Comparative Sensitivities of Sabin and Mahoney Poliovirus Type 1 Prototype Strains and Two Recent Isolates to Low Concentrations of Glutaraldehyde

MARTINE CHAMBON,1,* CHRISTINE JALLAT-ARCHIMBAUD,1 JEAN-LUC BAILLY,1 JEANNE MARIE GOURGAND,2 FRANCOISE CHARBONNE,1 CÉCILE HENQUELL,1 FLORENCE FUCHS,3 AND HELENE PEIGUE-LAFEUILLE1

Laboratoire de Bactériologie-Virologie, Faculté de Médecine et Pharmacie, 63001 Clermont-Ferrand Cedex,1 LIMOS, Université Blaise Pascal, 63177 Aubière Cedex,2 and Laboratoire National de la Santé, Faculté de Médecine Grange Blanche, 69373 Lyon Cedex,3 France

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Significant intratypic differences in the glutaraldehyde (GTA) sensitivity of echoviruses have been shown. While exploring ways to optimize the study of GTA sensitivity of enteroviruses, we also observed intratypic differences in poliovirus type 1 isolates collected in France. A suspension procedure was used for assessing the virucidal effect of GTA at low concentrations (≤0.1%) against purified viruses. Two recent isolates of poliovirus type 1 were first fully characterized by the PCR restriction fragment length polymorphism (RFLP) test. The RFLP pattern of clinical isolate 5617 was similar to that of poliovirus type 1 LS-c, 2ab (Sabin strain), confirming the vaccine origin of strain 5617. The RFLP pattern of strain 5915 recovered from sewage was different from that of the Mahoney strain, suggesting that the Mahoney strain was a wild isolate. We then analyzed under the same controlled conditions the GTA sensitivities of both isolates and their respective prototype strains. The wild Mahoney and 5915 strains exhibited significantly lower sensitivities to GTA than did the vaccine Sabin and 5617 strains. The inactivation rates of clinical isolates 5617 and 5915 were very similar to those of their corresponding reference Sabin and Mahoney strains. Both the conformational structure of the capsid of each strain and the amino acid constitution of structural polypeptides could be involved in the variations observed. The relevance of our comparative sensitivity studies to standardization of virucidal tests is discussed.

Enteroviruses, members of the Picornaviridae family, are divided into three major subgroups, the polioviruses, coxsackieviruses, and echoviruses, plus the more recently identified numbered enteroviruses. The intratypic variations in the antigenic properties of enteroviruses have been described in detail for polioviruses (8, 11, 16) and coxsackieviruses (19, 22) and more recently for echoviruses (12, 21).

There are three serotypes of polioviruses, which do not cross-react in neutralization tests with type-specific antisera. The neutralization epitopes of poliovirus were found to cluster in three antigenic sites (sites 1, 2, and 3) (reviewed by Minor [18]). Antigenic variability was observed in wild isolates of the three serotypes when they were tested by panels of neutralizing monoclonal antibodies (8, 17). Antigenic and genomic variability are strongly correlated. Many of the sequence variations occur in short segments of the three major viral capsid proteins (VP1, VP2, and VP3), which define major and minor neutralization antigenic sites on the surface of the virion (10, 20).

The aim of this study was to determine whether these antigenic differences are related to the different sensitivities of poliovirus type 1 (PV-1) strains to glutaraldehyde (GTA). In a previous study (7), we reported that two recent clinical isolates of echovirus type 25 differed from the reference strain of echovirus type 25 in their sensitivity to low concentrations of GTA. In the present study, we compared the level of sensitivity to GTA, under the same conditions, of the two PV-1 prototype strains (vaccine and wild strain) with those of two recent natural isolates of PV-1. We showed that PV-1 vaccine strains (prototype and clinical isolate) were more sensitive than the wild strains. The relevance of these results to the standardization of virucidal tests will be discussed.

MATERIALS AND METHODS

Virus strains and identification. Two PV-1 strains, Mahoney 1-397 and the corresponding attenuated Sabin LS-c, 2ab reference strains, and two natural isolates, 5617 and 5915, were used in this study. All these strains were obtained from the World Health Organization Collaborating Center, National Reference Center for Enterovirus, Lyon, France.

The two natural isolates of PV-1, 5617 and 5915, were recovered in 1992 and 1993 from the stools of an infant and from sewage, respectively. These isolates, identified by the National Reference Center for Enterovirus were defined as PV-1 by type-specific sera.

Tissue cultures. Vero cells, a line of African green monkey kidney cells, were obtained from Flow Laboratories, Inc. (McLean, Va.) and used for passages 154 to 158. MRC5 cells were obtained from Bio-Whittaker (Fontenay sous Bois, France) and used at passage 19. Monolayers of Vero or MRC5 cells were grown at 37°C in Eagle’s minimum essential medium (EMEM) (Bio-Whittaker), containing 7 or 10% (vol/vol) fetal calf serum (Boehringer-Mannheim, Meylan, France), respectively, and 1% glutamine.

Virus growth and purification. Vero cell monolayers were inoculated with each virus strain at a multiplicity of infection of 1 to 10 most probable number of cytopathic units (MPNCU) per cell. High-titer stocks of polioviruses were produced in Vero cells and purified by isopycnic centrifugation in CsCl as described previously (1) except for the desalting operation. The virus-containing fraction was not dialyzed against phosphate-buffered saline (PBS) for 24 h at 4°C but was concentrated to 100 μl with a Microcon-100 microcentrator (GrACE, Division Amicon, Epernon, France). The Microcon-100 microcentrator removed contaminants such as CsCl by repeated centrifugations and concentrated the virus-containing fraction. In addition, because the membrane in Microcon-100 microcentrators has a molecular mass cutoff of 100 kDa, low-molecular-mass contaminants, such as cellular proteins, were removed during successive concentrations. Virus titers were determined as described elsewhere (1).
protein concentration was determined by the procedure of Bradford (5) with the Bio-Rad Protein Assay (Bio-Rad, Irvine sur Seine, France).

Inactivation tests. GTA stock solutions (10% solutions) were prepared from a 25% solution (reagent grade I; Sigma OSI, Paris, France), stored, and controlled by UV absorption as described previously (1). Purified poliovirus preparations were mixed with appropriate GTA dilutions in PBS to obtain final GTA concentrations of 0.02, 0.05, and 0.10% (wt/vol) and incubated in a water bath at 25°C. The treated preparations were diluted 100-fold in ice-cold EMEM without fetal calf serum to stop the action of GTA on the virus. Viral infectivity was then determined by using the endpoint dilution method in MRC5 cells cultured in 96-well tissue culture plates (Falcon 3072; Becton Dickinson) for 48 h (1). An appropriate number of 10-fold dilutions was prepared from the virus suspensions in EMEM without fetal calf serum. The last 10-fold dilution was used to prepare seven fourfold dilutions. Aliquots (50 μl) of these eight dilutions were dispensed into the microtiter plate at the rate of 11 replicates per dilution. EMEM supplemented with 2% fetal calf serum was added (150 μl per well), and the plates were incubated under 5% CO2 at 37°C. The wells were scored microscopically as positive or negative for virus growth on days 4 and 5, and the most-probable-number method was used to evaluate the number of cytopathic units (1). The MPNCU was calculated with a computer program.

Preparation of viral RNA for reverse transcription and PCR amplification. Viral RNA was prepared as described elsewhere (2). Briefly, MRC5 cell monolayers were infected with each strain, and cytoplasmic RNAs were isolated at 4 h postinfection. Infected MRC5 cell monolayers were washed once with cold PBS and lysed in 200 μl of cold lysis buffer, which consists of 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, and 0.5% (vol/vol) Nonidet P-40. Cytoplasmic RNAs were extracted with chloroform. Complementary DNA was synthesized in a volume of 50 μl from 5 μg of RNA, with the StrataScript RT-PCR kit (Stratagene Cloning Systems) and oligo(dT) (0.1 μg/μl) as a primer. A 480-nucleotide fragment, spanning nucleotides 2402 to 2881 of the PV-1 genome, was amplified by PCR. This amplified 480-nucleotide sequence coded for the N-terminal half of the VP1 capsid polypeptide including antigenic site 1 (3). The amplification reaction was performed with 10 μl of the cDNA and with 1 μM each of the two following primers: the downstream primer UCl (5’-TTTGGTCAGCGTGTAATGA-3’) and the upstream primer UG1 (5’-TTTGGTCAGCGCTGGTAAATGA-3’) (3). In addition, the amplification reaction mixture contained 10 mM Tris-HCl (pH 8.3 at 20°C), 1.5 mM MgCl2, 50 mM KCl, 200 μM each of the four deoxynucleoside triphosphates, and 2.5 U of Tag DNA polymerase (Boehringer-Mannheim). Amplification was performed in 40 cycles. The first cycle consisted of denaturation for 5 min at 93°C, primer hybridization for 1 min at 46°C, and elongation for 1 min at 72°C. Each of the other 39 cycles consisted of 40 s at 93°C, 1 min at 46°C, and 1 min at 72°C. Samples were run on a Hybrid thermocycler. The amplification products were analyzed by electrophoresis on 1% agarose minigels.

Restriction enzyme mapping. The genomic variability of poliovirus isolates was analyzed by the restriction fragment length polymorphism (RFLP) assay by a previously described procedure (3). The amplified 480-bp fragment was separately digested with HaeIII, DdeI, or HpaII endonucleases (Boehringer-Mannheim). Digestion products were resolved by electrophoresis through 4% agarose in parallel with molecular weight markers (MW) (Bio Marker Low; ATGC Biotechnologie) and with an uncut (UC) DNA fragment derived from PV-1 strain Mahoney.

RESULTS

Determination of the precise phenotypes of the poliovirus strains. The initial characterization was performed by neutralization with strain-specific monoclonal antibodies and restriction growth at different temperatures (data not shown). The two isolates were weakly recognized by anti-Sabin monoclonal antibodies and anti-wild-strain monoclonal antibodies produced by the Institut Pasteur, Paris, France. Furthermore, examination of the growth properties of strains 5617 and 5915, analyzed at three temperatures (36.5, 39.2, and 40°C), showed that the former was slightly more temperature sensitive than the latter at 39°C but that at 40°C, both strains were temperature sensitive. Unfortunately, the vaccinal (attenuated) or wild (neurovirulent) origin of the two isolates could not be precisely determined by these means, so characterization of the two strains was carried out by RFLP (3). This test was chosen because it uses a genetic marker in the VP1 protein-coding region that characterizes the strains. Furthermore, the VP1 polypeptide was predicted to be involved in GTA binding (6, 7).

RFLP analysis. A 480-nucleotide fragment in the VP1 protein-coding region was amplified by PCR from the viral genome. RFLP patterns specific for the two isolates were generated by digestion of the PCR product with HaeIII, DdeI, and HpaII restriction enzymes and compared with the patterns obtained with the Sabin and Mahoney reference strains of PV-1. The results of the current study are presented in Fig. 1. The RFLP profiles of the isolate 5617, after cleavage with HaeIII, DdeI, and HpaII restriction enzymes, were identical to those obtained with the PV-1 Sabin strain (Fig. 1, lanes 5617 and S). The restriction profiles after cleavage with HaeIII and DdeI of the strain 5915, isolated from sewage, were similar to those obtained with PV-1 Mahoney strain. However, one exception was recorded. Cleavage with HpaII yielded DNA fragments with sizes different from those obtained with Mahoney strain (Fig. 1, lanes 5915 and M), which suggests genetic variation and molecular evolution of the genome of this strain.

Preparation of virus stocks. For each PV-1 strain tested, all the results were obtained with a single purified stock of virus. The comparison of the four virus stocks was based on two
criteria, infectious titer and total protein concentration, and the homogeneity of the stocks was estimated by the relative infectious titer. The stocks were stored at −80°C in 20-μl aliquots. Aliquots of the Mahoney prototype and 5617 stocks contained 10^7.9 MPNCU in 20 μl, and the protein concentrations were 89 and 86 μg/ml, respectively. The protein concentrations of Sabin prototype and 5915 stocks were 110 μg/ml. These stocks contained 10^{8.4} and 10^{8.2} MPNCU per 20-μl aliquot, respectively. Hence, the conditions of preparation of virus stocks described in Materials and Methods were reproducible, and the four virus stocks can be compared with one another. Therefore, the four stocks contained 7.7 to 8.0 log_{10} MPNCU/ml/μg of protein.

Inactivation kinetics of PV-1 strains by GTA. (i) Inactivation kinetics. Three kinetic experiments were performed to determine the inactivating processes of the four poliovirus strains with GTA at 0.02% (Fig. 2A), 0.05% (Fig. 2B), and 0.10% (Fig. 2C). All inactivation tests were carried out under the same conditions at 25°C and pH 7.2. Viral survival ratio was plotted versus time at the three GTA concentrations. Each plotted point is the mean of two or three survival ratios. The survival ratio is defined as the reduction in the viral concentration and is expressed as log_{10} (T/T₀), where T is the infectious titer at time t and T₀ is the initial infectious titer at time zero.

The kinetics of the activity of GTA on the two prototype strains of PV-1 showed striking differences. In all experiments and for the time periods and GTA concentrations tested, the Sabin strain was significantly more sensitive than the Mahoney strain. For instance, a reduction of about 4 log_{10} units in the infectious titer of the Sabin strain was obtained with 0.10% GTA in 15 min, whereas the reduction in the infectious titer of Mahoney strain was half as great (2 log_{10} units) in the same conditions (Fig. 2C). The natural isolates differed from each other, since strain 5617 was more sensitive than strain 5915 at the different concentrations of GTA tested. For instance, a decrease of 2.5 log_{10} units in the viral titer of strain 5617 was observed with 0.05% GTA after a 20 min-exposure time, whereas 70 min was required for strain 5915 (Fig. 2B).

At the different GTA concentrations tested, the Sabin and 5617 strains showed similar inactivation rates since the differences in reduction titers observed for each point and at different times were <0.5 log_{10} unit. Likewise, the infectivity of the two wild strains, Mahoney and 5915, decreased at similar rates. In all experiments, the Mahoney and 5915 strains were significantly less sensitive than the Sabin and 5617 strains. However, differences in the GTA sensitivities of strains were less significant when the GTA concentration increased. Hence, we were able to compare the two more-resistant strains and the two more-sensitive strains by using the differences observed between their specific inactivation rates at a defined time. For example, after 80 min of exposure time, differences ranged from 1.0 to 2.0 log_{10} units with 0.02% GTA, whereas they were between 0.5 and 0.9 log_{10} units with 0.10% GTA (compare Fig. 2A and C).

(ii) Statistical analysis. A regression analysis of inactivation rates at the different GTA concentrations against inactivation time was performed to determine which model fit the experimental data. The overall results of this analysis are summarized in Table 1. The results of the regression analysis, using a linear model, indicated that there was a statistically significant relationship at the 99% confidence level between inactivation rates with 0.02 and 0.05% GTA and inactivation time for the four strains. With 0.10% GTA, the results of the regression analysis of inactivation rates against time were more complex. The data for the inactivation process of Mahoney and 5915 strains fit a linear model at the 99% confidence level (Table 1). However, the correlation coefficients (−0.909 and −0.907) indicated a moderately strong relationship between the variables (Table 1). In contrast, for the other two strains, Sabin and 5617, the results with 0.10% GTA showed that the relationship between inactivation rates and time was not statistically significant at the 90% or higher confidence level. However, the relationship between inactivation rates and inverse of time (1/T) was statistically significant at the 99% confidence level, with correlation coefficients of −0.995 and −0.942 for Sabin and 5617 strains, respectively (data not shown), indicating a relatively strong relationship between the variables.

The calculated slopes of the plots are given in Table 1. The values obtained with 0.02 and 0.05% GTA showed that the inactivation rates of Sabin and 5617 strains were always higher than those of Mahoney and 5915 strains. The strains were not compared at 0.10% GTA because the inactivation process of the first two strains did not follow a linear model against time. The comparison also showed that the inactivation processes of the 5915 and 5617 strains were very similar to those of the Mahoney and Sabin strains, respectively. There was only one exception: the plotted curves of the inactivation processes of Sabin and 5617 strains with 0.02% GTA had very different slopes. Examination of the data showed that the absence of experimental results at the 120-min time point for the Sabin strain probably induced an overestimation of the slope with respect to the 5617 strain that would account for the observed difference.

Multiple range tests were performed for a more precise comparison of the experimental data. Fisher’s least-significant difference (LSD) procedure was used to discriminate among the 66 pairwise comparisons. Forty-four pairs showed statistically significant differences at the 95.0% confidence level (Table 1). When the two pairs of strains, strains Mahoney and 5915 and strains Sabin and 5617, were compared at the same GTA concentration, they were clearly distinguished (Table 2) because the differences observed between the strains of one pair were never statistically significant. In contrast, the difference was always significant when one of the strains of one pair was compared with the two strains of the other pair.

**DISCUSSION**

In a previous study, we reported that two recently isolated variants of echovirus type 25 exhibited significantly lower sensitivity to GTA than did their corresponding prototype strain PV-4 (7). In this study, we determined whether such differences in the sensitivity to GTA can be observed between PV-1 isolates currently circulating in the population and the reference strains. Two recent natural isolates of PV-1, 5617 and 5915, were selected because of difficulties encountered in their identification, which suggested that they were virulent. The isolates could not be classified as wild or vaccine strains by using the replicative capacity at elevated temperatures (rc marker test) (15) and strain-specific monoclonal antibodies.

The two PV-1 natural isolates were differentiated by PCR-RFLP (3). The poliovirus 5617 strain, isolated from stools of an infant, was closely related to PV-1 Sabin strain, as shown by its identical RFLP patterns obtained by cleavage with HaeIII, DdeI, and HpaII enzymes. This result confirms that, as shown by different researchers (3, 14), the Sabin profile is conserved during virus replication in humans. In contrast, the PV-1 5915 strain, isolated from sewage, appeared to be similar to wild PV-1 Mahoney strain by its HaeIII and DdeI profiles. However, these two wild strains can be differentiated from each other by their HpaII profile, which shows a genetic variation in the 5915
isolate. Because the RFLP test is a more powerful technique than antigenic analysis with neutralizing monoclonal antibodies to define the genetic origin of poliovirus isolates, we concluded that in the genomic region analyzed, isolate 5617 was a Sabin-like strain and isolate 5915 was a variant of the neurovirulent Mahoney strain. We therefore analyzed the GTA sensitivities of the two isolates and compared the isolates to their respective reference strains.

The comparative analysis of the inactivation kinetics showed that two groups could be differentiated from the strains of poliovirus serotype 1, with a significantly different level of sensitivity to GTA. One group, comprising the Mahoney and Mahoney-like strains, was more resistant than the other group, made up of the Sabin and Sabin-like strains. These results are supported by the statistical analysis of the experimental data.

The statistical analysis of the experimental data also provided information on the inactivation mechanism of virus infectivity by GTA. The results of the regression analysis of inactivation rates highlighted two distinct inactivating processes of polioviruses. The inactivation process of the more-resistant strains (group of wild polioviruses) followed a linear model against time, at all the concentrations tested. Up to a GTA concentration of 0.05%, the inactivation process of the more-sensitive strains (vaccine polioviruses) followed a linear model against time, during the period tested. At the highest GTA concentration tested (0.10%), a departure from linearity was observed for these two strains. We have already reported such departures from linearity in inactivation process with echovirus type 25 strains (7). We observed that the inactivation kinetics of the JV-4 reference strain differed strongly from those of the two recently isolated variants tested, which were more resistant. Differences in the inactivation process compared with the test virus have already been described with other enteric viruses and disinfectants (24, 25). One parameter involved in this process could be the presence of viral aggregates in suspension. In this case, the line of the survival curve could correspond to the inactivation of the monodispersed virus particles. Viral aggregates would then contribute to the changes of slope in virus survival curves and the loss of GTA efficiency as time is extended. Vanden Bossche et al. (24) have shown that with higher ozone levels, particles

![Diagram of inactivation kinetics](http://aem.asm.org/)
from rotavirus-seeded test suspensions tended to increase in size, and that as a result, virus-associated complex greatly influenced the inactivation kinetics. This hypothesis could explain both the rapid slope of the linear segment and the departure from linearity observed at 0.10% GTA for the more rapidly inactivated strains. We think that a departure from linearity would also be observed for the two more-resistant strains if the exposure time were extended beyond 120 min. However, the intrinsic mechanism of the GTA reaction with virions could also be responsible for the complexity of the kinetics and the differences observed between the vaccine and wild strains. It has been suggested that there is a consistent relationship between the amount of GTA combined with the proteins and the number of lysine residues in the proteins (13).

The three-dimensional structure of the poliovirus Mahoney strain (9) showed that the two top loops, one from VP1 (near the fivefold axis) and the other from VP3, both containing lysine groups, were prominently exposed on the surface of the shell and hence accessible to GTA. The top loop of the VP1 capsid protein of the Sabin prototype strain, which is more sensitive, contains one more lysine group than that of the Mahoney strain (9, 23).

A major concern of this study was to emphasize the necessity of always working in the same conditions to ensure repeatability and reproducibility of results. The results of the inactivation kinetics to GTA in the Sabin strain differed significantly (about 1.5 log$_{10}$ unit difference) from those obtained in a previous study (1). However, the methodology had been modified between the two test periods: the desalting operation of the viral preparation, the multiplicity of infection for the preparation of virus stocks, and the type of cell culture used for titration assays were different. Published reports have shown that, in suspension test methods for bactericidal activity of disinfectants, significant differences in microbicidal effect occurred both within and between laboratories (4). Therefore, virucidal tests must be performed under strictly controlled conditions to ensure accuracy and reproducibility of results. In this respect, it is important to use well-defined viral strains, which should come from an international test virus bank. The cell cultures, which should also belong to a cell bank, must be used in the same range of passages to minimize significant variations in the sensitivity of the cells and variability of titration assays. Likewise, the number of virus passages from original stock and the multiplicity of infection require rigorous monitoring. In order to achieve tests that can be used worldwide, the methods of preparation of virus stocks require more-rigorous standardization. Finally, the choice of the test enterovirus strain is very important. Hence, the Sabin strain of PV-1 is the most frequently chosen test virus because of its safety. However, our overall results raise doubts about whether this vaccine strain is still currently representative. Model strains must be carefully selected to ensure that they represent the group. Also, we think that the choice of an enterovirus more resistant than the PV-1 Sabin strain would be judicious for evaluating the efficacy of disinfectants.

The overall results of this study show that, as for echovirus type 25 strains, there are intratypic differences in the sensitivity to GTA in the PV-1 strains. However, the extent of intratypic divergence seems smaller than for echoviruses because differences are limited to the separation between Sabin-related strains and wild Mahoney-related strains. Comparative studies should now be extended to include clinical isolates of PV-2 and PV-3 and of echoviruses and coxsackieviruses that have been associated with serious clinical syndromes such as myocarditis, congenital malformations, or persistent infection.

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