약제학회지 (1986), 제 16 권 제 2 호
*J. Korean Pharm. Sci.*, Vol. 16, No. 2, 85~88 (1986)

# Studies on Effects of Antibiotics on Pyrogen Tests

**Kwang-Bum Shin, Young-Joon Song and Jung-Woo Kim**

*Chong Kun Dang Corporation*

(Received April 3, 1986)

To estimate the effect of some injectable antibiotics (ampicillin sodium, cefazolin sodium, cephaloridine, cefuroxime sodium and chloramphenicol sodium succinate) on pyrogen tests, the Limulus amebocyte lysate (LAL) test and an ultrafiltration technique were used. The rabbit pyrogen test was also used in the case of cafazolin sodium. At high antibiotic concentrations, these samples which were artificially contaminated with endotoxin inhibited the gelation reaction of LAL. But the gelation reaction occurred when most of the antibiotic was removed by ultrafiltration. Likewise, cefazolin sodium interfered not only with the LAL test but also with the rabbit pyrogen test. From these results it can be said that special modification to eliminate interference should be taken into consideration for valid method of pyrogen tests in the parenteral products containing these antibiotics.

Pyrogens may be responsible for fever and other toxic reactions in man, if they occur in sufficient amounts in parenterals.[1] It is generally believed that pyrogens are endotoxins, specially lipopolysaccharides, usually resulting from the autolysis of cell walls of gram-negative bacteria.[2-4] Both raw materials and inadequate manufacturing procedure might contribute to the endotoxin contamination, and the toxic activity of endotoxins is not easily removed by conventional method.[5]

Endotoxins are usually detected by the rabbit pyrogen test,[6,7] and by the Limulus amebocyte lysate (LAL) test specified in the USP XXI.[8]

The LAL test is based on the discovery of Levin and Bang that the amebocytes of the *Limulus polyphenmus* coagulate in the presence of endotoxin.[9] A number of later investigators have demonstrated that the amebocyte lysate contains a clottable protein which reacts specifically with trace amounts of endotoxin to form an opaque gel.[10,11]

The sensitivity of the LAL test, however, is affected by the presence of certain drugs.[12-14] The rise of the rabbits' body temperature caused by the endotoxin also is sometimes inhibited by the pharmacologic activity of the coexistent drugs.[15,16]

Newsome showed that penicillins inhibited the sensitivity of the LAL test.[17] Recently Takahashi *et al.* reported the detection of trace amounts of pyrogens in injectable ampicillin sodium preparations using ultrafiltration technique.[18]

This study reports the effects of some injectable antibiotics (ampicillin sodium, cefazolin sodium, cephaloridime, cefuroxime sodium, and chloramphenicol sodium succinate) on the sensitivity of the LAL test. In the case of cefazolin sodium, the effect on the rabbit pyrogen test is also reported in this paper.

## EXPERIMENTAL

### Samples and Reagents

Injectable ampicillin sodium, cefazolin sodium, cephaloridine, cefuroxime sodium and chloramphenicol sodium succinate preparations (Chong Kun Dang Co.) were used as the test samples. *E. coli* Endotoxin® and Pyrogent® were purchased from Malinckrodt. The Blue

Dextran® solution (2μg/ml of dye-combined dextran) was also purchased from Sigma Chemical Co., and it was used after confirmed to be pyrogen-free. Pyrogen-free distilled water was used as a solvent.

**Ultrafiltration Apparatus**

Ultrafilitration of the test solutions was performed with the use of Centricon-10 microconcentrator® (Amicon Co.) designed for use in a centrifuge. A diagramatic representation of this apparatus are shown in Fig. 1.

**Experimental Animals**

New Zealand white rabbits weighing 2.0-3.5kg were used in the experiment. They were bred and maintained in a constant temperature (25 ± 2°C) and relative humidity (50 ± 10%). Only those rabbits that showed good endotoxin sensitivity were conditioned for 3 hours prior to the pyrogen test by conducting sham tests (i.e. without injection).

**LAL Test**

A 0.1ml aliquot of the reconstituted lysate was added to the bottom of a number of pyrogen-free, 10 × 75mm test tubes sufficient to perform the necessary tests. A 0.1 ml aliquot of the sample solution was transferred to the tubes, which were stoppered and incubated for 1 hour in a 37 ± 1°C water bath. Special caution was taken not to disturb the tubes during this incubation period. The tubes were examined at the end of 1 hour by carefully inverting them 180 degrees. Each tube was judged using the following four grades : ( + + ) a solid gel was formed and it did not move when the tube was inverted; ( + ) although a gel was formed, it moved when the tube was inverted; ( ± ) a coarse granular gel was formed and the viscosity was increased; and ( − ) the media remained in the liquid state without any change.

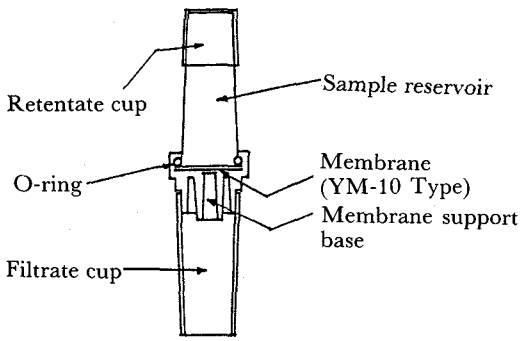


**Figure 1** — Diagramatic representation of ultrafiltration apparatus.

**Ultrafilitration**

All glass apparatus were depyrogenized by heating at 250°C for 2 hrs. The inside of ultrafiltration apparatus was washed three times with pyrogen-free distilled water, and the washings were confirmed to be pyrogen-free by the LAL test. The surface of the membrane was coated with 1 ml of dye-combined dextran (average molecular weight 2,000,000) solution to prevent adsorption of the endotoxin. The test sample was dissolved in pyrogen-free distilled water and diluted to the required concentrations, 100, 75 and 50 mg (potency)/ml, which were artificially contaminated with 10 ng/ml of endotoxin. Each 2 ml of these sample solutions was placed in the ultrafiltration apparatus, and the ultrafiltration was carried out by centrifugation at about 1200 G until the volume of the residual solution was reduced to one-third. After the addition of pyrogen-free distilled water to the original volume, filtration was repeated until a half of the original volume was remained. Finally pyrogen-free distilled water was added to the retentate to make the original volume, and then the retentate and filtrate were detected by the LAL test.

**Rabbit Pyrogen Test**

Rabbit rectal temperatures were measured using a thermoelectric couple-type thermometer (Type APT 75, Ellab Instruments, Denmark). Temperatures were measured twelve times at intervals of 15 min. prior to administration: the twelfth measurement was regarded as the control. Immediately thereafter, the sample solutions (2 ml/kg) were administered intravenously through the auricular vein, and rectal temperatures were measured at intervals of 15 min. for 3 hours. The difference between the highest temperature recorded after injection and the control was regarded as the rise in

**Table I** – *Influence of the Antibiotic Concentration on the LAL Test.*

| Sample | Concentration of the samples containing 10ng/ml of endotoxin[a], mg(potency)/ml | | | | | | |
|---|---|---|---|---|---|---|---|
| | 100 | 75 | 50 | 25 | 12.5 | 0 | Negative[b] control |
| Ampicillin sod. | − | − | + | ++ | ++ | ++ | + |
| Cephaloridine | − | − | ++ | ++ | ++ | ++ | + |
| Cefazolin sod. | − | − | ++ | ++ | ++ | ++ | + |
| Cefuroxime sod. | − | − | + | ++ | ++ | ++ | + |
| Chloramphenicol sod. succinate | − | − | ± | + | + | ++ | + |

[a] *E. coli* 055-B5. [b] LAL test for water for injection.

temperature.

## RESULTS AND DISCUSSION

The samples, which were dissolved in pyrogen-free distilled water, diluted to the required concentrations and artificially contaminated with 10 ng/ml of endotoxin, were tested by the LAL test. As shown in Table I, the gelation reaction by 10 ng/ml of endotoxin was inhibited by low concentration (50mg as potency/ml) of the antibiotic samples except chloramphenicol sodium succinate. Noticeably chloramphenicol sodium succinate inhibited the gel reaction even at relatively low concentration. In ampicillin sodium, Newsome[17] and Takahashi *et al.*[18] reported the same results.

**Table II** – *LAL Test of Antibiotics After Ultrafiltration.*

| Sample | | Concentration of the samples containing 10 ng/ml of endotoxin[a], mg(potency)/ml | | |
|---|---|---|---|---|
| | | 100 | 75 | 50 |
| Ampicillin sod. | Retentate | + | + | + |
| | Filtrate | − | − | ± |
| Cephaloridine | Retentate | ++ | ++ | ++ |
| | Filtrate | − | − | − |
| Cefazolin sod. | Retentate | ++ | ++ | ++ |
| | Filtrate | − | − | − |
| Cefuroxime sod. | Retentate | ++ | ++ | ++ |
| | Filtrate | − | − | − |
| Chloramphenicol sod. succinate | Retentate | + | + | ++ |
| | Filtrate | − | − | − |

[a] *E. coli* 055-B5

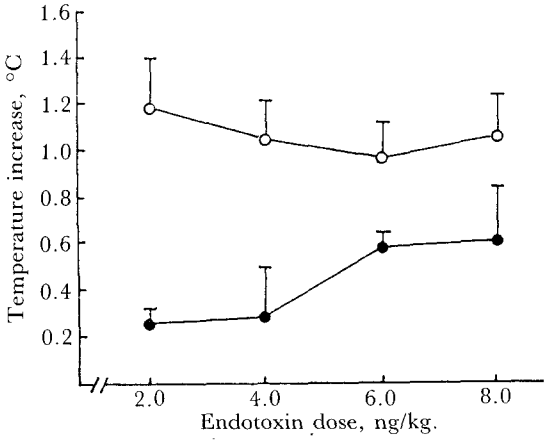


**Figure 2** — Dose-response curve of *E. coli* endotoxin in the rabbit pyrogen test.
Key: O——O, endotoxin solution without cefazolin sodium (n = 3); ●——●, endotoxin solution containing 400mg of cefazolin sodium (n = 3)

To separate antibiotics from endotoxin, the sample solutions which showed the inhibition of gel reaction were ultrafiltrated. After ultrafiltration the retentate and filtrate were tested by the LAL test, and the results are summarized in Table II.

Although the retentate of ampicillin sodium and chloramphenicol sodium succinate showed weak gel reaction, others exhibited strong gel reaction. In all the filtrate, however, no gel reaction was observed.

These results from Table I and Table II indicate that the gel reaction by the LAL test is inhibited by certain concentrations of the antibiotics used in this experiment.

On the other hand, to estimate the effect of antibiotics on the rabbit pyrogen test, we picked out cefazolin sodium and compared the rise of rabbits' body temperature after the administration of endotoxin solution with and without cefazolin sodium.

As shown in Fig. 2, although the data for the endotoxin solutions without cefazolin sodium did not show dose-dependent temperature increase, there was a clear difference in temperature

increase between endotoxin solution with and without cefazolin sodium.

These *in vivo* results showed that cefazolin sodium has an inhibitory effect on temperature rise in rabbit so that the sensitivity of the official pyrogen test might be decreased in the parenteral products containing certain antibiotics such as cefazolin sodium. These results were also consistent with the results for ampicillin sodium of Takahashi *et al.*[18]

The mechanism to inhibit the sensitivity of the LAL test and the rabbit pyrogen test is not known clearly. Newsome[17] reported that the inhibitory effects of penicillins was not mediated by chemical reaction of various penicillin decomposition products, nor was the penicillin $\beta$-lactam ring involved in inhibition of the LAL test. And he also reported that there was some correlation of serum binding potential of several penicillins with their ability to inhibit the Limulus reaction.

## CONCLUSION

It was found that three cephalosporins and two other antibiotics had an inhibitory effect on the gelation reaction of the LAL test, and cefazolin sodium interfered with the sensitivity of the rabbit pyrogen test. These resultls are summarized in the following:

1. Ampicillin sodium, cefazolin sodium, cephaloridine, cefuroxime sodium, and chloramphenicol sodium succinate inhibited the sensitivity of the LAL test.
2. These antibiotics could be separated from the endotoxin by ultrafiltration.
3. Cefazolin sodium interfered not only with the LAL test but also with the rabbit pyrogen test.
4. Special modification to eliminate the interference is necessary for valid method of pyrogen tests in these antibiotic parenterals.

## ACKNOWLEDGEMENTS

All the members of Chong Kun Dang Central Research Laboratory and Quality Control Department are acknowledged for their assistance.

## REFERENCES

1) G. Haska and R. Nystrand, *Appl. Environ. Microbiol.*, **38**, 1078 (1979)
2) C. Baggerman, C. Pathmamanoharan, F. Spies, J.G.H. Joosten and H.E. Junginger, *J. Pharm. Pharmacol.*, **37**, 521 (1985)
3) R.E. Wachtel and Kiyoshi, *Appl. Environ. Microbiol.*, **33**, 1265 (1977)
4) R.J. McAuley, R.D. Ice and E.G. Curtis, *Amer. J. Hosp. Pharm.*, **31**, 688 (1974)
5) K.J. Sweadner, M. Forte and L.L. Nelsen, *Appl. Environ. Microbiol.*, **34**, 382 (1977)
6) U.S. Pharmacopeia, 21st Rev., U.S. Pharmacopeial Convention, Inc., Rockville, Md., 1985, p. 1181.
7) Korean Pharmacopeia, 4th Rev., Korea Pharmaceutical Association, Seoul, 1982, p. 1165.
8) U.S. Pharmacopeia, 21st Rev., U.S. Pharmacopeial Convention, Inc., Rockville, Md., 1985, p. 1165.
9) J. Levin, and F.B. Bang, *Bull. Johns Hopkins Hosp.*, **115**, 265 (1964)
10) J.Y. Tai and T.Y. Liu, *J. Biol. Chem.*, **252**, 2178 (1977)
11) J.D. Sullivan, JR., and S.W. Watson, *Biochem. Biophys. Res. Com.*, **66**, 848 (1975)
12) D.R. Daoust, S.J. Orlowski, G. McMahon, C.K. Weber, W.C. Shaw, T.C. Fisher, C.R. Bennett and A. Gray, *Bull. Parenteral Drug. Assoc.*, **30**, 13 (1976)
13) J.D. Sullivan, JR., and S.W. Watson, *Appl. Microbiol.*, **28**, 1023 (1974)
14) T.J. Novitsky, S.S. Ryther, M.J. Case and S.W. Watson, *J. Parenteral Sci. Technol.*, **36**, 11 (1982)
15) S. Minami, N. Sakaguchi, and S. Shintani, *J. Takeda Res. Lab.*, **33**, 213 (1974)
16) K. Tsuji, K.A. Steindler and S.J. Harrison, *Appl. Environ. Microbiol.*, **40**, 533 (1980)
17) P.M. Newsome, *J. Pharm. Pharmacol.*, **29**, 704 (1977)
18) S. Takahashi, S. Yano, U. Nagaoka, K. Kawamura and S. Minami, *J. Pharm. Sci.*, **72**, 739 (1983)