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Interference of Peptides and Specific Antibodies with the Function of the *Actinobacillus pleuropneumoniae* Transferrin-Binding Protein

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Multiple-antigenic peptides (MAPs) containing transferrin-binding domains of the *Actinobacillus pleuropneumoniae* serotype 7-derived transferrin-binding protein (TfbA) (K. Strutzberg, L. von Olleschik, B. Franz, C. Pyne, M. A. Schmidt, and G.-F. Gerlach, Infect. Immun. 63:3846–3850, 1995) were constructed. It was found that the MAPs inhibited transferrin binding of the recombinant TfbA protein, whereas antibodies directed against transferrin-binding domains failed to do so.

Actinobacillus pleuropneumoniae is the etiological agent of porcine pleuropneumonia, a severe and highly contagious disease encountered worldwide (17). Due to the rapid onset of disease and an increasing occurrence of isolates with multiple-antibiotic resistance, therapeutic measures are difficult (8, 20). Vaccination strategies, on the other hand, are hampered by the apparent lack of cross-protection (11). Therefore, innovative strategies for therapeutic measures and vaccine formulations are required.

One possible strategy could target the iron uptake mechanism. *A. pleuropneumoniae*, like other species in the families *Pasteurellaceae* and *Neisseriaceae*, is able to express transferrin-binding proteins as a response to iron-limiting growth conditions as they occur in vivo (5, 6, 14, 16). One of these proteins, designated TfbA or Tbp2 protein, is recognized by convalescent-phase serum (3), and vaccination with this highly variable protein (4) induces a protective but serotype-specific immune response (15). However, porcine convalescent-phase serum did not inhibit receptor-transferrin binding (18).

Recently, the transferrin-binding domains have been characterized for the small transferrin-binding proteins of *A. pleuropneumoniae* and *Neisseria meningitidis*, and one domain with a high degree of homology has been identified (1, 18, 19). Now it remains to be investigated whether peptides containing these domains (i) can inhibit receptor-transferrin interaction and (ii) can be used to raise antibodies which—in contrast to convalescent-phase serum—might inhibit transferrin binding.

In this study, we have investigated the effects of monomeric and multimeric peptides (multiple-antigenic peptides [MAPs]) containing the transferrin-binding domain of the transferrin-binding protein (TfbA) of *A. pleuropneumoniae* serotype 7 and the effects of anti-transferrin-binding-domain antibodies on receptor-transferrin interaction. Designations and descriptions of the synthetic peptides used are given in Table 1, and the structure of a MAP is shown in Fig. 1. All peptides and MAPs (obtained from the Institut für Peptidforschung, Hannover, Germany) were readily soluble in distilled water and were stored at −20°C. Porcine transferrin was prepared as described by Niven et al. (13). Transferrin was iron depleted by the method of Mazurier and Spik (12), and the resulting apotransferrin was resaturated as described by Herrington and Sparling (10). For radioactive labelling of transferrin, iron-59 (^{59}Fe) was used, and excess of ^{59}Fe was removed by gel filtration on prepacked PD10 columns (Pharmacia, Freiburg, Germany). TfbA-containing membranes were prepared by inducing *Escherichia coli* HB101(pTF205/E2) transformants (15) (optical density at 660 nm, ≈0,3) with 10 μM (final concentration) isopropylthiogalactoside (IPTG). Two hours after induction, bacteria were harvested by centrifugation (at 3,000 × *g* for 15 min at 4°C), and membranes were prepared as described by Hancock and Nikaido (9).

In order to test the inhibitory potentials of MAPs and single peptides, these competitors and TfbA protein (as a control) were preincubated with ^{59}Fe-labelled transferrin in a separate radioimmunoassy module (Nunc, Wiesbaden, Germany) while the module with TfbA-containing membranes as the solid-phase antigen (coated for 1 h at 37°C in 50 mM carbonate-bicarbonate buffer [pH 9.6]) was blocked with TBST (15 mM Tris(hydroxymethyl)aminomethane hydrochloride–150 mM NaCl [pH 8.0])–0.5% Tween 80) containing 0.5% gelatin. A membrane preparation of vector-only-transformed *E. coli* served as a control. It was found that MAPs D1 and D3 caused 50% inhibition at concentrations below 100 pmol; similar results were obtained when biotinylated transferrin and a streptavidin-alkaline phosphatase-based detection system were used. For MAPs D2 and $D2_{15}$, and for recombinant TfbA protein, an approximately fourfold higher concentration was

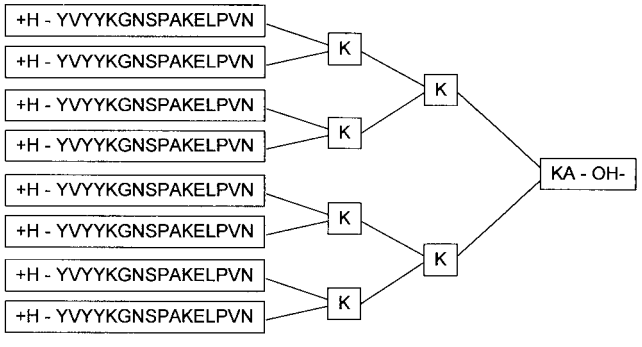

FIG. 1. Octameric structure of the MAP D2.

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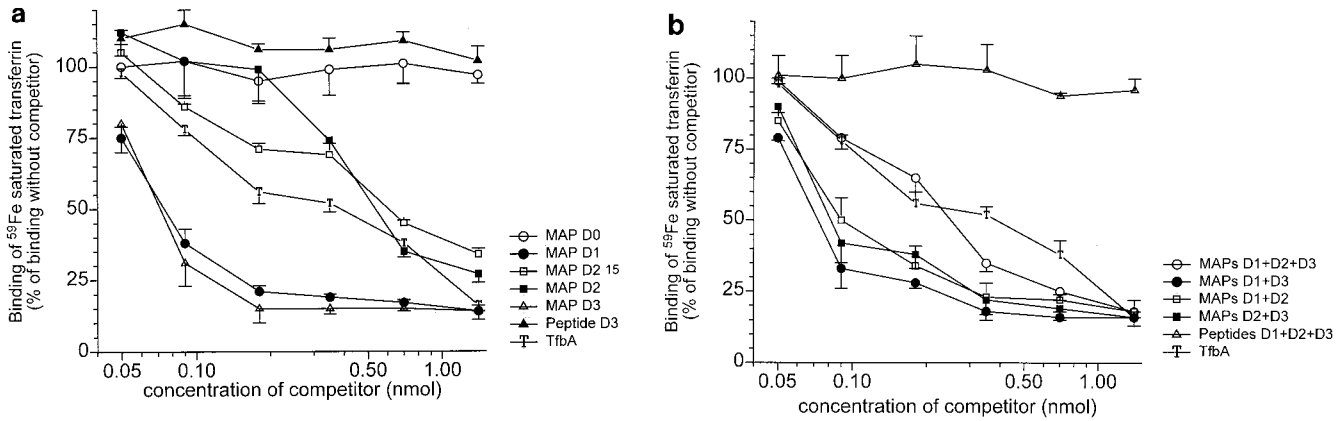

FIG. 2. Inhibition of transferrin binding to TfbA-containing *E. coli* membranes by MAPs and single peptides. Amounts of peptides added are indicated in nanomoles. (a) Inhibitory activity of each of the MAPs, with MAP D0 as a negative control and TfbA as a positive control. Results for peptide D3 are representative of those for all single peptides used. (b) Inhibitory activity of combinations of MAPs. The combination of all three single peptides is representative of all possible combinations of single peptides. The amount of bound ^{59}Fe-saturated porcine transferrin was determined in a γ counter and is expressed as the percentage of binding obtained without competitor. Results are arithmetic means of binding of ^{59}Fe-saturated porcine transferrin in three duplicate experiments ± standard deviations.

needed. MAP D0, as well as single peptides, did not interfere with transferrin binding (Fig. 2a). The combination of two MAPs appeared to have an additive effect, and a combination of all three MAPs (D1, D2, and D3) resulted in 50% inhibition at a concentration indistinguishable from that of recombinant TfbA protein. In contrast, a combination of single peptides did not interfere with TfbA-transferrin binding (Fig. 2b). This ability of MAPs D1, D2, and D3 to effectively inhibit receptor-transferrin binding proves their function as transferrin-binding domains (18); on the other hand, the total lack of inhibition by a combination of single peptides covering all three domains suggests the complex nature of the receptor-transferrin interaction.

Using an in vitro binding assay, we had previously found that porcine convalescent-phase serum does not inhibit transferrin binding (18). However, since the common domain D2 presented a potentially cross-protective vaccine component, we decided to investigate the immunogenicity of this domain as well as the inhibitory potential of domain-specific antibodies. Using MAP D2 in complete Freund's adjuvant for the repeated immunization of eight rabbits, we were not able to induce an immune response; therefore, subcutaneous implantation of cellulose-coupled D2 peptide (7) prepared according to the method of Frank (2) (kindly provided by L. von Olleschik, Zentrum für Molekularbiologie der Entzündung, Münster, Germany) was performed in order to successfully raise antibodies against the D2 peptide. Then the reactivity of this serum, as well as those of rabbit anti-TfbA serum and porcine convalescent-phase serum, was investigated in an enzyme-linked immunosorbent assay (ELISA) (Fig. 3). Here, Maxisorb plates (Nunc, Wiesbaden, Germany) were coated with MAPs at a concentration of 1 μg/ml for 1 h at 37°C by using a peptide coating buffer (0.5 M NaCl–50 mM sodium phosphate [pH 5.6]). The anti-D2 antiserum reacted with MAP D2 and, in addition, with MAP D1; this reaction, however, could not be inhibited by preincubation with D2 and therefore was considered to be caused by a different population of antibodies. The anti-TfbA antiserum—as expected—reacted with all MAPs. In contrast, the porcine convalescent-phase serum did not detect any of the MAPs, a result which is consistent with the observation that this serum reacts solely with the carboxy-terminal half of the TfbA molecule (18). In order to investigate the inhibitory potentials of the three sera, a competitive ELISA

TABLE 1. Designations and descriptions of synthetic peptides

Peptide	No. of residues	Amino acid sequence and chemical structure
D0	16	KEKEQLGELMEPALGY-amide
D1	16	VPYKANSSKYNYPDIK-amide
D2	16	YVYYYKGNSPAKELPVN-amide
$D2_{15}$	15	GYVYYYKGNSPAKELP-amide
D3	16	RPNYLNDDYYTKFIGK-amide
MAP D0	16[a]	$(KEKEQLGELMEPALGY)_8$ K_7A
MAP D1	16[a]	$(VPYKANSSKYNYPDIK)_8$ K_7A
MAP D2	16[a]	$(YVYYYKGNSPAKELPVN)_8$ K_7A
MAP $D2_{15}$	15[a]	$(GYVYYYKGNSPAKELP)_8$ K_7A
MAP D3	16[a]	$(RPNYLNDDYYTKFIGK)_8$ K_7A

[a] Without residues of the polylysine core structure.

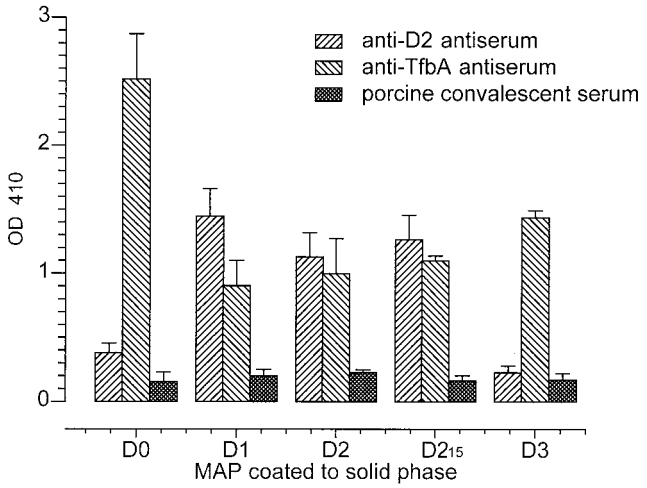

FIG. 3. Recognition of peptide antigens comprising the amino acid sequences of transferrin-binding domains by different sera. ELISA plates were coated with MAPs (Table 1). Antigens were incubated with serial twofold dilutions of sera; bound antibodies were detected as described in the text. Results are expressed as means of optical densities measured at 410 nm in three duplicate experiments ± standard deviations.

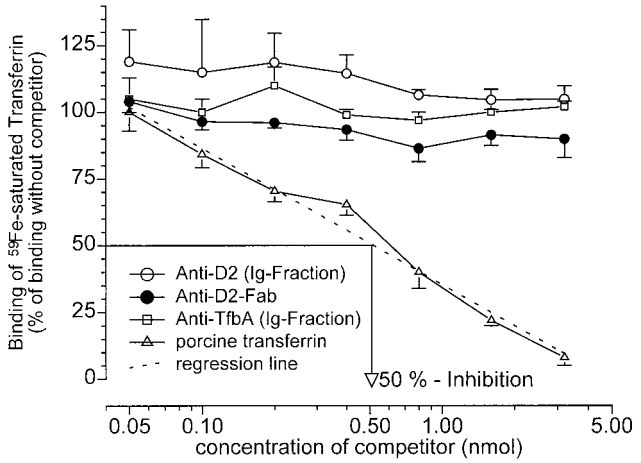

FIG. 4. Abilities of antibodies and Fab fragments to inhibit binding of ^{59}Fe-saturated porcine transferrin to TfbA-containing *E. coli* membranes. Amounts of competitors added are indicated in nanomoles. The amount of bound ^{59}Fe-saturated porcine transferrin was determined in a γ counter and is expressed as the percentage of binding obtained without competitor. The inhibition obtained with porcine transferrin serves as a control. Results are arithmetic means of the binding of ^{59}Fe-saturated porcine transferrin in three experiments performed in duplicate ± standard deviations.

was performed by inoculating plates coated with recombinant TfbA-containing *E. coli* membranes with different sera as well as Fab fragments prepared from anti-D2 antibodies; unlabelled transferrin served as a positive control. Surprisingly, none of the antibody preparations, including the D2-domain-specific Fab fragments, were able to inhibit transferrin binding (Fig. 4). To exclude low antibody affinity as a possible cause for this result, we investigated whether transferrin would inhibit binding of anti-TfbA or anti-D2 antiserum to MAP D2-coated plates; it was found that transferrin did not interfere with the binding of serum to MAP D2. These results indicate that transferrin and antibodies recognize different parts of the D2 domain.

In summary, the lack of interference of domain-specific antibodies with transferrin binding, as well as the apparent lack of immunogenicity of MAP D2, does not support a potential application of this domain as a vaccine component. In contrast, this lack of immunogenicity, paired with the clear inhibitory function of all MAPs containing transferrin-binding domains, suggests that investigation of the possible therapeutic value of MAPs as blockers of receptor-ligand interactions might be a promising approach.

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