Evaluation of Gentamicin for Use in Virology and Tissue Culture

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Data are presented comparing gentamicin to penicillin and streptomycin (Pen-Strep) in tissue culture medium with respect to a number of parameters associated with virology and tissue culture. Unlike Pen-Strep, gentamicin was stable at pH 2 to 10 for 15 days at 37 °C in tissue culture medium, and its activity was unaffected by the presence of serum. Moreover, it was stable to autoclaving. Twenty cell types replicated normally at the suggested concentration of 50 μg/ml, and all cells were unaffected by 20 times this concentration. Evidence for its practical use in virus studies was demonstrated in that (i) it was not viricidal to ribonucleic acid or deoxyribonucleic acid viruses at 40 times the suggested concentration at 37 °C, (ii) the size and number of plaques were not affected by 20 times the suggested concentration, (iii) interferon assays and production were unaffected by 20 times the suggested concentrations. Gentamicin may be uniquely useful for shipment of clinical specimens and long-term tissue culture and virus studies.

Penicillin and streptomycin (Pen-Strep) have been used in cell culture for years in spite of several undesirable characteristics of the mixture. Streptomycin is rapidly destroyed at alkaline pH, and penicillin is extremely unstable to both acid and alkaline pH. Neither antibiotic can withstand autoclaving, and the activity of penicillin is reduced in the presence of serum. Neither has as wide a spectrum of biological activity as gentamicin.

Occasionally antibiotics which exhibit low cytotoxicity are introduced for use in cell culture studies (e.g., tylosin, bacitracin, kanamycin). However, little effort has been directed towards determining the effects of these antibiotics on several important parameters often measured by the virologist. These parameters include the influence of the antibiotic on cell multiplication, virus replication, virus yield, virus infectivity, interferon production, interferon assays, and numerous other indexes of biological activity.

Gentamicin has biological and biochemical properties which may render it superior to penicillin alone, streptomycin alone, or the two in combination for use in tissue culture. It is active against strains of Proteus and Staphylococcus as well as numerous other gram-positive and gram-negative organisms (1). Moreover, unlike Pen-Strep, gentamicin is active against the ubiquitous Pseudomonas strains (1, 13). When used in tissue culture at the suggested concentration of 50 μg/ml (base equivalent), it is bactericidal for a wide variety of bacteria. Although it is not active against yeasts, fungi, or protozoa, it has been reported to be active against several strains of Mycoplasma (2, 9, 10). These characteristics make gentamicin a candidate for use in cell and tissue culture and also for transport of cell cultures, certain clinical specimens containing viruses, or specimens in which overgrowth by bacteria should be avoided.

The purpose of this study was to evaluate and compare gentamicin in cell cultures with Pen-Strep by studying the influence of these antibiotics on several parameters of common interest to those who employ tissue culture techniques. Data are also presented on the effects of gentamicin on cell morphology and multiplication, virus stability and replication, and the synthesis and assay of interferon. An investigation of the antibacterial activity of gentamicin was not conducted as such studies have been already reported (4, 6, 11).

MATERIALS AND METHODS

Gentamicin solution. Gentamicin solution was reconstituted in distilled water equivalent to 10,000 μg of gentamicin base per ml, passed through a 0.22-
Effect of gentamicin on interferon synthesis. Monolayers of L-929 cells were inoculated with egg-propagated NDV at a multiplicity of 10 plaque-forming units (PFU)/cell and adsorbed for 1 hr, and the residual inoculum was aspirated. Petri plates were incubated at 37 °C with 3.5 ml of MEM containing 0.5% bovine serum albumin and various concentrations of gentamicin or Pen-Strep. After 24 hr, the fluid was harvested, dialyzed against 20 volumes of HCl-KCl buffer at pH 2 for 5 days at 4 °C followed by dialysis against PBS at pH 7 for 3 days at 4 °C, passed through a 0.45-μm filter, and stored at −60 °C.

Effect of gentamicin on interferon assays. Interferon was assayed by the plaque inhibition test described by Wagner (12) employing L-929 cells and VSV. Interferon titers were expressed as the reciprocals of the dilution of 1.0 ml of fluid which inhibited 50% of VSV plaques. Assays were conducted in the presence and absence of gentamicin or Pen-Strep.

Stability of antibiotics at various pH levels. Either gentamicin (50 μg/ml final concentration) or Pen-Strep (100 units and 100 μg/ml final concentration, respectively) was added to a commonly used tissue culture fluid, Medium 199 containing 3% fetal calf serum. The pH was adjusted with 5 N NaOH or 6 N HCl and thoroughly mixed, and 1.5 ml of the complete medium was dispensed into glass ampoules, immediately flame-sealed, and placed at 37 °C. At various time intervals, ampoules were frozen at −60 °C pending assay for antibiotic activity.

Antibiotic assays. Gentamicin was quantitated by the method of Oden et al. (8). Penicillin and streptomycin were assayed by the methods described by Grove and Randall (5).

RESULTS

pH stability of several antibiotics in cell culture medium. The stability of gentamicin at various pH levels was determined and compared with pH stability of Pen-Strep under identical conditions in Medium 199 containing 3% fetal calf serum. Medium was adjusted to the indicated pH (Fig. 1), and antibiotic was added and dispensed as described (see above). Samples were analyzed for biological activity as described above.

Gentamicin was found to be completely stable at all pH levels. Streptomycin showed progressive loss of activity at pH 6.5, 7.0, 7.5, 8.0, and 10.0, and penicillin lost activity more rapidly than did streptomycin at all pH levels. Furthermore, it was observed that penicillin immediately lost at least 30% of its activity upon addition to the medium containing serum. The decrease of penicillin activity may be attributed to the complexing of the antibiotic with serum proteins.

Antibiotic stabilities to autoclaving. Since tissue culture medium which can be autoclaved is now available, it would be advanta-
To determine the influence of autoclaving on antibiotics, the following experiment was performed. Aqueous solutions of penicillin (100 units/ml), streptomycin (100 μg/ml), or gentamicin (50 μg/ml) were prepared. Half of each sample was stored at 4°C for control purposes, whereas the other half of each sample was autoclaved for 15 min at 121°C and 15 lb of pressure. Under these conditions, penicillin was almost completely inactivated (99%) and streptomycin activity was reduced by 50%. In contrast, gentamicin was completely stable.

**Cell tolerance to gentamicin.** Twenty cell types of various origins were passaged continuously at least 20 times in the presence of 50 μg of gentamicin per ml (Table 1). Usually, at each passage level, cells were fed with fresh medium two or three times at intervals of 1 to 4 days. No microscopically detectable changes in cell morphology nor changes in growth characteristics were observed.

In another series of studies, seven commonly used cells were selected to determine the maximum tolerated dose of gentamicin. Monolayers on 60-mm petri dishes were incubated with various concentrations of gentamicin or
with antibiotic-free medium and microscopically examined daily for morphological changes (Table 2). AC amnion cells incubated for 4 days with medium containing antibiotic showed no gross morphological changes. This concentration is 20 times the suggested dose of gentamicin for use in cell culture. As indicated in the last column, 6 mg of gentamicin per ml was required to produce gross morphological alterations of AC amnion cells by day 2.

Hence, the suggested concentration for tissue culture use was found to be well below the maximum tolerated dose for all seven cell types as evidenced by the lack or morphological alterations.

**Stability and replication of viruses in the presence of gentamicin.** Although gentamicin was well tolerated by cells, it was of interest to determine its effects on virus stability and replication. To this end, selected representatives of some of the major classes of RNA- and DNA-containing viruses were incubated with 2 mg of gentamicin per ml PBS, far in excess of the suggested concentration, or with PBS free from antibiotic at 37 and 4 C. Samples were removed at 1 and 4 hr after the addition of gentamicin, and virus infectivity was determined by plaque assay. The results shown in Table 3 indicate that the infectivity of all viruses tested was unaffected by 40 times the suggested concentration of gentamicin.

To determine the effect of the suggested concentration of gentamicin (50 µg/ml) upon virus replication, duplicate monolayers were infected with the viruses listed in Table 4 and incubated with medium containing Pen-Strep, gentamicin, or antibiotic-free medium. Cytopathology developed at the same rate in monolayers with antibiotic-free or antibiotic-containing medium. Supernatant fluids were harvested after most of the cells had been destroyed and virus yields were determined. It can be seen that gentamicin did not affect virus yields.

Other experiments demonstrated that 1 mg of gentamicin per ml incorporated into the agar overlay had no effect on the number or the size of VSV plaques in chick embryo fibroblasts. Size and number of plaques were markedly reduced only at extremely high concentrations (5 mg/ml).

### Table 1. Cell types continuously* passed in gentamicin

| Origin  | Cell line                                      |
|---------|------------------------------------------------|
| Human   | AC amnion                                      |
|         | Human fetal trachea                           |
|         | M-HeLa                                        |
|         | R-HeLa                                        |
|         | HEP-2                                         |
|         | U-amnion                                      |
|         | FS-1 amnion                                    |
| Hamster | BHK-9                                         |
|         | BHK-21/C13                                     |
|         | HKCC                                          |
|         | DON                                           |
|         | Ha K                                          |
| Mouse   | L-929                                         |
| Feline  | Feline lung                                    |
|         | CRFK Crandell feline kidney                   |
| Monkey  | MK 2                                          |
|         | Vero                                          |
| Fish    | Fat head minnow                               |
| Bovine  | MDBK                                          |
|         | EBTr                                          |

* Cells were passaged at least 20 times in gentamicin, 50 µg/ml.

### Table 2. Effect of gentamicin on cell cultures

| Cell culture                      | Nontoxic concn* (µg/ml) | Tolerated: suggested* dose | Toxic concn* (µg/ml) |
|----------------------------------|--------------------------|---------------------------|-----------------------|
| Human amnion (AC amnion)         | 1,000 (4)                | 20×                       | 6,000 (2)             |
| Chick fibroblast                 | 1,000 (5)                | 20×                       | 3,000 (3)             |
| Human foreskin (FS-1)            | 1,000 (6)                | 20×                       | 3,000 (2)             |
| Human carcinoma of cervix (HeLa)| 6,000 (3)                | 120×                      | 6,000 (2)             |
| Mouse fibroblast (L-929)         | 1,000 (3)                | 20×                       | 5,000 (2)             |
| Human amnion (U)                 | 6,000 (3)                | 120×                      |                       |
| Monkey kidney (Vero)             | 4,000 (4)                | 80×                       |                       |

* Number of days observed noted in parentheses.

* Tolerated dose was determined experimentally. The suggested dose was arbitrarily selected on the basis of in vitro antibacterial activity.

* Numbers in parentheses indicate day at which gross morphological changes were observed.
TABLE 3. Stability of RNA and DNA viruses to 2,000 μg of gentamicin per ml in phosphate-buffered saline (PBS)

| Virus                      | Plaque-forming units/ml |
|----------------------------|-------------------------|
|                            | 37 C/1 hr               | 4 C/1 hr               | 37 C/4 hr               | 4 C/4 hr               |
| RNA viruses                |                         |                        |                        |                        |
| Sindbis (HR)               |                         |                        |                        |                        |
| Gentamicin                 | $1 \times 10^3$         | $1.5 \times 10^3$      | $1.4 \times 10^4$      | $2.3 \times 10^4$      |
| PBS                        | $1.2 \times 10^3$       | $1.4 \times 10^4$      | $1.4 \times 10^4$      | $2.3 \times 10^4$      |
| Vesicular stomatitis virus |                         |                        |                        |                        |
| Gentamicin                 | $2.3 \times 10^4$       | $2.8 \times 10^4$      | $4.2 \times 10^4$      | $5.9 \times 10^4$      |
| PBS                        | $2.8 \times 10^4$       | $5.9 \times 10^4$      | $2.8 \times 10^4$      | $4.2 \times 10^4$      |
| Rhinovirus (HGP)           |                         |                        |                        |                        |
| Gentamicin                 | $6.2 \times 10^7$       | $6.4 \times 10^7$      | $6.7 \times 10^7$      | $1.1 \times 10^4$      |
| PBS                        | $9.5 \times 10^7$       | $9.6 \times 10^7$      | $6.7 \times 10^7$      | $1.1 \times 10^4$      |
| DNA viruses                |                         |                        |                        |                        |
| Herpes simplex             |                         |                        |                        |                        |
| Gentamicin                 | $1.8 \times 10^4$       | $1.3 \times 10^4$      | $1.1 \times 10^4$      | $1.8 \times 10^4$      |
| PBS                        | $1.1 \times 10^4$       | $1.7 \times 10^4$      | $1.1 \times 10^4$      | $1.8 \times 10^4$      |
| Vaccinia                   |                         |                        |                        |                        |
| Gentamicin                 | $2.8 \times 10^4$       | $2.4 \times 10^4$      | $2.3 \times 10^4$      | $2.6 \times 10^4$      |
| PBS                        | $2.7 \times 10^4$       | $2.8 \times 10^4$      | $2.3 \times 10^4$      | $2.6 \times 10^4$      |

TABLE 4. Effect of gentamicin on virus replication

| Virus                      | Virus yields (PFU/ml)* |
|----------------------------|------------------------|
|                            | No antibiotic | Pen (100 units/ml)-Strep (100 μg/ml) | Gentamicin (50 μg/ml) |
| RNA viruses                |              |                                       |                        |
| Rhinovirus (HGP)           | $1.6 \times 10^7$   | $1.7 \times 10^7$                     | $1.7 \times 10^7$      |
| Newcastle disease virus    | $2.0 \times 10^7$   | $2.1 \times 10^7$                     | $3.4 \times 10^7$      |
| Sindbis (HR)               | $2.9 \times 10^4$   | $4.4 \times 10^4$                     | $3.4 \times 10^4$      |
| Vesicular stomatitis virus | $7.3 \times 10^4$   | $4.9 \times 10^4$                     | $7.9 \times 10^4$      |
| DNA viruses                |              |                                       |                        |
| Herpes simplex             | $1.9 \times 10^7$   | $3.2 \times 10^7$                     | $1.4 \times 10^7$      |
| Vaccinia                   | $8.0 \times 10^5$   | $6.1 \times 10^6$                     | $7.6 \times 10^6$      |

* PFU, plaque-forming units.

Effect of gentamicin on interferon synthesis. L-29 cells were stimulated to synthesize interferon with live NDV as described (see above) in the presence and absence of gentamicin (1 mg/ml). Under these conditions, interferon yields were not affected.

Effect of gentamicin on interferon assays. The influence of gentamicin on the sensitivity of the interferon assay was studied by assaying mouse serum interferon in the presence of several concentrations of gentamicin. Serum containing interferon was diluted in medium with either 50, 100, 500, or 1,000 μg of gentamicin per ml and incubated with monolayers at 37 C. After 24 hr, the monolayers were washed three times and challenged with 50 to 100 PFU of VSVV and overlaid with agarose. Concentrations as great as 1,000 μg/ml had no effect on the assay results. Higher concentrations were not tested. Thus, the sensitivity of interferon assays was unaffected by 20 times the suggested concentration of gentamicin.

DISCUSSION

These and other studies indicate that gentamicin offers several advantages over Pen-Strep for use in cell culture. The data confirmed Casemore's observation (4) which showed that gentamicin was completely stable to autoclaving and thus could be added to media before sterilization. The microbiological activity of gentamicin was completely stable in cell culture medium over a wide range of pH and temperature. These characteristics are especially desirable since cells, viruses, and clinical
specimens may undergo wide fluctuations in pH and temperature while in transit or during long-term experiments. The stability of gentamicin at pH 2 is of added value, since interferon preparations are frequently subjected to pH 2 for 1 to 5 days to preferentially inactivate virus.

As much as 85% of some types of penicillin are bound by human serum (3). Similarly, 30% of the biological activity of penicillin G was lost immediately when it was added to tissue culture medium containing 3% fetal calf serum (Fig. 1). The biological activity of gentamicin was not reduced in the presence of serum.

An ideal antibiotic for cell culture should have broad-spectrum antibacterial and antimycoplasma activity as well as freedom from cell toxicity. Gentamicin approaches these requirements. Casemore (4) reported that it was not toxic to human amnion cells, rhesus monkey cells, or HeLa cells at a concentration of 50 μg/ml, and Rudin et al. (11) indicated that toxicity was not observed with African green monkey kidney cells or WI-38 cells at a concentration of 200 μg/ml. Results presented here extend these observations to 23 different cell types which are unaffected by 50 μg/ml. In another series of recent experiments, Litwin (7) compared the effect of pure reagent grade gentamicin and gentamicin for human use (Garamycin) on the growth of human diploid lung fibroblasts. He found the tissue culture grade (pure) antibiotic to be free from toxicity at 100 μg/ml. However, some additives of Garamycin (see above) proved to be detrimental to cell growth, suggesting that Garamycin should not be used for cell culture purposes.

Gentamicin is stable to extremes in pH and temperature, is free from apparent cytotoxicity at bactericidal and antimycoplasmal levels, does not affect stability or replication of viruses, and does not influence the detection or synthesis of interferon. These characteristics indicate that gentamicin may be uniquely suited for tissue culture and virus studies.

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LITERATURE CITED

1. Black, J., B. Calesnick, D. Williams, and M. J. Weinstein. 1964. Pharmacology of gentamicin, a new broad-spectrum antibiotic. Antimicrob. Ag. Chemother. 1963, p. 138-147.
2. Braun, P., J. Ő. Klein, and E. H. Kass. 1970. Susceptibility of genital Mycoplasma to antimicrobial agents. Appl. Microbiol. 19:62-70.
3. Busch, H., and M. Lame. 1967. Chemotherapy, an introductory text. p. 10. Year Book Medical Publishers, Inc., Chicago.
4. Casemore, D. P. 1967. Gentamicin as a bactericial agent in virological tissue culture. J. Clin. Pathol. 20: 288-299.
5. Grove, D. C., and W. A. Randall. 1955. Assay methods of antibiotics. A laboratory manual. Medical Encyclopedia, Inc., New York.
6. Laskey, R. A. 1970. The use of antibiotics in the preparation of amphibian cell cultures from highly contaminated material. J. Cell Sci. 7:653-659.
7. Litwin, J. 1970. The effect of commercial and pure gentamicin on the growth of human diploid lung fibroblasts. Acta Pathol. Microbiol. Scand. Section B 78: 273-276.
8. Oden, E. M., H. Stander, and M. J. Weinstein. 1964. Microbiological assay of gentamicin. Antimicrob. Ag. Chemother. 1963, p. 8-13.
9. Perlman, D., S. B. Rahman, and J. B. Semar. 1967. Antibiotic control of Mycoplasma in tissue culture. Appl. Microbiol. 15:82-85.
10. Rahman, S. B., J. B. Semar, and D. Perlman. 1967. Antibiotic resistance in Mycoplasma isolates from tissue culture. Appl. Microbiol. 15:970.
11. Rudin, A., A. Healey, C. A. Phillips, D. W. Gump, and B. R. Forsyth. 1970. Antibacterial activity of gentamicin sulfate in tissue culture. Appl. Microbiol. 20: 989-990.
12. Wagner, R. R. 1960. Virological interference. Some considerations of basic mechanisms and their potential relationship to host resistance. Bacteriol. Rev. 24:151-166.
13. Waitz, J. A., and M. J. Weinstein. 1969. Recent microbiological studies with gentamicin. J. Infec. Dis. 119: 355-360.
14. Weinstein, M. J., G. M. Luedemann, E. M. Oden, G. H. Wagman, J. P. Rosselet, J. A. Marquez, C. T. Coniglio, W. Charney, H. L. Herzog, and J. Black. 1963. Gentamicin, a new antibiotic complex. J. Med. Chem. 6:463-464.