Enhancement of Penicillin-Induced Lysis in Sarcina lutea by Lysozyme

JOSEPH M. KORN Feld

Laboratory Division, Connecticut State Department of Health, Hartford, Connecticut 06101

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ABSTRACT

Using either paper disc or cylinder plate methods for detection of penicillin, addition of egg-white lysozyme (N-acetylmuramide glycaneohydrolase, E. C. 3.2.1.17) to seeded agar caused production of larger zones of inhibition. The increased zone size enables detection of lesser quantities of the antibiotic. It is suggested that this modification be considered for use in detection of low levels of the antibiotic.

Under regulations of the United States Food and Drug Administration (FDA), presence of penicillin in milk constitutes adulteration. Since the current FDA regulations specify a zero-tolerance for the antibiotic in milk and milk products (4), it follows that any milk which contains any penicillin, no matter how little, is adulterated. From a practical point of view, however, it is the lowest concentration of the antibiotic which is detectable by the prevailing technology which determines the adulteration status.

For detection of penicillin in milk or other foods, the most widely used procedures are microbiological plate assays, employing either Sarcina lutea or Bacillus subtilis as the indicator organism. The use of B. subtilis enables more rapid detection, producing readable results in about 4-5 h. The level of sensitivity obtained with this organism, however, is such that it is difficult to detect concentrations below 0.03 unit/ml of milk. In contrast, the S. lutea method requires overnight incubation, but enables detection of the antibiotic at levels as low as 0.00125 u/ml. It has been claimed by Wright (8) that the S. lutea method was sensitive to 0.005 unit. However, in our experience, the method never detects so low a level, and even 0.0125 u/ml is not detected consistently. Nevertheless, the S. lutea - cylinder plate method has been adopted by the FDA as the official assay procedure for detection of penicillin in milk (3).

It is likely that no assay procedure will ever reach the zero tolerance adopted by the FDA. However, modifications of the assay system should enable detection of lower concentrations than current procedures allow. This paper presents evidence that lesser quantities of penicillin are detected when lysozyme is added to the detection system.

MATERIALS AND METHODS

The procedures used were modified from the established protocols for the assay of penicillin in various materials (2). Antibiotic Agar #4 (BBL) in 100-ml volumes was seeded with 0.03 ml of a suspension of S. lutea (ATCC 9341) prepared by washing the surface of an overnight slant culture with 5 ml of physiological saline. The seeded agar was agitated to ensure homogeneity and maintained at 49 C for not more than 30 min before use. Freshly prepared lysozyme solution (Egg white, Sigma, 3x crystallized) in phosphate buffer, pH 6.0 was added to 100 x 15 mm plastic petri plates and the total volume adjusted to 1 ml with additional buffer. In control plates, only buffer was added. The molten, seeded agar was then added to the petri plates in 4-ml volumes and rapidly mixed with the buffer and/or lysozyme. Originally, the prescribed base layer of 10 ml of Antibiotic Agar #1 was omitted to enable use of stainless steel cylinders. In the presence of both the base layer and cylinders, the petri dish cover could not rest on the lip of the petri dish base resting instead on the upper edges of the cylinders. Since then, it has been shown (5) that omission of the base layer enhances the zone size response to penicillin. After the plates solidified, standards and samples were applied to the surface. When cylinders were used, each contained eight drops of the appropriate penicillin solution; one cylinder always contained only the diluent in which the penicillin standards were prepared.

Penicillin standards were prepared by dissolving a 400,000-unit tablet of Penicillin G (Pfizer) in pH 6.0 buffer and diluting the suspension to give final concentrations of 0.1, 0.05, 0.03, 0.015, 0.0075, and 0.0035 unit/ml. The standards were maintained at 10 C and were discarded after 1 week.

All plates were incubated in an upright position at 32 C, and following overnight incubation, zones of inhibition were estimated.

RESULTS AND DISCUSSION

When lysozyme (0.5 mg) was incorporated into the seeded growth medium, zones of inhibition produced by commercially supplied penicillin discs were dramatically enlarged (Center, Fig. 1). It must be emphasized that in the experiment pictured, a single bottle of seeded agar was used to produce both plates, that both plates contained the same volume of medium, and that both penicillin discs were taken from a single package. The zones of inhibition are caused by the penicillin, but...
enhancement of the size of the zone may only be attributed to the lysozyme incorporated into the agar.

When the cylinder procedure for the assay of antibiotics was substituted for the disc procedure, results shown in Fig. 2 were obtained. Starting at the bottom of each plate and moving clockwise, the concentrations of penicillin G used were (in unit/ml) 0.05, 0.00 (diluent), 0.03, 0.015, 0.0075, and 0.005. Once again, both plates pictured were prepared from a single bottle of seeded agar. In the plate at the left, which contained no lysozyme and served as the control, distinct zones of inhibition are found around the sites of the cylinders which had contained 0.05, 0.03, and 0.015 unit of penicillin/ml. In the plate at the right, which contained 0.5 mg of lysozyme, zones of inhibition are found at all concentrations of penicillin except 0.005 u/ml. It is noteworthy that in the plate containing lysozyme, the dark background is visible through the agar at all the sites formerly occupied by the cylinders, with the sole exception of the diluent site. On the plate devoid of lysozyme, site transparency ends at 0.0075 u/ml. In the assay of penicillin diluted in milk, these transparent sites would not be observed since deposition of milk components would render them opaque. In addition to opacity, use of milk as diluent also causes a reduction in zone diameters, presumably because of the binding of penicillin to milk proteins with a concomitant reduction in the concentration of the free, diffusible antibiotic. Reduction of zone diameters because of protein binding has previously been reported for cefazolin diluted with a variety of body fluids (6). Notwithstanding any protein binding when milk serves as the diluent for penicillin, the zone sizes in the lysozyme plates are always larger than those on the control plate for equivalent penicillin concentrations.

In experiments typified by results in Fig. 2, diameters of zones of inhibition were plotted against the log of the penicillin concentration used to generate dose-response curves. Characteristic results are shown in Fig. 3. The two curves generally parallel each other, with the lysozyme curve displaced to the right. Since the cylinders themselves impose a limit on zone size, an intercept line has been drawn at 8 mm, the diameter of the cylinders.
The intercept of each curve with the 8-mm line indicates the minimum penicillin concentration detectable by each procedure. Although there is variation in the 8-mm intercept on a day to day basis, the addition of lysozyme to the agar always enables the detection of 0.0075 u/ml and frequently detects as little as 0.005 u/ml. Using the routine cylinder procedure, in which lysozyme is not added, both these concentrations would be missed.

Use of S. lutea as the indicator organism was particularly fortuitous. Since the use of lysozyme appeared to render it more sensitive to penicillin, it was thought that other organisms of either equivalent or lower sensitivity to penicillin might be made more sensitive in the presence of the enzyme. A number of other organisms were examined, including Micrococcus agilis, Micrococcus denitrificans, Micrococcus roseus, Micrococcus lysodeikticus, Micrococcus diversus, Micrococcus rubens, and Bacillus subtilis. Of these, some were insensitive to penicillin with no increased sensitivity in the presence of lysozyme and others were so sensitive to lysozyme that their use was contraindicated. Some organisms which were sensitive to penicillin showed no added sensitivity in the presence of lysozyme. To date, no organism which has been tested has proven superior to S. lutea as the indicator organism.

The evidence presented here clearly indicates that lysozyme enhances the effect of penicillin on this organism. However, the mechanism of the enhancement is obscure and the subject of speculation. Although the specifics of cell death mediated by antibiotics which interfere in cell wall synthesis are poorly understood, it appears that lysis results from the concerted action of two separate activities: (a) inhibition of the formation of peptide bridges between peptidoglycan strands, and (b) hydrolysis of specific bonds in the preexisting murein matrix, mediated by autolysins. Among the autolysins cited as involved in the hydrolysis are various peptidases, amidases, glucosaminidases, and glycosidases.

It is proposed that in the phenomenon reported here, the level of endogenous autolysin activity is sufficient to enable lysis only at the relatively high penicillin concentrations which exist within the lytic zone, but not at the lesser concentrations which obtain more distal to the center of diffusion. When lytic activity is augmented by the exogenous addition of glycosidases, lysozyme in this instance, additional glycosidic bonds in the cell walls of the bacteria within this region of low penicillin concentration are destroyed, weakening the cell walls and making them unable to withstand the osmotic force of the cell. Additional lysis then occurs in this area and the zone size is increased.

Irrespective of any rationale concerning the mechanism of the phenomenon, it is clear that use of exogenous lysozyme enhanced the sensitivity of S. lutea in the cylinder plate method. Consequently, its use is indicated in all systems which are concerned with the detection of extremely low levels of penicillin.

REFERENCES