Sensitivity of the Limulus Test and Inhibitory Factors in the Radiopharmaceuticals

Hajime Murata, Masayoshi Kobayashi,* Masahiro Iio, Hideo Yamada, Kazuo Chiba, Kengo Matsui, and Shinichiro Kawaguchi

Tokyo Metropolitan Geriatric Hospital, Tokyo, Japan

The basic sensitivity of the Limulus test and the inhibitory factors were examined in 21 radiopharmaceuticals commonly used in Japan. The sensitivity of the Limulus test using pre-gel was found to be 1 μg/ml for* Escherichia coli *endotoxin. This sensitivity is about ten times that of the rabbit test adopted by USP and JP. The Limulus test was applicable, with full sensitivity and without inhibitory reaction, for the evaluation of $^{99m}TcO_4^-$, ^{99m}Tc-albumin, ^{99m}Tc-MAA, ^{99m}Tc-Sn-colloid, ^{131}I-Hippuran, $Na^{131}I$, $Na_2{}^{51}CrO_4$, ^{67}Ga citrate, and ^{57}Co-bleomycin as commercially supplied. On the other hand, with ^{111}In-DTPA, ^{99m}Tc-phytate, ^{99m}Tc-pyrophosphate, ^{99m}Tc-DTPA, ^{131}I-polyvinylpyrrolidone (PVP), $^{59}FeCl_3$, $Na_2{}^{32}PO_4$, ^{198}Au colloid, and selenomethionine (Se-75), the pH required adjustment to avoid inhibition of the gelation reaction. Benzyl alcohol showed an inhibitory effect on the gelation reaction at concentrations of more than 1%. Iodine-131-Bromsulphalein (BSP) and ^{131}I-rose bengal showed intense inhibition of the gelation reaction.

J Nucl Med 17: 1088–1092, 1976

The specific gelation reaction between the Limulus lysate and the endotoxin of gram-negative bacteria reported by Bang et al. (*1*) in 1965 provides a sensitive method for the detection of these endotoxins. Recently, Cooper et al. recommended adopting the Limulus test for the detection of endotoxin in the radiopharmaceuticals used for cisternography. Occasional contamination of these agents with small amounts of endotoxin has caused adverse reactions following cisternography (*2*). A false-negative result may occur, however, if due attention is not paid to inhibitory factors in the test solution. Accordingly, in order to detect the presence of inhibitors in a given radiopharmaceutical, a preliminary Limulus test should be performed on the radiopharmaceutical to which the endotoxin is added at a detectable concentration.

The purpose of the present study is to examine factors that might cause inhibition of the Limulus test in 21 commonly used radiopharmaceuticals.

MATERIALS

Limulus lysate. Dry Limulus lysate was prepared from the amebocytes of the Japanese horseshoe crab *Tachypleus tridentatus*. This lysate is known as pre-gel.*

Endotoxin. *Escherichia coli* endotoxin 0111, B_4 (Difco lot No. 564-550) was used in this study.

Buffer. The pH of the test solution was adjusted with 0.2 *M* Tris–HCl buffer of pH 7.2 or 8.0.

Commercially available distilled water and saline solution, as permitted by *JP* for intravenous administration, were used in this study. The basic sensitivity of the Limulus test and inhibitory factors were examined in 21 radiopharmaceuticals as follows:

Received Sept. 25, 1975; revision accepted May 25, 1976.

For reprints contact: Hajime Murata, Tokyo Metropolitan Geriatric Hospital, 35-2 Sakae-cho, Itabashi-ku, Tokyo 173, Japan.

* Teikoku Hormone Mfg. Co., Kawasaki, Japan.

TABLE 1. OPTIMAL pH OF REACTION MIXTURE FOR GELATION REACTION

	pH of solution	Concentration of endotoxin (ng/ml)					
		10	5	2.5	1	0.5	0.25
	4.75	+	−	−	−	−	−
	5.00	+	±	−	−	−	−
	5.25	++	+	±	−	−	−
	5.50	++	+	±	−	−	−
	5.75	++	+	±	±	−	−
Optimal pH range	6.00	++	++	+	±	−	−
	6.25	++	++	+	+	±	−
	6.50	++	++	++	++	+	±
	6.75	++	++	++	++	+	±
	7.00	++	++	++	+	±	−
	7.25	++	++	+	+	±	−
	7.50	++	+	+	±	−	−
	7.75	++	+	±	−	−	−
	8.00	+	±	−	−	−	−
	8.25	+	−	−	−	−	−

1. Agents used for cisternography: ^{169}Yb-DTPA† and ^{111}In-DTPA.†
2. ^{99m}Tc-labeled agents: $^{99m}TcO_4^-$,† ^{99m}Tc-albumin,† ^{99m}Tc-MAA,‡ ^{99m}Tc-Sn-colloid,† ^{99m}Tc-phytate,† ^{99m}Tc-pyrophosphate,‡ and ^{99m}Tc-DTPA.†
3. ^{131}I-containing agents: $Na^{131}I$,† ^{131}I-Hippuran,† ^{131}I-rose bengal,† ^{131}I-polyvinylpyrrolidone (PVP),‡ and ^{131}I-Bromsulphalein (BSP).†
4. Miscellaneous agents: $Na_2{}^{51}CrO_4$,† ^{67}Ga citrate,† selenomethionine (Se-75),† ^{57}Co-bleomycin,†, $^{59}FeCl_3$,† $Na_2{}^{32}PO_4$,† and ^{198}Au colloid.†

METHODS

Limulus test. The pre-gel (400 μg of dry Limulus lysate) was dissolved in 0.1 ml of distilled water and added to 0.1 ml of the test radiopharmaceutical. Then this reaction mixture was incubated for 60 min at 37°C.

The quality of the resulting gel was graded as follows: (A) A gel retaining its solid formation when tilted at 45° was judged as 2+. (B) A gel that formed but was movable as a mass was judged as 1+. (C) A granular gel with high viscosity but still fluid when tilted was judged as equivocal (±). (D) Ungelled solution was judged as minus.

Acidity of reaction solution. When the pH of the test solution or reaction mixture was outside the optimal range for gel formation, the pre-gel was dissolved in 0.1 ml of Tris–HCl buffer instead of distilled water. In our laboratory the optimal pH range for the gelation reaction was found to be 6.0–7.5 (Table 1). To examine the sensitivity and inhibitory factors, preliminary Limulus tests were performed on the radiopharmaceuticals. These were added to seven known concentrations of *E. coli* endotoxin ranging from 0 to 10 μg/ml.

The results were compared with those of a control study using similar concentrations of endotoxin in saline. The sensitivity of the Limulus test and inhibitory factors in the radiopharmaceuticals were thus examined. Each of the radiopharmaceuticals was procured from at least two lots, and additional studies were performed on the materials suspected of endotoxin contamination.

RESULTS

Sensitivity. Test solutions of *E. coli* endotoxin were prepared in seven concentrations ranging from 0 to 10 μg/ml. The minimum concentration required to produce an incompletely solid gel (taken as the end point) was 1 μg/ml. In these studies this end point is used to assess the sensitivity of the present Limulus test reagent (at pH 6.3) for the positive control of *E. coli* endotoxin.

Inhibitory factors in the radiopharmaceuticals used for cisternography. The results of the Limulus test on ^{169}Yb-DTPA and ^{111}In-DTPA are shown in Table 2. Adjustment of the pH is needed to avoid inhibition of the gelation reaction because in both ^{169}Yb-DTPA and ^{111}Yb-DTPA the pH is below the optimal range.

TABLE 2. RADIOPHARMACEUTICALS FOR CISTERNOGRAPHY

Radiopharmaceuticals	pH of solution	Gel formation at various concentrations of added endotoxin (ng/ml)						
		10	5	2.5	1	0.5	0.25	0
^{169}Yb-DTPA	4.8	—	—	—	—	—	—	—
	7.2*	++	++	+	±	—	—	—
^{111}In-DTPA	5.5	++	++	+	—	—	—	—
	6.6*	++	++	++	++	±	—	—
Control	6.3	++	++	++	+	—	—	—

* pH adjusted.

TABLE 3. INHIBITION OF GELATION REACTION BY DTPA

	DTPA concentration* in reaction mixture	Gel formation at various concentrations of added endotoxin (ng/ml)						
		10	5	2.5	1	0.5	0.25	0
DTPA	10^{-2} M	−	−	−	−	−	−	−
	10^{-3} M	++	++	±	−	−	−	−
	10^{-4} M	++	++	++	+	−	−	−
	10^{-5} M	++	++	++	+	−	−	−
Control (saline)	0	++	++	++	+	−	−	−

* Dissolved in saline.

TABLE 4. $^{99m}TcO_4^-$ AND ^{99m}Tc-LABELED RADIOPHARMACEUTICALS

Radiopharmaceuticals	pH of solution	Gel formation at various concentrations of added endotoxin (ng/ml)						
		10	5	2.5	1	0.5	0.25	0
$^{99m}TcO_4^-$	6.2	++	++	++	+	−	−	−
^{99m}Tc-albumin	6.0	++	++	++	++	++	+	±
^{99m}Tc-MAA	6.0	++	++	++	+	−	−	−
^{99m}Tc-Sn-colloid	6.2	++	++	++	++	++	++	++
^{99m}Tc-phytate	5.4	++	++	+	−	−	−	−
	6.2*	++	++	±	−	−	−	−
^{99m}Tc-pyrophosphate	4.4	−	−	−	−	−	−	−
	7.4*	++	++	++	+	−	−	−
^{99m}Tc-DTPA	5.8	++	++	+	±	−	−	−
	6.6*	++	++	++	+	−	−	−
Control	6.3	++	++	++	+	−	−	−

* pH adjusted.

Since the ^{169}Yb-DTPA available in Japan contains 0.9% benzyl alcohol, Limulus tests were performed with several concentrations of benzyl alcohol in saline. Gelation was inhibited completely with 2% benzyl alcohol, and 1% showed slight inhibition. At 0.5%, however, benzyl alcohol was equal to that of control, and no inhibition was observed.

The chelating agent DTPA also inhibits the gelation reaction at concentrations more than 10^{-3} *M*, but no inhibition was observed in the present study because the commercially available radiopharmaceuticals contained less than 10^{-4} *M* DTPA (Table 3).

Inhibitory factors and endotoxin contamination in the technetium products. For these the results of the Limulus test are shown in Table 4. With commercially supplied $^{99m}TcO_4^-$, ^{99m}Tc-albumin, ^{99m}Tc-MAA, and ^{99m}Tc-Sn-colloid, the pH stayed in the optimal range and no adjustment was needed.

In $^{99m}TcO_4^-$ and ^{99m}Tc-MAA, gel formation paralleled that of the control, thus indicating the expected quantities of added endotoxin. It follows that $^{99m}TcO_4^-$ and ^{99m}Tc-MAA can be checked by the Limulus test with normal sensitivity and without inhibition.

In the gelation reactions with ^{99m}Tc-phytate and ^{99m}Tc-pyrophosphate, the pH was lower than optimal, probably due to chelating reactions between the phytate or pyrophosphate and the metallic ions in the pre-gel. Here inhibition of gel formation was observed.

After pH adjustment, however, ^{99m}Tc-pyrophosphate showed normal sensitivity in the Limulus test. On the other hand, even after pH adjustment, ^{99m}Tc-phytate suffered from minor inhibition in the Limulus test, with a sensitivity of 2.5 μg/ml of endotoxin.

Commercially supplied ^{99m}Tc-DTPA also showed moderate inhibition due to unsuitable pH, but this was overcome by pH adjustment. In this series, ^{99m}Tc-albumin and ^{99m}Tc-Sn-colloid showed endotoxin contamination.

^{131}I-containing radiopharmaceuticals. These were also evaluated and the results are shown in Table 5.

The Limulus test was found to be applicable to Na^{131}I and ^{131}I-Hippuran without pH adjustment, and endotoxin contamination was found in both. The ^{131}I-PVP required pH adjustment to make the test successful; however, ^{131}I-BSP and ^{131}I-rose ben-

TABLE 5. $Na^{131}I$ AND ^{131}I-LABELED RADIOPHARMACEUTICALS

Radiopharmaceuticals	pH of solution	Gel formation at various concentrations of added endotoxin (ng/ml)						
		10	5	2.5	1	0.5	0.25	0
$Na^{131}I$	6.6	++	++	++	++	++	++	++
^{131}I-Hippuran	6.2	++	++	++	++	++	±	±
^{131}I-rose bengal	6.4	−	−	−	−	−	−	−
^{131}I-PVP	5.2	−	−	−	−	−	−	−
	6.4*	++	++	+	+	±	−	−
^{131}I-BSP	5.8	−	−	−	−	−	−	−
	6.7*	−	−	−	−	−	−	−
Control	6.3	++	++	++	+	−	−	−

* pH adjusted.

TABLE 6. MISCELLANEOUS RADIOPHARMACEUTICALS

Radiopharmaceuticals	pH of solution	Gel formation at various concentrations of added endotoxin (ng/ml)						
		10	5	2.5	1	0.5	0.25	0
$Na_2{}^{51}CrO_4$	6.2	++	++	++	++	+	±	±
^{67}Ga citrate	6.6	++	++	++	+	−	−	−
^{57}Co-bleomycin	6.0	++	++	++	++	++	+	±
$^{59}FeCl_3$	5.4	±	−	−	−	−	−	−
	6.7*	++	++	++	+	−	−	−
$Na_2{}^{32}PO_4$	5.6	+	+	±	−	−	−	−
	6.6*	++	++	+	±	−	−	−
^{198}Au colloid	5.4	−	−	−	−	−	−	−
	6.0*	++	++	++	++	++	++	++
Selenomethionine (Se-75)	5.5	±	±	−	−	−	−	−
	6.6*	++	++	++	++	+	+	±
Control	6.3	++	++	++	+	−	−	−

* pH adjusted.

gal showed intense inhibition of the gelation reaction both before and after pH adjustment.

Miscellaneous radiopharmaceuticals (Table 6). Before testing, $^{59}FeCl_3$, $Na_2{}^{32}PO_4$, ^{198}Au colloid, and selenomethionine (Se-75) required pH adjustment. In this group, $^{59}FeCl_3$ showed the same end point as the control study and $Na_2{}^{32}PO_4$ showed slight inhibition in the Limulus test.

Endotoxin contamination in the radiopharmaceuticals. Limulus tests were performed in 1:10 and 1:100 dilutions of eight radiopharmaceuticals with suspected endotoxin contamination in the original concentration. Limulus tests were judged as minus in 1:10 dilutions of ^{99m}Tc-albumin, ^{131}I-Hippuran, $Na_2{}^{51}CrO_4$, ^{57}Co-bleomycin, and selenomethionine (Se-75).

In the radiopharmaceuticals that gave a 2+ result in the Limulus test in their original concentrations, 1:100 dilutions of ^{99m}Tc-Sn-colloid and $Na^{131}I$ resulted in negative Limulus tests. On the other hand, ^{198}Au colloid, whose Limulus test was rated 2+ in the original concentration, gave an equivocal result even in 1:100 dilution.

Limulus tests were performed, undiluted, on several lots of ^{198}Au colloid and ^{99m}Tc-Sn-colloid. In ten lots of ^{198}Au colloid, the results were judged positive in three, equivocal in one, and minus in six. In five lots of ^{99m}Tc-Sn-colloid, on the other hand, three were positive or equivocal and two were negative.

DISCUSSION

When gel formation does not occur in the preliminary (positive control) Limulus test on a specific radiopharmaceutical, to which endotoxin is added at a detectable concentration, it is concluded that inhibitory factors were contained in the radiopharmaceutical and that a false-negative result might occur in a routine Limulus test.

Inhibitory factors may include either inhibitory contaminants or an acidity (*3–5*) that lies outside the favorable pH range. In the latter case, the in-

hibitory reaction can be avoided by proper pH adjustment. In this study, inhibition due to pH was recognized in nine out of 21 radiopharmaceuticals, but no inhibition was observed after pH adjustment by Tris–HCl buffer.

Other inhibitory factors include substances such as some protein-denaturing agents (*6,7*), antiseptics (*7*), sulhydryl compounds (*7*), and chelates (*7–10*).

In the radiopharmaceuticals studied, ^{131}I-BSP and ^{131}I-rose bengal showed intense inhibition of gel formation both before and after pH adjustment. The inhibition is probably due to lysate inactivation by BSP and rose bengal. Nevertheless, the possibility of inactivation of the low levels of endotoxin by the dyes cannot be ruled out.

The importance of detecting the endotoxin in the agents used for cisternography is emphasized by the finding that the CSF circulating system is at least 1,000 times as sensitive to endotoxin as is the circulating blood (*2*). In the cat, direct injection of endotoxin into the cerebral ventricle also induces fever (*11*).

The routine Limulus test was found to be applicable to 19 out of 21 radiopharmaceuticals for the detection of endotoxin contamination. In some of the tracers studied it gave a positive reaction, indicating the presence of endotoxin contamination.

In the present study we did not compare the results of the Limulus test for endotoxin with the standard pyrogen test using rabbits. Good correlation has been found between the two tests by other workers (*3,5,12,13*).

If the sensitivity of the Limulus test to a given endotoxin is given the symbol L, and the corresponding sensitivity of the rabbit test is called R, the ratio L/R for various endotoxins ranges from 1 to 10 (*3,7*). For *E. coli* endotoxin (0111, B_4), L/R = 10, which means that the Limulus test will detect 1 ng/ml whereas the rabbit test requires 10 ng/ml. For other endotoxins, where L/R may be less than 10, the Limulus test may remain negative in spite of a positive rabbit test.

The Limulus test, where applicable, is simpler, more rapid, and more sensitive than the rabbit test. It is adaptable for use in nuclear medicine and commercial laboratories.

ACKNOWLEDGMENT

This study was presented in part at the 22nd Annual Meeting of the Society of Nuclear Medicine, held in Philadelphia, June 18, 1975.

FOOTNOTES

* Product of Teikoku Hormone Mfg. Co., Ltd., Japan.

† Indicates two joint-venture companies and manufacturing foreign and Japanese pharmaceuticals.

‡ Indicates two distributors of foreign pharmaceutical products.

REFERENCES

1. BANG FB: The bacterial disease of Limulus Polyphemus. *Bull Johns Hopkins Hosp* 98: 325–351, 1956

2. COOPER JF, HARBERT JC: Endotoxin as a cause of aseptic meningitis after radionuclide cisternography. *J Nucl Med* 16: 809–813, 1975

3. KOBAYASHI M, YAMAMOTO M: Preparation of pre-gel from the amebocytes of Japanese horseshoe crab (*Tachypleus tridentatus*) with the anti-aggregating effect of methylxanthins II. *Yakugaku Zasshi* 94: 298–303, 1974

4. LEVIN J, TOMASULO PA, OSER RS: Detection of endotoxin in human blood and demonstration of an inhibitor. *J Lab Clin Med* 75: 903–911, 1970

5. NIWA M, HIRAMATSU T, WAGURI O: The significance of Limulus test as a microassay of endotoxin. *Mod Media* 20: 137–152, 1974

6. YOUNG NS, LEVIN J, PRENDERGAST RA: An invertebrate coagulation system activated by endotoxin: Evidence for enzymatic mediation. *J Clin Invest* 51: 1790–1797, 1972

7. KOBAYASHI M, MORIMOTO T, YAMAMOTO M: Studies on the gelation reaction of Limulus lysate (pre-gel). III. Influences of various substances on the gelation reaction of Limulus lysate. *Yakugaku Zasshi* 95: 720–727, 1975

8. KOBAYASHI M, YAMAMOTO M: Studies on the gelation reaction of Limulus lysate (pre-gel). IV: Effect of alkaline earth metals and chelating agents on the gelation reaction of Limulus lysate. *Yakugaku Zasshi* 95: 959–965, 1975

9. LEVIN J, BANG FB: Clottable protein in Limulus: Its localization and kinetics of its coagulation by endotoxin. *Thromb Diath Haemorrh* 19: 186–197, 1968

10. LEVIN J, BANG FB: The role of endotoxin in the extracellular coagulation of Limulus blood. *Bull Johns Hopkins Hosp* 115: 265–274, 1964

11. CLARK WG, CUMBY HR, DAVIS HE: The hyperthermic effect of intracerebroventricular cholera enterotoxin in the unanaesthetized cat. *J Physiol* 240: 493–504, 1974

12. COOPER JF, HOCHSTEIN HD, SELIGMAN EB: The Limulus test for endotoxin (pyrogen) in radiopharmaceuticals and biologicals. *Bull Parenter Drug Assoc* 26: 153–162, 1972

13. COOPER JF, LEVIN J, WAGNER HN: Quantitative comparison of in vivo and in vitro methods for the detection of endotoxin. *J Lab Clin Med* 78: 138–148, 1971