The Modification of the Cytotoxic Effect of Leucocidin by *N*-Ethylmaleimide, Flavine Mononucleotide and Menadione

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1. The movement of the cytoplasmic granules in the leucocidin-treated leucocyte is prevented in the presence of *N*-ethylmaleimide or menadione. This effect follows a change of state in the cytoplasm. It may not be due to reaction with SH groups. When granule movement is prevented in this way the subsequent addition of Ca^{2+} and ATP does not induce the secretion of the proteins of the granules. 2. Menadione or iodoacetate stimulates some effects of suboptimum amounts of leucocidin. This effect probably follows a reaction with SH groups. 3. Flavine mononucleotide inhibits some effects of suboptimum amounts of leucocidin. 4. Leucocidin decreases the stimulation of glucose oxidation due to menadione but increases that due to flavine mononucleotide. Leucocidin decreases the adsorption of menadione by leucocytes but increases that of flavine mononucleotide. 5. The redox state of the nicotinamide-adenine nucleotide coenzymes is not altered during leucocidin action and flavine mononucleotide and menadione do not undergo significant continuous oxidation and reduction when added to the leucocyte.

When polymorphonuclear leucocytes are treated with leucocidin, restricted changes in the permeability of the cell occur and are accompanied by an accumulation of P_i and by alterations in the turnover of the cell nucleotides. If Ca^{2+} is present in the medium it is accumulated within the cell and induces the secretion of the contents of the cytoplasmic granules to the cell exterior. The secretion of the granule proteins proceeds by fusion of the membranes of the granule and the cell surface and it is facilitated if nucleoside phosphates are present. If leucocidin-treated cells are maintained in the presence of EDTA, secretion of the granule proteins does not occur but the cell becomes permeable to cytoplasmic constituents. Subsequent addition of Ca^{2+} and ATP induces the secretion of the granule proteins, and by adding inhibitors together with Ca^{2+} and ATP an inhibitory action on secretion can be distinguished from one on the earlier, direct effects of leucocidin. The permeability changes of the leucocidin-treated cell resemble those in excitable tissues during membrane depolarization. At the motor end-plate, for example, acetylcholine induces an increased permeability to ions and in the pancreas and adrenal medulla stimulates Ca^{2+} uptake and secretion of material contained in the cytoplasmic granules. In the living cell, changes in ion content are accompanied by increased respiration and are reversed; in the leucocidin-treated cell respiration does not increase and the ion changes

are not reversed. Thus, for the purposes of description, the leucocidin-treated cell can be regarded as a permanently depolarized, uncoupled, potentially secreting cell. From the analysis of the response of the leucocyte to leucocidin it was not clear if the primary change was with phosphorus metabolism or membrane structure. Reviews of the cytotoxic effect of leucocidin are given by Woodin & Wieneke (1964a) and Woodin (1965, 1966).

The analysis of the molecular mechanism of these cytotoxic changes will be facilitated by the discovery of inhibitors operating at different stages. The present paper describes the modification of the cytotoxic effect of leucocidin by *N*-ethylmaleimide, FMN and menadione. The second paper in this series (Woodin & Wieneke, 1966a) describes the interaction of leucocidin with the cell membrane and the third paper (Woodin & Wieneke, 1966b) chemical and enzymic studies on the isolated cell membrane of the leucocyte.

METHODS

Materials. Polymorphonuclear leucocytes (referred to in this paper as leucocytes), macrophages, Hanks medium and Krebs-Ringer media for suspending the cells were prepared as described by Woodin (1961) except that glucose was omitted from the suspending media. Albumin labelled with ^{131}I was obtained and used as described by Woodin & Wieneke (1963). The two crystalline components

of leucocidin (Woodin, 1960) were dissolved in 0.9% NaCl solution. In the experiments described in the present paper both components of leucocidin were present and the concentrations quoted refer to that of each component. FMN (British Drug Houses Ltd., Poole, Dorset) was dissolved in water, adjusted to pH 7.2 with NaOH and diluted to give a 50 mM solution. Menadione (2-methyl-1,4-naphthoquinone) was purchased from L. Light and Co. Ltd., Colnbrook, Bucks. It was used as a solution in ethanol or in Hanks medium. To prepare the solution in Hanks medium, 0.5 ml. of a saturated solution of menadione in ethanol was diluted with 40 ml. of Hanks medium and the mixture filtered. The concentration of the menadione dissolved in the filtrate was determined from $E_{265\text{ m}\mu}$. Menadione sodium bisulphite (sodium 2-methyl-1,4-naphthoquinone-2-sulphonate) was also obtained from L. Light and Co. Ltd. and was used as a solution in Hanks medium.

Inhibition of Ca^{2+} -induced β -glucuronidase extrusion in the leucocidin-treated cell. To determine the time-dependence of the inhibitory action of *N*-ethylmaleimide, suspensions of 5×10^7 leucocytes in 0.9 ml. of Ca^{2+} -free Hanks solution containing EDTA (magnesium salt) (0.3 mM) were incubated with 2 μg . of leucocidin at 37° for 5 min. and then 0.1 ml. of *N*-ethylmaleimide (10 mM) in 0.9% NaCl solution was added. After various periods 0.025 ml. of 40 mM- CaCl_2 and 0.025 ml. of 40 mM-ATP were added and incubation was continued for a further 10 min. The suspensions were cooled in ice and centrifuged at 1800g for 5 min. The β -glucuronidase in the cell supernatant was determined.

The effect of other substances was determined in the same way except that menadione was added as a 50 mM solution in 0.01 ml. of ethanol to 1 ml. of cell suspension. A control tube contained the same amount of ethanol. The substances listed in Table 1 were found not to change the small amount of β -glucuronidase released from normal cells in Ca^{2+} -free medium and did not affect the activity of β -glucuronidase.

*Morphological changes induced by *N*-ethylmaleimide.* To detect changes in the motion of the cytoplasmic granules, duplicate samples of leucocytes (5×10^7) in 1 ml. of Ca^{2+} -free Hanks solution containing EDTA (magnesium salt) (0.3 mM) were incubated with 4 μg . of leucocidin at 37° for 3 min. and then 0.1 ml. of 10 mM-*N*-ethylmaleimide was added to one sample of cells and 0.025 ml. of 40 mM- CaCl_2 plus 0.025 ml. of 40 mM-ATP to the other. Incubation was continued at 37° and the cells were examined with a phase-contrast microscope at 2 min. intervals.

*Effect of *N*-ethylmaleimide on isolated granules.* The effect of *N*-ethylmaleimide on the release of enzymes from isolated granules of the leucocyte was assessed with granules prepared from leucocytes by the method of Cohn & Hirsch (1960). The concentration of the granules was determined from $E_{600\text{ m}\mu}$. The unit mass of granules is the amount that, suspended in 1 ml. of 0.32 M-sucrose, would have $E_{1\text{ cm}}^{600\text{ m}\mu}$ 1.0 (Woodin & Wieneke, 1964b). Granules were suspended in duplicate to give 2.5 units/ml. in 2.0 ml. of the following media, one sample of each medium containing *N*-ethylmaleimide (1 mM): 0.32 M-sucrose; Hanks medium; Ca^{2+} -free Hanks medium; 0.32 M-sucrose-1 mM- CaCl_2 -1 mM-ATP. The suspensions of granules were incubated at 37° for 10 min. and centrifuged at 22000g for 20 min. and the β -glucuronidase and peroxidase in the supernatant were determined. The β -glucuronidase and peroxidase

activities of a suspension of frozen and thawed granules were not affected by 1 mM-*N*-ethylmaleimide.

Inhibition of adenosine triphosphatase by *N*-ethylmaleimide was tested by comparing the amount of P released in suspensions of 2.5 units of granules in 1.0 ml. of 1 mM-ATP-1 mM- CaCl_2 -0.05 M-tris buffer, pH 8.0, incubated at 37° for 10 min. alone or with *N*-ethylmaleimide (1 mM).

Modification of the cytotoxic effect of leucocidin by FMN and menadione. Leucocytes (5×10^7) in 0.5 ml. of Hanks medium containing bovine serum albumin (0.1%) were added to tubes containing 0.02 ml. of 50 mM-FMN. After mixing, serially diluted solutions of equal amounts of the two components of leucocidin in 0.5 ml. of Hanks medium containing bovine serum albumin (0.1%) were added and the suspensions incubated at 37° for 10 min. They were then centrifuged at 1800g for 5 min. at 0° and the supernatants retained. Further samples of leucocytes were treated in the same way except that FMN was omitted from the incubation mixtures but added after the supernatants had been removed from the cell pellets.

To determine the effect of FMN on the extrusion process duplicate mixtures of 0.5 ml. of leucocytes and 0.5 ml. of serially diluted leucocidin, both in Ca^{2+} -free Hanks medium containing bovine serum albumin (0.1%), were incubated at 37° for 10 min. and then to one set of mixtures 0.020 ml. of 50 mM-FMN was added. Then 0.025 ml. of 40 mM- CaCl_2 -40 mM-ATP was added to both sets of mixtures and incubation was continued at 37° for 10 min. The mixtures were centrifuged at 1800g and the supernatants retained. Then 0.020 ml. of 50 mM-FMN was added to the supernatant of the cells that had been incubated without it. The β -glucuronidase was determined in the four sets of cell supernatants.

A similar procedure was followed to determine the effect of promethazine hydrochloride, chlorpromazine hydrochloride or hydrocortisone on the release of β -glucuronidase from the leucocidin-treated cell. To determine the effect of menadione, ubiquinone or vitamin E they were dissolved in ethanol. In all cases before determining the β -glucuronidase, the appropriate test compound was added to the supernatants isolated from cells that had been incubated without it.

Effect of FMN on the composition of the leucocyte. Leucocytes (5×10^7) in various media were incubated at 37° for 10 min. alone or with leucocidin (0.4 μg /ml.), or with FMN (1 mM) or with leucocidin (0.4 μg /ml.) plus FMN (1 mM). The mixtures were centrifuged at 1800g and the cell pellet and supernatant solution separated. To determine the effect on β -glucuronidase extrusion 1 ml. of cells in Hanks medium was used and the β -glucuronidase in the supernatant determined. The effect on leakage of aldolase into the medium was determined by using 1 ml. cells in Ca^{2+} -free Hanks solution containing EDTA (magnesium salt) (0.3 mM). The Ca^{2+} , K^+ and Na^+ contents were determined on the pellets from 5 ml. of cells that had been maintained in Hanks medium containing Ca^{2+} (0.2 mM). To determine the effect on P_i production and ATP breakdown 10 ml. of cells in phosphate-free Krebs-Ringer bicarbonate solution were taken, the P_i content of 1 ml. of cell suspension was determined, and the remainder was centrifuged and the ATP content of the pellets determined.

Uptake of FMN by the leucocyte. Leucocytes (5×10^7 /ml.) in 6 ml. of Hanks medium were added to duplicate sets of

weighed tubes containing FMN in 0.3 ml. of 0.9% NaCl solution. They were incubated at 37° for 10 min. and 0.3 ml. of ^{131}I -labelled albumin solution (70000 counts/min.) was added to one set of tubes. Both sets were centrifuged at 1800g for 5 min. and the supernatants removed. The tubes were weighed. The tubes containing ^{131}I -labelled albumin were dried at 110° and weighed again, the solid was dissolved in 1 ml. of 0.1 N-NaOH and the radioactivity was determined. The radioactivity in the corresponding supernatant was also determined. The cell pellets in the tubes that had not been treated with radioactive albumin were extracted with 1 ml. of 10% (w/v) trichloroacetic acid solution and after about 30 min. the suspensions were diluted to 3 ml. with water and centrifuged. The FMN in the original cell supernatant and the soluble fraction of the extracted pellet were determined from $E_{455\text{ m}\mu}$.

From the radioactivity in the cell pellet and the cell supernatant the extracellular volume of the cell pellet was calculated and the amount of FMN adsorbed to the cell determined.

The destruction of FMN was determined by incubating 5×10^7 leucocytes in 1 ml. of Hanks medium containing FMN (0.1 mm) at 37° for 10 min., extracting the suspension with 1 ml. of 10% (w/v) trichloroacetic acid and centrifuging at 1800g for 20 min. The supernatant was then shaken five times with 3 vol. of ether, adjusted to pH 7.2 with 0.1 N-NaOH and the FMN determined by enzymic assay (Huennekens & Felton, 1955).

Uptake of menadione by leucocytes. The uptake of menadione was much greater than that of FMN and it could be measured by a simpler method. Suspensions of 5×10^7 leucocytes in 0.1 ml. of Hanks medium were added to tubes containing 0.9 ml. of serially diluted solutions of menadione in Hanks medium. After 10 min. at 37° the mixtures were centrifuged at 1800g and $E_{265\text{ m}\mu}$ of the supernatant was determined. The effect of treatment with *N*-ethylmaleimide (5 mm) or leucocidin (5 μg .) was assessed by incubating the leucocytes for 10 min. at 37° with these reagents before addition to the menadione solutions.

Spectroscopic methods. The absorption spectrum of 0.22 mm-FMN in Hanks medium was determined with Hanks medium as blank solution and that of 0.22 mm-FMN in Hanks medium containing leucocytes ($5 \times 10^7/\text{ml}$.) was determined with leucocytes in Hanks medium as blank suspension. When cells were present they were resuspended by means of a Pasteur pipette before each reading. The same procedure was used with 0.185 mm-menadione in Hanks medium or in Hanks medium containing leucocytes ($5 \times 10^7/\text{ml}$.). A Unicam SP.500 spectrophotometer and cells with 0.5 cm. light-path were used.

Electron-paramagnetic-resonance spectra were measured by Dr C. Gutch with a Varian V-4500 100 kcy./sec. spectrometer, with the aqueous-solution cells. Leucocytes (10^9 cells) in 1 ml. of Hanks medium were mixed with 0.01 ml. of 0.1 M-FMN in water or with 0.02 ml. of 50 mM-menadione in ethanol and immediately transferred to the cavity (capacity 0.06 ml.). Further samples of leucocytes (10^9) in 1 ml. of Hanks medium containing FMN (1 mm) plus leucocidin (4 μg .) or menadione (1 mm) plus leucocidin (20 μg .) were examined in the same way.

Oxidation of glucose by leucocytes. The conversion of [^{14}C]glucose into $^{14}\text{CO}_2$ was determined. Leucocytes or macrophages were washed with Krebs-Ringer phosphate solution or Ca^{2+} -free Krebs-Ringer phosphate solution and

2.7 ml. samples of the cell suspensions placed in the main compartment of Warburg flasks with two side arms. The centre well of the flask contained 0.2 ml. of 20% (w/v) KOH solution. One side arm contained 44 mg. of [^{14}C]glucose (1.9 μC) in 0.05 ml. of the appropriate Krebs-Ringer phosphate solution; the other side arm contained 0.3 ml. of 100% (w/v) trichloroacetic acid solution. The leucocytes were treated with 0.3 ml. of Krebs-Ringer phosphate solution containing, in some cases, leucocidin (10 μg .) or FMN (25 mm) or menadione (0.56 mm) or this amount of leucocidin together with either FMN (25 mm) or menadione (0.56 mm). The flasks were stoppered and incubated at 37° for 3 min. and then glucose was added from the side arm.

Incubation was continued for a further 10 min. and the trichloroacetic acid tipped in. The flasks were left at room temperature for 2-3 hr. Then the contents of the centre well were washed out with three 1 ml. portions of water, and 0.1 ml. of 2% (w/v) Na_2CO_3 solution was added followed by 1.0 ml. of 5% (w/v) BaCl_2 solution. The precipitate was collected on filter paper, dried and the radioactivity counted.

Action of N-ethylmaleimide, FMN and iodoacetate on leucocidin. Solutions (1 ml.) containing 12 μg . of leucocidin in 0.145 M-NaCl-0.05 M-sodium phosphate buffer, pH 7.2, were maintained at room temperature for 30 min., alone or with *N*-ethylmaleimide (5 mm), FMN (5 mm) or sodium iodoacetate (5 mm), and then 2 mg. of bovine serum albumin in 0.2 ml. water was added. Each mixture was passed down a column containing 2 g. of Sephadex G-50 in 0.145 M-NaCl-0.05 M-sodium phosphate buffer, pH 7.2, and eluted with that solvent. The leucocidin in the protein peak was determined by the macrophage method (Woodin & Wieneke, 1966a). No significant difference in the leucocidin activity/mg. of protein was found with the four preparations.

Chemical methods. NAD and NADH_2 were determined by the method of Vilee (1962). NADP and NADPH_2 were determined by the method of Glock & McLean (1955). The ATP and Ca^{2+} contents of the cell pellets and β -glucuronidase, aldolase or peroxidase present in the cell medium were determined as described by Woodin & Wieneke (1963). Phosphorus was determined by the method of Berenblum & Chain (1938). Sodium and potassium were determined by flame photometry on material ashed in HNO_3 (Woodin, 1961).

Determination of radioactivity. A mica end-window Geiger-Müller counter was used. With this detector 1 mc gave 2×10^8 counts/min. ^{131}I was counted at infinite thinness and ^{14}C at infinite thickness.

RESULTS

Inhibition of extrusion of the granule proteins by N-ethylmaleimide. If *N*-ethylmaleimide (1 mm) is added to leucocidin-treated cells prepared in the absence of external Ca^{2+} , the extrusion of the granule proteins induced by the subsequent addition of Ca^{2+} and ATP is greatly diminished. The effect is dependent on the time between adding *N*-ethylmaleimide and Ca^{2+} plus ATP (Fig. 1). The granules of the leucocidin-treated cell prepared in the absence of external Ca^{2+} are in Brownian motion (Woodin, French & Marchesi, 1963), and

Fig. 1. Dependence of the inhibitory action of *N*-ethylmaleimide on the time of incubation of leucocytes in Ca^{2+} -free solution. Suspensions of 5×10^7 leucocytes in 0.9 ml. of Ca^{2+} -free Hanks solution, containing EDTA (magnesium salt) (0.3 mM), were incubated with leucocidin for 5 min. and then 0.1 ml. of 10 mM-*N*-ethylmaleimide was added. At various times CaCl_2 and ATP (each at 1 mM final concentration) were added and incubation was continued for a further 10 min. The amount of β -glucuronidase released was determined.

this persists when *N*-ethylmaleimide is added until after 5–10 min. it abruptly stops and phase-contrast microscopy shows the cytoplasm has a refractile appearance (Fig. 2). This suggests that the effect of *N*-ethylmaleimide is to induce a change of state of the cytoplasm, possibly the formation of a gel.

When isolated granules from normal cells were incubated in sucrose solution or Hanks medium the amount of β -glucuronidase or peroxidase released did not differ from that found by Woodin & Wieneke (1964b, see Table 6 of that paper) and these amounts were not changed by 1 mM-*N*-ethylmaleimide. The adenosine-triphosphatase activity of the isolated granules was similar to that reported by Woodin & Wieneke (1964b) and was not changed in the presence of 1 mM-*N*-ethylmaleimide. If *N*-ethylmaleimide is added to leucocytes in the presence of Ca^{2+} at the same time as leucocidin the extrusion of β -glucuronidase is not inhibited and *N*-ethylmaleimide also increased the amount of β -glucuronidase released from 10^8 normal cells from 5–10 units to 20–40 units (Table 4). These results suggest that *N*-ethylmaleimide does not inhibit the changes that occur when contact of the granule and the cell surface is established but that it prevents this contact being made. The action of *N*-ethylmaleimide is slow (Fig. 1) and only manifested when the permeability

Table 1. Inhibition of β -glucuronidase extrusion by substances affecting granule movement

Duplicate samples of leucocytes (5×10^7 cells) in 0.5 ml. of Ca^{2+} -free Hanks medium containing EDTA (magnesium salt) (0.3 mM) were treated with 4 μg . of leucocidin at 37° for 2 min. and the test substance was added in 0.5 ml. of Hanks medium. Immediately, or 10 min. after the test substance, 0.025 ml. of 40 mM- CaCl_2 –40 mM-ATP was added and 10 min. later the β -glucuronidase in the cell supernatant determined. Bovine serum albumin (1 mg./ml.) was present in the Hanks medium.

Test substance	β -Glucuronidase released (units/ 10^8 cells)	
	Ca^{2+} -ATP added immediately after test compound	Ca^{2+} -ATP added 10 min. after test compound
None	105	88
<i>N</i> -Ethylmaleimide (5 mM)	65	5
<i>N</i> -Ethylmaleimide (1 mM)	80	10
Menadione sodium bisulphite (5 mM)	100	15
Menadione sodium bisulphite (1 mM)	105	25
Menadione sodium bisulphite (5 mM) and cysteine (5 mM)	92	20
Sodium iodoacetate (5 mM)	100	95
Ethanol (0.17 M)	100	83
Menadione (0.5 mM) and ethanol (0.17 M)	72	25

of the leucocidin-treated cell is increased by Ca^{2+} depletion. It is possible that *N*-ethylmaleimide acts directly on the ground substance of the cytoplasm.

Table 1 shows the effect of some analogous compounds on the extrusion of β -glucuronidase induced in the leucocidin-treated cell by Ca^{2+} and ATP. The activity of menadione sodium bisulphite is not prevented if it is added as a mixture with an equivalent amount of cysteine, and it is possible that combination with SH groups is not the basis of the effect of these compounds.

Modification of the cytotoxic action of leucocidin by FMN. Fig. 3 shows that FMN (1 mM) decreases the amount of β -glucuronidase released from the leucocidin-treated cell prepared in the presence of external Ca^{2+} . If leucocytes are treated with leucocidin in the absence of Ca^{2+} then the subsequent addition of Ca^{2+} plus ATP induces the usual release of β -glucuronidase when FMN is present. The inhibitory action of FMN is not due to its affecting the extrusion process. The inhibitory action of FMN is only observed at low leucocidin

Fig. 2. Effect of *N*-ethylmaleimide on the cytoplasm of the leucocyte. (a) Leucocidin-treated leucocytes 10 min. after adding *N*-ethylmaleimide. (b) Leucocidin-treated leucocytes 10 min. after adding CaCl_2 and ATP. Both samples were examined by phase-contrast microscopy.

concentrations (Fig. 3); high leucocidin concentrations can overcome the protective action of FMN. Table 2 shows that the leakage of aldolase that occurs in the leucocidin-treated cell maintained in the absence of extracellular Ca^{2+} is inhibited by FMN and that changes in the P_i content are also modified. Ca^{2+} and P_i accumulate in the normal

cell treated with FMN but it is not known if this is by the same mechanism that operates when leucocidin is present. The loss of K^+ and gain of Na^+ in the leucocidin-treated leucocyte are not prevented by FMN, which therefore is not a leucocidin inhibitor but prevents the development of certain cytotoxic effects after the primary change induced by leucocidin.

Recovery of FMN from leucocytes. With 1mM-FMN in Hanks medium, 10^8 normal cells took up

Fig. 3. Inhibition of β -glucuronidase release by FMN. Suspensions of leucocytes (5×10^7 /ml.) in Hanks medium alone or with FMN (1mM) were incubated with leucocidin as indicated for 10min. and the β -glucuronidase in the cell supernatant was determined. Further duplicate cell suspensions in Ca^{2+} -free Hanks solution were incubated with leucocidin for 10min. and then treated with 1mM- CaCl_2 -1mM-ATP or with 1mM-FMN-1mM- CaCl_2 -1mM-ATP. After 10min. incubation the β -glucuronidase in the cell supernatant was determined. ●, Cells in Hanks medium; ▲, cells in Hanks medium with FMN; ○, cells in Ca^{2+} -free Hanks medium; △, cells in Ca^{2+} -free Hanks medium, FMN added after 10min.

Fig. 4. Adsorption of FMN by leucocytes. Suspensions of leucocytes in 6ml. of Hanks medium containing FMN were incubated alone or with leucocidin (20 $\mu\text{g.}$) for 10min. at 37° and centrifuged. The pellets and supernatant were treated with 10% (w/v) trichloroacetic acid and the FMN in the extracts was determined. The wet weight, dry weight and the extracellular volume in the pellets were also determined and the apparent intracellular FMN concentration was determined. △, Normal cells; ○, leucocidin-treated cells.

Table 2. *Effect of FMN on the composition of leucocytes*

Suspensions of 5×10^7 leucocytes/ml. were maintained in various media alone, with FMN (1mM), with leucocidin (0.4 $\mu\text{g.}/\text{ml.}$) or with FMN (1mM) plus leucocidin (0.4 $\mu\text{g.}/\text{ml.}$). The β -glucuronidase and aldolase were determined in the supernatant and the Ca^{2+} , K^+ , Na^+ and ATP determined in the cell pellets. The P content is for the cell suspensions.

	β -Glucuronidase content (units/ 10^8 cells)	Aldolase content (unit/ 10^8 cells)	Ca^{2+} content of pellet ($\mu\text{g.}/10^8$ cells)	K^+ content of pellet ($\mu\text{equiv.}/10^8$ cells)	Na^+ content of pellet ($\mu\text{equiv.}/10^8$ cells)	ATP content of pellet ($\mu\text{mole}/10^8$ cells)	P_i content of cell suspension ($\mu\text{mole}/10^8$ cells)
Normal cells	6.5	0.004	1.2	1.4	10	0.22	0.51
Normal cells with FMN (1mM)	6.5	0.004	8.2	1.4	9.4	0.20	0.71
Leucocidin-treated cells	95	0.05	8.0	1.02	15	0.16	0.67
Leucocidin-treated cells with FMN (1mM)	21	0.002	7.5	1.02	14.5	0.18	0.70

12 μ moles and 10^8 leucocidin-treated cells took up 20 μ moles. This was largely due to adsorption on the surface and not penetration into the cyto-

Fig. 5. Effect of menadione on the release of β -glucuronidase from leucocytes. Suspensions of leucocytes (5×10^7 /ml.) were incubated in Hanks medium, alone or with menadione (0.5 mM), with leucocidin, as indicated, for 10 min. and the β -glucuronidase in the cell supernatant was determined. Further (duplicate) cell suspensions in Ca^{2+} -free Hanks medium were incubated with leucocidin, as indicated, for 10 min. and then treated with 1 mM- CaCl_2 -1 mM-ATP or 0.5 mM-menadione-1 mM- CaCl_2 -1 mM-ATP. After a further 10 min. incubation the β -glucuronidase in the cell supernatant was determined. ●, Cells in Hanks medium; ▲, cells in Hanks medium with menadione; ○, cells in Ca^{2+} -free Hanks medium; △, cells in Ca^{2+} -free Hanks medium, menadione added after 10 min.

plasm; Fig. 4 shows that an increase of the external FMN concentration is not accompanied by a corresponding increase in the concentration within the cells. Destruction of FMN by the cell occurred to only a small extent. When 5×10^7 leucocytes in 1.0 ml. of Hanks medium were incubated with FMN (0.1 mM) at 37° for 10 min. and precipitated with 10% (w/v) trichloroacetic acid, enzymic assay showed the presence of 0.086 μ mole in the extract. The absorption spectrum of 0.22 mM-FMN in Hanks medium and in Hanks medium containing 5×10^7 leucocytes/ml. was measured. There was a small decrease in extinction when leucocytes were present. In three determinations at 445 m μ the difference in extinction was 0.04, 0.045 and 0.065. The difference remained for 20 min. at room temperature and was not altered by the presence of leucocidin (2 μ g./ml.). The decrease in $E_{445 \text{ m}\mu}$ could be due to 3.5% of the FMN being in the reduced form; this corresponds to about 8 μ moles/ml. and is the same order of magnitude as the amount of adsorption on the cell surface. If FMNH_2 is present it probably does not arise from a rapid alternate oxidation and reduction. When 6×10^7 normal or leucocidin-treated leucocytes in 1 mM-FMN were examined in the electron-paramagnetic-resonance spectrometer no free radicals could be detected.

Modification of the cytotoxic effects of leucocidin by menadione. Fig. 5 shows the effect of menadione on the release of β -glucuronidase from the leucocidin-treated cell. At low concentrations of leucocidin the release is stimulated by menadione and at high concentrations of leucocidin the release is inhibited. It is probable that menadione has two separate actions on the leucocidin-treated cell. The inhibition of β -glucuronidase extrusion at high leucocidin concentrations is dependent on the time between the addition of menadione and Ca^{2+} (Table 1) and is probably due to inhibition of granule movement. The inhibitory action of menadione on the effects of leucocidin is only

Table 3. *Effect of menadione on the Ca^{2+} , phosphate and ATP contents of normal and leucocidin-treated cells*

To determine the changes in phosphorus compounds 16×10^8 cells in 5 ml. of phosphate-free Krebs-Ringer bicarbonate solution were incubated alone or with menadione (0.5 mM) or with leucocidin (72 μ g.) or with leucocidin (72 μ g.) plus menadione (0.5 mM) and centrifuged. The cell pellets were analysed for P_i and ATP. The Ca^{2+} content was determined on pellets from 3×10^8 cells treated with leucocidin or menadione in Hanks medium containing Ca^{2+} (0.3 mM).

	P_i content (μ mole/ 10^8 cells)	ATP content (μ mole/ 10^8 cells)	Ca^{2+} content (μ moles/ 10^8 cells)
Normal cells	0.29	0.148	1
Normal cells with menadione (0.5)	0.45	0.096	2
Leucocidin-treated cells	0.58	—	13
Leucocidin-treated cells with menadione (0.5 mM)	0.74	0.029	7

Table 4. *Effect of compounds reacting with SH groups on the extrusion of β -glucuronidase induced by leucocidin*

Leucocytes (5×10^7 cells) in 0.5 ml. of Hanks medium were added to 0.25 ml. of the test compounds in Hanks medium and then 0.25 ml. of leucocidin in Hanks medium was added. After 10 min. at 37° the β -glucuronidase in the supernatant was determined. The Hanks medium contained bovine serum albumin (1 mg./ml.).

Concn. of leucocidin (μ g./ml.)	β -Glucuronidase released (units/ 10^8 cells)			
	No inhibitor	Iodoacetate (0.25 mM)	N-Ethylmaleimide (0.25 mM)	Menadione sodium bisulphite (1 mM)
0	0	11	40	10
0.03	18	28	50	20
0.06	30	54	62	28
2.5	93	88	84	63

Fig. 6. Adsorption of menadione by leucocytes. Suspensions of leucocytes (5×10^7 /ml.) were incubated for 10 min. at 37° with menadione in Hanks medium and centrifuged. The concentration of menadione in the supernatant was determined from $E_{265 \text{ m}\mu}$.

Table 5. *Effect of N-ethylmaleimide and leucocidin on adsorption of menadione by leucocytes*

Leucocytes (5×10^7) in 0.1 ml. of Hanks medium were incubated alone or with leucocidin (5 μ g.) or with N-ethylmaleimide (5 mM) at 37° for 10 min. and then added to 0.9 ml. of Hanks medium containing menadione. After a further 10 min. incubation the mixtures were centrifuged and the menadione in the cell supernatant was determined.

	Menadione added (μ mole)	Menadione adsorbed (μ mole)
Normal cells	0.24 0.47	0.12 0.17
Normal cells with N-ethylmaleimide (5 mM)	0.24 0.47	0.05 0.07
Leucocidin-treated cells	0.24 0.47	0.07 0.11

noticed when the effects are assessed from the extrusion of the granule proteins. In general, menadione acts in an opposite sense from FMN. Table 3 shows that menadione induces an accumulation of P_i and hydrolysis of ATP in normal cells, but when leucocidin is present an additional accumulation of P_i occurs. Ca^{2+} is not accumulated in normal leucocytes in the presence of menadione.

Table 4 shows that iodoacetate can also stimulate the release of β -glucuronidase from the leucocyte when it is added immediately before leucocidin. Menadione sodium bisulphite does not affect the amount released and the data in Table 4 suggest that the stimulation is due to an effect on SH groups in the cell.

Recovery of menadione from leucocytes. Fig. 6 shows the uptake of menadione by the leucocyte; the shape of the curve resembles that of an adsorption isotherm. Table 5 shows that this uptake is

decreased if the cells are treated with leucocidin or with N-ethylmaleimide.

The position of the maxima and minima in the absorption spectrum of the supernatant of leucocytes that had been incubated for 10 min. at 37° with 0.5 mM-menadione did not differ from that of menadione. At 250 $\text{m}\mu$ and 265 $\text{m}\mu$ the ratio of the extinction of the cell supernatant to that of the added menadione was 0.78 and 0.73 respectively. This indicates that no reduced menadione is present in the cell supernatant. The absorption spectrum of 0.185 mM-menadione in Hanks medium and in Hanks medium containing 5×10^7 leucocytes/ml. was measured. When cells were present the extinction changed with time and after about 20 min. the suspensions were yellow. There was an increased extinction at 330 $\text{m}\mu$ when cells were present, but as increased extinctions also occurred at 370–550 $\text{m}\mu$ it cannot be concluded that the reduced form of menadione is formed. If it is present a cyclic

oxidation and reduction does not occur to any extent. When 6×10^7 normal and leucocidin-treated leucocytes in 1.0mm-menadione were examined in the electron-paramagnetic-resonance spectrometer no free radicals could be detected.

Failure of other reagents to modify the release of β -glucuronidase from the leucocyte. When tested under the conditions described in Fig. 3 the following compounds failed to modify the release of β -glucuronidase when added to the leucocyte 2–5 min. before adding leucocidin (final concentrations are given): ubiquinone (0.5mm); promethazine hydrochloride (0.5mm); chlorpromazine hydrochloride (0.2mm); vitamin E (5 or 0.5mm); hydrocortisone (0.5 or 5mm).

Oxidation of glucose in the leucocidin-treated cell. The effect of leucocidin on glucose oxidation in the leucocyte was investigated as glucose oxidation is greatly modified during phagocytosis (Karnovsky, 1962) and the degranulation phase of phagocytosis has features in common with protein extrusion in the leucocidin-treated leucocyte. Oxidation of glu-

cose in the normal leucocyte can also be modified by FMN and menadione (Iyer, Islam & Quastel, 1961).

Table 6 shows that, when the extrusion of the granule proteins in the leucocidin-treated cell is prevented by omitting extracellular Ca^{2+} , leucocidin treatment does not change the rate of oxidation of [$1\text{-}^{14}\text{C}$]glucose. This was also found when macrophages were treated with leucocidin. When Ca^{2+} is present leucocidin treatment stimulated the oxidation of [$1\text{-}^{14}\text{C}$]glucose in the leucocyte. It is noteworthy that a stimulation is also found in phagocytosis. Table 6 also shows that in the normal leucocyte and macrophage FMN and menadione stimulate the oxidation of [$1\text{-}^{14}\text{C}$]glucose and that when leucocidin is also present the extent of the stimulation is changed in an opposite sense with menadione and FMN.

Changes in the NAD and NADP contents of the leucocyte. The modification of the cytotoxic effect of leucocidin induced by FMN and menadione prompted measurements of the ratio of the oxidized

Table 6. *Oxidation of glucose in normal and leucocidin-treated cells*

Leucocytes (14×10^7) or macrophages (14×10^6) in Krebs–Ringer phosphate solution or Ca^{2+} -free Krebs–Ringer phosphate solution were incubated for 3 min. at 37° in Warburg flasks alone or with leucocidin ($10\mu\text{g.}$) or FMN (2.5mm) or menadione (0.056mm), and then [$1\text{-}^{14}\text{C}$]glucose was added from a side arm. After a further 10 min. incubation, trichloroacetic acid was added and the $^{14}\text{CO}_2$ determined.

	Concn. of Ca^{2+} (mm)	Total $^{14}\text{CO}_2$ produced (counts/min.)		
		Leucocytes with [$1\text{-}^{14}\text{C}$]glucose	Leucocytes with [$6\text{-}^{14}\text{C}$]glucose	Macrophages with [$1\text{-}^{14}\text{C}$]glucose
Normal cells	1.65	54	8	—
Leucocidin-treated cells	1.65	106	7	—
Normal cells	0	62		28
Leucocidin-treated cells	0	63		26
Normal cells with FMN	0	95		45
Leucocidin-treated cells with FMN	0	150		63
Normal cells with menadione	0	450		235
Leucocidin-treated cells with menadione	0	250		125

Table 7. *NAD and NADP contents of normal and leucocidin-treated cells*

Suspensions of leucocytes ($2 \times 10^8/\text{ml.}$) in Hanks medium, in some cases containing nicotinamide (0.1m), were incubated alone or with $10\mu\text{g.}$ of leucocidin for 10 min. at 37° and then 0.8ml. was added to 5ml. of 0.1N-HCl or 0.1N-NaOH at 100° . The coenzyme content of the soluble fraction was determined.

	Coenzyme content ($\text{m}\mu\text{moles}/10^8$ cells)			
	NAD	NADH_2	NADP	NADPH_2
Normal cells	7.9	2.0	3.3	< 0.05
Leucocidin-treated cells	4.1	1.2	3.3	< 0.05
Normal cells with nicotinamide (0.1m)	8.0	—	3.8	< 0.05
Leucocidin-treated cells with nicotinamide (0.1m)	8.6	—	3.5	< 0.05

and reduced forms of the respiratory coenzymes. Table 7 shows that the ratios are not changed when leucocytes are treated with leucocidin but that there is a decrease in the NAD content when leucocidin is present. This is inhibited by nicotinamide.

DISCUSSION

N-Ethylmaleimide and menadione inhibit the β -glucuronidase extrusion induced by Ca^{2+} in the leucocidin-treated cell. It has been shown that *N*-ethylmaleimide induces a change of state in the cell cytoplasm and it is suggested that protein extrusion does not occur because contact between the granule and cell membrane can no longer be made. The chemical basis of this phenomenon is not clear. It may not be due to interaction of the reagents concerned with SH groups. The different behaviour of iodoacetate and *N*-ethylmaleimide might be explained in terms of availability of SH groups to these reagents and, in the cell, menadione sodium bisulphite might be hydrolysed to menadione, but it is difficult to explain the finding that an equimolar mixture of menadione sodium bisulphite and cysteine is as effective as menadione itself, in terms of availability of SH groups. A phenomenon as complex as the inhibition of granule motion need not have a single chemical cause, however. A reaction that can be considered in addition to combination with SH groups is the Diels-Alder condensation of menadione and *N*-ethylmaleimide with molecules containing conjugated double bonds. The significance of the results reported in this paper is that a chemical basis is provided for further investigation of the conditions controlling movement of cytoplasmic granules. Though the physical state of the cytoplasm may be the controlling factor the possibility that water streaming is involved cannot be excluded.

The partial inhibition of β -glucuronidase extrusion when menadione is added to leucocytes before leucocidin is only observed at high leucocidin concentrations and very probably is due to an inhibition of granule movement. At low leucocidin concentrations, when menadione may not be able to penetrate the cell, menadione potentiates the effect of leucocidin. The chemical basis of this is clearer than that for the phenomenon described above. Potentiation of β -glucuronidase release induced by leucocidin in the leucocyte is shown by iodoacetate and menadione but not by menadione sodium bisulphite, and might result from an interaction with SH groups.

At low leucocidin concentrations FMN inhibits the release of β -glucuronidase and thus acts in an opposite sense to menadione. This antagonism is not restricted to β -glucuronidase extrusion; when

leucocidin is present the changes the two reagents make on glucose oxidation in macrophages are altered in opposite directions. Not all the effects of leucocidin are modified by FMN and menadione, and so the reagents act by preventing or facilitating some change that follows from the primary action of leucocidin but precedes protein extrusion.

Both FMN and menadione can undergo reversible oxidation and reduction. The very high concentration at which they are effective in the leucocidin-treated cell makes it improbable that their action is due to replacement of a metabolic intermediate, and the spectroscopic data indicate that continuous production of large amounts of the reduced forms of FMN and menadione does not occur. Thus though some reduced form of the two reagents may be present it will not function in a dynamic way. With menadione, as shown above, there is good evidence that the potentiation of leucocidin action is a consequence of combination with SH groups. It is not known if FMN interacts in a similar way with SH groups in the cell. It may be significant that leucocidin alters in opposite directions the amount of FMN and menadione adsorbed by the cell. The effect of leucocidin on the uptake of menadione suggests that there is a decrease in free SH groups on the cell during leucocidin action.

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