Detection of bacterial interaction with lactoferrin by an enzyme-linked ligand binding assay (ELBA)

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Summary. An enzyme-linked ligand binding assay (ELBA) was devised to measure the interaction between bacteria and human (H) or bovine (B) lactoferrin (Lf) linked to horseradish peroxidase. Reagents were calibrated for optimum colour development with *o*-phenylenediamine as chromophore and organisms that were either positive or negative in a radioisotope-labelled ligand binding assay (RLBA) with ^{125}I -Lf. Good correlation of Lf binding ($r = 0.89$) was found between ELBA and RLBA with 169 randomly selected strains of *Escherichia coli*. A semi-quantitative scoring system for ELBA, corresponding to a similar system for RLBA, was established and shown to be valid for 517 strains from seven species of bacterial pathogens. ELBA was used to measure bacterial Lf binding-saturation and displacement kinetics and shown to be comparable with RLBA. ELBA may be a suitable method for examining the binding of Lf to bacteria without the need for radioactive isotopes.

Introduction

Lactoferrin (Lf) is a mammalian iron-binding glycoprotein produced by polymorphonuclear leucocytes (PMNL) and exocrine glands, that occurs at a high concentration in milk and at mucosal surfaces.^{1–3} Since Lf binds to specific receptors on various eukaryotic cells,^{4–10} it has been suggested that it is involved in many physiological processes including intestinal iron absorption, amplification of inflammatory responses, antigen processing and antibody production.^{5, 6, 11–13} Lf also binds to various microbial pathogens,^{14–25} and this interaction seems to potentiate its antimicrobial action.^{26–29} However, certain micro-organisms seem to utilise this interaction to acquire Lf-bound iron for growth.^{23, 30, 31}

Studies on the interaction of Lf with eukaryotic or prokaryotic cells are generally performed with radioisotope-labelled ligand binding assays (RLBA) that require expensive equipment and strict radiation protection. Furthermore, isotope-labelled proteins are less stable because of continuous radioactivity-induced disintegration. Enzyme-linked Lf reagents have been used in Western blotting techniques to demonstrate specific Lf-binding proteins of bacteria.^{16, 20, 21} These reagents are easy to handle and are stable over a long period of storage. Therefore, an enzyme-linked ligand binding assay (ELBA) has been developed to measure the interaction of Lf with bacteria. This paper describes the standardisation of ELBA against an ^{125}I -labelled

Lf binding assay as a reference method, and the use of ELBA to measure saturation and binding-inhibition kinetics and the characterisation of the magnitude of Lf binding to various bacterial pathogens.

Materials and methods

Lactoferrins and horseradish peroxidase

Highly purified human Lf (HLf) from human milk, and bovine Lf (BLf) from bovine colostrum were obtained from US Biochemicals, Cleveland, OH, USA. Both Lf preparations were homogeneous when tested in ion-exchange (Mono-Q column, Pharmacia AB, Uppsala, Sweden) and in molecular-sieve (TSK G4000 SW, LKB Produkter AB, Bromma, Sweden) high-performance liquid chromatography. Horseradish peroxidase (HRPO) was purchased from Sigma.

Coupling of Lf to HRPO

HRPO was coupled to HLf and BLf by a periodate method.³² Briefly, 0.2 ml of 0.1 M sodium-m-periodate was mixed with HRPO 0.4% (final volume, 1 ml) with constant stirring for 20 min at room temperature. Excess periodate was removed by overnight dialysis at 4°C against 1 mM sodium acetate buffer, pH 4.4. After the addition of 20 μl of 0.2 M sodium carbonate-bicarbonate buffer, pH 9.5, the dialysate was mixed with 1 ml of HLf or BLf 0.8% (dissolved in 0.01 M sodium carbonate buffer, pH 9.5) and incubated for 2 h at room temperature with constant stirring. A 0.1-ml volume of fresh sodium borohydrate 0.4% was added and kept for a further 2 h at 4°C. Finally,

ovalbumin (Sigma) 2% in 0.1 ml of phosphate-buffered saline (PBS), pH 7.2, was added to stabilise the HRPO-Lf conjugate. This procedure yielded HRPO-Lf conjugates with high binding activity. After dialysis against PBS at 4°C overnight the conjugate preparation was mixed with an equal volume of glycerol 50% v/v and stored at -20°C.

Preparation of bacterial suspensions

Escherichia coli strains, previously characterised for interaction with Lf,²² were used for the standardisation of ELBA. Bacteria were grown on colonisation factor antigen (CFA) agar and fresh overnight cultures were subcultured on CFA agar for 24 h at 37°C. Bacterial growth was gently scraped with a sterile loop (Sarstedt, Numbrecht, Germany), mixed in PBS and thoroughly suspended with a vortex mixer (Vortex Genie 2, Scientific Industries, NY, USA). The suspension was centrifuged at 5300 *g* for 10 min and the bacteria were recovered as a pellet. The pellet was resuspended and washed twice in PBS. Finally, the bacterial density in PBS was optically adjusted to 10¹⁰ cells/ml (OD 0.125 at 600 nm; Varian DMS 100S) for binding studies. The cell density of selected samples was confirmed by counting stained bacteria in a Petroff-Hausser chamber.

Standardisation of Lf-ELBA

A suspension of 10⁹ bacteria in 0.1 ml of PBS was mixed with 0.1 ml of HRPO-Lf conjugate. After incubation at 37°C for 1 h, the mixture was washed three times by centrifugation at 5300 *g* for 10 min with ice-cold PBS containing Tween-20 0.05%. The supernate was aspirated carefully and the bacterial pellet was mixed with 0.5 ml of *o*-phenylenediamine substrate (30 mg in 75 ml of 0.1 M sodium acetate buffer, pH 5.0, containing 40 µl of H₂O₂, Sigma, 30%) and incubated for 10 min at room temperature. The reaction was terminated with 0.25 ml of 4 M H₂SO₄. A bacteria-free supernate from the reaction mixture was collected by centrifugation at 5300 *g* for 5 min and the absorbance was measured at 492 nm, in a Reader Microelisa system (Organon Technika NV, Turnhout, Belgium).

Standardisation of Lf-RLBA

HLf and BLf were labelled with Na¹²⁵I (specific activity 629 GBq/mg; DuPont Scandinavia AB, Stockholm, Sweden) with Iodobeads® (Pierce Chemicals Co., Rockford, IL, USA).³³ The ¹²⁵I-labelled Lf binding assay was used as a reference during standardisation of the ELBA, and done as described previously.^{18,19} Briefly, 10⁹ bacteria in 0.1 ml of PBS were mixed with 0.1 ml of PBS containing c. 8 ng of ¹²⁵I-Lf (specific activity 0.16 MBq/µg) and incubated at 37°C for 1 h. The binding reaction was terminated with 2 ml of ice-cold PBS containing Tween-20 0.1%,

Table I. Lf binding to an isogenic pair of *E. coli* strains by ELBA and RLBA

Strain	Lf-type	ELBA Absorbance (SD)	RLBA Percentage ¹²⁵ I-Lf binding (SD)
<i>E. coli</i> H10407	HLf	0.20 (0.03)	9 (2)
	BLf	0.19 (0.03)	7 (2)
<i>E. coli</i> H10407(Lf)	HLf	1.98 (0.20)	53 (5)
	BLf	1.96 (0.21)	41 (4)

and after centrifugation at 5300 *g* for 15 min, the supernate was aspirated. Radioactivity bound to the bacterial pellet was measured in a gamma counter (LKB Wallac Clinigamma 1272, Turku, Finland). Background radioactivity (from incubation mixtures without bacteria) was deducted, and the binding was expressed as a percentage calculated from the total labelled ligand added to the bacteria.

Comparison of ELBA and RLBA

The ELBA reagents were calibrated for optimal colour reactions with *E. coli* strains H10407 and H10407(Lf) that served as negative and positive controls respectively in Lf-RLBA. The strains were tested 10 times in both ELBA and RLBA, each time with fresh cultures. The binding results were comparable and reproducible in both assays (table I). ELBA was further evaluated for a correlation between absorbance values and ¹²⁵I-Lf percentage binding. For this purpose, 169 randomly selected strains of *E. coli* were examined. Parameters such as bacterial growth conditions, cell density and incubation conditions for binding were similar for both the assays. *E. coli* strain H10407 and variant H10407(Lf) were always included as negative and positive controls respectively.

Saturation kinetics and displacement of bound Lf

ELBA was used to determine the saturation kinetics of the Lf-bacteria interaction as a function of time. HRPO-labelled HLf or BLf was incubated with cells of strain H10407(Lf) for different periods, and the colour reactions were measured.

To determine the displaceability of the ligand-bacteria interaction with the same strain, increasing amounts of unlabelled ligand (in homologous and heterologous combinations) were added to the reaction mixture containing HRPO-labelled HLf or BLf and incubated for 1 h. The washing steps and the colour development were performed as described above.

Screening of bacteria for Lf binding

Clinical isolates of *Staphylococcus aureus* (43), *Aeromonas hydrophila* (40), *E. coli* (295), *Prevotella intermedia* (11), *Shigella flexneri* (35), *Salmonella*

Fig. 1. Comparison of binding of HLf to 169 strains of *E. coli* by ELBA (absorbance at 492 nm) and RLBA (percentage ^{125}I -Lf binding).

Table II. Classification of ELBA scores according to the RLBA categories

ELBA		RLBA	
Score	Absorbance interval	Category	Percentage binding interval
0	≤ 0.22	Negligible	≤ 10
1	0.23–0.44	Low	11–20
2	0.45–0.66	Moderate	21–30
3	≥ 0.67	High	≥ 31

typhimurium (37) and *Yersinia enterocolitica* (56) were examined. *S. aureus* strains isolated from patients with septicaemia, endocarditis or toxic shock syndrome were recovered and subcultured on Columbia agar.¹⁹ *P. intermedia* strains, from patients with periodontitis, were recovered and subcultured on modified Brucella blood agar.¹⁵ The enteropathogenic gram-negative bacteria isolated from the faeces of patients with gastroenteritis were recovered and subcultured on

CFA agar at 37°C for 24–72 h, according to the requirement of each bacterial species. Cell suspensions were prepared and ELBA was performed according to the procedures described.

Results

To establish a correlation between ELBA and RLBA, 169 randomly selected *E. coli* strains were examined for HLf and BLf binding by each assay. The results expressed as percentage binding for RLBA and as absorbance at 492 nm for ELBA showed a third order polynomial correlation with a correlation coefficient of 0.89 (fig. 1). On the basis of these results an ELBA absorbance of 0.22 corresponding to $\leq 10\%$ ^{125}I -Lf binding was chosen as a class interval to define ELBA scores further to simplify the test (table II).

E. coli strains were redistributed according to ELBA scores and percentage ^{125}I -Lf binding (fig. 2). The median values for HLf were 8% for score 0 and 16%,

Fig. 2. Comparison of percentage ^{125}I -Lf binding and ELBA scores: —, interpolation of the mean values of score classes 0, 1 and 2; ----, $\pm 10\%$ RLBA binding range of the mean interpolation value.

22% and 39% for scores 1, 2 and 3 respectively. Corresponding values for BLf were 7%, 17%, 23% and 29%. In *c.* 75% of the strains (126 of 169) the ELBA scores for the HLf coincided with the RLBA intervals. Eighteen strains were scored one class lower and 24 strains one class higher, but all deviations were within $\pm 10\%$ of the RLBA binding interval. The corresponding values for BLf were 64% (108 of 169) for absolute matching; 12 strains were scored one class lower and 45 strains one class higher. Five strains deviated by two score classes (two strains lower and three strains higher). A value of 10% ^{125}I -labelled Lf binding had previously been considered to be the break-point for differentiating non-binding (= negligible binding) and binding strains.^{18,19} Based on this criterion, the sensitivity and specificity values for HLf-ELBA were estimated to be 0.92 and 0.94, respectively. Corresponding values of 0.97 and 0.86 respectively, were obtained for BLf-ELBA.

The degree of HLf and BLf binding to 517 strains from seven bacterial species associated with various human infections were further examined with ELBA (table III). These ELBA score distributions corresponded well with RLBA results reported previously.^{15,16,19,22}

The value of ELBA for the characterisation of the Lf-bacteria interaction was evaluated. The ELBA reaction with *E. coli* strain H10407(Lf) was time-dependent and *c.* 3 h was required for complete saturation with both Lf conjugates (fig. 3), and unlabelled HLf and BLf inhibited the binding of HRPO-HLf or HRPO-BLf to the strain in a dose-dependent manner. About 150 μg and 100 μg of unlabelled ligand was required to give 50% inhibition (homologous or heterologous) of the HLf- and BLf-ELBA reactions respectively (fig. 4).

Discussion

ELBA may be considered to reflect Lf binding to approximately the same degree as RLBA. In addition, the four-class scoring system of ELBA will make it possible to develop a semi-quantitative test in which result will be read visually by comparing the colour

Table III. Percentage distribution of bacteria according to ELBA scores

Bacterial species (number of strains)	Percentage of strains that gave							
	ELBA scores for HLf				ELBA scores for BLf			
	0	1	2	3	0	1	2	3
<i>S. aureus</i> (43)	63	30	2	5	5	21	37	37
<i>A. hydrophila</i> (40)	0	10	22	68	0	8	27	65
<i>E. coli</i> (295)	39	41	5	15	40	42	6	12
<i>P. intermedia</i> (11)	0	0	9	91	0	0	18	82
<i>Sh. flexneri</i> (35)	23	46	3	28	0	43	26	31
<i>Sal. typhimurium</i> (37)	16	49	5	30	5	35	21	39
<i>Y. enterocolitica</i> (56)	20	18	44	18	12	9	9	70

Fig. 3. Time-dependent binding of HLf (○) and BLf (●) to *E. coli* H10407(Lf) as determined by ELBA.

intensity of the reaction against a colour chart, as previously described for other enzymatic reactions (API SYSTEM, La Balme les Grottes, France). Work is currently in progress to develop such a system.

Taken together, these results support the use of ELBA as an alternative to RLBA for measurement of the interaction of Lf with pathogenic micro-organisms. The standardised ELBA is reproducible and classifies the magnitude of Lf binding to bacteria with a sensitivity and specificity comparable to that of RLBA. ELBA may be particularly useful in laboratories that lack facilities for work with radioisotopes. Occasionally, interfering reactions that lead to increased background absorbance values with some bacteria may occur with the ELBA chromophore. Another possible limitation may be the large molecular size of the HRPO-Lf complex that may alter the binding to some

Fig. 4. Displacement of (a) HRPO-HLf and (b) HRPO-BLf binding from *E. coli* H10407(Lf) by unlabelled HLf (○) and BLf (●) as determined by ELBA.

cells and thus affect estimations of the receptor density and binding affinity constants in Scatchard-plot analyses. Work is in progress to evaluate modifications of ELBA to suit these conditions.

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