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NOTES

Development of Poliovirus Having Increased Resistance to Chlorine Inactivation

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A laboratory strain of poliovirus (LSc) became progressively more resistant to chlorine inactivation during a series of repeated sublethal exposures to the halogen.

It is well documented that viruses are more resistant than bacteria to chlorine inactivation (7, 9). Further, differences in resistance even exist among strains of the same virus, as well as between viruses of different taxonomic groups (8, 10). It is generally agreed that viruses are present in wastewaters, even after the usual chlorine disinfection treatments (14, 15). Surface waters exposed to the activities of man likewise contain viruses (1, 3), but to a markedly lower extent. Therefore, it is possible that drinking-water supplies developed from contaminated waters may contain viruses (15). Although it has been assumed that drinking-water treatment removes virus and that viruses cannot survive in the presence of free chlorine residuals in finished waters, there are several reported isolations of virus from such waters (7a, 11).

A comparative study of the relative resistance of 20 human enteric viruses to free chlorine has provided evidence to suggest that naturally occurring virus may be much more resistant to chlorination than virus prepared in the laboratory (10). A similar finding was recently reported (P. T. B. Shaffer, National Conference on Environmental Engineering, Vanderbilt University, Nashville, Tenn., July 1977) for two poliovirus isolates, which were shown to be several orders of magnitude more resistant to chlorine than two standard strains of poliovirus (Mahoney and LSc). Viruses are known to adapt to a variety of environmental factors such as pH, temperature, and cell culture type (16), frequently leading to stable genetic changes (4). However, development of increased resistance by a virus to chlorine inactivation has not been reported. The objective of this study was to determine if repeated sublethal exposures of a laboratory strain of poliovirus (LSc) could lead to the development of greater chlorine resistance through an evolutionary process of selection of the most resistant individual virions.

The Buffalo green monkey (BGM) cell line was obtained from the Carborundum Co. and maintained in minimum essential medium supplemented with fetal bovine serum (FBS). Type 1 poliovirus (strain LSc) was grown in BGM cell monolayers in 32-ounce (0.95-liter) bottles and constituted the seed virus used to inoculate cells for propagation and purification of virus for cycle 1.

The procedure for partial purification of the virus used in each cycle of chlorine inactivation was as follows. For cycle 1, the source of virus was the seed virus prepared as described above. For cycles 2 through 10, the source of virus was the pooled plaque material from survivor plaques following each cycle of chlorine inactivation. The preparation of partially purified virus was initiated by inoculating BGM cells in 32-ounce bottles with 2 ml of virus. The inoculated cell cultures were incubated for 24 to 36 h at 37°C or until advanced cytopathic effect was observed. After freezing and thawing, the cell debris was removed by centrifugation at 10,000 $\times g$ for 20 min and the supernatant fluids were collected. The virus was pelleted from the supernatant by centrifugation at 135,000 $\times g$ for 3 h. The virus pellet was resuspended in 1.8 ml of phosphate-buffered saline (0.1 M potassium phosphate–0.15 M sodium chloride [pH 7.0]) and dispersed by sonic oscillation. The virus sample was passed through a 0.45-μm cellulose nitrate membrane (Millipore Filter Corp., Bedford, Mass.), which had been pretreated with 5 ml of a 1:5 dilution of FBS in phosphate-buffered saline. After filtration, 0.2 ml of FBS was added to the virus sample, giving a final volume of 2 ml. The titer of the virus was determined by plaque assay. For plaque assays, BGM cells were

seeded in minimum essential medium in petri dishes (60 by 15 mm; 10^6 cells per dish) and used when the cells reached confluency. Serial 10-fold dilutions of the samples were prepared in minimum essential medium, and 0.25 ml of each dilution was inoculated onto separate plates. After adsorption for 30 min at 37°C, 5 ml of overlay medium consisting of 1× minimum essential medium, 1% Noble agar, and 5% FBS was added to each plate. The plaques were visualized at 48 h by staining the monolayers with neutral red.

The stock solution of chlorine was prepared from calcium hypochlorite (65% available chlorine) by adding distilled water to achieve a concentration of 200 mg/liter and was stored in an amber bottle at 4°C. Chlorine determinations were made with an amperometric titrator (Wallace and Tiernan) for free and combined available chlorine (as described in *Standard Methods* [2]). Chlorine was added to 450 ml of 0.1 M phosphate buffer in a 500-ml reactor flask (Fig. 1). After a 30-min prereaction period, 200 ml of buffer with halogen was removed and the chlorine residual was determined amperometrically. Sufficient chlorine dosage was made at 0 min to give a residual of 0.8 mg/liter at the end of the 30-min prereaction period. At this time the virus sample was added and a contact period of 30 min was allowed. At the end of the 30-min contact period (60 min from addition of the chlorine to the buffer at 0 min) a final chlorine determination was made and there was a 0.6-mg/liter residual. Chlorine additions and determinations were carefully controlled, and at each cycle of inactivation the values were 0.8 and 0.6 mg/liter at the end of the 30-min prereaction and 30-min contact periods, respectively. In all cases, the chlorine was in the free available form, since combined chlorine was not detected when the free and combined chlorine determinations were made. However, a decrease from 0.8 to 0.6 mg of chlorine residual per liter was observed immediately upon addition of the virus inoculum, as evidenced by comparison of chlorine determinations at the end of the 30-min prereaction period and immediately after addition of the virus at the beginning of the 30-min contact period.

Poliovirus LSc1 was exposed to 10 cycles of chlorine inactivation. All experiments were conducted in a constant-temperature room at 17°C. The glassware was thoroughly cleaned and rinsed with distilled, deionized water. Similarly, all buffer and solutions were prepared with distilled, deionized water. Preliminary experiments were performed to determine the concentration of chlorine that would give an inactivation rate with surviving virus at times intermediate in the 0- to 30-min sampling period. Once this concentration was determined, it was used throughout all cycles of inactivation to make comparisons of inactivation rates.

For each cycle, two sterile 500-ml flasks, containing 450 ml of phosphate buffer, pH 7.0, for the reactor flask and 250 ml of buffer for the control flask, were prepared (Fig. 1). Chlorine was added to the reactor flask and allowed to prereact for 30 min. After removal of 200 ml of buffer from the reactor flask for the chlorine determination, 0.2 ml of the virus sample was added to the reactor flask and the control flask. The virus sample was prepared as described above and was diluted with phosphate buffer so that 0.2 ml contained approximately 5×10^7 plaque-forming units. This dilution procedure reduced the demand of the FBS, which was added during purification to prevent aggregation of the virus. Samples were removed from the reactor flask at 20, 40, 60, and 90 s and 2, 2.5, 3, 4, 5, 10, 15, 20, 25, and 30 min after addition of the virus. Each 3.5-ml sample was rapidly deliv-

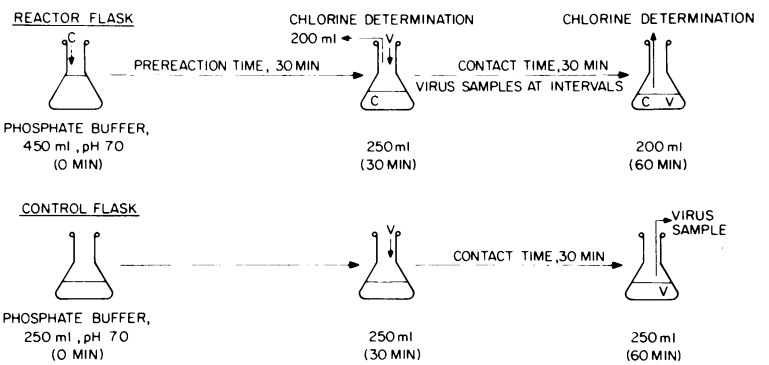

FIG. 1. *Diagram of experimental procedure for exposure of poliovirus LSc1 to chlorine. C, Chlorine; V, virus.*

ered into a tube containing sodium thiosulfate (10%) to neutralize the chlorine. A sample was collected at 30 min after addition of the virus to the control flask. At the end of the 30-min contact period, a sample of 200 ml of buffer was removed from the reactor flask for a chlorine determination. All virus samples were stored at 4°C. Plaque assays were done within 48 h to establish initial titers, utilizing a series of dilutions for each sample, and then five replicate assays were made at appropriate dilutions for each sample. Plaques on plates representing the longest time of survival were picked, combined, and inoculated onto a BGM cell monolayer. The virus was grown and partially purified as described above, and the next cycle of inactivation was initiated. The exposure of survivor virus to chlorine was repeated 10 times. In parallel with cycle 10 of inactivation, previously untreated virus (i.e., virus ready for cycle 1) was exposed to chlorine under identical conditions.

In all cycles, virus at approximately the same initial starting titer was exposed to the same concentration (0.8 mg/liter) and active species (free) of chlorine. The titers of virus that remained at each sampling interval after exposure to chlorine in each cycle are shown in Table 1. These data show that surviving virus populations were increasingly resistant to chlorine inactivation upon repeated exposure to the halogen. When comparing cycle 1 inactivation with successive cycles, two general observations can be made: (i) an increase in virus survival at longer periods of exposure and at higher titers occurred with repeated exposure of the virus to chlorine, and (ii) the rate of virus inactivation decreased with increasing cycles of exposure to chlorine.

In the early cycles of exposure of the virus to chlorine, a progression in the development of resistance was noted; however, there was some fluctuation in the length of time that virus survived and in the titers of virus detected (Table 1). The trend towards virus resistance was clearly established by cycle 5, and in subsequent cycles the trend towards greater resistance appears to continue. When cycles 1, 5, and 10 were compared (Fig. 2), it was quite apparent that the rate of virus inactivation decreased with increasing numbers of cycles of exposure of the virus to chlorine. For example, at 3 min in cycle 1, no virus survivors were detected. At the same sampling interval in cycle 10, a 2-log-higher titer of virus survived than at the same time in cycle 5 (Table 1).

It is particularly important to note that when cycle 1 performed at the beginning of the cyclic exposure series was compared to cycle 1 performed at the end of the series, the results were essentially identical (Table 1). The difference in resistance to chlorine by poliovirus LSc1, which had not been previously exposed to chlorine and virus after 10 repetitive exposure-replication cycles, was clearly evident when the results for cycles 1 and 10, run in parallel under identical conditions, were examined (Table 1; Fig. 2). In cycle 10 the virus survived for a longer period of exposure and at higher titers. Therefore, the inactivation rate of virus in cycle 10 was greatly decreased over that of cycle 1 virus.

Although viruses have been shown to adapt to a variety of environmental factors (16), the studies described here provide the first evidence for the development of resistance by a laboratory strain of virus to chlorine inactivation. A general progression in the development of resistance to chlorine was observed in early cycles of exposure of poliovirus LSc1 to chlorine. However, there was some fluctuation in the length of time virus survived and in the titer of virus detected in the early cycles of exposure. For example, virus in cycle 3 appears nearly as resistant to inactivation as virus in later cycles. Although the reason for this fluctuation is not directly apparent, it may have resulted from the probable heterogeneity of the resistance of virus in separate surviving plaques, which were picked and pooled to serve as the stock virus for the next cycle of inactivation. As the number of cycles of exposure increases, the virus of surviving plaques may be more homogenous in terms of resistance.

Resistance to inactivation or a decrease in inactivation rates is commonly seen in virus survival curves. This "tailing effect" is usually attributed to the presence of virus aggregates and other physical factors (6). The change in slope of survival curves as a result of a tailing effect may be gradual or abrupt. Since the poliovirus used in this study was prepared in a similar manner for each cycle of inactivation, it is unlikely that the decrease in inactivation rate with increasing numbers of cycles of inactivation was due to any significant differences in the physical state of the virus when exposed to chlorine.

Several recent studies have examined the influence of the physical state of poliovirus and reovirus on the kinetics of inactivation (5, 12, 13). It was clearly demonstrated in these studies that aggregated virus was substantially more resistant to disinfection than suspensions of single particles. In contrast to our findings with poliovirus, reovirus progeny grown from resistant plaque-forming units at the 10^{-4} survival level were no more resistant to bromine disinfection than parent virus cultures (12). It was concluded that the resistance of these plaque-forming units to bromine inactivation was due to

TABLE 1. *Titers of poliovirus LSc1 after repeated exposure to chlorine*

Time	Titer (PFU/ml)[a] at cycle:										
	1	2	3	4	5	6	7	8	9	10	1[b]
Control	2.2×10^5[c]	6.4×10^4	8.0×10^4	2.6×10^5	3.4×10^5	2.4×10^5	2.3×10^5	6.0×10^5	8.8×10^4	1.1×10^5	1.5×10^5
20 s	2.0×10^4	2.0×10^4	1.5×10^5	3.2×10^5	5.7×10^4	3.9×10^4	7.1×10^4	5.4×10^4	2.8×10^4	4.7×10^4	1.4×10^4
40 s	3.2×10^3	5.7×10^3	2.8×10^4	4.4×10^4	2.1×10^4	7.6×10^3	4.2×10^4	1.3×10^4	7.7×10^3	3.2×10^4	3.5×10^3
60 s	8.0×10^2	1.8×10^3	9.6×10^3	7.0×10^3	1.7×10^4	4.3×10^3	1.0×10^4	1.6×10^4	2.8×10^3	1.4×10^4	4.6×10^2
90 s	1.5×10^2	5.6×10^1	7.4×10^3	2.6×10^3	2.9×10^3	3.8×10^2	4.2×10^3	3.6×10^3	6.4×10^2	6.0×10^3	6.4×10^1
2 min	5.6×10^0	1.2×10^2	3.6×10^3	6.8×10^3	1.5×10^3	1.4×10^2	4.8×10^2	2.0×10^3	2.6×10^2	3.1×10^3	6.4×10^0
2.5 min	——[d]	6.7×10^1	1.2×10^3	4.0×10^2	3.8×10^2	6.0×10^1	7.1×10^2	6.4×10^2	1.1×10^2	1.8×10^3	3.2×10^0
3 min	—[e]	—	8.9×10^2	4.0×10^1	1.4×10^1	1.6×10^1	1.5×10^2	3.6×10^2	4.0×10^1	1.1×10^3	—
4 min	—	—	3.7×10^2	—	1.7×10^1	2.4×10^1	3.8×10^1	1.0×10^2	6.0×10^0	2.5×10^2	—
5 min	—	—	6.1×10^1	—	6.4×10^0	—	4.8×10^0	2.6×10^0	4.0×10^0	6.4×10^1	—
10 min	—	—	—	—	—	—	—	—	—	—	—
15 min	—	—	—	—	—	—	—	—	—	—	—
20 min	—	—	—	—	—	—	—	—	—	—	—
25 min	—	—	—	—	—	—	—	—	—	—	—
30 min	—	—	—	—	—	—	—	—	—	—	—

[a] PFU, Plaque-forming units.

[b] This cycle 1 was repeated at the same time as cycle 10.

[c] Except for cycles 4, 6, and 8, these values represent the averages of five replicates at the appropriate dilution. The values for cycles 4, 6, and 8 represent the averages of two or more plates at different dilutions.

[d] ——, Time was not sampled in this cycle.

[e] —, No plaques were detected at these sampling times.

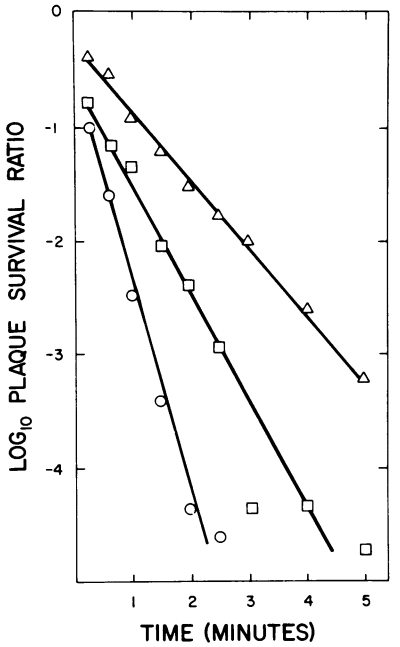

FIG. 2. *Rate of inactivation of poliovirus LSc1 during cyclic exposure to chlorine. Symbols: Cycle one* (○); *cycle 5* (□); *cycle 10* (△).

large aggregates present in the virus preparation. However, the virus in these survivor plaques was not repeatedly re-exposed to bromine to determine if resistance would develop or could be selected for in the virus population. The development of increased resistance by poliovirus to chlorine inactivation in our studies showed a gradual progression over several cycles of exposure rather than a single-step process. This gradual development of resistance suggests an evolutionary or adaptive alteration in the virus population after repeated sublethal exposures to chlorine. The molecular basis for this development of resistance is not known at this time.

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