Aminoglycoside and aminocyclitol antibiotics: hygromycin B is an atypical bactericidal compound that exerts effects on cells of *Escherichia coli* characteristic for bacteriostatic aminocyclitols

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(Received 10 July 1991; revised 11 October 1991; accepted 5 November 1991)

The effects of aminoglycoside and aminocyclitol antibiotics on intact cells of *Escherichia coli* were compared. The aminoglycosides streptomycin, gentamicin, kanamycin and neomycin had similar, but not identical, effects. They all caused misreading during protein synthesis, permeabilization of the cell membrane, inhibition of the initiation of DNA replication, and loss of cell viability. Cells treated with these antibiotics continued to synthesize two proteins (apparent molecular masses 72 and 60 kDa) that were not made by cells treated with the aminocyclitol hygromycin B, which did not cause misreading. Cells treated with the aminoglycosides regained their membrane tightness after residual protein synthesis in these cells had been inhibited by chloramphenicol, suggesting that under these conditions the mistranslated membrane proteins were rapidly degraded. The bacteriostatic aminocyclitols spectinomycin and kasugamycin did not cause membrane permeabilization, suggesting that these compounds do not cause misreading. Hygromycin B resembled these aminocyclitols in that it inhibited protein synthesis without causing misreading, membrane permeabilization or inhibition of initiation of DNA synthesis. However, hygromycin B also decreased cell viability. In minimal medium this lethal effect began late in comparison to the process of inhibition of protein synthesis. It is concluded that hygromycin B is an atypical bactericidal antibiotic that strongly resembles the bacteriostatic aminocyclitols spectinomycin and kasugamycin in its action.

Introduction

Aminoglycoside antibiotics and related compounds can be divided into two groups. The aminoglycosides streptomycin, neomycin, gentamicin and kanamycin belong to the first and most important group. Besides being inhibitors of protein synthesis these compounds are also bactericidal to many Gram-positive and Gram-negative bacteria. The lethal effect does not occur without inhibition of protein synthesis by these antibiotics (Hancock 1981b; Davis, 1987). Lethality may be related to the fact that these compounds also cause ‘misreading’ during translation and premature termination of protein synthesis followed by the incorporation of some of the aberrant proteins into the cytoplasmic membrane (Hancock, 1964; Davis *et al.*, 1986; Davis, 1987). The latter process causes a limited increase in the permeability of the cell membrane to several compounds (Dubin & Davis, 1961; Dubin *et al.*, 1963; Davis, 1987; Busse *et al.*, 1992) and promotes uptake of aminoglycoside antibiotics by the cell (Höltinge, 1978, 1979; Davis, 1987). The high concentration of the antibiotic in the cytoplasm causes complete blockage of protein synthesis (Davis, 1987). Under these conditions the cells may degrade their mistranslated (membrane) proteins (Busse *et al.*, 1992), leading to ‘caging’ of the antibiotic in the cells (Nichols & Young, 1985; Busse *et al.*, 1992) and to subsequent cell death (Davis, 1987). It has however also been observed that bactericidal aminoglycosides block the initiation of DNA replication (Tanaka *et al.*, 1984; Matsunaga *et al.*, 1986) and it has not been excluded that this effect is responsible for the lethal effect (Davis, 1988a).

The second group of antibiotics includes the (amino)cyclitols spectinomycin and kasugamycin. The major effect of these compounds on cells is inhibition of protein synthesis. They do not cause misreading, and act bacteriostatically (Hancock, 1981a, b; Davis, 1987).

Streptomycin is the best-studied compound of the first group of antibiotics. However, even for this compound it is a matter of debate whether its bactericidal action can be explained satisfactorily (Hancock, 1981b; Davis, 1987, 1988b; Nichols, 1989; Busse *et al.*, 1992). In addition, it is often assumed that observations made with...
streptomycin are also valid for other bactericidal aminoglycosides (Hancock, 1981a, b; Davis, 1987), but there are only few data that support this notion (Höltje, 1979). I compared the following effects of members from the two groups of antibiotics on cells: inhibition of protein synthesis, misreading, the increase in the permeability of the cytoplasmic membrane for K+ and for dihydrostreptomycin, the inhibition of the initiation of DNA synthesis, and loss of cell viability. My results indicate (i) that streptomycin, gentamicin, kanamycin and neomycin all exert similar, but not identical, effects; (ii) that these compounds may initiate the stress response of the cells; and (iii) that the bactericidal aminocyclitol hygromycin B shows many effects typical of other aminocyclitol antibiotics.

Methods

Bacterial strains and growth conditions. Escherichia coli K12 strains were used. Strain TK1110 F- thi lacZ yla SkdAPABC5 trkA405 (Rhoads et al., 1976) and AS-1 acrA (Hirota et al., 1981) were from our culture collection. Strain PC-1 (CGSC strain 5030) F- leuB6 x-thyA47 rpsL153 dnaC1 (Ts) deoC3 (Carl, 1970; Wechsler & Gross, 1971) was from Dr B. J. Bachmann, E. coli Genetic Stock Center, Yale University, New Haven, Connecticut, USA.

Cells of strain TK1110 were grown in minimal medium containing 0.5 mM-K+ ("K0.5-medium") as described in the preceding paper (Busse et al., 1992). For the [3H]dihydrostreptomycin-uptake experiments of strain AS-1 were grown aerobically at 37°C in nutrient broth medium (NB medium, containing, per litre 3 g Bacto-beef extract and 5 g Bacto-peptone; Difco). Strain PC-1 was grown in the semisynthetic medium described by Matsunaga et al. (1986).

Aminoglycoside treatment. The protocol for the addition of antibiotic to cells of strain TK1110 growing in minimal medium, the determination of the K+ content and the viable count of the aminoglycoside-treated cells is given in the preceding paper (Busse et al., 1992).

Labelling of protein with [35S]methionine. Samples (1 ml) of a culture of strain TK1110 growing in minimal medium were incubated for 2 min with 5–10 µCi [35S]methionine (500–1000 Ci mmol−1; 18.5–37 TBq mmol−1). Any further incorporation of radioactivity into macromolecules was then stopped by the addition of 0.1 ml 50% (w/v) trichloroacetic acid. The samples were transferred to 1.5 ml centrifuge tubes and incubated for 30 min on ice. The preceding paper (Busse et al., 1992) describes the electrophoretic separation of cell proteins by SDS-PAGE and the autoradiography of radioactively labelled proteins.

Dihydrostreptomycin uptake. The experiments were carried out with cells of strain AS-1 growing aerobically in NB medium at 37°C. An overnight culture was diluted with fresh medium to an OD670 of about 0.05. At an OD578 of 0.5 the cells received antibiotic; 25 min later a mixture of 400 nCi [3H]dihydrostreptomycin ml−1 (21.9 ng ml−1) and 2 µg streptomycin ml−1 was added to the cell suspension. Alternatively, the cells received at OD578 0.5 the mixture of [3H]dihydrostreptomycin and streptomycin. Samples (0.5 ml) of the cell suspension were filtered through Millipore HAWP2500 filters and the cells on the filters were washed three times with 2 ml 0.1 M-LiCl. The filters were dried, cut into mini-scintillation vials (Canberra Packard) and mixed with 5 ml scintillation fluid (Hydroluma, Baker). Radioactivity of the samples was determined in a liquid scintillation counter (type 460C, Canberra Packard).

Initiation of DNA synthesis. Reinitiation of DNA synthesis in the dnaC (Ts) strain was determined as described by Matsunaga et al. (1986). The extent of incorporation of [3H]thymidine into macromolecules was taken as a measure of DNA synthesis. The assay was carried out as described (Keweloh & Bakker, 1984).

Radiochemicals and antibiotics. [35S]Methionine, [3H]dihydrostreptomycin sesquisulphate (18.3 Ci g−1; 677 GBq g−1) and [3H]thymidine (50 Ci mol−1; 1850 GBq mol−1) were from Amersham-Buchler. Aminoglycosides were from Sigma. Hygromycin B was a gift from Professor Dr A. Böck, University of Munich, FRG.

Results

Effect of aminoglycoside antibiotics on cell K+

The addition of streptomycin causes net loss of K+ from cells of E. coli strain TK1110 growing at 0.5 mM-K+ (Busse et al., 1992; Fig. 1 a). The same phenomenon was observed with cells treated with gentamicin, neomycin or kanamycin (Fig. 1 b–d, Fig. 2 b, c, lower part). Note, however, that with neomycin the extent of K+ release was smaller than that observed with the other aminoglycosides (Figs 1 and 2). This might explain why this effect of neomycin has been missed before (Dubin & Davis, 1961).

Neither spectinomycin nor kasugamycin caused K+ loss from strain TK1110 (Fig. 1 e and f, respectively) under conditions where these bacteriostatic antibiotics inhibited cell growth completely (results not shown). Unexpectedly, the bactericidal compound hygromycin B also did not cause K+ loss (Fig. 2 d, lower part) under conditions where it inhibited cell growth almost immediately (results not shown). Note that the effect of this antibiotic on cell viability was much slower than that of the bactericidal aminoglycosides (Fig. 2, lower part).

Misreading

Due to misreading, proteins synthesized by streptomycin-treated cells occur as diffuse bands on SDS-polyacrylamide gels (Piepersberg, 1985; Davis et al., 1986). I tested whether the same is true for other bactericidal aminoglycosides. To this end, growing cells were treated with aminoglycoside antibiotics and pulse-labelled with [35S]methionine before or after the antibiotic had been added to the cell suspension. The pattern of the radioactively labelled proteins was analysed by SDS-PAGE and compared with the K+ content and the viability of the cells (Fig. 2). Streptomycin, gentamicin and neomycin exerted the following effects: (i) general inhibition of protein synthesis, as indicated by the extent of incorporation of radioactivity into proteins, which decreased with time (Fig. 2 a–c, upper part); (ii) preferential inhibition of the synthesis of large proteins,
Fig. 1. Effect of aminoglycoside and aminocyclitol antibiotics on K⁺ content of growing E. coli cells. Strain TK1110 was grown in K0-5 medium. At OD₅₇₈ 0.7, antibiotic was added at the concentrations (µg ml⁻¹) indicated in the figure. (a) Streptomycin; (b) gentamicin; (c) neomycin; (d) kanamycin; (e) spectinomycin; (f) kasugamycin. Open circles, controls; filled symbols, cells treated with antibiotic.

indicating premature chain termination; and (ii) a general haziness of the autoradiograms and diffuse instead of sharp bands, indicating misreading (Piepersberg, 1985; Davis et al., 1986; Busse et al., 1992). Compared to gentamicin and neomycin (Fig. 2b and c, upper part) streptomycin had the following specific effects: (a) protein synthesis continued longer; and (b) several proteins continued to be synthesized under conditions where the synthesis of others was almost completely inhibited. The molecular masses of these persistent proteins were about 80, 72 (band 1), 60 (band 2), 55, 45 (band 3, EF₅ₙ?), 30 (band 4, OmpA?) 27, 25 and 22 kDa (Fig. 2a, upper part). All of these proteins were among the latest still synthesized by cells treated with gentamicin or neomycin (Fig. 2b, c).

Reversal of K⁺ loss by chloramphenicol

Since the pattern of proteins synthesized by gentamicin- or neomycin-treated cells was different from that of streptomycin-treated cells, the question arose whether rapid degradation of mistranslated proteins as observed with streptomycin (Busse et al., 1992) also occurs in cells treated with gentamicin or neomycin. As an indication of protein degradation I used the phenomenon of reaccumulation of K⁺ by cells observed after the addition of chloramphenicol to cells treated with streptomycin (Busse et al., 1992). Fig. 3 shows that chloramphenicol does restore membrane tightness of gentamicin- or neomycin-treated cells, suggesting that these cells also exert a high rate of proteolysis.

Hygromycin B does not cause misreading

The addition of hygromycin B caused rapid inhibition of protein synthesis (Fig. 2d, upper part). The proteins that were still synthesized formed sharp bands (Fig. 2d, upper part), indicating that in intact cells the antibiotic did not cause misreading. Except for the 72 kDa (band 1) and 60 kDa (band 2) proteins, cells in which protein synthesis was already partially inhibited by hygromycin B made the same proteins as streptomycin-treated cells (Fig. 2a and d, upper part, respectively).

Uptake of [³H]dihydrostreptomycin

Treatment of streptomycin-insensitive cells with kanamycin permeabilizes them to (dihydro)streptomycin (Hölter, 1979). This observation has been interpreted to mean that the mistranslated membrane proteins allow
enhanced uptake of other aminoglycosides by the kanamycin-treated cells (Davis, 1987). Fig. 4 shows that cells that were preincubated with streptomycin, neomycin or kanamycin all took up ([3H]dihydro)streptomycin faster than did non-pretreated cells, supporting the view that these compounds cause membrane permeabilization. Once again the effect of neomycin was smaller than that of the other two compounds (cf. Allen et al., 1987, and the above). By contrast, and in agreement with previous data (Allen et al., 1987), pretreatment with chloramphenicol or with hydromycin B did not lead to enhanced uptake of radioactivity, indicating that neither of these antibiotics causes misreading to such an extent that the cell membrane becomes permeable to (dihydro)-streptomycin.

Control experiments showed that at 20 μg ml⁻¹ hygromycin B still did not increase the permeability of the membrane to (dihydro)streptomycin. In the NB medium used for these experiments 20 μg of hygromycin ml⁻¹ decreased cell viability by a factor of 1000 within 20 min.

Effect on initiation of DNA replication

Bactericidal aminoglycosides inhibit the initiation of DNA replication in a temperature-sensitive dnaC strain (Tanaka et al., 1984; Matsunaga et al., 1986). In order to obtain synchronization, cells were first preincubated for 60 min at the non-permissive temperature. The cells were then shifted back to the permissive temperature and the incorporation of radioactive thymidine into acid-precipitable macromolecules was measured as a function of time (Matsunaga et al., 1986). Fig. 5 shows that in a dnaC (Ts) strain gentamicin blocked the initiation of replication completely. Kanamycin exerted the same effect and streptomycin was not inhibitory, since the strain used was rpsL and therefore insensitive to this antibiotic (results not shown). Remarkably, preincubation of the cells with 200 μg hygromycin B ml⁻¹ caused partial inhibition of incorporation of the [14C]thymidine into DNA. Since chloramphenicol causes the same effect (Fig. 1 in Matsunaga et al., 1986),

Fig. 2. Effect of streptomycin (a), gentamicin (b), neomycin (c) and hygromycin B (d) on protein synthesis (upper part), K⁺ content (lower part, ◆) and cell viability (lower part, ♦) of growing cells of E. coli. Antibiotics were added at zero time to cells of strain TK1110 growing in K0-5 medium. (a) Streptomycin, added at 50 μg ml⁻¹; (b) gentamicin, added at 10 μg ml⁻¹; (c) neomycin, added at 15 μg ml⁻¹; (d) hygromycin B, added at 200 μg ml⁻¹. Lanes 1–12 and 13–24 of the upper part of the figure are each from a single autoradiogram. Lanes 4–6, 9–12, 15–18, 20–24 were exposed about three times as long as lanes 1–3, 7, 8, 13, 14 and 19. The bands marked with numbered arrows are discussed in the text.
Fig. 3. Reversal of K⁺ loss of gentamicin- or neomycin-treated cells after the addition of chloramphenicol. Neomycin or gentamicin was added at zero time at a concentration of 10 µg ml⁻¹ to cells of strain TK1110 growing in K0.5 medium. Chloramphenicol was added at t = 25 min at a concentration of 100 µg ml⁻¹. Control; ○, cells treated with chloramphenicol; □, cells treated with neomycin; ■, cells treated with neomycin and chloramphenicol; ▲, cells treated with gentamicin; ▼, cells treated with gentamicin and chloramphenicol.

Fig. 4. Hygromycin B does not stimulate the uptake of (dihydro)streptomycin by E. coli cells. Cells of strain AS-1 were grown in NB medium (see Methods). Filled symbols, cells preincubated for 25 min with the following antibiotics: streptomycin (2 µg ml⁻¹, ●), kanamycin (5 µg ml⁻¹, ▼), neomycin, (1 µg ml⁻¹, ▲), chloramphenicol, (100 µg ml⁻¹, ▲) or hygromycin B (5 µg ml⁻¹, ◦). At zero time the cells received a mixture of 400 nCi [³H]dihydrostreptomycin ml⁻¹ (22 ng ml⁻¹) and 2 µg of streptomycin ml⁻¹. Control cells (○) were not preincubated with antibiotic and received the mixture of radioactive dihydrostreptomycin and streptomycin together at zero time. Uptake data were calculated with the assumption that non-radioactive streptomycin behaved as radioactive dihydrostreptomycin.

Fig. 5. Hygromycin B does not inhibit the initiation of DNA replication in E. coli. Cells of strain PC1 were grown at 28 °C in the semi-synthetic medium of Matsunaga et al. (1986). At zero time the culture was shifted to 38 °C and shaken for 60 min at this temperature. At t = 60 min the culture was shifted back to 28 °C. Thymidine uptake by the cells was determined as a function of time. ○, Control cells (no antibiotic present); □, 10 µg gentamicin ml⁻¹ added at t = 55 min; ◦, 100 µg hygromycin B ml⁻¹ added at t = 55 min.

we conclude that unlike bactericidal aminoglycosides hygromycin B does not inhibit the initiation of DNA replication.

Discussion

Hygromycin B is an aminocyclitol antibiotic (Umezawa et al., 1985). It inhibits protein synthesis by blocking ribosomal translocation (Cabanas et al., 1978) and by preventing A-site occupation during the elongation cycle (Hausner et al., 1988). In addition, hygromycin B causes misreading of the genetic code in an in vitro system (Davies et al., 1965) and thereby exerts an effect typical for bactericidal aminoglycosides like streptomycin (Davies et al., 1965; Gorini, 1974). Approximately the same concentration of hygromycin B inhibits protein synthesis in the in vitro system as is required for causing misreading (Cabanas et al., 1978), suggesting that both effects are equally important for the understanding of the mechanism of action of the antibiotic. My results indicate, however, that with hygromycin B the in vivo situation is very different from that in vitro: the main effect of the antibiotic on growing cells was its rapid inhibition of protein synthesis. Neither with a direct assay nor with several indirect ones did I obtain any evidence for hygromycin B causing misreading in vivo.
(Figs 2d and 4; Allen et al., 1987). Hence, in its mode of action hygromycin B resembles the bacteriostatic aminocyclitols spectinomycin and kasugamycin, which inhibit protein synthesis without causing misreading and subsequent permeabilization of the cell membrane (Fig. 1e, f). In addition, unlike the bactericidal aminoglycosides gentamicin or kanamycin, hygromycin B did not block the initiation of DNA synthesis and behaved in this assay in a way similar to the inhibitor of protein synthesis chloramphenicol (Fig. 5; Matsunaga et al., 1986). Thus, my results with cells suggest that hygromycin B belongs to the bacteriostatic aminocyclitols group rather than to that of the bactericidal aminoglycosides. This conclusion is not amazing in view of the fact that on the basis of its structure hygromycin B is thought to be an aminocyclitol rather than an aminoglycoside antibiotic (Umezawa et al., 1985). However, the question remains unanswered why hygromycin B is lethal to cells. In the minimal medium loss of cell viability was a late event when compared to inhibition of protein synthesis (Fig. 2d) or inhibition of growth (results not shown), suggesting that hygromycin-B-induced lethality represents a side effect. However, in nutrient broth cell viability decreased much more rapidly than it did in the minimal medium (Results). But even in nutrient broth cells were not permeabilized by the antibiotic, suggesting that a large extent of misreading is not a prerequisite for the bactericidal effect of hygromycin B. Nevertheless, it cannot be excluded that this lethal effect requires misreading of the genetic code at a level that was too low to be detected by the methods I used.

The comparison of the effects that some commonly used bactericidal aminoglycoside antibiotics exert on cells showed that these compounds affect E. coli cells in a similar, but not identical manner: streptomycin, gentamicin, neomycin and kanamycin all caused membrane permeabilization (Figs 1, 2 and 4). However, in streptomycin-treated cells the synthesis of several proteins continued longer than in cells treated with gentamicin or neomycin (Fig. 2a–c). Most of the proteins that were still produced by streptomycin-treated cells belong to the group of major cell proteins (Fig. 2a) and were initially also made by cells treated with gentamicin, neomycin or hygromycin B (Fig. 2b–d). Apparently, these proteins are not only synthesized with high speed, but also with a high fidelity, since they form sharp bands (Fig. 2) and are relatively insensitive to proteolysis after the addition of chloramphenicol to streptomycin-treated cells (cf. Fig. 8a in Busse et al., 1992). Two of these proteins are probably EF-Tu (band 3 in Fig. 2) and OmpA (band 4 in Fig. 2). More interestingly, cells treated with streptomycin, gentamicin or neomycin also continue to synthesize two proteins (bands 1 and 2 in Fig. 2, approximate molecular masses 72 and 60 kDa, respectively) that were not produced by cells treated with hygromycin B (Fig. 2d). I propose that these proteins are ‘stress’ proteins. They may be identical to the two large proteins produced in E. coli after heat shock (Goff & Goldberg, 1985) and might be DnaK and GroEL, the two most abundant heat-shock proteins of the E. coli cell (Neidhardt & VanBogelen, 1987; Craig & Gross, 1991). Both DnaK and GroEL bind proteins during heat shock and may prevent them from unfolding (Landry & Gierasch, 1991).

Under a variety of stress conditions, including streptomycin-induced mistranslation, cells produce Lon and other proteases (Goff & Goldberg, 1985; Neidhardt & VanBogelen, 1987). This phenomenon might explain why cells treated with streptomycin and chloramphenicol exert such a high protease activity on mistranslated proteins (Fig. 3; Busse et al., 1992). It remains unclear, however, why this protease activity is only switched on after the addition of chloramphenicol (or other antibiotics that inhibit translation completely) to the cells (Busse et al., 1992). Streptomycin-treated cells have normal ATP levels (Goss et al., 1988) and one may expect that the addition of chloramphenicol will not affect the ATP level of the cells drastically. It is therefore unlikely that the ATP-hydrolysing Lon protease alone is responsible for the high protease activity of the cells. The same conclusion has been drawn for the protease activity of cells submitted to heat shock (Neidhardt & VanBogelen, 1987).

In contrast to the bactericidal aminoglycosides gentamicin, habekacin or kanamycin, hygromycin B did not inhibit the initiation of DNA replication (Tanaka et al., 1984; Matsunaga et al., 1986; Fig. 5). Since hygromycin B is the only member of this group that does not cause misreading in intact cells, this result might suggest that misreading during translation is responsible for the inhibition of the initiation of DNA replication. It is, however, not possible to decide whether this inhibition or the enhanced uptake of the antibiotic followed by ‘caging’ of the compound inside the cytoplasm (Davis, 1987; Busse et al., 1992) or both phenomena cause the lethal effect of these aminoglycosides.

I thank H.-J. Busse for helpful discussion and for drawing my attention to hygromycin B, A. Böck for sending me this compound, C. Wöstmann for carrying out some initial experiments and E. Limpinsel for expert technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (SFB171, Teilprojekt Cl), the Fonds der Chemischen Industrie and the European Community, contract SC-1-0334-C(A).

References

Bactericidal aminoglycoside antibiotics


