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Systemic or local co-administration of lactoferrin with sensitizing dose of antigen enhances delayed type hypersensitivity in mice

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Abstract

Lactoferrin (LF), a major defense protein synthesized and stored in granulocytes has been implicated in maintaining immune homeostasis during an insult-induced metabolic imbalance. In this study, we demonstrated that lactoferrin augments the delayed type hypersensitivity (DTH) response to specific antigens in mice. Lactoferrin (LF) was given to mice orally or intraperitoneally (i.p.) at the time of immunization, or subcutaneously (s.c.) in a mixture with the immunizing doses of the following antigens, sheep red blood cells (SRBC), Calmette–Guerin bacillus (BCG) or ovalbumin (OVA). A DTH reaction was determined 24 h after administration of an eliciting dose of antigen as a specific increase in foot pad swelling. Lactoferrin enhanced DTH reaction to all studied antigens in a dose-dependent manner. Lactoferrin (LF) given to mice in conjunction with antigen administered in an incomplete Freund's adjuvant induced the DTH response at the level of control mice given antigen in a complete Freund's adjuvant. In addition, LF remarkably increased DTH response to a very small, otherwise non-immunogenic SRBC dose. The increase in DTH response was less pronounced for orally administered LF than for any other routes of administration, however, statistically significant augmentation was demonstrated for each antigen studied. Although the costimulatory action of LF was accompanied by the appearance of bovine lactoferrin-specific cellular responses in mice, it is very unlikely that such responses will be generated in humans, since bovine lactoferrin is a dietary antigen to which a tolerance has been acquired. Considering the involvement of LF in generation of stimulatory signals during the induction phase of an antigen specific immune responses, we suggest that LF may be useful for development of safer and more efficacious vaccination protocols. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Lactoferrin; Adjuvant; Vaccine; Immunoregulation

1. Introduction

Generation and maintenance of specific and long-lasting cellular immune responses is crucial for resistance of animals and humans to pathogens. This can be achieved by multiple vaccination with killed or attenuated pathogens together with adjuvants (reviewed in references [1,2]). Adjuvants are included in vaccine formulations to make the immune response more effective, or in some cases, to make an immune response occur. Classical adjuvants, e.g. Freund's complete adjuvant, consist of a vehicle (a mixture of mineral oil and detergent) and a co-stimulatory factor (suspension of killed Guerin–Calmette bacillus or protein A isolated from the cell wall of these bacteria). The role of a vehicle is to disperse antigen and to prolong its exposure to the antigen-processing cells. The costimulatory molecules, in turn, activate antigen-presenting cells to more efficient antigen processing and secretion of cytokines relevant in cell-to-cell cooperation during the

Abbreviations: BCG, Calmet–Guerin bacillus; cFA, complete Freund's adjuvant; DTH, delayed type hypersensitivity; iFA, incomplete Freund's adjuvant; i.p., intraperitoneally; s.c., subcutaneously; SRBC, sheep red blood cells.

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early steps of an immune response [3]. Crude preparations of bacterial antigens, have been, so far, proven to be most efficient co-stimulatory molecules [1–3]. Their use, however, is limited due to inflammatory side-ef fects. More purified fragments of bacterial cell walls or synthetic molecules are devoid of undesirable side effects, their effectiveness at the same time, measured by the augmentation of the immune response is much smaller [4]. So far, the only approved adjuvant for use in humans is aluminum hydroxide (alum). Application of alum allowed to diminish many times otherwise toxic doses of immunogens (diphtheria–pertusis–tetanus vaccine).

For the reasons mentioned above, there is a high demand for safe, biologically degradable, co-stimulatory molecules which can replace classical adjuvants. Evidence is accumulating that cytokines may be effective in elevating specific immune resistance to pathogens [5,6]. Our interest in lactoferrin, a multifunctional defense protein, often regarded as cytokine (reviewed in references [7–10]), has prompted us to investigate its potential in generation of the cellular immune response, during the induction phase of an antigen specific response in mice. LF is a 80 kDa glycoprotein involved in iron metabolism [10]; it is present in mucosal secretion of mammals as well as in secondary granules of granulocytes [7,8]. Lactoferrin (LF) exhibits antimicrobial [12–14], antifungal [15], antiviral [16] and antitumor [17,18] properties, it also activates macrophages [19,20] and natural killer cells [21]. Lactoferrin (LF) exhibits immunoregulatory properties; it may be prooxidant [22] and pro-inflammatory [19,23], or antioxidant [24,25] and anti-inflammatory [11,26–28]. The protein was shown to promote maturation of T [29] and B [30] cells and inhibits the effector phase of the cellular immune response [31]. The immunoregulatory action of LF was recently demonstrated in vivo in endotoxemic mice [32], as well as in vivo [33] and in vitro [34] in the human model. The regulatory action on cells of the immune system may be direct [35] or indirect via cytokines induced by LF in target cells [11,19,32]. The cell receptors for LF were found on various cell types including brush border cells [36], monocytes [37] and activated lymphocytes [38].

Taking into account the desirable features of LF such as, presence of specific cell receptors [36–38], ability to transduce activation intracellular signals [35] induction of cytokines [19,28], upregulation of adhesion molecule expression [39], biodegradability [40–42], and its effectiveness at oral administration [33,40–45], our objective was to substantiate the potential use of lactoferrin as an adjuvant for future vaccination in humans and other animals. We tested our hypothesis in mice using three different antigens — sheep red blood cells, bacterial antigen, Guerin–Calmette bacillus, and a soluble protein ovalbumin.

2. Materials and methods

2.1. Animals

CBA mice (8–12-week old) of both sexes were delivered by the Animal Facility of the Institute of Immunology and Experimental Therapy, Wroclaw, Poland. Mice were fed commercial, pelleted food and water ad libitum.

2.2. Antigens, adjuvants and reagents

Sheep red blood cells were delivered by the Wroclaw Agriculture Academy. Sheep red blood cells (SRBC) were kept in Alsever's medium and washed three times with PBS before use. Lyophilized BCG (lot 360388/s) and old tuberculin (tuberculin pristinum concentratum) lot 10173, were purchased from Biomed, Cracov. Low endotoxin bovine milk lactoferrin (<1 E.U./mg, $<25\%$ iron saturated), ovalbumin lot 43H7010, complete Freund's adjuvant (cFA) lot F-4258, and incomplete Freund's adjuvant (iFA), lot F 5506 were purchased from Sigma Chemical Company, MO, USA.

2.3. Generation of the cellular immune response to sheep red blood cells

Mice were immunized with 10^8 SRBC in 0.1 ml cFA into tail base. After 4 days, the delayed type hypersensitivity reaction was elicited by subcutaneous (s.c.) administration of 10^8 SRBC in 0.05 ml PBS in both hind foot pads. Following the next 24 h, the foot-pad swelling was measured. The antigen specific reaction to SRBC was calculated by subtracting SRBC-elicited reaction in nonsensitized mice from SRBC-elicited reaction of sensitized animals.

2.4. Generation of the cellular immune response to BCG

Mice were immunized s.c. into tail base with 0.1 ml of Freund's complete adjuvant containing 0.1 mg of lyophilized BCG. After 4 days, the delayed type hypersensitivity reaction was elicited by s.c. administration of 0.05 ml of old tuberculin (OT) — final concentration, 1:500 — emulsified in Freund's incomplete adjuvant into one hind foot pad. The other foot pad (control) was injected with iFA only. The foot-pad swelling was measured 24 h later with a caliper (accuracy 0.05 mm). The antigen specific reaction was calculated by subtracting OT-elicited reaction of non-sensitized mice from BCG-sensitized mice.

2.5. Generation of the cellular immune response to ovalbumin

Mice were immunized s.c. into tail base with 5 μg ovalbumin (OVA) in cFA or iFA. Lactoferrin (LF; 10–250 μg) was admixed with the immunizing dose of OVA in iFA and delivered intraperitoneally (i.p.) or it was given per os (1–10 mg) at the time of immunization. After 4 days, the DTH reaction was elicited by s.c. injection of 50 μg OVA in iFA into hind feet. Specific DTH reaction was calculated by subtracting the foot pad thickness of naive mice given eliciting dose of antigen from DTH reaction of sensitized mice.

2.6. Statistics

All data are expressed in DTH units (1 unit = 0.1 mm) as mean values from ten determinations ± standard error (S.E.). Differences between groups were analyzed by the Student unpaired t-test when two groups were analyzed and analysis of variance (ANOVA) when more than two groups were analyzed.

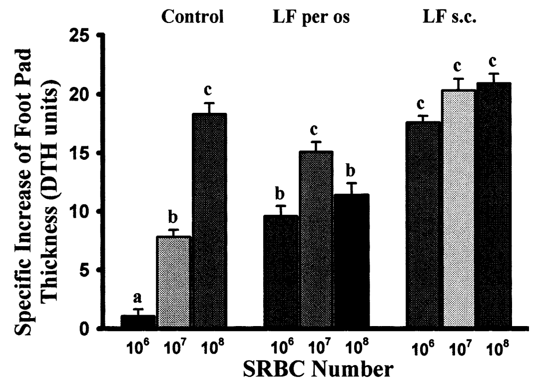

Fig. 2. Co-stimulatory effects of LF on SRBC-induced delayed type hypersensitivity-suboptimal dose of SRBC. Mice were immunized with SRBC in iFA s.c. into tail base (control). Lactoferrin (LF) was given per os at the time of immunization (5 mg) or was admixed with the immunizing dose of SRBC (250 μg). Four days later the DTH reaction was elicited by s.c. administration of 10^8 SRBC in iFA. Antigen-specific response was calculated by subtracting nonspecific reaction caused by injection of the eliciting dose of antigen into foot pads of naive mice. Any two means within a given experimental setting not identified by the same letter are significantly different ($P < 0.05$).

3. Results

3.1. Stimulation of SRBC-induced delayed type hypersensitivity by LF

Effects of LF given to mice i.p., per os or s.c., on SRBC-induced DTH are presented in Fig. 1. The results are expressed as specific increase of foot-pad thickness 24 h following elicitation of the reaction. The foot-pad reaction was moderately increased when LF was administered i.p. or per os (5-mg dose) but a significantly higher increase was noted when LF (50 or 250 μg) was admixed with the immunizing dose of antigen and given s.c. Also, LF given to mice per os, or admixed with the sensitizing suboptimal dose of antigen and given s.c., greatly enhanced DTH response, the effects being more evident in the latter case (Fig. 2). The most spectacular effect of LF occurred at minimal antigen dose (10^6 SRBC), normally non-immunogenic (1.07 DTH units) to mice. In this case, DTH responses were nine- to seventeen-fold augmented by lactoferrin given orally or s.c., respectively. Also, LF given to mice with suboptimal dose of antigen (10^7 SRBC), significantly enhanced DTH response. Lactoferrin given to mice with the optimal dose of immunizing agent (10^8 SRBC) did not stimulate DTH responses (s.c.) or even suppressed this reaction (oral LF).

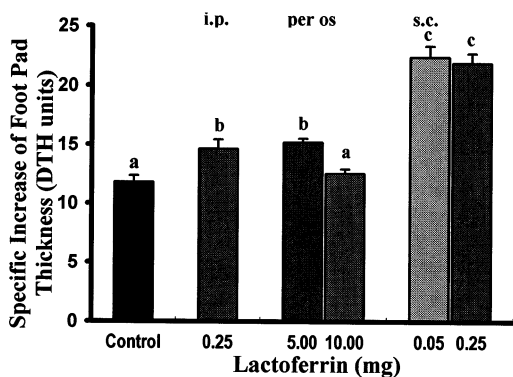

Fig. 1. Co-stimulatory effects of LF on SRBC-induced delayed type hypersensitivity. Mice were immunized s.c. in tail base with 10^8 SRBC in cFA (control). Lactoferrin (LF) was given i.p. or per os at the time of immunization, or it was administered s.c. together with the sensitizing dose of antigen. DTH reaction was elicited in hind foot pads by injection of 10^8 SRBC in PBS. The foot-pad swelling was measured after 24 h and expressed in DTH units. Any two means within a given experimental setting not identified by the same letter are significantly different ($P < 0.05$).

In addition, lactoferrin used as adjuvant to augment SRBC-induced response, developed a concomitant LF-specific response (Fig. 7).

3.2. Stimulation of BCG-induced delayed type hypersensitivity by LF

The effects of LF given to mice per os or s.c., on BCG-induced DTH are presented in Figs. 3 and 4, respectively. The stimulatory activity of LF in this antigen model was similar to that described for SRBC. It was dose-dependent and more efficient in the case of s.c. administration.

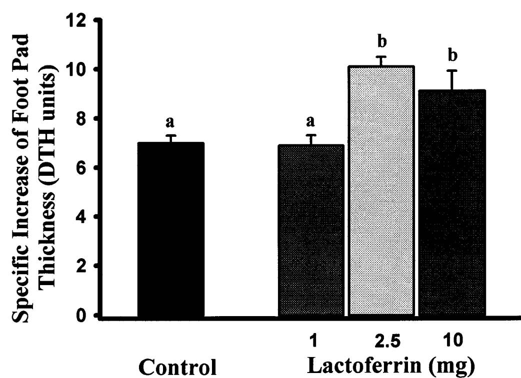

Fig. 3. Co-stimulatory effects of LF on BCG-induced delayed type hypersensitivity. Mice were immunized s.c. into tail base with 0.1 mg BCG in cFA. Lactoferrin (LF) was given per os at the time of immunization with BCG in cFA. Four days later, the DTH reaction was elicited by administration of 0.05 ml of OT (1:500) emulsified with iFA. Control foot pads received iFA only. The reaction was elicited analogously in control, naive (nonimmunized mice). The nonspecific reaction caused by OT irritation in control mice was subtracted. Any two means within a given experimental setting not identified by the same letter are significantly different ($P < 0.05$).

3.3. *Stimulation of ovalbumin-induced delayed type hypersensitivity by LF*

Fig. 5 shows that LF admixed with the sensitizing dose of OVA at the concentration 0.05 or 0.250 mg, and emulsified in Freund's incomplete adjuvant, enhanced DTH responses to the level of control group receiving OVA in complete Freund's adjuvant. Similar results were obtained for lactoferrin given i.p. or per os at the time of immunization (Figs. 6 and 7).

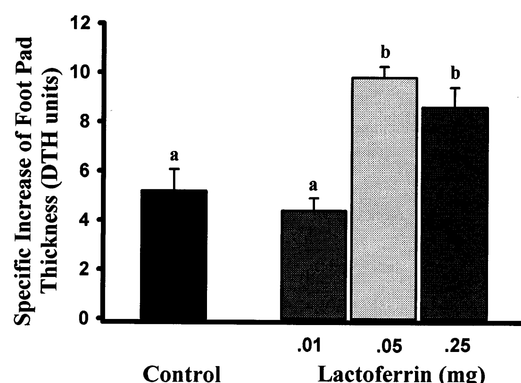

Fig. 4. Co-stimulatory effects of LF on BCG-induced delayed type hypersensitivity. Mice were immunized s.c. into tail base with 0.1 mg BCG in cFA. Lactoferrin (LF) was given s.c. together with BCG in cFA. Four days later the DTH reaction was elicited by administration of 0.05 ml of OT (1:500) emulsified with iFA. Control foot pads received iFA only. The reaction was elicited analogously in control, naive (nonimmunized mice). The nonspecific reaction caused by OT irritation in control mice was subtracted. Any two means within a given experimental setting not identified by the same letter are significantly different ($P < 0.05$).

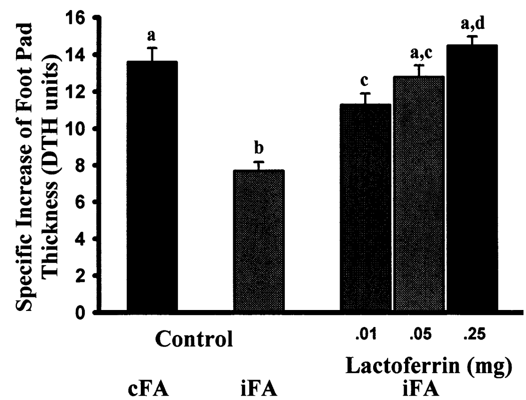

Fig. 5. Co-stimulatory effects of LF on ovalbumin-induced delayed type hypersensitivity. Mice were immunized s.c. into tail base with 5 μg OVA in cFA or iFA (Controls). Lactoferrin (LF) was admixed with the immunizing dose of OVA in iFA (10–250 μg per dose) and delivered s.c. into mice tail base. After 4 days the DTH reaction was elicited by s.c. injection of 50 μg OVA in iFA into hind feet. Any two means within a given experimental setting not identified by the same letter are significantly different ($P < 0.05$).

4. Discussion

The results presented in this study revealed stimulatory properties of lactoferrin in generation of cellular immune response in mice. The immune response was measured by the delayed type hypersensitivity induced by specific antigen. Lactoferrin (LF) was able to amplify the immune response when admixed with the sensitizing dose of antigen or when given orally to mice at the time of immunization. Lactoferrin (LF) exhibited remarkable co-stimulatory properties in augmentation of the immune response to sub-optimal doses of antigen. In addition, non-immunogenic doses of antigen induced normal immune response when co-administered with LF. The results presented in this study

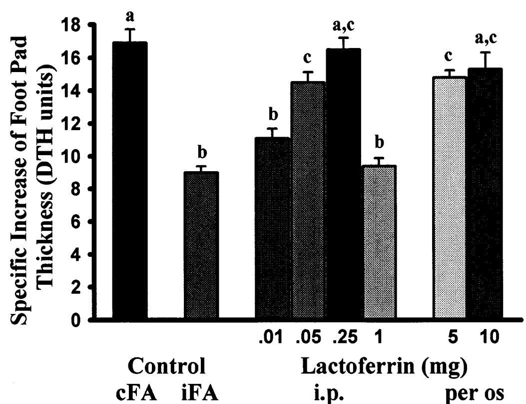

Fig. 6. Co-stimulatory effects of LF on ovalbumin-induced delayed type hypersensitivity. Mice were immunized s.c. into tail base with 5 μg OVA in cFA or in iFA (Controls). Lactoferrin (LF) was administered i.p. or per os at the time of immunization. After 4 days the DTH reaction was elicited by s.c. injection of 50 μg OVA in iFA into hind feet. Any two means within a given experimental setting not identified by the same letter are significantly different ($P < 0.05$).

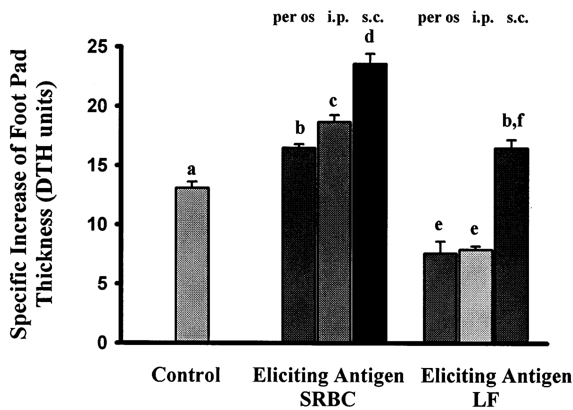

Fig. 7. Co-stimulatory effects of LF associated with the induction of LF-specific delayed type hypersensitivity. Mice were immunized with 10^8 SRBC in 0.1 ml iFA into tail base and treated with bovine LF per os (5 mg), i.p. (250 μg), or s.c. (50 μg). In the case of s.c. administration, LF was admixed with SRBC emulsified in iFA. After 4 days, each experimental group was divided into two subgroups and the delayed type hypersensitivity reaction was elicited either by s.c. administration of 10^8 SRBC in iFA or bovine LF (50 μg) in iFA into hind foot pads. Following next 24 h, the foot pad swelling was measured. The antigen specific responses were calculated by subtracting SRBC- or LF-elicited reaction in non-sensitized mice from SRBC- or LF-elicited reaction of sensitized animals. Any two means within a given experimental setting not identified by the same letter are significantly different ($P < 0.05$).

provide strong evidence that LF administered orally or peripherally can act as an immunological adjuvant.

The concept of common mucosal immune system has been established previously and relies on the migration of lymphocytes from the mucosal inductive sites to the effector tissues [46]. Oral administration of LF was shown to stimulate gut associated lymphoid tissue and systemic immune response [43]. Lactoferrin can cross the gut barrier [13,42,43] in a form of various degradation products [40,41] and also in part as an intact molecule [42], thus enabling interaction with lymphoid cells from Peyer's patches and mesenteric lymph nodes. It is also possible that LF can bind LPS in the gut since it shows a high affinity to form complexes [47] and in such a form to interact with immunocompetent cells. We recently showed (unpublished data) that less purified batches of LF, presumably not completely devoid of LPS, were more efficient in protecting mice against sublethal doses of LPS, than those purified to the homogeneity. Such LF preparations were also able to elicit the appearance of serum pro-inflammatory cytokines when injected intravenously to mice [28]. Increased resistance of infants, fed LF, to infections [43], and rats to cholera toxin [45], further indicates the role of oral treatment in enhancing immunity. Co-stimulatory effects of LF at i.p. administration may be associated more directly with interaction of LF with peritoneal macrophages. Lactoferrin (LF) can induce, by itself, production of TNF-α, IL-8, IL-6 and NO in monocytes [19]. The presence of immunostimulatory sequences located at the N-terminal of lactoferrin molecule, as well as the fact that LF cross reacts with HSP65 [48], may also account for the co-stimulatory effects of LF.

In addition to the mechanisms described above, we propose that other properties of LF may be relevant in stimulation of the cellular immune response at intradermal administration of LF together with the sensitizing dose of antigen. By induction of pro-inflammatory mediators and increase of capillary-vessel permeability, LF may facilitate migration of dermal Langerhans cells into adjacent lymph nodes. Such a notion can be supported by our recent results from the experiment in which we demonstrated a significant increase in proliferation of popliteal lymph node cells from mice treated with lactoferrin (data not shown). In addition, LF may augment the presentation of antigen to T cells by increasing the expression of an accessory adhesion molecule like LFA-1 [39]. Finally, LF may interact directly with T cells by transducing mitogen-like signal [35].

The co-stimulatory action of LF in the generation of the immune response is not restricted to the cellular response only. Parallel studies have shown that LF could also stimulate development of the humoral immune response to sheep red blood cells and ovalbumin measured as the number of antibody-forming cells and antibodies, respectively. The stimulation of the humoral immune response to SRBC induced by suboptimal doses of antigen, was statistically significant (six-fold) attaining the level of response elicited by optimal, ten times higher dose of antigen (manuscript in preparation). This suggests that LF affects activation pathways common for induction of both types of the immune response. Our unpublished data also showed that LF did not preferentially direct generation of a particular type of the immune response when an immunization protocol was used to induce low, concomitant humoral and cellular responses to SRBC. The co-stimulatory action of LF, however, has some limitations due to the immunoregulatory property of this protein, and strictly depends on the magnitude of the DTH response in a given experiment. In one experiment (not shown), where a maximal DTH reaction to OVA (16.3 units) was induced, we observed no effect or some decrease of the DTH reaction by LF. Another limitation may be associated with immunogenicity of LF in mice and possibly in other experimental animals. However, use of Freund's adjuvant also generates specific response to its constituents. One of the advantages of bovine lactoferrin is that it does not elicit inflammatory responses when administered intradermally into guinea pigs (unpublished data). More importantly, potential use of bovine LF to increase effectiveness of vaccination in humans, particularly by oral route, should not result in

development of bovine LF-specific response since this protein is a dietary antigen.

Of particular importance is the property of LF to stimulate the immune response when administered per os at the time of immunization. The described property of LF may be also of value to increase antibody titers in animals for commercial use as well as to augment effectiveness of vaccination for domestic and farm animals.

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