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Effects of Ozone, Chlorine Dioxide, Chlorine, and Monochloramine on *Cryptosporidium parvum* Oocyst Viability

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Purified *Cryptosporidium parvum* oocysts were exposed to ozone, chlorine dioxide, chlorine, and monochloramine. Excystation and mouse infectivity were comparatively evaluated to assess oocyst viability. Ozone and chlorine dioxide more effectively inactivated oocysts than chlorine and monochloramine did. Greater than 90% inactivation as measured by infectivity was achieved by treating oocysts with 1 ppm of ozone (1 mg/liter) for 5 min. Exposure to 1.3 ppm of chlorine dioxide yielded 90% inactivation after 1 h, while 80 ppm of chlorine and 80 ppm of monochloramine required approximately 90 min for 90% inactivation. The data indicate that *C. parvum* oocysts are 30 times more resistant to ozone and 14 times more resistant to chlorine dioxide than *Giardia* cysts exposed to these disinfectants under the same conditions. With the possible exception of ozone, the use of disinfectants alone should not be expected to inactivate *C. parvum* oocysts in drinking water.

Cryptosporidium parvum, a coccidian intestinal parasite, infects humans and may cause gastroenteric disease. The life cycle is completed in the terminal portions of the small intestine. An environmentally resistant oocyst stage is produced and passed into the environment with host feces. Unlike other coccidia, the *C. parvum* oocyst is fully sporulated and ready to initiate infection upon excretion (8). Numerous cases of cryptosporidial infection have been reported in otherwise healthy people since the first report of an infected three-year-old child in 1976 (19). Immunodeficient individuals, acquired immunodeficiency syndrome patients, and the immunologically naive are especially vulnerable to persistent infection (7).

Waterborne transmission of *C. parvum* recently has been documented (10, 12; B. A. Rush, P. A. Chapman, and R. W. Ineson, Letter, Lancet **ii**:632, 1989). In 1987, *Cryptosporidium* contamination of a chlorinated and filtered water supply led to an estimated 13,000 cases of gastroenteritis in Carrollton, Georgia (14). Outbreaks of *Cryptosporidium*-related diarrheal illness in Ayershire, Scotland, and Oxfordshire-Swindon, England, recently have been attributed to consumption of contaminated surface water containing oocysts (H. V. Smith, R. W. A. Girdwood, W. J. Patterson, R. R. Hardie, L. A. Green, C. Benton, W. Turloch, J. C. M. Sharp, and G. I. Forbes, Letter, Lancet **ii**:1484, 1988; P. McIntyre and J. Day, *Oxford Mail*, Oxford, England, 23 Feb. 1989).

Cryptosporidium oocysts are extremely resistant to most commonly used disinfectants. Viability was not affected by exposure to 1.05 and 3% chlorine as sodium hypochlorite for up to 18 h. Long-term exposure to 10% Formalin, 5 to 10% ammonia, and 70 to 100% bleach was deemed necessary to completely eliminate infectivity (6, 21). Thirty minutes of exposure to temperatures above 65°C or below freezing also arrests oocyst infectivity (24). Although the studies of these exposures did not specifically address the use of chlorine in drinking water treatment, it is probable that chlorine disinfection alone is not sufficient to prevent *Cryptosporidium* infection. A recent study which examined the influence of ozone and chlorine dioxide on the viability of *Cryptosporidium parvum* oocysts in drinking water showed that treating demand-free water with 1.11 ppm of ozone (1.11 mg/liter) completely eliminated infectivity in 5 min and that 15 to 30 min of exposure to 0.43 ppm of chlorine dioxide significantly reduced infectivity as measured by oocyst production by mice inoculated intragastrically with 1,000 treated oocysts (20).

Disinfection kinetics of most chemicals commonly used to ensure the safety of drinking water have been reasonably well established for bacteria, viruses, and *Giardia* spp. (16, 26). Little, however, has been reported on the effects of such chemicals on the viability of *C. parvum* oocysts. Using neonatal mouse infectivity and in vitro excystation as measures of viability, we have investigated the effects of ozone, chlorine dioxide, chlorine, and monochloramine on the survival of *C. parvum* oocysts suspended in demand-free buffer in order to estimate the disinfectant concentrations and exposure times needed for oocyst inactivation.

MATERIALS AND METHODS

Oocysts. Contaminant-free *C. parvum* oocysts were recovered from the feces of experimentally infected 2- to 5-day-old Holstein calves by employing sequential discontinuous sucrose gradients followed by isopycnic Percoll gradients (2). The recovered oocysts were suspended in 2.5% (wt/vol) aqueous potassium dichromate solution, counted with a hemacytometer by using a bright-field phase-contrast microscope, and stored at 4°C until needed (8). A volume of the dichromate solution containing 10^8 oocysts was withdrawn for each experiment. Oocysts were used from a given stock suspension as long as excystation remained above 60%. The oocysts were washed three times with 0.025 M phosphate-buffered saline, centrifuged (1,500 × *g* for 10 min), suspended in oxidant-demand-free 0.01 M phosphate-buffered water, and treated with ozone, chlorine dioxide, chlorine, or monochloramine. Oocyst viability was assessed by excystation and neonatal mouse infectivity. The fluorogenic vital dyes fluorescein diacetate and propidium iodide were also tested as indicators of oocyst viability (22). They were not deemed useful for this purpose (D. G. Korich, J. R. Mead, M. S. Madore, N. A. Sinclair, and C. R. Sterling, unpublished data).

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Mouse inoculation and infection evaluation. Litters of 3- to 6-day-old neonatal BALB/c mice maintained with their dams were infected per os with disinfectant-exposed oocysts suspended in 2 to 4 μl of NANOpure water. Litter sizes varied naturally, and no attempt was made to standardize the number of mice used in the experiments. Generally, more mice were employed for examining the effects of the more effective disinfectants such as ozone, and fewer were used for the less effective agents such as monochloramine. Washed oocysts recovered from the disinfectant reaction flasks were suspended in NANOpure water, and the oocyst concentration was determined by hemacytometer count. Suspensions containing appropriate concentrations of oocysts were prepared by dilution. A volume calculated to contain the required dose of oocysts was withdrawn and fed to the mice by means of a micropipette. A new pipette tip was used for each different dose administered, but a single pipette tip was used, without flushing, for all mice receiving the same dose. Doses were 600, 6,000, and 60,000 oocysts per neonatal mouse. These doses, which represented multiples of 10, 100, and 1,000 of a previously determined mean infectious dose (ID_{50}) of 60 oocysts, allowed estimates of oocyst inactivation levels of at least 90, 99, and 99.9%, respectively (Korich et al., unpublished data). Oocyst doses fed to the mice were adjusted by an amount based on the control percent excystation observed for each sample of oocysts in order to minimize variation. For example, the oocyst dose was increased by 20% for a control excystation of 80%. This same procedure was employed in the trials used to determine the ID_{50}. Infected animals were sacrificed 7 days postinoculation. Since the ileum is the first region of the gut to be colonized by *Cryptosporidium* spp. and always harbors the greatest number of parasitic developmental stages, approximately 3 cm of the terminal ileum was removed, fixed in 5% Formalin, embedded in paraffin, and sectioned (9). Hematoxylin- and eosin-stained paraffin sections were examined microscopically for evidence of parasites in the microvillous region of villous enterocytes (8). The tissue sections invariably exhibited an all-or-none pattern of infectivity. Tissue specimens from mice that had received at least the ID_{50} contained many *Cryptosporidium* developmental stages, while there were no parasites in specimens from mice that had received less than the ID_{50}. There was never any doubt as to how a given specimen should be scored. Specimens with parasitic stages present were scored as positive; those without were scored negative. Positive specimens always showed numerous parasitic stages (at least 50 to 60 per ×100 microscope field), while no parasites could be found on any sections taken from negative tissue samples. Mouse litters were selected randomly during the course of the experiments to serve as uninfected controls to verify that the colony remained free of incidental *C. parvum* infection. These animals were sacrificed, and the terminal ilea were prepared and examined as above.

Excystation procedure. Washed oocysts were suspended in 0.5 ml of tissue culture phosphate-buffered saline (8% NaCl, 1.15% Na_2HPO_4, 0.2% KCl, 0.2% KH_2PO_4). This suspension was mixed with an equal volume of excysting fluid (0.5% trypsin, 1.5% sodium taurocholate, in tissue culture phosphate-buffered saline) and incubated for 60 min in a 37°C water bath (11). Excysted sporozoites, intact oocysts, and oocyst shells in the suspension were counted under ×400 magnification by using a hemacytometer and phase-contrast microscopy. Microscopic counts performed after in vitro excystation of untreated oocysts often yielded a variable number of sporozoites per shell. This variation appeared to depend on the age (storage time) of the oocyst stock, duration of incubation, composition of the excystation medium, and activity of the trypsin used in the excystation procedure. Therefore, a control excystation with complete counts, with untreated oocysts, was performed in conjunction with each series of disinfectant treatments in order to establish a baseline for calculating and correcting the percent excystation of the treated oocysts. Excystation levels were determined according to a formula adapted from one used for computing excystation of *Giardia* spp. (5): percent excysted = $100 \times S/(S + I)$, and $S = S_1/S_2$. In these equations, S_1 is the number of excysted sporozoites in the treated sample, S_2 is the number of excysted sporozoites per shell in the control, and I is the number of intact oocysts in the treated sample. Percent excystation of the treated oocysts was corrected against the control baseline percent excystation as follows (17): corrected percent excysted = $E \times (100/C)$, where E is the percent excystation in the treated sample and C is the mean percent excystation in the control.

Ozone treatment of oocysts. Ozone was generated with a P-series model P-20 ozonator (Ozone Technology, Inc., Tyler, Tex.). Ozone-demand-free water was prepared by ozonating NANOpure water for 1 h at 2 mg/liter. The water was then boiled for 1 h to remove the ozone and stored in sealed ozone-demand-free glass containers until needed. The glassware was soaked in water containing 2 mg of ozone per liter for 1 h and then dried at 110°C for 5 h to satisfy the ozone demand (25). Experiments were conducted with constant stirring at 25°C in borosilicate glass reactors containing 350 ml of ozone-demand-free water buffered to pH 7 with 0.01 M phosphate and seeded with 10^8 oocysts (2.8×10^5 oocysts per ml). Ozone was bubbled continuously into the water at a rate predetermined to achieve and maintain the desired equilibrium ozone concentration. Washed oocysts suspended in 1 ml of ozone-demand-free buffered water were added after equilibrium was achieved as indicated by no change in ozone concentration during three successive measurements over a 15-min period. Ozone concentration was measured spectrophotometrically by the decolorization of indigo trisulfonate (3). Samples of 50 ml each were withdrawn at 0, 1, 3, and 5 min into centrifuge tubes containing 1 ml of 10% (wt/vol) sodium thiosulfate. The oocysts were then washed and prepared for either excystation or mouse inoculation as described above. At first, a duplicate reactor was simultaneously operated with air rather than ozone. This was abandoned during subsequent experiments, however, when it was determined that there was no difference between untreated control and control reactor excystation rates.

Chlorine treatment of oocysts. Chlorine solutions (pH 7) were prepared with reagent grade sodium hypochlorite in pH 7 demand-free 0.01 M phosphate buffer. The concentration of free chlorine in a freshly prepared standard solution was accurately measured with a Fisher amperometric titrimeter. Dilutions of this standard were used to construct a standard curve for measuring the concentrations of working solutions by the Hellige DPD (*N*, *N*-diethyl-*p*-phenylenediamine) method with a DU-6 spectrophotometer (Beckman Instruments, Inc.) (15). Chlorine-demand-free water was prepared by adding 3 ml of commercial bleach containing approximately 5% active chlorine to 6 liters of NANOpure water in a closed container. The water was held at least 24 h at room temperature (25°C) and then placed in direct sunlight for 6 h. Free chlorine, as determined by the Hellige DPD method, was not detected in chlorine-demand-free water prepared in this manner. All glassware was soaked for 24 h in deionized

water containing 50 mg of free chlorine per liter and was thoroughly rinsed with chlorine-demand-free water prior to use (13). Glass flasks (250 ml) containing 250 ml of chlorine solution were inoculated with 10^8 washed oocysts suspended in 1 ml of demand-free 0.01 M phosphate buffer (4×10^5 oocysts per ml) and stirred constantly. Five 40-ml samples were withdrawn at timed intervals into centrifuge tubes containing 2 ml of 10% $Na_2S_2O_3$. The oocysts were then processed as described above for excystation or inoculation.

Chlorine dioxide treatment of oocysts. A stock solution of pure chlorine dioxide was prepared by reacting 20 ml of 4 N sulfuric acid with a solution containing 10 g of sodium chlorite in 750 ml of distilled water. The gases produced in the reaction were scavenged with a stream of clean compressed air, passed through a saturated solution of $NaClO_2$, and dissolved in 1 liter of chlorine-demand-free water. Chlorine dioxide concentration in the stock solution was measured by titrating iodine released by the ClO_2 from an acidic solution of potassium iodide against a 0.005 N standard solution of $Na_2S_2O_3$ (1). Working solutions of chlorine dioxide were prepared by diluting samples of the stock solution with demand-free phosphate buffer (pH 7). The ClO_2 concentrations in the working solutions were determined by spectrophotometric measurements by the Hellige DPD method in the presence of glycine against a standard curve obtained by appropriate dilutions of the stock solution (1, 15). Measurements made by this method were confirmed by the decolorization of acid chrome violet K (18). The two methods were found to be comparable for measuring pure solutions of chlorine dioxide, but the acid chrome violet K method was easier to use and produced more consistent results than the DPD method did. The experimental design was similar to that described for chlorine with the exception that the reactor vessels were stirred only during sample withdrawal in order to minimize the loss of ClO_2 from the solution.

Monochloramine treatment of oocysts. Fresh stock solutions of monochloramine were prepared for each experiment by mixing equal volumes of a chlorine solution (pH 9 to 10) containing the equivalent of 2 g of HOCl per liter and an $(NH_4)_2SO_4$ solution (pH 9 to 10) containing 31 g/liter (4). Monochloramine concentrations were determined by the Hellige DPD method with the standard curve derived for chlorine. The experimental design was identical to that described for chlorine.

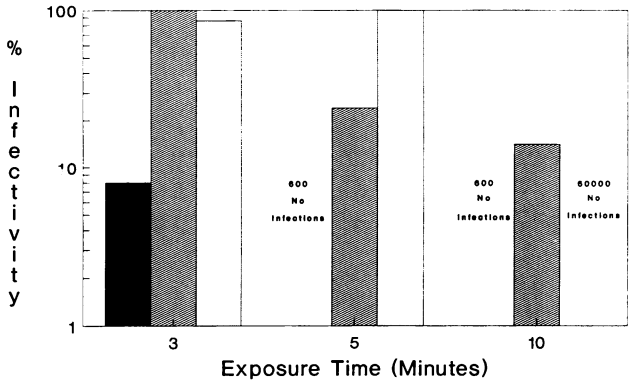

FIG. 2. Loss of infectivity in neonatal mice for *Cryptosporidium* oocysts exposed to 1 ppm of ozone at 25°C. Doses: ■, 600 oocysts; ▨, 6,000 oocysts; □, 60,000 oocysts.

RESULTS

Ozone. Excystation of oocysts exposed to 1 ppm of ozone decreased from 84% upon initial exposure to 0% after 5 min (Fig. 1). The rate of decrease was highest for the first minute of exposure and declined over the next 4 min even though ozone was maintained at a constant 1 ppm. Results of mouse infectivity were similar (Fig. 2). For example, 5 of 21 mice (24%) and 5 of 36 mice (14%) became infected when dosed with 6,000 oocysts exposed to ozone for 5 and 10 min, respectively. All 11 mice dosed with 60,000 oocysts exposed to ozone for 5 min became infected, while none of 21 mice receiving the same number of oocysts exposed to ozone for 10 min became infected. Thus, at least 90% of the oocysts were inactivated when exposed for 3 min, between 90 and 99% were inactivated when exposed for 5 min, and between 99 and 99.9% were inactivated when exposed for 10 min. Based on these data, the approximate $C \cdot t'$ (concentration times time [in minutes]) value for 99% inactivation would be between 5 and 10.

Chlorine dioxide. Excystation of oocysts exposed to 1.3 ppm of chlorine dioxide decreased from 87% upon initial exposure to 5% after 1 h (Fig. 3). Mouse infectivity showed a similar decline (Fig. 4). Two of nine mice (22%) and none of eight mice became infected when dosed with 600 oocysts exposed for 45 and 60 min, respectively, while eight of ten mice (80%) became infected after inoculation with 6,000

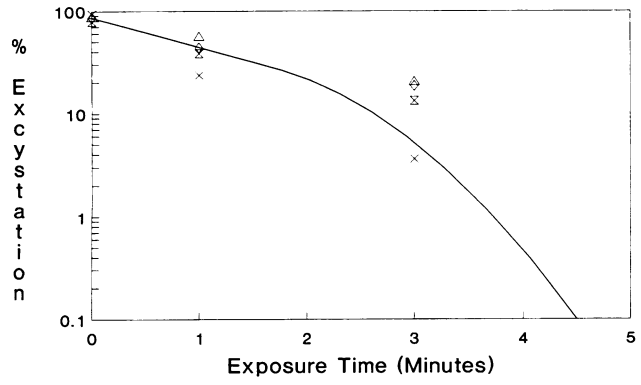

FIG. 1. Decline in mean percent excystation of *Cryptosporidium* oocysts exposed to 1 ppm of ozone at 25°C. Symbols show results of independent trials.

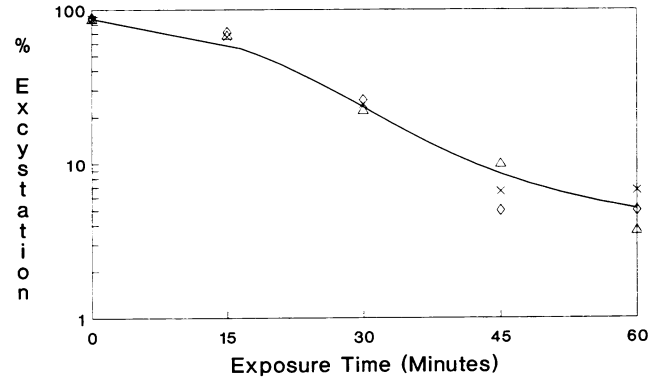

FIG. 3. Decline in mean percent excystation of *Cryptosporidium* oocysts after exposure to 1.3 ppm of chlorine dioxide (measured as parts per million of ClO_2) at 25°C. Symbols show results of independent trials.

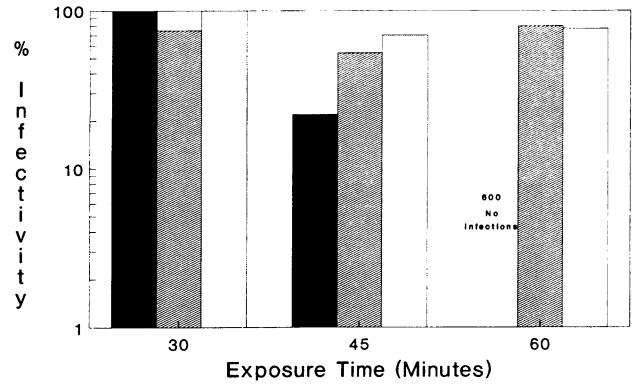

FIG. 4. Infectivity of *Cryptosporidium* oocysts exposed to 1.3 ppm of chlorine dioxide. Doses: ■, 600 oocysts; ▨, 6,000 oocysts; □, 60,000 oocysts.

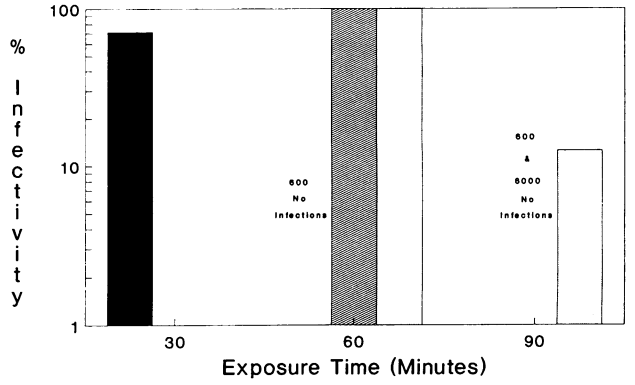

FIG. 6. Infectivity of *Cryptosporidium* oocysts exposed to 80 ppm of chlorine at 25°C. Doses: ■, 600 oocysts; ▨, 6,000 oocysts; □, 60,000 oocysts.

oocysts exposed for 1 h. Thus, approximately 90% of these oocysts were inactivated after 1 h of exposure. A $C \cdot t'$ value of 78 was calculated based on these data. Excystation of oocysts exposed to 0.6 ppm of chlorine dioxide decreased by only 40% after 1 h of exposure. Measurements showed a rapid decrease in chlorine dioxide concentration after the reaction flask was inoculated with 10^8 oocysts. The ClO_2 concentration dropped from 0.6 ppm to 0.2 ppm after 5 min of exposure. Losses as high as 0.9 ppm occurred during the 1 h that oocysts were exposed to 1.3 ppm of ClO_2.

Chlorine. Excystation of oocysts exposed to 80 ppm of free chlorine decreased from 80% upon initial exposure to 0% after 2 h (Fig. 5). After 1 h, only 20% of oocysts had excysted. Mouse infectivity data were similar (Fig. 6). Five of seven mice (71%) became infected at the 600-oocyst dose level after 30 min of exposure. All mice inoculated with 6,000 or 60,000 oocysts which had been exposed to chlorine for 60 min became infected. Only one of eight mice (12.5%) inoculated with 60,000 oocysts exposed to chlorine for 90 min became infected. There were no infections noted in 22 mice inoculated after 2 h of chlorine exposure. Inactivation after 90 min of chlorine exposure was at least 99%. Based upon these data, a $C \cdot t'$ value of 7,200 was calculated.

Monochloramine. The decline in excystation for oocysts exposed to 80 ppm of monochloramine was similar to that observed with free chlorine (Fig. 7). Excystation decreased from 91% upon initial exposure to 2% after 2 h. Only 10% of the oocysts exposed to 80 ppm of monochloramine excysted after 1 h. Mouse infectivity also showed a similar decline (Fig. 8). Five of eleven (45%), one of ten (10%), and zero of three (0%) mice became infected when dosed with 600 oocysts exposed to monochloramine for 30, 60, and 90 min, respectively. High infection rates were observed in mice inoculated with 6,000 and 60,000 oocysts exposed to monochloramine over the same time periods. Oocyst viability decreased at least 90% over 90 min of monochloramine exposure. A $C \cdot t'$ value of 7,200 was calculated from these data.

DISCUSSION

Measurements of oocyst viability, determined by both excystation and mouse infectivity, indicate that ozone and chlorine dioxide are many times more effective than free chlorine and monochloramine for inactivating *C. parvum* oocysts. Similar results have been obtained for oocyst inactivation by using chlorine with excystation as the sole measure of viability (23). Oocysts treated with 2.25 ppm of ozone for 8 min showed a 99% reduction in oocyst output in mice initially infected with 10^5 oocysts (20). This result, based on ozone levels achieved at the beginning of the experiment but not maintained, may be comparable to our finding that constant exposure of oocysts to 1 ppm of ozone for 10 min reduced infectivity by approximately the same

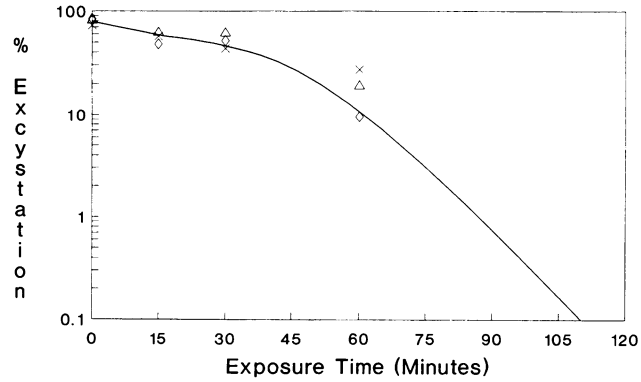

FIG. 5. Decline in mean percent excystation of *Cryptosporidium* oocysts after exposure to 80 ppm of chlorine (measured as parts per million of Cl_2) at 25°C. Symbols show results of independent trials.

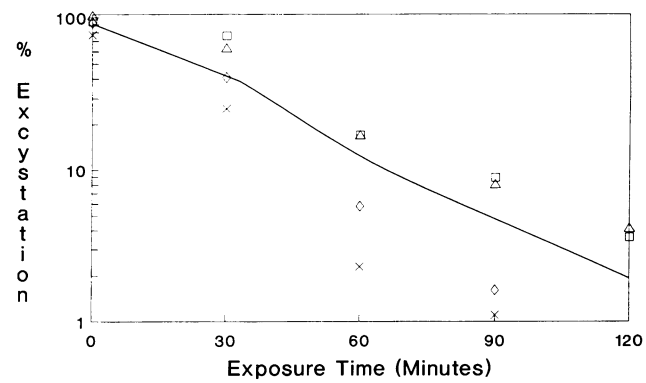

FIG. 7. Decline in mean percent excystation of *Cryptosporidium* oocysts exposed to 80 ppm of monochloramine at 25°C. Symbols show results of independent trials.

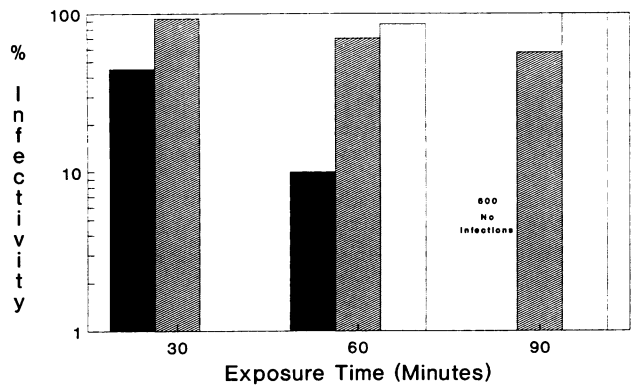

FIG. 8. Infectivity of *Cryptosporidium* oocysts exposed to 80 ppm of monochloramine at 25°C. Doses: ■, 600 oocysts; ▨, 6,000 oocysts; □, 60,000 oocysts.

amount. Data reflecting oocyst inactivation by chlorine dioxide, however, do not agree. Mice infected with oocysts exposed to 0.43 ppm of chlorine dioxide for 30 min showed a 94.3% reduction in oocyst output in another study, while we were only able to observe 90% oocyst inactivation as measured by mouse infectivity after exposure to 1.3 ppm for 1 h, i.e., roughly the same level of inactivation by three times the concentration for twice as long an exposure time (20).

Any test for disinfectant effectiveness must ultimately depend upon some measure of oocyst viability. Unfortunately, a quick, easy, reliable, and completely reproducible test is not yet available. We assessed inactivation by uptake of the vital dyes fluorescein diacetate and propidium iodide, excystation, and mouse infectivity. The vital dyes proved to be totally unreliable for predicting *C. parvum* oocyst viability even though they have been used successfully for determining *Giardia* viability (22; D. G. Korich, J. R. Mead, M. S. Madore, C. R. Sterling and N. A. Sinclair, poster session, American Society for Microbiology annual meeting, 1989). Furthermore, we observed a large apparent increase in excystation after there should have been none. These anomalous observations proved to be an artifact of the counting procedure. Oocysts exposed to high disinfectant concentrations or for long times at lower concentrations appeared to have excysted, i.e., were empty, when in fact their contents had been destroyed by the disinfectant. Sporozoites were conspicuously absent from such suspensions. To avoid subsequent errors, we counted and factored in the number of sporozoites produced. This revised procedure gave reasonably good agreement between excystation and infectivity as indicators of viability. Even so, viability estimates based on excystation alone should be regarded with caution, since the sensitivity of the estimate depends not only on the accuracy of the counting procedure but also on the age (storage time) of the oocysts. When excystation is compared with infectivity as an indicator of oocyst inactivation after treatment with 1 ppm of ozone, note that excystation (Fig. 1) showed 100% inactivation after 5 min of exposure while infectivity (Fig. 2) showed at least 99 to 99.9% inactivation after 10 min. Infection occurred with oocysts treated with ozone for 5 min even though excystation indicated otherwise. Similar comparisons may be made with the other disinfectant treatments used. Oocysts stored in dichromate solution longer than 6 months cannot reliably be used for disinfection studies because low levels of excystation (below 60%) are frequently observed. Mouse infectivity was the most sensitive indicator of oocyst inactivation under all the experimental conditions tested. Since some 20% of the oocysts produced during *C. parvum* infections are autoinfective, only a small dose of oocysts is required to produce a conspicuous infection (9). Microscopic examination of terminal ileum tissues, therefore, provides unequivocal evidence of infection.

The strategy for estimating disinfectant $C \cdot t'$ value was based on our determination that the ID_{50} for neonatal BALB/c mice was approximately 60 oocysts. Inability to produce infection with an incremental $\log_{10}$-increasing dose of the ID_{50} implied that level of oocyst inactivation. An absence of mouse infection with a dose of 600 oocysts equates to at least a 90% level of inactivation, no infection with 6,000 oocysts equates to a 99% level, and no infection with 60,000 oocysts equates to at least 99.9% inactivation. The $C \cdot t'$ values expressed herein must be regarded as estimates because they are based on only a single disinfectant concentration. A more accurate treatment, strictly applying the Watson equation ($k = C^n t$), requires using the results of a number of experiments with different disinfectant concentrations to find the value of n. When plotted on double logarithmic paper, the concentrations (C) and the times (t) needed for a given inactivation level result in a straight line with slope n (16). By assuming that $n = 1$, however, it is possible to make useful comparisons between these $C \cdot t'$ values and those for *Giardia* spp. Such comparisons show that *C. parvum* is many times more resistant than *Giardia* spp. are to these commonly used drinking water disinfectants. The $C \cdot t'$ value of 5 to 10 obtained for 99% ozone inactivation of *C. parvum*, for example, is at least 30 times greater than the $C \cdot t'$ of 0.18 ($n = 1.1$) required to achieve the same inactivation level of *Giardia lamblia* (26), while the $C \cdot t'$ value of 78 obtained for 90% inactivation of *C. parvum* oocysts by ClO_2 is at least 14 times higher than the 5.3 ($n = 1.37$) reported for 99% inactivation of *Giardia muris* (J. G. Leahy, M.S. thesis, Ohio State University, Columbus, 1985). Further infectivity studies need to be performed at different disinfectant concentrations in order to confirm the $C \cdot t'$ values. It is evident, however, that with the possible exception of ozone, the use of disinfectants alone cannot be expected to inactivate *C. parvum* oocysts.

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