Mechanisms of diarrhea in the interleukin-2-deficient mouse model of colonic inflammation

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Barmeyer, C., M. Harren, H. Schmitz, U. Heinzel-Pleines, J. Mankertz, U. Seidler, I. Horak, B. Wiedenmann, M. Fromm, and J. D. Schulzke. Mechanisms of diarrhea in the interleukin-2-deficient mouse model of colonic inflammation. Am J Physiol Gastrointest Liver Physiol 286: G244–G252, 2004; 10.1152/ajpgi.00141.2003.—Colitis in interleukin-2-deficient (IL-2−/−) mice resembles ulcerative colitis in humans. We studied epithelial transport and barrier function in IL-2−/− mice and used this model to characterize mechanisms of diarrhea during intestinal inflammation. [22Na] and [36Cl] fluxes were measured in proximal colon. Net Na+ flux was reduced from 4.0 ± 0.5 to 0.8 ± 0.5 μmol·h−1·cm−2, which was paralleled by diminished mRNA and protein expression of the Na+/H+ exchanger NHE3. Net Cl− flux was also decreased from 2.2 ± 1.6 to −2.7 ± 0.6 μmol·h−1·cm−2, indicating impaired Na+-Cl− absorption. In distal colon, aldosterone-induced electrogenic Na+ absorption was 6.1 ± 0.9 μmol·h−1·cm−2 in controls and was abolished in IL-2−/− mice. Concomitantly, mRNA expression of β- and γ-subunits of the epithelial sodium channel (ENaC) was reduced. Epithelial barrier was studied in proximal colon by impedance technique and mannitol fluxes. In contrast to ulcerative colitis, epithelial resistance was increased and mannitol fluxes were decreased in IL-2−/− mice. This was in accord with the findings of reduced ion transport as well as increased expression of tight junction proteins occludin and claudin-1, -2, -3, and -5. In conclusion, the IL-2−/− mice develop normally before they become severely compromised with systemic disorders of the hematopoietic and immune system, including lymphocytic hyperplasia, progressive loss of B cells, disturbances in bone marrow hemopoietic cells, and anemia. About 50% of the mice die within 9 wk. Thereafter, intestinal inflammation develops with striking clinical and histological similarities to UC (39). The colitis is mediated by thymus-dependent CD4+ T cells invading the colon and bone marrow (27). Antigenic stimulation by nonpathogenic intestinal flora is essential, because mice raised under specific pathogen-free and germ-free conditions show later onset or attenuation of intestinal inflammation (46). Many of the alterations in IL-2−/− animals are also found in UC, e.g., high numbers of activated T and B cells in the colonic mucosa, elevated proinflammatory cytokine patterns, the relative reduction of γδ TCR+ T cells, highly elevated IgG1, and expression of major histocompatibility complex class II molecules on enterocytes (16, 25, 29, 33, 49). The importance of IL-2 in UC has also been demonstrated recently by two studies with anti-IL-2 receptor antibodies, daclizumab and basiliximab, indicating a potential benefit for patients with refractory UC (11, 52).

However, epithelial transport and barrier properties of the inflamed colon in IL-2−/− mice have not as yet been studied in detail. The striking similarities between UC and colitis in IL-2−/− mice suggest that these mucosal dysfunctions and their underlying regulation in IBD can be studied in the IL-2−/− model. Thus in the present study we aimed to identify diarrheal mechanisms during intestinal inflammation by using the IL-2−/− model in a search for factors in the pathogenesis of mucosal dysfunction in IBD. Here we report that colonic inflammation in IL-2−/− mice causes a decrease in electroneutral Na+/Cl− absorption and active electrogenic Na+ absorption and that this is due to impaired function and expression of the transporters Na+/H+ exchanger 3 (NHE3) and epithelial sodium channel (ENaC), whereas epithelial barrier dysfunction did not contribute to diarrhea.

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MATERIALS AND METHODS

Animals and tissue preparation. IL-2−/− mice bred on C57BL/6 × 129/Ola genetic background were generated by gene targeting as previously described (43). The animals had free access to food and water.

After they developed intestinal inflammation between 12 and 14 wk of age, animals were killed by CO2 inhalation. The colon was excised after the abdomen was opened; colon was then opened along the mesenteric insertion and flushed free of intestinal contents before it was mounted in Ussing-type chambers without stripping. Control measurements were performed on normal colon from wild-type littermates (IL-2+/+).

The average weights of IL-2−/− and IL-2−/+ mice were 14.7 ± 0.6 and 24.9 ± 1.1 g, respectively. Measurements were performed in the proximal colon up to 3 cm from the ileocecal valve and in the last 0.6 and 24.9 cm of the colon, respectively. Inflammation of the colon in IL-2−/− mice revealed a severe inflammatory process restricted to the proximal colon up to 3 cm from the ileocecal valve and in the last 0.6 and 24.9 cm of the colon.

Addition, amiloride (10−4 M) was added to the mucosal compartment (CVC 6; Fiebig, Berlin, Germany). Before each single experiment, the resistance of the bathing fluid between the voltage-sensing electrodes was determined and taken into account as described by Tai and Tai (51). Data were given in micromoles per hour per square centimeter. The exposed tissue area was 0.28 cm². For calculating net fluid absorption.

Electrogenic Na+ absorption. In the distal colon, electroneutral NaCl transport and paracellular solute transport in the proximal colon, measurements of unidirectional fluxes with 22Na+, 36Cl−, and [3H]mannitol were performed. All experiments were performed under short-circuit conditions as described previously (47). Voltage, short-circuit current (Isc), and electrical resistance were measured by using a computer-controlled voltage clamp device (CVC 6; Fiebig, Berlin, Germany). Before each single experiment, the resistance of the bathing fluid between the voltage-sensing electrodes was determined and taken into account as described by Tai and Tai (51). Data were given in micromoles per hour per square centimeter. The exposed tissue area was 0.28 cm². For calculating net fluxes, tissues were matched with a difference in conductance of not more than 10%.

Electrogenic Na+ absorption. In the distal colon, electroneutral NaCl transport and paracellular solute transport in the proximal colon, measurements of unidirectional fluxes with 22Na+, 36Cl−, and [3H]mannitol were performed. All experiments were performed under short-circuit conditions as described previously (47). Voltage, short-circuit current (Isc), and electrical resistance were measured by using a computer-controlled voltage clamp device (CVC 6; Fiebig, Berlin, Germany). Before each single experiment, the resistance of the bathing fluid between the voltage-sensing electrodes was determined and taken into account as described by Tai and Tai (51). Data were given in micromoles per hour per square centimeter. The exposed tissue area was 0.28 cm². For calculating net fluid absorption.

Alternating current impedance analysis. Alternating current impedance analysis the epithelial (Rf) and subepithelial (Rw) portion of the total wall resistance (Rw) can be differentiated based on the three-parameter model of the colonic wall (15, 22, 21). In this model, the colonic wall is described as an electrical equivalent circuit with a resistor and a capacitor in parallel representing the epithelium followed by a resistor in series representing the subepithelium. After application of 48 discrete frequencies of an effective sine-wave alternating current of 35 μA/cm² ranging from 1.3 to 65 kHz, changes in tissue voltage were detected by phase-sensitive amplifiers (1250 frequency response analyzer and 1286 electrochemical interface; Solartron Schlumberger, Farnborough, England). Complex impedance values were calculated and corrected for the resistance of the bathing solution and the frequency behavior of the measuring device for each frequency. The impedance for each frequency was then plotted in a Nyquist diagram by least square analysis. Because of the frequency-dependent electrical characteristics of the epithelium, Rw is obtained at low frequencies, whereas Rw is obtained at high frequencies. Rw was calculated as Rw = Rf − Rw.

Western blot analysis. To determine NHE3 and tight junction protein expression, Western blot analysis was performed from membrane extracts of colonic tissue from the proximal colon. Membrane proteins were extracted by performing three freeze-thaw cycles and homogenization with a nerve grinder (27.5 G) on lysate buffer containing 20 mM Tris (pH 7.4), 5 mM MgCl2, 1 mM EDTA, 0.3 mM EGTA, 1 μl/ml aprotinin, 16 μg/ml benzamidine-HCl, 10 μg/ml phenanthroline, 10 μg/ml leupentin, 10 μg/ml pepstatin, 1 mM PMSF, 210 μg/ml sodium fluoride, 2.16 mg/ml β-glycerophosphate, 18.5 μg/ml NaVO₃, and 1 μl/ml trypsin inhibitor (all from Sigma). To remove insoluble material, the extract was centrifuged at 200 g for 5 min at 4°C. The supernatant was then centrifuged at 43,000 g for 30 min at 4°C. The pellet representing a crude membrane fraction was resuspended in lysate buffer.

After protein concentrations were determined by Pierce BCA protein assay, aliquots of 2.5 μg protein were separated by polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). Blots were blocked for 2 h in 5% milk powder before being incubated with primary mouse monoclonal IgG antibody against NHE3 (kindly provided by Dr. Daniel Biemesderfer, Yale University, New Haven, CT) and rabbit polyclonal IgG antibodies against claudin-1, -2, -3, and -5 and occludin. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG antibodies and the chemiluminescence detection system Lumi-LightPLUS Western blotting kit (Boehringer Mannheim, Mannheim, Germany) were used to detect bound antibodies. Antibodies against claudin-1, -2, -3, and -5 and occludin were kindly provided by Zymed Laboratories (South San Francisco, CA). Synthesis and test of specificity of these antibodies were recently described by Rahner and coworkers (34). Intensity of protein expression was quantified with luminescent imaging (LAS-1000; Fuji Film) by using AIDA software (Raytest).

Northern blot analysis. For determining ENaC α-, β-, and γ-subunit mRNA, RNA was prepared from the same tissue specimens of distal colon immediately after determination of electronegative Na+ absorption. The area exposed in the Ussing chamber was cut off, and RNA was isolated as described previously (9). Aliquots of 12–15 μg were separated on 1.5% agarose gels. Nucleic acids were transferred to nylon membranes (Boehringer Mannheim), UV cross-linked, and hybridized. Hybridization was performed with radioactively labeled probes generated from cDNA of α-, β-, and γ-subunits of rat ENaC, which are 95, 96, 97, and 98% identical in amino acid sequences with mouse α-, β-, and γ-ENaC and GAPDH (1). The probes were labeled with α-[32P]dCTP by using a random-priming DNA labeling system (GIBCO-BRL). After hybridization, membranes were exposed for 3–6 days at −20°C (Kodak XOMAT LS film).

RT-PCR. Abundance of NHE3 was determined in the proximal colon according to a protocol described previously (38). Briefly, RNA was isolated as described by Chomczynski and Sacchi (9). The total RNA was reverse transcribed into cDNA with the Superscript preamplification system for the first-strand cDNA synthesis kit (GIBCO-BRL) according to the manufacturer’s recommendations. Five micro-liters of cDNA were used as template in a 100-μl PCR containing 10X PCR buffer, Q solution, nucleotides, MgCl2, Taq polymerase, and primer for 18s rRNA and NHE3. The amplification was performed for 14–24 cycles for 18s rRNA and 24–34 cycles for NHE3. PCR products were separated on a 1.5% agarose gel, and the optical density of the ethidium bromide-stained bands was measured by using the ImageMaster VDS system and software (Pharmacia, Freiburg, Germany). The amplification efficiency of NHE3 and 18s rRNA was determined by calculating the slope following semilogarithmic plot.
ting of the values against the cycle (38). The virtual relationship between integrated optical density (IOD) of NHE3 after 26 cycles vs. OD1 of 18s rRNA after 16 cycles was calculated. The IOD of 18s rRNA was compared with NHE3 amplified with a similar number of cycles.

**Morphometry of the mucosal surface area.** A morphometry of the mucosa was performed as described earlier by using combined microdissection procedures and conventional histology (10, 32, 47). Tissues from the proximal colon were immediately fixed in 10% buffered formalin after impedance measurements. Morphometry was performed on digitally stored light micrographs. Inner crypt diameter was measured in cross sections of the same tissues that had been embedded in paraffin for at least 24 h and stained with hematoxylin and eosin. Microdissection technique was used to determine crypt length and the number of crypts per serosal area. For these measurements, the tissues were fixed at the same degree of stretch as in the electrophysiological experiments.

**Statistical analysis.** Results are given as means ± SE. Significance was tested by the two-tailed Student’s t-test. P < 0.05 was considered significant.

**RESULTS**

**Na⁺ and Cl⁻ flux measurements in proximal colon.** The data are presented in Table 1. In IL-2⁺/⁺ mice, net Na⁺ and Cl⁻ absorption was identified (4.0 ± 0.5 and 2.2 ± 1.6 μmol·h⁻¹·cm⁻², respectively). Net Cl⁻ absorption was lower than net Na⁺ absorption and is most likely due to a small active secretory component of Cl⁻ counteracting electroneutral Na⁺-Cl⁻ absorption. This small active secretory component of Cl⁻, together with the residual flux, is the basis of the Jnet of 4.5 ± 0.5 μmol·h⁻¹·cm⁻². The residual flux, which is usually assumed to represent HCO₃⁻ secretion, was 2.7 ± 1.2 μmol·h⁻¹·cm⁻² in IL-2⁺/⁺ mice.

In IL-2⁻/⁻ mice, there was a parallel decrease in net Na⁺ absorption (4.0 ± 0.5 to 0.8 ± 0.5 μmol·h⁻¹·cm⁻²; P < 0.001) and in net Cl⁻ transport (2.2 ± 1.6 to −2.7 ± 0.6 μmol·h⁻¹·cm⁻²; P < 0.05), which indicates impaired electroneutral Na⁺-Cl⁻ absorption in the IL-2⁻/⁻ mouse model.

There was also a decrease in Jnet in IL-2⁻/⁻ mice by 47% (2.4 ± 0.5 vs. 4.5 ± 0.5 μmol·h⁻¹·cm⁻²; P < 0.05). This is mainly due to a decrease in the residual flux (from 2.7 ± 1.2 in IL-2⁺/⁺ mice to −1.2 ± 0.4 μmol·h⁻¹·cm⁻² in IL-2⁻/⁻ mice; P < 0.05). Thus there is no evidence for the activation of active anion secretion in the colon of IL-2⁻/⁻ mice.

To assess the contribution of NHE3 to the overall Na⁺ absorption in IL-2⁻/⁻ mice, Na⁺ fluxes were measured before and after the addition of 700 μM DMA, a highly specific NHE blocker at this concentration (28). Net Na⁺ absorption was reduced by DMA from 4.4 ± 0.4 to 1.4 ± 0.3 μmol·h⁻¹·cm⁻² (n = 6; P < 0.05; Fig. 1), supporting a role of NHE3 as predominant transport protein in murine proximal colon, as also supposed by others (45).

At the end of each flux experiment, the Iₛₑc response to 1 mM theophylline was determined. Both groups showed a significant increase in Iₛₑc after the addition of theophylline to the serosal compartment. However, the change in Iₛₑc in the IL-2⁻/⁻ group was only 64% of that in the control group [4.7 ± 0.5 (n = 13) vs. 7.4 ± 0.9 μmol·h⁻¹·cm⁻² (n = 15); P < 0.05].

**mRNA and protein expression of the electroneutral NHE3.** To study NHE3 mRNA expression in the proximal colon of IL-2⁻/⁻ mice, RT-PCR was used to amplify the gene of interest. As shown in Fig. 2A, the relation of NHE3 to 18s rRNA in IL-2⁻/⁻ mice was about half of that in wild-type mice (mean ratio NHE3/18s rRNA ± SE was 1.20 ± 0.13 in IL-2⁻/⁻ (n = 3) and 2.04 ± 0.22 in IL-2⁺/⁺ mice (n = 4); P < 0.05). Thus NHE3 mRNA expression is diminished in the inflamed colonic epithelium.

To investigate NHE3 protein expression, Western blot analyses were performed. A typical blot is shown in Fig. 2B. Densitometric analysis revealed that protein expression of NHE3 was reduced to 76.5 ± 8.3% (n = 5) compared with that in IL-2⁺/⁺ mice (n = 5; P < 0.05; Fig. 2C).

**Electrogenic Na⁺ absorption in distal colon.** Data are presented in Fig. 3. After the addition of 3·10⁻⁹ M aldosterone in vitro, Iₛₑc started to increase in IL-2⁺/⁺ mice after ~0.5–1 h and reached a maximum of 7.1 ± 1.0 μmol·h⁻¹·cm⁻² after 6–8 h. Eight hours after aldosterone, 10⁻⁴ M amiloride was added. After the addition of amiloride, Iₛₑc decreased by 6.1 ± 0.9 μmol·h⁻¹·cm⁻² in IL-2⁺/⁺ mice (n = 20). This drop in Iₛₑc has been shown to be fully attributed to electronegative Na⁺ absorption (14). In contrast, IL-2⁻/⁻ mice did not exhibit an increase in Iₛₑc after exposure to aldosterone (from 1.9 ± 0.5 to 1.1 ± 0.4 μmol·h⁻¹·cm⁻²).

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**Table 1. Na⁺ and Cl⁻ fluxes in proximal colon from IL-2⁺/⁺ and IL-2⁻/⁻ mice**

<table>
<thead>
<tr>
<th></th>
<th>Jnet</th>
<th>Jn</th>
<th>Jκ</th>
<th>Jh</th>
</tr>
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<tbody>
<tr>
<td>R, Ω·cm⁻²</td>
<td>73 ± 5</td>
<td>4.5 ± 0.5</td>
<td>10.6 ± 0.4</td>
<td>6.6 ± 0.5</td>
</tr>
<tr>
<td>Iₛₑc, μmol·h⁻¹·cm⁻²</td>
<td>4.0 ± 0.5</td>
<td>5.5 ± 0.7</td>
<td>4.7 ± 0.4</td>
<td>-2.7 ± 0.6</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.05</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
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</table>

Values are means ± SE. IL-2⁺/⁺, wild type; IL-2⁻/⁻, IL-2 deficient. R, tissue resistance; Iₛₑc, short circuit current; J, flux; m, mucosa to serosa; sm, serosa to mucosa; R, residual.

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**Fig. 1.** Effect of 700 μM 5-(N,N-dimethyl)-amiloride (DMA) on Na⁺ absorption (Jnet) in proximal colon of IL-2 wild-type (IL-2⁺/⁺) mice. Na⁺ absorption in the DMA-treated group was only 32% of that observed in the untreated group (control). Data are means ± SE for 6 IL-2⁺/⁺ mice. *P < 0.05 vs. control.
A series of experiments was performed with 10 IL-2 mice (in IL-2 mice) and 4 per group). RT-PCR analysis. Data are means ± SE of 4 IL-2+/+, and 3 IL-2+/− mice. B: representative Western blot of NHE3 protein expression. Lanes 1 and 2 are samples from IL-2+/+ and lanes 3-5 are samples from IL-2−/− mice. C: densitometric analysis of NHE3 protein expression. Control was set as 100%. Data are means ± SE of 5 IL-2+/+ and 5 IL-2−/− mice. *P < 0.05 vs. IL-2+/+.

μmol·h⁻¹·cm⁻² after 8 h). Furthermore, the drop in Iₛ after the addition of amiloride was minimal [0.8 ± 0.4 μmol·h⁻¹·cm⁻² (n = 16)], indicating a severe dysfunction of aldosterone-dependent electrogenic Na⁺ absorption during intestinal inflammation.

To exclude the possibility that spontaneous electrogenic Na⁺ transport was present before the addition of aldosterone, a series of experiments was performed with 10⁻⁴ M amiloride in both groups. However, Iₛ did not change after amiloride either in IL-2+/+ or in IL-2−/− mice (in IL-2+/+, 0.9 ± 0.8 μmol·h⁻¹·cm⁻²; in IL-2−/−, −0.2 ± 0.2 μmol·h⁻¹·cm⁻²; n = 4 per group).

Northern blot analysis of the ENaC α-, β-, and γ-subunit mRNA expression. After the electrophysiologic experiments, in five tissues from each group mRNA expression of the ENaC α-, β-, and γ-subunits was measured and correlated with the measured electrogenic Na⁺ absorption values. The data are presented in Fig. 4. In IL-2+/+ mice not treated with aldosterone, only the α-subunit mRNA expression was detectable (constitutive expression). Eight hours after the addition of aldosterone, β- and γ-subunit mRNA were also expressed.

Fig. 3. Time course of aldosterone action, given as short-circuit current (Iₛ). In IL-2+/+ mice, Iₛ started to increase after 1.5–2 h and reached a maximum between 6 and 8 h. This increase was missing in IL-2−/− mice. Electrogenic Na⁺ absorption (Jₑ) was determined as amiloride-sensitive Iₛ 8 h after the addition of 10⁻⁴ M amiloride. The drop of Iₛ is totally accounted for by aldosterone-induced Na⁺ absorption.

Fig. 4. Effect of aldosterone on epithelial sodium channel (ENaC) subunit mRNA expression in distal colon of IL-2+/+ and IL-2−/− mice. RNA was extracted immediately after determination of Na⁺ absorption. Each lane in the blot represents 1 IL-2+/+ and 1 IL-2−/− mouse. The distal colon was divided into 2 parts, which were both incubated in the Ussing chamber. One was treated with aldosterone (+Aldo), the other not (control). Blots are representative of at least 5 Northern blots detecting the single subunits. GAPDH was used as internal standard.
These results are consistent with data recently published in rats late distal colon (12).

In IL-2−/− mice not treated with aldosterone, α-subunit mRNA expression was also detectable although less intense than in IL-2+/+ mice. In contrast to IL-2+/+ mice, the β and γ signal was not identified after aldosterone stimulation in IL-2−/− mice. Thus severe dysfunction of aldosterone-induced electrogenic Na+ absorption in IL-2−/− mice was paralleled by an impaired upregulation of β- and γ-subunit mRNA expression.

Alternating current impedance analysis and mannitol fluxes. Typical impedance locus plots of colon specimens from IL-2+/+ and IL-2−/− mice are shown in Fig. 5. The data are presented in Table 2. In the proximal colon of IL-2+/+ mice, Rε and Rsub were 36 ± 3 Ω cm² (n = 13) and 22 ± 2 Ω cm² (n = 16), respectively. Thus Rε contributes 62% to the total transmural resistance. In IL-2−/− mice, Rε was 1.6-fold higher than in IL-2+/+ mice [57 ± 4 Ω cm² (n = 16) vs. 36 ± 3 Ω cm² (n = 13)]. Also, Rsub was increased in IL-2−/− mice [39 ± 4 Ω cm² (n = 16) vs. 22 ± 2 Ω cm² (n = 13)], the structural correlate of which was thickening of the subepithelium due to edema of the lamina propria and submucosa with a dense inflammatory cell infiltrate.

Before active transport rates of IL-2+/+ and IL-2−/− mice can be compared, measured Iε values and net fluxes have to be corrected for the resistance of the respective subepithelial layers (15, 48). This is necessary whenever, in addition to the Rε, significant nonepithelial series resistances are present between the voltage-sensing electrodes. This correction, applied to bath resistance, is well accepted, but it is also necessary to correct Iε and net fluxes for the Rsub of intestinal preparations. The implications of this correction have previously been provided (15, 22, 47, 48, 51). Generally, after correction for the bathing solution, the true active transport rate becomes underestimated by a factor that is given by the ratio of Rε and Rsub. This factor was 1.7 ± 0.1 (n = 13) in IL-2+/+ mice and 1.8 ± 0.1 (n = 16) in IL-2−/− mice. Because these factors were not significantly different, Iε values and net fluxes of both groups were compared without further correction.

To further characterize the increase in Rε, mannitol fluxes (JMan) were performed (Table 2). JMan was 167 ± 36 nmol h⁻¹cm⁻² in IL-2+/+ mice (n = 4). In IL-2−/− mice, a 67% reduction in JMan to 55 ± 14 nmol h⁻¹cm⁻² (n = 4; P < 0.05) was observed.

Morphology of the inflamed colon in IL-2−/− mice. Morphometry of both surface and crypt mucosa revealed an altered surface geometry of inflamed colonic tissues in IL-2−/− mice. The data are provided in Table 3. Most prominently, crypts were elongated (266 ± 34 vs. 122 ± 6 μm) and inner crypt diameter increased in IL-2−/− mice (from 13.0 ± 0.7 to 22.7 ± 1.4 μm). Concomitantly, there was a decreased number of crypts in IL-2−/− mice compared with IL-2+/+ mice (133 ± 16 vs. 382 ± 22/mm² serosal area). Because these alterations in surface geometry compensated each other, mucosal surface area per serosal area was not changed in IL-2−/− mice compared with IL-2+/+ mice. Therefore, surface geometry cannot explain the increase in Rε observed in the inflamed mucosa of IL-2−/− mice.

Western blot analysis of tight junction proteins claudin-1,-2,-3, and -5 and occludin. To investigate further whether altered tight junction structure contributed to the increased Rε and reduced mannitol fluxes in IL-2−/− mice, Western blot analyses were performed on claudin-1, -2, -3, and -5 and occludin.

Table 2. Epithelial and subepithelial contributions to transmural resistance in proximal colon of IL-2+/+ mice

<table>
<thead>
<tr>
<th></th>
<th>JMan, nmol h⁻¹cm⁻²</th>
<th>Rε, Ω cm²</th>
<th>Rsub, Ω cm²</th>
<th>Rε/Rε</th>
<th>Rsub/Rε</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2+/+</td>
<td>167 ± 36</td>
<td>36 ± 3</td>
<td>22 ± 2</td>
<td>58 ± 3</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>IL-2−/−</td>
<td>55 ± 13</td>
<td>57 ± 4</td>
<td>39 ± 4</td>
<td>96 ± 5</td>
<td>1.8 ± 0.1</td>
</tr>
</tbody>
</table>

P < 0.05 < 0.001 < 0.001 < 0.001 n.s.

Values are means ± SE. Results of mannitol fluxes (JMan) and alternating current impedance measurements (R) in unstripped proximal colon of IL-2+/+ mice and corresponding controls. Epithelial and subepithelial resistance (Rε and Rsub) were obtained as described in MATERIALS AND METHODS. Total resistance (Rε) represents Rε + Rsub. Rε/Rε is the correction factor of active transport rates (Iε or net fluxes) for Rsub contributions (see RESULTS). n.s., not significant.

Table 3. Morphometry of the proximal colon mucosa of IL-2−/− mice

<table>
<thead>
<tr>
<th></th>
<th>Crypt length, μm</th>
<th>Inner crypt diameter, μm</th>
<th>Crypts per serosal area, mm⁻²</th>
<th>Mucosal area per serosal area, mm²/mm⁻²</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2+/+</td>
<td>122 ± 6</td>
<td>13.0 ± 0.7</td>
<td>382 ± 22</td>
<td>2.9 ± 0.5</td>
<td>8</td>
</tr>
<tr>
<td>IL-2−/−</td>
<td>266 ± 34</td>
<td>22.7 ± 1.4</td>
<td>133 ± 16</td>
<td>3.3 ± 1.0</td>
<td>9</td>
</tr>
</tbody>
</table>

P < 0.01 < 0.001 < 0.001 n.s.

Values are means ± SE. Analysis was performed on tissues that underwent alternating current impedance analysis. After the electrical measurement, the tissue was fixed when still in the chamber, guaranteeing the same degree of stretch as during the electrophysiological experiment. The number of crypts per square millimeter of serosal area, crypt length, and diameter were determined as described in MATERIALS AND METHODS.
Because these proteins are integral membrane proteins forming tight junction strands, membrane extracts were used for these studies. As shown in Fig. 6, claudin-1, -2, -3, and -5 as well as occludin expression was increased in IL-2+/− mice. The most pronounced alteration was observed for claudin-1 and -2 (+668 ± 118 and +787 ± 42%, respectively; Fig. 6B). For detection of claudin-2 in IL-2+/− mice, expression was too low at 2.5 μg protein but could be seen by increasing protein load to 5 μg.

For occludin, three different bands were detected, with the most intense form at 65–67 kDa and less intense bands at 51–56 and 39–41 kDa. The less intense forms are more pronounced in lanes 1 and 5–7 of Fig. 6. However, all forms are seen in IL-2+/+ as well as in IL-2+/− mice, indicating that the increased intensity in at least three out of four IL-2+/− mice is due to higher expression and not due to a degradation or cleavage phenomenon.

When up- or downregulation of tight junction molecules is considered, one has to keep in mind that quantification is performed with reference to the protein content of the membrane fractions in both groups. Importantly, mucosal surface area did not differ in the two groups. However, the mucosa of IL-2−/− mice showed a dense infiltration with immune cells, which would probably contribute to the protein content of the membrane extract, but it does not possess tight junction proteins. Thus the upregulation of tight junction proteins observed in Fig. 6, if anything, underestimated the true difference between IL-2−/− mice and controls.

**DISCUSSION**

\( \text{Na}^+ \) and \( \text{Cl}^- \) transport. Relatively little is known about electrolyte transport across inflamed colonic mucosa of mice. To date, only a few data are published regarding electrolyte transport in normal mice colon. The most complete studies were performed by Charney et al. (8). In proximal colon, they observed net \( \text{Na}^+ \) absorption and a low rate of \( \text{Cl}^- \) absorption in \( \text{HCO}_3^- \)-containing solution. These observations were confirmed by our flux measurements. However, in the inflamed proximal colon of IL-2−/− mice a marked decrease in absorption of \( \text{Na}^+ \) and \( \text{Cl}^- \) was demonstrated. The epithelium almost lost its ability to absorb these ions, which is the main pathomechanism for diarrhea in IL-2−/− mice. Furthermore, a negative residual flux was observed, suggestive of net \( \text{HCO}_3^- \) absorption. These alterations could reflect reduced \( \text{Cl}^-/\text{HCO}_3^- \) exchange on the apical membrane. Another possible explanation is that basal \( \text{HCO}_3^- \) secretion, which is normally present, is reduced or even absent. This could be due to a reduction in the expression of an anion exchanger, the most likely candidates of which are the putative \( \text{Cl}^-/\text{HCO}_3^- \) exchangers downregulated in adenoma and anion exchanger 1 (31, 35).

To determine the nature of the observed defect in \( \text{Na}^+ \) absorption, it is necessary to identify apical transport mechanisms mediating basal \( \text{Na}^+ \) absorption in the proximal colon. So far, three different \( \text{Na}^+/\text{H}^- \) exchangers have been described in murine colonic epithelial cells, namely NHE1, NHE2, and NHE3. NHE1 is predominantly located on the basolateral membrane and therefore is unlikely to contribute to apical electroneutral \( \text{Na}^+/\text{Cl}^- \) exchange. NHE2 and NHE3 are both located on the apical membrane of surface epithelial cells and are possible candidates. NHE2, however, has been shown not to play a major role in vectorial \( \text{Na}^+ \) absorption in the colon (44). Mice deficient for NHE2 showed no signs of an intestinal absorptive defect and exhibited no changes in blood pH, \( \text{HCO}_3^- \), \( \text{Na}^+ \), or aldosterone levels. NHE3, in contrast, has been shown to play a key role in mouse colon. Schultheis and co-workers (45) concluded on the basis of defective \( \text{Na}^+ \) absorption in NHE3-deficient mice that NHE3 is the major transporter for \( \text{Na}^+ \) absorption in mouse colon. Based on this observation, we determined the relative contribution of NHE3 to net \( \text{Na}^+ \) absorption in mouse proximal colon by using the NHE isoform blocker DMA. At the concentration of 700 μM that was used in our protocol, DMA inhibits all three NHE

**Fig. 6.** Expression of tight junction proteins in IL-2+/+ and IL-2−/− mice. Western blots were performed on 4 IL-2+/+ and 4 IL-2−/− mice. Each lane represents 1 animal. A: lanes 1–4 are samples from IL-2+/+ mice, and lanes 5–8 are samples from IL-2−/− mice. The most pronounced alteration was observed for claudin-2. At 2.5 μg, protein sensitivity was too low to detect claudin-2 in controls, but by increasing protein amounts weak signals could be seen (not shown). B: intensity of protein expression as detected by luminescent imaging. Protein expression in IL-2−/− mice are given as %control (control set as 100%). A marked increase of protein expression in IL-2−/− mice was seen for claudin-1, -2, -3, and -5 and occludin. Data are means ± SE of 4 IL-2+/+ and 4 IL-2−/− mice. **p < 0.05 vs. IL-2+/+; ***p < 0.01 vs. IL-2+/+; ****p < 0.001 vs. IL-2+/+.
isofoms (28) but, as pointed out above, NHE1 and NHE2 are not contributing to Na\(^+\) absorption to a significant degree, and therefore the observed alterations in Na\(^+\) absorption after 700 μM DMA can be attributed to NHE3. Thus our results indicate that NHE3 contributes ~70% to net Na\(^+\) absorption in the basal state, and this means that NHE3 is in fact the major transporter involved in electroneutral Na\(^+\)-Cl\(^-\) anion exchange. To further characterize the decrease in sodium absorption in IL-2\(^-/-\) mice, NHE3 mRNA and protein expression was studied.

Expression of the electroneutral NHE3. One of the main findings of the present study was the inhibition of NHE3 mRNA expression in IL-2\(^-/-\) mice. This could be due to a release of inflammatory mediators such as prostaglandins or proinflammatory cytokines. In line with this assumption, inhibition of NHE3 has been observed in Caco-2/bbe cells and rat intestine after treatment with IFN-γ (37). Therefore, it may be reasonable to speculate on a similar process of downregulation in the inflamed colon of IL-2\(^-/-\) mice because IFN-γ is upregulated in this model (4).

Reduced mRNA expression was accompanied by reduced NHE3 protein expression, which explains reduced Na\(^+\) absorption at least in part. However, net Na\(^+\) absorption was reduced by 80%, whereas NHE3 mRNA and protein expression were reduced by only 41 and 24%, respectively. Therefore, the reduction in net Na\(^+\) absorption is not solely a result of a reduction in NHE3 function. Other explanations for the substantial decrease in net Na\(^+\) absorption include 1) a reduction in Cl\(^-\) anion exchange, which is coupled to NHE3 activity; 2) a reduced Na\(^+\)-K\(^+\)-ATPase activity that provides the driving force for Na\(^+\) uptake; 3) contribution of another Na\(^+\)-mediated transport process that remains unidentified, and/or 4) regulation of NHE3 by endocytosis (24). Evidence for a reduction in Cl anion exchange was provided by the reduction in net Cl\(^-\) absorption and a negative residual flux (see Table 1). A decrease in Na\(^+\)-K\(^+\)-ATPase could also contribute, because it has recently been demonstrated that IFN-γ can decrease Na\(^+\)-K\(^+\)-ATPase activity and expression in a dose- and time-dependent manner (48).

An argument that has also been discussed for inflammation-associated transport dysfunction is changes in the maturity and viability of epithelial surface cells. It is reasonable to speculate that in a state of inflammation, maturity and/or viability of the surface epithelial cells, which are the major site for absorption, are disturbed. However, expression of NHE3 protein was reduced by only 24%. Thus it is rather unlikely that reduced maturity and/or viability are the primary explanations for impaired Na-Cl absorption in IL-2\(^-/-\) mice.

Electrogenic Na\(^+\) transport. After the cDNAs of the three subunits forming the pore of the ENaC (termed α, β, and γ) were cloned (7), several previous studies examined the molecular regulation of aldosterone on these subunits in mammalian distal colon (for review, see Ref. 6). Many of them were performed on rat distal colon in vivo (3, 26, 36). In one of these studies, transport measurements demonstrated a parallel increase in electronegenic Na\(^+\) absorption and γ-ENaC mRNA expression after a 14-day period of salt deprivation (26). Subsequently, we demonstrated a time correlation of the induction of β- and γ-ENaC mRNA expression and electronegenic Na\(^+\) absorption, suggesting that the early aldosterone effect is entirely due to transcriptional upregulation of the β- and γ-ENaC mRNA expression (12).

In contrast, no information is available at present on mRNA expression of the three ENaC subunits in inflamed tissue. Here we report that aldosterone stimulation of electronegenic Na\(^+\) transport failed to increase electronegenic Na\(^+\) absorption in IL-2\(^-/-\) mice. Concomitantly, there was no increase in the expression of the β- and γ-ENaC mRNA. A defect in activating this transport system by aldosterone is of pathophysiological relevance because this aldosterone-sensitive Na\(^+\)-absorptive process as well as the upregulation of the Na\(^+\)-H\(^+\)-ATPase acts as one of the major compensatory mechanisms for reduced Na\(^+\) absorption (45).

When looking for the pathomechanisms of this transport defect, TNF-α and IL-1β could be candidates that may alter gene expression. Very recently, we found that these cytokines can impair electronegenic Na\(^+\) transport and prevent upregulation of the ENaC β- and γ-subunit mRNA in rat late distal colon in vitro (unpublished data). Indeed, these cytokines are elevated in IL-2\(^-/-\) mice (4). Identification of the molecular mechanism of these cytokine effects on ENaC β- and γ-subunit mRNA expression, however, would require cloning of the ENaC β- and γ-subunit promoters with subsequent expression analysis in reporter gene assays.

Epithelial barrier function. Because the epithelium represents the main barrier between the intestinal lumen and the vascular compartment, alternating current impedance analysis was necessary to discriminate pure R\(^c\) and the resistance of the different subepithelial tissue layers between the voltage-sensing electrodes in the Ussing chamber. As described by Sadlack and co-workers (39), IL-2\(^-/-\) mice develop a colitis resembling that in UC. Recent data from our own group (42) showed an epithelial barrier disturbance in UC with concomitant tight junction alterations. However, so far no studies exist on epithelial barrier function in IL-2\(^-/-\) mice.

Because the colon is an intermediate tight epithelium, transepithelial transport takes place mainly via the transcellular route. In an intact epithelium, changes in the overall epithelial permeability (transcellular + paracellular permeability) are also dependent on activation or inhibition of membrane transport mechanisms. The present study showed that R\(^c\) was not impaired but was increased 1.5-fold in the inflamed colon of IL-2\(^-/-\) mice. Since mannitol fluxes were also decreased by 66% in IL-2\(^-/-\) mice, the increase in R\(^c\) cannot be explained solely by reduced transepithelial transport. Other mechanisms that could explain this finding include 1) a reduction in mucosal surface area and/or 2) alterations in the molecular composition of the tight junction meshwork. For this purpose, we determined the geometry of the mucosal surface area and the expression of different tight junction proteins.

Mucosal surface area. Resistance values and transport rates are usually related to the serosal surface area as defined by the opening of the Ussing chamber. However, R\(^c\) and active transport rates are dependent on the mucosal surface area. Chronic inflammation in IL-2\(^-/-\) mice leads to alterations in mucosal surface geometry (39). As a consequence of this, over- or underestimation of epithelial properties by measured parameters were possible. However, the mucosal surface morphology revealed no change in mucosal surface area in IL-2\(^-/-\) mice. A slight increase in the mean value (+14%) was not significant and functionally not relevant compared with the striking changes in transport and barrier functions. Furthermore, an increase in mucosal surface area would have led to a
decrease in $R^e$ and an increase in active transport rates. Instead, $R^e$ was increased and active transport decreased in IL-2−/− mice. Thus increased $R^e$ and diminished active transport are not due to alterations in mucosal surface area but reflect changes in tight junction structure and cellular dysfunction.

Expression of tight junction proteins. The structural correlate for paracellular resistance and ion-selective permeability are tight junction strand molecules (17, 19, 2). Before claudins were discovered, data seemed to support a predominant role of occludin (5, 30, 54). For example, Wan and co-workers (53) showed occludin cleavage with a consecutive breakdown of barrier function in confluent airway epithelial cells by Der p 1. Other studies, however, indicated that occludin is not a condition sine qua non for their formation (5, 54). For example, Saitou and co-workers (40, 41) reported that embryonic occludin-deficient stem cells can differentiate into polarized epithelial cells that form tight junction strands, and intestinal barrier function was found not to be impaired in occludin-deficient mice, which can be explained by substitutional redundancy. For claudin-1, Inai et al. (23) reported an increase in barrier function when it is overexpressed in MDCK cells. Therefore, it is likely that claudin-1 and occludin have fusing properties within the tight junction barrier. Investigations by Furuse et al. (20) revealed that heterogeneous claudin species can interact within and between tight junction strands, and the speculation arose that individually paired tight junction strands differ in their tightness depending on their combination and mixing ratios. This was emphasized by the observation of a decreased transepithelial resistance in MDCK I cells by introducing claudin-2 and led to the concept that claudin-2 is involved in the formation of conductive pores within tight junction strands (18).

One of the important findings of the present study was an increase in claudin-1, -2, -3, and -5 and occludin expression that was observed in the IL-2−/− mouse model paralleling the increase in $R^e$. As already mentioned in Epithelial barrier function, part of the increase in $R^e$ could be attributed to the decrease in transcellular transport sites. However, it seems reasonable to assume that the increase in tight junction molecule expression in IL-2−/− mice as, for example, for occludin and claudin-1 also contributed to this increase. However, this is speculative since it is known that in the intestine, tight junction strands are composed of a subset out of >20 different claudin species and our understanding of their individual function is just at the beginning (34). Furthermore, for example, claudin-2 possesses pore-forming properties, making this system even more complex (2). But together, the data on tight junction protein expression in this model of intestinal inflammation provides both understanding of the increase in resistance in IL-2−/− mice and also important information on the functional role of tight junction proteins in general.

A further question is why tight junction proteins are expressed more intensively in the inflamed colon of IL-2−/− mice. It is possible that in an early stage of the inflammatory process low levels or a distinct pattern of proinflammatory cytokines exclusively influence transporter expression and even permit the overexpression of tight junction proteins in an adaptive attempt by the mucosa to compensate for the loss in active transport function. Then, only in later stages of inflammation, e.g., in UC (42), defects in epithelial barrier function do occur.

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