Numerous reports have implicated *Aeromonas* species as a cause of human enteric disease in children as well as in adults (for reviews, see references 3, 22, 23, 28, and 36). However, although a large body of literature on *Aeromonas* virulence factors has been accumulated (for a review, see references 9 and 22), the mechanisms of the diarrheic action of *Aeromonas* are not completely understood. As for other pathogens, *Aeromonas* virulence factors can be divided into cellular properties such as adherence, invasiveness, motility, and extracellular factors secreted by pathogenic aeromonads (22). Evidence for the enteropathogenic potential of these virulence factors was obtained from their epidemiological association with diarrheal disease (3, 10, 24), from their ability to induce enteral fluid secretion in animal models, and from the observation of effects linked to enteral fluid secretion elicited in various animal and cell culture systems (see, for example, references 4, 7, 29, and 33). Surprisingly, then, the precise mechanism for how *Aeromonas* infection causes intestinal fluid secretion has not yet been elucidated.

From a general pathophysiological point of view, intestinal pathogens can induce diarrhea by induction of active ion secretion and/or by impairment of the epithelial barrier function of the intestine. In support of the latter mechanism, certain *Aeromonas* toxins were shown to be cytotoxic for intestinal cells, as well as for other mammalian cells (1, 6, 19, 29). However, it is questionable whether massive physical disruption of the epithelial barrier as the result of necrosis of enterocytes is the predominant mode of action in *Aeromonas* enteritis, which usually presents as watery diarrhea but only rarely as dysenteric disease (3). Therefore, it seemed to be necessary to take a closer look at the interaction of the bacteria and their toxins with the intestinal epithelium in order to define the mechanisms of diarrhea caused by aeromonads. However, to the best of our knowledge, at present there is only one study addressing the issue of intestinal barrier impairment by an *Aeromonas* toxin at the enterocyte level (2). In that study, *Aeromonas* beta-hemolysin caused a decrease in transepithelial resistance in polarized intestinal epithelial cells, but no attempt was undertaken to characterize the mechanism underlying this resistance drop, and no data referring to transepithelial movement of ions or other solutes were provided. Therefore, it is currently not known whether *Aeromonas* induced diarrhea is due solely to passive solute flux after damage of the epithelial barrier or also to active ion secretion. Furthermore, the effector mechanism of the *Aeromonas* induced barrier impairment remains to be identified.

In order to gain more insight into the epithelial effects of *intestinal Aeromonas* infection, we performed electrophysiological studies in monolayers of HT-29/B6 cells. These cells represent a stable, highly differentiated subclone derived from wild-type HT-29 cells by glucose deprivation (27). When grown on permeable supports, they form polarized monolayers with
intact regulation of tight junction formation, which is the key determinant of epithelial barrier function (38). In addition, HT-29/B6 monolayers are capable of regulated vescicular Cl− and mucin secretion (12, 13). Thus, this highly differentiated cell line possesses most of the features of native intestinal epithelia necessary for maintenance of intestinal barrier function, as well as for the regulation of intestinal ion transport.

For these reasons, HT-29/B6 cells have been used as model epithelium for mechanistic investigations of altered intestinal ion secretion and barrier function in many studies before (see, for example, references 12, 13, 31, and 38).

In the present study, we investigated the ability of aeromonads to stimulate ion secretion and to impair epithelial barrier function of HT-29/B6 monolayers. In an initial set of experiments, an (apparently) enteropathogenic Aeromonas strain, isolated as the only enteropathogen from a patient with acute watery diarrhea, was compared to an (apparently) non-enteropathogenic strain that was isolated from normal stool of a patient without any symptoms of diarrhea. Since only the enteropathogenic strain altered the transport and barrier properties of the monolayers, further experiments focused on the mechanisms and signal transduction of the effects elicited by the pathogenic strain and its secretory products.

MATERIALS AND METHODS

Bacterial strains and plasmids. All Aeromonas strains were obtained from the Department of Microbiology of the Freie Universität Berlin. Aeromonas sp. strain Sb was isolated from a patient with acute watery diarrhea. Other than Aeromonas, no other enteropathogens were found in the stool specimens of this patient. Strain Ha has been isolated from the normal stool of a patient without diarrhea. Sequencing of the 16S rRNA subunit identified strain Sb as genotype Aeromonas hydrophila and strain Ha as genotype Aeromonas veronii. Escherichia coli DH5α and TOP10F (Invitrogen, Karlsruhe, Germany) were used for plasmid expression. Plasmid pBR322 encoding for ampicillin and tetracycline resistance (R\text{A}) was used to test for confluence. After confluence, monolayers of HT-29/B6 cells form an epithelial barrier between the apical compartment and the basolateral compartment outside. Monolayers were used for the experiments 7 or 8 days after seeding, when R\text{A} values were between 300 and 500 Ω cm⁻².

Measurements in human colon. Segments of macroscopically normal colon were obtained from five patients undergoing resective surgery for colon carcinoma. As described earlier (11), the colon epithelium was mounted into Ussing-type chambers. Then, electrophysiological studies were performed as described below for the cell culture experiments.

Electrophysiological studies. Intact culture plate inserts carrying confluent filter grown monolayers of HT-29/B6 cells were placed into modified Ussing-type chambers as described previously (12). Briefly, the mucosal and the serosal compartment were filled with 10 ml of a modified Ringer solution (bathing solution) containing 151 mM Na⁺, 5 mM K⁺, 1.7 mM Ca²⁺, 0.9 mM Mg²⁺, 130.4 mM Cl−, 28 mM HCO₃⁻, 1 mM H₂PO₄⁻, 0.9 mM SO₄²⁻, and 25 mM β-(l)-glucose. The solution was stirred and oxygenated via bubble lift. The pH was 9.15 ± 0.05. The bathing solution was restored by replacing the solution at a flow rate of 5.0 ml/min at room temperature. Measurements in human colon were performed with a computerized automatic clamp device (Fiebig Hard- and Software, Berlin, Germany). Mouse colonic epithelial mucosa was excised as described previously (39). Ion transport was measured by means of unidirectional (i.e., mucosal-to-serosal [ms] and serosal-to-mucosal [sm], respectively) ⁵¹Na and ⁴⁰Cl fluxes (DuPont, Bad Homburg, Germany). For calculating net fluxes, monolayers were matched for conductance. All flux experiments were performed under short-circuit conditions. For ion replacement studies, the monolayers were stimulated with Aeromonas supernatant as described above. When submaximum chloride values were reached, 5 ml of the bathing solution was cautiously removed and replaced by a modified bathing solution free of Cl⁻ ions (see below). In this manner, the barrier-lift concentration of the bathing solution was unanticipated and repeated three times. By then, the Cl⁻ content of the bathing solution was reduced to 16.3 mM. I\text{sc} values were determined after a 10-min equilibration period. Then, a high Cl⁻ concentration within the bathing solution was restored by repeated (three times) partial exchange of the bathing solution with standard solution again. The Cl⁻ solution was composed of 60 mM Na₂SO₄, 2.7 mM K₂SO₄, 1.2 mM MgSO₄, 7 mM H₂O, 1.2 mM CaSO₄, 2.9 mM HEPES, and 25 mM β-(l)-glucose. The pH of the solution was adjusted to 7.4 by titration with HSO₄⁻. For all electrophysiological experiments, independent controls were processed in parallel.

Immunofluorescence. In order to test for the integrity of the tight junctional membrane, the tight junction protein ZO-1 was visualized after 2 h of Aeromonas exposure, when R\text{A} had leveled off at minimum values. Monolayers were removed from the Ussing chamber washed with phosphate-buffered saline (PBS) and fixed in ice-cold methanol for 10 min. The cells were then washed with PBS and permeabilized by incubation with 0.5% Triton X-100 for 5 min. Reduced unspecific binding sites, cells were incubated with 0.5% goat serum for 30 min at room temperature. The cells were then incubated with mouse anti-ZO-1 antibodies (1:50; Zymed, San Francisco, Calif.) for 30 min at room temperature, followed by a wash with 0.5% goat serum. Alexa Fluor immunofluorescence antibodies (Molecular Probes Europe, Leiden, The Netherlands) Alexa Fluor-594 goat anti-rabbit immunoglobulin G and Alexa Fluor-594 goat anti-mouse immunoglobulin G (1:50) were used to visualize the samples. After washing the samples were washed and mounted in ProTaqs MountFluor medium (Biocytex, Luckenwalde, Germany). Immunofluorescence microscopy was performed.

LDH release assay. Lactate dehydrogenase (LDH) release from HT-29/B6 cells was measured according to the method of Madara and Stafford (30). Briefly, the LDH content in the supernatant of controls and of cells treated with Aeromonas supernatant was determined and compared to the total LDH content of the residual cells which was determined after detergent extraction with 2% Triton X-100 for 30 min. LDH release was expressed as a percentage of total LDH released into the supernatant.

Measurement of hemolytic activity. To assess the hemolytic activity of bacterial lysates or culture supernatants, a serial twofold dilution of the respective samples (in PBS) was incubated with 0.5% sheep erythrocytes (diluted with PBS containing 10 mM dithiothreitol) in microtiter trays at 37°C. Sodium dodecyl sulfate was used as a positive control, and PBS served as a negative control. A hemolytic reaction was considered positive if lysis of erythrocytes was evident on inspection after 4 h of incubation. The hemolytic activity of a sample was expressed as the reciprocal of the highest dilution that still gave a positive hemolytic reaction.

Cloning of the beta-hemolysin gene of strain Sb. Total DNA was extracted from strain Sb with 0.05 N NaOH at 95°C, followed by neutralization with 1 M Tris-HCl and removal of cell debris by centrifugation. The beta-hemolysin gene was then amplified by PCR. Seven different primer pairs derived from Aeromonas beta-hemolysin sequences published in GenBank were tested. The 303 bp amplification product amplified with the primers SFLA (5'-GCGATGGATACACATCCGGAAAG-3') and SE-R (5'-CTATGAAAGGGGCTGCGG-3') was based on an aerolysin-related beta-hemolysin of Aeromonas hy-

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Aeromonas sp. strain Sb was isolated from a diarrheal isolate. Overnight cultures (250 ml) were harvested, washed with PBS, and resuspended in 10 ml of ice-cold bathing solution. The intracellular content of the bacteria was released into the bathing solution after cell wall disrupture with a hydraulic press. Fifty white colonies were picked and plated on kanamycin-containing blood agar plates. A colony showing a distinct zone of beta-hemolysis was selected and cultured overnight in Luria-Bertani medium containing ampicillin. The plasmid DNA was then isolated with a commercially available kit (GFX Micro Plasmid Prep Kit; Amersham Biosciences, Freiburg, Germany), and the insert was analyzed by sequencing.

Lysozymes from E. coli, E. coli DH5α, or TOP10F’ cells as indicated were harvested from overnight cultures (250 ml), washed with PBS, and resuspended in 10 ml of ice-cold bathing solution. The intracellular content of the bacteria was released into the bathing solution after cell wall disruption with a hydraulic press (Thermo Spectronic, Cambridge, United Kingdom). After cell lysis, cell debris and cell membrane remnants were removed by centrifugation (20,000 × g for 30 min at 4°C), and the lysate was used for electrophysiological experiments and hemolysin titer tests.

RESULTS

Effect of coinoculation with Aeromonas on transport and barrier properties of HT-29/B6 monolayers. Bacteria were cultured in RPMI 1640 and added to the mucosal compartment of HT-29/B6 cells mounted in Ussing chambers to yield an initial concentration of 10^5 CFU/ml. After 90 min post addition of the pathogenic strain Sb (diarrhetic isolate), the ISc started to increase and the Rt values started to decline (Fig. 1). Maximum ISc values of 39 ± 3 μA/cm² were reached after 140 min of coinoculation. At this time point Rt had dropped to ca. 50% of its initial value. Thereafter, the ISc gradually dropped to −20 μA/cm². Rt values, on the other hand, decreased continually until they leveled off after 200 min at a residual Rt of −24 Ω cm² equaling 6.8% of the initial Rt (354 ± 4 Ω cm²). In contrast to strain Sb, the apathogenic strain Ha (derived from an asymptomatic patient without gastrointestinal symptoms) did not evoke any effects on ISc and Rt over more than 5 h, although both strains grew well during the experiment. Thus, after 3 h of incubation in the bathing solution, serial dilutions revealed an estimated bacterial number of 4 × 10^8 CFU/ml for strain Sb compared to 8 × 10^7 CFU/ml for strain Ha. The respective numbers after 5 h of incubation were 10^6 CFU/ml for strain Sb and 8 × 10^7 CFU/ml for strain Ha.

Effect of Aeromonas supernatant on transport and barrier properties of HT-29/B6 monolayers. In order to determine whether the effects observed were due to direct interaction of the bacteria with the epithelial cells or rather caused by a toxin secreted into the bathing medium, monolayers were coinoculated with Aeromonas sp. strain Sb as for the experiments described in Fig. 1. After a 100-min lag time, the ISc started to increase. When maximum ISc values were reached (Fig. 2A; ISc = 41 ± 2 μA/cm² and Rt = 63% ± 4% of initial Rt [Rt data not shown]), the mucosal bathing solution was removed and cleared from bacteria by centrifugation and sterile filtration. By addition of the bacterium-free solution to the mucosal side of new monolayers mounted in Ussing chambers, without any latency, the Aeromonas effect on ISc and Rt could be restored (Fig. 2B, maximum ISc = 29 ± 1 μA/cm² and Rt after 120 min = 68% ± 9% of initial Rt). Obviously, the active component within the bathing solution was independent from the presence of bacteria and, as a consequence, the ISc and Rt responses must have been caused by the action of a soluble factor secreted by the aeromonads. Subsequent experiments were therefore performed with bacterium-free supernatant.

In Fig. 3, dose-response data obtained with different concentrations of Aeromonas supernatant are depicted. Both the maximum ISc values and the Rt drop induced clearly correlated to the concentration used. Next, we tested whether the monolayers were also sensitive to serosal addition of Aeromonas sp. strain Sb supernatant. In fact, the epithelial resistance decreased faster with serosal versus mucosal addition of the supernatant (Rt at 50% of initial value, 22 min after serosal addition versus 54 min after mucosal addition, respectively). Interestingly, after serosal addition the basal short circuit current was completely abolished, indicating cessation of all active electrogenic ion transport processes (Fig. 4).

Effect of Aeromonas supernatant on ion transport of native human colon epithelium. In a limited set of experiments we tested the effect of Aeromonas sp. strain Sb supernatant on short circuit current of native human colon epithelium. As depicted in Fig. 5, after an initial ISc peak, immediately after addition of the supernatant, the Sb supernatant elicited a prolonged and marked increase in ISc. Thus, native human colon was responsive to Aeromonas sp. strain Sb supernatant as well.
Apart from the initial peak, the $I_{SC}$ response observed in native colon epithelium was quite similar to that of HT-29/B6 monolayers.

**Ion and mannitol fluxes induced by Aeromonas supernatant and ion replacement studies.** In order to identify the ion transport processes responsible for the $I_{SC}$ induced by *Aeromonas* supernatant, unidirectional NaCl fluxes before (control) and after mucosal addition of *Aeromonas* supernatant (strain Sb) were performed (Table 1). All unidirectional fluxes increased after addition of the supernatant, indicating a significant increase in paracellular permeability induced by the supernatant. Unidirectional ms and sm fluxes were used to calculate net fluxes reflecting vectorial ion transport. Under control conditions, there were no significant net fluxes. However, after addition of the supernatant, the net Cl$^-$/H$^+$ flux (representing chloride secretion), significantly increased, whereas sodium absorption (net Na$^+$ flux) and bicarbonate secretion (negative residual flux) remained unchanged. Quantitatively, there was an excellent correlation between $I_{SC}$ (1.52 ± 0.08 μmol h$^{-1}$ cm$^{-2}$) and Cl$^-$/H$^+$ secretion (1.58 ± 0.52 μmol h$^{-1}$ cm$^{-2}$). The ionic basis of the $I_{SC}$ induced by *Aeromonas* supernatant was further investigated by ion replacement studies. Reducing the Cl$^-$ concentration to 12.5% of the standard bathing solution (16.3 mM) by serial dilution with Cl$^-$-free solution led to a 40% decrease of supernatant-induced $I_{SC}$ (14.9 ± 0.9 μA/cm$^2$ versus 35.5 ± 1.5 μA/cm$^2$ [$P < 0.001$, $n = 6$ for each group]), and subsequent elevation of the Cl$^-$ concentration within the bathing solution restored the $I_{SC}$ to 89% of the initial value (32.0 ± 3.3 μA/cm$^2$, Fig. 6). Taken together, both experiments indicate that the $I_{SC}$ induced by the *Aeromonas* sp. strain Sb supernatant was mainly accounted for by electrogenic chloride secretion.

In order to further characterize the drop in epithelial resistance caused by *Aeromonas* supernatant, experiments with labeled mannitol (a marker of paracellular permeability) were performed. After 3 h of mucosal incubation with *Aeromonas* supernatant, the transepithelial resistance of the monolayers had decreased to $<10\%$ of the initial value. At the same time, unidirectional mannitol fluxes revealed a 4.6-fold increase of mannitol permeation ($P < 0.001$), indicating a significant increment of paracellular permeability (Table 2).

**Structural alterations of monolayers after treatment with Aeromonas supernatant.** In order to test for disrupture of the epithelial barrier after damage of epithelial cells, monolayers were incubated with *Aeromonas* sp. strain Sb supernatant in the Ussing chamber and fixed in methanol after the transepithelial resistance had dropped to $<20\%$ of the initial value. Visualization of the tight junction protein ZO-1 showed an intact meshwork pattern with no gaps or other visible differences to controls processed in parallel (Fig. 7).

Furthermore, in independent experiments, LDH release from *Aeromonas* supernatant-treated HT-29/B6 monolayers was determined again, after the $R^2$ had dropped to $<20\%$ of the initial value. Since the percentage of LDH released into the supernatants was equal in controls and *Aeromonas* supernatant-treated cells (1.6% ± 0.2% versus 1.2% ± 0.1% [not
significantly, n = 6 for each group), the supernatant induced decrease in R' was obviously not a consequence of necrosis of epithelial cells.

**Signal transduction of Aeromonas sp. strain Sb supernatant-induced I_sc.** Since it has been shown that protein kinase A, protein kinase C, and elevation of intracellular Ca\(^{2+}\) comprise the most important stimulatory signal transduction pathways of chloride secretion (5, 13), inhibitors of protein kinase A (H8), protein kinase C (chelerythrine), and a membrane-permeable Ca\(^{2+}\) chelator (BAPTA-AM) were tested for their inhibitory action on the I_sc induced by Aeromonas supernatant, as was a tyrosine kinase inhibitor (genistein). Prior to these experiments, all inhibitors were tested with respect to their action on HT-29/B6 cells. Thus, H8 inhibited the stimulatory effect of the cyclic AMP mobilizer forskolin (1 \(\mu\)M) on ion secretion of HT-29/B6 cells, chelerythrine inhibited ion secretion induced by the phorbol ester phorbol myristate acetate (5 nM), and BAPTA-AM inhibited the ion secretion elicited by the Ca\(^{2+}\)-dependent agonist carbachol (100 \(\mu\)M). Since a tyrosine kinase-dependent agonist inducing chloride secretion in HT29-B6 cells is not available, genistein could not be tested in the same way as the other inhibitors. However, it was shown previously that genistein inhibited the drop in R' induced by tumor necrosis factor alpha in HT-29/B6 cells (38). Thus, we included genistein in our experiments in order to investigate the involvement of tyrosine phosphorylation in the barrier effect elicited by the Aeromonas supernatant. For quantitative comparison, the I_sc induced by Aeromonas supernatant after preincubation with the respective inhibitor was related to the I_sc induced by Aeromonas supernatant without inhibitor as determined in parallel experiments. As shown in Fig. 8, inhibition of protein kinase C by chelerythrine significantly inhibited the chloride secretion induced by strain Sb supernatant (I_sc induced 32 \(\pm\) 2 \(\mu\)A/cm\(^2\) with chelerythrine versus 42 \(\pm\) 2 \(\mu\)A/cm\(^2\) without chelerythrine [n = 6, P < 0.005]), whereas inhibition of protein kinase A or tyrosine kinase, as well as intracellular Ca\(^{2+}\) chelation, had no effect. In contrast to the I_sc, the drop in R' was not significantly affected by any of the inhibitors used (data not shown). Taken together, activation of
protein kinase C obviously plays a role in the secretory action of the Aeromonas sp. strain Sb supernatant, whereas protein kinase A and intracellular Ca²⁺, which are commonly regarded as the most important regulatory signaling pathways of epithelial chloride secretion, do not seem to be involved.

Selected physicochemical properties of the putative Aeromonas toxin secreted into the supernatant. For a size estimate of the active compound, aliquots of Aeromonas supernatant were ultrafiltrated by using filters with different exclusion sizes. The potential of the different fractions to induce chloride secretion and to impair the transepithelial resistance of HT-29/B6 monolayers was then investigated in the Ussing chamber. As shown in Fig. 9, the size of the active compound could be narrowed down to between 30 and 100 kDa.

Since both heat-labile and heat-stable exotoxins have been described for Aeromonas, the activity of the supernatant was tested for heat sensitivity. After mild heat treatment (10 min at 56°C), the effects of the Aeromonas supernatant on I_SC and transepithelial resistance were completely abolished (i.e., an I_SC increase after the addition of untreated supernatant of 26.8 ± 0.7 μA/cm² versus –0.43 ± 0.7 μA/cm² after the addition of heat-inactivated supernatant). Heat sensitivity (56°C, 10 min) has been described for Aeromonas beta-hemolysin, a 50-kDa toxin actively secreted by many Aeromonas strains and considered to be the single most important virulence factor of Aeromonas (3). Interestingly, when grown on blood agar, plates, only pathogenic strain Sb but not apathogenic strain Ha produced broad zones of beta-hemolysis, and the supernatant of Sb cultures grown overnight were positive in the hemolysis titer test (between 8 and 16 hemolysis units) compared to no measurable activity for the Ha supernatant. It was therefore hypothesized that the epithelial effects observed were caused by beta-hemolysin secreted by pathogenic aeromonads. Since zinc has been reported to inhibit oligomerization and channel function of this pore-forming toxin (40), we performed inhibition experiments with zinc. Zinc indeed significantly inhibited the effects of Aeromonas sp. strain Sb supernatant on ion secretion and barrier function of HT-29/B6 monolayers (Fig. 10) since, in the presence of zinc, maximum supernatant-induced I_SC amounted to <50% of the I_SC obtained without zinc in parallel experiments (supernatant-induced I_SC of 42.1 ± 1.7 μA/cm² in the presence of zinc versus an I_SC of 97.2 ± 6.8 μA/cm² without zinc).

Effects of recombinant Aeromonas beta-hemolysin on ion secretion and barrier function of HT-29/B6 monolayers. In order to obtain conclusive evidence that Aeromonas beta-hemolysin can cause intestinal ion secretion and barrier impairment, we cloned the beta-hemolysin gene from strain Sb. The complete coding sequence can be retrieved from GenBank (accession number AJY611033). Sequencing of the gene revealed a high homology to known Aeromonas beta-hemolysin sequences. The highest homology (98% identity at the amino acid level) was found in relation to Aeromonas sobria beta-hemolysin, which has been shown to cause fluid accumulation.

### Table 1. Bidirectional NaCl fluxes after addition of Aeromonas supernatant in HT-29/B6 monolayers

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean value (μmol h⁻¹ cm⁻²) ± SEM</th>
<th>Mean R² (Ω cm⁻²) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.14 ± 0.02 1.10 ± 0.09 0.93 ± 0.11 0.18 ± 0.15</td>
<td>0.71 ± 0.07 0.65 ± 0.03 0.05 ± 0.07</td>
</tr>
<tr>
<td>Supernatant</td>
<td>1.52 ± 0.08** 2.47 ± 0.33* 1.66 ± 0.36* 0.81 ± 0.52†</td>
<td>2.18 ± 0.27* 3.77 ± 0.44* -1.58 ± 0.52* -0.87 ± 0.74</td>
</tr>
</tbody>
</table>

*Strain Sb supernatant (1:10) was added to the mucosal compartment of monolayers mounted in Ussing chambers. Under control conditions, there were no significant net fluxes. After the addition of the supernatant, all unidirectional fluxes increased significantly due to the parallel decrease in Rₑ. In addition, an increase of net Cl⁻ flux (i.e., Cl⁻ secretion) could be observed, whereas there were no significant changes in net Na⁺ and residual fluxes. Numerically, there was excellent agreement between the Cl⁻ secretion and the I_SC induced by the supernatant. All data are means ± the SEM (n = 6 monolayers). **P < 0.01 versus unidirectional and versus 0 for net fluxes; †, Not significantly different from zero. J, flux value; net, total.

### Table 2. Mannitol fluxes after addition of Aeromonas supernatant in HT-29/B6 monolayers

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean value ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>51 ± 0.64</td>
</tr>
<tr>
<td>Supernatant</td>
<td>235 ± 8.4*</td>
</tr>
</tbody>
</table>

*Strain Sb supernatant (1:10) was added to the mucosal compartment of monolayers mounted in Ussing chambers. After the transepithelial resistance (Rₑ) had dropped to <10% of its initial value, [³H]mannitol was added to the mucosal compartment, and the transepithelial permeation of the label was quantified by beta counting. Monolayers treated with carrier only served as controls. Values are means ± the SEM (n = 6 monolayers for each group). **P < 0.01 versus controls treated with carrier only.
lation in the mouse intestinal loop assay without cellular damage as visualized by histopathologic examination (16) (GenBank accession number AY157998.1). Less but still high homology was found with respect to the sequence of aerolysin, an *Aeromonas* beta-hemolysin sequenced in the late 1980s as the first of the *Aeromonas* beta-hemolysins. Thus, the *Aeromonas* strain Sb beta-hemolysin showed 68% (20) (GenBank accession number M16495) and 60% (21) (GenBank accession number Y00559) amino acid identity to the aerolysin precursor (preproaerolysin) and 71% (20) and 64% (21) identity to the mature aerolysin that emerges from preproaerolysin after intracellular and extracellular cleavage of N- and C-terminal peptides (21).

To functionally characterize the *Aeromonas* strain Sb beta-hemolysin, cell lysates from overnight cultures of E. coli TOP10F* transformed with the beta-hemolysin gene from strain Sb were taken for electrophysiological experiments. Lysates from TOP10F* cells transformed with the vector alone served as controls. For comparison, experiments with transformed DH5α cells with or without the beta-hemolysin (aerolysin) gene previously identified (21) were performed in an analogous manner. As shown in Fig. 11A, cell lysate of untransformed TOP10F* cells did not exert any effect on the transport and barrier properties of HT-29/B6 cells. If, however, the TOP10F* cells contained the *Aeromonas* beta-hemolysin gene, the cell lysates evoked a strong secretory response, as well as a rapid decline of transepithelial resistance. Identical results were obtained with DH5α cells transformed with or without the *Aeromonas* beta-hemolysin (Fig. 11B). As ex-

![FIG. 7. Immunofluorescence localization of ZO-1 in HT-29/B6 monolayers.](image)

![FIG. 8. Effect of inhibitors of *Aeromonas* supernatant induced I_{sc}.](image)

![FIG. 9. Effect of size-fractionated *Aeromonas* Sb supernatant on the I_{sc} value.](image)
 Unexpectedly, the cell lysates exerted strong hemolytic activity with 16 hemolytic units for TOP10F’ cells harboring the *Aeromonas* beta-hemolysin gene and 256 hemolytic units for DH5α cells harboring the aerolysin gene. Lysates of TOP10F’ or DH5α cells transformed with the respective vectors alone did not show any hemolytic activity in the titer test (data not shown).

**DISCUSSION**

Data from numerous epidemiological, clinical, and experimental studies provide strong evidence that pathogenic aeromonads can cause diarrheal disease. However, how they do this is not known, i.e., it has not been determined whether intestinal fluid secretion in *Aeromonas* enteritis is only caused by an impaired epithelial barrier function or whether active ion secretion is also activated. Furthermore, there is little information about the role of *Aeromonas* virulence factors in this context. This lack of information is due to the heterogeneity of the genus *Aeromonas*, the great variety of *Aeromonas* virulence factors, and the loose association between virulence factors and the phenotype and genotype (3, 22, 23). For these reasons, a straightforward correlation with a single virulence factor and the pathogenic effects of *Aeromonas* enteritis has not been demonstrated thus far. As a consequence, many researchers...
have proposed a multifactorial pathogenesis with the involvement of a number of cellular and extracellular virulence factors (9, 19).

In order to study the events leading to intestinal fluid secretion in enteral Aeromonas infection, we established a cell culture infection model that allows investigation of the epithelial effects of aeromonads (and other intestinal pathogens) with high resolution. Although the findings obtained with this model do not, of course, reflect all aspects of the pathology of intestinal Aeromonas infections, this approach offers some important advantages. The basic setting can be seen as a reductionist approximation with an intestinal epithelium exposed to a luminal (i.e., mucosal) pathogen. Therefore, chances are good to detect effects resulting from the interplay of different virulence factors expressed from the same pathogen. In addition, differentiation between cellular (e.g., adherence and invasiveness) and extracellular virulence factors secreted from the bacteria can easily be achieved. Furthermore, our cell culture infection model allows real-time observation of functional epithelial effects elicited by enteropathogens, and interpretation of these effects is straightforward compared to that of pathogenic effects observed in more complex organ model or in vivo systems. Finally, the biological significance of effects observed in our cell culture model was corroborated by experiments with native human colonic epithelium that showed a qualitatively similar response to Aeromonas supernatant such as the monolayers.

The first important finding of our study was that a pathogenic Aeromonas strain was capable of inducing both active ion secretion and significant barrier impairment. Although adherence and invasiveness are known virulence factors of certain Aeromonas strains (22, 25, 33), a direct interaction between bacteria and epithelium was obviously not a prerequisite for the effects of strain Sb on ion secretion and barrier function. On the contrary, all epithelial effects observed in our model could likewise be induced by sterile Aeromonas supernatant. Moreover, several lines of evidence leave no doubt that the effects elicited by Aeromonas strain Sb supernatant were caused by a secretory beta-hemolysin. First, we observed a correlation between the capacity of the Aeromonas supernatant to induce ion secretion and barrier impairment and its hemolytic activity, whereas an apathogenic strain with no hemolytic activity in the titer test did not exert any effects on ion secretion or barrier properties of the HT-29/B6 monolayers. Furthermore, considering known Aeromonas virulence factors, the physicochemical properties (molecular weight, heat sensitivity, and inhibition by zinc) of the supernatant’s active compound leave an aerolysin-related beta-hemolysin as the main suspect and, most strikingly, the beta-hemolysin cloned from Aeromonas strain Sb and expressed in E. coli exerted identical effects with the HT-29/B6 cells as with supernatant from Aeromonas strain Sb. Finally, the beta-hemolysin obtained from A. hydrophila strain Sb was highly homologous to other aerolysin-related Aeromonas beta-hemolysins, and the aerolysin cloned previously from an Aeromonas trota strain (21) also caused effects in our experiments identical to those of the beta-hemolysin of strain Sb. Taken together, our data prove that aerolysin and related Aeromonas beta-hemolysins induce active chloride secretion and significant barrier impairment in the intestinal epithelium. Although adherence to the epithelium may play a role in vivo by increasing the local Aeromonas density, the beta-hemolysin obviously sufficed to elicit the epithelial effects observed without the involvement of additional virulence factors.

This finding underlines the significance of the aerolysin family of beta-hemolysins, which have been suspected for a long time to be the major virulence factor of Aeromonas enteritis (9). Aerolysin is a pore-forming toxin first identified by Bernheimer and Avigad in 1974 (6). Later, a number of Aeromonas beta-hemolysins with similar properties (molecular weight, heat sensitivity, etc.) were identified and now more than 10 full-length sequences of aerolysin-related Aeromonas beta-hemolysins can be retrieved from GenBank that share between 30 and 99% homology with each other. A great diversity of pathogenic effects have been described for these beta-hemolysins, such as fluid accumulation in intestinal loops (4, 14), release of inflammatory mediators from granulocytes (37), and induction of apoptosis in human lymphoma cells (32). The data explaining the mechanism of the putative diarrheic action of this toxin are sparse, however. Only recently, one study was published investigating the interaction of aerolysin with its primary target in enteric disease: the intestinal epithelium (2). In this case, aerolysin caused a dose-dependent drop in transepithelial resistance, indicating barrier impairment by aerolysin. Our results confirm and extend the findings of that study.

According to our data, both passive solute and water secretion secondary to barrier impairment and active chloride secretion contribute to the fluid secretion in Aeromonas-induced diarrhea. Thus, we demonstrate for the first time that aerolysin-related Aeromonas beta-hemolysins not only cause a decrease of transepithelial resistance but also induce active chloride secretion. This finding came as a surprise because many researchers interpreted the diarrheic action to result from intestinal barrier damage after the killing of epithelial cells by membrane permeabilization (15, 35). However, despite the marked effects on epithelial permeability, we did not find any disrupt of the epithelial monolayer, as visualized by ZO-1 immunofluorescence staining. Furthermore, LDH release from the epithelial cells was not enhanced by treatment with Aeromonas supernatant. These results are in line with other studies showing that, depending on the toxin concentration, mammalian cells survive several hours of aerolysin treatment (1) and that mouse intestinal loops treated with an Aeromonas beta-hemolysin closely related to the hemolysin obtained from strain Sb (98% sequence homology at amino acid level) developed significant fluid secretion without histopathologic evidence for epithelial cell damage or mucosal inflammation (16). Furthermore, this Aeromonas beta-hemolysin stimulated cyclic AMP production in T84 cells without causing cell death, as evidenced by LDH release assay and trypan blue exclusion (17), and aerolysin triggered G-protein activation and Ca2+ release from intracellular stores but not cell lysis in human granulocytes (26). Taken together, there is strong evidence that Aeromonas beta-hemolysins of the aerolysin family can trigger complex cellular events independently from cell lysis. This concept is corroborated by demonstration of active chloride secretion induced by Aeromonas beta-hemolysin, which cannot be explained by cell death.

However, what precisely is the particular mechanism of Aeromonas beta-hemolysin induced chloride secretion? The
general scheme of chloride secretion in intestinal epithelial cells involves basolateral chloride entry by the Na⁺/K⁺/2Cl⁻ symporter NKCC1, potassium recycling via basolateral potassium channels, and apical chloride outflow via apical chloride channels like, e.g., the cystic fibrosis transmembrane conductance regulator (CFTR) (5). The energy is supplied by the Na⁺/K⁺-ATPase, which promotes sodium coupled chloride entry by maintaining a low intracellular sodium concentration. The apical chloride outflow is generally considered the rate-limiting step, and activation of protein kinase A and elevation of intracellular Ca²⁺ have been identified as the most prominent stimulating pathways (5). In addition, it has been shown that protein kinase C and tyrosine kinases are also potential activators of apical chloride channels (5). In our study, only the protein kinase C inhibitor chelerythrine inhibited Aeromonas beta-hemolysin induced chloride secretion. Therefore, it is possible that activation of protein kinase C is involved in the secretory action of the beta-hemolysin, although we did not explore the signal transduction involved in this stimulation. In searching for a potential link between protein kinase C and apical chloride channels, we became aware of a chelerythrine-sensitive, calcium-independent protein kinase C activation of the CFTR in polarized airway epithelium in response to apical ATP (via purinergic receptors) (34). Thus, protein kinase C activation of CFTR could occur also in HT-29/B6 cells in response to Aeromonas. The initial event would then be apical release of ATP, as recently described for an aerolysin-like hemolysin from A. sobria in T84 cells (18).

However, since chelerythrine only inhibited a minor part of the overall secretion, the major pathway of Aeromonas hemolysin-induced chloride secretion seems to be independent from protein kinase C, as well as from protein kinase A or calcium. According to extensive experimental evidence in the literature, Aeromonas beta-hemolysin effects are generally thought to follow pore formation by insertion of aerolysin oligomers into the membrane of the target cell (see, for example, references 2, 8, 14, 26, and 32; for reviews, see references 15 and 35). Even intracellular events triggered by Aeromonas beta-hemolysin have been assumed to occur secondary to formation of the hemolysin pore within the cell membrane of the target cell (1, 15). Therefore, we hypothesize that the effects observed in our cell model were also caused by membrane insertion of Aeromonas hemolysin. Furthermore, it has been shown in patch clamp studies that the pore formed by the Aeromonas beta-hemolysin is an unselective ion channel with a slight preference for anions such as chloride (8, 40). When the basolateral components of epithelial chloride secretion are constitutively active, insertion of an apical chloride channel completely suffices to elicit active chloride secretion. From these findings, it seems likely that, once the aerolysin pore has been inserted into the apical membrane, Cl⁻ leaves the cell along its electrochemical gradient via the hemolysin pore, leading to active Cl⁻ secretion.

As for the inhibitory effect on I_SC of Aeromonas hemolysin applied serosally, the reasoning presented above and the velocity of the inhibition strongly support the notion that this effect may be caused by basolateral insertion of the hemolysin pore. Since the aerolysin pore is an unselective ion channel, the cessation of all electrogenic ion movement can easily be explained by the breakdown of the basolateral membrane potential abolishing the driving force for all active ion transport processes. We propose, on the basis of these results, that chloride secretion induced by aerolysin-related beta-hemolysins involves protein kinase C activation, as well as chloride outflow through the ion pores formed by the hemolysin inserted into the apical membrane, although it should be noted that direct evidence for the latter hypothesis of functionally active channel insertion into the apical membrane is difficult, if not impossible, to obtain in this experimental system.

Regarding the effect of Aeromonas on the epithelial barrier, we observed a marked decrease in transepithelial resistance induced by Aeromonas beta-hemolysins similar to the effect of aerolysin on Caco-2 cells described by Abrami et al. (2). Thus, evidence is mounting that beta-hemolysins of the aerolysin family can cause severe impairment of the intestinal epithelial barrier. Although the aerolysin pore itself may contribute to this process to some degree, our flux data indicate that an increase in paracellular rather than transcellular permeability is the main correlate for the resistance decrease observed. In principle, an increment of paracellular permeability can occur either after disintegration of the epithelial monolayers after the death of epithelial cells or after structural alteration of the paracellular seal, the tight junction. Although there is little doubt that aerolysin, depending on the locally active concentration, can cause cell death by osmotic lysis (1), our data, surprisingly, seem to point to alteration of the tight junction and away from cell necrosis as the main mechanism of the aerolysin-induced barrier impairment observed in our experiments, since with the aerolysin concentration and the incubation time used in our experiments we did not find any evidence of death of HT-29/B6 cells. In agreement with the results of others (17), there was no increase in LDH release after hemolysin treatment, and, morphologically, Aeromonas hemolysin-treated monolayers did not show any leaks or disruptions of the epithelial monolayer. Furthermore, the resistance values did not decrease completely but remained stable at a low level for a prolonged period of time. However, a direct effect on tight-junction strand-forming proteins, such as occludin, claudin-1, and claudin-4, was not quantitatively investigated in the present study.

In summary, we have demonstrated for the first time that beta-hemolysins of the aerolysin family not only induce impairment of the intestinal epithelial barrier but also stimulate active chloride secretion. Although our data are compatible with chloride secretion via the hemolysin pore inserted into the apical membrane and to some extent by protein kinase C activation, the hemolysin-induced barrier impairment seems to be due to an indirect hemolysin-mediated increase in paracellular permeability.

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