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Full Length Research Paper

Effect of iron limitation on the production of Streptolysin S (SLS) of *Streptococcus pyogenes* Group A

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The effect of iron on the growth of *Streptococcus pyogenes* Group A and its influence on Streptolysisn S (SLS) production was investigated. Two strains of *S. pyogenes* used in this study C203S and 55903 M were grown in iron-restricted medium. The iron chelator, (Ethylenediamindi- (*o*- hydroxyphenyl-acetic acid) (EDDA) was added at a concentration giving a 10-fold molar excess over Fe. Successful growth of strains of *S. pyogenes* was obtained suggesting no absolute requirement for iron in the growth and the yield of SLS was not affected by the concentration of iron in the growth medium.

Key words: Streptolysin S, *Streptococcus pyogenes* Group A, iron requirement.

INTRODUCTION

Microbes, except for *Lactobacillus plantarum* (Archibald, 1986) and *Borrelia burgdorferi* (Verstraeta et al., 1989), as for other living tissues, require iron for their cellular functions. This essential micro-nutrient is involved in many biochemical processes in microorganisms serving as catalysts, or enzymes or engaged in electron transport processes. Several effects including changes in growth rate, growth efficiency (mitochondrial functions, cytochromes, etc.) and growth yields are associated with growth of microorganisms under iron limiting conditions (Messenger and Ratledge, 1986).

Haemolysins are virulence factors for many micro-rganisms and it has been suggested that stimulation of bacterial growth might be due to the increase of available iron produced by the lysis of erythrocytes by haemolysins (Martinez et al., 1990). Extremely low concentrations of available iron may trigger production of virulence factors such as the shiga toxin of *Shigella dysenteriae* (Dubos and Geiger 1946), diphtheria toxin of *Corynebacterium diphtheriae* (Pappenheimer, 1955), shiga-like toxin I of enterohaemorrhagic *Escherichia coli* (Calderwood and Mekalanos, 1987) and exotoxin A of *Pseudomonas aeruginosa* (Bjorn et al., 1978; 1979).

Many different phenotypic changes occur in bacteria during growth under iron-limiting conditions (Weinberg, 1978). The activity of membrane transport systems for iron and the production of extracellular or cell associated iron–complexing compounds called siderophores are usually increased dramatically by iron deprivation (Neilands, 1981).

S. progenies Group A is a major etiological agent causing a variety of human diseases ranging from pharyngitis to severe and life threatening invasive diseases, such as toxic shock- like syndrome (TSLS) and necrotizing fasciitis (Goldmann et al., 2004).

Two major cytolytic toxins are produced by Group A Streptococci (GAS), these are the oxygen-labile streptolysin O (SLO) and the oxygen-stable streptolysin S (SLS) (Ginsburg, 1970). Streptolysin S is a potent cytolytic toxin responsible for the zone of -haemolysis surrounding GAS colonies grown on blood agar, and SLS production is now linked with severe infections including toxic shock syndrome and necrotizing fasciitis (NF), in which GAS can invade skin or soft tissues and even destroy limbs.

SLS is only active when compared with certain carrier substances or stabilizers, such as RNA-core, serum components, nonionic detergents or bisazobenzidine

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dyes (Taketo and Taketo, 1986, 1987). This exotoxin has a membrane damaging effect not only on erythrocytes, but also on lymphocytes (Hryniewicz and Pryjma, 1977) neutrophils, platelets (Ginsburg, 1972), tumour cells (Taketo and Taketo, 1966) and subcellular organelles (Bernheimer and Schwartz, 1964; Weismann, 1964). Elias et al. (1966) found that the treatment of erythrocytes and their ghosts with phospholipase C, followed by exposure to SLS, resulted in diminished binding of SLS suggesting a role for membrane phospholipids in SLS action.

Lai et al. (1978) reported that the active peptide of SLS consisted of 32 amino acid residues comprising tyrosine and phenylalanine, but was deficient in histidine, valine, leucine, cysteine, and methionine and arginine residues. According to Loridan and Alouf (1986), carrier free SLS is basic (pI 9.2) and the molecular weight of the denatured peptide is about 1,800 Da. More recently, Betschel et al. (1998) and Nizet et al. (2000) identified the genes encoding SLS production and showed that a gene *saga* encoded a 53 amino acid prepropolypeptide that was thought to be proteolytically cleaved to produce SLS as a 30 amino acid peptide.

The object of current study was to investigate the effects of iron limitation on the yield of SLS.

MATERIALS AND METHODS

Strain

Two strains of *Streptococcus pyogenes* examined for Streptolysin S (SLS) production in this study C203S and 55903M were grown in iron-restricted and iron-depleted medium. Strain C203S, obtained from Professor Joseph E. Alouf, Institute Pasteur, Paris, was selected because it produces a high level of SLS (Loridan and Alouf, 1986). The strains were stored lyophilized or as broth culture supplemented with 20% glycerol at -20°C.

The medium used in this study was a complex medium BHI- BM (Difco). The iron content of this medium was 39 nmole /ml as measured by the iron Binding Capacity Kit (Sigma). (Ethylenediamindi-(*o*- hydroxyphenyl-acetic acid) (EDDA) was added at a concentration giving a 10-fold molar excess over Fe and broth was store at 4°C for 24 h in order to allow further binding of Fe in the medium. After 6 h growth, the cells (grown in iron – restricted and iron-replete medium) were harvested, washed and suspended in induction buffer (Loridan and Alouf, 1986). After induction with RNA-core, the supernate material was collected and examined fore haemolytic activity.

Toxin production and purification

The methods used for toxin production and purification were essentially as described by Loridan and Alouf (1986). All steps were done at 4°C. The purified product had a specific activity 3.5×10^5 haemolytic units (mg protein)$^{-1}$.

Assays

Haemolytic assay

Haemolytic activity was determined using two-fold or ten fold dilutions of SLS in 150 mM sodium phosphate buffer (pH 6.8) in tubes or microtiter trays (Sterilin). Defibrinated sheep blood (Becton Dickinson) was centrifuged at 5000 g for 5 min and the sedimented erythrocytes washed three times in 150 mM sodium phosphate buffer (pH 6.8). Sheep red blood cells (SRBC) were suspended (about 2% v/v in 150 mM sodium phosphate buffer) such that a 30 fold dilution of this suspension in distilled water gave A_{514} of 0.16. This standard sheep red blood cells (SRBC) suspension (about 4.3×10^7 cells/ml) was kept at 4°C and used within 4 days. For the tube assay, 0.9 ml diluent (150 mM PBS) was placed in tubes and 0.1 ml of purified toxin was added to the first tube (10^{-1}) and was mixed gently. Diluted toxin (0.1 ml) was transferred to tube 2(10^{-2}) and mixed well and in this way a sample was transferred to tubes 3(10^{-3}) and 4(10^{-4}). To all these tubes, 0.5 ml of 2% SRBC was added. The tubes were incubated at 37°C for 45 min and then briefly centrifuged at 5000 g (Biofuge A, Heraeus Sepatech or Mistral 6 L) for 5 min. The percentage of released haemoglobin was estimated by the A_{541} of the supernatant fluid. One haemolytic unit (HU) was defined as the amount of test material which causes release of 50% of the haemoglobin. Values were estimated graphically. Controls were done by mixing the test material with cholesterol (10 µg/ml), which inhibit haemolytic activity of SLO and not that of SLS (Loridan et al., 1986) or with trypan blue (13 µg/ml) which inhibits SLS. The RNA-core alone and mixtures with 30% maltose plus 100 mM ammonium acetate did (used as induction buffer) not exhibit any haemolysis up to 10 mg ml^{-1}.

RESULTS

The supernatant fluids obtained from both induced cells (grown in iron-restricted and iron-replete medium) gave the same haemolytic titre (1000 HU/ml) and, also, it was observed from experiments on the growth in the presence of EDDA (Figures 1 and 2) that there was no absolute requirement for iron in the growth of *S. pyogenes* group A. These findings also showed that SLS is not regulated by iron nor was there any dependence of SLS production on iron.

DISCUSSION

The present study clearly shows that this potential pathogen can proliferate *in vitro* under iron-depleted conditions and has no need for iron or production of siderophores. Studies of Francis et al. (1985) found no requirement or transport system for Fe in *S. pyogenes* group A. Hence, these pathogenic bacteria may establish infection in mammalian hosts irrespective of levels of free iron, which is known to be a limiting factor in the virulence of pathogenic bacteria. Also, the ability to thrive either in oxygen rich or oxygen free conditions adds further to the versatility of this organism.

Griffiths and McClain (1988) reported that Fe-limitation stimulated SLS production in *S. pyogenes* . Reports of toxin synthesis being derepressed by Fe have been made for diphtheria toxin in *C. dipthheriae*; also Fe is known to affect the levels of Shiga toxin production *S. dysenteriae.* All the data on growth and SLS production in C203S suggest that SLS is not regulated by iron and nor there any evidence for a dependence of SLS production

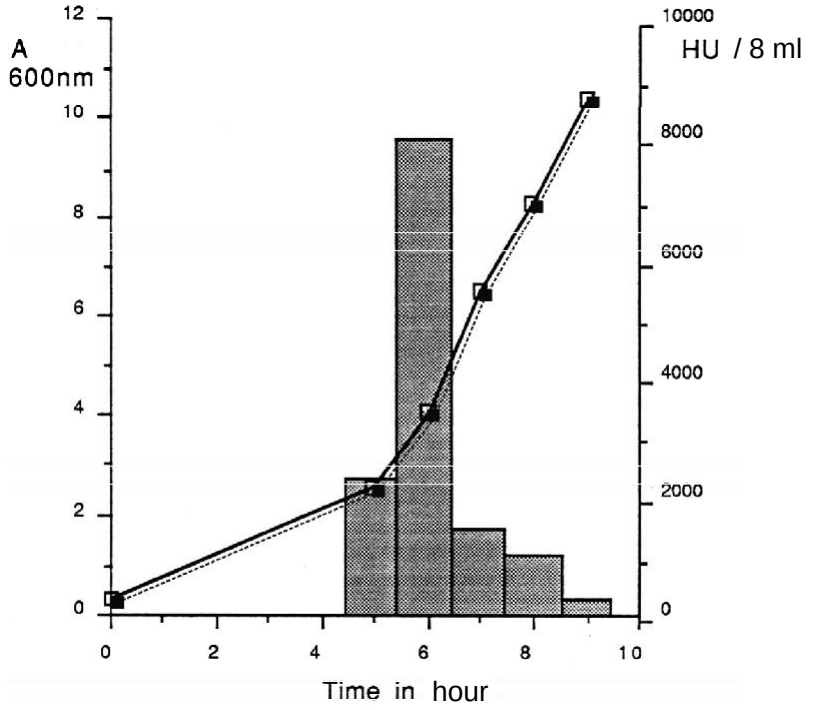

Figure 1. Key: the line shows the absorbance (at 600 nm) of culture (control, no addition of Fe), … the dots shows addition of 10:1 molar excess of EDDA over Fe. The histograms show the haemolytic activity of the supernatant fluids. SLS production in relation to growth rate strain C203S.

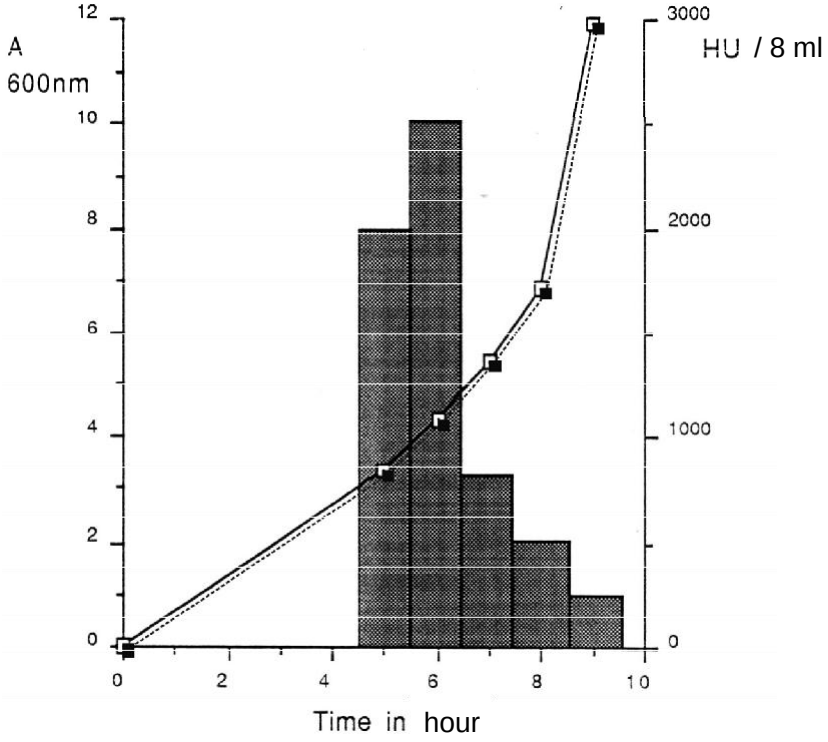

Figure 2. Key: . The line shows the absorbance (at 600 nm) of culture (control, no addition of Fe), ….. the dots shows addition of 10:1 molar excess of EDDA over Fe The histograms show the haemolytic activity of the supernatant fluids. SLS production in relation to growth rate strain 55903M.

on iron. Addition of EDDA at a 10 molar is over Fe while in BHI-BM, it did not restrict the growth of C203S and the level of haemolysin (SLS) production was not affected.

Metal ions such as manganese, copper**,** iron**,** cobalt, and zinc are essential trace elements but are also potentially harmful, which necessitates careful regulation of metal homeostasis (Nelson, 1999). Several species of streptococci can grow in the absence of iron, and it has been proposed that Mn can replace iron (Archibald, 1986; Jakubovics et al., 2000; Niven et al., 1999). A connection between Mn homeostasis and sensitivity to oxidative stress has been reported (Kehres et al., 2000; Martin et al., 1984). *S. pyogenes* lacks catalase but produces a Mn-dependent SOD (Gerlach et al., 1998; Gibson and Caparon 1996). In contrast to many other species which have several SODs, with different cofactors (Mn, Fe, and Cu/Zn), it has been suggested that *Bacillus subtilis* and most streptococci and enterococci mainly utilize the Mn SOD (Inaoka et al., 1999).

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