Bovine lactoferrin induces both mucosal and systemic immune response in mice

By HAJER DEBBABI, MICHEL DUBARRY*, MICHÈLE RAUTUREAU and DANIEL TOMÉ

Unité INRA de Nutrition Humaine et de Physiologie Intestinale, Institut National Agronomique Paris-Grignon, 16 rue Claude Bernard, F-75005 Paris, France

(*Received 16 June 1997 and accepted for publication 21 October 1997*)

Summary. Lactoferrin (Lf) is a milk iron-binding glycoprotein that plays a role in iron transport and acts as both a bacteriostatic and a growth modulating agent. The aim of this study was to investigate the nature of immune responses induced by repeated oral administration of bovine milk Lf in mice. Groups of ten female BALB/c mice were fed daily for 4 weeks with two doses of protein antigen: a low (0·05 mg/g body weight per d) or high (1 mg/g body weight per d) dose of Lf, or water as a control. A fourth group was immunized intramuscularly with 0·01 mg Lf in complete Freund's adjuvant. Anti-Lf IgA and IgG were detected in the intestinal fluid and serum of mice given Lf. Total immunoglobulins were higher in the intestinal fluid in Lf groups than in the control group. No difference could be detected in the serum. IgA and IgG secretion was enhanced in Peyer's patches and spleen from Lf-fed mice, in comparison with controls. [^{3}H]thymidine uptake into Peyer's patch and spleen cells from both control and Lf-fed mice was enhanced by 75 μg Lf/ml *in vitro*, but Lf groups had a greater proliferation rate than the control group. These findings suggested that Lf could act as an immunostimulating factor on the mucosal immune system and that activation of the mucosal immune system is dependent on the ability of Lf to bind to the intestinal mucosa.

Food proteins are ingested daily in quantities that should result in induction of immune responses since small intact antigenic fractions can cross the intestinal epithelium and can be detected in serum (Marcon-Genty *et al*. 1989; Caillard & Tomé, 1994). Low levels of antibodies to milk protein antigens have been detected in serum (Host *et al*. 1992). Administering protein antigens generally induces oral tolerance, defined as a systemic unresponsive state to parenteral immunization (Mowat, 1987, 1994). Some authors have reported both a systemic suppression and mucosal antibody response (Challacombe & Tomasi, 1980), whereas others have discussed the poor capacity of protein antigens given orally to stimulate local immunity (Mowat, 1987).

Lactotransferrin or lactoferrin (Lf) is an 80 kDa iron-binding glycoprotein found in milk and other external secretions and to a lesser degree in specific granules of neutrophils. It is involved in iron uptake by the intestinal mucosa and acts as an antibacterial factor (Iyer & Lönnerdal, 1993; Lönnerdal & Iyer, 1995). As a result of experiments on the effects of bovine Lf supplementation in children and in mice,

* For correspondence and reprints.

https://doi.org/10.1017/S0022029997002732 Published online by Cambridge University Press

it has been suggested that Lf may influence the ecosystem of the infant and the mouse and may also be involved in iron absorption (Chierici *et al.* 1992; Roberts *et al.* 1992; Teraguchi *et al.* 1995). Lf plays a role in inflammatory processes (Lönnerdal & Iyer, 1995). Furthermore, its ability to bind to lymphocytes and monocytes or macrophages has been associated with other functions (Bennett & Davis, 1981; Sato *et al.* 1990; Eda *et al.* 1997). For instance, Lf may affect various aspects of the immune system, by inducing a humoral immune response (Zimecki *et al.* 1991), the maturation of T and B cells (Zimecki *et al.* 1991, 1995) and the proliferation of lymphocytes (Hashizume *et al.* 1983).

The aim of this study was to investigate the nature of humoral responses induced by repeated oral administration of the milk-derived protein Lf. As bovine Lf is poorly digested in the gastrointestinal tract (Spik *et al.* 1982) and is able to bind to murine intestinal receptors (Hu *et al.* 1988), we examined the immune response elicited by the prolonged oral administration of bovine Lf in mice. Most studies have examined the systemic and mucosal responses to dietary antigens separately. We chose to examine the capacity of Lf to modulate both systemic and mucosal antibody responses. Experiments were performed using two daily doses of protein antigen: a low dose of oral Lf (0·05 mg/g body weight), which is comparable to the physiological concentration of Lf in mouse milk, and a considerably larger amount to elicit an immune response (1 mg/g body weight).

Materials and methods

Animals, oral administration and immunization

The female BALB/c mice used in this study were 10–15 weeks old and were obtained from Charles River France (F-76410 Saint-Aubin-lès-Elbeuf, France). Bovine lactoferrin (17% iron saturation; LactoBretagne, F-35460 Saint-Brice-en-Coglès, France) was dissolved at 1 or 20 mg in 0·5 ml sterile water. The mice were allocated to four groups of ten. To the mice in the two Lf groups 0·5 ml Lf preparation (0·05 or 1 mg/g body weight per d and per mouse) was administered through a stainless steel feeding tube for 5 consecutive days each week for 4 weeks. A control group was given sterile water under the same conditions. The fourth group of mice was immunized intramuscularly with 0·01 mg Lf in complete Freund's adjuvant (Sigma, F-38297 Saint-Quentin Fallavier Cedex, France) and 3 weeks later received an intramuscular injection of 0·01 mg Lf in incomplete Freund's adjuvant. Body weight was measured and routine clinical observations carried out regularly throughout the experiments. There was no significant difference in live body weight of control, Lf-fed and immunized mice, and no clinical abnormalities were observed in mice during the experiments.

Collection of saliva, intestinal secretions, serum and intestinal mucosal surface

Before collection, mucosal secretions were induced by intraperitoneal injection of 0·1 mg pilocarpine-HCl (Sigma) in 0·1 ml 0·01 M-phosphate-buffered saline, pH 7·0 (PBS: 80·0 g NaCl, 11·6 g Na_2HPO_4, 2·0 g KH_2PO_4, 2·0 g KCl plus H_2O to 10 l). Saliva was collected by inserting a small cotton pad into the mouth for 1–2 min until it was saturated, as described by Haneberg *et al.* (1994). The proteins extracted were stored at −20 °C until assay. Intestinal fluids were collected as described by Elson *et al.* (1984). Mice were given 0·5 ml (48 mmol/ml) polyethylene glycol MW 4000 (Prolabo, F-75011 Paris, France) every 15 min for 1 h. At 30 min after intraperitoneal injection of pilocarpine-HCl, intestinal contents were collected in 3 ml of a cold PBS

https://doi.org/10.1017/S0022029997002732 Published online by Cambridge University Press

solution containing $1{\cdot}5 \times 10^{-2}$ M-di-isopropylfluorophosphate (Sigma). The resulting material was clarified by two successive centrifugations (1500 ***g***, 4 °C, 15 min) and stored frozen at −70 °C until assay. Mice were bled routinely from the retro-orbital plexus under light ether anaesthesia to collect sera, ~ 150 μl blood being collected from each mouse. After centrifugation (1000 ***g***, 4 °C, 15 min) the serum was stored at −70 °C until assay. Secretions from the mucosal surfaces of the small intestine were collected from animals killed by cervical dislocation. The small intestine was removed and rinsed in cold RPMI 1640 medium (Gibco Life Technologies, F-95613 Cergy Pontoise Cedex, France). The tissue was opened along the mesenteric border and the mucosal surface scraped into a tube with a glass slide. After addition of phenylmethylsulphonyl fluoride (1 mmol/ml, Sigma), the mucosal material was shaken vigorously, centrifuged and stored in 1 ml PBS containing 10 g bovine serum albumin (BSA)/l at −20 °C until assay.

Preparation of cell suspensions from Peyer's patches and spleen and cell cultures

Mice were killed by intramuscular injection of pentobarbital sodium (Sanofi, F-33501 Libourne Cedex, France). Peyer's patches (PP) were carefully excised from the wall of the small intestine, slit by a surgical blade and finely minced in RPMI 1640 medium. The cell suspension was passed through a cell strainer (Falcon; Polylabo, F-67023 Strasbourg, France), then a sterilized gauze band to remove cell debris and finally it was washed twice with cold RPMI 1640 medium. PP cells ($2 \times 10^6 - 5 \times 10^6$ cells/ml) were obtained from each mouse (viability > 90 %). Spleens were aseptically removed, slit by a surgical blade and finely minced in RPMI 1640 medium. Single cells were separated using a cell strainer. Red blood cells were removed by incubating spleen cells with NH_4Cl solution (8·4 g/l) at 4 °C for 10 min and cell suspensions were washed twice with cold RPMI 1640 medium. Cell yield from spleen was $0{\cdot}5 \times 10^8 - 1 \times 10^8$ cells/ml (viability > 90 %).

Samples (100 μl) of spleen and PP cell suspensions from mice given both water and Lf were cultured in a total volume of 200 μl in 96 well plates (Falcon) in complete medium consisting of RPMI 1640 supplemented with (per ml) 292 μg L-glutamine, 100 i.u. penicillin, 0·1 mg streptomycin, 0·2 mg gentamycin, 3·9 μg 2-mercapto-ethanol and 100 μl heat-inactivated fetal calf serum (Gibco) at a density of 4×10^6 cells/ml. For antigen stimulation, cultures were supplemented with 75 μg Lf/ml. For polyclonal stimulation, 4 μg concanavalin A/ml (ConA, Sigma) or 8 μg lipopoly-saccharide/ml from *Escherichia coli* serotype O111:B4 (LPS, Sigma) was added to each well. These conditions provided good cell proliferation rates (results not shown). Control cultures received complete medium only. All cultures were incubated at 37 °C in a humidified atmosphere of air–CO_2 (95:5). Cells were harvested at 6 d and supernatants were collected for measurement of immunoglobulin (Ig) production.

Measurement of total IgA and IgG

Total IgA and IgG were detected in sera and mucosal secretions with a sandwich type enzyme-linked immunosorbent assay (ELISA). Microtiter plates (Maxisorp Nunc, Polylabo) were coated overnight at 4 °C with 2 μg anti-mouse IgA/ml (Sigma) or 2 μg anti-mouse IgG/ml (Sigma) in PBS. After blocking nonspecific protein sites with PBS containing 30 g BSA/l, samples of serum or mucosal secretions were incubated at 37 °C for 1·5 h. After washing with PBS containing 0·5 ml Tween 20/l, plates were incubated for 1·5 h at 37 °C with biotin-conjugated anti-IgA (Sigma) or anti-IgG (Sigma), both diluted 1:20000 in PBS containing 10 g BSA/l and 0·5 ml Tween 20/l. After washing, 1:5000 ExtrAvidine peroxidase (Sigma) was added for

https://doi.org/10.1017/S0022029997002732 Published online by Cambridge University Press

30 min incubation at 37 °C. H_2O_2 (300 ml/l at 0·25 ml/l, Sigma) and *o*-phenylenediamine (0·5 mg/ml, Sigma) were used as substrates and the plates were read at 490 nm in an automatic ELISA reader (EL-309; Bio-Tech, OSI, F-75015 Paris, France). The antibody concentration was calculated by comparison of A_{490} values with a reference curve obtained from a purified monoclonal mouse IgA or IgG2a (Sigma). Results are expressed as μg/ml. The sensitivity was < 2 ng/ml for both assays.

Measurement of Lf-specific antibodies

IgA and IgG antibodies to Lf in intestinal fluids and serum were quantified by a sandwich ELISA as described by Le Magnen *et al.* (1989). Plate wells were coated with 2 μg Lf/ml in 0·1 M-$NaHCO_3$ buffer, pH 9·6. After blocking, samples were titrated in two step dilutions and incubated on the plates overnight at 4 °C. The sequence described earlier for total Ig was used. Results are given as the reciprocal logarithmic dilution of the last sample dilution that gave an A_{490} of $> 0{\cdot}1$ units. The values obtained with preimmune sample were $< 0{\cdot}1$ units.

Proliferative cell responses

A single-cell suspension of PP or spleen cells was seeded in a flat-bottomed 96-well culture plate (Falcon) at 4×10^6 cells/ml in complete RPMI 1640 medium. Triplicate wells were challenged with 75 μg Lf/ml or 4 μg ConA/ml or complete medium. Incorporation of [6-^{3}H]desoxythymidine (specific activity 185×10^9 Bq/mmol; Amersham, F-91944 Les Ulis Cedex, France) was used as a measurement of DNA synthesis. The cells were pulsed with $2{\cdot}77 \times 10^4$ Bq per well during the last 18 h of incubation periods (24, 48, 72 and 144 h) before freezing. After thawing, the cells were harvested on filter paper and the [^{3}H]thymidine uptake was determined with an automatic filter counting system (INB-384; Inotech, CH-5605 Dottikon, Switzerland). The results are expressed as mean counts/min $\pm$ SE of six means of triplicate cell cultures.

Statistical analysis

Results were expressed as means $\pm$ SE. Differences between experimental and control groups were analysed using analysis of variance (ANOVA). When the treatment effect was significant by ANOVA ($P < 0{\cdot}05$), the significant difference between groups was determined by the Fisher PLSD test.

RESULTS

Total IgA and IgG in saliva, intestinal fluid and serum in mice given Lf

Saliva, intestinal secretions and serum were sampled weekly for 4 weeks from mice given no Lf (water controls), low Lf (0·05 mg Lf/g body weight per d), high Lf (1 mg Lf/g body weight per d) or immunized intramuscularly with Lf in Freund's adjuvant. The samples collected were analysed for total IgA and IgG antibodies (Table 1). As expected, total antibodies were higher in intestinal fluids than in saliva and were highest in serum. After 2 or 3 weeks both IgA and IgG were higher in intestinal fluid and saliva from both low Lf and high Lf mice than in control animals. The concentration of IgG in the intestinal secretions of mice immunized intramuscularly was significantly higher than in those from controls. The concentration of IgA was not significantly different from that of the controls. No significant difference was detected in the serum for IgG or IgA concentrations between the different groups.

https://doi.org/10.1017/S0022029997002732 Published online by Cambridge University Press

Table 1. *Effect of oral or parenteral administration of bovine lactoferrin on total antibody (immunoglobulins A and G) production in saliva, intestinal fluid and serum of mice*

(Values are immunoglobulin concentration, μg/ml, means ± SE for $n = 6$–10)

Sample	Time on diet, d	Mouse group: Water	Low Lf†	High Lf‡	Immunized Lf§
		Total immunoglobulin A			
Saliva	7	0·082 ± 0·008[a]	0·88 ± 0·009[a]	0·102 ± 0·022[a]	—
	14	0·113 ± 0·017[a]	0·117 ± 0·034[a]	0·122 ± 0·023[a]	—
	21	0·109 ± 0·009[a]	0·184 ± 0·025[b]	0·195 ± 0·020[b]	—
	28	0·126 ± 0·007[a]	0·170 ± 0·010[b]	0·206 ± 0·011[b]	Not done
Intestinal fluid	7	14·6 ± 1·1[a]	19·9 ± 1·9[c]	11·6 ± 1·1[a]	—
	14	11·4 ± 0·5[b]	15·0 ± 0·7[d]	16·1 ± 0·3[d]	—
	21	10·6 ± 0·5[b]	12·9 ± 0·9[b, d]	12·5 ± 0·5[a, b]	—
	28	10·7 ± 0·5[b]	13·4 ± 0·3[d]	14·3 ± 0·7[a, d]	12·8 ± 2·1[b]
Serum	7	82·2 ± 10·2[a]	66·2 ± 12·8[a]	72·9 ± 11·3[a]	—
	14	75·6 ± 10·1[a]	67·0 ± 13·1[a]	83·5 ± 6·7[a]	—
	21	66·7 ± 9·2[a]	67·1 ± 14·4[a]	66·2 ± 11·1[a]	—
	28	73·9 ± 10·4[a]	68·1 ± 11·5[a]	71·8 ± 11·4[a]	71·3 ± 4·7[a]
		Total immunoglobulin G			
Saliva	7	0·082 ± 0·016[a]	0·197 ± 0·029[a]	0·133 ± 0·058[a]	—
	14	0·155 ± 0·019[a]	0·183 ± 0·025[a]	0·133 ± 0·033[a]	—
	21	0·126 ± 0·022[a]	0·235 ± 0·035[a]	0·207 ± 0·069[a]	—
	28	0·114 ± 0·021[a]	0·268 ± 0·047[b]	0·310 ± 0·066[b]	Not done
Intestinal fluid	7	4·9 ± 0·8[a]	7·6 ± 1·6[a]	4·6 ± 0·2[a]	—
	14	7·3 ± 1·2[a, b]	7·5 ± 2·1[a, b]	11·6 ± 1·2[b]	—
	21	4·5 ± 0·1[a, b]	15·4 ± 4·8[a, b]	8·1 ± 1·4[b]	—
	28	4·9 ± 0·4[a]	12·7 ± 2·5[c]	13·1 ± 1·4[c]	12·74 ± 0·85[c]
Serum	7	2900 ± 600[a]	4200 ± 800[a]	1900 ± 500[a]	—
	14	3400 ± 800[a]	5100 ± 1200[a]	2200 ± 500[a]	—
	21	3800 ± 1200[a]	4200 ± 800[a]	2700 ± 600[a]	—
	28	3100 ± 600[a]	4700 ± 800[a]	2700 ± 500[a]	5700 ± 950[a]

Lf, lactoferrin.

† Low Lf mice were fed with 0·05 mg Lf/g body weight per d for 4 weeks.

‡ High Lf mice were fed with 1 mg Lf/g body weight per d for 4 weeks.

§ Immunized Lf mice were immunized intramuscularly with 0·01 mg of Lf in complete Freund's adjuvant and 3 weeks later received an intramuscular injection of 0·01 mg Lf in incomplete Freund's adjuvant.

[a, b, c, d] For each antibody (immunoglobulins A and G) and for each sample (saliva, intestinal fluid and serum) values without a common superscript letter were significantly different: $P < 0·05$ (ANOVA and Fisher PLSD tests).

Specific anti-Lf IgA and IgG in saliva, intestinal fluid and serum in mice given Lf

Specific IgA and IgG anti-Lf antibodies were also analysed weekly in saliva, intestinal fluid and serum during the 4 week experiment. Neither IgA nor IgG anti-Lf antibodies could be detected in saliva. Increasing concentrations of both anti-Lf IgA and anti-Lf IgG antibodies were found in intestinal secretions during the 4 weeks of feeding (Table 2). Specific antibodies were also detected in the intestinal secretions from mice immunized intramuscularly, but at lower levels than those from low and high Lf animals. Mice given Lf for 4 weeks also had serum IgG and IgA anti-Lf antibody responses (Table 3). The anti-Lf IgA response in the serum of mice given Lf orally appeared at the end of the second week of feeding and did not differ between low and high dose mice. After 4 weeks feeding, anti-Lf IgA response was close to the response observed with mice immunized intramuscularly. The serum anti-Lf IgG response showed a characteristic production at 2 weeks that did not differ significantly between low and high dose groups. In immunized mice, this response was significantly higher when compared with mice given Lf orally.

https://doi.org/10.1017/S0022029997002732 Published online by Cambridge University Press

Table 2. *Effect of oral or parenteral administration of bovine lactoferrin on specific immunoglobulin A and G immune responses to lactoferrin in intestinal fluids of mice*

(Values are the reciprocal logarithmic dilution of the last sample dilution that gave an A_{490} of $> 0{\cdot}1$ units, means ± SE for $n = 6$–10)

Immunoglobulin isotype	Time on diet, d	Mouse group: Water	Low Lf†	High Lf‡	Immunized Lf§
Anti-lactoferrin immunoglobulin A	0	ND	ND	ND	ND
	7	ND	0·2 ± 0·8	2·1 ± 0·2	—
	14	ND	1·7 ± 1·0	2·7 ± 0·5	—
	21	ND	5·1 ± 0·8	6·7 ± 0·3	—
	28	ND	6·4 ± 0·2	6·8 ± 0·6	8·3 ± 0·9
Anti-lactoferrin immunoglobulin G	0	ND	ND	ND	ND
	7	ND	2·3 ± 0·5	2·3 ± 0·7	—
	14	ND	7·8 ± 0·7	9·2 ± 0·8	—
	21	ND	10·5 ± 0·6	11·2 ± 0·7	—
	28	ND	10·8 ± 0·2	11·5 ± 0·3	7·8 ± 1·1

Lf, lactoferrin; ND, not detected.
† Low Lf mice were fed with 0·05 mg Lf/g body weight per d for 4 weeks.
‡ High Lf mice were fed with 1 mg Lf/g body weight per d for 4 weeks.
§ Immunized Lf mice were immunized intramuscularly with 0·01 mg Lf in complete Freund's adjuvant and 3 weeks later received an intramuscular injection of 0·01 mg Lf in incomplete Freund's adjuvant.

Table 3. *Effect of oral or parenteral administration of bovine lactoferrin on specific immunoglobulin A and G immune responses to lactoferrin in serum of mice*

(Values are the reciprocal logarithmic dilution of the last sample dilution that gave an A_{490} of $> 0{\cdot}1$ units, means ± SE for $n = 6$–10)

Immunoglobulin isotype	Time on diet, d	Mouse group: Water	Low Lf†	High Lf‡	Immunized Lf§
Anti-lactoferrin immunoglobulin A	0	ND	ND	ND	ND
	7	ND	5·7 ± 0·3	0·1 ± 1·2	—
	14	ND	6·2 ± 0·5	6·8 ± 0·6	—
	21	ND	8·6 ± 0·5	10·5 ± 0·6	—
	28	ND	11·1 ± 0·3	11·0 ± 0·4	7·8 ± 1·0
Anti-lactoferrin immunoglobulin G	0	ND	ND	ND	ND
	7	ND	0·05 ± 0·01	0·05 ± 0·01	—
	14	ND	5·2 ± 0·6	6·4 ± 0·8	—
	21	ND	7·1 ± 0·7	8·8 ± 0·3	—
	28	ND	9·8 ± 0·4	10·3 ± 0·9	16·5 ± 1·2

Lf, lactoferrin; ND, not detected.
† Low Lf mice were fed with 0·05 mg Lf/g body weight per d for 4 weeks.
‡ High Lf mice were fed with 1 mg Lf/g body weight per d for 4 weeks.
§ Immunized Lf mice were immunized intramuscularly with 0·01 mg of Lf in complete Freund's adjuvant and 3 weeks later received an intramuscular injection of 0·01 mg Lf in incomplete Freund's adjuvant.

In vitro *IgA and IgG production by PP and spleen cells after oral administration of Lf*

To study the effect of orally administered Lf in antibody production (IgA, IgG) by antigen or mitogen primed cells, PP and spleen cells isolated from control mice and from those given Lf for 4 weeks were restimulated *in vitro* with Lf or LPS. Table 4 shows the IgA and IgG production in PP and spleen culture supernatants after 6 d. No significant increase in IgA and IgG production by PP cells from Lf mice was found after antigen-specific stimulation in comparison with controls. In contrast, we

https://doi.org/10.1017/S0022029997002732 Published online by Cambridge University Press

Table 4. *Effect of oral administration of bovine lactoferrin on total immunoglobulin A and G antibody responses in Peyer's patches and spleen of mice after 6 d incubation in medium alone or in the presence of lactoferrin or lipopolysaccharide*

(Values are immunoglobulin concn, ng/ml, means ± SE for $n = 6$)

Antigen in culture	Mouse group	Peyer's patches		Spleen	
		IgA	IgG	IgA	IgG
Medium	Water	320 ± 116[a]	938 ± 140[a]	807 ± 41[a]	4175 ± 440[a]
	Low Lf†	261 ± 48[a]	1324 ± 510[a]	853 ± 93[a]	4682 ± 1300[a]
	High Lf‡	183 ± 26[a]	756 ± 44[a]	452 ± 32[a]	3291 ± 760[a]
Lactoferrin, 75 μg/ml	Water	282 ± 51[a]	1238 ± 731[a]	1891 ± 188[a]	7240 ± 1300[a]
	Low Lf†	234 ± 35[a]	1941 ± 640[a]	3176 ± 331[b]	14220 ± 1590[b]
	High Lf‡	224 ± 40[a]	1733 ± 782[a]	3621 ± 300[b]	13250 ± 1600[b]
Lipopolysaccharide, 8 μg/ml§	Water	1535 ± 410[b]	7645 ± 1470[b]	3463 ± 176[b]	12370 ± 990[b]
	Low Lf†	1716 ± 340[b]	14390 ± 2500[c]	3212 ± 243[b]	15100 ± 1200[b]
	High Lf‡	772 ± 240[b]	7060 ± 480[b]	4008 ± 155[b]	14261 ± 180[b]

Lf, lactoferrin.
† Low Lf mice were fed with 0·05 mg Lf/g body weight per d for 4 weeks.
‡ High Lf mice were fed with 1 mg Lf/g body weight per d for 4 weeks.
§ Lipopolysaccharide from *Escherichia coli* O111:B4.
[a, b, c] For each site (Peyer's patches and spleen) and for each antibody (immunoglobulins A and G), values without a common superscript letter were significantly different: $P < 0·05$ (ANOVA and Fisher PLSD tests).

found an increase in IgG concentrations in LPS-stimulated PP cell cultures from mice given low doses. Lf-stimulated splenocytes from Lf mice produced IgA and IgG levels that were significantly higher than those from control animals. Ig production by spleen cells was not significantly enhanced in LPS-stimulated cell cultures from mice given Lf in comparison with controls. Specific antibodies could not be detected in supernatants from either PP or spleen cell cultures.

Proliferative response of PP and spleen cells after oral administration of Lf

In order to determine the effect of feeding Lf on PP and spleen cell proliferation, [^{3}H]thymidine incorporation was measured after 1, 2, 3 or 6 d of culturing PP and spleen cell preparations in the absence (medium alone) or presence of either 75 μg Lf/ml or 4 μg ConA/ml (Table 5). For each group of mice, the proliferation of PP cells was significantly higher in the presence of 75 μg Lf/ml as compared with the control group without Lf, whereas thymidine incorporation in spleen cells was enhanced in the presence of Lf after 1, 2 and 3 d culture, as compared with the control group without Lf. In addition, polyclonal stimulation of spleen cells by 4 μg ConA/ml resulted in intense proliferative responses in all groups when compared with cells incubated in the presence or absence of 75 μg Lf/ml. When cells were incubated 1–3 d without Lf (medium), the proliferation of spleen cells was significantly higher in the oral Lf group when compared with control mice. This effect was not found for PP cells incubated under the same conditions without Lf. When PP and spleen lymphoid cells were incubated (1, 2 or 3 d) with 75 μg Lf/ml, proliferation was significantly higher in the group of mice given Lf orally in comparison with the control group. No significant difference was detected between all groups after 6 d. Polyclonal stimulation of spleen cells by 4 μg ConA/ml resulted in significantly higher proliferation in Lf groups than in the mice given water.

https://doi.org/10.1017/S0022029997002732 Published online by Cambridge University Press

Table 5. *Effect of oral administration of bovine lactoferrin on cell proliferative responses in Peyer's patches and spleen of mice after incubation in medium alone or in the presence of lactoferrin or concanavalin A*

(Values are [^{3}H]thymidine uptake, counts/min, means ± SE for $n = 6$)

Antigen in culture	Mouse group	Culture time, d			
		1	2	3	6
		Peyer's patches			
Medium	Water	124 ± 10[a]	471 ± 10[a]	2592 ± 374[a]	245 ± 6[a]
	Low Lf†	185 ± 28[a]	715 ± 118[a]	2996 ± 305[a]	343 ± 6[a]
	High Lf‡	164 ± 4[a]	529 ± 62[a]	3139 ± 354[a]	696 ± 352[a]
Lactoferrin, 75 μg/ml	Water	246 ± 16[b]	1790 ± 126[b]	3914 ± 129[b]	1682 ± 361[b]
	Low Lf†	274 ± 37[a, b]	2367 ± 30[c]	4953 ± 502[c]	1828 ± 41[b]
	High Lf‡	243 ± 9[b]	2177 ± 15[c]	4513 ± 191[b]	1978 ± 325[b]
		Spleen			
Medium	Water	419 ± 61[a]	1883 ± 80[a]	3660 ± 984[a]	7704 ± 1265[a]
	Low Lf†	690 ± 88[b]	3197 ± 425[b]	3981 ± 515[a]	2710 ± 435[a]
	High Lf‡	841 ± 42[b]	3602 ± 340[b]	9058 ± 1265[b]	4676 ± 2134[a]
Lactoferrin 75 μg/ml	Water	662 ± 28[b]	2915 ± 551[b]	7700 + 672[b]	9437 ± 1621[a]
	Low Lf†	1150 ± 130[c]	4162 ± 712[b]	13144 ± 1981[c]	9647 ± 2318[a, b]
	High Lf‡	1169 ± 101[c]	4645 ± 284[b]	17596 ± 1222[c]	5782 ± 2136[a]
Concanavalin A, 4 μg/ml	Water	2146 ± 419[d]	42475 ± 5060[c]	27631 ± 7746[c]	16526 ± 4538[b]
	Low Lf†	4357 ± 710[e]	60127 ± 4502[d]	42002 ± 12149[c]	18691 ± 2068[b]
	High Lf‡	3770 ± 538[d]	64162 ± 3850[d]	42062 ± 2637[d]	10846 ± 1158[b]

Lf, lactoferrin.

† Low Lf mice were fed with 0·05 mg Lf/g body weight per d for 4 weeks.

‡ High Lf mice were fed with 1 mg Lf/g body weight per d for 4 weeks.

[a, b, c, d, e] For each site (Peyer's patches and spleen) and for each time (1–6 d), values without a common superscript letter were significantly different: $P < 0·05$ (ANOVA and Fisher PLSD tests).

DISCUSSION

In the present investigation, we have shown that repeated Lf given orally as an antigen could lead to the biosynthesis of specific A and G isotype antibodies in both intestinal secretions and serum. In addition, the present results provide strong evidence that bovine Lf given orally acted as a growth modulating agent of the intestinal immune system in mice.

Other studies have shown little or no specific IgA antibodies in intestinal secretions against BSA or keyhole limpet haemocyanin with one oral administration (Elson & Ealding, 1984; De Aizpurua & Russell-Jones, 1988). However, specific antibody responses to oral keyhole limpet haemocyanin antigens have been detected in the intestinal secretion from mice that were given the antigen more than once (Van der Heijden *et al.* 1991). Whey milk protein antigens can induce an immune response because their degradation is reduced in the gastrointestinal tract and absorption can occur as an intact antigenic form (Coste *et al.* 1992). Bovine Lf (specially iron-saturated Lf) is relatively resistant to proteolysis *in vivo* (Spik *et al.* 1982) and *in vitro* (Brines & Brock, 1983). Degradation by enzymic digestion in the gastrointestinal tract could give immunogenic forms that have the ability to provide an antigenic stimulus. The transport of the immunogenic forms of Lf could occur at two sites, through specialized M cells and/or enterocytes. Hu *et al.* (1988) demonstrated the presence of Lf receptors on mouse intestinal epithelial cells. These receptors are able to bind both mouse and bovine Lf. The induction of immune responses seems to be mediated through the binding of Lf to its intestinal receptors, since sera from mice fed with modified Lf (Lf covalently coupled to α-lactalbumin) had lower levels of

https://doi.org/10.1017/S0022029997002732 Published online by Cambridge University Press

serum specific IgG antibodies, compared with those from mice fed with native Lf (results not shown). These findings are in agreement with those of De Aizpurua & Russell-Jones (1988), who concluded that the binding of mucosal immunogens such as lectin-like proteins to the intestinal epithelium is a prerequisite for eliciting immune responses. Mikogami *et al.* (1994) showed that a small fraction ($\sim 10\%$) of human immunogenic Lf is transported across HT-29 intestinal epithelial cells. In addition, the induction of oral tolerance by protein antigens depends on the way they are taken up and processed by both intestinal cells and antigen-presenting cells (macrophages, B cells and dendritic cells) within the immune system (Mowat, 1987; Panja & Mayer, 1994). Specific binding of Lf has been reported for intestinal epithelial cells, B lymphocytes and macrophages or monocytes (Bennett & Davis, 1981; Hu *et al.* 1988; Sato *et al.* 1990; Eda *et al.* 1997). Lf protein antigen could possibly stimulate the activity of antigen-presenting cells, which would affect the outcome of the systemic antibody responses.

Bovine Lf protein antigen promoted specific humoral responses by two routes of administration (oral *v.* peripheral). Systemic immunization with Lf mainly induced IgA and IgG anti-Lf antibody response in serum. Intestinal fluid response remained low. In agreement with current studies, the route of antigen administration, the number of feedings and the nature of the protein seemed critical factors for eliciting local and peripheral responses, and greatly modulate the immune responses. The presence of serum specific IgG antibodies following repeated ingestion of Lf corroborates previous observations obtained with milk proteins in infants and rats (Host *et al.* 1992; Enomoto *et al.* 1993). This systemic stimulation after oral administration of Lf raises the hypothesis that an antigenic fraction of either the absorbed Lf or Lf-activated lymphocytes reaches the systemic circulation. The concept of a 'common mucosal immune system' is well known (Mestecky *et al.* 1994). Lymphocytes stimulated in mucosal inductive sites (e.g. PP) migrate to mucosal effector tissues via the systemic circulation. The stimulation of a specific site, i.e. mucosal *v.* systemic, could also in part be related to the direct exposure to the antigen of each specific inductive site, i.e. PP, spleen and lymph nodes. The low level of intestinal response induced by intramuscular administration of Lf in Freund's adjuvant could be related to a limited access of Lf to the PP. In contrast, mucosal and peripheral humoral stimulation produced by oral administration of Lf could originate from direct stimulation by Lf of both PP and lymph nodes as mucosal and systemic inductive sites respectively. Under these conditions, we can speculate that Lf given orally could gain access to the systemic circulation. In fact, it seems likely that both mechanisms, i.e. direct Lf antigen circulation as well as enteroenteric circulation of activated lymphocytes, were involved in the immune response to orally administered Lf. In the mucosal (PP) and peripheral lymphoid tissues (lymph nodes, spleen), lymphocytes that carry B cell or T cell receptors with the appropriate specificity will bind to free antigen or to fragments presented by macrophages. In this case, they become activated and differentiate into mature anti-Lf cells: helper T cells and B cells that collaborate to promote an antibody response. Specific anti-Lf antibodies and their plasma cells (mature B lymphocytes) leave the lymphoid organs and enter the blood stream. The lack of detection of specific antibodies in *in vitro* PP and spleen cell cultures stimulated with Lf or LPS originated from the low Ig concentrations in cell culture supernatants.

This study also suggested that Lf was able to induce nonspecific activation of both intestinal and peripheral lymphocytes. Indeed, the amounts of total IgA and IgG were increased in intestinal fluids. These findings should not be taken to apply

https://doi.org/10.1017/S0022029997002732 Published online by Cambridge University Press

to the production of specific antibodies, since other workers did not find any change in total IgA in intestinal fluids from mice given keyhole limpet haemocyanin or cholera toxin, while specific antibodies have been detected in intestinal secretions (Elson *et al*. 1984). In addition, and in agreement with previous reports (Hashizume *et al*. 1983), we have also observed that bovine Lf is able to stimulate *in vitro* murine lymphocyte cell proliferation and Ig production. In contrast, Shida *et al*. (1992) reported that bovine whey proteins did not induce *in vitro* IgA production in PP cell cultures in mice. We have also shown that Lf given orally could act as a factor inducing proliferation of PP and spleen cells. These observations provide evidence that Lf interacted with B and/or T cells in mucosal inductive sites (e.g. PP), and probably in systemic lymphoid tissue (e.g. spleen). The stimulatory effect of Lf has also been observed with human phytohaemagglutinin-activated lymphocytes and is related to the iron-binding activity of this protein (Mazurier *et al*. 1989). This effect may be mediated by its specific receptor on the cell surface and/or by its potential direct transcriptional function of Lf (He & Furmanski, 1995). The specific role of Lf in cellular responses is controversial and its mechanisms have not been elucidated. The present study extends the properties of Lf to include an immunostimulating activity on the mucosal immune system, by leading to activation of immunocompetent cells (B or T lymphocytes).

REFERENCES

Bennett, R. M. & Davis, J. 1981 Lactoferrin binding to human peripheral blood cells: an interaction with a B-enriched population of lymphocytes and a subpopulation of adherent mononuclear cells. *Journal of Immunology* **127** 1211–1216

Brines, R. D. & Brock, J. H. 1983 The effect of trypsin and chymotrypsin on the *in vitro* antimicrobial and iron-binding properties of lactoferrin in human milk and bovine colostrum: unusual resistance of human apolactoferrin to proteolytic digestion. *Biochimica et Biophysica Acta* **759** 229–235

Caillard, I. & Tomé, D. 1994 Modulation of β-lactoglobulin transport in rabbit ileum. *American Journal of Physiology* **266** G1053–G1059

Challacombe, S. J. & Tomasi, T. B. 1980 Systemic tolerance and secretory immunity after oral immunization. *Journal of Experimental Medicine* **152** 1459–1472

Chierici, R., Sawatzki, G., Tamisari, L., Volpato, S. & Vigi, V. 1992 Supplementation of an adapted formula with bovine lactoferrin. 2. Effects on serum iron, ferritin and zinc levels. *Acta Pœdiatrica* **81** 475–479

Coste, M., Huneau, J.-F., Mahé, S. & Tomé, D. 1992 Interactions between milk protein peptides and intestinal mucosa. In *Foods, Nutrition and Immunity*, vol. 1, pp. 96–103 (Eds M. Paubert-Braquet, C. Dupont and R. Paoletti). Basel: Karger

De Aizpurua, H. J. & Russell-Jones, G. J. 1988 Oral vaccination: identification of classes of proteins that provoke an immune response upon oral feeding. *Journal of Experimental Medicine* **167** 440–451

Eda, S., Kikugawa, K. & Beppu, M. 1997 Characterization of lactoferrin-binding proteins of human macrophages membrane: multiple species of lactoferrin-binding proteins with polylactosamine-binding ability. *Biological and Pharmaceutical Bulletin* **20** 127–133

Elson, C. O. & Ealding, W. 1984 Generalized systemic and mucosal immunity in mice after mucosal stimulation with cholera toxin. *Journal of Immunology* **132** 2736–2741

Elson, C. O , Ealding, W. & Lefkowitz, J. 1984 A lavage technique allowing repeated measurement of IgA antibody in mouse intestinal secretions. *Journal of Immunological Methods* **67** 101–108

Enomoto, A., Konishi, M., Hachimura, S. & Kaminogawa, S. 1993 Milk whey protein fed as a constituent of the diet induced both oral tolerance and a systemic humoral response, while heat-denatured whey protein induced only oral tolerance. *Clinical Immunology and Immunopathology* **66** 136–142

Haneberg, B., Kendall, D., Amerongen, H. M., Apter, F. M., Kraehenbuhl, J.-P. & Neutra, M. R. 1994 Induction of specific immunoglobulin A in the small intestine, colon-rectum, and vagina measured by a new method for collection of secretions from local mucosal surfaces. *Infection and Immunity* **62** 15–23

Hashizume, S., Kuroda, K. & Murakami, H. 1983 Identification of lactoferrin as an essential growth factor for human lymphocytic cell lines in serum-free medium. *Biochimica et Biophysica Acta* **763** 377–382

He, J. L. & Furmanski, P. 1995 Sequence specificity and transcriptional activation in the binding of lactoferrin to DNA. *Nature* **373** 721–724

Host, A., Husby, S., Gjesing, B., Larsen, J. N. & Lowenstein, H. 1992 Prospective estimation of IgG, IgG subclass and IgE antibodies to dietary proteins in infants with cow milk allergy. Levels of antibodies to whole milk protein, βLG and ovalbumin in relation to repeated milk challenge and clinical course of cow milk allergy. *Allergy* **47** 218–229

https://doi.org/10.1017/S0022029997002732 Published online by Cambridge University Press

Hu, W.-L., Mazurier, J., Sawatzki, G., Montreuil, J. & Spik, G. 1988 Lactotransferrin receptor of mouse small-intestinal brush border. Binding characteristics of membrane-bound and Triton X-100-solubilized forms. *Biochemical Journal* **249** 435–441

Iyer, S. & Lönnerdal, B. 1993 Lactoferrin, lactoferrin receptors and iron metabolism. *European Journal of Clinical Nutrition* **47** 232–241

Le Magnen, C., Rainard, P., Maubois, J. L., Paraf, A. & Phan Thanh, L. 1989 Enzyme linked immunosorbent assay for bovine lactoferrin titration. *Lait* **69** 23–32

Lönnerdal, B. & Iyer, S. 1995 Lactoferrin: molecular structure and biological function. *Annual Review of Nutrition* **15** 93–110

Marcon-Genty, D., Tomé, D., Dumontier, A. M., Kheroua, O. & Desjeux, J.-F. 1989 Permeability of milk protein antigens across the intestinal epithelium *in vitro*. *Reproduction, Nutrition, Development* **29** 717–723

Mazurier, J., Legrand, D., Hu, W. L., Montreuil, J. & Spik, G. 1989 Expression of human lactotransferrin receptors in phytohemagglutinin-stimulated human peripheral blood lymphocytes. Isolation of the receptors by antiligand-affinity chromatography. *European Journal of Biochemistry* **179** 481–497

Mestecky, J., Abraham, R. & Ogra, P. L. 1994 Common mucosal immune system and strategies for the development of vaccines effective at the mucosal surfaces. In *Handbook of Mucosal Immunology*, pp. 357–372 (Eds P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. R. McGhee and J. Bienenstock). San Diego, CA: Academic Press

Mikogami, T., Heyman, M., Spik, G. & Desjeux, J.-F. 1994 Apical-to-basolateral transepithelial transport of human lactoferrin in the intestinal cell line HT-29cl.19A. *American Journal of Physiology* **267** G308–G315

Mowat, A. McI. 1987 The regulation of immune responses to dietary protein antigens. *Immunology Today* **8** 93–98

Mowat, A. McI. 1994 Oral tolerance and regulation of immunity to dietary protein antigens. In *Handbook of Mucosal Immunology*, pp. 185–201 (Eds P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. R. McGhee and J. Bienenstock). San Diego, CA: Academic Press

Panja, A. & Mayer, L. 1994 Diversity and function of antigen-presenting cells in mucosal tissues. In *Handbook of Mucosal Immunology*, pp. 177–183 (Eds P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. R. McGhee and J. Bienenstock). San Diego, CA: Academic Press

Roberts, A. K., Chierici, R., Sawatzki, G., Hill, M. J., Volpato, S. & Vigi, V. 1992 Supplementation of an adapted formula with bovine lactoferrin. 1. Effect on the infant faecal flora. *Acta Pœdiatrica* **81** 119–124

Sato, K., Shinomoto, H., Tanimoto, M., Dosako, S. & Nakajima, I. 1990 Uptake and re-secretion of human lactoferrin by B lymphocytes. *Agricultural and Biological Chemistry* **54** 1275–1279

Shida, K., Nagami, K. & Takamizawa, K. 1992 Effect of milk components on IgA production in Peyer's patch cell cultures from mouse. *Bioscience Biotechnology Biochemistry* **56** 1874–1875

Spik, G., Brunet, B., Mazurier-Dehaine, C., Fontaine, G. & Montreuil, J. 1982 Characterization and properties of the human and bovine lactotransferrins extracted from the fæces of newborn infants. *Acta Pœdiatrica Scandinavica* **71** 979–985

Teraguchi, S., Shin, K., Ogata, T., Kingaku, M., Kaino, A., Miyauchi, H., Fukuwatari, Y. & Shimamura, S. 1995 Orally administered bovine lactoferrin inhibits bacterial translocation in mice fed bovine milk. *Applied and Environmental Microbiology* **61** 4131–4134

Van der Heijden, P. J., Bianchi, A. T. J., Dol, M., Pals, J. W., Stok, W. & Bokhout, B. A. 1991 Manipulation of intestinal immune responses against ovalbumin by cholera toxin and its B subunit in mice. *Immunology* **72** 89–93

Zimecki, M., Mazurier, J., Machnicki, M., Wieczorek, Z., Montreuil, J. & Spik, G. 1991 Immunostimulatory activity of lactotransferrin and maturation of CD4-CD8-murine thymocytes. *Immunology Letters* **30** 119–124

Zimecki, M., Mazurier, J., Spik, G. & Kapp, J. A. 1995 Human lactoferrin induces phenotypic and functional changes in murine splenic B cells. *Immunology* **86** 122–127

https://doi.org/10.1017/S0022029997002732 Published online by Cambridge University Press