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IRON ENHANCES THE BACTERICIDAL ACTION OF STREPTONIGRIN*

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SUMMARY: The lethal action of streptonigrin on strains of Escherichia coli is greatly enhanced by citrate (10^{-2} M). Desferrioxamine (2×10^{-4} M), when added with streptonigrin and citrate, eliminates the citrate enhancement. These observations point to a role for iron in the bactericidal mechanism of streptonigrin. Extracellular citrate is known to promote the acquisition of iron by E. coli by delivering it as a ferric citrate complex to a specific transport apparatus on the cell envelope. Therefore, it may promote action of streptonigrin by increasing the intracellular concentration of available iron. Desferrioxamine, which forms a much stronger complex with ferric ion than does citrate, would be expected to suppress the ferric citrate effect, and this was observed.

INTRODUCTION

Streptonigrin is a quinone antitumor agent that causes DNA breakage in Escherichia coli. It requires both a supply of intracellular electrons and the presence of oxygen for the greatest expression of its bactericidal effect[1,2]. When reduced in vitro, streptonigrin autoxidizes to produce the superoxide anion radical, an agent that is known to bring about DNA strand breakage indirectly. Therefore it has been proposed that streptonigrin is intracellularly reduced and autoxidized in a cyclic process that produces a flux of superoxide and other active oxygen species (such as hydroxyl radical). DNA strand breakage would result from attack by some form of active oxygen[3,4].

Certain heavy metal divalent cations such as Co^{++}, Mn^{++}, or Zn^{++} may play a role in the action of streptonigrin, since they greatly enhance its bactericidal action at 10^{-4} to 10^{-5} M. They also form complexes with streptonigrin and promote binding of the antibiotic to DNA in vitro, an observation of uncertain significance[5].

We report here experiments suggesting that the availability of intracellular iron is an important factor in the action of streptonigrin.

MATERIALS AND METHODS

Streptonigrin was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. Desferrioxamine is a

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product of Ciba-Geigy Corp. E. coli strain JG151 is a derivative of strain 15T⁻ in which the defective lysogenic phage is not expressed [6]. Strain AN193 is an entA derivative of AB1515, obtained from the E. coli Genetic Stock Center, Yale University School of Medicine, New Haven CT. Experiments were carried out on aerated log phase cultures growing on an M9 medium [7] fortified with growth requirements and a micronutrient mixture [8]. Samples for viability determinations were diluted serially in saline and plated on Difco nutrient agar. The concentration of iron in the M9 buffer was determined by measuring the absorbance at 535 nm of the complex of bathophenanthroline sulfonate (Sigma) with iron in the presence of dithionite [9].

RESULTS

The bactericidal action of streptonigrin on E. coli strain JG151 was potentiated by simultaneous addition of ferric iron (10^{-5} M). Identical results were obtained using a freshly-prepared solution of Fe $NH_4(SO_4)_2$ or a year-old suspension of ferric hydroxide prepared by dissolving $FeCl_3$ (Fig. 1A). The action of streptonigrin was also enhanced by simultaneous addition of citrate (10^{-2} M), as seen in Figure 1B. After 40 min the fraction of surviving cells had declined 3 decades further in the presence of citrate than in its absence. In contrast the presence of desferrioxamine (2×10^{-4} M), when added immediately before streptonigrin, was strongly protective, even in the presence of citrate. Desferrioxamine is a powerful chelating agent whose effect here is to make extracellular iron unavailable for forming a ferric citrate complex.

There appears to be a brief lag in the potentiating action of citrate, suggesting that synthesis of a protein must be induced before citrate can exert

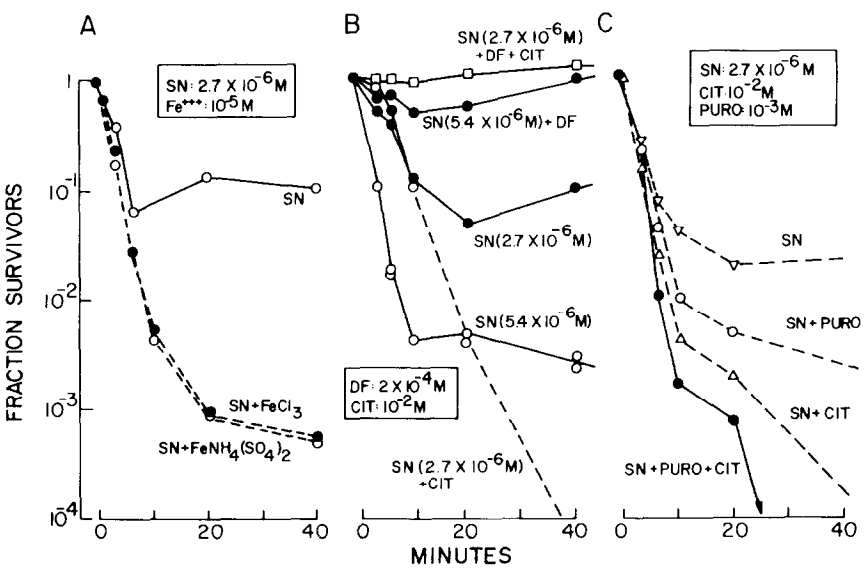

Figure 1. Effects of iron, citrate and desferrioxamine on the lethal action of streptonigrin on E. coli strain JG151. Inhibitors were added to log phase cells (10^8/ml) at 0' except in panel C, where puromycin was added at -5 min, streptonigrin and citrate at 0 min. Abbreviations: SN, streptonigrin; DF, desferrioxamine; PURO, puromycin; CIT, citrate.

Table I. Effects of citrate and desferrioxamine on strains of E. coli

Expt. #	Bacterial Strain	Conc. streptonigrin ($Mx10^6$)	Other agents	Fraction viable (time)
1[a]	JG151	5.5	Desferrioxamine ($2x10^{-4}$ M)	0.13 (40')
2	B/r	11.0	-	0.19 (40')
	"	11.0	Na citrate (0.01 M)	$8.1x10^{-5}$ (40')
3[b]	K12 (AN193)	40	-	0.17 (70')
	"	40	Na citrate (0.01 M)	$6.0x10^{-5}$ (70')

[a] Desferrioxamine was added 5 hr before streptonigrin.

[b] The growth medium contained casamino acids, tryptophan and thiamine in addition to salts and glucose.

its effect. However, this is apparently not the case, as can be seen in the experiment of Figure 1C. Here protein synthesis was inhibited by the addition of puromycin (0.5 mg/ml) five minutes before the addition of other agents. The inhibition of protein synthesis itself enhanced the lethal action of streptonigrin in agreement with previous observations in which chloramphenicol was observed to have this effect[1]. When citrate was also added, there was a further enhancement of the lethal action, showing that in strain JG151 the effect of citrate did not require synthesis of new protein.

Desferrioxamine had little if any effect on log phase cultures of strain JG151 (not shown). When cells were preincubated with desferrioxamine, the latter offered no protection against the lethal action of streptonigrin (Table I).

The citrate effect could also be demonstrated in other strains of E. coli, such as B/r and K12 (Table I).

We sought to demonstrate the existence of a complex between ferric ion and streptonigrin by studying the effect of ferric hydroxide suspensions on the 365 nm peak of streptonigrin. We did not observe any effect between pH 5 and 7. Nor did ferric hydroxide have any effect on the spectrum of the Zn^{++}-streptonigrin complex, which is readily detected spectrophotometrically[5].

DISCUSSION

Iron is present in our M9 growth medium as an impurity at a concentration of $1.5x10^{-6}$ M. When this concentration was raised by 10^{-5} M the lethal action of streptonigrin was enhanced. This experiment by itself does not distinguish iron from other metals that promote the action of streptonigrin at this concentration[5]. However, the potentiating action of citrate suggests that iron

plays a unique role in the action of streptonigrin. Although citrate cannot penetrate the permeability barrier of E. coli, it is known to promote the uptake of iron by forming a chelate complex with ferric ion, which normally exists at neutral pH as polymeric, highly insoluble ferric hydroxide. The ferric citrate complex interacts with a specific receptor on the outer membrane, and the iron atom is transported into the cell[10,11]. This is the only known direct effect of citrate on aerobically growing E. coli. Therefore we presume that citrate promotes streptonigrin lethality by making intracellular iron more available. This is achieved at an extracellular iron concentration of 1.5×10^{-6} M, or about an order of magnitude lower than the synergistic effects of other heavy metal ions. Therefore it appears that the action of iron is in some way special.

The effect of citrate in enhancing the lethal action of streptonigrin is dependent on the availability of extracellular iron, since the effect is eliminated by the presence of desferrioxamine, a stronger iron-chelating agent ($K_f = 10^{30.6}$) than citrate ($K_f = 10^{12}$)[12]. It is conceivable that the effect of desferrioxamine is to keep streptonigrin out of the cell, since one might imagine that streptonigrin enters the cell as a complex with ferric ion. However, our spectrophotometric studies did not reveal the existence of such a complex. Furthermore the experiment of Table I shows that streptonigrin is still lethal, and so must enter the cell, even when the presence of desferrioxamine makes extracellular iron unavailable for interacting with streptonigrin.

The effect of citrate may be a direct result of the rapid introduction of iron into the cell. If this is the case the ferric citrate transport apparatus must be present constitutively in strain JG151 to some extent. The influx need not be great provided the iron is in a form that can readily participate in the streptonigrin mechanism. A less obvious possiblity is that the association of a ferric citrate complex with the cell membrane triggers the intracellular release of stored iron. The growth of avirulent E. coli in siderophore-containing serum has been ascribed to such a mechanism rather than to the direct provision of extracellular iron by the siderophore[13].

Iron may promote the action of streptonigrin by participating in the formation of a species of active oxygen (such as hydroxyl radical), which in turn damages DNA. Iron has been shown to promote DNA damage in vitro when streptonigrin is reduced in the presence of DNA[4]. Iron catalyzes the production of hydroxyl radicals from superoxide and hydrogen peroxide[14], both of which result from the autoxidation of streptonigrin. However, the role of iron in the action of streptonigrin may be more complicated. A complex between ferrous ion and streptonigrin can be demonstrated spectrophotometrically[1], and such a complex would be expected to bind to DNA as do other metal-streptonigrin complexes Hence iron may assure that active oxygen is formed close to the target DNA.

The manner in which other heavy metal ions such as Zn^{++}, Co^{++}, Mn^{++}, and Cd^{++} enhance the bactericidal action of streptonigrin remains to be clarified.

It is possible that some of them simply make iron more available, either by an intracellular or extracellular mechanism. Alternatively, they may have an independent action, arising (for example) from their ability to promote the binding of streptonigrin to DNA.

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