Capsid Functions of Inactivated Human Picornaviruses and Feline Calicivirus

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The exceptional stability of enteric viruses probably resides in their capsids. The capsid functions of inactivated human picornaviruses and feline calicivirus (FCV) were determined. Viruses were inactivated by UV, hypochlorite, high temperature (72°C), and physiological temperature (37°C), all of which are pertinent to transmission via food and water. Poliovirus (PV) and hepatitis A virus (HAV) are transmissible via water and food, and FCV is the best available surrogate for the Norwalk-like viruses, which are leading causes of food-borne and waterborne disease in the United States. The capsids of all 37°C-inactivated viruses still protected the viral RNA against RNase, even in the presence of proteinase K, which contrasted with findings with viruses inactivated at 72°C. The loss of ability of the virus to attach to homologous cell receptors was universal, regardless of virus type and inactivation method, except for UV-inactivated HAV, and so virus inactivation was almost always accompanied by the loss of virus attachment. Inactivated HAV and FCV were captured by homologous antibodies. However, inactivated PV type 1 (PV-1) was not captured by homologous antibody and 37°C-inactivated PV-1 was only partially captured. The epitopes on the capsids of HAV and FCV are evidently discrete from the receptor attachment sites, unlike those of PV-1. These findings indicate that the primary target of UV, hypochlorite, and 72°C inactivation is the capsid and that the target of thermal inactivation (37°C versus 72°C) is temperature dependent.

Human enteric viruses are recognized as leading causes of food-borne and waterborne disease in the United States. Norwalk-like viruses (NLVs) and hepatitis A virus (HAV) are leading agents among known food-borne pathogens (22).

Poliovirus (PV) and HAV are the type species of the genera *Enterovirus* and *Hepatovirus*, respectively, in the *Picornaviridae* family; NLVs are in the family *Caliciviridae*. These human enteric viruses share simple morphological structures: one molecule of linear, positive-sense, single-stranded RNA, covered by capsid protein without an envelope (naked). The nucleic acid has a small, genome-linked virion protein (VPg) and a poly(A) tract covalently attached to the 5' and 3' ends of the single-stranded RNA genome, respectively. Arrangements of capsid protein subunits are determined by genetic economy and functionality (3). The capsid of the human enteric viruses serves some crucial functions: RNase protection, attachment to host cell receptors (as part of the entry process), and also interaction with the host cellular immune system (12).

A primary function of the capsid is to protect the viral genome from environmental conditions and ultimately to deliver the genome to the interior of a homologous host cell. The exceptional stability of viruses transmitted via food and water most likely resides in the capsid, in that the genomes of other viruses are no less stable than the RNA of these agents. Extreme conditions of heat, UV, chlorine, or acidity are required to inactivate enteric viruses rapidly and efficiently in food or water (7, 9, 18, 20, 27, 31, 38). When the viral RNA is inside the

intact capsid of a native picornavirus, the infectivity of the virus particle is a million times more resistant to RNase than that of the unprotected RNA genome. The enteric virus genome itself is extremely susceptible to RNase (<10 ng/ml), which appears to be ubiquitous and unavoidable (11). Once the enteric viruses get inside a host's body, they must, without fail, encounter and resist stomach acid and pepsin, as well as intestinal proteases. Earlier experiments have revealed that a combination of proteinase K (PK) and RNase yields a negative reverse transcription (RT)-PCR with inactivated PV, HAV, and feline calicivirus (FCV) that have been inactivated by high temperature, UV, or chlorine (25). Evidently the stability of the viral capsid is critical for protecting the viral genome in the harsh environment outside of host cells.

Once inside the host's body, enteric viruses attach specifically to a homologous host cell. Thus, the capsid is also necessary to supply the attachment (receptor-binding) site to react with specific homologous receptors on the host cell and trigger the virion entry process. Many factors influence the attachment of enteric viruses to cell receptors. Unlike those of HAV and the NLVs, the attachment and the receptor of PV are well documented (11). The Frp/3 cell receptor for HAV may consist of phospholipids, protein, and galactose (32), whereas a recent study showed that the African green monkey kidney cell receptor of HAV is a mucin-like class I integral membrane glycoprotein (17).

The infectivity of enteric viruses requires the functional integrity of both the viral RNA and capsid. At the protein structural level, conformational changes in the capsid, which may diminish viral stability or affect attachment to a cell receptor, directly jeopardize viral infectivity. Even though the antigenicity of a virus is not an infectivity determinant, changes in

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antigenic function may provide insight into the capsid conformational changes that accompany inactivation.

The principal viruses transmitted via food and water are detectable only by molecular methods that do not distinguish between virus that is infectious and virus that has been inactivated. Furthermore, evaluations of processes intended to eliminate viral hazards cannot generally be done by infectivity tests because laboratory hosts for these viruses do not exist and human subject trials are problematic. The present study examined capsid functions in representative viruses because this is where the relative stability of these agents resides. The results were expected to point to further ways to modify molecular tests so as to obtain positive results only from infectious virus (25). Further, where capsid modifications are a demonstrated mode of inactivation, it should be possible to evaluate virus inactivation treatments without conducting infectivity tests.

The objective of the present study was to demonstrate the functional changes of capsids of viruses that had been inactivated by UV, hypochlorite, and high-temperature (72°C) and physiological-temperature (37°C) heat. These modes of inactivation were chosen because UV light and hypochlorite are used to disinfect water, as well as food surfaces and food-contact surfaces, and heat is commonly used in food preparation and processing and, in emergencies, with water. Inactivation at 37°C was intended to represent the fate of the majority of viruses that lose infectivity in the environment, without human intervention. These inactivating agents were expected to represent different modes of attack: high-temperature heat principally attacks the viral coat protein (4), UV predominantly targets the viral RNA, depending on the dose (14, 39), and hypochlorite is supposed to affect both the coat protein and the RNA (1, 26, 40). At moderate temperatures ($\leq 37^\circ\text{C}$), the RNA is probably the labile moiety. The model viruses comprised a vaccine strain of PV type 1 (PV-1), along with a cytopathic, laboratory strain of HAV. Since no laboratory host for the NLVs has yet been identified and since FCV and the NLVs belong to the same virus family (36), FCV has been used in other studies as a surrogate for NLVs (10), as was done in the present study.

MATERIALS AND METHODS

Viruses and cell cultures. PV-1 strain CHAT was obtained from the American Type Culture Collection (ATCC VR-192; Manassas, Va.). HAV strain HM175/18f (HM175 cytopathic clone B) is a cell culture-adapted cytopathic variant, kindly provided by Marylynn Yates, University of California, Riverside. FCV vaccine strain was kindly provided by Niels Pedersen, Center for Companion Animal Health, School of Veterinary Medicine, University of California, Davis. FRhK-4, a continuous line of fetal rhesus monkey kidney cells, was also contributed by M. Yates, and the Crandell Reese feline kidney (CRFK) cell line was contributed by N. Pedersen. Both cell lines were grown in a medium composed of Dulbecco's modified Eagle's medium powder containing 4,500 mg of D-glucose and L-glutamine/liter, 110 mg of sodium pyruvate/liter, and pyridoxine hydrochloride (Gibco BRL, Life Technologies, Grand Island, N.Y.) and supplemented with 10% fetal bovine serum (Sigma, St. Louis, Mo.), 0.1 mM nonessential amino acids (Gibco BRL), and 44 mM NaHCO_3 (Mallinckrodt AR, Paris, Ky.). The maintenance medium was like the growth medium but contained only 5% fetal bovine serum. Cells for virus propagation and assay were grown in polystyrene flasks (Corning Glass Works, Corning, N.Y.).

Virus preparation. PV-1 and HAV were propagated in FRhK-4 cells and FCV was propagated in CRFK cells, in maintenance medium at 37°C in a conventional incubator without supplemental carbon dioxide. When cytopathic effects were complete, medium was collected from the flask and eventually mixed with lysate of the cells, obtained by treatment with 0.4% (wt/vol) sodium dodecyl sulfate and

phosphate-buffered saline (PBS) (ratio, 1:1). The pooled supernatant and cell lysate were passed through a series of polycarbonate filters of porosity 0.4 to 0.2 μm (Gelman Sciences, Ann Arbor, Mich.), dispensed into small-volume tubes, and kept at -70°C until used.

Virus assay (plaque technique). Tenfold virus dilutions were inoculated (0.5 ml) on confluent monolayers of cells in 25-cm² flasks. The control flasks were inoculated with 0.5 ml of viral diluent. The viral diluent was PBS (Sigma) (137 mmol of sodium chloride, 2.7 mmol of potassium chloride, and 10 mmol of phosphate buffer). Flasks were incubated and rocked at 37°C for 1 h. Without pipetting off the inocula, 5 ml for PV-1 and FCV but 10 ml for HAV of overlay medium at 45°C was added. The overlay medium was the maintenance medium plus a final concentration of 0.75% agarose (agarose type II medium EEO; Sigma). All flasks were incubated cell-side-up at 37°C, for 3 days for PV-1, 14 days for HAV, and 2 days for FCV. Following the incubation period, cell monolayers were fixed with formaldehyde solution and stained with crystal violet solution as described previously (25). Virus titer was recorded as the number of PFU per milliliter of virus suspension inoculated.

Inactivation methods. The inactivation methods selected for study were UV, hypochlorite, and high-temperature (72°C) and low-temperature (37°C) heat, with PBS as the viral diluent. Preliminary inactivation studies established the minimal treatment required to eliminate all viral infectivity, starting with $\sim 10^3$ PFU/ml. This working titer was chosen so that the last of the infectivity could be eliminated without the earlier-inactivated virions having sustained excessive repetitions of the event by which they had been inactivated. Although precise quantification was not needed, the inactivation curves were monitored for signs of viral aggregation.

The sensitivity of the RT-PCR detection method (see below) is such that it can amplify a few infectious virus particles in a mixed population. Therefore, a positive RT-PCR test might result from the presence of a few infectious particles, rather than a reaction by the inactivated virus. In most instances, when no residual PFU could be detected, a further sample of the final suspension was tested in cultures under fluid medium to verify the absence of any residual infectivity.

UV inactivation. A low-pressure mercury vapor discharge lamp (germicidal lamp TUV 30 W; Phillips, Holland) was used for this study. The germicidal lamp, with tubular glass envelope, emits short-wavelength UV radiation with the peak (monochromatic) at 253.7 nm, with only about 1% of other wavelengths. The intensity of the UV radiation was measured by using a digital UVX radiometer (UVP, San Gabriel, Calif.). The UV dose is defined and usually measured as incident energy (not absorbed energy), which is the product of constant UV intensity or dose rate in units of milliwatts per square centimeter and time in units of seconds. The range of UV doses used in the experiment was $\leq 125 \text{ mW} \cdot \text{s}/\text{cm}^2$ ($\leq 1,250 \text{ J}/\text{m}^2$). The continuous ventilation in the biosafety cabinet and the glass of the lamp tube filtering out 185-nm-wavelength radiation prevented ozone formation in the air between the UV source and sample. The UV irradiation effect could have been confounded by that of ozone, which could dissolve in the sample virus suspension and be harmful to the viruses (13, 15, 37). The average UV intensity was about 1.60 to 1.70 mW/cm^2 . The stock virus suspensions were diluted 10-fold with PBS to prepare the working virus suspension, essentially to eliminate UV absorption by any proteins left over from the cell culture maintenance medium. It has been shown that phosphate buffer solution has only slight absorption of wavelengths $>220 \text{ nm}$, even with solutions 1 cm deep (16). Virus suspensions, ca. 1 to 3 ml, were dispensed to form a layer of fluid $<2 \text{ mm}$ deep in a round, flat petri dish of 4-cm diameter. The samples were not stirred because the thin layer of virus suspension made this unnecessary; however, vibration from the fan in the biological safety cabinet also agitated the virus suspensions at all times. The UV inactivation doses required were determined by the 90% (1-log) inactivation dose and the initial titer of virus suspension (14, 38).

Photoreactivation. The photoreactivation experiment was done only with PV-1. The PV-1 suspension was divided into three samples. One was tested for the initial titer. The other two samples were irradiated with UV at $75 \text{ mW} \cdot \text{s}/\text{cm}^2$ ($750 \text{ J}/\text{m}^2$). One irradiated sample was kept under aluminum foil in darkness, and the other was exposed to white light (fluorescent lamp). After 2 h, a virus assay was done to determine the residual infectivity of these last two samples.

Hypochlorite inactivation. The working concentration of free chlorine (FC) was either 1.20 or 1.25 mg/liter (ppm). The FC concentration was measured by the *N,N*-diethyl-*p*-phenylene diamine colorimetric method, using a portable microprocessor chlorine colorimeter (HI 93701) plus free chlorine reagent (HI 93701-0; Hanna Instruments, Woonsocket, R.I.). The stock solution of 5% NaOCl (Sigma) was added directly to the working virus suspension, and the concentration of FC was measured directly from the suspension. The amount of NaOCl to be added is dependent upon the chlorine demand of the virus sus-

TABLE 1. PCR primers used in this study

Virus	Primer	Sequence 5'-3'	Amplicon (bp)
HAV	HAV1(+)	GTT TTG CTC CTC TTT ACC ATG CTA TG	247
	HAV2(-)	GGA AAT GTC TCA GGT ACT TTC TTT G	
PV-1	EV1(+)	CAA GCA CTT CTG TTT CCC CGG	435
	EV2(-)	ATT GTC AAC CAT AAG CAG CCA	
FCV	FCV1(+)	GTC CCA TGA CTA AGT TAT	386
	FCV2(-)	TTT TTT CCC TGG GGT TAG GC	

pension and container. Inactivation was done at 5°C; pH was approximately 7. To neutralize the FC activity, 0.1 ml of a 1.20- or 1.25-g/liter solution (0.12 or 0.125%) of sodium thiosulfate ($\text{Na}_2\text{O}_3\text{S}_3$) was added to 10 ml of virus suspension, to a final concentration of 12.0 to 12.5 mg/liter (ppm), whereby the neutralizer concentration was 10 times the initial FC concentration. At pH ca. 7.0, the $C \times T$ (concentration $\times$ time) values (in milligrams per liter per minute) to inactivate 90% of PV-1, HAV, and FCV are 0.717 (29, 30), 7.0 (34), and 0.4 (10), respectively.

Thermal inactivation. The high-temperature thermal inactivation temperature was 72°C, to avoid complete destruction of the viral coat protein. For all three viruses, PBS was preheated in the water bath at 72°C and the stock virus suspension was diluted 10-fold into the preheated diluent and incubated for the selected time. When the selected time had elapsed, the treated virus suspension was diluted 10-fold in prechilled diluent. This method minimized time spent by the virus at temperatures other than what was selected. At 72°C, PV-1 and HAV require 5.44 and 18.35 s (21) to inactivate 90% of PFU/ml, respectively. The physiological-temperature thermal inactivation was done at 37°C. The HAV suspension and a mixture of PV-1 and FCV were kept in the incubator and tested for infectivity weekly.

Composite enzymatic digestion. PK (Sigma) was dissolved in PBS and prepared freshly for each experiment (25). Tris-EDTA buffer (Sigma) was diluted 100-fold to obtain 1.0 M Tris-HCl, 0.1 M EDTA, pH 8.0. RNase (Boehringer Mannheim, Indianapolis, Ind.) was diluted in the Tris-EDTA buffer and kept at -20°C. Both PK, 20 U, and RNase, 100 ng, were added to the inactivated and infectious control viruses and incubated at 37°C for 30 min. RNase inhibitor solution (Perkin-Elmer, Foster City, Calif.), 40 U, was then added to the suspension. After this digestion step, the virus suspension was subjected to RNA extraction and routine RT-PCR.

Attachment to cell monolayers. Confluent cell monolayers were washed once with 5 ml of Dulbecco's PBS (Gibco BRL) including calcium and magnesium ions, aspirated, and then inoculated with 0.5 ml of virus suspension. After interaction of virus with the cell monolayer for 1 h at 37°C for HAV and FCV but at room temperature for PV-1, the cell monolayer flasks were washed three times with 1 ml of maintenance medium. The washing fluids, including the inoculum, were pooled. The cell monolayers were scraped off with a sterile dSPo cell scraper (American Scientific Products, MacGaw Park, Ill.). The pooled washing medium and the scraped-off cell suspension were assayed separately for the virus by RT-PCR.

RNA extraction. The QIAamp viral RNA mini kit (Qiagen, Valencia, Calif.) was used to extract the viral RNA from the virus suspensions after digestion, according to the manufacturer's directions. Briefly, the process entails extracting the RNA chemically from the virus and loading it on a small chromatographic column in a microcentrifuge tube. After two washings, the RNA is eluted and ready for analysis or RT-PCR. The extracted viral RNA is stable for up to 1 year when stored at lower than -70°C.

AC-PCR. The present antigen-capture PCR (AC-PCR) procedure was slightly modified from that described previously (8). Mouse PV-1 monoclonal antibody (Chemicon International, Temecula, Calif.) is a type-specific reagent for the presumptive identification of PV-1 obtained from cell culture. Monkey PV-1, PV-2, and PV-3 (Sabin) immunoglobulin with neutralizing antibody titers of 1:8,192, 1:4,096, and 1:4,096, respectively, were kindly provided by David Schnurr, Viral and Rickettsial Disease Laboratory, California Department of Health Services, Richmond, Calif. Rabbit anti-HAV and rabbit anti-FCV were raised by the Animal Resources Service, School of Veterinary Medicine, University of California, Davis. For the antigen-capture step, sterile 200- μ l microcentrifuge tubes were coated with 100 μ l of antiserum diluted 1:1,000 in 50 mM sodium carbonate buffer, pH 9.6, and incubated at 37°C for more than 4 h. The antiserum in the tube was aspirated off and replaced by 150 μ l of 1% bovine serum albumin in 20 mM sodium carbonate buffer, pH 9.6, and the tubes were again incubated at 37°C for 1 h. After the bovine serum albumin was removed,

the now-antibody-coated tubes were washed three times with 200 μ l of PBS supplemented with a final concentration of 0.05% (vol/vol) Tween 80. The virus suspension (100 μ l) was added to the antiserum-coated tubes to be captured for at least 15 h (or overnight) at 4°C. The virus suspensions were aspirated off from the now-antigen-antibody complex-coated tubes, and 150 μ l of TKM buffer (20 mM Tris [pH 8.4], 75 mM KCl, and 2.5 mM MgCl_2) was used to wash the tubes three times. For the RNA extraction, 250 μ l of lysis buffer was added directly into the washed, antigen-antibody complex-coated tubes to release viral RNA into the buffer. Then the aforementioned RNA extraction procedure (QIAamp viral RNA mini kit) was followed. The extracted RNA of each virus was subjected to the appropriate RT-PCR.

RT-PCR. (i) PV-1. For RT, extracted RNA (20 μ l) was added to 30 μ l of RT mix. The RT mix had 5 μ l of 10 $\times$ PCR buffer II (Perkin-Elmer), 2.0 mM MgCl_2 (Perkin-Elmer), a 0.3 mM concentration of each deoxynucleoside triphosphate (dNTP; Perkin-Elmer), 1.2 μ M EV2 primer (100 μ M; Operon), 50 U of Maloney murine leukemia virus reverse transcriptase (50 U/ μ l; Perkin-Elmer), 20 U of RNase inhibitor (20 U/ μ l; Perkin-Elmer), and 8.0 μ l of diethyl pyrocarbonate-treated water. The enterovirus primer sequences are shown in Table 1. The mixture was incubated for 30 min at 42°C and 5 min at 95°C and then cooled to 4°C using the GeneAmp PCR system 9700 (PE Applied Biosystems). For PCR, the PCR mix (50 μ l) was added to the RT reaction tubes. The PCR mix had 44 μ l of deionized water, 5 μ l of 10 $\times$ PCR buffer II (Perkin-Elmer), 1.2 μ M EV1 primer (100 μ M; Operon), and 2.5 U of AmpliTaq DNA polymerase (50 U/ μ l; Perkin-Elmer). This reaction mixture was prepared for PCR at 95°C (1 min), 60°C (1 min) and 72°C (1 min), and then subjected to 40 cycles of denaturation at 95°C (50 s), annealing at 60°C (50 s), and extension at 72°C (50 s), with an additional 7-min extension at 72°C, using the GeneAmp PCR system 9700 (PE Applied Biosystems).

(ii) HAV. The process for HAV was generally the same as that described for PV-1, except that HAV primers were used (Table 1) and the RT mixture included a 0.6 mM concentration of each dNTP. The reaction mixture was prepared for PCR at 94°C (5 min), 55°C (1 min), and 72°C (1 min) and then subjected to 35 cycles of denaturation at 94°C (20 s), annealing at 55°C (20 s), and extension at 72°C (20 s), with an additional 7-min extension at 72°C (8), using the GeneAmp PCR system 9700 (PE Applied Biosystems).

(iii) FCV. The FCV process was generally the same as that described for PV-1, except that FCV primers were used (Table 1) and the RT mixture included 3.0 mM MgCl_2 and a 0.4 mM concentration of each dNTP. The reaction mixture was prepared for PCR at 94°C (30 s) and then subjected to 40 cycles of denaturation at 94°C (1 min), annealing at 55°C (45 s), and extension at 72°C (1 min), with an additional 7-min extension at 72°C (33), using the GeneAmp PCR system 9700 (PE Applied Biosystems).

Analysis of PCR products. Agarose was added to 150 ml of TAE electrophoresis buffer (2 M Tris acetate, 0.05 M EDTA [pH 8.3]; catalog no. 5302-844314; 5 Prime $\rightarrow$ 3 Prime, Inc.) to a final concentration of 2% and dissolved in a microwave oven. Ethidium bromide (7.5 μ l of a 10-mg/ml solution in water; Gibco BRL) was added and mixed thoroughly. The gel was poured, loaded, and run for 30 to 40 min at 110 V. Digital images of the gel electrophoresis were obtained with the Gel Doc 1000 gel documentation hardware system and Quantity One software (Bio-Rad Laboratories, Inc.).

RESULTS

Virus inactivation. Each of the three viruses was completely inactivated by each of the four methods. No photoreactivation of UV-inactivated PV-1 was detected.

Capsid protection against RNase. We had determined earlier that viruses which had been inactivated with UV, chlorine,

FIG. 1. Agarose gel analysis of RT-PCR assay of 37°C-inactivated viruses treated with PK and RNase. (A) HAV assay. Lanes 1 to 4, HAV treated with PK plus RNase (lane 1), PK (lane 2), or RNase (lane 3), or untreated (lane 4). Lanes 5 and 6, native HAV treated with PK plus RNase (lane 5) or untreated (lane 6). (B) PV-1 assay. Lanes 1 to 4, PV-1 treated with PK plus RNase (lane 1), PK (lane 2), or RNase (lane 3), or untreated (lane 4). Lanes 5 and 6, native PV-1 treated with PK plus RNase (lane 5) or untreated (lane 6). (C) FCV assay. Lanes 1 to 4, FCV treated with PK plus RNase (lane 1), PK (lane 2), or RNase (lane 3), or untreated (lane 4). Lanes 5 and 6, native FCV treated with PK plus RNase (lane 5) or untreated (lane 6). Lanes M, 100-bp DNA ladder (Gibco BRL). Arrows indicate the amplicon of interest.

and 72°C yielded a true-negative RT-PCR after they had further undergone a composite enzymatic digestion (25). This finding provides a simple tool not only to probe the conformational change of the capsid with only a single step added to the routine RT-PCR detection method, but also to eliminate the false-positive RT-PCR detection of inactivated viruses. In the present study, composite enzymatic digestion with both PK and RNase was applied only to PV-1, HAV, and FCV that had been inactivated at 37°C. The capsids of these inactivated viruses were still capable of protecting viral genome from RNase, as shown by the positive RT-PCR (Fig. 1).

Attachment of inactivated viruses to cell monolayers. (i) PV-1. RT-PCR of cellular total RNA using enterovirus primer sets was performed beforehand, as a background or control to detect any cellular amplicons that otherwise could mask the PV-1 amplicon. Cellular nucleic acid moved ahead of the viral amplicons in the gels; for example, no cellular amplicon was found in the same position as the PV-1 amplicon (Fig. 2A). Native PV-1 attachment to FRhK-4 (homologous) cell receptors was specific (Fig. 2B, lane 1), and only a small portion of the viruses was washed off (Fig. 2B, lane 2). The interaction of native PV-1 with CRFK (heterologous) cell receptors did not prevent the PV-1 from being entirely washed off the cell mono-

layer (Fig. 2B, lanes 4 to 6). All inactivated PV-1 entirely failed to attach to the homologous cell monolayers (Fig. 2C).

(ii) HAV. RT-PCR of cellular total RNA using HAV primer sets was performed beforehand, as a background or control to detect any cellular amplicons that otherwise could mask the HAV amplicon. No cellular amplicon or DNA was found in the same position as the HAV amplicon (Fig. 3A). Native HAV attachment to FRhK-4 (homologous) cell receptors was specific and successfully held during the washing step (Fig. 3B, lanes 1 to 3). The interaction of native HAV with CRFK (heterologous) cell receptors did not hold the HAV to the cell monolayer (Fig. 3B, lanes 4 to 6). All inactivated HAV entirely failed to attach to the homologous cell monolayers, except that UV-inactivated HAV partially retained the attachment capability (Fig. 3C).

(iii) FCV. RT-PCR of cellular total RNA using FCV primer sets was performed beforehand, as a background or control to detect any cellular amplicons that otherwise could mask the FCV amplicon. No cellular amplicon was found in the same position as the FCV amplicon (Fig. 4A). Native FCV attachment to CRFK (homologous) cell receptors was specific and successfully held during the washing step (Fig. 4B, lanes 1 to 3). The interaction of native FCV with FRhK-4 (heterologous)

FIG. 2. Agarose gel analysis of RT-PCR assay of attachment of inactivated PV-1 to cell monolayer (receptors). (A) Lane 1, FRhK-4 cell lysate amplified with enterovirus primer set; lane 2, CRFK cell lysate with enterovirus primer set; lane 3, native PV-1 without cell lysate. (B) Lanes 1 to 3, native PV-1 attachment to FRhK-4 (homologous) cell receptors in lysate of FRhK-4 cells inoculated with PV-1 and washed (lane 1), wash-off PV-1 inoculum from lane 1 (lane 2), or lysate of FRhK-4 cells inoculated with PV-1 and not washed (lane 3). Lanes 4 to 6, native PV-1 attachment to CRFK (heterologous) cell receptors in lysate of CRFK cells inoculated with PV-1 and washed (lane 4), wash-off PV-1 inoculum from lane 4 (lane 5), or lysate of CRFK cells inoculated with PV-1 and not washed (lane 6). Lane 7, PV-1 inoculum alone. (C) Lanes 1 to 8, inactivated PV-1 attachment to FRhK-4 (homologous) cell receptors (subsequently washed) in lysate of cells inoculated with UV-inactivated PV-1 (lanes 1 and 2), lysate of cells inoculated with hypochlorite-inactivated PV-1 (lanes 3 and 4), lysate of cells inoculated with 72°C-inactivated PV-1 (lanes 5 and 6), or lysate of cells inoculated with 37°C-inactivated PV-1 (lanes 7 and 8). Lane 9, lysate of cells inoculated with native PV-1 and washed. Lanes M, 100-bp DNA ladder (Gibco BRL); lane M1, 1-kb DNA ladder (Gibco BRL). Arrows indicate the amplicon of interest.

FIG. 3. Agarose gel analysis of RT-PCR assay of attachment of inactivated HAV to cell monolayer (receptors). (A) Lane 1, FRhK-4 cell lysate amplified with HAV primer set; lane 2, CRFK cell lysate with HAV primer set; lane 3, native HAV without cell lysate. (B) Lanes 1 to 3, native HAV attachment to FRhK-4 (homologous) cell receptors in lysate of FRhK-4 cells inoculated with HAV and washed (lane 1), wash-off HAV inoculum from lane 1 (lane 2), or lysate of FRhK-4 cells inoculated with HAV and not washed (lane 3). Lanes 4 to 6, native HAV attachment to CRFK (heterologous) cell receptors in lysate of CRFK cells inoculated with HAV and washed (lane 4), wash-off HAV inoculum from lane 4 (lane 5), or lysate of CRFK cells inoculated with HAV and not washed (lane 6). Lane 7, HAV inoculum alone. (C) Lanes 1 to 8, inactivated HAV attachment to FRhK-4 (homologous) cell receptors in lysate of cells inoculated with UV-inactivated HAV and washed (lane 1), wash-off inoculum from lane 1 (lane 2), lysate of cells inoculated with hypochlorite-inactivated HAV and washed (lane 3), wash-off inoculum from lane 3 (lane 4), lysate of cells inoculated with 72°C-inactivated HAV and washed (lane 5), wash-off inoculum from lane 5 (lane 6), lysate of cells inoculated with 37°C-inactivated HAV and washed (lane 7), or wash-off inoculum from lane 7 (lane 8). Lanes 9 and 10, native HAV attachment to FRhK-4 in lysate of cells inoculated with native HAV and washed (lane 9) or wash-off inoculum from lane 9 (lane 10). Lanes M, 100-bp DNA ladder (Gibco BRL). Arrows indicate the amplicon of interest.

cell receptors did not hold the native FCV to the cell monolayer (Fig. 4B, lanes 4 to 6). All inactivated FCV entirely failed to attach to the homologous cell monolayers (Fig. 4C).

Capture of inactivated viruses by antibodies. (i) **PV-1.** Native PV-1 was captured by mouse monoclonal anti-PV-1 (homologous antibody), but not by rabbit anti-HAV or rabbit anti-FCV (heterologous antibodies) (Fig. 5A). PV-1 inactivated by UV, hypochlorite, and 72°C were not captured by monkey polyclonal anti-PV-1 (homologous antibody) (Fig. 5B, lanes 1 to 3), and only a small portion of 37°C-inactivated PV-1 particles were captured (Fig. 5B, lane 4). PV-1 inactivated by UV, hypochlorite, 72°C, and 37°C were again not captured by monkey anti-PV-1, anti-PV-2, and anti-PV-3 (Fig. 6).

(ii) **HAV.** Native HAV was captured by rabbit anti-HAV (homologous antibody) but not by rabbit anti-FCV, mouse monoclonal anti-PV-1, monkey anti-PV-1, monkey anti-PV-2, or monkey anti-PV-3 (heterologous antibodies) (Fig. 7A). HAV inactivated by UV, hypochlorite, and 72°C heat were captured by rabbit anti-HAV (homologous antibody), whereas

HAV inactivated at 37°C was still antigenically intact (Fig. 7B and C).

(iii) **FCV.** Native FCV was captured by rabbit anti-FCV (homologous antibody) but not by rabbit anti-HAV or mouse monoclonal anti-PV-1 (heterologous antibody) (Fig. 8A). FCV inactivated by UV, hypochlorite, 72°C heat, and 37°C heat were still antigenically intact (Fig. 8B).

DISCUSSION

Enteric viruses, such as HAV and the NLVs, enter the host by ingestion (11) and so must withstand the acidity and proteolytic enzymes in the stomach and small intestine, respectively, in order to infect their eventual host cells. This exceptional property, incidentally, also confers stability to the viral infectivity in harsh environments outside the host's body, including in food and water (7, 9, 18, 20, 27, 31, 38). Both the viral RNA and capsid must be functional for infection to occur. The genome of most RNA viruses is quite resistant to external

FIG. 4. Agarose gel analysis of RT-PCR assay of attachment of inactivated FCV to cell monolayer (receptors). (A) Lane 1, FRhK-4 cell lysate amplified with FCV primer set; lane 2, CRFK cell lysate with FCV primer set; lane 3, native FCV without cell lysate. (B) Lanes 1 to 3, native FCV attachment to CRFK (homologous) cell receptors in lysate of CRFK cells inoculated with FCV and washed (lane 1), wash-off FCV inoculum from lane 1 (lane 2), or lysate of CRFK cells inoculated with FCV and not washed (lane 3). Lanes 4 to 6, native FCV attachment to FRhK-4 (heterologous) cell receptors in lysate of FRhK-4 cells inoculated with FCV and washed (lane 4), wash-off FCV inoculum from lane 4 (lane 5), or lysate of FRhK-4 cells inoculated with FCV and not washed (lane 6). Lane 7, FCV inoculum alone. (C) Lanes 1 to 8, inactivated FCV attachment to CRFK (homologous) cell receptors in lysate of cells inoculated with UV-inactivated FCV and washed (lane 1), wash-off inoculum from lane 1 (lane 2), lysate of cells inoculated with hypochlorite-inactivated FCV and washed (lane 3), wash-off inoculum from lane 3 (lane 4), lysate of cells inoculated with 72°C-inactivated FCV and washed (lane 5), wash-off inoculum from lane 5 (lane 6), lysate of cells inoculated with 37°C-inactivated FCV and washed (lane 7), or wash-off inoculum from lane 7 (lane 8). Lanes 9 and 10, native FCV attachment to CRFK in lysate of cells inoculated with native FCV and washed (lane 9) or wash-off inoculum from lane 9 (lane 10). Lanes M, 100-bp DNA ladder (Gibco BRL). Arrows indicate the amplicon of interest.

FIG. 5. Agarose gel analysis of AC-PCR assay (capture) of native and inactivated PV-1 by antibodies. (A) Lanes 1 to 8, capture of native PV-1 by homologous and heterologous antibodies, including rabbit anti-HAV (lanes 1 and 2), rabbit anti-FCV (lanes 3 and 4), mouse monoclonal anti-PV-1 (lanes 5 and 6), or no antibody (lanes 7 and 8). Lane 9, RT-PCR of native PV-1; lane 10, negative control of RT-PCR without PV-1. (B) Lanes 1 to 4, capture of inactivated PV-1 by monkey anti-PV-1 following UV inactivation (lane 1), hypochlorite inactivation (lane 2), 72°C inactivation (lane 3), or 37°C inactivation (lane 4). Lanes 5 and 6, capture of native PV-1 by monkey anti-PV-1 (lane 5) or no antibody (lane 6). Lanes M, 100-bp DNA ladder (Gibco BRL). Arrows indicate the amplicon of interest.

influences, as long as the capsid protects it from ubiquitous RNase (11). This protective property is usually limited in viruses that are transmitted by aerosol, sexual contact, or a vertical route, such as with rhinovirus, human immunodeficiency virus, and herpes simplex virus. It appears that the essence of this stability lies in the capsid structure, since the genomes of all viruses comprise essentially the same nucleic acids (either RNA or DNA).

The immune response of an animal toward a protein, including a viral capsid, evokes antibody against that protein. This antibody plays an important role in memorizing the first entry of a virus and specifically binds that serotype of virus in the next entry. Since antibodies may recognize multiple configurations of a protein, interactions of capsid epitopes and antibodies may not all cause neutralization (28). That is, the main (or any) epitope may not be associated with viral infectivity (19, 28).

The selection of modes of virus inactivation in the present study was based on the viruses' transmission in the environment via water (UV and hypochlorite) and food (thermal pro-

cessing). Inactivation at 37°C was intended to represent the fate of most virus produced in the human body, which spontaneously loses infectivity with time without human agency. HAV and PV have often been transmitted via food and water (5), and FCV is the best available surrogate for the NLVs (10), which are leading causes of food-borne and waterborne disease in the United States. As was stated earlier, the choice of inactivating agents for this study was based partly on the knowledge that UV predominantly targets the viral RNA, depending on the dose (14, 39), and hypochlorite is supposed to affect both the coat protein and the RNA (1, 26, 40). It was somewhat surprising to find that UV, hypochlorite, and 72°C inactivation significantly attack the capsid, whereby the capsid becomes susceptible to RNase in conjunction with PK (also representing the proteolytic enzymes in the intestine) and can no longer protect the viral RNA (25). Hypochlorite-inactivated PV-1 and FCV yielded negative RT-PCR results even without enzyme treatment, but hypochlorite-inactivated HAV required treatment with both enzymes to yield a negative RT-PCR. This indicates that hypochlorite is more potent than other virus-

FIG. 6. Agarose gel analysis of AC-PCR assay (capture) of native and inactivated PV-1 by three serotypes of polyclonal monkey antibodies. Lanes 1 to 4, capture of native PV-1 by homologous and heterologous antibodies, including monkey anti-PV-1 (lane 1), nonspecific rabbit serum (lane 2), monkey anti-PV-2 (lane 3), or monkey anti-PV-3 (lane 4). Lanes 5 to 8, capture of inactivated PV-1 by monkey anti-PV-1 following UV inactivation (lane 5), hypochlorite inactivation (lane 6), 72°C inactivation (lane 7), or 37°C inactivation (lane 8). Lanes 9 to 12, capture of inactivated PV-1 by monkey anti-PV-2 following UV inactivation (lane 9), hypochlorite inactivation (lane 10), 72°C inactivation (lane 11), or 37°C inactivation (lane 12). Lanes 13 to 16, capture of inactivated PV-1 by monkey anti-PV-3 following UV inactivation (lane 13), hypochlorite inactivation (lane 14), 72°C inactivation (lane 15), or 37°C inactivation (lane 16). Lanes M, 100-bp DNA ladder (Gibco BRL). Arrows indicate the amplicon of interest.

FIG. 7. Agarose gel analysis of AC-PCR assay (capture) of native and inactivated HAV by antibodies. (A) Capture of native HAV by homologous and heterologous antibodies, including rabbit anti-HAV (lanes 1 and 2), rabbit anti-FCV (lanes 3 and 4), mouse anti-PV-1 (lanes 5 and 6), monkey anti-PV-1 (lanes 7 and 8), monkey anti-PV-2 (lanes 9 and 10), and monkey anti-PV-3 (lanes 11 and 12). (B) Lanes 1 to 6, capture of inactivated HAV by rabbit anti-HAV following UV inactivation (lanes 1 and 2), hypochlorite inactivation (lanes 3 and 4), or 72°C inactivation (lanes 5 and 6). Lanes 7 and 8, capture of native HAV by rabbit anti-HAV. Lane 9, RT-PCR of native HAV. (C) Lanes 1 and 2, capture by rabbit anti-HAV of native HAV (lane 1) or HAV following 37°C inactivation (lane 2). Lane 3, RT-PCR of native HAV without antigen capture. Lanes M, 100-bp DNA ladder (Gibco BRL). Arrows indicate the amplicon of interest.

inactivating agents and acts differently, since in some instances hypochlorite can also inactivate viruses via the viral RNA, as well as the capsid. However, susceptibility of the capsid to composite enzymatic digestion did not result from 37°C inactivation of PV-1, HAV, and FCV (Fig. 1). Therefore, the mode of attack appeared to be temperature dependent, with only slight modification of the viral capsid with 37°C inactivation among the viruses tested. Inactivation by acid pH, representing conditions in the host's stomach, should be considered in future studies.

RT-PCR assay of virus attached to the cell monolayer is a sensitive (Fig. 2A, 3A, and 4A) and specific (Fig. 2B, 3B, and 4B) test of the ability of a virus to attach to cell receptors. The results suggested that the cell-attachment ability of PV-1, HAV, and FCV was lost regardless of virus inactivation method, except for UV-inactivated HAV. This points to the receptor attachment site on the capsid surface as the principal target of these modes of inactivation and recalls that the binding of HAV to cell surfaces shows evidence of positive cooperativity (6). It suggests that inactivation required a conformational change of only a few receptor attachment sites to prevent attachment of the whole inactivated virus particle and may explain why the results obtained were generally all or none. However, the partial loss of attachment of the UV-

inactivated HAV suggested that the capsid (especially the receptor attachment site) of HAV (17) was different from that of PV-1 (23) and FCV. These results imply that virus inactivation is almost always accompanied by loss of the virus' ability to attach to homologous cell receptors.

Both HAV and FCV were captured by homologous antibodies, even though most of the virions were not able to attach to host cell receptors. This indicates that the receptor attachment sites are discrete from the antigenic epitopes on the capsid. Considerable evidence suggests that the immunodominant neutralizing antigenic site of HAV is in VP3 (2). The present result is consistent with previous evidence indicating that the immunodominant neutralization antigenic site of HAV is not directly involved in cell attachment (35).

Among the best-known picornaviruses, the neutralizing antigenic site of PV is determined by the prominent connecting loops and C termini on the surface of VP1 near the vertices of fivefold axes (23, 24). The canyon (surface architecture on the capsid) of PV has been shown to be the receptor attachment site; the inside of the canyon, to which the PV receptor penetrates, is surrounded by parts of VP1, VP2, and VP3 (11). The antigenicity of inactivated PV-1 did not shift from serotype specific to PV group specific, since no inactivated PV-1 was captured by monkey anti-PV-2 or monkey anti-PV-3 (Fig. 6).

FIG. 8. Agarose gel analysis of AC-PCR assay (capture) of native and inactivated FCV by antibodies. (A) Lanes 1 to 8, capture of native FCV by homologous and heterologous antibodies, including rabbit anti-HAV (lanes 1 and 2), rabbit anti-FCV (lanes 3 and 4), or mouse monoclonal anti-PV-1 (lanes 5 and 6), or no antibody (lanes 7 and 8). Lane 9, RT-PCR of native FCV; lane 10, negative control of RT-PCR without FCV. (B) Lanes 1 to 8, capture of inactivated FCV by rabbit anti-FCV following UV inactivation (lanes 1 and 2), hypochlorite inactivation (lanes 3 and 4), 72°C thermal inactivation (lanes 5 and 6), or 37°C thermal inactivation (lanes 7 and 8). Lanes 9 and 10, RT-PCR of native FCV. Lanes M, 100-bp DNA ladder (Gibco BRL). Arrows indicate the amplicon of interest.

The loss of the ability of inactivated PV-1 to attach to homologous cell receptors was accompanied by a loss of ability to be captured by neutralizing homologous antibody in all inactivated PV-1 except that inactivated by 37°C. This suggests that UV, hypochlorite, and 72°C inactivation caused a conformational change of VPs that, though perhaps slight, affected both the neutralizing antigenic site and receptor attachment site and enormously diminished these functions of the capsid. These results may be applicable in developing further treatments to yield negative RT-PCR test results with inactivated virus and in evaluating processes for virus inactivation, without having to test directly for infectivity.

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