Elimination of Mycoplasma Contaminants from Virus Stocks by Treatment with Nonionic Detergents

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Five nonionic detergents (Tweens 20, 40, 60, and 80, and Triton WR-1339) were tested for their ability to inactivate four Mycoplasma species which are common contaminants of animal cell cultures. Tween 20 was found to be the most effective, in that a concentration of 2.5 mg/ml completely inactivated cultures of M. hominis, M. hyorhinis, and Acholeplasma laidlawii within 1 hr and a culture of M. orale within 3 hr. The other detergents exhibited various degrees of activity against the different mycoplasmas, with Triton WR-1339 being the least effective. The virucidal activity of the detergents was determined for six viruses. All four Tween compounds were highly virucidal for herpes simplex virus. Tween 20 also exhibited virucidal effects against vesicular stomatitis virus, California encephalitis virus, and Newcastle disease virus, and Tween 80 was found to be active against California encephalitis and Newcastle disease viruses. Detergent treatment procedures were effective in two instances in eliminating mycoplasma contaminants from virus preparations while the preparations retained most of the viral infectivity. The limitations of this technique for routine use are discussed.

MATERIALS AND METHODS

Mycoplasmas. M. hominis (PG-21), M. orale (CH-19), and the GDL strain of M. hyorhinis were obtained from the National Institutes of Health Reference Laboratory, Rockville, Md. Acholeplasma laidlawii (PG-8) was kindly supplied by R. A. Del-Guidice, Baltimore Biological Laboratories, Cockeysville, Md. All strains of Mycoplasma were propagated in broth media composed of eight parts mycoplasma broth base, one part unheated agamma horse serum from Microbiological Associates, Inc. (MBA), Bethesda, Md., and one part yeast extract (MBA). The agar medium employed for plate cultures consisted of nine parts Columbia agar base (BBL) and one part unheated agamma horse serum (MBA).

Viability counts were done by plating 0.1 ml of each test sample onto agar media. Each sample was tested in duplicate and incubated at 37 C for 2 to 7 days in a humidified atmosphere. M. hominis, M. hyorhinis, and A. laidlawii were cultured and incubated under aerobic conditions; M. orale was grown in an atmosphere of 5% CO2 and 95% N2.

Cell cultures. The baby hamster kidney (BHK-21) cell line was obtained from MBA, and the primary human embryonic lung cells were obtained from Flow Laboratories, Rockville, Md. Both cell types were propagated with the minimal essential medium of Eagle with Earle balanced salt solution (EMEM) supplemented with 10% fetal bovine serum. The maintenance medium employed was EMEM plus 2% fetal bovine serum. To test for cytoxicity, monolayer cultures were exposed to various concentrations of the detergents contained in maintenance medium. Five tubes were used per detergent concentration, and the tubes were read daily for cytopathology for 5 days.

Detergents. Tweens 20, 40, 60, and 80 were ob-
tained from Atlas Chemical Industries, Inc., Wilmington, Del., and Triton WR-1339 was obtained from the Ruger Chemical Co., Irvington, N.Y. Stock solutions of each detergent were prepared in distilled water and sterilized by filtration.

**Effect of detergents on mycoplasma.** Log-phase cultures of the Mycoplasma species were diluted 1:10 with maintenance medium. To 1 ml of the mycoplasma suspension was added an equal volume of detergent to give a final concentration of 2,500 μg/ml. The mixtures were incubated in a water bath at 37°C for 1 hr, after which they were serially diluted in maintenance medium and plated for viability. At the time of plating, 0.1-ml portions of each mycoplasma-detergent combination were inoculated into tubes of broth media. These cultures were incubated for 5 days, at which time samples were removed and plated onto agar. Controls consisted of the mycoplasma suspensions handled in an identical manner but with detergent omitted.

**Viruses.** Vaccinia virus, vesicular stomatitis virus (VSV), Newcastle disease virus (NDV), California encephalitis virus (CEV), poliomyelitis virus type 1, and herpes simplex virus were all obtained from stocks maintained in this laboratory. The host cell system used for vaccinia virus, VSV, and CEV was the BHK-21 cell line; human embryonic lung cell cultures were used for propagating poliovirus and herpes simplex virus. NDV stocks were prepared and titrated in the allantoic cavities of 9-day-old embryonated chicken eggs obtained from Duckworth Hatchery, Hanover, Md. Titers of all virus pools were determined by assaying 10-fold virus dilutions in their respective host systems, with the use of a total of five hosts (eggs or cell cultures) per dilution of virus.

**Testing of detergents for virucidal activity.** For determining the virucidal activity of the detergents, the same treatment procedure as described for the mycoplasma studies was employed. After the 1-hr incubation at 37°C, a sample was removed and titrated in its appropriate host system. Controls consisted of the following: (i) titration of the stock virus before and after the 1-hr incubation; (ii) cell cultures inoculated with the various detergent dilutions alone; and (iii) cell cultures receiving maintenance medium only.

**Effect of detergents on mycoplasma and virus combinations.** To test the applicability of the treatment procedures, it was decided to use virus stocks already known to be contaminated with mycoplasmas, even though the mycoplasma involved was known in only one instance. The procedures used were the same as described for the testing of mycoplasma-detergent or virus-detergent mixtures.

**RESULTS**

**Effect of detergents on mycoplasma.** The GDL strain of *M. hyorhinis* was completely inactivated by treatment for 1 hr at 37°C with Tween 20, 40, 60, and 80 at a concentration of 2,500 μg/ml (Table 1). Triton was found to be the least effective in reducing the titer of this organism at this concentration; however, it did reduce the viability count approximately 400-fold when compared to the control. This is in agreement with the results of Reynolds and Hetrick (6), who demonstrated that Tween 20, 40, 60, and 80, and Triton all killed mycoplasmas, with the Tweens being more active than Triton on the GDL strain of *M. hyorhinis*.

It was of interest to determine the minimal time required for complete inactivation of the organism. As shown in Table 2, Tween 60 and 80 were effective within 15 min; although Tween 20 and 40 had reduced the viable count approximately one million-fold in 15 min, viable organisms were still detected. However, after a 30-min treatment, all four Tween compounds had completely inactivated the organism.

| Table 1. Effect of treatment with nonionic detergents on the viability of several Mycoplasma species |
|---|---|---|---|---|
| Detergent* | *M. hyorhinis* C | *M. hominis* C | *M. orale* C | *A. laidawai* C |
| None | 6.3 | 6.5 | 5.8 | 7.9 |
| Tween 20 | 0* | 0 | 2.5 (0)* | 4.1 (0.7) |
| Tween 40 | 0 | 5.6 | 5.8 | 7.3 |
| Tween 60 | 0 | 5.5 | 5.3 | 7.0 |
| Tween 80 | 0 | 2.7 | 1.2 (0) | 4.7 (1.2) |
| Triton WR-1339 | 3.7 | 5.8 | 5.7 | 7.8 |

* Final concentration of each detergent was 2,500 μg/ml.
* Titers are expressed as log₁₀ of the number of colony-forming units per milliliter after a 1-hr exposure at 37°C.
* No colonies were observed on plates inoculated with the undiluted test sample.
* Numbers in parentheses indicate the results after a 3-hr treatment at the same concentration.

| Table 2. Rate of inactivation of the GDL strain of *M. hyorhinis* by various nonionic detergents |
|---|---|---|
| Detergent* | Time of exposure (min) |
| | 1 | 15 | 30 |
| Tween 20 | 6.3* | 0.8 | 0* |
| Tween 40 | 6.5 | 0.5 | 0 |
| Tween 60 | 6.3 | 0 | 0 |
| Tween 80 | 6.0 | 0 | 0 |

* Final concentration of each detergent was 2,500 μg/ml.
* Log₁₀ of colony-forming units per milliliter. In a control with no detergent, tested at 0 min, the value was 6.3.
* No colonies were observed on plates inoculated with the undiluted detergent-mycoplasma samples. Results at 45 and 60 min were the same as those at 30 min.
To determine whether the effect of the various detergents was lethal or just inhibitory, samples of detergent-treated mycoplasma were inoculated into tubes of broth which diluted the detergent concentration to less than 5 μg/ml. The tubes were incubated for 5 to 6 days at 37°C and then plated for viable organisms. If the detergent effect was merely inhibitory, colonies should have appeared when the cultures were plated; however, since the results of these tests were all negative, it appears that the detergent treatment was lethal.

*M. hominis* (PG-21) was more resistant to the lytic action of the various detergents than *M. hyorhinis*; Tween 20 was the only detergent which completely inactivated *M. hominis* within 1 hr (Table 1). Tween 80 also exhibited a marked lytic effect, as it reduced the titer of *M. hominis* approximately 10,000-fold, but viable organisms remained. Tweens 40 and 60, together with Triton, were the least effective; in all cases, the reduction of titer was only 10-fold or less.

For *M. orale* (CH-19), Tweens 20 and 80 were again the most effective, in that these two detergents reduced the viable count by approximately 95%, whereas Tweens 40 and 60, together with Triton, had little or no effect on this organism. For Tweens 20 and 80, it was of interest to determine the effect on *M. orale* of an exposure time of 3 hr to the same concentration of detergent. The results shown in Table 1 indicate that both Tween 20 and Tween 80 were effective in eliminating this organism after a 3-hr treatment, as no viable cells were detected in the undiluted samples. *A. laidlawii* proved to be the most resistant organism tested. Tweens 20 and 80 significantly reduced the titer of the organism within 1 hr, but even after a 3-hr treatment viable organisms remained. Tweens 40 and 60 and Triton were again almost totally ineffective.

**Cytotoxicity results.** Human embryonic lung cells, BHK-21 cells, and 9-day-old embryonated eggs were all killed by each of the detergents at a concentration of 2,500 μg/ml. None of the detergents was toxic at a concentration of 250 μg/ml or lower for any of the host systems, except that Triton produced cytopathic changes in human embryonic lung cells after a 48-hr incubation. Since a 1:10 dilution of the detergent concentration employed for treatment in these studies would represent 250 μg/ml, the toxicity of the detergents for the various host systems should pose no problem in their use for treating virus stocks because they could readily be diluted to a non-toxic level before the virus titer would be lost.

**Virucidal studies.** The results of the virucidal studies are shown in Table 3. Triton was the least virucidal of the detergents tested, as it did not significantly reduce the titers of vaccinia virus, VSV, herpes simplex virus, or NDV; however, it did reduce the titer of CEV by 1.5 logs. Tween 20 was the most virucidal compound tested, as it completely inactivated both herpes simplex and CEV, and it reduced the titers of VSV and NDV by 3.5 and 1 log, respectively. It had no virucidal effect on vaccinia virus or poliovirus. Tween 80 was virucidal for CEV and herpes simplex virus, but had little or no effect upon the other viruses. Tweens 40 and 60 caused no reduction in infectivity of vaccinia virus, VSV, NDV, and poliovirus, but they did reduce the titer of CEV and herpes simplex virus significantly.

**Effects of detergent treatment on mycoplasma-contaminated virus stocks.** Stocks of VSV, vaccinia virus, and poliovirus were determined by preliminary testing to be contaminated with mycoplasma. Table 4 shows the effects of detergent treatment on both virus and mycoplasma titers. Tween 20 was effective in eliminating the contaminants from the polio and vaccinia stocks without appreciably diminishing the virus titer.

The fact that Tween 20 completely eliminated *A. laidlawii* from the virus stock is probably due to the fewer number of organisms present. In the previous study, in which Tween 20 did not completely inactivate *A. laidlawii* even after a 3-hr treatment, the number of organisms present was much greater (approximately 25,000 times as many). Although it markedly reduced the titer of the contaminant in the VSV stock, Tween 20 also reduced the virus titer 10,000-fold.

Tween 80 significantly reduced the titers of the contaminants in all three virus stocks, but in no case did the 1-hr treatment eliminate

| Detergent    | Vaccinia | VSV | Herpes Simplex | CEV | NDV | CEV Type 1 |
|--------------|----------|-----|----------------|-----|-----|------------|
| Control      | 4.5      | 8.0 | 5.8            | 5.8 | 6.5 | 7.0        |
| 37°C control | 4.5      | 8.0 | 5.8            | 5.5 | 6.5 | 6.8        |
| Tween 20     | 4.5      | 4.8 | <0.5           | <0.5| 5.0 | 6.7        |
| Tween 40     | 4.3      | 8.0 | <0.5           | 4.0 | 5.0 | 6.7        |
| Tween 60     | 4.2      | 8.0 | <0.5           | 4.5 | 5.5 | 6.8        |
| Tween 80     | 4.3      | 7.7 | <0.5           | <0.5| 5.0 | 6.9        |
| Triton WR-1339 | 4.5   | 8.0 | 5.8            | 4.0 | 6.0 | 7.0        |

*Log_{10} of number of TCID_{50} per milliliter (egg LD_{50} in the case of NDV).
TABLE 4. Effect of nonionic detergents on mycoplasma contaminants in virus preparations

| Detergenta | Poliomyelitis stock | Vaccinal virus stock | VSV stock |
|------------|---------------------|----------------------|----------|
|            | Mycoplasma titer    | Virus titer          | Mycoplasma titer | Virus titer |
| None       | 3.5<sup>b</sup>     | 6.0                  | 4.6       | 5.6       | 4.3 | 8.2 |
| Tween 20   | 0<sup>a</sup>       | 5.8                  | 0         | 5.4       | 0.8 | 4.2 |
| Tween 40   | 3.2                 | 6.0                  | 4.3       | 5.6       | 4.1 | 8.0 |
| Tween 60   | 3.0                 | 6.0                  | 3.8       | 5.3       | 3.8 | 7.8 |
| Tween 80   | 0.8                 | 5.8                  | 2.1       | 5.5       | 2.2 | 7.5 |

<sup>a</sup> Final concentration of each detergent was 2,500 μg/ml; treatment was for 1 hr at 37°C.

<sup>b</sup> This contaminant was identified as *A. laidlawii* by Joseph Tully, National Institute of Allergy and Infectious Diseases, Bethesda, Md.

<sup>c</sup> Mycoplasma species not identified.

<sup>d</sup> Log<sub>10</sub> of number of colony-forming units per milliliter.

<sup>e</sup> No colonies observed on plates inoculated with the undiluted sample.

them completely. In contrast to Tween 20, the virus titers were relatively unaffected. Treatment with Tweens 40 and 60 had little or no effect on the mycoplasma contaminants.

**DISCUSSION**

This study was initiated because of the lack of virucidal activity previously reported for Triton WR-1339 (4, 5, 7). However, the results reported here indicate that treatment of virus stocks with nonionic detergents for the purpose of eliminating mycoplasma contaminants has definite limitations. Although the ability of nonionic detergents to lyse mycoplasma in vitro was definitely established, the virucidal activity of these same compounds minimizes their usefulness in this regard. It is true that most of the viruses tested contained essential lipids; indeed, they were selected for this reason, as treatment with ether and other organic solvents is quite effective in “cleaning up” preparations of the non-lipid-containing viruses.

Triton WR-1339 had little or no virucidal activity against the agents tested; however, this detergent proved to be the least effective of those tested for activity against mycoplasma. Conversely, Tween 20 was the most effective detergent insofar as lysing mycoplasma was concerned, but it also showed marked virucidal activity for four of the six viruses tested.

Although some successes were obtained with this detergent-treatment method, several factors would have to be considered for each case in which this approach was contemplated. These include (i) the virucidal activity of the selected detergent(s), (ii) the number of mycoplasmas present in the sample, which would influence the length of treatment required, (iii) the drop in virus titer that is tolerable, assuming the detergent was not totally virucidal under the treatment conditions employed, and (iv) the nature of the contaminant itself, because mycoplasmas vary in their sensitivity to different detergents. In cases where other methods prove to be unsatisfactory for eliminating mycoplasmas, a successful detergent-treatment procedure could probably be developed. However, because of the variables described, it was not possible to define a single detergent-treatment procedure that could be routinely used to rid virus stocks of mycoplasma.

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