Improved Rubella Hemagglutination Inhibition Test: Inactivation of Non-Immunoglobulin Hemagglutination Inhibitors by Phospholipase C

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Received for publication 26 July 1976

A method using phospholipase C (PL-C) for removing nonspecific inhibitors (NSI) of rubella virus hemagglutinin is described. PL-C was found to hydrolyze NSI without altering the hemagglutination inhibition (HI) activity of the specific antibody and could be used to remove NSI in the rubella HI test by using formalinized erythrocytes, which resisted the enzymatic action; fresh erythrocytes were lysed by PL-C. The HI test using PL-C-treated sera gave true measurements of actual rubella antibody content, and HI titers of PL-C-treated sera were identical or equivalent (± 1 dilution) to those of sera treated with dextran sulfate and CaCl_2 (DS-C). Thus, the PL-C method gave results as reproducible and reliable as the DS-C method and was more convenient.

The hemagglutination inhibition (HI) test for rubella antibody developed by Stewart et al. (21) has proved to be a valuable tool in the serological diagnosis of rubella. The HI test is more sensitive and easier to perform than neutralization and complement fixation tests (11); however, the non-immunoglobulin inhibitors of rubella hemagglutinin (HA) present in essentially all human sera may give false positive results in the measurement of rubella HI antibody. These nonspecific inhibitors (NSI) are β -lipoproteins (1, 3, 4). To remove NSI, treatment of serum with kaolin (8, 21) or heparin- MnCl_2 (2, 12) is widely used, and another method, using dextran sulfate- CaCl_2 (DS-C), has also been described (12). The DS-C treatment is reproducible and reliable, but is as laborious as the others for large-scale serological surveys.

The present paper reports a new and simple method using phospholipase C (PL-C) for removal of NSI.

MATERIALS AND METHODS

PL-C. PL-C, prepared from *Clostridium perfringens*, was purchased from P-L Biochemicals, Inc., Milwaukee, Wis. The enzyme preparation was assayed for specific activity by the method of Macfarlane and Knight (14), and the specific activity was determined to be 1.2 units/mg. One unit frees 1 μmol of phosphorylcholine per min from egg lecithin at pH 7.3, 37°C.

α -Chymotrypsin. α -Chymotrypsin, prepared from bovine pancreas, was purchased from Sigma Chemical Co., St. Louis, Mo. The enzyme preparation was assayed for specific activity using benzoyl-

L-tyrosine ethylester and was determined to contain 42 units/mg. One unit hydrolyzes 1 μmol of benzoyl-L-tyrosine ethylester per min at pH 7.8, 25°C.

Formalinized erythrocytes. Blood collected from unfed chicks by heart puncture within 24 h after hatching was pooled in acid-citrate-dextrose solution. The 1-day-old chick erythrocytes (CRBC) were washed three times with phosphate-buffered saline, pH 7.2 (5), and then erythrocyte hemoglobin was converted into carboxyhemoglobin by exposure of the blood to carbon monoxide. The intensely colored erythrocytes were suspended to a concentration of 10% in phosphate-buffered saline with 10% formalin and allowed to stand at 4°C for 7 days for fixation. They were then washed six times with 20 volumes of distilled water to remove unabsorbed formalin and suspended in distilled water to a concentration of 10% (9).

Rubella HA. Stem cells (PS-Y 15) of porcine kidney origin were inoculated with the M33 strain of rubella virus. Medium containing virus was harvested after 7 days and freeze-dried according to the procedures of Ito et al. (10).

Serum specimens. Randomly coded human sera were supplied by the Osaka Clinic of Takeda Chemical Industries, Ltd. All sera were stored at -20°C until tested.

HI test. HI tests were performed by the microtiter method of Sever (20), using V plates of the permanent type (Tomii Seiko Co., Tokyo, Japan) and spiral loops (Microbiological Associate Inc., Bethesda, Md.). HEPES-saline-albumin-gelatin (HSAG) buffer, pH 6.2 (consisting of 0.025 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], 0.14 M NaCl, 10^{-3} M CaCl_2 , 0.2% bovine serum albumin, and 0.0025% gelatin), was used throughout as a diluent for serum, HA, and CRBC suspensions (12, 18, 19). Formalinized CRBC were used as 0.3%

suspensions, and fresh CRBC were used as 0.25% suspensions.

Pretreatment of the sera to be tested was as follows. (i) For PL-C treatment, 0.1 ml of unheated serum was mixed with 0.2 ml of HEPES-buffered saline, pH 7.3 (consisting of 0.002 M HEPES, 0.14 M NaCl, and 10^{-3} M CaCl_2), and to the diluted serum was added 0.1 ml of PL-C solution (0.06 unit/ml). The mixture was incubated at 37°C overnight and then inactivated by heating at 56°C for 30 min. (ii) For kaolin treatment, serum diluted 1:3 was mixed with an equal volume of a 25% acid-washed kaolin (Fisher Scientific Co., Pittsburgh, Pa.) suspension in 0.005 M phosphate-buffered saline, pH 7.2. After being shaken at room temperature for 30 min, the mixture was centrifuged at 2,000 rpm for 20 min and the treated serum was removed. (iii) For DS-C treatment, 0.2 ml of serum was mixed with 0.4 ml of HSAG and to the diluted serum was added 0.1 ml of 1% dextran sulfate (molecular weight, 500,000; Pharmacia, Uppsala, Sweden) and 0.1 ml of 0.3 M CaCl_2 . After incubation at 4°C for 1 h, the precipitate was removed by centrifugation at 2,000 rpm for 15 min. This method differs little from that described by Liebhaber (12).

To remove nonspecific HA by absorption, each pretreated serum was mixed with 1/8 volume of a 10% erythrocyte suspension. After being allowed to stand at 4°C for 1 h, the erythrocytes were removed by centrifugation.

Preparation of lipoproteins from sheep erythrocytes and their enzymatic hydrolysis. Erythrocyte lipoproteins were prepared from sheep erythrocytes according to the method of Morgan and Hanahan (16). They were assayed for rubella HI activity before and after enzymatic hydrolysis by either PL-C or α -chymotrypsin. For hydrolysis by PL-C, the lipoproteins were dissolved in HEPES-buffered saline (pH 7.3) as a 0.1% (wt/vol) solution and incubated with an equal volume of the PL-C solution, of various concentrations, at 37°C overnight. For α -chymotrypsin digestion, the lipoproteins were dissolved in tris(hydroxymethyl)aminomethane-buffered saline (pH 7.8) containing 10^{-3} M CaCl_2 as a 0.1% (wt/vol) solution and incubated with an equal volume of enzyme solution at 37°C for 30 min. After the enzymatic reaction, each solution was assayed for rubella HI activity.

Diethylaminoethyl-cellulose column chromatography. Test serum was fractionated on a column of diethylaminoethyl-cellulose according to the method of Fahey and Horbett (6). The column (60 by 1.4 cm) was equilibrated with phosphate-buffered 0.01 M NaCl, pH 8.0 (starting buffer). A 1.0-ml sample of serum that had been dialyzed overnight against 300 volumes of starting buffer was run into the column. Elution was started immediately with a gradient of 0.01 to 0.5 M NaCl, buffered throughout with 0.01 M phosphate, pH 8.0. The eluate was continuously monitored at a wavelength of 280 nm. Each 10-ml fraction was dialyzed twice against 100 volumes of water, lyophilized, and then redissolved in 1.0 ml of HEPES-buffered saline.

Immunoelectrophoresis. Qualitative analysis of human serum fractionated on a diethylaminoethyl-

cellulose column was carried out by immunoelectrophoresis in starch gels (Difco, Noble) according to the method of Grabar et al. (7), using antisera specific for human whole serum and immunoglobulins (Ig) (IgG + IgA + IgM; Miles Laboratories, Inc., Elkhart, Ind.).

RESULTS

Effect of PL-C on the HA test. To determine if PL-C inhibited the HA reaction between rubella HA and formalinized CRBC, HA tests were performed in the presence of PL-C (Table 1). PL-C solutions were incubated at 37°C overnight and then heated at 56°C for 30 min, as in the case of PL-C treatment of test sera.

The PL-C solutions thus treated were added to the antigen solutions, which had been serially diluted in twofold steps, and then a fresh or formalinized CRBC suspension was added to the mixture. The HA patterns formed with fresh CRBC were generally poor, due probably to the hemolysis elicited by more than 0.06 unit of PL-C per ml. By contrast, formalinized CRBC formed clear patterns, showing no signs of hemolysis even in the presence of 0.12 unit of PL-C per ml.

These results showed that PL-C had little effect on the reaction between rubella HA and formalinized CRBC.

Enzymatic hydrolysis of erythrocyte lipoproteins. Erythrocyte stromal lipoproteins prepared according to the method of Morgan and Hanahan are nearly comparable to serum low-density lipoproteins (β -lipoproteins) (16). These lipoproteins showed a rubella HI titer of 64, which was completely inactivated by 0.06 unit of PL-C per ml, whereas chymotrypsin diges-

TABLE 1. Effect of PL-C concentration on HA titer and erythrocyte pattern in rubella HA tests

PL-C concn (unit/ml)	CRBC ^a	HA titer ^b	RBC pattern ^c
None	Fresh	32	Excellent
	Formalinized	32	Excellent
0.01	Fresh	32	Excellent
	Formalinized	32	Excellent
0.03	Fresh	32	Good
	Formalinized	16	Excellent
0.06	Fresh	— ^d	Poor
	Formalinized	32	Excellent
0.12	Fresh	— ^d	Poor
	Formalinized	32	Good

^a One-day-old CRBC.

^b Reciprocal of rubella HA titer.

^c The RBC pattern was evaluated by the contrast between agglutinated and nonagglutinated RBC and by the stability of the agglutination pattern.

^d Fresh CRBC were lysed by PL-C.

tion resulted in an increase of HI titer from 64 to 4,096 (Table 2).

Because of the heat stability of PL-C, a test of whether the complete inactivation of the lipoproteins by PL-C was due to the PL-C or to some other enzymes contaminating the PL-C preparation was done by heating a solution of the enzyme (0.2 unit/ml) for 15 min at 70°C. This heated PL-C solution completely inactivated the lipoproteins.

These results suggested that the β -lipoprotein rubella HI activity in human serum might also be inactivated by PL-C.

Removal of non-immunoglobulin HA inhibitors by PL-C. Serum specimens were assayed for rubella HI activity before and after treatment with PL-C, kaolin, or DS-C. Table 3 shows HI titers of the representative sera. Titers of sera treated with PL-C or DS-C were the same or within a twofold agreement.

To determine if PL-C inactivated only NSI

and not specific antibody, test serum was separated by diethylaminoethyl-cellulose chromatography. The elution pattern is shown in Fig. 1. HI activity was eluted in two peaks. Each fraction of peaks 1 and 2 was treated with PL-C and assayed for rubella HI activity. The fractions of each peak were then pooled, concentrated by pervaporation, and analyzed by immunoelectrophoresis in starch gels. Peak 1 material, with maximum HI activity, eluted at 0.03 M NaCl in the buffer and was confirmed to be IgG. The immunoelectrophoretic pattern of the concentrated pool of peak 2, representing material eluted at 0.1 to 0.5 M NaCl, showed several precipitin lines, including that of IgA. The high levels of HI activity associated with the non-IgG protein fractions (fractions 19 to 23) were abolished by PL-C treatment, whereas the HI activity of the IgG fractions (fractions 9 to 11) was not altered by PL-C (Fig. 1). These results show that PL-C inactivated only NSI

TABLE 2. HI activity^a of erythrocyte stromal lipoproteins before and after enzymatic hydrolysis

Enzyme	Concn (unit/ml)	HI titer ^b
Control		64
L-C	0.006	32
	0.06	<2
	0.12	<2
α -Chymotrypsin	2	256
	10	4,096
	50	2,048

^a Rubella HI tests were carried out with 0.3% suspensions of formalinized erythrocytes.

^b Reciprocal of the dilution of lipoprotein solutions.

TABLE 3. HI activity^a of human sera before and after treatment with PL-C, kaolin, or DS-C

Serum no.	HI titer ^b			
	Untreated	PL-C treated	Kaolin treated	DS-C treated
101	512	64	32	64
324	2,048	<8	<8	<8
209	2,048	64	16	32
456	512	16	8	16
501	1,024	64	32	32
107	256	<8	<8	<8
455	512	64	32	64
311	256	<8	<8	<8
158	256	32	32	32
279	512	32	32	32
112	1,024	<8	<8	<8
310	1,024	128	128	128

^a All rubella HI tests were carried out with 0.3% suspensions of formalinized erythrocytes.

^b Reciprocal of the serum dilution.

FIG. 1. Diethylaminoethyl-cellulose column chromatography of untreated human serum. Protein concentration in the column eluate is indicated by the curve of ultraviolet absorbancy. Immunoglobulin content of each peak was determined qualitatively by immunoelectrophoresis in starch gels. The distribution of immunoglobulins found in the peaks is indicated by solid bars. Each fraction was assayed for HI activity before and after treatment with PL-C.

and that the remaining HI activity was due solely to the presence of specific antibody.

Effect of PL-C concentration on rubella HI activity. The concentrated pools of peaks 1 and 2, respectively, were assayed for HI activity using various concentrations of PL-C (Table 4). The HI titer of peak 1 was independent of the PL-C concentrations used. The HI titer of peak 2 was reduced to a great extent but was not abolished by treatment with 0.04 unit of PL-C per ml. This was probably due to rubella IgA or IgM antibodies, or to contamination with IgG, since other experiments verified that the PL-C treatment completely inactivated lipoprotein inhibitors.

About 0.06 unit of PL-C per ml was found to be sufficient for inactivating NSI with a titer of 2,048.

Comparison of PL-C and DS-C treatments. The set of 72 serum specimens was treated according to the PL-C method described in Materials and Methods and by the slightly modified method of Liebhaver (12). Both sets of treated sera were assayed in parallel for HI activity using formalinized CRBC. The results are compared in Fig. 2. Titers were identical or within one dilution of each other by both methods in more than 90% of the specimens. These results show that the PL-C method is as reliable as the DS-C method.

Reproducibility of the HI test using PL-C treatment. The reproducibility of the PL-C method was evaluated by comparing the HI titers obtained by duplicate testing of 70 specimens (Fig. 3). HI titers were identical or equivalent (± 1 dilution) for 66 of the 70 duplicate sera. Thus, the reproducibility of these results

FIG. 2. Comparison of PL-C treatment and DS-C treatment. Seventy-two serum specimens were treated with DS-C and assayed for rubella HI antibody by the method of Liebhaver, slightly modified. Samples of the same sera were treated with PL-C and assayed for antibody according to the method detailed in the text. Titers obtained for PL-C-treated sera were plotted against those obtained for the corresponding sera treated with DS-C.

was about 95% in duplicate serum specimens treated with PL-C.

DISCUSSION

The present study has shown that PL-C can inactivate non-immunoglobulin inhibitors of rubella HA in human sera and reduce their HI titers to an undetectable level, without interfering with the activity of specific antibody. PL-C treatment was comparable to DS-C treatment in giving reproducible and precise rubella-specific antibody titers.

Kaolin has been widely used as an absorbent for removing NSI of rubella HA. However, selective removal of NSI from serum may be difficult, since kaolin may absorb immunoglobulins at neutral pH. In the present study, some sera were treated with kaolin in phosphate-buffered saline at pH 7.2 and assayed for HI activity. A few showed lower titers as compared to those of sera treated with PL-C or DS-C. In this respect, kaolin treatment should be carried out in borate-buffered saline at pH 9.0 to minimize absorption of specific antibody, as pointed out by Schmidt and Lennette (19).

Various methods for specifically removing NSI were introduced after the identification of NSI as β -lipoproteins (1, 3, 4). Mann et al.

TABLE 4. Effect of PL-C concentration on HI activity^a of the fractions obtained from diethylaminoethyl-cellulose column chromatography (cf. Fig. 1)

Peak	Fraction	Immunoglobulin present in fractions	PL-C concn (unit/ml)	HI titer ^b
1	9-11	IgG	0	256
			0.02	256
			0.04	128
			0.06	256
			0.12	128
2	19-23	IgA, IgM	0	2,048
			0.02	128
			0.04	16
			0.06	16
			0.12	16

^a All rubella HI tests were carried out with 0.3% suspensions of formalinized erythrocytes.

^b Reciprocal of the dilution of the pooled solution.

FIG. 3. Reproducibility of rubella HI antibody titers in PL-C sera. Antibody levels were determined by simultaneous titration of 70 duplicate serum specimens after treatment with PL-C.

developed a method for precipitating NSI of reovirus HA with heparin and MnCl_2 (15). Liebhaber showed that DS-C could be utilized as a specific remover of NSI (12). Unlike kaolin, DS and CaCl_2 are chemically defined, and the specificity of their reaction for low-density β -lipoproteins has been well documented. Moreover, DS and CaCl_2 more completely remove the β -lipoproteins from serum than do heparin and MnCl_2 . However, DS and CaCl_2 are used preferably as rather concentrated solutions to insure complete removal of NSI, and such high concentrations frequently produce unreadable hemagglutination patterns, especially at serum dilutions below 1:8.

On the contrary, the new procedure using PL-C gave clear hemagglutination patterns at low serum dilutions. This procedure also permits complete inactivation of the NSI in sera by enzymatic hydrolysis and does not require separating the NSI from specific antibody by centrifugation. Thus, it can be said that the present PL-C method is simple and less laborious than those using kaolin, heparin and MnCl_2 , or DS-C.

Since they are comparable to serum β -lipoprotein inhibitors in nature (16), the erythrocyte stromal lipoproteins were used in the present study for some investigations on inhibitory activity for rubella HA. It was found that the stromal lipoproteins possessed a strong inhibitory activity for rubella HA, which was reduced to an undetectable level by treatment with PL-

C whereas it was increased by treatment with chymotrypsin. These results suggest that phospholipids in a β -lipoprotein molecule are essential parts in the manifestation of the HI activity and, further, that the active phospholipid moieties masked by the protein moieties in the β -lipoprotein molecule become accessible to rubella HA as a result of chymotrypsin digestion.

Schmidt et al. (17) found that PL-C inactivated serum inhibitors of echovirus and reovirus HA when it was used in combination with chymotrypsin. But the present study showed that NSI for rubella virus HA could be completely inactivated by treatment with PL-C alone. The probable reasons are that the NSI of rubella virus HA differ from echovirus and reovirus HA inhibitors and perhaps that the incubation time for enzymatic hydrolysis used here was much longer than that adopted by Schmidt et al. In fact, inhibitory activity of rubella virus HA inhibitors was not abolished by treatment with PL-C for 2 h. Thus, the present method using PL-C requires an overnight incubation to insure complete hydrolysis of NSI.

The PL-C method should be used in combination with formalinized CRBC so as not to lyse the erythrocytes. Formalinized CRBC, which can be freeze-dried and stored for more than 1 year, provide a sensitivity comparable to that of fresh cells (9). Thus, PL-C treatment in combination with formalinized CRBC has proved useful for large-scale serological surveys and for the standardization of rubella HI tests to detect specific antibody in sera.

ACKNOWLEDGMENTS

We are grateful to Homu Ito for valuable discussions and support of this work. We also thank Isamu Yoshida and Mitsugu Nakata for excellent technical assistance.

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