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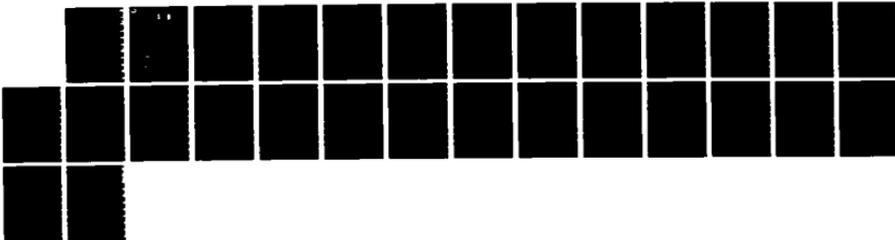
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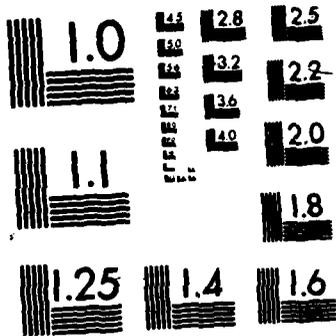
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THE ETIOLOGY AND PATHOGENESIS OF VIRAL GASTROENTERITIS

Annual Progress Report

by

Neil R. Blacklow, M.D.

31 July, 1984

(For the Period 1983 - 1984)

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ected against the group specific antigen shared by mammalian rotaviruses. The monoclonal antibody reagent has been adapted to an enzyme-linked immunosorbent assay (EIA), making it available for use in field studies. This monoclonal EIA has considerably improved the sensitivity and specificity of rotavirus EIA detection efforts with neonatal stool samples and adult stool specimens. We are also continuing studies to develop monoclonal antibodies against the to-date noncultivable Norwalk virus both by production of human-mouse heterohybridomas and by immunization of mice with extensively purified Norwalk virus. Success in these efforts, as well as with our attempts to cultivate Norwalk virus, will readily permit recognition of these viruses in nature. Additional studies have to date failed to reveal any antigenic relatedness between Norwalk virus and feline calicivirus. Recently, we have initiated efforts to develop a much needed convenient, quantitative immunoassay for the study of enteric adenoviruses (types 40 and 41) which have been associated with diarrheal disease in spite of current difficulties in their recognition in vitro. Such an immunoassay will advance understanding of the biology and epidemiology of enteric adenoviruses.

Studies have continued to be performed on the etiology and epidemiology of gastroenteritis in several populations. We have reported severe diarrhea produced by rotavirus in adults requiring hospitalization in Thailand, as well as evidence implicating rotavirus as a cause of waterborne gastroenteritis in the U.S. Six outbreaks of gastroenteritis in the U.S. associated with the consumption of raw and undercooked clams and oysters have been shown to be produced by Norwalk virus; Also, one Norwalk virus outbreak has been associated with eating food from a salad bar. Finally, two collaborative studies in U.S. Peace Corps volunteers in Thailand have revealed Norwalk virus to be implicated in 3 to 7 percent of cases of diarrhea.

Studies on the pathogenesis of viral gastroenteritis have indicated that absence of detectable antibody to Norwalk virus in acute phase sera was not statistically associated with subsequent seroconversion or susceptibility to illness in a naturally occurring outbreak of Norwalk virus gastroenteritis among American teenagers. Serum IgM antibody to Norwalk virus was also detected for the first time in most ill persons in this naturally occurring outbreak. Finally, studies of the role of cell-mediated immune responses in the pathogenesis of and recovery from rotavirus infections have been initiated. An H-2 defined cell line infected with rotavirus has been developed, now permitting its use as a defined target cell for cytotoxicity assays.



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SUMMARY

The purpose of this project is to identify, cultivate, and characterize etiologic agents of viral gastroenteritis of man, and to study the epidemiology and pathogenesis of infection in order to provide information necessary to attain the ultimate goals of prevention and cure of this common syndrome. Progress achieved during the present contract year can be summarized as follows. An improved method to identify human rotavirus in stool samples has been developed through our preparation and use of a monoclonal antibody reagent directed against the group specific antigen shared by mammalian rotaviruses. The monoclonal antibody reagent has been adapted to an enzyme-linked immunosorbent assay (EIA), making it available for use in field studies. This monoclonal EIA has considerably improved the sensitivity and specificity of rotavirus EIA detection efforts with neonatal stool samples and adult stool specimens. We are also continuing studies to develop monoclonal antibodies against the to date noncultivable Norwalk virus both by production of human-mouse heterohybridomas and by immunization of mice with extensively purified Norwalk virus. Success in these efforts, as well as with our attempts to cultivate Norwalk virus, will readily permit recognition of these viruses in nature. Additional studies have to date failed to reveal any antigenic relatedness between Norwalk virus and feline calicivirus. Recently, we have initiated efforts to develop a much needed convenient, quantitative immunoassay for the study of enteric adenoviruses (types 40 and 41) which have been associated with diarrheal disease in spite of current difficulties in their recognition in vitro. Such an immunoassay will advance understanding of the biology and epidemiology of enteric adenoviruses.

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BACKGROUND INFORMATION ON VIRAL GASTROENTERITIS

This research program is designed (a) to identify, cultivate, and characterize etiologic agents of viral gastroenteritis, and (b) to study the epidemiology and pathogenesis of infection in order to provide information necessary to achieve goals of disease prevention and cure.

Acute viral gastroenteritis is an extremely common illness that affects all age groups and occurs in both epidemic and endemic forms (1). It is second in frequency only to the common cold among illnesses affecting United States families under epidemiological surveillance. It is also responsible for some of the common travelers' diarrhea encountered in Latin America, Africa, and Asia. The illness varies in its clinical presentation, but in general it is self limited, begins with an explosive onset, and consists of varying combinations of diarrhea, nausea, vomiting, low grade fever, abdominal cramps, headache, anorexia, myalgia and malaise. It can be severe, indeed fatal, in the elderly, infant, debilitated or malnourished patient.

Viral gastroenteritis occurs primarily in two epidemiologically distinct clinical forms (1). One entity is characteristically epidemic and is responsible for family and community-wide outbreaks of gastroenteritis among older children and adults. The older medical literature gives a variety of descriptive labels to this one to two day illness, such as winter vomiting disease, epidemic collapse, viral diarrhea, epidemic diarrhea and vomiting, and acute infectious nonbacterial gastroenteritis. In recent years, one agent, Norwalk virus, has been shown to be responsible for about forty percent of these disease outbreaks in the United States. Other Norwalk-like viruses have also been discovered such as Hawaii agent, and although they have not yet been studied epidemiologically, they are likely to be responsible for many more epidemic cases of this illness.

The second clinical entity is usually sporadic and occasionally epidemic and it occurs predominantly in infants and young children (1). However, as noted below it can occur in adults. This form of illness typically produces severe diarrhea that commonly lasts for five to eight days and is usually accompanied by fever and vomiting. Rotavirus, which was discovered during the 1970's, is responsible for approximately one half of the cases of this clinical entity requiring hospitalization. Although the major target of rotavirus is the very young, it can produce surprising severe clinical disease in adults (1).

Despite the frequency of viral gastroenteritis syndromes, the etiology of these illnesses remained obscure until the 1970's. The principal investigator began his studies into the etiology and pathogenesis of this illness in 1970, with the initial goal of development of materials and methodology necessary for an understanding of this disease. Initially, he transmitted enteritis to healthy adult volunteers by the oral administration of bacteria-free, toxin-free stool filtrates derived from several outbreaks of the disease. These studies led to the discovery of the first major group of agents responsible for viral diarrhea, the Norwalk-like viruses (2).

1. Cukor, G., and Blacklow, N.R.: Human viral gastroenteritis. Microbiological Reviews 48:157-179,1984.
2. Dolin, R., Blacklow, N.R., DuPont, H. et al: Transmission of acute infectious nonbacterial gastroenteritis by oral administration of stool filtrates. J.Inf.Dis. 123:307-312,1971.

The prototype Norwalk virus, which is still currently noncytopathic in vitro and not disease producing for experimental animals, was initially described by the investigator and colleagues as a small lipid-free virus (3). It was later visualized in infectious stool filtrates and partially characterized by immune electron microscopy (IEM) and ultracentrifugation (4). Other 27nm sized Norwalk-like viruses, such as Hawaii and Ditchling viruses, have been uncovered by similar techniques but appear to be immunologically distinct based on IEM studies (1).

The investigators have shown that the Norwalk and Hawaii agents both produce a mucosal lesion of the proximal human small intestine, the likely site for replication of these viruses (5,6). This lesion is accompanied by transient small intestinal malabsorption, and also by delayed gastric emptying despite normal gastric morphology and secretory function (7).

The investigator has also established that clinical immunity to Norwalk virus in volunteers is novel and fails to fit immunologic concepts traditionally associated with common human viral illnesses (8): pre-existing serum antibody is paradoxically associated with the development of illness in volunteers, and lack of pre-challenge antibody is found in volunteers who remain well after exposure to the virus and also fail to seroconvert to the agent (8-10). In addition, antibody

1. Cukor, G., and Blacklow, N.R.: Human viral gastroenteritis. Microbiological Reviews 48:157-179,1984.
3. Dolin, R., Blacklow, N.R., DuPont, H. et al: Biological properties of Norwalk agent of acute infectious nonbacterial gastroenteritis. Proc.Soc. Exp. Biol. & Med. 140:578-583,1972.
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9. Greenberg, H.B., Wyatt, R.G., Valdesuso, J. et al: Solid-phase microtiter radioimmunoassay for detection of the Norwalk strain of acute nonbacterial, epidemic gastroenteritis virus and its antibodies. J.Med.Virol. 2:97-108,1978.
10. Blacklow, N.R., Cukor, G., Bedigian, M.K. et al: Immune response and prevalence of antibody to Norwalk enteritis virus as determined by radioimmunoassay. J.Clin. Micro. 10:903-909,1979.

to Norwalk virus in prechallenge intestinal fluids has been found predominantly in those volunteers who subsequently developed illness. At least 2 forms of clinical immunity exist for Norwalk virus: one group of subjects (persistently lacking antibody) maintains long-term immunity to the virus as shown by lack of illness after initial challenge and after rechallenge up to 34 months later. A second group of volunteers (persistently possessing antibody) is susceptible to infection both upon initial exposure and again upon rechallenge 27 to 42 months later. Short term immunity exists to the virus when all subjects are reexposed after 6 to 14 weeks.

In recent years, investigators at the National Institutes of Health, as well as the principal investigator, have developed a radioimmunoassay (RIA) technique for the detection of Norwalk virus in diarrheal stools and for quantitation of antibody to the agent (9,10). The RIA represents a major advance in the study of this virus, and now provides a laboratory handle for studies to cultivate the virus in vitro. The principal investigator has already used the RIA to study forms of clinical immunity to Norwalk virus (10) (also see the preceding paragraph), and to show that Norwalk RIA serum antibody prevalence levels rise during adolescence in the U.S.(10). It has also been observed that antibody to Norwalk virus is acquired at a significantly earlier age in less developed and tropical areas than in more developed and nontropical areas (11,12). The RIA test has also been used to show that Norwalk virus is responsible for approximately 40 percent of viral gastroenteritis epidemics that occur in the United States(13). Recently, the principal investigator has developed an RIA test for IgM antibody to Norwalk virus (14). This test indicates that, with volunteer sera, virus-specific IgM is not necessarily indicative of primary infection with Norwalk virus inasmuch as reinfection produces enhancement of the IgM response. Furthermore, these IgM responses in volunteers appear not to be associated with subclinical illness.

9. Greenberg, H.B., Wyatt, R.B., Valdesuso, J. et al: Solid-phase microtiter radioimmunoassay for detection of the Norwalk strain of acute nonbacterial, epidemic gastroenteritis virus and its antibodies. J. Med. Virol. 2:97-108, 1978.
10. Blacklow, N.R., Cukor, G., Bedigian, M.K. et al: Immune response and prevalence of antibody to Norwalk enteritis virus as determined by radioimmunoassay. J. Clin. Micro. 10:903-909, 1979.
11. Greenberg, H.B., Valdesuso, J., Kapikian, A.Z., et al: Prevalence of antibody to the Norwalk virus in various countries. Infect. & Immunity. 26:270-273, 1979.
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It should be noted that the RIA test for Norwalk virus and its antibody is currently available in only a few research laboratories including that of the principal investigator. This is because the procedure requires the use of precious limited human volunteer materials (stools and sera). The Norwalk RIA procedure for the first time permits the large-scale rapid testing of stool and serum specimens from individuals for evidence of infection with Norwalk virus. Such studies have already shown the epidemiologic importance of Norwalk virus in various parts of the world, including its involvement in waterborne, foodborne, and shipborne outbreaks of acute gastroenteritis (13,15-18). In addition, the investigator and colleagues have shown Norwalk virus to be a cause of travelers' diarrhea in Mexico and Thailand (19,20).

During the past decade, a second viral enteric pathogen of man has been identified and is now known to be a major cause of diarrhea in young children (21,22). It can also produce illness in adults (23-25). This pathogen, rotavirus, has been

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identified by electron microscopy in stool filtrates derived from ill individuals (21,22). Serologic assay techniques have been developed for this agent by our laboratory and others and can detect antibodies in human sera (26,27). In addition, rotavirus has been identified by our laboratory and others in diarrheal feces by RIA or enzyme-linked immunosorbent assay (ELISA) techniques (28-30). Laboratory techniques now permit in vitro study of the biologic properties of rotavirus. We have already used these methods to establish the role of this virus in diarrhea in several nations around the world, including travelers' diarrhea experienced by U.S. military populations overseas (19,31-35). During 1981, Japanese scientists successfully cultivated human rotavirus in rotated cell cultures by incorporating low concentrations of trypsin into the culture medium (36).

19. Kesswick, B.H., Blacklow, N.R., Cukor, G., DuPont, H.L. and Vollett, J.J.: Norwalk virus and rotavirus in travellers' diarrhoea in Mexico. The Lancet 1:109-110, 1982.
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34. Echeverria, P., Ramirez, G., Blacklow, N.R. et al: Travelers' diarrhea among United States Army troops in South Korea. J. Inf. Dis. 139:215, 1979.
35. Echeverria, P., Blacklow, N.R., Vollett, J.J. et al: Etiology of gastroenteritis among Americans living in the Philippines. Am. J. Epid. 109:493, 1979.
36. Urasawa, T., Urasawa, S., and Taniguchi, K.: Sequential passages of human rotavirus in MA-104 cells. Microbiol. Immunol. 25:1025-1035, 1981.

Studies reveal that mechanisms of clinical immunity to rotavirus are complex (1). It seems likely that serum antibody to the virus is associated with protection from illness, and perhaps, local intestinal tract antibody as well. However, interpretation of studies is complicated by the presence of 4 human serotypes and 2 (perhaps 3) subgroups of the virus (1). Immune responses are heteroserotypic and heterosubgroup in nature, and various human and animal rotaviruses are closely related both by serotype and subgroup. A European group has administered calf rotavirus as a potential immunogen to adults and children (37). This "vaccine" has demonstrated homotypic immunogenicity with lesser degrees of heterotypic responses, and has shown some protective clinical effects in a small field trial of children who were later naturally exposed to wild rotavirus. Of particular note in the field of rotavirus pathogenesis and immunity is the nearly complete absence of any information concerning cellular immune responses to infection, either in man or animal model systems.

During the past few years, several other potential agents of viral gastroenteritis have been described, including enteric adenovirus, calicivirus, enteric coronavirus and astrovirus (1). Among these agents, the evidence seems strongest that "enteric" adenoviruses are medically important pathogens like rotavirus and Norwalk virus. These adenoviruses differ from the well characterized conventional serotypes of adenoviruses which are readily propagated in standard tissue cultures and are not commonly associated with gastroenteritis. The "enteric" adenoviruses are recognized by electron microscopy in stools and cultivatable in an adenovirus transformed cell line, Graham 293. Two such "enteric" serotypes (types 40 and 41) have been identified and in the few studies performed to date, have been highly associated with gastroenteritis in infants and young children and much less commonly found in asymptomatic children (1). The potential role of enteric adenoviruses in travelers' diarrhea or in disease in adults has not been studied. In addition, convenient and specific immunoassays to identify enteric adenoviruses are greatly needed.

The following Annual Progress Report covers only work accomplished during the period of August 1, 1983 - July 31, 1984

IDENTIFICATION, CHARACTERIZATION AND GROWTH OF GASTROENTERITIS VIRUSES

During the current contract year, we have taken several approaches to develop and improve methods to recognize gastroenteritis viruses. The results of these approaches are detailed below. Considerable effort has been made in the development of monoclonal antibodies against gastroenteritis viruses including rotavirus, Norwalk virus, and enteric adenovirus. In the case of Norwalk virus, successful development would permit a detailed analysis of the biochemical nature of the virus, provide a potential diagnostic probe for a possible group-specific antigen for the Norwalk-like virus group of agents, and also provide a highly sensitive and specific handle to detect Norwalk virus in inoculated cell cultures. In order to gain detailed experience with this methodology, we first developed monoclonal antibodies

1. Cukor, G., and Blacklow, N.R.: Human viral gastroenteritis. Microbiological Reviews 48:157-179, 1984.
37. Vesikari, T., Isolauri, E., D'Hondt, E., Delem, A., Andre, F.E. and Zissis, G.: Protection of infants against rotavirus diarrhea by RIT4237 attenuated bovine rotavirus strain vaccine. The Lancet 1:977-981, 1984.

against rotavirus, since laboratory handles for this virus are well-defined and readily exploited, in contrast to Norwalk virus and enteric adenovirus. As a result, we were able to develop new rapid detection assays for rotavirus using monoclonal antibody and to apply them with increased sensitivity for the detection of infections in certain age groups.

1. Monoclonal Antibody Studies With Rotavirus

In June of 1984, we published data with the use of a monoclonal antibody reagent directed against the group-specific antigen shared by mammalian rotaviruses (38). This reagent was used to detect rotavirus rapidly in human stools, with increased sensitivity and specificity compared with the currently widely used commercial assay (Rotazyme). A monoclonal antibody, 3F7, that reacts with the common rotavirus antigen on the sixth viral gene product was prepared against purified murine rotavirus (EDIM), a strain which lacks rotavirus subgroup prejudice since it contains the broadly cross-reactive mammalian rotavirus group-specific antigen without sharing either subgroup 1 or 2 specificity with various human rotavirus strains. The monoclonal antibody was used in a direct monoclonal antibody radioimmunoassay (RIA) as a diagnostic reagent for detection in 3.5 h, of rotavirus in human pediatric stool specimens. In the 177 samples tested, a concordance of 96% was seen between the monoclonal RIA and the well-established and commonly used commercially available Rotazyme test. Six discrepant specimens that were positive by monoclonal RIA but negative by Rotazyme were shown to be positive by either electron microscopy or confirmatory blocking immunoassay. A seventh discrepant specimen was positive by Rotazyme and negative by monoclonal RIA as well as by both direct and immune electron microscopy. The monoclonal RIA test is highly sensitive and specific, and is a rapid, convenient, diagnostic assay that can reduce currently encountered problems associated with diagnosing rotavirus infection by immunoassay (39).

Recently, we have adapted the rotavirus monoclonal antibody reagent to an enzyme-linked immunosorbent assay (EIA), and used the antibody as a detector reagent (in contrast to its use as a capture reagent in the RIA procedure). The expertise in immunoassay technology of Dr. John Herrmann (who has recently joined our research unit) has greatly aided this effort. The monoclonal rotavirus EIA is readily adaptable to use in field studies. Most important, it has greatly improved rotavirus EIA detection efforts with neonatal stool samples and adult stool specimens, both of which pose problems when tested by Rotazyme. Our studies will be presented at the 1984 ICAAC meeting and include Drs. Krause and Ogra as collaborators (40). The data, in summary, are as follows: the monoclonal EIA (M-EIA) test was compared with Rotazyme for rotavirus diagnosis in 163 stool specimens collected from children, adults and neonates. Discrepant results were resolved by either electron microscopy (EM), seroconversion, or confirmatory blocking EIA. Of 124 specimens from children, 61 were positive by both tests, 57 negative, and 6 positive by M-EIA only. These 6 were positive by EM and/or confirmatory EIA. In 28 neonatal samples, one was positive by EM, M-EIA and Rotazyme. There were 17 additional Rotazyme positive samples that were EM and M-EIA negative, giving a low

38. Cukor, G., Perron, D.M., Hudson, R., and Blacklow, N.R.: Detection of rotavirus in human stools using monoclonal antibody. J.Clin.Micro. 19:888-892, 1984.
39. Brandt, C.D., Kim, H.W., Rodriguez, W.J., Thomas, L., Yolken, R.H., Arrobio, J.O., Kapi-
kian, A.Z., Parrott, R.H., and Chanock, R.M.: Comparison of direct electron micro-
scopy, immune electron microscopy, and rotavirus enzyme-linked immunosorbent
assay for detection of gastroenteritis viruses in children. J.Clin.Micro. 13:
976-981, 1981.
40. Herrmann, J.E., Blacklow, N.R., Cukor, G., Perron, D., Krause, P.J., Hyams, J.S., Ogra, P.L.
and Barrett, H.: Monoclonal antibody enzyme immunoassay for detection of rota-
virus antigen in stool specimens. To be presented at 24th Annual ICAAC Meeting
October, 1984.

specificity (10/27) for Rotazyme in this group. Of 11 adult samples positive by seroconversion, all were positive by M-EIA and 6 by Rotazyme. We conclude that use of this monoclonal antibody eliminates false positives in neonates and provides greater sensitivity for detecting rotavirus in stools from both adults and young children.

2. Monoclonal Antibody Studies With Norwalk Virus

We are endeavoring to build upon the experience gained with our monoclonal antibody studies with rotavirus to develop monoclonal antibodies against Norwalk virus. To date we have been confronted with several logistical problems previously not presented with our rotavirus studies. With the noncultivable Norwalk virus, mice need to be immunized with virus, albeit partially purified from feces, that is nonetheless still surrounded by numerous fecal antigens. The only screening test available for monoclonal antibody to Norwalk virus is RIA which uses stool containing virus as antigen. Thus, most monoclonal antibodies that are produced following immunization with the Norwalk fecal inoculum will react with extraneous stool components. Furthermore, stool also contains human immunoglobulins (coproantibodies). As a result, many monoclonal antibodies that are produced react with the human serum which must be used as the coating reagent in the RIA test which screens for antibody to Norwalk virus. In addition, the presence of human immunoglobulins in the Norwalk fecal inoculum also results in the development of antihuman immunoglobulin serum antibodies by the mice. These antibodies preclude the possibility of monitoring mice for their development of Norwalk specific serum antibody in order to ascertain adequate and optimal schedules of immunization.

Despite these logistical problems, we have made progress in solving them. We have empirically determined an immunization schedule and dose which does not result in death of mice (which are immunized with human stool derived material). Efficiency in producing hybridomas has been increased by careful screening of lots of fetal calf serum and use of a growth factor in culture media. Our murine immunization studies designed to produce monoclonal antibodies specific for Norwalk virus are to date negative, using virus-containing fecal filtrate as the inoculum. We are currently subjecting our Norwalk virus inoculum to an extensive purification process (involving ion exchange, size exclusion and immunoaffinity chromatography) prior to inoculation into mice. This type of an approach has been published by others in the successful preparation of monoclonal antibody to hepatitis A virus derived from feces (41).

Another approach that we are using is the production of human-mouse heterohybridomas to Norwalk virus, an approach that obviates the problems encountered with immunization with stool derived material. These studies involve heparinized peripheral blood collected from volunteers convalescing from induced Norwalk illness at the University of Texas Health Science Center at Houston (Dr. Herbert DuPont collaborator). The human studies are conducted by Dr. DuPont as part of a program unrelated to this Army Contract. At various times during the current contract year, we have received blood collected from 10 ill volunteers 5 to 8 days after the onset of illness and again 2 to 4 weeks after illness. Enriched B lymphocyte cell fractions have been extracted from these blood samples and then fused with mouse myeloma cells. Large numbers of human-mouse heterohybridomas have been prepared. These have been expanded, cloned and stored in liquid nitrogen. We have screened hybridoma fluids and selected those with anti-human IgG secreting activity. These selected hetero-

41. MacGregor, A., Kornitschuk, M., Hurrell, J.G.R., Lehmann, N.I., Coulepis, A.G., Locarnini, S.A. and Gust, I.D.: Monoclonal antibodies against hepatitis A virus. J. Clin. Micro. 18:1237-1243, 1983.

hybridomas are now in the process of being tested for their antiviral specificity by using them as "coating" reagents in our Norwalk virus radioimmunoassay.

3. Adaptation of Norwalk Virus Radioimmunoassay to an Enzyme Immunoassay

To date, immunoassay studies with Norwalk virus have relied upon the radioimmunoassay (RIA) methodology. The recruitment of Dr. John Herrmann to our research unit in April, 1984 has brought expertise in enzyme immunoassay (EIA) techniques. Early experiments trying to adapt the Norwalk virus immunoassay to an EIA are encouraging. We are now testing panels of the defined human stool and paired serum reagents reported by us previously (10) by EIA. If the Norwalk assay can be adapted to an EIA, it will make the test available for performance in the field.

4. Cultivation Studies with Norwalk Virus.

Cell lines, selected because of their ability to support the growth of hepatitis A virus and rotavirus (36,42-45), have been inoculated with Norwalk virus and passaged 12 times on a roller apparatus at 36 °C in the presence of either trypsin or pancreatin. These cell lines are MA 104, FrhK4, FrhL2, BSC-1 and two continuous human fibroblast lines, HEL299 and HFF. Cell extracts and media are negative for Norwalk virus by RIA, and by immunofluorescence examination using human serum possessing high titers of Norwalk RIA antibody. In addition, human diploid cells have been inoculated with Norwalk virus and maintained at low (room) temperature, without production of Norwalk antigen detectable by RIA. Also, five cell lines of intestinal origin (46) (kindly supplied by Drs. Weisz and Burke of the Mallory Institute of Pathology at Boston City Hospital) have been tested for their capabilities to support the growth of Norwalk virus, as measured by RIA, and have showed no evidence of viral growth. These cell lines are colonic adenocarcinomas, epithelial-like in morphology, and in varying degrees of differentiation.

10. Blacklow, N.R., Cukro, G., Bedigian, M.K. et al: Immune response and prevalence of antibody to Norwalk enteritis virus as determined by radioimmunoassay. J. Clin. Micro. 10:903-909, 1979.
36. Urasawa, T., Urasawa, S., and Taniguchi, K.: Sequential passages of human rotavirus in MA-104 cells. Microbiol. Immunol. 25:1025-1035, 1981.
42. Provost, P.J. and Hilleman, M.R.: Propagation of human hepatitis A virus in cell culture. Proc. Soc. Exp. Biol. & Med. 160:213-221, 1979.
43. Flehmig, B.: Hepatitis A-Virus in cell culture. I. Propagation of different hepatitis A-virus isolates in a fetal rhesus monkey kidney cell line (FrhK-4). Med. Microbiol. Immunol. 168:239-248, 1980.
44. Lemon, S.M., Binn, L.N. and Marchwicki, R.H.: Radioimmunofocus assay for quantitation of hepatitis A virus in cell cultures. J. Clin. Micro. 17:834-839, 1983.
45. Siegl, G., Frosner, G.G., Gauss, V. et al: The physicochemical properties of infectious hepatitis A virions. J. Gen. Virol. 57:331-341, 1981.
46. Dexter, D.L.: Cancer Research 39:1020-1025, 1979.

Currently ongoing efforts to cultivate Norwalk virus rely upon two new approaches: (a) inoculation of human monocyte cultures, available to us through the courtesy of Dr. Francis Ennis and (b) inoculation of Norwalk virus into gnotobiotic calves through collaboration with Dr. Janice Bridger (Institute for Research on Animal Diseases, Compton, England).

5. Studies on Possible Relationship of Norwalk Virus and Feline Calicivirus

It has been suggested that Norwalk virus may be a member of the RNA-containing calicivirus group, based on its morphology, buoyant density, and presence of a single structural protein of a molecular weight of 66,000(47). We therefore examined the possibility that Norwalk virus might cross-react serologically with a well described cultivatable member of the calicivirus group, feline calicivirus. Through the courtesy of Dr. Leonard Binn, Walter Reed Army Institute of Research, we obtained a panel of hyperimmune goat anti-calicivirus sera and tested them for their reactivities in our standard blocking radioimmunoassay (RIA) for antibody to Norwalk virus. All were negative by failing to meet our criterion of a greater than 50% reduction in RIA counts. However, a few of the sera reduced counts by 40%, suggesting that maybe low levels of antibody could be present and nondetectable by our direct RIA blocking test. Therefore, we retested these sera in a modified indirect RIA test in the hope that this would provide added sensitivity. In this modified test, we replaced our usual labeled detector antibody with a radiolabeled antigoat serum designed to react with any goat anti-calicivirus antibody attached to Norwalk virus. Tested in this way, these serum specimens remained negative. Thus, there is no evidence of at least a one way serologic cross-reactivity between Norwalk virus and feline calicivirus. We are currently in the process of examining the question of a potential reciprocal serologic relationship---i.e., whether defined human sera possessing Norwalk antibody react with feline calicivirus strains (cultivated in Crandell feline kidney cells).

6. Enteric Adenovirus Studies

As outlined above in the "Background Information on Viral Gastroenteritis," the newly discovered "enteric" adenoviruses types 40 and 41 have been associated with gastroenteritis in infants and young children (1). Although there now seems to be good evidence for their medical importance in the pediatric age group, no studies have been performed on the role of these agents in travelers' diarrhea or in disease in adult age groups. The "enteric" adenoviruses are fastidious in their growth in cell culture, unlike the well described conventional serotypes that are established causes of acute respiratory tract disease (but not gastroenteritis). Conventional adenoviruses are commonly shed in the stool without gastrointestinal symptoms, so electron microscopic exam of feces cannot distinguish "enteric" adenoviruses from non-enteric strains. Adenoviruses types 40 and 41 can be cultivated in Graham 293 cells, a line of adenovirus type 5 transformed cells. However, their growth is inefficient, and uncomplicated specific quantitative methods for their identification and characterization are lacking. Currently, they can be identified and characterized by a combination of techniques such as electron microscopy, growth

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47. Greenberg, H.B., Valdesuso, J.R., Kalica, A.R., Wyatt, R.G., McAuliffe, V.J., Kapikian, A.Z., and Chanock, R.M.: Proteins of Norwalk virus. J. Virol. 37:994-999, 1981.

in Graham 293 cells, and detection in a cumbersome immunoassay (48). The latter relies upon hyperimmune polyclonal anti-adenovirus serum which is employed in an immunoassay after the serum has been adsorbed repeatedly with representative strains of other (non-"enteric") adenovirus subgroups. Adsorptions are performed in order to try to remove antibodies reactive with components of the non-enteric adenovirus strains.

These procedures clearly are not ideal, and a reliable, simplified, quantitative method for the study of enteric adenovirus is greatly needed. We have initiated efforts described below to develop such a method. Successful development will enable us to study and advance the understanding of the biology and epidemiology of the enteric adenoviruses.

Our plan is to develop an immunoassay for enteric adenoviruses, using monoclonal antibodies prepared against purified virions and adenovirus pentons (the structural component of adenovirus conferring type-specificity). We have already cultivated adenovirus types 40 and 41 in Graham 293 cells (49), purified each virus by cesium chloride density gradient ultracentrifugation, and inoculated mice with the intent of preparing monoclonal antibodies. In addition, we have isolated and purified pentons from each virus and inoculated mice with these preparations, also with the intent of preparing monoclonal antibodies. For reference polyclonal antibody reagents against adenoviruses 40 and 41, we have been kindly supplied with adsorbed rabbit antisera by Drs. S. Straus and H. Takiff (N.I.H.), R. Yolken (Johns Hopkins) and G. Wadell (Sweden). Successful development of specific immunoassays against enteric adenoviruses will enable us to examine stored specimens from our previously published studies of diarrheal disease in the U.S. and overseas. In some of these studies, stool specimens have shown adenovirus particles by electron microscopy. In addition, diarrheal stools from different age groups containing adenovirus particles are being collected for our use by two collaborators, Drs. P. Ogra (Buffalo) and P. Echeverria (U.S. Armed Forces Institute of Medical Sciences, Bangkok).

ETIOLOGY AND EPIDEMIOLOGY OF GASTROENTERITIS IN VARIOUS POPULATIONS

Because of the development of new laboratory techniques, it has become possible in recent years to assess the role of newly discovered viral agents in outbreaks of infectious nonbacterial gastroenteritis. These laboratory assays also enable us to study for the first time prevalence of these agents in different areas of the world and in various age groups. In collaboration with Dr. Peter Echeverria we have previously published several studies on the role of rotavirus in diarrhea among either American soldiers or native populations in South Korea, United States, Taiwan and

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49. Takiff, H.E., Straus, S.E., and Garon, C.F.: Propagation and in vitro studies of previously noncultivable enteral adenoviruses in 293 cells. The Lancet 2:832-834, 1981.

the Philippines (27,31-35,50-52). Also, with Dr. Echeverria we have examined the antibody prevalences to Norwalk virus in the Philippines, Taiwan and the United States (12) and in rural Thailand (53) and the potential role of Norwalk virus in diarrhea among Peace Corps volunteers who are newly arrived in Thailand (20). Also, with the University of Texas Medical Center at Houston group, we have shown roles for both rotavirus and Norwalk virus in travelers' diarrhea among American student travelers to Mexico (54) and have shown Norwalk virus to be responsible for at least a small proportion of family outbreaks of diarrhea in Texas (55). Clearly, based on the

12. Cukor, G., Blacklow, N.R., Echeverria, P., Bedigian, M.K., Puruggan, H. and Basaca-Sevilla, V. Comparative study of the acquisition of antibody to Norwalk virus in pediatric populations. Inf. & Immunity 29:822-823, 1980.
20. Echeverria, P., Blacklow, N.R., Sanford, L.B. and Cukor, G.: Travelers' diarrhea among American Peace Corps volunteers in rural Thailand. J. Inf. Dis. 143:767-771, 1981.
31. Echeverria, P., Ho, M.T., Blacklow, N.R. et al: Relative importance of viruses and bacteria in the etiology of pediatric diarrhea in Taiwan. J. Inf. Dis. 136:383, 1977.
32. Echeverria, P., Blacklow, N.R., Vollett, J.J. et al: Reovirus-like agent and enterotoxigenic Escherichia coli infections in pediatric diarrhea in the Philippines. J. Inf. Dis. 138:326, 1978.
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50. Echeverria, P., Blacklow, N.R., Smith, D.H. Role of heat-labile toxigenic Escherichia coli and reovirus-like agent in diarrhea in Boston children. The Lancet 2:1113-1116, 1975.
51. Echeverria, P., Blacklow, N.R. Ho, M.T. et al: Age distribution of antibody to reovirus-like agent in children in Taiwan. J. Ped. 91:960-962, 1977.
52. Cukor, G., Blacklow, N.R. Capozza, F.E. et al. Persistence of antibodies to rotavirus in human milk. J. Clin. Micro. 9:93-97, 1979.
53. Echeverria, P., Burke, D.S., Blacklow, N.R., Cukor, G., Charoenkul, C. and Yanggratoke, S.: Age specific prevalence of antibody to rotavirus, Escherichia coli heat-labile toxin, Norwalk virus, and hepatitis A virus in a rural community in Thailand. J. Clin. Micro. 17:923-925, 1983.
54. Kesswick, B.H., Blacklow, N.R., Cukor, G., DuPont, H.L., and Vollet, J.J.: Norwalk and rotavirus in travelers' diarrhea in Mexico. The Lancet 1:109-110, 1982.
55. Pickering, L.K., Blacklow, N.R. DuPont, H.L. and Cukor, G. Diarrhea due to Norwalk virus in families. J. Inf. Dis. 146:116-117, 1982.

studies to date, rotavirus and Norwalk virus need to be added to the list of pathogens responsible for diarrhea in different populations, with varying roles for each pathogen in different population groups. Additional data were collected and published during the current contract year, as outlined below.

1. Rotavirus A Cause of Severe Diarrhea in Adults in Rural Thailand

During the current contract year, we published in collaboration with Dr. Peter Echeverria a study of severe diarrhea produced by rotavirus in adults in rural Thailand (56). The data in summary were as follows: rotavirus was identified as the only etiological agent in 5% of adults (28 of 526) with diarrhea who were admitted to Bamrasnaradura Hospital in Nonthaburi, Thailand during a 1-year period. Infection was determined by detection of rotavirus in diarrheal stools by enzyme-linked immunosorbent assay accompanied by a greater than fourfold rise in serum complement fixation and radioimmunoassay antibody titers to rotavirus. Adults with clinical rotavirus infections were as severely ill as patients with most bacterial enteric infections; only patients with cholera passed more watery stools and were more dehydrated than those with rotavirus infections. Only 2 of the 28 adults with rotavirus infections had known recent contact with young children with diarrhea. Rotavirus infections in these adults occurred most frequently in the cooler, drier months in Thailand than during the rest of the year. We have concluded that in some settings rotavirus should be considered in the differential diagnosis of severe diarrhea in adults as well as in young children.

Specimens from many of these 526 Thai adults are currently being studied for evidence of infection with Norwalk virus and will also be studied for enteric adenoviruses when we have developed appropriate immunoassays to detect these agents.

2. Rotavirus Associated With Waterborne Illness in the U.S.

During the current contract year, we published in collaboration with Dr. Hopkins of the Colorado Department of Health a study implicating rotavirus as a cause of waterborne gastroenteritis (57). Norwalk virus is a well known cause of waterborne disease (13), but documentation of rotavirus as the etiologic agent in such outbreaks has been infrequent and inconclusive to date. Our published data in summary were as follows: A community waterborne nonbacterial gastroenteritis outbreak occurred in Eagle-Vail, Colorado in March, 1981. Illness (defined as vomiting and/or diarrhea) was statistically associated with water consumption (x_2 for linear trend = 7.07, $p < .005$). Five of seven persons associated with the outbreak were infected with rotavirus as shown by virus detection or serological methods. Bacterial pathogens, *Giardia lamblia*, and Norwalk virus were excluded as responsible agents. We have concluded that rotavirus should be looked for as a cause of waterborne gastroenteritis outbreaks.

13. Kaplan, J.E., Gary, G.W., Baron, R.C. et al: Epidemiology of Norwalk gastroenteritis and the role of Norwalk virus in outbreaks of acute nonbacterial gastroenteritis. Ann. Int. Med. 96:756-762, 1982.
56. Echeverria, P., Blacklow, N.R., Cukor, G., Vibulbandhitkit, S., Changehawalit, S. and Boothai, P.: Rotavirus as a cause of severe gastroenteritis in adults. J. Clin. Micro. 18:663-667, 1983.
57. Hopkins, R.S., Gaspard, B.G., Williams, F.P., Karlin, R.J., Cukor, G. and Blacklow, N.R.: A community waterborne gastroenteritis outbreak: Evidence for rotavirus as the agent. Amer. J. Public Health 74:263-265, 1984.

3. Shellfish Associated Gastroenteritis in the U.S. Due to Norwalk Virus

In collaboration with Drs. D. Morse, J. Hanrahan and R. Deibel of the New York Department of Health, we have linked Norwalk virus to several outbreaks of gastroenteritis induced by the ingestion of inadequately cooked shellfish (clams and oysters). The New York Department of Health studied 103 such outbreaks associated with shellfish during 1982. Twenty-two outbreaks occurred during the summer and 81 during the winter. At least two people were ill during each outbreak and some affected over 100 individuals. Appropriate acute and convalescent phase serum specimens were collected from 7 outbreaks; Norwalk virus was incriminated in 5 of these outbreaks (2 summer and 3 winter outbreaks) based on seroconversions by RIA to the virus. In addition, IgM antibody to Norwalk virus was found in individuals from all 5 outbreaks. Clams from several outbreaks have been studied for the presence of Norwalk virus antigen by RIA, and preliminary data have revealed Norwalk virus in some clams. The New York state outbreaks of shellfish-associated gastroenteritis during 1982 are impressive because of the large numbers of affected individuals. A preliminary report of some of the clinical and epidemiological features of these outbreaks appeared in the CDC Morbidity and Mortality Weekly Report of August 27, 1982. A manuscript providing details of these outbreaks and their link to Norwalk virus is currently in preparation.

During the spring of 1983, several gastroenteritis outbreaks were noted in Hawaii associated with consumption of undercooked or raw clams. In collaboration with Dr. J. Greenspan of the Hawaii Department of Health, 8 paired sera were available for study from these outbreaks. Three of the 8 seroconverted to Norwalk virus by RIA and the remaining 5 showed high titers in both acute and convalescent specimens (all 5 "acute" sera were drawn at least a week after disease onset). As is the case with the New York State outbreaks, preliminary data have revealed Norwalk virus antigen in some clams by RIA.

4. Food-Borne Gastroenteritis Due to Norwalk Virus

In collaboration with Dr. Jenkins of C.D.C., we have linked Norwalk virus to an outbreak of gastroenteritis that occurred during the fall of 1982 at a college campus. Symptoms and clinical course were compatible with gastroenteritis caused by Norwalk virus and 7 out of 9 paired sera from symptomatic people demonstrated seroconversions by RIA to Norwalk virus. Eating food from a salad bar in the campus dining room was significantly associated with illness ($p=0.0016$). Deficiencies in kitchen sanitation and a salad maker who worked while she had diarrhea may have contributed to the outbreak. These data are being prepared for publication.

5. Peace Corps Volunteer Study in Thailand

We have previously reported (20), in collaboration with Dr. Peter Echeverria, on the role of multiple pathogens in travelers' diarrhea occurring in Peace Corps volunteers who are newly arrived in Thailand. Norwalk virus was associated with 3% of illnesses in that report. We have had the opportunity to participate in a similar study performed in another group of Peace Corps volunteers entering Thailand. In this second study, 2 ill of 62 volunteers seroconverted to Norwalk virus by RIA and none to rotavirus during their first five weeks in Thailand. This second study was also performed in collaboration with Dr. R.B. Sack, and provided further supportive evidence of Aeromonas hydrophila as an enteric pathogen as part of a trial for prophylactic doxycycline in travelers' diarrhea. A summary of this second study, which

20. Echeverria, P., Blacklow, N.R., Sanford, L.B. and Cukor, G.: Travelers' diarrhea among American Peace Corps volunteers in rural Thailand. J. Inf. Dis. 143:767-771, 1981.

is to be published in the American Journal of Epidemiology (58), is as follows: A randomized double blind study to determine the efficacy of doxycycline (100 mg daily) in preventing travelers' diarrhea was performed among 63 American Peace Corps volunteers during their first five weeks in Thailand. Eight (24%) of 33 volunteers taking placebo and three (10%) of 30 taking doxycycline developed travelers' diarrhea ($p=0.12$) while nine (30%) of 30 taking doxycycline and ten (30%) of 33 taking placebo developed mild diarrhea during their first three weeks in the country. Aeromonas hydrophila was isolated from four of eight volunteers with travelers' diarrhea in the placebo group, but none of three with travelers' diarrhea in the treatment group ($p=0.21$). In those with mild diarrhea, A. hydrophila was isolated from four of ten of the placebo group, and one of nine in the treatment group ($p=0.18$). Enterotoxigenic Escherichia coli were isolated from only one volunteer with travelers' diarrhea in the placebo group. Doxycycline prophylaxis of travelers' diarrhea, but not mild diarrhea, appeared to be due partially to prevention of A. hydrophila rather than enterotoxigenic E. coli infections. This experience further supports the role of A. hydrophila as an enteric pathogen.

Recently, in collaboration with Dr. Echeverria, we have examined paired sera for antibodies to Norwalk virus from a third group of Peace Corps volunteers during their first 5 weeks in Thailand (August-September, 1983). Three of 35 individuals studied seroconverted to Norwalk virus by RIA (2 individuals with diarrhea), a proportion similar to that reported in the two earlier studies. This third study revealed that the overall illness attack rate, the bacterial isolation rate, and the variety of pathogens was similar to previous studies. However, in this study, Salmonella was as frequently isolated as the etiologic agent as was heat labile toxigenic E. coli, and Shigella was notably missing from the list of pathogens. In this study, in retrospect, the use of antibiotics would have been of little help in preventing these episodes of diarrhea.

PATHOGENESIS STUDIES OF VIRAL GASTROENTERITIS

1. IgM Responses to Norwalk Virus in Naturally Occurring Illness

We have previously reported that there is an IgM serum antibody response to Norwalk virus in volunteers following primary infection as well as reinfection (14). It has not been known, however, whether this response may have diagnostic utility in assessing naturally occurring disease outbreaks by examining single serum samples. We attempted to answer this question, in collaboration with Dr. R. Baron, C.D.C., through the study of a Norwalk virus gastroenteritis outbreak that occurred among teenagers at a camp in Brevard, North Carolina in the fall of 1979. Twenty-one teenagers who were exposed to a common source (contaminated water) during this outbreak were tested for seroconversion by RIA to Norwalk virus. Serum pairs were collected within 72 hours of exposure and four weeks later. Each of the 11 subjects who developed symptoms and 5 persons who remained well demonstrated a total serum antibody response to Norwalk virus by RIA, while 5 non-ill subjects failed to seroconvert. Absence of detectable antibody in acute phase serum specimens was not statistically associated with subsequent seroconversion or susceptibility to illness. These findings underscore the view, previously derived by us from volunteer studies (8,10), that immunity to Norwalk virus is not determined by serum antibody and support the concept that susceptibility may be determined by Norwalk-specific intestinal receptor sites.

14. Cukor, G., Nowak, N. and Blacklow, N.R.: Immunoglobulin M responses to the Norwalk virus of gastroenteritis. Infection and Immunity 37:463-468, 1982.
58. Echeverria, P., Sack, R.B., Blacklow, N.R., Bodhidatta, P., Rowe, B., McFarland, A.: Prophylactic doxycycline for travelers' diarrhea in Thailand: Further supportive evidence for Aeromonas hydrophila as an enteric pathogen. Amer. J. Epidem., In Press.

Norwalk specific IgM responses were detected in only 7 of the 11 persons with illness (64%) and in 9 of the 16 subjects who seroconverted (56%). Detection of an IgM response was favored in persons who had evidence of preexisting antibody and were probably experiencing a secondary infection. It may be that primary Norwalk IgM responses, which peak two weeks after exposure and fall rapidly thereafter (14) were missed in several persons by our collection of convalescent-phase specimens at 4 weeks. The diagnostic utility of IgM in the evaluation of naturally occurring Norwalk disease may be improved by collecting convalescent-phase serum specimens at a shorter time interval. These data will be published in the Journal of Infectious Diseases in October, 1984 (59).

2. Cell Mediated Immune Responses to Rotavirus Infection

Nearly all of the published data concerning the immune responses to rotavirus infections concern antibody responses in animals and man, following either experimental or natural infection (1). Data on the role of humoral immune responses in protection from or cure of rotavirus infection, although plentiful, are somewhat contradictory (1). Local antibodies (intestinal IgA and IgG) in one report correlated with protection from and cure of rotavirus illness, whereas in other published reports no such correlation could be demonstrated in human volunteers or mice. In one report concerning adult volunteers, resistance to illness and/or infection correlated with the level of existing serum antibody. However, recent data do not suggest a primary protective role for the systemic antibody response in children. Little obvious correlation was noted between seroconversion after administering an experimental vaccine and protection to natural infection (37). It is generally recognized that both experimental and natural rechallenge studies indicate that prior infection significantly ameliorates subsequent clinical symptoms associated with reinfection which occurs in nature with the same or different viral strain, but the mechanism(s) of protection are unknown (1).

In view of the above, it is important to assess the role of cell mediated responses to rotavirus infections, the immune responses to which are likely to be more complicated than previously imagined. That few data have been available concerning cell mediated immune (CMI) responses is probably due to two major obstacles: (a) difficulties in establishing expertise in a suitable animal model to obtain a thorough understanding of the pathogenesis and recovery from infection, and (b) fastidiousness of rotavirus in cell culture, hindering the development of suitable virus infected target cells. The murine model of this infection seems ideal for the dissection of the immune responses to rotavirus infection, first, because we and others have previously published several of the clinical and pathological features of this infection in mice (60). Furthermore, evaluation of effector lymphocyte responses can be performed in H-2 defined inbred mouse

1. Cukor, G., and Blacklow, N.R.: Human viral gastroenteritis. Microbiological Reviews 48:157-179, 1984.
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strains. Finally, in collaboration with Dr. Francis Ennis of our infectious diseases research unit and postdoctoral fellow, Dr. Andre Dascal, we have now adapted murine rotavirus to cell culture for the first time; we have developed early results arising in an H-2 defined murine cell line infected with rotavirus indicating that it or alternative murine cell cultures should be suitable for use as a defined target cell for cytotoxicity assays.

Our data will be presented at the October, 1984, meeting of the Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) (61). They may be summarized as follows: Murine rotavirus, heretofore noncultivable, was serially passaged in enzyme treated and rotated MA104 cells. CD-1 suckling mice were infected with stool derived murine rotavirus, sacrificed, and filtered gut preparations used to inoculate MA104 cells. Proteolytic enzymes (trypsin and pancreatin) were added to cell culture media, and were required for production of viral CPE. Serial passage of murine rotavirus was monitored by CPE, indirect FA, and by solid phase RIA and monoclonal EIA of supernates which yielded P/N values of about 20. The RNA electropherotypes of stool and culture derived murine rotavirus were indistinguishable, and differed from that of SA11 (simian) rotavirus. MA104-produced murine rotavirus was used to infect an H-2 defined (H-2b) continuous murine cell line, Mc57G (C57b1), a suitable target for detecting rotavirus-specific cytotoxic T-lymphocyte responses. Infected Mc57G cells demonstrated rotavirus-specific cytoplasmic antigen by indirect FA. Mc57G should be useful as infected murine target cells for the analysis of various CMI responses to murine rotavirus infection.

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PUBLICATIONS OF WORK SUPPORTED BY THIS CONTRACT FOR THE CONTRACT YEAR 1983-1984

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