TOXICITY OF ANTIBIOTICS AND ANTIFUNGALS ON CULTURED HUMAN CORNEAL CELLS: EFFECT OF MIXING, EXPOSURE AND CONCENTRATION

M. BERRY, A. GURUNG and D. L. EASTY
Bristol

SUMMARY

Toxic effects of topical drugs may be masked by manifestations of the disease they cure. The toxicity of drug mixtures has not been thoroughly studied. We therefore investigated cytopathic effects on primary cultures of human corneal cells of six topical antimicrobials singly and in combinations of any two, to determine the combined toxicity ranking and the interaction between duration of exposure and concentration. Preconfluent cultures were exposed to fixed dilutions of single drugs, or to equal-dilution mixtures of two drugs, for 7 and 14 days. Diminishing concentrations of single drugs were applied sequentially to cultures for 14 days. The number of metabolically competent cells was assessed by measuring hexosaminidase and total protein. Toxic effects depended on substance, concentration and exposure. The scale of toxicity determined for single drugs after 7 days of exposure was: gentamicin > econazole ⩾ methicillin ⩾ clotrimazole ⩾ miconazole ⩾ chloramphenicol. After 14 days this order changed: in particular chloramphenicol showed a highly increased toxicity. The order of diminishing effects was: gentamicin > chloramphenicol ⩾ methicillin > miconazole > econazole > clotrimazole. A clear reduction in cytopathic effects was observed when drug concentration was decreased progressively only in cultures treated with gentamicin or methicillin. All drug combinations were more toxic than their components at equal dilution. Combinations containing chloramphenicol ranked most toxic overall, those containing econazole least. A tapering off combination regime did not improve cell survival. These *in vitro* toxicity data complement clinical studies and suggest ways in which topical drugs can be chosen to minimise toxic effects to corneal surface.

Topical antimicrobial drugs are a widespread and effective treatment of bacterial and fungal conjunctivitis or keratitis. The external eye surface may be exposed to broad spectrum drugs or drug combinations to control multiple or unidentified infective agents. Appearance of local toxic effects of the medication, often difficult to distinguish from the disease process itself,[1] may prompt a diminution of drug concentration or frequency or a change of medication.

Toxicity of drug combinations, resulting from a summation or potentiation of individual adverse effects, has been insufficiently studied. It is this relationship between the toxicity of a drug mixture and that of its components that is the subject of this study.

The number of all possible combinations of drugs is too large to be exhaustively tested. We tested, singly and in combinations of any two, three antibiotics and three antifungals most frequently prescribed in the Bristol Eye Hospital: chloramphenicol, gentamicin, methicillin, clotrimazole, econazole and miconazole.

An *in vitro* system was chosen to isolate drug toxicity from cytopathic effects of a microbial infection. Human corneal epithelial cell cultures were exposed to antimicrobial drugs for a period commensurate with clinical use. They were exposed to fixed and to sequentially decreasing concentrations of test substance, the latter mimicking a tapering off regime.

Cytopathic effects depended on the drug(s), concentration and duration of exposure. Rankings of single drugs and combinations were not correlated. Exposure to diminishing drug concentrations did not always improve the outcome.

MATERIALS AND METHODS

Selection of Topical Drugs

Twenty-four questionnaires were sent to consultants, registrars and senior house officers in the Bristol Eye

Correspondence to: Dr M. Berry, University of Bristol, Department of Ophthalmology, Bristol Eye Hospital, Lower Maudlin Street, Bristol BS1 2LX, UK.

Hospital. They were asked to list in order of preference drugs (and combinations) for treating suspected bacterial and fungal conjunctivitis or keratitis.

Tissue Culture

Epithelial cell cultures were established from human corneas unsuitable for transplantation from the UKTSSA Eye Bank in Bristol. Epithelium and a thin layer of underlying stroma was separated from the main bulk of the stroma by blunt dissection. Explants 2–4 mm^2 were plated on tissue culture flasks in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco), streptomycin, penicillin, amphotericin mixture (Sigma) and RPMI nonessential amino-acids (Sigma). When epithelial growths had been established explants were removed to minimise fibroblast content of the cultures. After confluence cultures were passaged and the cells used in this study. All experiments were carried out in six replicates in tissue culture medium containing 5% fetal bovine serum.

Experimental Design

Effects on Cell Proliferation. A total of 5×10^3 cells per well were seeded in 96-well tissue culture plates (Costar), allowed to adhere overnight at 35 °C, and exposed to a fixed concentration of test substance for 7 or 14 days. Water-soluble topical solutions were diluted in tissue culture medium, lipid-soluble ones in arachis oil (Hill Cross Pharmaceuticals). The latter were always applied to the cell layer before tissue culture medium to ensure penetration. Cells treated with arachis oil and tissue culture medium served as controls for each experiment. Medium and test substance were renewed twice a week. The number of cells was evaluated by measuring hexosaminidase activity[2] and total protein content (BCA Protein Assay, Pierce). The results are presented as optical densities, with standard curve number of cells corresponding to that optical density on the right-hand vertical axis.

Effects on Cell Proliferation and Migration. Cells were plated overnight either in a dense plaque (5×10^3 cells per well) in the centre of a 1.5 cm diameter well (Costar), or in an annulus (15×10^3 cells per well) around its perimeter, and exposed to test solutions for 14 days. The number of cells was evaluated by measuring hexosaminidase activity and total protein, and well cover visualised by staining with haematoxylin.

Effects of Decreasing Concentration. Step decreases in the concentration of the test substance (single drugs or combinations) were compared with exposure to fixed concentrations. Cells plated in 96-well plates were exposed to a highest concentration (C) of test substance for 3 days. On day 3 the concentration was halved ($C/2$) for all but 6 wells, which were exposed to the initial concentration for the remainder of the experiment, as a fixed concentration comparator. On day 6 the concentration was halved again ($C/4$) for all but another block of 6 wells which continued to be exposed at half the initial concentration ($C/2$). This process was repeated every 3 days, resulting in different *concentration* × *step length* regimes. The number of metabolically competent cells was determined in all the cultures on day 21.

Analysis of variance (ANOVA), *post hoc* tests and non-parametric analysis of variance by ranks (Kruskal–Wallis test) were performed with Stat-View 4.02 (Abacus Concepts, Berkeley, CA).

RESULTS

Thirteen of 24 questionnaires were returned. The unanimous first choice of drug for the treatment of suspected bacterial conjunctivitis was chloramphenicol 0.5%. No drug combinations were envisaged for this condition. For suspected bacterial keratitis gentamicin (1.5%) or a combination of gentamicin (1.5% or 0.3%) and methicillin 2% were equal first preferences. Suspected fungal keratitis would most often be treated with either miconazole 1%, or a mixture of two antifungals (e.g. econazole plus miconazole), or a mixture of an antibiotic and an antifungal (e.g. econazole plus chloramphenicol, or clotrimazole plus gentamicin forte).

In accordance to these preferences we chose to study the effects of chloramphenicol 0.5% (Scherring-Plough), gentamicin forte 1.5% (Moorfields Eye Hospital) and methicillin 2% (Bristol Eye Hospital). The antifungals chosen were econazole 1% (Moorfields Eye Hospital), miconazole 1% (Moorfields Eye Hospital) and clotrimazole 1% (Moorfields Eye Hospital).

The number of metabolically competent cells as evaluated by the hexosaminidase assay paralleled closely the results obtained by measuring total protein. Hexosaminidase results had, however, a wider distribution, i.e. a larger difference in optical density between the most and the least populated wells (Fig. 1). We chose the latter to represent the effects of treatment on cell numbers.

Effect on Cell Proliferation

After exposure to antimicrobials the number of cells depended on test substance, concentration and duration of exposure (Kruskal–Wallis analysis of variance by ranks, $p<0.001$; Figs. 2 and 3).

Fourteen days of exposure to antibiotics brought a further decrease in population size compared with the shorter exposure (Fig. 3). This effect was not seen with antifungals.

After 7 days of exposure topical solutions ranked

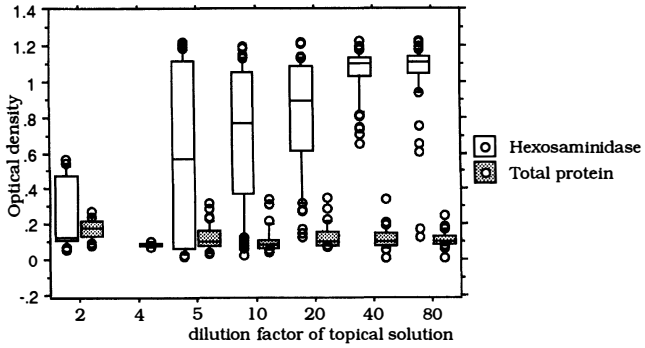

Fig. 1. *Comparison between hexosaminidase and total protein assays for evaluating population size. The box plots show the distribution of all optical densities obtained in one experiment by either method. The boxes showing the means contain 95% of the distribution; outliers are plotted individually. The spread of values obtained by measuring hexosaminidase activity is larger than for total protein and therefore this method was chosen to represent population size.*

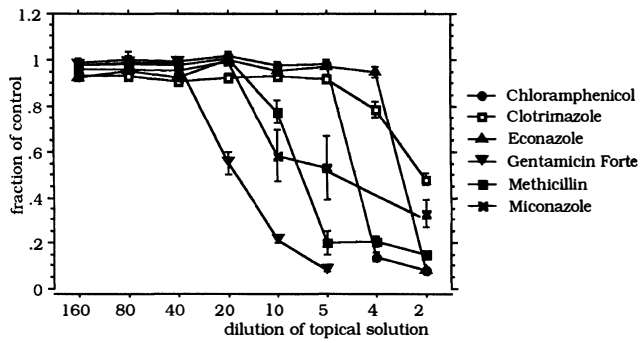

Fig. 2. *Dependence of effect on substance and concentration. Analysis of variance (optical densities expressed as fraction of appropriate controls) showed a significant difference between population sizes depending on test substance and concentration ($p<0.001$). Kruskal–Wallis ranking: gentamicin > clotrimazole ⩾ econazole ⩾ methicillin > miconazole > chloramphenicol.*

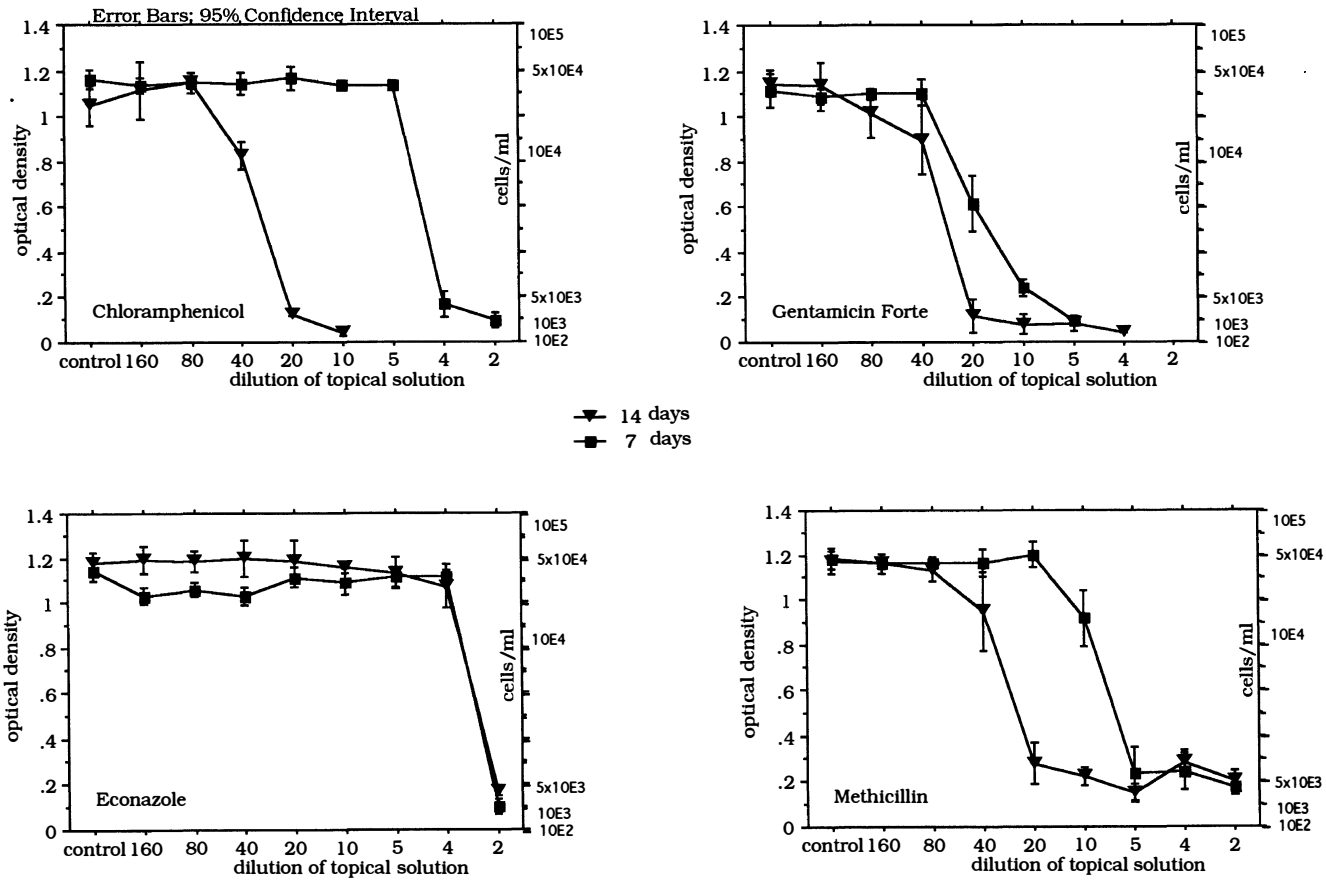

Fig. 3. *Dependence of effects on duration of exposure. Fourteen days of exposure increased the toxic effects of antibiotics but not of antifungals. Kruskal–Wallis ranking: gentamicin > chloramphenicol ⩾ methicillin > miconazole > econazole > clotrimazole.*

in diminishing order of effects: gentamicin > clotrimazole ⩾ econazole ⩾ methicillin > miconazole > chloramphenicol.

After 14 days of exposure gentamicin still ranked most toxic, but the subsequent order was: chloramphenicol ⩾ methicillin > miconazole > econazole > clotrimazole.

Exposure to combinations of two drugs (at equal dilution of the topical preparation) led to a dose-dependent decrease in cell numbers compared with

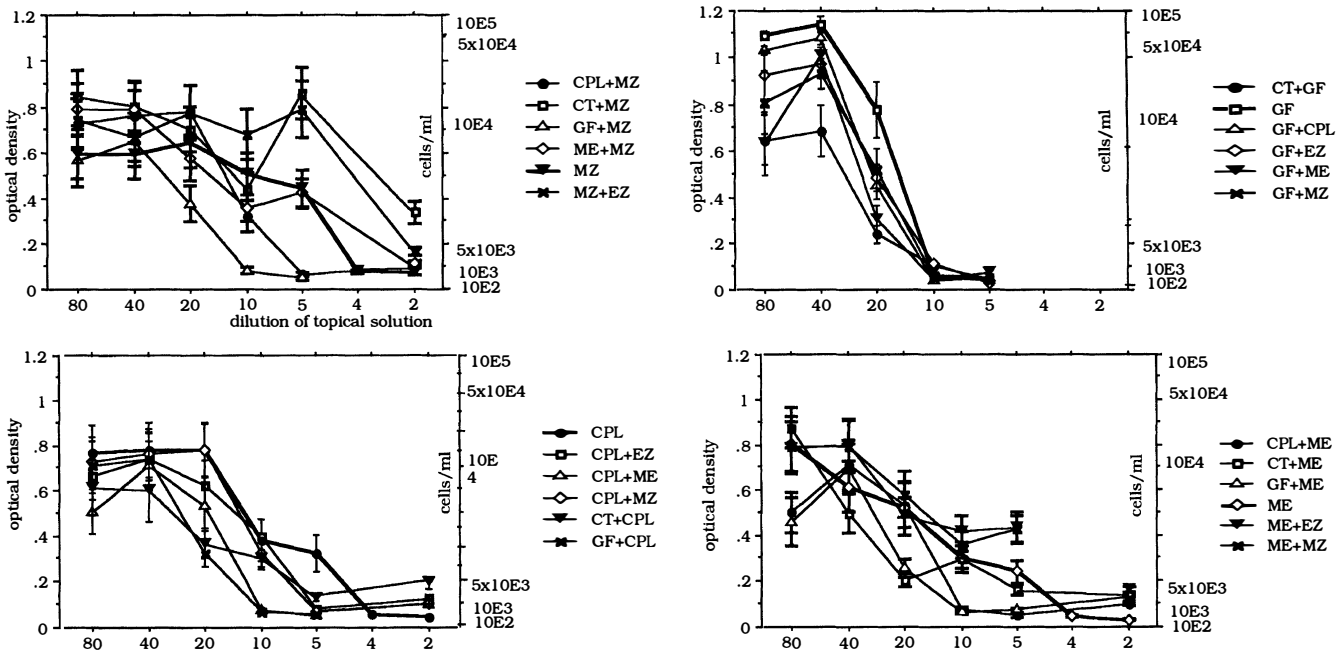

Fig. 4. *Effect of mixtures of topical solutions. Exposure to combinations of two solutions resulted in a smaller population than after exposure to either solution alone. Population size depended on treatment and concentration (ANOVA* $p<0.001$*). Kruskal–Wallis ranking of combinations was: chloramphenicol (CPL) + X > gentamicin (GF) + X ⩾ methicillin (ME) + X > clotrimazole (CT) + X > miconazole (MZ) + X > econazole (EZ) + X, where X represents all topical solutions tested.*

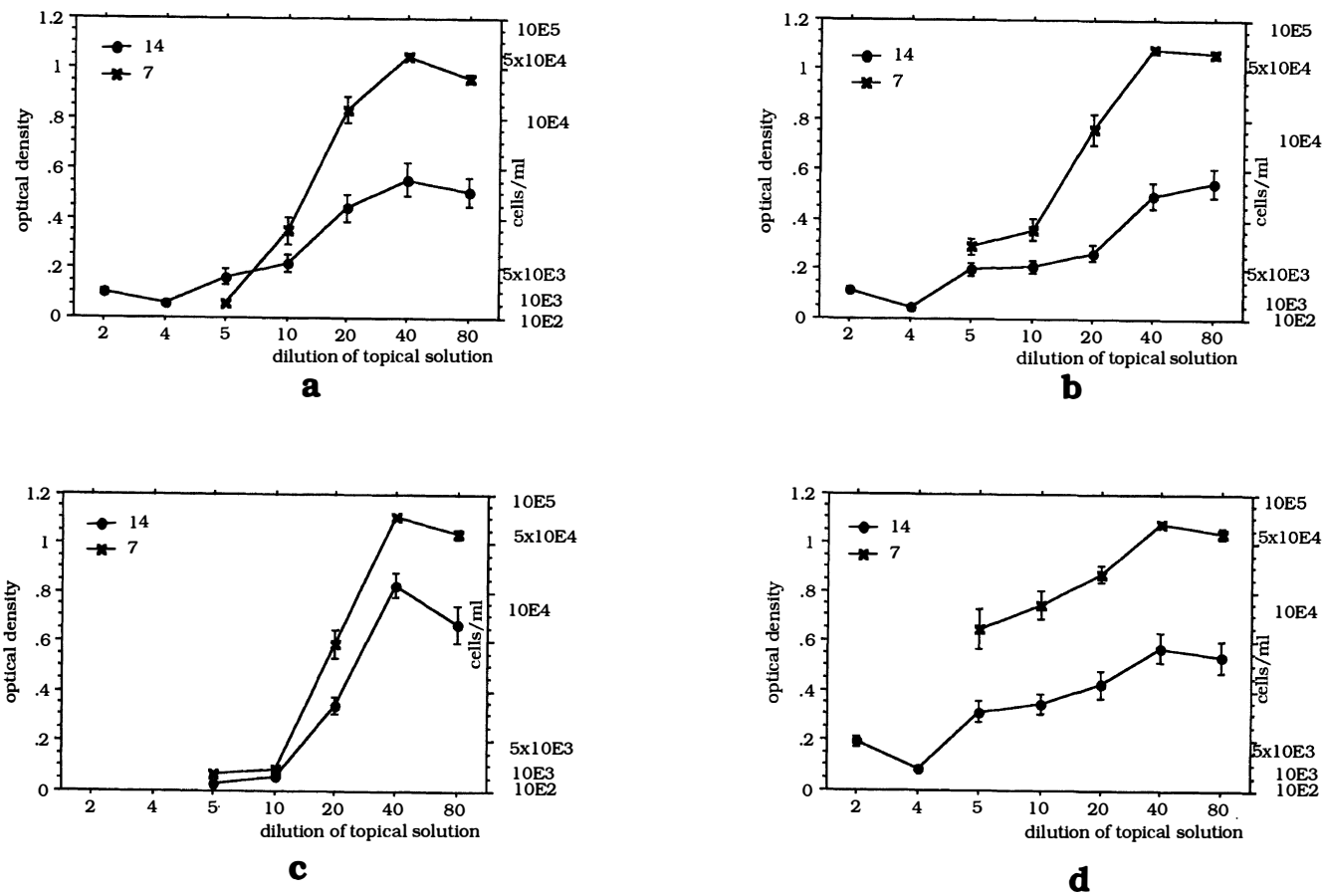

Fig. 5. *Combination of topical solutions: dependence on duration of exposure. Combinations with (a) chloramphenicol, (b) methicillin, (c) gentamicin, (d) econazole. Fourteen days of exposure increased cytopathic effects in a dilution- and substance-dependent manner (ANOVA* $p<0.001$*). Population sizes were significantly different after 7 and 14 days of exposure* ($p<0.001$).

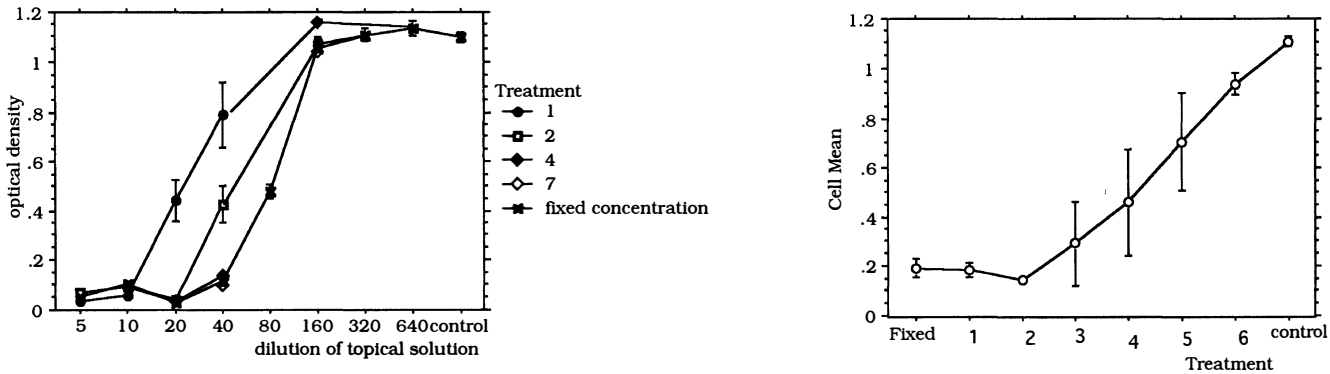

Fig. 6. *Effect of diminishing concentrations of gentamicin (left) and methicillin (right) on cytopathic effects. A stepwise decrease in concentration was chosen to mimic a tapering off regime. All wells were exposed to the highest concentration tested for 3 days. Every 3 days the concentration was halved for all but a block of replicates which continued to be exposed to the respective higher concentration for the rest of the experiment.*

control ($p<0.001$, ANOVA; Fig. 4), except for the combination econazole plus miconazole (Fisher's PLSD, $p = 0.05$).

Mixtures were more toxic than either topical preparation alone: all combinations containing gentamicin were more toxic than gentamicin at the same concentration. Summing the ranks, combinations containing chloramphenicol were more toxic than mixtures with gentamicin (Kruskal–Wallis ANOVA by ranks). Gentamicin-containing combinations ranked equal to those with methicillin. Less toxic were ranked combinations with clotrimazole, then mixtures including miconazole, and last econazole-containing solutions.

Duration of exposure significantly affected population levels in cultures exposed to drug combinations (Fig. 5).

Effect on Cell Migration and Proliferation

Cells plated in a restricted area of the well covered the naked areas and proliferated to an extent depending on the test substance and concentration (ANOVA, $p<0.001$). Where enough cells remained there was no indication of restricted growth or preferential well cover. Overall there was no significant difference between effects depending on plating pattern (Mann–Whitney *U*-test, $p = 0.08$). Ranking of groups of combinations was as follows: chloramphenicol $\geqslant$ gentamicin $>$ methicillin $\geqslant$ econazole $>$ clotrimazole $\geqslant$ miconazole.

Effect of Stepped Decreasing Concentrations

There was a significant difference between cultures exposed to fixed and decreasing concentrations of gentamicin and methicillin (Fig. 6). The shorter the time of exposure to high concentrations, the greater the cell survival. Other drugs and drug combinations did not show this pattern.

DISCUSSION

Dependence on test substance, concentration and duration of exposure are essential requirements for a toxicity test. Effects on the number and metabolic competence of corneal epithelial cells are relevant in the selection of a topical antimicrobial solution.

It was outside the scope of this study to disentangle effects of pure antimicrobials from those of their vehicles and preservatives; the study was limited to 'off the shelf' topical drugs.

The migration–proliferation test presented here lacks the element of trauma of *in vitro* wound-healing tests,[3] and improves control over initial cell numbers. Comparison of effects of antiviral drugs in the two tests showed no additional effect of wounding on toxicity levels.[4] The antimicrobials tested ranked unchanged in the proliferation and proliferation–migration test, suggesting that the main effect is on cellular proliferation.

Econazole, miconazole and clotrimazole appeared least toxic in all *in vitro* tests. They are usually well tolerated by the corneal epithelium even after weeks-long therapy.[5,6]

Chloramphenicol, considered a most effective 'routine' topical antibiotic,[7,8] produced low toxicity after 7 days of exposure. A longer exposure, however, caused a large increase in toxic effects, mirrored in the toxicity rank change.

Stepwise reduction in concentration did not produce an increase in cell survival. This is consistent with the relative insensitivity to concentration seen in cultures treated with chloramphenicol for 7 days. Combinations with any other antimicrobial were remarkably toxic, suggesting potentiation rather than summation of effects.

Very short exposures to gentamicin impair cell division.[9] Wound healing *in vivo*[10] and *in vitro*[11] is also slowed by this drug in a dose-dependent manner. *In vitro* the effects of gentamicin show a strong dependence on concentration and duration of exposure, suggesting possibilities of fine tuning of therapy to individual responses.

Consistent with clinical experience that gentamicin dose reduction after bacterial keratitis speeds recovery, the tissue culture assays showed a net improve-

ment in population size, i.e. a diminished toxic effect, with diminishing concentrations of gentamicin and methicillin. The absence of this effect in the other regimes implies that the product duration × concentration is not a constant, even when effects of substances tested show dependence on dose and exposure time.

The limited *in vitro* testing of three antibiotics, three antifungals and their combinations showed that lengthening exposure increases toxicity in a substance-dependent fashion, that toxicity of combinations is not a linear combination of individual toxicities, and that diminishing the dose does not necessarily lessen cytopathic effects.

Quantitative *in vitro* toxicity data usefully complement clinical toxicity studies and may guide the clinician in how best to use available drugs.

Key words: Chloramphenicol, Clotrimazole, Cornea, Econazole, Epithelium, Gentamicin forte, Human, *In vitro*, Methicillin, Miconazole, Toxicity.

REFERENCES

1. Pfister RR. Clinical measures to promote epithelial healing. Acta Ophthalmol (Copenh) Suppl 1992;202: 73–83.
2. Landegren U. Measurement of cell numbers by means of the endogenous enzyme hexosaminidase: applications to detection of lymphokines and cell surface antigens. J Immunol Methods 1984;67:379–85.
3. Jumblatt MM, Neufeld AH. A tissue culture assay of corneal epithelial wound closure. Invest Ophthalmol Vis Sci 1986;27:8–13.
4. Berry M, Easty DL, de Clercq E. Effect of antivirals on human corneal cells *in vitro*. Toxic In Vitro 1994; 8:727–9.
5. Jones BR, Clayton YM, Oji EO. Recognition and chemotherapy of oculomycosis. Postgrad Med J 1979; 55:625–8.
6. Foster CS. Miconazole therapy for keratomycosis. Am J Ophthalmol 1981;91:622–9.
7. Seal DV, Barret SP, McGill JI. Aetiology and treatment of acute bacterial infection of the external eye. Br J Ophthalmol 1982;66:357–60.
8. Fraunfelder FT. Drug-induced ocular side effects and drug interactions. 2nd ed. Philadelphia: Lea and Febiger, 1982:20.
9. Lass JH, Mack RJ, Imperia PS, Mallick K, Lazarus HM. An *in vitro* analysis of aminoglycoside corneal epithelial toxicity. Curr Eye Res 1989;8:299–304.
10. Petroutsos G, Guimares R, Giraud J, Pouliquen Y. Antibiotics and corneal epithelial wound healing. Arch Ophthalmol 1983;101:1775–8.
11. Nelson JD, Silverman V, Lima PH, Beckman G. Corneal epithelial wound healing: a tissue culture assay on the effect of antibiotics. Curr Eye Res 1990;9:277–85.