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False-Positive Result in *Limulus* Test Caused by *Limulus* Amebocyte Lysate-Reactive Material in Immunoglobulin Products

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Limulus **amebocyte lysate (LAL)-reactive material other than endotoxin was detected in the plasma and urine of patients after intravenous immunoglobulin therapy. Thirty-seven vials of six different immunoglobulin products were analyzed for the LAL-reactive material by combined use of a conventional chromogenic *Limulus* test and a chromogenic endotoxin-specific test. The amount of LAL-reactive material in reconstituted immunoglobulin solutions ranged from a mean (standard deviation) of 10.2 (2.1) to 2,448.1 (988.9) pg/ml, and there were statistically significant differences among the six brands. The levels of LAL-reactive material in plasma increased in proportion to the amounts contained in the immunoglobulin products administered. The material accumulated in the blood with repeated administration. Urinary excretion of the material was less than 5% of the total amount administered. Such material seems to be derived from the cellulose-based membranes used during preparation of the blood products. Thus, interpretation of *Limulus* test results of patients receiving immunoglobulin therapy requires special consideration.**

A chromogenic substrate was used in a quantitative *Limulus* test (3), and this test enabled objective diagnosis of endotoxemia with excellent sensitivity (10). By combined use of a conventional chromogenic *Limulus* test (CCLT) and a newly developed chromogenic endotoxin-specific test (EST) (7), we have demonstrated that the difference between the two assays (CCLT value minus EST value) is useful for detection of fungal polysaccharides in plasma and reflects the severity of candidal infection (2).

We have used these two assays with immunocompromised hosts for diagnosis of endotoxemia and invasive candidiasis. However, false-positive results occurred in pediatric patients with no evidence of fungal infection. We reviewed all of the clinical records and found that intravenous immunoglobulin products were administered to the patients just before or during the assay. Therefore, the immunoglobulin products were considered to be responsible for misleading results which caused increased CCLT values accompanied by low EST values. Since CCLT contains two independent pathways that activate the proclotting enzyme in the *Limulus* amebocyte lysate (LAL), while EST contains only an endotoxin-sensitive pathway, immunoglobulin products would contain LAL-reactive material other than endotoxin. Hence, we analyzed commercially available intravenous immunoglobulin products and found LAL-reactive material in those products.

MATERIALS AND METHODS

Immunoglobulin products. Thirty-seven vials of six immunoglobulin products commercially available in Japan (Tables 1 and 2) were quantitatively analyzed for LAL-reactive material. Two of them are also available in the United States and three are available in Europe under different brand names. No fewer than two lots of each product were analyzed. Immunoglobulin products except Polyglobin were supplied in lyophilized form and reconstituted with pyrogen-free distilled water as described in the package inserts.

Patients. Eight consecutive pediatric patients admitted to our hospital were included in the study (Table 1). They had 11 episodes of neutropenia (neutrophils fewer than 500/mm^3) because of cytotoxic therapy. Intravenous immunoglobulin therapy (doses ranged from 120 to 240 mg/kg per day) was performed during each episode. These are the usual doses administered to such patients. Blood samples for LAL-reactive material assay and bacterial cultures were drawn from these patients. Renal function was normal in all of the patients.

Levels of LAL-reactive material in plasma were measured before and 1 day after immunoglobulin administration. Serial measurements were made for 6 successive days in three patients (no. 1, 6, and 8) who received Gamma-Venin intravenously daily for 3 days.

To determine the excretion of LAL-reactive material introduced into the blood with immunoglobulin products, Gamma-Venin (10 g) was administered intravenously to a 19-year-old male patient (40-kg body weight). Gamma-Venin was selected because it contained the highest level of LAL-reactive material among all of the brands. The patient had a moderate head injury and was otherwise healthy and had normal renal function. Blood and urine samples were taken from the patient at 0.5, 1, 2, 3, 5, 7, 9, 11, 14, 18, 26, and 30 h after administration.

Written informed consent was obtained from all subjects before the study.

Quantitative analysis of LAL-reactive material. We used CCLT (Toxicolor; Seikagaku Kogyo Co., Ltd., Tokyo, Japan) and EST (Endospecy; Seikagaku Kogyo) to quantify LAL-reactive material (2, 4–7, 9). Values of CCLT, EST, and the difference between the two tests were referred to *Escherichia coli* O111:B4 endotoxin (Westphal type).

Immunoglobulin products and plasma samples were treated with perchloric acid (6, 9) and measured as reported previously (2).

To avoid bacterial contamination, urinalysis was performed for the adult patient with a head injury who had been catheterized for measurement of hourly urine output. Urine samples were diluted by distilled water and measured with each reagent.

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TABLE 1. Pediatric patients and immunoglobulin products administered

Patient no.	Age (yr)	Sex[a]	Body wt (kg)	Underlying disease	Commercial name of product	Dose(s) (g/kg per day)
1	8	M	32.4	Acute lymphoblastic leukemia	Gamma-Venin[b]	0.15, 0.16
2	10	F	41.2	Acute lymphoblastic leukemia	Venilon[c]	0.12
3	3	F	18.0	Neuroblastoma	Polyglobin[d]	0.13, 0.14
4	1.3	M	10.4	Acute myelomonocytic leukemia	Venilon	0.24
5	4.1	M	14.1	Chronic myelomonocytic leukemia	Venilon	0.18
6	5	F	18.6	Acute lymphoblastic leukemia	Gamma-Venin	0.13
7	4	F	15.3	Ganglioneuroblastoma	Polyglobin	0.16
8	2	M	17.7	Neuroblastoma	Polyglobin	0.22
					Gamma-Venin	0.22

[a] M, Male; F, female.
[b] Behringwerke AG, Marburg/Lahn, Federal Republic of Germany.
[c] The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan.
[d] Miles Laboratories, Inc., Elkhart, Ind.

The data are shown as means with standard deviations in parentheses. Statistical tests comparing the values obtained before and after immunoglobulin therapy were done with the Mann-Whitney U test, and the differences among immunoglobulin products were examined by Kruskal-Wallis one-way analysis of variance. Significance was defined at the 5% level.

RESULTS

LAL-reactive material in immunoglobulin products. The EST values of immunoglobulin products ranged from 0 to 17.4 pg/ml (Table 2). There were no significant differences among the brands. On the other hand, statistically significant differences among CCLT values were found, ranging from 12.0 to 4,260.0 pg/ml. The difference between the two assays ranged from 7.8 to 4,252.2 pg/ml. These data indicate that all of the immunoglobulin products tested contained LAL-reactive material and that the levels of LAL-reactive material were significantly different among the brands.

Levels of LAL-reactive material in plasma. The data collected were based on 11 doses administered to eight patients (Table 1) who received Gamma-Venin, Venilon, or Polyglobin. The difference between the two assays increased from 4.0 (3.6) to 17.8 (19.3) pg/ml after a single dose of an immunoglobulin product, and the degree of this increment was proportional to the amount of LAL-reactive material found in the immunoglobulin product (Fig. 1). Normal values of CCLT, EST, and the difference between the two assays for plasma obtained from 20 healthy adults (10 males and 10 females) who received no immunoglobulin product were 7.4 (1.5), 0.5 (0.5), and 7.0 (1.5) pg/ml, respectively. No patients had any evidence of bacterial or fungal infection during 11 episodes of neutropenia.

TABLE 2. Mean values of immunoglobulin products

Commercial name	No. of vials	Mean (SD) level (pg/ml) of:		
		CCLT	EST	Difference
Gamma-Venin[a]	10	2,454.0 (990.5)	5.9 (5.3)	2,448.1 (988.9)
Venoglobulin-I[b]	3	936.0 (60.9)	5.2 (0.7)	930.8 (60.8)
Venilon	4	624.6 (29.0)	4.7 (3.8)	620.0 (30.7)
Polyglobin[a]	14	221.3 (108.0)	3.9 (1.8)	217.4 (107.8)
Sandoglobulin[c]	2	127.2 (41.6)	8.4 (0.8)	118.8 (42.4)
Glovenin-I[d]	4	16.5 (4.6)	6.3 (2.6)	10.2 (2.1)

[a] Significant differences were found among lots of this brand.
[b] Green Cross Co., Ltd., Osaka, Japan.
[c] Sandoz Ltd., Basel, Switzerland.
[d] Nihon Pharmaceutical Co., Ltd., Tokyo, Japan.

EST values were almost unchanged during serial measurement in three patients, while CCLT values increased significantly as immunoglobulin therapy was repeated (Fig. 2). The CCLT values returned to the initial level in 3 days (patients 1 and 8) or 4 days (patient 6; data not shown) after discontinuation of immunoglobulin therapy. The result suggests accumulation of LAL-reactive material during therapy.

Excretion of LAL-reactive material. Gamma-Venin was administered to an adult patient over a 1-h period. Ten grams of this product contained 488,520 pg of LAL-reactive material. The CCLT value in plasma increased rapidly from normal to 155.4 pg/ml just after intravenous administration, and then the value decreased, with a half-life of about 2 h (Fig. 3). However, the level of LAL-reactive material in plasma remained significantly high (28.8 pg/ml), even at 30 h after administration. The EST value in plasma remained normal throughout the test.

LAL-reactive material was detected in urine for only 2 h after administration (Fig. 3). Normal values of CCLT, EST, and the difference between the two tests for urine samples from the 10 males were 8.8 (3.4), 5.2 (3.3), and 3.7 (2.0) pg/ml, respectively. Total urinary output of the material was 23,520 pg. This means that no more than 5% of the total amount administered was excreted in urine.

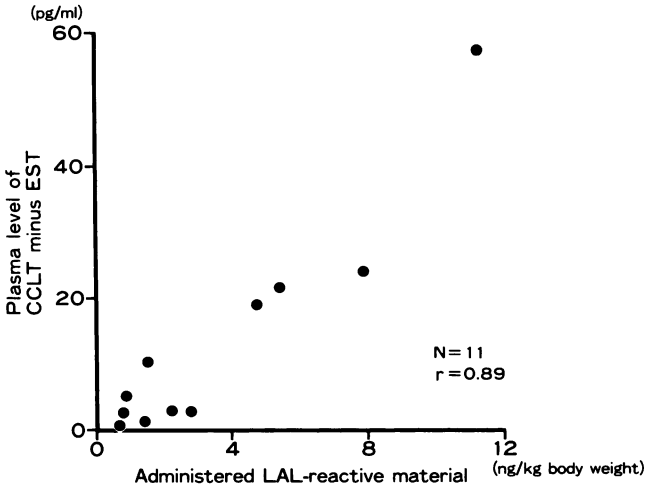

FIG. 1. Relationship between administration of an immunoglobulin product and level of LAL-reactive material in plasma.

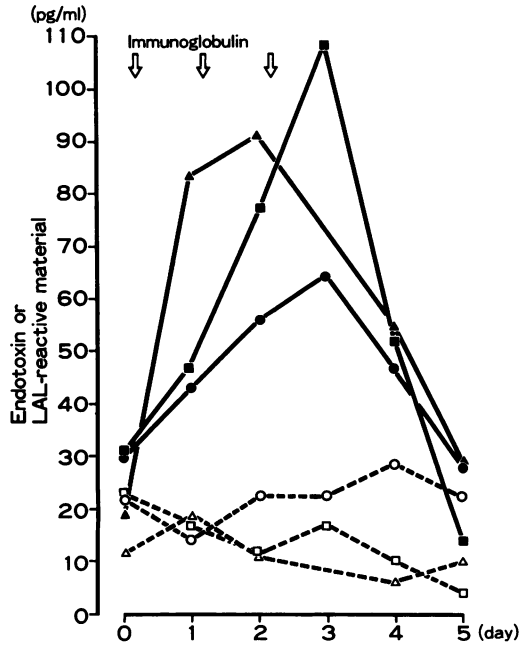

FIG. 2. CCLT (solid lines) and EST (broken lines) values obtained from patients administered Gamma-Venin for 3 days. Symbols: ○ and ●, patient 1; △ and ▲, patient 6; □ and ■, patient 8.

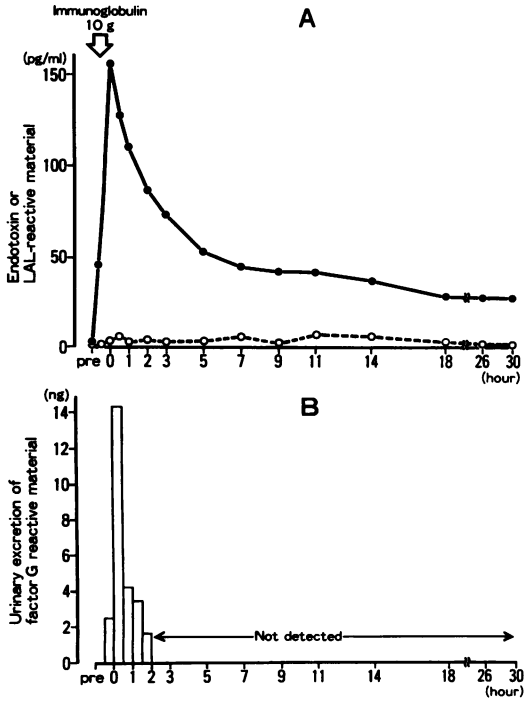

FIG. 3. Endotoxin and LAL-reactive material in an adult patient who received Gamma-Venin intravenously. Blood and urine samples were taken before, during, and after administration. (A) Values of CCLT (●) and EST (○) in the blood; (B) urinary excretion of LAL-reactive material other than endotoxin, that is, factor G-reactive material. Time zero is immediately after immunoglobulin administration.

DISCUSSION

CCLT (Toxicolor) contains two independent factors which activate the proclotting enzyme in LAL, i.e., factors C and G, while EST (Endospecy) contains only factor C. Since factor C is activated by endotoxin (5) and factor G is activated by β-1,3-glucan (4), the difference between the two assays would reflect the concentration of factor G-reactive material which corresponds to LAL-reactive material other than endotoxin (2). Thus, by using these two assays, we established the differential diagnosis of endotoxemia and candidal infection (2). It was also revealed that false-positive results associated with hemodialysis were attributable to LAL-reactive material from cupro-ammonium rayon membrane used in hemodialysis (11). In this study, we demonstrated that immunoglobulin products are another source of false-positive results.

Quantitative analysis of intravenous immunoglobulin products revealed that they all contained LAL-reactive material. The amounts of reactive material in those products varied significantly among the brands. Among the three immunoglobulins tested, the highest levels of reactive material in plasma were found in patients who received Gamma-Venin. Those levels were high enough to lead to a misdiagnosis of fungal infection. Estimates based on the amounts of reactive material in immunoglobulin products and the body weights of the patients indicate that the three brands that contain higher levels of reactive material (Table 2) would cause false-positive results immediately after administration of the single usual dose (between 120 and 240 mg/kg).

The LAL-reactive material found in immunoglobulin products cannot be endotoxin, and it is a factor G-reactive material, since the EST values remained low. Since β-1,3-glucan is the only factor G-reactive material reported, this LAL-reactive material would probably be β-1,3-glucan. If so, enzymatic degradation of this LAL-reactive material would not be accomplished in vivo, since β-glucanase does not exist in humans. That might explain the slow elimination of the material from plasma (Fig. 3). Although it remains to be determined whether this material is toxic, no clinical changes that seemed attributable to the material were observed.

Levels of LAL-reactive material in plasma increased further with repeated immunoglobulin administration (Fig. 2), as expected on the basis of the slow excretion. The duration and extent of that elevation of the material depended on the brands, lots, and doses of immunoglobulin products administered.

The results of this study are clinically important. Patients with leukopenia are susceptible to both bacterial and fungal infections. Immunoglobulin products are often administered to these patients to prevent or treat infections. Since factor G is contained in all conventional *Limulus* tests, we must recognize that intravenous immunoglobulin therapy may hamper diagnoses based on conventional *Limulus* tests. To achieve correct differential diagnosis of endotoxemia and candidal infection in such conditions, it is essential to assay plasma simultaneously with both CCLT and EST. It is recommended that the amount of LAL-reactive material in the immunoglobulin product administered and the levels in plasma before and several days after therapy be measured.

The origin of LAL-reactive material is of interest. This study indicates that immunoglobulin products prepared with nylon-based filter membrane (1) contain less LAL-reactive material than products prepared with cellulose-based filter membrane (Tables 2 and 3). We could not identify the material of the membrane used for Gamma-Venin, because we did not receive information from the manufacturer. This

TABLE 3. Preparative processes of the immunoglobulin products studied[a]

Commercial name	Filter membrane base used for:	
	Fractionation	Removal of bacteria
Gamma-Venin	NA[b]	NA
Venoglobulin-I	Cellulose	Cellulose
Venilon	Cellulose	Cellulose
Polyglobin	Cellulose	Cellulose
Sandoglobulin	None	Nylon
Glovenin-I	None	Nylon

[a] These data were obtained by an inquiry directed to the manufacturers.
[b] NA, No answer was obtained from the manufacturer.

LAL-reactive material seems to have originated from cuproammonium rayon or cellulose-based membrane (8, 11), and the significant differences among the materials of the different brands are attributable to the differences among the membranes used for preparation. However, sources other than membranes are not completely excluded.

In summary, we demonstrated by combined use of CCLT and EST that intravenous immunoglobulin products contain LAL-reactive material and that this material causes false-positive results in *Limulus* tests. Therefore, special consideration is required in the diagnosis of patients who receive immunoglobulin products.

LITERATURE CITED

1. **Cohn, E. J., L. E. Strong, W. L. Hughes, D. J. Mulford, J. N. Ashworth, M. Melin, and H. L. Taylor.** 1946. Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. J. Am. Chem. Soc. **68**:459–475.
2. **Ikegami, K., K. Ikemura, T. Shimazu, M. Shibuya, H. Sugimoto, T. Yoshioka, and T. Sugimoto.** 1988. Early diagnosis of invasive candidiasis and rapid evaluation of antifungal therapy by combined use of conventional chromogenic limulus test and a newly developed endotoxin specific assay. J. Trauma **28**:1118–1126.
3. **Iwanaga, S., T. Morita, T. Harada, S. Nakamura, M. Niwa, K. Takada, T. Kimura, and S. Sakakibara.** 1978. Chromogenic substrates for horseshoe crab clotting enzyme. Haemostasis **7**:183–188.
4. **Morita, T., S. Tanaka, T. Nakamura, and S. Iwanaga.** 1981. A new (1→3)-β-D-glucan-mediated coagulation pathway found in limulus amebocytes. FEBS Lett. **129**:318–321.
5. **Nakamura, T., T. Morita, and S. Iwanaga.** 1986. Lipopolysaccharide-sensitive serine-protease zymogen (factor C) found in Limulus hemocytes: isolation and characterization. Eur. J. Biochem. **154**:511–521.
6. **Obayashi, T.** 1984. Addition of perchloric acid to blood samples for colorimetric limulus test using chromogenic substrate: comparison with conventional procedures and clinical applications. J. Lab. Clin. Med. **104**:321–330.
7. **Obayashi, T., H. Tamura, S. Tanaka, M. Ohki, S. Takahashi, M. Arai, M. Masuda, and T. Kawai.** 1985. A new chromogenic endotoxin-specific assay using recombined limulus coagulation enzymes and its clinical applications. Clin. Chim. Acta **149**: 55–65.
8. **Obayashi, T., H. Tamura, S. Tanaka, M. Ohki, S. Takahashi, and T. Kawai.** 1986. Endotoxin-inactivating activity in normal and pathological human blood samples. Infect. Immun. **53**: 294–297.
9. **Tamura, H., T. Obayashi, K. Takagi, S. Tanaka, C. Nakahara, and T. Kawai.** 1982. Perchloric acid treatment of human blood for quantitative endotoxin assay using synthetic chromogenic substrate for horseshoe crab clotting enzyme. Thromb. Res. **27**:51–57.
10. **Van Deventer, S. J. H., H. R. Buller, J. W. Ten Cate, A. Sturk, and W. Pauw.** 1988. Endotoxaemia: an early predictor of septicaemia in febrile patients. Lancet **i**:605–609.
11. **Yoshioka, T., K. Ikegami, K. Ikemura, S. Shiono, M. Uenishi, H. Sugimoto, and T. Sugimoto.** 1989. A study on limulus amebocyte lysate (LAL) reactive material derived from dialyzers. Jpn. J. Surg. **19**:38–41.