Differential Effect of Phenethyl Alcohol on Mycoplasmas and Enveloped Viruses

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Mycoplasmas are totally inactivated by a 20-min treatment with 1% phenethyl alcohol, whereas suspensions of enveloped viruses resist the same treatment. This treatment has proved successful in eliminating mycoplasma contamination of a virus suspension.

Contamination of virus suspensions with mycoplasmas is a recurring problem in virology laboratories. In the case of nonenveloped viruses, selective destruction of mycoplasma contamination is readily accomplished by extraction with lipid solvents such as chloroform or ether. Such solvents, however, totally inactivate enveloped viruses. Nonionic detergents have been employed with limited success in the elimination of mycoplasmas from virus stocks (7). To our knowledge, however, there have been no prior reports of a quick effective method for the elimination of mycoplasma contamination from preparations of enveloped viruses. We present here studies showing that treatment of virus suspensions with 1% phenethyl alcohol (PEA) can perform this function.

PEA has been shown to have a reversible effect on a number of membrane-associated events in bacteria, including sporulation and DNA synthesis (1, 5). A recent report demonstrated that low concentrations of PEA are capable of lysing enterococcal L-forms (3); electron microscopy revealed that treatment with 0.1 to 0.6% PEA produces lysis without the membrane disaggregation commonly observed with detergent treatment. This report prompted us to investigate whether PEA could selectively inactivate mycoplasmas while preserving the functional integrity of enveloped viruses.

We initially compared the PEA sensitivity of an enveloped C-type virus, the AKR-L1 strain of murine leukemia virus (AKR-MLV) and two mycoplasma species (*Acholeplasma laidlawii* and *Mycoplasma pneumoniae*). Stock solutions of 0.2, 1.0, and 2.0% PEA (Eastman Kodak Co., Rochester, N.Y.) in phosphate-buffered saline (PBS), pH 7.4, were prepared; at the higher concentrations the PEA did not fully dissolve, and the suspensions were shaken vigorously before mixing with the virus. The clarified virus

suspension and broth cultures of the mycoplasmas were diluted in an equal volume of the stock PEA solutions or PBS. After 20 min at room temperature, with occasional agitation, the specimens were serially diluted and assayed. The virus was assayed on NIH mouse embryo cultures (6), and the mycoplasmas by colony formation on agar. Final concentrations of PEA greater than 0.1 to 0.25% were toxic to tissue culture cells. As shown in Fig. 1, exposure to 1% PEA reduced the titer of the AKR-MLV by only 2 log₁₀, but completely inactivated the mycoplasmas. *M. pneumoniae* was more resistant than *A. laidlawii* to the lower concentrations of PEA, which may correlate with its known resistance to osmotic lysis (R. M. Chanoock; personal communication).

The effect of 1% PEA on a number of viruses and mycoplasma species was then examined. Mycoplasma species grown in broth and capable of colony formation on agar under aerobic conditions were employed. Treatment conditions were as described above. The results are shown in Table 1. Human cytomegalovirus, respiratory syncytial virus, parainfluenza 3, and lymphocytic choriomeningitis virus proved highly resistant to PEA while the mycoplasmas were completely inactivated.

As a practical test of the usefulness of PEA for eliminating mycoplasma contamination, we treated Ad 169 human cytomegalovirus which had been passaged 330 times through WI38 cells in this laboratory and was known to have a long standing contamination with an untyped mycoplasma species capable of aerobic growth on agar. The contaminated virus suspension was treated with 1% PEA for 20 min, inoculated into mycoplasma-free WI38 cultures, and carried for three passages in the absence of antibiotics. None of the cultures showed the graininess characteristic of the mycoplasma-contaminated

FIG. 1. Inactivation of two mycoplasma species, *A. laidlawii* and *M. pneumoniae*, and a C-type RNA virus, AKR-MLV, by PEA. Initial titer and assay conditions are given in Table 1 and the text.

cultures, and repeated attempts to culture mycoplasma, both aerobically and anaerobically, were negative.

These results indicate that 1% PEA may prove generally effective in eliminating mycoplasma contaminants of enveloped viruses. We should caution, however, that we have not investigated the ability of PEA to eliminate anaerobic mycoplasmas or mycoplasmas unable to grow on agar. The marked resistance of many of the tested viruses to PEA suggests that they may withstand more extended or repeated PEA treatments if necessary. This technique may also prove useful in differentiating viruses from mycoplasmas by a simple inactivation assay.

The biochemical basis for the differential inactivation of mycoplasmas and enveloped viruses by PEA is unknown. PEA has been shown to lyse L-forms without producing membrane disaggregation detectable by electron microscopy (3). It is possible that the virion envelope is similarly affected by PEA, but its structure is preserved to the extent that absorption and penetration can occur; also increased

TABLE 1. Sensitivity of viruses and mycoplasmas to 1% PEA^a

Agent	Titer (log ₁₀)		
	Un-treated	PEA-treated (1%, 20 min)	Reduction (log ₁₀)
Virus			
Murine leukemia virus ^b	6.3	4.3	2.0
Human cytomegalovirus	4.7	≥4.0	≤0.7
Respiratory syncytial virus	6.5	6.6	0
Parainfluenza 3 virus	5.6	5.5	0.1
Lymphocytic choriomeningitis virus	3.0	2.0	1.0
Mycoplasma			
<i>A. laidlawii</i> ^b	7.0	— ^c	>7.0
<i>M. pneumoniae</i> ^b	6.4	—	>6.4
<i>M. orale</i>	6.1	—	>6.1
<i>M. arginini</i>	6.2	—	>6.2

^a With the exception of LCM virus, all virus stocks were grown in tissue culture. AKR-MLV was titrated by adding 0.2 ml of virus dilutions to secondary NIH mouse embryo cell cultures as previously described (6). Human cytomegalovirus, strain Ad 169, was titrated by adding 0.2 ml to tubes of WI38 cells (HEM Research Inc., Rockville, Md.). The cells were maintained for 2 weeks in McCoy 5A medium plus 2% fetal calf serum and standard concentrations of penicillin and streptomycin (GIBCO, Grand Island, N.Y.). They were observed twice weekly for the development of viral CPE. Respiratory syncytial virus was human strain A2 and was titrated by adding 0.1 ml to HEp-2 cells in micro-wells (Flow Labs., Rockville, Md.). After a 1-h absorption period, the cells were overlaid with medium containing methyl cellulose, and plaques were counted after 3 days as previously described (2). Parainfluenza type 3, Mills strain, was assayed in the same manner as respiratory syncytial virus. All tissue cultures were maintained at 37°C in a 5% CO₂ atmosphere. Titers of AKR-MLV, respiratory syncytial virus and parainfluenza 3 are expressed as plaque forming units per ml. The titer of Ad 169 is expressed as the TCID₅₀ per 0.2 ml. LCM virus was strain CA 1371 as 10% mouse brain extract. Residual virus was titrated by inoculating weanling NIH Swiss mice intracerebrally with 0.03 ml., using five mice per dilution. Mice injected with 0.01% PEA alone, the concentration in the lowest virus dilution tested, showed no signs of toxicity. Titers are expressed as LD₅₀ per 0.03 ml. Mycoplasma species were produced in broth culture and grown on PPLO agar (Microbiological Associates, Bethesda, Md.) for colony counts. Plates were incubated at 37°C and observed for 7 days, except for *M. pneumoniae*, which was observed for 14 days. Colonies were counted under a dissecting microscope. Titers are expressed as colonies per 0.1 ml.

^b Data from experiments shown in Fig. 1.

^c —, Negative.

permeability of a viral envelope might not result in loss of critical virion components. Alternatively, the membrane constituting the viral envelope may be resistant to PEA. The viral envelope membrane possesses unique structural features (4) that may stabilize it and render it more resistant than cellular or mycoplasma membranes to various membrane-active agents.

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