Immunological Cross-Reaction between Lactoferrin and Transferrin

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ABSTRACT. Hardly any or only a weak immunological cross-reaction was found between native lactoferrin (Lf) and transferrin (Tf). However, when these iron-binding proteins were denatured with sodium dodecyl sulfate and dithiothreitol, a definite immunological cross-reaction was detected between them. These results indicate that although Lf and Tf are immunologically quite different from each other in their native forms, they have the common antigenic determinant(s) in their unfolded forms.—KEY WORDS: cross-reaction, lactoferrin, transferrin.

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Lactoferrin (Lf) and transferrin (Tf) are iron-binding glycoproteins having similar molecular weights of about 80 kDa [6]. There is a considerable amino acid sequence homology between these two proteins: 59% between human Lf and Tf [13] and 60% between porcine Lf and Tf [1]. Both Lf and Tf consist of two similar globular lobes, each of which carries one iron-binding site [6].

Montreuil *et al.* [14] and Blanc and Isliker [4] found that human Lf was immunologically different from Tf in the immunodiffusion test. On the contrary, Hetherington *et al.* [7] reported that human Tf cross-reacted with the anti-human Lf antiserum in an enzyme-linked immunosorbent assay (ELISA), and Janatova *et al.* [8] found the cross-reaction between anti-human Tf antibody and Lf in immunoblotting. As Lf and Tf co-exist in some mammalian physiological fluids such as plasma [7], milk [17], and tears [5], the immunological methods, such as the ELISA and immunoblotting, using the strictly specific antibodies to Lf or Tf have to be employed for analyzing the two iron-binding proteins in these fluids. However, different results have been reported with regard to the specificity of the antibodies produced against Lf and Tf as described above. Thus, in the present study, the immunological properties of Lf and Tf were reinvestigated in detail.

Lf was purified from bovine mature milk essentially according to the procedure of Bläckberg and Hernell [3], who used the heparin-Sepharose affinity chromatography for purifying the protein. Because heparin bound to Lf, but not to Tf, Tf present in milk [17] was separated from Lf by this chromatographic step. Tf was purified from bovine serum bearing homozygous phenotype D_2 as described previously [21]. Lf present in serum [7] was separated from Tf by the Cibacron Blue F3GA-Sepharose affinity chromatography, because this dye bound to Lf [18], but not to Tf. Figure 1 shows the patterns of purified Lf and Tf on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Purified Lf showed a single band with a molecular weight of 80 kDa. Purified Tf showed two bands as reported by Tsuji *et al.* [19], and the molecular weights of the slower and the faster bands were 75 kDa and 70 kDa, respectively. From the results of Maeda *et al.* [12], it is considered that the faster band is derived from the molecule which has the cleavage of the peptide bond between residues 55 and 54 from the C-terminus of the whole protein chain (the slower band). The small peptide (6 kDa [21]) derived from cleaved Tf co-migrated with the marker dye, bromophenol blue, under the conditions of SDS-PAGE described below. A single precipitin line was formed between rabbit antiserum to purified Lf and bovine whey or purified Lf, as well as between antiserum to purified Tf and bovine serum or purified Tf in the double immunodiffusion test (data not shown). From these results, purified Lf and Tf were considered to be homogeneous.

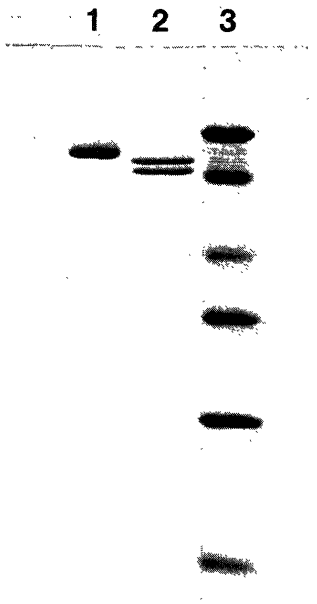

Fig. 1. SDS-PAGE of purified Lf and Tf. Samples are 1) Lf (2 μg), 2) Tf (2 μg), and 3) marker proteins (2 μg each): phosphorylase b (92.5 kDa), serum albumin (66.3 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (36.0 kDa), adenylate kinase (21.7 kDa), cytochrome *c* (12.4 kDa).

Protein concentration was determined by the method of Lowry *et al.* [11] using bovine serum albumin (BSA; Boehringer Mannheim, F.R.G.) as a standard.

To get the denatured antigens, four milligrams of purified Lf or Tf were heated at 95°C for 5 min in 1 m*l* of 50 mM Tris/HCl buffer (pH 7.4) containing 2% SDS and 20 mM dithiothreitol (DTT), and then dialyzed against 100 m*l* of 50 mM Tris/HCl (pH 7.4) containing 0.1% SDS

and 2 mM DTT.

Antisera to native Lf and Tf and to denatured Lf and Tf were produced in female Japanese white rabbits (Clea Japan, Tokyo) using the immunization protocol described elsewhere [15].

SDS-PAGE was carried out using 4.5% polyacrylamide stacking gel and 10% polyacrylamide running gel with the buffer system of Laemmli [10]. The samples were heat-treated at 95°C for 5 min with a sample buffer containing 50 mM Tris/HCl (pH 6.8), 2% SDS, 40 mM DTT, 0.002% bromophenol blue, and 5% (v/v) glycerol.

After SDS-PAGE, proteins were transferred from the gels to polyvinylidene difluoride membranes (Atto, Japan) using a blotting buffer containing 100 mM Tris, 192 mM glycine, and 5% (v/v) methanol, pH 9.0, according to the method of Kyhse-Andersen [9]. The membranes were blocked with 1% BSA in Tris-buffered saline (TBS: 20 mM Tris/HCl, 150 mM NaCl, pH 7.6), and then incubated with antiserum diluted 1:10,000 with TBS containing 0.1% BSA and 0.1% (v/v) Tween 20. The membranes were washed with TBS containing 0.1% (v/v) Tween 20, and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Tago, Inc., U.S.A.). After washing, the membranes were incubated in a developing solution containing 13 mM H_2O_2, 0.67 mM 3,3'-diaminobenzidine tetrahydrochloride, and 100 mM Tris/HCl (pH 7.6). The enzymatic reaction was stopped by washing the membranes with distilled water, and the membranes were air-dried.

A double-antibody ELISA was performed as described previously [20] except that one hundred microliters of 10 μg/m*l* native Lf or Tf in phosphate-buffered saline (PBS: 20 mM sodium phosphate, 150 mM NaCl, pH 7.2) or denatured Lf or Tf in PBS containing 0.1% SDS and 2 mM DTT were added to each well of the ELISA microtiter plates, and that PBS containing 0.1% gelatin, 0.1% Tween 20, and 0.02% sodium azide was used as an ELISA buffer.

Rabbit antisera to native bovine Lf and Tf were examined for their specificity and cross-reactivity by the double immunodiffusion test (data not shown), by the double-antibody ELISA (Fig. 2), and by immunoblotting (Fig. 3). In the double immunodiffusion test, while a single, intense precipitin line was formed between anti-native Lf or Tf serum and the homologous antigen, the two antisera showed no cross-reaction with the heterologous antigens. The antiserum to each of the native proteins showed a strong reaction with the homologous antigen in the ELISA, while hardly any or only a weak cross-reaction was detected between the antiserum and the heterologous antigen. The results of immunoblotting demonstrated that the antisera to native Lf and Tf recognized only the homologous antigens.

The antisera to denatured Lf and Tf were examined for their cross-reactivity by the double-antibody ELISA (Fig. 4) and by immunoblotting (Fig. 3). In both methods, each of the antisera showed a strong reaction with the homologous antigen, and showed a definite cross-reaction

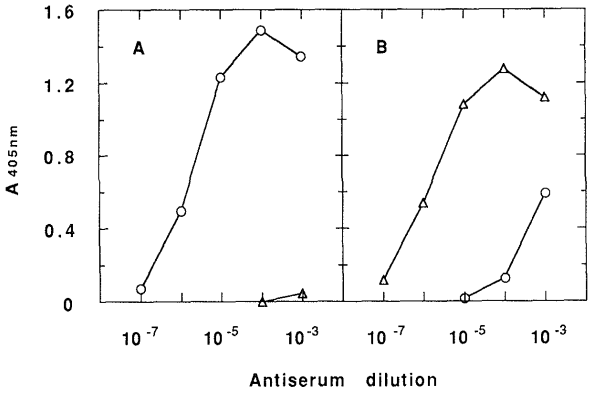

Fig. 2. The reactions of antisera to native Lf and Tf with the native antigens in a double-antibody ELISA. Anti-native Lf (A) and anti-native Tf (B) antisera serially diluted (10-fold) were incubated with native Lf (○) and Tf (△).

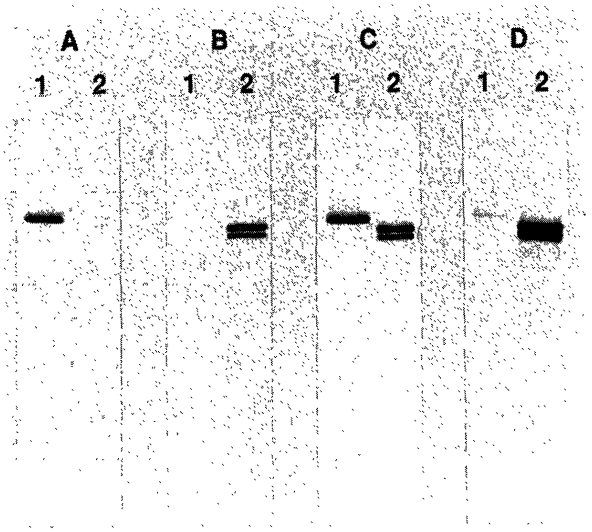

Fig. 3. Immunoblotting of Lf and Tf with the antisera to the native and denatured antigens. The membranes containing the purified antigens (100 *n*g each), Lf (1) and Tf (2), were incubated in the antisera to native Lf (A), native Tf (B), denatured Lf (C), and denatured Tf (D).

with the heterologous antigen. Neither anti-denatured Lf serum nor anti-denatured Tf serum reacted with BSA treated with SDS and DTT in the ELISA, indicating that the cross-reactivity between denatured Lf and Tf is not attributable to antibodies directed toward SDS. The double immunodiffusion test could not be performed to detect the cross-reaction between the denatured antigens because SDS and DTT in the antigen solutions hindered the antigen-antibody reaction on the agar plate.

It is known that immunological cross-reaction is detected between lysozyme and α-lactalbumin [2], between serum albumin and α-fetoprotein [16], or between mitochondrial and cytosolic adenylate kinases [22] when they are modified by reduction and alkylation, although these homologous proteins are non-cross-reactive in their

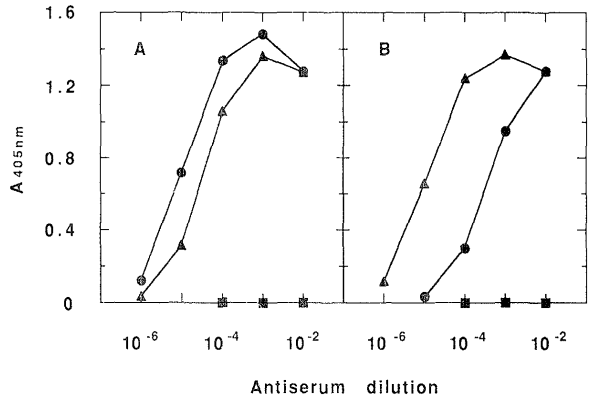

Fig. 4. The reactions of antisera to denatured Lf and Tf with the denatured antigens in a double-antibody ELISA. Anti-denatured Lf (A) and anti-denatured Tf (B) antisera serially diluted (10-fold) were incubated with denatured Lf (●), Tf (▲), and BSA (■).

native forms. In this study, it was shown that there was hardly any or only a weak immunological cross-reactivity between native Lf and Tf, and that these iron-binding proteins became immunologically cross-reactive when denatured with SDS and DTT. These results indicate that although native Lf and Tf are immunologically quite different from each other, there is (are) the common antigenic determinant(s) in their unfolded forms. Therefore, if Lf and Tf are partially (if not completely) denatured in the process of purifying them, during the storage of them, or in the course of emulsifying them with the adjuvant for immunization, they might elicit the cross-reacting antibodies when injected into the animals.

The Lf- and Tf-specific antibodies in the antisera to native Lf and Tf, respectively, can be used for analyzing these proteins in the biological fluids of the mammalian species. The cross-reacting antibodies in the antisera to denatured Lf and Tf are considered to be useful to search the new member(s) of a family of iron-binding proteins including Lf, Tf, ovotransferrin (conalbumin) and melanotransferrin [6].

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