Neutrophil depletion prevents intestinal mucosal permeability alterations in burn-injured rats

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Sir, Özcan, Nadeem Fazal, Mashkoor A. Choudhry, Richard L. Gamelli, and Mohammed M. Sayeed. Neutrophil depletion prevents intestinal mucosal permeability alterations in burn-injured rats. Am J Physiol Regulatory Integrative Comp Physiol 278: R1224–R1231, 2000.—Cutaneous thermal injury increases intestinal mucosal permeability. The mechanisms of this functional disturbance are not fully understood. We investigated whether accumulation of neutrophils in the intestine contributes to the increase in mucosal permeability. Labeled and unlabeled lactulose and mannitol were infused into a segment of rat ileum or jejunum. Blood concentrations of [3H]lactulose and [14C]mannitol were measured after 30, 60, and 90 min. On day 1 postburn, lactulose permeability increased fourfold in the ileum and twofold in the jejunum compared with sham-burned rats; mannitol permeability increased twofold in the ileum and 1.5-fold in the jejunum. A greater increase in permeability occurred on day 3 postburn in the ileum, but not in the jejunum. The depletion of neutrophils in burned rats prevented the increase in permeability in both segments on day 1 postburn. Histological studies of intestines from burned, with or without neutrophil depletion, and sham-burned rats showed similar morphology. However, numerous neutrophils were found in the extravascular compartment in day 1 postburn, but not in neutrophil-depleted and sham-burned rats. These findings support the concept that the burn-induced increase in mucosal permeability is produced during the accumulation of neutrophils in the intestine and can be abrogated by the depletion of neutrophils.

jejunum; ileum; epithelial paracellular transport; bacterial translocation; lactulose; mannitol; antineutrophil antibody

 GREATER THAN 40% MORTALITY in critically injured patients suffering from burns, multiple trauma, or hemorrhage appears to be due to multiple organ dysfunction (MOD) (3, 23). The gut is considered to be the “motor” for MOD as it allows for translocation of bacteria/endotoxin to activate submucosal cells, which, in turn, might release deleterious mediators and cause mucosal tissue structural and/or functional alterations (2, 5). Such mucosal tissue alterations are apparently accompanied by an increase in gut permeability after a severe burn and trauma (30, 31). The increase in gut permeability could aggravate the translocation of bacteria/endotoxin and enhance the risk of a sepsis syndrome in the injured host (9, 27). With the use of the carbohydrates lactulose and mannitol as markers of mucosal permeability, it is shown that patients with burn injuries have significantly increased intestinal permeability (8). However, there is controversy whether a burn injury alone can induce intestinal permeability alterations; a number of clinical and experimental studies indicates that intestinal permeability is increased only when a burn injury is accompanied by infection (24, 38).

Previous studies (5, 16) have implicated the roles of submucosal leukocytes in general and of macrophages in particular in the increase of gut permeability. Although some studies (21) have indicated a role for neutrophils in the observed postinjury alterations in gut permeability, some others (6, 35) have not supported an intestinal accumulation of neutrophils in burn-injury conditions. An initial occurrence of inflammation in the bowel after a burn injury could result in an accumulation and infiltration of neutrophils into the intestine (18). Such neutrophil accumulation and attendant release of neutrophil products (e.g., superoxide anion and proteases) may affect gross alterations in gut solute permeability. In this study, we evaluated intestinal mucosal permeability to nonabsorbable carbohydrates lactulose and mannitol in neutrophil-depleted rats subjected to burns.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (250–275 g) purchased from Harlan (Indianapolis, IN) were housed in plastic cages and had free access to water and chow. The rats were acclimatized in the animal quarters for 3 days before their use. The care of the animals was in accordance with the guidelines set forth by Loyola University Chicago Medical Center Animal Care and Use Committee.

Thermal injury protocol. As in Ref. 31a, the animals were anesthetized with an intraperitoneal injection of pentobarbital sodium, 45 mg/kg body wt. The hair on the animals’ backs was clipped off. The animals were then placed in a supine position in a plastic template that exposed 25–30% of the total body surface area. In the burn group rats, full thickness skin scalds were inflicted by immersing the back of the animal in 95°C water for 10 s. In the sham-burned group, the exposed backs were immersed for 10 s in a room temperature water bath. Rats were quickly dried after the exposure to hot water to avoid additional injury. The animals were resuscitated with 15 ml ip of normal saline. No mortality was observed in sham-burn rats. Three groups were compared for
Depletion of neutrophils. Rabbit anti-rat polymorphonuclear neutrophil antiseraum (0.3 ml; catalog no. A51140, Accurate Chemicals & Scientific, Westbury, NY) diluted in 1 ml of normal saline was injected intravenously into the rats 12–18 h before subjecting them to thermal injury. Blood samples from neutrophil-depleted rats were withdrawn before injecting antineutrophil antibody, immediately before the burn injury and 24 h postburn to assess the presence of neutrophils. The air-dried blood films were stained with Giemsa Stain for 2 min. The slides were placed in deionized water for 3 min and air dried again. Neutrophil counts were performed by means of light microscopy (×40 magnification). The number of neutrophils was counted among a total of 100 cells on each slide made from each blood sample. In blood samples from five rats taken before the administration of the antibody, the means ± SE value of the neutrophil counts were 27 ± 3. Blood samples taken from these rats just before the burn (12–18 h after the neutrophil antibody administration) showed a complete absence of neutrophils in two of five samples, a total of two neutrophils in two samples, and a single neutrophil in one sample. The blood smear of the rats 24 h after the burn also showed the sparse presence of neutrophils (0–2 neutrophils per 100 cells counted per slide).

Determination of intestinal mucosal permeability to lactulose and mannitol. After the rats were anesthetized, bilateral lumbar incisions were performed. Both kidneys were ligated and placed back in the retroperitoneal cavity, and the muscles and skin were closed subsequently. A 2.5 in. midline laparotomy incision was performed and a 20-cm segment of the ileum or jejunum wasatraumatically clamped off. A 3-ml blood sample was withdrawn immediately before the infusion, a 1-ml blood sample was drawn before intestinal infusion, and a 3-ml blood sample was drawn after their passage from the ileum or jejunum. Unlabeled lactulose and mannitol (both 11 mM) were dissolved in PBS (310 mosM) along with additions of trace quantities of [3H]lactulose (1 µCi, specific activity 20 Ci/mmol) and [3H]mannitol (0.5 µCi, specific activity 53 mCi/mmol) (American Radiolabelled Chemicals, St. Louis, MO). Intestinal infusions were carried out, taking care to avoid bowel distension. Immediately after the infusion, 5 ml of NaCl was added into the abdominal cavity. The intestine was placed back into the abdomen, and the abdominal wall was sutured. The right femoral vein was exposed and catheterized using PE-50 tubing (Becton Dickinson). At time 0, just before intestinal infusion, a 1-ml blood sample was drawn from the femoral catheter and plasma was collected. This procedure was repeated at 30, 60, and 90 min after the infusion. During the 90-min period, the animal was kept anesthetized by giving intraperitoneal anesthesia as needed. Five milliliters of liquid scintillant (ICN Biomedical) were added to 0.3-ml aliquots of the collected plasma. [3H]lactulose and [3H]mannitol radioactive activity were counted by using a two-channel liquid scintillation counter (Beckman Coulter). Lactulose and mannitol concentrations in plasma were calculated by multiplying the plasma-to-perfusate radioactivity ratios (dpm/ml) with lactulose or mannitol concentrations in the mucosal perfusate (mmol/l). To check the reliability of kidney ligation, urine radioactivity was counted before and after the intestinal perfusion.

Histology of intestinal mucosal tissue. For paraffin sections, 1-cm-long intestinal segments (ileum, jejunum) were fixed in 10% Formalin in PBS and 6–10 sections, 4 µm thick, were cut and mounted. Standard hematoxylin-eosin staining was performed to determine intestinal morphology.

Immunohistochemical determination of neutrophil presence in intestinal tissue. Segments of distal ileum and proximal jejunum, ~1 cm long, were frozen in liquid nitrogen and fixed in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC). Frozen intestinal segments were cut in a cryostat at 10 µm thickness and mounted on slides. All subsequent procedures were carried out at room temperature using Vectastain avidin-biotin complex (ABC) kit (Vector Laboratories). Nonspecific protein-binding sites were blocked during a 30-min incubation of tissue sections with goat antiserum. The sections were then incubated for 60 min with the primary antibody against a neutrophil α-nicotinamide adenine dinucleotide phosphate (reduced form) component protein p47phox (a generous gift from Dr. G. Knaus, Dept. of Immunology, The Scripps Institute, La Jolla, CA), followed by three washes with PBS (BSA free), and reincubated for 30 min with biotinylated secondary goat anti-rabbit antibody. After washing and endogenous peroxidase quenching with 0.3% H2O2 in PBS for 15 min, slides were rinsed again and incubated with ABC reagent for 30 min. The slides were washed, and immunoperoxidase staining was performed using avidin-biotin peroxidase complex (ABC) kit, Sigma Immuno Chemicals, St. Louis, MO). Finally, the slides were rinsed in water, counterstained for 30 s with Gill no. 2 hematoxylin, and mounted.

Statistical analysis and data presentation. All statistical analyses and linear regressions were carried out using the Statistical Package, Social Sciences Software Program (SPSS, SigmaStat version 2.0, Chicago, IL). To determine inter- and intragroup differences between variables, a one-way repeated-measures ANOVA, followed by a pairwise multiple comparison procedure (Tukey's post hoc test), was performed. The statistical analysis of the different experimental groups included the comparison of sham-burned (n = 5), day 1-postburn (n = 4), day 3-postburn (n = 5), and neutrophil-depleted burn rats (n = 5). Statistical significance was assigned at P values <0.05. All results in Figs. 1-4 and the text are expressed as means ± SE.

RESULTS

Lactulose and mannitol permeability. Figure 1 shows the time course of the increase in plasma concentrations of lactulose and mannitol after their infusion into the ileal lumen (Fig. 1A). Similar data from measurements of plasma lactulose and mannitol after their infusion into the jejunal lumen are shown in Fig. 1B. The time-dependent increases in plasma lactulose and mannitol concentrations after their transfer from the ileum were of significantly greater magnitude in both day 1- and day 3-postburn groups compared with the sham-burned group. However, the transfer of lactulose was higher than that of mannitol in both day 1- and day 3-postburn groups. The data from the measurements of lactulose and mannitol transfer from the jejunum also showed significantly higher rates of transfer in the day 1- and day 3-postburn groups compared with the sham-burned group, but there were no significant differences between the day 1 and day 3 groups.

The linearity of the accumulations of lactulose and mannitol in blood, after their passage from the ileum or jejunum [regression coefficient (r²) > 0.9, shown in Fig. 1], allowed us to determine the permeabilities to these solutes from the slopes of the regressed lines. The calculated values of the slopes, in terms of changes in solute concentrations per unit time (µM/min), are re-
ferred to as permeability indexes and shown in Fig. 2. The ileal lactulose permeability index was fourfold higher in day 1-postburn rats than in the sham-burned group and 8.6-fold higher in the day 3-postburn group. The ileal mannitol permeability index was comparable to that of lactulose in the sham-burned rats; its increase in day 1- and day 3-postburn groups, respectively, was threefold and fourfold compared with the values from the sham-burned rats. Thus, although the permeability to lactulose was comparable to that of mannitol in the ileum of sham-burned rats, the permeabilities of the two solutes were differentially affected in the burn rat groups.

Like the findings in the ileum, the jejunal permeability indexes were comparable for lactulose and mannitol in the sham-burned group. Also, the jejunal permeability indexes for both lactulose and mannitol were significantly higher in the postburn groups. As for lactulose, the jejunal permeability index in both the day 1- and day 3-postburn groups was about twofold greater than the values of the sham-burned rats. The increase in mannitol permeability in both burn groups was ~1.5-fold greater than in the sham-burned group. Thus compared with the increases in lactulose and mannitol permeabilities in the ileum of day 1-postburn rats, the increases in permeabilities of the two solutes in the jejunum were significantly lower (P < 0.05). Another difference noted in the permeabilities to the two solutes in the ileum versus the jejunum was a further increase from day 1 to day 3 postburn in the ileum but not in the jejunum.

Effect of antineutrophil antibody pretreatment on ileal and jejunal mucosal permeability. Figure 2 also shows the effect of antineutrophil antibody pretreatment on ileal and jejunal lactulose permeability in day 1-postburn rats. As can be seen in the figure, when rats were pretreated with the antineutrophil antibody, the burn injury-induced increase in ileal lactulose and mannitol permeabilities were completely abolished. Jejunal lactulose and mannitol permeability changes in thermally injured rats were also completely prevented by the antibody pretreatment. Thus antineutrophil antibody pretreatment was effective in both intestinal segments in preventing the burn injury-induced in-
crease in intestinal permeability to lactulose and mannitol.

Histological examination of intestine. Figure 3 shows the hematoxylin-eosin staining of paraffin sections of the intestinal segments. There were no demonstrable histological differences between the sham-burned (Fig. 3A) and day 1-postburn (Fig. 3B) groups. Both groups showed similar normal morphology in the villous lamina propria and submucosal regions of the intestine.

Immunohistochemical neutrophil staining in intestine. Figure 4 shows the distribution of neutrophils in the intestinal tissue as evidenced by immunostaining. A clustered appearance of reddish brown precipitates seen in the burned rat intestinal section indicates the presence of neutrophils (Fig. 4B). The tissue sections of the sham-burned (Fig. 4A) and neutrophil-depleted (Fig. 4C) rats are devoid of such neutrophil immunoreactivity. Although these particular sections of the intestines from the sham-burned and neutrophil-depleted rats showed an absence of neutrophils, in some other sections we could identify a few neutrophils.

**DISCUSSION**

This study indicates that an absence of neutrophils in burn-injured rats prevents adverse alterations in intestinal mucosal permeability, which are induced in the early periods after a burn. Several previous studies (30, 31) show burn-related increases in mucosal permeability, in both patients and animal models of injury. It is reasonable to assume that such alterations cannot only disturb gut nutrient absorptive functions, but also play some role in the translocation of gut bacteria to extraintestinal sites in the injured host (9, 21, 27, 29). The translocation of the indigenous gut bacteria can contribute to and/or exacerbate the systemic inflammatory response after a burn injury (11, 13). The intestinal mucosal permeability alterations are attributed to splanchnic hemodynamic/microvascular alterations occurring after burns (19, 33). It is not definitively known whether the influx of neutrophils into the splanchnic vascular bed and tissues, which is shown to occur after burns (18), is in some manner responsible for the development of intestinal mucosal functional and/or structural alterations causing mucosal permeability disturbances.

An influx of neutrophils into the intestine in response to a burn injury can either potentiate a microvascular blockade, leading to tissue hypoperfusion, or cause neutrophil infiltration into extracellular spaces in the submucosal region, leading to neutrophil-mediated submucosal tissue damage. The latter tissue damage could presumably involve neutrophils' release of reactive oxygen species and proteolytic enzymes (28, 32). Both tissue hypoperfusion and submucosal tissue injury can potentially affect alterations in mucosal permeability to solutes. Previous studies (10) show that the prior depletion of neutrophils in rats was not effective in preventing either the hemorrhage-induced gut translocation of bacteria or the associated disruption of intestinal mucosal morphology. These findings implied that

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**Fig. 2.** Ileal and jejunal solute permeability indexes in burn- and in neutrophil-depleted burn rats. Permeability indexes (in µM/min) for ileum and jejunum were calculated as slopes of regression lines in Fig. 1. Values represent means ± SE; n = number of animals; *P < 0.05 compared with sham-burned rats; **P < 0.05 compared with day 1 postburn; #P < 0.05 compared with sham-burned rats; ##P > 0.05 NS compared with day 1 postburn; †P > 0.05 NS compared with sham-burned rats.
neutrophils caused neither the gut translocation nor the mucosal structural damage in hemorrhage-injured animals. The rat burn injury protocol employed in the present study evidently did not cause any gross disruption of intestinal mucosal structures (Fig. 3), although it clearly resulted in overt mucosal functional alterations leading to increased mucosal permeability to carbohydrate solutes, i.e. disaccharide lactulose and monosaccharide mannitol.

The measurements of intestinal mucosal permeabilities to lactulose and mannitol have frequently been carried out in animal models and in patients (8, 30, 37). Such measurements, however, rely on assessments of urinary excretion of the solutes after they are adminis-
tered orally. Although intestinal permeability to the solutes may be rate limiting in the recovery of the solutes in urine, several other factors could additionally influence the rate of solute excretion, for example, gastric-emptying rate, intestinal transit time, hepatic and renal clearances of the solutes, and tissue perfusion in the liver and kidney. The aforementioned factors may be altered in injured animals, such as those employed in this study, and can thus influence the urinary recovery of solutes after their oral administration independently of alterations in intestinal mucosal permeability characteristics. We have, therefore, employed an intestinal permeability assessment procedure involving in situ infusion of solutes into a segment of the bowel, in bilaterally kidney-ligated rats, and monitored appearances of solutes in the blood. The appearances of solutes in the blood in our studies were found to be linear over a 90-min time period, indicating a zero-order kinetics of the transport of solutes (Fig. 1). Such kinetic behavior of the solute transport allowed us to make valid estimations of the rate of passage of solutes across the intestinal mucosa, dependent primarily on the permeability characteristics of the intestinal wall and relatively independent of potential alterations in volume and solute concentration in solutions present in the mucosal lumen.

The higher permeability for both solutes in the jejunum over that in the ileum in sham-burned rat intestines, as observed in this study, is in keeping with the well-known differences in the transepithelial barrier properties in these two regions (15, 25). As originally shown by Fordtran et al. (15), the portals for entry into paracellular passages (namely tight junctions) are presumably of relatively larger radii in the jejunum than in the ileum. Thus if lactulose and mannitol are to cross the epithelial layer mainly through the paracellular routes, a higher rate of their passage would be expected in the jejunum than in the ileum. As for lactulose, there is greater certainty that it passes primarily through the paracellular route (34). In the case of mannitol, although some investigators (4) postulated it to be transported transcellularly across the apical membrane, numerous studies (25, 34) in a variety of tissues have indicated it to be restricted to the extracellular spaces. Thus a number of investigators (25, 34) presume mannitol to also pass mainly through paracellular routes in the intestine. A notable finding in this study, which we are unable to explain, is the absence of any differences in the permeabilities to lactulose and mannitol within a given intestinal region in the sham-burned rats.

The concept of differences in the sizes of the tight junctions leading to paracellular routes in the epithelial villar tips versus crypt regions may provide some explanation for the permeabilities to lactulose and mannitol within a given intestinal segment. The villar tips are presumably studded with many more junctions of relatively smaller diameter than in the crypt region (25, 26, 34). Whereas the junctions of larger diameter in the crypts, albeit fewer in number, may allow for the passage of both the larger lactulose as well as the smaller mannitol molecules in the crypt surface, the smaller diameter villar junctions may only allow the smaller mannitol to permeate. Thus the total quantity of mannitol transported would include some molecules passing through the few crypt junctions and some through the many villar junctions. If lactulose and mannitol were to be present at comparable concentrations in the mucosal lumen, it is conceivable that mannitol may pass through the villar junctions without any competition with lactulose, and it tends to pass across crypt junctions only after competition with lactulose. Such a competition at the crypt surface is likely to lead to a quantitatively greater transport of lactulose than mannitol because of lower concentrations of mannitol in the vicinity of crypt junctions resulting from its prior passage through the villar tip junctions. Overall, although a majority of mannitol molecules is likely to pass through the villar surface and lactulose passes through the crypt surface, the net transport of the molecules may remain comparable. This could apply to both ileal and jejunal regions.

The present study demonstrated that not only was there an increase in mucosal permeability to both lactulose and mannitol after a burn, but unlike the comparable permeability for the two solutes in the two intestinal regions in the control rats, there was also a greater increase with a burn injury in the permeability to lactulose than to mannitol in both of the intestinal regions. This may well be due to a burn injury causing a selective increase in permeability to lactulose through the larger diameter junctions in the ileal and jejunal crypts. The increase in permeability to mannitol may also be due to its enhanced permeability through the junctions in the crypts. We do not have dyes as to whether the changes in mannitol permeability may also be due to alterations in the villar tip junctions. The greater increase in permeability to lactulose in the ileum than in the jejenum supports the concept of a greater sensitivity of the ileal crypt region to a burn injury.

Another presently observed difference in mucosal solute permeability between the ileal and jejunal segment was the progression in permeability changes from day 1 to day 3 postburn. The presence of such a progression beyond day 1 postburn in the ileum, but not in the jejenum, may be due to a continued presence of a tissue injury signal in the ileum through day 3 postburn. In the jejenum, such an injury signal may have exerted a maximum effect by day 1 which is then maintained through day 3. Alternatively, a secondary injury signal arising in the jejenum sometime before or after the initial 24 h may superimpose itself on the initial primary signal. Several previous studies show an initial tissue perfusion deficit to occur in the splanchnic vascular bed after trauma (13), hemorrhage (36), and burn injury (20). Such a tissue perfusion disturbance likely occurs due to a vasocostrictive response of the splanchnic vasculature (22) and is likely accompanied by splanchnic intravascular accumulation of neutrophils and their infiltration into intestinal mucosal tissue (19). As noted above, an intravascular accumula-
tion of neutrophils might cause hypoxia in the intestinal tissue, and infiltration of neutrophils into the tissue could lead to O$_2$ and protease release within the mucosa (32). Tissue hypoxia or neutrophil oxidant and/or protease production could have an adverse effect on the structural and functional integrity of the mucosal barrier. The absence of gross histological mucosal changes on day 1 postburn in this study supports the occurrence of intestinal mucosal permeability dysfunction without mucosal structural disruption. The role of neutrophil influx in contributing to mucosal functional alterations is supported by our observation that the depletion of neutrophils in the burned rats prevented the mucosal solute permeability alterations observed on day 1 postburn. The abovementioned considerations assert that the intestinal tissue hypoperfusion, neutrophil oxidants, and/or proteases may serve as a primary injury signal for the mucosal permeability disturbance in the initial 24 h after a burn and that such a primary signal can be abrogated if the neutrophils are prevented from accumulating in the bowel.

Because the ileum is a more likely site of bacterial translocation after a burn than the jejunum (1), a progressive increase in the ileal mucosal permeability, such as observed in this study, could be in some manner related to or associated with bacterial translocation. The translocation of bacteria and/or their products may cause some form of injury to mucosal tissue and potentiate the neutrophil influx-related tissue functional derangement to contribute to a heightening of ileal solute permeability on day 3 postburn. This would also imply that bacterial translocation may serve as a secondary injury signal. Alternatively, neutrophil influx itself may cause a greater tissue derangement in the ileum than in the jejunum, not only to exacerbate mucosal permeability changes, but also to promote bacterial translocation. Another study from our laboratory (14) indicated that the translocation of bacteria in rats after a burn is dependent on accumulation of neutrophils in the extravascular spaces of the intestine. Recent studies have implicated other causative factors such as activation of mucosal inducible nitric oxide synthases (7) and T lymphocytes (17) as playing roles in the induction of bacterial translocation. Thus factors in addition to neutrophils can cause intestinal barrier dysfunction. In summary, our study has suggested that neutrophil influx-related mucosal tissue damage after a burn injury includes an increased solute permeability through mucosal paracellular routes and that such alterations are more pronounced in the ileum than in the jejunum.

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