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The Role of Copper on Ethambutol's Antimicrobial Action and Implications for Ethambutol-induced Optic Neuropathy

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The principal side effect of the antimycobacterial agent ethambutol (EMB) is an optic neuropathy with clinical features very similar to a mitochondrial hereditary optic neuropathy (Leber's). The mechanism of EMB-induced optic neuropathy may be EMB's chelation of copper, thereby precluding normal cytochrome c *oxidase activity and mitochondrial metabolism in the optic nerve. Before attempting to use therapeutic copper to replenish endogenous stores in an attempt to preclude EMB-induced optic neuropathy, we wished to determine whether EMB is still effective against mycobacteria in the presence of copper. EMB and copper, alone and in combination, were tested against six strains of* Mycobacterium tuberculosis *and five strains of* Mycobacterium avium *using a radiometric broth macrodilution assay. Copper did not effect EMB's antimicrobial actions against either species of mycobacteria. This in vitro study suggests that if copper were given to patients to prevent EMB-induced optic neuropathy, it would not compromise EMB's bacteriostatic properties.* © *1998 Elsevier Science Inc.*

INTRODUCTION

Ethambutol (EMB) is a potent synthetic antimycobacterial agent introduced in 1961 as a treatment for patients with tuberculosis (TB). The principle side effect of EMB is an optic neuropathy often incorrectly described as a retrobulbar optic neuritis. Because the incidence of EMB-induced optic neuropathy in standard dose is 1 to 5%, it was proposed 10 years ago to bar EMB as an antituberculosis compound (Smith 1987). However, due to the emergence of multidrug-resistant TB in immunocompromised and immunocompetent patients, as well as *Mycobacterium avium* complex (MAC) infections in AIDS patients, EMB has once again become a key agent in combination drug regimens (Alvarez and Krop 1993). The drug is considered primary therapy against *M. tuberculosis* and has synergistic actions, when combined with other agents, against *M. avium* (Inderlied and Salfinger 1995). Therefore, it is prudent to investigate EMB-induced optic neuropathy further.

EMB-induced optic neuropathy exists in two types. The more common form is a noninflammatory (although common, it is an error to regard this as an optic neuritis) axial fiber disease involving central fibers of the optic nerve. It results in the loss of green or red color vision, diminished visual acuity, and

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bilateral central or cecocentral scotoma (Alvarez and Krop 1993; Leibold 1966). The more unusual (<1%) form is periaxial ocular toxicity involving peripheral fibers of the nerve. It results in the constriction of peripheral visual fields, with normal color discrimination and visual acuity. Like Leber's hereditary optic neuropathy, a mitochondrial DNA inherited disorder, EMB-induced optic neuropathy has a predilection for the papillomacular bundle of the retinal nerve fiber layer making the optic nerve head changes subtle (mild temporal pallor) and easy to miss on funduscopic examination. Although EMB-induced optic neuropathy is often reversible, permanent damage may occur even at standard doses of EMB (15–25 mg/kg/day) (Alvarez and Krop 1993).

The mechanism(s) of EMB-induced optic neuropathy has yet to be elucidated. However, there is evidence suggesting that EMB may be chelating the copper ion of the enzyme cytochrome-c oxidase in mitochondria of optic nerve axons (Buyske et al. 1966; Cole et al. 1981; Prohaska and Wells 1975). This may impair normal cytochrome-c oxidase activity and mitochondrial function, which may result in this type (papillomacular) of optic neuropathy (Sadun and Rubin 1996). Before attempting to use therapeutic copper to replenish endogenous stores to preclude EMB-induced optic neuropathy, we wished to determine if EMB is still active against mycobacteria in the presence of copper in vitro.

MATERIALS AND METHODS

Materials

EMB and $CuCl_2$ were tested against six strains of *M. tuberculosis* isolated from patients with clinical TB and five strains of *M. avium* isolated from AIDS patients with disseminated MAC disease. EMB (Lederle Laboratories, Pearl River, NY) solutions were prepared fresh in sterile distilled water and diluted in Middlebrook 7H9 broth. The following concentrations of EMB were tested: 1, 2, 4, 8, 16, and 32 μg/mL against both *M. tuberculosis* and *M. avium*.

$CuCl_2$ (Sigma Co., St. Louis, MO) solutions were prepared fresh in sterile distilled water and diluted in Middlebrook 7H9 broth. The following concentrations of $CuCl_2$ were tested: 1, 10, 100, 500, and 667 μg/mL. Each *M. tuberculosis* and *M. avium* strain was initially tested at 1 to 500 μg/mL to define the MIC for $CuCl_2$ alone. Copper is rarely used as an antimicrobial agent. This concentration range was chosen for in vitro testing based in part on the study by Duguid (1983). In this study, it was shown that 130 μg/mL $CuCl_2$ inhibited the growth of the tested bacterial species and 13 μg/mL $CuCl_2$ had no effect on bacterial growth (Duguid 1983).

Methods

The radiometric broth macrodilution assay (BACTEC method) was used for testing the susceptibilities of *M. tuberculosis* and *M. avium* to EMB and $CuCl_2$ (Inderlied and Salfinger 1995). This method is a standardized method for testing *M. tuberculosis* (Inderlied and Salfinger 1995). Although there is no standardized method for in vitro susceptibility testing of MAC isolates, radiometric broth macrodilution is commonly used and considered reliable (Inderlied and Salfinger 1995).

For both *M. tuberculosis* and *M. avium* testing, controls included no drug and the inoculum was diluted 1:100. The latter control defined the threshold for growth inhibition as 99% of the starting inoculum. Therefore, the MIC for EMB and $CuCl_2$ was defined as the lowest concentration of compound that inhibited the growth of either the *M. tuberculosis* or *M. avium* strains to an extent equivalent to the growth of the inoculum, in the absence of compound, diluted 1:100 (i.e., 99% inhibition). There is no defined "critical concentration" for $CuCl_2$, and the use of a critical concentration for EMB was inappropriate given the nature of this study. Furthermore, there is no defined "critical concentration" for EMB when tested against *M. avium* strains.

EMB and $CuCl_2$ were tested alone and in combination to determine if there was a synergistic effect or antagonistic effect between EMB and $CuCl_2$ on the growth of *M. tuberculosis* or *M. avium* based on changes in the MIC for EMB.

A modified checkerboard assay was used to test combinations of EMB and $CuCl_2$. The concentrations of these drugs tested in combination were based on initial separate measurements of the MICs for EMB and $CuCl_2$ alone. The objective of combination testing was to determine if $CuCl_2$ decreased (synergistic effect) or increased (antagonistic effect) the MIC for EMB. Therefore, $CuCl_2$ was tested at three concentrations (1, 10, and 100 μg/mL) against *M. tuberculosis* strains and two concentrations (500 and 667 μg/mL) against *M. avium* strains, because these concentrations best reflected the MICs for $CuCl_2$ alone. A synergistic or antagonistic effect was defined as a $\geq$2 dilution difference in the MIC for EMB in combination with $CuCl_2$ compared with the MIC for EMB alone.

RESULTS

EMB and $CuCl_2$ Alone

When tested against *M. tuberculosis* strains, the MIC for EMB alone was $\leq$2 μg/mL for five of six strains; for the sixth strain (CDC-J), the MIC for EMB was 4

μg/mL (Table 1). The MIC for $CuCl_2$ alone was 500 μg/mL for five of six strains; the sixth strain (4321-G) grew at this highest concentration.

When tested against *M. avium* strains, the MIC for EMB alone was 4 to 8 μg/mL for all five strains. The MIC for $CuCl_2$ alone was greater than 500 and 667 μg/mL (data not shown) for all five strains.

EMB and $CuCl_2$ in Combination

For the *M. tuberculosis* strains tested, the combination of 1 μg/mL $CuCl_2$ with EMB increased the MIC for EMB by one dilution for two of six strains compared with EMB alone. For all six strains, when EMB was combined with 10 μg/mL $CuCl_2$, the MIC for EMB was either equal to or less than EMB alone. In combination with 100 μg/mL $CuCl_2$, the MIC for EMB decreased for all six strains compared with EMB alone. The *M. tuberculosis* strains proved to be susceptible to EMB at all $CuCl_2$ concentrations tested compared with EMB alone. Based on our definition, there was no synergistic or antagonistic effect of $CuCl_2$ on the MICs for EMB against *M. tuberculosis*.

For four of five *M. avium* strains tested in the presence of 500 μg/mL $CuCl_2$, the MIC for EMB was either equal to or less than EMB alone; the MIC for the fifth strain (116) increased one dilution. In combination with 667 μg/mL $CuCl_2$, the MIC for EMB increased one to four dilutions for four of five strains; the MIC decreased for the fifth strain (101). Only at 667 μg/mL $CuCl_2$ was there an antagonistic effect (≥2 dilution difference in MIC) of $CuCl_2$ on the MICs for EMB compared with EMB alone.

DISCUSSION

The BACTEC method was used to determine the antimicrobial susceptibilities of *M. tuberculosis* and *M. avium* to EMB in the presence of therapeutic levels of copper. Our results indicate that $CuCl_2$ has no antagonistic effect on the activity of EMB against *M. tuberculosis*. Likewise, $CuCl_2$ has no antagonistic effect on the activity of EMB against *M. avium*, except at levels well above the therapeutic range (667 μg/mL). Therapeutic levels of copper may only need to approximate normal serum values (0.7–1.5 μg/mL) (Sass-Kortsak 1965). Thus, the present results suggest that if copper were given to patients as treatment for EMB-induced optic neuropathy, it would not compromise EMB's antimicrobial actions.

EMB-induced optic neuropathy is a dose and treatment duration-related ocular toxicity (Alvarez and Krop 1993; Leibold 1966). At 15 mg/kg/day EMB (standard low dose), or when used for less than 2 months duration, the incidence of neuropathy is only ~1%. At 25 mg/kg/day EMB (standard high dose) or when used for more than 2 months' duration, the incidence increases to 5% (Alvarez and Krop 1993). Moreover, EMB is more likely to cause an optic neuropathy in mycobacterial infections that require a much longer duration of therapy (months to

TABLE 1 Activity of EMB and $CuCl_2$, Alone and in Combination, against *M. tuberculosis* and *M. avium* Strains

	MIC (μg/mL)[a]		Combination Assays MIC for EMB (μg/mL)[b]				
	EMB	$CuCl_2$	at a $CuCl_2$ Concentration (μg/mL)[c]				
Strain			1	10	100	500	667
M. tuberculosis							
4829-K	<1	500	2	1	1	nd[d]	nd
4675-K	2	500	2	1	1	nd	nd
4696-K	2	500	2	2	1	nd	nd
4650-K	2	500	2	2	1	nd	nd
4321-SG	2	>500	1	1	1	nd	nd
CDC-J	4	500	8	4	1	nd	nd
M. avium							
100	8	>500	nd	nd	nd	8	32
101	8	>500	nd	nd	nd	2	<2
108	8	>500	nd	nd	nd	8	32
109	4	>500	nd	nd	nd	4	8
116	4	>500	nd	nd	nd	8	16

[a] MIC in μg/mL.
[b] EMB = ethambutol.
[c] *M. tuberculosis* and *M. avium* strains were tested against EMB in combination with $CuCl_2$ using $CuCl_2$ concentrations based on the MICs for $CuCl_2$ alone (i.e., <500 μg/mL and ≥500 μg/mL, respectively).
[d] nd = not done.

years), such as MAC infections in AIDS patients, multidrug-resistant TB, and particularly in renal TB, which through decreased kidney function leads to increases in the drug's serum concentration and risk of toxicity (Alvarez and Krop 1993). In addition, the optic neuropathy may be unpredictable and catastrophic, leading to permanent ocular damage even at standard doses (Alvarez and Krop 1993; Leibold 1966). Therefore, it is generally recommended to use EMB at a dose of 15 to 25 mg/kg/day for the first 2 months of therapy and then decrease to 15 mg/kg/day, as well as to immediately discontinue the drug upon the development of any visual symptoms (Alvarez and Krop 1993).

EMB-induced toxicity in animals, as well as in humans, first localizes in the visual pathway before other structures in the central nervous system. When high levels of EMB are given to monkeys (Schmidt 1966) or rats, focal axonal dilatation occurs in the optic nerve and optic chiasm without producing other central nervous system lesions (Lessell 1976).

Leber's hereditary optic neuropathy and toxic/nutritional/metabolic optic neuropathies show an indistinguishable and exquisite predilection for pathology to occur in the very thin nerve fibers of the retinal papillomacular bundle (Sadun and Rubin 1996). There is recent evidence that this family of diseases may be related, as they all show similar clinical features (dyschromatopsia, visual loss from cecocentral scotoma), similar pathologies (to the papillomacular bundle), and microscopic evidence of impairment to the axonal transport system (Sadun and Rubin 1996; Sadun et al. 1994). Mitochondrial insufficiency in the optic nerve fibers probably underlies this impairment.

The exact mechanism(s) of EMB-induced optic neuropathy has yet to be ascertained. However, there is evidence suggesting that EMB may be chelating copper in the optic nerve fibers. EMB and its metabolite, ethylenediiminodibutyric acid, are strong chelators of metal ions such as copper, and have the ability to alter copper metabolism via chelation (Cole et al. 1981; Solecki et al. 1984). Indeed, EMB therapy most often induces a decrease in copper levels in the eyes of monkeys and in the eyes of rats (Buyske et al. 1966).

Copper is required as a cofactor of the enzyme cytochrome *c* oxidase in mitochondria as part of the electron transport chain and cellular oxidative metabolism. Without sufficient levels of copper, cytochrome *c* oxidase is unable to maintain adequate production of cellular energy in the form of adenosine triphosphate. When looking at animal models, copper deficiency in the developing rat brain produces an 80% decrease in cytochrome *c* oxidase activity (Prohaska and Wells 1975). Furthermore, with EMB treatment, there is a decrease in copper levels, as well as a concomitant decrease in cytochrome *c* oxidase activity, in the liver of rats and the myocardium of dogs (Buyske et al. 1966). EMB's copper chelating properties could be affecting the function of cytochrome *c* oxidase in the human retinal nerve fiber layer, resulting in decreased adenosine triphosphate production and subsequent axonal pathology.

There is evidence that copper deficiency such as that brought about by ethambutol therapy produces mitochondrial insufficiency similar to Leber's hereditary optic neuropathy and similar to toxic/nutritional/metabolic optic neuropathies (Sadun and Rubin 1996; Sadun et al. 1994). EMB's chelation of copper may result in decreased cytochrome-c oxidase activity and mitochondrial insufficiency leading to EMB-induced optic neuropathy.

The present in vitro study suggests that copper does not compromise EMB's usefulness in the treatment of TB or MAC infections. There remains the need to investigate whether therapeutic levels of copper can actually attenuate EMB-induced optic neuropathy in an animal model. If so, our findings suggest that if copper were given to patients as treatment for EMB-induced optic neuropathy, it would probably not compromise EMB's antimicrobial actions.

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