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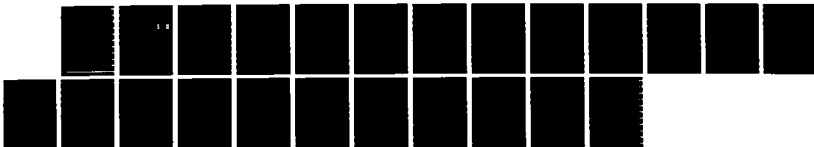
THE ETIOLOGY AND PATHOGENESIS OF VIRAL GASTROENTERITIS
(U) MASSACHUSETTS UNIV MEDICAL SCHOOL WORCESTER
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THE ETIOLOGY AND PATHOGENESIS OF VIRAL GASTROENTERITIS

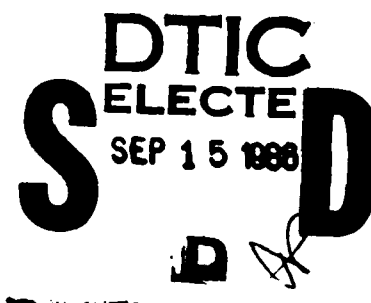
Annual Progress Report

by

Neil R. Blacklow, M.D.

31 July, 1983

(For the Period 1982 - 1983)



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stools. These tests provide shortened performance times as well as increased sensitivity and specificity compared to the currently commercially available assay. We are now building upon this experience gained with rotavirus to develop monoclonal antibodies against Norwalk virus, which is to date noncultivable and fails to produce illness in animal model systems. Approaches are being taken to circumvent logistical problems that have developed in the preparation of monoclonal antibodies to stool derived Norwalk virus. When produced, these antibodies will permit analysis of the biology of Norwalk virus, provide a potential diagnostic probe for a possible group-specific antigen common to the noncytopathic Norwalk-like virus group, and permit recognition of these viruses in nature. In addition, a series of experiments designed to cultivate Norwalk virus in vitro have been undertaken. Initial results using radioimmunoassay analysis of inoculated trypsin treated virus and cultures are negative with additional cell culture studies and immunofluorescence analysis in progress.

We have continued studies on the etiology and epidemiology of gastroenteritis with data acquired in several study populations. No evidence was found for a Norwalk virus etiology of acute diarrheal disease experienced in Egypt by U.S. Naval personnel of the U.S.S. Dwight D. Eisenhower. Norwalk virus was shown to be the likely cause of at least a small proportion of endemic family diarrhea in Texas. A rapid development of antibody to Norwalk virus was found during early childhood among inhabitants of a rural Thailand village. Severe clinical gastroenteritis in hospitalized adults attributable to rotavirus was noted in a prospective diarrheal disease study in Thailand. Findings consistent with a rotaviral etiology were obtained in a waterborne gastroenteritis outbreak in Colorado. Finally, we have implicated Norwalk virus as the cause of several gastroenteritis outbreaks in the U.S., including its role in widespread illness produced by consumption of raw and undercooked clams.



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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. A monoclonal antibody reagent has been developed that is directed against the group specific antigen shared by mammalian rotaviruses; this reagent has then been used to develop two novel rapid diagnostic tests for rotavirus in human stools. These tests provide shortened performance times as well as increased sensitivity and specificity compared to the currently commercially available assay. We are now building upon this experience gained with rotavirus to develop monoclonal antibodies against Norwalk virus, which is to date noncultivable and fails to produce illness in animal model systems. Approaches are being taken to circumvent logistical problems that have developed in the preparation of monoclonal antibodies to stool derived Norwalk virus. When produced, these antibodies will permit analysis of the biology of Norwalk virus, provide a potential diagnostic probe for a possible group-specific antigen common to the noncytopathic Norwalk-like virus group, and permit recognition of these viruses in nature. In addition, a series of experiments designed to cultivate Norwalk virus *in vitro* have been undertaken. Initial results using radioimmunoassay analysis of inoculated trypsin treated virus and cultures are negative with additional cell culture studies and immunofluorescence analysis in progress.

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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, a newly discovered 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, the 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

1. Blacklow, N.R. and Cukor, G.: Viral gastroenteritis. New Eng. J. Med. 304:397-406, 1981.

major group of agents responsible for viral diarrhea, the Norwalk-like viruses (2). 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 similar Norwalk-like viruses, such as Hawaii and Ditchling viruses, have been uncovered by similar techniques; these two agents appear to form (with Norwalk virus) three immunologically distinct agents 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 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

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1. Blacklow, N.R. and Cukor, G.: Viral gastroenteritis. New Eng. J. Med. 204:397-406,1981.
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 8. Parrino, T.A., Schreiber, D.S., Trier, J.S., Kapikian, A.Z., Blacklow, N.R.: Clinical immunity in acute gastroenteritis caused by Norwalk agent. New Eng. J. Med. 297:86-89,1977.

seroconvert to the agent (8-10). In addition, antibody to Norwalk virus in pre-challenge 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 ill subjects are reexposed after 6 to 14 weeks.

During the past 5 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 viruses inasmuch as reinfection produces an enhancement of the IgM response. Furthermore, these IgM responses in volunteers appear not to be associated with subclinical illness.

8. Parrino, T.A., Schreiber, D.S., Trier, J.S., Kapikian, A.Z., Blacklow, N.R.: Clinical immunity in acute gastroenteritis caused by Norwalk agent. New Eng. J. Med. 297:86-89,1977.
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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.

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 identified

13. Greenberg, H.B., Valdesuso, J., Yolken, R.H. et al: Role of Norwalk virus in outbreaks of non-bacterial gastroenteritis. J.Inf.Dis. 139:564-568,1979.
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17. Gunn, R.A., Terranova, W.A., Greenberg, H.B. et al: Norwalk virus gastroenteritis aboard a cruise ship: An outbreak on five consecutive cruises. Am.J.Epid. 112 :820-827,1980.
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21. Bishop, R.F., Davidson, G.P., Holmes, I.H., et al: Detection of a new virus by electron microscopy of fecal extracts from children with acute gastroenteritis Lancet 1:149-151,1974.
22. Kapikian, A.Z., Kim, H.W., Wyatt, R.G. et al: Human reovirus-like agent as the major pathogen associated with "winter" gastroenteritis in hospitalized infants and young children. New Eng. J. Med. 294:965-972, 1976.
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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 cell culture by incorporating low concentrations of trypsin into the culture medium(36).

19. Kesswick, B.H., Blacklow, N.R., Cukor, G., DuPont, H.L. and Vollet, J.J.: Norwalk virus and rotavirus in travellers' diarrhoea in Mexico. The Lancet 1:109-110, 1982.
21. Bishop, R.F., Davidson, G.P., Holmes, I.H., et al: Detection of a new virus by electron microscopy of fecal extracts from children with acute gastroenteritis. Lancet 1:149-151, 1974.
22. Kapikian, A.Z., Kim, H.W., Wyatt, R.G. et al: Human reovirus-like agent as the major pathogen associated with "winter" gastroenteritis in hospitalized infants and young children. New Eng. J. Med. 294:965-972, 1976.
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33. Echeverria, P., Hodge, F.A., Blacklow, N.R. et al: Travelers' diarrhea among United States Marines in South Korea. Amer. J. Epid. 108:68, 1978.
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., Vollet, 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.

During the past few years, several other potential agents of viral gastroenteritis have been described, including enteric adenovirus, calicivirus, enteric coronavirus and astrovirus (1). The extent of the medical importance of these agents is for the most part uncertain, in contrast to Norwalk virus and rotavirus.

The following Annual Progress Report covers only work accomplished during the period of August 1, 1982 - July 31, 1983.

IDENTIFICATION, CHARACTERIZATION AND GROWTH OF GASTROENTERITIS VIRUSES

During the current contract year, considerable effort has been made to develop monoclonal antibodies against gastroenteritis viruses. In the case of Norwalk virus, successful development would permit for the first time 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 experience with this methodology, we decided first to develop monoclonal antibodies against rotavirus, since laboratory handles for this agent are well-defined and readily exploited, in contrast to Norwalk virus. In the process of using this methodology, we were able to develop new rapid detection assays for rotavirus using monoclonal antibody.

1. Rapid Detection Assays for Rotavirus Using Monoclonal Antibody

We have developed a monoclonal antibody reagent directed against the group-specific antigen shared by mammalian rotaviruses. This reagent has then been used to develop two novel rapid detection tests for rotavirus in human stools; these tests provide shortened performance times as well as increased sensitivity and specificity compared to the currently widely used commercially available assay.

Preparation of antibody used in these assays has been as follows: Monoclonal antibody. BALB/c mice were immunized with sucrose gradient purified murine rotavirus (EDIM). EDIM does not share either subgroup 1 or 2 specificity with human rotavirus but does contain the broadly cross-reacting mammalian rotavirus group-specific antigen. Spleen cells from immunized mice were fused with SP2 mouse myeloma cells and hybridoma antibodies of interest were selected by screening for reactivity in an RIA test that employs simian rotavirus, SA-11. Monoclonal cultures were established after double cloning by limiting dilutions. One monoclonal antibody, 3F7 was selected for use in the assay based on its uniformly strong reactivity with murine, simian, bovine and human rotaviruses. 3F7 is an IgG2 kappa antibody which we have shown by polyacrylamide gel electrophoretic studies to react with VP6 of SA-11 (37). Large quantities of purified 3F7 IgG were prepared by affinity chromatography of culture supernate on protein A-Sepharose followed by ammonium sulfate precipitation. Rabbit antiserum. New Zealand white rabbits, pre-selected for low rotavirus antibody titers, were immunized with gradient purified SA-11. High titered purified IgG fraction of rabbit serum was prepared by ammonium sulfate precipitation and ion exchange chromatography on DEAE-cellulose.

1. Blacklow, N.R. and Cukor, G.: Viral gastroenteritis, New Eng. J. Med. 304:397-406, 1981.
37. Mason, B.B., Graham, D.Y., and Estes, M.K.: In vitro transcription and translation of simian rotavirus SA11 gene products. J. Virol. 33:1111-1121, 1980.

Our antisera have then been used to establish the following RIA diagnostic test procedures:

(a) Multideterminant one-step RIA. This procedure takes advantage of the fact that while monoclonal antibody only reacts with a single viral epitope, conventional rabbit antiserum reacts with several antigenic determinants. Test stool samples are mixed with monoclonal antibody, allowed to incubate for 15 minutes, and then added to microtiter plates precoated with rabbit antiserum. Radioactive anti-mouse immunoglobulin is then used as the detection antibody. The total performance time of this test is approximately 4 hours.

(b) Monoclonal antibody capture assay. In this test configuration, microtiter plates are coated with purified 3F7 IgG. Test stool samples are added and ¹²⁵I-labelled rabbit IgG is then used as the detection antibody. Performance time for this test is approximately 3 hours. This test as well as the multideterminant one-step RIA test, has been carefully optimized as to concentration of reagents and incubation times and temperatures.

Our two RIA monoclonal antibody diagnostic test procedures have been evaluated with 177 human stool samples. 82 of these samples are known to be positive for rotavirus by a commercial ELISA test (Rotazyme), and 95 are negative. With but one exception, all Rotazyme positive stools are positive by both monoclonal antibody assays. This one specimen likely represents a false-positive Rotazyme test since it is negative for rotaviral particles by direct EM. A few Rotazyme false-negative samples also have been found, which are positive by both monoclonal antibody assays and by direct EM. We have also shown by antigen titration studies that our two new assays detect rotaviral antigen to equivalent or greater titers than does Rotazyme. Also, the P/N values in the 2 new RIA tests are considerably higher than in previously described RIA tests for rotavirus. Thus, use of our monoclonal antibody diagnostic test procedures provides (1) results obtained more rapidly; (2) more sensitive tests and (3) a high degree of specificity that avoids well described problems with immunoassays for rotavirus (38). Our data have recently been submitted for publication.

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

38. Brandt, C.D., Kim, H.W., Rodriguez, W.J., Thomas, L., Yolken, R.H., Arrobio, J.O., Kapikian, A.Z., Parrott, R.H., and Chanock, R.M.: Comparison of direct electron microscopy, immune electron microscopy, and rotavirus enzyme-linked immunosorbent assay for detection of gastroenteritis viruses in children. J.Clin. Micro. 13:976-981, 1981.

immunoglobulins (copro-antibodies). 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 achieved progress in solving them. We have empirically determined an immunization schedule and dose which does not result in the death of the animals (which are immunized with human stool derived material). Our efficiency in producing hybridomas has been increased by including a growth factor in the culture media and by carefully selecting the lot of fetal calf serum used. In our initial studies, reported upon in last year's Progress Report, we used as our immunizing antigen stool filtrate that contains Norwalk virus and that is not used as a reagent in our RIA tests. A hybridoma (and 4 clones derived from it) was first isolated which produced monoclonal antibody apparently specific for Norwalk virus by the criteria that it reacted in RIA only with a Norwalk virus containing stool and not with a panel of Norwalk virus-negative stools (including a pre-Norwalk challenge volunteer stool (RIA-negative) from the same individual who provided the Norwalk positive stool). Our RIA procedure tested for reactivity of monoclonal antibody against known Norwalk virus positive and negative stools which were all used at a 1:20 or a 1:50 dilution (the dilutions for stool used in RIA diagnostic tests for Norwalk virus). However, when our 5 monoclonal antibody preparations were retested against undiluted Norwalk virus-containing and control stool samples, they proved not to be specific for Norwalk virus but were apparently reacting with a stool component which is bound to the solid phase in our RIA and that is present in variable amounts in some stool specimens---i.e., detectable at a 1:1 but not at a 1:20 dilution.

Subsequent to the above observation, we have prepared three additional monoclonal antibodies with the same experience of a differential reactivity between undiluted control stool and feces diluted to 1:20 or 1:50. Currently, we have 6 additional monoclonal antibodies that are still under investigation and which we hope will yield specificity for Norwalk virus.

In addition, we have been using an altered Norwalk virus-containing inoculum that is used to immunize mice in an effort to purify further the inoculum free of extraneous fecal antigenic components. Mice have been inoculated with Norwalk antigen-antibody complexes prepared in two ways: (a) Complexes have been attached to Sepharose beads (initially attached with high titered Norwalk antibody containing human serum that then was linked to Norwalk virus containing stool as shown by RIA) and used to inoculate mice by the intraperitoneal route. Mice have tolerated the inoculation with the antigen containing beads and will soon provide splenic cells for hybridoma experiments. (b) Another set of mice has recently received ultracentrifuge pelleted Norwalk antigen-antibody complexes without beads, prepared in the manner described for immune electron microscopy (IEM) (4).

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It should be noted that animals inoculated with antigen-antibody complexes can be expected to produce monoclonal antibodies to human serum. To overcome initial interference by these antibodies in the selection of useful hybridomas, all monoclonal antibodies will be diluted in Norwalk antibody-negative human serum prior to their testing in our RIA test.

Finally, we are planning to use two additional approaches to prepare monoclonal antibody to Norwalk virus, which are described in detail in our Contract Renewal Application: (a) In vitro immunization to produce monoclonal antibody, using Norwalk antigen affinity purified from Sepharose beads and (b) Production of human hybridoma antibody to Norwalk virus, by using peripheral blood lymphocytes derived from volunteers (at the University of Texas Medical Center, Dr. Herbert DuPont) convalescing from Norwalk illness and fusing these lymphocytes with myeloma cells to produce human hybridoma antibody. This latter human hybridoma antibody approach seems to us to provide a good possibility for success since it has already been used successfully to prepare human hybridoma antibody to tetanus toxin (39) and since it obviates the problems that we have encountered with immunizations with stool derived material.

3. Cultivation Studies With Norwalk Virus

Efforts to try to grow the currently noncultivable Norwalk virus have been intensified and accelerated because of the convenient laboratory handle that now exists to identify the virus, RIA. Cell cultures inoculated with Norwalk virus can be monitored by RIA for evidence of noncytopathic viral replication. During this contract year, we have inoculated 6 cell lines with Norwalk virus (four continuous simian lines, MA104, FrhK4, FrhL2, BSC-1, and two continuous human fibroblast lines, HEL 299 and HFF). These cell lines have been chosen, in part, because they have supported the replication of hepatitis A virus, and rotavirus, previously noncultivable agents (40-). Cell extracts and media have been tested for Norwalk virus by RIA with negative results, even after 12 passages. Cells and virus have also been treated with trypsin (1ug/ml and 10ug/ml, respectively), incubated on a roller apparatus at 36°C, and then tested after 12 passages by RIA, with negative results. Finally, Norwalk virus has been centrifuged on to cells, passaged 12 times, again with no evidence of viral replication by RIA. In all series of experiments, coverslips have been collected and stored for subsequent examination by immunofluorescence for Norwalk viral antigen, using human serum possessing high titers of Norwalk RIA antibody. These immunofluorescence analyses are currently in progress.

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4. Radioimmunoassay for Study of Hawaii Virus

The Hawaii virus is a Norwalk-like virus, 27nm in diameter, which has been second to the Norwalk virus as the best studied Norwalk-like virus in human volunteers and by immune electron microscopy. During the current contract year, we have attempted to establish an RIA test for the Hawaii virus using human reagents. We have followed our RIA procedure for Norwalk virus (30). We have been unsuccessful in establishing this RIA test using the limited numbers of stored human stool and serum samples from previously studied Hawaii virus infected individuals. This is not surprising since in order to establish the Norwalk virus RIA test, it was necessary to painstakingly search through many human specimens for the right match of human serum and stool combinations. Our future plans to try to establish an RIA test for Hawaii virus are to use new stool and serum materials collected from volunteers to be inoculated at the University of Texas Medical Center (Dr. Herbert DuPont) as part of their ongoing program of Norwalk-like virus volunteer studies.

ETIOLOGY AND EPIDEMIOLOGY OF GASTROENTERITIS IN VARIOUS POPULATIONS

Because of the development of new laboratory techniques, including RIA, it has become possible in recent years for the first time 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 the Philippines (27,31-35,44-46).

27. Blacklow, N.R., Echeverria, P., Smith, D.H.: Serologic studies with reovirus-like enteritis agent. Infect. Imm. 13:1563-1566, 1976.
30. Blacklow, N.R. and Cukor, G.: Viral gastroenteritis agents. Manual of Clinical Microbiology, Third Edition, Ed. Lennette, E. et al (American Society for Microbiology) 891-898, 1980.
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More recently with Dr. Echeverria we have examined the antibody prevalences to Norwalk virus in the Philippines, Taiwan and the United States (12) and the potential role of Norwalk virus in diarrhea among Peace Corps volunteers who are newly arrived in Thailand (20). Also, recently, 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 (47). Clearly, based on the 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. Norwalk Virus Studies in U.S. Naval Personnel in Egypt

During the current contract year, we have examined 45 stool samples and 48 paired (acute and convalescent) serum specimens collected by Dr. Louis Bourgeois of NAMRU3, Cairo, Egypt, from crew members of the U.S.S. Dwight D. Eisenhower. Most of these individuals experienced acute diarrheal disease during or immediately following an Alexandria, Egypt port visit in May of 1982. Studies for bacterial (including toxigenic E. Coli and Campylobacter) and parasitic pathogens performed by NAMRU3 were negative. All stool samples were negative for Norwalk virus by RIA, and only one serum pair showed a 4 fold or greater rise in antibody titer to Norwalk virus (1:50 to 1:400 change in titer). Interestingly, the individual who seroconverted did not experience diarrheal illness. Future collaborative studies with Dr. Bourgeois are planned.

2. Norwalk Virus Studies in Texas Families with Diarrhea

In collaboration with Dr. Herbert DuPont and associates of the University of Texas Health Science Center at Houston, we have published during the current contract year a report on the role of Norwalk virus in endemic diarrhea among families under epidemiological surveillance (48). Although Norwalk virus is known to be an important cause of epidemic diarrhea, the importance of its role in endemic diarrhea is uncertain. Of 28 families under surveillance for 2 years following 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.
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birth of a newborn infant, 14 families developed outbreaks of diarrhea. Sero-logic evidence for a Norwalk virus etiology was found in 2 of the 14 outbreaks (14%). This initial study demonstrated that Norwalk virus is the likely cause of at least a small proportion of family outbreaks of diarrhea.

3. Antibody Prevalences to Norwalk Virus and Rotavirus in a Rural Community in Thailand

In collaboration with Dr. Peter Echeverria of AFRIMS, Bangkok, Thailand, we have published during the current contract year a report on the age specific prevalences of antibody to rotavirus, E. Coli heat-labile enterotoxin, Norwalk virus and hepatitis A in the rural Thai community of Soongnern (240 km northeast of Bangkok) (49). Most inhabitants acquired RIA antibody to rotavirus between the ages of 6 months and 6 years, 60% developed RIA antibody to Norwalk virus by the ages of 4 and 5 years, and most to hepatitis A between the ages of 6 and 35 years. Antibody to E. Coli heat-labile toxin was most prevalent between 1 and 4 years and 18 and 25 years of age. The 60 percent rate of antibody positivity to Norwalk virus reached by age 5 is only reached in United States populations by adulthood. The different patterns of antibody acquisition to Norwalk and hepatitis A viruses suggests that the epidemiology of infections with these two viral pathogens may be different. It is already known that both viruses are transmitted by the fecal-oral route and that Norwalk infection can also be transmitted by a waterborne route.

4. Rotaviral Illness Among Adults in Thailand

In collaboration with Dr. Peter Echeverria, we have demonstrated rotavirus to be a cause of severe gastroenteritis in adults with diarrhea admitted to Bamrasnradura Hospital in Nonthaburi, Thailand. During a 1 year period (10/1/80-9/30/81), 526 patients over 18 years of age admitted to the hospital were evaluated prospectively for the etiology of their diarrhea. Rotavirus infections occurred in five percent (28/526) of the patients. Infection was determined by detection of rotavirus in diarrheal stools by ELISA accompanied by a greater than fourfold rise in serum CF and RIA 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 two of the 28 adults with rotavirus infections had known recent contact with young children with diarrhea. Rotavirus infections in these adults occurred more frequently in the cooler, drier months in Thailand than during the rest of the year. In some settings, then, rotavirus should be considered in the differential diagnosis of severe diarrhea in adults as well as in young children. These data have recently been accepted for publication in the Journal of Clinical Microbiology.

5. Rotavirus Associated With Waterborne Illness In U.S.

Although Norwalk virus is well known to be spread frequently by a waterborne route, this is not clearly established to be the case for rotavirus. In collaboration with Dr. Richard Hopkins of the Colorado Department of Health, we have studied

49. Echeverria, P., Burke, D.S., Blacklow, N.R., Cukor, G., Charoenkul, C. and Yanggratoke, S. Age specific prevalence of antibody to rotavirus, Norwalk virus, hepatitis A virus and Escherichia coli heat-labile toxin in a rural community in Thailand. J.Clin.Micro. 17:923-925, 1983.

the etiology of a community waterborne nonbacterial gastroenteritis outbreak that occurred in Eagle-Vail, Colorado in March, 1981. The clinical syndrome among affected individuals, with fever rare, duration short, and vomiting prominent was characteristic of viral gastroenteritis. Illness (defined as vomiting and/or diarrhea) was statistically associated with water consumption ($p \leq 0.005$). Five of seven persons associated with the outbreak were infected with rotavirus as shown by virus detection (EM) or serological (RIA) methods. Parasitic and bacterial pathogens (including heat-labile and heat-stable toxigenic *E. Coli*) were excluded as responsible agents, as was Norwalk virus. Although this outbreak has not been conclusively shown to be caused by rotavirus, the data are consistent with a rotaviral etiology. Certainly, rotavirus should be carefully looked for in the future as a cause of waterborne outbreaks of gastroenteritis. These data have recently been accepted for publication (50).

6. Norwalk Virus Gastroenteritis Outbreaks in the U.S.

During the current contract year, we have implicated Norwalk virus as the etiology of three outbreaks of gastroenteritis in the United States. These studies are helpful to us in two ways: (a) they add to our understanding of the epidemiology of Norwalk virus infections; and (b) they provide our research laboratory with valuable samples to study the immunoglobulin responses to naturally occurring Norwalk virus infections (see our Contract Renewal Application).

The first outbreak is actually a series of outbreaks of gastroenteritis associated with the ingestion of raw clams by individuals in New York State, the clams having been harvested from Rhode Island and Massachusetts waters. These outbreaks have been studied by Drs. John Hanrahan, Dale Morse and Rudolf Deibel of the New York Department of Health, Albany, who have sent specimens to us for Norwalk virus studies. (The New York State Laboratory has ruled out bacterial and parasitic agents). Some of the clinical and epidemiological features of these outbreaks were reported in the C.D.C. Morbidity and Mortality Weekly Report of August 27, 1982. Although a few cases of hepatitis A have occurred in these outbreaks, most cases have been clinically those of viral gastroenteritis not followed by hepatitis A illness. It thus appears that clams may contain multiple enteric pathogens, in view of the different forms of clinical illness produced in these outbreaks. To date, we have studied specimens from four of the summer outbreaks of gastroenteritis in New York State, and have associated Norwalk virus with two outbreaks, based on strong sero-conversions to the virus in 2 of 4 and 4 of 8 individuals, respectively. In addition, during the spring of 1983, viral gastroenteritis outbreaks have been noted in Hawaii, again epidemiologically associated with the consumption of clams harvested in Rhode Island and Massachusetts. Dr. Joel Greenspan of the Hawaii Department of Health has recently sent to our laboratory a set of paired sera from affected individuals (as well as the implicated raw clams) for Norwalk virus analysis which will be performed shortly.

The second outbreak is from Hood College, Maryland, in which gastroenteritis was associated with a point source, likely salad. Samples were sent to us by Drs. J. Mehse Joseph and Suzanne Jenkins of the Maryland Department of Health and C.D.C. Seven of nine paired serum specimens tested showed seroconversions to Norwalk virus.

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50. 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, In Press.

The third outbreak is from a summer boys camp in Maryland in which no common source for gastroenteritis could be implicated and in which primary person-to-person spread is hypothesized. Samples were sent to us by Drs. Joseph Horman and Ebenezer Israel of the Maryland Department of Health and Dr. Suzanne Jenkins of C.D.C. In this outbreak 8 of 9 paired serum specimens tested showed seroconversions to Norwalk virus.

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PUBLICATIONS OF WORK SUPPORTED BY THIS CONTRACT FOR THE CONTRACT YEAR 1982-1983

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