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NOTES

Interaction of Ruminant Transferrins with Transferrin Receptors in Bovine Isolates of *Pasteurella haemolytica* and *Haemophilus somnus*

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The interactions of ruminant transferrins with receptors on bovine isolates of *Pasteurella haemolytica* and *Haemophilus somnus* were compared by growth studies and direct and competitive binding assays. Isolates of *P. haemolytica* were capable of utilizing and binding transferrin from sheep, goat, or cattle, whereas isolates of *H. somnus* were capable of utilizing and binding only bovine transferrin.

Because of the iron sequestering effects of transferrin and lactoferrin, the availability of iron in the extracellular environment of the host is limited (22). Therefore, successful bacterial pathogens require mechanisms for obtaining iron from the transferrin pool. One mechanism for utilization of transferrin iron involves direct binding of transferrin by surface receptors on the bacterium and, by an as yet unknown process, removal of iron from transferrin and uptake of the iron into the cell (7, 19–21). This receptor-mediated type of iron acquisition has been demonstrated in a variety of bacterial pathogens from human and animal hosts and characteristically involves specific binding of transferrin from the natural host (9, 14–16, 18, 20). Although there is considerable structural and sequence homology among transferrins (1, 2, 5, 6, 13), immunological studies indicate considerable variability in the surface epitopes of these proteins (3), which may explain the strict specificity observed with bacterial transferrin receptors. In contrast, the mammalian receptor is capable of binding to transferrins from a variety of species, indicating that it binds to a region of the transferrin surface that is relatively conserved among different host species (4).

The bacterial pathogens that have been shown to possess the receptor-mediated type of iron acquisition system characteristically have a limited host range for disease causation, which correlates with in vitro growth studies demonstrating that these pathogens are capable of utilizing only the host's transferrin as a source of iron for growth (9, 14, 15, 20). The proposal that these bacteria may be solely dependent upon their transferrin receptors for iron acquisition in vivo (17) implies that they can cause disease only in those hosts whose transferrin is recognized by the surface receptors. Previous studies have demonstrated that two important bovine pathogens, *Pasteurella haemolytica* and *Haemophilus somnus*, possess surface receptors capable of binding bovine transferrin (bTf) but not transferrin from other, nonruminant host species (14, 15). However, these two pathogenic species differ in their ability to cause infection in other ruminant species. *H. somnus* is a significant pathogen in cattle but has not been reported to cause infection in other ruminants (11). In contrast, strains of *P. haemolytica* have been reported to cause infection in cattle, sheep, and goats (8). These observations raised the question of whether the host range for these bovine pathogens is reflected in the specificities of their surface transferrin receptors.

To address this question, bovine disease isolates of *H. somnus* (14) and *P. haemolytica* (15), along with the control human, equine, and porcine pathogens *Neisseria meningitidis* (20), *Actinobacillus* (*Haemophilus*) *equuli* (provided by Sharon Lundberg, Animal Health Laboratory, Alberta Agriculture, Airdrie, Alberta, Canada), and *Actinobacillus pleuropneumoniae* (9), were analyzed for their interactions with ruminant transferrins. Since sheep transferrin (oTf) and goat transferrin (gTf) were not available from commercial sources, it was necessary to isolate these transferrins from serum. A purification scheme involving iron saturation, ammonium sulfate fractionation (35 to 70%), and a combination of hydrophobic exchange (phenyl-Sepharose), gel exclusion (Sephacryl S200HR), and ion-exchange (DEAE-Sepharose) chromatography was used to purify oTf, gTf, and horse transferrin (eTf) from their respective sera (GIBCO Canada). The resultant transferrin preparations were comparable in purity to commercial preparations of human transferrin (hTf; Sigma), bTf (Sigma), and porcine transferrin (pTf; The Binding Site Ltd.) (Fig. 1). The identity of the purified proteins as transferrins was based on their iron binding and spectral properties, lectin binding properties, molecular weight, and ability to bind to bacterial transferrin receptors.

It is also evident in Fig. 1 that the commercial preparation of bTf and our preparation of oTf contained more than one protein band. Heterogeneity of the different transferrin preparations was even more evident when the samples were not exposed to a reducing agent and were not boiled prior to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis (data not shown). Attempts to separate the two forms of bTf by a variety of chromatographic techniques were unsuccessful. Even when mono- to octosialic acid forms of transferrin were separated by high-pressure liquid chromatography chromatofocusing, each form was shown to consist of the two protein bands. Enzymatic deglycosylation experiments indicated that the difference between the two bands was not due to glycosyl-

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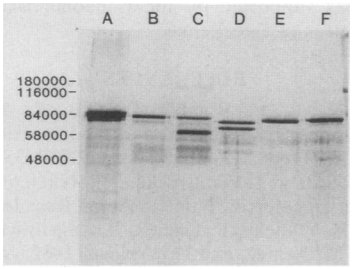

FIG. 1. SDS-PAGE analysis of transferrin preparations. Samples containing 1 μg of transferrin in sample buffer were electrophoresed on an SDS–8% polyacrylamide gel, and the gel was silver stained as described in the text. Numbers refer to molecular weights of standard proteins. Lanes: A, eTf; B, gTf; C, oTf; D, bTf; E, pTf; F, hTf.

ation. Peptide analysis by cyanogen bromide cleavage indicated that these different forms of transferrin were very similar (data not shown).

The ability of iron-deficient cells to utilize transferrin iron for growth was assessed by two different growth assays (14, 15). The results demonstrated that isolates of *P. haemolytica* were capable of utilizing iron from bTf, oTf, or gTf for growth, whereas *H. somnus* was capable of utilizing only bTf (Table 1).

Binding of transferrins by immobilized intact cells was assessed by a simple binding assay (20) after preparation of horseradish peroxidase (HRP) conjugates of the purified transferrins (9). Representative strains were grown under iron-limited conditions to induce expression of transferrin binding activity, and suspensions of the iron-limited cells were immobilized onto paper prior to exposure to mixtures containing the HRP-transferrin conjugates. As illustrated in Fig. 2, strains of *P. haemolytica* were capable of binding all three ruminant transferrins, whereas strains of *H. somnus* were capable of binding only bTf. Results for control isolates of the human pathogen *N. meningitidis*, the horse pathogen *A.* (*H.*) *equuli*, and the pig pathogen *A. pleuropneumoniae* demonstrated that the failure to bind the conjugates of hTf, eTf, and pTf was not due to inadequacies of the reagents.

The observation that isolates of *P. haemolytica* were capable of binding all three ruminant transferrins whereas isolates of *H. somnus* were capable of binding only bTf suggested that *P. haemolytica* either had a receptor with broader specificity or had additional receptors for oTf and gTf. To address this question, a competition binding assay using unconjugated transferrins and HRP-bTf, HRP-oTf, or HRP-gTf was established. The results in Fig. 3 illustrate that bTf, oTf, and gTf were capable of blocking binding of either of the labelled ruminant transferrins. Thus, it is evident that bTf, oTf, and gTf bind to the same receptor in *P. haemolytica* h44 and that this receptor is different in specificity from the bTf receptor in *H. somnus* h74. These observations were essentially identical when the additional strains illustrated in Fig. 2 were tested in a competitive binding assay.

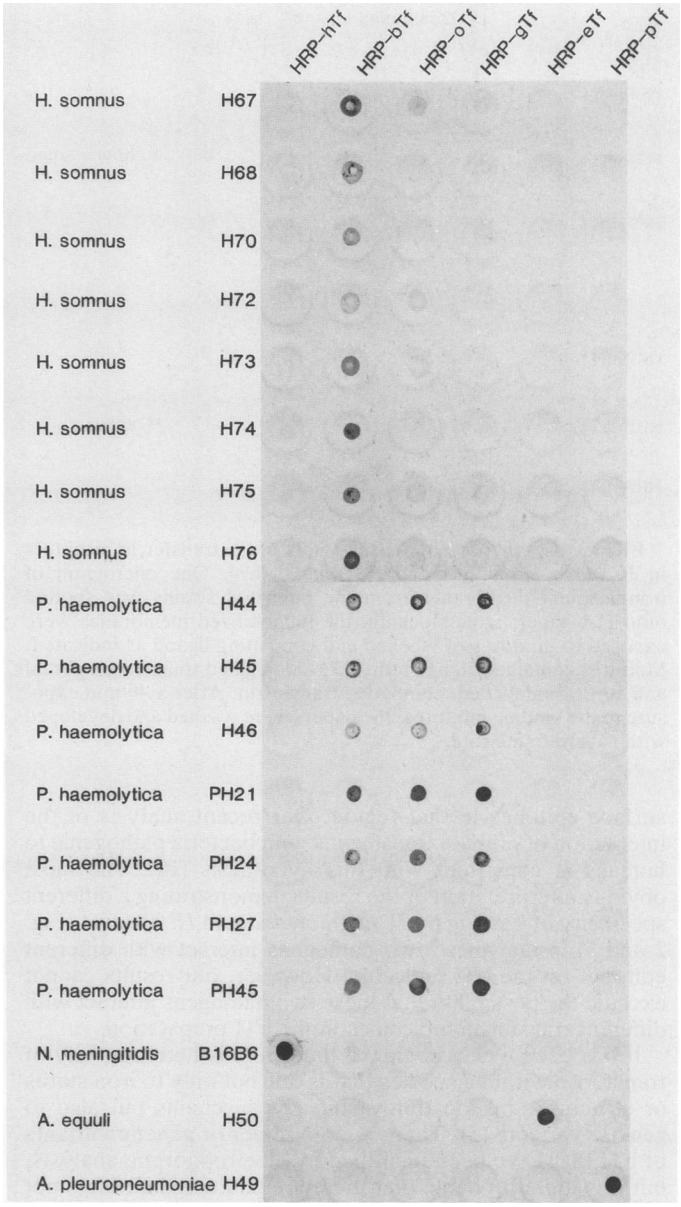

FIG. 2. Detection of transferrin binding activity in iron-deficient cells. Intact bacterial cells from the indicated species and strains grown on iron-deficient plates were spotted onto HA paper and exposed to binding mixtures containing the indicated HRP-conjugated transferrin prior to washing and development with an HRP substrate mixture. The concentration of the conjugated transferrin in the binding mixtures was 100 ng/ml.

TABLE 1. Growth of iron-deficient cells on different sources of iron[a]

Bacterial strain	Iron source[b]				
	$FeCl_3$	bTf	oTf	gTf	hTf
P. haemolytica h44	+	+	+	+	−
H. somnus h74	+	+	−	−	−
N. meningitidis B16B6	+	−	−	−	+

[a] Growth experiments were performed as described previously (15).
[b] +, evident growth; −, no growth detected.

If the transferrin-bacterial receptor interaction were highly conserved, one might anticipate that receptors from different bacteria would interact with the same region of the transferrin molecule. This notion would predict that all bacterial pathogens that interact with a given transferrin will have the same specificity for binding of related transferrins, since these transferrins would have either similar or different

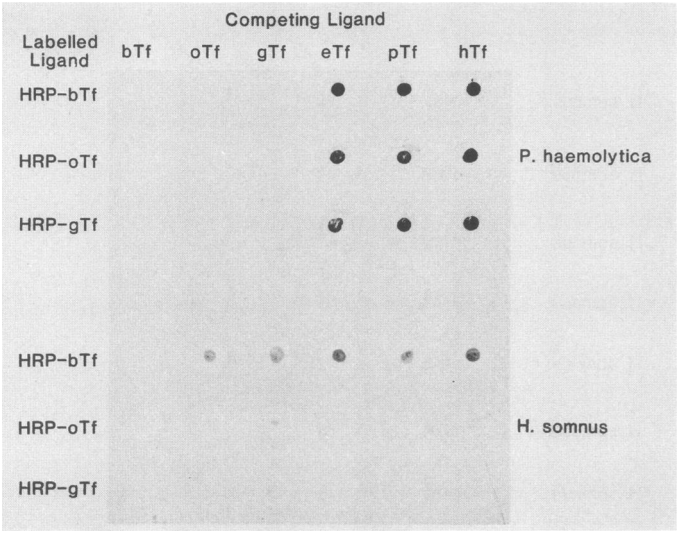

FIG. 3. Comparison of the specificity of the transferrin receptors in *P. haemolytica* h44 and *H. somnus* h74. One microgram of iron-deficient membranes from the indicated strains was spotted onto HA paper; after blocking, the immobilized membranes were exposed to mixtures of labelled and competing ligand as indicated. Mixtures contained 50 ng of the HRP-conjugated transferrins per ml and 10 μM unlabelled, competing transferrin. After a 40-min exposure to the binding mixtures, the papers were washed and developed with substrate mixture.

surface epitopes in that region. Our recent analysis of the interaction of primate transferrins with bacteria pathogenic to humans is consistent with this hypothesis (10). The most obvious interpretation of the results demonstrating a different specificity of binding by *P. haemolytica* and *H. somnus* (Fig. 2 and 3) is that these two pathogens interact with different epitopes on the bTf molecule. However, our results cannot exclude the possibility that these two pathogens interact with different transferrin molecules in the bTf preparation.

It has been well recognized that there is heterogeneity of transferrin within a species that is due not only to iron status or structure and location of the glycan chains but also to genetic variants (5). There are a number of genetic variants of bTf that have been identified by electrophoretic analysis, but it is quite probable that there are additional variants not detected by this approach. The presence of two protein bands upon SDS-PAGE analysis of the commercial bTf preparation (Fig. 1) that has been attributed to an internal cleavage of the transferrin polypeptide by an age-dependent serum proteolytic activity (12) may also represent variants that differ in susceptibility to the proteolytic cleavage. Our inability to separate these two forms of bTf by a variety of chromatographic approaches illustrates the potential difficulties in utilizing biochemical approaches to obtain purified preparations of transferrin variants. Since the type of transferrin produced by a given host may contribute to the host's susceptibility to certain bacterial pathogens, it is interesting to speculate on how the apparent heterogeneity of transferrin molecules may affect the host-pathogen interaction. The prospect that allelic variants of bTf in different breeds of animals or in different individuals contribute to susceptibility to infection by potential pathogens has many interesting implications. Further analysis of the interaction of bTf with these two bovine pathogens may provide insight into the transferrin-receptor interaction, but it may be necessary to use genetic approaches to ultimately obtain definitive results.

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