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Report

A Method for Quantifying Particle Absorption from the Small Intestine of the Mouse

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We have developed a method for quantifying the absorption of model fluorescent latex particles from the mouse small intestine into Peyer's patches, mesenteric lymph nodes, and spleen. The procedure combines a simple and exhaustive particle recovery technique with a highly sensitive particle counting technique. Mice were orally gavaged with fluorescent polystyrene latex suspensions, and at various time points Peyer's patches, normal absorptive small intestinal tissue, mesenteric lymph nodes, and spleen were collected. The tissue samples were solubilized using an aqueous potassium hydroxide and surfactant solution and particles were counted using a flow cytometer. Using this method we were able to detect and quantify small numbers of particles, measure the course of uptake and clearance, and determine the tissue distribution of absorbed particles. Data generated using this technique indicate that particle absorption depends on the dose level, particle size, and fed state of the animals.

KEY WORDS: Peyer's patches; particle absorption; particle quantitation; M cells.

INTRODUCTION

Intestinal absorption of particulate matter and macromolecules as a potential means of immunization (1,2), of macromolecular drug delivery (3,4), and of entry of pathogens (5,6) and toxins (7) has been studied. Experiments indicate that the primary portals for entry of particulate material are the gut-associated lymphoid tissues (GALT), represented by the Peyer's patches (8,9). The follicle-associated epithelium (FAE) overlying the Peyer's patches contains a population of phagocytic cells called M cells that are responsible for absorbing particulates. Particles absorbed into Peyer's patches proceed via the mesenteric lymph to the systemic circulation and are distributed, depending on their size and physical properties, to the various organs of the reticuloendothelial system (RES).

Numerous methods for measuring absorption of particulates, which include bacteria (10), viruses (11), polymer latex (12), and other particulates (13), by Peyer's patches have been reported. Direct counting of particles in tissue via microscopy is the most sensitive method, however, because the particles are not uniformly distributed within the follicles of Peyer's patches or within other organs, the amount of tissue sectioning required for exhaustive particle counting is prohibitive. Macroscopic methods such as recovery of particles from digested tissue using gradient centrifugation or filtration are limited by their sensitivity and thus require chronic feeding models rather than a single oral dose. This limits the ability to use quantitative dosing regimens. The use of radiolabeled particles is limited by the stability of the label and the availability of test particles. In addition, high radiation doses are required to obtain measurable numbers of particles in Peyer's patches because of the very low efficiency of uptake. For example, with a detection limit of 10 particles, using particle doses equal to those in the experiments described here would require up to 1 mCi of a high-efficiency gamma emitter per mouse per dose. The method of particle quantitation that we have developed combines a simple and exhaustive particle recovery technique with a highly sensitive particle counting technique. Mice were orally gavaged with polystyrene latex suspensions, and at various time points Peyer's patches, normal absorptive intestinal tissue, mesenteric lymph nodes, and spleen were collected. Tissue samples were solubilized using an aqueous potassium hydroxide and surfactant solution, and the particles were counted using a flow cytometer. Using this method we were able to detect and quantify small numbers of particles, measure the time course of uptake and clearance, and determine the tissue distribution of absorbed particles.

MATERIALS AND METHODS

Particle Dosing and Tissue Collection

Young adult female BALB/c mice were used for all of the particle uptake experiments. Five groups of three mice were given a single oral gavage of 0.2 ml of particle suspension, the concentration of which depended on the size of the particles and the intended dose. Fasted mice, as defined here, were housed in wire cages and were deprived of food for 12 hr prior to dosing and 1 hr postdosing. Fed mice were permitted to feed ad libitum. At 1, 2, 4, 7, and 14 days after dosing, groups were sacrificed and dissected. Mesenteric lymph nodes and spleen were removed prior to excising the

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small intestine to prevent contamination of the samples with the luminal contents. Peyer's patches and a 3-cm length of normal absorptive small intestinal tissue that contained no Peyer's patches were removed, washed with 0.85% saline, and suspended in 0.85% saline for approximately 1 hr to remove adherent particles. The particles did interact closely with the mucus layer associated with the intestinal tissues. On day 1 adherent fluorescent particles were visibly detectable even after washing, and thus day 1 results are not reported for these samples. Fluorescence microscopy of the excised tissue showed that adherent fluorescent particles were not present from day 2 onward. Tissues from the three mice in each group were pooled and processed as one sample. All samples were frozen until further processing.

Particles

Fluorescent, monodisperse, polystyrene latex spheres (Polysciences) were used for all particle uptake experiments as models for nonspecifically absorbed inert materials. The particles were obtained as 2.5 (w/v%) aqueous suspensions and were diluted with distilled water to the desired latex concentrations. Gravimetric determinations of the weight fraction of particles agreed with the manufacturer's stated value.

The particles exhibited yellow-green fluorescence with a maximum excitation wavelength of 458 nm and maximum emission at 540 nm. Fluorescence enabled us to distinguish the test particles from a variety of particulate material contained in the tissue samples. The individual particles showed uniform levels of dye content which facilitated the counting using the flow cytometer. The measured coefficient of variation (CV) of the fluorescence, as determined by the manufacturer, was less than 5%. Consistency of dye content was determined using a fluorescence-activated cell sorter by demonstrating the equivalency of the light-scattering and fluorescence distributions of untreated particles and particles that had been subjected to the tissue dissolution procedure described below. Two particle sizes, 2.65- and 9.13-μm diameter, were used in the experiments. The measured CV of the diameters, as determined by the manufacturer, was less than 3%.

Tissue Dissolution

Tissue samples were cut into small pieces and dissolved by adding 2 ml of a solution of 1% Triton X-100 and 1% KOH and heating to 60°C for 72 hr. Dissolved samples were diluted to 10 ml with 1% Triton X-100 and centrifuged (Sorvall RL-5B, HS-4 rotor) at 25,000*g* for 4 hr to pellet the latex particles. Nine milliliters of the supernatant was pipetted off and the pellet was resuspended in 9 additional ml of surfactant solution. Centrifugation and decantation were repeated and the particles were finally suspended in 3 ml of 1% Triton X-100. Microscopy and flow cytometry showed that the size, shape, and spectral properties of the fluorescent particles were unaffected by the tissue dissolution and particle recovery process.

Particle Counting

Particle counting was accomplished using a Beckton–Dickinson FACStar fluorescence-activated cell sorter (FACS). In the flow cytometer the sample, a uniform particle suspension, was passed as a fine jet through a narrow beam of laser light of the excitation wavelength of the fluorescent particles. The intensity of fluoresced light that results from the excitation of the particles within the sample stream was detected with a photomultplier situated at 90° to the angle of incidence. Forward scattering intensity, which was indicative of particle size, shape, and refractive index, was also detected. The forward scattering (FS) and green fluorescence (FL) signals were used to differentiate between the test particles and other particles in the treated samples. The uniformity in size and fluorescent dye content of the latex test particles resulted in a narrow distribution in the resulting forward scattering and fluorescence signals. Minimum and maximum FS and FL values were used to define a gate in the FL versus FS space which included the latex particles, and all signals falling within that gate were counted as test particles. Approximately 20% of the 3-ml volume of each sample suspension was analyzed using the FACS and the particle counts obtained from the fraction analyzed were multiplied by an appropriate factor to obtain the total number of particles contained in the sample. The multiplying factor was determined by weighing the sample feed container before and after the analysis to calculate the fraction of the suspension that had actually been analyzed. Calibration samples were periodically run to identify and correct for signal drift and variations in the fluidics. A schematic of graphical output from the flow cytometer is shown in Fig. 1. The points within the square gate represent fluorescent latex particles. Other particulate material detected in the samples generally exhibited low fluorescence and was recorded along the forward scattering axis of the plot.

The efficiency of the particle dissolution and counting process was determined by adding approximately 2200 test particles to Peyer's patch, normal intestine, spleen, and mesenteric lymph node tissue samples. The tissue samples with added particles were treated with the dissolution and counting procedure and the recovery was 94 ± 8% of the added particles. Analysis of tissues that contained no test particles resulted in an apparent number of particles of 4 ± 1 per sample for 2.65-μm particle detection settings and 0 for 9.13-μm particle detection settings. As a result four particles per sample was considered background for 2.65-μm particle

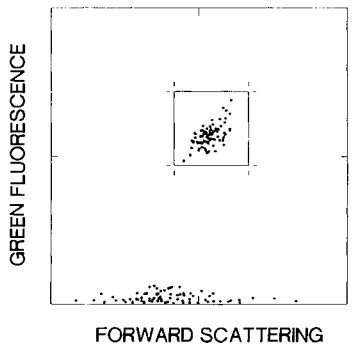

Fig. 1. Graphical output from the flow cytometer. The points within the square gate represent fluorescent latex particles. Other particles within the tissue are nonfluorescent and thus the points from the resulting signal appear near the forward scattering axis.

analysis, while the background for 9.13-μm particle analysis was zero.

RESULTS AND DISCUSSION

We were able to measure small numbers of particles in Peyer's patches, spleen, and mesenteric lymph nodes following a single oral gavage of particle suspension. As discussed in the particle dosing and tissue collection section, the samples were treated and analyzed as pools from three mice. All data are reported on a per-animal basis. The results from fasted mice that were dosed 10^8 of 2.65-μm-diameter particles are shown in Fig. 2. The data for Peyer's patches and normal small intestinal tissue on day 1 are not included because a disproportionate number of particles were found. Large numbers of particles were visibly detectable in the lumen of the small intestine on day 1 after dosing and the high particle counts were attributed to an inability to wash completely these particles from the tissues prior to processing. The long residence time of latex particles in the lumen of the mouse small intestine following oral dosing has been observed by other investigators (14). The gradual decrease in the number of particles found in the Peyer's patches and normal small intestinal tissue from day 2 onward was typical for these experiments. While particles were detected in normal small intestinal tissue, the number on a per-gram of tissue basis was generally an order of magnitude less than the number found in Peyer's patches. Fluorescent light microscopy of fixed tissue samples indicated that particle absorption via normal absorptive epithelium did not occur. Particle counts from normal absorptive epithelium were close to background and any counts above background were likely the result of adsorbed or physically entrapped material. The maximum number of particles found in the spleen usually occurred on day 1, with a comparable number on day 14. A minimum in the number of particles in the spleen between day 4 and day 7 was consistently observed. The minimum may have been the result of more than one mechanism of particle transport to, uptake by, or clearance from the spleen. The number of particles found in mesenteric lymph nodes was low and fluctuated with time but the average number was consistent with the levels found in the Peyer's patches and spleen.

Given the general observations cited above about the time course of tissue particle levels, the dose, fed state, and size dependence of absorption data are presented in terms of the maximum number of particles found in Peyer's patches and spleen. The maximum number of particles found is reported as a measure of uptake since the time course of absorption, distribution, and elimination of the particles varied with the dose, size, and fed state of the animals. The dose response of absorption into Peyer's patches and spleen for fasted mice given 10^6, 10^7, and 10^8 of 2.65-μm-diameter particles is shown in Fig. 3. While the maximum number of particles found increased with increasing dose, the number found was not linear in dose. The absorption mechanism, however, did not appear to be saturated over this dose range. The difference in absorption into Peyer's patches and spleen between fed and fasted mice dosed 10^8 of 2.65-μm-diameter particles is shown in Fig. 4. A greater maximum number of particles was detected in fed mice but the mechanism for the effect is unknown. Possibilities include an increase in gastrointestinal transit time or change in the residence time distribution and an increase in M-cell activity because of the presence of food.

There are several reports in the literature on the size dependence of particle absorption from the small intestine (7,12,14). It has been hypothesized that particles gain access to the mesenteric lymph by passing through the Peyer's patches, then proceed to the systemic circulation via the lymph and thoracic duct and lodge in the organs of the reticuloendothelial system (RES), which include the spleen. In addition, it has been shown that particles greater than 5 μm in diameter cannot escape the Peyer's patches and are therefore not distributed systemically (15). We experimented with two particle sizes, one above the 5-μm limit and one below. The results from fasted mice dosed 10^7 of 2.65- or 9.13-μm-diameter particles are shown in Fig. 5. Greater numbers of smaller particles than larger particles were found in Peyer's patches and the 9.13-μm particles were not found in the spleen or the mesenteric lymph nodes. These results

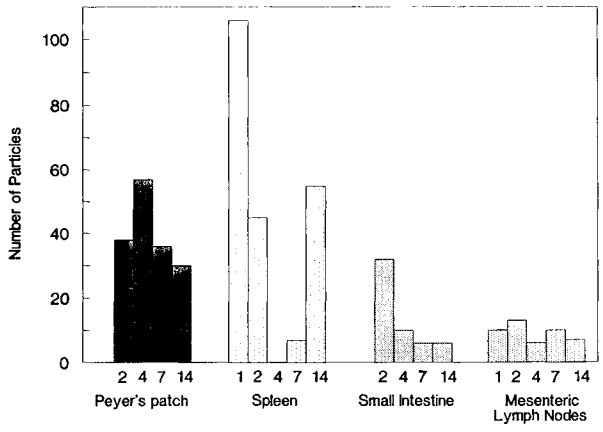

Fig. 2. Time dependence of the number of particles found in Peyer's patches, spleen, normal absorptive small intestinal tissue, and mesenteric lymph nodes following a single oral gavage of 10^8 2.65-μm-diameter particles. The time points are 1, 2, 4, 7, and 14 days after dosing.

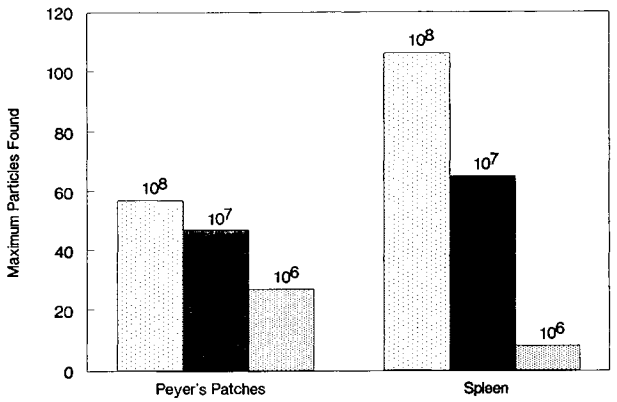

Fig. 3. Dose dependence of the maximum number of particles found in Peyer's patches and spleen. Mice were dosed 10^6, 10^7, and 10^8 2.65-μm-diameter particles by a single oral gavage. The absorption mechanism does not appear to be saturated over this dose range.

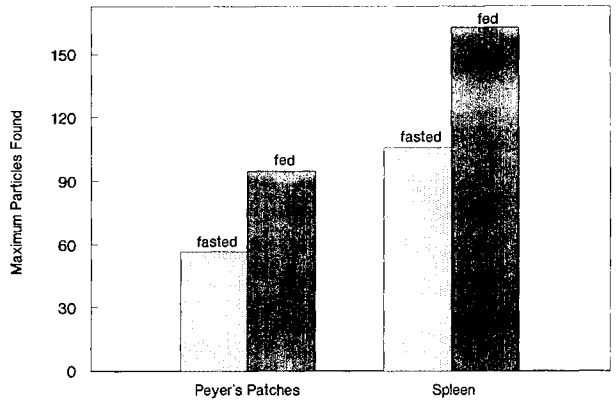

Fig. 4. The effect of fed state on the maximum number of particles found in Peyer's patches and spleen. Mice were dosed 10^8 2.65-μm-diameter particles by a single oral gavage. Mice were fasted for 12 hr prior to dosing. Fed mice were allowed to feed ad libitum.

agree with previous findings on the size dependence of absorption.

The examples given here demonstrate a quantitative method of measuring the number of particles absorbed into the Peyer's patches, small intestinal tissue, mesenteric lymph nodes, and spleens of mice following oral dosing of fluorescent latex. While we were interested in Peyer's patch absorption as a means of achieving oral immunization and thus examined particle uptake by the spleen, extension of the method to include the liver, lungs, and kidneys should be straightforward. The data generated using the technique indicate that particle absorption did depend on the dose level, particle size, and fed state of the animals. Although total-body counts of particles were not conducted, the results indicate that less than 0.01% of doses in the 10^6 to 10^8 particle number range are absorbed. Whereas this would seem to preclude delivery of systemic drugs, the level of absorption may be adequate for oral immunization.

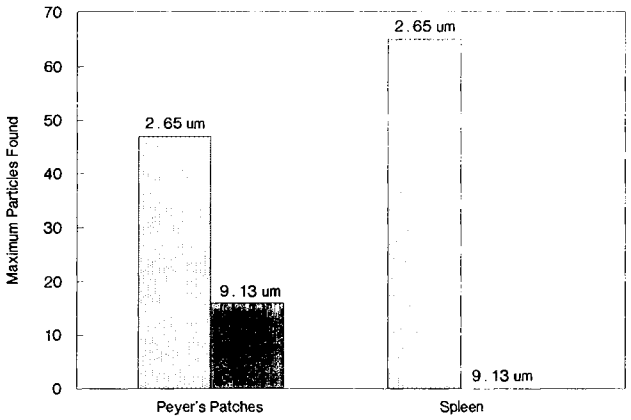

Fig. 5. Size dependence of the maximum number of particles found in Peyer's patches and spleen. Mice were dosed 10^7 2.56- or 9.13-μm particles by a single oral gavage. No 9.13-μm particles were found in the spleen.

The method efficiently provides information about particle absorption from the small intestine of mice. The procedure is as accurate as, and less labor intensive than, direct optical counting in fixed tissue. Optical counting in fixed tissue, however, does provide information on the spatial distribution of the particles. The technique provides a potential means to examine closely the effects of particle size and surface properties such as charge polarity, charge density, and surface energy on absorption. Future work should examine the kinetics of particle absorption and elimination, include other organs of the RES to quantify better the extent of absorption and distribution, and extend the dose range to lower doses to determine the maximum absorption efficiency.

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