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Protective effect of tissue ferritins in experimental *Escherichia coli* infection of mice *in vivo*

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Summary. The effect of ferritins from horse (FH) and bovine (FB) spleen and murine liver (FM) on the survival rate of CFW mice lethally infected with *Escherichia coli* (strain 844 0-78 K 80/B) was evaluated. Ferritins given intravenously 24 h before intravenous inoculation of bacteria, protected mice most effectively from death due to infection. The effect was dose dependent. At 500 μg of ferritin per mouse, the maximum survival rates were 86% (FH), 81% (FM) and 79% (FB), while only 5% of the control mice survived up to the 30th day. The survival rates of animals injected with bovine serum albumin (BSA) and heat-inactivated FB were 8 and 25%, respectively. Intraperitoneal injection of FB was as effective as intravenous in enhancing the resistance of mice against bacteria. These data provide evidence for the beneficial role of tissue ferritins in nonspecific antibacterial resistance.

Keywords: ferritins, protective effect, mice, *Escherichia coli*, experimental infection

Ferritin (FR) is a ubiquitous, and highly conserved iron-containing protein which plays a central role in intracellular iron metabolism. FR is known to sequester and thus detoxify iron that is taken up by cells. However, under conditions of iron need FR iron can be released for cellular utilization (reviewed by Crichton & Charloteaux-Wauters 1987). Compared with the large amounts of intracellular FR, serum FR is an extremely small fraction (Jacobs 1977).

Infection and inflammation strongly affect the metabolism of iron and of iron-binding proteins. The decrease in serum iron and iron-withholding are believed to be nonspecific defence against infection (reviewed by Weinberg 1984). A rise in serum FR levels during infection and aseptic inflammation has been reported by several authors (Elin *et al.* 1977; Birgegard *et al.* 1979; Zdravkovic 1987) but the source of FR in serum during inflammation is still uncertain. Cells of reticuloendothelial origin have been proposed (Birgegard & Caro 1984; Schiaffonati *et al.* 1988). It is probable that elevated serum FR observed in a number of inflammatory and infectious diseases is a consequence of an increased FR synthesis in inflammatory cells, followed by its increased release to the serum (Birgegard & Caro 1984). The augmentation of FR synthesis in inflammation occurs probably in response to an increased concentration of intracellular iron due to an impaired outflow of this metal from the

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reticuloendothelial system cells (Konijn & Hershko 1977). However, there is at least one more possible mechanism for explaining the stimulation of FR synthesis and its elevation in serum during inflammation. The time course of serum FR (a high proportion of which is present in glycosylated form (Birgegard 1980)) elevation parallels that of other glycoproteins, so-called acute phase proteins, most of which are synthesized under nonspecific stimulation of inflammatory mediators (Morimoto *et al.* 1989). In recent papers the evidence of an increased, iron-independent synthesis of FR *in vitro* in response to tumour necrosis factor and interleukin-1 has been demonstrated (Torti *et al.* 1988; Rogers *et al.* 1990). Although the mechanism of an increased FR synthesis in inflammation is partially explained, the biological role of this event is to be elucidated.

There is increasing evidence that the biological activity of iron-binding proteins is not limited to iron transport and storage. For example, lactoferrin, transferrin and acidic isoferritins have been proposed to be physiological regulators of myelopoiesis (Broxmeyer *et al.* 1983). It has been very recently suggested that the in-vitro and in-vivo myelopoietic suppressive effect of ferritins is linked to their ferro-oxidase activity (Broxmeyer *et al.* 1991). Furthermore, it has been suggested that FR is implicated in cellular differentiation (Fibach *et al.* 1985; Rhyner *et al.* 1985), e.g. differentiation of monocytes to macrophages (Andreesen *et al.* 1984), a process of fundamental importance for the functional activity of the immune system.

We have recently reported that lactoferrin when given to mice *in vivo* can protect a considerable proportion against a lethal bacterial infection (Zagulski *et al.* 1989). In the present study we examined the ability of tissue ferritins from different animal species to enhance antimicrobial resistance in the same experimental model of the lethal *Escherichia coli* infection. The data presented here may be helpful for an understanding of the biological role of FR in inflammation.

Materials and methods

Mice

Male and female CFW mice were obtained from the Department of Animal Breeding, Polish Academy of Sciences, Lomna-Las. They were fed a standard diet (Murigram) and had access to water, *ad libitum*. They were used at the age of 8–10 weeks, at which time they weighed 25–30 g. Mice were routinely acclimatized for 2 weeks prior to use in experiments.

Preparation and use of tissue ferritins

Bovine (FB) and murine (FM) ferritin were isolated from bovine spleen and murine livers (obtained from 50 CFW mice), respectively according to the method described previously (Cetinkaya *et al.* 1985). The purity of FB and FM preparations, as checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis was about 95 and 90%, and iron-saturation was about 23 and 11%, respectively. Horse spleen ferritin (FH), 46% iron-saturated, was purchased from Sigma Chemical Co. (St Louis), purity 98%. All ferritin preparations contained less than 8 ng endotoxin/g protein, as determined by the *Limulus* assay for endotoxin (Gardi & Arpagaus 1980, Sigma Chemical Co. St Louis). Heat-inactivated FB was prepared by heating it at 100°C for 5 min. In all experiments, appropriate dilutions of tissue ferritins were made in 0.1 ml of pyrogen-free phosphate buffered saline, pH7.2 (PBS) and injected into mice prior to infection by different routes: intravenously (i.v.), intraperitoneally (i.p.), intramuscularly (i.m.), and subcutaneously (s.c.). Detailed protocols of experiments are presented in the legend to each table and figure.

Bovine serum albumin (BSA)

This was purchased from SERVA, purity 98%. BSA was given to mice as control protein at a dose of 10 mg/mouse in 0.1 ml of PBS.

Preparation and use of bacteria

Two enterotoxigenic strains 844 0-78 K 80/B and 1338 0-9 K 38 of *Escherichia coli* were used for experimental infection. The protective effect of FB against the latter strain was examined in only one experiment. Both strains were obtained from the Pulawy Veterinary Institute, Veterinary Museum of Microorganisms. For all experiments the bacteria were prepared as described previously (Zagulski *et al.* 1986). Mice were injected intravenously (into the lateral tail vein) with a single dose of living *E. coli* containing 2×10^8 cells suspended in 0.1 ml of PBS. Mice were infected 24 h after tissue ferritins or BSA administration.

Statistical analysis

Data are presented as the mean ± standard deviation of survival rates of mice from three separate experiments (three separate *E. coli* inoculations). The determination of statistical significance between the survival rates of mice from different groups (20–70 mice per group) utilized the chi-squared test.

Results

Dose-dependent protective effect of tissue ferritins

There were no apparent toxic effects of tissue ferritins in mice. The dose-dependence of the protective effect of tissue ferritins on the lethality of *Escherichia coli* (strain 844 0-78 K 80/B) in experimentally infected mice is shown in Table 1. The maximum survival rate observed was 89%, when mice were stimulated with 1000 μg FB per mouse, while only 5% of the control animals receiving *E. coli* alone survived up to 30 days after infection. At 500 μg of FB per mouse the protective effect was less pronounced but not significantly. All tissue ferritins given at a dose of 500 μg per mouse had almost the same ability to protect mice against infection. Highly reduced protection of mice from death due to infection was observed after stimulation of animals with 100 μg of FB or FM per mouse. In all experiments thereafter, FB being the most available tissue ferritin in our laboratory was used at a dose of 250 μg per mouse. We found this dose as the least that gave the maximum protective effect.

Table 1. Tissue ferritins were given to mice 24 h before inoculation. The control group received no treatment; survival rate, 5 ± 3%. The survival rate was observed for 30 days after inoculation

Tissue ferritin	Dose (μg/mouse)	No. of exp./mice	Survival rate (%)
FB	1000	3/39	89 ± 5
	500	5/58	79 ± 7
	250	4/47	72 ± 9
	100	4/34	47 ± 10
FM	500	3/33	81 ± 2
	250	4/47	74 ± 5
	100	4/47	47 ± 10
FH	500	4/36	86 ± 3

FB, bovine spleen ferritin; FM, murine liver ferritin; FH, horse spleen ferritin.

Specificity of the protective effect of FB

A single intravenous dose of 250 μg of FB per mouse 24 h before challenge by *E. coli* (strain 844 0-78 K 80/B) protected 73% of mice throughout the 30-day observation period (Fig. 1). The survival rate of FB-stimulated mice was almost unchanged from day 7 (survival rate, 75%) to 30 days post infection. Survival curves of control mice and mice pretreated with BSA rapidly decline on day 1 (survival rate about 20%), and on day 30 after infection reach levels of 6 and 8%, respectively. Mice receiving an injection of heat-inactivated FB showed increased survival rates, when compared with controls and BSA pretreated mice. However, the mortality in this group from day 1 until the end of the observation period was signifi-

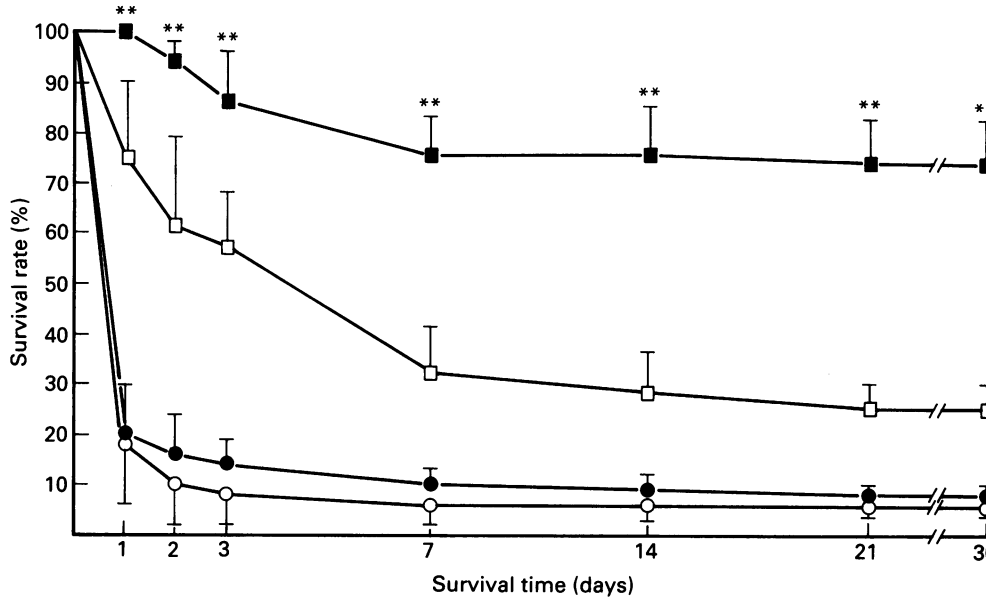

Fig. 1. FB and heat-inactivated FB were given to mice i.v. at a dose of 250 μg per mouse 24 h before inoculation of *E. coli*. BSA was given to mice in the same way as FB. The number of experiments and of animals in each group is shown in parentheses. ■, FB + *E. coli*, (6/66); □, heat-inactivated FB + *E. coli*, (3/28); ●, BSA + *E. coli*, (6/54); ○, *E. coli*, (6/79). ** $P<0.01$, significantly different from the value for the group of mice treated with heat-inactivated FB.

cantly higher ($P<0.01$), when compared with FB-stimulated mice. About 17% of control mice infected with a less virulent strain (1338 0-9 K 38) of *E. coli* survived the challenge (Fig. 2). The protective effect of FB

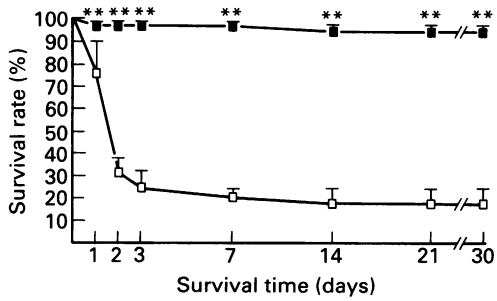

Fig. 2. FB was given to mice i.v. at a dose of 250 μg per mouse 24 h before inoculation with *E. coli*. The number of experiments and of animals in both groups is shown in parentheses. ■, FB + *E. coli*, (3/38); □, *E. coli*, (3/29). ** $P<0.01$, significantly different from the value for the group of control mice.

in mice infected with this strain resulted in the final survival of 98% of mice.

Time-dependent protective effect of FB

FB most effectively increased the survival rate of infected mice, when given between 12 and 72 h before *E. coli* infection (Fig. 3). The maximum protection (survival rate on day 30, 77%) was observed after stimulation of mice with FB 24 h prior to infection. The effects of i.v. administration of FB to mice more than 72 h or less than 12 h before *E. coli* infection were significantly decreased ($P<0.01$), when compared with 24 h stimulation. This significant decrease was observed from day 1 until the end of experiment.

Route-dependent protective effect of FB

FB given to mice i.v. or i.p. at a dose of 250 μg per mouse 24 h before infection with *E. coli*

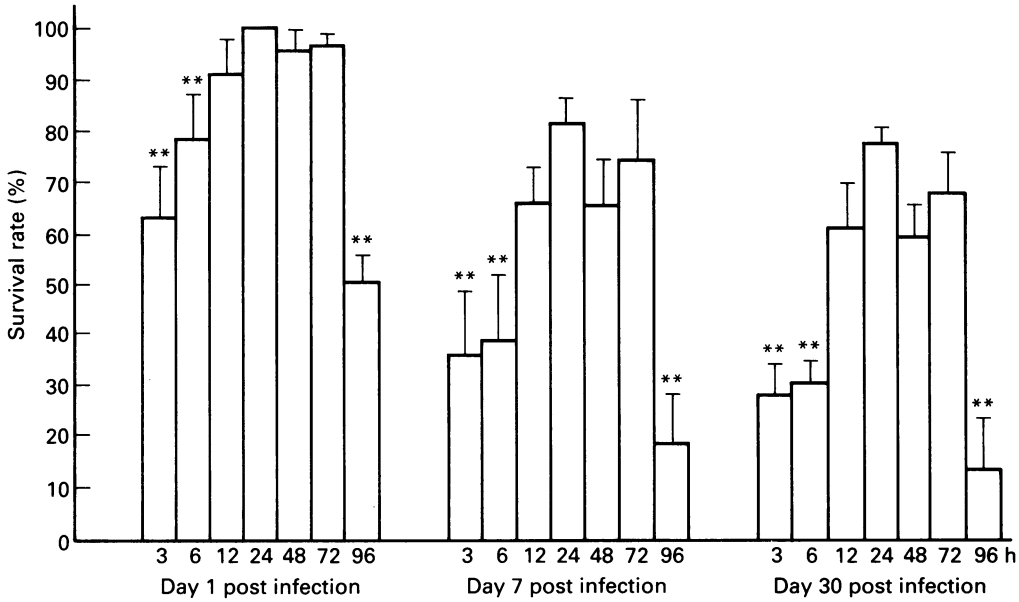

Fig. 3. FB was given to mice at a dose of 250 μg per mouse at periods of 3–96 h before inoculation with *E. coli*. The control group received no treatment; final survival rate, 5±2%. **, $P<0.01$, significantly different from the value for the group of mice treated with FB 24 h before inoculation with *E. coli*.

(strain 844 O-78 K 80/B) protected more than 70% of animals from death (Table 2). The administration of FB i.m. or s.c. resulted in a reduction in the final survival rates of infected mice compared with the i.v. route. A relatively high protective effect was observed when control protein, BSA, was given to mice. There were no significant differences between survival rates of mice stimulated i.m. or s.c. with FM or BSA.

Table 2. FB was given to mice at a dose of 250 μg per mouse 24 h before inoculation with *E. coli*. The control group received no treatment; survival rate, 6±2%. The survival rate was observed for 30 days after inoculation

Route	Group	No. of exp./mice	Survival rate (%)
i.v.	FB+*E. coli*	6/66	73±10**
	BSA+*E. coli*	6/54	8±2
i.p.	FB+*E. coli*	3/40	72±10**
	BSA+*E. coli*	3/39	18±3
i.m.	FB+*E. coli*	2/20	30±0
	BSA+*E. coli*	3/36	25±9
s.c.	FB+*E. coli*	4/56	46±10
	BSA+*E. coli*	3/30	33±5

** $P<0.01$, significantly different from the value for the group of mice treated with BSA.

Discussion

In the present study we demonstrated the protective role of i.v. administered tissue ferritins using the experimental model of a lethal systemic infection of mice with *E. coli* (strain 844 O-78 K 80/B). Tissue ferritins isolated from three animal species significantly increased antibacterial resistance of mice in a dose-dependent manner (Table 1). All tissue ferritins showed almost the same protective effect, when given to mice at a dose of 500 or 250 μg per mouse (Table 1). The ineffectiveness of BSA given i.v. in our experimental model (Fig. 1) and high protection of mice obtained with FM (Table 1) suggest that the protective effect of tissue

ferritins given i.v. is specific for these proteins and cannot be considered as a nonspecific effect of foreign proteins. The relatively high survival rates of mice pretreated with heat-inactivated FB (Fig. 1) may be due to incomplete inactivation of the protein. The heat-resistance of the FR molecule was known long ago (Granick 1946). However, survival rates of mice pretreated with heat-inactivated FB were significantly lower ($P<0.01$), when compared with those of FB-stimulated mice. Although the highest final survival rate (89%) in our experiment was obtained when mice were stimulated with 1000 μg of FB per mouse, we have found the dose of 250 μg sufficient for significant stimulation of antibacterial resistance in mice. The protective effect of FB in experimental infection of mice with the strain 844 0-78 K 80/B was confirmed in the same experimental conditions but with a less virulent (final survival rate of controls, 17%) strain 1338 0-9 K 38 of *E. coli*. Pretreatment of mice with FB resulted in the protection of almost all animals infected with this strain (Fig. 2.).

Taking advantage of the results obtained in our previous experiments with lactoferrin (Zagulski *et al.* 1989), we stimulated mice with tissue ferritins 24 h prior to bacterial infection, and we found the same protective effectiveness of both iron-binding proteins given to mice i.v. at this time. Furthermore, we demonstrated in the present study that the protective effect of FB was almost unchanged, when treatment preceded infection by 12–72 h (Fig. 3). Pretreatment of infected mice with FB by different routes showed ineffectiveness of intramuscular and subcutaneous administration (Table 2). It is not excluded, however, that on the other schedule and at a dose higher than 250 μg per mouse, FB could be more effective, when given to mice i.m. or s.c.

On the basis of our findings, it remains unresolved whether the biological activity of tissue ferritins observed in our experimental model may be exhibited by FR naturally released to the serum during inflammatory and infectious diseases. FR circulating *in vivo* appears to have little or no iron (Worwood *et al.* 1975). In contrast, ferritins used by us were more or less iron-saturated. Furthermore, we failed to protect mice against infection with bovine apoferritin (apoFB) obtained by dialysis against thioglycollic acid (data not shown). It can not be excluded, however, that apoFB obtained by a less drastic method could have a similar effect to FB. No differences in the clearance rate were observed between apoFR prepared by high-speed centrifugation and whole FR derived from rat liver (Halliday *et al.* 1979).

The major part (70–80%) of serum FR is glycosylated unlike intracellular FR (Birgegard 1980). The rate of glycosylation of FR molecule seems to be decisive for the clearance of FR from the serum into the intracellular compartment (Halliday *et al.* 1979). When tissue ferritins were injected i.v. into animals from different species (Pollock *et al.* 1978; Halliday *et al.* 1979; Simon *et al.* 1987; Frenkel *et al.* 1987), they were cleared from circulation with a half-life of between 2 and 10 min. Although the clearance rate of glycosylated FR is significantly prolonged (Halliday *et al.* 1979), the time needed for the stimulation of host defence of mice in our experimental model (12–72 h) allows us to suppose that the glycosylated form of FR could be equally effective in our experimental model as the non-glycosylated. It is probable that in our study we mimic the effect exhibited by tissue ferritins leaked in increased amounts from cells damaged during infectious and inflammatory diseases. These iron-rich ferritins, whose iron can be easily accessible for invading pathogens, have to be quickly cleared from the circulation and at the same time can be responsible for the rapid stimulation of host resistance. This can be continued by glycosylated ferritins naturally released from the reticuloendothelial cells.

An approximate calculation of FR concentration in the serum of mice after injection of 250 μg of protein indicates that the serum FR level exceeds about tenfold the highest FR levels found in the serum of patients with

acute inflammatory diseases (Birgegard *et al.* 1979; Zdravkovic 1987). However, the model of a strong, lethal infection practically never occurs during normal conditions in ontogenic life, so the host resistance would not be enhanced as dramatically as it was in the case of mice used in our experimental model.

On the basis of our results it is difficult to offer a mechanistic explanation for the mode of action of tissue ferritins in antibacterial resistance. Some cytokines are capable of stimulating resistance against experimental bacterial infections in mice in a similar way as tissue ferritins in our experimental model (Ozaki *et al.* 1987; Weyand *et al.* 1987; Blanchard *et al.* 1988). A direct stimulation of phagocyte function has been proposed for explaining the beneficial role of interleukin-1 (Ozaki *et al.* 1987) and tumour necrosis factor (Blanchard *et al.* 1988) during experimental bacterial infection in mice. We suppose that a similar mechanism can be taken into account in the case of tissue ferritins. In accordance with the previous observations (Lagunoff & Curran 1972), FR is found to bind to coated pits on the surface of macrophages. It has been demonstrated that macrophage plasma membrane is equipped with binding sites for FR and that FR is internalized by receptor-mediated endocytosis (Thyberg *et al.* 1985). Organ distribution of radiolabelled tissue ferritins injected i.v. into dogs (Pollock *et al.* 1978), rats (Halliday *et al.* 1979) and guinea-pigs (Simon *et al.* 1987) indicates that ferritins are accumulated mainly in liver and spleen—major sites of reticuloendothelial system.

Results of the previous work (Laohapand *et al.* 1985) have demonstrated a generalized leucocyte mobilization, sequestration and tissue infiltration after intravenous injection of horse spleen ferritin into rats. The authors suggest a major systemic activation of a factor during circulation and removal of foreign protein (horse ferritin), but in the light of the present results the inflammatory-like effect observed by them can be considered as specific for FR.

The mechanism of the protective effect of tissue ferritins in experimental *E. coli* infection in mice is under investigation in our laboratory.

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