Restitution of single-cell defects in the mouse colon epithelium differs from that of cultured cells

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Günzel, D., P. Florian, J. F. Richter, H. Troeger, J. D. Schulzke, M. Fromm, and A. H. Gitter. Restitution of single-cell defects in the mouse colon epithelium differs from that of cultured cells. Am J Physiol Regul Integr Comp Physiol 290: R1496–R1507, 2006. First published January 5, 2006; doi:10.1152/ajpregu.00470.2005.—Integrity of colon crypt to villus axis and shedding of apoptotic cells into the lumen prevents the accumulation of carcinogenic DNA lesions (6, 39), plays a major role in the process that follows, the restitution of the injured intestine.

THE EPITHELIAL LINING OF THE INTESTINE forms a barrier against the external environment. The cells are the target for mechanical damage, toxic food ingredients, and the bacterial flora (32, 35), against which the integrity of the barrier must be maintained. Thus the intestinal epithelium is injured and continuously restored during the normal course of digestion and absorption of a meal (26, 30). Furthermore, continuous renewal along the crypt to villus axis and shedding of apoptotic cells into the lumen prevents the accumulation of carcinogenic DNA lesions (21). Hence, cells may be lost after damage, or constitutively in the regular turnover. Not only the loss of artificially damaged cells leads to a potentially dangerous leak in the intestinal epithelium (28, 35, 47) but also spontaneous or cytokine-induced apoptosis (18). To prevent the entry of antigens, rapid repair even of minor defects is mandatory.

In native intestinal epithelium, the closure of large defects proceeds via two mechanisms: restitution and regeneration. Epithelial restitution is the coverage of superficial lesions. In rabbit and human colon, first morphologic evidence of multicellular restitution becomes visible 15 min or 30 min after injury, respectively (13). Restitution is based on the migration of intact cells (41) and is complete within a few hours (23, 33). In contrast, regeneration, the healing of deep wounds, occurs within days and, in addition to cell migration, requires cell proliferation (7, 47).

Intestinal wound healing is a crucial event in inflammatory bowel diseases (IBD) like Crohn’s disease and ulcerative colitis. In these diseases, proinflammatory cytokines such as the TNF-α and IFN-γ play a key role. In IBD, cytokine concentrations are raised (29) and, at pathological concentrations, they impair the barrier function of cultured (9, 15, 39) and native intestinal epithelia (14, 40). Data on the effect of TNF-α on cell migration and thus on restitution of large epithelial defects are conflicting. Although restitution is impaired by TNF-α in cultured mouse intestinal epithelial cells (10), this is not the case in the intestinal epithelial cell line-6 (IEC-6) (12). In contrast, IFN-γ accelerates restitution of large epithelial defects in IEC-6 epithelia (12), as well as in Calu-3 human lung epithelial cells (2) and lung type II epithelial cells (27).

Repair after the loss of a single or very few cells (single/oligo-cell restitution) has not yet been explored in native intestinal epithelium, and the present investigation shows this process for the first time. It was found to proceed considerably faster than in the cultured colon-derived cell lines HT29/B6 (16) and Caco-2 BBe (38) and differed with respect to the effects of cytokines. The proinflammatory cytokine IFN-γ, but not TNF-α, delayed the restitution. Thus IFN-γ, which exerts smaller impairing effects on active transport or barrier function than TNF-α (6, 39), plays a major role in the process that follows, the restitution of the injured intestine.

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The importance of rapid single-cell restitution is emphasized by the finding that the supernatant of the hemolytic *E. coli* O4 culture delays the restitution of such lesions and may thus provide the bacteria with an access route into the body across the epithelial barrier during pathogenic processes.

**MATERIALS AND METHODS**

**Tissue preparation.** Untreated male and female NMRI mice (30–35 g), which had free access to a standard diet (Altromin 1320) and to tap water ad libitum, were anesthetized and killed by inhalation of 100% CO$_2$. This is in accordance with the guidelines of GV-SOLAS (Gesellschaft für Versuchstierkunde - Society for Laboratory Animal Science). Late distal colon was dissected and subepithelial layers were removed using the following procedure. The gut was cut open along the mesenteric insertion, and the tissue was placed mucosal side down on a plastic plate. The muscularis propria together with the submucosa and parts of the muscularis mucosae were removed using fine forceps, so that epithelium, lamina propria and the outer layer of the muscularis mucosae remained. The tissue was mounted in a container system (43), which could be inserted into the electrophysiological setups.

Human biopsy specimens were obtained endoscopically from the sigmoid colon at 30 cm from the anus with a 3.9-mm forceps. Human rectal cancer. Specimens were “partially stripped” i.e., the muscularis propria was removed. The mucosa appeared macroscopically normal. The study design was approved by the local ethics committee.

**Cell cultures.** Two-colon-derived cell lines, HT-29/B6 and an enhanced green fluorescent protein (EGFP)/β-actin fusion protein expressing Caco-2 BBe cell line ([38]; generous gift by J. R. Turner, Department of Pathology, University of Chicago, Chicago, IL), were cultured on Millicell-PCF support filters (PITP 01250, Millipore, Bedford, MA). As previously described (19), the apical compartment of each chamber was filled with 0.5 ml culture medium; the basolateral compartment contained 10 ml. The media (RPMI 1640, Biochert KG, Berlin, Germany and DMEM, Gibco/Invitrogen, Karlsruhe, Germany, respectively) contained 2% stabilized L-glutamine and were supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and, for Caco-2 BBe cells, 1 µg/ml blasticidine. Culture was performed at 37°C in a 95% air-5% CO$_2$ atmosphere. After 7 days, confluent monolayers were either used in a conventional Ussing chamber setup or, similar to the colon epithelium, mounted in a container system and used for conductance scanning experiments as described below.

**Drugs and solutions.** Cytochalasin D was purchased from Sigma Chemical (St. Louis, MO), and a stock solution was made with 250 μg/ml in dimethyl sulfoxide. The final concentration used was 10 μg/ml (mucosally). In control experiments, the same amount of dimethyl sulfoxide (25 μl/10 ml) was added mucosally. Mouse colon was incubated in conventional Ussing chambers for 90 min before the experiments in the conductance scanning setup.

TNF-α was purchased from Biomol (Hamburg, Germany), and a stock solution was made with 10 μg/ml. The final concentration used was 100 ng/ml. Recombinant rat IFN-γ (Biotrend Chemikalien GmbH, Cologne, Germany) was diluted into a stock solution of 10,000 units/100 μl. The final concentration was 1,000 units/ml. Conventional Ussing chambers were used to incubate the tissue with cytokines for a time period of 17 h. Both half-chambers were continuously perfused with Ringer solution, which was constantly equilibrated with 95% O$_2$-5% CO$_2$ and preheated to 39°C to obtain a chamber temperature between 36°C and 37°C. The composition of the solution was (in mmol/l): 140 Na⁺, 123.8 Cl⁻, 5.4 K⁺, 1.2 Ca$^{2+}$, 1.2 Mg$^{2+}$, 2.4 HPO$_4^{2-}$, 0.6 H$_2$PO$_4^{-}$, 21 HCO$_3^{-}$, 10 D(+)-glucose, 10 D(+)-mannose, 2.5 glutamine, 0.5 β-OH-butyrate; pH 7.4. The antibiotics Pipercillin 50 mg/l (Hexal AG, Holzkirchen, Germany) in combination with Zienam 10 mg/l (MSD Sharp & Dohme GmbH, Haar, Germany) were added to the solution to effectively inhibit bacterial growth. Before adding the cytokines to the serosal side of the tissue, the surface of the water-jacketed gas lifts containing the Ringer solution was saturated with 10% fetal calf serum (FCS; Biochrom, Berlin, Germany) to avoid the adhesion of cytokines on the glass surface. After 17 h, the tissue was moved to the conductance-scanning setup.

**Viability assay.** Ussing chamber experiments were performed on mouse colon under control conditions and in the presence of the proinflammatory cytokines TNF-α (100 ng/ml) and/or IFN-γ (1,000 units/ml) for up to 17 h. At the beginning of each experiment, the tissue was allowed to equilibrate for about 30 min. Then, 10% fetal calf serum, and after 15 min, the cytokines were added to the serosal compartment. Transepithelial resistance was recorded online over 17 h, and representative values at certain time points are shown. To check the ability to respond to secretagogues, all epithelia were exposed to forskolin (10$^{-5}$ M stock solution in DMEM, final concentration 10$^{-3}$ M, Sigma Chemical) on the serosal side of the epithelium. DMEM alone (10 μl/10 ml) was tested in other experiments and showed no effect.

**Induction of lesions.** Lesions were induced by iontophoretic injection of Ca$^{2+}$ from a conventional glass microelectrode filled with 0.1 mol/l CaCl$_2$ using current pulses of 1.5 μA amplitude and 0.5 to several seconds duration (16). Purely mechanical destruction of a single cell was more difficult, but if successful, it caused the same result. If lesions were to be investigated by confocal laser scanning microscopy (CLSM), the electrode was filled with 30 μM of the fluorescence dye DAPI (4′-6-diamidino-2-phenylindole dihydrochloride) in addition to CaCl$_2$. As DAPI is positively charged it is co-injected with Ca$^{2+}$ and specifically stains the nuclei of the damaged cells. This made it easier to find the lesions in the subsequent CLSM studies.

**Conductance scanning.** The magnitude of the leak caused by single/o mega-cell lesion was quantified by measurement of its electrical conductance (Fig. 1), as described previously (16, 19, 20). In brief, sinusoidal electric current (100 μA/cm$^2$, 24 Hz) was clamped across the epithelium. Use of alternating current avoided polarization effects and made synchronous demodulation techniques possible for im-
provement of the signal-to-noise ratio. Control experiments, as described previously (17), excluded the possibility that the data were affected by amplitude or frequency of the alternating current applied. The electric field ($\Delta V/\Delta z$) generated in the mucosal bath solution was measured with a mobile probe positioned at a constant distance $z_0$ above the mucosal surface (Fig. 1). This distance was readjusted at every position of measurement by lowering the probe until it barely touched the surface and then elevating it exactly 25 $\mu$m by means of a computer-controlled, stepping motor-driven micromanipulator. The apparent conductivity $G_A$ was calculated from the scanning signal ($\Delta V/\Delta z$) applying the equation

$$G_A = \left(\frac{\Delta V}{\Delta z}\right) \rho \cdot U$$

where $\rho$ is the specific resistivity of the Ringer solution ($53.7 \, \Omega \cdot \text{cm}$); and $U$ is the transepithelial voltage, induced by the transepithelial electric current.

To measure the spatial distribution of $G_A$, an area of about $50 \times 50 \, \mu$m was chosen on the surface epithelium, as far as possible from crypts and without any visible apoptotic events or lesions. The resulting $G_A$ value represented the conductivity of the intact epithelium, $G^{\text{intact}}$.

After inducing a lesion, the injection microelectrode was retracted, and the scanning probe was advanced to the site of the lesion. A single-cell lesion created a local leak with an increased $G_A$ in close proximity.

In experiments on cultured cells, the probe was placed directly above the lesion ($x=0$) and then moved parallel to the epithelial surface, along a line ($x$ axis) to points at $x = 10, 20, 40, 70,$ and $100 \, \mu$m to determine the spatial distribution of $G_A$. From these data, the conductance associated with a single leak, $g^{\text{leak}}$, was calculated by integration of $G_A - G^{\text{intact}}$ assuming rotational symmetry of the leak current around the lesion (16).

Because of the fast repair in colon epithelia, a spatial scan of $G_A$ was not feasible. Because geometry ($G_A$ measured 25 $\mu$m above the leak in a flat epithelium) and bulk conductivity of the saline were the same as in cell cultures, we estimated the leak conductance $g^{\text{leak}}$ from the linear relation of $G_A - G^{\text{intact}}$ and $g^{\text{leak}}$ found in cultured cells. Thus we calculated the time course of the leak, $g(t)$. After inducing the lesion, measurements were performed between $t=1.5 \, \text{min}$ and $t=4 \, \text{min}$ postlesion. The leak conductance $g^{\text{leak}}$ declined rapidly (approximately exponentially).

**Video microscopy and histology.** The living tissue was observed during restitution using an upright microscope with a water immersion objective lens ($\times 40$ W IR-Achroplan, numerical aperture 0.80, working distance 3.61 mm; Zeiss, Oberkochen, Germany). After lesions were induced, the tissue was either fixed directly, or conductance scanning was carried out and the tissue subsequently fixed with 10% formalin. Thereafter, either conventional histology was performed using series transversal sections of paraffin-embedded tissues stained with formaldehyde and eosin (H&E) or staining of actin with Alexa Fluor 594 phalloidin for CLSM studies was carried out.

**TUNEL assay.** To determine the apoptotic ratio of epithelial cells, terminal deoxynucleotidyl transferase-mediated dNTP-biotin nick end labeling (TUNEL) assay was performed with fresh tissue and after 17 h of incubation in Ussing chambers with control Ringer solution with or without cytokines. Deparaffinized sections (1 $\mu$m thick) of mouse distal colon were digested with protease K solution (Gibco BRL, Grand Island, NY) ($20 \, \mu$g/ml) for 20 min at 37°C in a humidified chamber. Slides were rinsed in PBS (Dulbecco’s PBS, PAA Laboratories GmbH, Pasching, Austria) and blocked in 5% BSA (bovine serum albumin, Merck, Darmstadt, Germany) and 20% FCS (pH 7.5) diluted in PBS for 30 min at room temperature. After washing with PBS test slides were incubated in terminal deoxynucleotidyl transferase (TdT) (Roche, Mannheim, Germany, diluted 1:10 enzyme solution in label solution) for 60 min in a dark humidified chamber. After washing, slides were mounted in ProTaqs Mount Fluor (Biocyc, Luckenwalde, Germany), and images were subsequently analyzed on a personal computer (analySIS 3.0, Soft Imaging System, Münster, Germany). Epithelial apoptosis was determined as the number of apoptotic nuclei per total number of cells in the visual fields (apoptotic ratio).

**Staining procedures for confocal laser scanning microscopy studies.** Fluorescence staining of actin and of the integral tight junction protein occludin in mouse colon was carried out according to the following protocol. Tissues were washed twice with PBS and fixed with methanol at $-20^\circ$C for 15 min (occludin) or with 10% formalin for 6 h (actin). Tissues were washed again in PBS and permeabilized with 0.5% and 1% Triton X-100 in PBS for 10 and 60 min, respectively. To block nonspecific binding sites, cells were then bathed in PBS containing 10% (vol/vol) goat serum (blocking solution) and 0.1% Triton X-100 for 60 min. All following washing procedures were performed with this blocking solution. For staining of actin, the samples were incubated in Alexa Fluor 594 phalloidin (Molecular Probes, Carlsbad, CA; 1:200 in blocking solution) and then washed and mounted as described below. For occludin staining, the cells were incubated with a primary polyclonal antibody against occludin (rabbit anti-occludin, Zymed Laboratories, San Francisco, CA) diluted 1:50 in DAKO antibody diluent with background reducing components (No. S3022, DAKO, Carpinteria, CA) overnight at 4°C. After four washes (2, 10, 20, and 25 min), tissues were incubated with Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probes; diluted 1:1000 in blocking solution with 0.1% Triton X-100) for another 3 h at room temperature. After washing with PBS, the tissue was mounted in ProTaqs Mount Fluor (Biocyc, Luckenwalde, Germany).

**EGFP/β-actin expressing Caco-2 BBe were fixed with 3.7% paraformaldehyde in PBS, washed in PBS, and either directly mounted in ProTaqs Mount Fluor, or additionally stained with Alexa Fluor 594 phalloidin, as in many cases, the GFP signal appeared patchy, probably due to different levels of expression in the observed cells.**

Preparations were viewed under a Zeiss LSM 510 Meta confocal laser scanning microscope.

**Preparation of E. coli O4 filtrate.** Overnight bacterial cultures of E. coli O4 were diluted to an optical density (OD) of 0.4 in LB (Luria-Bertani, BIO 101 Carlsbad, CA) broth. After aerobic growth at 37°C to an OD of 1.2, bacteria were washed and resuspended into cell culture medium to a final OD of 0.5. Sterile filtered supernatant was prepared when cultures had reached an OD of 1.2. Bacteria-containing medium was centrifuged 7,500 g for 5 min at 4°C, and supernatant was filtered through a filter with a pore size of 0.22 $\mu$m (Sterivex, Millipore Schwabach, Germany), which had previously been blocked with BSA to reduce loss of hemolytic activity. Then the filtrate was stored at $-20^\circ$C until usage.

**Statistics.** All values are given as means ± SE. The significance of differences between control and test groups ($P < 0.05$) was evaluated using the $U$-test of Wilcoxon, Mann and Whitney, with Bonferroni correction for multiple testing. $m$ represents the number of animals, and $n$ represents the number of lesions.

**RESULTS**

**The time course of restitution.** Local conductance changes within an otherwise homogenous area of an epithelium or of a layer of cultured cells were measured by means of the conductance scanning technique. The conductivity of intact surface epithelium ($G^{\text{intact}}$) was $3.74 ± 0.35 \, \text{mS/cm}^2$ ($m = 16$ colon specimens) and thus was highly significantly larger ($P < 0.002$) than the 1.29 ± 0.10 $\text{mS/cm}^2$ ($n = 17$ different filter supports) previously found in HT-29/B6 cell layers (16).

DeSTRUCTION of single or few ($\leq 5$) cells caused sharp increases in conductance, indicating the presence of a local leak. 1.5 min after inducing such lesions in mouse colonic surface...
epithelium, these leaks had a mean conductance of $1.73 \pm 0.36 \mu S$ ($n = 16$ lesions, $m = 6$ colon specimens) and were thus more than three times larger than a single-cell leak in a layer of HT-29/B6 cells [$0.48 \pm 0.05 \mu S$, $n = 17$, 2 min postlesion (16)].

The time course of restitution was determined from subsequent decreases in local conductance. Simultaneously, the mucosal side of mouse colon was viewed by means of video microscopy. At low magnification, it was possible to distinguish between surface epithelium and crypts in the mouse colon (Fig. 2A). At higher magnification, the white boxed area of undisturbed epithelium. A few minutes later, a small gap remained visible only if the plane of the apical membrane was focused (Fig. 2B), but not in the plane of the basal membrane.

The conductance of the leak decreased exponentially with time, $t$. From 1.5 min postlesion (first measurement), to 2.5 min after setting the lesion, the conductance of the leak decreased by $91\% \pm 1\%$ (from $1.73 \pm 0.36 \mu S$ to $0.19 \pm 0.06 \mu S$, $n = 16$ lesions, $m = 6$ colon specimens). On the basis of an exponential time course of repair, a time constant $\tau$ was calculated (see Fig. 3 and 5). Under control conditions (after 17 h sham incubation), the leak sealed with $\tau = 0.28 \pm 0.03$ min ($n = 16$ lesions, $m = 6$ colon specimens, Table 1). With proceeding time of repair, $g_{\text{leak}}$ decreased toward zero. Because repair resulted in an even distribution of epithelial conductivity, the repaired epithelium assumed a tightness similar to that of undisturbed epithelium. Hence, the new tight junctions around the leak formed the same barrier to ions as those in the undisturbed epithelium. This indicates the existence of a control system of the junctional barrier that works (at least) as rapid as the morphological closure.

For studying restitution in the CLSM, both human biopsies ($n \leq 2$) resectate ($n = 1$) and murine colonic epithelium ($n = 3$) were used. In each specimen, four lesions were induced surrounding one crypt. The time gap between two lesions was 45 s; time between the induction of the fourth lesion and fixing of the tissue was 15–30 s. Under control conditions, only one or two of the four lesions could be detected (mouse colon, Fig. 4A; human biopsy, Fig. 4D), indicating that repair in both species was complete after $\sim 2$ min. However, unlike observa-

![Image](http://ajpregu.physiology.org/)

Fig. 2. Video microscopy of the native mouse colon. The mucosal surface of the colonic epithelium with a single-cell lesion under control conditions, as seen by intravital video microscopy in the perfused miniaturized Ussing chamber. $A$: surface epithelium and crypts viewed with a $\times 40$ water immersion-objective lens. $B$: higher magnification of the recorded image shows the defect (arrow) 5 min postlesion.

![Diagram](http://ajpregu.physiology.org/)

Fig. 3. Blockage of actin polymerization by cytochalasin D. $A$: effect of cytochalasin D on transepithelial resistance. Ussing chamber-mounted mouse colon was allowed to equilibrate for at least 15 min. Cytochalasin D was added to mucosal reservoirs at a final concentration of 10 $\mu g/ml$ ($n = 3$). Cytochalasin D caused a drop in transepithelial resistance ($R^*$) of 30% in a time of 90 min compared with control tissue ($n = 3$). Data represent mean values $\pm$ SE. $B$: conductance of the leak, $g_{\text{leak}}$, of single-cell defects in mouse colonic epithelium. Time course of functional restitution (sealing of the leak), under control conditions ($\bullet, n = 5$, $m = 3$) and after incubation for 90 min with cytochalasin D ($\square, n = 10$, $m = 4$) in a conventional Ussing chamber from the mucosal side of the tissue. Restitution was slower after incubation with cytochalasin D. Within the first 2 min after setting the lesion (first measurement after 1.5 min compared with 3.5 min), 91% $\pm$ 2% were repaired under control conditions in contrast to 74% $\pm$ 3% ($P < 0.05$, $U$-test) after cytochalasin D preincubation. Control measurements after 17 h Ussing chamber incubation revealed a similar time course ($\pm 91\% \pm 1\%, n = 16$, $m = 4$), underlining the fact that both tissues were of similar viability. Data represent mean values $\pm$ SE, *$P < 0.05$; **$P < 0.01$; $U$-test of Wilcoxon, Mann and Whitney, with Bonferroni correction for multiple testing. 

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Only 75% contrast, IFN-γ conductance during restitution

Table 1. Conductivity of the intact epithelium and leak conductance during restitution

<table>
<thead>
<tr>
<th>Condition</th>
<th>$G_{	ext{intact}}$ (mS/cm$^2$)</th>
<th>τ (min)</th>
<th>n (lesions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (17 h sham)</td>
<td>3.74 ± 0.35</td>
<td>0.28 ± 0.03</td>
<td>16 (6)</td>
</tr>
<tr>
<td>17 h TNF-α</td>
<td>4.27 ± 0.81 n.s.</td>
<td>0.28 ± 0.03</td>
<td>7 (4)</td>
</tr>
<tr>
<td>17 h IFN-γ</td>
<td>3.08 ± 0.69 n.s.</td>
<td>0.49 ± 0.07*</td>
<td>13 (6)</td>
</tr>
<tr>
<td>17 h TNF-α + IFN-γ</td>
<td>6.08 ± 0.83 n.s.</td>
<td>0.59 ± 0.06*</td>
<td>10 (4)</td>
</tr>
<tr>
<td>Control (90 min sham)</td>
<td>4.94 ± 0.53</td>
<td>0.36 ± 0.10</td>
<td>5 (3)</td>
</tr>
<tr>
<td>90 min cytochalasin D</td>
<td>6.93 ± 0.61*</td>
<td>0.69 ± 0.10*</td>
<td>9 (4)</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. Conductivity of intact epithelium ($G_{\text{intact}}$) was measured apart from apoptosis or edge damage, before inflicting a lesion. The time constant τ of the exponential decrease in conductance of induced leaks was used to quantify the speed of restitution. n is the number of lesions, m is the number of colon specimens; n.s., not significantly different vs. control, $P > 0.05$; *$P < 0.05$; †$P < 0.01$.

Inclusions in cultured cells, neither human nor mouse colonic specimens showed any evidence of actin accumulation around the lesions. In cultured cells, this actin ring has been postulated to be part of the “purse string” mechanism for restitution (11, 16, 28, 38) and its formation was confirmed in the present study, using Caco-2 BBc cells expressing EGFP-linked β-actin (Fig. 4E).

Inhibition of actin polymerization. To further evaluate a potential involvement of actin in the mechanisms contributing to single-cell restitution in native mouse colon, experiments were carried out with cytochalasin D, an inhibitor of actin polymerization. Preincubation of mouse colon in Ussing chambers with cytochalasin D caused a decrease of transepithelial resistance within 60–90 min after adding the drug (Table 1, Fig. 3A). This is in accordance with other studies using intestinal tissue from guinea pigs (3). After preincubation with cytochalasin D, the tissue was mounted horizontally in the conductance scanning setup to study single-cell restitution. Control tissue (after 90 min Ussing chamber) revealed a fast repair of single-cell lesions with a time constant τ = 0.36 ± 0.10 min, which did not differ significantly from that recorded after 17 h incubation in the Ussing chamber (Table 1, Fig. 3B). In contrast to that, specimens incubated with cytochalasin D showed a delay in repair (Fig. 3B). τ increased significantly to 0.69 ± 0.10 min ($P < 0.05$ vs. control, Table 1). Delay in repair could also be demonstrated by CLSM, in which all four lesions surrounding a crypt were clearly visible, indicating that lesions were not closed after 3 to 4 min (n = 3, Fig. 4B). These results demonstrate that in spite of the lack of evidence for an active purse string mechanism, the repair of single-cell lesions in native tissue strongly depended on actin.

Effect of the proinflammatory cytokines TNF-α and IFN-γ. To study the effect of proinflammatory cytokines (TNF-α, IFN-γ), epithelial tissue samples and cultured HT-29/B6 cells were incubated with the serosal side in conventional Ussing chambers for 17 h and 7 h, respectively. After incubation with TNF-α (100 ng/ml) or IFN-γ (1,000 units/ml) alone or with both cytokines together, the conductivity of intact colonic surface epithelium ($G_{\text{intact}}$) was not significantly different from controls (Table 1).

In colonic epithelium, the time course of repair did not change after TNF-α preincubation alone ($τ = 0.28 ± 0.03$ min, n = 7 lesions, m = 4 colon specimens, Table 1, Fig. 5A). In contrast, IFN-γ alone caused a delay in single-cell restitution. Only 75% ± 3% ($τ = 0.49 ± 0.07$ min, n = 13 lesions, m = 6 colon specimens) of the defect was repaired within the time course from 1.5 min to 2.5 min after setting the lesion (Table 1, Fig. 5B). After combined incubation with TNF-α and IFN-γ, repair time was also higher than in controls and similar to results after incubation with IFN-γ alone (Table 1, Fig. 5B). Only 68% ± 3% ($τ = 0.59 ± 0.06$ min, n = 10 lesions, m = 4 specimens) of the defect was repaired within the time course from 1.5 min to 2.5 min after setting the lesion (Table 1, Fig. 5B). In the same time interval, more than 90% of the defect was repaired under control conditions and after TNF-α incubation alone.

These results differed from the findings in HT-29/B6 cells. In these cells, incubation with 100 ng/ml TNF-α caused an increase in $G_{\text{intact}}$ from 1.3 ± 0.1 mS/cm$^2$ (n = 17) to 8.4 ± 1.3 mS/cm$^2$ (n = 9) and greatly delayed restitution, as indicated by an increase of τ from 5.73 ± 0.35 [n = 17; (16)] in controls to 35.7 ± 7.9 min (n = 9) in the presence of TNF-α (Fig. 5, C and D). The assembly of the actin ring surrounding the lesion was not affected by the presence of TNF-α (not shown), indicating that some later process leading to restitution in cultured cells must be impaired.

After conductance scanning, tissues were stained with H&E. Figure 6 shows samples under control conditions (Fig. 6A), incubated with TNF-α alone (Fig. 6B), incubated with IFN-γ alone (Fig. 6C), and incubated with TNF-α in combination with IFN-γ (Fig. 6D). After histological examination of three independent colon specimens from each condition, we found no evidence of necrosis or multicellular erosions after 17 h in the Ussing chamber.

Apoptotic ratio of epithelial cells. The identical specimens used in the conductance scanning setup were analyzed for epithelial apoptosis using a TUNEL assay (Table 2). Fresh tissue revealed an apoptotic ratio of 1.24%. After 17 h in the Ussing chamber (sham incubation), the ratio, 1.30%, was not significantly altered. Tissues incubated either with TNF-α alone, 2.74%, or with IFN-γ alone, 2.21%, or with TNF-α in combination with IFN-γ, 2.58%, showed about doubled apoptotic ratio. Representative images are shown in Fig. 7 (arrows indicate apoptotic nuclei).

Viability assay after 17 h in the Ussing chamber. Because 17 h incubation in the Ussing chamber raises the question of long-term survival of the tissues, three parameters of viability were tested, active, forskolin-stimulated ion transport, transepithelial resistance, and the meshwork distribution of a tight junction protein. After 17 h in the Ussing chamber forskolin (10−5 M) induced active ion transport [reported to be due to chloride secretion, (46)], which was detected by an increase of short circuit current ($ΔI_{sc}$) in a control group (n = 4) and in colon specimens incubated with TNF-α + IFN-γ (n = 5). In controls, forskolin induced an increase of $ΔI_{sc}$ by 178 ± 33 μA/cm$^2$ (n = 4). The cytokine incubated colon specimens show an increase of $ΔI_{sc}$ by 61 ± 15 μA/cm$^2$ (n = 5). The stimulation of the cytokine-incubated specimens was thus smaller than in control tissues, but both showed the ability to respond to secretagogues. Although it cannot be ruled out that the cytokines had a direct antisercretory effects in murine colon, the differences in $ΔI_{sc}$ after cytokine incubation are roughly proportional to changes in overall tissue geometry (i.e., number of crypt and depth of crypt) and may, therefore, be predominantly attributed to the decreased cell density per unit gross.
tissue area. No dramatic change in epithelial integrity was detected in the H&E-stained tissue sections.

The transepithelial resistance, $R_t$, was measured in conventional Ussing chambers (Table 3). All tissues, controls, and samples incubated with proinflammatory cytokines, showed an initial increase of $R_t$ within the first 6 h, which decreased over time. Incubated tissues did not show a significant difference compared with controls. All tissues remained on high $R_t$ values, indicating intact barrier function after 17-h Ussing chamber incubation.

Conversion of the $R_t$ values given in Table 3 into conductivities result in somewhat higher values than the corresponding values obtained during conductance scanning experiments (cf. Table 1). This is due to the fact that during scanning, a small, intact area of the tissue well away from the edges of the chamber is evaluated, whereas values obtained from Ussing...
chamber experiments refer to the whole tissue samples mounted in the chamber and thus include all leaks present in this sample, such as apoptoses or leaks along the edge of the chamber.

To visualize the intact barrier after incubation for 17 h in Ussing chambers, the integral tight junction protein occludin was stained in control specimens. In Fig. 6, a representative confocal laser scanning image is shown, demonstrating an intact tight junction meshwork between epithelial cells. Thus, the functional analysis with forskolin, high \( R_t \) values after 17 h in Ussing chambers, and the intact barrier shown by occludin are significant arguments supporting the fact that tissue incubated for 17 h in Ussing chambers remains viable.

Effect of supernatant of hemolytic and nonhemolytic \textit{E. coli} cultures. To investigate the relevance of single-cell lesions in pathogenic processes, restitution was investigated in the presence of the supernatant of the hemolytic \textit{E. coli} strain O4 and a nonhemolytic mutant of the same strain. As the supernatant of the hemolytic \textit{E. coli}, O4 may cause direct damage to the epithelium; time of exposure was minimized by adding the supernatant to the mucosal side of the preparation just before inducing four lesions around a crypt at 45-s intervals and then immediately fixing the preparation in 10% formalin. Subsequently, preparations were stained for actin, and restitution was evaluated by the number of lesions that could still be identified by CLSM. The site of lesions was identified by the staining of cell nuclei with the fluorescence dye DAPI, which was co-injected together with \( \text{Ca}^{2+} \) during the iontophoretic induction of the lesions. In the presence of the supernatant of the nonhemolytic \textit{E. coli}, O4 mutant restitution was comparable to control tissue: a maximum of two of the four lesions could be identified, as disruptions of the otherwise intact epithelial structure (“chicken-wire”-like appearance), whereas the DAPI staining showed all 4 sites of injection. In contrast, the supernatant of the hemolytic \textit{E. coli} O4 strain effectively prevented restitution—all four lesions were clearly visible, whereas the surrounding tissue appeared to be intact (Fig. 4C).

DISCUSSION

Under normal physiological conditions, superficial damage causing minor breaks in epithelial continuity may be associated with intestinal motility (16, 30, 47). Loss of a single cell, caused by exogenous injury or spontaneous apoptosis (37), disturbs epithelial barrier function, enabling antigens to permeate the epithelial barrier into the mucosa. Hence, rapid repair is necessary, because even small defects may allow pathogens/antigens to enter the organism and trigger inflammatory responses (34) that may contribute to the pathogenesis of inflammatory bowel disease (20).

Recently, we characterized mechanisms of restitution in single-cell defects induced in HT-29/B6 monolayers (16), an established cell culture model of colonic epithelium (25), and in Caco-2 BBe cells (38). Single-cell defects are rapidly...
patched by flattening of the neighboring intact cells, without migration of distant cells. Actin forms a purse string suture around the gap that, with myosin, pulls taut the edges and serves as a base for new tight junction proteins (45). Concurrently, the local conductive leak associated with the defect decreases with an exponential time course, and functional sealing is accomplished in 20 min. This is much faster than the restitution of multicellular defects, which may take several hours (13, 47).

So far, nothing is known about restitution after the loss of a single cell in native intestinal epithelium. Iontophoretic injection of Ca\(^{2+}\) visibly damaged the injected cell and caused the

Table 2. Apoptotic ratio of analyzed colon specimens

<table>
<thead>
<tr>
<th></th>
<th>Control, 0 h</th>
<th>Control, 17 h</th>
<th>TNF-(\alpha), 17 h</th>
<th>IFN-(\gamma), 17 h</th>
<th>TNF-(\alpha) + IFN-(\gamma), 17 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptoses</td>
<td>124</td>
<td>89</td>
<td>96</td>
<td>89</td>
<td>146</td>
</tr>
<tr>
<td>Cells counted</td>
<td>9975</td>
<td>6846</td>
<td>3510</td>
<td>4036</td>
<td>5564</td>
</tr>
<tr>
<td>%apoptoses</td>
<td>1.24%</td>
<td>1.30%</td>
<td>2.74%</td>
<td>2.21%</td>
<td>2.58%</td>
</tr>
</tbody>
</table>

Apoptotic ratio was analyzed using the terminal deoxy nucleotidyl transferase-mediated dUTP-biotin nick end labeling assay. Specimens from animals directly after death (Control, 0 h) were compared with tissues incubated for 17 h in a conventional Ussing chamber (Control, 17 h; with TNF-\(\alpha\), INF-\(\gamma\), or TNF-\(\alpha\) + IFN-\(\gamma\)); in each condition; data from 3 independent colon specimens were pooled.
adjacent cells to spread to fill the lesion. This indicates that the artificial injury applied here was not restricted to the apical membrane (32) but destroyed the whole cell, or, as found when investigating the defect by CLSM, often also a few neighboring cells. This finding was supported by measuring the resulting conductive leaks using the conductance scanning technique (17, 18, 20). The leaks were found to be even larger than in cultured cells; their value was equivalent to about 10,000 large

Table 3. Transepithelial resistance of colonic epithelium during incubation

<table>
<thead>
<tr>
<th></th>
<th>$R_t \Omega \text{cm}^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$t = 1 \text{ h}$</td>
</tr>
<tr>
<td>Control</td>
<td>129.8±4.3</td>
</tr>
<tr>
<td>TNF-α</td>
<td>148.7±15.1</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>146.6±14.4</td>
</tr>
<tr>
<td>TNF-α + IFN-γ</td>
<td>146.8±7.7</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. Transepithelial resistance of colonic epithelium ($R_t$) was measured in conventional Ussing chambers during a time course of 17 h. m is the number of colon specimens. None of the values for TNF-α, IFN-γ, and TNF-α + IFN-γ were significantly different from control. Statistical analysis, U-test of Wilcoxon, Mann and Whitney with Bonferroni correction for multiple testing.
ion channels of 100-pS mean conductance, e.g., Maxi-K⁺-channels. Hence, rapid repair of the native epithelium appears to be important for the maintenance of barrier function.

Our results show that in native colon epithelium, single/oligo-cell defects close with a time constant of about 0.28 min. The repair process observed here is thus the fastest observed so far in any epithelial restitution. This explains why defects are rarely found in histological sections of healthy colonic surface epithelium (5). Restitution was more than 10 times faster than in the cell cultures investigated previously (16, 38). Reasons for these differences in time-course between colonic epithelium and cultured cells are unknown. They are not due to a difference in species, as human biopsies or resectates showed the same rapid repair as murine colonic epithelium. It cannot be ruled out that the differences are due to the different origin of the cells (both HT29/B6 cells and Caco-2 BBt cells used in the present study are derived from tumor cells); however, this only adds additional stress to the fact that results obtained from cell cultures must be interpreted with care.

Indeed, speed was not the only difference between restitution of a native colonic epithelium and of cultured cells. In cultured cells, actin plays a key role in the purse string mechanism of restitution (4, 8, 11, 38). In contrast, in native epithelium we were unable to detect any actin accumulation surrounding the lesion during the repair process. This is not surprising, as in cultured cells, actin ring assembly itself only begins within the first 2 min after wounding and a complete ring is found after about 8 min (38), and thus at a time when in the native epithelium the wound has long been closed. Nevertheless, cytochalasin D, a membrane permeant fungal toxin that blocks actin polymerization (42), inhibited repair of single-cell lesions not only in cell culture (16) but also slowed it down significantly in native colon. In addition, cytochalasin D caused a drop in transepithelial resistance, in accordance to a study of intestinal tissue from guinea pigs (3), indicating that actin is of general, major importance for the formation of an intact epithelial barrier. It is therefore hypothesized that actin may either be needed directly for cell motility during the restitution process or indirectly to accelerate TJ assembly and thus barrier formation.

In a further set of experiments the question was raised whether restitution is affected by the presence of cytokines (TNF-α, IFN-γ) that are released during inflammation. Disturbance of epithelial barrier function by proinflammatory cytokines is of great pathophysiological importance. Several mechanisms have been identified such as apoptosis (1, 18), increased paracellular permeability (9, 39), and cell lesions and erosions (20). The possible effect of proinflammatory cytokines on the restitution of multicellular epithelial lesions has been investigated only in cell cultures and nonintestinal tissue. IFN-γ stimulates migration in IEC-6 cells (12) and in lung epithelial cells (2) but had no effect in primary ooyctic cultures (24). Recently, it was shown that TNF-α regulates intestinal epithelial migration by receptor-dependent mechanisms (10). Pathological levels (100 ng/ml) decrease migration through TNF receptor 1. Our study is the first that investigates the effect of these cytokines on restitution in native intestinal epithelium.

In the present study, TNF-α was used at a concentration of 100 ng/ml and thus at a concentration showing maximum effect on barrier function in HT-29/B6 cells (39). This concentration is well above serum concentrations in patients with Crohn’s disease (31) and close to concentrations found in patients dying from septic shock (44). IFN-γ was used at a concentration of 1,000 units/ml. Grotjohann et al. (22) found a strong effect on barrier function of rat colonic epithelium when applying IFN-γ at this concentration together with 100 ng/ml TNF-α.

Because of the long incubation times for TNF-α and IFN-γ, the viability of the epithelium had to be tested during prolonged (17 h) preservation in Ussing chambers, before analyzing the influence of cytokines on epithelial restitution (22). Viability of the tissue was demonstrated by measurements of transepithelial resistance and the response to a secretagogue, by the absence of necrosis and by staining of occludin, an integral membrane protein of the tight junction, to visualize continuity of the tight junctions. A further indication for viability of the tissue was the unchanged time course of restitution after 90 min and 17 h (compare controls in Fig. 3B).

Single-cell restitution in native epithelium was unchanged after incubation with TNF-α, but after incubation with IFN-γ, the duration of repair almost doubled. Coincubation of TNF-α and IFN-γ did not show any significant additional effects. In contrast, TNF-α greatly inhibited restitution in HT-29/B6 cell layers. This again indicated that restitution processes in cultured cells and in native epithelia differ with respect to the underlying mechanism. The findings are also in contrast to the effect of TNF-α and IFN-γ on active transport barrier function, where TNF-α plays the major role (6, 39). In conclusion, the proinflammatory cytokine IFN-γ, but not TNF-α, impairs the repair of single-cell wounds in native epithelia. This may suggest a new mechanism aggravating the loss of barrier function in the inflamed intestine in IBD by slowing down its repair.

Finally, to demonstrate the relevance of small, single or oligo-cell lesions in pathogenic processes, the effects of sterile filtered supernatants of hemolytic and nonhemolytic E. coli O4 strains were tested. Prolonged exposure of epithelia to the hemolytic E. coli O4 strain is known to directly damage the epithelium (36). Exposure time was therefore kept to a minimum (<5 min), by adding the supernatant just before inducing four lesions every 45 s and then immediately fixing the tissue by the application of 10% formalin. However, even this brief time was sufficient to effectively block restitution, while the surrounding cells looked completely undamaged. In contrast, the supernatant of the nonhemolytic strain had no apparent effect on wound repair. This finding indicates that some bacteria may use preformed lesions (e.g., due to apoptosis) as potential sites for entry from the gut into the organism by arresting restitution. This finding again emphasized the importance of very fast and efficient repair mechanisms even for smallest injuries of the epithelial barrier.

In conclusion, this study presents a histological and functional description of repair of single- or oligo-cell wounds in native colon epithelia. Different agents such as proinflammatory cytokines or factors produced by pathogenic E. coli are able to impair the repair of these wounds. This may suggest a new mechanism interfering with the barrier function of inflamed intestine in IBD by slowing down its repair and supporting pathogens in the process of entering the body. The differences in observations between cell culture model analyses and native tissue measurements underline the necessity for in vivo studies.
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