An Innate Natural Antibody Is Reactive with a Cryptic Sequence of Lactoferrin Exposed on Sperm Head Surface (44189)

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Abstract. **Lactoferrin (LF), an 80-kDa glycoprotein of ubiquitous occurrence in body fluids, is multifunctional and capable of assuming different configurations to serve those functions. The capacity of LF to undergo endocytosis and the recent demonstration of LF binding to sequence specific DNA indicate that a function or capability of LF, in addition to iron chelation, bacteriostasis, and receptor-specific lymphocyte binding, may be that of gene activation or silencing. The data of this report present a human physiological system, that of sperm entry into the oocyte in performance of fertilization in which, since LF is a component of the sperm protein coat, that capability could be expressed. However, the configuration of LF in that locus is one in which a revealed cryptic sequence provides the specific binding site for a natural antibody present in the fertilization milieu. The presence of that antibody suggests that a system of control of the potential interaction of LF with the intra-ooplasmic DNA, that of gametes or pronuclei, is operative. The configuration of LF on the sperm surface and designation of the reactive site for the natural antibody were enabled by a monoclonal antibody secreted by a hybridoma derived from a human cord blood B cell. Thus, in addition to information concerning the molecular flexibility of LF, these observations support the proposition that the repertoire of natural antibodies provides an innate homeostatic system, with each antibody serving a specific role.**

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Lactoferrin (LF) has long been considered to be a component of the protein coat of the spermatozoon head (1, 2). In this study, we have identified a natural antibody, invariably present in normal human sera (3, 4), that is specifically reactive with a sequence revealed *in situ* in the configuration of LF present on the sperm head. In other forms of native LF (e.g., those present in plasma and milk), that sequence is not revealed and reactivity with the natural antibody is not displayed *in vitro* or, presumably, *in vivo*. The dependence of the natural antibody epitope upon a cryptic sequence of LF displayed in the configuration of LF in the sperm coat is supported by demonstration of serum IgM reactivity with denatured, but not with native, LF isolated from human milk or seminal plasma. We have localized the reactive site, revealed by denaturation, to a 10-kDa fraction of LF consisting of two peptides, 81 and 88 residues, and we have shown that all of a large cohort of normal human sera contain an IgM antibody reactive with that fraction (4). Further, the characterization of the natural antibody of human serum and its specific recognition of LF in the sperm coat has been verified by a monoclonal antibody, secreted by a hybridoma derived from a human cord blood B cell, that is specifically reactive with that 10-kDa fraction.

Natural antibodies are those for which no exogenous source of induction is apparent and to which benign, rather than pathologic, function is attributed (5, 6). Continued

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study and identification of specific natural antibodies support the concept that such antibodies have arisen and, by virtue of contribution to homeostasis, have become established in the innate repertoire. The occurrence of a natural antibody capable of recognizing LF in the specific configuration in which it occurs in the sperm coat may indeed be an example of the protective function of a natural antibody. Recent studies (7, 8) have shown that LF may enter cells, be transported to the nuclei, and interact with DNA. Since the sperm protein coat is shed and dispersed as the fertilization-bent sperm undergoes capacitation, acrosome reaction, and penetration of the protective zona pellucida surrounding the oocyte (9, 10), the endocytosis-capable LF could gain entry to the fertilized oocyte and carry out interaction with the DNA of the gametes in their state of transition to the pronuclei of the embryo. Thus, the presence of the LF-reactive natural antibody, in the plasma present in the ducts of the female reproductive tract, may be a fundamental mechanism of protection of the nascent genetic complement.

Materials and Methods

LF Proteins. Human milk lactoferrin, obtained from Sigma Chemical Co. (L3770; St. Louis, MO) is designated LF(M). Seminal plasma LF was isolated from pooled specimens of semen, from clinically normal volunteer donors. Following liquefaction, sperm-free plasma was obtained by centrifugation and separated by DEAE ion exchange chromatography (11) into a pool of basic and a pool of acidic fractions. Each pool was subjected to gel filtration (Sephacryl S 300 HR; Pharmacia, Piscataway, NJ) and the first fraction of each pool was resolved at 80 kDa and designated SP80-basic and SP80-acidic, respectively.

Cyanogen Bromide Cleavage and SDS-PAGE. CNBr treatment of SP80-basic, SP80-acidic, and LF(M) was carried out as described (12). Briefly, a 10-mg/ml 70% formic acid solution of each protein was incubated with CNBr (200-fold molar excess) at room temperature for 18–24 hr. Following lyophylization, the cleavage mixtures were electrophoresed on an SDS-PAGE (Fig. 1). For enhanced resolution of the low–molecular weight fractions (Fig. 2) electrophoresis was carried out on a 16.5% tricine gel (13).

Characterization of Fraction 7B. Fraction 7B was excised from the gel and extracted with H_2O. SDS was precipitated by addition of KCl and the component peptides of 7B were purified by dialysis against PBS (pH 7.2). Untreated sperm-free seminal plasma proteins and native LF were PBS solutions. Determination that LF fraction 7B consisted of two peptides was carried out by the Laboratory of Mass Spectrometry at Rockefeller University, utilizing matrix-associated laser desorption/ionization mass spectrometry (14). N-terminal sequencing of the peptides of LF fraction 7B was carried out by the Protein Sequencing Facility at Rockefeller University, utilizing repeated cycles of Edman degradation followed by PTH analysis with microbore HPLC (15).

Immunoreactivity. Western blot was performed on Immobilon-P (Millipore, Bedford, MA) transfers of the electropherograms of LF(M) and acidic and basic SP80 and visualized by chemiluminescence. Enzyme-linked immunosorbent assay (ELISA) was carried out by standardized methodology (16–18). Sera were those of a rabbit immunized with human LF(M), a rabbit immunized with SP80 (acidic and basic combined), and human sera selected at random from a group of discards from clinical laboratories, identified by gender, age, and "no clinical findings." Reactivity by all human sera was solely with fraction 7 of the PAGE (Fig. 1) and resolved at a distinct band designated 7B (Fig. 2).

Monoclonal Antibody Specific for Fraction 7B. Mononuclear cells were isolated from cord blood of a normal neonate by density gradient centrifugation using Ficoll-Pague (Pharmacia) and transformed with Epstein-Barr virus (19). Fusion with the parental cell line HMMA, utilizing standard procedures (20), resulted in a set of IgM-secreting hybridomas for which monoclonality was established by limiting dilution. Since reactivity of serum with denatured milk LF(M) and SP80 was confined to a single PAGE fraction (Fig. 2) that fraction was isolated from the gel and utilized, together with a set of proteins and peptides for which specific reactivity by other human natural antibodies has been established (16, 18), as antigens in ELISA to screen those monoclonal antibodies (mAbs) for exclusive reactivity with fraction 7B.

Sperm Coat Protein Fraction. A fraction containing the components of the sperm coat was obtained by induction of the acrosome reaction (21) in a suspension of spermatozoa: the swim-up sperm were gently washed with PBS, collected, and suspended in Ca medium: 2 m*M* $CaCl_2$, 10 m*M* ionophore A23187 (Calbiochem, La Jolla, CA), 1 m*M* PMSF (Sigma) and incubated 4 hr at room temperature. The sperm cells were pelleted by low-speed centrifugation and the resultant supernatant cleared of particles by high-speed centifugation followed by dialysis overnight at 4°C. The supernatant was tested by ELISA, for reactivity with human sera and with the mAb reactive with LF fraction 7B (Fig. 3).

Cytologic Localization of LF/SP80 in Sperm Heads. A fraction of swim-up human sperm was obtained from spontaneously liquified seminal plasma, washed three times with phosphate-buffered saline (PBS), and finally suspended in either human serum diluted 1:500 in PBS or in PBS solution of the purified mAb, followed by overnight incubation at 4°C. Each suspension was washed three times with PBS and the collected sperm incubated in FITC-labeled anti-human IgM (Sigma) for 1 hr. The sperm were washed with PBS, and a drop of the suspension placed on a slide, examined, and photographed, utilizing FITC-specific filters (Fig. 4).

Results

The data reported here confirm previous studies indicating that an 80-kDa protein of human seminal plasma is

homologous with LF (1, 2). Fractionation of sperm-free seminal plasma by DEAE ion exchange chromatography (not shown) confirmed that the 80-kDa protein is present in two forms: basic and acidic, which contains the glycan moiety (22). CNBr cleavage fractions on SDS gels were identical for both forms of SP80 as well as for LF derived from human milk (Figs. 1A and 2A). Also, the pattern of immunoreactivity of those fractions with serum of a rabbit immunized with SP80 (Fig. 1) or with LF from human milk (not shown) are correspondingly identical. Similarly, prior reports (4) that normal human sera show no immunoreactivity with native LF from milk or with SP80 isolated from, or in the context of, seminal plasma are confirmed (Fig. 3). Especially significant is the confirmation (Figs. 1 and 2) that a natural antibody, identified in normal human sera (3, 4), is reactive with a cryptic sequence of LF and SP80 that is revealed upon denaturation of those proteins (Figs. 1 and 2). That sequence is segregated in fraction 7B from the PAGE of CNBr cleavage products of LH(M) and SP80 (Fig. 2). The innate occurrence of the natural antibody is stikingly demonstrated by the derivation of a hybridoma from a cord blood cell which secretes an IgM/*K* that is specifically reactive with a component of fraction 7B (Figs. 2 and 3).

Mass spectrometry revealed that fraction 7B contains two peptides, of 10 and 9 kDa. N-terminal sequencing identified DKVER for the 10-kDa major peptide and SLDGG for the 9-kDa peptide. Upon the assumption that CNBr cleavage of LF is at methionine residues and by reference to the published structure of LF (12), the sequence of each of the two peptides was derived and localized to the C lobe. A set of 12 residue peptides, with 5 residue overlaps, comprising the derived linear sequences of the 2 peptides, was created (Table I). Thus far, specific reactivity of human serum IgM has not been identified with any one of those

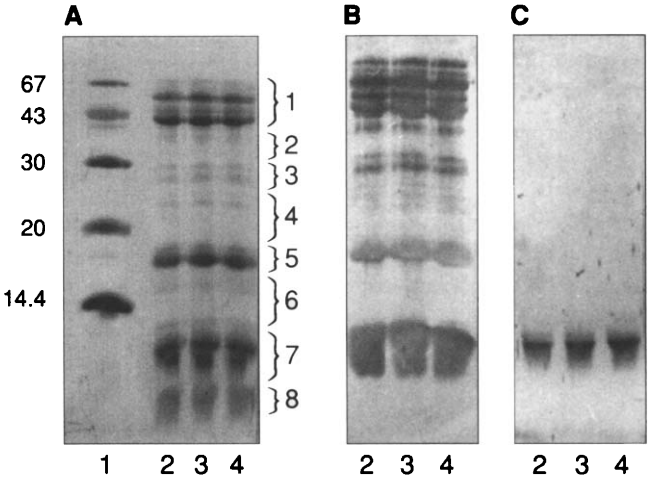

Figure 1. SDS-PAGE of CNBr cleaved LF and SP80 (A) Protein stain: 1, molecular weight markers; 2, LF(M); 3, SP80-basic; 4, SP80-acidic. All three proteins (2, 3, and 4) show identical cleavage fractions 1–8. (B) Immunotransfer with serum of rabbit immunized with SP80 (acidic and basic) showing multiplicity of reactive sites and homology of reactivity of LF(M) and SP80. (C) Immunotransfer with normal human male serum showing reactivity solely with fraction 7 of each of the three proteins.

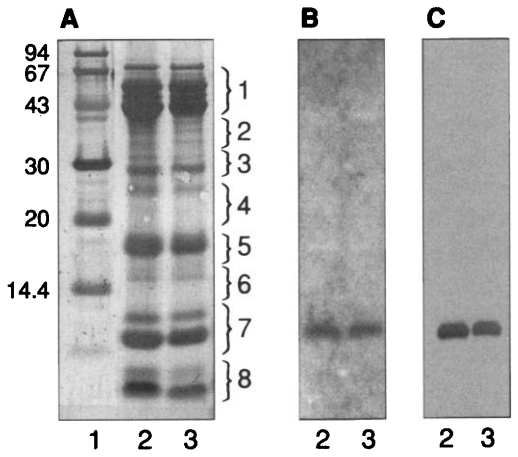

Figure 2. Tricine SDS-PAGE. (A) Protein stain: 1, molecular weight markers; 2, LF(M); 3, SP80 (acidic and basic). Resolution of fraction 7 shows two distinct bands. (B) Immunotransfer with normal human male serum showing reactivity specifically localized in fraction 7B. (C) Immunoreactivity with fraction 7B of an Mab IgM from a human B cell derived hybridoma.

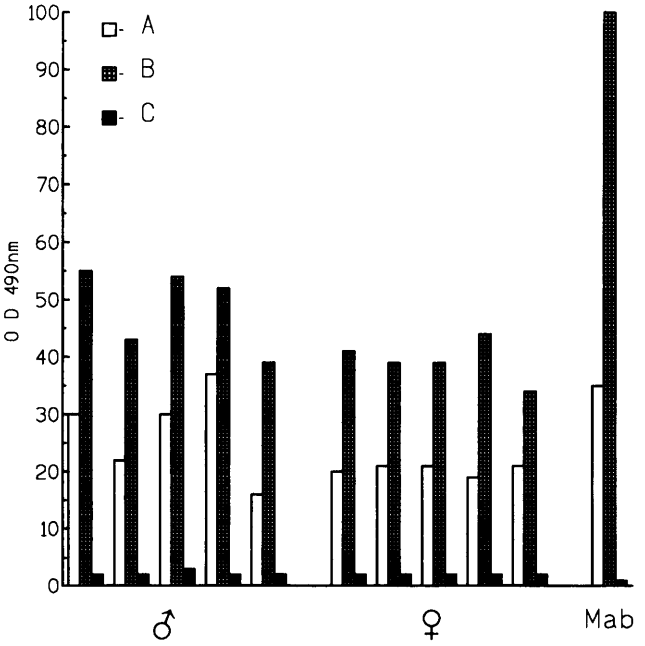

Figure 3. Reactivity, by ELISA, of serum (1:100) of each of five males, five females, and the mAb with: (A) 10 μg/ml of the complement of sperm coat proteins released following induction of the acrosome reaction in a suspension of swim-up spermatozoa; (B) 10 μg/ml of purified fraction 7B LF(M); (C) 10 μg/ml native (nondenatured) LF(M). The relative reactivities of Bars A and B indicate that a serum antibody and the mAb are reactive with a specific component, but not all, of the sperm coat complement. The lack of reactivity with native LF(C) verifies that the natural antibody of serum and the mAb are reactive with a site of LF that is not revealed in its native state.

peptides tested singly, indicating that the fundamental epitope for the natural antibody, although embodied in LF fraction 7B, is conformation dependent.

The localization of that epitope *in situ,* in the sperm head, is demonstrated by cytoimmunoreactivity of human serum and by the mAb specifically reactive with LF(M)/SP80 fraction 7B (Fig. 4). Further evidence that LF is pres-

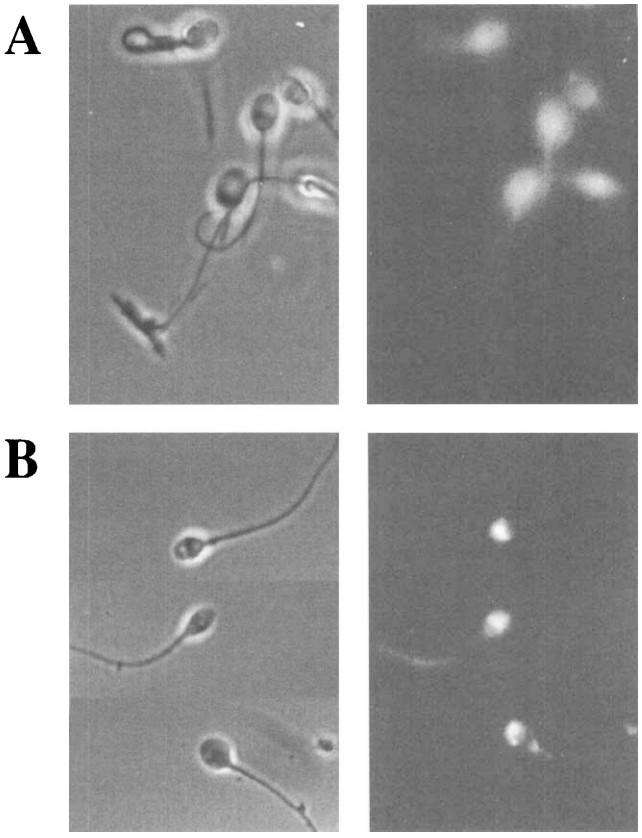

Figure 4. *In situ* immunoreactivity, displayed by FITC-labeled anti-human IgM, of a component of human sperm heads with: (A) human serum; (B) mAb reactive with LF fraction 7B.

ent in the sperm coat proteins, in that configuration in which the natural antibody epitope is revealed, is provided by Figure 3. Following induction of the acrosome reaction (21), resulting in dispersion of the protein coat/plasma membrane ensemble overlying the acrosomal region of the sperm head, reactivity of a component of the coat with human serum IgM and with the mAb was shown (Fig. 3). Thus, Figures 3 and 4 provide evidence that, following the sequence of capacitation and acrosome reaction *in vivo,* the LF shed from the sperm coat may be available for entry into the sperm-penetrated oocyte. However, since the complete immunoglobulin repertoire of plasma is present in the female reproductive tract (23) that availability may be inhibited by the natural antibody.

Table I. Overlapping Duodecapeptides Comprising the Components of LF Fraction 7B

A.	B.
D K V E R L K Q V L L H	S L D G G Y V Y T A C K
K Q V L L H Q Q A K F G	V Y T A C K C G L V P V
Q Q A K F G R N G S D C	C G L V P V L A E N Y K
R N G S D C P D K F C L	L A E N Y K S Q Q S S D
P D K F C L F Q S E T K	S Q Q S S D P D P N C V
F Q S E T K N L L F N D	P D P N C V D R P V E G
N L L F N D N T E C L A	D R P V E G Y L A V A V
N T E C L A R L H G K T	Y L A V A V V R R S D T
R L H G K T T Y E K Y L	V R R S D T S L T W N S
T Y E K Y L G P Q Y V A	S L T W N S V K G K K S
G P Q Y V A G I T N L K	
G I T N L K K C S T S P	
K C S T S P L L E A C E	
S P L L E A C E F L R K	

Note. A = 10 kDa; B = 9 kDa. As noted (Results) reactivity of human serum IgM or of the mAb was not displayed against any of the peptides, indicating that the epitope is conformational.

Discussion

We have identified an IgM characterized as a natural antibody since it has been shown to be present in a large cohort of normal human sera, and for which no pathologic role or association is apparent. The reactive site for that natural antibody has been shown previously (3), and confirmed here, to be present in the plasma membrane complex of the sperm head. These studies, designed to establish the molecular identity of that reactive site, have confirmed that an approximately 72-6-kD protein present in seminal plasma (2), accurately determined here as 80 kD, is also present in the protein coat of the sperm head and that 80-kDa protein is homologous with, and in fact is, lactoferrin. We show here that the noted natural antibody is specifically reactive with LF in a configuration other than that of the LF ubiquitous in body fluids. That configuration and the natural antibody reactivity is revealed *in vitro* (Figs. 2 and 3), following denaturation of native circulating LF and is revealed *in vivo* (Fig. 4) in the LF incorporated in the protein coat of the human sperm head. LF is present in seminal plasma in the native configuration and, by a mechanism not yet determined, the antibody recognition form is assumed when it is deposited in the spermatozoal membrane/coat complex. The transition to that form and deposition in the sperm surface coat presumably take place during the period of spermatogenic maturation in the seminiferous tubules of the testes. It is relevant, therefore, to note that large molecules such as immunoglobulins, particularly IgM, are excluded from the lumina of the seminiferous tubules (24) and, therefore, from immunoreactivity with sperm components during spermiogenesis. That barrier, however, does not exist in the female reproductive tract, where the full complement of circulating antibodies is present (23). Therefore, the LF reactive natural antibody is available for immunoreactivity with the LF of the sperm coat, following ejaculation into the female reproductive tract. That interaction may take place in the sperm coat *in situ* as shown (Fig. 4) and is definitely capable of taking place with the LF released, along with other coat and plasma membrane components (Fig. 3) as the sperm undergoes the sequence of capacitation and acrosome reaction, which facilitate passage of the sperm through the protective zona pellucida surrounding the oocyte, and subsequent entry into the oocyte (9, 10). Since the acrosome reaction involves fusion of the acrosomal membrane with the plasma membrane, the components of the overlying protein coat are dispersed. Thus, the released LF could have ready access to the ooplasm were it not for the presence, in the fertilization milieu, of the natural antibody capable of immunological nullification of the ability of that LF to en-

docytose through the oocyte membrane and, subsequently, to interact with the DNA of the gametes or pronuclei.

The salutory effect of that restriction of LF entry may be particularly relevant since there is good evidence that, in mammals, the transition of the male gamete chromosomal complement to the pronucleus of the embryo includes a brief interim period when the DNA is "naked"—that is, the interval between shedding of spermatozoal specific protamines and replacement with the characteristic somatic complement of histones (25, 26). In that interval, intraooplasmic LF could have ample opportunity to interact with the paternal DNA.

Among the many functions and interactions defined for LF, its capacity to be endocytosed and interact with DNA is of increasing interest (7, 8, 27–29). Particularly interesting are the recent reports that the interaction of LF with DNA is marked by sequence specificity (7). The underlying molecular bases for that specificity have not been defined, but it is reasonable to expect that, if LF/DNA interaction occurs *in vivo,* it does so within a defined control system. It is logical, also, to propose that such a system exists in the organized chromosomal complement of somatic cells, but not in the nascent undifferentiated complements of the pronuclei. Thus, in that context the postulated control of sequence specificity in interaction of LF with DNA may not be operative. The presence of a natural antibody selectively reactive with LF in the specific configuration in which it exists in the sperm coat, but not with LF in its ubiquitous circulating form, may represent a fortuitous natural selection mechanism on two bases: (i) inhibition of LF interaction with the DNA complements of the fertilized oocyte and (ii) restriction of immunoreaction by the circulating natural antibody with LF at other loci, in its more prevalent, important function-serving forms. The innate occurrence of that natural antibody is verified since the hybridoma secreting the mAb, utilized to provide significant data of this study, was derived from a human cord blood B cell.

The capacity of LF to assume different configurations to serve its different functions has been imaginatively expressed as "the jaws of lactoferrin" (30) and less whimsically, but cogently, described in recent analyses of its physical properties (31–33). In this study, we present evidence for a functional involvement of lactoferrin that requires the opening of a previously unrecognized "set of jaws" or a novel configuration.

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