Evidence for the Phagocytic Transport of Intestinal Particles in Dogs and Rats

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Fluorescent latex beads of two different colors were implanted into separate intestinal segments in individual dogs and rats. Mesenteric lymph node phagocytes subsequently contained multiple beads of one or the other color but rarely both colors, indicating that intestinal phagocytes transported the latex beads to the draining lymph node. Fluorescent labeled *Escherichia coli* was implanted into rat ligated intestinal segments, and rare mesenteric lymph node phagocytes subsequently contained fluorescent bacteria, suggesting that intestinal bacteria might be transported in the same manner as inert latex beads.

The intestinal tract is thought to be a portal of entry for many human pathogens. The mechanism by which this mucosal surface is penetrated and bacteria are transported into the circulation system is unknown. Furthermore, there is growing evidence that certain species of normal intestinal bacteria can be absorbed under conditions of stress or pharmacological manipulation; such organisms are then found to be viable within the mesenteric lymph nodes, spleen, liver, or paraenteric inflammatory foci (2, 3, 7, 13, 17, 21, 23). Because both motile and nonmotile translocating organisms have been observed, we hypothesized that intestinal macrophages could ingest particles and then transport them via lymphatic channels to the mesenteric lymph nodes (22). If, under conditions of stress or intrinsic resistance, phagocytic killing of the ingested bacteria were delayed or impaired, viable organisms could invade the body via this route. In support of these speculations, several species of facultative intracellular bacteria, namely, *Salmonella* spp. (16, 19) and *Listeria monocytogenes* (18), have been observed within phagocytic cells beneath the surface of the intestinal epithelium. In addition, Harmsen et al. (6) recently described the use of fluorescent latex beads of two colors to show that lung alveolar macrophages transported particles to the draining tracheobronchial lymph nodes. In initial experiments, we attempted to modify the method of Harmsen et al. (6) to challenge the hypothesis that macrophages play a key role in the transport of intestinal particles.

Experiments were designed with latex beads of two different colors (fluorescein labeled and rhodamine labeled) that were implanted into separate intestinal segments of mongrel dogs. After a suitable time period, the common draining mesenteric lymph node was excised to look for latex beads within phagocytic cells. If phagocytic cells contained only fluorescein-labeled beads or only rhodamine-labeled beads, this would indicate that intestinal phagocytes transported the particles to the draining lymph node. Conversely, if the phagocytes contained both bead colors, this would indicate that the beads migrated to the draining lymph node and were subsequently phagocytosed in situ. The mesenteric lymph node was chosen because it is the obvious draining lymph node and because it has been shown that intestinal bacteria can enter the mesenteric lymph node

before dissemination to other otherwise sterile tissues, such as the liver and the spleen (for a review, see reference 1).

Four mongrel dogs were used, each of which had a single 50-cm isolated jejunal loop constructed with each end existing as a stoma on the skin. The mesentery and vascular supply were undisturbed. The continuity of the remaining intestinal tract was restored after the isolated loop was constructed. After several months of recovery, the experiments reported herein were performed. On different days, three of the four dogs (A, B, and C) were anesthetized and a midline incision was made to expose the small intestine. The jejunal loop was located, and the stoma were closed with sutures. This jejunal segment was then cut in half; one half was filled with 10 ml (4.5×10^{10} beads per ml) of 1.09- μ m-diameter fluorescein-labeled latex beads (Polysciences, Inc., Warrington, Pa.), and the other half was filled with 10 ml (4.5×10^{10} beads per ml) of 0.89- μ m-diameter rhodamine-labeled latex beads (Polysciences). The open end of each jejunal segment was then carefully closed with sutures, taking care to avoid contaminating the peritoneal cavity with latex beads. The abdomen was closed and, after recovery from anesthesia, each dog was given food and water ad libitum. Seven days after bead implantation, the dogs were again anesthetized. Through a midline incision, the isolated intestinal segments were examined and the main mesenteric lymph node draining the small intestine was excised. The histology of the isolated jejunal segments was evaluated by frozen section and by hematoxylin and eosin staining. By light microscopy, normal intestinal histology was noted in all jejunal loops, although more subtle pathology cannot be excluded by this methodology. With frozen sections, it was verified that the jejunal segment implanted with fluorescein-labeled beads still contained only fluorescein-labeled beads and that the jejunal segment implanted with rhodamine-labeled beads still contained only rhodamine-labeled beads.

After excision, each mesenteric lymph node was immediately processed into a single-cell suspension (gentle crushing of the lymph node in RPMI 1640 [GIBCO Laboratories, Grand Island, N.Y.]) and cytospin centrifuge preparations were then viewed directly with a fluorescence microscope. The leukocytes appeared to be normal cells of the morphology expected in lymph node tissue; i.e., most cells appeared to be small lymphocytes, whereas other cells (0.1 to 0.01%) appeared to be larger phagocytic cells, a finding which is consistent with macrophages. Only rare cells (approximately

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1 of 1,000 to 1 of 10,000) contained latex beads, and these cells were the larger cells in the cell population; typically, multiple beads were present in these cells. Nearly all phagocytes (containing at least two latex beads) contained latex beads of only one color, indicating that intestinal phagocytes transported the particles to the draining mesenteric lymph node (Table 1). Examples of these phagocytic cells are presented in Fig. 1A and 1B. The occasional phagocyte containing both bead colors can be explained in one of two ways. (i) Free beads may have migrated to the mesenteric lymph node and were then engulfed by lymph node phagocytes. (ii) Lymph node phagocytes containing beads died and liberated free beads which were then picked up by another phagocytic cell.

Similar results were noted with dogs A, B, and C (Table 1), i.e., nearly all mesenteric lymph node phagocytes with latex beads contained either fluorescein- or rhodamine-labeled beads but not both. (A much greater number of cells were examined from dog A than from subsequent dogs because dog A was the first dog examined and we wished to be confident of the results.) By using the binomial distribution and assuming each phagocyte had an equal opportunity to ingest beads of each color, the probability is <0.0001 of having beads of a single color in 122 of 130 phagocytes, 24 of 25 phagocytes, and 23 of 25 phagocytes, as noted with dogs A, B, and C, respectively (Table 1). This data strongly indicated that the mesenteric lymph node phagocytes did not have an equal opportunity to ingest beads of both colors and most likely ingested the beads in the only site where the colors were separate, i.e., the intestinal tract.

To confirm these observations, the fourth dog (D) was treated as follows. The jejunal loop in dog D was sutured closed at the two stoma, but the loop was not cut in half. Before closing the stoma, a mixture of 10 ml of 4.5×10^{10} of fluorescein-labeled beads per ml and 10 ml of 4.5×10^{10} of rhodamine-labeled beads per ml was implanted into the loop. The dog was sacrificed 7 days later. Once again, the jejunal segment was grossly and histologically (light microscopy) normal. The mesenteric lymph node cells were excised and examined as previously described. As expected, nearly all phagocytes with beads contained latex beads of both colors. Only 1 of 25 sequentially observed phagocytes with at least two latex beads contained beads of a single color (Table 1). Again, by using the binomial distribution and assuming each phagocyte had an equal opportunity to ingest beads of each color, the probability of 1 of 25 phagocytes containing beads of one color is 0.9998, or essentially 100%. Thus, in this instance, mesenteric lymph node phagocytes most likely had an equal opportunity to ingest beads of both colors, and

indeed in this dog (dog D), the bead colors were mixed in the intestinal segment. As further statistical analysis of the results (Table 1), a chi-square analysis of the data from dogs A, B, C, and D shows that, for each of the dogs with the two bead colors implanted into different intestinal segments (dogs A, B, and C), the numbers of phagocytes containing latex beads of a single color of latex bead are statistically different from those of dog D with the bead colors mixed in a single intestinal segment, with $P < 0.005$ in each instance.

By changing the focus of the microscope, it was clear that the fluorescent beads were intracellular and not merely adherent to the cell surface. Consecutive examination of cells with Nomarski differential interference contrast optics and epifluorescence revealed the fluorescent beads to be in optimum focus when the cells were optically sectioned through the cytoplasm as seen by differential interference optics; focusing with differential interference contrast optics at the apical or basal surface of the cells resulted in a defocused image of the fluorescent beads. Electron micrographs of air-dried lymph node cells coated with gold palladium and observed under 20 kV showed that the beads were clearly within the cytoplasm of the phagocyte (Fig. 2).

It should be noted that the dog intestinal segments used were all jejunal segments and careful inspection showed that they were devoid of Peyer's patches (although the existence of less organized lymphoid follicles could not be excluded). This could be important because there is considerable evidence that Peyer's patches, with their phagocytic M cells, are the primary site of penetration of inert particles, such as latex beads and carbon (4, 9–11), as well as certain species of pathogenic intestinal bacteria (5, 8, 12, 15). The present study did not rule out the role of the Peyer's patch in particle uptake, but it did indicate that particle transport through the intestinal mucosa need not involve a grossly visible Peyer's patch. It is also important to point out that, although isolated intestinal segments were histologically normal by light microscopy, one cannot exclude the possibility that leukocyte activity was modulated in the artificial environment of the isolated intestinal segment. However, assuming this intestinal segment had such abnormalities as increased local inflammation and increased local pressure, this would not be incompatible with a number of clinical situations. Using this animal model, we can, therefore, document one method by which intestinal particles might be transported to the draining lymph node. However, the results presented herein must be challenged by other experimental models.

The results of the above experiments indicated that inert intestinal particles could be transported to the draining mesenteric lymph node by phagocytic cells. (However,

TABLE 1. Numbers of fluorescein- or rhodamine-labeled latex beads in 130 sequential phagocytes containing at least two beads in the mesenteric lymph nodes of dogs^a

Total no. of beads/phagocyte	No. (%) of phagocytes with beads											
	Fluorescein only				Rhodamine only				Fluorescein and rhodamine			
	Dog A	Dog B	Dog C	Dog D	Dog A	Dog B	Dog C	Dog D	Dog A	Dog B	Dog C	Dog D
2–3	23	3	1	0	35	8	7	1	2	1	2	14
4–6	10	2	3	0	13	8	7	0	1	0	0	4
7–10	10	1	1	0	6	0	0	0	2	0	0	2
>10	12	2	2	0	13	0	0	0	3	0	0	4
Total no. of phagocytes	55 (42)	8 (32)	9 (36)	0 (0)	67 (52)	16 (64)	14 (56)	1 (4)	8 (6)	1 (4)	2 (8)	24 (96)

^a Dogs A, B, and C each had two isolated jejunal segments, each implanted with either fluorescein- or rhodamine-labeled latex beads. Dog D had a single isolated jejunal segment implanted with a mixture of fluorescein- and rhodamine-labeled beads. See text for statistical analyses of the data.

FIG. 1. Cytospin centrifuge preparations of unstained mesenteric lymph node cells recovered after implantation of latex beads into isolated dog jejunal segments. Phagocytic cells contain either 1.09- μm -diameter fluorescein-labeled latex beads (A) or 0.89- μm -diameter rhodamine-labeled latex beads (B). The fluorescein-labeled beads appear clear, and the rhodamine-labeled beads appear black. Also shown are fluorescein-labeled *E. coli* before injection into rat ligated ileal loops (C) and a rare mesenteric lymph node phagocyte with intact fluorescein-labeled *E. coli* (D). Bar, 5 μm .

FIG. 2. Electron micrographs of lymph node cells from a dog who had a single isolated jejunal segment implanted with a mixture of fluorescein- and rhodamine-labeled latex beads. (A) Mesenteric lymph node cells and a cell containing multiple spherical intracellular latex beads lying under the cell membrane. (B) Ileal lymph node cells from the same dog for contrast; here no intracellular fluorescent beads were detected, and a mixture of fluorescein- and rhodamine-labeled beads was subsequently added to the cell preparation to demonstrate the appearance of extracellular latex beads. Bar, 2 μ m.

neither the initial site of particle entry into the intestinal mucosa nor the initial site of particle ingestion by macrophages was clarified.) It would be interesting to know if the same process of phagocytic transport might apply to live intestinal bacteria. Unlike fluorescein-labeled latex beads, it is relatively difficult to study live fluorescein-labeled bacteria that replicate, degrade, lyse, or otherwise dilute the label. In addition, phagocytosis of fluorescein-labeled *E. coli* might be expected to cause degradation of the fluorescein label within phagolysosomes, producing "glowing" phagocytes that would be indistinguishable from the autofluorescence liberated by dead cells. To study intestinal transport of *E. coli*, a rat model was used. As preliminary experiments, three 5-cm ligated, sequential rat ileal segments were prepared in each of three 200-g male Sprague-Dawley rats (Bio-Lab Corp., St. Paul, Minn.). Each outer segment was injected with 0.7 ml of either 4.5×10^{10} fluorescein- or rhodamine-labeled latex beads per ml; 6 h later, the draining mesenteric node contained occasional phagocytes with beads. For example, as a typical result from one rat, of 130 phagocytes with beads, 50 cells contained from 4 to 40 beads per cell; 77 cells contained only rhodamine-labeled beads, 53 cells contained only fluorescein-labeled beads, and only 5 cells contained both bead colors. These preliminary experiments documented that transport of intestinal latex beads could be detected in only 6 h in rat ligated ileal loops (although the results were not as dramatic as in the dog model), and it might be possible to view undegraded fluorescein-labeled *E. coli* in that time span. As a final experiment, 3 ml containing 1.5×10^{10} saline-washed, fluorescein-labeled (14) *E. coli* was injected into 35-cm rat ligated ileal segments (proximal to

ileocec valve). After 6 h, the single draining mesenteric lymph node was excised, and single-cell suspensions were viewed under a fluorescence microscope. Compared with similar control rat mesenteric lymph node cells, occasional lymph node cells appeared to glow and could not be distinguished from dead or dying cells. Also, very occasionally, cells had what appeared to be glowing phagolysosomes, with no distinguishable intracellular bacteria. However, rare cells appeared to contain fluorescein-labeled *E. coli* that could still be seen as typical rod-shaped bacteria (Fig. 1C and 1D); these bacteria-laden phagocytes were extremely rare, i.e., approximately 30 to 100 such cells per mesenteric node preparation (approximately 10^8 cells) from any given rat ($n = 4$). Because of the extreme rarity of mesenteric lymph node cells associated with live bacteria, we could not confirm the intracellular nature of the bacteria by electron microscopy. However, the extreme rarity of these mesenteric lymph node cells, coupled with the relatively large number of bacteria associated with an individual leukocyte, indicated (but did not prove) that the phagocytic transport of live bacteria might be analogous to that of inert latex beads.

It is known that once a particle enters the mesenteric lymph node, it can then enter the blood stream (and thence, seed other tissues) via the thoracic duct. We do not yet know if translocation of intestinal particles occurs as an ongoing process in a normal host. It is possible that potentially pathogenic microbes are frequently engulfed by phagocytes and transported to the draining lymph node while intracellular killing occurs. Therefore, in immunocompromised patients, the frequency of systemic infection with normal intestinal flora (14, 20) could reflect a failure of the normal

mechanism of intracellular killing by phagocytic cells. The present study is an important first step in clarifying these issues because it provides evidence that intestinal particles can be transported to the draining lymph node by phagocytic cells.

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