Int. J. Exp. Path. (1998), **79,** 117–123

Antibacterial system generated by lactoferrin in mice *in vivo* is primarily a killing system

TADEUSZ ZAGULSKI, PAWEŁ LIPIŃSKI, ALINA ZAGULSKA AND ZOFIA JARZĄBEK

Polish Academy of Sciences, Department of Molecular Biology, Institute of Genetics and Animal Breeding, Mrokow, Poland

Received for publication 23 June 1997
Accepted for publication 9 February 1998

Summary. A single dose of bovine lactoferrin (BLF) was given intravenously (i.v.) to CFW mice 24 hours (h) prior to the i.v. injection of the *E. coli* lethal dose (LD_{100}). BLF strongly accelerated the clearance rate of *E. coli* from the blood as well as its killing rate in the liver, lungs, spleen and kidney. The highest clearing and killing rate was found 5 h after *E. coli* LD_{100} injection. The most intensive killing in the organs examined was found in the lungs and kidney. Analysis of organs of i.v. BLF-stimulated mice which survived up to day 30 after the infection by *E. coli* showed that not all animals were definitely pathogen-free. It was concluded that the defense system generated by BLF in mice *in vivo* is primarily a bacteria-killing one. The participation and cooperation of reticulo-endothelial (RE)-macrophages and granulocytes in the phagocytosis and killing of *E. coli* may thus be related to the protective activity of LF.

Keywords: E. coli, lactoferrin, clearing rate, killing rate, mouse, *in vivo*

Lactoferrin (LF) is an iron-binding, multifunctional glycoprotein found in mammalian tissues of ectodermal origin, as well as in their secretions and in secondary granules of neutrophils (Masson *et al*. 1966, 1969). A substantial part of the biological role of LF appears to be connected with natural antimicrobial defense. By means of its capacity to bind iron strongly, LF (iron-free and/or partially saturated with iron) is able to scavenge iron from the serum (together with serum transferrin) or other biological fluids, deprive micro-organisms of the metal, inhibit their growth and thus to generate the microbiostatic defense mechanism in the host (Emery 1980; Bullen *et al*. 1991; Weinberg 1992). This mechanism is efficient enough to eliminate approximately 90% of micro-organisms from the pathogenic or potentially pathogenic being not able to compete for iron with the iron-binding proteins of the host.

Correspondence: Dr T. Zagulski, Polish Academy of Sciences, Institute of Genetics and Animal Breeding Jastrzębiec, 05-551 Mroków, Poland. Fax: +48 22 7561699, E-mail: panighz@atos.warman.com.pl or zagulski@yahoo.com

However, some pathogens are well adapted to acquire iron in the presence of LF or other iron-binding proteins by various biological mechanisms described earlier (Brock *et al*. 1983; Stoebner & Payne 1988; Yang *et al*. 1993; Tigyi *et al*. 1992). In such a case iron deprivation generated by LF may only delay the microbial growth *in vivo*. To eliminate the micro-organisms definitely the host has to develop a powerful bacteria-killing mechanism(s).

We have previously reported that bovine LF (BLF) given i.v. to mice protects a high number of animals against death caused by *Escherichia coli* lethal infection *in vivo* and that supplementation of mice with iron did not change the survival rate of animals (Zagulski *et al*. 1989).

Over the last few years LF has been shown to display various immunoregulatory functions (Bagby *et al*. 1981;

Zimecki *et al.* 1991; Machnicki *et al.* 1993; Zimecki & Machnicki, 1994).

The aim of this study was to determine directly the nature of the antimicrobial mechanism(s) generated by BLF in mice *in vivo*.

Materials and methods

Animals

Male and female (50%/50%) CFW mice were bred in the Institute of Genetics and Animal Breeding (Jastrzębiec). The animals had free access to a standard laboratory food and water and were kept at 24–25°C with a light-dark cycle of 12 hours. Mice were used at the age of 8–10 weeks, weighing 25–30 g.

Preparation and use of BLF

BLF was isolated from milk according to the method described earlier (Zagulski *et al.* 1979). The purity of BLF preparations, as checked by SDS-PAGE and immunoelectrophoresis, was not less than 99% with iron saturation of about 95%. LPS contamination was removed from BLF preparations according to Karpulus *et al.* (1987). Briefly, BLF was passed through the Polymyxin B-Sepharose 4B (Sigma, St Louis, MO) preparative affinity chromatography column. After eluting from the column BLF solution was dialysed against LPS-free water and recovered by lyophilization. After passing through the column BLF contained from 3 to 9 pg LPS/1 mg protein depending on the preparation used. To determine the contamination of BLF preparations with LPS the Quantitative Chromogenic *Limulus amebocyte* Lysate assay (QCL-1000, BioWhitaker, Qalkersville, MD) was used.

For all experiments BLF was diluted in pyrogen-free phosphate-buffered saline (PBS), pH 7.2 and was given to mice i.v. or subcutaneously (s.c.), 24 hours before inoculation with *E. coli*, at a dose of 10 mg/mouse in a total volume of 0.1 ml.

Preparation and use of bacteria

Enterotoxigenic strain of *E. coli* 844 0-78K 80/B was obtained from National Veterinary Research Institute, Pulawy, Poland, Veterinary Museum of Microorganisms. For all experiments the bacteria were prepared as described earlier (Zagulski *et al.* 1986). Mice were injected i.v. (into the lateral tail vein) with a single dose of living *E. coli* containing 2×10^8 cells suspended in 0.1 ml PBS. The dose given i.v. was lethal in control mice.

Human serum albumin (HSA)

HSA (Sigma) was low endotoxin, of 99% purity, used as a control protein injected to mice at a dose of 10 mg in a volume of 0.1 ml of PBS. All other chemicals used were obtained from well-known commercial firms (mainly Serva, Heidelberg and Sigma) and were of the highest commercially available grade.

Experimental design

In the first series of experiments blood and tissues from mice given i.v. BLF, HSA or PBS (controls) and infected 24 hours later with a lethal dose of *E. coli* were analyzed for the presence of living *E. coli* cells at different times after infection as indicated in the legend to each figure. Other series of experiments was performed according to the same protocol except that BLF, HSA, or PBS were injected to mice s.c., and that the presence of living *E. coli* cells was only evaluated in organs of mice injected with BLF or PBS (controls).

Determination of the number of living E. coli cells in blood and tissues

The mice were killed by cervical dislocation at different times after infection. Blood samples from individual animals were collected asseptically from the portal vein. Serial tenfold dilutions of blood in sterile PBS were plated in a volume of 0.1 ml on McConkey agar (Difco Lab., Detroit, MI). The plates were incubated at 37°C for 18 hours. Colonies were counted and expressed as the log number of the colony forming units (CFU) per 1 ml blood. After blood collection, spleen, liver, lungs, and kidney were removed from mice, weighed and homogenized in sterile PBS (1 g wet tissue for 25 ml of PBS) with Glas-Coll TRI-R K41 homogenizer, 3×10 s at 6000 cycles/min using a glass tube and teflon pestle. Colonies were expressed as the log CFU per 1 g wet tissue.

On day 30 after lethal *E. coli* infection a group of mice stimulated with BLF was examined for the presence of living bacteria in the blood and organs as described.

Statistical analysis

Analysis of variance using the SAS General Linear Model was made for clearing and killing rates to establish significant differences ($P < 0.01$ and $P < 0.05$) between means in different experimental groups.

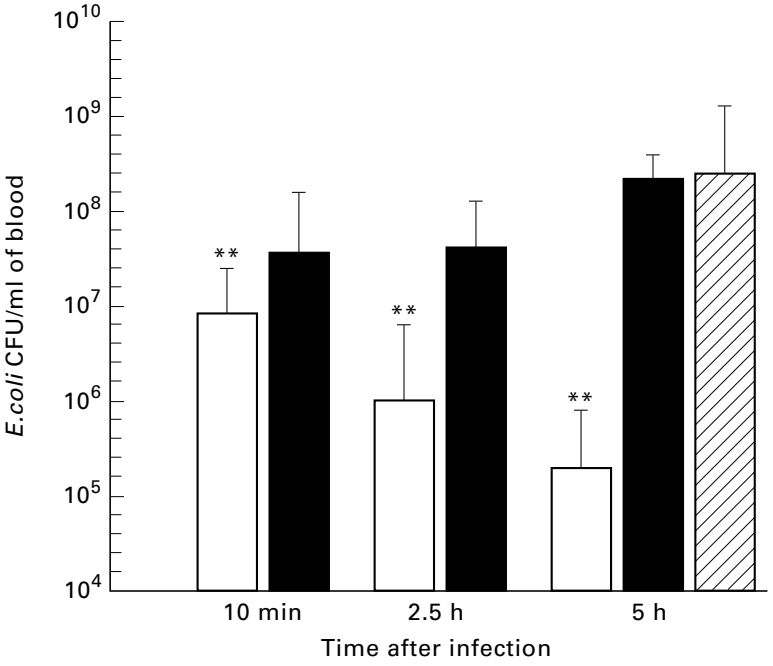

Figure 1. Clearing rate of *E. coli* cells from blood of lethally infected mice, intravenously pretreated with (□) BLF; (■) PBS (controls); (▨) HSA. Blood samples were collected from individual mice 10 min, 2.5 and 5 h after infection. In each treatment group blood from 12 mice was analyzed. Results are expressed on logarithmic scale as a mean ± SEM. ** Means differ significantly from the control group, $P \leqslant 0.01$.

Results are presented as means from at least 3 parallel experiments.

Results

Effect of BLF on clearing of E. coli cells from the blood of lethally infected mice

Accelerated clearing of bacteria from the blood of mice stimulated i.v. with BLF was a very quick and dynamic process (Figure 1). The significant effect of BLF was already seen 10 min after infection. The number of living *E. coli* cells gradually decreased, the difference in the bacterial count per 1 ml of blood between BLF-stimulated and control mice being wider than 3 orders of magnitude as measured 5 h after infection. In controls the number of living *E. coli* cells remained at constant high level up to 2.5 h post infection and increased at 5 h post-infection. The number of living *E. coli* cells in the blood of HSA-treated mice was similar to that of controls. BLF was much less effective in accelerating clearance of bacteria from the circulation when injected to animals s.c. (Figure 2). Although the number of living *E. coli* cells was significantly lower in the blood of s.c. BLF-stimulated mice than in that of controls, at 10 min and at 5 h post-infection, the absolute number of living *E. coli* cells found in the blood of mice from this group increased with time passing from infection.

The blood of BLF-stimulated mice which survived up to day 30 after infection was free of living *E. coli* (Table 1).

Effect of BLF on killing of E. coli cells in organs of lethally infected mice

BLF when injected to mice i.v. dramatically stimulated

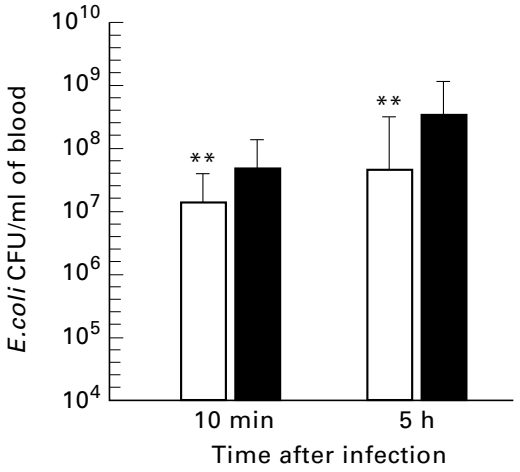

Figure 2. Clearing rate of *E. coli* cells from blood of lethally infected mice, subcutaneously pretreated with (□) BLF and (■) PBS (control). Blood samples were collected from individual mice 10 min and 5 h after infection. In both treatment groups blood from 12 mice was analyzed. Results are expressed on logarithmic scale as a mean ± SE. **Means differ significantly from control group, $P \leqslant 0.01$.

Table 1. Presence of bacteria in the blood and organs of BLF-stimulated mice lethally infected with *E. coli*

Tissue	Number of mice*	
	Free of *E. coli*	Infected with *E. coli*
Blood	12	0
Liver	11	1
Lung	9	3
Kidney	11	1
Spleen	11	1

*Blood and organs from 12 mice were analyzed on day 30 after *E. coli* infection. The number of living *E. coli* cells ranged from 120 to 30000 per 1 g of wet tissue.

killing of bacteria in four examined organs (Figure 3). The differences in the bacterial count per 1 g wet tissue between BLF-stimulated and control mice ranged from 1.5 to 3 orders of magnitude being highest in lungs and in kidney.

The absolute number of living *E. coli* cells in organs of BLF-stimulated mice did not decrease in the course of time after infection, except in the lungs (data not shown). However, the number of bacteria in organs of control mice simultaneously increased up to 1 order of magnitude. Moreover, the analysis for the presence of living *E. coli* cells in organs of BLF-stimulated mice which survived up to day 30 after infection showed that 75% of animals were free of pathogens, in remaining 25% a small number of living *E. coli* was found in different organs (Table 1). HSA has no significant effect on killing of bacteria in examined organs (Figure 3).

Unlike in the case of clearing of bacteria from the blood, BLF when s.c. injected to mice efficiently stimulated killing of bacteria in internal organs in a manner similar to that found for i.v. injected protein (Figure 4).

Discussion

The present results show that the protective system generated in mice by lactoferrin against *E. coli in vivo* (Zagulski *et al.* 1989) is primarily killing of the organisms. These are the first results which clearly demonstrate that native lactoferrin can generate a powerful, nonspecific, killing, antibacterial system in mammals *in vivo*. The

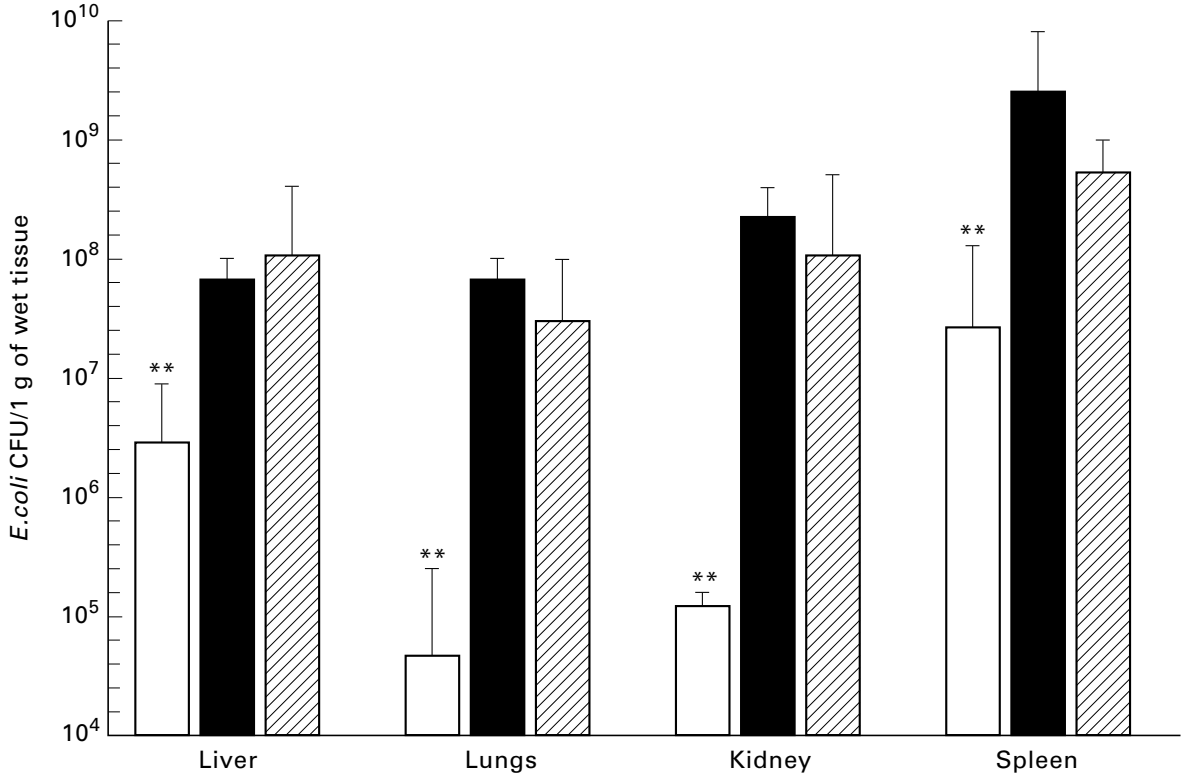

Figure 3. Killing of *E. coli* cells in livers, lungs, kidneys and spleens from lethally infected mice, intravenously pretreated with (□) BLF; (■) PBS (controls); (▨) HSA. Organs from individual mice were analysed 5 h after infection. In each treatment group organs from 12 mice were analyzed. Results are expressed on logarithmic scale as a mean ± SE. **Means differ significantly from control group, $P \leqslant 0.01$.

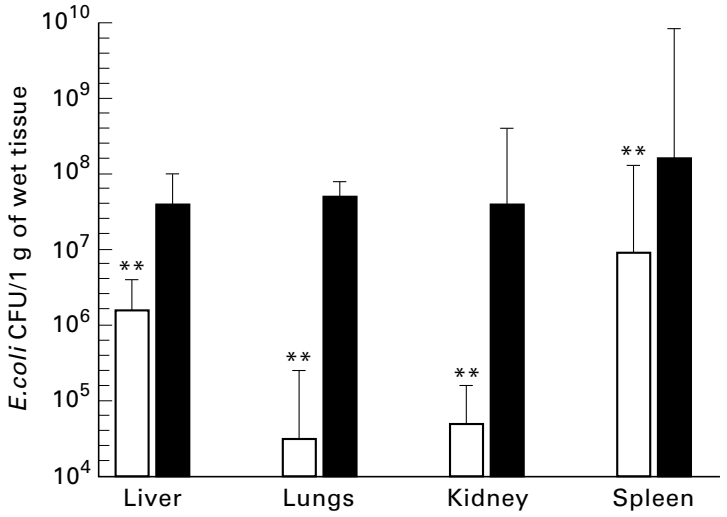

Figure 4. Killing of *E. coli* cells in livers, lungs, kidneys and spleens from lethally infected mice, subcutaneously pretreated with (□) BLF and (■) PBS (controls). Organs from individual mice were analysed 5 h after infection. In both treatment groups organs from 12 mice were analysed. Results are expressed on logarithmmic scale as a mean ± SE. **Means differ significantly from control group, $P \leqslant 0.01$.

system is based on a dramatic acceleration of the clearing rate of live *E. coli* from the blood, which strongly suggests the stimulation by LF of expression of the receptors of *E. coli* on the surface of phagocytosing (and killing) cells and the killing rate of *E. coli* in the four organs examined. The results also suggest that BLF given to mice by the s.c. route stimulates only the second part of protecting system, i.e. accelerates killing rate of *E. coli* in the internal organs of mice, to an extent comparable to that shown for BLF given by i.v. route; s.c. BLF did not stimulate clearing of *E. coli* from the blood. This corroborates our previous results which showed that when BLF was given by s.c. route only 26% of animals survived on day 30 after receiving *E. coli*, compared to the i.v. route, where more than 72% of animals survived (Zagulski *et al*. 1997). Taking both results jointly, we conclude that for survival of animals cooperation between both parts of the protective system is important.

It is well known that during growth in an iron-limited environment *E. coli* synthesize siderophores - enterochelin and/or aerobactin (Miles & Khimji 1975; Shand *et al*. 1985). Like other siderophore-synthesizing microorganisms (Trivier & Courcol 1996) *E. coli* can easily grow in the serum at extremely low iron level.

We conclude that *E. coli* multiply in both BLF-stimulated and BLF-unstimulated animals, although in the first group the multiplication rate may have been slightly lower because of lowering of the serum iron levels. The animals from the BLF-stimulated groups survived (Zagulski *et al*. 1989) because of the dramatic acceleration of clearing and killing activity, stimulated by BLF, which strongly exceeded the multiplication rate of *E. coli*.

In our earlier survival experiments with BLF (free of iron) in rabbits the serum iron levels were measured in the blood of individual animals. No correlation was observed between the serum iron level and the survival time of the animals (Zagulski *et al*. 1986). Iron (Fe^{+3}) given to mice in a single dose sufficient for full saturation of serum transferrin or in three doses given at three different times after *E. coli*, did not change the survival rate of animals (Zagulski *et al*. 1989).

A difference between iron-free and iron-saturated LF in protecting animals against *E. coli* has not been seen previously (Zagulski *et al*. 1985, 1986, 1989). The protective activity of LF in mice against *E. coli* was dramatically reduced, however, after reticulo-endothelial system (RES) blockade by dextran sulphate, particularly if the blocking agent was given before *E. coli* injection (Zagulski *et al*. 1997). This confirms that LF generates not only bacteriostatic but also a powerful killing system *in vivo*. Both systems should act together and cooperate *in vivo*. In the present study it is difficult to determine whether the low levels of iron in serum helps to eliminate the *E. coli* from blood and internal organs of BLF-stimulated animals.

The mechanism of the generation of the serum iron-dependent microbiostatic system by lactoferrin differs markedly from that found in other biological fluids (e.g. milk or saliva), in which the system is 'passive' and dependent on the iron-chelating ability of iron-free lactoferrin only. In serum, lactoferrin (iron-free or iron-saturated) acts by blocking the release of iron from the reticulo-endothelial (RE)-cells to serum. Simultaneously, iron from serum transferrin is quickly used for heme group synthesis (Latendre & Holbein 1983, 1984; Torrance *et al*. 1977; Bothwell 1978). The plasma transferrin iron pool of normal mice is very dynamic, with a

half-life of iron in the pool of 0.7 h (Latendre & Holbein 1983). This leads to short-term hypoferraemia.

In the liver (Perry & Ofek, 1984) and of other organs (Koch *et al*. 1993) there is non-immune rapid clearance of *E. coli* from blood. LF also binds with granulocytes (PMN) (Boxer *et al*. 1982; Maneva *et al*. 1983), and for stimulation and augmentation of phagocytosis PMN requires LF (Hansen *et al*. 1976; Bullen & Armstrong 1979; Gresham *et al*. 1988; Gahr *et al*. 1991). The regulatory role of LF in the migratory response of PMN during inflammation has also been described (Kijstra & Broersma 1984). Some authors postulate that the activated PMN leave the circulation (de Haas *et al*. 1994). Systemic infusion of LF induced rapid reduction in the number of circulating granulocytes within the first 2 min (Boxer *et al*. 1982).

Summarizing we suggest that in the powerful killing system generated by LF, two types of killing cels cooperate – macrophages and granulocytes. Cooperation of these types of cells in relation to the role of LF in the host defence system against intracellular parasites and other pathogens has been suggested previously (Byrd & Horowitz, 1991).

The interrelationship between the *in vitro* killing ability of LF (or its fragments) (Arnold *et al*. 1981, Bellamy *et al*. 1994) and the killing, protecting system generated by lactoferrin in mammals *in vivo* (Zagulski *et al*. 1989) remains unknown. We believe that in mammals this protecting system is active under normal physiological conditions, and its persistent activity may be supported by the LF continuously released into the blood from broken down (aged) polymorphonuclear leucocytes. In our experiment conditions the basal level of its activity was too low to clear off or kill the lethal dose of *E. coli* and the injection of endogenous BLF strongly activated the microbicidal capacity of the RE-system. We suggest therefore that the protecting system generated *in vivo* by BLF in mice against *E. coli* is primarily of a killing nature.

References

Arnold R.R., Russell J.E., Champion W.J. & Gauthier J.J. (1981) Bactericidal activity of human lactoferrin: influence of physiological conditions and metabolic state of the target microorganism. *Infect. Immun.* **32,** 655–660.

Bagby G.C., Vasiliki J., Rigas D. & Bennett R.M. (1981) Interaction of lactoferrin, monocytes, and T lymphocyte subsets in the regulation of steady-state granulopoiesis *in vitro*. *J. Clin. Invest.* **68,** 56–63.

Bellamy W.R., Yamauchi K., Wakabayashi H. & Takase M. (1994) Antifungal properties of lactoferricin B, a peptide derived from the N-terminal region of bovine lactoferrin. *Lett. Appl. Microbiol.* **18,** 230–233.

Bothwell T.H. (1978) The mechanisms of endotoxin-induced hypoferraemia. *Scand, J. Haematol.* **21,** 403–410.

Boxer L.A., Haak R.A., Yang H.H. & Wolach J.B. (1982) Membrane-bound lactoferrin alters the surface properties of polymorphonuclear leukocytes. *J. Clin. Invest.* **70,** 1049–1057.

Brock J.H., Pickering M.G., McDowall M.C. & Deacon A.G. (1983) Role antibody and enterobactin in controlling growth of *Escherichia coli* in human milk and acquisition of lactoferrin- and transferrin-bound iron by *Escherichia coli*. *Infect. Immun.* **40,** 453–459.

Bullen J.J. & Armstong J.A. (1979) The role of lactoferrin in the bactericidal function of polymorphonuclear leucocytes. *Immunology* **36,** 781–791.

Bullen J.J., Ward C.G. & Rogers H.J. (1991) The critical role of iron in some clinical infections. *Eur. J. Clin. Microbiol. Infect. Dis.* **10,** 613–617.

Byrd T.F. & Horwitz M.A. (1991) Lactoferrin inhibits or promotes *Legionella pneumophila* intracellular multiplication in non-activated and interferon gamma-activated human monocytes depending upon its degree of iron saturation. Iron-lactoferrin and nonphysiologic iron chelates reverse monocyte activation against *Legionella pneumophila*. *J. Clin. Invest.* **88,** 1103–1112.

De Haas M., Kerst, J.M.., Van Der Schoot C.E., Calafat J., Hack C.E., Nuijens J.H., Roos D, Van Oers R.H. & Von Dem Borne A.E. (1994) Granulocyte colony-stimulating factor administration to healthy volunteers: analysis of the immediate activating effects on circulating neutrophils. *Blood* **84,** 3885–3894.

Emery T. (1980) Iron deprivation as a biological defence mechanism. *Nature* **287,** 776–777.

Gahr M., Speer C.P., Damerau B. & Sawatzki G. (1991) Influence of lactoferrin on the function of human polymorphonuclear leukocytes and monocytes. *J. Leukoc, Biol.* **49,** 427–433.

Gresham H.D., McGarr J.A., Shackleford P.G. & Brown E.J. (1988) Studies on the molecular mechanisms of human Fc receptor-mediated phagocytosis. Amplification of ingestion is dependent on the generation of reactive oxygen metabolites and deficient in polymorphonuclear leukocytes from patients with chronic granulomatous disease. *J. Clin. Invest.* **82,** 1192–1201.

Hansen N.E., Karle H., Andersen V., Malmquist J. & Hoff G.E. (1976) Neutrophilic granulocytes in acute bacterial infection. Sequential studies on lysozyme, myeloperoxidase and lactoferrin. *Clin. Exp. Immunol.* **26,** 463–468.

Karpulus T.E., Ulevitch R.J. & Wilson C.B. (1987) A new method for reduction of endotoxin contamination from protein solutions. *J. Immunol. Methods* **105,** 211–220.

Kijlstra A. & Broersma L. (1984) Lactoferrin stimulates the production of leucocyte migration inhibitory factor by human peripheral mononuclear leucocytes. *Clin. Exp. Immunol.* **55,** 459–464.

Koch T., Duncker H.P., Axt R., Schiefer H.G., Van Ackern K. & Neuhof H. (1993) Alterations of bacterial clearance induced by endotoxin and tumor necrosis factor. *Infect. Immun.* **61,** 3143–3148.

Latendre E.D. & Holbein B.E. (1983) Turnover in the transferrin iron pool during the hypoferremic phase of experimental *Neisseria meningitidis* infection in mice. *Infect. Immun.* **39,** 50–59.

Latendre E.D. & Holbein B.E. (1984) Mechanism of impaired iron

release by the reticuloendothelial system during the hypoferremic phase of experimental *Neisseria meninitidis* infection in mice. *Infect. Immun.* **44,** 320–325.

MACHNICKI M., ZIMECKI M & ZAGULSKI T. (1993) Lactoferrin regulates the release of tumor necrosis factor alpha and interleukin 6 *in vivo*. *Int. J. Exp. Path.* **74,** 433–439.

MANEVA A.I., SIRAKOV L.M. & MANEV V.V. (1983) Lactoferrin binding to netrophilic polymorphonuclear leukocytes. *Int. J. Biochem.* **15,** 981–984.

MASSON P.L., HEREMANS J.F. & DIVE C. (1966) An iron binding protein common to many external secretion. *Clin. Chim. Acta* **14,** 735–739.

MASSON P.L., HEREMANS J.F. & SCHONNE E. (1969) Lactoferrin, an iron-binding protein in neutrophilic leucocytes. *J. Exp. Med.* **130,** 643–658.

MILES A.A. & KHIMJI P.L. (1975) Enterobacterial chelators of iron: their occurrence, detection, and relation to pathogenicity. *J. Med. Microbiol.* **8,** 477–490.

PERRY A. & OFEK I. (1984) Inhibition of blood clearance and hepatic tissue binding of *Exherichia coli* by liver lectin-specific sugars and glycoproteins. *Infect. Immun.* **43,** 257–262.

SHAND G.H., ANAWAR H., KUDURUGAMUWA J., BROWN M.R.W., SILVERMAN S.H. & MELLING J. (1985) *In vivo* evidence that bacteria in urinary tract infection grow under iron-restricted conditions. *Infect. Immun.* **48,** 35–39.

STOEBNER J.A. & PAYNE S.M. (1988) Iron-regulated hemolysin production and utilization of heme and hemoglobin by *Vibrio cholerae*. *Infect. Immunity* **56,** 2891–2895.

TIGYI Z., KISHORE A.R., MAELAND J.A. FORSGREN A. & NAIDU A.S. (1992) Lactoferrin-binding proteins in *Shigella flexneri*. *Infect. Immunity* **60,** 2619–2626.

TORRANCE J.D., CHARLTON R.W., SIMON M.O., LYNCH S.R., VAN SNICK K.L., MARKOWETZ B. & MASSON P.L. (1977) The ingestion and digestion of human lactoferrinn by mouse peritoneal macrophages and the transfer of its iron into ferritin. *J. Exp. Med.* **146,** 817–827.

TRIVIER D. & COURCOL R.J. (1996) Iron depletion and virulence in *Staphylococcus aureus*. *FEMS Microbiol. Letters* **141,** 117–127.

WEINBERG E.D. (1992) Iron depletion: A defense against intracellular infection and neoplasia. *Life Sci.* **50,** 1289–1297.

YANG H., KOOI C.D. & SOKOL P.A. (1993) Ability of *Psuedomonas pseudomallei malleobactin* to acquire transferrin-bound lactoferrin-bound and cell-derived iron. *Infect. Immun.* **61,** 656–662.

ZAGULSKI T., JARZĄBEK Z., ZAGULSKA A. & JĘDRA M. (1979) A simple method of obtaining large quantities of bovine lactoferrin. *Pr. Mater. Zootech.* **20,** 87–102.

ZAGULSKI T., ZAGULSKA A., JĘDRA M. & JARZĄBEK Z. (1985) Rabbit plasma iron level after *in vivo* administration of lactoferrin. *Pr. i Mater. Zootech.* **36,** 95–105.

ZAGULSKI T., JĘDRA M., JARZĄBEK Z. & ZAGULSAK A. (1986) Protective effect of lactoferrin during a systemic experimental infection of rabbits with *Escherichia coli. Anim. Sci. Pap. Rep.* **1,** 59–75.

ZAGULSKI T., LIPIŃSKI P., ZAGULSKA A., BRONIEK S. & JARZĄBEK Z. (1989) Lactoferrin can protect mice against a lethal dose of *Escherichia coli* in experimental infection *in vivo*. *Br. J. Exp. Path.* **70,** 697–704.

ZAGULSKI T., LIPIŃSKI P., ZAGULSKA A. & JARZĄBEK Z. (1997) Bovine lactoferrin protect mice against *Escherichia coli*: comparison of different ways of its application and effect of reticuloendothelial system blockade. *Animal Sci Papers and Reports* **15,** 183–193.

ZIMECKI M., MAZURIER J., MACHNICKI M., WIECZOREK Z., MONTREUIL J. & SPIK G. (1991) Immunostimulatory activity of lactoferrin and maturation of CD4− CD8− murine thymocytes. *Immunol. Letters* **30,** 119–124.

ZIMECKI M. & MACHNICKI M. (1994) Lactoferrin inhibits the effector phase of the delayed type hypersensitivity to sheep erythrocytes and inflammatory reactions to *M. bovis* (BCG). *Arch. Immunol. Ther. Exp.* **42,** 171–177.