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The Role of Copper and Zinc Toxicity in Innate Immune Defense against Bacterial Pathogens*

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Zinc (Zn) and copper (Cu) are essential for optimal innate immune function, and nutritional deficiency in either metal leads to increased susceptibility to bacterial infection. Recently, the decreased survival of bacterial pathogens with impaired Cu and/or Zn detoxification systems in phagocytes and animal models of infection has been reported. Consequently, a model has emerged in which the host utilizes Cu and/or Zn intoxication to reduce the intracellular survival of pathogens. This review describes and assesses the potential role for Cu and Zn intoxication in innate immune function and their direct bactericidal function.

Six first row *d*-block metal ions, manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), and zinc (Zn), are essential micronutrients in living organisms. Investigations and discussions of the roles of these trace metals in biology often relate to their acquisition, storage, and incorporation into enzymes. However, conditions where these metal ions are present in excess or are found in the wrong location, resulting in toxicity, have also been described. Thus, there are also systems that sequester, export, and detoxify excess trace metal ions. Today, the concept of trace metal homeostasis, in which various cellular actions maintain the fine balance between nutrition and toxicity, is well developed.

Recently, the concept of “nutritional immunity” has emerged in the context of host defense against pathogens. This envisages a role for mechanisms that protect the host from invading pathogens by restricting their ability to acquire key transition metal ions. One example involves lipocalin, which binds siderophores and thereby prevents Fe acquisition by bacterial pathogens (1). Another is calprotectin, which restricts acquisition of Zn or Mn (2). However, what if the host was able to harness the toxic properties of transition metal ions and use them as bactericides? This review explores the evidence for an antimicrobial

role for Cu and Zn in the host defense against bacterial pathogens.

The Importance of Cu and Zn for Innate Immune Function

The innate immune system is a collection of cells and proteins that are functionally diverse and that defend against invasion by foreign organisms. At sites of infection, epithelial cells act as the first and highly effective layer of barrier, but if they are breached, a rapid influx of phagocytes such as neutrophils and macrophages will assist in curbing the initial progress of infection. Engulfment of pathogens by these phagocytes helps activate adaptive immunity, which leads to a permanent resolution of the infection.

The link between Cu and innate immune function has been recognized for decades. Early literature recorded that mild Cu deficiency in humans and animals was often characterized by neutropenia (3, 4). This condition was associated partly with a decrease in the number of circulating neutrophils, and thus a role for Cu in leukocyte differentiation, maturation, and proliferation has been proposed (5). Of interest in this review are the observations that Cu deficiency also led to impaired neutrophil function. Neutrophils isolated from hypocupremic subjects displayed reduced phagocytic capacity and/or diminished bactericidal activity, but these actions were restored readily upon dietary supplementation with Cu (6, 7). Cu deficiency had similar effects on macrophage function, and macrophages isolated from Cu-starved rats had a reduced Cu content and a decreased antimicrobial activity (8).

It thus followed that dietary manipulations of Cu levels influenced the susceptibility of animal hosts to bacterial infections. Cu-deprived diets typically sensitized the animal to infection, prolonged the duration of infection, and increased mortality rates (9–11). These phenotypes were suppressed by increasing Cu intake (12, 13). This apparent Cu requirement for optimal innate immune function has been verified recently using cell culture models of mouse macrophages. Pretreatment with added Cu boosted macrophage antibacterial activity and enhanced intracellular killing of *Escherichia coli* (K12) (14). Conversely, Cu starvation as induced by the non-permeable Cu(I) chelator bathocuproine disulfonate led to a decline in bactericidal function and an increase in the intracellular survival of the pathogen *Salmonella enterica* serovar Typhimurium (15). The effect was again reversible by the readdition of Cu into the culture medium.

Like Cu, appropriate levels of Zn are required for the proper functioning of the immune system. The importance of Zn in immunity is notably illustrated by the high occurrences of infectious diseases in developing nations due to Zn-poor diets (16–18). Zn is essential for the normal development and activity of cells involved in both innate and acquired immunity. Recently, there have been reports that Zn might function as a signaling molecule (19, 20). Of relevance to this review, studies of Zn-deficient animals and human conditions of Zn deficiency have shown that, although there was no loss of total neutrophil

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numbers, there was a decrease in their chemotactic response (21–23). Zn deficiency also led to a reduced chemotactic response of monocytes and macrophages, resulting in impaired phagocytosis and intracellular killing of pathogens (24–27). These effects were readily reversible by Zn supplementation.

Antibacterial Effects of Cu and Zn

The precise roles for Cu in innate immune function remain to be fully explored, but the current hypothesis postulates that Cu ions may be harnessed directly by phagocytes as an antibacterial agent, and this model has gained significant traction in recent years (28–30). The antibacterial effects of excess Cu ions are quite well understood. Within the reducing bacterial cytoplasm, Cu exists exclusively as Cu(I), and its level is buffered by Cu(I)-specific, high-affinity sites (31). However, the Cu(I)/Cu(II) redox couple remains biologically accessible. Thus, excess or “unbuffered” (weakly bound) Cu(I) ions are poised to catalyze gratuitous electron transfer. In the context of host-pathogen interactions, this action of Cu(I) as a redox catalyst is considered to be of critical relevance. The activities of the host NADPH oxidase during the initial respiratory burst and inducible NOS during the late phase of infection generate a potent mixture of antimicrobials, namely O_2^- and NO^* , respectively (32). If Cu(I) is also mobilized to this environment, it would potentiate the redox cycling of these reactive oxygen and nitrogen species (33, 34). This cycling may produce additional redox-active species and concomitantly deplete crucial bacterial antioxidants such as thiols, causing severe oxidative or nitrosative stress and ultimately bacterial death (35, 36).

Excess Cu(I) may also exert a bacteriotoxic effect via alternative mechanisms that are redox-independent. Cu(I) may out-compete other *d*-block metal micronutrients from their binding sites in metalloproteins, especially those that contain soft thiolate ligands, as the affinities of such sites to transition metals tend to follow the Irving-Williams series. One well characterized example is the displacement of Fe(II) from solvent-exposed Fe-S clusters by Cu(I) (37–41). The consequence is a systemic disruption of bacterial metabolic pathways that rely on the activity of Fe-S cluster enzymes. This may have downstream repercussions on intracellular survival in the host. For instance, inhibition of coproporphyrinogen(III) oxidase in the pathway for heme biosynthesis in *Neisseria gonorrhoeae* led to defects in heme-dependent defenses against H_2O_2 and NO^* (33, 40).

In contrast to Cu, Zn is not usually regarded as having the same level of toxicity. Zn is present in up to 10% of proteins in the human proteome, and computational analysis predicted that ~30% of these ~3000 Zn-containing proteins are crucial cellular enzymes, such as hydrolases, ligases, transferases, oxidoreductases, and isomerases (42, 43). This suggests that Zn is well tolerated or, more correctly, that the cell has mechanisms to control homeostasis of this metal ion.

Like Cu, Zn is regulated tightly within cells by high-affinity sites, and intracellular “free” Zn is kept well below the pM range (44). However, like Cu, Zn lies at the top of the Irving-Williams series, and there is evidence that Zn exerts an antimicrobial effect by antagonizing the uptake of other key trace metal nutrients. In *Streptococcus pneumoniae*, excess Zn was shown to

prevent the uptake of Mn(II) by binding irreversibly to the extracellular Mn(II) solute binding protein PsaA (45, 46). Consequently, *S. pneumoniae* becomes hypersensitive to oxidative stress (45, 46). Zn may also bind adventitiously to other sites that do not normally contain a metal ion and thereby inhibit key enzymes in cells (35, 47–49). We have addressed this in the case of the human bacterial pathogen *Streptococcus pyogenes* and shown that exposure to elevated Zn results in inhibition of glycolytic enzymes and phosphoglucosyltransferase (50). The latter is a key enzyme in the biosynthesis of capsule.

Evidence for a Direct Role of Cu and Zn in the Control of Bacterial Infection

The Role of Bacterial Cu and Zn Detoxification Systems in Virulence

The bulk of the evidence for a role of Cu as a host-derived antibacterial has been inferred from phenotypic characterization of bacterial mutants that are impaired in key functions associated with Cu detoxification. These mutants typically display reduced virulence in animal and macrophage model hosts, but the tolerance determinant varies and notable exceptions do exist (Table 1).

Bacterial Cu detoxification systems are diverse and are often found in some degree of redundancy, as best exemplified by the dual CueR/CusRS systems in *E. coli* (51), the CsoR/RicR regulons in *Mycobacterium tuberculosis* (52, 53), and the SctR/GolS systems in *S. Typhimurium* (54). These redundancies may also be a strategy to fine-tune the response to fluctuating Cu levels in the environment. Other detoxification systems are considerably more straightforward, such as the Cop regulon in *S. pneumoniae* (55) and the single-component CopA system in *N. gonorrhoeae* (33). Based on current understanding of these systems, the minimum requirement for Cu tolerance appears to be a membrane-bound, Cu(I)-effluxing ATPase that extrudes Cu(I) ions from the cytoplasm, usually designated as CopA. Inactivation of the *copA* gene invariably leads to trapping of Cu in the cytoplasm. Predictably, this failure to remove cytoplasmic Cu renders *copA* mutants sensitive to *in vitro* killing by Cu salts (33, 55, 56). *copA* usually operates under the control of Cu(I)-sensing transcription factors, which may also activate the expression of accessory genes that encode for cytoplasmic or periplasmic Cu chaperones and periplasmic cuprous oxidases (CueO),³ all of which are necessary for optimal Cu tolerance *in vitro* (57, 58).

If Cu acts as a host bactericide, inactivation of *copA* should suppress virulence of the invading pathogen. This was indeed the case in several, but not all, medically significant pathogens. The *copA* mutant strains of *Pseudomonas aeruginosa* (*cueA*) (59) and *S. pneumoniae* (55, 60) were less invasive in murine host models of systemic infection. The *copA* mutant strain of *N. gonorrhoeae* also had impaired survival within cervical epithelial cell host (33). By contrast, the *copA* mutants of *Listeria monocytogenes* (61, 62), *Vibrio cholerae* (63), and *S. Typhimurium* (56) displayed no loss of pathogenic fitness in animal or macrophage infection models.

³ The abbreviations used are: CueO, cuprous oxidase; ZnT, zinc transporter.

TABLE 1
 Phenotypic characterization of bacterial mutants that are impaired in Cu detoxification (export)

Bacterium	Strain	Export determinant	Host model (infection mode)	Phenotype relative to the wild type strain	Reference
<i>E. coli</i>	K12	<i>copA</i>	RAW264.7	Decreased intracellular survival.	[14]
	CFT073	<i>cusFCBA</i>	Mice (urethra)	Decreased bacterial burden in the urinary tract/urine.	[12]
	UTI89, EC958	<i>copA</i>	RAW264.7, BMMs Human neutrophils	No difference in intracellular survival. No difference in survival.	McEwan laboratory, unpublished
<i>L. monocytogenes</i>	EGDe	<i>copA</i>	Mice (oral)	No difference in mice mortality rate. No difference in bacterial burden in the gall bladder, spleen, liver.	[60]
	DRDC8	<i>ctpA</i>	Mice (intravenous-tail)	Faster clearance of bacteria from the liver.	[61]
			J774 macrophages	No difference in clearance from the spleen. No difference in intracellular survival.	[61]
		<i>ctpV</i>	Guinea pigs, mice (inhalation)	No difference in bacterial burden in the lung; decrease tissue damage	[63]
<i>M. tuberculosis</i>	H37Rv	<i>ctpV</i>	Mice (inhalation)	Decreased mortality rate of mice (not restored by genetic complementation) Decreased host immune response.	[63]
		<i>mctB</i>	Guinea pigs, mice (inhalation)	Decreased bacterial burden in the lung, lymph nodes, and spleen. Decreased tissue damage.	[13]
<i>N. gonorrhoeae</i>	1291	<i>copA</i>	Human cervical epithelial cells	Decreased intracellular survival.	[33]
<i>P. aeruginosa</i>	PAO1	<i>cueA</i>	Mice (intraperitoneal)	Decreased competitive index in the spleen. No difference in competitive index in the liver. Increased LD ₅₀ dose required to achieve killing of mice.	[58]
			Mice (intranasal)	Decreased mortality rate of mice. Decreased bacterial burden in the nasopharynx and blood.	[54], [59]
<i>S. pneumoniae</i>	D39, TIGR4	<i>copA</i>	Murine lung macrophages	Decreased adherence and survival.	[59]
			Human neutrophils	No difference in survival.	McEwan laboratory, unpublished
<i>S. Typhimurium</i>	SL1344	<i>cueO</i>	Mice (oral)	Decreased bacterial burden in the liver and spleen. No difference in bacterial burden in mesenteric lymph nodes or Peyer's patches	[56]
			RAW264.7	No difference in intracellular survival.	[56]
		<i>copA</i>	RAW264.7	No difference in intracellular survival.	[55]
		<i>copA/golT</i>	RAW264.7 Mice (oral)	Decreased intracellular survival at t = 12, 24 h. No difference in bacterial burden in the liver or spleen.	[55] [55]
<i>V. cholerae</i>	C7258	<i>copA</i> , <i>copA/copG</i>	Mice (intra-gastric)	No difference in mice mortality rate. No difference in bacterial burden in the intestines.	[62]

Here, it is important to recognize that some prokaryotes possess more than one Cu(I) efflux pump, and these may be able to compensate for the loss of CopA. For *S. Typhimurium*, a phenotype for the *copA* mutant in terms of survival within macrophages was observed only upon simultaneous inactivation of *golT* (56), which encodes for a second Cu(I)-exporting pump in this bacterium. Nonetheless, virulence of the *copA/golT* double mutant was maintained in a mouse model host (56). For *M. tuberculosis*, inactivation of the *copA* homologue *ctpV* had no clear effect on mycobacterial pathogenesis in mice or guinea pigs (64), but disruption of an alternative Cu(I) export pump encoded by *mctB* was detrimental to survival (13). Likewise, for *L. monocytogenes*, only the plasmid-borne *ctpA* gene but not the chromosomal *copA* gene was essential for long-term colonization of mice (62). However, the behavior of the *ctpA* mutant in isolated macrophages was indistinguishable from that of the parent strain (62).

Although the above examples suggest that pathogenic success depends on an efficient removal of excess Cu from the bacterial cytoplasm, the importance of controlling Cu in the bacterial envelope or periplasm must not be overlooked. Mutation of the *cusFCBA* operon was recently shown to suppress colonization by uropathogenic *E. coli* in a mouse model of urinary tract infection (12). The *cus* operon encodes for a secondary Cu(I) efflux system in *E. coli* that protects against periplasmic Cu toxicity under anaerobic conditions (51, 65). The effect of *copA* mutation in this genetic background is not known, but

inactivation of *copA* in the laboratory strain K12 was shown to impair its ability to colonize mouse macrophages (14). Similarly, a *cueO* mutant strain of *S. Typhimurium* lacking the periplasmic cuprous oxidase was attenuated in mouse liver and spleen (57), although it showed no phenotype in isolated macrophages. The results with *cueO* were different from those generated with the *copA/golT* double mutant, which showed reduced survival only in macrophages but not in mice (56). At present, a rationale for this apparent discrepancy is not available, but it must be mentioned that inactivation of *cueO* in *E. coli* (K12 and uropathogenic strain) caused pleiotropic effects that were unrelated to Cu tolerance (66, 67).

To some extent, bacterial Zn detoxification systems appear simpler when compared with those of Cu. They typically consist of a transcriptional response regulator paired with the Zn efflux transporter, although there may be some redundancies in these exporters. The best exemplified Zn export systems in the literature are the ZntR/ZntA regulator/P-type ATPase Zn efflux system in *E. coli* (68, 69) and the GczA (SczA)/CzcD regulator/cation diffusion facilitator system in *S. pyogenes* and *S. pneumoniae* (70, 71).

In relation to infection, it has been established that Zn deficiency in animal models promotes susceptibility to bacterial pathogens such as *L. monocytogenes* (72) and *M. tuberculosis* (73). Until recently, all evidence had pointed to Zn deprivation as the key antimicrobial strategy in innate immune cells, and reports of bacterial Zn export as a virulence determinant had

TABLE 2
Phenotypic characterization of bacterial mutants that are impaired in Zn detoxification (export)

Bacterium	Strain	Export determinant	Host model (infection mode)	Phenotype relative to the wild type strain	Reference
<i>E. coli</i>	BW25113 EC958	<i>zntA</i>	Human macrophages Human neutrophils	Decreased intracellular survival Decreased intracellular survival	[73] McEwan laboratory, unpublished
<i>H. pylori</i>	P149	<i>cznABC</i>	Gerbil (oral-gastric)	Decreased bacterial load in the stomach	[74]
<i>M. tuberculosis</i>	GC1237	<i>ctpC</i>	Human macrophages Mice (intranasal)	Decreased intracellular survival No difference in bacterial burden in the lung; no difference in survival	[73] [73]
<i>S. pyogenes</i>	5448	<i>czcD</i>	Human neutrophils Mice (subcutaneous)	Decreased survival Decreased survival	[70] [70]

been limited (Table 2). In 2011, Botella *et al.* (74) demonstrated that a mutant strain of *M. tuberculosis* that lacks the Zn-effluxing ATPase CtpC was attenuated in a human-derived macrophage model of infection. However, there was no observable loss of virulence in mice (74). Similarly, a *zntA* deletion mutant of *E. coli* also had reduced survival within human macrophages when compared with the wild type strain (74). These results are clearly consistent with the idea that, like Zn starvation, Zn excess may also play an antimicrobial role. Subsequently, it was shown that inactivation of *czcD*, which encodes for the Zn-specific cation diffusion facilitator in *S. pyogenes*, led to a decreased survival in human neutrophils and a reduced virulence in a mouse model of infection (71). Similarly, deletion of *cznABC*, a broad-spectrum ATP-binding cassette (ABC) transport system that is thought to play a role in the export of Zn, resulted in decreased virulence of *Helicobacter pylori* in a gerbil model of gastric infection (75).

Alterations in Host Cu and Zn Status in Response to Bacterial Infection

Additional support for the involvement of Cu in the clearance of intracellular pathogens is available from studies that examine the host responses to bacterial infection. In response to inflammation by *S. Typhimurium* or the proinflammatory agent *Salmonella* LPS, expression of ATP7A, which encodes for the ATP-dependent Cu(I) transporter ATP7A, was elevated in mouse macrophages (15). ATP7A is required for the biosynthesis of cuproenzymes, and so it usually resides in the Golgi apparatus. This expression of ATP7A was accompanied by robust increases in the expression of *CTR1* and *CTR2*, which encode for transporters for the import of Cu(I) into the cytoplasm, and *CP*, which encodes for ceruloplasmin (15).

These alterations in the expression of genes involved in Cu metabolism may signify a concerted boosting of Cu uptake into the host cell. One hypothesis proposes that Cu is trafficked specifically to phagosomes to enhance killing of intracellular bacteria. Indeed, activation of mouse macrophages by IFN- γ was shown to trigger partial trafficking of ATP7A to phagosomal compartments (14). Consistent with this hypothesis, RNAi silencing of *ATP7A* diminished the bactericidal activity of these macrophages against engulfed *E. coli* K12 (14). However, direct detection of Cu levels within phagosomes, whether before, during, or after infection, has not yet been confirmed. Elemental analyses of mouse macrophages by

x-ray fluorescence detected an apparent increase in the amounts of Cu in extracellular *M. tuberculosis* when compared with those in internalized bacteria within mycobacterium-containing phagosomes, but this difference was *not statistically significant* (76). A separate imaging study of mouse macrophages using the fluorescent sensor CS-1 also showed that although Cu ions were mobilized to discrete vesicles in response to inflammation by *S. Typhimurium*, these Cu-rich vesicles did *not* co-localize with *Salmonella*-containing vacuoles (15).

Nevertheless, there was activation of the *copA* promoter of *S. Typhimurium* during interactions with mouse macrophages (56). This requirement for CopA function certainly implies a situation of Cu excess. However, it should be noted that the *copA* promoter was activated only after 12–24 h after infection (56). This timing coincides nicely with the expression of Cu homeostasis genes in macrophages, which peaked at 14 h after infection (15). This apparent elevation of Cu does not coincide with the timing of the oxidative burst in macrophages. Instead, it is temporally more coincident with the onset of nitric oxide production.

Mammalian Zn homeostasis is highly complex. It can involve 10 ZnT (SLC30) family and 14 ZIP (SLC39) family transporters, as well as metal transcription factors (MTF-1/2) and Zn storage proteins (77). Prior to the findings by Botella *et al.* (74), there was an emphasis on Zn deprivation as the key antimicrobial strategy in innate immune cells, based on inference from the function of metallothionein and calprotectin. Recently, the use of gene expression studies in conjunction with total metal analysis and fluorescent microscopy has allowed better detection of Zn and its mobilization within innate immune cells. Immunofluorescence microscopy of macrophages infected with *M. tuberculosis* indicated that Zn was released from intracellular stores (metallothionein) and trafficked into mycobacterial-containing phagosomes (74). Likewise, internalization of *S. pyogenes* correlated with the release of Zn within human neutrophils (71).

This increase in Zn within innate cells has also been observed during fungal infections. Metal analysis demonstrated that total Zn content was increased within activated macrophages infected with the fungal pathogen *Histoplasma capsulatum* (78). This was the result of increased Zn import via the ZIP2 importer, but the Zn ion was then distributed to intracellular

organelles such as the Golgi apparatus via Zn transporters ZnT4 and ZnT7 or bound to metallothionein (78).

By contrast, LPS binding to the TLR4 receptor does not trigger Zn import into the macrophage. Instead, it rapidly increases the expression of the ZIP8 importer located on the lysosomal membrane (79, 80). However, this action also elevates the free Zn levels in the cytosol, which can trigger the production of inflammatory cytokines, resulting in T-cell proliferation (81, 82). Despite occurring through different mechanisms, both bacterial and fungal infections result in increased free Zn within innate immune cells. As prolonged inflammatory response can be detrimental to the host, it must be regulated to minimize tissue damage. Recently, this role of Zn as a signaling molecule has been further defined by demonstrating that the elevated Zn within monocytes and macrophages results in dampening of the inflammatory response via a negative feedback loop (80). The elevated cytosolic Zn within monocytes and macrophages (imported via ZIP8) was shown to suppress NF- κ B signaling, leading to decreased expression of cytokines such as IL-6 and TNF- α , which in turn leads to decreased inflammation (80). Thus, it is clear that Zn is central to the control of the inflammatory response in response to infection.

Trafficking of Cu and Zn to Sites of Infections

The biodistribution of Cu in whole animals and humans has been studied extensively following exogenous administration of synthetic Cu salts and complexes, although not always coincident with bacterial infection (83, 84). In most cases, the Cu was trafficked to the liver and kidney, as would be expected from our current understanding of mammalian Cu transport and metabolism (85). By contrast, little is known regarding the status of *endogenous* Cu at sites of infection.

Direct evidence that Cu may be mobilized to sites of infection is provided by two recent studies. These detected an increased Cu concentration in granulomatous lesions from guinea pigs that were infected with *M. tuberculosis* when compared with the uninfected control (13), and a significant accumulation of Cu in urine of patients with urinary tract infection by uropathogenic *E. coli* when compared with urine from healthy subjects (12). In retrospect, the latter is not altogether surprising. It is well documented that Menkes patients are subject to recurring urinary tract infections (86, 87). Menkes disease is a disorder of Cu metabolism due to genetic defects in *ATP7A*, and clinical manifestations of this disease often mimic Cu deficiency.

Gene expression analyses of bacteria that were isolated from infected niches in the animal host have provided further support that Cu is trafficked to sites of infection. Expression of the *cop* operon was up-regulated in *S. pneumoniae* isolated from the murine nasopharynx (55). Expression of the secondary Cu(I) efflux operon *cusFCBA* from uropathogenic *E. coli* was induced in uropathogenic *E. coli* isolated from the urine of infected mice (12). Consistently, a *cusFCBA* mutant strain was attenuated in this animal model (12). An increase in the expression of *copA* was also detected (12), but the role of this gene in the virulence of uropathogenic *E. coli* has not been established.⁴ Similarly, expression of *ctpV* from *M. tuberculosis* was highly

up-regulated in mice lungs. By contrast, there was little change in *mctB* expression under the same conditions (88). This contradicts results from phenotypic characterization, which found only *mctB* and not *ctpV* to be essential for virulence (13, 64). These findings indicate that gene expression patterns do not always correlate with the importance of the gene in pathogenesis and may point to a complex role for Cu that is yet to be fully understood.

As described earlier, the main consideration with regard to Zn at the host-pathogen interface has been the role of nutritional immunity. In this context, the innate immune system prevents Zn acquisition by a bacterial pathogen (2). In this process, Zn is redistributed to various tissues such as the liver, resulting in decreased serum Zn levels. This process can occur through the IL-6-dependent up-regulation of ZIP14, the Zn transporter responsible for accumulating Zn in hepatocytes (89). Plasma Zn is also depleted, and the mechanism appears to involve metallothionein, which is accumulated in the liver in its Zn-bound form following stimulation by inflammatory cytokines such as IL-1 (90).

Zn nutritional immunity is also exemplified by the action of the human protein calprotectin, which sequesters Zn and Mn and starves the bacterial pathogen of these trace metal nutrients (91). Calprotectin is found abundantly in neutrophil extracellular traps, which represent a key mode of extracellular bactericidal killing by neutrophils (92). Here it needs to be noted that calprotectin probably only exerts its effect once it is released from the neutrophil (93, 94). Nevertheless, it may be the case that there are specific niches where local elevation of Zn is linked to its antimicrobial action. For example, it has been shown that Zn is elevated in the nasopharynx upon a challenge by *S. pneumoniae* (45). Thus, the antimicrobial action of the elevated Zn may be associated with mucosal surfaces.

Conclusions

This review presents a now considerable body of data consistent with the hypothesis that Cu and Zn toxicity can contribute to the clearance of bacterial pathogens. This has led to the intriguing concept of the “brass dagger” in innate immunity (30), which then raises the question of how such a bactericidal mechanism could be integrated into the complex immune system of mammals. However, we suggest that the mechanisms behind the antibacterial actions of Cu and Zn may be more complicated than the simplistic view proposed by this model.

The systemic movements of Cu, Zn, and Fe in response to bacterial infection are well documented. In the case of Zn, it is clear that movement of this ion is central to the control of inflammation and innate immune function. Trafficking of Zn to intracellular locations may enhance the bactericidal capability of macrophages and neutrophils. In such circumstances, robust defenses against elevated Zn would be a requirement for a successful intracellular bacterial pathogen. However, the question of the bacterial niche still needs to be considered. Locally elevated levels of Zn may well play a role in controlling growth of extracellular mucosal pathogens, as already evidenced in the case of *S. pneumoniae* in the nasopharynx (45).

The situation is more complex for Cu as increases in Cu levels in the serum or at sites of infection are usually associated

⁴ M. E. Achard and A. G. McEwan, unpublished results.

with an increase in the production of ceruloplasmin (95, 96). Ceruloplasmin is a ferroxidase and is a key component of Fe homeostasis, but it is also a major Cu store in cells as it binds six Cu ions in the active site. Fe transport and regulation are linked intimately with the inflammatory response, where removal *away* from sites of infection is the norm. Based on our current understanding of eukaryotic Fe homeostasis, movement of Fe out of cells depends on the efflux of ferrous iron via ferroportin, and this requires the ferroxidase activity of ceruloplasmin (97, 98). Assembly of holo-ceruloplasmin in the host endoplasmic reticulum or Golgi relies on the transport of Cu via the Cu-transporting ATP7B (99). As ATP7B is not found outside the liver, in macrophages, this Cu transporter is presumed to be ATP7A. Thus, although it is not surprising that engulfed pathogens encounter elevated Cu in macrophages, it is difficult to separate a direct bactericidal effect of Cu ions from their key role in facilitating the removal of Fe from the macrophage.

Whatever the relative importance of the two processes, it does follow that possession of a Cu tolerance system would enhance bacterial survival. In the case of some pathogens such as *S. pneumoniae*, it appears that loss of *copA* is sufficient to reduce survival within macrophages and in an animal model (Table 1). However, professional intracellular pathogens such as *S. enterica* and *M. tuberculosis* have more robust defenses against Cu, and thus the loss of *copA* does not always have a clear effect on their pathogenesis (Table 1). These inconsistent phenotypes clearly illustrate the need to examine precisely *when*, *where*, and *how* Cu exerts its effects on the innate immune system. Central to future studies, at least in macrophages, is the requirement to *directly* determine whether Cu is elevated in the same phagosomal compartment where the bacterial pathogen is located. The systematic cataloguing of virulence phenotypes of Cu detoxification mutants in various infection models and at various infection niches, even when there are no clear effects, will also be increasingly important.

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