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Review

Pathogenicity of *Erysipelothrix rhusiopathiae*: virulence factors and protective immunity

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ABSTRACT – *Erysipelothrix rhusiopathiae* is the causative agent of erysipelas in animals and erysipeloid in humans. In the absence of specific antibodies, the organism evades phagocytosis by phagocytic cells, but even if phagocytized, it is able to replicate intracellulary in these cells. In this review, recent advances in our understanding of the pathogenicity of *E. rhusiopathiae* and its protective immunity are described. © 2000 Éditions scientifiques et médicales Elsevier SAS

Erysipelothrix rhusiopathiae **/ pathogenicity / capsule / opsonic antibody / cell-mediated immunity**

1. Introduction

Erysipelothrix rhusiopathiae, the causative agent of erysipelas, is a Gram-positive, non-spore-forming, non-acid-fast, rod-shaped bacterium. *E. rhusiopathiae* is classified in the genus *Erysipelothrix* together with *E. tonsillarum* and two other unnamed species [1].

E. rhusiopathiae is ubiquitous in nature and has been isolated from many species of wild and domestic mammals and birds as well as reptiles, amphibians, and fish [2]. The organism causes a variety of diseases in many species of birds and mammals, including humans, but it is most important as the causative agent of swine erysipelas [2]. Swine erysipelas usually results from ingestion of contaminated food or water. It has been reported that approximately 30–50% of apparently healthy swine carry the organism in their tonsils and other lymphoid tissues of the alimentary canal, and such bacteria in the tissues are thought to gain entry to the deeper body tissues or bloodstream [2].

Swine erysipelas may occur as an acute septicemic disease or chronic disease typically characterized by endocarditis and polyarthritis [2]. The chronic arthritis in swine resembles rheumatoid arthritis in many respects, making erysipelas polyarthritis an interesting model for studying the pathomechanisms of chronic inflammation. It also causes polyarthritis in sheep and lambs and serious death losses in turkeys. In humans, the organism causes erysipeloid, a local cutaneous lesion usually on the fingers or hands, and rarely causes endocarditis or acute septicemic disease [2, 3].

The vaccine against swine erysipelas was first developed by Pasteur and Thuillier in 1883, and live attenuated vaccines or bacterins have long been used to control the disease in pigs and turkeys; however, the mechanisms of the pathogenicity of the organism and the basis of acquired protective immunity remain poorly understood. The application of modern genetic methods have only recently provided new insights into them, and it was revealed that *E. rhusiopathiae* has a capsule which is important in regard to its virulence. This article reviews the pathogenesis of *E. rhusiopathiae* infection, focusing on the capsule, and describes the mechanisms of protective immunity against infection.

2. Virulence factors

2.1. Neuraminidase and hyaluronidase

E. rhusiopathiae produces various enzymes which have been suggested to be involved in the pathogenicity of bacteria. The organism produces neuraminidase (sialidase) which can release terminal sialic acid residues from glycoproteins, glycolipid, and oligosaccharides expressed on host cells. In a study of mice and pigeons that had been subcutaneously inoculated with virulent *E. rhusiopathiae* strain, it was shown that the injected bacteria were usually intracellular of endothelial cells lining the veins and capillaries and in endothelial cells free in the bloodstream [4]. Müller and Krasemann [5] demonstrated a correlation between the virulence of *E. rhusiopathiae* strains and the amount of neuraminidase produced, and showed that antibodies to the enzyme are protective in mice, suggesting a role of the enzyme in adherence or antiphagocytic function. Nakato et al. [6, 7] reported that neuraminidase may be involved in the pathogenesis of arteritis and throm-

bocytopenia in rats experimentally infected with the organism. They observed that in vivo distribution of bacterial invasion was always concomitant with that of desialated sites of arterial regions, and that in vitro adhesion of bacteria to aortic endothelial cells was inhibited by addition of *N*-acetylneuramin-lactose, a substrate of bacterial neuraminidase. Thus, it was demonstrated that neuraminidase of *E. rhusiopathiae* plays an important role in bacterial attachment and the subsequent invasion into host cells.

The organism also produces hyaluronidase, a spreading factor which facilitates the dissemination of pathogens into tissues. The importance of the enzyme in the pathogenesis of the infection has been suggested by Mann [8]. However, Nørrung [9] examined the virulence and hyaluronidase production of 62 isolates from joints or regional lymph nodes of pigs with arthritis, and concluded that there was no association between the organisms' hyaluronidase production and their virulence. Thus, the role of the enzyme in the pathogenesis of the infection remains controversial.

To clarify the role of hyaluronidase in virulence, transposon mutagenesis with Tn*916* was used to construct mutants deficient in hyaluronidase production. Approximately 10 000 transposon mutants were generated from a highly virulent strain, *E. rhusiopathiae* Fujisawa-SmR, and screened for loss of hyaluronidase production. The results showed that the virulent Fujisawa-SmR strain generated hyaluronidase-negative mutants at a high frequency, with this generation correlating with a change in colony morphology; colonies of the mutants displayed circular flat edges in contrast to those of the parent strain, which were convex and irregular in shape [20]. Interestingly, most of the hyaluronidase-negative mutants were avirulent for mice, but one hyaluronidase-negative mutant (AST121) which had the same colony morphology as the parent strain, although it had two copies of Tn*916* in the chromosome, exhibited the same level of virulence for mice as its parent strain. It was later discovered that all of the avirulent mutants had also lost their capsules (refer to section 2.3), whereas the highly virulent mutant (AST121) still possessed the capsule (unpublished data). These results raise the possibilities that both hyaluronidase and capsule production may be regulated by a common genetic locus, and that in these hyaluronidase-negative mutants, with the exception of AST121, insertion of Tn*916* occurred in this regulatory region, resulting in the loss of both hyaluronidase and capsule production (phenotypes of the transposon mutants are summarized in *table I*). Thus, the data suggested that the lack of virulence of most of the hyaluronidase-negative mutants could be attributed to a loss of the capsule, and that hyaluronidase is not essential for the pathogenesis of infection in mice. However, since the organism causes a variety of diseases other than septicemia, the enzyme may be important in the pathogenesis of infection in other hosts.

Table I. Phenotypes of *E. rhusiopathiae* strains.

Strain	*Hyaluronidase*	*Capsule*	*Virulence for mice*
Parent			
Fujisawa-SmR	+	+	Virulent
Mutant			
AST121	–	+	Virulent
47B12	–	–	Avirulent
566	–	–	Avirulent
736	–	–	Avirulent
942	–	–	Avirulent
293	–	–	Avirulent
33H6	+	–	Avirulent

2.2. Surface proteins of *E. rhusiopathiae*

Surface proteins of Gram-positive bacteria play pivotal roles in virulence, interacting in various ways with the environment in the host [10]. Takahashi et al. [11] reported that a heat-labile and trypsin-sensitive surface component(s) of *E. rhusiopathiae* is important in bacterial adherence to host cells; however, this component has not yet been identified.

In *E. rhusiopathiae* bacteria, only a few surface proteins have been defined. Lachmann and Deicher [12] have shown that the 66- to 64-kDa antigen and its 43-kDa derivative in cell surface extracts solubilized with detergents are the most prominent antigens reacting with a rabbit antiserum against heat-killed *E. rhusiopathiae*. The surface location of these antigens was demonstrated, but the role of the antigens in virulence has not been tested. Gálan and Timoney [13] identified and cloned a 5.4-kb *Eco*RI DNA fragment containing a protective gene from a virulent strain. They found that guinea pig antiserum raised against the recombinant clones is reactive with the 66- to 64-kDa and 43-kDa antigen in Triton X-100 extracts and that these antigens are expressed less on strains of low or moderate virulence than on highly virulent strains. However, neither the DNA sequence of the gene nor the function of this protein was described.

SpaA protein, a protective antigen of *E. rhusiopathiae*, is also a surface protein [14, 15]. In strain Fujisawa (serovar 1a), *spaA* encodes a mature protein with a molecular mass of 69 kDa [15]. SpaA is very similar in its structure and the amino acid sequences of its C-terminal region to those of choline-binding proteins [16–18] of *Streprococcus pneumoniae*. These results imply that SpaA, like the *Streptococcus* proteins, also plays a specific role in virulence; however, this role also remains to be elucidated.

Recently, Makino et al. [19] cloned the gene encoding a surface protein with a molecular mass of 19 kDa. It was shown that this protein itself has no haemolytic activity, but when cloned in *Escherichia coli*, it facilitates the haemolytic activity of the host bacterium. Neither the function nor the role in the virulence of this protein are known, however.

2.3. Capsule

2.3.1. Resistance to phagocytosis

Lachmann and Deicher [12] first reported that *E. rhusiopathiae* has a capsule and that it may be important in the pathogenesis of infection. They analyzed the surface components of the organism by sulfate-polyacrylamide gel electrophoresis and immunoblotting, and concluded

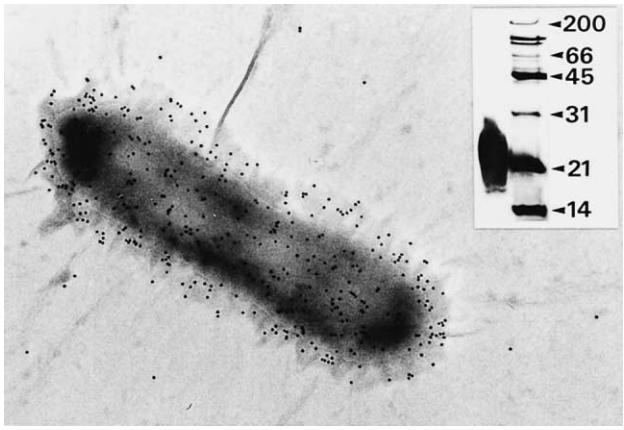

Figure 1. Immunogold labelling of a capsule of *E. rhusiopathiae* Fujisawa-SmR strain using monoclonal antibody (mAb) ER21. The inset depicts the immunoblotting of the capsular antigen with mAb ER21.

that the major nonprotein antigen with a molecular mass of 14 000 to 22 000 is the capsular polysaccharide antigen.

To study the potential role of the capsule in virulence, Tn*916*-generated mutants which changed colony morphology on agar plates were selected and examined by electron microscopy for their cell surface structure and virulence in mice [20]. Electron microscopy studies demonstrated the presence of an external layer resembling a capsule on the parent strain (Fujisawa-SmR) *(figure 1)*, as well as its absence on all the mutants. These mutants were found to be totally avirulent for mice (10 000-fold increase in intraperitoneal 50% lethal dose [LD_{50}]), suggesting that the capsule structure on the cell surface is responsible for the virulence. Phagocytosis assay studies revealed that, in the presence of normal serum, the virulent parent strain resisted phagocytosis by murine polymorphonuclear leukocytes (PMNs), whereas all the mutants were susceptible to phagocytosis *(figure 2)*. Furthermore, a revertant strain resisted phagocytosis and reverted to virulence following acquisition of the capsule when the transposon was lost by spontaneous excision. It was revealed that the avirulent mutants are devoid of the capsular antigen, whereas the revertant strain expresses the antigen as does the parent strain (unpublished data). Thus, it was found that the virulence of *E. rhusiopathiae* is at least associated with resistance to phagocytosis by PMNs and that this property is a function of the capsule.

2.3.2. Intracellular survival

Early studies reported by Timoney [21, 22] have provided evidence of the importance of intracellular survival of *E. rhusiopathiae* within professional phagocytes for pathogenicity of the organism. In these studies, a significant number of virulent *E. rhusiopathiae* bacteria survived within macrophages from unimmunized mice and within PMNs of pigs affected with erysipelas polyarthritis. To examine whether the capsule of the organism plays a role in mediating intracellular survival, phagocytosis by murine macrophages and subsequent intracellular survival were examined [23]. Phagocytosis assay studies showed that although enhanced phagocytosis was observed in the presence of immune serum, the virulent *E. rhusiopathiae* Fujisawa-SmR strain and its acapsular mutants were both ingested even in the presence of normal serum; however, the number of ingested bacteria was three- to fourfold greater in the case of the acapsular mutants than in the case of the parent strain, showing that, relative to the mutants, the virulent strain resists phagocytosis by macrophages. In the presence of normal serum, the phagocytosis index of macrophages for the virulent Fujisawa-SmR strain was 35 times greater than that of PMNs. These results are consistent with other studies of mice and pigeons [4], and of swine [24, 25], in which phagocytosis of the bacteria was carried out primarily by macrophages, not PMNs. The reason for the difference in these cells' ability to phagocytize the organism is currently unknown. Thus, it was found that even in the presence of normal serum, macrophages can phagocytize the virulent parent strain to some extent.

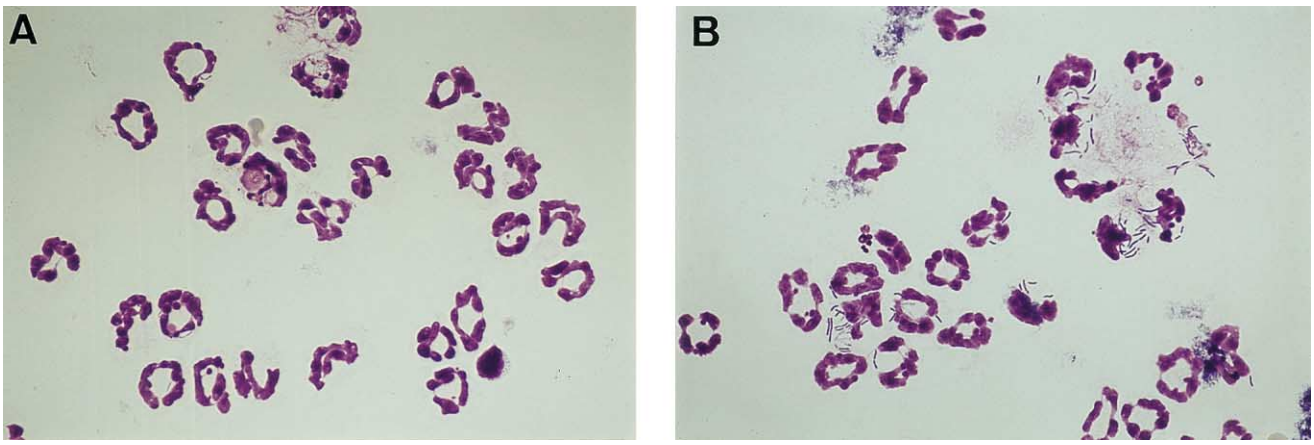

Figure 2. Phagocytosis of *E. rhusiopathiae* strains by murine PMNs in the presence of normal serum. **A.** Fujisawa-SmR strain; **B.** 33H6 strain.

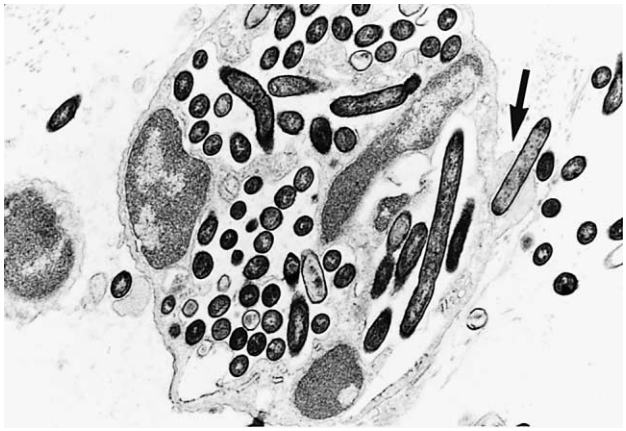

Figure 3. Extensive proliferation of *E. rhusiopathiae* Fujisawa-SmR strain within a phagocytic cell at the skin lesion of an infected mouse. Note that the cells has not been killed and appear functionally normal, as evidenced by the process of phagocytosis (arrow). EM was prepared by Dr. S. Tanaka.

To further examine whether the *E. rhusiopathiae* bacteria ingested by macrophages can survive and replicate within the cells, an intracellular killing assay was performed. The results showed that when ingested in the presence of normal serum, the number of viable virulent parent (Fujisawa-SmR) bacteria decreased only minimally during the first 2 h and then began to multiply, with an approximately twofold increase at 3 h of incubation. In contrast, the numbers of the mutant strains decreased significantly during the observation period. When ingested in the presence of immune serum, both the parent and mutant strains were readily killed. It was found that the virulent parent strain ingested by PMNs in the presence of normal serum also survives and replicates intracellulary (unpublished data). An electron micrograph obtained 6 h after infection showed that the virulent Fujisawa-SmR strain vigorously replicates within phagocytic cells in vivo, as visualized by the presence of many morphologically intact organisms, some of which are undergoing division *(figure 3)*. Thus, it was revealed that in the presence of normal serum, the virulent Fujisawa-SmR strain, but not the acapsular mutants, ingested by macrophages or PMNs survives and replicates within these professional phagocytic cells.

2.3.3. Intracellular survival strategies

What is the strategy by which *E. rhusiopathiae* evades the bactericidal activities of phagocytic cells? To test the hypothesis that, in the presence of normal serum, the virulent parent strain does not stimulate phagocytic cells during phagocytosis, oxidative burst responses of macrophages were examined by monitoring chemiluminescence (CL) responses to *E. rhusiopathia*e strains [23]. The results showed that when macrophages were incubated with the bacteria preopsonized with normal serum, the CL response was much lower for the parent strain than for the mutant strains, but when macrophages were incubated with the bacteria preopsonized with immune serum, both the parent and the mutant stains induced a strong CL response.

Since CL responses provide no information with regard to the responses of individual cells to the bacteria, an intracellular nitro blue tetrazolium (NBT) reduction assay, in which NBT is reduced by reactive oxidative metabolites to a visible intracellular formazan precipitate, was performed. After it was confirmed that most of the cell-associated bacteria were internalized by macrophages, the percentage of formazan-stained bacteria was determined microscopically. The results showed that in the presence of normal serum, 84% of the ingested acapsular mutants were formazan stained, versus 26% of those of the parent strain. In the presence of immune serum, most of the parent and mutant strains in the cells were formazan stained. *Figure 4* shows the differences between the parent and mutant strains in the presence of normal serum. It was thus revealed that, when ingested in the presence of normal serum, the virulent Fujisawa-SmR strain does not induce reactive oxidative metabolites from macrophages.

How does the virulent *E. rhusiopathiae* strain resist inducing the oxidative burst from macrophages? Are the macrophages damaged or killed by infection with the virulent *E. rhusiopathiae* strain? It was found that the macrophages that had phagocytized the virulent parent strain opsonized with normal serum could release vigorous oxidative metabolites in response to the stimulus with fresh serum-opsonized zymosam (unpublished data). This suggests that infection with the virulent strain does not affect the viability of the macrophages. There are at least two possible explanations, not necessarily mutually exclusive, for the reduced production of reactive oxidative metabolites when the virulent strain enters macrophages. First, *E. rhusiopathiae* may quench oxidative metabolites by enzymes such as superoxide dismutase (SOD) and catalase. *E. rhusiopathiae* produces SOD but not catalase. However, the results showing that the addition of exogenous SOD could not prevent the intracellular killing of the mutants by macrophages, and that both the parent and mutant strains produced SOD (unpublished data), suggest that the role of the enzyme in evading bactericidal mechanisms by oxidative metabolites may be limited. However, it is possible that once the mutants are ingested and, as described below, the macrophage becomes activated, the effect of SOD may not be detected. A second hypothesis is that receptors involved in phagocytosis of the virulent parent strain, in the presence of normal serum, do not stimulate the oxidative burst of macrophages, whereas those involved in phagocytosis of the mutant strain do. It has been reported that the receptor used for phagocytosis may influence the intracellular fate of such bacteria as *Salmonella* spp. [26] and *Listeria monocytogenes* [27]. It has also been reported that the killing of organisms by phagocytes is closely related to the cell surface properties of the organism [28–30]. These findings strongly suggest that the intracellular fate of the organism is dependent upon the microbe's surface molecules, which determine this fate either by themselves or by binding to specific host receptor(s) that can influence the host cell response. In a similar fashion, in the absence of specific antibodies, virulent *E. rhusiopathiae* bacteria may use certain host receptor(s) for successful intracellular infection, and in such cases the capsule will directly or indirectly affect the

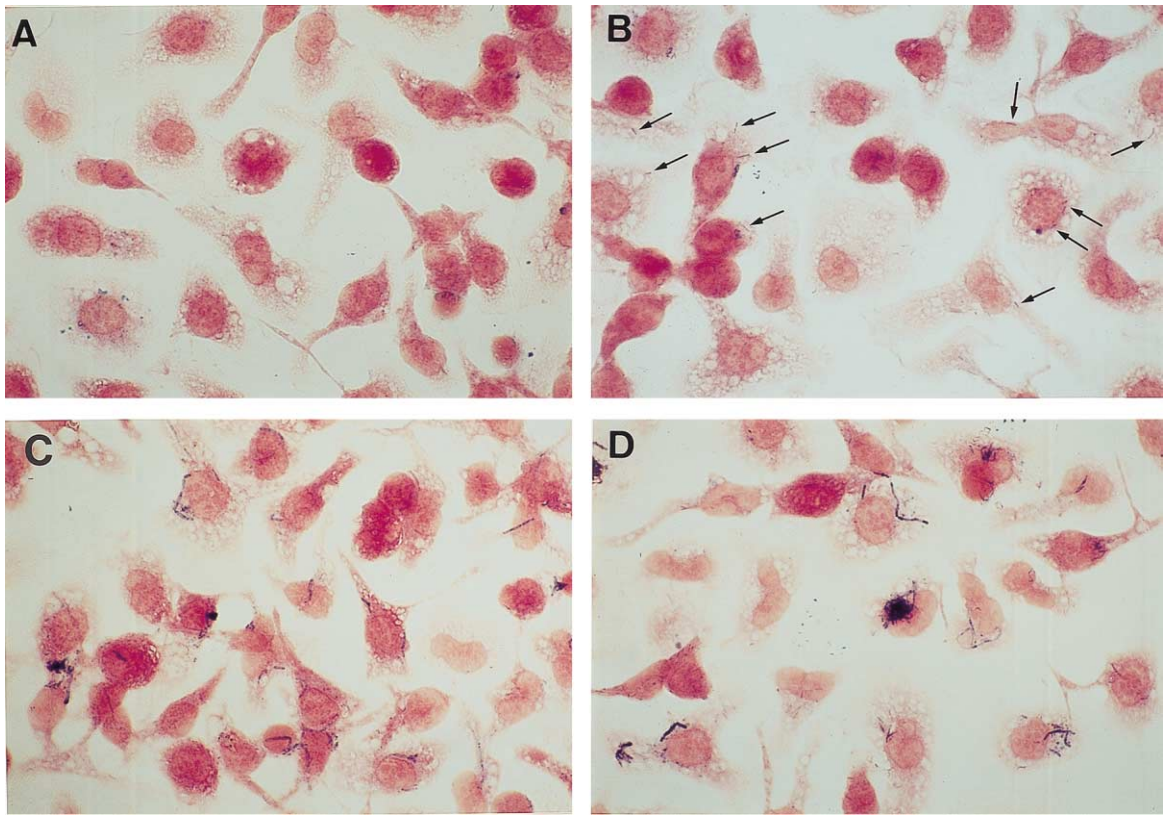

Figure 4. Intracellular reduction of NBT within macrophages in the presence of normal serum. Macrophage monolayers were incubated with *E. rhusiopathiae* strains in the presence of NBT. **A.** Bacterium-free NBT control; **B.** Fujisawa-SmR, the parent strain; **C.** 33H6, transposon acapsular mutant; **D.** 28G12, transposon acapsular mutant. Note that in panel B, the bacteria are not stained or only partially stained (arrows), but the majority of the bacteria are stained deep blue by formazan precipitates in panels C and D. Reproduced from Infection and Immunity [23] with permission of the publisher.

ligand-receptor interactions that mediate phagocytosis of the bacteria.

It is recognized that several intracellular pathogens use the complement receptors to gain entry into phagocytic cells. Binding to these receptors is able to promote phagocytosis, but does not result in triggering of the oxidative burst that accompanies Fc gamma receptor (FcγR)-mediated phagocytosis [31, 32]. Then, do virulent *E. rhusiopathiae* bacteria use complement receptors for entry into phagocytic cells? Timoney [33] reported the importance of complement in protection against *E. rhusiopathiae* infection in mice. In this study, mice were decomplementated by injection of heat-aggregated sheep γ-globulin and challenged with the bacteria. The results showed that the decomplementated mice died earlier than did normal control mice. Furthermore, when challenge bacteria were preopsonized with fresh mouse serum, the decomplementated mice survived a lethal challenge, and heat treatment of the mouse serum at 56 °C abolished its protective activity. Thus, it appears that *E. rhusiopathiae* bacteria opsonized with complement components are easily killed by phagocytic cells and cleared from the host. It has also been reported that *E. rhusiopathiae* Fujisawa strain activates the alternative pathway of the complement, resulting in opsonization with C3b [34] and presumably iC3b. However, the organism activates the alternative pathway of the complement system and exploits it for successful intracellular parasitism; this would certainly appear paradoxical. Further, it has been reported that the protective activity of antiserum to *E. rhusiopathiae* for mice was found only in the IgG fraction, and not in the IgM fraction [35]. Assuming that, after activation of the complement system (the classical pathway), IgM-mediated phagocytosis occurs depending exclusively on the use of complement receptors, it would seem that phagocytosis of the organism via complement receptors results in successful intracellular survival of the organism. Whether *E. rhusiopathiae* bacteria use complement receptors to invade the host macrophage should be examined in future studies.

3. Mechanism of acquired immunity

3.1. Role of opsonic antibody

In erysipelas infection, both humoral and cell-mediated immunity play an important role in host defense. A protective role of the specific antibody in the infection has long been suggested by the fact that immunization with bacterins or treatment with antiserum is widely used for disease control [2]. It has also been shown that *E. rhusio-*

pathiae bacteria opsonized with immune serum were readily eliminated by PMNs [20, 36] and by macrophages [23]. These findings strongly suggest that the protective activity of immune serum is, as shown above, probably the opsonic activity of IgG antibodies in type I phagocytosis, in which FcγR which triggers oxidative burst and other important intracellular bactericidal mechanisms is mediated [37]. It was found that mice immunized with purified capsular antigen of *E. rhusiopathiae* were not protected from lethal challenge and that mAbs raised against purified capsular antigen were exclusively IgM isotypes (unpublished data). Thus, the capsular antigen alone cannot be a protective antigen, indicating that molecule(s) on the cell surface other than the capsular antigen are important in inducing protective IgG antibodies.

One of such antigens on the bacterial cell surface is the SpaA antigen [14, 15]. This protein is found in all the strains of *E. rhusiopathiae* tested [14], suggesting that this is a species-specific protective antigen [38]. It has been demonstrated that antisera raised against purified SpaA protected mice from challenge with a homologous virulent strain [15]. It has also been shown that purified SpaA can elicit protection in pigs by inducing opsonic antibodies [39]. The protective epitope of SpaA has been shown to be located within the 20-kDa N-terminal segment, which is composed of amino acids 12 to 195 of SpaA [15]. This suggests that the N-terminal end region of the protein might be surface exposed, being extended into and through the capsule, demonstrating therefore that the antibodies to this region have opsonic activity.

The 66- to 64-kDa protein in Triton X-100 extracts of the organism is also a protective antigen [13, 40]. As already described, the protein has been suggested to be located on the cell surface, and mice immunized with a recombinant 66- to 64-kDa protein fused with β-galactosidase were partially protected [13]. In this study, however, whether this protein elicits opsonic protective antibodies was not investigated, leaving the possibility that it induces a cell-mediated immune response.

3.2. Cell-mediated immunity

The role of cell-mediated immunity in protection was demonstrated by the study using acapsular *E. rhusiopathiae* YS-1 strain [41]. In this study, the acapsular YS-1 strain was generated using a novel mechanism after Tn*916* has excised from the chromosome of a transposon mutant 33H6, and its protective capability as a live vaccine was evaluated in mice. The results showed that although the acapsular YS-1 strain was unable to persist in vivo (cleared within 72 h after immunization), it elicited a complete long-lasting protection in mice. It was demonstrated that in the YS-1 immunized-mice, protective antibodies were elicited and spleen cells obtained at days 7, 14, and 21 postimmunization proliferated significantly in response to *E. rhusiopathiae* antigens, showing that specific cell-mediated immune response was induced. Moreover, at day 7 postimmunization, growth inhibition in liver and spleen of the antigenically unrelated intracellular parasite *L. monocytogenes* was observed in these mice, indicating that nonspecific resistance to heterologous bacteria had been induced. This cross-protection has been known as an indicator of cell-mediated immunity in intracellular bacterial infection [42, 43], thus indicating that in the YS-1-immunized mice, both arms of the immune system contribute to immunity to *E. rhusiopathiae* infection. The bacterial antigen(s) involved in inducing this cell-mediated immunity remains, however, unidentified.

4. Conclusion

The pathogenicity of *E. rhusiopathiae* appears to be related mostly to the intracellular survival properties of the bacterium. The bacterium probably utilizes several different host cell receptors to gain access to the host intracellular niche. The receptor-ligand interactions involved in entry into macrophages constitute a complex but provocative area of research. A clear understanding of the molecular basis of cell surface molecule(s) of the bacterium should enable investigators to identify the receptor(s) involved in entry of the organism into the cells, and to clarify the receptor-ligand interactions that decide the fate of the organism.

Acknowledgments

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