Ultrastructural effects of intravascularly injected polyethylene glycol-hemoglobin in intestinal mucosa

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Baldwin, Ann L., Lisa M. Wilson, and J. Edward Valeski. Ultrastructural effects of intravascularly injected polyethylene glycol-hemoglobin in intestinal mucosa. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H615–H625, 1998.—Polyethylene glycol (PEG)-conjugated Hb (PEG-Hb) is being considered as a blood substitute. Previously, we showed that PEG-Hb extravasates rapidly from the intestinal mucosa and causes transient epithelial sloughing, resulting in temporary unimpeded passage of material between the intestinal lumen and the microcirculation. The present study quantifies the time course of factors related to this disturbance. Anesthetized Sprague-Dawley rats (350–450 g) were injected with a bolus of PEG-Hb (10 mg/ml) in saline. Control animals received saline, alone or with Dextran 70 (5 mg/ml). After 2, 8, 15, 60, or 90 min, the small intestine was perfusion fixed for microscopy (4 animals for each time point). Epithelial cell detachment and mucosal mast cell degranulation peaked at 2 and 8–15 min, respectively, but by 90 min were back to normal. Goblet cell secretion increased with time up to 8–15 min, after which it leveled off. Mean interstitial width was significantly greater 8 min after injection than for controls and continued to increase with time. In capillaries, endothelial fenestral diaphragms were replaced by thick, amorphous structures. Mesenteric mast cell degranulation was significantly greater 60–90 min after injection compared with controls. We propose that these results are consistent with intravascular injection of PEG-Hb invoking a transient inflammatory response in the intestine.

Two necessary requirements for Hb-based blood substitutes are 1) that they remain in the circulation for an appropriate time period and 2) that when they do leave the circulation they do not cause tissue damage. A problem with Hb-based blood substitutes is that they may leave the circulation and produce cytotoxic side effects. One approach that is being taken to reduce extravasation is to bind Hb to a substance such as polyethylene glycol (PEG), in which case five molecules of PEG bind to each Hb molecule. This procedure increases the molecular Stokes-Einstein radius from 50 to 250 Å. However, we have shown that after bolus injection, PEG-Hb extravasates rapidly from intestinal mucosal capillaries and causes epithelial detachment in villi near Peyer’s patches (2). These ultrastructural effects are transient, and the epithelium starts to repair after 60–90 min. The fact that PEG-Hb causes epithelial detachment is an important observation with regard to its use as a Hb-based oxygen carrier, because loss of gut mucosal integrity allows translocation of bacteria and endotoxins into the circulation, resulting in a systemic inflammatory response. The intestine is also important with respect to the immune system because the mucosa contains Peyer’s patches, or organized aggregates of lymphoid tissue between the villi. Peyer’s patches play a key role in the initiation and expression of mucosal immunity.

In our previous study (2) with electron microscopy, we showed, using a low dose of PEG-Hb (5 ml of 2 mg/ml), that PEG-Hb extravasates from intestinal mucosal capillaries via widened endothelial junctions and causes various ultrastructural changes in the intestinal mucosa and accompanying capillaries. Apart from epithelial detachment, these changes include mast cell degranulation, goblet cell secretion, and tissue edema. Mast cell degranulation is important because it results in release of inflammatory mediators such as histamine, leukotrienes, prostaglandin D2, and cytokines, which increase microvascular permeability, causing edema, and recruit and activate inflammatory cells such as eosinophils and neutrophils (12). Goblet cell secretion is important because it is a defense response of the intestinal mucosa to oxidant stress (19).

In the present study we compare our previous results to those obtained using a fivefold higher concentration, which has been used in transfusion experiments (8), and we quantitatively determine the time course of mast cell degranulation and goblet cell secretion. Using these new data, we propose a model to explain the mechanisms by which intravenously injected PEG-Hb transiently disrupts the tissue separating the mucosal microcirculation from the intestinal lumen.

Materials and Methods

PEG-Hb. The PEG-Hb was formulated in (mmol/l) 5 NaHCO3, 4 Na2HPO4, and 150 NaCl [methemoglobin < 5%, endotoxin < 0.5 endotoxin units (EU)/ml] at a concentration of 60 mg/ml and stored in 1-ml aliquots at −20°C in plastic microcentrifuge tubes. This product has a longer half-life in the circulation of dogs (58.3 h; Ref. 7) when the solution is exchange transfused 30% than does Hb (4.0 h; Ref. 15). Other details regarding PEG-Hb have been described elsewhere (15). Immediately before use, one vial was thawed at 4–8°C, diluted to 2 or 10 mg/ml with HEPES-buffered saline (HBS), and warmed to 37°C by placing the container in a water bath, and the pH was measured to ensure that it had not drifted from 7.4.

Experimental design. The following study was performed to characterize intestinal tissue damage caused by intravenous injection of PEG-Hb. A 5-ml bolus of 10 mg/ml PEG-Hb was injected via the aorta and allowed to circulate for 2, 8, 15, 60, or 90 min before we perfusion fixed the small intestine and prepared tissue samples for light and electron microscopy. Four animals were used for each time point. This number of

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animals was justified to be sufficient by utilizing a sample-size nomogram in conjunction with estimates of the difference in means that needed to be detected and the mean standard deviation for each parameter (21). The numbers of animals used for low-dose (2 mg/ml) experiments for 2, 8, 15, 60, and 90 min were 4, 6, 4, 4, and 4, respectively. Rats injected with either 5 ml of HBS (n = 33) or 5 ml of 5 mg/ml Dextran 70 in HBS (n = 5) and perfused fixed 2 min later served as controls. Clinical studies have proved that dextran solution is safe and effective as a plasma substitute (17). The colloid osmotic pressure (COP) of 10 mg/ml PEG-Hb was measured using a colloid osmometer (Wescor, Logan, UT) and was found to be 3 mmHg. In control experiments, Dextran 70 was used at a concentration of 5 mg/ml in PBS, which provided a COP of 2.8 mmHg as measured using the colloid osmometer. The viscosities of 10 mg/ml PEG-Hb and 5 mg/ml Dextran 70 were measured at 37°C using an Ostwald microviscometer (Cannon Instrument, State College, PA) and were found to be 0.94 and 0.81 cP, respectively. These values compare with a plasma viscosity of 1.2 cP.

To determine the effects of endotoxin, which was present at very low concentration (0.5 EU/ml) in the PEG-Hb samples, five further experiments were performed. A 5-ml bolus of HBS containing 0.5 EU/ml endotoxin was injected, and the intestinal tissue was fixed for light and electron microscopy 15 min later.

Surgical procedures. Male Sprague-Dawley rats (350–450 g) were anesthetized with pentobarbital sodium (6 mg/100 g body wt ip). In each rat, the aorta was cannulated just downstream from the superior mesenteric artery in a retrograde direction. The free end of the catheter tubing was connected to a reservoir of HBS, pH 7.4, 37°C, that could be pressurized to 100 mmHg to allow for perfusion and thus check that the cannulation had been successful. A loop of intestinal ileum, close to the cecum, was pulled outside the body cavity and arranged on a Plexiglas pillar attached to the plastic stage on which the rat was situated. PEG-Hb (50 mg; 10 mg/ml) in HBS was injected through a 0.2-µm filter via the aortic cannula and allowed to circulate with the blood for 2, 8, 15, 30, 60, or 90 min. In control rats, HBS or 5 mg/ml Dextran 70 (Sigma, St. Louis, MO) replaced PEG-Hb. After the chosen circulation time, the aorta was clamped proximal to (upstream from) the superior mesenteric artery and the intestinal circulation was perfused with Karnovsky’s fixative in phosphate buffer, pH 7.4, at 4°C. When perfusion was complete, the inlet pressure was dropped to 40 mmHg and the portal vein was clamped. The animal was killed with an intravenous injection of Beuthanasia. Fixation continued for 60 min, and fixative was also applied to the outside of the intestinal segment. After fixation, the intestinal loop was excised and cut into several segments, each ~1 cm long, some containing a Peyer’s patch; these segments were washed in buffered saline. Each segment was opened longitudinally and divided into squares a few millimeters long. Two squares were kept from each segment (one with a Peyer’s patch) and processed as described in Tissue preparation for light and electron microscopy. In 16 of the rats (6 low dose, 10 high dose), one portion of the mesentery was selected (close to the cecum for reproducibility), excised, spread flat on a microscope slide, and suffused with 1% crystal violet, then 15 min before being flushed with HBS and mounted. This procedure stains mast cells so that the number of degranulated cells may be counted.

Tissue preparation for light and electron microscopy. Tissue squares were immersed in diaminobenzidine (DAB) overnight in the dark to stain specific granules in immune cells and thus make the cells easier to identify. The DAB was prepared as follows (13). DAB (0.1 g) was added to 50 ml of 0.1 M monobasic phosphate buffer, and the pH was adjusted to 7.2 very gradually with concentrated NH4OH. The solution became a light tannish-pink color. Next, the tissue squares were rinsed in distilled water. Meanwhile, 25 ml of DAB solution were added to 1.66 ml of 3% H2O2, to give a final concentration of 0.2%. The tissue was placed in this solution for 60 min and then rinsed three times in 0.15 M sodium cacodylate buffer. Finally, the tissue was dehydrated in increasing concentrations of ethanol and embedded in Spurr’s resin. The pieces of tissue were oriented in the resin so that the blocks could be sectioned perpendicular to the villus plane. Thick sections (2 μm) were cut for light microscopy (Zeiss Axioplan), mounted on slides, and stained with toluidine blue. Ultrathin sections were cut for electron microscopy (Phillips CM12). Before we examined the sections under electron microscopy, the grids were stained with lead citrate and uranyl acetate.

Data acquisition and analysis. Thick sections, cut longitudinally through the intestinal villi, were examined under light microscopy to assess the integrity of the epithelium (detachment and cell separation or intact), the presence or absence of edema in the interstitium, the degree of distension of the lymphatics (collapsed or distended), and the numbers of degranulated mast cells and secreting goblet cells per villus cross section. Mast cells were categorized as degranulated if they exhibited empty vacuoles (see Fig. 3). Edema was quantified by measuring the width of villus interstitium...
between the epithelium on each side of the villus section. Each measurement was taken midway down the length of each villus. Only villus sections that contained a central lacteal were included, because these villi were centrally sectioned. For each parameter, four thick sections were examined from each experiment from two different regions of the tissue sample. Each section usually contained ~10 villi. Because our previous study (2) indicated that tissue alterations were most marked at Peyer's patches, we limited this investigation to villi surrounding Peyer's patches. Sections from the low-dose study were also included in this analysis because they had not previously been used for quantitative evaluation but only for descriptive observation. Degranulated mesenteric mast cells, identified by the presence of intracellular granules released into the surrounding tissue, were counted within each circular ×20 field of view of a Zeiss light microscope (field area 1.13 mm²). Rows of fields were counted systematically from left to right. Cells located in the periphery of the field were only counted if at least one-half of the cell area was within the field. The error of repeat counting was <2%. About 30 fields were counted for each mesenteric window.

Table 1. Measurements of villus interstitial width after injection of PEG-Hb

<table>
<thead>
<tr>
<th>Group</th>
<th>Villus Interstitial Width, µm</th>
<th>No. of Measurements</th>
<th>No. of Rats</th>
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<tbody>
<tr>
<td>Dextran control</td>
<td>44 ± 1</td>
<td>120</td>
<td>5</td>
</tr>
<tr>
<td>8 min, 2 mg/ml PEG-Hb</td>
<td>57 ± 1</td>
<td>180</td>
<td>4</td>
</tr>
<tr>
<td>60 min, 2 mg/ml PEG-Hb</td>
<td>52 ± 2</td>
<td>106</td>
<td>4</td>
</tr>
<tr>
<td>8 min, 10 mg/ml PEG-Hb</td>
<td>55 ± 1</td>
<td>156</td>
<td>4</td>
</tr>
<tr>
<td>60 min, 10 mg/ml PEG-Hb</td>
<td>148 ± 5</td>
<td>71</td>
<td>4</td>
</tr>
</tbody>
</table>

Values for villus interstitial widths are means ± SE. PEG-Hb, polyethylene glycol-conjugated Hb.
Fig. 3. A: light micrograph of section through part of an intestinal villus to show toluidine blue-stained degranulated mast cells (MC). Empty vacuoles are indicated by arrowheads. Scale bar, 25 µm. B: electron micrograph of section through intestinal villus to show degranulated mast cell (arrowhead). Scale bar, 5 µm. Note similarity between degranulated mast cells as seen by light and electron microscopy.

Fig. 4. Time course of mast cell degranulation after low-dose (A) and high-dose (B) injections of PEG-Hb. Error bars represent SE. *Statistically significantly different from control ($P < 0.05$).

Fig. 5. Part of mesentery shown en face to demonstrate degranulated connective tissue mast cells (DMC), as identified by presence of intracellular granules that have been released into surrounding cytoplasm. Intact mast cells (arrowhead) can also be seen. Scale bar, 25 µm.
RESULTS

Light microscopy. Control preparations showed an epithelium that was intact, or in which the cells were slightly separated from each other, at the basal region of the intercellular junctional membrane, at villus tips (Fig. 1). The interstitium was tight and nonedematous, the lymphatics were collapsed (Fig. 1), and the goblet cells were not usually secreting. Animals that had received a bolus injection of Dextran 70 showed intestinal mucosal villi similar to those of saline controls. Rats that had received a bolus injection of PEG-Hb showed time-dependent alterations in villus morphology. These changes were similar whether a high or a low dose had been administered. Light micrographs from low-dose experiments are shown in Fig. 2. Some villi are shown sectioned longitudinally (Fig. 2, A–C) and one transversely (Fig. 2D). However, similar characteristics can be observed regardless of the specific sectioning plane. After 2 min, the epithelial cells were detaching from the basement membrane in some regions, usually at the villus tip (Fig. 2A) and separating from each other in other regions (Fig. 2C). The degree of separation was much greater than that sometimes seen at villus tips in control preparations. This response gradually became less pronounced with time, until after 90 min the condition of the epithelium was almost restored to normal (Fig. 2D). After 2 min, the central villus lymphatic vessels were distended and the interstitium was edematous (Fig. 2A). Mean values of interstitial width are given in Table 1. Values obtained 8 and 60 min after injection of a low or a high dose of PEG-Hb were significantly greater than those from dextran controls. Goblet cells continued to secrete mucin, regardless of the time that had elapsed since injection of PEG-Hb (Fig. 2D). Animals that received 0.5 EU/ml endotoxin showed a small degree of epithelial detachment in some villi after 15 min. However, the extent of detachment was minor compared with that observed in animals fixed 15 min after receiving PEG-Hb.

Degranulated mast cells. Mucosal degranulated mast cells (DMC) were easy to identify by light microscopy because they stained intensely with toluidine blue and demonstrated empty vacuoles as shown in Fig. 3A. An electron micrograph of a mucosal DMC is shown in Fig. 3B to demonstrate the similarity between the cells as seen by light and electron microscopy. The electron micrograph of the DMC is typical of those shown in the literature (see, e.g., Ref. 3). The time course of mast cell degranulation after injection of PEG-Hb is shown in Fig. 4, A and B (low dose and high dose, respectively). Values at zero time are from dextran controls. The number of villi in which counts were made for low-dose experiments were 166, 34, 43, 32, 78, and 68 for control, 2, 8, 15, 60, and 90 min, respectively. Corresponding values for high-dose experiments (not including controls) were 63, 93, 96, 57, and 80. In both cases, mast cell degranulation peaked at 8–15 min and dropped at 60 min. The high dose showed a further increase of degranulation at 90 min. All measurements are significantly greater than control values, except for those taken at 60 min after injection for both doses and at 90 min for the low dose. The values obtained at a given time point are not significantly different from each other.

<table>
<thead>
<tr>
<th>Group</th>
<th>DMC per Field of View</th>
<th>No. of Views</th>
<th>No. of Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextran control</td>
<td>1.4 ± 0.2</td>
<td>105</td>
<td>4</td>
</tr>
<tr>
<td>60 min, 2 mg/ml PEG-Hb</td>
<td>3.9 ± 0.8</td>
<td>107</td>
<td>4</td>
</tr>
<tr>
<td>60 min, 10 mg/ml PEG-Hb</td>
<td>12.7 ± 1.5</td>
<td>118</td>
<td>4</td>
</tr>
</tbody>
</table>

Values for nos. of degranulated mast cells (DMC) per field of view are means ± SE.
other when low and high doses are compared except at 90 min, when the high dose gives a significantly higher value. The three rats that received endotoxin showed $0.80 \pm 0.12$ (SE) ($n = 70$) DMC per villus, which was significantly less than for specimens fixed 15 min after injection of PEG-Hb [$1.32 \pm 0.14$ (SE)].

Connective tissue mast cell degranulation in the mesentery was easily visualized after injection of PEG-Hb (Fig. 5). Mean values of numbers of degranulated mast cells per field of view are shown in Table 2.

Mast cell degranulation 60 min after injection of 2 mg/ml PEG-Hb was significantly greater than for dextran controls. Degranulation 60 min after injection of 10 mg/ml PEG-Hb was significantly greater than for the lower dose. Thus concentration of PEG-Hb appears to make a difference in degranulation of mesenteric mast cells.

Secreting goblet cells. The time course of goblet cell secretion is shown in Fig. 6, A and B (low dose and high dose, respectively). In both cases, numbers of secreting...
goblet cells per villus section increased significantly, compared with control values, by 8–15 min and then gradually leveled. Between 8 and 90 min the low dose of PEG-Hb caused significantly more goblet cell secretion than the high dose.

Electron microscopy. Figure 7, A and B, shows typical electron micrographs of an HBS control and a dextran control, respectively. The epithelial cells are firmly attached to the basement membrane, and tight epithelial junctions can be seen. Intraepithelial lymphocytes are positioned between the epithelial cells, usually close to their basal aspect. Transverse sections through mucosal capillaries demonstrate that the endothelium, subjacent to the epithelium, is thin and contains fenestrae. The interstitium shows an abundance of apparently normal lymphoid cells and extracellular collagen fibers.

Electron micrographs of preparations from animals injected with PEG-Hb for 2, 8, 15, and 90 min are shown in Fig. 8, A–D, respectively. Because there were no noticeable differences between the ultrastructural effects of high and low doses of PEG-Hb, we have pooled the photographs; Fig. 8, A, C, and D, are from high-dose experiments, and Fig. 8B is from a low-dose experiment. After PEG-Hb has circulated for 2 min (Fig. 8A), the epithelium is disrupted and the cells show many elongated pseudopodia. After 8 and 15 min (Fig. 8, B and C), the epithelium is clearly becoming detached from the basement membrane. In Fig. 8B, an epithelial cell can be seen that has detached, and a migrating cell, probably a leukocyte, has taken its place. Very little contact is apparent between neighboring epithelial cells. In Fig. 8C, a cell that is probably a mast cell as identified by its granules, which are stained intensely with DAB, is sandwiched between an intraepithelial lymphocyte and two detaching epithelial cells. A degranulating mast cell is visible in Fig. 8A within the villus interstitium. Although the interstitium is still fairly tight just beneath the epithelium where the mucosal capillaries are located, it is very edematous deeper within the villi (see Fig. 8, A and B). Very often, groups of collagen fibers were seen oriented circumferentially around mucosal capillaries in the subepithelial region in a highly organized fashion (see Fig. 8B). Such dense arrays of circumferentially arranged collagen fibers are demonstrated in Fig. 9, 15 min after PEG-Hb injection. Control preparations did not show such preferential orientation of collagen fibers; the fibers were arranged more randomly (Fig. 7, A and B). Previous authors (14) have suggested that collagen-epithelial interactions are important in restitution after injury and that collagen can be produced locally and rapidly at the site of injury to allow restitution of the villus tip epithelium to proceed. However, because we observed alterations in collagen fibers only 2 min after injection of PEG-Hb, it is highly unlikely that extra collagen was synthesized. Probably, the collagen fibers were just rearranged.

With regard to mucosal capillaries, a thickening of the endothelium was often visible in the subepithelial region. Plasmalemmal vesicles could be seen in the thickened region (Figs. 8B and 9). Normally, the subepithelial region of the endothelium is very thin and fenestrated and does not possess many vesicles (Fig. 7, A and B). This gradual disappearance of normal fenestrae and thickening of the endothelium apposing the disrupted epithelium are consistent with other studies.

Fig. 9. Electron micrograph of transverse section through intestinal mucosa of rat injected with PEG-Hb allowed to circulate for 15 min. Note dense array of circumferentially arranged collagen fibers (arrows). Scale bar, 2 µm.

Fig. 10. Electron micrograph of transverse section through intestinal mucosa of rat injected with PEG-Hb allowed to circulate for 60 min. Note eosinophil (arrow) positioned between 2 epithelial cells (E). In mucosal capillary, portion of endothelium closest to epithelium is thicker than normal (arrowhead). Scale bar, 2 µm.
that have demonstrated similar fenestral changes accompanying epithelial damage. For example, in an ultrastructural study of the guinea pig vas deferens and ureter, the authors observed fenestrated capillaries only near the epithelium in both organs and noted that the fenestrae tended to occupy the side of the capillary facing the epithelium (6). When the mucosa of the vas deferens or the ureter was transplanted, fenestrated capillaries were seen only in transplants containing epithelium. Capillaries in transplants stripped of epithelium lost their fenestrae.

Ninety minutes after injection of PEG-Hb, a more intact epithelium was evident; the epithelial cells formed a continuous layer and showed a greater number of intercellular connections (Fig. 8D). However, the interstitium was usually fairly edematous, degranulated mast cells were sometimes evident, and the endothelium was usually still thickened at the subepithelial aspect.

All preparations that had been subjected to intravenous injection of PEG-Hb showed occasional eosinophils in the villus interstitium. These cells were easily identified by their bilobed nucleus and elliptical DAB-stained granules, each of which was bisected by a darker band along the major axis. An example of an eosinophil, from a preparation fixed 60 min after injec-

Fig. 11. Electron micrographs of transverse sections through intestinal mucosal capillaries of rats injected with HBS (A), PEG-Hb for 15 min (B), and PEG-Hb for 90 min (C). In A and C, junctions (J) show focal contacts. Such contacts are not visible in B. Scalebar, 1 µm.

Fig. 12. Electron micrographs of transverse sections through intestinal mucosal capillaries of rats injected with PEG-Hb allowed to circulate for 2 (A) and 8 (B) min. Endothelial fenestrae (arrowheads) show single diaphragms in A, but in B, fenestral diaphragms have been replaced by a thicker, fuzzy layer. L, lumen. Scale bar, 2 µm.
tion of PEG-Hb, is shown in Fig. 10. This eosinophil is positioned between two epithelial cells. The thickened subepithelial endothelium of a mucosal capillary can also be seen.

Endothelial junctions and fenestrae. As reported in our previous study (2), endothelial junctions, which usually possess focal contacts (zonulae adherens) as shown in Fig. 11A, lost these contacts after injection of PEG-Hb (Fig. 11B), thus opening the junctions to the basement membrane, resulting in increased capillary permeability. Ninety minutes after PEG-Hb injection, there was some evidence that the zonulae adherens were reforming (Fig. 11C).

The fenestrae in the endothelium of mucosal capillaries gradually changed in appearance after injection of PEG-Hb. This alteration was not observed as rapidly as some of the other changes, such as epithelial disruption and edema, and 2 min after injection of PEG-Hb the fenestrae looked normal (Fig. 12A). After 15 min (low dose) or 8 min (high dose), single-diaphragmed fenestrae

![Fig. 13. Electron micrograph of a control preparation (HBS) to show multidiaphragmed fenestrae (arrowheads). Scale bar, 1 µm.](image)

![Fig. 14. Time course for fenestral changes after low-dose injection of PEG-Hb. A: number of single-diaphragmed fenestrae. B: number of multidiaphragmed fenestrae. C: number of transition fenestrae. Error bars represent SE. *Significantly different from control (P < 0.05).](image)

![Fig. 15. Time course for fenestral changes after high-dose injection of PEG-Hb. A: number of single-diaphragmed fenestrae. B: number of multidiaphragmed fenestrae. C: number of transition fenestrae. Error bars represent SE. *Significantly different from control (P < 0.05).](image)
trae started to disappear as the fenestrated portion of the endothelium thickened. The single diaphragm was replaced by a thicker, fuzzy layer, which we called a “transition” fenestra (Fig. 12B). Sometimes double- and triple-layer fenestrae were seen, but they were also present in controls (Fig. 13). Fenestrae were grouped according to these three categories, and their relative numbers per capillary cross section were recorded for each time point. The results are shown in Figs. 14 (low dose) and 15 (high dose). By 60 min, the numbers of single-diaphragmed fenestrae per capillary cross section were significantly lower than control for both doses of PEG-Hb. Multilayered fenestrae were less abundant than single-diaphragmed fenestrae in control preparations. Low-dose PEG-Hb had little effect on numbers of multilayered fenestrae until 90 min after injection, but high-dose PEG-Hb reduced their numbers further. Numbers of transition fenestrae significantly increased after both low and high doses of PEG-Hb.

DISCUSSION

This study has shown that intravascular injection of PEG-Hb causes cellular and interstitial transient responses in the intestinal mucosa and mesentery. The effects on intestinal ultrastructure and mucosal mast cell degranulation were similar whether 2 or 10 mg of PEG-Hb are contained in the 5-ml bolus injection. However, goblet cell secretion was less marked with the higher dose, whereas villus interstitial edema and mesenteric connective tissue mast cell degranulation were more marked with the higher dose. The responses we observed are similar to those produced by inflammation of the intestine and of the airways (4, 16, 18). Inflammation of the intestine causes infiltration of intestinal tissues by lymphocytes, eosinophils, and neutrophils (9) as well as increased mast cell counts (1, 5). In addition, enlargement of the central lymphatic capillary at the core of the villi and edema of the lamina propria are observed 6 days after trinitrobenzenesulfonic acid-induced gut inflammation (11). In a model of chronic granulomatous colitis in rats, epithelial sloughing was observed 3 wk after induction of inflammation, producing direct contact between the intestinal lumen and the interstitium. Large accumulations of mucus were also seen (20).

Asthma and related allergic diseases cause similar responses in airways. Mast cells, basophils, lymphocytes, eosinophils, and endothelial cells are involved. In the tissues, the eosinophils degranulate, releasing toxic proteins that damage the respiratory epithelium. The airways are blocked by mucus. Collagen is deposited beneath the membrane (16). The airway and intestinal epithelia share the property that they are uniquely vulnerable because of their potential for exposure to both endogenous and exogenous agents, and so it is not surprising that they demonstrate similar responses when irritated. The important point to note is that injection of PEG-Hb produces responses that are almost identical to those seen in inflammation of the intestine and the airways but that these responses are activated much more rapidly, i.e., within minutes rather than days or weeks.

Recently, it has been suggested that airway inflammation is generated by reactive oxygen species (ROS) (4, 18). It is possible that the responses of the rat intestinal mucosa to intravascular bolus injection of PEG-Hb are also caused by an oxidant-antioxidant imbalance. Some of the responses that we observed after injection of PEG-Hb are consistent with those that are known to be produced by excess ROS. For example, goblet cells in the epithelium of conducting airways are stimulated by ROS to secrete vast quantities of mucus in fractions of a second (19). Secretion of mucus is a protective mechanism, because it has been shown that mucus has antioxidant activity (10). Experiments are now underway to test the hypothesis that the intestinal inflammatory response produced by intravascular injection of PEG-Hb is triggered by generation of excess ROS.

The disruption of the tissue between intestinal capillaries and the intestinal lumen by a bolus injection of PEG-Hb, although apparently transient, has important implications regarding its use as a blood substitute. This response, which lasts for at least 90 min in rats, enhances extravasation of the PEG-Hb and also increases transport of plasma proteins from the bloodstream into the intestinal lumen. Thus transport of nutrients and drugs between blood and tissue will also be compromised during this time period. Because the damage is resolved in a few hours, it may easily remain undetected in routine preclinical safety studies. However, the interaction of PEG-Hb with pathological states such as prolonged ischemia and hemorrhagic or septic shock is unknown. Such pathological states will often be evident in patients in need of transfusions. Therefore, it is vital to develop protocols to reduce intestinal extravasation and subsequent inflammation.

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