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6. Immunoblotting was as described [N. M. Hunkeler, J. Kullman, A. M. Murphy, *Circ. Res.* **69**, 1409 (1991) (*3*, *4*)], except that the cardiac muscle was homogenized in 100 mM potassium phosphate buffer (pH 7.8) with 1 mM dithiothreitol, 2 mM EDTA, 1% Triton with protease inhibitors, then pelleted and resuspended in sample buffer.
7. Transgenic mice had a higher incidence of sudden death with anesthesia.
8. Mice were sedated with methoxyflurane and placed on a warming pad. Echocardiograms were obtained with a 15-MHz vascular probe with a standoff from the chest using an Acuson ultrasound machine.
9. RNA was prepared using Trizol reagent (GIBCO). Dot blots were prepared and hybridized with end-labeled transcript-specific oligonucleotide probes as described [W. K. Jones *et al.*, *J. Clin. Invest.* **98**, 1906 (1996); A. Sanbe *et al.*, *J. Biol. Chem.* **274**, 21085 (1999)], and expression was quantified using a Packard phosphorimager. Relative expression was normalized to expression of glyceraldehyde 3-phosphate dehydrogenase. The results from three nontransgenic mice were compared by *t*-test to the results from nine transgenic mice.
10. Mice were euthanized by deep methoxyflurane anesthesia. Tissues were fixed in formalin (Sigma), and paraffin-embedded sections were stained with hematoxylin and eosin, and with Masson's trichrome. A cardiac pathologist blinded to the identity of the mice reviewed multiple sections from base to apex of hearts from three nontransgenic and three transgenic mice. No differences in cellular histology or fibrosis could be distinguished.
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Role of the Enteric Nervous System in the Fluid and Electrolyte Secretion of Rotavirus Diarrhea

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The mechanism underlying the intestinal fluid loss in rotavirus diarrhea, which often afflicts children in developing countries, is not known. One hypothesis is that the rotavirus evokes intestinal fluid and electrolyte secretion by activation of the nervous system in the intestinal wall, the enteric nervous system (ENS). Four different drugs that inhibit ENS functions were used to obtain experimental evidence for this hypothesis in mice in vitro and in vivo. The involvement of the ENS in rotavirus diarrhea indicates potential sites of action for drugs in the treatment of the disease.

Rotavirus is the major cause of infantile gastroenteritis worldwide and is associated with about 600,000 deaths every year, predominantly in developing countries (*1*). Although two decades of research have significantly increased our understanding of virus immunology and have led to the development of an oral vaccine, our knowledge of the mechanisms that induce rotavirus diarrhea, nausea, and vomiting remains limited.

Rotavirus infects the mature enterocytes in the mid and upper villous epithelium of the small intestine, ultimately leading to cell death and villus atrophy. A striking observation in both animals and humans is that only a few percent of the mature villus epithelial cells and no crypt cells seem to be infected (*2*–*5*). Fluid secretion is usually ascribed to an imbalance between the secretory crypt cells and the mature absorptive villous epithelium. The death of the villus cells leads to a repopulation of the epithelium with immature secretory type cells. Mechanisms proposed to explain the rotavirus-induced intestinal secretion of fluid and electrolytes include villous ischemia (*5*) and a toxin-like effect by a nonstructural virus protein, NSP4 (*6*).

A localized systemic response triggered by rotavirus-enterocyte interaction has been proposed previously (*4*). According to Ste-

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phen and collaborators, rotavirus induces ischemia in villi before significant virus replication occurs in epithelial cells. These changes in the microcirculation are believed to be of pathophysiological importance and to be mediated by endogenous, neuroactive, hormonal substances (*5*).

During the last two decades it has become increasingly apparent that intestinal secretion of fluid and electrolytes evoked by luminal agents is often induced by activation of the nervous system located in the intestinal wall, the enteric nervous system (ENS) (*7–9*). Here we have investigated the role of the ENS in the fluid secretion evoked by rotavirus in newborn mice.

In Ussing chamber experiments (*10*), transmural potential difference (PD) was monitored continuously in mice intestines in vitro. The intestinal segment was voltage-clamped at 0 V and the short circuit current (SCC) determined at various intervals, which allowed us to estimate tissue electrical resistance or conductance. The nearly twofold increase in PD in infected (1.89 ± 0.15 mV; $n = 27$) relative to uninfected (0.90 ± 0.07 mV; $n = 21$) animals was significant by the Mann-Whitney U test ($P < 0.0001$).

Two types of Ussing chambers were used to measure the SCC. In one type, the electrical field was homogeneous, so that it was possible to reliably quantify tissue conductance. In the virus-infected tissue, conductance was significantly higher than in the noninfected tissue [28.6 ± 2.7 mS cm^{-2} ($n = 13$) versus 18.3 ± 1.1 mS cm^{-2} ($n = 12$); $P < 0.01$]. The corresponding values of PD in these experiments were 1.91 ± 0.15 mV versus 0.79 ± 0.06 mV ($P < 0.0001$).

Two drugs that specifically abolish nerve action potentials were tested, tetrodotoxin (TTX; blocker of sodium channels in excitable tissues) and lidocaine (local anesthetic). Neither drug significantly affected tissue conductance. Addition of TTX to the serosal and the mucosal compartments in infected and noninfected animals attenuated the PD in a dose-dependent manner (Fig. 1A). The decrease in PD was significantly larger in the infected than in the noninfected animals ($P < 0.01$).

In initial series of experiments, lidocaine was applied to both sides of the intestinal wall at three concentrations (0.4, 4, and 40 mM). When the intestinal mucosa was exposed to theophylline at the end of those experiments (to test the viability of the tissue), no change in PD was observed, suggesting that lidocaine had affected enterocyte function. In a second series of experiments lidocaine was therefore only administered to the serosal side at two concentrations (0.4 and 4 mM). Theophylline evoked a significant increase in PD in these experiments. Lidocaine attenuated the PD in infected and noninfected intestines in a dose-dependent manner (Fig. 1B), with a significantly larger decrease seen in infected than in control animals ($P < 0.05$).

Hexamethonium, which inhibits synaptic transmission by blocking the nicotinic receptor, had no effect (2, 20, and 200 μM) (*11*). Because hexamethonium is a highly polar compound and therefore not readily soluble in lipids, we investigated the effects of a more lipophilic nicotinic receptor blocker, mecamylamine. Mecamylamine tested at the same concentrations as hexamethonium decreased the PD in infected relative to noninfected intestinal segments without affecting tissue conductance (Fig. 1C).

In perfusion experiments (*12*) we monitored the PD and net fluid transport (NFT), which was measured with ^{14}C-labeled polyethylene glycol as a nonabsorbable radioactive marker (Fig. 2). In experiments to test the viability of the intestinal segments, PD and NFT remained relatively constant in the noninfected intestines, but changed significantly in the intestines exposed to virus. Because the differences in NFT and PD were not significant at 60 min, the results reported below are based on experiments lasting only 40 min.

The PD measured in infected animals was significantly higher than in noninfected mice (Fig. 2B). This was also apparent in a different series of experiments comparing the PD in infected and noninfected animals during the control period (*10*) (control animals: 2.71 ± 0.10 mV; $n = 24$; infected animals: 4.24 ± 0.20 mV; $n = 27$; $P < 0.0001$). Similarly, in the control period, the amount of fluid secreted in the infected animals (28.8 ± 4.9 μl $hour^{-1}$ cm^{-1}; $n = 26$) was significantly different from the fluid absorption observed in the noninfected animals (1.1 ± 2.6 μl $hour^{-1}$ cm^{-1}; $n = 22$, $P < 0.0001$).

When the serosal surface of the perfused segment was exposed to a TTX concentration of 0.1 μM, there was a significant decrease in the PD of virus-infected segments, whereas the PD of control animals was not affected. Concomitantly, net fluid secretion in the infected intestines was completely inhibited.

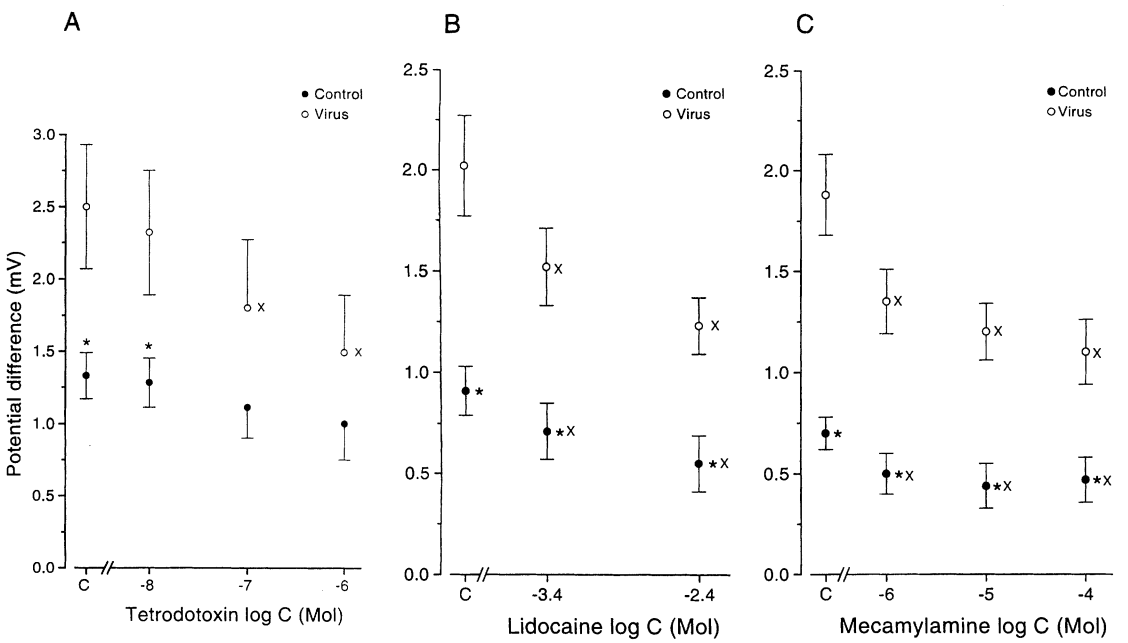

Fig. 1. Results obtained in the Ussing chamber experiments. **(A)** The effect of increasing concentrations of TTX on the transepithelial PD in control intestinal segments ($n = 5$) and segments exposed to rotavirus ($n = 6$). TTX was administered both to the mucosal and serosal compartments. **(B)** The effect of lidocaine (0.4 and 4 mM) on PD in control ($n = 5$) and virus-infected ($n = 6$) intestinal segments. Only the serosal surface was exposed to the drug. **(C)** The effect of increasing concentrations of mecamylamine on the PD in control ($n = 5$) and virus-infected ($n = 6$) intestinal segments. The drug was administered both to the serosal and mucosal compartments. Crosses indicate statistically significant differences compared with control observations (C). Asterisks indicate statistically significant differences between control and virus groups.

NFT in the noninfected intestines changed from a small net secretion to net absorption (Fig. 3). The changes caused by TTX were much larger in the infected than in the control intestines (Table 1). Exposure of the intestinal serosa to a 4 mM solution of lidocaine evoked a response similar to that seen with TTX (Fig. 4 and Table 1).

Hexamethonium at a concentration of 20 μM significantly decreased the PD in the virus-infected segments but not in control segments (Fig. 5B). Similarly, net fluid secretion was attenuated in the infected segments, whereas in the control segments no significant effect was detected (Fig. 5A). The changes in PD and NFT were larger in the infected than in the control intestines (Table 1).

A third type of experiment was carried out on 4- to 6-day-old awake animals. Diarrhea was determined by judging the stool according to a scoring system (*13*) after gently pressing the abdomen. Only lidocaine given intraperitoneally (i.p.) was tested in these experiments, because the effect of hexamethonium or mecamylamine given intravenously (i.v.) lasts for only 1 to 2 hours (*14*). Lidocaine (25 mg/kg, dissolved in 50 μl of physiological saline) was injected twice daily after giving rotavirus orally. About 48 hours after rotavirus administration the incidence of diarrhea peaked in the control group, with 14 of 15 mice exhibiting diarrhea. In the lidocaine group, 6 of 14 mice showed signs of diarrhea. The difference between the two groups was highly significant ($P < 0.005$; Fisher's exact probability test).

The observation that all four drugs significantly attenuated the intestinal secretory response to rotavirus strongly suggests that the ENS participates in the virus-induced electrolyte and fluid secretion, as has been shown for bacterial enterotoxins (*7–9*). The involvement of the ENS may explain how comparatively few virus-infected cells at the villus tips can cause the intestinal crypt cells to augment their secretion of electrolytes and water. The enhanced fluid secretion may serve as a defense mechanism against the potentially harmful mucosal influence. In the case of the fluid secretion caused by cholera toxin and bile salt, it has been shown that the secretory response is accompanied by a motility response that enhances the luminal transport in an aboral direction (*15*).

We used the TTX data to estimate the extent of ENS involvement in the intestinal fluid and electrolyte secretion evoked by rotavirus, given that TTX probably is the most investigated and most specific blocker of nervous activity among the drugs used in the present study (*16*). In the Ussing chamber experiments, 67% of the virus effect was mediated by the nervous system. A similar calculation for the effect of TTX on NFT in the perfusion experiments indicates that 85% of the virus response can be ascribed to neuronal involvement. The PD measurements in the perfusion experiments indicate than the virus effect is entirely due to nervous system activity. However, the latter calculation seems less reliable because the PD measurements in the noninfected segments were abnormally high. We conclude from these calculations that at least two-thirds of the secretory response to rotavirus is mediated by the ENS.

Intestinal inflammation has been shown to evoke fluid secretion by activation of the ENS (*9*). The results of the present study are consistent with these observations. Inflammation is accompanied by an increased tissue concentration of a large number of biologically highly active compounds that alone or together may activate enteric neurons (*17*, *18*). In the case of rotavirus, ENS activation may also occur by other mechanisms. It has been shown that rotavirus can infect primary neurons (*19*). Furthermore, it was recently suggested that rotavirus exerts its effects through a "toxin-like" protein (NSP4), a nonstructural protein produced by the

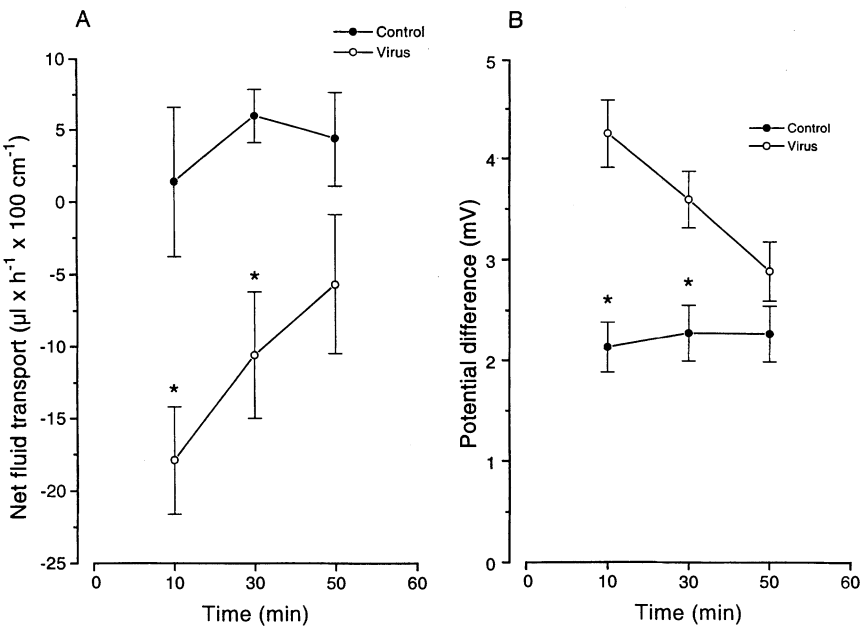

Fig. 2. Viability of the intestinal preparation perfused in vitro. The PD was monitored continuously for 60 min, and NFT was measured during three consecutive 20-min periods. (**A**) Changes in NFT during perfusion of infected ($n = 7$) and noninfected ($n = 5$) intestinal segments. Negative values indicate net fluid secretion. (**B**) Changes in the PD during perfusion of control segments ($n = 6$) and in intestinal segments exposed to rotavirus ($n = 7$). Both the NFT and PD remained constant in the control series, but decreased in the virus group. For technical reasons NFT was not recorded in one experiment. Asterisks indicate statistically significant differences between control and virus groups.

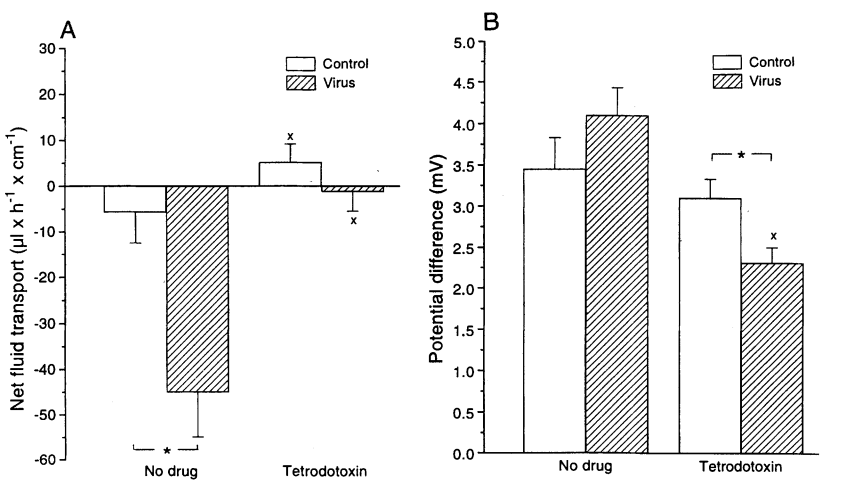

Fig. 3. (**A**) NFT and (**B**) PD before and after exposure of the intestinal serosa to TTX (10^{-7} M). Perfusion experiments were performed on control segments ($n = 6$) and on segments infected with rotavirus ($n = 6$). Crosses indicate statistically significant differences compared with the corresponding No drug observations. Asterisks indicate statistically significant differences between control and virus groups.

virus (*6*), thereby increasing intracellular calcium concentrations. Such calcium increases may trigger the release of amines or peptides from the endocrine cells of the gut to stimulate denDrites or free nerve endings located underneath the epithelial layer, thereby activating secretory nervous reflexes (*20*).

The replacement of fluid losses in diarrhea with an oral solution of glucose and sodium chloride represented a major therapeutic advance when it was first introduced. The effect is dependent on an intact absorptive capacity of the intestinal epithelium. Combining the oral glucose-salt solution with a drug that attenuates the intestinal secretion of fluid has the potential to enhance this effect. The results of this and earlier studies (*8*) strongly suggest that in most, if not all, intestinal secretory states, nerve reflexes in the ENS are stimulated to cause intestinal fluid losses. This implies new potential sites of action for drugs in the treatment of diarrhea. The results with nicotinic receptor blockers indicate that the secretory nervous reflexes contain at least one synapse. Synaptic transmission may in principle be influenced by drugs other than the nicotinic receptor blockers reported here. For example, it may be possible to target the system presynaptically (*21*) or to blockade neurotransmitter receptors on the enterocytes. Finally, intestinal secretion evoked by diarrheal agents that activate ENS via the intestinal endocrine cells can be significantly inhibited by L-type calcium channel blockers, which markedly attenuate the release of amines and peptides from these cells (*20*).

Table 1. Differences in transmural epithelial potential difference (PD) and net fluid transport (NFT) between control measurements and measurements made 20 min after exposure of the intestinal mucosa to the drug tested. In the "No drug" experiments, the change in NFT between the first and second 20-min period was determined. The number of observations is indicated in parentheses.

Drug	PD (mV)		NFT (μl min^{-1} cm^{-1})	
	Control	Virus	Control	Virus
No drug	0.13 ± 0.07 (6)	−0.66 ± 0.13† (7)	4.6 ± 4.3 (5)	7.3 ± 6.2 (7)
Tetrodotoxin	−0.36 ± 0.22 (6)	−1.79 ± 0.20*† (6)	10.8 ± 3.3 (6)	43.8 ± 7.9*† (6)
Lidocaine	−1.36 ± 0.19* (6)	−2.77 ± 0.41*† (8)	15.0 ± 3.8 (6)	29.3 ± 12.3* (8)
Hexamethonium	−0.18 ± 0.29 (6)	−1.22 ± 0.26*† (6)	9.2 ± 4.2 (5)	25.2 ± 3.7*† (6)

*Statistically significant compared with the "No drug" experiments. †Statistically significant compared with the corresponding control experiments (Mann-Whitney U test; $P < 0.05$).

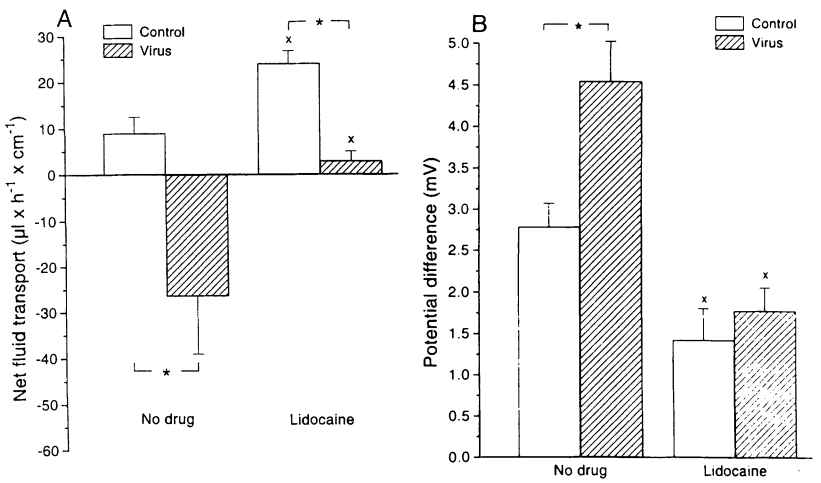

Fig. 4. The effect of lidocaine (1.6×10^{-3} M) on (**A**) NFT and (**B**) PD. Control experiments were performed on six segments, and rotavirus experiments on eight segments. Crosses indicate statistically significant differences compared with the corresponding No drug observations. Asterisks indicate statistically significant differences between control and virus groups.

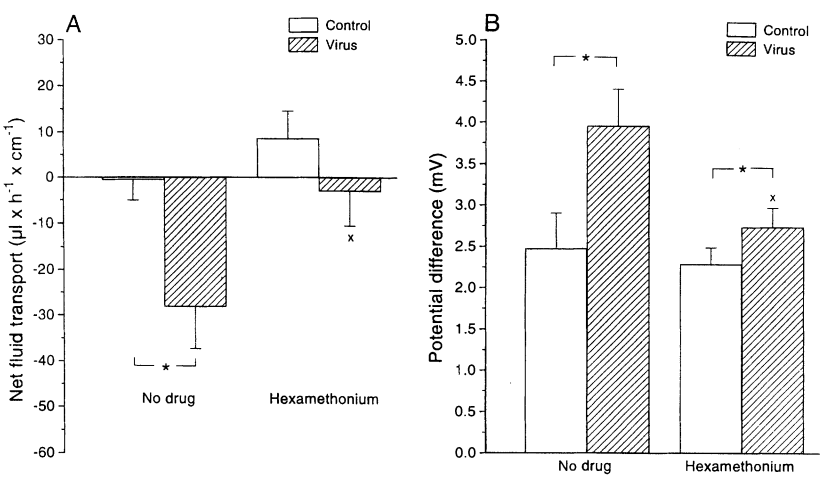

Fig. 5. (**A**) NFT and (**B**) PD before and after exposure of the intestinal serosa to hexamethonium (2×10^{-5} M). Perfusion experiments were performed on control segments ($n = 5$ or 6) and on segments infected with rotavirus ($n = 6$). For technical reasons, NFT was not determined in one experiment. Crosses indicate statistically significant differences compared with the corresponding No drug observations. Asterisks indicate statistically significant differences between control and virus groups.

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10. Pregnant Balb/c mice were purchased from B&K, Sollentuna, Sweden. Experiments were performed on newborn mice at 11 to 13 days of age. Plaque-purified Rhesus rotavirus (RRV) was prepared for administration as described (*22*). Briefly, RRV was grown on MA-104 cells until complete cytopathogenic effect was observed, followed by freeze-thawing and titer determination (*23*). Litters of mice, seronegative for rotavirus, were infected by oral administration of 10 μl of 2×10^7 plaque-forming units of RRV 9 to 11 days after birth. The experiments were performed 48 to 60 hours after inoculation when intestines exhibited the classical charac-

teristics of inflammation, oedema, and vasodilatation. Experiments were also performed on age-matched control animals. Mice were anesthetized with ether or tribromoethanol (125 mg/kg). Intestinal segments from the middle third of the small intestine were extirpated and immediately stored in ice-cold phosphate-buffered saline. A segment (1 to 2 cm long) was opened along the antimesenteric border and pinned as a flat sheet (area 0.24 cm^2) between the two halves of an Ussing chamber, which contained a modified Krebs-Henseleit solution (5 ml; 114 mM NaCl, 25 mM $NaHCO_3$, 5 mM KCl, 1.65 mM NaH_2PO_4, 1.1 mM $MgCl_2$, 2.5 mM $CaCl_2$). The serosal bathing fluid contained glucose (10 mM), and the mucosal compartment mannitol (10 mM). The solutions were continuously bubbled with CO_2 (5%) in oxygen at 37°C. The transmural PD was monitored with a pair of calomel electrodes. Two types of Ussing chambers were used. In one type, the electrical field of the voltage clamp was perpendicular to the intestinal segment, which made it possible to estimate tissue resistance in absolute terms by using an automatic voltage clamp device to measure SCC. PD and SCC were recorded on a polygraph. Values of PD and SCC reported in the text, tables, and figures are given as the mean ± SEM. After mounting the tissue, the PD was allowed to stabilize for about 30 min. In control experiments, PD and SCC remained constant in infected and noninfected animals after this stabilizing period (*24*). For testing of drugs, each tissue was exposed to one drug in increasing concentrations with a 10-min interval between administrations. The drugs used were administered in volumes of 20 μl. At the end of the experiments, 200 μl of theophylline solution was added to the mucosal and serosal solutions to a final concentration of 1 mM to test tissue viability. If theophylline increased the PD by less than 40% of the control value, the experiment was discarded.

11. O. Lundgren *et al.*, data not shown.
12. The perfusion technique used was that described by Starkey *et al.* (*25*). Mice were anesthetized with tribromoethanol (125 mg/kg, i.p.). The abdomen was opened and a jejunal segment 4 to 6 cm long was chosen for the experiment. The intestinal segment was flushed with physiological saline and then placed in an organ bath containing a modified Krebs-Henseleit solution with glucose (122 mM NaCl, 25 mM $NaHCO_3$, 3.5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 2 mM $CaCl_2$, 30 mM glucose) continuously oxygenated with 5% CO_2 in oxygen at 37°C. The intestinal segment was perfused at a constant rate (990 μl $hour^{-1}$) through plastic tubes in both ends. The perfusion solution was identical to the solution in the organ bath except that glucose was substituted with mannitol. The perfusion solution also contained a radioactively labeled nonabsorbable marker (^{14}C-polyethylene glycol 4000; specific activity, 10 to 20 mCi g^{-1}; Amersham Pharmacia Biotech, Buckinghamshire, U.K.) at a concentration of ~0.07 μCi ml^{-1}. The tracer and plastic tubing were immersed in a solution containing nonlabeled polyethylene glycol 4000 (2 g ml^{-1}) for at least 24 hours before use. In control experiments this procedure prevented any adsorbance of the tracer to the plastic tubing. After placing the segment in the organ bath, the intestine was perfused for 20 min without sampling. Then 20-min samples were collected in tubes at the outflow end of the perfusion system. In one series of experiments (Fig. 2), in which the viability of the intestinal preparation was tested, the perfusion fluid was sampled during three consecutive 20-min periods. For reasons given in the text, only two samplings were performed in the experiments with drugs. Drugs were administered to the organ bath between the first and second sampling period. Radioactivity in duplicate samples was measured from the inflow and outflow solutions. The sample (100 μl) was mixed with 3.0 ml of scintillation fluid (Ultima Gold XR, Packard). At least 10,000 decays were measured in a Packard scintillation counter (Packard 1900 TR Liquid scintillation analyzer). Net fluid transport was estimated from the rate of perfusion, and the radioactivity of the solutions entering and leaving the intestine was determined (*26*). The transmural PD of the intestinal segment was recorded by two electrodes. One electrode was connected by a T-tube to the inlet of the perfusion system and the other was in contact with the solution in the organ bath. The PD was continuously recorded on a polygraph.
13. Diarrhea in the mice was judged by a scoring system, with a score of 1 indicating unusually loose yellow stool and a score of 4 a completely loose stool. A score of ≥2 (mucous with liquid stool, some loose but solid stool) was considered to indicate diarrhea. The scoring was performed by the same individual twice daily after administration of the rotavirus.
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A Role for Histone Acetylation in the Developmental Regulation of V(D)J Recombination

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V(D)J recombination is developmentally regulated in vivo by enhancer-dependent changes in the accessibility of chromosomal recombination signal sequences to the recombinase, but the molecular nature of these changes is unknown. Here histone H3 acetylation was measured along versions of a transgenic V(D)J recombination reporter and the endogenous T cell receptor α/δ locus. Enhancer activity was shown to impart long-range, developmentally regulated changes in H3 acetylation, and H3 acetylation status was tightly linked to V(D)J recombination. H3 hyperacetylation is proposed as a molecular mechanism coupling enhancer activity to accessibility for V(D)J recombination.

V(D)J recombination is initiated by recombinase activating gene–1 (RAG-1)– and RAG-2–mediated cleavage between T cell receptor (TCR) and immunoglobulin coding gene segments (V, D, and J) and flanking recombination signal sequences (RSSs) (*1*). Chromosomal and nucleosomal RSSs are refractory to RAG-mediated cleavage relative to naked DNA (*2*, *3*), and V(D)J recombination is thought to be regulated in vivo by enhancer- and promoter-dependent changes in chromatin structure that provide RAG proteins access to specific RSSs (*4*). However, the nature of chromatin structural modifications associated with accessibility to RAG proteins is not known. Recent studies indicate that enhancers and promoters can direct the hyperacetylation of core histones (*5*) due to histone acetyltransferase activity of transcriptional coactivators (*6*). Moreover, histone hyperacetylation alters chromatin structure, as suggested by increased general sensitivity to endonucleases (*7*) and increased binding of transcription factors (*8*). Hyperacetylation of histones therefore provides a potential mechanism linking enhancer and promoter activity to RSS accessibility to RAG proteins.

We initially addressed the relation between histone acetylation and accessibility for V(D)J recombination by studying a TCRδ minilocus V(D)J recombination reporter in thymocytes of transgenic mice (Fig. 1, A and B). This reporter contains unrearranged human Vδ, Dδ, Jδ, and Cδ gene segments. With a functional enhancer in the Jδ3-Cδ intron, V, D, and J gene segments are all accessible to RAG proteins, and fully rearranged products (VDJ) are efficiently generated (*9*, *10*). With the enhancer deleted or mutated, only V-to-D rearrangement is observed (*9*, *11*, *12*). The failure of VD-to-J rearrangement without an enhancer reflects an inability of RAG proteins to access and cleave J segment RSSs (*13*). Because V and D accessi-

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