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LISTERIA MONOCYTOGENES - MICROCALORIMETRIC INVESTIGATIONS REGARDING THE ANTIBACTERIAL EFFICIENCY OF CHEMOTHERAPEUTICS

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SUMMARY

The technique of microcalorimetry enables one to distinguish between the effects of bactericidal and bacteriostatic antibiotics, despite the well known antibiotic tolerance shown by Listeria monocytogenes. Synergism between ampicillin and gentamicin could not be shown. Despite a fall in viable counts in the kill-kinetic tests aminoglycosides in inhibitory concentrations do not lead to a corresponding reduction in thermal output. The bactericidal effect of ampicillin in high doses in the traditional broth dilution test must be regarded as an artefact.

INTRODUCTION

The technique of microcalorimetry enables one to observe the effects of chemotherapeutic agents on bacterial metabolism without interruption. The heat thus measured correlates well with bacterial growth; bactericidal drugs show their effect by a fall in the thermal output while bacteriostatic drugs lead to plateau formation in the thermogram (ref. 1).

Here we have investigated microcalorimetrically the effectiveness of various drugs which are recommended for the treatment of listeriosis. Listeria monocytogenes is a gram-positive rod which causes illness in warm-blooded animals and man. Most antimicrobial drugs which are bactericidal cannot be shown to have a cidal effect on Listeria monocytogenes.

METHODS

Microcalorimeter

We used two LKB calorimeters 2107/210 (Flow System). The heat produced by the growth of the bacteria is transformed into thermal currents by semiconductors situated in measuring cells each with a capacity of 0,7 ml. Our machines were adjusted to measure over a range of 0 - 30 μV.

Bacterial strains

The strain Listeria monocytogenes No.: A957/86 was isolated from a newborn child at the State Bacteriological and Serological Laboratory Innsbruck (

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SEMENITZ E.) and is listed in the Special Listeria Culture Collection (=SLCC) as SLCC 7315 (Serovar 1/2a). The strains SLCC 1191 (3a), 5591 (3b), 2715 (1/2a) and 5025 (1/2a) were kindly donated by SEELIGER H.P.R.

Experimental method

In order to demonstrate the microcalorimetric pattern produced by the growing bacteria 5ml of bacterial suspension (grown up from a single colony incubated for sixteen hours in 5 ml broth) were inoculated into 500 ml Columbia Broth (GIBCO EUROPE), yielding an initial density of 10^6 bacteria/ml. The broth was then stirred continuously with a magnetic stirrer while incubating in a waterbath (35°C). After passing through the microcalorimeter at a rate of 25 ml/h the broth was collected in a separate container.

Test substances

Test substances from the following firms were used: penicillin G (BIOCHEMIE), ampicillin (BIOCHEMIE), imipenem (MERK,SHARP & DOHME), erythromycin (ABBOTT), clindamycin (UPJOHN), tetracycline (BIOCHEMIE), chloramphenicol (BAYER), gentamicin (BIOCHEMIE), netilmicin (ESSEX), streptomycin (BIOCHEMIE), penicillinase 10.000.000 IU/ml (Penase Concentrate, DIFCO), quaternary (Zephirol°, BAYER), formaldehyde (Formaldehyde solution, MERK).

RESULTS

On microcalorimetry Listeria monocytogenes produces a double-peaked thermogram. The addition of inhibitory concentrations of antimicrobial drugs gives rise to the formation of a plateau form : penicillin G 0,06 units/ml, ampicillin 0,2 µg/ml, imipenem 0,02 µg/ml, gentamicin 1,5 µg/ml, netilmicin 1,5 µg/ml, streptomycin 10 µg/ml. A gradual change from the double-peaked pattern to the plateau form was found with the following antibiotics: chloramphenicol 4 µg/ml, tetracycline 1 µg/ml, erythromycin 0,25 µg/ml and clindamycin 2 µg/ml. Figs. 1 and 2 show the thermograms on addition of penicillin G and gentamicin, and Figs. 3 and 4 on addition of tetracycline and erythromycin.

The addition of combinations of the above mentioned antibiotics in inhibitory concentrations did not cause any further reduction in thermal output beyond that shown by the plateau formation (Fig. 5 (3)).

The addition of disinfectants such as Zephirol° ad 1%, a quaternary agent, and formaldehyde ad 0.5% caused an immediate cessation of thermal output (Fig. 5).

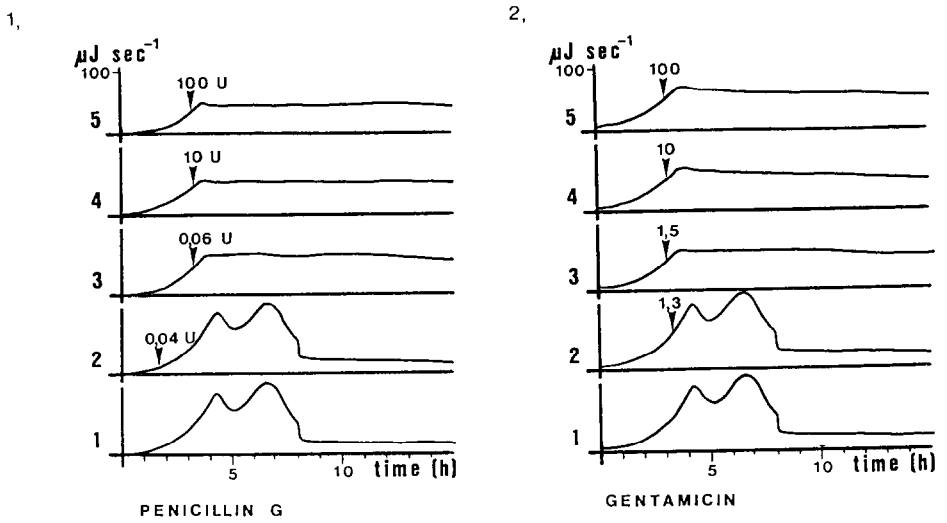

Fig.1. Thermograms of L.m. SLCC 7315 (1) with varying concentrations of penicillin G: 0,04 units/ml (2), 0,06 units /ml (3), 10 units/ml (4), 100 units/ml (5).

Fig.2. Thermograms of L.m. SLCC 7315 (1) with varying concentrations of gentamicin: 1,3 μg/ml (2), 1,5 μg/ml (3), 10 μg/ml (4), 100 μg/ml (5).

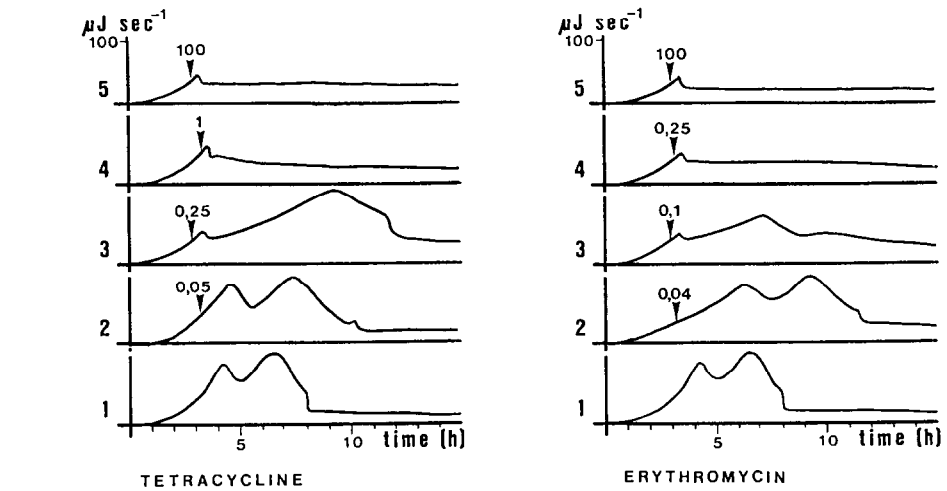

Fig.3. Thermograms of L.m. SLCC 7351 (1) with varying concentrations of tetracycline: 0,05 μg/ml (2), 0,25 μg/ml (3), 1 μg/ml (4), 100 μg/ml (5).

Fig.4. Thermograms of L.m. SLCC 7351 (1) with varying concentrations of erythromycin: 0,04 μg/ml (2), 0,1 μg/ml (3), 0,25 μg/ml (4), 100 μg/ml (5).

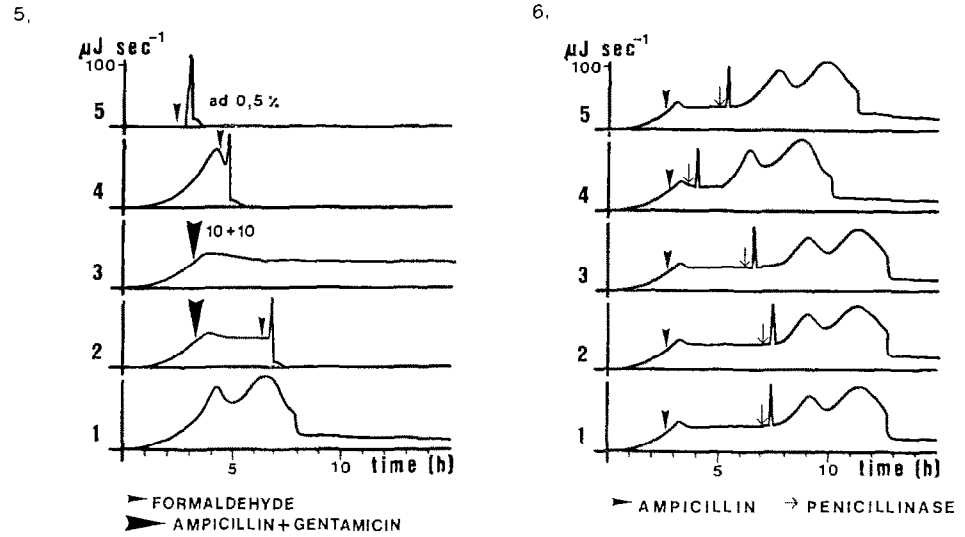

Fig.5. Thermograms of L.m. SLCC 7315 (1), after addition of ampicillin 10 µg/ml + gentamicin 10 µg/ml (2,3) followed by formaldehyde ad 0.5% at 6.5 hours in (2), and after addition of formaldehyde ad 0,5% alone (4). Thermogram of sterile broth after addition of formaldehyde ad 0.5% (5).

Fig.6. Thermograms of different L.m.-strains after addition of 500 µg/ml ampicillin followed by 200 million unitis penicillinase: SLCC 7315 (1), SLCC 5025 (2), SLCC 2715 (3), SLCC 1191 (4), SLCC 5591 (5).

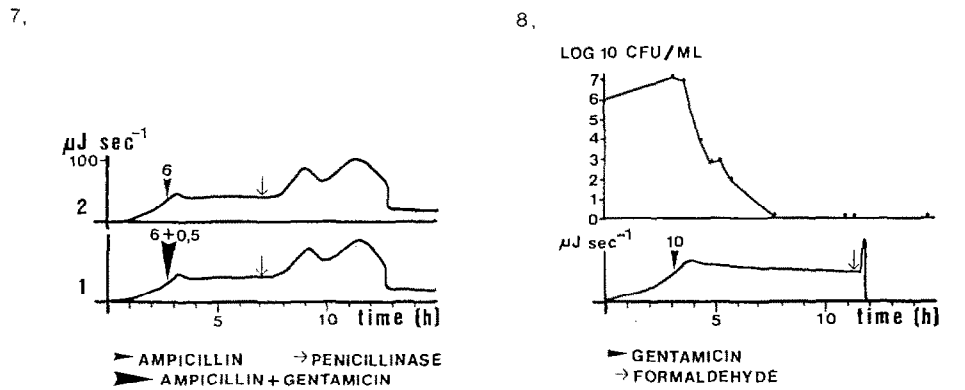

Fig.7. Thermograms of L.m. SLCC 7315 after addition of ampicillin 6 µg/ml + gentamicin 0,5 µg/ml (1) and after addition of ampicillin 6 µg/ml alone (2) followed in both (1,2) by 200 million units penicillinase.

Fig.8. Thermogram and kill kinetic of L.m. SLCC 7315 after addition of 10 µg/ml gentamicin followed by formaldehyde ad 0.5%.

The inactivation of high concentrations of ampicillin through the addition of penicillinase led to regrowth of the test strains both in the broth dilution test and in the microcalorimetric tests (Fig. 6). Adding subinhibitory concentrations of gentamicin to the ampicillin before addition of the penicillinase had no effect on the form or time-course of the curves (Fig. 7).

Whereas in the conventional kill-kinetic tests gentamicin causes a rapid fall in the viable counts, it shows no corresponding fall in thermal output on microcalorimetry; the thermal activity is completely stopped by adding disinfectants (Fig. 8).

DISCUSSION

Listeria monocytogenes shows the distinguishing feature of antibiotic tolerance. This is a condition in which a microorganism can survive in the presence of the antibiotic and recommence growth when the antibiotic is removed; thus bactericidal antimicrobial drugs only show a bacteriostatic effect. A number of methods for determining antimicrobial susceptibility is available. Usually quantitative data are provided by methods that incorporate serial dilutions of antimicrobials in agar-containing or broth culture media. The lowest concentration of the antimicrobial agent that prevents visible growth after an 18- to 24-hour incubation period is known as the minimal inhibitory concentration (MIC). The minimal bactericidal concentration (MBC) may be determined in broth dilution tests by subculturing the containers that show no growth onto antibiotic-free agar-containing media. The lowest concentration of antimicrobial that totally suppresses growth on antibiotic-free media (or results in a 99.9 percent or greater decline in colony count) after overnight incubation is known as the MBC (ref. 2).

In contrast to the traditional methods of MIC and MBC determination microcalorimetry enables one to differentiate between antimicrobials with a bactericidal and bacteriostatic mode of action in spite of antibiotic tolerance. The addition of incremental doses of classically bactericidal antibiotics, in this case penicillin G, ampicillin, imipenem, gentamicin, netilmicin and streptomycin, leads to an abrupt change in the form of the curve in the thermogram, namely plateau formation, once an inhibitory concentration of antibiotic is reached. Classically bacteriostatic drugs, such as in this case chloramphenicol, tetracycline, erythromycin and clindamycin, also show plateau formation on addition of incremental doses, but in contrast to the abrupt change shown by bactericidal drugs there is a gradual change over several increments from the normal double-peak in the thermogram to the plateau, which is caused by the bacteriostasis. Thus with bacteria which show antibiotic tolerance this microcalorimetric technique of inhibitory concentration determination shows a distinct advantage over the traditional

methods, which in these cases cannot distinguish between bactericidal and bacteriostatic antibiotics.

Even the addition of very high doses of ß-lactams (e.g. 1000 µg/ml ampicillin) did not show the typical picture of a bactericidal effect on microcalorimetry. The cause of antibiotic tolerance is thougt to be the deficiency of autolytic enzymes in the cells (ref.3). It is known that lysis of the antibiotic-treated bacteria is essential for the cidal effect of the antibiotics and that this lysis is brought about by endogenous autolytic enzymes that essentially cause the inflicted organism to commit suicide. Assuming that this theory is correct, then the lack of a bactericidal effect even with high doses of ß-lactams is understandable. On the other hand several authors have described a bactericidal action of ampicillin in high concentrations on listeria (ref. 4). HANDWERGER and TOMASZ have already highlighted the problem of antibiotic carry-over with the traditional broth dilution test (ref. 5). "When a loop full of bacteria from the drug-containing MIC tube is transferred to the surface of drug-free agar, dilution of the drug by the volume of the agar medium does not necessarily occur. Adding penicillinase to cultures prior to agar plating for viabiltiy or MBC determinations may be necessary to reliably determine tolerance in some strains." Adding penicillinase to the subcultures in the broth dilution test confirms that even high doses of antibiotic do not have a bactericidal effect. With this microcalorimetric technique this source of error does not exist; bacteriostasis and bacterial cell kill can be distinguished from one another directly from the pattern of thermal output, without needing to inactivate the antimicrobial drugs.

Inhibitory concentrations of gentamicin cause a significant drop in the viable counts in kill-kinetic tests. However, on microcalorimetry there is no corresponding decrease in thermal activity. Thus aminoglycosides cause an irreversible inhibition of bacterial multiplication in Listeria monocytogenes, but they do not kill the bacteria. Aminoglycosides belong to that group of chemotherapeutic agents with a bactericidal mode of action, but it is not clear why they act in that way (ref. 5). The inhibition of protein synthesis appears insufficient to explain the bactericidal effect of aminoglycosides. Other antibacterial agents inhibit protein synthesis at least as effectively as do aminoglycosides and yet fail to cause a lethal event, that is, produce only bacteriostasis. Similarly, the production of "fraudulent" proteins correlates imperfectly with lethality. Until now aminoglycosides have been regarded as the only antibiotics which retain their bactericidal action against Listeria monocytogenes. Our microcalorimetric tests show, however, that aminoglycosides produce, in their action against listeria, a pattern of behaviour considerably different to that caused by bactericidal drugs on non-tolerant bacteria.

In vitro a synergistic effect of gentamicin and ß-lactam antibiotics has been observed (refs. 6-9). SCHRÖTER et al. have described a synergistic effect between ampicillin and subinhibitory concentrations of gentamicin (ref. 10). We were able to show microcalorimetrically that despite the presence of subinhibitory concentrations of gentamicin, the inactivation of the ß-lactam antibiotic led to the recurrence of the normal listeria-curve in the thermogram, and that therefore synergism did not occur.

The lack of synergism of ampicillin and gentamicin in experimental listeriosis was reported by HOF and GÜCKEL recently using mice (ref. 11). Our clinical observations in the course of a listeria epidemic in Austria in 1986 also did not prove that there was an advantage in the combination of ampicillin and gentamicin versus ampicillin alone (in preparation). Various antimicrobial agents have been used in treatment of listeriosis; however, there is uncertainty as to what regimen constitutes the most effective therapy. We believe the ultimate test of superiority of a drug must come from clinical studies; however, these microcalorimetric studies provide insights into the mode of action of antimicrobial drugs on Listeria monocytogenes which have not been apparent using traditional methods.

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