Development of Candidate Rotavirus Vaccines Derived from Neonatal Strains in India

Roger I. Glass,[1] Maharaj K. Bhan,[4] Pratima Ray,[4] Rajiv Bahl,[4] Umesh D. Parashar,[1] Harry Greenberg,[2] C. Durga Rao,[5] Nita Bhandari,[4] Yvonne Maldonado,[2] Richard L. Ward,[3] David I. Bernstein,[3] and Jon R. Gentsch[1]

[1]Respiratory and Enteric Viruses Branch, Centers for Disease Control and Prevention, Atlanta, Georgia; [2]Stanford University Medical School, Palo Alto, California; [3]Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio; [4]Department of Pediatrics, All India Institute of Medical Sciences, New Delhi, and [5]India Institute of Science, Bangalore, India

The need for a rotavirus vaccine in India is based on the enormous burden associated with the >100,000 deaths due to rotavirus diarrhea that occur annually among Indian children. Two rotavirus strains identified during nosocomial outbreaks of rotavirus infection in New Delhi and Bangalore, India, more than a decade ago are being developed as live oral vaccines. Infected newborns had no symptoms, shed virus for up to 2 weeks after infection, mounted a robust immune response, and demonstrated protection against severe rotavirus diarrhea after reinfection. The 2 strains are naturally occurring bovine-human reassortants. The New Delhi strain, 116E, is characterized as having a P[11],G9 genotype, and the Bangalore strain, I321, is characterized as having a P[11],G10 genotype. The strains have been prepared as pilot lots for clinical trials to be conducted in New Delhi. This unique project, which is developing a new rotavirus vaccine in India with the use of Indian strains, an Indian manufacturer, and an Indian clinical development program, aims to expedite introduction of rotavirus vaccines in India.

In India, diarrhea is a leading cause of illness and death among children <5 years old. Efforts to decrease the number of cases of diarrheal disease through programs of home-based treatment with oral rehydration therapy and improvements in water and sanitation, although partially successful, have so far fallen short of their goals. This may be due, in part, to the observations that (1) ~25%–35% of childhood hospitalizations for severe diarrhea result from infection with rotavirus, a pathogen that is not affected by improvements in clean water and hygiene, and (2) attempts to use rehydration therapy for patients with rotavirus are most likely to fail because vomiting, which is a main cause of treatment failure, is also a common presenting symptom of rotavirus gastroenteritis [1].

Because every child in India will become infected with rotavirus during the first few years of life, it is estimated that 25 million children born in India each year will experience ~20 million episodes of rotavirus diarrhea by 5 years of age, with ~400,000 hospitalizations and 100,000 deaths resulting from these episodes [2]. The 100,000 deaths due to rotavirus diarrhea represent ~5% of all deaths occurring among children <5 years of age and ~1 death among every 250 children born. The development and introduction of a rotavirus vaccine have become a national priority. The national immunization program in India currently reaches >70% of all children with their first vaccination. If a rotavirus vaccine could be introduced into the routine schedule of childhood immunization through the expanded program of immunization, it could have an immediate effect by reducing, within 2 or 3 years, the burden associated with diarrhea, the number of diarrhea-related hospitalizations, and, perhaps, the number of deaths due to diarrhea. Few other health interventions could have such an immediate and visible effect.

The process for making a rotavirus vaccine uses technology appropriate for manufacturers in the developing world. All rotavirus vaccines developed to date have been based on live rotavirus strains that have been iso-

The Journal of Infectious Diseases 2005;192:S30–5

lated from humans or animals and that have been adapted to cell culture, formulated for oral delivery, and tested in clinical trials. Both monovalent human strains and multivalent animal-based strains have demonstrated efficacy as candidate vaccines. Two vaccines, Rotarix (GlaxoSmithKline) and Rotateq (Merck), are the most advanced in their development; RotaShield (Wyeth-Ayerst) was licensed but then was withdrawn. Rotarix is derived from a single serotype G1 human rotavirus strain attenuated by multiple passages in cell culture. Like natural infection in children, Rotarix appears to induce cross-protection against G9 strains; this finding suggests that Rotarix will be suitable against a range of the common serotypes [3]. Vaccines derived from animal strains of simian (RotaShield) or bovine (Rotateq) origin appear to require a combination of reassortant strains to ensure adequate protection against the common serotypes [4], although candidate monovalent vaccines developed from a single animal strain have been effective in some settings [5].

In the absence of good correlates of protection, field trials remain the only method by which to evaluate the efficacy of a new vaccine, a process that can take several years to complete. During the past 2 decades, much experience has been gained in conducting clinical trials of various candidate rotavirus vaccines. The trials have often been successful, and, in a trial performed in Finland (the one country where nearly every candidate vaccine has been tested), all live oral vaccines tested to date, with the exception of one, demonstrated >80% efficacy against severe disease [6].

Most vaccines currently administered to Indian children and much of the childhood vaccines tendered by the United Nations Children's Fund are manufactured in India. New vaccines against hepatitis B virus and *Haemophilus influenzae* type b, produced by multinational vaccine manufacturers, have been introduced into the market in the private sector; however, the fact that uptake of these vaccines has been slow had raised clear questions of equity. Children with the heaviest burden of disease, the highest associated mortality rate, and the least access to care are the last to receive benefit from new vaccines. As Indian manufacturers have begun producing vaccines against hepatitis B virus, the supply has increased, the associated cost has plummeted, and the vaccine has finally become part of the programs of routine childhood immunization in several states. If a rotavirus vaccine were developed and manufactured in India, it might also be introduced directly into a program of routine childhood immunization. This could lead to an earlier, positive benefit with regard to a reduction in the number of cases of disease among children who are at greatest risk for hospitalization or death associated with diarrhea. In the present study, we review previously reported information on the isolation of 2 naturally attenuated rotavirus strains from Indian newborns, describe the rationale for using these strains to develop candidate vaccines, present new data on the early clinical testing of these candidate vaccines, and outline plans for further evaluation of these vaccines in India.

ORIGIN AND PROPERTIES OF THE INDIAN NEONATAL ROTAVIRUS STRAINS

In 1985, an "outbreak" of asymptomatic rotavirus infections was observed in the newborn unit of the All India Institute of Medical Sciences (AIIMS) in New Delhi, India; this outbreak was similar to that previously observed by Bishop et al. [7] in Australia. The infections were clearly nosocomial; the rate of infection was lowest for newborns discharged after 1 day of hospitalization, and it reached 50% for newborns hospitalized for 3 days and 75% for newborns hospitalized for a full week [8, 9]. None of these newborns developed symptoms, and the source of the infection was never determined. Among multiple isolates, all 11 gene segments of the neonatal strains appeared to be identical on the basis of the results of electrophoresis, confirming a point-source nosocomial infection with a single strain at AIIMS [9]. We never identified the source of infection, but the outbreak of infection among newborns persisted for several years.

When we attempted, by means of reverse-transcription polymerase chain reaction (PCR), to type the strain (now known as "strain 116E") that was the source of this outbreak, we were, at first, unsuccessful. A major effort to decipher the identity of these neonatal strains from New Delhi by means of sequence analysis led to clarification of the problem: the sequence of the VP7 gene was homologous to G9, a rare strain that, at the time, had been seen only twice previously in children in Philadelphia and Japan [10, 11]. The sequence of the VP4 gene was homologous to P[11], a genotype commonly found in cattle [12]. Strain 116E appeared to be a natural bovine-human reassortant typed as P[11],G9. We then performed genogrouping to identify the origin of the different gene segments in the new strain. Strain 116E was a human G9 strain into which a single bovine VP4 gene segment had been introduced.

The finding of a bovine-human reassortant added fuel to the argument that reassortment between animal and human strains was a plausible mechanism for the evolution of rotaviruses in nature. Furthermore, because mothers would not be expected to have antibodies to bovine strains, the presence of a bovine outer capsid gene could explain why the strain replicated so well in the infant gut. The infants mounted a readily detectable immune response to rotavirus in their serum and saliva, suggesting that their infections might protect against severe disease after reinfection [13]. Moreover, although rotavirus infection rarely occurs among infants during the first few months of life, these infants shed virus in their stools for up to 2 weeks after infection.

A study conducted by researchers in Bangalore, India, and at Stanford University (Palo Alto, CA) followed a similar pattern of discovery [14]. A nosocomial outbreak of infection at

a maternity center in Bangalore led to the identification among neonates of another "outbreak" due to a strain (known as "strain I321") that was also a bovine-human reassortant strain. Unlike strain 116E, strain I321 had a base of 9 bovine gene segments, and only gene segments 5 and 7, which encoded nonstructural proteins 1 and 3, were of human origin [15]. A strain with the same G and P characteristics as strain I321 has emerged as a cause of diarrhea in children in Vellore, India; however, it is unclear whether this virulent strain is identical to the original neonatal isolate [16].

These 2 Indian neonatal strains have interesting differences in their genomic structure (figure 1). Strain 116E is primarily a human strain with a single bovine VP4 gene, making it similar to the human vaccine strain being developed by GlaxoSmith-Kline. The difference lies in its bovine VP4 gene, which may improve its ability to colonize the infant gut in the presence of maternal antibody and to induce an immune response. Presumably, the bovine gene is responsible, at least in part, for attenuation. The I321 strain is composed primarily of bovine genes and has only 2 segments that are of human origin, making it more similar to the strain used in the Merck vaccine but without the serotype-specific gene products.

RATIONALE FOR DEVELOPING CANDIDATE ROTAVIRUS VACCINES BASED ON INDIAN NEONATAL STRAINS

The ability of these rotavirus strains to replicate in infected newborns without causing disease in the presence of high titers of maternal antibody suggested that the strains were unusual and, perhaps, naturally attenuated. Our research to explore their potential as vaccine candidates went in several directions.

We first followed a cohort of infants with or without documented neonatal infection with strain 116E, to determine whether these infants were indeed protected against rotavirus diarrhea after reinfection [17]. This study demonstrated that neonatal rotavirus infections occurred in 60% of newborns by day 4 of life and that they conferred protection against rotavirus diarrhea at a level similar to that observed by Bishop et al. [7] in Australia. The 148 children who had a neonatal infection experienced 0.23 episode of rotavirus diarrhea/child-year, compared with 0.42 episode/child-year for the 56 children without a neonatal infection. During the follow-up, approximately one-third of the control subjects (i.e., infants who had not been infected in the newborn unit) developed a 4-fold increase in antibodies to rotavirus, indicating either a neonatal infection that had been missed or an infection that occurred by 3 months of age. Nonetheless, the data supported a protective role for neonatal infection against subsequent and more-severe disease. We then tried to determine whether this neonatal strain was circulating in the community and was causing disease in older children [18]. A survey of strains from around India failed to identify this strain in children with diarrhea, but it found that other serotype G9 strains were the most common rotavirus strains detected in children with diarrhea in India. Since then, G9 strains have emerged as new strains in the United States and many other countries [19–22].

A recent 2-year follow-up study of a cohort of infants who had been infected with I321 rotaviruses during the first 2 weeks of life suggested that this group of infants was similarly protected against disease [23]. One (2%) of 44 neonates infected with strain I321 developed rotavirus diarrhea, compared with 11 (39%) of 28 neonates who were not infected with strain I321. A significant decrease in the number of rotavirus infection–associated admissions to the hospital was noted in parallel with a high (i.e., 25%–50%) rate of asymptomatic neonatal infections. Collectively, these findings suggested that asymptomatic neonatal infections with these rotavirus strains are common in Indian infants and that they confer protection against symptomatic rotavirus disease dur-
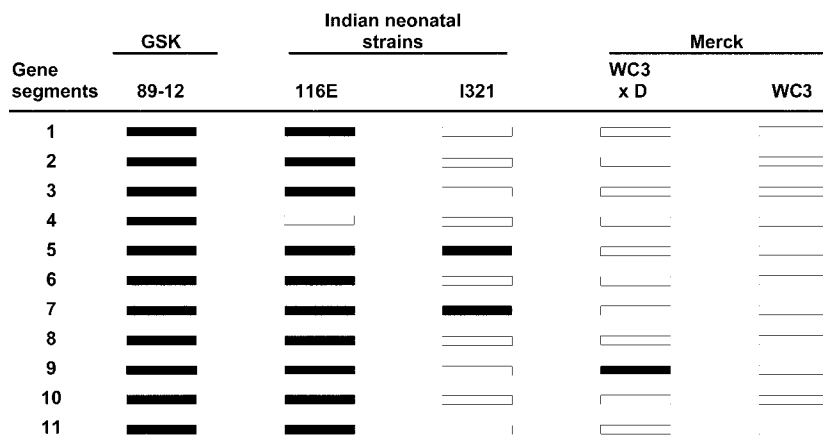

Figure 1. Gene origin of rotavirus vaccine strains. *Shaded bars,* genes of human origin; *unshaded bars,* genes of bovine origin. GSK, GlaxoSmithKline; WC3, bovine rotavirus strain; WC3 × D, bovine-human reassortant strain.

ing the first 2 years of life, prompting us to develop candidate vaccines based on these strains. All stages of development of these vaccine candidates have been approved by the ethical review committees at each of the collaborating institutions.

PROGRESS WITH VACCINE DEVELOPMENT AND EARLY CLINICAL TESTING

The 2 Indian neonatal strains each appeared to be attractive as future candidate rotavirus vaccines, and we had no basis, a priori, to decide that one strain would prove to be better than the other (table 1). The strains colonized the infant gut extremely well, perhaps because maternal antibody does not have neutralizing activity against this bovine outer capsid VP4. They caused no disease after infection, were shed for >1 week (which induced a robust immune response despite the presence of maternal antibody), and were each associated with protection against disease after reinfection.

Both strains were prepared as pilot vaccine lots for challenge studies involving humans. For the I321 strain, the virus was adapted to growth in cell culture by 2 passages in primary African green monkey kidney (AGMK) cells, followed by 3 passages in MA104 cells. The virus was then plaque purified 3 times in MA104 cells, and a final plaque was used to infect another flask of MA104 cells. After 1 more passage in these cells to make a large preparation, the virus was frozen at −70°C in aliquots and was stored. This virus went through 10 passages, including the plaque purification steps. In 1996, these strains were prepared as pilot vaccine lots at Flow Laboratories (L. Potash, personal communication). The pilot lots were prepared by using serially passaged AGMK cells. The final preparations at passage 19 were thoroughly tested for use in humans and were approved for clinical studies.

Two phase 1 clinical trials were initiated in the Vaccine Trial and Evaluation Unit at the Cincinnati Children's Hospital (Cincinnati, OH), beginning with safety studies involving adults and seropositive children (age, 2–12 years). Both studies were randomized placebo-controlled trials. In each, stool specimens were obtained to determine levels of virus excretion, and serum samples were analyzed to determine changes in rotavirus antibody titers. In the initial study, 30 adults with antibody to rotavirus were equally divided into 3 groups of the same size to receive a single oral dose of 10^5 ffu of strain I321, strain 116E, or a placebo after stomach acids were buffered. No vaccine recipient shed detectable levels of rotavirus, and, in these seropositive adults, the vaccines were poorly immunogenic. Only 2 subjects had a 4-fold increase in antibody titer, and both received the 116E strain. Side effects were rare and mild, although 2 subjects who received strain 116E developed mild diarrhea for 1 day.

In the second study, 30 healthy children aged 2–12 years who had rotavirus antibody received either the 116E or I321 vaccine (10^5 ffu) or placebo after stomach acids were buffered. One vaccine recipient shed detectable levels of rotavirus, and 4 had seroconversion; all had received strain 116E. Side effects were rare and mild; no subject developed diarrhea or vomiting, but, 22 days after immunization, 1 recipient of strain 116E who did not shed rotavirus or who did not experience seroconversion developed ileus that was thought to be unrelated to the vaccine.

Both vaccines appeared to be attenuated, compared with wild-type strains. They replicated poorly in adults and children with preexisting immunity to rotavirus, and they appeared to be safe. The 116E strain replicated somewhat more efficiently and therefore was more immunogenic than the I321 strain.

PLANNED STUDIES IN INDIA

Further clinical studies are under development in India and will progress through safety trials involving adults, children, and infants. The safety trials involving infants will be the first to determine whether these neonatal strains that were so successful in infecting newborns at AIIMS and in Bangalore will continue

Table 1. Comparison of Indian neonatal rotavirus strains.

Characteristic	New Delhi strain 116E	Bangalore strain I321
Origin	All India Institute of Medical Sciences, New Delhi, India, 1985	Indian Institute of Science, Bangalore, India, 1988–1991
Type	P8[11],G9	P8[11],G10
Reassortment	Human rotavirus with a single bovine gene (VP4)	Bovine rotavirus with 2 human genes encoding NSP1 and NSP3
Passage history	Two passages in primary AGMK cells	Two passages in primary AGMK cells
	⇓	⇓
	Seven passages in Ma104 cells	Eight passages in Ma104 cells
	⇓	⇓
	Ten passages in SpAGMK cells	Eight passages in SpAGMK cells
Subjects in US clinical studies, no.		
Adults	10	10
Children	10	10

NOTE. AGMK, African green monkey kidney; NSP1, nonstructural protein 1; NSP3, nonstructural protein 3; SpAGMK, specially passaged AGMK.

to be avirulent and highly infectious and whether they will induce both shedding and a good serum immune response after administration of a single dose. Proof of the efficacy of these strains will require several years of further testing.

One additional improvement in this candidate vaccine has been the adaptation to growth in Vero cells, a cell line more commonly used and widely available as a vaccine substrate. Once pilot lots of these candidate vaccines have been prepared at Bharat Biotech in Hyderabad, India, and have been approved for use in humans by the Drug Controller General of India, we anticipate a series of phase 1 and bridging studies that will ultimately lead to a clinical trial of the use of at least 1 of these preparations for Indian children.

DISCUSSION

This project is an alternative approach for the development of vaccines to ensure the survival of children in developing countries. A great impetus to push this effort forward was the untimely withdrawal of RotaShield from the market as the result of a detectable risk of intussusception developing in the 2 weeks after vaccination. At the time of its withdrawal, RotaShield was undergoing a clinical trial in New Delhi. It was hoped that a successful trial outcome would lead to rapid introduction of the vaccine. Indian authorities had been primed to consider the need for the vaccine, to permit the importation of Rota-Shield, and to approve its testing under the auspices of the World Health Organization. Although concerns about the safety of RotaShield in the United States led to its withdrawal, in India, the high mortality associated with rotavirus disease, as well as the rarity of the development of intussusception and the low risk attributed to the vaccine, might still have made this vaccine a lifesaver. However, it was considered politically difficult and imprudent to consider the further development and testing in India of a vaccine withdrawn from the US market.

These collaborative studies, conducted over more than a decade by 2 independent groups of Indian and American researchers, have identified several rotavirus strains isolated from newborns without diarrhea that may be suitable as future candidate rotavirus vaccines. The strains are each bovine-human reassortants that grow well in the newborn, do not cause diarrhea, are shed for a prolonged period, induce a good immune response, and appear to protect infants against severe disease after reinfection. Whether these strains will perform the same after adaptation to growth in Vero cells remains to be determined. Clinical trials will be required to determine whether these vaccines are safe and effective. Many hurdles lie ahead in the development process.

These collaborations have changed our understanding of the epidemiological profile of rotavirus infections in the developing world. First, attempts to characterize rotavirus strains from many sites in India let us appreciate not only that the newborn strains are really unique to newborns and are not found in children with diarrhea but, also, that India has a greater diversity of strains in circulation than do most countries. For example, our first small survey of 25 rotavirus specimens from Lucknow, India, identified 8 different strains of rotavirus, more than had ever been seen, at the time, in the rest of the world. Mixed infections with >1 strain were common, as were infections with bovine-human reassortants, a finding that encouraged us to think of genetic exchange between human and bovine strains of the virus. Neonatal infections that are uncommon in industrialized countries appear to be common in India and could provide another focus for routine immunization of infants at birth. Finally, our studies determined that 70%–80% of all rotavirus disease–associated hospitalizations in India occur among children <1 year of age, which is earlier than such hospitalizations occur among children in the United States. This means that vaccines must be administered to children before the age of 3 months if they are to be effective.

India has never developed, on its own, a new vaccine from primary material obtained in India, with first manufacture in India, and with the full clinical development program in an Indian population. The rotavirus vaccine development program is now funded by multiple donors within and outside India. The goal remains to make a vaccine in India for India. The consequences of this program could be the more rapid national introduction of a vaccine for a disease that is already considered to be a national priority, as well as a relationship with a local producer committed to providing vaccine for the national immunization program at an affordable price.

There remain many hurdles in bringing this program to successful completion, including the uncertainties associated with determining whether the vaccine will have the efficacy anticipated by the natural-history experiments. Many years of study and research remain before the results of this experiment are known. The goal of preventing 100,000 deaths/year in India is certainly worth this sustained effort.

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