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# Rotavirus Diarrhea Is Caused by Nonreplicating Viral Particles

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**Rotaviruses infect the villous epithelium of the small intestine and cause severe diarrhea in young children. The mechanism by which rotavirus causes diarrhea has not been elucidated. It has been hypothesized that rotavirus replication in the intestinal epithelium causes a loss of viable absorptive cells, leading to an imbalance of intestinal secretion and absorption. Cell destruction has generally been thought to result from rotavirus transcription and replication. However, the widely used heterologous murine model of rotavirus infection demonstrates minimal viral replication and histological changes limited to epithelial vacuolation on the distal villus despite the simultaneous occurrence of voluminous liquid diarrhea. We have genetically inactivated rotaviruses to test the importance of viral replication in the pathogenesis of rotavirus-induced diarrhea. We present direct evidence that transcription- and replication-defective rotaviruses cause diarrhea in an animal model. These findings suggest that rotavirus attachment or entry into cells is sufficient for the induction of diarrhea. The mechanism of rotavirus-induced diarrhea is therefore consistent with a viral toxin-like effect exerted during virus-cell contact.**

Rotaviruses are members of the *Reoviridae* family and contain 11 double-stranded RNA segments inside a 70-nm triple-layered protein capsid. VP2 proteins form the core of the virus, which is surrounded by VP6 proteins to form characteristic single-shelled viral particles. The icosahedral outer capsid of rotavirus is composed of proteins VP4 and VP7. Functions attributed to the outer capsid proteins are viral virulence, host range restriction, hemagglutination, serotype specificity, neutralization, disease prevention, and cell attachment and penetration. The VP4 protein is involved in cellular attachment and in particular contains a putative fusion region that may facilitate cell penetration. The involvement of VP4, VP7 or other rotavirus proteins in inducing diarrhea has not been defined.

The murine model of rotavirus disease has been used to define the rotavirus proteins involved in neutralization and permitted the correlation of viral neutralization with disease protection (12, 15, 16, 18). In the murine model, large doses of rotaviruses isolated from other species are administered to infant mice to induce infection and diarrhea (17). The rhesus monkey-derived rotavirus RRV is a human vaccine strain that was evaluated extensively in this model. Murine (homologous) rotaviruses have also been studied in this model, but difficulties in growing murine strains to high titers in tissue culture have limited their use in studying rotavirus disease.

Two observations on RRV and murine rotavirus disease in mice prompted the described experiments to determine the role of viral replication in diarrheal disease. First, while murine rotaviruses replicate efficiently in mice, simian rotavirus (RRV) titers are not significantly increased during infection even though mice consistently have diarrhea (7, 19). Second, RRV induces diarrhea in mice only when administered at high doses ($>10^6$ PFU), while murine strains cause diarrhea at very low doses (about $10^1$ PFU) (7). Therefore, we hypothesized that rotavirus replication is not a fundamental requirement for the induction of disease. Instead, the interaction of a threshold quantity of nonreplicating viral capsids with enterocytes may initiate a disease-inducing chain of events. We have tested this hypothesis by studying the ability of genetically inactivated rotaviruses to induce diarrhea in mice (8).

## MATERIALS AND METHODS

**Virus preparation.** RRV was grown in MA104 cell monolayers in medium M199 including 0.2 μg of trypsin per ml as previously described (22). Virus was purified by a previously published protocol (21), using fluorocarbon extraction and rate-zonal ultracentrifugation on a 20 to 40% sucrose gradient. After purification, virus was dialyzed against a Tris-saline buffer (pH 7.4) with 2 mM $CaCl_2$. The osmolarity of the final virus preparation was 211 mOsmol/kg; that of the inactivated preparation was 216 mOsmol/kg. The osmolarity of the Tris-saline-calcium buffer was 209 mOsmol/kg.

**Virus inactivation.** The genetic inactivation of tissue culture-derived RRV was accomplished through a recently described process using psoralen and long-wave UV light that irreversibly cross-links viral RNA but does not alter the hemagglutination function or antigenic characteristics of rotavirus proteins (8, 9). A single pool of virus was divided, and one portion was subjected to psoralen-UV treatment. Briefly, virus was mixed with 4′-aminomethyl-4,5′,8-trimethylpsoralen at 50 μg/ml and exposed to UV light at 365 Å (36.5 nm) for 45 min as previously described (8). The condition of the inactivated virus was assessed as described below.

**Immunohistochemical detection of rotavirus antigen in MA104 cell monolayers.** The assay was performed to quantify infectious virus in the original sample and verify inactivation after psoralen treatment (21). Briefly, we exposed the monolayer to the virus for 1 h, washed it, and replaced the growth medium for 12 h. The monolayer was incubated at 37°C in 5% $CO_2$ and then fixed with ice-cold methanol. Antigen detection was accomplished by the sequential use of guinea pig anti-RRV hyperimmune antiserum, goat anti-guinea pig immunoglobulin G (IgG) conjugated to horseradish peroxidase (KPL, Inc., Gaithersburg, Md.), and the peroxidase substrate aminoethylcarbazole (Sigma Chemical Co.). The monolayers were photographed on a Nikon inverted microscope at a magnification of ×200 to ×400 with Kodachrome 200 film.

**In vitro transcription assay.** The method used was modified from the standard procedure (2, 6). CsCl gradient-purified RRV and inactivated RRV were treated with 10 mM EGTA for 30 min at 37°C to remove the outer capsid. Single-capsid particles were mixed on ice with a mixture of nucleotides including [α-$^{32}$P]UTP, and the polymerase reaction was initiated by shifting the mixture to 42°C. Aliquots were removed at 0, 5, 10, 15, 30, and 60 min and then hourly until 15 h. Incorporation of [α-$^{32}$P]UTP into acid-precipitable counts was monitored by scintillation of nitrocellulose-filtered precipitates. Background counts were established by counting trichloroacetic acid-precipitable samples from which either virus, ribonucleoside triphosphates or [α-$^{32}$P]UTP had been omitted or by the addition of RNase after the reaction. Addition of RNase to samples from active RRV transcription reactions reduced precipitable counts to background levels.

**Further characterization of the virus inoculum.** Inactivated and intact RRV preparations to be used for murine inoculation were compared by spectrophotometric $A_{260}$ and $A_{280}$ measurements to ensure that similar viral masses were

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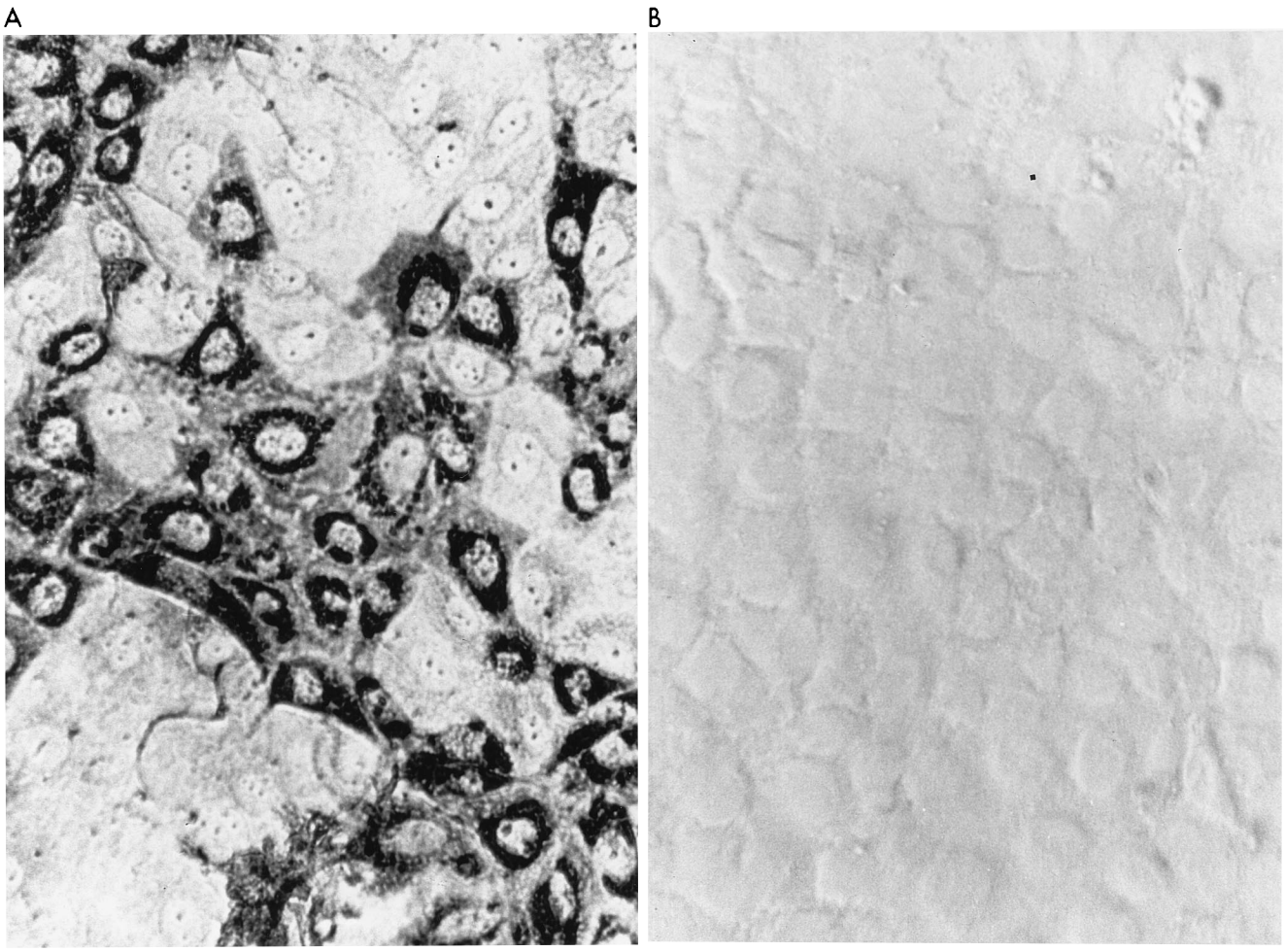


FIG. 1. RRV infection of MA104 monolayers before (A) and after (B) psoralen inactivation. Immunohistochemical assay of rotavirus antigen in MA104 cell monolayers was performed. Distinctive red-brown-staining cytoplasmic accumulations of viral antigen are readily seen in virtually every cell inoculated with the original preparation of RRV (A). Panel B shows the outcome of the same assay when psoralen-UV-treated virus was inoculated in the same amount as used for panel A. Viral antigen was not detected, confirming that all virus was rendered noninfectious by the treatment.

still present after inactivation. Hemagglutination assays (10) provided a continuing measure of the integrity of the VP4 protein on the outer capsid of RRV and thus outer capsid integrity.

**Inoculation of mice with RRV or inactivated RRV.** Pregnant seronegative dams were obtained from Taconic Farms (Germantown, N.Y.) in isolator cages under a protocol approved by the Institutional Animal Care Committee. They were housed in microisolator cages and provided sterile food and water. Cages were not changed until after rotavirus inoculation to discourage cannibalism. Infant mice were inoculated by gavage at 8 to 9 days of age, at which time they were administered by gavage 50 μl of either RRV ($3 \times 10^8$ focus-forming units/ml), inactivated RRV (equivalent to the RRV dose prior to inactivation), or control (M199 alone, or Tris-saline-calcium chloride buffer used in a sham fluorocarbon extraction). Mice were examined daily for signs of diarrhea (abdominal bloating, fecal staining of skin), and stool was expressed if possible by gentle abdominal palpation. A five-point rating system was used to characterize diarrhea: 0, no stool expressed, no fecal staining; 1, brown formed stool expressed; 2, soft brown stool expressed; 3, soft yellow stool expressed; 4, liquid yellow stool expressed. Fecal staining was usually seen in conjunction with category 4 stool, as was abdominal bloating. Both findings were seen occasionally in mice inoculated with RRV or inactivated RRV. No mice died as a result of the diarrhea.

**Enzyme-linked immunosorbent assay (ELISA) of intestinal IgA specific for RRV.** The assay has been previously described (20). RRV-specific IgA was used in these experiments as a marker of the virus-specific intestinal immune response. Intestinal secretions were harvested at sacrifice between 21 and 35 days after inoculation.

## RESULTS

Figure 1 demonstrates the effectiveness of psoralen inactivation in a histochemical infectious focus assay. The absence of rotavirus antigen in infected cells following inoculation with psoralen-inactivated RRV (PI-RRV) is clearly demonstrated in comparison with cells exposed to untreated RRV. The infectious titer of viral pools before psoralen exposure was $6 \times 10^9$ focus-forming units/ml. Reciprocal hemagglutination titers of RRV and PI-RRV were between $1 \times 10^5$ and $2 \times 10^5$.

Replication of rotavirus RNA was evaluated by measuring endogenous RNA polymerase activity in an in vitro transcription assay. In duplicate reactions, RRV incorporated 561,000 cpm, while a matched aliquot of PI-RRV did not incorporate counts above the background level (<3,000). The addition of RNase reduced RRV counts to background levels, while the PI-RRV counts remained unchanged. Thus, psoralen inactivation resulted in the complete elimination of incorporation of label into new viral RNA.

Eight- to nine-day-old infant CD2F1 mice from seronegative dams (Taconic Farms) were inoculated with $3 \times 10^8$ focus-forming units of virus (RRV or an equivalent amount of PI-

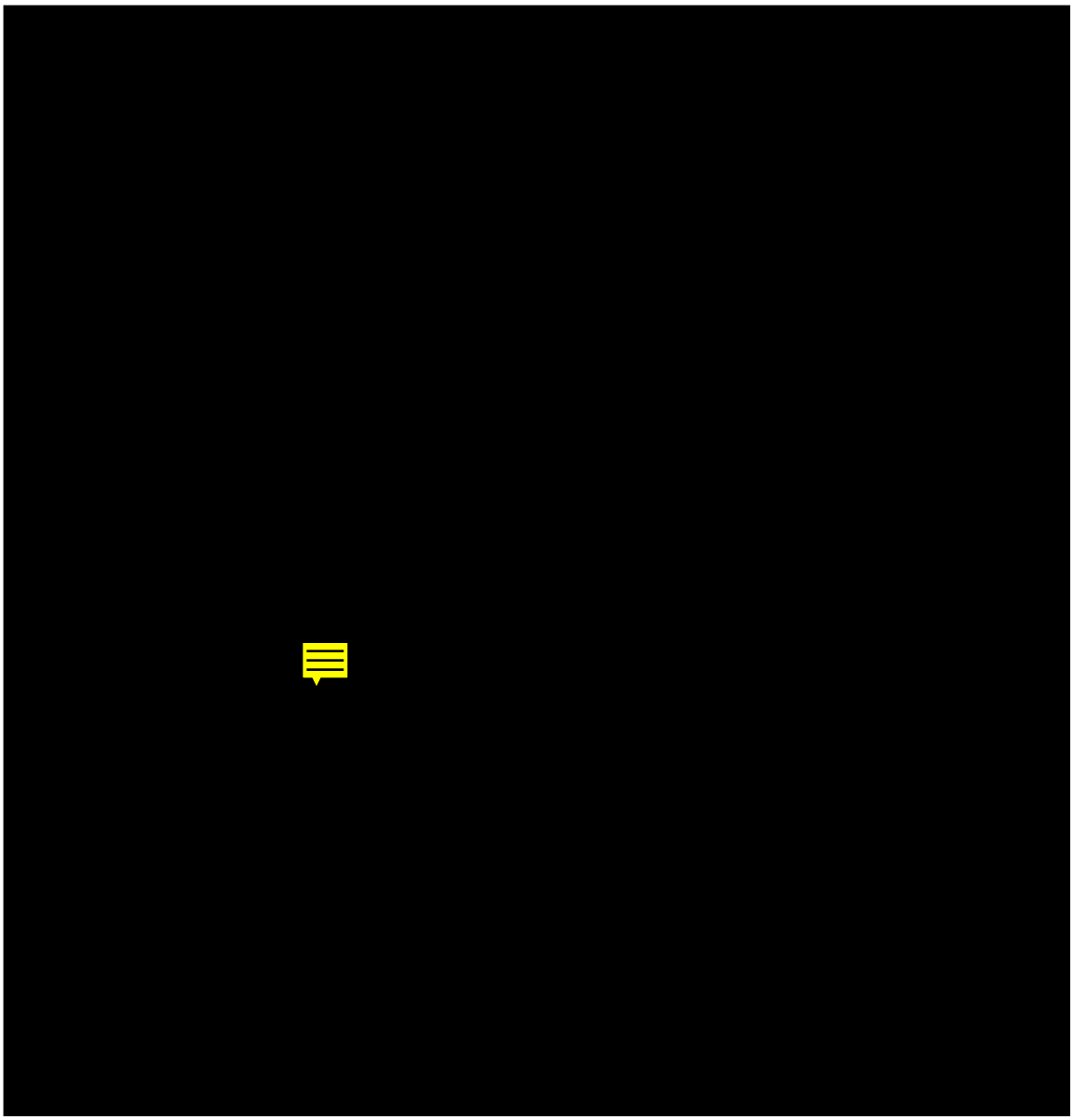

FIG. 2. Mouse diarrhea photo scoring system. A negative control is shown in panel a. Category 2 stool (as defined in Materials and Methods) was expressed from the mouse in panel b; liquid yellow stool was expressed from a mouse inoculated with RRV (c), and similar stool was expressed from a mouse inoculated with PI-RRV (d).

RRV) in 50-μl aliquots by gavage. The dose of infectious RRV that results in diarrhea in greater than 50% of infant mice is in our hands between $10^6$ and $10^7$ PFU or focus-forming units (14), and in all experiments, doses administered readily exceeded that value. Diarrheal stools from mice inoculated with RRV or PI-RRV are shown in Fig. 2; the quantitative diarrhea scoring system used is described in Materials and Methods. Signs of diarrhea were completely and reproducibly absent from all negative controls ($n = 35$). Controls included sham inoculations with a mock viral purification from a sucrose gradient, Tris-saline-calcium buffer, or MA104 cell lysate that was exposed to fluorocarbon extraction and psoralen-UV treatment (see the legend to Fig. 1). Purified virus was dialyzed against Tris-saline-calcium buffer (209 mOsM) to remove sucrose, and the final osmolarity of viral solutions was shown to be between 209 and 216 mOsM and therefore incapable of inducing osmotic diarrhea in mice.

All of 11 mice inoculated with infectious rotavirus developed moderate to severe diarrhea. PI-RRV was inoculated into 13 mice; 11 of the 13 developed moderate to severe diarrhea, and 2 had minimal symptoms (1+; see Materials and Methods and the legend to Fig. 2). Several repetitions of this experiment were performed, each with a freshly prepared viral pool (78 mice in total). The data were not merged so that the documentation of the specific properties of the viral inoculum pool in this experiment could be shown. One experiment was performed with BALB/c mice, one was performed with virus that was harvested from culture medium but not gradient purified, and one was performed with CsCl-purified virus. In each case, genetically inactivated RRV induced diarrhea when administered in the described doses.

Intestinal fluid RRV-specific IgA was obtained from mice inoculated with RRV and those inoculated with PI-RRV. The mean reciprocals of the terminal dilutions exceeding by 2 stan-

dard deviations the magnitude of the mean control wells are as follows: RRV ($n = 5$), 1,706 ± 269; and PI-RRV ($n = 7$), 37 ± 10. Values for sham-inoculated controls were not significantly different from those for PI-RRV-infected mice, while the difference between either controls or PI-RRV-inoculated mice compared with infectious virus-inoculated mice is highly significant ($P < 0.05$).

## DISCUSSION

The potential mechanisms by which rotavirus may induce diarrhea have been often discussed but not rigorously tested. Adenylate and guanylate cyclase second-messenger systems that mediate the intestinal effects of cholera toxin and *Escherichia coli* enterotoxins are not altered during murine rotavirus infection (3). Rotavirus infection may lead to increased epithelial cell turnover and a reduction in mature epithelial cells and their digestive and absorptive systems, a condition that may promote malabsorption; however, malabsorption has not been demonstrated to be important in extensive murine studies of rotavirus disease (3). Acute inflammatory cells (especially polymorphonuclear leukocytes) and mucosal ulceration (observed in cows and piglets) are not seen in the mucosa of infected individuals, nor are they seen in heterologous murine infections (17, 23). Natural infection of humans has been associated with blunting of villi and disorder of microvilli, as well as epithelial vacuolation. While these findings have been observed during homologous EDIM infection of mice, heterologous exposures in mice have shown little disturbance of villous structure (11, 17). Immunoperoxidase studies of viral antigen in the intestine of heterologously infected mice have shown little viral antigen (5% of epithelial cells stained) (17). A similar tissue distribution of antigen has also been reported in homologous EDIM infection (4). Whether any particular histological alteration is the cause of diarrheal disease, a manifestation of viral replication, or both is not known. In the study described in this report, routine histological sections from the proximal one-third and distal one-third of small intestine mice 2 and 3 days after inoculation demonstrated normal villous structure and absence of an inflammatory infiltrate in all cases regardless of whether mice were inoculated with RRV or PI-RRV (data not shown).

Diarrhea resulting from replication-incompetent RRV may be analogous to the condition caused by enteroadherent strains of *E. coli*, in which cellular morphological changes and diarrhea are associated merely with the attachment of bacteria to the mucosa. Viral functions of cell attachment and entry are mediated by outer capsid proteins unaltered by genetic inactivation. Therefore, it is plausible that genetically inactivated rotaviruses may stimulate epithelial responses.

A further indication that PI-RRV did not replicate in mice is the clear distinction of the immune response between PI-RRV- and RRV-inoculated mice. Dramatically different intestinal antibody responses, as measured by an RRV-specific IgA ELISA, occurred 21 to 41 days after inoculation in mice inoculated with otherwise identical RRV or PI-RRV. The results in RRV-inoculated mice are consistent with prior studies (14, 20). The dosage of PI-RRV was increased fourfold to simulate a mass effect of RRV replication in the gut, but this did not alter the response. The dramatic differences in immune responses observed between mice inoculated with RRV and those inoculated with PI-RRV further supported the finding that PI-RRV was truly inactivated and did not cause disease by replication.

While these studies have demonstrated that replication is not necessary to produce diarrhea, they support prior evidence that replication does appear to be an important adjuvant of intestinal antibody production. Prior studies quantitating the intestinal antibody response to RRV in the murine model documented a remarkable antigenic potency of replication-competent RRV, which contrasts sharply with the negligible ELISA response noted with PI-RRV (1, 14, 20). Improved access to antigen-presenting cells, more efficient surface display of viral antigens, or enhanced cytokine secretion supportive of antibody production are among possible mechanisms by which replication may augment the intestinal immune response.

Furthermore, Feng et al. (5) also recently noted that immunogenicity and pathogenicity were not well correlated. They noted that relatively large amounts of RRV or the bovine rotavirus Newcastle disease virus caused diarrhea but not immune protection from challenge, while extremely small amounts of a avirulent (but probably still in vivo replication-competent) homologous murine rotavirus did not cause diarrhea but effectively induced protection. A related observation was recently reported by McNeal and Ward (13), who noted that replication was important to the induction of intestinal immunity in a homologous murine model in which the immunogenicities of reassortant viruses of different replication capacities were compared.

Although no evidence indicating that rotaviruses or any other gastroenteritis viruses may act as diarrheal toxins has been previously reported, these data are supportive of the hypothesis that a viral toxin-like effect is exerted during virus-cell contact. The particular importance of virus-cell attachment and entry in the disease process is yet to be defined, as are the specific viral protein mediators of the apparent PI-RRV toxin-like effect. This study indicates that a novel mechanism of viral toxigenic diarrhea is probable and suggests new possibilities for therapeutic intervention.

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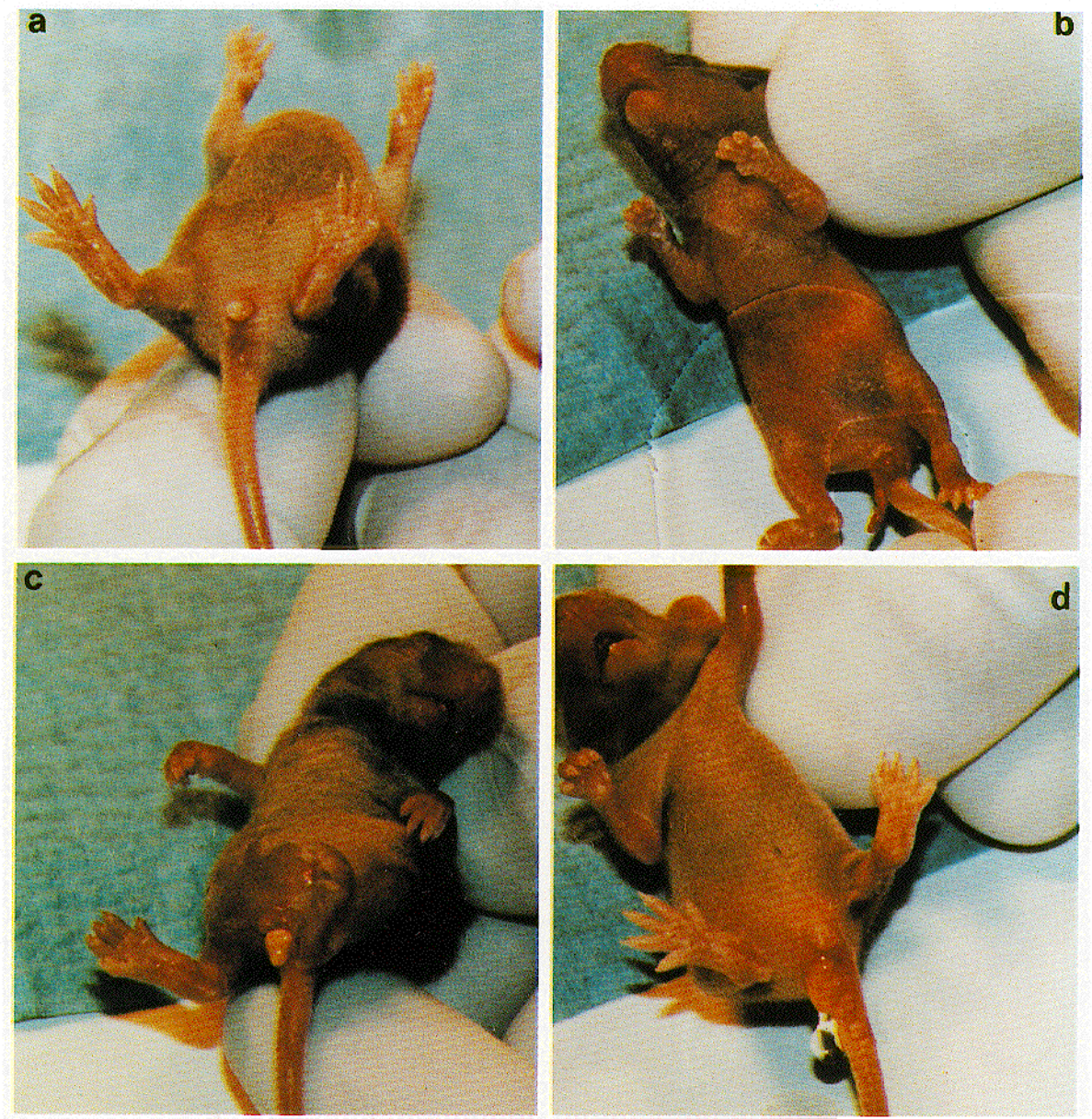
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