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Development of an enzyme immunoassay for the Hawaii agent of viral gastroenteritis

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Summary

The Hawaii agent is a Norwalk-like virus of acute gastroenteritis in humans which is antigenically distinct from the prototype Norwalk agent. We established a solid phase sandwich type microtiter enzyme immunoassay (EIA) for Hawaii antigen employing sera and stools from experimentally challenged volunteers as reagents. This assay detected the Hawaii agent in stools from 3 of 8 volunteers who were ill after oral challenge with the Hawaii agent, including one specimen which was positive to a dilution of 1/320. Virus shedding occurred on days 3 to 7 after challenge. The Hawaii-positive stools did not react in the EIAs for Norwalk and Snow Mountain agent (SMA), nor did Norwalk or SMA-positive stools react in the Hawaii EIA. Human rotavirus, enteric adenovirus, feline calicivirus, and several enteroviruses also did not react in the Hawaii EIA. A blocking EIA to detect serum antibody to the Hawaii agent was also developed employing a diarrheal stool containing Hawaii as a source of antigen. Serum antibody rises were detected in 15 of 16 individuals with experimentally induced illness after challenge and in 3 of 5 individuals who remained well after challenge. The EIA for the Hawaii agent should permit epidemiologic studies of the Hawaii agent to be carried out as well as allow further characterization of the Hawaii virion.

Gastroenteritis; Enzyme immunoassay; Hawaii agent; Norwalk-like agent

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These studies were approved by the Committee on Investigations Involving Human Subjects of the University of Rochester and all subjects gave written informed consent prior to participation.

Introduction

The Hawaii agent is a 26–29 nm virus that was associated with a family outbreak of gastroenteritis in Honolulu, Hawaii, and which has subsequently been shown to induce acute gastroenteritis in normal volunteers following experimental challenge (Dolin et al., 1975; Thornhill et al., 1977). Morphologically, the Hawaii agent resembles the Norwalk agent and is referred to as a member of the Norwalk-like viruses (Dolin et al., 1987). In addition, the Hawaii and Norwalk agents have similar densities in cesium chloride (Thornhill et al., 1977; Greenberg et al., 1981), induce similar illnesses in normal volunteers, and cause similar reversible proximal small intestinal lesions in normal volunteers (Dolin et al., 1975). However, the Hawaii agent is antigenically distinct from the Norwalk agent by cross challenge studies in volunteers (Wyatt et al., 1974) and by immune electron microscopy (Thornhill et al., 1977), and from another Norwalk-like virus, the Snow Mountain agent (SMA) by immune electron microscopy (Dolin et al., 1982). The development of rapid microtiter radio and enzyme immunoassays for the Norwalk (Greenberg et al., 1978; Gary et al., 1985; Herrmann et al., 1985) and Snow Mountain agents (Dolin et al., 1986; Madore et al., 1986) has facilitated further study of these agents, but to date only the more cumbersome technique of immune electron microscopy has been available for the Hawaii agent. In this report, we describe the establishment of a solid phase microtiter enzyme immunoassay for detection of the Hawaii agent in diarrheal stools, and for the detection of anti-Hawaii antibody in serum specimens.

Materials and Methods

Challenge of normal volunteers with the Hawaii agent

Because in vitro culture or animal model systems are not available for the Hawaii agent, only human serum and stool specimens are available as sources of Hawaii antigen and antibody. In order to obtain a sufficient quantity of such specimens with which to establish the assay, we challenged healthy adult volunteers with the Hawaii agent. Ten volunteers with unremarkable medical histories and physical examinations and normal values for hematocrit, white blood cell count and liver function were challenged with 1 ml of the Hawaii inoculum after alkalinization of stomach contents as previously described (Thornhill et al., 1977; Dolin et al., 1972). This inoculum containing the Hawaii agent was derived from a volunteer who had been challenged with a stool filtrate from the original outbreak and has been designated 21 Ki (Thornhill et al., 1977). Volunteers were housed in group isolation at the University of Rochester, and pre- and 3-week postchallenge serum specimens as well as the entire contents of all stools passed for 1 week after challenge were obtained. Additional sera from volunteers challenged with the Hawaii agent in a previous study (Dolin et al., 1975) were also tested.

For tests of the reaction of other agents in the Hawaii EIA, stool specimens from volunteers previously challenged with the Norwalk (Dolin et al., 1972) or Snow

Mountain agent (Dolin et al., 1982) were used. Stools containing rotavirus were obtained from children with gastroenteritis in Rochester, NY. Human enteric adenovirus type 41 was obtained from S. Strauss, Bethesda, MD, and tissue culture-adapted human rotavirus types 1–4 were obtained from Y. Hoshino, Bethesda, MD. Coxsackievirus A9 and B1, and echovirus 5 were clinical isolates obtained by R. Dolin, and feline calicivirus Bolin strain was obtained from the American Type Culture Collection, Rockville, MD.

Enzyme immunoassay for Hawaii antigen

The method for this assay was adapted from our previously described radioimmunoassays and enzyme immunoassays for Norwalk and SMA (Dolin et al., 1986; Madore et al., 1986). Alternate wells of the inner 60 wells of a 96-well U-bottom polystyrene plate (Serocluster, Costar, Cambridge, MA) were coated with pre- or postchallenge serum from a volunteer challenged with the Hawaii agent. One hundred microliters of a 1:2500 dilution of these sera in PBS, pH 7.4, were added to each well, and the plates were incubated at room temperature for 6 h. Excess antibody was removed by washing twice with washing buffer (0.5 M NaCl 0.1M Tween 20 PBS) and the plates were incubated overnight at 4°C with 250 μl of blocking buffer (1% FCS 0.5% gelatin 0.5 M NaCl PBS). Excess blocking buffer was removed, and 25 μl fetal calf serum was added to each well, followed by 25 μl of the sample added to duplicate pre- and postchallenge wells. The plates were incubated at 37°C for 2 h, and then washed six times with washing buffer.

As indicator antibody, we employed IgG purified from the convalescent sera of a second volunteer challenged with the Hawaii agent. The IgG was purified from these sera by DI amine chromatography (Zetachrome, MF Meriden, CT) and biotinylated by reaction with biotin-*N*-hydroxysuccinimide (Jackson et al., 1982) for 4 h at room temperature in the dark. Fifty microliters of indicator antibody diluted in dilution buffer (10% FCS 0.5% gelatin 0.5 M NaCl PBS) was added to each well and the plates incubated at 37°C for 2 h. After six washings with washing buffer, avidin linked to horseradish peroxidase (Tago, Burlingame, CA) diluted in dilution buffer was added for 30 min at 37°C. After six washings, the reaction was developed by addition of 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma Chemical, St. Louis, MO) per well (0.1 mg/ml TMB in 0.1 M citrate buffer, pH 5.0, 0.006% hydrogen peroxide). To stop the reaction, 50 μl of 1 N HCl was added per well, and the optical density at 450 nm read in a micro ELISA minireader MR590 (Dynatech Instruments, Santa Monica, CA). Background activity in wells without antigen was subtracted from each reading and samples which had an optical density in wells coated with postchallenge serum twice that in wells coated with prechallenge serum were considered positive ($P/N \geq 2$). Samples with a P/N ratio of 2 to 3 but with an optical density of less than 0.2 in the postchallenge wells were considered borderline and repeated. Enzyme immunoassays for the Norwalk and Snow Mountain agents were performed as previously described (Madore et al., 1986).

Blocking EIA for antibody to the Hawaii agent

We used a method similar to that previously described for the Norwalk and SMA EIAs (Madore et al., 1986) to develop a blocking EIA for antibody to the Hawaii agent. The inner 60 wells of the 96-well plate were coated with postchallenge serum and blocked with blocking buffer as described above. Ten control wells coated with pre- and postchallenge serum were also included with each performance of the test, and four each of these wells served as negative control (antigen absent) wells. Twenty-five microliters of a stool sample containing Hawaii antigen at a dilution which resulted in a *P/N* ratio of 4 in the standard antigen assay were added to 25 μl of FCS in each well and incubated overnight at 4°C. After six washings, 50 μl of serial four-fold dilutions of the test sera beginning at 1:100 were added in duplicate to wells, incubated at 37°C for 2 h and washed six times. Finally, 50 μl of biotinylated indicator antibody were added, and the remaining steps of the assay performed as described above. After subtraction of the value of the optical density at 450 nm of the negative control (antigen absent) wells from all of the remaining wells, the result for each antibody dilution was calculated as the % OD compared with unblocked antigen wells coated with postchallenge serum (typically 0.80–1.20). The antibody titer was defined as the reciprocal of the highest dilution of serum that resulted in a reduction in OD of 40% compared with control. Enzyme immunoassays for antibody to Norwalk and SMA were performed as previously described (Madore, 1986). Antibody to human rotavirus was determined by microneutralization as described previously (Christy et al., 1986).

Results

Challenge of normal volunteers with the Hawaii agent

Eight of ten of the experimentally challenged volunteers developed a typical acute gastrointestinal illness consisting of nausea, vomiting, chills and myalgias. Seven volunteers had mild diarrhea, (2–3 stools/day), and 3 had lowgrade fever. The median incubation period of illness was 36 h (range 32–40 h), and the duration of illness was 24–48 h.

Detection of the Hawaii agent in stool samples

The Hawaii agent was detected in 5/41 stools from the 8 ill individuals from the current challenge study, and in 0/9 of the stools from the 2 volunteers who remained well. The 5 stool samples positive for Hawaii antigen were obtained from 3 volunteers in whom 1/5, 2/7, and 2/6 samples were positive. Serial two-fold dilutions of the positive Hawaii samples were then tested to determine the titer of virus antigen present. One sample had a titer 1:320 while the other four had titers which ranged from 1:20 to 1:80. However, the 21 Ki inoculum used to infect the volunteers was not positive in the Hawaii EIA even at a 1:2 dilution.

In order to determine the specificity of the Hawaii EIA, we tested stool samples from volunteers challenged with Norwalk, Hawaii, or SMA in the Hawaii antigen EIA and also in the EIAs for the Norwalk and Snow Mountain agent. These re-

TABLE 1

Specificity of Hawaii agent EIA

Agent[a]	N	Number positive in EIA for		
		Norwalk	Hawaii	SMA
Norwalk	5	5	0	NT
Hawaii	5	0	5	0
SMA	7	NT	0	7

[a] Stool specimens from volunteers positive by EIA were assayed at a 1:2 dilution in the indicated EIAs as described in Materials and Methods. NT = not tested.

sults are shown in Table 1. The Hawaii antigen EIA was specific for the Hawaii agent, and did not detect samples containing the Norwalk or Snow Mountain agent. In addition, samples containing the Hawaii agent also did not react in the Snow Mountain or Norwalk EIAs. The Hawaii EIA did not react with 6 stools known to contain rotavirus from children with naturally acquired rotavirus gastroenteritis. The EIA for the Hawaii agent did not react either with tissue culture grown coxsackie virus A9, B1, echovirus 5, human enteric adenovirus type 41, or human rotavirus types 1–4, or feline calicivirus.

Of the 45 stool samples from volunteers challenged with the Hawaii agent which were negative in the Hawaii antigen assay, 13 had high background at 1:12 dilution (optical density in the prechallenge wells of over 1.00) and were retested at higher dilution (1/10–1/50). None of these samples were positive for the Hawaii agent at any dilution. In addition, 2 samples had borderline results, i.e., had P/N values of > 2 but P values less than 0.20. Both were consistently negative on repeat testing at dilutions of 1:2 and greater.

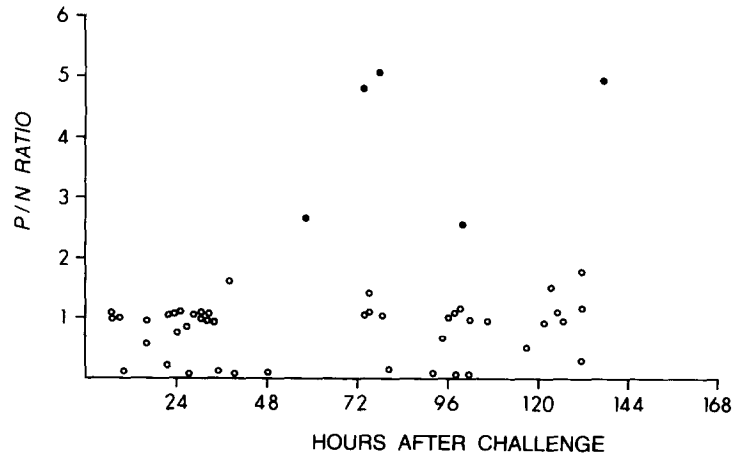

Fig. 1. Relationship between the P/N ratio of stool samples tested at a 1:10 dilution in the Hawaii antigen EIA and the time after virus challenge that they were collected. ●, positive samples ($P/N \geq 2$); ○, negative samples ($P/N < 2$).

Fig. 1 depicts the relationship between the *P/N* ratio of stool samples from the volunteers challenged with the Hawaii agent and the time after challenge that the samples were collected. The onset of virus shedding as determined by a *P/N* 2 was 18 h after onset of illness, or 54 h after challenge. None of the samples collected before the onset of illness was positive.

Blocking assay for serum antibody to the Hawaii agent

The results of the blocking assay for serum antibody to the Hawaii agent are shown in Table 2. Eight of eight ill volunteers who had been challenged with the Hawaii agent in the current study demonstrated four-fold or greater antibody titer rises by EIA, as did one of two volunteers who remained well. In addition, 7/8 ill volunteers from a previous challenge study had four-fold titer rises to the Hawaii agent by blocking EIA, as did 2/3 who remained well after challenge. Volunteers who manifested significant antibody rises had similar degrees of rise regardless of the presence of illness, although when the entire group was considered, those with illness had a greater degree of rise.

Discussion

This report describes the development of a rapid, specific EIA for the Hawaii agent of gastroenteritis. This assay is based on the method used in previously described assays for the Norwalk and Snow Mountain agent (Dolin et al., 1986; Madore et al., 1986) and utilizes the biotin-avidin system which takes advantage of the high affinity of biotin for avidin (Guesdon et al., 1979). Unlike the IEM procedure used in previous studies of the Hawaii agent (Thornhill et al., 1977), the enzyme immunoassay for Hawaii can be performed in a microtiter format, allowing processing of multiple samples, and is more conservative of reagents than IEM. The Hawaii antigen detection EIA was specific for the Hawaii agent. Samples containing Norwalk and Snow Mountain agent did not react in the Hawaii EIA, and the 5 Hawaii positive samples did not react in either the Norwalk or SMA EIA. This confirms previous reports that the Hawaii agent can be distinguished from the

TABLE 2

Analysis of serum pairs from volunteers challenged with the Hawaii agent for an antibody rise by Hawaii blocking EIA[a]

Illness	Four-fold antibody rise	*N*	Geometric mean titer	
			Prechallenge	Postchallenge
yes	yes	15	158	15,500
yes	no	1	3,200	6,400
no	yes	3	631	20,400
no	no	2	562	562

[a] Pre- and 3-week postchallenge serum pairs were assayed by blocking EIA as described in Materials and Methods.

Norwalk and Snow Mountain agents antigenically (Wyatt et al., 1974; Dolin et al., 1986; Madore et al., 1986). In addition, the Hawaii antigen EIA did not detect rotaviruses, several enteroviruses, enteric adenovirus type 41, or feline calicivirus.

The pattern of shedding of the Hawaii agent detected by EIA was similar to that previously described for the Norwalk (Thornhill et al., 1977; Gary et al., 1987) and Snow Mountain agents (Dolin et al., 1982) in that virus shedding was observed after the onset of illness and lasted for 2–3 days. Antigen was detected in 3/8 (38%) of ill volunteers and in 5/41 (12%) of stool specimens from ill volunteers. By comparison, we detected Norwalk antigen in 3/6 (50%) of ill volunteers and 8/28 (29%) of stool specimens from ill Norwalk volunteers with the Norwalk EIA (Madore et al., unpublished results). In separate volunteer challenges, SMA antigen was detected in 2/9 (22%) and 9/9 (100%) of ill volunteers and in 12/45 (27%) and 22/43 (51%) of stool specimens from ill SMA volunteers with the SMA EIA (Dolin et al., 1982; Madore et al., 1986, and unpublished observations). Since the Hawaii, SMA and Norwalk EIAs are similar in format and type of reagents, the low level of Hawaii antigen positive stools may reflect a low level of virus shedding in this particular challenge or possibly a low assay sensitivity.

The Hawaii EIA was modified to a blocking format for detection of serum antibody to the Hawaii agent. The blocking assay detected serum antibody rises to the Hawaii agent in 15/16 volunteers ill after challenge with the Hawaii agent and also detected asymptomatic antibody rises in 3/5 volunteers who remained well after Hawaii challenge. Asymptomatic antibody rises have been previously reported in both experimentally induced (Greenberg et al., 1978; Herrmann et al., 1985) and naturally acquired infection (Baron et al., 1984) with the Norwalk agent.

In summary, the Hawaii agent EIA described above represents an efficient method of detection of Hawaii antigen and antibody which is less cumbersome and time consuming than immune electron microscopy. This assay should facilitate epidemiologic studies of the Hawaii agent and will aid in further characterization of the Hawaii virion.

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