THE ACTION OF STREPTOLYSIN S ON MOUSE-LIVER MITOCHONDRIA

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THE significance in disease of the several extracellular toxic factors elaborated by *Streptococcus pyogenes*—the group-A streptococcus—is not yet fully understood. However, study of the mode of action of individual toxic factors may provide a basis for clearer understanding of the pathogenicity of streptococci.

The cytolytic activity of whole living group-A streptococci on mammalian cells closely resembles the effect of the oxygen-stable haemolysin streptolysin S (SLS; Havas, Donnelly and Porreca, 1963; Taketo and Taketo, 1966; Quinn and Lowry, 1967). This cytolytic toxin damages a wide range of animal cells and therefore probably acts on a basic structure common to all sensitive cells. Its primary action is now generally believed to be an alteration of the permeability of cell membranes. SLS also disrupts membrane-bound organelles such as lysosomes, nuclei and mitochondria (Weissmann, Keiser and Bernheimer, 1963; Bernheimer and Schwartz, 1964; Keiser, Weissmann and Bernheimer, 1964). This damage to membranes probably initiates secondary metabolic changes in cells which in turn result in cell death.

At present SLS is thought to be a peptide of about 28 amino-acids which forms part of a carrier-toxin complex (Ginsburg, 1970); the carrier may be RNA, serum albumin, detergent or the streptococcal cell. In most studies of the cytolytic properties of SLS the carrier used was a yeast RNA core (Bernheimer, 1949). We also used RNA-streptolysin S (RNA-SLS) in the present study.

In a previous publication (Symington and Arbuthnott, 1969) we reported that RNA-SLS stimulates the succinic oxidase activity of Krebs-2 ascites-tumour cells. This observation might be explained by damage to the membrane by SLS removing the permeability barrier to succinate, which is then metabolised in the mitochondria. However, isolated mitochondria from these cells, when treated with RNA-SLS, showed impaired succinic oxidase activity. This confirmed the antimitochondrial activity of RNA-SLS (Keiser *et al.*) and suggested that the toxin, when acting directly on isolated mitochondria, impairs electron transport. The action of SLS on mitochondria and its site of attack on the electron-transport chain have been studied in greater detail and our findings are reported in this paper. Mouse-liver mitochondria were

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used throughout, because liver tissue presents a rich source of mitochondria, and because they were employed in similar studies of staphylococcal succinic-oxidase factor (Lominski, Gemmell and Arbuthnott, 1968).

MATERIALS AND METHODS

Toxin preparation

Streptolysin S was prepared by a modification of the method described by Bernheimer. Strain C203S of *S. pyogenes* was grown for 5 hr in Difco Brain-Heart Infusion Broth containing 0.1 per cent. maltose until the end of the logarithmic phase was reached. The cells were harvested by centrifugation at 15,000*g* for 10 min. and washed once in phosphate-buffered saline (0.85 per cent. sodium chloride with 0.14*M* sodium phosphate at pH 7.0). These resting cells were then incubated at 37°C for 3 hr in a suspending medium prepared according to the method of Bernheimer; this contained "Active Factor" (a ribonuclease-resistant yeast-RNA core commercially prepared by Worthington Biochemicals Ltd, Freehold, New Jersey, USA). After they had been allowed to stand overnight at 4°C, the cells were removed by centrifugation and the supernatant fluid was titrated against 0.8 per cent. human erythrocytes. Titres of between 2560 and 5120 haemolytic units (HU) per ml were consistently obtained. Control extracts from strain C203S were prepared from resting cells in a suspending medium lacking Active Factor; the supernatant fluid contained 80 HU per ml compared with 2560 HU per ml when the cells were incubated in the complete suspending medium. Extracts were also prepared from resting cells of strain C203U, a mutant deficient in SLS, incubated in complete suspending medium; these contained no detectable haemolytic activity.

Titration of haemolytic activity

The haemolytic activity of SLS was titrated by a modification of the method described by Bernheimer and Schwartz (1963) for measuring the haemolytic activity of staphylococcal α -toxin. Graded dilutions of SLS in 1-ml volumes were made in isotonic saline. A standard 0.8 per cent. erythrocyte suspension was prepared by adjusting the concentration of washed human group-O erythrocytes so that the sample, after haemolysis with saponin and addition of an equal volume of saline, gave an absorbance of 0.8 at 545 nm in a Pye Unicam SP 600 spectrophotometer with glass cells of 10 mm light path. Standard erythrocyte suspension was added in 1-ml amounts to each toxin dilution, and the reaction mixtures were incubated in a waterbath at 37°C for 30 min. after which tubes were centrifuged at 1000*g* for 2 min. The haemoglobin content in the supernatant fluid from each tube in the series was assayed by reading the extinction value at 545 nm (E_{545}). A standard for 50 per cent. haemolysis was prepared by adding 1 ml of standardised erythrocyte suspension to 3 ml of distilled water. By plotting the E_{545} against the toxin dilution, the concentration of toxin causing 50 per cent. haemolysis could be determined by interpolation.

Preparation of mitochondria and assay of enzymes

Mouse-liver mitochondria were prepared freshly each day by the method of Lominski, Gemmell and Arbuthnott. The buffer used to suspend the mitochondria was 0.25*M* sucrose containing 0.033*M* tris-HCl, pH 7.3.

The functioning of various regions of the succinic-oxidase enzyme-complex of mitochondria can be assayed by means of different electron donors or acceptors, as illustrated in fig. 1.

Succinic dehydrogenase was measured by assessing the reduction of methylene blue by mitochondria, with sodium succinate as substrate, in Thunberg tubes. The main vessel of the tube contained 0.5 ml mitochondrial suspension, 0.5 ml toxin or control fluid and 1.4 ml buffered sucrose; the side arm contained 0.3 ml 0.2*M* sodium succinate and 0.3 ml 0.1*M* methylene blue. The tubes were evacuated for 5 min., by means of an Edwards High

Vacuum Pump (model 2SC 50), with constant shaking to facilitate removal of dissolved oxygen. The contents of the side arm were tipped into the main well and the time taken to decolorise 90 per cent. of the dye (assessed visually) at 37°C was taken as a measure of dehydrogenase activity.

Succinic oxidase was measured manometrically in the Warburg apparatus. The substrate, sodium succinate, was present at a final concentration of 0.02M in each flask that contained 0.5 ml mitochondrial suspension. The reaction volume was 3 ml and flasks were shaken at 80–100 cycles per min. at 37°C. Sucrose tris-HCl buffer, pH 7.3, was used to make up

FIG. 1.—Model of the electron-transport chain of mitochondria; $\leftrightarrow$ indicates the enzyme systems studied.

all solutions and assays were carried out in duplicate. Control fluids included heat-inactivated toxin, the suspending fluid used to extract SLS, and extracts from strain C203U.

Cytochrome-oxidase activity was measured manometrically by the method of Lominski, Gemmell and Arbuthnott with paraphenylene diamine (PPHDI, Koch-Light Ltd) as substrate. The mitochondrial suspension oxidised this substrate in the absence of cytochrome C.

RESULTS

Impairment of succinic-oxidase activity

Preparations of streptolysin S had no detectable activity on the functioning of actively respiring mitochondria that were utilising succinate. However, if the mitochondria were incubated with SLS before succinate was added, marked impairment of oxygen uptake was observed (fig. 2). By varying the time of preincubation it was found that the degree of impairment increased with time, reaching a maximum at 20 min.

Preparations of mitochondria varied from day to day in their susceptibility to the same number of HU of toxin (table I). This made it difficult to define a standard unit of mitochondrial impairment or to attribute an absolute impairment value to a given concentration (in HU) of toxin. Consequently the minimum amount of toxin required to cause observable impairment was a function of the mitochondrial preparation used. Above this threshold level, the percentage inhibition of oxygen uptake was dependent on the number of HU present (fig. 3). The maximum inhibition observed with most mitochondrial

preparations was between 70 and 80 per cent.; 100 per cent. inhibition was never obtained. A minimal level of 100 HU per ml was required to cause consistent impairment of mitochondrial activity.

Properties of the inhibitory factor

RNA-SLS has been reported to be free of other extracellular streptococcal toxic factors (Bernheimer). However, Bernheimer and Ruffier (1951) showed

FIG. 2.—Effect of adding RNA-streptolysin S (RNA-SLS) at a concentration of 56 haemolytic units (HU) per ml before and after the addition of substrate on the succinic-oxidase activity of mitochondria; ○—○, mitochondria to which SLS was added 20 min. after sodium succinate; ●—● respiring mitochondria to which control fluid was added at this time; ■—■ mitochondria preincubated with SLS for 30 min. before the addition of sodium succinate.

that resting streptococci elaborated extracellular DNAase when incubated under the conditions for RNA-SLS production and also when a suspending medium lacking Active Factor was employed. In the present study only extracts prepared in the presence of Active Factor impaired mitochondrial respiration. Also pancreatic DNAase at a concentration of 33 μ g per ml did not inhibit respiration. These findings exclude the possibility that the observed

TABLE I

Variation in susceptibility of the succinic-oxidase activity of mitochondria to inhibition by streptolysin S (SLS)

Experiment number	Concentration of SLS (HU per ml)	Percentage inhibition of mitochondrial uptake of O ₂
1	167	45
2	167	71
3	167	60
4	167	89
5	417	76
6	417	52
7	417	74
8	417	65
9	417	32

FIG. 3.—Influence of RNA-SLS concentration on the impairment of mitochondrial succinic-oxidase activity. Mitochondria were preincubated with toxin for 15 min. before the addition of sodium succinate.

effect was due to DNAase. Also, the influence of streptococcal NADase can be excluded because this enzyme is specific for NAD, which is not a cofactor of succinic oxidase. Moreover, toxin preparations from strain C203U, a mutant that does not produce SLS, did not impair mitochondrial respiration. It is notable that both the haemolytic and mitochondrial inhibiting activities of the toxin were destroyed by heating at 56°C for 60 min. and could not be separated by gel filtration on Sephadex G50 or G75. It seems therefore that the haemolytic and mitochondrial damaging activities are effects of SLS or a closely related product.

Site of action of SLS

When methylene blue was used as hydrogen acceptor to assay succinic dehydrogenase activity we found, in three experiments, that 167 HU per ml

TABLE II
Impairment of succinic and cytochrome oxidase of mitochondria by streptolysin S (SLS)

Experiment number	Concentration of SLS (HU per ml)	Percentage impairment of	
		succinic oxidase	cytochrome oxidase
1	267	56	31
2	267	50	45
3	267	44	27
4	249	50	40
5	416	45	44
6	207	45	22

RNA-SLS caused no increase in the time taken to cause 90 per cent. decolorisation of the dye. This amount of toxin was sufficient to cause 50 per cent. inhibition of succinic oxidase activity of the same mitochondrial preparation. It was concluded that SLS had no detectable effect on succinic dehydrogenase activity of mitochondria.

On the other hand, when paraphenylene diamine was used as an electron donor for mitochondria, pretreatment of these organelles with SLS resulted in a marked impairment of oxygen uptake (table II). Although the degree of impairment was on average one-third less than for succinic oxidase, these results suggested that mitochondrial impairment was due in part to blocking of the terminal part of the respiratory chain between cytochrome C and oxygen.

To specify the site of action of SLS on the electron-transport chain more precisely, attempts were made to restore the activity of toxin-impaired mitochondria by adding constituents of the electron transport chain. The addition of ubiquinone at a final concentration of 100 µg per ml did not significantly restore the activity of succinic oxidase impaired by SLS. This finding supported the conclusion that the site of action of SLS did not lie to the left of the cytochrome C locus in the respiratory chain (fig. 1). In contrast, strong evidence

Fig. 5.—Stimulation of cytochrome-oxidase activity by cytochrome C (2.4 mg per ml) in mitochondria impaired by preincubation for 30 min. with 533 HU per ml RNA-SLS; ○ —○, toxin-treated mitochondria; ● —●, control mitochondria.

Fig. 4.—Stimulation of succinic-oxidase activity by cytochrome C (2.4 mg per ml) in mitochondria impaired by preincubation for 30 min. with 70 HU per ml RNS-SLS; ○ —○, toxin-treated mitochondria; ● —●, control mitochondria.

was obtained to indicate immediate restoration of respiration of SLS-impaired mitochondria after the addition of cytochrome C (figs. 4 and 5). The addition of cytochrome C to toxin-damaged mitochondria increased the succinic- and cytochrome-oxidase activity to that of the controls or greater. Also, the presence of soluble cytochrome C at low concentration during preincubation of mitochondria with SLS, completely abolished observable inhibition of succinic- and cytochrome-oxidase activities. These results indicate that RNA-SLS inhibits cytochrome-oxidase activity in respiring mouse-liver mitochondria, and that the inhibition of succinic-oxidase activity is a reflexion of this. Mitochondrial damage by this toxin appears to cause dislocation of the electron-transport chain at the cytochrome C locus, an effect that may be masked by the presence *ab initio* of exogenous cytochrome C.

DISCUSSION

The finding that preparations of RNA-SLS impair isolated mammalian mitochondrial succinic-oxidase activity shows that the action of this cytolytic agent results in the disruption of respiration, an essential cellular function. Group-A streptococci produce also a NADase that inhibits the oxidation of substrates entering the respiratory chain at the level of NAD (Bernheimer *et al.*, 1957; Carlson *et al.*, 1957); this factor does not affect the oxidation of succinate (Carlson *et al.*, 1956), which enters the electron-transport chain at the level of a flavoprotein dehydrogenase. Pathogenic streptococci may therefore attack the respiratory chain at two points, and under certain conditions SLS and NADase might act synergistically.

Other bacterial extracellular toxins that have been shown to impair mitochondrial function include *Clostridium welchii* α -toxin (Edwards and Ball, 1954; McFarlane and Datta, 1954), staphylococcal succinic-oxidase factor (Lominski *et al.*, 1964), and *Pasteurella pestis* murine toxin (Ajl *et al.*, 1958). From the available evidence it seems that each toxin has a specific site of action but that all in some way disrupt the essential spatial configuration of the components of the electron-transport chain.

Assaying different regions of the succinic-oxidase chain showed that succinic dehydrogenase was resistant to attack by SLS but that cytochrome-oxidase activity was impaired. This, together with the fact that ubiquinone failed to restore impaired mitochondria, clearly indicates that SLS causes functional impairment of electron transport between cytochrome C and oxygen. That toxin-impaired succinic-oxidase and cytochrome-oxidase activity can be restored by the addition of exogenous cytochrome C suggests strongly that SLS causes dislocation of electron transport at the cytochrome-C locus. However, the restoration of activity after the addition of cytochrome C to toxin-impaired mitochondria probably does not result from a reversal of the action of RNA-SLS on the mitochondrion; it is more likely that exogenous cytochrome C provides a bypass around the toxin-damaged areas or replaces cytochrome C that has leaked from the mitochondria as a result of altered permeability.

These experiments pinpoint only a secondary functional lesion within the mitochondrion resulting from damage by RNA-SLS and do not provide

evidence for the primary site of action of the toxin, but the investigation of such secondary effects may increase our understanding of the pathogenesis of streptococcal disease.

In a previous study of the action of RNA-SLS on Krebs-2 ascites-tumour cells (Symington and Arbuthnott, 1969) we suggested that the toxin causes an alteration in the permeability of the cell membrane but does not reach the interior of the cell. Our present findings with isolated mitochondria are relevant to the action of SLS produced within cells by streptococci that have been phagocytosed. If produced in significant amount under these conditions, the effect of SLS on the essential cellular function of respiration may be to impair the cellular defence mechanisms of the host.

SUMMARY

We studied the action of streptolysin S on the respiration of mouse-liver mitochondria. Preparations of RNA-streptolysin S (RNA-SLS) impaired the succinic- and cytochrome-oxidase activity of these organelles. The ability to damage mitochondria could not be separated from the haemolytic activity of the toxin.

The presence of exogenous cytochrome C prevented impairment of these enzymatic activities by RNA-SLS and reversed the observable effects of the toxin on them. Thus it appeared that streptolysin S causes dislocation of the electron-transport between cytochrome C and oxygen. The ability of group-A streptococci to impair mitochondrial respiration may be of importance in the mechanism of pathogenicity.

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