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Penicillins and the limulus amoebocyte lysate test for endotoxin

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The use of the limulus amoebocyte lysate test for the detection of pyrogens has recently been reviewed by Cooper (1975). Eibert (1972) reported that 84 samples of injectable drugs gave consistent results when tested by the official rabbit test or the limulus test. Nguyen & Greppin (1974), using a nephelometric adaptation of the test of Eibert reported that a number of injectable products inhibited the reaction with limulus amoebocyte lysate. I have used a technique similar to that described by Eibert to establish (a) the causes and extent of penicillin inhibition of the limulus test, and (b) the usefulness of the test with penicillins compared with the official rabbit pyrogen test.

Limulus polyphemus amoebocyte lysate, as 0·1 ml limulus reagent from Mallinckrodt Chemical Co., or from Associates of Cape Cod Inc. was added to 0·1 ml of test solution in 7 mm diameter glass test tubes and incubated for 60 min at 37°. Tubes were then gently inverted and read as follows: positive (+) = firm gel adhering to bottom of tube; negative reaction (−) = clear solution with no gelation, running freely from tube; intermediate reaction (±) = viscous or flocculent tube contents deforming on inversion of tube. Positive (endotoxin in water) and negative (water for injection) controls were run in parallel with all tests.

S. minnesota R595 glycolipid endotoxin was prepared according to Galanos, Luderitz & Westphal (1969). Purified *E. coli* lipopolysaccharide was obtained from Associates of Cape Cod Inc. Benzyl penicilloate, BRL 1071 and Penicillin G polymer were prepared in these laboratories according to Clark, Johnson & Robinson (1949), Doyle & Nayler (1961) and Smith & Marshall (1971). Other materials were from commercial sources.

The effect of penicillins at various concentrations on the sensitivity of the limulus test for a purified endotoxin obtained from *S. minnesota* was examined. Results for methicillin (Table 1) showed that in the concentration range 0–10 mg ml^{-1} methicillin had no effect on the gelation reaction, so that the sensitivity of that test for pyrogen in the antibiotic could be increased by increasing the antibiotic concentration. Above 10 mg ml^{-1} methicillin inhibited the limulus gelation reaction such that the sensitivity of the test decreased with increasing antibiotic concentration. For subsequent limulus tests the optimum concentration of methicillin (10 mg ml^{-1}) was used. Qualitatively similar results were obtained with other penicillins, but the highest concentration of antibiotic that failed to inhibit gelation (i.e. the optimum test concentration) varied with the structure of the penicillin; the optimum test concentrations and levels of

pyrogen detection for some clinically important penicillins are given in Table 2. The limit of pyrogen detection for each of these materials was in the parts per thousand million range (pptm).

Several authors (Eibert, 1972; McAuley, Ice & Curtis, 1974; Cooper, 1975) have attempted to control for drug inhibition of the limulus reagent gelatin by the addition of a single 'spike' concentration of endotoxin to the sample under test. The results in Table 1 show this to be unsatisfactory, since a reduction in sensitivity of the test may only be revealed by comparative titration of the pyrogen both in the drug solution and in water.

The limitations of the Pharmacopoeial rabbit pyrogen test as a quantitative test have been reviewed by Bangham (1971). Estimates of the pyrogenic threshold of this test are of the same order as results in Table 2, but the published figures do not permit accurate comparison of the two tests. A direct comparison of the *in vitro* and *in vivo* tests was therefore made by comparing the results of the two tests perfomed in parallel on a substantial number of penicillin samples. Limulus tests were at the optimum concentration for each antibiotic, so that those on methicillin were more sensitive than those on cloxacillin. Rabbit pyrogen tests were performed in accordance with the U.S.P. (1975) using antibiotic at 100mg kg^{-1}.

As tests on penicillins from a commercial plant are of limited value, since the materials are almost always pyrogen-free, some experimental batches of antibiotic, prepared in the laboratory without regard to endotoxin contamination, were also included. Comparative testing results for samples of methicillin are presented in Fig. 1. A good correlation was observed between the two testing procedures for 100 methicillin samples, indicating that the limulus assay was more sensitive than the rabbit test for detection of the pyrogen in these samples ($P < 0.01$ Matnimar Test). The opposite was true for the cloxacillin samples ($P < 0.01$); only samples that were highly pyrogenic in the rabbit caused gelation of limulus reagent whilst some samples that passed the limulus test caused pyrogenic responses in rabbits. These gelled limulus reagent if the penicillin was removed by dialysis. The inferiority of the limulus test for cloxacillin samples was consistent with the relatively high limit of pyrogen detection for this antibiotic as shown in table 2.

Table 1. *Sensitivity of the limulus test in the presence of various concentrations of methicillin.* Serial dilutions of *S. minnesota* R595 glycolipid were performed in 0·9% sodium chloride solution containing the specified concentrations of methicillin and the results of limulus tests on these dilutions were expressed as described in the text.

Methicillin (mg ml^{-1})	Pyrogen concentration (g ml^{-1})5 × 10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}	0	Pyrogen detected *
100	—	—							>50 000
20			+	+	—	—	—	—	250
10				+	+	±	—	—	5
1			+	+	+	±	—	—	50
0		+	+	+	+	±	—		

* g g^{-1} penicillin.

Table 2. *Maximum concentrations of penicillins and 6-aminopenicillanic acid which were observed not to inhibit the limulus assay.*

Material tested	Maximum conc. at which interference absent (mg ml^{-1})	Limit of pyrogen detection pptm*
Methicillin	10	5
Cloxacillin	2	25
Ampicillin	10	5
6 APA	5	10

* Parts per thousand million of *S. minnesota* R595 glycolipid pyrogen in the antibiotic.

Two gel-forming components of limulus amoebocyte lysate have been characterized as proteins; Young, Levin & Prendergast (1972) have described a clotting enzyme which is activated by endotoxin, whilst Solum (1973) has described a second 'coagulogen' protein which is gelled under the influence of activated clotting enzyme.

Since Young & others (1972) have shown that the clotting enzyme is sensitive to mercurial reagents that modify thiol groups, the inhibitory effect on the limulus gelation reaction of some chemically reactive thiol and aldehyde decomposition products of the penicillins

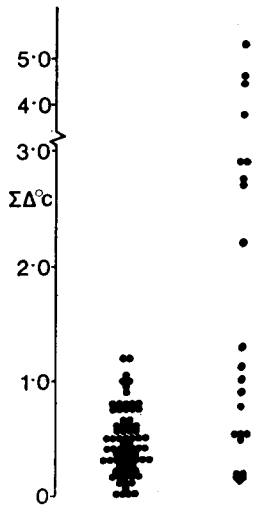

FIG. 1. Scatter diagram showing the distribution of pyrogenic responses for 77 methicillin samples which passed the limulus test (left hand column) and 23 which failed the limulus test (right hand column). Pyrogen tests were performed using 100 mg kg^{-1} in groups of three rabbits. Limulus tests were performed on the same samples using 10 mg ml^{-1} test solutions. All samples which passed the limulus test showed a low pyrogenic response which was within the range specified by the U.S.P. Ordinate: summed rectal temperature increase in three rabbits (°C).

Table 3. *Inhibition of the gelation reaction between limulus reagent and* E.coli *endotoxin in the presence of various penicillin decomposition products and related compounds.* The compounds were tested as 300 mM solutions in the presence of 10^{-7} g ml^{-1} of purified *E. coli* endotoxin.

Inhibitory	Non inhibitory
Penicillin G	Sodium chloride
Benzyl penicilloate	Sodium phenylacetate
Sodium 6-amino-penicillanic acid	Aminoacetaldehyde
Penicillin G Polymer	1-Thiazolidine-4-carboxylic acid
	Penicillamine
	2-Mercaptoethanol
	Ethanal

Table 4. *Correlation of serum binding potential of several penicillins with their ability to inhibit the limulus reaction.* Figures for serum binding were provided by Dr D. Merrikin of these laboratories and were measured for mouse serum. Spearman rank correlation coefficient = 1·0, $P < 0{\cdot}01$.

Pencillin	Protein binding %	Maximum concn at which a positive gelation reaction observed (mM)
Ampicillin	22	150
Methicillin	34	120
Pencillin G	44	60
Penicillin V	73	30
Cloxacillin	80	15
BRL 1071	94	1·5

were examined. The results (Table 3) indicate that the inhibitory effects of penicillins are not mediated by chemical reaction of such compounds with the limulus amoebocyte lysate, nor is the penicillin β-lactam ring, which is essential for the inhibitory effect of the penicillins towards enzymes of the bacterial cell wall, involved in inhibition of the limulus gelation reaction.

In the search for an explanation of the greater inhibitory effect of cloxacillin relative to the other antibiotics, it was noted that cloxacillin is relatively highly serum bound (Rolinson & Sutherland, 1965). The concentration of a penicillin necessary to inhibit limulus gelation was therefore compared with the ability of that penicillin to bind to serum proteins. A statistically significant correlation was obtained for the five commercially important antibiotics tested ($P < 0{\cdot}01$) (Table 4). The correlation was confirmed by testing the inhibitory effect of triphenylmethyl penicillin, BRL 1071, which is very heavily serum bound and thus showed the predicted strong interference in the limulus test by completely inhibiting the reaction with 1 ng of *E. coli* lipopolysaccharide at a 1·5 mM concentration. This correlation is of practical value in predicting the probable level of interference of other penicillins in the limulus reaction, since serum binding of these antibiotics has been widely studied. Whether this type of correlation holds for structurally dissimilar molecules was not investigated, but it is known from competitive binding experiments that structurally unrelated drugs, such as sulphadimidine, sodium salicylate and novobiocin have affinity for the same protein binding sites as penicillins (Rolinson & Sutherland, 1965). Nguyen & Greppin (1974) have reported that cephelothin inhibits limulus gelation and have suggested that this may be due to an osmotic or redox effect. The present studies do not support this view and suggest that cephelothin, like the penicillins, inhibits the gelation reaction by virtue of its capacity for protein binding (Barker & Prescott, 1973).

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