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Effect of Disinfection of Drinking Water with Ozone or Chlorine Dioxide on Survival of *Cryptosporidium parvum* Oocysts

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Demineralized water was seeded with controlled numbers of oocysts of *Cryptosporidium parvum* purified from fresh calf feces and subjected to different treatments with ozone or chlorine dioxide. The disinfectants were neutralized by sodium thiosulfate, and neonatal mice were inoculated intragastrically and sacrificed 7 days later for enumeration of oocyst production. Preliminary trials indicated that a minimum infection level of 1,000 oocysts (0.1-ml inoculum) per mouse was necessary to induce 100% infection. Treatment of water containing 10^4 oocysts per ml with 1.11 mg of ozone per liter (concentration at time zero [C_0]) for 6 min totally eliminated the infectivity of the oocysts for neonatal mice. A level of 2.27 mg of ozone per liter (C_0) was necessary to inactivate water containing 5×10^5 oocysts per ml within 8 min. Also, 0.4 mg of chlorine dioxide per liter (C_0) significantly reduced infectivity within 15 min of contact, although some oocysts remained viable.

Cryptosporidium parvum is a protozoan belonging to the class *Sporozoasida*, which infects primarily the small intestines of most mammalian species. The parasite is a common etiologic agent associated with diarrhea in neonatal calves (26). Infected animals excrete large numbers of oocysts (11, 12, 22), which may soil drinking water resources (24). Surface water running through cattle pastures was shown to contain up to 6×10^3 oocysts per liter (15).

The first documented cases of human cryptosporidiosis were reported in 1976 (19, 20), but relatively few were subsequently diagnosed until cryptosporidiosis was reported to be a life-threatening disease in patients with acquired immune deficiency syndrome (8, 14, 23). Now cryptosporidiosis is accepted as a common cause of acute, self-limiting diarrheal disease in immunocompetent hosts worldwide (7). Fecal-oral spread among humans and animals and ingestion of contaminated water appear to be the principal modes of transmission (5, 10, 24).

The fact that surface and ground water may be contaminated by cryptosporidial oocysts and the lack of effective treatment (7) stress the importance of techniques to clear drinking water of this parasite. Oocysts of *Cryptosporidium* spp. may remain viable in aqueous suspensions for up to 12 months at 4°C (4). Few commercial disinfectants have been found to be effective in penetrating oocysts of the parasite (1, 2, 21), and neither the usual chlorination of drinking water nor normal water filtration systems remove oocysts effectively (10). Because ozone and chlorine dioxide have been used as alternatives to hypochlorite to disinfect water, we decided to study the possible influence of these disinfectants on the viability of cryptosporidial oocysts in drinking water.

MATERIALS AND METHODS

Animals and husbandry. A total of 39 coccidium-free litters of Swiss OF1 mice maintained with the dams in separate litters were used during the experiments. All animals were kept at 23°C in plastic cages (North Kent Plastic Cages Ltd., Dartford, Kent, England) with wire mesh tops and wood shavings for bedding and received a commercial all-mash pelleted feed (AO3; Usine d'Alimentation Rationnelle, Villemoisson, France) and water ad libitum.

Purification of oocysts. Two- to three-week-old calves from a large fattening plant were screened individually for excretion of *C. parvum* oocysts by carbol fuchsin stain (9). Fresh fecal material was collected by rectal sampling of calves excreting large numbers of *C. parvum*. The material was suspended immediately 1:1 in cold 5% (wt/vol) aqueous potassium dichromate solution and stored at 4°C until purification within 48 h. All purification steps were done at 4°C. Feces were washed through sieves (Endocotts Ltd., London, England) with meshes of 150 and 45 μm, respectively, and the resulting fluid was centrifuged at $500 \times g$ for 5 min. The sediment was suspended in 20 ml of water and 20 ml of diethyl ether after three washings with distilled water. The tube was then shaken in an inverted position for 30 s with a Heidolph shaking device. Subsequently, the suspension was spun down at $500 \times g$ for 10 min and the top three layers were decanted. This step was repeated until the sediment was free of lipids. After further washing at $500 \times g$ for 5 min, the sediment was suspended in distilled water. One-milliliter samples of the resulting suspension were then passaged on a discontinuous Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient constituted of four 2.5-ml layers with densities of 1.13, 1.09, 1.05, and 1.01 g/ml and centrifuged at $650 \times g$ for 15 min. The band containing purified oocysts was then removed, washed twice at $500 \times g$ for 5 min, and suspended in distilled water. The number of oocysts present in 1 mm^3 of suspension was counted in a modified Neubauer hemacytometer after 0.2 ml of suspension was mixed with 0.8 ml of malachite green (malachite green, 0.16 g; sodium dodecyl sulfate, 0.1 g; distilled water, 100 ml). The suspension was then diluted to the desired concentrations. The total organic carbon content of the suspension was determined with a Technicon AutoAnalyzer II by industrial method 451-76W.

Homogenization procedure. The effects of different homogenization methods on the recovery of oocysts were evaluated. Therefore, 8×10^5 oocysts were mixed with the guts of

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2-day-old mice in 5 ml of phosphate-buffered saline. Intestines were minced with a pair of scissors, followed by repeated passage of the mixture through 18- and 19-gauge needles, or they were subjected to ultrasonication, or they were homogenized by Ultra-Turrax (Janke & Kunkel KG, Staufen, Federal Republic of Germany) (three times for 10 s each time). Passage through 18- and 19-gauge needles did not result in destruction of intestinal villi sufficient to remove most oocysts. Ultrasonication disrupted villi but resulted in loss of ca. 38% of the oocysts and indentation of the walls of others. Homogenization resulted in loss of up to 41% of the oocysts but was adopted for the studies described below because the oocysts appeared microscopically normal.

Ozonization procedure. Ozone was generated in an Ozonlab CFD device (Chemie und Filter GmbH, Heidelberg, Federal Republic of Germany) and bubbled through demineralized water (Milli-Q; specific resistance, 10 MΩ/cm; Millipore Corp., Bedford, Mass.) for 30 min. The concentration of O_3 was determined iodometrically. Ten milliliters of a buffered potassium iodide solution (20 g of KI, 13.6 g of KH_2PO_4, and 14.2 g of Na_2HPO_4 made up to 1 liter) was placed in a volumetric flask and brought to 250 ml with the ozonized water sample. Titration was started with 0.005 N $Na_2S_2O_3$ and ended with 2 ml of 5 N H_2SO_4. After iodometry, 14.4 ml of ozonized water was mixed with 1.6 ml of oocyst suspension in a quartz spectrophotometric cell. At 1-min intervals during exposure of the oocysts, the remaining O_3 was evaluated by measuring the UV absorption of the solution at 254 nm in a Perkin-Elmer λ1 spectrophotometer.

Production of chlorine dioxide. Chlorine dioxide was generated by reaction of diluted sodium chlorite (1 g of $NaClO_2$ per liter) in bidistilled water with acetic anhydride, as described previously (17). The stock solution, containing approximately 50 mg of ClO_2 per liter, was titrated iodometrically first in a neutral environment and then in an acid environment as described previously (18a). Subsequently, a working solution was prepared by diluting the stock solution immediately before adding oocysts. ClO_2 concentrations before, during, and after treatment were determined spectrophotometrically with acid chrome violet at 548 nm in a Perkin-Elmer λ1 spectrophotometer (16).

Experimental infection and enumeration of oocysts. Two- to six-day-old neonatal mice were inoculated intragastrically with 0.1-ml oocyst suspensions by using a 16-mm-long 26-gauge needle fitted with plastic tubing. At 7 days postinfection (p.i.), the mice were sacrificed. The total small and large intestines were recovered in 5 ml of cold phosphate-buffered saline containing 1.0 mg of streptomycin per ml and 1.0×10^3 IU of penicillin per ml and stored at 4°C for a maximum of 48 h. The total oocyst contents of the intestine were evaluated after the guts were homogenized with an Ultra-Turrax (three times for 10 s each time). Subsequently, the number of oocysts present was counted in a hemacytometer as described above. The sediment of negative samples was reexamined by carbol fuchsin staining following centrifugation.

Experimental design. (i) Experiment 1. Five litters of neonatal mice were inoculated with 0, 10, 10^2, 10^3, or 10^4 *C. parvum* oocysts, respectively. The same protocol was repeated with another group of five litters. At 6 to 9 days p.i., all mice were sacrificed for oocyst counts.

(ii) Experiments 2 to 5. Oocyst suspensions containing 10 times the desired end concentrations were mixed in a proportion of 1:10 with demineralized water containing O_3 or ClO_2. The different oocyst concentrations, the O_3 or ClO_2 dosages at the start of exposure, and the exposure times used are listed in Table 1. At the end of exposure, the remaining O_3 or ClO_2 was neutralized by adding 0.03 ml of a 0.1 N solution of $Na_2S_2O_3$ (Tritrisol 9950; E. Merck AG, Darmstadt, Federal Republic of Germany). Subsequent litters of neonatal mice were inoculated with 0.1 ml of exposed or nonexposed suspensions. At 7 days p.i., mice were sacrificed for oocyst counts.

Statistics. Mean oocyst output was tested by the nonparametric Wilcoxon Mann-Whitney U test, whereas the numbers of infected mice were analyzed by the χ^2 test (25).

RESULTS

Experiment 1. Experimental infection of suckling mice with different numbers of oocysts resulted in detectable infection only after gavage of 10^3 or more oocysts. The highest oocyst numbers were detected at 7 days p.i., when 100% of the mice were found to be infected. At 6 days p.i., 4 of 7 mice inoculated with 10^4 oocysts were positive, and only 4 of 12 mice inoculated with 10^3 oocysts were positive at 8 days p.i. Therefore, all suckling mice were sacrificed at 7 days p.i. in subsequent experiments.

Experiments 2 to 5. During the first minute during which the oocyst suspensions were exposed to ozone, the ozone concentration dropped by 12 to 26%, depending on the oocyst suspensions. During the next 5 to 7 min, another drop of 8 to 33% was observed. As for chlorine dioxide, a 43% drop was established during the first minute of contact. The different initial and endpoint concentrations of ozone and chlorine dioxide are listed in Table 2, as are the total organic C values of the concentrated oocyst suspensions before treatment.

Both ozone and chlorine dioxide reduced the infection ratio of *C. parvum* oocysts considerably ($P < 0.05$). The effects of different exposure periods and treatments on the infectivity of *C. parvum* oocysts are shown in Table 1. The data of experiment 2 show that the effect depended on the number of oocysts present. Addition of 2.25 mg of O_3 per liter to water containing 10^4 oocysts per ml resulted in infection of only 1 of 9 mice after 2 min of contact, whereas similar treatment of water containing 5×10^5 oocysts per ml was not able to prevent infection (experiments 2 and 3). After longer contact periods, only ozone was capable of rendering contaminated water noninfectious to mice. A concentration of 1.1 mg/liter for 6 min was sufficient to disinfect water containing 10^4 oocysts per ml (experiment 4), and 2.25 mg/liter for 8 min served to disinfect water containing 5×10^5 oocysts per ml (experiments 2 and 3). Chlorine dioxide reduced the infectivity of contaminated water considerably, but some oocysts remained viable after 30 min of contact (experiment 5).

DISCUSSION

Cryptosporidiosis affects a wide range of mammals, birds, fish, and reptiles. In contrast to other coccidia, *C. parvum* lacks host specificity and animal strains may infect humans as well. Infected animals excrete large numbers of oocysts, which soil drinking water resources. In the United States, about 15,000 human cases have been attributed to consumption of contaminated drinking water (13). The presence of oocysts was also shown in a drinking water reservoir which caused an outbreak in 104 human patients in England (24).

Important to the understanding of why a small number of oocysts can cause severe infections in humans are some particular aspects of the life cycle of cryptosporidia which

TABLE 1. Oocyst production in neonatal mice inoculated with different numbers of *C. parvum* oocysts exposed or not exposed to ozone or chlorine dioxide

Expt no.	No. of oocysts/ml	Treatment and (dosage [mg/liter] at time zero)	Exposure time (min)	No. of positive mice/total[a]	Mean ± SEM $\log_{10}$ oocyst production[a]	% Reduction of oocyst production
2	1×10^4	Ozone (2.25)	0	6/7, a	5.23 ± 1.35, a	0
			2	1/9, b	<4, b	>98
			8	0/8, b	0, b	100
	1×10^5	Ozone (2.25)	0	4/5, a	5.20 ± 1.50, a	0
			2	3/5, ac	4.11 ± 1.25, b	81
			8	1/7, bc	<4, b	>99
	5×10^5	Ozone (2.25)	0	5/5, a	6.52 ± 0.10, a	0
			2	4/4, a	4.67 ± 0.85, b	93
			8	0/7, b	0, c	100
3	5×10^5	None		8/8, a	6.82 ± 0.16, a	0
		Ozone (0.59)	2	7/8, a	5.10 ± 1.44, b	75
			5	3/8, b	<4, c	>99
			8	6/10, b	4.42 ± 1.31, c	96
		Ozone (1.06)	2	13/13, a	6.20 ± 0.35, b	72
			5	3/4, a	4.48 ± 1.02, c	99
			8	5/9, b	4.17 ± 1.27, c	95
		Ozone (2.27)	2	7/7, a	5.40 ± 1.06, b	86
			5	2/10, bc	<4, c	99
			8	0/4, c	0, c	100
4	1×10^4	None		7/7, a	5.82 ± 0.67, a	0
		Ozone (1.11)	2	4/9, b	<4, bc	92
			4	2/6, bd	<4, bc	99
			6	0/7, cd	0, b	100
		Chlorine dioxide (0.31)	8	5/9, b	4.11 ± 1.13, c	88
			16	6/11, b	<4, c	97
5	1×10^4	Chlorine dioxide (0.43)	0	13/13, a	6.35 ± 0.58, a	0
			5	13/13, a	5.58 ± 1.04, a	48.6
			15	4/11,b	<4,b	93.5
			30	4/13,b	<4,b	94.3

[a] Means or numbers followed by different lowercase letters are significantly different ($P < 0.05$).

differs from that of most other mammalian and avian coccidia by two important features (3). (i) *Cryptosporidium* oocysts sporulate within the host and thus are infective when passed into the feces. Besides, about 20% of cryptosporidial oocysts are thin walled, which allows sporozoites to be released endogenously and to reinitiate a new development cycle. (ii) First-generation meronts do not always immediately generate second-generation meronts but may recycle and cause autoinfection. Both of these features explain why a small number of oocysts can cause severe infection and why it is necessary to clear drinking water of viable *Cryptosporidium* oocysts. Moreover, no effective treatment for cryptosporidiosis exists (7) and until now, few commercial disinfectants, such as 10% formol or 5% ammonia, were able to inactivate oocysts of *Cryptosporidium* spp. (1, 2, 21). Normal chlorination levels in drinking water have been shown to be ineffective, even after 18 h of contact. Only sand filtration may reduce oocyst concentrations, but it does not eliminate them completely (15).

The results of the above-described experiments indicate that both ozone and chlorine dioxide constitute a means of disinfecting drinking water from *Cryptosporidium* oocysts. During our trials, a minimum infection level of 10^4 oocysts per ml was used. This was necessary because the preliminary data from trial 1 indicated that a minimum of 10^3 oocysts (0.1-ml inoculum) was required to establish infection in all of the mice inoculated. This is similar to the findings of Ernest et al. (6), who also found a 100% infectious dose of 500 to 1,000 oocysts. This level is a factor of 1,000 higher than the levels detected in surface waters (15).

Several water-supplying companies use ozone and chlorine dioxide to disinfect water. They maintain a minimal residual ozone concentration of 0.4 mg of O_3 per liter for 6 min by injecting 1.5 to 4 mg of O_3 per liter of water. This is similar to our experimental conditions and is sufficient to disinfect water containing less than 10^4 oocysts per ml. The operating costs range from 20 to 25 Wh/g of O_3. Chlorine dioxide is applied for postdisinfection of drinking water and

TABLE 2. Total organic carbon contents of oocyst suspensions and initial and end concentrations of disinfectants used

Expt no.	Total organic C of oocyst suspension (mg/liter)[a]	Treatment	Maximal exposure time (min)	Disinfectant concn (mg/liter)	
				Start	End
2	3.5	Ozone	8	2.25	1.80
3	14.5	Ozone	8	0.59	0.24
			8	1.06	0.51
			8	2.27	1.49
4	1.5	Ozone	6	1.11	0.77
		Chlorine dioxide	16	0.31	0.07
5	2.0	Chlorine dioxide	30	0.43	0.22

[a] Before dilution of the suspensions 1 to 10 in the respective disinfectants.

maintenance of an active residual oxidant in the water distribution system (18b). The operating costs of ClO_2 generation range from 0.3 to 0.6 U.S. cents per gram. In Belgium, the residual concentration of ClO_2 allowed is 0.25 mg/liter, and in the Federal Republic of Germany it is 0.20 mg/liter while the United States recommends a limit of 1 mg/liter for chlorine dioxide, chlorite, and chlorate together. Since treatment of drinking water with chlorine dioxide permits an active residual concentration for several hours, it seems reasonable to assume that the product might kill all oocysts of *C. parvum* present in slightly contaminated water. This should be confirmed by further experiments.

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