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Research Article

# Cooperative Cytotoxins: A New Look at an Old Observation of Bacterial Crosstalk

**Regina Linder[1*], Camille D. McIntyre[2]**

*[1]Department of Medical Laboratory Sciences, Hunter College (CUNY), New York, NY, USA; [2]Department of Medical Laboratory Sciences, Hunter College (CUNY), New York, NY, USA*

## ABSTRACT

Cooperative (or synergistic) hemolysis, the ability of two bacterial species to jointly lyse erythrocytes, has long been recognized as a helpful tool in the identification of common pathogens (i.e. the CAMP reaction between *Streptococcus agalactiae* and *Staphylococcus aureus*). However, to examine these biological partnerships separately from their use in the diagnostic lab provides new perspectives on toxicity to host tissue during infections and in health. Many examples of such pairings exist, and typically reflect the sequential action of a phospholipase (e.g., PLC from *Staphylococcus aureus* or *Clostridium perfringens*), followed by a second bacterial toxin acting on the altered membrane, e.g. the CAMP protein of group B *streptococci*, or the cholesterol oxidase of *Rhodococcus equi*. Commonly occurring cooperative cytotoxic partnerships are reviewed, along with their biochemical mechanisms of action. Newly reported is the ability of hemolytic collaborations to accommodate a midcourse change in conditions of oxygenation. Thus, erythrocytes altered by PLC of *C. perfringens* grown anaerobically, are lysed following exposure to the strict aerobe *R. equi*, in air. Why does this matter? Microbial communities on tissue (i.e. the microbiome) are increasingly understood to impact the health of hosts. Pathogenesis, especially in anaerobic infections, often reflects the combined actions of microbial pathogens, commensal (resident) microorganisms, and metabolites from the host. Products of some cooperative reactions (i.e., ceramide and oxysterol) are directly toxic, e.g., to the immune system. Host environments include a range of oxygenation not intuitively evident, i.e., extreme anaerobiosis in the mouth, creating ideal conditions for cooperative cytotoxicity to occur *in vivo*. To appreciate the impact of common hydrolytic enzymes and other proteins from diverse sources deepens our understanding of the host and its complex microbial community.

**Keywords:** Cooperative (synergistic) Hemolysis; Bacterial Cytotoxins; Microbiome; Crosstalk

## INTRODUCTION

Beginning with the CAMP reaction of group B *streptococci* (GBS), and named for the investigators who described it in 1944 [1], many common bacterial pathogens and commensals were found to participate in "cooperative" cytotoxicity or "synergistic" hemolysis. The reaction is typically visualized as a zone of enhanced hemolysis between two plated species on blood agar (often sheep blood), but not seen around either organism separately. Alternatively, some bacterial pairs may cause inhibition of lysis of one species by the other [2]. Mammalian cells other than erythrocytes can be damaged [3]. The biochemical mechanisms underlying many of these relationships were characterized in earlier decades following the purification of the individual cytotoxins, and usually involve enzymatic hydrolysis of (or binding to) membrane lipid components of the target cells (phospholipids or sterols) with paired agents, acting sequentially [2,3].

Typically one of the agents (the phospholipase) is considered to hydrolyze a superficial membrane component, sphingomyelin or phosphatidylcholine (lecithin). Sphingomyelin predominates in the outer leaflet of sheep erythrocyte membranes [4], allowing access of the partner toxin to its molecular target residing deeper

**Correspondence to:** Regina Linder, Department of Medical Laboratory Sciences, Hunter College (CUNY), New York, NY, USA, Tel: 212 396 7167; E-mail: rlinder@hunter.cuny.edu

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in the bilayer [5]. Table 1 illustrates some key examples, together with their known mechanisms of action. These commonly observed *in vitro* partnerships can be considered together with current concepts of the microbiome (the resident microbial communities on human tissues) [6] thus highlighting cooperative cytotoxicity as a means of inter-species communication in hosts.

Tissue and wound infections often reflect mixed flora with varied relationships to oxygen, and include known cooperative (and inhibitory) partner species [7,8] similarly providing a plausible model for *in vivo* activity. Based on typical environments of participating species, and known mechanisms of toxin action, collaborative membrane damage among resident and pathogenic microorganisms can be inferred to be active in humans and animals, in health and disease.

Results demonstrate that cooperative and inhibitory hemolysis among species does not require that both strains grow concomitantly. Pre-grown strict anaerobes (*clostridia*) collaborate to lyse erythrocytes with strict aerobes grown the following day, after transfer to air incubation. Facultative species collaborate following sequential or concomitant growth, in either anaerobic or aerobic conditions. This plasticity reflects the ubiquity of many of these microorganisms as normal resident flora on tissues, as well as isolates from a wide range of infectious disease scenarios [9].

## MATERIALS AND METHODS

### Bacterial strains; growth and maintenance

Bacterial strains used in this study derive either from long maintained culture collections of the authors [3], the Medical Laboratory Sciences Dept. of Hunter College, or clinical strains (designated KS) isolated from patient specimens at the VANY Harbor Healthcare System, by our late colleague Dr. Kuldip Sandhu, in 2007-8. Where strains were derived from public culture collections, i.e. ATCC, the Collection's designation is retained. These include *Rhodococcus equi* 33701 and *Corynebacterium pseudotuberculosis* 19140. Strains are stored as suspensions in rabbit blood at -80°C. Routine growth of strains is in trypticase soy broth or agar (Difco), at 37°C in air, or Gas Pak (BBL) for *Clostridium perfringens*.

### Demonstration of cooperative hemolysis or inhibition

Cross-streaking conditions have been described [2,3]. Cooperative cytotoxicity between wells inoculated with suspensions of known colony forming units (cfu) were carried out following inoculation as indicated and incubation in stated conditions. Documentation follows incubation, by digital photography.

## RESULTS

Enhanced as well as inhibitory hemolytic patterns of some pathogenic and commensal bacterial pairs, following sequential aerobic and anaerobic incubation are illustrated in Figure 1, a diameter streak of *Clostridium perfringens* (A) was pre-incubated overnight anaerobically, followed by perpendicular application of the test species [1-5] and continued incubation in air (24 hrs, 37°C). For B, *Staphylococcus aureus* is the diameter streak, and was plated concurrently with test strains 1-5, followed by 24 hrs at 37°C in air. The active toxin of *C. perfringens* is phospholipase C (α- toxin, lecithinase), and for *S. aureus*, sphingomyelinase C (β-toxin), and similar patterns of hemolysis are typically shown for both conditions and phospholipid sources [4]. The *coryneform* bacteria, *C. glucuronolyticum* and *R. equi*, together with *S. agalactiae* (GBS) showed enhanced hemolysis, in distinctive shapes. *A. haemolyticum* and *C. pseudotuberculosis*, were somewhat inhibitory to partial hemolysis by the diameter streak.

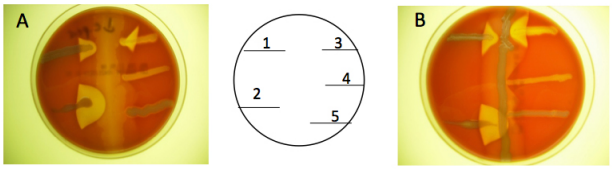


**Figure 1:** Cooperative hemolysis by human and animal pathogens grown in proximity to diameter plating on sheep blood agar of (A) *C. perfringens* (after prior anaerobic cultivation), and (B) *S. aureus* (plated concurrently in air): 1. *Corynebacterium glucuronolyticum*, 2. *Rhodococcus equi*, 3. *Streptococcus agalactiae* (GBS), 4. *Arcanobacterium haemolyticum*, 5. *Corynebacterium pseudotuberculosis*.

Hemolytic enhancement can also be viewed when standard bacterial inocula (2-3 × $10^4$ cfu) were placed in agar wells from which diffusible proteins interact on erythrocytes. The resulting hemolytic zones can be compared in a semi-quantitative fashion. Figure 2 shows the aerobic collaboration between *S. aureus* (β-toxin+ in central wells, panel (A) and three strains of GBS isolated from clinical specimens (diabetic foot ulcers) with co-pathogen, *Staphylococcus aureus*. The size of hemolytic zones were qualitatively similar (ranking KS17>KS7>KS15) to those that developed when the same strains were incubated anaerobically opposite *Clostridium perfringens* (panel B). In neither case was *S. aureus* (included as a control, KS13) active in enhanced hemolysis.

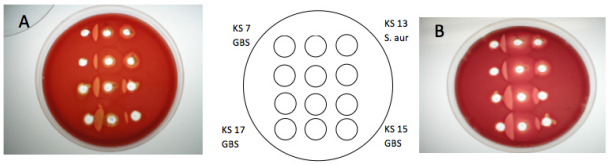


**Figure 2:** Cooperative hemolysis between (A) *Staphylococcus aureus* (central wells) or (B) *Clostridium perfringens* (central wells) and duplicate peripheral wells containing three strains of GBS (KS7 top left, KS17 bottom left, KS15 bottom right) incubated in (A) Air and (B) Anaerobically. A clinical strain of *S. aureus* (KS13, top right) serves as a negative control.

Figure 3 shows cooperative hemolysis between *C. perfringens* (a strict anaerobe) and *R. equi* (a strict aerobe) when central wells inoculated with the *clostridium* (2-3 × $10^4$ cfu, panel A) were pre-incubated anaerobically for 24 hrs, 37°C, followed by addition of 4 strains of *R. equi*, and incubation continued in air for an additional 24 hrs. In panel B, the same peripheral strains of *R. equi* are inoculated and incubated in air for 24 hrs 37°C, followed by inoculation of *C. perfringens* in central wells, and anaerobic incubation continued for 24 hrs at 37°C. Degree of

hemolysis is similar for the two sequences, although the shape of hemolytic zones varies. The weakest cytotoxin of the *R. equi* strains (7502) did not produce visible hemolysis when anaerobic cultivation preceded air incubation.

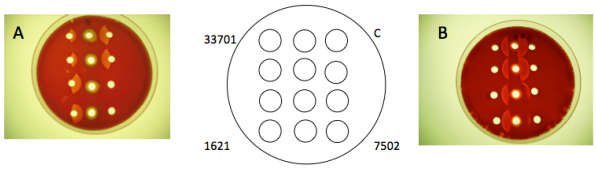


**Figure 3:** Cooperative hemolysis between *Clostridium perfringens* (central wells) and 4 strains of *Rhodococcus equi* in duplicate peripheral wells (33701 top left, 1621 bottom left, C top right, 7502 bottom right), following inoculation of (A) Central wells anaerobically followed by peripheral wells in air; and (B) Peripheral wells inoculated and incubated in air prior to addition of *C. perfringens* (central) and anaerobic incubation.

Taken together, Figures 1-3 demonstrate the plasticity of cooperative cytotoxicity with regard to sequence of growth and oxygenation. Toxins (phospholipases, cholesterol oxidase, CAMP factor) released into the agar retain their activity and ability to collaborate, even when conditions are changed to prevent continued growth of the bacteria that produced them. Thus, PLC secreted from *C. perfringens* during anaerobic incubation retains its robust ability to lyse erythrocytes when exposed to the cholesterol oxidase of *R. equi* grown subsequently in air (Figure 3) as shown in early studies [4]. It should be noted that facultative bacteria (i.e. GBS clinical isolates and *coryneforms*) retained the ability to cooperate even when all strains were cultivated anaerobically, and when growth was sparse (Data not shown).

## DISCUSSION

From its first description in 1944, the cooperative cytotoxic relationship between *S. aureus* and GBS (the CAMP reaction) proved a valuable diagnostic test to quickly categorize hemolytic *streptococci* important in both human and animal health [1,2]. Similar observations followed among other pathogens and commensals, providing assistance with presumptive lab identifications. However, little attention has been directed to the function or evolutionary benefit to the organisms that participate. Considering the large number of such relationships, as well as the property of these reactions to occur across the barrier of anaerobiosis, it is proposed that cooperative cytotoxicity be considered an example of bacterial "crosstalk", the complex array of microbial, immunological and environmental factors that impact host outcomes [9,10].

In considering the identity of organisms that participate in cooperative relationships themes emerge: *Coryneform* bacteria (the genus *Corynebacterium*, and related Gram-positive pleiomorphic rods), are associated with ruminant and other animal infections, as well as opportunistic disease in humans (see Table 1). An example is *R. equi*, a frank pathogen of foals, is able to infect immunocompromised humans exposed to equine environments [11].

Infections typically occur on mucosal surfaces with a mixed resident flora, including *S. aureus* and *C. perfringens*. *Listeria monocytogenes* is isolated from a wide variety of animals, birds and fish. Infection in humans is typically foodborne, and potentially serious in high risk hosts. *Acinetobacter* is a diverse genus increasingly recognized as a hospital opportunist, and resistant to multiple antibiotics. In general, pathogenesis among these species is opportunistic, and may derive from the commensal flora or from exogenous exposure to animals or soil. Infection most often develops on contaminated, rather than sterile, sites [12].

Cooperative cytotoxicity is not limited to bacteria. The observation that common dermatophyte fungi display synergistic hemolysis in partnership with staphylococci and Listeria [13] expands the potential for co-toxicity. The PLD of *C. pseudotuberculosis* is identical with the toxin of the brown recluse spider [14] introducing the opportunity for communication with sources other than microorganisms. Cytotoxins with activity profiles similar to those of bacteria have been identified from a range of biological sources, including snakes, insects, and sea anemones [15].

Microbial pathogenesis involving strictly anaerobic bacteria, including *Clostridium perfringens* often depends upon collateral tissue damage or co-infection. When colonized tissue (e.g. intestinal, oral, and skin) is damaged by mechanical force or infection with aerobic or facultative bacteria, the reduced oxygen tension that results supports growth of resident anaerobes, out of proportion to their usually balanced numbers [12].

Polymicrobial isolations are commonplace, and include bacteria with varied relationships to oxygen [9]. Molecular analysis of rRNA and similar markers from infected and uninfected chronic wounds reveals highly diverse flora, with dozens of species, facultative and anaerobic, and often including *S. aureus* [10]. The potential for cooperative interactions is extensive, albeit refractory to direct demonstration.

We suggest that cooperative cytotoxicity be considered among mechanisms of inter-species communication, providing enhanced ability to damage host tissue, increase inflammation, and introduce specific metabolic products associated with harm to humans. For example, ceramide (exposed on target cell membranes when sphingomyelin is hydrolyzed) is associated with an increasing range of physiological harm, including to mitochondrial function [16], and is recognized in the pathogenesis of Parkinsonism [17]. Oxidized sterols, a product of the sequential action of phospholipase and cholesterol oxidase have long been implicated in complex cardiovascular pathology [18]. In an earlier experimental protocol, cholesterol oxidase was directly lethal to rabbits rendered atherosclerotic by diet [19].

It should also be noted, in the literature and in our hands, weak cooperative hemolysis is a wider phenomenon than the well characterized robust cytotoxic pairings here discussed. Many *streptococci*, including group A and the *pneumococcus* are examples [3,4]. While not deemed useful in the identification of these species, cytotoxicity should not be ruled out among the numerous virulence determinants of these pathogens. Further, it is significant to recall that red blood cells in these studies are a

convenient model for plasma membranes of tissues and organs that may be exposed. The combined action of paired toxins was shown to act on cultured mammalian cells [5].

Cytotoxic partnerships among microorganisms are examples of the recently articulated concept, the "pathobiome" [20]. According to Bass et al., it is the complex sum of interactions among commensal, disease producing and host elements that influence the incidence and outcome of infectious diseases in humans, plants and animals [20]. Consistent with this approach, the microbiome, the mixture of microbial residents deemed critical for maintaining good health [21] has been shown to extend to infected tissue. In a recent study of diabetic wounds, Kalan et al., using molecular analysis, demonstrated the microbial diversity of persistent lesions, including aerobes and anaerobes, *corynebacteria* and very commonly *S. aureus* [9].

**Table 1:** Cooperative/Inhibitory cytotoxins of some common bacterial pathogens.

| Source | Agent/biochemical activity | Typical pathology in humans (animals) | References |
|---|---|---|---|
| *Clostridium perfringens* | PLC broad spectrum/ lecithinase C | Exogenous and endogenous tissue destruction | 2,4,12 |
| *Staphylococcus aureus* | β-hemolysin, sphingomyelinase C | Local and disseminated infection, opportunist | 2,4,12 |
| *Streptococcus agalactiae* (GBS) | CAMP factor, binding to ceramide | Wounds, neonatal meningitis (bovine mastitis) | 2,6,22 |
| *Corynebacterium pseudotuberculosis* (form. ovis) | PLD/ sphingomyelinase D | Rare opportunist (ruminant lymphadenitis) | 2,3,12,23 |
| *Arcanobacterium haemolyticum* | PLD/ sphingomyelinase D | Pharyngitis, soft tissue | 2,3,12,23 |
| *Rhodococcus equi* | Cholesterol oxidase | Lung opportunist/ (equine sepsis) | 2,3,7,12 |
| *Corynebacterium glucuronolyticum* | N/A | Genitourinary infection, granulomatous mastitis | 2,12 |
| *Listeria monocytogenes* | PLC broad spectrum | Foodborne bacteremia, stillbirth | 2,12 |
| *Acinetobacter baumanii* | PLC and PLD (not shown associated with cooperative hemolysis) | Nosocomial lung and bacteremia; multidrug resistance. | 2,12 |

Table 1 illustrates a range of bacterial species that participate in robust cooperative and inhibitory cytotoxic partnerships, together with the enzymatic or binding products that have been shown to mediate the reactions. In order to highlight the significance of cytotoxic cooperation, the typical pathology of species is indicated.

## CONCLUSION

Thus, the numerous examples of cooperative (and inhibitory) cytotoxicity described decades ago highlight a subset of microbial and mammalian interactions that reflect contemporary concepts of health and disease.

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