Conjugation of Hydroxyethyl Starch to Desferrioxamine (DFO) Modulates the Dual Role of DFO in \textit{Yersinia enterocolitica} Infection

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The iron chelator desferrioxamine (DFO) B is widely used in the therapy of patients with iron overload. As a side effect, DFO may favor the occurrence of fulminant \textit{Yersinia} infections. Previous work from our laboratory showed that this might be due to a dual role of DFO: growth promotion of the pathogen and immunosuppression of the host. In this study, we sought to determine whether conjugation of DFO to hydroxyethyl starch (HES-DFO) may prevent exacerbation of \textit{Yersinia} infection in mice. We found HES-DFO to promote neither growth of \textit{Yersinia enterocolitica} nor mitogen-induced T-cell proliferation and gamma interferon production by T cells in vitro. Nevertheless, in vivo HES-DFO promoted growth of \textit{Y. enterocolitica} possibly due to cleavage of HES and release of DFO. The pretreatment of mice with DFO resulted in death of all mice 2 to 5 days after application of a normally sublethal inoculum of \textit{Y. enterocolitica}, while none of the mice pretreated with HES-DFO died within the first 7 days postinfection. However, some of the HES-DFO-treated mice died 8 to 14 days postinfection. Thus, due to the delayed in vivo effect HES-DFO failed to trigger \textit{Yersinia}-induced septic shock, which accounts for early mortality in DFO-associated septic mouse. Moreover, our data suggest that DFO needs to be taken up by host cells in order to exert its immunosuppressive action. These results strongly suggest that HES-DFO might be a favorable drug with fewer side effects than DFO in terms of DFO-promoted fulminant infections.

\textit{Yersinia enterocolitica} is a gram-negative bacterium which is pathogenic for humans and rodents (23, 25). Infection with this pathogen causes a wide range of clinical manifestations including enterocolitis and mesenteric lymphadenitis (28). In immunocompromised patients or patients with iron overload, \textit{Yersinia} causes systemic infections with abscesses in spleen and liver (27, 33, 37).

Previous work from this laboratory showed that desferrioxamine (DFO) may play a dual role in pathogenesis of \textit{Yersinia} infection: growth and virulence promotion of \textit{Y. enterocolitica} by iron provision to the pathogen and immunosuppression of the host. In fact, iron-loaded DFO (ferrioxamine [FO]) can be taken up and used as an iron source by \textit{Yersinia} (16, 36). The genes encoding FO uptake have been characterized and are considered part of the virulence factors required for high-level pathogenicity of \textit{Yersinia} (10, 11).

On the other hand, DFO exerts effects on various components of the immune system of the host. DFO inhibits proliferation of T and B lymphocytes and cytokine production of macrophages and modulates interaction of polymorphonuclear leukocytes with \textit{Yersinia} (3, 21). In keeping with these observations, we and others have demonstrated that DFO increases pathogenicity of \textit{Y. enterocolitica} in mice, resulting in fatal septicemia and shock (5, 39, 40). Moreover, fatal septicemia with \textit{Yersinia} and other microorganisms including the fungus \textit{Rhizopus} sp. has been reported for patients undergoing DFO therapy (13, 15, 40).

In an attempt to find drugs with comparable iron binding capacity but reduced \textit{Yersinia} virulence-enhancing properties, DFO B has been compared with DFO G in terms of its biological properties for bacteria and host cells (3, 5). DFO G was found to have fewer immunosuppressive properties and to exert less enhancement of virulence of \textit{Yersinia} in vivo (5). Thus, DFO G might be a favorable alternative to DFO B in clinical DFO therapy.

Moreover, studies have been conducted with DFO bound to hydroxethyl starch (HES) and have indicated that HES-DFO improves safety without interference with the iron binding efficacy of DFO (22, 29, 32). In accordance with these results, HES-DFO was found to significantly attenuate systemic oxidant injury, resulting in less toxicity to the lung and kidney in early sepsis (30, 34). Therefore, this study is focused on the immunological effects of HES-DFO on T cells and the virulence-modulating effect of HES-DFO on \textit{Y. enterocolitica} in vitro and in vivo.

**MATERIALS AND METHODS**

**Mice.** Female BALB/c or C57BL/6 mice aged 6 to 8 weeks (Charles River Wiga, Sulzfeld, Germany) were kept under specific-pathogen-free conditions (positive-pressure cabinet) and provided food and water ad libitum.

**Bacteria.** \textit{Y. enterocolitica} serotype O3 strain Y-108 (yersiniabactin-negative wild-type strain) (23) and \textit{Y. enterocolitica} serotype O8 strain WA-314 (yersiniabactin-positive wild-type strain), both harboring the virulence plasmid pYV, were passaged in mice and cultured as described previously (6). The WA-fax4 mutant strain, which lacks the fax4 gene encoding the FO receptor FoxA, was derived from the WA-314 strain (11). The \textit{Escherichia coli} strain HK97 (aroB fua4 fisnu4 E:Ap lac Mu; enterobactin-negative mutant with an insertationally inactivated gene of the FO E receptor) (20, 43, 44) and plasmid pFU2 encoding the DFO receptor FoxA of WA-314 (11) were kindly provided by K. Hanterke (Tübingen, Germany).

**Siderophores.** DFO (DFO mesylate; Desferal) was donated by Novartis (Basel, Switzerland), and HES-DFO was provided by Biomedical Frontiers, Inc. (Minneapolis, Minn.). HES-DFO consists of DFO that has been covalently attached to HES (22). The resulting polymeric iron chelator is polydisperse with an average molecular mass of 70,000 Da. The aqueous solution of HES-DFO was at a total chelator concentration of 40 mM (pH 6.0 to 6.6). This is equivalent to 26 mg of DFO/ml in chelating capacity. Both DFO and HES-DFO were dissolved in distilled water and sterile filtered prior to use. Mice were injected intraperitoneally with 1.0 ml of 8 mM DFO or HES-DFO 1 h prior to challenge with \textit{Y. enterocolitica} as described previously (6, 40). Control mice were injected with phosphate-buffered saline (PBS) at pH 7.4.

**Biassay for utilization of DFO B (DFO) and HES-DFO.** To determine the ability of the bacteria \textit{Y. enterocolitica} O8 strains WA-314 and WA-fax4; \textit{Y. enterocolitica} O3 strain Y-108; and \textit{E. coli} HK97(pFU2)) to utilize DFO and HES-
DFO as an iron carrier in vitro, the strains were grown in NB medium (8 g of nutrient broth and 5 g of NaCl per 1 liter of distilled water) to an optical density of 0.5 at a wavelength of 600 nm. Thirty microliters of the bacteria was seeded in 10 ml of 0.6% H2O top agar on 1% NB agar, both containing the iron chelator α-dipiridyl at a concentration of 200 μM (24). The iron-chelating compounds were provided by filter papers soaked with 12 μl of a solution containing 4 mM DFO or HES-DFO. The filter papers were placed on the agar surface, and the diameters (mean values of five separate determinations) of the zone of enhanced bacterial growth around the filter paper were determined after 24 h of culture at 26°C (Yersinia) and 37°C (E. coli), respectively. Additionally, iron-loaded chelators FO B (FO) and FO B-HES (HES-FO) were used in the same way as DFO.

The presence of iron-loaded chelators in sera of DFO- and HES-DFO-treated mice was monitored. For this purpose, mice were killed 1, 4, and 12 h after injection with DFO and HES-DFO, respectively. Sera were prepared and used in the bioassay described above to determine the in vivo feeding properties of the serum after injection of DFO and HES-DFO in order to reveal the presence of DFO in the serum.

Animal infection. For infection of mice, frozen stocks of *Y. enterocolitica* O3 strain *O3/Y-108* were thawed and diluted in PBS to the appropriate concentration as stated below. Suspensions containing various numbers of bacteria were administered intravenously to mice 1 h after pretreatment with 1 ml of 8 mM DFO, HES-DFO, or PBS. Briefly, three groups of mice (eight per group) were challenged with a normally sublethal inoculum of *Y. enterocolitica* O3 strain *Y-108* (0.2% 50% lethal dose = 1.2 × 10⁶ CFU). The optimization of the various compounds used in these experimental settings has been described previously (5). The actual number of bacteria administered was determined by plating 0.1 ml of serial dilutions of the inoculum on Mueller-Hinton agar and counting CFU after a 36-h incubation at 26°C. The survival of mice was observed for 12 days. In parallel experiments, mice were killed at days 2, 4, and 12 postinfection and the numbers of bacteria reisolated from spleen were determined. For this purpose, the spleen was aseptically removed and homogenized in sterile PBS-bovine serum albumin-Tergitol. Serial 1:10 dilutions of the homogenate were plated on Mueller-Hinton agar, and after a 36-h incubation at 26°C, CFU were counted.

Cell culture medium. Cells were cultured in Click/RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 2 mM L-glutamine (Life Technologies GIBCO BRL, Berlin, Germany), 10 mM HEPES (Biochrom), 5 × 10⁻³ M 2-mercaptoethanol, 100 μg of streptomycin per ml, 100 U of penicillin (Biochrom) per ml, and 10% heat-inactivated fetal calf serum (Biochrom).

Cell suspensions and culture conditions. Splenocytes from mice were removed aseptically, and single-cell suspensions were prepared; 2 × 10⁶ splenic mononuclear cells (SMNC) were cultured in rounded-bottom microtiter plates (Nunc, Wiesbaden, Germany) and incubated with serial dilutions of either DFO, HES-DFO, FO, or HES-FO. Simultaneously, 3 μg of concanavalin A (ConA; Pharmacia, Uppsala, Sweden) per ml of medium was added to the wells for mitogenic induction of T-cell activation and proliferation at 37°C in a humidified atmosphere of 5% CO₂.

Proliferation assay. Triplicate cultures of SMNC were pulsed with 1 μCi of [³H]thymidine (ICN Biochemicals, Eschwege, Germany) per well for 6 h after 24 days of incubation. The samples were collected using a cell harvester (Harvester 3, Chrom) per ml, and 10% heat-inactivated fetal calf serum (Biochrom).

Mass culture. Growth of different strains was promoted by iron-loaded FO (halo diameter of enhanced bacterial growth; 44.8 ± 2.6 mm). In contrast, DFO (halo diameter of 3.5 mm) suppressed growth of the strain *W. WA-314* defective in the FO B receptor FoxA but still able to take up iron by the yersiniabactin siderophore system. Growth of the *Y. enterocolitica* strain was promoted by iron-loaded FO (halo diameter of enhanced bacterial growth; 44.8 ± 3.0 mm) as well as by iron-free DFO (39.8 ± 2.6 mm). In contrast, HES-FO and HES-DFO suppressed growth of the *Y. enterocolitica* strain under test conditions (halo diameter of growth inhibition; 9.8 ± 1.3 (5 M μM; 2.3 mm, respectively).

**RESULTS**

Promotion of *Y. enterocolitica* growth by DFO (FO) and HES-DFO (HES-FO). HES-DFO represents a high-molecular-weight form of the siderophore DFO B (DFO), generated by covalent binding of DFO to HES (22). The different molecular

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Diam (mm) of growth enhancement zone (+)</th>
<th>Diam (mm) of growth inhibition zone (−)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Y. enterocolitica</em></td>
<td></td>
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<tr>
<td>WA-314</td>
<td>+39.6 ± 2.6*</td>
<td>−11.6 ± 2.3*</td>
</tr>
<tr>
<td>FO</td>
<td>−21.0 ± 2.5*</td>
<td>−14.0 ± 3.5*</td>
</tr>
<tr>
<td>HES-FO</td>
<td>−26.2 ± 3.3*</td>
<td>−9.8 ± 1.8*</td>
</tr>
<tr>
<td><em>E. coli</em> HK97</td>
<td>+32.4 ± 3.0*</td>
<td>0</td>
</tr>
</tbody>
</table>

* Results are means ± standard deviations of five independent investigations. The asterisks indicate statistically significant differences (*P* < 0.05) between DFO and HES-DFO for each strain. Strains were grown on α-dipiridyl-containing NB agar to restrict iron supply. Diameters of zones of enhanced or decreased growth around siderophore-containing filter papers were determined after 24 h of culture. Supplemental iron was provided by filter papers soaked with 12 μl of 4 mM solutions of siderophores FO or HES-FO, respectively. DFO and HES-DFO are the corresponding iron-free derivatives of these siderophores.

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Y. enterocolitica O3 strain Y-108 was used as a Yersinia strain that lacks the yersiniabactin siderophore system while still being able to take up FO by the FO receptor FoxA. Growth of the Y-108 strain was promoted by FO (30.2 ± 3.3 mm) and DFO (26.2 ± 3.3 mm), whereas both HES-FO and HES-DFO exhibited a slight inhibition of growth of the Y-108 strain (9.8 ± 1.8 and 8.2 ± 1.5 mm, respectively).

E. coli strain HK97(pFU2) has been used as an additional indicator strain for a selective FO uptake, as reported previously (5). Under the test conditions, both FO and DFO showed growth enhancement (halo diameter of enhanced bacterial growth, 31.4 ± 3.7 and 32.4 ± 3.0 mm, respectively) on E. coli strain HK97(pFU2), whereas HES-FO and HES-DFO exhibited no effect on growth enhancement.

The DFO-FO bioassay using E. coli strain HK97(pFU2) as indicator strain was used to detect DFO-FO in sera of mice after DFO treatment. To investigate the effect of parenterally administered DFO in comparison to HES-DFO on bacterial growth, we determined whether sera taken from mice injected with DFO or HES-DFO could provide iron to bacteria by using the bioassay described above (Table 2). The bioassay was done with E. coli strain HK97(pFU2), because both Yersinia strains (WA-314 and Y-108) showed some background growth when incubated with sera from mice, as reported previously (5). As shown in Table 2, serum obtained from mice 1 h after treatment with HES-DFO showed a significantly smaller growth-promoting effect on bacteria (23.8 ± 2.3 mm) than did serum from mice injected with DFO (42.4 ± 2.6 mm). As in vitro DFO-FO, but not HES-FO–HES-F, promotes growth of E. coli HK97(pFU2), the results suggest the presence of free DFO-FO in serum from mice injected with HES-DFO. Moreover, while DFO-FO was no longer detectable after 2 to 4 h, it was still present in sera of mice 24 h after injection with HES-DFO (Table 2).

DFO-modulated proliferation and cytokine production of T cells. In accordance with previous results (5), ConA-induced proliferation of T cells could be entirely inhibited in a dose-dependent manner by DFO as determined by [3H]thymidine uptake (Fig. 1). Blocking of ConA-stimulated proliferation by DFO could be reversed by the addition of equimolar concentrations of ferric iron (data not shown). In contrast, inhibition of T-cell proliferation was not observed with HES-DFO or HES-FO (Fig. 1).

The effect of DFO, FO, HES-DFO, and HES-FO on IFN-γ production by T cells was investigated by ELISA-based determination of IFN-γ concentrations in supernatants of ConA-stimulated T cells. The data depicted in Fig. 2 show that DFO at a 100 μM concentration inhibited the ConA-induced IFN-γ production of T cells by 90% (P < 0.001). As observed for T-cell proliferation, the addition of an equimolar concentration of ferric ions abolished this effect (data not shown). In contrast, HES-DFO showed only an insignificant effect on ConA-induced IFN-γ production by T cells (Fig. 2).

Influence of DFO and HES-DFO on virulence of Y. enterocolitica. In order to analyze the effects of DFO and HES-DFO on virulence of Y. enterocolitica for mice, BALB/c mice were sublethally infected with Y. enterocolitica strain 108-P 1 h after administration of DFO, HES-DFO, or PBS. As shown in Fig. 3, all mice injected with DFO before Y. enterocolitica infection died by days 2 to 5 from fulminant Yer ninia infection leading to septic shock with necrosis of liver tissue (data not shown). In contrast, none of the controls (PBS) or HES-DFO-treated mice died during the early phase of the infection. However, at days 7 to 12 postinfection some mice injected with HES-DFO died from severe Yersinia infection leading to formation of macroabscesses in liver, lung, and spleen, while all of the control mice survived (Fig. 3).

In an attempt to explain this different kinetics of survival after Yersinia infection in DFO- or HES-DFO-treated mice, additional experiments were conducted in which mice were killed at various intervals after the infection and the numbers of yersiniae in spleens were determined. As shown in Fig. 4, mice treated with DFO had significantly higher bacterial counts in the spleen compared to controls (P < 0.001) or HES-DFO-treated mice (P < 0.05) on day 4 postinfection.
DISCUSSION

Iron restriction encountered in the body fluids of mammals by invading microorganisms is part of the nonspecific host defense against bacterial pathogens. A highly efficient iron acquisition system is therefore an essential feature for successful multiplication of pathogenic bacteria in the host. Several species of pathogens use low-molecular-weight iron-chelating compounds (siderophores) such as DFO for iron uptake (8, 17, 19). As DFO is widely used in deferration therapy in humans with iron overload (31), a severe side effect of DFO treatment is an increased susceptibility of the patients to microbial infections (13, 15, 18, 39, 40, 47). In this study, we compared the effects of the high-molecular-weight form of DFO (HES-DFO) and DFO on bacterial growth, cellular immune response in terms of T-cell proliferation, and cytokine production in vitro. Moreover, we determined the effects of both siderophores on *Yersinia* infection in vivo.

For DFO and HES-DFO, the results of the in vitro siderophore feeding experiments demonstrate a different impact on growth of the *Yersinia* strains. *Y. enterocolitica* O8 strain WA-314 is able to utilize iron-loaded DFO (FO) as an iron source via the FO receptor FoxA (11, 42). Moreover, the WA-314 strain expresses an endogenous siderophore-mediated iron uptake system, the versiniabactin system (12, 35, 38). WA-foxA lacks the FO receptor but is still able to take up iron via the versiniabactin system. In contrast, *Y. enterocolitica* O3 strain Y-108-P is able to utilize FO but is devoid of the versiniabactin system. Consistently, the results of the in vitro feeding experiments indicate that HES-FO cannot be used as an iron source via FoxA by *yersinia*, whereas unmodified FO is able to provide iron to those strains expressing the FO receptor FoxA. This is most probably due to blocking of transmembranous DFO-FO uptake by conjugation to HES. Moreover, the results obtained with the mutant strain WA-foxA incubated either with FO or with HES-FO showed impaired bacterial growth when HES-FO was applied. As WA-foxA is not able to take up FO but still can utilize the endogenous siderophore versiniabactin, the results may suggest that WA-foxA is provided with iron by versiniabactin competing with iron-loaded FO. Moreover, it is tempting to speculate that versiniabactin is hindered in chelating iron from FO if the HES moiety is linked to the FO molecule.

Further feeding experiments were performed using serum of mice previously treated with either HES-DFO or DFO. In DFO-injected mice, serum exhibited strong growth promotion (42.4 ± 2.6-mm feeding halo) as revealed by the in vitro feeding assay. However, this effect could be observed only transiently at 1 h after DFO injection, suggesting that free DFO-FO is available only during a short time. In contrast, serum of HES-DFO-treated mice initially exhibited a significantly lower promotion of growth of *yersiniae* (23.8 ± 2.3-mm feeding halo) 1 h after injection compared to serum of DFO-injected mice. Moreover, this growth-promoting effect was observed for more than 24 h, suggesting that in HES-DFO-treated mice DFO is released from HES-DFO during a period of at least 24 h, probably due to cleavage of HES, e.g., by amylase present in normal serum (45).

Besides growth-promoting effects on *yersiniae*, we investigated the suppression of T-cell activation by HES-DFO. Inhibition of ConA-stimulated T-cell proliferation and IFN-γ production in vitro was exclusively caused by DFO, not by HES-DFO. IFN-γ is the most crucial and dominant cytokine required for control of *Yersinia* infection (2). However, we cannot exclude the possibility that production of other cytokines might also be affected by DFO.

Using primary cultures of rat proximal tubular cells, Pallier and Hedlund (34) have shown HES-DFO in contrast with DFO to be exclusively confined to the extracellular compartment. In line with this observation and our previous results (3–5), the bioassays argue for an intracellular target of DFO for the impairment of T-cell function. The short half-life of DFO in serum is due to the rapid renal clearance of this compound (1) and possibly due to the intracellular accumulation (14). Both mechanisms may not be true for DFO bound to HES. Thus, conjugation of DFO to HES affects both parts of the dual role of DFO in *Yersinia* infection, bacterial growth, and immunosuppression.

In vivo DFO induced a septic shock in *Yersinia*-infected mice leading to death after 2 to 5 days, as suggested by the marked hepatocellular necrosis observed in these mice. Moreover, bacterial counts were dramatically increased in infected organs (e.g., spleen) of DFO-treated mice compared to controls. More strikingly, HES-DFO-treated mice did not develop septic shock after *Yersinia* infection, as indicated by the survival for more than a week after the infection. Nevertheless, probably due to the release of DFO from HES-DFO and deposition of HES-DFO in certain organs such as the liver, *Yersinia* infection was finally exacerbated in some HES-DFO-treated mice, in which bacterial counts significantly increased compared to those in control mice. The majority of the HES-DFO-treated mice survived at least 14 days postinfection, and no bacteria could be detected in spleens of those mice.

From these results, we conclude that DFO released from HES-DFO is taken up by host cells and bacteria after injection (26) and thus promotes growth of *yersiniae* and immunosuppression of the host. Consequently, these events lead to an acute exacerbation of the *Yersinia* infection, resulting in early mortality. In fact, DFO-treated mice injected with 0.2 50% lethal dose died after 2 to 3 days. In contrast, the early fulmi-
nant course of infection is prevented by HES-DFO, possibly due to the lack of high concentrations of free DFO in serum. Nevertheless, due to cleavage of free DFO in HES-DFO-treated mice, a minor but, in the long run, detrimental virulence-enhancing effect on Yersinia also occurs in HES-DFO-treated mice.

On the other hand, additional mechanisms might account for the failure of HES-DFO to promote Yersinia-induced lethal shock. Thus, in other models of septic shock HES-DFO, but not HES alone, prevents early septic shock (30). In fact, HES-DFO significantly attenuates systemic oxidant injury (the degree of protection being most impressive in the lungs and kidneys) by diminishing iron as a catalytic mediator in the production of hydroxyl radicals (·OH) (34, 41). Activity of the serum amylase on HES-conjugated molecules depends on the molar substitution ratio between the proportions of hydroxyethyl-ether and glucose within the HES macromolecule (9). Therefore, in future studies we intend to investigate HES-DFO molecules with other molar substitutions of the HES moiety to analyze their possible effect on Yersinia infection. Moreover, a more rapid deposition of HES-DFO in liver tissue may influence its bioactivity and possibly the effect on yersinia virulence (42).

Taken together, these results argue for the possibility of modifying DFO in order to generate a drug with comparable iron-binding capacity but fewer side effects on the host and infectious pathogens such as Yersinia.


