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Antigenic Relatedness Among the Norwalk-Like Agents by Serum Antibody Rises

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The Norwalk, Snow Mountain (SMA), and Hawaii agents are etiologically associated with separate outbreaks of acute viral gastroenteritis. Previous cross-challenge of volunteers, immune electron microscopy, and/or enzyme-immunoassay analysis suggested that these agents are antigenically distinct. We examined paired sera from human volunteers challenged with these agents for the presence of homologous and heterologous serum antibody titer rises to the agents. Two-way cross-reactions occurred between Hawaii agent and SMA. A one-way cross-reaction occurred between Norwalk agent and SMA, as volunteers challenged with Norwalk agent had heterologous serum antibody titer rises to SMA, but the reverse did not occur. The Norwalk and Hawaii agents had minimal cross-reaction, with only one volunteer challenged with Hawaii agent having a heterologous rise to Norwalk agent. These observations indicate varying degrees of antigenic relatedness among these agents.

KEY WORDS: gastroenteritis viruses, serology, cross-reactions

INTRODUCTION

The Norwalk-like agents are a major cause of acute viral gastroenteritis, a self-limiting, acute viral illness [Dolin et al., 1971; Blacklow et al., 1972; Dolin, 1978; Greenberg et al., 1979]. The viruses are shed in only limited quantities in ill individuals and have not yet successfully been cultivated [Blacklow et al., 1972; Dolin et al., 1972]. These small, round viruses have a similar amorphous surface structure, size range (28–40 nm), and buoyant density in CsCl (1.36–1.41 g/cm^3) [Caul and Appleton, 1982; Appleton, 1987]. A single virion-associated protein of 59 kDa and 62 kDa on the Norwalk and Snow Mountain agents (SMA), respectively, is similar in size to that described for members of the family Caliciviridae [Greenberg et al., 1981; Madore et al., 1986a; Schaffer, 1979; Terashima et al., 1983].

The most extensively studied of these agents, the Norwalk, SMA, and Hawaii agents, are antigenically distinct by immunoelectron microscopy (IEM), radioimmunoassay (RIA), and enzyme immunoassay (EIA) [Kapikian et al., 1972; Thornhill et al., 1977; Dolin et al., 1982; Dolin et al., 1986; Madore et al., 1986b; Treanor et al., 1988]. The Norwalk and Hawaii agents are antigenically distinct by cross-challenge of volunteers [Wyatt et al., 1974].

On the other hand, in several outbreaks of gastroenteritis, homologous and heterologous serum antibody rises to Norwalk agent and SMA have been observed in ill individuals [Guest et al., 1987; Truman et al., 1987; Treanor et al., 1985]. The interpretation of these results, however, has been complicated by uncertainty regarding the specific agent involved or the possibility of dual infections in these outbreaks. We therefore sought to examine the antigenic relatedness of the Norwalk-like agents by analysis of homologous and heterologous serum antibody rises in human volunteers following experimental challenge with well characterized inocula containing the Norwalk, SMA, or Hawaii agents.

MATERIALS AND METHODS

Viral Antigens and Sera From Volunteers

The stool specimens and prechallenge and 2–3 weeks postchallenge sera were obtained from six separate volunteer challenge studies with Norwalk agent [Dolin et al., 1972; Dolin et al., unpublished], SMA [Dolin et al., 1982; Dolin et al., unpublished], and Hawaii agent [Treanor et al., 1988; Dolin et al., 1975]. Crude antigen-positive 2% (wt/vol) stool homogenates were made up in 0.5% bovine serum albumin (BSA)-veal infusion broth, aliquoted, and stored at −70°C. The inocula for the challenge studies were derived from human volunteers and were positive only for Norwalk, SMA or Hawaii agent by IEM, RIA, and EIA.

Blocking EIAs for Serum Antibody Rises

The double-sandwich blocking EIAs were performed as previously described [Madore et al., 1986b]. Briefly,

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the test wells (polystyrene 96-well tissue culture plates) were coated with capture antibody (2–3 weeks postchallenge positive serum) followed by blocking buffer (phosphate-buffered saline [PBS] with 0.5 M NaCl 0.5% (wt/vol) gelatin, 1% fetal bovine serum [FBS]), and Norwalk, SMA, or Hawaii antigen (2% crude stool homogenate) prepared in dilution buffer (PBS with 0.5M NaCl, 0.5% (wt/vol) gelatin, 10% FBS) to give a positive/negative (P/N) ratio of ≥4. Negative control (buffer, antigen absent) wells of postchallenge capture antibody were included.

For the blocking step, serum pairs to be titered were diluted to 1/100 followed by 4 fourfold dilutions. Duplicate aliquots of these dilutions and buffer only (unblocked antigen wells) were incubated in the antigen-positive wells. Subsequently, indicator antibody (biotinylated IgG from positive postchallenge serum), avidin-horseradish peroxidase, and TMB reaction mixture were added as previously described [Madore et al., 1986b]. The endpoint was defined as the highest dilution of serum that reduced the OD_{450} of the wells by 40% compared with the unblocked antigen wells (100% = OD_{450} of unblocked antigen wells minus OD_{450} of the negative control wells).

RESULTS

Challenges of Volunteers With Norwalk, SMA, and Hawaii Agents

Virus-positive specimens from the original Norwalk, SMA, and Hawaii outbreaks subsequently caused similar illnesses when administered to volunteers. Inocula derived from these volunteers were used in two separate challenge studies each for the Norwalk, SMA, and Hawaii agents. The challenge studies for each agent occurred in 1974–1978 and 1985–1986, approximately 8–12 years apart. The volunteers were healthy adults 18–35 years old. The attack rate for each agent was similar in the earlier (1974–1978) and later (1985–1986) challenges. The number of volunteers that became ill in the two challenges were 16/20 (80%) for SMA, 16/20 (80%) for Norwalk agent, and 16/21 (76%) for Hawaii agent.

The onset and duration of illness, virus shedding in stools, and seroconversions were similar in all instances and have been described for Norwalk, SMA, and Hawaii agents [Dolin et al., 1972, 1975, 1982]. We tested for the possibility of mixed infection in the challenge studies by assaying the inocula for the challenge studies and positive stools from the volunteers in EIAs specific for the Norwalk, SMA, and Hawaii agents. No evidence for mixed infection could be found [Madore et al., 1986b; Treanor et al., 1988; data not shown].

Homologous and Heterologous Serum Antibody Rises in Volunteers

Prechallenge and postchallenge serum pairs from the volunteers challenged with Norwalk, SMA, and Hawaii agents were assayed in the appropriate blocking EIAs for homologous serum antibody rises. The serum pairs were also assayed by blocking EIA for heterologous serum antibody rises. The seroresponses to the homologous agents from the 1974–1978 Norwalk, SMA, and the 1974–1978 and 1985–1986 Hawaii challenge studies have been reported [Madore et al., 1986b; Treanor et al., 1988]. Table I represents pooled seroresponses from two separate challenge studies for each agent, as the seroresponses in the two challenge studies for each agent were essentially the same. Serum pairs from volunteers clinically ill and well after challenge with the respective agents are included in the analysis.

A majority of the volunteers had homologous fourfold or greater serum antibody titer rises against the challenge agent. Thus, 90% (18/20) of the SMA volunteers had serum antibody rises to SMA, 75% (15/20) of the Norwalk volunteers had serum antibody rises to Norwalk agent, and 86% (18/21) of the Hawaii volunteers had serum antibody rises to Hawaii agent.

A variable pattern of heterologous fourfold or greater serum antibody titer rises against the nonchallenge agents was seen. Of the volunteers challenged with SMA, 50% (10/20) had rises to Hawaii agent. Conversely, 62% (13/21) of the volunteers challenged with Hawaii agent had rises to SMA. None of the volunteers challenged with SMA had antibody titer rises to the Norwalk agent, but 40% (8/20) of the volunteers challenged with Norwalk agent had antibody titer rises to SMA. None of the volunteers challenged with the Norwalk agent had antibody titer rises to the Hawaii agent, and only 5% (1/21) of the volunteers challenged with the Hawaii agent had rises to the Norwalk agent. In all instances, heterologous serum antibody titer rises were seen only in individuals who also had a significant homologous seroresponse to the challenge agent.

Differentiating between the clinically ill and well volunteers, a consistent pattern of seroresponse to the three challenge agents was observed. The seroresponse of the clinically ill volunteers was greater to the homologous (81%–100%) and heterologous (50%–70%) challenge agents than that of the well volunteers to the homologous (50%–60%) and heterologous (0–25%) challenge agents (data not shown).

TABLE I. Serum Antibody Rises in Volunteers Challenged With Norwalk-Like Agents

Challenge agent	No. of serum antibody rises (%)[a] by blocking EIA[b]		
	SMA	Norwalk	Hawaii
SMA	18/20 (90)	0/20 (0)	10/20 (50)
Norwalk	8/20 (40)	15/20 (75)	0/20 (0)
Hawaii	13/21 (62)	1/21 (5)	18/21 (86)

[a]Represents serum pairs from two separate challenge studies from each agent, including data from previous challenge studies [Dolin et al., 1972, 1975, 1982].
[b]Blocking EIA was performed on prechallenge and post-challenge serum pairs as described in Materials and Methods. Data represents ≥ fourfold serum antibody rises.

The extent of the homologous and heterologous serum antibody responses in volunteers was compared by calculating the geometric mean titers (GMT) of the prechallenge and postchallenge sera and the magnitude of the serum antibody rises (Table II). The magnitude of homologous rises was greater than that of heterologous rises. For example, the average antibody rise was 11-fold to SMA but threefold to the Hawaii agent in volunteers challenged with SMA. Conversely, volunteers challenged with Hawaii agent had a 45-fold rise to Hawaii agent and a fivefold rise to SMA. Similarly, in Norwalk volunteers, the rise to Norwalk agent was 22-fold and the heterologous rise to SMA was only threefold. This pattern was also evident in the seroresponse of volunteers challenged with different agents, but whose seroresponse was assayed in the same EIA. Thus, volunteers challenged with Hawaii agent or SMA had 45-fold and threefold serum antibody rises, respectively, to Hawaii agent, and volunteers challenged with SMA or Hawaii agent had 11-fold and fivefold antibody rises, respectively, to SMA.

The relationship of homologous and heterologous serum antibody rises to illness in volunteers after challenge was examined. Homologous serum antibody rises correlated with illness in volunteers challenged with SMA ($P=0.03$) and Hawaii agent ($P=0.05$), but not Norwalk agent (Table III). Heterologous serum antibody rises to SMA correlated with illness in Hawaii volunteers ($P=0.05$), but ill volunteers challenged with SMA or Norwalk agent did not have significant seroresponses to the heterologous agents.

The relation of preexisting homologous and heterologous serum antibody to protection from or development of illness upon challenge also was examined (Table IV). A titer of ≥ 100 was considered indicative of preexisting antibody. No correlation of preexisting homologous serum antibody to protection from or development of illness upon challenge with SMA, Norwalk, or Hawaii agents was evident. Preexisting heterologous serum antibody to SMA, however, appeared to correlate with development of illness after challenge with Norwalk agent ($P=0.01$). No other correlations of preexisting heterologous serum antibody were observed.

DISCUSSION

This report describes the antigenic relatedness of the Norwalk, SMA, and Hawaii agents of acute gastroenteritis by examining the frequency and magnitude of homologous and heterologous serum antibody titer rises in adult human volunteers challenged with these agents. Both homologous and heterologous serum antibody titer rises, as measured by blocking EIA, were observed in volunteers following the challenges with

TABLE II. Serum Antibody Geometric Mean Titer (GMT) and Rise in Volunteers*

Challenge agent	Blocking EIA					
	SMA		Norwalk		Hawaii	
	Pre	Post	Pre	Post	Pre	Post
SMA						
GMT	79	832	68	62	543	1840
Fold rise	11		1		3	
Norwalk						
GMT	119	373	104	2290	646	671
Fold rise	3		22		1	
Hawaii						
GMT	74	400	77	107	253	11,400
Fold rise	5		1		45	

*Serum pairs from all volunteers, ill and well, that were challenged with SMA, Norwalk, or Hawaii agents were tested in the EIAs for SMA, Norwalk, and Hawaii agents as described in Materials and Methods.

TABLE III. Relationship of Homologous and Heterologous Serum Antibody Rises to Illness in Volunteers After Challenge*

Challenge agent	No. of serum antibody rises (%)			
	Homologous		Heterologous	
	Ill	Well	Ill	Well
SMA	16/16 (100)	2/4 (50)[a]	9/16 (56)	1/4 (25)[b]
Norwalk	13/16 (81)	2/4 (50)	8/16 (50)	0/4 (0)[c]
Hawaii	16/16 (100)	3/5 (60)[a]	12/15 (75)	1/5 (20)[a,c]

*The groups of volunteers with only one or no heterologous serum antibody rises were not included.
[a]Correlation of serum antibody rise to illness in volunteers, $P \leq 0.05$, Fisher's exact test, two tail.
[b]Rises to Hawaii agent.
[c]Rises to SMA.

TABLE IV. Relationship of Preexisting Homologous and Heterologous Serum Antibody to Illness in Volunteers After Challenge

Challenge agent	No. of volunteers ill (%) Preexisting serum antibody[a] by blocking EIA					
	SMA		Norwalk		Hawaii	
	Yes	No	Yes	No	Yes	No
SMA	5/6 (83)	11/14 (79)	3/5 (60)	13/15 (87)	3/17 (76)	3/3 (100)
Norwalk	12/12 (100)	4/8 (50)[b]	10/11 (91)	6/9 (67)	14/17 (82)	2/3 (67)
Hawaii	7/7 (100)	9/14 (64)	6/7 (86)	10/14 (71)	12/16 (75)	4/5 (80)

[a]Titer $\geq$ 100.
[b]Correlation of preexisting antibody to illness in volunteers, $P = 0.01$ by Fisher's exact test, two tail. All other differences were $P > 0.05$.

respective agents. The frequency and magnitude of the homologous rises was consistently greater than that of the heterologous rises.

The blocking EIAs used to measure the serum antibody rises have previously been shown to be specific for these particular agents [Madore et al., 1986b; Treanor et al., 1988]. Analysis of the inocula and of the stool specimens derived from the volunteers subsequent to the challenges [Madore et al., 1986b; Treanor et al., 1988; data not shown] revealed no evidence of mixed infection. We therefore interpret the heterologous serum antibody titer rises seen in this study as an indication that some antigenic relatedness exists among the Norwalk-like agents. The extent of the antigenic relatedness of one agent to another is variable. The SMA and Hawaii agents, with two-way cross-reactivity, appear to be more closely related than the SMA and Norwalk agents, which have only one-way cross-reactions. The Norwalk and Hawaii agents appear to be the most distantly related, with little if any cross-reactivity.

Previous reports indicated that the Norwalk and Hawaii agents were antigenically distinct by IEM [Dolin et al., 1982], EIA [Treanor et al., 1988] and cross-challenge studies in volunteers [Wyatt et al., 1974]. Our findings of many homologous rises but only one heterologous rise among volunteers challenged with these agents is consistent with these reports, and suggests little antigenic relatedness between these agents.

SMA was shown to be antigenically distinct from the Norwalk and Hawaii agents by IEM, RIA, and EIA [Dolin et al., 1982; Madore et al., 1986b; Treanor et al., 1988]. The observation of homologous serum antibody titer rises coupled with heterologous rises of less frequency and magnitude suggests the existence of both antigenically distinct and cross-reactive domains between SMA and the Norwalk and Hawaii agents.

Serum antibody rises to both SMA and Norwalk agent have been observed in several outbreaks of acute gastroenteritis. In an outbreak associated with the eating of raw clams and attributed to SMA by isolation of the agent in stool specimens, serum antibody rises to SMA and to the Norwalk agent [Truman et al., 1987] occurred in some individuals. Another outbreak in a high school cafeteria had six serum antibody rises to SMA and two to Norwalk agent [Guest et al., 1987]. In the above outbreaks, the SMA EIA and Norwalk RIA were performed by different laboratories. This laboratory, however, recently detected serum antibody rises to both SMA and Norwalk agent with our blocking EIAs in two nursing home outbreaks [Treanor et al., 1985]. The serology of the above outbreaks suggests that two-way cross-reactions may exist between Norwalk agent and SMA, or that mixed infections occurred in the outbreaks. If two-way cross-reactions between Norwalk agent and SMA do exist, the one-way cross-reactions seen in the volunteers (Table I) may reflect different histories of exposure to these agents.

With regard to the cross-reactivity between SMA and Hawaii agent, as manifested by heterologous serum antibody titer rises in the volunteers, natural outbreaks have not yet been examined due to the only recent development of the Hawaii EIA [Treanor et al., 1988].

An antigenic relationship between the Norwalk-like agents and human enteric caliciviruses (HCV) has been established. Cubitt et al. [1987] described a one-way serologic cross-reaction between HCV strains UK4 and UK2 and Norwalk agent by Norwalk blocking RIA. Hayashi et al. [1989] isolated and characterized a small, round-structured virus (SRSV-9) associated with acute gastroenteritis outbreaks in Japan. On Western-blot analysis, the single structural protein (63 kDa) reacted with antiserum to Hawaii agent, but not with antiserum to Norwalk agent. The SRSV-9, however, did not react with anti-Hawaii or anti-Norwalk sera by IEM. The Norwalk-like agents also appear to be related to the human caliciviruses by biochemical criteria, particularly the existence among these agents of a single virion-associated protein of similar size [Greenberg et al., 1981; Madore et al., 1986a; Schaffer, 1979; Hayashi et al., 1989].

A practical consideration derived from our observations is that designation of a specific Norwalk-like agent as the cause of an acute gastroenteritis outbreak on the basis of serodiagnosis alone may be insufficient. Our analysis of the seroresponses of ill volunteers after

challenge (Table III) indicated that seroresponse does not consistently correlate with the challenge agent. In particular, ill volunteers challenged with Hawaii agent had significant seroresponses to both Hawaii agent and SMA. Conversely, the seroresponses to Norwalk agent did not correlate well in ill volunteers challenged with Norwalk agent. Detection of the agent(s) by specific assays (IEM, EIA, RIA) in stool specimens from affected individuals can help to confirm the serodiagnosis.

We also examined the relationship of preexisting serum antibody to protection from or development of illness upon challenge. Preexisting homologous antibody did not appear to correlate with protection from or development of clinical illness upon challenge with SMA, Norwalk, or Hawaii agents. Preexisting heterologous serum antibody against SMA, however, appeared to correlate with development of clinical illness in the volunteers challenged with Norwalk agent, but similar correlations were not observed in volunteers challenged with SMA or Hawaii agents.

Similar paradoxical observations on the role of preexisting serum antibody in protection from or the development of illness have been reported. Blacklow et al. [1979], in volunteer studies with Norwalk agent, observed an apparent correlation of preexisting serum antibody (RIA) with development of illness in volunteers challenged with Norwalk agent. Ryder et al. [1985], however, reported that preexisting serum antibody (RIA) to Norwalk agent appeared to correlate with protection from reinfection by Norwalk agent in Panamanian Indians. Preexisting antibody (RIA) to human calicivirus in infants also appeared to correlate with protection from illness upon reinfection of the infants with HCV [Nakata et al., 1985].

These observations indicate that factors such as the sensitivity and specificity of the blocking EIAs and RIAs, antigenic cross-reactivity of the agents, and exposure history of the subjects to these agents must be considered in defining the roles of humoral antibody, gut secretory antibody, and cell-mediated immunity in protection from or development of gastrointestinal illness caused by the Norwalk-like agents or human caliciviruses.

This report indicates the existence of varying degrees of antigenic relatedness among the Norwalk-like agents manifested by heterologous serum antibody rises in volunteers. To facilitate a more precise antigenic characterization of these agents, improved reagents such as hyperimmune sera from animals or monoclonal antibodies are needed. The location of possible type-specific or common antigenic determinants on virion-associated and/or soluble proteins also remains to be determined. Finally, the clinical significance of the heterologous rises seen in these challenge studies should be studied in cross-protection experiments. Should significant cross-protection exist, it may have important implications for future vaccine development.

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