Antigenic studies of animal rotaviruses: Relationships by virus neutralization *in vitro* and cross-protection in gnotobiotic piglets

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ABSTRACT

The serotype and cross-protection properties of rotaviruses isolated from canine, simian, bovine, porcine and human species were compared. The bovine strain B:USA:78:1A and canine strain C:USA:81:2 were adapted to cell culture and cloned in this study. The simian strain S:USA:79:2, porcine OSU (Ohio State University) strain P:USA:77:1 and the human WA strain had been adapted to cell culture previously by others but were further cloned for this work. To classify the viruses into different serotype groups, the serum neutralization test was used. Viruses exhibiting a greater than 20-fold difference in neutralization titer were placed into different serotype groups. Four major serotypes were found and were represented by the bovine, human, porcine, and canine-simian strains. These serotype differences were found to be significant in the cross-protection study. With the exception of the porcine virus, none of the strains protected against a challenge with virulent porcine rotavirus. Also, the canine virus did protect piglets against a challenge with simian virus. From these findings, it was concluded that only viruses belonging to the same serotype group can confer cross-protection and therefore vaccines should be made using the serotypes to which an animal is likely to be exposed.

INTRODUCTION

Studies using electron microscopy and specific pathogen-free animals in recent years have demonstrated the presence of several enteric viruses in association with diarrheal disease, including: rotaviruses, coronaviruses, parvoviruses, astroviruses, the Norwalk and related agents, calicivirus-like agents, adenoviruses (enteric types), and the newly reported "Breda" virus. Rotavirus is the virus most frequently associated with neonatal diarrhea of humans and some species of animals, including calves, horses, and lambs and is common in the pig. Despite their importance as a disease causing agent, the discovery and characterization of rotaviruses is comparatively recent, largely as a result of difficulties associated with tissue culture isolation using conventional methods.

Several methods have been developed for diagnosing rotavirus infections in animals, including electron microscopy, cell culture isolation and various antigenic tests. Electrophoresis of rotaviral RNA has proved to be a sensitive "finger-print" method for identifying a rotavirus from the presence of an eleven segmented genome and also for subtyping rotaviruses. Despite the presence of certain antigens common to all isolates of rotavirus, the widespread distribution of these viruses in human and all animal species studied including poultry, suggested the probability that antigenic subtypes

exist. As a result of much investigative work by several laboratories, recognition of strain differences has been achieved. Antigenic characterization proceeded at a rapid pace following the development of techniques to adapt many strains to replicate in cell culture. It has been shown that rotaviruses possess a common group antigen demonstratable by immunofluorescence, complement fixation or enzyme-linked immunosorbant assay (ELISA), and that serotype-specific antigens are demonstratable by serum neutralization, complement fixation and ELISA. Different studies on cross-protection among the rotaviruses have produced conflicting results. Some reports find cross-protection occurs between rotaviruses of different animal species whereas other reports fail to demonstrate cross-protection. However, antigenic differences by neutralization *in vitro* would suggest that serotypically different rotaviruses would not show optimal cross-protection *in vivo*. If such optimal cross-protection does not occur between members of different serotype groups, any potentially successful vaccines should be made against all the serotypes of rotavirus to which an animal is likely to be exposed.

This study is concerned with the antigenic characterization of several different animal rotaviruses (simian, canine, bovine and porcine). The purpose was to determine to what degree cross-neutralization *in vitro* correlates with cross-protection *in vivo*.

Isolates from different species were selected because these have been shown to possess major antigenic differences. From this work, evidence was found to support the view that rotaviruses possessing a 20-fold or greater difference between homologous and heterologous serum neutralization titers do not cross-protect in vivo. However, where there are minor differences (8-12 fold) cross-protection may occur.

LITERATURE REVIEW

Historical

Current work in rotavirus research did not begin until the late 1960s with the work of Dr. C. Mebus and his colleagues on a diarrheal disease of calves. A rotavirus was associated with this disease and was named the neonatal calf diarrhea virus (NCDV).[84] Interest in the rotaviruses greatly increased as workers in the medical field began describing similar viruses associated with human diarrheal disease and it thus seemed likely that many previously unexplained outbreaks of diarrhea may have had a viral origin. Although NCDV was not the first rotavirus discovered, it became recognized (although not officially) as the type species for the genus. NCDV has been well-characterized and it is with this virus that most new isolates were compared. The official type species for the genus is now listed as human rotavirus. As it is now known that different human rotaviruses exist, the type species will have to be further defined. In retrospect, earlier studies such as those of Light and Hodes[67] which involved the oral inoculation of calves with human diarrheic material with the subsequent development of diarrhea, and those of Cheever and Mueller[23] on epidemic diarrheal disease of suckling mice (EDIM), have been shown to have involved rotaviruses.[105] Therefore, several historic studies, besides those involving NCDV, deserve

mention. These studies were concerned with the EDIM virus, the SA.11 virus (a simian strain) and the "O" or offal agent (of unknown origin).

Research on infantile diarrhea in mice was first reported by Cheever and Mueller in 1947.[23] In their paper, they described epizootic diarrhea of infant mice or EDIM. Lizbeth Kraft later, through transmission experiments, demonstrated a viral cause for the disease.[61] A number of early studies were done on the EDIM virus including descriptions of the disease[22,23,90] and cellular pathology.[91,92] The disease was not necessarily fatal. It affected primarily young mice between 11 and 15 days of age[4] and was associated with a severe, yellowish, watery diarrhea. The incubation period was reported as varying from 40 hours to 10 days and the virus was highly infectious. The EDIM virus was found to be both heat- and ether-resistant and had an approximate diameter of 65-75 nm.[1,61,62] Replication of the virus was entirely cytoplasmic.[2] A study done by Banfield et al.[6] pointed out the EDIM viruses' similarity to reovirus and further characterization of this virus found that it contained RNA.[88] Early attempts to propagate the virus in cell culture were unsuccessful.[101]

Two of the first rotaviruses to be successfully propagated in cell culture, although they were not recognized as rotaviruses when isolated, were the simian agent SA.11 virus and "O" agent. The SA.11 virus was isolated from a rectal swab of a clinically normal

vervet monkey in 1958.[69] The "O" agent was isolated from intestinal washings from a slaughterhouse in South Africa.[69] Both of these viruses were cytopathic in primary vervet monkey kidney cells and were later shown to be morphologically indistinguishable by Els and Lecatsas in 1972.[32] They showed that these viruses possessed an inner capsid layer which resembled bluetongue virus and an outer defined capsid layer. Neither the "O" agent nor the SA.11 virus were associated with diseased animals. The history of the SA.11 virus is not fully known and the species of origin of the "O" agent also is uncertain. Later work has established that the SA.11 and "O" viruses are indeed rotaviruses.[63,105]

As was stated previously, the work of Mebus and others in 1969 on a calf diarrhea virus (NCDV) marks the beginning of current work on the rotaviruses. For the first time, gnotobiotic animals were used in the study of neonatal diarrhea. Mebus successfully transmitted infection into gnotobiotic calves by oral inoculation of bacteria-free filtrates of diarrheic feces. The use of gnotobiotic animals in diarrhea research was an important breakthrough as the ubiquitous nature of the rotaviruses and other enteric pathogens is now known. Thus, the study of these viruses in animals would be very difficult without the use of gnotobiotic animals.

The Mebus virus was first described as 'reovirus-like'. It measured 65 nm in diameter and after many attempts was adapted to grow in cell cultures of bovine embryo kidney cells.[37,83,130] It was also found that infected cells in feces could be immuno-fluoresced to identify virus positive cases in field outbreaks. This work on NCDV in the United States was confirmed by studies done in England in which a reovirus-like agent was isolated from diarrheic calves and two strains were adapted to cell culture.[16,133]

The work on calf diarrhea virus done by Mebus received little attention until about 1973 when Bishop and co-workers found viral particles, which they suggested may be orbiviruses, in intestinal epithelial cells of biopsy material taken from children suffering from acute non-bacterial gastroenteritis.[9] Also in 1973, Flewett et al. found large numbers of virus particles in the diarrheic feces of young children by electron microscopy.[38] These viruses had a double layered capsid with a defined outer rim. Both reports of Bishop and Flewett were confirmed by others. Middelton et al.[86] found that most adults had antibody to a virus similar to those described above. This he did by an indirect fluorescent antibody test using a virus positive biopsy material. He was also able to infect a sero-negative adult with a virus positive fecal filtrate.

Soon after the reports of these viruses, in association with human diarrhea, studies were published showing similarities between

the bovine (NCDV) and human viruses.[40,58] Flewett et al. reported cross-reactions between the bovine and human viruses by immuno-electron microscopy and immunofluorescence and suggested that a common antigen exists.[40] They also noted differences from reoviruses and suggested that these viruses be grouped together under the name of rotavirus.

Since this initial work, numerous studies have been published naming rotaviruses as among the major known agents associated with infantile gastroenteritis in many areas of the world.[29,57,59] Rotavirus research has increased at a rapid pace so that today much is known about their structure, chemical properties, antigenic composition and in vitro culture requirements. However, much work remains to be done in the area of antigenic characterization, especially with regard to in vivo work on cross-protection and virulence.

Classification, Structure and Chemical Properties

Several names including reovirus-like,[57,60] infantile gastro-enteritis virus,[94] duovirus,[29] and rotavirus[40,41] have been proposed for this group of enteric viruses. Of the suggested names, rotavirus has been accepted by the International Committee on the Nomenclature of Viruses[73] and Rotaviruses are now classified as a separate genus within the family Reoviridae.[29,73] With the inclusion of the rotaviruses as a new genus, there are now three genera of animal viruses within

the reoviridae; the reoviruses (reovirus types 1, 2 and 3), the orbiviruses (bluetongue), and the rotaviruses (NCDV). Viruses classified within the reoviridae have a segmented, double-stranded RNA genome, are nonenveloped and have cubic symmetry. The rotaviruses have been shown to be antigenically distinct from the other two genera[16,55,130,132] and yet share a common antigen among themselves thus making them an interrelated group.

Morphologically, the rotaviruses resemble the other members of the reoviridae but the bilayer appearance of complete particles makes them very distinct. The complete rotavirus particle has been described as double-shelled[45] or smooth.[135] The center of the virion measures approximately 38 nm and its geometry is hexagonal having a 5-3-2 symmetry which is characteristic of an icosahedron.[89] Surrounding this electron dense inner core is a translucent layer which gives the virus a wheel-like appearance, having a large central hub, short spokes and an outer rim. It is because of its appearance that the name, rota (which is the Latin word for wheel), was suggested.[40] When the outer layer is removed, the particles resemble orbiviruses. The incomplete, single-shelled, or rough particles measure 55-65 nm in diameter and are about 10 nm smaller than the complete particles which measure 65-75 nm.[32,45,70,80,89,135] Both complete and incomplete particles can usually be seen in electron micrographs of infected fecal or cell culture material. These

two types of particles can be separated by cesium chloride centrifugation.[17] The buoyant density of single-shelled particles is 1.38 g/cm^3 and 1.36 g/cm^3 for double-shelled particles.[56] The outer coat can be removed by chemical treatment with trypsin but not by treatment with chymotrypsin.[70,89] Bridger[12] found that type-specific antigens are associated with the outer shell whereas group antigens are associated with the inner core. The presence of a hemagglutinin has been reported for some rotaviruses but has not been found to be a characteristic of the genus as a whole.[36,49,112]

The rotaviruses have been found to be very stable and resistant viruses. However, extensive purification may lead to instability. A human rotavirus stored for nine years at -20° C and another rotavirus lyophilized for 30 years could be recognized as rotaviruses by their morphology.[3,44] Palmer et al. have published a report on the stability of these viruses.[89] Their study found that the morphological appearance of human rotavirus remained unchanged after the following treatments: heat (56° C for 1 hour), centrifugal force (100,000 x g), high salt concentration, pH 3 and 10, treatment with enzymes (chymotrypsin, papain, and pepsin), and nonionic detergents. They also found that rotaviruses were extremely labile to trypsin-versene and after exposure to a 0.125% concentration of trypsin-versene for 2 hours at 37° C they were unrecognizable. Ultraviolet light has been found to inactivate rotavirus.[133]

Rotaviruses have a segmented genome consisting of eleven segments of double-stranded RNA which can be separated by gel electrophoresis.[51,52,99,120,121] The segments have been reported to range in molecular weight from 0.2×10^6 to 2.2×10^6.[53] Different migration patterns of the RNA segments of rotavirus from different species and within the same species have been observed and these characteristic patterns have been shown to be a fingerprint useful for identifying different isolates.[51,53,97,98,106,120,121,122,129]

An RNA dependent RNA polymerase has been isolated from human, calf and simian rotaviruses.[25,48,71,113] It was found in rough or incomplete particles but smooth particles had to be treated with a chelating agent to uncover the enzyme.[25,26]

Rotaviruses have been reported to contain eleven polypeptides.[71,75] Recently, much interest has been placed on determining the functions of the various polypeptides and determining which RNA segment codes for a particular polypeptide.[71,75,76,108] Conflicting data have been reported in this area. Much of the gene coding work has been done with the SA.11 rotavirus.[31,108] The coding assignments of RNA segments 1-6 have been determined for the SA.11 virus and these assignments agree with coding assignments of RNA segments 1-6 of the UK strain of bovine rotavirus.[76] Conflicting data have been published on the remaining segments. RNA segments 7, 8 and 9 have been particularly difficult to assign to proteins because of their close

and sometimes indistinguishable electrophoretic migration patterns.[108] It also appears that in the human virus segments 10 and 11 can be reversed in the migration patterns of RNA from different strains. Thus, segment 10 would code for the protein coded for by segment 11 in another strain.[31]

Clinical Symptoms and Pathology

Rotaviruses can cause an acute infection of the small intestine and the disease is most evident in infant or young animals and humans.[84,132] Asymptomatic infections do occur.[69] Clinically, a short incubation period is followed by anorexia, occasional vomiting and diarrhea. In piglets, the incubation period but not the severity of infection is dependent upon the dose of virus given.[134] Mortality is highly variable and in economic terms, one of the most important aspects of the disease is the body weight loss followed often by an extended period of failure to gain weight. The conditions which determine the severity of the disease depend upon the virulence of the virus, the susceptibility of the animal, environmental conditions, and supportive therapy. Dehydration therapy has proven to be a successful treatment of the disease.[20] In humans, rotaviruses have been associated with up to 50% of the hospitalized cases of infants and small children in temperate climates[57] and the disease is more prevalent during the colder months.

The pathology of the disease in gnotobiotic piglets has been fully described by Crouch and Woode[28] and by McAdaragh et al.[74] Other descriptions of pathology have also been reported.[21,65,117,134] Briefly, McAdaragh[74] reported the pathology of the disease in piglets as follows. The cytoplasm of villous absorptive cells in the duodenum and upper jejunum immunofluoresced at 12 hours and rotavirus-specific fluorescence was most intense at 24-46 hours in the upper jejunum and middle small intestine. Virus replication resulted in the loss of the villous absorptive cells in the lower jejunum and upper ileum at 24 hours. Crypt hyperplasia was evident and by scanning electron microscopy, villous fusion, villous atrophy and exposure of the lamina propria occurred.

Another study, that of Crouch and Woode,[28] provided an even more complete picture of the disease. They reported the results of a serial study of rotavirus infection in gnotobiotic piglets. Seven-day-old piglets were infected with a pig rotavirus and the infection was followed for 21 days. The piglets developed the symptoms of anorexia, depression and diarrhea. Weight losses of about 15% were reported over 24-36 hours. By immunofluorescent staining, infected villous epithelial cells were detected in the middle and distal portions of the small intestine. The number of fluorescent cells greatly decreased by 48 hours. Virus titrations were done on contents taken from the gut at various positions and times post-

infection. Titers of the virus increased until 22 hours and then remained at a constant reduced level for 48 hours before gradually decreasing until virus disappeared at about 5 days. Intestinal lesions were found mainly in the middle and distal portions of the small intestine. Damage first appeared by 36 hours and was at a maximum by 46-65 hours. Lesions noted were; reduced number of villi, stunted villi, and some villous fusion. By five days post infection, the villi appeared normal although the villous length: crypt length ratios were smaller than in control piglets. In summary, Crouch and Woode concluded that recovery from infection appeared to consist of two phases. The first phase occurred 22 hours after infection. It was suggested that this first phase was apparently non-immune in nature and was due to the loss of viral receptor sites by the destruction of susceptible epithelial cells and possibly to the production of interferon or other non-specific inhibitors. The second phase occurred at about 90 hours. This phase eliminated the virus and was thought to be due to an antibody response.

In vitro Culture

Rotaviruses have been difficult to multiply to high titer in cell culture. The first viruses successfully adapted were the SA.11 virus and "O" agent,[69] but as these viruses were not associated with disease the study did not receive much attention. The next rotaviruses

successfully isolated were three bovine isolates including NCDV[16,66,77,78] but most early attempts at culture failed. However, even though most infections were abortive after 2-3 passages, the cultures could be used for diagnostic purposes by immunofluorescence.[16]

A major advance occurred when it was discovered that with the addition of proteolytic enzymes (trypsin or pancreatin) to the culture media, porcine and bovine isolates adapted to culture much more readily[5,116] and high titer virus could be produced.[24,35] Kidney cell lines are currently used for culturing purposes. Two monkey kidney cell lines are the most commonly used. These are lines from African green monkey kidneys (BSC-1) and from fetal rhesus kidneys (MA-104). It is possible that structural similarities to the gut epithelium make these cells, probably derived from kidney tubule cells, particularly susceptible (i.e., the presence of microvilli, similar membrane bound enzymes, and presumably viral receptors). Human rotaviruses have been much more difficult to grow in culture. Wyatt et al.[139] successfully adapted a human virus (strain WA) to grow in African green monkey kidney cells but this occurred only after first passing the virus eleven times through gnotobiotic pigs. Recently, two research groups have reported the successful <u>in vitro</u> culture of human rotavirus.[104,128] Briefly, the procedure used by Urasawa et al.[128] is as follows. Human fecal virus was pre-incubated with trypsin, adsorbed onto roller or stationary cultures of MA-104, passaged by

freeze-thawing and each passage pelleted by ultracentrifugation (100,000 x g) before inoculated onto fresh cultures. The adaptation of the rotaviruses to cell culture has enabled the production of high titer virus for vaccine development and further studies.

Antigenicity

Rotaviruses have been isolated from many species and are assumed to be ubiquitous. Species from which rotaviruses have been isolated include: calves,[84,133] cats,[47,109] deer,[124] dogs,[33,42] foals,[27,39,54,127] humans,[10,38,58,115] lambs,[78,111] mice,[61] monkeys,[69,114] pigs,[65,82,96,134] pronghorn antelope,[95] rabbits,[19,93] turkeys and chickens.[8,50,79,80] Rotaviruses share a common antigen which can be demonstrated by immunodiffusion, immunofluorescence, complement fixation, immune-electron microscopy, gel diffusion, and enzyme-linked immunosorbant assay.[41] The rotaviruses do not share common antigens with the orbiviruses or reoviruses.[16,55] The antigenic relationships between bovine and human viruses was first demonstrated by immune-electron microscopy and immunofluorescence.[40] Common antigens have been reported to exist in rotaviruses from humans, calves, pigs, lambs, rabbits, foals, mice, the SA.11 virus and "O" agent[55,119,135] as well as from dogs.[42] In addition to common group antigens, there are species-specific antigens which are demonstrated by the serum neutralization test, complement fixation and ELISA.[7,105,119,135,141,144] Flewett et al.[40] in 1974 found that even though human sera

contained antibody to the common group antigen, not all of this sera would neutralize bovine rotavirus. This work has been confirmed by other studies.[34,105,119,134,135] Thouless[119] and co-workers demonstrated differences by serum neutralization among rotavirus isolates from human, calf, piglet, foal, lamb, mouse, and rabbit species. Other studies point to differences by agglutination using immune-electron microscopy.[16,105] Estes and Graham[34] demonstrated differences between simian, porcine and bovine rotaviruses by plaque reduction. Thus, it is now well-established that there are group and species-specific antigens in the rotaviruses. The species-specific antigens allow the categorization of rotaviruses according to serotype.

Recently, there have been several reports of a virus morphologically similar to rotavirus in pigs[11,13,15,103] and one in chickens,[81] which lack the common group antigen. These viruses have been referred to as pararotavirus.[11] The significance and classification of these viruses is only beginning to be studied.

Cross-protection

Once it had been established that there are different antigens present on rotaviruses (i.e., group and species-specific antigens) the question arose as to whether infection with one rotavirus would protect an animal against infection with another rotavirus regardless of the viruses' serotype. Before such studies could begin, it had to

be determined whether rotaviruses would infect different species other than those from which they were isolated. Several studies have determined that cross-species infections can occur. The earliest studies that indicated that cross-infections may occur were those of Light and Hodes in 1943 and 1949.[67,68] They infected calves with a filtrate of human diarrheic feces. These feces were later shown to contain rotavirus although whether the virus inoculation was actually a human strain is not certain. Flewett and others in 1974 reported an unsuccessful attempt to infect calves with a human virus.[40] However, later studies did report the successful infection of calves with human rotavirus.[85] The human virus has since been reported to experimentally infect piglets without clinical signs[18,136] and with clinical signs,[55,123,126,142] to infect monkeys and cause diarrhea,[140] and to infect dogs asymptomatically.[125] Pigs have been successfully infected with calf rotavirus,[132,136] lamb rotavirus,[136] and foal rotavirus.[127,136] Also, the presence of neutralizing antibody to pig rotavirus in cow colostrum could be indirect evidence for the infection of cattle with porcine rotavirus.[14] Thus, it has been experimentally determined that cross-species infections can occur.

It was hoped that heterologous protection in the rotaviruses would be successful. This would allow the use of less virulent viruses from other species to be used as vaccines thereby saving on the cost of attenuation of a virulent homologous strain.

Studies on cross-protection have produced conflicting results. It appears that poor or only partial cross-protection will occur across serotypes.[131] Studies with the human virus indicate that sequential infections can occur between types I and II.[100] Bovine rotavirus as a vaccine did not protect piglets against challenge with porcine rotavirus.[64] In a study done by Woode and others, only approximately a 30% cross-protection rate was achieved when using foal or human virus as vaccine and bovine rotavirus as challenge.[131] Tzipori et al.[126] reported that piglets vaccinated with human rotavirus were protected against clinical disease but not against virus shedding when challenged with porcine rotavirus. Wyatt et al.[138] described a similar situation when calves vaccinated in utero with bovine rotavirus were protected against clinical disease but did shed virus when challenged with human rotavirus. Although heterologous cross-protection studies continue, this work and the work of others indicate that this is a less than perfect approach to vaccination.

METHODS

Animals

The National Animal Disease Center, Ames, Iowa supplied the guinea pigs used for the production of hyperimmune antisera.

Gnotobiotic animals are necessary in rotavirus research due to the ubiquitous nature of the viruses. Most of the materials used for the isolators were purchased from Standard Safety Equipment Company, Palatine, IL and from Allied Fabricating, Columbus, OH. The isolators were assembled and maintained by I. Zook. In preparation to receive the animals, isolators were washed with a 10% Wescodyne Solution (American Sterilizer Company, Erie, PA) and sterilized by fogging with a 2% peracetic acid solution (peracetic acid, water, and a few drops of liquid detergent). Portions of the cages were sterilized by autoclaving. Those materials which were heat-labile were gas-sterilized with ethylene oxide. Once in operation, any materials entering the isolators were handled with gloves and sprayed with the peracetic acid solution. The entry port was also sprayed and the material remained in the port for a minimum of thirty minutes before passing through to the inside.

Gnotobiotic rats were purchased from Charles Rivers Laboratories, Willmington, MA. When received, they were aseptically transferred into a prepared sterile isolator. Sterile feed and litter were

purchased from Charles Rivers and water for the rats was autoclaved. Gnotobiotic pigs were derived according to the method of Miniats and Jol[87] and gnotobiotic calves were derived following the procedure of Matthews et al.[72] The sows or cows were placed under general anesthesia and the young removed by Caesarean section. Dr. L. Evans was responsible for the surgical procedures. The calves and piglets were immediately passed through a germicidal trap filled with a 10% Wescodyne solution into sterile gnotobiotic isolators where they were resuscitated. Gnotobiotic piglets were fed canned evaporated milk (Carnation, Los Angeles, CA) or SPF-Lac (Borden Labs, Elgin, IL). The calves were fed evaporated milk diluted 1:1 with a mineral water supplement. The calves were fed the evaporated milk prepared as for the pigs.

Cell Culture

Three cell lines were used in this study; MDBK, BSC-1, and MA-104. MA-104 cells were originally developed by M. A. Bioproducts, Walkersville, MD and were kindly supplied by Dr. M. Cholmley. This is an established cell line of fetal rhesus monkey kidney cells. BSC-1 cells[46] are an established line of African green monkey kidney cells and were supplied by Dr. L. A. Babiuk. The MDBK cells (Madin Darby Bovine Kidney) are an established line of bovine kidney cells and were kindly provided by Dr. K. Theil.

Both MA-104 and BSC-1 cell lines were used for virus culture. Cultures of MA-104 or BSC-1 cells were prepared in 96-well microtiter plates (Costar, Cambridge, MA), in 75 or 25 cm^2 flasks (Linbro, Hamden, CT), and for large scale antigen production, in 850 cm^2 roller bottles (Corning Glass Works, Corning, NY). The growth medium consisted of Eagles Minimum Essential Medium (MEM) supplemented with 0.25% lactalbumin hydrolysate (Difco Laboratories, Detroit, MI), penicillin (100 IU/ml), streptomycin (100 µg/ml), amphotericin B (5 µg/ml), fungizone (E. R. Squibb and Sons Inc., Princeton, NJ) and 10% fetal bovine serum (GIBCO Laboratories, Grand Island, NJ). At two days of age, the exhausted medium was removed from the culture flasks and was replaced with MEM containing 5% fetal bovine serum. After the medium change, the cells could be maintained for up to three weeks by removing the exhausted medium and replacing it with serum-free MEM (SFM) at two day intervals. MA-104 or BSC-1 cells were passed into flasks, microtiter plates or culture tubes when they were 3-7 days of age. The cells from one 75 cm^2 flask were trypsinized and resuspended in 60 ml of MEM containing 10% fetal bovine serum. Approximately 100 µl of this suspension were added to each well of the microtiter plates, 2 ml were added to each culture tube, and 20 ml were added to each 75 cm^2 flask.

MDBK cells were grown in the same medium as described above. These cells were passed every 3 days by adding 60 ml of MEM

containing 10% fetal bovine serum to the trypsinized cell monolayers of one 75 cm^2 flask and dispensing 20 ml of the cell suspension into each 75 cm^2 flask. For plaquing, confluent cell cultures were passed undiluted following trypsinization and 1 ml of the cell suspension was added per well of an 8 well culture plate (Lux brand, Flow Laboratories Inc., McLean, VA).

Rotavirus Strains and Isolates

The rotavirus strains used in this study are designated using the terminology suggested by Dr. Woode of the FAO/WHO Comparative Virology Program (rotavirus subgroup) and first published in a report by Stuker et al.[114] The nomenclature lists in order, separated by colons: the first letter of the species from which the virus was isolated, the country in which the isolation took place, the year the isolate was discovered, and finally the number of the isolate. The porcine rotavirus (OSU strain, P:USA:77:1) was kindly supplied by Dr. E. Bohl at passage 32 in MA-104 cells[116] and the OSU vaccine strain was supplied by Ambico Inc., Dallas Center, IA. The OSU strain of porcine rotavirus was originally isolated from a 2-week-old conventional pig with clinical diarrhea at Ohio State University and subsequent passages in gnotobiotic piglets demonstrated virulence.[102] This strain is generally accepted as the type strain of porcine rotavirus. Dr. N. Schmidt kindly provided the simian rotavirus

(S:USA:79:2) at passage 15 in cell culture and plaque purified.[114] This simian rotavirus was isolated from a 3.5-month-old rhesus monkey with diarrhea.[49] The animal was raised at the California Primate Research Center, University of California, Davis, and thus was exposed to human contact. The possibility thus exists that this virus could possibly be a human strain which infects monkeys. The virus is antigenically closely related to SA.11[49] but its history is better documented. Dr. R. Wyatt supplied the WA strain of human rotavirus (serotype 2) at passage 16 in cell culture and plaque purified.[139] This strain was originally isolated from a pediatric patient (WA) with diarrhea.[139] It was passaged eleven times in gnotobiotic piglets and then adapted to grow to relatively high titer in primary cell cultures of African green monkey kidney cells. The canine isolate (LSU:79C-36,C:USA:81:2) was isolated from a two-day-old puppy with diarrhea.[42] The bovine rotavirus (B14 isolate, B:USA:79:1A, antigenically related but distinguishable from NCDV, B:USA:72:1) was isolated from a diarrheic calf in Iowa. The canine and bovine isolates were cloned by limiting dilutions after adaptation to cell culture and the canine isolate was further cloned by plaque selection. Rotavirus isolates B641, B681, B720, and B756 were detected in the diarrheic feces of calves from Florida, Iowa, Pennsylvania, and Iowa, respectively.

Preparation of Intestinal Contents or Fecal Specimens

Intestinal contents or fecal specimens were diluted 1:3 by volume in phosphate buffered saline (PBS) at pH 7.2, mixed well, and centrifuged at 7,500 RPM (6,000 x g) for 1 hour. The supernatant was removed and used for cell culture isolation or electron microscopy.

Indirect Immunofluorescent Antibody Test (IFAT) for Detection of Rotavirus Antigen

Rotavirus infected and uninfected control cultures in microtiter plates or coverslips were wet-fixed with acetone before CPE developed. To avoid opacity developing in the plastic of the microtiter plates due to the acetone treatment, the acetone was diluted with phosphate buffered saline (PBS) to 80% and stored and used at -24° C. The glass coverslips were fixed with undiluted acetone. After the preparations were dry, they were stored at -24° C or used immediately. They were rehydrated with PBS for two minutes and then the PBS was discarded. Two drops of gnotobiotic calf convalescent antiserum at a 1:40 dilution in PBS were added to each well (or coverslip) and the plates were then incubated for one hour at room temperature. The plates were then washed five times with PBS and two drops of fluorescein conjugated rabbit anti-bovine gamma globulin were added per well (or coverslip) and the cultures were again incubated for one hour. The preparations were then washed five times with PBS, shaken

free of fluid and 1 drop of 90% glycerin in PBS was added per well. The plates were inverted and read with a vertically transmitted ultraviolet light microscope through the 10X objective. A similar procedure was followed with the coverslip preparation. These, however, could be viewed through a 10X or 25X objective and gave better resolution. As some brands of microtiter plates did not transmit ultraviolet or excited light only only the Costar brand plates were used.

Rotavirus Isolation and Culture

Cell culture infectivity assay of fecal or intestinal rotavirus

Fecal or intestinal content supernatants were treated with an equal volume of 0.01% EDTA-free trypsin (1:250 Difco Laboratories, Detroit, MI) giving a final concentration of 500 µg/ml. This mixture was incubated for 30 minutes at 37° C and then diluted 1:10 in SFM containing 0.1% pancreatin (GIBCO Laboratories, Grand Island, NJ). MA-104 cells, 4-7 days of age, in 96 well microtiter plates were washed with SFM containing 0.1% pancreatin and 100 µl of the fecal dilution was added to each of 8 wells. The plates were then incubated for 24 hours at 37° C. After the incubation period, the media was removed from the plates and the cells were fixed with cold (-24° C) 80% acetone for 10 minutes. The acetone was then removed and the plates dried completely and immunofluoresced or stored at -24° C until stained.

Cell culture adaptation of fecal or intestinal rotavirus

Virus from some rotavirus positive feces (as detected by the cell culture infectivity assay) were adapted to cell culture. The same procedure was used for adaptation as was used for the cell culture infectivity assay described previously except that 25 cm^2 flasks (or test tube cultures) of MA-104 cells replaced the microtiter plates. Confluent cultures of MA-104 cells were 4-7 days of age when used. A negative control flask was always passed immediately prior to the passage of the virus flask to check for possible cross-contamination from other rotaviruses. The virus present in the supernatant fluids (and cells and cell debris) was passed by freeze-thawing, or by the addition of trypsin to strip the monolayer, every 24-48 hours if cytopathic effect (CPE) was not 100%. Cells from the control and virus flasks were used to check for the presence of rotavirus by IFAT. Generally, if CPE was observed during the early passages, the flasks were allowed to incubate until CPE was 100% (not more than 3 days). When viral CPE was 100% within 24-48 hours, the titer of the virus was generally high enough (10^5-10^7/50% tissue culture infective doses) for the virus to be used in various assays and tests. Each passage of the virus and its control were aliquoted and stored at -70° C.

In vitro culture and assay of cell culture adapted rotavirus

Rotavirus isolates were considered to be adapted to growth in cell culture by the third or fourth passage at which time their titer was 10^5 to 10^7 50% tissue culture infective doses ($TCID_{50}$), clearly demonstrating that replication of the virus had occurred in vitro. Cell culture adapted rotavirus was propagated in 75 cm^2 flasks of MA-104 cells with SFM containing 0.1% pancreatin and passed when CPE was 100% [usually within 24 hours post-infection (PI)]. For assay of the virus ten-fold dilutions were made in the above medium and 100 µl of each dilution added to each well of a microtiter plate of MA-104 cells (3-5 days of age); which had been rinsed two times with SFM and 0.1% pancreatin. The cultures were incubated 4-5 days at 37° C. The endpoint was read by CPE using an inverted microscope. The cultures were fixed with 10% phosphate buffered formalin for about one hour, stained with 1% crystal violet for ten minutes, rinsed with water, dried and stored at room temperature.

Virus cloning

Viruses were cloned by limiting dilutions or by plaque selection. Cloning by limiting dilutions was done by making ten-fold dilutions of the virus in SFM containing 0.1% pancreatin and inoculating these dilutions onto flying coverslip cultures of MA-104 or BSC-1 cells. The highest dilution at which infected cells could be detected by IFAT was designated a clone. This clone was passed once into a flask

of cells, incubated for 24-48 hours and the procedure repeated for a total of three clonings. For cloning by plaque selection, dilutions of the virus were made as described above and 1 ml of each dilution was added to each well of an 8 well tissue culture plate containing a 2-3 day old confluent monolayer of MDBK cells. The virus was allowed to adsorb for 1 hour at 37° C. The inoculum was then removed and an overlay consisting of equal volumes of 2x MEM and 2x oxoid agar (Oxoid Ltd., London, England), and 0.1% pancreatin and 0.003% neutral red, was added. The cultures were incubated at 37° C with minimal light exposure as neutral red is phototoxic. At about three days when plaques were visible, a well with less than about five plaques was selected and one plaque of infected cells, separated from other plaques was removed by scraping with a bent pasteur pipet. This plaque was then inoculated into a flask of MA-104 cells and grown as described previously. This procedure was repeated for further purification.

Virus Purification and Vaccine Preparation

Rotavirus grown in MA-104 or BSC-1 cells (usually in roller bottle culture) was harvested by freeze-thawing two times. The rotaviruses generally grew to a titer of 10^5-10^7 $TCID_{50}$/100 µl in roller bottle culture. To remove the cell debris, the media and cells were centrifuged at 6,000 xg and the supernatant virus was

pelleted at 80-100,000 xg for 1.5 hours in a Beckman L-65 ultracentrifuge, resuspended in PBS and extracted with an equal volume of trichlorotrifluoroethane (DuPont Co., Wilmington, DE). The aqueous phase was pelleted through 40% sucrose at 80-100,000 xg for 4 hours. The virus-containing pellet was resuspended in a small volume of PBS and stored at 4° C.

The purified virus pellet was used to vaccinate guinea pigs in order to produce hyperimmune sera. Non-inactivated rotavirus from 3-6 roller bottle cultures was pelleted and diluted approximately 1:50 in PBS for vaccination purposes.

Fecal rotavirus used for oral vaccination (or for challenge) of the gnotobiotic animals was prepared by diluting the rotavirus containing feces 1:50 in SFM, then mixing and centrifuging at low speed (6,000 xg). The supernatant was removed and filtered through a 0.45 μm filter. One drop of the filtrate was sterility-checked on blood agar plates 24-48 hours prior to inoculation. Cell culture adapted rotavirus was used for vaccination at approximately 10^7 $TCID_{50}$ per 100 μl, and was filtered (0.45 μm) and sterility-checked. The inocula (from feces or cell culture) were assayed for cell culture infectivity using the method described previously.

Serology

Convalescent antiserum

Convalescent antiserum was obtained from gnotobiotic pigs, calves, and rats which had been inoculated orally and/or nasally with rotavirus vaccines (prepared as described previously) and bled 2-3 weeks post-inoculation. Most of the animals received either cell culture adapted rotavirus or rotavirus from fecal samples collected in the field. Some of the animals received a gnotobiotic pass of the cell culture or fecal virus as a vaccine.

Convalescent antiserum was raised in gnotobiotic rats as follows. The rats (six females) were six weeks of age when received. They were allowed an adjustment period of one week. At the end of that time, one rat (Rat A) was removed from the isolator and bled out for a negative prevaccination control serum. The remaining five rats received an oral inoculation of approximately 1 ml of canine rotavirus at passage 8 in cell culture (approximately 10^7 $TCID_{50}$/100 µl) and had a low dilution of the virus in their drinking water for 24 hours. Fecal samples were collected daily and the animals were bled out three (Rats B and C) or four (Rats D, E, and F) weeks post-infection (PI).

Convalescent antiserum was prepared in gnotobiotic calves by the following method. The calves were vaccinated at one day of age with an oral-nasal inoculation of 5 ml of cell culture or fecal rotavirus prepared as described previously. The animals were bled three weeks

PI. Antiserum in gnotobiotic pigs was prepared similarly except that the animals received 1 ml of vaccine.

Hyperimmune antiserum

Hyperimmune antisera were obtained from guinea pigs which had been inoculated in the footpad twice at three week intervals with 0.1 ml of rotavirus in Freund incomplete adjuvant. The inoculum was prepared by emulsifying equal volumes of purified non-inactivated rotavirus and Freund incomplete adjuvant. Some of the guinea pigs received virus in Freund complete adjuvant for the first inoculation but this procedure was discontinued because the footpads became very swollen and developed open sores. The animals were bled three weeks after the last injection.

Detection of rotavirus antibody in serum

Adaptations of a method described by Woode et al.[135] were used to detect rotavirus antibody in serum by immunofluorescence (IF). Sera were screened for the presence of rotavirus antibody (IF) at a 1:10 dilution in PBS. The dilution was added to a rotavirus antigen plate which was made as follows: microtiter plates of MA-104 cells were inoculated with 100 µl/well of 100-1000 $TCID_{50}$/100 µl of canine rotavirus (in SFM with 0.1% pancreatin), incubated for 24 hours at 37° C, fixed with 80% cold (-24° C) acetone for 10 minutes, dried

and stored at -24° C. The remainder of the test was the same as that described for an IFAT except that the anti-gamma globulin fluorescein conjugate varied according to the species from which the serum to be screened originated. In some cases, the sera were titrated by 2-fold dilutions in PBS and the endpoint was recorded as a 50% decrease in fluorescence from one dilution to the next.

Serum neutralization test (SN)

Cell culture adapted rotavirus was diluted to 100-1000 $TCID_{50}$/ 100 µl in SFM containing 0.2% pancreatin. Serial 2-fold dilutions of serum were prepared in SFM. An equal volume of serum and virus dilutions was incubated at 37° C for 1.5 hours and then 100 µl of the preparation was added to each of 4-8 wells of a microtiter plate containing monolayers of 3-5 day-old MA-104 cells. The cultures were incubated for five days at 37° C, then fixed with formalin and stained with crystal violet as described previously.

Virulence of Rotaviruses in Gnotobiotic Piglets

All piglets in the experiments were weighed and observed daily for changes in their condition. Rotaviruses orally inoculated into gnotobiotic piglets were judged to be virulent based upon the following parameters. Clinical signs of disease included, fecal soiling of the skin and cage area, depression, refusal to feed, vomiting, diarrhea,

weight loss (or failure to gain weight), and sometimes death. Diarrhea was recognized as a sudden change in fecal color from the normal dark brown to light yellow or white, a marked increase in the fluid nature of the feces, and an increased volume. Fecal samples from clinically normal pigs were often small and difficult to obtain. However, fecal samples from pigs with diarrhea were easily obtained in relatively large amounts. Piglets were weighed daily using a spring balance to the nearest 25 g $\pm$ 5 g. Each litter of piglets used consisted of at least two piglets for controls. A comparison of weight curves of control vs. experimental animals, and of pre- and post-challenge animals was made to determine whether weight loss or a failure to gain weight had occurred.

Cross-Protection Studies in Gnotobiotic Piglets

After initial experimentation to determine the virulence of various rotavirus isolates to the pig, the gnotobiotic pigs were used as the animal model for the cross-protection studies. One way cross-protection work was done in the pigs using the bovine, canine, and simian viruses as vaccines and the OSU virulent strain of porcine rotavirus as the challenge virus. Pigs vaccinated with simian and canine viruses were challenged with the simian virus. For control of these comparative cross-protection experiments, some pigs received cell culture adapted and attenuated OSU rotavirus as a vaccine and

virulent OSU rotavirus as a challenge virus. Control pigs which received no vaccines but were challenged were included in every litter. Most of the animals received either cell culture adapted rotavirus or rotavirus from feces collected in the field. However, the OSU porcine rotavirus used as the challenge virus in the cross-protection studies was a fecal filtrate of a gnotobiotic passage in piglets of virulent OSU porcine rotavirus which was prepared as follows. Virulent rotavirus (gnotobiotic pig fecal filtrate provided by Dr. E. Bohl) was inoculated intranasally into two 26-day-old gnotobiotic piglets. Upon the onset of diarrhea, the intestinal contents were harvested, pooled, diluted to approximately $10^{4.1}$ $TCID_{50}$/ml, filtered, and stored at -70° C.

Piglets received 1 ml of inoculum intranasally-orally for vaccination or challenge. They were usually vaccinated at birth and challenged at two weeks of age. Fecal samples were collected daily after vaccination for one day before challenge and for several days after challenge. The piglets were weighed daily during the early portion of the experiments to determine the virulence of the vaccine virus but often as the pigs grew the weight recording was inaccurate and was not recorded during the period following challenge. Animals were observed daily for clinical signs of disease. The pigs were bled prior to the challenge and sometimes at the termination of the experiment. The sera were checked for anti-rotavirus antibody by IFAT or SN.

The absence or presence of fecal rotavirus post-challenge was used to indicate cross-protection or the lack of cross-protection. The presence or absence of diarrhea following challenge was viewed as a very subjective judgment although in most susceptible pigs anorexia and diarrhea were readily observable. Excretion or the lack of virus in the feces was thought to be the best indicator of whether or not cross-protection had occurred.

RESULTS

Convalescent Antisera

The comparative SN titers of convalescent antisera from orally inoculated animals are given in Table 1. Some sera showed 16- to 128-fold differences between homologous and heterologous titers, e.g., the OSU SN titer of antiserum to OSU (H1808) and the convalescent dog serum to a canine rotavirus. The gnotobiotic piglet from which the OSU antiserum (H1808, provided by Dr. E. Bohl) was collected was a convalescent animal that was also hyperimmunized. This serum clearly differentiated (i.e., > 20-fold difference) between porcine and human, bovine, and simian but only demonstrated a 16-fold difference between porcine and canine. The only strictly convalescent serum which clearly differentiated among the viruses was that from a conventionally reared dog convalescent to a natural infection with a canine rotavirus. This serum demonstrated a difference of 80-fold between canine and porcine, simian, and bovine rotaviruses. Other convalescent sera failed to distinguish between the different rotavirus isolates, e.g., gnotobiotic calf and pig antisera convalescent to B14 rotavirus.

Hyperimmune Guinea Pig Antisera

As preliminary data with the convalescent antisera did not definitively distinguish among all of the rotavirus isolates,

Table 1. Comparative SN titers of convalescent and hyperimmune sera

Vaccine virus[a]	Animal	SN titers to the following viruses				
		OSU	Canine	SIM	B14	WA
OSU	gnoto pig H1808[b]	6400	400	160	100	50
	gnoto pig III, 1	$\geq$ 800	400	100	ND[c]	ND
Canine	dog 38[d]	$\leq$ 10	800	$\leq$ 10	$\leq$ 10	ND
	gnoto pig II, 4	200	800	800	40	ND
	gnoto pig II, 5	200	1600	1600	100	ND
	gnoto rat B	< 40	800	200	< 10	ND
	gnoto rat C	< 20	800	800	< 10	ND
	gnoto rat D	< 40	400	200	< 20	ND
	gnoto rat E	< 40	800	100	< 10	ND
	gnoto rat F	< 20	800	200	< 40	ND
Sim	gnoto pig III, 3	20	$\geq$ 800	400	ND	ND
	gnoto pig VIII, 5	ND	200	200	ND	ND
B14	gnoto calf GC 5[e]	1600	800	800	1600	400
	gnoto pig I, 4	80	80	100	100	ND
	gnoto pig I, 6	40	80	80	100	ND

[a]Cell culture rotavirus.

[b]Convalescent animal also hyperimmunized by intramuscular inoculation of virus without adjuvant.

[c]ND, not done.

[d]Convalescent serum from a dog naturally infected with a canine rotavirus.

[e]Convalescent animal also hyperimmunized by one intramuscular inoculation of virus mixed with Freund incomplete adjuvant.

hyperimmune antisera were produced and subsequently were shown to be able to clearly differentiate the isolates. All of the guinea pigs used to produce hyperimmune antisera had been exposed to rotavirus (unknown strain) prior to immunization. In Table 2 are recorded the IF titers of the guinea pig sera to canine rotavirus antigen prior to hyperimmunization (which ranged from 10-160) and after hyperimmunization. The IF antigen represents the antigen common to all rotaviruses. There was a significant rise in IF titer in all the animals except those with high initial titers. Despite this previous exposure, highly specific antisera were acquired from most of the guinea pigs used. Table 3 lists the comparative homologous and heterologous SN titers of the guinea pig hyperimmune antisera. Differences of 20-500 fold were observed between homologous and heterologous SN titers. It was arbitrarily decided that a 20-fold difference between homologous and heterologous SN titers was the minimum difference used for distinguishing serotypes. However, lower differences are still significant. From the results in Table 3, the isolates of rotaviruses studied could be classified into four distinct serotype groups on the basis of differences in SN titers of 20-fold or greater. The four groups are represented by the porcine rotavirus (OSU), the bovine rotavirus (B14), the human rotavirus (WA), and the two isolates of simian (S:USA:79:2) and canine (C:USA:81:2). Canine and simian rotaviruses showed identical or less than 20-fold

Table 2. IF titers of guinea pig sera pre- and post-hyperimmunization

Animal no.	Vaccine virus[a]	Pre-hyperimmunization IF titer	Post-hyperimmunization IF titer
17	Canine	160	640
18	Canine	10	800
19	Canine	160	ND[b]
34	Simian	40	800
35	Simian	10	800
36	Simian	10	800
37	Bovine (814)	10	400
38	Bovine	10	800
39	Bovine	10	800
40	Bovine	10	400
41	Bovine	20	200
42	Porcine (OSU)	10	200
43	Porcine	20	400
44	Porcine	10	200

[a]Cell culture rotavirus.

[b]Not done.

Table 3. SN titers of guinea pig sera hyperimmune to various rotaviruses

Vaccine virus[a]	Animal #	SN titers to the following viruses					
		Canine	Simian	B14	OSU	B641	WA
Canine	17	51,200	12,800	800	400	ND[b]	ND
Canine	18	102,400	12,800	400	< 100	ND	ND
Canine	19	102,400	6,400	200	< 100	ND	ND
Simian	33	12,800	12,800	400	400	ND	ND
Simian	34	6,400	12,800	< 100	< 100	ND	ND
Simian	35	256,000	12,800	200	< 100	ND	ND
Simian	36	12,800	12,800	< 100	< 100	ND	ND
B14	37	< 100	< 100	12,800	< 100	ND	ND
B14	38	< 100	< 100	6,400	< 100	ND	ND
B14	39	< 100	< 100	25,600	< 100	ND	ND
B14	40	< 100	< 100	12,800	< 100	ND	ND
OSU	41	< 100	< 100	< 100	6,400	ND	ND
OSU	42	< 100	< 100	< 100	1,600	ND	ND
OSU	43	< 100	< 100	< 100	3,200	ND	ND
OSU	44	< 100	< 100	< 100	6,400	ND	ND
B641	45	< 100	< 100	3,200	< 100	1,600	100
B641	46	< 100	< 100	3,200	< 100	3,200	< 100
B641	47	< 100	< 100	6,400	< 100	3,200	< 100
B641	48	< 100	< 100	3,200	< 100	1,600	< 100
WA	49	< 100	< 100	< 100	< 100	ND	400
WA	50	< 100	< 100	< 100	< 100	ND	3,200
WA	51	< 100	< 100	< 100	< 100	ND	1,600
WA	52	< 100	< 100	< 100	< 100	ND	3,200

[a]Cell culture rotavirus.

[b]ND, not done.

differences with the exception of a convalescent dog serum which showed an 80-fold difference (Table 1). One guinea pig serum (serum no. 19) showed an approximate 16-fold difference between canine and simian. In contrast, the four serotype groups showed SN titer differences varying from each other by 32-fold (serum no. 43) to a maximum of approximately 500-fold (serum no. 19). There was marked variation observed in the immune responses between the different guinea pigs inoculated with the same antigen, particularly among the simian and human rotavirus inoculated guinea pigs. It should be noted that some sera failed to differentiate clearly between the groups (sera nos. 49, 51, 52), but these sera also showed low homologous titers. As these guinea pigs were all inoculated with the same antigen preparation (human rotavirus, WA strain), it is probable that the poor responses were the result of a relatively low concentration of antigen in the vaccine. However, none of the sera were tested below a 1:100 dilution so it is possible that the heterologous titers could be less than 50 which would demonstrate the 20-fold difference required to show that the viruses belong in different serotype groups.

Virulence of Rotaviruses in Gnotobiotic Piglets

The clinical signs associated with a virulent rotavirus infection are anorexia, diarrhea, occasional vomiting, an associated weight loss,

a period of failure to gain weight and sometimes death. However, the mortality rate is variable. Using the above criteria but excluding mortality as a required property, rotaviruses were judged to be virulent or avirulent to the gnotobiotic pig.

Vaccinated and control piglets which remained clinically normal continued to gain weight at an average rate of approximately 100g/day. These animals did not show more than one day of failure to gain weight. Piglets which demonstrated clinical signs of disease following vaccination or challenge showed a weight loss or failure to gain weight for three or more days. This failure to gain weight resulted in a 25-40% reduction of their potential weight 3-5 days post-infection, which was determined by extrapolation from their own weight curve before disease and in comparison to control curves. Based on weight gain failure and clinical signs, the virulent OSU rotavirus, canine rotavirus and bovine rotavirus strains B681 and B756 were judged to be virulent in these experiments.

OSU virulent fecal rotavirus when inoculated orally into 2-week-old piglets produced a severe diarrhea within 24 hours. The feces were very watery and contained yellow flecks. There was rapid development of dehydration and an accompanying weight loss. The piglets also vomited. When the OSU virulent strain was fed to two 3-day-old piglets, both died after 2-3 days of diarrhea.

Because the effect of the OSU rotavirus on the piglets was so pronounced (i.e., virulent) it was selected as the challenge strain in the cross-protection work. This virus was the most virulent to the piglets and therefore presented the most severe challenge to any vaccine used.

The canine tissue culture adapted rotavirus caused detectable signs of disease in four out of six piglets. These signs included, loss of appetite and weight loss or a failure to gain weight and diarrhea.

The bovine isolates B681 and B756 were also judged to be virulent on the basis of the criteria described previously, although only one pig out of two inoculated showed a noticeable difference in weight gain.

Cross-Protection

The results of the cross-protection studies in gnotobiotic piglets are recorded in Table 4. Pre-vaccination sera were negative for rotavirus antibody at a 1:10 dilution by IF. All rotavirus isolates used as vaccines infected, multiplied and caused seroconversion in the piglets. Of the vaccines used, only the OSU serotype vaccine virus protected the piglets against challenge with virulent OSU rotavirus as determined by a lack of clinical signs of

Table 4. Cross-protection studies in gnotobiotic piglets

No. of animals	Before challenge: Age vaccinated (days)	Before challenge: Vaccine virus[a]	Before challenge: No. of animals with diarrhea	Before challenge: Virus excretion[b]
2	7	OSU[d]	0	+
4	1	OSU[d]	0	+
4	1	B14[d]	0	+
2	7	B720[g]	0	+
2	7	B641[g]	0	+
2	1	B641[g]	0	+
2	1	B681[g]	2	+
2	1	B756[g]	2	+
4	1	Canine[d]	2	+
4	1	Simian[d]	0	+
10	NA[i]	Controls	0	-
2	NA	Controls	0	-
2	1	Canine[d]	2	+
2	1	Simian[d]	0	+
2	NA	Controls	0	-

[a]The virus was administered at 10^7 50% tissue culture infective doses.

[b]Cell culture isolation from feces detected by IF for at least 3 days after vaccination.

[c]Cell culture isolation from feces detected by IF for at least 3 days after challenge.

[d]Cell culture rotavirus.

[e]ND, Not done.

[f]OSU virulent virus.

[g]Fecal filtrates of bovine rotavirus.

[h]Serum was IF positive at 1:10 dilution to canine rotavirus anitgen.

[i]NA, Not applicable.

NT titer of prechallenge sera to following virus:			After challenge			
Vaccine	OSU	Simian	Age challenged (weeks)	Challenge virus	No. of animals with diarrhea	Virus excretion[c]
≥ 800	≥ 800	ND[e]	3	OSU[f]	0	-
≥ 800	≥ 800	ND	2	OSU[f]	0	-
40-160	≤ 10	ND	2	OSU[f]	4	+
ND[h]	10-20	ND	4	OSU[f]	2	+
ND[h]	10-20	ND	4	OSU[f]	2	+
ND[h]	≤ 10	ND	2	OSU[f]	2	+
ND[h]	≤ 10	ND	2	OSU[f]	2	+
ND[h]	≤ 10	ND	2	OSU[f]	2	+
160-180	≤ 10	ND	2	OSU[f]	4	+
80-400	≤ 10	ND	2	OSU[f]	4	+
NA	≤ 10	ND	2	OSU[f]	10	+
NA	≤ 10	ND	4	OSU[f]	2	+
400-800	ND	200-400	2	Simian[d]	0	-
200-400	ND	200-400	2	Simian[d]	0	-
NA	ND	≤ 10	2	Simian[d]	0	+

disease and the absence of virus in the post-challenge feces. All of the other isolates used failed to protect the piglets against both clinical signs of disease and virus shedding.

The canine and simian rotaviruses which possessed minimal antigenic differences by SN, showed cross-protection between them when the simian virus was used as the challenge virus and either the canine or simian viruses were used as the vaccine. The cell culture adapted simian rotavirus used for challenge was not found to be virulent to the piglets and thus protection was determined by a lack of virus shedding after challenge in the vaccinated animals.

Virulence of the vaccine virus did not appear to unduly influence the immune response. Two out of four of the bovine isolates and the canine cell culture adapted rotavirus caused diarrhea with an accompanying loss in body weight or a failure to gain weight for two to three days in some or all of the piglets. These viruses were judged to be virulent. However, despite this severity of infection, none of these piglets were protected against challenge with virulent OSU rotaviruses.

It was considered possible that a lack of cross-protection between the vaccine strains and OSU was due to a reduced ability of the vaccines isolated from species other than the pig to replicate efficiently enough in the intestine of the pig to generate a protective immune response. Therefore, vaccine virus shed in the feces was

assayed by infectivity in cell culture for a measure of the degree of viral multiplication occurring in the gut. The number of immunofluorescent cells (IC) in cell cultures inoculated with fecal supernatants and fixed at 24 hours varied widely among the piglets and appeared to correlate with the rotavirus serotype and to the number of days after vaccination. A fecal sample, from each of two piglets (inoculated with cell culture adapted vaccines) which had the highest number of IC at a 6×10^{-1} dilution, was selected and assayed for infectivity by counting the number of IC. The titers (IC per field) of the vaccine cell culture viruses were: bovine, 18×10^{1}; OSU, 31×10^{3}; simian, 21×10^{5}; and canine, 13×10^{4}. Cell culture adapted bovine rotavirus vaccines were detected only in low titers in the feces of inoculated piglets. However, the piglets which developed diarrhea after being inoculated with bovine fecal rotavirus vaccines shed virus at titers of approximately 10^{4}-10^{5} IC/field. Based on these results, no correlation was detected between the titers of the vaccine virus shed in the feces and protection against challenge with virulent OSU rotavirus.

DISCUSSION

It is generally accepted that major antigenic differences are required to discriminate among different serotypes within a virus genus. However, the definition of major antigenic differences is not uniform for all viruses. While in this study a 20-fold difference in serum neutralization titer was required to differentiate serotypes, much smaller differences are reported as acceptable for differentiating other viruses (e.g. influenza A viruses). The influenzas, like the rotaviruses, have a segmented genome and are well-known for their ability to reassort. As in the rotaviruses, problems have been encountered in deciding what degree of antigenic difference is required for the recognition of distinct serotypes of influenza A viruses. Many isolates have minor antigenic differences as measured by comparative homologous versus heterologous titers of antiserum. For this reason, an arbitrary distinction was made between serotypes in that only a 4-fold difference in hemagglutination titer was considered significant.[30] Although some work in this area has been done,[107] this recognition of serotypes remains to be confirmed by cross-protection studies. Indeed, according to the World Health Organization, the influenza A viruses are divided into subtypes - not serotypes, based upon the antigenic specificity of the hemagglutinin and neuraminidase antigens as determined by the

double immunodiffusion test.[137] Therefore, based upon the rotaviruses and influenzas alone, much work remains to be done in the area of viral taxonomy concerning the significance or degree of importance of antigenic differences.

The serum neutralization test has been shown to be able to detect species-specific antigens of rotaviruses as opposed to the group antigens which are demonstrated by complement fixation or immunofluorescence.[12,119,135] For this reason, the serum neutralization test was selected as the method of choice for differentiating rotaviruses in vitro and correlating the in vitro differences with in vivo studies on cross-protection. To permit the identification of different serotypes with a minimum of cross-reactivity, an arbitrary decision was made to accept a difference of no less than 20-fold between homologous and heterologous SN titers as being significant. As differences of this magnitude were not often observed in 3-4 week convalescent serum, antisera from hyperimmunized animals (guinea pigs) were used for serotyping.

The greater ability of hyperimmune as compared to convalescent antisera to demonstrate antigenic differences among rotaviruses confirms work done by others.[34] Interestingly, the guinea pigs used for producing hyperimmune antisera had all been previously exposed to a rotavirus (or rotaviruses) of unknown origin, as rotavirus-specific antibody was detected by IF in pre-hyperimmunization sera,

although the titers were low in most animals. Despite this previous exposure, the guinea pigs produced highly specific antisera.

In contrast to the hyperimmune antisera, the convalescent antisera were more cross-reactive and most were unable to discriminate among the rotavirus isolates. However, a dog convalescent to a natural infection of a canine rotavirus, and five gnotobiotic rats convalescent to an oral inoculation of canine rotavirus produced highly specific antisera. The reasons for these differences in specificity among the convalescent antisera are not known. The differences in specificity could possibly be due to the antigenic structure of the particular rotavirus or to the immune response of the animal species used for antiserum production. The heterologous titers, reported in Tables 1 and 4, of sera from pigs convalescent to canine rotavirus are different (i.e., those in Table 4 are more specific) presumably because the animals were bled at different times after inoculation. The animals were bled 3 weeks p.i. for the serum neutralization studies (Table 1) and 2 to 3 weeks for the cross-protection studies. Generally, antisera from 2 week convalescent animals demonstrated a narrower specificity, whereas specificity appeared to have broadened in 3 week convalescent animals. One pig which was orally inoculated with OSU rotavirus and also hyperimmunized, differentiated among OSU, B14, and simian by greater than 20-fold but did not differentiate clearly between OSU and canine rotavirus, i.e., less than 20-fold.

The differences between the isolates studied were most easily demonstrated by the guinea pig hyperimmune antisera (20-500 fold) and as these differences were reflected in a lack of cross-protection between each serotype and OSU they therefore are of importance. It should be noted that not every guinea pig inoculated produced antisera that was clearly able to differentiate among the rotaviruses. Generally, those producing the lower homologous titers were inoculated with rotaviruses which did not multiply to high titer in cell culture (i.e., B14 and WA) whereas those with high homologous titers were inoculated with viruses which multiplied to high titer in cell culture (i.e., canine rotavirus). Therefore, the reason these animals produced less specific antisera may have been due to a lower immunizing dose, a failure of an individual animal to mount an efficient immune response or a fault in the immunization procedure.

To conduct the cross-protection studies, an animal model had to be selected. The animals investigated to serve as models included pigs, calves, and rats. Initial experiments were done using the rat to determine if this animal could be used. However, due to the expense of the animals, the small amount of fecal material and blood that could be collected, and the fact that the canine rotavirus failed to cause any detectable clinical signs of disease and could not be detected in the feces, the gnotobiotic rats were discontinued in favor

of gnotobiotic calves and pigs which were more readily available and did not have to be derived elsewhere. Gnotobiotic pigs were selected for the cross-protection studies over calves because of (a) their comparatively low cost, (b) a number of experiments including controls could be done with one litter, and (c) a reliably virulent strain of porcine rotavirus (OSU) was available for challenge purposes. No such reliably virulent strain was available for the calves.

Several of the isolates studied were found to be virulent to the pig. Of these rotaviruses, the porcine strain (OSU) caused the most severe disease. It was for this reason that the OSU strain was selected as the challenge strain for the cross-protection studies. Similar studies done in calves with a virulent bovine rotavirus showed that cross-protection was less likely to occur if the challenge virus was virulent as opposed to a challenge virus of low or avirulence.[131,138] Homologous vaccine protection in these studies and others[64,126] prevented both clinical disease and virus shedding in the feces. However, heterologous vaccine protection, though reported to have prevented clinical signs of disease, did not prevent shedding of the challenge virus in the feces. In 1980, Tzipori et al.[126] reported that piglets vaccinated with human rotavirus and challenged with porcine rotavirus were protected from clinical disease but did become infected and did shed virus in the feces

after challenge. It was reported that the animals developed serum neutralizing antibody to SA.11 but the response to the porcine or human virus was not reported nor were the serological relationships of the viruses used. Therefore, it could not be determined if the piglets inoculated with the human virus actually developed neutralizing antibody to the porcine strain and thus it was not determined if the specificity or lack of specificity of the immune response influenced cross-protection. Another study appearing to point to successful heterologous cross-protection was done by Bridger and Brown.[14] They reported that bovine colostrum protected piglets from porcine rotavirus challenge. However, the colostrum was shown to possess neutralizing antibodies against the porcine virus. Another study however seemed to demonstrate that milk has poor protective properties in cattle.[110] Wyatt et al.[138] also reported heterologous cross-protection when calves vaccinated in utero with bovine rotavirus were protected against clinical disease when challenged with human rotavirus. However, cross-protection did not occur with respect to viral shedding in the feces post-challenge. As the human virus used in these studies is of low virulence (or even avirulence in some studies, G. Woode, Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, personal communication), it is reasonable to conclude that the calves did not show full cross-protection. Like Tzipori et al.'s study,[126] they did not report on the immune response of the animals to the challenge virus. In contrast,

Woode et al.[131] reported only a 30% cross-protection rate when foal or human virus (provided by Dr. Wyatt) was used to vaccinate calves and bovine rotavirus (British isolate) was used as challenge.

In this study, cross-protection between viruses of the same serotype resulted in a lack of clinical signs of disease and a lack of virus excretion following challenge. However, this only occurred when the homologous vaccine/challenge system was used with OSU porcine rotavirus. The apparent cross-protection between the antigenically closely related but not identical viruses, simian and canine, may have been a function of the lack of virulence of the simian virus used for the challenge.

Several studies have been published reporting the existence of antigenically different rotaviruses (different serotypes) in calves, piglets, mice, humans, monkeys, and foals[40,105,119,135] and also 2-3 different serotypes in humans.[118,143] Two viruses (canine and simian) in this study belong to the same serotype based on serum neutralization and cross-protection. Although this observance is unusual, there have been reports indicating that this is not a precedence. These reports suggest natural infections of animals or humans with rotaviruses from other species such as, children infected with a bovine serotype,[18] calves infected with a porcine serotype,[14] and pigs infected with a bovine serotype (G. Woode, personal communication). These reports are based on serological evidence. This study shows that the canine and simian

rotaviruses belong to the same serotype. Although great care was taken in the laboratory to avoid cross-contamination among the isolates studied, the possibility that cross-contamination had occurred between the simian and canine strains was considered. This possibility was soon eliminated. Differences between the two isolates could be detected by some of the antisera used, although these differences did not meet the criteria required for the two viruses to be placed in different serotypes. One convalescent dog sera did discriminate well between them but it is not known if this animal was infected with the same strain of canine rotavirus as was cultured in the laboratory. The RNA segments of these two isolates have been compared by coelectrophoresis.[43] The migration patterns differed in at least eight segments (1, 2, 4, 5, 6, 8, 9, 10, and possibly 3). These two rotaviruses are very interesting in that despite possessing such marked differences in RNA migration patterns they are very closely related antigenically and by cross-protection. From this data and from previously published studies on other rotaviruses, it is predicted that RNA segment 11 codes for the major serum neutralizing antigen and segment 2, 3 and 9 code for minor and less important neutralizing antigens.[43] It should be noted that serotype does not correlate with virulence (e.g., canine was judged to be pig virulent, simian was not). Thus, from this limited study, the coding for virulence must reside in the RNA segments which are different between canine and simian.

CONCLUSIONS

This work and other published studies confirms that there are different serotypes of rotavirus as judged by the serum neutralization test, that the serum neutralization test has proven to be a reliable and easy method for serotyping, and that these different serotypes are likely to show poor cross-protective properties. The gnotobiotic pig has served well as an animal model for cross-protection work because of the number of experiments one litter provides, their relatively low cost, and the animals' susceptibility to many different strains of rotavirus. From this study, it has also been shown that the same serotype of rotavirus can be found naturally in different species of animals and that the same serotype can be possessed by two very different (as judged by RNA coelectrophoresis) viruses.

In conclusion, since homologous vaccines provide the best protection against challenge, rotavirus vaccines should be made against all the serotypes to which an animal is likely to be exposed. The discovery of two different viruses, canine and simian that possess the same serotype raises the possibility of using a virus from one animal species as a vaccine for another animal species as long as the same serotype exists in both species. It is possible that the vaccine virus originating from another species would be of lower

virulence for the vaccinated animal. Until studies are done to test the feasibility of such an approach or to make viruses through genetic reassortment, homologous vaccines appear to be the best approach for successful protection.

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APPENDIX

Solutions and Reagents

Eagles' Minimum Essential Medium (MEM)

22.0 g $NaHCO_3$

96.06 g Eagles' Minimum Essential Medium powder (GIBCO, Grand Island, NY)

10 l H_2O

The solution was prepared, pH adjusted to 6.7 and filter sterilized using a 0.45 µm and 0.22 µm filter. The media was stored at 4° C.

X-ATV (for cell culture passing)

8.0 g Trypsin (DIFCO, Detroit, MI)

32.0 g NaCl

0.8 g KH_2PO_4

4.6 g Na_2HPO_4

4.0 g EDTA (ethylenediamine tetraacetic acid) (Sigma, St. Louis, MO)

4 l H_2O

The solution was prepared, filter sterilized and stored frozen (-24° C) until used.

2 X Oxoid Agar (1.2%)

6.0 g Oxoid agar (Oxoid Ltd., London, England)

500 ml H_2O

The solution was prepared, heat-stabilized and stored at 4° C for use.

Phosphate Buffered Saline (PBS)

85.07 g NaCl

1.22 g KH_2PO_4

1.20 g Na_2HPO_4

10 l H_2O

The solution was prepared and pH adjusted to 7.2. It was heat-sterilized and stored at 4° C for diluting purposes. For washing purposes, PBS was not sterilized and was stored at room temperature.

Phosphate Buffered Formalin

59.5 g NaCl

45.5 g Na_2HPO_4

28.0 g NaH_2PO_4

6.3 l H_2O

0.7 l 37.5% Formaldehyde

The solution was prepared and stored at room temperature.

Crystal Violet Stain (1%)

25	g	crystal violet
500	ml	absolute alcohol
2	l	distilled water

The stain was prepared and stored at room temperature.

Concentrated Mineral Supplement for Gnotobiotic Calves and Piglets

49.8	g	$FeSO_4 - 7H_2O$
3.9	g	$CaSO_4 - 5H_2O$
3.6	g	$MnCl_2 - 4H_2O$
0.26	g	KI
1	l	H_2O

If a precipitate formed, 2 ml HCl was added.

Dilutions

for piglets

5.3 ml mineral concentrate

1 l H_2O

for calves

3.53 ml mineral concentrate

1 l H_2O

The supplement was heat-sterilized.