Comparative Sensitivity of the Echovirus Type 25 JV-4 Prototype Strain and Two Recent Isolates to Glutaraldehyde at Low Concentrations

MARTINE CHAMBON,* JEAN-LUC BAILLY, AND HÉLÈNE PEIGUE-LAFEUILLE
Laboratoire de Bacteriologie-Virologie, Faculté de Médecine et Pharmacie, 63001 Clermont-Ferrand Cedex, France

Received 28 July 1993/Accepted 10 November 1993

The sensitivity of two recently isolated antigenic variants of echovirus type 25 (Montpellier 76-1262 and Thionville 86-222) to glutaraldehyde (GTA) at low concentrations was compared with that of the JV-4 prototype strain. The purified viruses were treated under the same conditions with GTA at concentrations ranging from 0.002 to 0.10%. The wild strains exhibited significantly lower sensitivity to GTA than did the prototype strain; with 0.10% GTA, a 2 log10 unit reduction was obtained in 5 min for JV-4 and in 60 and 80 min for Montpellier 76-1262 and Thionville 86-222, respectively. A comparison with previous results obtained with poliovirus type 1 showed that the inactivation rates of echovirus type 25 wild strains were fivefold lower than those of the poliovirus type 1 Sabin strain. The comparative electrophoretic and immunoblot analyses showed differences in the results of GTA binding with capsid proteins of the viruses. Unlike in the poliovirus type 1 Mahoney strain and in the echovirus type 25 JV-4 reference strain, GTA produced only minor intramolecular cross-linkings in the viral particles of the two wild strains of echovirus type 25. Our results suggest that there are both intertypic and intratypic differences in the GTA sensitivities of enterovirus strains. They are of relevance to disinfection procedures in digestive endoscopy and to the choice of the enterovirus strain used for evaluating the efficacy of disinfectants.

Because of their resistance under environmental conditions, enteroviruses are widely used to test the efficacy of disinfectants against viruses. Reference strains have been used in studies of the virucidal activity of glutaraldehyde (GTA) against enteroviruses. Vaccine strains of polioviruses were the most frequently used (2, 14, 20, 23), particularly the Sabin strain, which is known to be very resistant to GTA-containing disinfectants (23). There have been few reports of the use of wild strains of coxsackievirus type B3 (21, 22). In some studies the origin (prototype or clinical isolates) of the test virus strains was not specified (4, 5, 14).

However, clinical isolates and reference strains are known to differ in their sensitivities to antiviral compounds (6). No comparative study of the activity of GTA against wild clinical isolates and the corresponding prototype virus strains has been performed. However, different sensitivities could have direct effects on GTA concentrations and on contact times with disinfectant solutions used in endoscope disinfection procedures.

The intratypic variations of the antigenic properties of enteroviruses have been extensively described for polioviruses (10, 13) and coxsackieviruses (19). In a recent study (18), we extended these findings to echovirus type 25 wild strains. We have also shown that echovirus type 25 wild strains may exhibit differences in their structural proteins (17). In addition, the antigenic characterization of 28 wild strains collected in France showed that, even in the same area, many major antigenic variants sometimes coexisted (16).

The aim of this study was to determine whether these biological, antigenic, and structural heterogeneities are related to different sensitivities of echovirus type 25 strains to GTA. We compared the level of sensitivity to GTA, under the same conditions, of the echovirus type 25 JV-4 prototype strain with those of two antigenic variants of recent isolates of echovirus type 25. We also investigated the effect of GTA on the structural polypeptides of wild strains by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

MATERIALS AND METHODS

Virus strains and identification. The echovirus type 25 JV-4 reference strain was obtained from the World Health Organization Collaborating Center, National Reference Center for Enterovirus, Lyon, France. Two echovirus type 25 wild strains, Thionville 86-222 and Montpellier 76-1262, were recovered from rectal swabs of infants and children admitted to French hospitals. Montpellier 76-1262 was chosen because it exhibited the pattern most different from that of JV-4 in immunoblot analysis, and Thionville 86-222 was chosen because it had been shown to present the greatest antigenic variation (17, 18). Each wild strain was designated by (i) the city where it was isolated, (ii) the last two digits of the year of isolation, and (iii) a number given by the laboratory in which it was isolated. Echovirus isolates were identified by seroneutralization tests with the Lim-Benyes-Melnick antisera pools, as described elsewhere (18).

Tissue cultures. MRC5 cells (Laboratoire Bio-Mérieux, Marcy-L’Etoile, France), used for passages 28 to 38, were grown in Eagle’s minimum essential medium (EMEM) (Flow Laboratories, Inc., McLean, Va.) supplemented with 1% glutamine and 10% fetal calf serum (Boehringer, Meylan, France).

The HT 29-18 cell line, a clone derived from the HT 29 human colon adenocarcinoma cell line, was a kind gift from E. Coudrier and D. Louvard, Département de Biologie Moléculaire, Institut Pasteur, Paris, France (9). HT 29-18 cells were...
propagated with RPMI 1640 medium (Flow Laboratories) supplemented with 15% fetal calf serum (Boehringer) and human transferrin (1 mg/100 ml) (Sigma, Paris, France).

Kinetics of inactivation. (i) Virus growth. High-titer stocks of echoriavirus were produced in HT 29-18 cells and purified by isopycnic centrifugation in CsCl, and their titers were determined as described previously (1). All purified virus suspensions were diluted in phosphate-buffered saline (PBS), divided into aliquots, and stored at −80°C until used in inactivation assays. The protein concentration was determined by the procedure of Lowry et al. (12).

(ii) Inactivation tests. GTA stock solutions (10% solutions) were prepared, stored, and controlled by UV absorption as described previously (1). All tests were performed in PBS at 25°C. After incubation, the treated preparations were diluted 100-fold in cold EMEM without fetal calf serum (0°C). Virus infectivity was then determined by using the diluted preparations with MRCS cells in microtitration plates. Details of methods and of the evaluation of titers have been described elsewhere (1).

Analysis of labeled capsid proteins after GTA treatment. (i) Labeling of viruses. Confluent monolayers of MRCS cells were used to prepare stocks of [35S]methionine-labeled echoriaviruses. Briefly, MRCS cells were washed with PBS and infected with each virus strain at a multiplicity of infection of 50 MPNCU (most probable number of cytopathic units) per cell. The inoculum was removed after 3 h of incubation and replaced by methionine-free EMEM (Select-Amine Kit; Gibco-BRL) containing 15 µCi of [35S]methionine per ml (Amershams, Les Ulis, France) (3). The supernatants were collected when the cells showed a marked cytopathic effect and centrifuged at 20,000 × g for 30 min at 4°C. The virus was pelleted at 125,000 × g for 4 h at 4°C and suspended in PBS. The virus suspension was finally purified by isopycnic centrifugation in CsCl at 165,000 × g for 40 h (1), and [35S]-labeled virus preparations were aliquoted (50,000 cpm per aliquot) and stored at −20°C until inactivation tests were performed.

(ii) Inactivation tests. Samples of [35S]methionine-labeled echoriavirus, stored at −20°C in 1.5-ml polypropylene microcentrifuge tubes, were incubated at 25°C with appropriate GTA concentrations in a final volume of 170 µl. At the end of the incubation, the reaction mixture was transferred to an ultracentrifuge tube, and virus particles were collected by ultracentrifugation at 133,000 × g in an 18°C A-100 fixed-angle rotor (Beckman) and an Airfuge (air-driven ultracentrifuge; Beckman). The supernatant was removed from the ultracentrifuge tube, mixed with 1 volume of Laemmli's sample buffer (0.0625 M Tris-HCl [pH 6.8], 2% [wt/vol] SDS, 5% [vol/vol] 2-mercaptoethanol, 10% [vol/vol] glycerol), and stored. The pellet was suspended in 30 µl of sample buffer and stored. The polypropylene tubes in which the reactions were performed were thoroughly rinsed by vortexing, with 30 µl of sample buffer to desorb the viral material from the surface of the tubes. The supernatants, pellets, and rinsing buffers were then analyzed by SDS-PAGE.

(iii) SDS-PAGE. Electrophoresis under denaturing conditions was performed by the method of Laemmli (11) under previously described conditions (3). Samples were heated at 100°C for 5 min and run at 6 mA for 18 h. Gels were fixed, soaked in Amplify (Amersham), and subjected to autoradiography (Hyperfilm MP; Amersham). Four cloned molecular weight standards were obtained from Amersham.

Immunoblots. After inactivation, the virus particles were collected by ultracentrifugation and the pellets were mixed with the rinsing buffers and analyzed by SDS-PAGE, as described above. Proteins were transferred to nitrocellulose sheets (pore size, 0.45 µm; Schleicher & Schuell, Inc., Dassel, Germany) at 12 V for 10 min and then at 24 V for 50 min, with a transblot cell apparatus (Biocom, Dardilly, France). The membranes were treated for 1 h at room temperature and on a rocking table with Tris-buffered-saline (TBS; 10 mM Tris base [pH 7.4], 0.9% [wt/vol] NaCl) containing 0.05% (vol/vol) Tween 20 (TBS-T) and 10% (wt/vol) nonfat dried milk. Membrane-bound proteins were detected with a polyclonal rabbit serum raised against the JV-4 reference strain, diluted (1:100) in TBS containing 5% (wt/vol) nonfat dried milk. The reactivity of antiserum has been described elsewhere (17). The membranes were washed once each in TBS-T-5% nonfat dried milk, TBS-T, and TBS for 10 min each. Specifically bound antibodies were detected first by reaction with anti-rabbit immunoglobulin G IgG conjugated to peroxidase at a 1:1,500 dilution (TEBU S.A., Le Perray, France) and then by treatment with a solution containing 4-chloro-1-naphthol (3 mg/ml in methanol; Sigma) and H2O2 in TBS (100 µl of a 30% [vol/vol] solution in 100 ml of TBS) (5).

RESULTS

Preparation of virus stocks. In a previous study we reported that HT 29-18 cells were highly permissive for echoriavirus type 25 field-collected strains and supported virus growth to a high titer (18). We also observed that the rise in infectious titer on MRCS cells after one passage on HT 29-18 cells was statistically significant (data not shown). HT 29-18 cells were therefore chosen to produce stocks of echoriaviruses of high titer for purification. However, MRCS cells were used for all titer determinations because they exhibited a more typical cytopathic effect than HT 29-18 cells did.

For each echoriavirus type 25 strain tested, all the results presented in Fig. 1 were obtained with a single purified stock of virus. Aliquots of the JV-4 prototype and Thionville 86-222 stocks contained 108.1 MPNCU in 20 µl. The protein concentrations were 230 and 530 µg/ml, respectively. The stock of Montpellier 76-1262 contained 108.6 MPNCU per 20-µl aliquot, and the protein concentration was 375 µg/ml. The three stocks therefore contained 7.1 to 7.7 log10 MPNCU/ml/µg of protein. The stock of poliovirus type 1 Sabin LS-c, 2ab strain, used in a previous study (1) contained 7.1 log10 MPNCU/ml/µg of protein. Hence, the results of kinetics experiments performed with the three echoriaviruses can be compared with one another (Fig. 1) and with those of the experiments with poliovirus (Fig. 2).

Echoriavirus type 25 inactivation kinetics with low concentrations of GTA. Kinetics experiments were performed with GTA at 0.002, 0.005, 0.01, 0.05, and 0.10% to determine the inactivating processes of the echoriavirus type 25 JV-4 reference strain (Fig. 1A) and of the two wild strains, Thionville 86-222 (Fig. 1B) and Montpellier 76-1262 (Fig. 1C). All inactivation tests were carried out at 25°C and pH 7.2. Each plotted point is the mean of two or three survival ratios. The survival ratio is defined as the reduction of the viral concentration and is expressed as $\log_{10} (T/T_0)$, where $T$ is the infectious titer at time $t$ and $T_0$ is the initial infectious titer at time zero.

The kinetics of the JV-4 reference strain indicated that this virus was sensitive to very low GTA concentrations. A 2 log10 unit reduction in the infectious titer was obtained with 0.005% GTA in 90 min, with 0.01% GTA in 15 min, and with 0.10% GTA in less than 5 min (Fig. 1A). In contrast, the results showed that higher GTA concentrations were necessary to inactivate the two wild strains tested under the same conditions. No significant decrease in the infectious titers of the Thionville 86-222 and Montpellier 76-1262 strains upon expo-
FIG. 1. Kinetics of inactivation of echovirus type 25 strains by GTA. (A) JV-4 prototype strain; (B) Thionville 86-222 wild strain; (C) Montpellier 76-1262 wild strain. The purified virus-containing samples were incubated at 25°C in PBS with GTA at 0.002% (+), 0.005% (○), 0.01% (-), 0.05% ( ), and 0.10% (■) for various times. Inactivation was stopped by 100-fold dilution in cold EMEM. The survival ratio is the reduction of the viral concentration and is expressed as log₁₀ (T/T₀), with T being the titer at elapsed time t and T₀ being the titer at the outset of exposure to GTA (time zero).

sure to 0.005% GTA was noted. The 2 log₁₀ unit reduction was obtained with 0.05% GTA in 120 min and 0.10% GTA in 60 min for Montpellier 76-1262 (Fig. 1C) and with 0.10% GTA in 80 min for Thionville 86-222 (Fig. 1B).

The log₁₀ reduction in the concentration of the Montpellier 76-1262 (Fig. 1C) and Thionville 86-222 (Fig. 1B) strains was a linear function of the exposure time for the periods and concentrations tested, whereas the inactivation of the JV-4 reference strain was a more complex process. The curves for inactivation of the JV-4 strain by 0.05 and 0.10% GTA were drawn assuming that the process of the reaction was nonlinear. However, the possibility that the kinetics are two-component in shape cannot be excluded.

The inactivation rates were calculated by linear regression of the survival ratio values versus time and were plotted as a function of GTA concentration (Fig. 2). For the JV-4 reference strain, the inactivation rate at 0.01% GTA was determined between exposure times 0 and 15 min and the inactivation rate at 0.10% GTA was determined between 0 and 5 min. The value of the latter was 0.61 log₁₀ MPNCU/min but was not plotted on the graph, to make Fig. 2 easier to read. The inactivation rates of the two echovirus type 25 wild strains increased linearly with the GTA concentration under the conditions described. The results obtained in the previous study made with poliovirus type 1 Sabin LS-c, 2ab strain (1), were transferred to the graph to make comparison easier.

SDS-PAGE analysis of echovirus wild strain capsid proteins after GTA treatment. The patterns of [³⁵S]methionine-labeled capsid proteins of the Montpellier 76-1262 and Thionville 86-222 strains were analyzed by SDS-PAGE after GTA treatment of virus particles (Fig. 3).

Viral material was collected mainly in the pellets and after rinsing of the reaction tubes with the electrophoresis denaturing buffer. In all experiments, the electrophoresis patterns of polypeptides were the same whether viral particles were recovered in the pellets or in the rinsing buffers. These results are in agreement with previously reported findings (3) and indicate that nonspecific adsorption of viruses occurs on the surfaces of the tubes with and without GTA.

The effect of 0.005% GTA on the capsid protein of Thionville 86-222 strain was tested after a prolonged exposure (360 min). The electrophoresis patterns of polypeptides were not significantly modified in comparison with those of controls (Fig. 3C, lanes 1, 2, 11, and 12). The same results were obtained with the Montpellier 76-1262 strain (data not shown).

No significant modification in the electrophoresis patterns of the capsid proteins of Montpellier 76-1262 and Thionville 86-222 was observed after treatment with 0.05% GTA (Fig. 3A and C). One additional protein of about 67 kDa was detected in the samples of the Thionville 86-222 strain, both in those treated with GTA and in the control (Fig. 3C). This polypeptide was therefore of cellular origin and contaminated the viral preparation. A newly formed protein product of about 65 kDa was seen in viral particles of the Montpellier 76-1262 strain incubated with 0.10% GTA for 120 min (Fig. 3B, lanes 3 and 4). However, in all experiments the autoradiographic signal of this additional protein was weak, even when the GTA concen-
GTA treatment was greatly increased (up to 1% [results not shown]). At
the GTA concentrations and for the period tested, the auto-
radiographic signals of the three major polypeptides, VP1, VP2, and VP3, remained unchanged for the two wild strains.

Immunoblotting analysis of echovirus proteins after GTA treatment. The products of the reaction between GTA and echovirus type 25 proteins were analyzed by immunoblot with a rabbit polyclonal immune serum raised against the JV-4 prototype strain (17). The immunoblot pattern of proteins of the JV-4 prototype strain treated with 0.005% GTA for 60 min revealed reactivity against VP1 (Fig. 4, lane 2). In a previous study, we reported that at least three high-molecular-weight products (HMWP) were formed in virus particles after GTA treatment of the JV-4 strain (3). These products were also detected in the present study (HMWP 1, 3, and 4). The immunoblotting assays in the two studies were performed with the same virus stock. However, one additional product was detected in the present study (HMWP 2), probably because the concentration of viral proteins was twice as high as that used in the previous work.

The immunoblotting pattern of Thionville 86-222 proteins treated with 0.005% GTA for 60 min showed that one HMWP was formed, which was detected with a very weak signal (Fig. 4, lane 5). When the virus preparations were treated with 0.05% GTA for 60 min, a great decrease in the immunoblotting signals of VP1 was observed for the three strains of echovirus (Fig. 4, lanes 3, 6, and 8), and neither HMWP was detected.

**DISCUSSION**

In previous experiments we investigated the virucidal activity of low concentrations of GTA on poliovirus type 1 and the echovirus type 25 JV-4 prototype strain (1, 3). In this study, using the same methods, we compared the sensitivity to GTA of two recent echovirus type 25 isolates with that of the JV-4 strain.

The kinetics of the activity of GTA on the two echovirus type 25 wild strains Montpellier 76-1262 and Thionville 86-222 showed that the infectivity of both strains decreases with a similar timing: the virucidal activity of GTA against both wild strains followed a first-order reaction, with a linear relationship between the inactivation rate and the GTA concentration, at least for the GTA concentrations tested in this study. In contrast, the activity of GTA against the JV-4 strain followed a first-order reaction only at GTA concentrations of 0.002 and
0.005%; above this range of concentrations, the kinetics were nonlinear, indicating a change in the inactivation process. A decrease in the inactivation rate as the time is extended is commonly observed in virus survival curves. The change may be abrupt (two-component curves) or gradual (multicomponent curves). Such departures from first-order kinetics during the inactivation of viruses have been observed after treatment with both physical and chemical agents and have already been reviewed (8). We attributed a multicomponent character to the curves describing the inactivation kinetics of the JV-4 strain with 0.01 and 0.05% GTA, but the possibility that the function is in fact strictly biphasic and that the curves have a two-component character cannot be ruled out. It is not possible to explain these changes of slope without a greater knowledge of the virus-inactivating process of GTA. However, the inactivation kinetics of the JV-4 strain with GTA concentrations greater than 0.005% showed that the dose (concentration × time) of GTA could be underestimated if extrapolations are based on the first segments of the kinetics, although this strain was more sensitive than the wild strains.

The comparative analysis of the inactivation kinetics showed that the two wild strains exhibit significantly lower sensitivity to GTA than does the prototype strain at the GTA concentrations used and for the period tested. Thus, a 2 log₁₀ unit decrease in the viral titer of the JV-4 strain was observed with 0.10% GTA after a short exposure time (5 min), whereas 60 and 80 min were required for the Montpellier 76-1262 and Thionville 86-222 strains, respectively. The inactivation rates of wild strains of echovirus type 25 were 10-fold lower than those of the JV-4 reference strain (in the range of GTA concentrations in which the inactivating reaction follows first-order kinetics) and 5-fold lower than those of the poliovirus type 1 Sabin LS-c, 2ab strain.

These results raise doubts about how far the test virus strains used to evaluate and standardize the virucidal activity of disinfectants are representative and add to our misgivings about the use of disinfectants containing low concentrations of GTA in gastrointestinal endoscope disinfection procedures.

The action of GTA at low concentrations against the capsid proteins of the two echovirus type 25 clinical isolates was studied so that we might have a fuller understanding of the mode of virucidal action of this compound. Previously published findings (3) indicated that the reference strain of echovirus type 25 and poliovirus type 1 underwent cross-linking of their capsid polypeptides at GTA concentrations of 0.005 and 0.05%, respectively. For these two strains, such cross-linking was identified by autoradiography, which showed the presence of HMWP on the patterns of polypeptides after GTA treatment. Surprisingly, GTA did not greatly modify the electrophoretic patterns of the capsid proteins of the two echovirus type 25 wild strains, even after prolonged incubations with 0.05 and 0.10% GTA. Nevertheless, those GTA concentrations strongly decreased the infectivity of both viruses. Likewise, no significant HMWPs were observed by immunoblot analysis of the capsid proteins of wild strain particles treated with GTA. The only exception was a product at 65 or 67 kDa, observed with a very faint signal in all experiments.

Analysis of the electrophoretically fractionated products resulting from the treatment of wild strains of echovirus type 25 with 0.05% GTA, with a polyclonal antiserum against echovirus type 25 VP1, revealed that the detection signal of VP1 was greatly altered. In contrast, VP1 protein was clearly detected on the autoradiographic patterns, thus proving that the reduction in the immunoblot signal of VP1 cannot be attributed to a decrease in the amount of viral material collected to perform the electrophoresis. This repeatedly observed alteration is probably due to a decrease in the ability of polyclonal antibodies after GTA treatment to recognize the epitopes of the protein. This suggests that GTA does in fact bind to the structural proteins of wild strains Montpellier 76-1262 and Thionville 86-222, producing only minor intermolecular cross-linking in the virus particles, in contrast to the JV-4 strain. It remains to be determined whether GTA produces intramolecular cross-links and whether they account for the inactivation of the virus infectivity of the two wild strains. Nevertheless, the overall results of this study clearly show that the wild strains Montpellier 76-1262 and Thionville 86-222 differ from the reference strain of echovirus type 25 and from poliovirus type 1 in their sensitivity to low concentrations of GTA. In addition, the biochemical events that lead to the inactivation of virus infectivity are different depending on whether GTA reacts against the wild strains or the JV-4 strain of echovirus type 25. In previous studies, we reported that the wild strains of echovirus type 25 collected in France, and particularly Montpellier 76-1262 and Thionville 86-222, differed widely from the JV-4 strain in their antigenic properties (16, 18) and in the molecular weight of the structural proteins VP1 and VP3 (17). In the polioviruses, these antigenic differences are due to amino acid substitutions (13, 15). Furthermore, mutations in the capsid proteins of human rhinovirus 14 have been shown to confer antiviral drug resistance (7). Such molecular differences in the capsid proteins of virus particles may account for the intertypic and intratypic differences observed in the GTA sensitivity of enterovirus strains.

ACKNOWLEDGMENTS

We express our appreciation to Madeleine Trimolet for helpful technical assistance. We thank Jeffrey Watts for the revision of the English version of the manuscript.

REFERENCES


