Anti-bovine β-lactoglobulin antibodies react with a human lactoferrin fragment and bovine β-lactoglobulin present in human milk

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Summary. Human milk samples react against anti-bovine β-lactoglobulin rabbit antibodies, as measured by a competitive radioimmunoassay. Immunoreactivity was positive even in milk from mothers consuming a diet free of cow's milk. An increase with a diet rich in cow's milk proteins was detected by immunoelectrophoresis. The human milk fraction cross-reacting with anti-bovine β-lactoglobulin antibodies corresponds to the 20 kDa fragment from the N-terminal end of human lactoferrin. Three regions of this fragment exhibit sequence homology with a sequence contained in cow's β-lactoglobulin (between residues 124 and 141).
Key words. Human milk; β-lactoglobulin; lactoferrin; sequence; immunochemistry.

β-Lactoglobulin (β-LG), which is the most abundant cow's milk whey protein and is considered to be a major allergen in infantile hypersensitivity to cow's milk, has never been clearly demonstrated in human milk. Nevertheless, human milk reacts with rabbit antibodies raised against cow's milk β-LG, and there are three possible explanations for this reactivity: a) the antibodies react with cow's milk proteins ingested by the mother[1,2]; b) rabbit antibodies cross-react with a human milk whey protein different from bovine β-LG[3]; or c) β-LG is an endogenous human milk protein[4]. We observed that the immunochemical response of human milk samples to anti-β-LG increased significantly when cow's milk whey proteins were introduced into the mother's diet; however, with a competitive radioimmunoassay a positive response was obtained even when cow's milk proteins were completely eliminated from the donor's diet. We purified a protein fragment of about 20 kDa reacting with anti-β-LG antibodies from the milk serum of mothers whose diet was free of cow's milk. A structural study allowed us to demonstrate that it corresponded to one half of the N-lobe of human lactoferrin (HLF)[5].

Experimental. Milk samples. Human milk samples were obtained from four women aged between 23 and 26 years, who gave birth at term and were submitted to the following diet; for three days they abstained from consuming cow's milk and dairy products. On the third day, milk samples were collected, and from the fourth day the donors drank a solution of cow's milk whey concentrate containing about 4 g of β-LG; breast milk samples were taken 4 h after ingestion of the cow's milk protein solution. For chromatographies and immunoelectrophoresis, caseins were sedimented by acidification of the defatted milk samples with 1 M HCl at pH 4.6 followed by centrifugation at 25,000 × g during 3 h at 25 °C.

Radioimmunoassay (RIA) and immunoelectrophoresis (IE). Antisera used in the immunochemical tests were obtained after injection of thrice-crystallized bovine β-LG in New Zealand White rabbits and purified by affinity chromatography. The solid phase RIA was an adaptation of the procedure described by Catt and Tregear[6]. The polyvinyl cups (Dynatech) were coated with 100 ng of bovine β-LG in 10 mM bicarbonate buffer, pH 9.6. Protein A (Pharmacia) was iodinated with ^{125}I-Bolton & Hunter reagent (Amersham). The competition RIA was performed on the defatted milk samples serially diluted with 0.1 M Tris-HCl buffer, pH 8.0, containing 1 mg/ml of bovine serumalbumin.

The IE was performed according to Scheidegger[7] on a 2117 Multiphor II (LKB) apparatus with a veronal buffer (50 mM 5,5-diethylbarbituric acid sodium salt, 5 mM oxalic acid and 2 mM citric acid), pH 8.3. For IE, human milk whey was reconstituted with water, after lyophilization, at a concentration of 300 mg/ml.

Chromatographic methods and sequence determination. Human milk whey was filtered on Sephadex G-100 using a 20 mM Tris-HCl, 1 M NaCl buffer, pH 8.0. The fractions eluted between 25 and 14.6 kDa were pooled, dialyzed and chromatographed on a FPLC (Pharmacia) apparatus equipped with a Mono-S column under the following conditions; buffer A was 20 mM Tris-HCl, pH 7.2 and buffer B was buffer A containing 1.5 M NaCl. The N-terminal amino acid sequence of the fragment purified by FPLC was established using a 470 A gas-phase sequencer (Applied Biosystems). The phenylthiohydantoin amino acid derivatives were automatically identified with an Applied Biosystems 120 A phenylthiohydantoin amino acid derivatives analyzer used on-line with the sequencer.

Results and discussion. RIA and IE analysis of milk samples. Before re-introduction of cow's milk proteins in the mother's diet, the amount of β-LG equivalents determined by RIA varied between 0.6 and 1 ng/ml, increasing to between 6 and 415 ng/ml following ingestion of cow's whey milk concentrate. No precipitation arcs were observed by IE with milk samples obtained from women submitted to a diet free of cow's milk proteins; however, a net reaction was observed at the position of β-LG when the cow's milk whey proteins were ingested by the donors. This observation indicates that the intact or almost intact β-LG molecule crossed the mother's intestinal barrier and was concentrated in her milk (fig. 1).

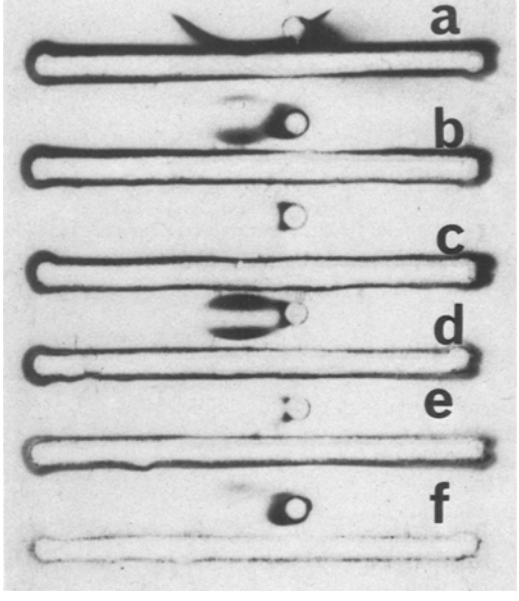

Figure 1. Immunoelectrophoresis of human milk whey samples (*c* and *e*) during a cow's milk free diet. *b*, *d* and *f*, samples after ingestion of cow's whey milk proteins concentrate. *a*, bovine β-LG.

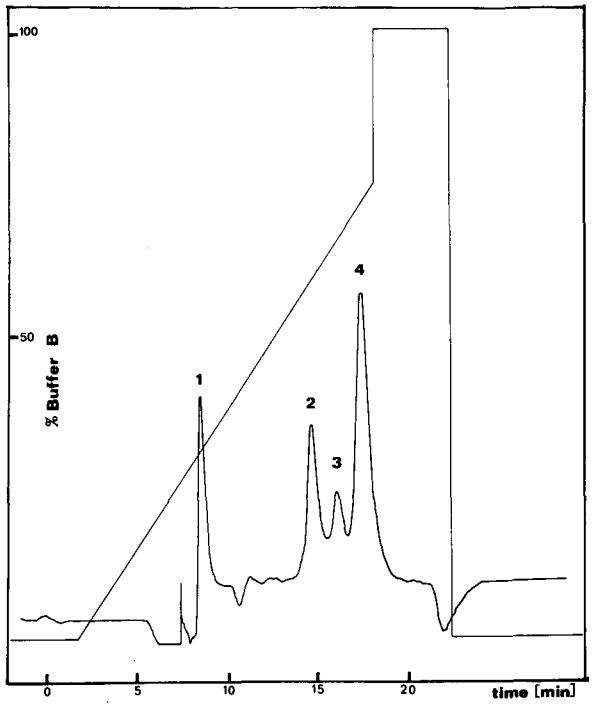

Figure 2. FPLC pattern of the human milk whey fractions obtained on Sephadex G-100. Peaks 2 and 4 gave a positive response against anti-bovine β-LG antibodies in the RIA.

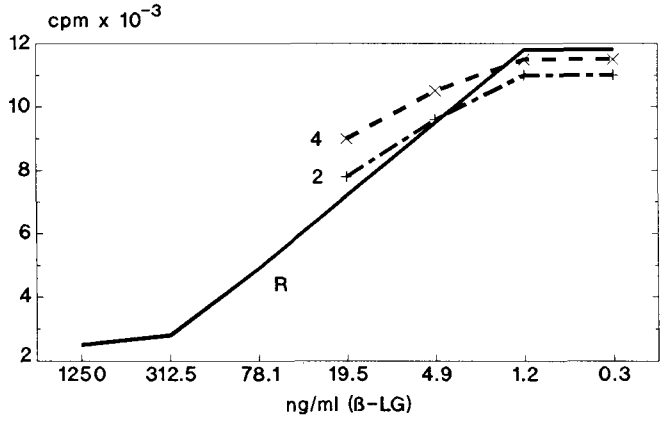

Figure 3. RIA of fractions 2 and 4 (fig. 2) obtained by FPLC.

Analysis of the fractions obtained by chromatographic methods. The fraction obtained by gel filtration on Sephadex G-100 gave rise to four fragments by FPLC (fig. 2); two of them, peaks 2 and 4, gave a positive response in the competition RIA (fig. 3). Peak 4 was clearly identified as a fragment of HLF: indeed its sequence was determined (table) and corresponded to the N-terminal sequence of HLF [9]. This fragment had a molecular mass of about 20 kDa determined by SDS-PAGE (data not shown). The structure of peak 2, which also reacted with anti-β-LG antibodies, could not, so far, be elucidated.

Structural homology between human lactoferrin (HLF) and cow's β-LG. The amino acid sequence of bovine β-LG [8] was compared with that of HLF [9]. Three regions of the 20 kDa HLF fragment exhibit homologies with one β-LG sequence (residues 124–141, fig. 4). The latter is a potential antigenic determinant scored at 70–100% according to Fraga's prediction method [10]; this can explain the cross-reactivity with the 20 kDa HLF fragment.

Amino acid sequence of peak 4 (fig. 2) determined with a gas-phase sequencer

Step	Residue	pmole
1	Gly	164
2	Arg	55
3	Arg	58
4	Arg	64
5	Arg	61
6	Ser	35
7	Val	50
8	Gln	47
9	Trp	23
10	(Cys-)*	–
11	Ala	44
12	Val	30
13	Ser	13
14	Gln	26
15	Pro	22

* Determination carried out on a non-reduced sample.

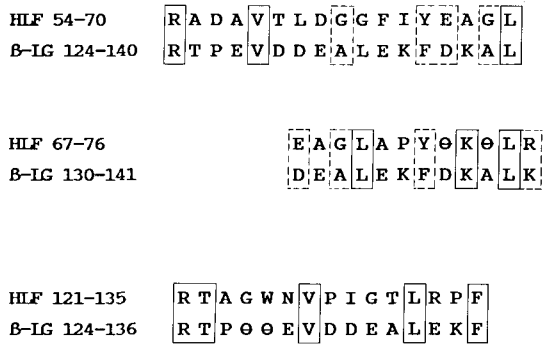

Figure 4. Sequence homologies observed between a bovine β-LG fragment and three sequences contained in the N-terminal lobe of HLF. Identical (—) and homologous (---) residues are boxed. (Θ), deletion.

Conclusion. We showed in our study that the immunoreactivity of human milk with anti-bovine β-LG antibodies is due to two different causes: a) specific reaction with bovine β-LG ingested by the mother [1, 2], and b) cross-reactivity with a part of the N-terminal lobe of HLF [5], presenting sequence homology with β-LG. Cross-reactivity of intact HLF with anti-bovine β-LG antibodies was previously reported by Ribadeau-Dumas et al. [3]: With our immunochemical methods we were unable to obtain a positive response with intact HLF, possibly due to the antigen presentation in the commercial preparations. The presence of foreign proteins in human milk is now generally accepted; therefore, the mother's diet must be controlled if the newborn who is breastfed is atopic.

Abbreviations. β-LG, β-lactoglobulin; HLF, human lactoferrin; RIA, radioimmunoassay; IEF, immunoelectrophoresis; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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Role of thiols in human peripheral blood Natural Killer and Killer lymphocyte activities

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Summary. The thiol reagents, dithiothreitol, diethyldithiocarbamate and reduced glutathione were each found to inhibit Natural Killer and Killer lymphocyte-mediated cytotoxicities. A biphasic aspect to the inhibition with increasing concentration was observed with diethyldithiocarbamate and reduced glutathione. The inhibition observed in response to reduced glutathione, a non-permeant compound, suggests that cell surface thiols may be critical functional groups in the processes of NK and K lymphocyte-mediated cytotoxicities.
Key words. Natural Killer lymphocyte; Killer lymphocyte; thiols; inhibition; cell surface.

Over recent years several studies have investigated the role of thiols in lymphocyte-mediated cytotoxicity with the suggestion that membrane thiol groups are involved in T lymphocyte-mediated cytotoxicity[1-3]. Similarly, studies have indicated the importance of membrane thiols in Natural Killer (NK) lymphocyte-mediated cytotoxicity[4-8]. Studies showing inhibition of NK activity by zinc and cadmium can also lead to such conclusions[9-11]. Other investigations have examined the role of the endogenous intracellular thiol, glutathione, in the process[8,12,13]. One of these[12], while focussing on endogenous GSH, presents evidence that could indicate a role for membrane thiol groups in the function of Killer lymphocytes which effect the process termed antibody-dependent cell-mediated cytotoxicity (ADCC). However, examination of surface thiol groups has not been specifically addressed for NK or K cell activity. Therefore, the present study was undertaken to compare NK and K cell functions in response to thiol-modulating agents and to determine if surface sulfhydryl groups are of importance.

Materials and methods. Thiol reagents used in this study include dithiothreitol (DTT) (BDH Chemicals, England), diethyldithiocarbamate (DEDTC) (Sigma, St. Louis) and reduced glutathione (GSH) (Calbiochem, San Diego). All other chemicals used were of the highest grade commercially available.

Human peripheral blood lymphocytes and target cells were prepared for the cytotoxicity assay as previously described[14]. The cytotoxicity assay itself followed the procedure described in a recent study[15]. The thiol reagents were added at various final concentrations ranging up to 5 mM. Examination of cell viability by trypan blue exclusion and by release of ^{51}Cr showed no evidence of any chemically-induced cytotoxicity.

The single cell assay to determine conjugates formed was carried out according to the method of Roozemond and Bonavida[16].

Results. Percent cytotoxicities in control incubates for NK and K cells were 34.0 ± 2.2 and 41.3 ± 1.6, respectively. The effects of DTT on both NK and K cell functions are clearly shown in figure 1, with inhibition being evident at 1 mM. Diethyldithiocarbamate was found to be inhibitory at concentrations of 1 µM and greater (fig. 2). At the highest concentration tested (1 mM) there was evidence of a biphasic pattern of response. As shown in figure 3, a similar biphasic effect was observed with GSH, where concentrations of 0.5 mM and greater were inhibitory. For each of the thiols the pattern of inhibition was the same for both NK and K cell functions.

Concentrations of each of the thiol reagents which clearly inhibited lymphocyte-mediated cytotoxicity were tested for ability to inhibit conjugate formation in a single cell assay. The data in the table show no evidence for a decrease in conjugates of effector cells with either target cell (K 562 or antibody-coated Chang).

Discussion. The results clearly demonstrate that thiol reagents have an inhibitory effect on NK and K lymphocyte-mediated cytotoxicities. The near identical responses for the two functions suggest that thiol groups may play a similar role in the cytotoxic processes. Furthermore, the agents used

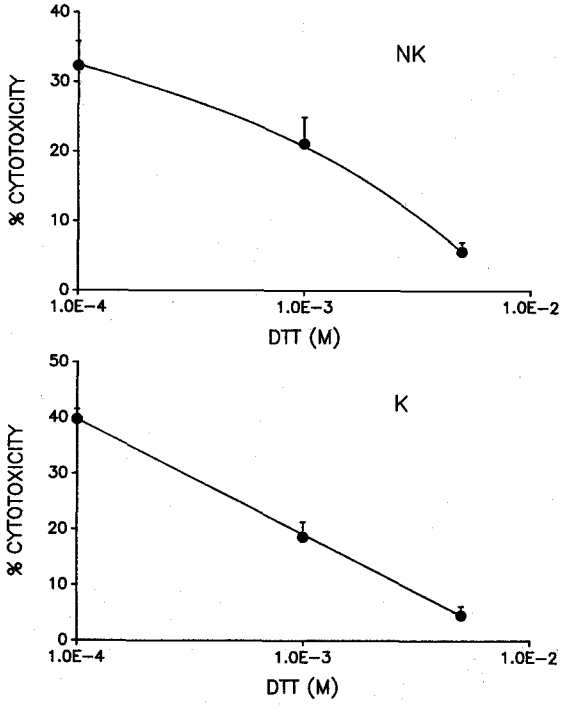

Figure 1. Effects of dithiothreitol (DTT) (100 µM–10 mM) on Natural Killer (NK) and Killer (K) cell-mediated specific cytotoxicity. Points are means and bars the SEM; N = 5.