The Participation of Phospholipids in the Interaction of Leucocidin and the Cell Membrane of the Polymorphonuclear Leucocyte

By A. M. WOODIN AND ANTONNETTE A. WIENEKE
Sir William Dunn School of Pathology, University of Oxford

(Received 14 April 1967)

1. The interaction of the two components of leucocidin with various lipids has been studied by sedimentation, flotation, light-scattering and changes in the biological activity of leucocidin. 2. Phosphatidylserine, phosphatidylcholine, diphosphoinositide, triphosphoinositide and phosphatidic acid, but not phosphatidylethanolamine, lysophosphatidylcholine, cerebrosides, gangliosides or tristearin, induce aggregation of the F component of leucocidin. 3. The S component of leucocidin does not interact directly with these phospholipids, but interacts with the F component of leucocidin after its modification by lipids. 4. The increased sedimentation or light-scattering induced by low phospholipid concentrations is reversed at higher phospholipid concentrations. 6. The aggregates formed by phospholipids and leucocidin are due, not to adsorption of leucocidin alone, but also to the formation of leucocidin polymers. 7. It is concluded that the aggregation is due to the interaction of the F component with the fatty acid side chains in the lipid micelle. 8. The S component of leucocidin is inactivated by triphosphoinositide at physiological ionic strength; the F component of leucocidin is inactivated at low ionic strength by triphosphoinositide and remains inactive when the ionic strength is increased. 9. It is suggested that in the leucocyte cell membrane the S component of leucocidin interacts with the polar hydrophilic groups of triphosphoinositide and that the F component of leucocidin interacts with the hydrophobic parts of triphosphoinositide.

Woodin & Wieneke (1966) found that leucocidin could interact with the surface of the polymorphonuclear leucocyte in three ways. Large amounts of cells or isolated cell membranes could completely inactivate the two components of leucocidin at physiological salt concentrations. Most of the inactivated leucocidin remained in solution and the second interaction, adsorption of leucocidin, resulted in a maximum uptake of 20% of the S component of leucocidin and less of the F component of leucocidin. At low ionic strength a third interaction occurred, leading to an increased polymerization of leucocidin. A maximal effect was achieved with tenfold smaller amounts of cell membrane than were needed to inactivate leucocidin and the process did not proceed to completion, 20–30% of a given leucocidin concentration remaining unpolymerized. As adsorption and facilitated polymerization do not proceed to completion the leucocidin remaining unadsorbed or unpolymerized is different from that originally added to the cells or membranes, and it follows that leucocidin can adopt different isomeric forms.

Some of these properties of leucocyte membranes

were also exhibited by a lipid fraction isolated from the membrane. The present paper describes the interaction of leucocidin with purified phospholipids.

METHODS

Preparation of lipids

Leucocyte lipids. These were prepared from polymorphonuclear leucocytes (referred to in this paper as leucocytes) isolated from ascitic fluid (Woodin, 1961). The cells were centrifuged at 3000g for 10 min. and the pellets extracted with 20 vol. of chloroform-methanol (2:1, v/v) at room temperature for 30 min. The suspension was filtered and the filtrate shaken with 0.2 vol. of 0.145 M-NaCl solution. The lower layer was dried *in vacuo* and the solid dissolved in chloroform, forming the 'total leucocyte lipid' fraction.

Separation into acidic and neutral phospholipid fractions was achieved by adsorbing a sample of total lipids from 10^{10} cells on a column containing 10 g. of silicic acid, washing the column with 200 ml. of chloroform to remove lipids other than phospholipids and eluting the acidic phospholipids with 200 ml. of chloroform-methanol (9:1, v/v). The neutral phospholipids were then eluted with 200 ml. of methanol. The two fractions were dried *in vacuo* and dissolved in chloroform.

Ox brain lipids. Crude phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine, sphingomyelin, cerebrosides, gangliosides and lysocleithin were bought from Koch-Light Laboratories Ltd. (Colnbrook, Bucks.).

To purify crude phosphatidylserine, 350 mg. in 10 ml. of chloroform was adsorbed on a column containing 15 g. of silicic acid, which was then washed with 200 ml. of chloroform. Chloroform-methanol (9:1, v/v) was then applied to the column, the material eluted by 200 ml. collected and the phosphatidylserine it contained purified further on DEAE-cellulose by the method of Rouser, Kritchevsky, Heller & Lieber (1963). The product is referred to as 'partially purified phosphatidylserine'. Thin-layer chromatography on Kieselgel G with chloroform-methanol-7 N-NH₃ (12:7:1, by vol.) showed the presence of four components. The major component was eluted and is referred to as 'purified phosphatidylserine'. It had a P/amino N molar ratio 1.00:1.00, contained less than 0.004 mole of choline or 0.02 mole of inositol/g. atom of P and paper chromatography of an acid hydrolysate showed the presence of serine but less than 1% of ethanolamine.

Crude phosphatidylethanolamine was purified by chromatography on silicic acid, DEAE-cellulose and Kieselgel G in a similar way. The product had a P/amino N molar ratio 1.00:0.94. It contained less than 0.03 mole of choline or 0.02 mole of inositol/g. atom of P. Less than 1% of the amino N was derived from serine.

To purify crude phosphatidylcholine 700 mg. was adsorbed on 15 g. of silicic acid, impurities were removed by washing with 200 ml. of chloroform and elution was carried out with 200 ml. of chloroform-methanol (2:3, v/v). The product was purified further on DEAE-cellulose by the method of Rouser *et al.* (1963). Thin-layer chromatography on Kieselgel G with chloroform-methanol-7 N-NH₃ (12:7:1, by vol.) showed a single component in the purified phosphatidylcholine. It had a P/choline molar ratio 1.0:1.1 and contained less than 0.02 mole of inositol or 0.005 g. atom of amino N/g. atom of P.

Triphosphoinositide was prepared from ox brain by the method of Dittmer & Dawson (1961), except that after treatment with acetone and then ethanol the crude triphosphoinositide in the acid extract was shaken with N-HCl and the lower layer shaken with chloroform-methanol-N-HCl (3:48:47, by vol.) and then four times with chloroform-methanol-5 mm.-CaCl₂ (3:48:47, by vol.). The calcium salt of triphosphoinositide in the lower layer was diluted with 10 vol. of chloroform to decrease the methanol concentration and applied to a column of silicic acid (1 g./mg. of P). Impurities were removed by washing with chloroform and the triphosphoinositide was eluted with chloroform-methanol (9:1, v/v), 20 vol./g. of silicic acid being used. (Quantitative elution of the calcium salt of triphosphoinositide was obtained, but the sodium salt was irreversibly adsorbed.) The product is referred to as 'crude triphosphoinositide'. In some cases the crude triphosphoinositide was converted into the free acid, dissolved in chloroform and fractionated with methanol by the procedure of Dittmer & Dawson (1961). The material precipitated as the ammonium salt is referred to as 'triphosphoinositide (fraction B)'. Preparations of triphosphoinositide (fraction B) analysed by the method of Dawson & Eichberg (1965) contained 15-20% of diphosphoinositide. In some cases the triphosphoinositide (fraction B) was further purified by chromatography on formaldehyde-treated paper with the upper

phase of butan-1-ol-acetic acid-water-ether (20:5:25:6, by vol.) as solvent. The position of the separated components was determined by staining strips of the chromatogram with Rhodamine B (0.001% in water); the triphosphoinositide was eluted with chloroform-methanol (2:1, v/v) containing 0.05% of conc. HCl and converted into the ammonium salt. The product is referred to as 'highly purified triphosphoinositide'. This material contained 2-10% of diphosphoinositide.

Diphosphoinositide. This was prepared from 258 g. of ox brain that had been treated with acetone at room temperature for 3 hr. The crude diphosphoinositide was prepared from the acetone-insoluble residue by the method of Folch (1949). The product, containing 11 mg. of P, was converted into the calcium salt (see above), dissolved in chloroform and adsorbed on a column containing 8 g. of silicic acid in chloroform. After the silicic acid had been washed with chloroform the diphosphoinositide fraction was eluted with 250 ml. of chloroform-methanol (9:1, v/v) and converted into the free acid. This was dissolved in chloroform and fractionated with methanol in the same way as the preparation of the triphosphoinositide (fraction B) (Dittmer & Dawson, 1961). The diphosphoinositide was then isolated by chromatography on formaldehyde-treated paper (see above).

The diphosphoinositide contained less than 0.01 g. atom of amino N/g. atom of P, and after hydrolysis 95% of the phosphorus was recovered as inositol diphosphate when analysed by the method of Dawson & Eichberg (1965).

Phosphatidic acid. This was prepared by the method of Chibnall & Channon (1927). It was partly purified by adsorbing a sample, containing 0.5 mg. of P, on a column containing 2 g. of silicic acid. Impurities were removed by washing with chloroform, and the phosphatidic acid was eluted with chloroform-methanol (9:1, v/v). The product contained less than 0.01 g. atom of amino N/g. atom of P.

Phosphatidylinositol preparation. This was prepared by chromatography on DEAE-cellulose by the method of Hendrickson & Ballou (1964). The preparation contained 35% of phosphatidylserine.

Interaction of lipids with leucocidin

Preparation of solutions. Lipids dissolved in chloroform were dried *in vacuo* and suspended ultrasonically in water. The solutions were adjusted to pH 7.2 with 4% (w/v) NaHCO₃ solution and the concentration was determined from the phosphorus content. The two crystalline components of leucocidin were dissolved in and dialysed against 0.145 M-NaCl to give solutions containing 2-5 mg./ml. and maintained at 2°. Before use in the determination of the effect of phospholipids on the sedimentation or light-scattering of leucocidin, the leucocidin solutions were centrifuged at 30000g for 30 min. The concentrations were determined from the ultraviolet adsorption.

Sedimentation. Mixtures of lipid and leucocidin (in some cases labelled with ¹³¹I) were maintained in thick-walled glass tubes at room temperature for 1 hr. and centrifuged at room temperature for 30 min. at 7500g (8000 rev./min.) in the angle head of the MSE no. 17 centrifuge. The radioactivity or the protein in the mixtures before sedimentation and in the supernatant after sedimentation were determined.

To determine the distribution of labelled leucocidin in a sucrose gradient, mixtures of leucocidin and phospholipid in

0.5 ml. of 42% (w/v) sucrose and 1 mM-EDTA in 10 mM-tris buffer, pH 7.2, were maintained at room temperature for 30 min. and then placed on top of 2.25 ml. of a 60–45% (w/v) sucrose gradient. A further 2.25 ml. of a 40–30% (w/v) sucrose gradient was added and the mixtures were centrifuged at 39000 rev./min. (130000g) for 1 hr. in the SW 39 rotor of the Spinco model L ultracentrifuge. The bottom of the tube was pierced and 0.5 ml. fractions were collected. The radioactivity was determined.

Light-scattering. Mixtures of lipids and leucocidin (100 μ g./ml. in 0.20 ml. of 1 mM-EDTA in 10 mM-tris buffer, pH 7.2) were maintained at room temperature for 1 hr. and the intensity at 575 m μ of light scattered at 90° was observed when the suspensions were irradiated with light at 550 m μ . A Locarte fluorimeter was used and standardized with suspensions of polystyrene spheres (diam. 0.088 μ). Cylindrical silica cells with internal diam. 1 mm. were used. The intensity of light scattered by the phospholipids in the absence of leucocidin was determined and subtracted from that for the mixtures. The light scattered by leucocidin alone was less than 0.3 unit. The intensity of light scattered by a suspension of the polystyrene spheres (50 μ g./ml.) was given the value 14 fluorimeter units.

Modification of the biological activity of leucocidin by phospholipids. Solutions containing 0.6 μ g. of either or both components of leucocidin in 0.002 ml. of 0.145 M-NaCl were added to 0.05 ml. of the phospholipid suspension in 0.02 M-tris buffer, pH 7.2. After 30 min. at room temperature the mixtures were diluted with 0.5 ml. of 0.145 M-NaCl in 0.05 M-phosphate buffer, pH 7.2, containing 1 mg. of bovine serum albumin/ml. The leucocidin activity was determined. The effect of doubling dilutions of the phospholipids was investigated in the range 0.5–0.007 mM. In some cases an equimolar amount of CaCl₂ or EDTA (final concn. 20 mM) was added to the phospholipids before dilution. The effect of triphosphoinositide was determined in the same way and also with dilutions of the phospholipid made in 0.145 M-NaCl in 0.05 M-tris buffer, pH 7.2. Triphosphoinositide concentrations in the range 5.0–0.001 mM were investigated.

Recovery of triphosphoinositide after treatment with the S component of leucocidin. A sample of triphosphoinositide (fraction B) (1 μ mole in 1.0 ml. of 0.145 M-NaCl in 0.05 M-tris buffer, pH 7.2) was treated with 280 μ g. of the S component of leucocidin in 0.01 ml. of 0.145 M-NaCl. After 30 min. at room temperature and mixture was freeze-dried. The solid was extracted with 1.0 ml. of chloroform-methanol-water (75:25:2, by vol.) and centrifuged at 3000g for 5 min. The supernatant was dried *in vacuo* and the solid suspended ultrasonically in water. The phosphorus content and the ability to inactivate the S component of leucocidin under the conditions described above were determined. A control sample of triphosphoinositide was treated in the same way except that no leucocidin was added. The recovery of the triphosphoinositide phosphorus was 92% in each case.

Other methods

Determination of leucocidin activity. The method of Woodin & Wieneke (1966) involved the use of antitoxin, but, as the conformational changes of leucocidin described in the present paper could affect the avidity of the interaction of leucocidin with antibody, this was omitted. To the serially diluted leucocidin solutions were added 0.02 μ g. of the S component of leucocidin (to determine the F

component of leucocidin) or 0.02 μ g. of the F component of leucocidin (to determine the S component of leucocidin) in 0.025 ml. of gelatin-phosphate buffer, pH 7.2 (Woodin, 1961). The development of the end points then followed the method of Woodin & Wieneke (1966). The activity of leucocidin is expressed in units, the number of units in a leucocidin preparation being numerically equal to the dilution (in ml.) at the end point.

Chemical methods. These were: for phosphorus (Berenblum & Chain, 1938); for inositol (Norris & Dabre, 1956); for lipid amino N (Lea & Rhodes, 1954); for choline (Appleton, La Du, Levy, Steele & Brodie, 1953); for protein (Lowry, Rosebrough, Farr & Randall (1951)).

Determination of radioactivity. Radioactive samples were counted at infinite thinness with a Nuclear-Chicago gas-flow counter. Under the conditions used in this work, 1 mc gave 3.9 $\times 10^8$ counts/min.

Buffers. Phosphate buffers were potassium salts, tris buffers were chlorides and the EDTA solutions were sodium salts.

RESULTS

Sedimentation of leucocidin induced by leucocyte lipids at low ionic strength. Table 1 shows that the sedimentation of ¹³¹I-labelled leucocidin closely follows the sedimentation of total protein. The increased sedimentation of the S component of leucocidin is only found when the F component is also present, and the presence of the S component increased the amount of the F component sedimented when lipids are present. Fig. 1 shows the effect of a wide range of lipid concentrations. The increased sedimentation induced at low lipid concentrations is reversed at higher concentrations. The activity possessed by the total lipids of the leucocyte is also shown by the acidic phospholipid fraction; the degree of increased sedimentation induced by the neutral lipids is much smaller. The leucocidin that spontaneously polymerizes at low ionic strength has varied from 0 to 15% of a given preparation, although the increment induced by phospholipids has been more constant. However, the variation in the amount of spontaneously polymerizing leucocidin has resulted in some lack of precision in assessing the effect of lipids. This defect was subsequently overcome by studying the interaction by light-scattering.

Sedimentation of leucocidin induced by purified phospholipids. Fig. 2 shows the effect of phosphatidylserine and phosphatidylethanolamine on the sedimentation of leucocidin. Highly purified phosphatidylethanolamine has no effect, but with highly purified phosphatidylserine the induced sedimentation at low phosphatidylserine concentrations is reversed at higher concentrations. The reversal is not due to an impurity present in the phosphatidylserine preparation. Fig. 2 shows that the partially purified phosphatidylserine preparation shows a slightly smaller reversal. The effect of phosphatidylserine on leucocidin sedimentation was

Table 1. *Sedimentation of leucocidin by leucocyte lipids*

Leucocyte phospholipids were maintained with 100 μ g. of either or both components of leucocidin/ml., sometimes labelled with ^{131}I , in 10mM-NaCl and 1mM-EDTA in 5mM-phosphate buffer, pH 7.2, for 1 hr. and centrifuged. The amount of protein and radioactivity lost from the supernatant were determined.

Concn. of lipid (μ g. of P/ml.)	Leucocidin sedimented (% of amount added)							
	^{131}I -labelled F component only		^{131}I -labelled S component only		^{131}I -labelled S component and F component		^{131}I -labelled F component and S component	
	^{131}I	Protein	^{131}I	Protein	^{131}I	Protein	^{131}I	Protein
0	0	0	9	8	2	2	2	1
2.2	19	16	5	6	44	54	39	29
5.6	26	22	0	5	67	62	58	49

Fig. 1. Sedimentation of leucocidin by lipid fractions from the leucocyte. Solutions containing 50 μ g. of the F component of leucocidin/ml. and 50 μ g. of the S component of leucocidin/ml. labelled with ^{131}I (18000 counts/min./ml.) and lipid fractions as indicated were maintained in 10mM-NaCl and 1mM-EDTA in 5mM-phosphate buffer, pH 7.2, containing 1mg. of bovine serum albumin/ml., for 1 hr. at room temperature and centrifuged. The radioactivity remaining in the supernatant was determined. ●, Total leucocyte lipid; ○, acidic phospholipid fraction from leucocytes; ▲, neutral phospholipid fraction from leucocytes.

Fig. 2. Sedimentation of leucocidin induced by phosphatidylserine and phosphatidylethanolamine. Solutions of phospholipids and 100 μ g. of the F component of leucocidin/ml. and 100 μ g. of the S component of leucocidin/ml. labelled with ^{131}I (35000 counts/min./ml.) were maintained in 10mM-NaCl and 1mM-EDTA in 5mM-phosphate buffer, pH 7.2, containing 1mg. of bovine serum albumin/ml., at room temperature for 1 hr. and centrifuged. The radioactivity remaining in the supernatant was determined. ●, Partially purified phosphatidylserine; ○, highly purified phosphatidylserine; ▲, crude phosphatidylethanolamine; ▽, highly purified phosphatidylethanolamine.

not inhibited by a 100-fold molar excess of phosphoserine. Fig. 3 shows the effect of crude phosphatidylcholine, sphingomyelin, cerebroside and tristearin. The last three compounds are without effect, but phosphatidylcholine shows a small increased sedimentation that is reversed at higher phosphatidylcholine concentrations. It is probable that this effect is a function of the phosphatidylcholine content of this preparation, since a similar effect of highly purified phosphatidylcholine is found on the light-scattering of leucocidin solutions.

Light-scattering. The amount of light scattered by leucocidin solutions at low ionic strength was very small and this enabled the effects of phospholipids to be investigated with greater precision. Fig. 4 shows that some phospholipids over a

certain concentration range can increase the amount of light scattered by leucocidin solutions. At higher concentrations of phospholipid the effect is reversed. The reversal is not an artifact due to the presence of an inhibitor. If phosphatidylserine

Fig. 3. Effect of phosphatidylcholine, sphingomyelin, cerebrosidcs and tristearin on the sedimentation of leucocidin. Solutions of the lipids and 100 μ g. of the F component of leucocidin/ml. and 100 μ g. of the 131 I-labelled S component of leucocidin/ml. (35 000 counts/min./ml.) were maintained at room temperature for 1 hr. in 10 mM-NaCl and 1 mM-EDTA in 5 mM-phosphate buffer, pH 7.2, containing 1 mg. of bovine serum albumin/ml., and centrifuged. The radioactivity remaining in the supernatant was determined. ∇ , Crude phosphatidylcholine; $\bullet$, cerebrosidcs; Δ , sphingomyelin; $\circ$, tristearin.

in increasing amounts is added sequentially to a leucocidin solution the light scattered is similar to that described by Fig. 4(c). Fig. 4 also shows that with phosphatidylcholine and triphosphoinositide the increased light-scattering by the F component of leucocidin is greater in the presence of the S component but that little increase is found in the light scattered by the S component of leucocidin alone. This synergism was also observed when 20 μ g. of each or both components of leucocidin/ml. was treated with phosphatidylserine. Thus the effects of phospholipids on light-scattering by leucocidin parallel those on leucocidin sedimentation.

Fig. 4 shows that different phospholipids produce different maximal intensities of light-scattering and that the concentration of phospholipid required to produce a maximal effect varies. An increased light-scattering was also produced by phosphatidic acid and by diphosphoinositide, but phosphatidylethanolamine (0.03–0.5 mM) at pH 5, 6, 7 or 9 was without action. At pH 7.2 under the conditions described in Fig. 4 no increased light-scattering was found when leucocidin was mixed with sphingomyelin or lysolecithin. Brain gangliosides (0.03–0.5 mM) did not increase the light scattered by the F component of leucocidin. The increased light-scattering induced by triphosphoinositide was not inhibited by an eightfold molar excess of inositol triphosphate nor the increase due to phosphatidylserine by a 25-fold molar excess of serine phosphate. Calcium ions inhibited the action of both phospho-

Fig. 4. Increased light-scattering by leucocidin induced by phospholipids. Phospholipids in 0.18 ml. of 1 mM-EDTA in 0.01 M-tris buffer, pH 7.2, were added to 0.02 ml. of 0.145 M-NaCl containing 20 μ g. of one or both components of leucocidin. Further samples of phospholipid were added to 0.02 ml. of 0.145 M-NaCl. After 1 hr. at room temperature the intensity of the scattered light was determined. The values for the phospholipids alone were subtracted from those for the mixtures with leucocidin. $\circ$, Both components of leucocidin present; Δ , F component of leucocidin present; $\bullet$, S component of leucocidin present. (a) Triphosphoinositide, 1 min. ultrasonic treatment. (b) Phosphatidylcholine, 2 min. ultrasonic treatment. (c) Phosphatidylserine, 1 min. ultrasonic treatment.

tidylserine and triphosphoinositide (Figs. 5a and 5b).

Complex-formation between leucocidin and phospholipids. Fig. 6 shows the amount of leucocidin sedimented as a function of the leucocidin in the supernatant and does not resemble a Langmuir adsorption isotherm. In the experiment recorded in Fig. 6 all the phosphatidylserine was sedimented in the presence of leucocidin, but in its absence less than 10% was sedimented and this was not increased by doubling the sedimentation time. The lowest protein concentration recorded in Fig. 6 gave a protein/phospholipid ratio 1.5 : 1.0 (assuming phosphatidylserine to have molecular weight 800), which is insufficient for the increased sedimentation

Fig. 5. Inhibition by Ca^{2+} ions of the increased light scattered by leucocidin-phospholipid mixtures. Mixtures of phospholipids and leucocidin were treated as described in Fig. 4. (a) Phosphatidylserine, 1 min. ultrasonic treatment: ▲, 1 mM-EDTA present; △, 1 mM- CaCl_2 present. (b) Triphosphoinositide, 10 sec. ultrasonic treatment: ○, 1 mM-EDTA present; ●, triphosphoinositide/ Ca^{2+} molar ratio 1:1.

Fig. 6. Effect of leucocidin concentration on the sedimentation induced by phosphatidylserine. Mixtures of equal amounts of each component of leucocidin and phosphatidylserine (5.2 $\mu\text{g.}$ of P/ml.) in 1 mM-EDTA in 10 mM-tris buffer, pH 7.2, were maintained at room temperature for 30 min. and then centrifuged at 8000g for 30 min. The protein lost from the supernatant and the phosphorus in the pellet were determined.

to be explained by adsorption of leucocidin on the surface of the phospholipid micelle. It appears that leucocidin polymers are also produced. Table 2

describes a similar experiment with triphosphoinositide. Alone this was not sedimented during 4 times the centrifugation time used in the experiment recorded in Table 2 but in the presence of 5.5 times its mass of leucocidin all was sedimented.

The complexes produced between leucocidin and phospholipids are weak and readily dissociate. A mixture of 200 $\mu\text{g.}$ of each component of leucocidin/ml. and 3.0 $\mu\text{g.}$ of phosphatidylserine P/ml. was opalescent, and when centrifuged in a sucrose gradient the opalescence was lost with recovery of phospholipid, but not leucocidin, in the top of the tube. With a smaller leucocidin/phospholipid concentration ratio there was evidence for transfer of some leucocidin to regions of lower density (Fig. 7).

Inactivation of leucocidin in solution by phospholipids. Leucocytes and leucocyte cell membranes inactivate leucocidin in solution at physiological ionic strength. This does not result solely as a consequence of the polymerization reactions described above. Exposure of leucocidin at low ionic strength to phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid, diphosphoinositide or the phosphatidylinositol preparation as the calcium salts or in the presence of EDTA did not decrease the leucocidin activity found when the ionic strength was subsequently increased to physiological values.

Triphosphoinositide inactivated the S component of leucocidin at physiological ionic strength (Fig. 8). Expressed in terms of the triphosphoinositide content, similar activities were found with crude triphosphoinositide (fraction B) and the latter material further purified by chromatography on formaldehyde-treated paper. The effect of crude triphosphoinositide required traces of bivalent cations and was inhibited by 50 mM-EDTA. Four preparations of triphosphoinositide (fraction B) have inactivated the S component of leucocidin at similar concentrations, and a sample of the ammonium salt dissolved in chloroform-methanol-water (75:25:2, by vol.) and stored at -70° during 3 months has given preparations that inactivated the S component of leucocidin at similar concentrations. Some preparations have shown a reversal of the inactivation of the S component of leucocidin at high triphosphoinositide concentrations (Fig. 8). This reversal has been observed with eight suspensions made from four different preparations of triphosphoinositide (fraction B); it was apparently absent from 15 suspensions. After several days at 0° some suspensions of triphosphoinositide had become converted into a form that activated leucocidin (Table 3). This treatment did not degrade the triphosphoinositide; when the preparation was treated with 20 vol. of chloroform-methanol-conc. hydrochloric acid (800:400:3, by vol.) and 0.4 vol.

Table 2. *Sedimentation of mixtures of leucocidin and triphosphoinositide at low ionic strength*

Mixtures of equal amounts of each component of leucocidin and triphosphoinositide in 3.0 ml. of 1 mM-EDTA in 10 mM-tris buffer, pH 7.2, were maintained at room temperature for 30 min. and centrifuged at 8000g for 30 min. The phosphorus lost from the supernatant and the protein present in the pellet were determined.

Triphosphoinositide added ($\mu\text{g. of P}$)	Triphosphoinositide in supernatant ($\mu\text{g. of P}$)	Leucocidin added ($\mu\text{g. of protein}$)	Leucocidin in pellet ($\mu\text{g. of protein}$)
4.6	4.6	0	—
2.3	2.3	0	—
4.6	1.2	350	250
2.3	0.4	350	250
4.6	0.3	700	520
2.3	0.2	700	250

Fig. 7. Flotation of leucocidin in the presence of phosphatidylserine. Mixtures of equal amounts of the two components of leucocidin (labelled with ^{131}I ; 5300 counts/min.) were mixed with 1.5 $\mu\text{g.}$ of phosphatidylserine in 0.5 ml. of 42% (w/v) sucrose and 1 mM-EDTA in 10 mM-tris buffer, pH 7.2, and inserted in a 60–30% (w/v) sucrose gradient. After centrifugation at 114000g for 1 hr. the distribution of radioactivity in fractions withdrawn from the tubes was determined. (a) 200 $\mu\text{g.}$ of leucocidin protein present. (b) 33 $\mu\text{g.}$ of leucocidin protein present.

Fig. 8. Inactivation of the S component of leucocidin by triphosphoinositide. Mixtures of 0.6 $\mu\text{g.}$ of the S component of leucocidin and triphosphoinositide in 0.05 ml. of 0.145 M-NaCl in 0.05 M-tris buffer, pH 7.2, were maintained at room temperature for 30 min. and then the leucocidin activity was determined. ▲, Crude triphosphoinositide, triphosphoinositide/ Ca^{2+} molar ratio 1:1; Δ, crude triphosphoinositide, 50 mM-EDTA present in all mixtures; ●, triphosphoinositide (fraction B); ○, triphosphoinositide (fraction B) showing reversal of the inactivation of the S component of leucocidin.

of N-hydrochloric acid no phosphorus could be found in the upper layer. In a similar experiment a sample of triphosphoinositide (2 mM in water) was maintained for 11 days at 0°. Tested under the conditions of Fig. 8, 0.3 mμmole of the preparation inactivated 0.6 $\mu\text{g.}$ of the S component of leucocidin initially, but after 4 and 11 days the amounts required were 2 and 50 mμmoles respectively. The esterified fatty acids were determined by gas-liquid chromatography after methanolysis (Hendrickson & Ballou, 1964) and found not to differ significantly from those reported to be present in triphosphoinositide by Hendrickson & Ballou (1964). Thus

under some conditions the interaction of leucocidin and triphosphoinositide is altered without a change in the covalent structure of triphosphoinositide. It is possible that, in water, triphosphoinositide can adopt different micellar forms and that the proportions of these determine the change produced in leucocidin. Woodin & Wieneke (1966) found that the two components of leucocidin can adopt different isomeric forms, and it is possible that the interaction with triphosphoinositide also depends on the proportions of these in a given preparation of the S component.

The inactivation of increasing amounts of the S component of leucocidin requires increasing amounts of triphosphoinositide (Table 4). This is not a result of reversible adsorption of leucocidin on triphosphoinositide, for a tenfold increase in the volume of the system does not affect the amount of leucocidin inactivated. Table 5 shows that the S component of leucocidin and triphosphoinositide sediment independently; stable complexes are not present. When triphosphoinositide (1 μ mole) was treated with 18000 activity units of leucocidin for 30 min. at room temperature this activity was decreased to 1800 units; but the triphosphoinositide could be recovered by extraction into chloroform-methanol solution, and on subsequent suspension in water it inactivated leucocidin at the same concentration as the starting material. The action of leucocidin on triphosphoinositide is thus reversible.

The F component of leucocidin partly reverses the loss of activity of the S component of leucocidin induced by triphosphoinositide (Table 6). The activity of the F component of leucocidin is

irreversibly decreased by triphosphoinositide at low ionic strength (Fig. 9), and under these conditions the ability of triphosphoinositide to inactivate the S component is decreased fivefold (cf. Fig. 9). The sodium, potassium and ammonium salts of triphosphoinositide behaved identically in inactivating leucocidin at low ionic strength.

DISCUSSION

Two types of interaction between leucocidin and phospholipids are described in the present paper. The inactivation of the F and the S components of leucocidin is a specific effect of triphosphoinositide. This can be the basis of the inactivation of leucocidin by the cell membrane. The second interaction occurs with certain glycerophospholipids at low ionic strength and results in the production of aggregates. This can be involved in the elimination of leucocidin by leucocyte membranes at low ionic strength (Woodin & Wieneke, 1966).

Aggregation. The aggregation reaction involves

Table 3. *Effect of aging on the action of triphosphoinositide suspensions on the activity of the S component of leucocidin*

A sample of triphosphoinositide (2 mm in water) was maintained for the time indicated and then 2 μ moles were treated with 0.3 μ g. of the S component of leucocidin in 0.05 ml. of 0.145 M-NaCl in 0.05 M-tris buffer, pH 7.2, for 30 min. The activity of the S component of leucocidin was determined.

Age of triphosphoinositide suspension	Ratio: $\frac{\text{S component activity withtriphosphoinositide}}{\text{S component activity alone}}$
30 min.	0.025
2 hr.	0.025
4 days	2
7 days	2.5

Table 5. *Sedimentation of leucocidin and triphosphoinositide*

The ^{131}I -labelled S component of leucocidin (24 μ g.; 40000 counts/min.) was maintained in 1.5 ml. of 0.145 M-NaCl in 0.05 M-tris buffer, pH 7.2, alone or with 0.41 mm-crude triphosphoinositide, for 30 min. at room temperature. Either 0.02 mm- CaCl_2 or 50 mm-EDTA was also present. The mixtures were centrifuged at 102000g for 3 hr. and the phosphorus and radioactivity in the supernant determined.

Addition	% of P sedimented	% of radioactivity sedimented
CaCl_2 + triphosphoinositide	64	13
CaCl_2	—	18
EDTA + triphosphoinositide	62	15
EDTA	—	18

Table 4. *Dependence of the inactivation of the S component of leucocidin on the amount of triphosphoinositide*

Serial dilutions of triphosphoinositide were made in 0.05 ml. (A) or 0.5 ml. (B) of 0.145 M-NaCl in 0.05 M-tris buffer, pH 7.2, and then the amounts of leucocidin indicated were added in 10 μ l. of 0.145 M-NaCl. After 30 min. at room temperature the leucocidin activity was measured. The results for the two conditions A and B were identical.

S component added (μ g.)	Triphosphoinositide required to produce inactivation (μ moles)			
	95% inactivation	85% inactivation	60% inactivation	20% inactivation
0.3	1.7	1.1	1.1	1.1
0.9	5.9	4.5	3.9	2.6
2.7	20	17	12	8.8

Table 6. *Reversal by the F component of leucocidin of the inactivation of the S component of leucocidin induced by triphosphoinositide*

Mixtures containing triphosphoinositide and the F component of leucocidin as indicated were maintained with 0.6 $\mu\text{g.}$ of the S component of leucocidin in 0.05 ml. of 0.145 M-NaCl in 0.05 M-tris buffer, pH 7.2, for 30 min. and then the S component activity was determined.

Triphosphoinositide present (m μ moles)	F component of leucocidin present ($\mu\text{g.}$)	Activity of S component (activity units)
0	0	14
4	0	< 0.25
4	0.6	0.5
4	3.0	2.0
4	6.0	8.0

Fig. 9. Inactivation of leucocidin by triphosphoinositide at low ionic strength. Mixtures of triphosphoinositide and leucocidin (0.3 $\mu\text{g.}$ of each component) were maintained in 0.05 ml. of 0.5 mM-tris buffer, pH 7.2, for 30 min. at room temperature, diluted with 0.5 ml. of 0.145 M-NaCl in 0.05 M-tris buffer, pH 7.2, containing 1 mg. of bovine serum albumin/ml., and the leucocidin activity was determined. $\circ$, F component of leucocidin only present; $\bullet$, F component activity in the presence of both components of leucocidin; Δ , S component of leucocidin only present.

primarily the F component of leucocidin; the S component is not polymerized by phospholipids directly but reacts with the F component after its modification by the phospholipids. Stable complexes between leucocidin and phospholipids are not produced, but there is evidence for the formation of readily dissociated complexes. The properties of mixtures of leucocidin and phospholipids are not due to adsorption of leucocidin on the surface of a phospholipid micelle alone; leucocidin polymers are also produced. The increased light-scattering

and sedimentation induced in leucocidin solutions is reversed at higher phospholipid concentrations. This could result from the adsorption of phospholipid on leucocidin polymers at low phospholipid concentrations and, at higher concentrations, the existence of phospholipid micelles with adsorbed monomeric leucocidin.

The polymerization of leucocidin is induced by ionized glycerophospholipids that possess two fatty acid side chains. Sphingomyelin, cerebroside, tristearin, gangliosides and lysophosphatidylcholine are inactive. All the acidic phospholipids tested and phosphatidylcholine induce polymerization and there is no specificity in the structure of the polar parts of the lipids. This suggests that the F component of leucocidin interacts with the esterified fatty acids and not the polar regions. Consistent with this is the failure of serine phosphate to inhibit phosphatidylserine, inositol triphosphate to inhibit triphosphoinositide or phosphate to inhibit the leucocyte lipids. The need for the low ionic strength in the aggregation reaction can arise because only under these conditions will the distance between the polar parts of the phospholipid in the micelle be large enough to permit entry of the relevant part of the F component of leucocidin. Cross-linking of the polar regions by Ca^{2+} would be expected and Ca^{2+} inhibits the polymerization of leucocidin. The failure of phosphatidylethanolamine to induce polymerization is anomalous, but Wren & Merryfield (1966) have produced evidence that the uncharged tautomer contributes to the structure of this lipid.

Inactivation. The inactivation of increasing amounts of leucocidin requires increasing amounts of triphosphoinositide, yet alteration of the covalent structure of triphosphoinositide or firm binding to the S component of leucocidin does not occur. This suggests that leucocidin induces a conformational change in the micelle. It has also been suggested in the present paper that the reversal of the inactivation of the S component of leucocidin with high triphosphoinositide concentrations and the alteration in the properties of triphosphoinositide during storage in water also indicate that triphosphoinositide can adopt different forms. Supporting evidence that this is true for triphosphoinositide is not available, but there is evidence from the properties of other lipids to suggest that it is feasible. Thus van Deenen (1966) has described the dependence of the size and shape of phosphatidylcholine micelles on the nature of the esterified fatty acids. Chapman (1966) has described variations in the dispersibility in water and the swelling pressure in monolayers of lipids with different esterified fatty acids. It has also been shown by van Golde & van Deenen (1967) that natural phosphatidylethanolamine consists of a mixture of

molecules containing different esterified fatty acids. This molecular heterogeneity can be the basis of variations in the form of the micelle. A further source of the heterogeneity of the phospholipid micelles used in the present work is the very short period of ultrasonic treatment. Attwood & Saunders (1965) have shown that, even after 1 hr. ultrasonic treatment, phosphatidylcholine micelles were poly-disperse.

The inactivation of the F component of leucocidin by triphosphoinositide proceeds only at low ionic strength; this is consistent with the conclusion drawn above that the interaction of the F component of leucocidin with phospholipids involves contact with the esterified fatty acids. With the leucocyte cell membrane inactivation of the F component of leucocidin occurs at physiological ionic strength, but that is not evidence that the process does not involve triphosphoinositide; it suggests that in the membrane the esterified fatty acids are exposed. Woodin & Wieneke (1966) found the inactivation of leucocidin by leucocyte membranes was prevented by treating the membranes with a phospholipase A preparation.

It will not be possible with aqueous suspensions of triphosphoinositide to reproduce the conditions in the leucocyte membrane where simultaneous access of the S component of leucocidin to the polar regions and of the F component of leucocidin to the hydrophobic regions of triphosphoinositide at physiological ionic strength could occur. The molecular basis of the synergism between the two components of leucocidin is thus not clear.

We thank Mr H. Showell for excellent technical assistance. Both authors are members of the External Staff of the Medical Research Council. Dr E. Adams kindly made the gas-liquid chromatography measurements.

REFERENCES

- Appleton, H. D., La Du, B. N., Levy, B. B., Steele, J. M. & Brodie, B. B. (1953). *J. biol. Chem.* **205**, 803.
Attwood, D. & Saunders, L. (1965). *Biochim. biophys. Acta*, **98**, 344.
Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
Chapman, D. (1966). *Ann. N.Y. Acad. Sci.* **137**, 717.
Chibnall, A. C. & Channon, H. J. (1927). *Biochem. J.* **21**, 717.
Dawson, R. M. C. & Eichberg, J. (1965). *Biochem. J.* **96**, 634.
Dittmer, J. C. & Dawson, R. M. C. (1961). *Biochem. J.* **81**, 535.
Folch, J. (1949). *J. biol. Chem.* **177**, 497.
Hendrickson, H. S. & Ballou, C. E. (1964). *J. biol. Chem.* **239**, 1369.
Lea, O. H. & Rhodes, D. N. (1954). *Biochem. J.* **56**, 613.
Lowry, C. H., Rosebrough, N. J., Farr, L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Norris, F. W. & Darbre, A. (1956). *Analyst*, **81**, 394.
Rouser, G., Kritechsky, G., Heller, D. & Lieber, E. (1963). *J. Amer. Oil Chem. Soc.* **40**, 425.
van Deenen, L. L. M. (1966). *Ann. N.Y. Acad. Sci.* **137**, 717.
van Golde, L. M. G. & van Deenen, L. L. M. (1967). *Chem. Phys. Lipids*, **1**, 164.
Woodin, A. M. (1961). *Biochem. J.* **80**, 562.
Woodin, A. M. & Wieneke, A. A. (1966). *Biochem. J.* **99**, 479.
Wren, J. J. & Merryfield, D. S. (1966). *Nature, Lond.*, **209**, 1315.