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ROLE OF PHOSPHOLIPASE A AND C IN MAST CELL DEGRANULATION INDUCED BY NON-PURIFIED *CLOSTRIDIUM WELCHII* TOXIN

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Abstract—From the observation that toxin from *Clostridium welchii* causes degranulation of mast cells it has been concluded that phospholipase C is responsible for this effect.[6,7] In the present study it has been shown that the mast cell degranulating agent is not phospholipase C but probably phospholipase A, originating from the sterile culture medium.

IN SOME recent papers from our laboratories it has been suggested that synthetic polymer amines such as compound 48/80, "histamine liberators" isolated from *Ascaris suis* and *Cyanea capillata*, and antigen induce degranulation of rat mast cells by activating a lytic enzymatic process in the mast cell.[1–5] For reasons mentioned in these papers the enzyme was thought to be a phospholipase, related to or identical with phospholipase A.

It was observed[6,7] that mast cells were degranulated when exposed to *Clostridium welchii* toxin, and concluded that the degranulation was caused by α-toxin, the toxin assumed to be identical with phospholipase C. Some of our observations, e.g. the action of various enzyme inhibitors on mast-cell disruption, did not lend weight to the assumption that phospholipase C was the enzyme in question, but rather contradicted it.

The aim of the present study was to investigate the nature of the mast cell degranulating factor in the *Cl. welchii* toxin.

MATERIALS AND METHODS

Preparation of crude toxin

Medium for cultivation of *Cl. welchii*[8] contained pancreatic digest of casein, Na_2HPO_4, KH_2PO_4, $MgSO_4$, dextrin and thioglycollic acid. For sterilization the medium was heated to 100 °C for 20 min and to 120 °C for 30 min.

To 155 ml of this medium, the pH of which had been adjusted to 7·5, was added 0·5 ml of a *Cl. welchii* stock culture (strain CN 1491). The inoculated broth was kept at 35 °C for 16 hr and in the refrigerator for another 8 hr. Some 25 ml of this seed culture was inoculated into the vessel used for toxin production. The same medium was used, and the culture was kept at 35 °C for 16 hr. The culture was then run through a Seitz filter. The filtrate was concentrated by freeze-drying, dialysed against distilled water, then freeze-dried again. Finally it was dissolved in distilled water.

Fractionation of mast-cell degranulating factor

A sample of 3 g of IRC 50 (XE-64) was buffered with O.I.M. H_4NOH–HAc to pH 7·6. A toxin preparation (9 ml) was carefully stirred with the resin for 10 min and allowed to stand at 4 °C for 1 hr. It was then charged into a chromatogram tube of 10 mm diameter, washed with 10 ml distilled, demineralized water, then eluted with 0·1 N H_4NOH.

Fractionation of α-toxin

Cl. welchii α-toxin was prepared by ammonium sulphate fractionation and nucleic acid precipitation.[9] In order to obtain a stable preparation it was dialyzed against glycerol at 3 °C.

Phospholipase A. Prepared from bee venom.

Observations on mast cells

The technique was that described by Högberg, and Uvnäs[1] with the modification that the mesentery was incubated in a buffered solution (pH 7·0) containing $1{\cdot}6 \times 10^{-1}$ M NaCl, 3×10^{-3} M KCl, 9×10^{-4} M $CaCl_2$ (anhydrous) and 10 per cent Sörensen phosphate buffer (3×10^{-2} M Na_2HPO_4, 2 H_2O + $3{\cdot}5 \times 10^{-2}$ M KH_2PO_4).

Haemolysis

The haemolytic activity was determined on washed erythrocytes of rat. The buffered solution described in the last preceding section was used. Each test tube contained 0·4 ml of the solution to be tested, 0·4 ml of inhibitor (or buffer) and 3·2 ml of the erythrocyte suspension (about 2 per cent). The tubes were incubated at 37 °C for 3 hr. The reaction was stopped by centrifuging at 5000 rev/min for 5 min. The extinction was determined in a Hilger Biochem Colorimeter at 5500 Å, using 13 mm round cuvettes.

Manometric procedure

The reactivity of phospholipase A and C can be measured by manometric procedures.[10,11] The reaction mixture in our experiments had the following composition: 2·0 ml 4% lecithin (or phosphatidyl ethanolamine) in water; 0·3 ml M $NaHCO_3$; 0·3 ml distilled water; 0·3 ml enzyme solution, diluted in 1% albumin and 0·004 M $CaCl_2$. The flasks were filled with 100% CO_2. In experiments with antitoxin the toxin and antitoxin were pre-incubated 30 min before being put into the substrate-containing mixture.

RESULTS

Well-known effects of phospholipase C are the hydrolysis of lecithin and the (direct) lysis of erythrocytes. From Fig. 1 and 2 it is evident that our *Cl. welchii* toxin has both

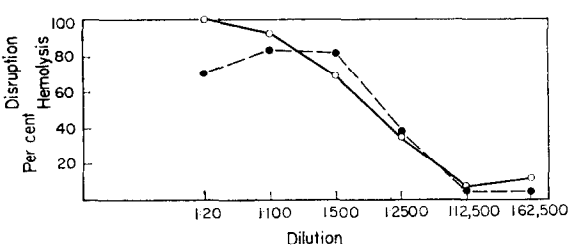

FIG. 1. Haemolysis ● —— ● —— ● and mast cell degranulation ○——○——○ caused by crude *Clostridium welchii* toxin.

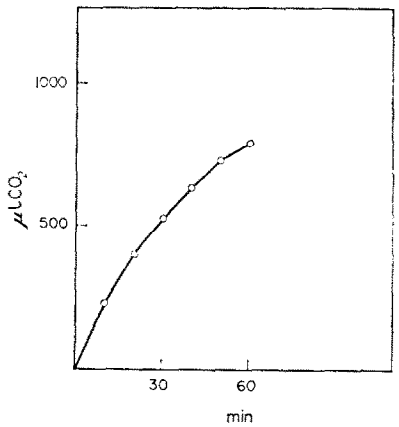

FIG. 2. Hydrolysis of lecithin caused by crude *Clostridium welchii* toxin.

of these properties and in addition degranulates mast cells. The apparent correlation between these actions is consonant with the idea that they might be due to one and the same factor, i.e. phospholipase C. However, several arguments can be presented against this conclusion:

(1) Antitoxin against *Cl. welchii* inhibited the haemolytic action of the toxin but not its *mast cell degranulating* action (Fig. 3).

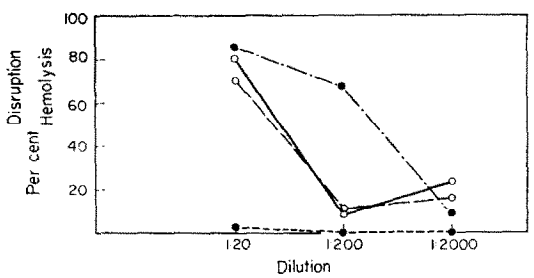

FIG. 3. Inhibition by *Clostridium welchii* antitoxin of *haemolysis* caused by crude toxin. ● - . - . - ● - . - . - ● without antitoxin; ● . . . . . ● . . . . . ● with antitoxin. Note the absence of inhibition of mast-cell degranulation, ○ —— ○ —— ○ without antitoxin; ○ - - - ○ - - - ○ with antitoxin.

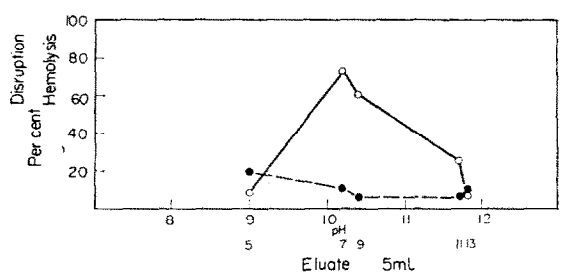

FIG. 4. Separation of mast-cell degranulating factor ○——○——○ from haemolytic factor ● - - - ● - - - ● in crude *Clostridium welchii* toxin. Small figures refer to number of the elutate. Dilution 1:40.

(2) Using Amberlite IRC 50 (XE-64), the mast cell degranulation factor in the toxin preparation was separable from the haemolytic one (Fig. 4). This latter factor was not recovered from the resin. Possibly it was denatured.[12]

(3) With salt fractionation and nucleic acid precipitation it was possible to obtain fractions with high haemolytic and lecithin splitting activities. These fractions did not hydrolyse phosphatidyl ethanolamine, nor did they degranulate mast cells. The hydrolytic activity was blocked by *Cl. welchii* antitoxin (Figs. 5a, b and c).

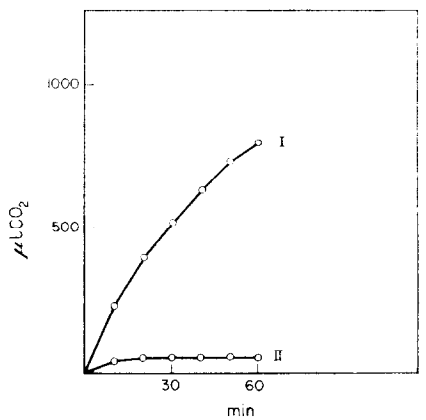

FIG. 5(a). Hydrolysis of lecithin produced by partly purified *Clostridium welchii* toxin (I). Note the absence of phosphatidyl ethanolamine hydrolysis (II).

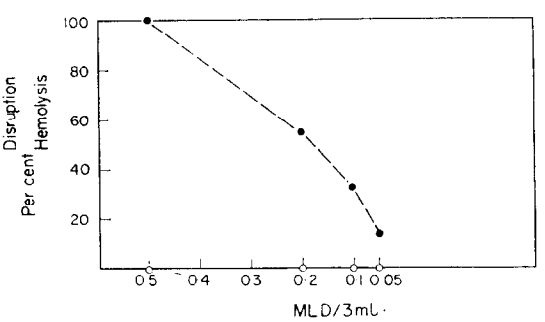

FIG. 5(b). Haemolytic action of partly purified *Clostridium welchii* toxin ●----●----●. Note the absence of mast cell degranulation ○——○——○.

(4) It was observed that even the sterile culture medium contained a mast cell degranulating factor. There was no increase of the degranulating activity after the growth of *Cl. welchii* (Fig. 6).

The above observations militate against the supposition that the mast cell degranulation is due to phospholipase C in the toxin. It is more likely to be due to the presence of phospholipase A in the culture medium, since:

(a) Ox pancreas, an organ rich in phospholipase A, was one of the raw materials for the culture medium. It is true that the broth was sterilized by heating to 120 °C,

but phospholipase A is known to be unusually heat resistant. In control experiments extracts of ox pancreas heated to 120 °C still showed mast-cell disrupting activity (Fig. 7).

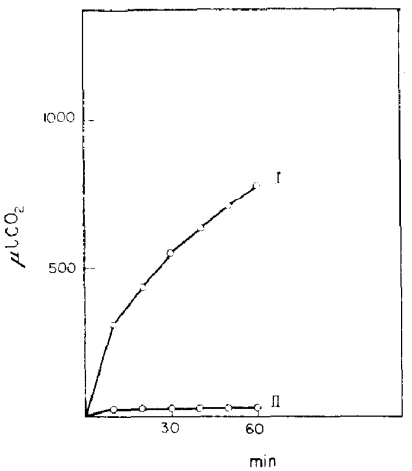

FIG. 5(c). Inhibition of lecithin hydrolysing action of *Clostridium welchii* toxin by *Clostridium welchii* antitoxin. I: Without antitoxin. II: With antitoxin.

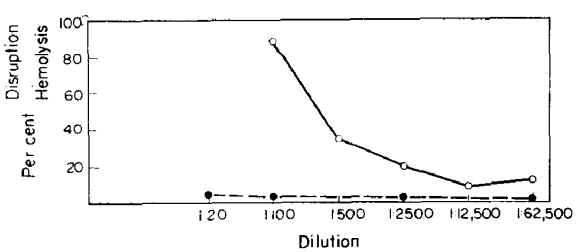

FIG. 6. Haemolysis ●- - -●- - -● and mast-cell degranulation ○——○——○ caused by sterile culture medium.

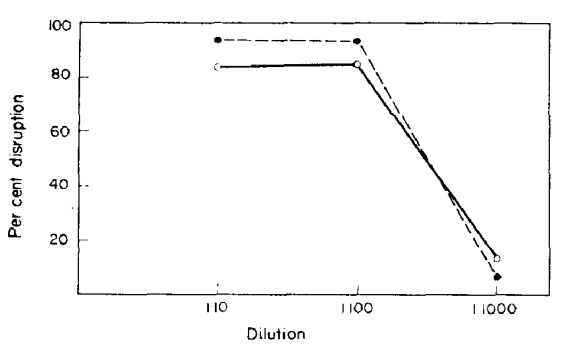

FIG. 7. Heat resistance of mast-cell degranulating factor in extract of ox pancreas. ○——○——○ untreated medium; ●- - -●- - -● sterilized medium.

(b) Phospholipase A acts on e.g. phosphatidyl ethanolamine; phospholipase C does not. With the Warburg technique it was found that the culture medium was able to split both lecithin and phosphatidyl ethanolamine (Fig. 8).

(c) Phospholipase A is able to lyse red blood corpuscles (e.g. from the rat) only in the presence of lecithin. In our experiments the sterile culture medium required lecithin for its haemolytic action. This is in contrast to the direct haemolytic activity of the toxin preparation.

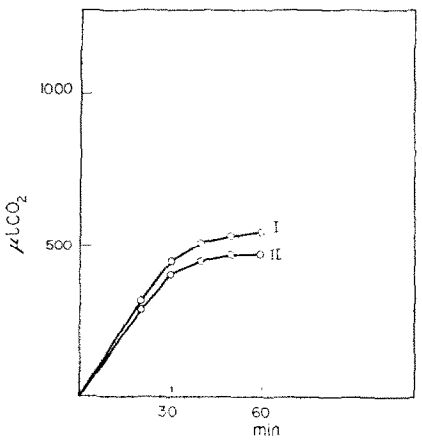

FIG. 8. Hydrolysis of lecithin (I) and phosphatidyl ethanolamine (II) produced by sterile culture medium.

DISCUSSION

Some properties of phospholipase A and C, including the influence of *Cl. welchii* antitoxin, are listed in the Table 1. The data indicate that the mast-cell degranulating

TABLE 1. SOME PROPERTIES OF PHOSPHOLIPASE A AND PHOSPHOLIPASE C

	Hydrolysis of lecithin	Hydrolysis of phosphatidyl ethanolamine	Direct haemolysis	Indirect haemolysis	Mast-cell degranulation	Inhibition by *Cl. welchii* antitoxin
Phospholipase A	+	+	0	+	+	0
Phospholipase C	+	0	+	−		+

factor in our toxin preparation was not phospholipase C: The mast cell degranulation caused by the toxin was not inhibited by *Cl. welchii* antitoxin. Fractions causing haemolysis and hydrolysis of lecithin *in vitro* did not degranulate mast cells and did not attack phosphatidyl ethanolamine. The haemolytic and lecithin-splitting actions were blocked by *Cl. welchii* antitoxin. Evidently it is possible to separate a phospholipase C fraction showing no mast-cell degranulating ability.

In fact the mast-cell degranulating factor does not even seem to be formed by the bacteria. It already occurs in the sterile culture medium, and the observations are

consistent with the assumption that it is phospholipase A: It contains a principle that splits phosphatidyl ethanolamine, and it requires lecithin for its haemolytic action.

Högberg and Uvnäs[1] suggested that the degranulation of mast cells induced by compound 48/80, agents extracted from *Ascaris suis* and *Cyanea capillata*, and antigen was due to an enzymatic process localized in the mast cell, involving the activation of a lytic enzyme attacking the phospholipids of the membranes. The influence of inhibitors suggested that the enzyme is related to phospholipase A but not to phospholipase C. The observation that the mast-cell degranulating factor in the crude *Cl. welchii* toxin is not phospholipase C but probably phospholipase A tends, in our opinion, to support our theory.

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