The binding of human milk lactoferrin to immunoglobulin A

Toshiteru Watanabe, Hiroshi Nagura*+, Keiichi Watanabe* and William R. Brown†

*Cell Biology Research Laboratory and *Department of Pathology, Tokai University School of Medicine, Isehara, Kanagawa 259-11, Japan* and †*Department of Medicine, University of Colorado, Health Science Center and VA Hospital, Boulder, CO 80302, USA*

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It was recognized that in human milk some amounts of lactoferrin (LF) were naturally bound to secretory IgA (sIgA). Since not only secretory component (SC) but also LF was released from sIgA by disulfide bond cleavage, it is conceivable that LF is naturally bound to IgA as well as SC. An in vitro binding test to LF and IgA was performed and the binding was confirmed by the use of an IgA–Sepharose 4B affinity column.

Lactoferrin *Secretory IgA* *Secretory component* *IgA affinity column*

1. INTRODUCTION

Lactoferrin (LF) is an iron-binding glycoprotein [1] that is present in most of the mucous fluids similarly to sIgA, and is involved in the bacteriostatic effect of human milk on *Escherichia coli* and other bacteria [2,3]. It has also been shown that the bacteriostatic activity is due to the combined effect of LF and IgA [4,5]. However, the mechanism by which IgA participates in the antibacterial function of LF is not clear.

During an attempt to purify SC and sIgA from human milk and to make antibodies against these proteins, anti-LF as well as anti-SC and anti-IgA antibodies were unexpectedly obtained by immunization with purified sIgA of rabbits [6].

Since not only SC but also LF was released from purified sIgA by disulfide bond cleavage with 20 mM dithiothreitol, 1 M propionic acid and 40 mM iodoacetamide, it is conceivable that LF is naturally bound to IgA as well as SC. This study was also designed to determine whether LF could bind to an IgA-affinity column in vitro and possible implications of the observed binding phenomenon are discussed.

2. MATERIALS AND METHODS

Human colostrum was obtained from the department of Obstetrics and Gynecology, Tokai University Hospital, and stored frozen at −20°C until use. Sera from patients with IgA myeloma, containing dimeric IgA, were obtained at autopsy.

Secretory IgA and myeloma IgA were isolated and purified from pooled human colostrum and serum by combined 50% ammonium sulfate precipitation, DEAE–cellulose column [stepwise elution, 0.01 M phosphate buffer (pH 7.2), 0.05 M NaCl and 0.2 M NaCl] and Sephacryl S-200 gel filtration (Pharmacia, Uppsala). Purity was confirmed by Ouchterlony double gel immunodiffusion and immunoelectrophoresis using antibodies to whole human serum (Medical Biological Laboratory, Nagoya), anti-LF (Dako, Kyowa Medex, Tokyo) and anti-IgA (Medical Biological Laboratory, Nagoya).

+ Present address: Institute of Disease Mechanism, Laboratory of Germfree Life Research, Nagoya University School of Medicine, Nagoya, Japan

Abbreviations: LF, lactoferrin; sIgA, secretory IgA; SC, secretory component; PAGE, polyacrylamide gel electrophoresis

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Fractions of milk sIgA and myeloma IgA eluted by 0.05 M NaCl on a DEAE–cellulose column were gel filtered on Sephacryl S-200 to obtain IgA of homogeneous molecular mass and to remove free LF. The purity of sIgA was examined by 7% PAGE and immunoelectrophoresis. Antibodies against the sIgA were prepared in rabbits by subcutaneous injections of the purified sIgA preparation in Freund's complete adjuvant (Difco, MI) every 2 weeks (5 injections in total), each injection containing 0.25 mg sIgA.

The fraction containing SC and LF was isolated from human colostrum by combined gel filtration and DEAE–cellulose chromatography as in [7].

IgA affinity columns were prepared as follows. Myeloma IgA and serum IgA eluted from DEAE–cellulose were coupled to AH-Sepharose 4B (Pharmacia) by means of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl. One hundred mg of dimeric IgA and monomeric IgA each were covalently bound to 12 g AH-Sepharose 4B.

Binding experiments of LF to the IgA affinity column were performed as in [8]. Briefly, a crude LF fraction of human milk was first dialysed at 4°C against phosphate-buffered saline (pH 7.2), containing 5 mM each of EDTA and iodoacetamide, and 1% sodium azide. They were then passed through the IgA-Sepharose-4B column equilibrated with the same buffer at room temperature. Non-bound proteins were first washed off from this column with the same buffer. A further wash of loosely bound proteins was performed by the addition of 0.5 M NaCl in the same buffer. Finally, bound proteins were eluted with the same buffer containing 1.5 M ammonium thiocyanate, 5 mM EDTA and 5 mM iodoacetamide. Eluted proteins were estimated from the absorbance at 280 nm, dialysed against ion-exchanged water and concentrated by lyophilization. Aliquots of eluted fractions were determined by the Ouchterlony double immunodiffusion method against anti-LF antibody.

The molecular mass of each fraction was estimated by 0.1% SDS–7% PAGE [9,10] and the amount in each fraction quantitated by single radial immunodiffusion (SRID) using anti-LF antibody.

3. RESULTS AND DISCUSSION

The elution patterns of colostrum sIgA on DEAE–cellulose are illustrated in fig.1. All 3 peaks contained immunologically identified sIgA, but peaks 2 and 3 contained several other proteins. Peak 1 was used for immunization to raise anti-sIgA antibodies. Purified sIgA separated by Sephacryl S-200 from peak 1 was also used for immunization.

The antisera obtained revealed 3 precipitation lines against human milk. These correspond to those revealed by IgA, SC and LF by using specific antibody as shown in fig.2.

The first question was whether the free LF was contaminated in the purified sIgA fractions used for immunization. Since the M_r of free LF is 75000–95000 [11,12], it was easily separated from sIgA on Sephacryl S-200. In addition, a single band is obtained by the application of the purified sIgA by Sephacryl S-200 on 7% PAGE (fig.3).

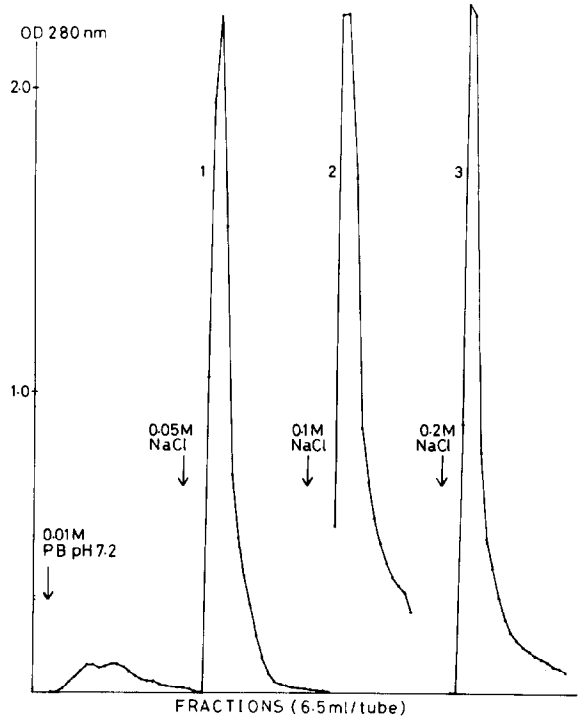

Fig.1. Elution patterns of colostrum sIgA on DEAE–cellulose column; all 3 peaks were immunologically sIgA, but peaks 2 and 3 contained additional proteins. Peak 1 was used for immunization to raise anti-sIgA antibodies.

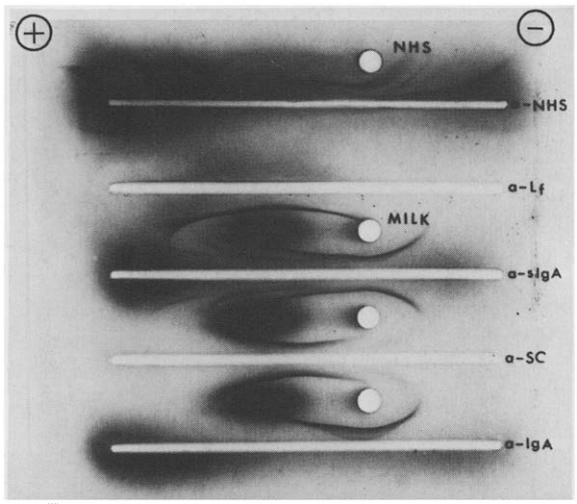

Fig.2. Immunoelectrophoresis pattern of serum from rabbit immunized with purified sIgA. Relative mobility of proteins was estimated by NHS (normal human serum) and anti-NHS (a-NHS). The anti-sIgA antibody (a-sIgA) in this study was examined on human milk. Three precipitation lines were observed, two of which fused to anti-LF (a-LF) and anti-SC (a-SC).

However, LF is immunologically detected in these purified sIgA samples by the Ouchterlony double immunodiffusion method. It indicates that no free LF is present in the sIgA used for immunization, but bound LF might be detected.

An experiment was performed to establish whether LF or other proteins were separated after reduction and alkylation of the purified sIgA on Sephacryl S-200 with 20 mM dithiothreitol, 1 M propionic acid and 40 mM iodoacetamide. The gel filtration patterns of the treated sIgA on Sephacryl S-200 are illustrated in fig.4; IgA, LF, SC and J chain were detected by using specific antibodies by the Ouchterlony double gel diffusion method.

From these results, we speculate that a part of LF might be naturally bound to milk sIgA, probably through disulfide bonds similarly to SC binding to J chain-linked dimeric IgA [13].

Experiments to determine whether LF bound to IgA in vitro were then performed using a dimeric myeloma IgA affinity column. The LF bound to dimeric IgA in the column was eluted by the corresponding buffer. LF was not observed to bind to the column, the fraction loosely bound to dimeric IgA being eluted by 0.5 M NaCl and the tightly

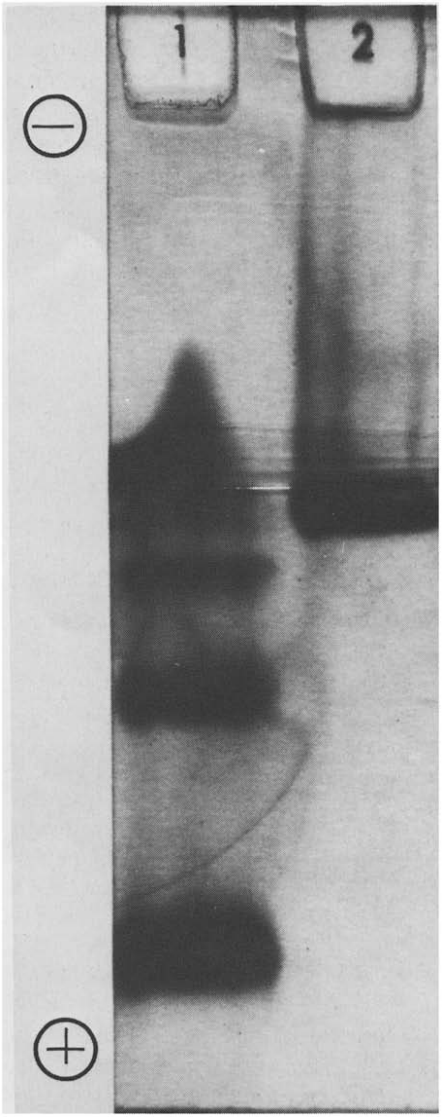

Fig.3. Profile of the repurified sIgA by Sephacryl S-200 gel filtration examined in 7% PAGE. A single band is obtained (track 2), showing no other contaminating proteins; NHS in track 1.

bound fraction eluted by 1.5 M NH_4SCN as shown by Ouchterlony double gel diffusion against monospecific anti-LF antibody (fig.5).

The molecular mass of the eluted LF was estimated in 0.1% SDS–7% PAGE to be approx. 85 kDa (fig.6). An M_r of approx. 68000, which corresponds to SC, was also observed. The LF binding to monomeric IgA was also examined using a monomeric IgA affinity column. The bind-

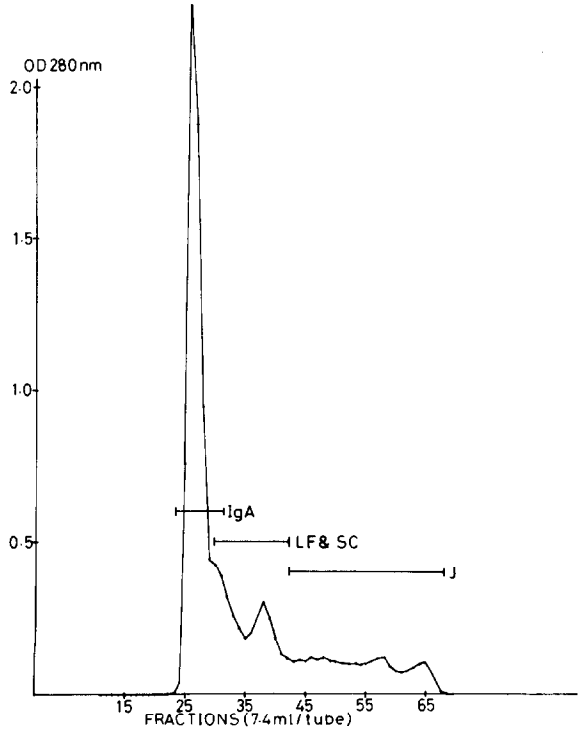

Fig.4. Gel filtration profile of the reduced and alkylated sIgA on Sephacryl S-200. Eluted fractions were estimated from the absorbance at 280 nm. IgA, LF, SC and J chain were detected by using relevant antibody by the Ouchterlony double gel diffusion method as illustrated. sIgA, 152 mg; DTT, 20 mM; propionic acid, 1 M; iodoacetic acid, 40 mM. Sephacryl S-200, 2.5 × 91 cm; eluant, 20 mM DTT containing 0.1 M Tris buffer (pH 8.0).

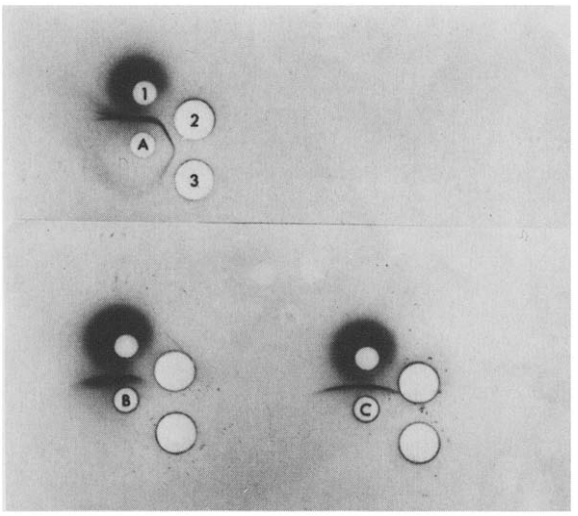

Fig.5. The LF bound to the IgA affinity column was eluted by corresponding buffers and examined by Ouchterlony double gel diffusion. LF was not observed to bind to the column (1), 0.5 M NaCl eluted loosely bound fraction (2) and 1.5 M NH_4SCN eluted tightly bound fraction as shown by the precipitation line against anti-LF (A). Small amounts of SC were observed against anti-SC (B) but no binding of anti-α_1-antichymotrypsin was observed in the eluate (C).

ing to monomeric IgA or sIgA was also observed. Only a trace amount of LF bound to Sepharose 4B not coupled to dimeric IgA or monomeric IgA was observed.

The binding of albumin [13,14] and α_1-antitrypsin [15] to IgA has been reported. It could be that their bioavailability to target organs or microbes might be more effective in a complexed form, however there is as yet no direct proof of this. Also, in the case of LF, antibacterial activities have been reported to be enhanced when coexisting with IgA molecules [5]. Whether these binding properties of LF to IgA have any connection with its physiological function is a matter for further investigation.

Authors in [16] reported a purification method for free LF using a copper-chelate gel, since the LF is able to bind copper as well as iron. In that system an admixture of sIgA in the free LF fraction was also noted; one may speculate that the binding of sIgA to the copper-chelate gel could have occurred through the LF which was attached to the sIgA molecules.

It was demonstrated [14] that LF tended to form associations with acidic proteins such as albumin, casein and lactoalbumin. However, the binding was dissociated by the sucrose density gradient technique.

The experiments described here revealed that LF and SC were separated from sIgA by reduction with 20 mM dithiothreitol and 1 M propionic acid. This indicates that the binding of LF to IgA might have occurred through formation of disulfide bonds. However, in an in vitro binding experiment with a myeloma IgA affinity column, the binding of LF to IgA was dissociated with increasing molarity of salt, and thus the ionic binding is also involved in both bindings.

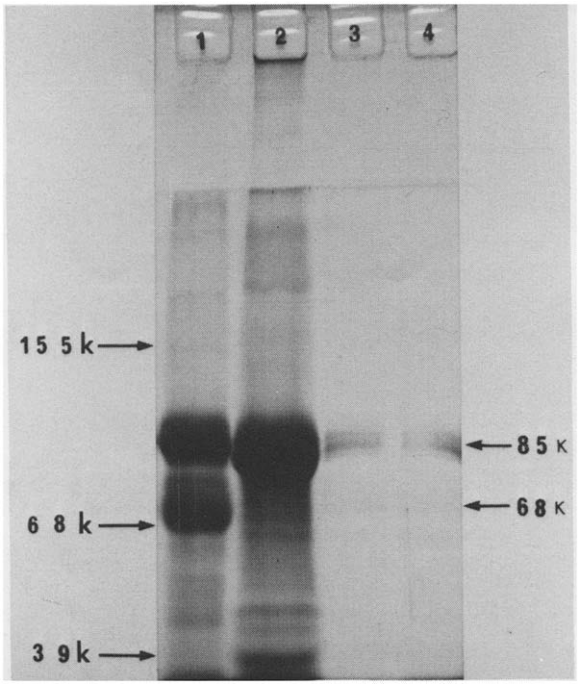

Fig.6. Estimation of molecular mass of bound LF to IgA affinity column. The loosely bound and tightly bound LF were estimated in 0.1% SDS–7% PAGE. Molecular masses of approx. 85 kDa and 68 kDa were revealed on Coomassie brilliant blue staining of the PAGE. 1, human milk; 2, eluted LF fraction; 3, loosely bound LF and 4, tightly bound LF. K, kDa.

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