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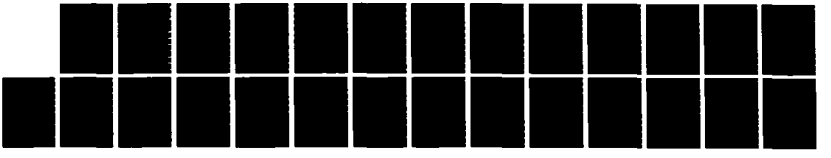

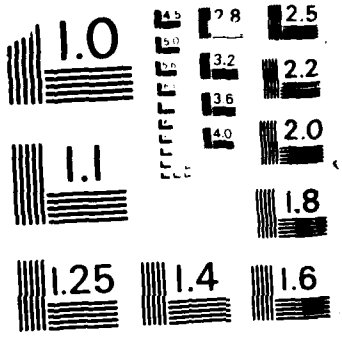

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THE ETIOLOGY AND PATHOGENESIS OF VIRAL GASTROENTERITIS

Final Report

Neil R. Blacklow, M.D.

August 1986

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This project has attempted to enhance our understanding of the etiology, epidemiology, and pathogenesis of human viral gastroenteritis. Efforts have been directed at the identification and characterization of etiologic agents, and towards an understanding of the epidemiology and pathogenesis of the infections they produce. ~~Considerable~~ effort has been devoted to the development ~~and utilization~~ of immunoassays to detect etiologic agents, with the preparation and use of monoclonal antibody reagents where possible. A monoclonal antibody based enzyme immunoassay (EIA) for rotavirus detection was developed, which greatly improves the sensitivity and specificity of diagnosis in neonatal and adult stool specimens over that previously available with polyclonal antibodies. Our older radioimmunoassay procedure for Norwalk virus was adapted to an EIA format, permitting a more convenient and more sensitive assay for epidemiological studies of naturally occurring infections with this virus. Of considerable interest ~~was our~~ finding that we were able to demonstrate an immunological relatedness between Norwalk virus and the enteric human →

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calicivirus. Humans experiencing naturally occurring calicivirus gastroenteritis usually developed seroconversions to Norwalk virus, indicating at least one-way serological cross-relatedness between the two viruses. In other studies, monoclonal antibodies were prepared against adenovirus serotype 41, as well as against an epitope shared by adenovirus serotypes 40 and 41 (and to a lesser degree by group C adenoviruses). Used in an EIA format, these monoclonal antibodies detected all of 10 stools tested known to contain enteric adenovirus serotypes 40 or 41. These kinds of enteric adenovirus reagents for the first time offer promise for the rapid, simple, serotype-specific identification of enteric adenoviruses in stool samples. Continuing epidemiological studies with collaborating colleagues of the role of viral agents in gastroenteritis in several populations revealed: the ~~important~~ role for Norwalk virus in ~~widespread~~ outbreaks of clam and oyster associated gastroenteritis ~~in the U.S.~~ and a role for Norwalk virus in 3 to 10% of travelers' diarrhea in Peace Corps volunteers in Thailand, and in U.S. servicemen in Panama, Honduras, and Egypt. In studies of disease pathogenesis, we were able to demonstrate serum IgM antibody to Norwalk virus among most young people in a naturally occurring disease outbreak. This outbreak also gave further support for the view that immunity to Norwalk virus is not determined by serum antibody. Another collaborative study demonstrated the localization of rotavirus to the small intestine as indicated by analysis of fluid specimens obtained by string capsule; this was in contrast to the absence of bacterial enteric pathogens isolated from the same site.

SUMMARY

This project has attempted to enhance our understanding of the etiology, epidemiology, and pathogenesis of human viral gastroenteritis. Efforts have been directed at the identification and characterization of etiologic agents, and towards an understanding of the epidemiology and pathogenesis of the infections they produce. Considerable effort has been devoted to the development and utilization of immunoassays to detect etiologic agents, with the preparation and use of monoclonal antibody reagents where possible. A monoclonal antibody based enzyme immunoassay (EIA) for rotavirus detection was developed, which greatly improves the sensitivity and specificity of diagnosis in neonatal and adult stool specimens over that previously available with polyclonal antibodies. Our older radioimmunoassay procedure for Norwalk virus was adapted to an EIA format, permitting a more convenient and more sensitive assay for epidemiological studies of naturally occurring infections with this virus. Of considerable interest was our finding that we were able to demonstrate an immunological relatedness between Norwalk virus and the enteric human calicivirus. Humans experiencing naturally occurring calicivirus gastroenteritis usually developed seroconversions to Norwalk virus, indicating at least a one-way serological cross-relatedness between the two viruses. In other studies, monoclonal antibodies were prepared against enteric adenovirus serotype 41, as well as against an epitope shared by adenovirus serotypes 40 and 41 (and to a lesser degree by group C adenoviruses). Used in an EIA format, these monoclonal antibodies detected all of 10 stools tested known to contain enteric adenovirus serotypes 40 or 41. These kinds of enteric adenovirus reagents for the first time offer promise for the rapid, simple, serotype-specific identification of enteric adenoviruses in stool samples. Continuing epidemiological studies with collaborating colleagues of the role of viral agents in gastroenteritis in several populations revealed: the important role for Norwalk virus in widespread outbreaks of clam- and oyster-associated gastroenteritis in the U.S.; and a role for Norwalk virus in 3 to 10% of travelers' diarrhea in Peace Corps volunteers in Thailand, and in U.S. servicemen in Panama, Honduras, and Egypt. In studies of disease pathogenesis, we were able to demonstrate serum IgM antibody to Norwalk virus among most young people in a naturally occurring disease outbreak. This outbreak also gave further support for the view that immunity to Norwalk virus is not determined by serum antibody. Another collaborative study demonstrated the localization of rotavirus to the small intestine as indicated by analysis of fluid specimens obtained by string capsule; this was in contrast to the absence of bacterial enteric pathogens isolated from the same site.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

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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 begins with an explosive onset, and consists of varying disabling 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. In recent years, one agent, Norwalk virus, has been shown to be responsible for about 40 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, occasionally epidemic, and 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 5 to 8 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 surprisingly severe clinical disease in adults (1,2).

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 (3).

The prototype Norwalk virus, which is currently noncultivatable in vitro and not disease producing for experimental animals, was initially described by the investigator and colleagues as a small lipid-free virus (4). It was later visualized in infectious stool filtrates and partially characterized by immune electron microscopy (IEM) and ultracentrifugation (5). Other 27 nm 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 small intestine, the likely site for

replication of these viruses (6,7). This lesion is accompanied by transient small intestinal malabsorption, and also by delayed gastric emptying despite normal gastric morphology and secretory function (8).

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 (9): 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 (9-11). In addition, antibody 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 ill subjects are reexposed after 6 to 14 weeks.

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 (10,11). The RIA represents a major advance in the study of this virus, and provides a laboratory handle for studies to cultivate the virus in vitro. The principal investigator has used the RIA to study forms of clinical immunity to Norwalk virus (11) (also see the preceding paragraph), and to show that Norwalk RIA serum antibody prevalence levels rise during adolescence in the United States (11). It has also been observed that antibody to Norwalk virus is acquired at a signfiicantly earlier age in less developed and tropical areas than in more developed and nontropical areas (12,13). 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 (14). The principal investigator has also developed an RIA test for IgM antibody to Norwalk virus (15). 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.

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 permits the large-scale rapid testing of clinical specimens from individuals for evidence of infection with Norwalk virus. Its drawbacks, however, are the requirement for radioactive reagents and a low efficiency in detecting virus in stools from natural disease outbreaks. Epidemiologic studies have shown the importance of Norwalk virus in various parts of the world, including its involvement in waterborne, foodborne, and shipborne outbreaks of acute gastroenteritis (1). In addition, the investigator and colleagues have shown Norwalk virus to be a cause of travelers' diarrhea in Mexico and Thailand (16,17).

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 infants and young children (18,19). It can also produce illness in adults (2). This

pathogen, rotavirus, has been identified by electron microscopy in stool filtrates derived from ill individuals. Serologic assay techniques have been developed for this agent by our laboratory and others to detect antibodies in human sera (20). In addition, rotavirus has been identified by our laboratory and others in diarrheal feces by RIA or enzyme-linked immunosorbent assay (EIA) techniques (20,21). Further, we have recently developed a monoclonal antibody based RIA technique to enhance the sensitivity and specificity of detection of rotavirus (22). We have used immunassay methodologies to establish the role of rotavirus in diarrhea in several nations around the world, including travelers' diarrhea experienced by U.S. military populations overseas (16,23-27). During 1981, Japanese scientists successfully cultivated human rotavirus in rotated cell cultures by incorporating low concentrations of trypsin into the culture medium. This finding has been confirmed by many laboratories; unfortunately, however, the in vitro cultivation of this virus is inefficient.

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 four human serotypes and two (perhaps three) 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 potential immunogen to adults and children (28). This "vaccine" has demonstrate 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. Rhesus rotavirus vaccine has been shown more recently to be more immunogenic than the bovine strain, and a suitable dose is being sought that eliminates concurrent side effects.

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 inefficiently in an adenovirus transformed cell lines, Graham 293. Two "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. Convenient and specific immunoassays to detect enteric adenoviruses are greatly needed, and would permit an understanding of their epidemiology as has already occurred with the use of immunoassays to study rotavirus and Norwalk virus.

IDENTIFICATION, CHARACTERIZATION, AND GROWTH OF GASTROENTERITIS VIRUSES

We have employed several approaches to develop and improve methods to recognize and characterize gastroenteritis viruses. The results of these studies are detailed below in this section. By way of background information, we have emphasized the development of monoclonal antibodies against gastroenteritis viruses including Norwalk virus, enteric adenovirus and

rotavirus. In the case of Norwalk virus, successful development would permit analysis of the biochemical nature of the virus, provide a potential diagnostic probe for group-specific antigens shared by the Norwalk-like virus group of agents, and also provide a sensitive and specific handle to detect Norwalk virus in inoculated cell cultures. In the case of enteric adenovirus (serotypes 40 and 41), monoclonal antibodies would greatly enhance our understanding of the biology and epidemiology of these agents, which are currently identified by inefficient and cumbersome methods. Initially, in order to gain expertise with hybridoma technology, we developed monoclonal antibodies against rotavirus as we have reported (22). Rotavirus was selected because laboratory assays for this virus are well-defined and readily exploited, in contrast to Norwalk virus and enteric adenovirus. We then have used monoclonal antibody against rotavirus in an enzyme-immunoassay (EIA) format to enhance the sensitivity and specificity of detection of this virus in certain age groups (29). Subsequently, we have adapted our radioimmunoassay (RIA) for Norwalk virus to an EIA format, which permits a simplified and more sensitive method for testing for this agent in stool samples (30). Finally, recent studies in our laboratory have revealed an important serological relatedness between Norwalk virus and the cultivatable human enteric calicivirus reported by Cubitt (31). Details of our studies on the identification, characterization, and growth of gastroenteritis viruses are now provided.

1. Monoclonal Antibody EIA for Detection of Rotavirus in Stools

We previously developed and used a monoclonal antibody reagent to detect rotavirus rapidly in human stools (22). This antibody reacts with the common rotaviral antigen (VP6) that is shared by mammalian rotaviruses. This reagent, used in an RIA procedure, showed increased sensitivity and specificity compared with a currently widely used commercial assay (Rotazyme). We have adapted this rotavirus monoclonal antibody reagent to an EIA format that is readily adaptable for 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 monoclonal EIA studies have been published by The Journal of Infectious Diseases (29), and are summarized as follows.

An EIA which uses monoclonal antibody as the detector reagent was compared with electron microscopy (EM) and a commercial polyclonal EIA (Rotazyme) for detection of rotavirus in stools from 141 neonates, children, and adults. Forty samples were positive by the standard reference test, EM. The monoclonal EIA detected 100% of the EM positive samples compared with 77.5% for the polyclonal EIA. In 17 samples from neonates which gave false positive results by the polyclonal EIA, none were positive in the monoclonal EIA. Also, the monoclonal EIA test was far more sensitive than the polyclonal test for detecting EM-positive samples from children and adults. Based on confirmatory tests, there were no false positive or false negative results with the monoclonal EIA. Compared with EM only, the sensitivity of the assay was 100%, the specificity was 97.0%, and the agreement with EM was 97.4%.

In addition to showing greater accuracy for diagnosing rotavirus infections, our data have demonstrated that monoclonal antibodies can be used as detector reagents in EIA tests that provide greater sensitivity as well as greater specificity for direct detection of infectious agents in clinical samples. Furthermore, the rotavirus monoclonal EIA now permits for the first time accurate screening by immunoassay for rotavirus infection in neonates and adults. This is important for infection control in hospital environments, and

will be useful in epidemiological studies.

One practical example of the utility of the monoclonal antibody EIA is a collaborative study we performed with Dr. Stephen Hoffman of the U.S. Naval Medical Research Unit Number 2 in Jakarta, Indonesia. Dr. Hoffman studied hospitalized patients with diarrhea, and generated confusing nonreproducible and "borderline" results on stool samples tested by the commercially available Rotazyme test. We examined by our monoclonal EIA 1.. such stools sent to us by Dr. Hoffman and found 17 of them to be Rotazyme false-positive reactions.

2. Comparison of Monoclonal Antibody EIA for Rotavirus with Polyclonal Assays

We have applied our monoclonal antibody EIA for rotavirus to the study of 176 human fecal specimens which were also evaluated by available polyclonal assays. The monoclonal EIA agreed with results obtained by IEM and/or RNA gel electrophoresis, confirming the highly sensitive and specific characteristics of the assay. Other assays evaluated were the original polyclonal antibody enzyme immunoassay marketed by Abbott Laboratories, Chicago, IL (Rotazyme I); a modification of this assay which is now commerically-available (Rotazyme II); and a latex agglutination test (Rotalex) recently introduced by Medical Technology Corporation, Somerset, NJ. Forty specimens were positive using the monoclonal antibody enzyme immunoassay; 136 were negative. Using the results obtained with this procedure as the reference standard, the sensitivities of the Rotazyme I, Rotazyme II and Rotalex tests were 97.4, 100, and 81.6%, respectively. The specificities of these three procedures were 88.8, 83.9 and 100%, respectively. These data have been published in the Journal of Clinical Microbiology (32).

3. Detection of Norwalk Virus in Stools by Enzyme-Immunoassay

Initially, immunoassay studies with Norwalk virus relied upon radioimmunoassay (RIA) methodology. Based on our successful experience with rotavirus detection in an EIA format, we adapted our Norwalk RIA to an EIA. In the process, we developed an antigen detection test that appears to be more sensitive than RIA, that is more consistent and uses stable reagents, and that can be performed in more convenient settings. The Norwalk virus EIA test now permits rapid testing of stool samples for epidemiological studies, a feature that heretofore has been lacking with the RIA test. The latter procedure has been of low efficiency in detecting virus in stools from natural disease outbreaks and therefore has been utilized almost exclusively to measure seroconversion to Norwalk virus as an indicator of infection. Our Norwalk virus EIA studies have been published by The Journal of Medical Virology (30), and are summarized as follows.

The EIA was compared with our RIA for detection of Norwalk virus antigen in stored stool samples previously obtained from 30 volunteers who received Norwalk virus. The EIA detected viral antigen in stools from 17 of the volunteers and the RIA detected viral antigen in 15. Seroconversion was a more sensitive indicator of infection in some patients. However, two samples from volunteers who were clinically ill but did not show seroconversion to Norwalk virus were positive for Norwalk virus antigen by both immunoassays. This indicates that antigen detection may be important for use in epidemiological studies. Neither of the immunoassays gave positive reactions for stools known to contain enteric adenovirus, rotavirus, or Hawaii virus, or in stools from patients with acute diarrhea of unknown cause. The stability of the EIA reagents and ease of use should provide a means for more extensive testing for Norwalk virus in outbreaks of gastroenteritis. Also, the EIA

antigen test appeared to be more sensitive than RIA in the detection of Norwalk virus in stools from symptomatic volunteers. Finally, we noted that antigen detection is a more sensitive means for diagnosing infection in some volunteers than is serology, much as the converse is also true.

4. Diagnosis of Naturally Occurring Norwalk Virus Gastroenteritis by Antigen Detection

Naturally occurring Norwalk virus disease outbreaks previously relied exclusively upon demonstration of seroconversion to the virus for diagnosis. This is because tests for antigen detection in stools of patients by RIA or immune electron microscopy (IEM) were either unable to detect antigen or were of low efficiency for antigen detection in naturally occurring disease cases. Thus, the diagnosis of Norwalk disease had been retrospective only, requiring collection of a convalescent serum specimen 3 weeks after illness. Furthermore, serological techniques may have limitations in terms of specificity for Norwalk virus. Recent studies (see section 5 below) we have done in collaboration with W.D. Cubitt have shown that acute and convalescent sera collected from patients with calicivirus infection usually seroconvert to Norwalk virus as well as calicivirus. To date, we have not demonstrated that seroconversion to calicivirus in human stools occurs with acute and convalescent Norwalk sera. This suggests that antigen testing may be more specific for Norwalk virus than seroconversion.

We therefore have evaluated our recently developed EIA test for Norwalk virus antigen (see Section 3 above) with samples obtained in a naturally occurring outbreak of gastroenteritis, and evaluated the method in conjunction with seroconversion for investigating the role of Norwalk virus in gastroenteritis. Collaborators were Drs. George Kent and Jack Brondum of the Rhode Island Department of Health who studied epidemiologically an outbreak of non-bacterial gastroenteritis that occurred in a nursing home. Of 22 patients involved in the outbreak, paired sera or stool samples were available from 10. There were five patients for which only stool samples were available, three in which only paired sera were available and two patients for which both stool and paired sera were available. By RIA antibody tests, three of five patients seroconverted to Norwalk virus. The three patients who seroconverted had clinical symptoms of gastroenteritis, as did one of two patients who did not seroconvert. Norwalk virus antigen was detected by EIA in four of seven patients, three of whom had clinical symptoms. There was one case that was seroconversion positive in which antigen could not be detected.

This study, which has recently been accepted for publication in the Journal of Infectious Diseases (33), indicates that antigen detection by EIA for diagnosis of naturally occurring Norwalk virus infection is not only a useful adjunct to serological tests, but also is sufficiently sensitive to be used alone. In addition, the EIA antigen test permits diagnosis within 1 day after specimen receipt, and will expedite study of the epidemiology of Norwalk virus infection.

5. Immunological Relatedness of Norwalk Virus and Human Calicivirus

We have recently demonstrated an immunological relatedness between Norwalk virus and the human calicivirus described by Cubitt (31). It has been known that Norwalk virus contains a single structural protein of molecular weight 59,000, which is characteristic of the RNA-containing calicivirus group. These viruses also share properties of size and buoyant density with Norwalk virus. Early in this project, we found that hyperimmune anti-feline calicivirus sera failed to react with Norwalk virus by RIA. We also found

that defined human sera possessing Norwalk antibody failed to react by immunofluorescence with feline calicivirus strains cultivated in NLFK cells. Of considerable importance, however, has been our recent finding of a serological relationship between Norwalk virus and the human calicivirus (HCV) described by Cubitt.

In collaboration with Dr. Cubitt (London), we have studied serum antibody responses to Norwalk virus produced by patients with HCV gastroenteritis. Acute and convalescent serums were tested under code from 18 adults in 4 HCV outbreaks, all of whom seroconverted to HCV by immune electron microscopy. Seven of ten paired sera from two HCV type UK4 outbreaks demonstrated $\geq$ 4-fold antibody rises to Norwalk virus by radioimmunoassay (RIA); one outbreak produced seroconversion in 5/7 patients and the other in 2/3 individuals. Two of eight paired sera from HCV type UK2 outbreaks seroconverted by RIA; one outbreak caused seroconversion in 2/3 patients and the other in 0/5 persons. Titers obtained were of a magnitude similar to those that are observed in well-defined outbreaks of Norwalk disease. These data provide evidence for at least a one-way serological cross-relatedness between Norwalk virus and calicivirus, and were reported at the A.S.M. National meeting in 1986 (34).

6. Monoclonal Antibody and Cultivation Studies With Norwalk Virus

We have previously reported the production of monoclonal antibody against the common antigen shared by mammalian rotaviruses, which we have used as an effective reagent to detect rotavirus in human stools with enhanced sensitivity and specificity (22,29). We are endeavoring to build upon our experience gained with rotavirus to develop monoclonal antibodies against the noncultivatable Norwalk virus which would greatly aid studies on the biology of this agent and its recognition in nature. Two approaches have been taken: (a) production of human-mouse heterohybridomas and (b) production of mouse monoclonal antibody.

We were unsuccessful in preparing human-mouse heterohybridomas reactive with Norwalk virus. We obtained blood from 10 volunteers ill with Norwalk virus at the University of Texas Health Science Center at Houston (Dr. Herbert DuPont collaborator) and fused enriched B lymphocyte cell fractions from these blood samples with mouse myeloma cells. Heterohybridoma fluids possessing anti-human IgG secreting activity were tested for their antiviral specificity by using them as coating reagents in our Norwalk virus radioimmunoassay. No Norwalk-specific monoclonal antibodies were detected by this assay.

We are also attempting to produce murine monoclonal antibody to Norwalk virus. The major limitation to this effort is the amount and purity of virus available. In order to increase our chances of successful antibody production, we have purified virus from stools we have previously collected from volunteers and stored at -70^{o}C. We followed a method of purification that has been successfully used to produce monoclonal antibodies to hepatitis A virus from virus-containing stool material (35). The development of our Norwalk antigen EIA permits rapid monitoring of the purification process. Initial purification has involved virus concentration and DEAE column purification with enhancement of Norwalk antigen titer. Inoculated mice have, however, failed to mount significant antibody titers to Norwalk virus by antibody EIA. Current efforts are underway to modify the purification technique.

Our efforts to cultivate Norwalk virus in the past have not met with success. However, the recent reported cultivation in vitro of human calicivirus by Cubitt (36) in manipulated human embryonic kidney (HEK) cells

has given us renewed hope to be able to cultivate Norwalk virus in view of the immunological relatedness between the two viruses (see above #5). With Norwalk virus, we have followed Cubitt's method of cultivation, namely, use of trypsin exposed HEK cells. Our efforts in serially passaged inoculated cultures have not met with success to date, as judged by absence of cultivated Norwalk virus antigen (by EIA) and failure to detect fluorescent-stainable cells (following reaction with convalescent human serum containing Norwalk antibody). We are currently studying Norwalk virus inoculated dolphin kidney cells inasmuch as Cubitt has indicated they also support the growth of the virus.

7. Monoclonal Antibodies to Enteric Adenoviruses: Characterization and and use in Immunoassays

Enteric adenoviruses include adenovirus (Ad) types 40 and 41 and are frequently found in stools of children with diarrhea (37). These Ad strains differ from other Ad serotypes in that they are not common causes of acute respiratory tract disease and do not replicate readily in cell culture. They can be cultivated relatively inefficiently in Graham 293 cells (38), a line of adenovirus five transformed cells, as can other adenovirus serotypes. Unfortunately for diagnostic purposes, however, many "non-enteric" Ad types are often shed asymptomatically for prolonged periods in feces. Thus, detection of enteric Ad strains requires serotype specific identification of the agent in stools. To date, this has been accomplished by cumbersome methods such as identification of viral DNA electropherotype or polyclonal EIA tests rendered "specific" by first absorbing out nonenteric Ad serotypic cross-reactions in polyclonal test sera (39). Clearly, the study of the enteric adenoviruses has been slowed because of the lack of a rapid, specific, sensitive and convenient way to identify them.

In order to expedite the study of enteric Ad types 40 and 41, we developed two monoclonal antibody-producing hybridomas for use in detecting enteric Ad in stool specimens by enzyme immunoassay (EIA). One, designated clone 9B9, produces IgG2A which is specific for Ad41, reacts with a type-specific component of adenovirus hexon antigen as shown by immunoblotting, and neutralizes virus infectivity. The antibody does not cross-react with Ad40 or non-enteric Ad types. The other, designated clone 9D4, produces IgG1 which is specific for Ad40 and Ad41 and does not neutralize virus. However, data with 9D4 shows that there is a low level cross-reaction by EIA with cell culture grown group C Ad types, but none with other non-enteric Ad types. Of 49 stools shown to contain Ad by electron microscopy, 10 have been shown to contain enteric Ad by gel electrophoresis of viral DNA (six were Ad40, four were Ad41). All 10 were correctly identified by monoclonal antibody EIA tests. These monoclonal antibodies described offer promise for the rapid, type-specific identification of enteric Ad in stool samples for clinical diagnosis and epidemiological studies. However, we first need to develop a monoclonal antibody specific for adenovirus 40 and non-reactive with all other serotypes (including group C viruses) before we have the capability to diagnose adenovirus 40 and 41 infections simply and efficiently. Our adenovirus monoclonal antibody data were presented at the A.S.M. National meeting in 1986 (40).

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 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 and colleagues, we have previously published several studies on the role of rotavirus in diarrhea among either American soldiers or native populations in South Korea, the United States, Taiwan, the Philippines, and Thailand (2,23-27,41-44). Also, with Dr. Echeverria and colleagues we have examined the antibody prevalences to Norwalk virus in the Philippines, Taiwan, and the United States (13) and in rural Thailand (45) and the potential role of Norwalk virus in diarrhea among Peace Corps volunteers who are newly arrived in Thailand (17). 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 (16) and have shown Norwalk virus to be responsible for at least a small proportion of family outbreaks of diarrhea in Texas (46). 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 during the course of this Contract and are outlined below.

1. Widespread Outbreaks of Clam- and Oyster-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 outbreaks of gastroenteritis caused by ingestion of inadequately cooked clams and oysters. Consumption of raw shellfish has long been known to be associated with individual cases and sporadic outbreaks of enteric illness. However, during 1982, outbreaks of raw shellfish-associated gastroenteritis reached epidemic proportions in New York state. Between May 1 and December 31, there were 103 well documented outbreaks in which 1,020 persons became ill: 816 cases were clam related, 204 were oyster related. The most common symptoms were diarrhea, nausea, abdominal cramps, and vomiting. Incubation periods were generally 24-48 hours with a duration of 24-48 hours. Ten cases of hepatitis A were identified in persons from five outbreaks, leading to subsequent recommendations for prophylactic use of immunoglobulin. Determination of shellfish source was not always possible, but northeastern coastal waters were implicated. Stool and shellfish specimens were negative for bacterial pathogens. Seroconversions to Norwalk virus were detected on paired sera from persons in five (71%) of seven outbreaks in which testing was done. Furthermore, specific anti-Norwalk IgM antibodies, previously shown by us to be indicative of recent infection with Norwalk virus (15), were detected in 22 of 25 serum specimens tested. We were also able to detect Norwalk virus antigen by radioimmunoassay in clam and oyster specimens from two of the outbreaks. Our results implicate Norwalk virus as the predominant etiologic agent in these outbreaks. In addition, the magnitude, persistence, and widespread nature of these outbreaks raise questions about the safety or wisdom of consuming raw shellfish. Our data were recently published in the New England Journal of Medicine (47).

2. Peace Corps Volunteer Travelers' Diarrhea Studies in Thailand

We have previously reported (17), in collaboration with Dr. Peter Echeverria et al., 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 also had the opportunity to participate in similar studies performed with two other groups of Peace Corps volunteers entering Thailand. One study was published in The Lancet (48). In this study, of 35 Peace Corps volunteers in Thailand, 20 (57%) had a total of 30 episodes of diarrhea during their first 6

weeks in the country (August-September, 1983). Enteric pathogens were associated with 90% of the episodes. A single pathogen was identified in 17 (57%) episodes, 2-4 pathogens were identified in 10 (33%) episodes, and there were 15 symtomless infections. Enterotoxigenic Escherichia coli was identified in 37% of these episodes, and various salmonella serotypes were isolated in 33%. Infections with other enteric pathogens were also identified, including Norwalk virus and rotavirus. Two ill patients demonstrated seroconversion to Norwalk virus by RIA (representing 7% of episodes of diarrhea) and one ill individual seroconverted to rotavirus by RIA (representing 3% of episodes of diarrhea). One asymptomatic person also seroconverted to Norwalk virus. This study has reinforced the concept of a polymicrobial etiology of travelers' diarrhea, in which viral agents play a role.

The results of a second Peace Corps volunteer study in Thailand were published in The American Journal of Epidemiology (49). In this study, two ill patients of 62 volunteers (3%) 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.

3. Diarrhea Among U.S. Military in Central America

Through Dr. Samuel Formal at Walter Reed, we received 40 washings of stool swab samples from U.S. servicemen in Panama and Honduras who had experienced febrile diarrheal illness. Campylobacter, salmonella, and shigella were ruled out as causes of their illness. All 40 fecal swab washings were negative for rotavirus by monoclonal antibody EIA testing. However, four specimens (10 percent) were positive for Norwalk virus antigen by EIA, indicating that at least some of these illnesses may have been due to Norwalk virus infection. The failure to detect Norwalk virus antigen in most stool swab washings may have been due to the very limited amount of fecal material (washings of fecal swabs) that our laboratory was provided for testing. Acute and convalescent phase serum samples were not available for serological testing. Additional studies of a prospective nature are planned.

4. Travelers' Diarrhea Among U.S. Navy and Marine Corps Personnel Visiting Egypt

In collaboration with Dr. A.L. Bourgeois of the U.S. Naval Medical Research Unit No. 3, Cairo, Egypt, we had the opportunity to study for the presence of viral agents specimens collected from U.S. Navy and Marine Corps personnel visiting Egypt in the spring of 1982. In this study group, 24.9% of 189 individuals with documented exposure ashore, developed travelers' diarrhea. Recognized enteric pathogens were isolated from less than 50% of episodes examined. Enterotoxigenic E. coli was the most frequent etiologic agent identified (15.6% of the episodes). Regarding viral agents, it is interesting that rotavirus was not detected by Rotazyme in Egypt in any of 107 diarrheal stools examined. A subset of 30 stools from patients with acute diarrhea negative for other enteropathogens as well as stools from 15 asymptomatic individuals were sent to our laboratory for Norwalk virus testing by immunoassay. All stools were negative for Norwalk virus. We also examined 30 acute and convalescent serum pairs from illness cases and 8 pairs from exposed asymptomatic persons and detected one subclinical seroconversion to Norwalk virus (titer rise of <1:50 to 1:400). It was therefore clear that rotavirus and Norwalk virus were not associated with diarrhea in this study.

It is interesting that 28 of 38 individuals studied possessed pre-exposure serum antibody to Norwalk virus, which is a somewhat high prevalence of antibody to this agent among young adult Americans. This finding suggests that this military study group may have had increased exposure to Norwalk virus prior to this study. It is possible that some of the diarrheal illness in this study may have been due to viral agents such as the calicivirus reported by Cubitt, enteric adenovirus and astrovirus.

5. An Outbreak of Norwalk Virus Gastroenteritis at a Boys Camp

In collaboration with Dr. S. Jenkins, C.D.C., we have showed Norwalk virus to be the cause of an acute gastrointestinal illness affecting boys and staff at a summer camp in the Catoctin Mountains of Maryland. This study was published by the American Journal of Diseases of Children (50). In summary, illness affected 213 (52%) of 407 campers and 64 (52%) of 121 staff at the camp. Nausea was the predominant symptom for ill campers and staff (73%), but more staff members experienced diarrhea (49%) than did campers (9%). Eight of nine paired blood specimens from ill staff members showed 4- to 16-fold increase in antibody titer to Norwalk virus by RIA. Environmental inspections and laboratory tests failed to implicate a common source of exposure, and a person to person route of infection may have been the primary mode of spread. This study reemphasizes the importance of a camp environment for the occurrence of epidemic illness due to Norwalk virus among both children and adults.

PATHOGENESIS STUDIES OF VIRAL GASTROENTERITIS

In addition to our efforts to detect and characterize gastroenteritis viruses and to assess their roles in disease in various populations, we continue to be interested in the pathogenesis of illness produced by these agents. Work covered during this Contract is summarized below.

1. Serological Responses Among Teenagers After Natural Exposure to Norwalk Virus

We have previously reported that there is an IgM serum antibody response to Norwalk virus in volunteers following primary infection as well as reinfection (15). 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. Twenty-one teenagers exposed to a contaminated water supply during an outbreak of gastroenteritis were tested for seroconversion to Norwalk virus. Serum specimens were collected within 72 hours of exposure and 4 weeks later. Each of the 11 individuals who developed symptoms and 5 of the 10 who remained well had a whole antibody response in serum. None of the remaining five teenagers became ill or seroconverted. Neither seroconversion nor susceptibility to illness was associated with an absence of detectable antibody from acute phase serum specimens. These findings support the view that immunity to Norwalk virus is not determined by serum antibody. Furthermore, the results are consistent with the possibility, suggested by previous studies in volunteers (9), that susceptibility is determined by Norwalk virus-specific intestinal receptor sites. IgM responses to Norwalk virus were detected in only seven persons who became ill (64%) and nine who seroconverted (56%). The seroassay for the Norwalk IgM component might have proved a more sensitive diagnostic tool in this outbreak if convalescent-phase specimens had been collected sooner than 4 weeks after the onset of illness. These data

were published during the current reporting year in the Journal of Infectious Diseases (51).

2. Identification of Rotavirus in the Small Intestine of Children with Diarrhea

In collaboration with Dr. Peter Echeverria of the Armed Forces Research Institute of Medical Sciences and Children's Hospital, Bangkok, Thailand, we analyzed duodenal specimens obtained with a string capsule for the presence of rotavirus in children with diarrhea. Rotavirus was identified in the stools of 43 of 100 children, and was recovered from the small intestine from nine (21%) children who were excreting this virus in the feces. In contrast to rotavirus, bacterial enteric pathogens were not isolated from the small intestine, although they were recovered from the stool. The localization of rotavirus to the small intestine by string capsule confirms early findings of rotavirus in small intestinal tissue by electron microscopy. Although rotavirus clearly causes disease by its effect on the small and not large intestine, examining the small intestine with a duodenal string capsule did not identify rotavirus as frequently as examining stool. These data, which reemphasize the small intestinal site for replication of rotavirus, were published in Diagnostic Microbiology and Infectious Disease (52).

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