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Characterization of a protective protein antigen of *Erysipelothrix rhusiopathiae*

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SUMMARY

Although vaccination is widely practiced against infection by *Erysipelothrix rhusiopathiae* in pigs and turkeys, the protective antigen(s) involved have not been fully characterized or purified to homogeneity. Antigens of *E. rhusiopathiae* strain T28, serotype 2b, and of FRANKFURT XI, serotype N, in culture supernatant and in extracts made with hot acid, 10 mM NaOH, ultrasound or EDTA were compared by SDS-PAGE and immunoblotting and in a mouse protection test. EDTA and 10 mM NaOH yielded highly protective extracts; culture supernatant was less protective and ultrasonic or hot acid extracts stimulated little or no protection in mice. Protective antisera from swine, horses and mice recognized prominent bands of molecular mass (m.m.) of 66–64 and 40–39 kDa in EDTA and 10 mM NaOH extracts. Mice immunized with preparations of the 66–64 kDa band purified by preparative electrophoresis were protected. Both antigens were trypsin sensitive, contained no detectable polysaccharide, and showed a marked tendency to aggregate in the absence of SDS.

INTRODUCTION

Erysipelothrix rhusiopathiae is a widely distributed commensal of the oral, pharyngeal and intestinal mucosa of mammals, birds and fishes [1–4]. Exposure to stress or reduction in local resistance may result in spread of *E. rhusiopathiae* into the regional lymphatics and blood leading to potentially-fatal septicaemia with complications such as endocarditis, arthritis and skin lesions. A variety of manifestations have been reported in pigs, lambs, birds, dogs, horses, cattle and man [4–6].

The economic importance of erysipelas in swine and turkeys is well illustrated by the fact that in the United States in 1980 approximately 80 million doses of *E. rhusiopathiae* bacterin and attenuated vaccines were used [7]. The extensive use of vaccines has probably contributed to the low incidence of the acute form of erysipelas, despite the wide distribution of the causative organism. However,

chronic swine erysipelas characterized by polyarthritis is still frequently diagnosed in vaccinated herds and this raises the question as to whether vaccination leads to excessive local immune responsiveness of the synovial tissue to infection derived from field exposure [8]. The resemblance of porcine erysipelas polyarthritis to human rheumatoid arthritis has also inspired the suggestion that the arthritic pig might serve as a model for the study of the disease in humans [9–12].

E. rhusiopathiae stimulates humoral immunity in swine and mice and convalescent or commercially available hyperimmune serum can be used for passive protection or therapy. Antibodies are assayed by the agglutination test using formalinized cells or by growth inhibition of the bacteria *in vitro* [13–16]. However, titres of these antibodies do not always correlate with protection [17]. most probably because *in vitro* assays do not distinguish between protective antibodies and antibodies to non-protective, type-specific polysaccharide antigens. Non-protective *E. rhusiopathiae* antigens in hot acid extracts are predominantly polysaccharides and have been used to classify strains into 22 different serotypes [5, 18–21]. Immunity to *E. rhusiopathiae* is stimulated by vaccination with attenuated or formalized cells, by culture supernatant [22, 23] and by crude extracts obtained by ultrasound or 10 mM NaOH [24]. Cross protection between culture supernatants of different serotypes [25–27] suggests that a protective antigen is species-specific. However, attempts to purify this antigen by anion exchange or gel filtration chromatography were unsuccessful and resulted in a reduction of its protective activity [24, 28, 29]. More recently, the major Triton X extracted antigen of 65 kDa from an *E. rhusiopathiae* type 1 strain was cloned and expressed in *Escherichia coli* [30]. Mice immunized with the recombinant protein survived longer and in higher numbers than control. The incomplete protection stimulated by the recombinant protein was unexplained but may have been due to conformational changes resulting from its fusion to β-galactosidase.

The objective of our study was to gain more information about the 'protective antigen(s)' of *E. rhusiopathiae*. We compared antigens of strain T28, serotype 2b and of strain FRANKFURT XI, serotype N, following extraction by acid, alkali, EDTA and ultrasound.

MATERIALS AND METHODS

Bacteria

E. rhusiopathiae strains used were: strain T28, serotype 2b, obtained from Professor Dr Böhm, Institut für Bakteriologie der Tierärztlichen Hochschule, Hannover; strain FRANKFURT XI, serotype N, the official German challenge strain for vaccine testing; and strain E1-6P, serotype 1a, obtained from the late Dr R. D. Shuman, National Animal Disease Laboratory, Ames, Iowa.

Culture media

Batches (5L) of serum free broth [31] were inoculated with 10 ml log phase starter cultures of the respective strains and grown for 18 h at 37 °C. Bacteria were harvested by centrifugation at 14000 g for 25 min and washed three times in distilled water.

Sera and conjugates

Commercially available hyperimmune sera from horses and pigs against *E. rhusiopathiae*, serotype 2 (WDT, Hoyerhagen, Fed. Rep. Ger.) were used. Sera from convalescent pigs were obtained after an experimental *E. rhusiopathiae* infection with strain E1-6P. Mice vaccinated with a commercially available bacterin (Erysorb, Behringwerke, Marburg, Fed. Rep. Ger.) were bled after 14 days and again 14 days after a challenge infection with strain FRANKFURT XI and their sera combined in 7 pools, each pool representing 10 mice. All mouse sera were titrated for antibody activity by the killed antigen agglutination test [13].

Rabbit anti-horse IgG (RAH, H + L-chain specific, Dianova, Hamburg, Fed. Rep. Ger.), goat anti-mouse IgG (GAM, H + L-chain specific, Dunn, Asbach, Fed. Rep. Ger.) and Protein A conjugated to horse radish peroxidase (Biorad, Munich, Fed. Rep. Ger.) served as conjugates for immunoblotting.

Hot acid extraction [*32*]

Five gram wet bacteria were suspended in 50 ml phosphate buffered saline, pH 7·2 (PBS), acidified to pH 2·4 and boiled for 12 min at 95 °C. After neutralization, the suspension was centrifuged at 18000 g for 40 min and supernatants pooled and concentrated by ultrafiltration through a YM 10 membrane (Amicon, Witten, Fed. Rep. Ger.) to a volume of 2 ml.

EDTA extraction [*33*]

Five gram wet bacteria were suspended in 50 ml 10 mM TrisHCl buffer, pH 7·2, containing 1 mM EDTA and incubated for 30 min at 37 °C. The cells were then removed by centrifugation at 18000 g for 25 min and the supernatant concentrated by ultrafiltration to a volume of 2 ml and stored for further analysis. The cell pellet was further extracted with alkali as described below.

Alkaline extraction [*24*]

Five gram wet bacteria were suspended in 100 ml of 10 mM NaOH and incubated with constant stirring for 18 h at 4 °C. Following neutralization with acid the suspension was centrifuged and the supernatant concentrated to a volume of 2 ml.

Ultrasonic extraction [*24*]

Five gram wet bacteria in 20 ml distilled water were cooled on ice and sonicated for 5 min (Branson Ultraschall-Sonifier B15, G. Heinemann, Schwäbisch-Gmünd, Ged. Rep. Ger.). Cell debris was removed by centrifugation at 18000 g for 60 min and the supernatant concentrated to a volume of 2 ml.

Culture supernatant

The supernatants of the cultures were filtered through 0·45 μm filters and concentrated by ultrafiltration through a hollow fibre cartridge H1P10-8 (Amicon, Witten, Fed. Rep. Ger.) to a volume of approximately 2 ml. The concentrates were dialysed against 10 mM TrisHCl, pH 8·0, and their volumes adjusted to 1/100th that of the original volume. Protein concentrations and sugar content were

determined [34, 35]. Amounts of protein or sugar in supernatants were expressed in mg per g wet bacteria.

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Culture supernatants and the various extracts of *E. rhusiopathiae* were mixed with equal volumes of sample buffer (2% SDS, 10% glycerine, 0·001% bromphenol blue in 60 mM Tris phosphate buffer) and incubated for 30 min at 56 °C. Care was taken to adjust the amounts of protein loaded. Samples were electrophoretically separated by SDS-PAGE according to Laemmli [36] on 1·5 mm 12 or 15% gels (LKB, Bromma, Sweden) at a current of 35 mAmp. Gels were stained by Coomassie blue and/or silver [37]. Polysaccharides were stained by Schiff's reagent according to the method of Fairbanks and colleagues [38]. After electrophoretic transfer of the bands to nitrocellulose (Schleicher and Schuell, Dassel, Fed. Rep. Ger.) in a transblot cell at 70 V for 7 h, (Biorad, Munich, Fed. Rep. Ger.) the nitrocellulose membranes were immunoblotted [39] as described below.

For staining of hexoses, gels were fixed in 10% glacial acetic acid and 40% methanol in distilled water for at least 48 h with four changes of fluid. Following sequential incubation for 2 h in 0·5% periodic acid, 1 h in a solution of 0·5% sodium arsenite in 5% glacial acetic acid, two washes of 30 min each in a solution of 0·1% sodium arsenite in 5% glacial acetic acid and immersion for 30 min in 5% glacial acetic acid, the hexoses were coloured with Schiff's reagent for 12 h. The reaction was stopped and the gels fixed in 0·1% sodium metabisulphite in 10 mM HCl for 6 h with four changes of fluid. Molecular masses (m.m.) were determined using low m.m. markers (Biorad, Munich) stained with Coomassie blue.

Preparative electrophoresis

Proteins separated on a 10% SDS-PAGE gel in a slab gel apparatus were harvested by elution in a stream of distilled water run through a channel in the centre of the gel. Eluted protein bands were lyophilized and tested for reactivity by SDS-PAGE and immunoblotting with hyperimmune serum [40].

Immunoblotting

Immunoblots were prepared by blocking the nitrocellulose membranes with 1% gelatine in PBS, followed by a 2 h incubation with the respective sera diluted 1:100 in PBS with 0·1% Tween 20. After three washes with PBS with 0·1% Tween 20 the sheets were incubated for 90 min with the appropriate peroxidase conjugate diluted in PBS with 0·01% Tween 20 as follows: rabbit anti-horse IgG, 1:8000; goat anti-mouse IgG, 1:2000; Protein A, 1:2000. The blots were then washed three times in pure PBS with 1% Tween 20, three times in pure PBS and then developed with 0·05% 4-chlor-1-naphthol (Fluka AG, Buchs, Swiss) in 20% methanol and 0·0001% hydrogen peroxide. Lanes containing low molecular mass standard proteins were separately stained with amido black to calibrate the blots.

Tryptic digests

Samples (30 μl) of alkaline extract of strain T28 containing 135 μg protein each were incubated with tenfold dilutions of bovine trypsin (1 mg/ml to 1 ng/ml,

Boehringer, Mannheim, Fed. Rep. Ger.) for 30 min at 37 °C. Following the addition of sample buffer for electrophoresis [36] the reaction was stopped by boiling for 5 min. The proteins in the digests were separated by SDS-PAGE and immunoblotted with equine hyperimmune serum to *E. rhusiopathiae*.

Production of antisera to the 66–64 kDa antigen

Proteins (1·5 mg) in an alkaline extract of strain T28 were separated on a 10 cm SDS-PAGE gel (12%/1·5 mm thick) and transferred to nitrocellulose. Strips (3 mm) were removed at each side and blotted with equine hyperimmune serum to *E. rhusiopathiae* to locate the immunologically reactive 66–64 kDa band. The area corresponding to this band on the untreated sheet was cut out, shredded into small pieces and injected subcutaneously (s.c.) into rabbits (Chinchilla Random, Ivanovas, Kissleg, Fed. Rep. Ger.). Each dose of antigen consisted of the band harvested from one run. The rabbits received booster injections 16 and 32 days later. Sera obtained 48 days after primary vaccination were tested in a mouse protection test.

Mouse protection tests

In experiment 1, vaccines were prepared from EDTA, EDTA-alkaline, alkaline, ultrasonic and hot acid extracts as well as from culture supernatant protein according to the method of White and Verwey [22] and adsorbed to aluminum hydroxide. Aliquots (0·5 ml) of these suspensions containing 0·5 mg protein/dose were inoculated subcutaneously into 16–18 g female NMR1 mice. The mice were challenged after 21 days by intraperitoneal (i.p.) injection of approximately 1000 LD_{50} of *E. rhusiopathiae* strain FRANKFURT XI. Deaths were recorded over the next 10 days.

The protective activity of the electroeluted 66–64 kDa antigen was tested in Experiment 2 by vaccinating mice three times subcutaneously with 20 μg of this antigen adsorbed to aluminum hydroxide on days 1, 28 and 56. The mice were challenged with a dose of one LD_{50} of *E. rhusiopathiae* E1-6P 84 days after primary vaccination and mortality was recorded over the following 7 days.

Determination of LD_{50}

Groups of ten 16–18 g female NMR1 mice were inoculated intraperitoneally with tenfold dilutions (10^{-6} to 10^{-9}) of overnight cultures of *E. rhusiopathiae* E1-6P and FRANKFURT XI in serum free broth [31]. Mortality after 7 days was recorded and the LD_{50} of each strain calculated from the formula of Reed and Muench [41]. These determinations were conducted in the Paul-Ehrlich-Institute, Langen, Germany and at Cornell University, Ithaca, NY.

Statistical methods

The significance of the difference in survival between immunized and control mice was determined using adjusted Chi-square analysis.

RESULTS

E. rhusiopathiae grew well in serum-free Feist medium and produced yields of approximately 4·0 g and 2·2 g wet weight per litre of broth for strains T28 and FRANKFURT XI respectively. Major amounts of low molecular weight

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Table 1. *Protective properties of extracts and of purified 66–64 kDa protein of* Erysipelothrix rhusiopathiae *strain T28 following immunization of mice and challenge infection*

Exp.	Vaccine	Mice (*n*)	Survivors	Percentage survival	Chi-square*
I†	EDTA	16	16	100	21·95 ($P < 0·01$)
	EDTA-10 mM NaOH	16	16	100	21·95 ($P < 0·01$)
	10 mM NaOH	16	13	81	13·16 ($P < 0·01$)
	Culture filtrate	16	9	56	6·30 ($P < 0·05$)
	Ultrasonic	16	2	13	0·17 (NS)
	Hot Acid	16	0	0	< 0·1 (NS)
	Bacterin	16	16	100	21·95 ($P < 0·01$)
	Neg. control	10	0	0	
II§	66–64 kDa protein	40	40	100	17·66 ($P < 0·01$)
	Neg. control	19	10	53	

* Adjusted Chi-square value for difference in mortality between immunized and control mice.
† Challenged with approximately 1000 LD_{50} of strain FRANKFURT XI.
§ Challenged with approximately one LD_{50} of strain E1-6P.
NS, Not significant.

polysaccharides were removed by ultrafiltration from crude extracts generated by EDTA, hot acid, 10 mM NaOH, EDTA-10 mM NaOH, ultrasound and by concentration of filtered culture supernatant. As shown in Table 1, significant levels of mouse protection ($P < 0·01$) against strain FRANKFURT XI (serotype N) were stimulated after 21 days by 10 mM NaOH and by EDTA extracts of strain T28, serotype 2b. An EDTA-10 mM NaOH extract of bacteria previously treated with EDTA was also highly protective. Culture supernatant protected 56% of the mice ($P < 0·05$), but extracts obtained by ultrasound and hot acid were not protective.

The various extracts were subjected to further biochemical and immunological investigations to determine the chemical basis of the antigens involved in protection. Protein released by 10 mM NaOH or EDTA was at relatively low levels, i.e. approximately 1 mg protein per g wet cells of strains T28 and FRANKFURT XI, while culture supernatants of strains T28 and FRANKFURT XI contained 4·3 mg and 6·3 mg protein respectively. Lysates obtained by ultrasound contained the greatest amounts of protein per g wet cells, i.e. 35·9 mg for strain T28 and 46·7 mg for strain FRANKFURT XI, respectively. Polysaccharide yields were highest in culture filtrates of strains FRANKFURT XI and T28, the latter producing about ten times the amount produced by the FRANKFURT XI strain (10·3 mg/g wet cells). EDTA, EDTA-10 mM NaOH and 10 mM NaOH extracts contained less than 0·2 mg hexoses per g wet cells.

Proteins in extracts separated by SDS-PAGE and stained by Coomassie blue had molecular masses of 92, 66–64, 40–39 and approximately 35 kDa. In addition, several closely spaced bands of approximately 30 kDa were prominent in the alkaline, EDTA and ultrasonic extracts. The hot acid extract showed no banding above 30 kDa and culture filtrate showed only two weak reactions at around 64–66 kDa. Gels stained by periodate acid Schiff (Figure 1) showed major amounts of low molecular mass polysaccharides in all samples. Culture supernatants

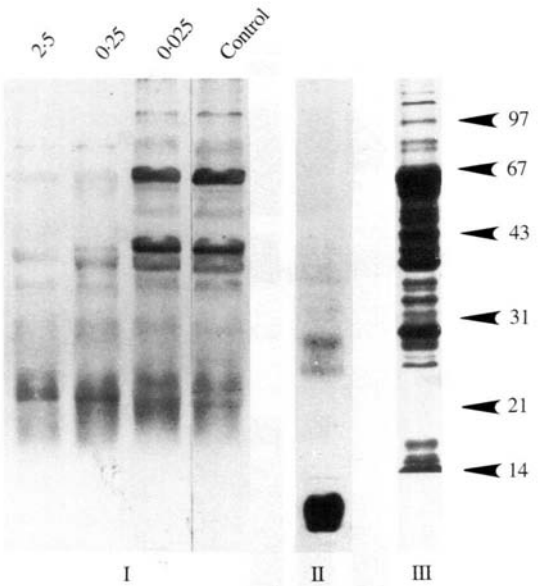

Fig. 1. An alkaline extract of *E. rhusiopathiae* strain T28 separated by SDS-PAGE and stained by Coomassie blue (lane III) and Periodic Acid Schiff (lane II). The trypsin sensitivity of antigens in alkaline extracts is shown in panel I. A trypsin digest of a 10 mM NaOH extract of strain T28 was immunoblotted with equine antiserum to *E. rhusiopathiae* followed by peroxidase conjugated rabbit anti-horse serum. The figures on top are the amounts of trypsin in mg added to the extract. The control lane contained undigested 10 mM NaOH extract.

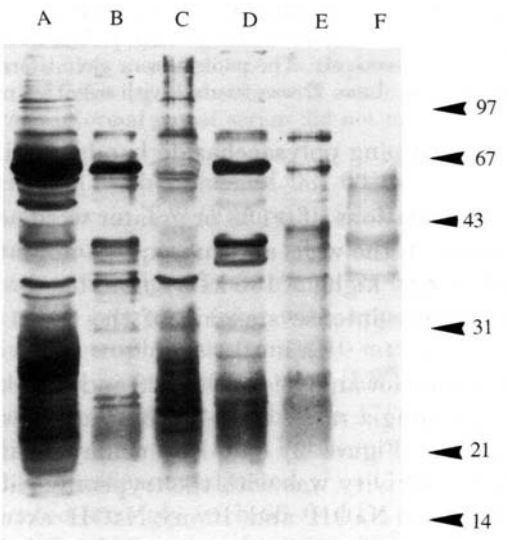

Fig. 2. Immunoblot analysis of different extracts of *E. rhusiopathiae* strain T28. Lanes: A, EDTA; B, EDTA-0·01 N NaOH; C, ultrasound; D, 0·01 N NaOH; E, culture filtrate, and F, hot acid extract. The amount of protein solution loaded was proportional to the wet weight of extracted bacteria. Proteins were separated on a 12% SDS-PAGE gel, transferred to nitrocellulose and treated with equine hyper-immune serum followed by rabbit anti-horse serum conjugated to horseradish peroxidase and the reaction developed with 4-chloro-1-naphthol. A panel treated with normal horse serum did not react with any band in the extracts.

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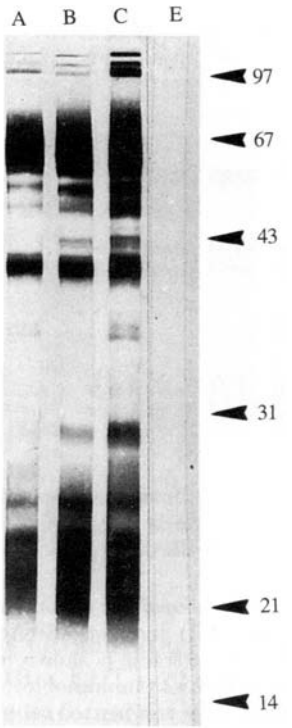

Fig. 3. Antigens in a 10 mM NaOH extract of *E. rhusiopathiae* strain T28 following separation by SDS-PAGE and immunoblotting with pools of sera from vaccinated mice after a challenge infection with 1000 LD_{50} of strain FRANKFURT XI (A, B, C). Peroxidase conjugated goat anti-mouse serum and 4-chloro-1-naphthol served as conjugate and substrate respectively. The pools of sera gave titers of 1:8–1:64 in the killed cell agglutination test. Lane E was treated with negative control serum.

showed a variety of overlapping polysaccharide bands ranging from 66 to above 300 kDa.

In general, SDS concentrations of 0·1 % or greater were necessary to minimize aggregation of the sample in the wells and subsequent migration of the aggregate into the top of the gel as very high (> 100 kDa) m.m. bands. Moreover, the use of 0·1 % SDS resulted in more intense staining of the 66–64 and the 40–39 kDa bands.

SDS-PAGE and immunoblot analysis of the T28 and Frankfurt strains revealed multiple reactivities spanning a range of 92 to 25 kDa following immunoblotting with hyperimmune horse (Figure 2) and hyperimmune and convalescent pig antisera. The strongest reactivity was with the trypsin sensitive 66–64 kDa band (Figure 1) in EDTA-10 mM NaOH and 10 mM NaOH extracts and in culture supernatants. This band was weaker in extracts made with EDTA or ultrasound and was very faint in the hot acid extract of strain T28. A trypsin sensitive 40–39 kDa band was also prominent in extracts made with EDTA-10 mM NaOH or 10 mM NaOH alone and was very faint or absent in all the other extracts. Normal horse and pig sera did not react with the 66–64 kDa band.

Similarly, serum pools from mice collected following vaccination with a commercial type 2 bacterin and pools collected after challenge of these mice with

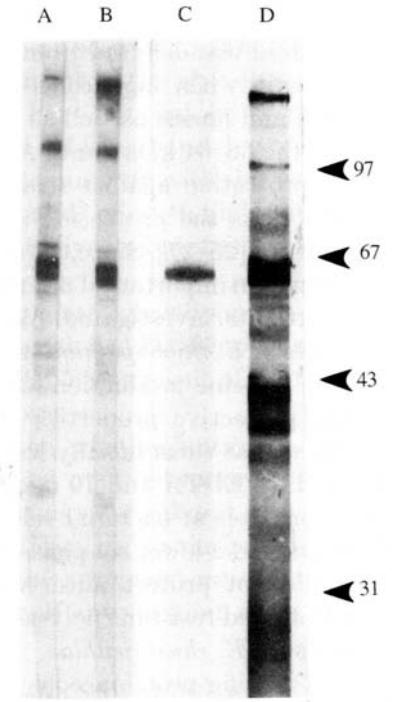

Fig. 4. Immunoblot showing the reactivities of pools of sera from mice immunized with commercial bacterin and (tracks A and B) and from mice hyperimmunized with the 66–64 kDa antigen (track C) as purified by preparative SDS-PAGE (10%) from a 10 mM NaOH extract (track D) of *E. rhusiopathiae* strain T28. Tracks A and B were developed with peroxidase conjugated goat anti-mouse serum and 4-chloro-1-naphthol as substrate. Tracks C and D were developed with peroxidase conjugated protein A and 4-chloro-1-naphthol following incubation with serum from a pig hyperimmunized against *E. rhusiopathiae*. Normal mouse serum did not react.

the type N strain, reacted with bands of 98, 66–64, 40–39 and 26–23 kDa in 10 mM NaOH extracts of strain T28 (Figure 3). Sera of non-immune control mice did not react with these bands.

The protective capacity of antigen in the range 66–64 kDa was demonstrated by active immunization of a group of mice with approximately 3 mg of antigen purified by preparative electrophoresis from a 10 mM NaOH extract of T28 (Fig. 4). A group of 40 mice vaccinated 3 times with this antigen and challenged with strain E1-6P serotype 1a was protected whereas 47% of mice in a control group died (Table 1). The difference in mortality between the groups of vaccinated and control mice was highly significant (adjusted $\chi^2 = 17{\cdot}66$, $P < 0{\cdot}01$). Pre-challenge sera of these mice reacted strongly with 66–64 kDa antigen in alkaline extracts and with two other bands of 98 and > 100 kDa. They reacted very weakly with the 39–40 kDa antigen.

DISCUSSION

The data presented in this paper demonstrate that antibodies against protein in the range 66–64 kDa are involved in protective immunity to *E. rhusiopathiae*. This protein is present in EDTA, EDTA-10 mM NaOH, and 10 mM NaOH extracts as

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well as in ultrasonic lysates and culture filtrates, but is not detectable in hot acid extracts. The presence of protein was deduced from its sensitivity to trypsin and its presence in PAGE gels only when high concentrations of SDS were used. Hyperimmune sera from pigs and horses as well as convalescent sera of pigs and mice reacted uniformly with the 66–64 kDa band. Antigen derived from serotype 2b (strain T28) stimulated protection against strains of serotype N (FRANKFURT XI) and 1a (E1-6P). Thus the protection stimulated was not serotype-specific confirming earlier reports [25–27]. Since the 66–64 kDa protein is common to these serotypes it is seemingly an important if not the major immunostimulating antigen in the protective extracts investigated. We also found large amounts produced in detergent extracts of *E. rhusiopathiae* strain SE-9 which is frequently used in the United States for vaccine production [31].

Correlation of the mouse protective properties of crude extracts with their chemical composition revealed that substantially less polysaccharide and protein contaminants were extracted by EDTA and 10 mM NaOH. These contaminants were most obvious in ultrasonicates of bacterial cells and were recognizable as a stratification into an upper gel and a lower sol phase. Hot acid extracts which are used for seroclassification did not protect mice, which is consistent with the generally held assumption that acid resistant polysaccharide antigens do not play a significant role in immunity to *E. rhusiopathiae*.

The existence of a species-specific proteinaceous 'protective antigen' distinct from type-specific polysaccharide antigens has been repeatedly mentioned by earlier investigators [18, 32, 42]. A generic antigen extracted from cells of *E. rhusiopathiae* by acetone and purified by ion exchange chromatography [20, 43] was composed of four serologically identical antigens of which one fourth was larger than 200 kDa as measured by analytical centrifugation [44]. White and Verwey [22] examined crude culture supernatants of *E. rhusiopathiae* and, based on molecular sieve exclusion chromatography, concluded that the mouse protective antigen was a glycolipoprotein complex of 200 kDa. It was sensitive to trypsin and muramidase but not to ribonuclease or lipase; was solubilized by high concentrations of SDS and precipitated in ammonium sulphate (50%). Erler's studies [24] showed that the protective antigens of *E. rhusiopathiae* were sensitive to pepsin, to exposure to 1 M HCL or trichloroacetic acid and to temperatures above 60 °C. Also, a pH of less than 7 led to irreversible aggregation and precipitation in 10 mM NaOH extracts. Care was taken to avoid any of these conditions in our study. Lachmann and Deicher [33] also working with strain T28 and using affinity chromatography and immunoblotting, demonstrated major immunologically reactive proteins of 60–75 and 40–50 kDa in EDTA, CHAPS, Triton X and SDS extracts of *E. rhusiopathiae*, but did not test the protective activities of these extracts. Recently, the structural gene of the major antigen in Triton X extracts of *E. rhusiopathiae* type 1a strain E1-6P of 65 kDa was cloned and expressed in *Escherichia coli* as a protein fused to β-galactosidase [30]. Mice immunized with the recombinant fusion protein were poorly protected when subsequently challenged with 100 LD_{50} of strain E1-6P. The result of the present study using native antigen from an *E. rhusiopathiae* type 2b strain provides better evidence for the protective properties of the 66–64 kDa protein.

The tendency of the 66–64 kDa protein to aggregate unless solubilized in SDS,

together with the trypsin sensitivity, mouse protectivity, and reactivity with hyperimmune protective antisera, suggest that this protein in our study might be a detergent solubilized constituent of the glycolipoprotein complex described by White and Verwey [23]. In Fig. 4 it can be seen that the serum from mice hyperimmunized with the 66–64 kDa band harvested by preparative electrophoresis (track C) reacted predominantly with this band in a 10 mM NaOH extract but also with two prominent bands of much higher molecular mass that probably represent larger fractions of this glycolipoprotein complex. The superior protective ability of crude extracts may be due to an adjuvant effect of polysaccharide antigens in this molecular mass glycolipoprotein complex that stimulate immunological responses to the 66–64 kDa component. Furthermore there may be additional antigenic sites available as a result of the association of the protein and polysaccharide in the complexes.

Further studies will focus on whether antibody titres to 66–64 kDa antigen correlate with protection in mice, pigs and turkeys *in vivo*. Eventually, this should lead to the creation of a new and safer subunit vaccine against erysipelas as well as to an economic *in vitro* alternative to the mouse infection model for vaccine testing. Characterization of the antigen(s) involved in protective responses will facilitate studies on their role, if any, in joint sensitization.

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