintained in animal care facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care. The research described in this report was supported by the U.S. Department of Energy under Contract DE-AC02-76CH-00016 and the U.S. Environmental Protection Agency under Contract 79-D-X-05333 and research Grant R810140.

0037-9727/85 $1.50

tles equipped with sipper tubes. The suspension was mixed by frequent manual shaking of the bottles and by the escape of bubbles as the mice drank. Ingestion was terminated after 15 days, and the mice were maintained for 3 days more on sterile distilled water to clear the intestine of the majority of free particles. Another group of germfree mice (Group C) was maintained on sterile distilled water throughout the experiment. Groups A and B drank the latex suspensions readily, and the three groups ingested similar amounts of fluid. The mice were fed pelleted rodent food (Charles River RMH 3500), autoclaved shortly before transfer into the isolators. Fecal samples from the two germfree groups were cultured biweekly and were always negative for both aerobic and anaerobic bacteria. All mice were active and appeared healthy.

Two separate experiments were conducted a year apart. Numbers of mice were as follows: Experiment 1: Group A, 3; Group B, 4; Group C, 4. Experiment 2: Group A, 5; Group B, 5; Group C, 5. The findings of the two studies were similar and the results were pooled.

At the termination of the experiments, the mice were removed from their isolators, killed immediately by ether overdose, and weighed. Weights ±SD were Group A, 29.88 ± 3.51 ($n = 8$); Group B, 29.61 ± 2.72 ($n = 9$); Group C, 30.44 ± 2.61 ($n = 9$). The intestine was removed and extended to its full length on a piece of paper. Peyer's patches were located and their positions marked on the paper. Four patches located as close as possible to 5, 20, 30, and 45 cm from the cecum were rapidly excised and placed in 70% ethanol. The remaining patches were placed in 10% acetate-buffered Formalin or other fixative. Each germfree mouse had a grossly enlarged cecum, hallmark of the germfree state in rodents (9, 10). In addition, the intestines of germfree mice were more flaccid and the contents more fluid than those of their conventional counterparts.

Particle quantitation in cleared Peyer's patches. Histological clearing of alcohol-fixed Peyer's patches was accomplished by treating them with potassium hydroxide and glycerol as described previously (1). After clearing, the patches were placed mucosal side up in a depression slide and examined by light microscopy. The cleared patches were transparent, but the epithelial surface and major structures such as crypts and villi could be discerned. Follicle diameter was obtained by averaging two right-angle diameters measured *en face* between bordering crypts with an ocular micrometer.

As illustrated in a previous publication from this laboratory (1), latex particles were found throughout Peyer's patches of latex-fed mice, but were most abundant immediately beneath the epithelial layer in the center of the dome (the apical portion of the follicle that projects into the intestinal lumen). Latex was sampled by mapping out 0.0144 mm^2 on an optical plane through this region and counting the particles within the area. The area was delineated at 430× by an eyepiece reticule; the optical plane was obtained by advancing the microscope focus beyond the epithelial layer in the center of the dome to the first position at which latex particles were visible in all parts of the reticule. The particles were distinguished by their size, shape, and refractility. For each dome two independent counts were carried out and the results averaged. Counting was done without knowledge of patch identity.

Light microscopy. Formalin-fixed Peyer's patch tissue was embedded in Ladd's low-viscosity Epon. Sections were cut at 1 μm with a glass knife, stained with azure II-methylene blue, and mounted in immersion oil. For comparison, latex particles were embedded in agar and processed as tissue.

Results. The number and distribution of Peyer's patches along the intestine were determined for the three groups involved in the study (Table I). Conventional mice had more patches than either germfree group, but the differences were not significant. To determine patch distribution, the number of patches per 10-cm segment was converted to percentage of the total for the intestine. Patches were most abundant in the distal intestine of all groups; no significant differences appeared when patch abundance in the two germfree groups was compared with that of the conventional group at the same level in the intestine (Table I).

Sections of jejunal Peyer's patches of latex-fed mice were examined by light microscopy. In both conventional and germfree mice,

TABLE I. NUMBER AND DISTRIBUTION OF PEYER'S PATCHES

Group	*N*	Total patches[a]	% of total in segment[b] 0–10[a]	10.1–20[a]	20.1–30[a]	30.1–40[a]	40.1 Junction[a,c]
Conventional latex fed	8	9.9 ± 1.6	32.5 ± 7.3	20.1 ± 6.9	18.0 ± 5.4	11.6 ± 3.7	17.9 ± 5.0
Germfree latex fed	9	9.6 ± 0.9	29.2 ± 6.8	21.9 ± 7.4	16.2 ± 7.3	14.9 ± 4.5	17.7 ± 8.2
P		>0.6	>0.3	>0.5	>0.5	>0.1	>0.9
Germfree control	9	9.3 ± 1.5	28.7 ± 6.9	22.0 ± 8.6	16.5 ± 4.5	11.8 ± 6.4	21.0 ± 10.0
P		>0.4	>0.2	>0.6	>0.5	>0.9	>0.4

Note. *N* = number of mice. The significance of the difference between means was calculated by Student's *t* test for unpaired values. *P* values compare means for germfree mice with the corresponding mean for conventional mice.

[a] Values are means ± SD for *N*.

[b] Column headings delineate the segment in terms of cm from ileocecal junction.

[c] Gastroduodenal junction. Length of this segment varied.

latex particles were not seen within the epithelial layer, but they were present in subepithelial dome macrophages (Fig. 1) and rarely in macrophages in the middle or basal regions of follicles. Subepithelial macrophages in both conventional and germfree mice had similar morphology. As described previously for conventional mice (11), the macrophages contained pale cytoplasm, numerous inclusions, and a large round nucleus with one or more prominent nucleoli. The patch epithelium in both conventional and germfree mice contained occasional degenerating epithelial cells, but these bore no apparent relation to latex particles within the patch.

Cleared whole Peyer's patches consisted of 3 to 11 follicles, each follicle demarcated by rows of crypts. Latex particles were observed beneath the dome surface in Peyer's patches of germfree mice as described previously for conventional mice (1–3). Latex particles were assayed in four widely spaced, cleared patches from each mouse. No particles resembling 2-μm latex were present in cleared Peyer's patches of non-latex-fed control mice.

To test the reliability of the particle counting procedure, randomly selected cleared patches containing a total of 49 follicles were requantified after 7 months. The counts in the assayed area were expressed as percentage of the value obtained 7 months earlier. Mean percentage ±SD was 96.4 ± 8.2 (n = 49), indicating good reproducibility.

Figure 2 diagrams two representative intestines, and illustrates the type of data obtained. Peyer's patches are depicted by groups of circles, each circle representing a follicle. Particle counts are given for the assayed area of each follicle; the mean for all assayed areas of the patch is given in the first row below. To facilitate analysis of particle abundance as a function of location in the intestine, the patch means were converted to percentages. For each mouse the four means were totaled and the individual means were expressed as percentage of the sum. This "patch percentage" is also given in the illustration.

Table II summarizes results for Peyer's patches from conventional and germfree mice without regard to the location of the patch. Follicle diameter was significantly smaller in both germfree groups than in the conventional group. The number of follicles per patch was less in germfree than in conventional mice, but the differences were not significant. Despite large variation, latex-particle abundance in Peyer's patches was significantly less in germfree latex-fed than in conventional mice ($P < 0.01$).

The overall summary given in Table II does not effectively describe differences in latex distribution revealed by the experiments and suggested in Fig. 2. Conventional mice tended to have the lowest concentration of particles in proximal patches, whereas the opposite was true of germfree mice. Figure 3 shows the results of linear regression analysis of the relationship between latex abundance (expressed as the previously described patch

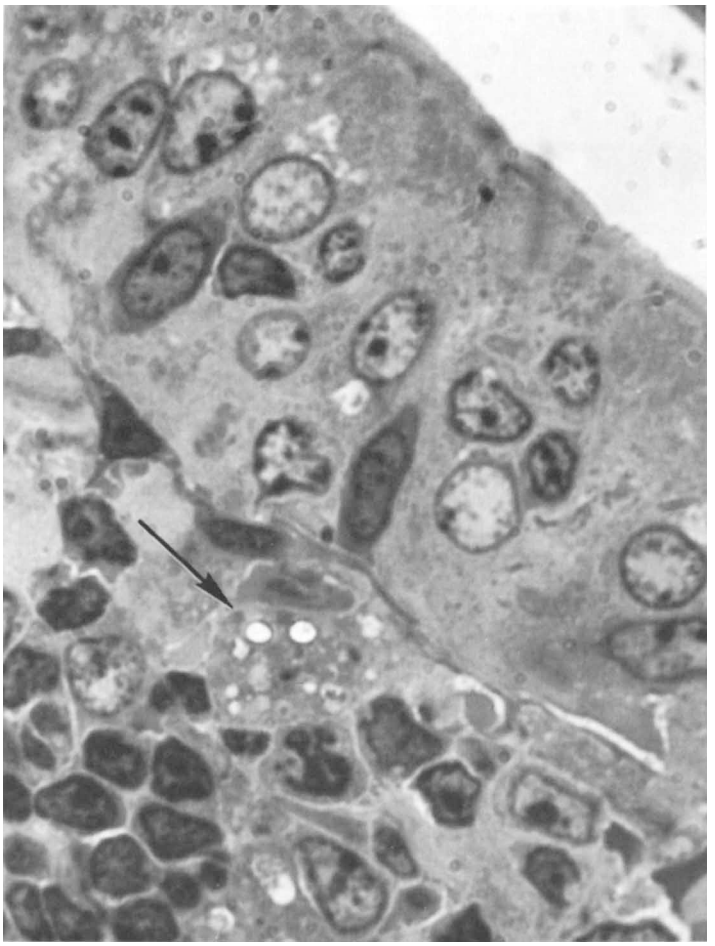

FIG. 1. Epon-embedded Peyer's patch tissue showing epithelial layer and two 2-μm latex particles in a subepithelial macrophage (arrow). Germfree mouse. (×1125).

percentage) and patch location for all latex-fed conventional and germfree mice. Correlation coefficients for the relationship were 0.56 ($P < 0.01$) and 0.80 ($P < 0.001$) for germfree and conventional mice, respectively. The slopes of the two lines differed significantly ($P < 0.001$). To see if the findings were correlated with follicle-size distribution, mean follicle diameter for each patch was similarly plotted against patch location for all latex-fed conventional and germfree mice (not shown). The two lines were nearly horizontal, and no significant difference was found between their slopes ($P > 0.3$).

Discussion. Particle uptake into Peyer's patches is an intriguing phenomenon which merits quantitative assessment. Detailed quantitation of the phenomenon is difficult, however, because the number of follicles making up patches is not uniform (Fig. 2) nor is the distribution of particles within follicles (1–3). We previously described the recovery of 2-μm polyvinyltoluene particles from whole patches by gradient centrifugation of alkali-solubilized tissue (2). Quantitative recovery of 5.7-μm styrene-divinylbenzene particles from acid-solubilized Peyer's patches by a filtration process was subsequently reported (3). Both these methods had drawbacks. The former could not be

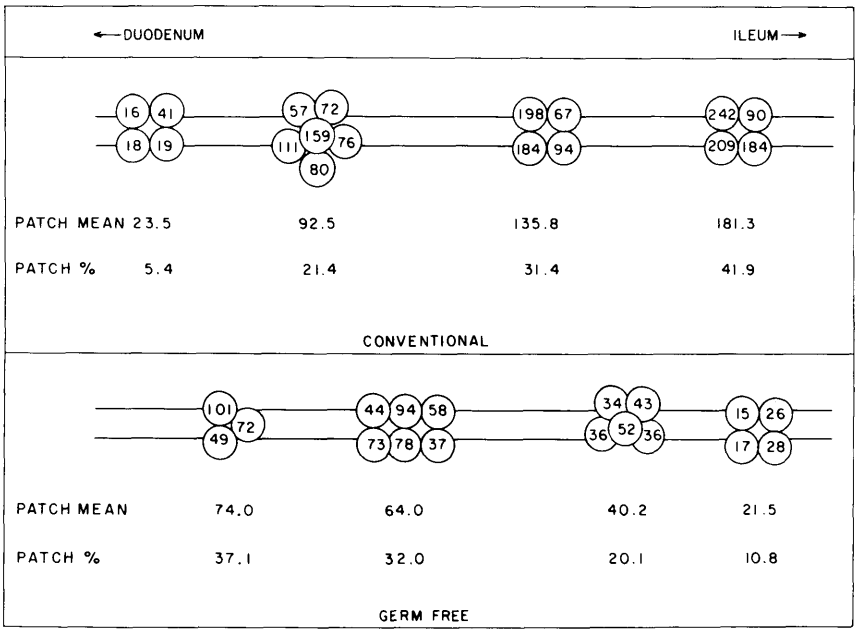

FIG. 2. Diagrammatic depiction of findings from individual conventional (top) and germfree (bottom) mice. Four Peyer's patches composed of varying numbers of follicles (circles) are shown for each intestine. Numbers within the circles indicate the number of particles counted in an area of 0.0144 mm^2 in the follicle dome. The first row of numbers gives the mean count in the 0.0144-mm^2 area for the follicles of each patch (patch mean). The second row expresses the mean as percentage of the sum of the means (patch percent). Differences in follicle size are not illustrated.

utilized for small numbers of particles, and the latter was unsatisfactory for particles degraded by strong acid (e.g., PVT particles). The use of radioactively labeled microspheres to measure uptake was also unsatisfactory (12; LeFevre ME, unpublished experiments).

TABLE II. PEYER'S PATCH CHARACTERIZATION AND PARTICLE ABUNDANCE IN CONVENTIONAL AND GERMFREE MICE

Group	*N*	Follicles per patch[a]	Follicle diameter (mm)[a]	Particles per follicle area[a,b]
Conventional latex fed	8	6.19 ± 2.32 (32)	1.18 ± 0.19 (197)	89.44 ± 86.33 (197)
Germfree latex fed	9	5.58 ± 1.75 (36)	0.83 ± 0.12 (202)	61.48 ± 49.81 (202)
P		>0.2	<0.001	<0.01
Germfree control	9	5.75 ± 2.06 (36)	0.81 ± 0.13 (207)	0 (207)
P		>0.4	<0.001	

Note. *N* = number of mice. *P* values compare means of germfree groups with the corresponding mean for conventional mice. The significance of the difference between means was calculated by Student's *t* test for unpaired values.

[a] Values are means ± SD (based on number of observations). Number of observations given in parentheses.

[b] Follicle area = 0.0144 mm^2.

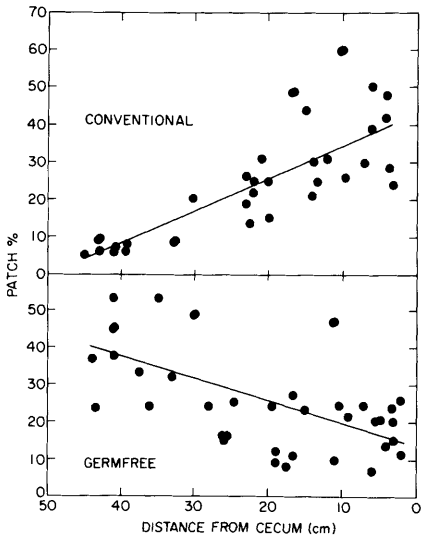

FIG. 3. Latex abundance as a function of patch location for latex-fed conventional and germfree mice. Numbers of mice: conventional, 8; germfree, 9. Each mouse generates four points; each point represents a single Peyer's patch. Abscissa shows linear distance along the intestine; duodenum is at left, ileum at right. Ordinate gives latex abundance as patch percentage (see legend to Fig. 2).

The present paper presents a fourth method for quantitating latex-particle uptake into murine Peyer's patches, the optical counting of particles *in situ*. The method is reliable and repeatable and can be applied to small numbers of particles. In the present work it was used to assess particle accumulation in Peyer's patches of mice that ingested the particles for 15 days.

The results show that latex-particle accumulation takes place in Peyer's patches of germfree mice, indicating that an intestinal flora is not essential for the occurrence of the phenomenon. (However, both conventional and germfree mice were fed autoclaved rodent pellets which contain dietary antigens; thus the absence of an intestinal flora should not be equated with the absence of all antigenic stimulation.) Nevertheless, the sampled patches from germfree mice contained significantly less latex than those from conventional mice (Table II). Factors that might contribute to the difference are villus size and motility, mucus composition and abundance, and epithelial turnover time, all of which differ in germfree and conventional mice (9, 10). Another possibility that cannot be assessed at present is that uptake was as great in germfree as in conventional mice, but that retention was less. Accumulated latex particles gradually disappear from Peyer's patches (1, 13), but their rate of loss and eventual fate have not been determined.

Uptake of macromolecules and particulate antigens into Peyer's patches has been ascribed to the activities of specialized cells (M cells) whose functions are important in the initiation of intestinal immune responses (14–17). M cells also phagocytose *Vibrio cholerae* (18) and are sites of adherence and penetration for type 1 reovirus (19). Distribution of M cells over the Peyer's patch dome has been described (20) and their presence in germfree mice confirmed (6). It is not at all certain, however, that particles as large as the 2-μm particles used in the present experiments penetrate the Peyer's patch epithelium via the M-cell route. Owen (21) has suggested that the large protozoan *Giardia muris* penetrates the epithelium through gaps left by degenerating columnar epithelial or M cells. Since the distribution of M cells could not be determined in the cleared preparations used for the present work, the role of M cells in latex-particle uptake remains uncertain.

We found that relative particle abundance was greatest in proximal patches of germfree mice, whereas the opposite was true in conventional mice (Fig. 3). Increased uptake might result from increased exposure time of dome surfaces to particles. However, the duration of exposure of patches to particles in various regions of the intestines of the two groups is difficult to assess. Overall transit of food through the gastrointestinal tract is retarded in germfree rodents, but most of the delay is accounted for by increased residence time of digesta in the enlarged cecum (10). Nevertheless, loss of smooth-muscle tone (9) and increased cecal pressure against the lower small bowel may contribute to keeping the proximal intestine, empty much of the time in conventional mice, filled with digesta in germfree mice. This could lead to longer exposure of dome surfaces to particles in the proximal intestine and an increase in particle uptake. Whatever the explanation for the

qualitative and quantitative differences between germfree and conventional mice, the findings of the present study suggest that uptake of 2-μm latex particles reflects an intrinsic property of Peyer's patches—one that is independent of the presence of bacteria in the gastrointestinal tract.

The authors gratefully acknowledge the assistance of Ms. Angela Boccio, Ms. Joanne Leverah, Mr. William Maston, Mr. Howard Pate, and Mr. Richard Ruffing.

1. LeFevre ME, Olivo R, Vanderhoff JW, Joel DD. Accumulation of latex in Peyer's patches and its subsequent appearance in villi and mesenteric lymph nodes. Proc Soc Exp Biol Med **159**:298–302, 1978.
2. LeFevre ME, Vanderhoff JW, Laissue JA, Joel DD. Accumulation of 2-μm latex particles in mouse Peyer's patches during chronic latex feeding. Experientia **34**:120–122, 1978.
3. LeFevre ME, Hancock DC, Joel DD. The intestinal barrier to large particulates in mice. J Toxicol Environ Health **6**:691–704, 1980.
4. LeFevre ME, Joel DD. The Peyer's patch epithelium: An imperfect barrier. In: Schiller CM, ed. Toxicology of Intestinal Function. New York, Raven Press, pp45–56, 1984.
5. Abrams GD. Microbial effects on mucosal structure and function. Amer J Clin Nutr **30**:1880–1886, 1977.
6. Owen RL, Nemanic P. Antigen processing structures of the mammalian intestinal tract: An SEM study of lymphoepithelial organs. SEM, Vol II:pp367–378, 1978.
7. Khoury KA, Floch MH, Hersh T. Small intestinal mucosal cell proliferation and bacterial flora in the conventionalization of the germfree mouse. J Exp Med **130**:659–670, 1969.
8. Crabbé PA, Nash DR, Bazin H, Eyssen H, Heremans JF. Immunohistochemical observations on lymphoid tissues from conventional and germfree mice. Lab Invest **22**:448–457, 1970.
9. Gordon HA, Pesti L. The gnotobiotic animal as a tool in the study of host microbial relationships. Bacteriol Rev **35**:390–429, 1972.
10. Wostman BS. The germfree animal in nutritional studies. Annu Rev Nutr **1**:257–279, 1981.
11. Hammer R, Joel DD, LeFevre ME. Ultrastructure of macrophages of the murine Peyer's patch dome. Exp Cell Biol **51**:61–69, 1983.
12. LeFevre ME, Joel DD, Laissue JA, El-Aasser MS, Vanderhoff JW. Stability of ^{125}I label after intragastric or intravenous administration of radioiodinated latexes to mice. J Reticuloendothel Soc **22**:189–197, 1977.
13. LeFevre ME, Hammer R, Joel DD. Macrophages of the mammalian small intestine: A review. J Reticuloendothel Soc **26**:553–573, 1979.
14. Bockman DE. Range of function of gut-associated lymphoepithelial tissue. In: Solomon JB, ed. Aspects of Developmental and Comparative Immunology. Oxford, Pergamon, Vol 1:pp273–277, 1980.
15. Cebra JJ, Kamat R, Gearhart P, Robertson SM, Tseung J. The secretory IGA system of the gut. In: Ciba Foundation Symposium, Immunology of the Gut. Amsterdam, Elsevier, pp1–29, 1977.
16. Kagnoff MF. Functional characteristics of Peyer's patch lymphoid cells. J Immunol **118**:992–997, 1977.
17. Owen RL. Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine. Gastroenterology **72**:440–451, 1977.
18. Owen RL. Autoradiographic analysis of M cell uptake and transport of cholera vibrios into follicles of rabbit Peyer's patches. Gastroenterology **84**:1267, 1983.
19. Wolf JL, Rubin DH, Finberg R, Kauffman RS, Sharpe AH, Trier JS, Fields BN. Intestinal M cells: A pathway for entry of reovirus into the host. Science (Washington, DC) **212**:471–472, 1981.
20. Smith MW, Peacock MA. "M" cell distribution in follicle-associated epithelium of mouse Peyer's patch. Amer J Anat **159**:167–175, 1980.
21. Owen, RL. Macrophage function in Peyer's patch epithelium. Adv Exp Med Biol **149**:507–513, 1982.

Received January 17, 1985. P.S.E.B.M. 1985, Vol. 179.
Accepted April 4, 1985.