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INHIBITION OF IRON OVERLOAD TOXICITY IN RAT HEPATOCYTE CULTURES BY PYOVERDIN Pf, THE SIDEROPHORE OF *PSEUDOMONAS FLUORESCENS*

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Abstract—The effect of the pyoverdin Pf (an iron chelating agent isolated and purified from *Pseudomonas fluorescens* CCM 2798) was studied on iron overloaded rat hepatocyte cultures. Iron overload was obtained by addition of 5–80 μM ferric nitrilotriacetate to the culture medium. Twenty-four hours after iron treatment, a significant increase in aspartate aminotransferase and lactate dehydrogenase in the culture medium was observed. This corresponded to intracellular decrease in the activity of these two enzymes and correlated with a decrease in albumin secretion and an increase in total free malondialdehyde production. The iron toxicity was inhibited by desferrioxamine B. Pyoverdin Pf added to the hepatocyte cultures served as an effective agent to prevent iron toxicity induced in overload. The observed effect of the pyoverdin Pf was as potent as that of desferrioxamine B.

Genetic as well as secondary hemochromatosis cause iron overload leading to hepatotoxicity. The major clinical outward sign of chronic iron overload in the human is the development of hepatic fibrosis. This depends on combined effects of hepatic iron concentration and duration of iron overload [1]. After many years of excess iron deposit, cirrhosis may occur [2, 3] and about 10–15% of the cirrhotic patients develop a hepatocellular carcinoma [4]. Desferrioxamine B (DFO¶) or Desferal, a siderophore isolated from *Streptomyces pilosus*, is currently used in the case of secondary hemochromatosis because of its high specificity for Fe^{3+} and for its ability to facilitate the removal of this metal under a non toxic form. In secondary hemochromatosis, iron chelation therapy has been reported to achieve negative iron balance [5] as well as decreased liver fibrosis [6]. However, DFO half-life is very short and this iron chelator is ineffective when administered orally. Thus there is a need for a better iron chelator. The present work was undertaken to study the effect on iron overloaded hepatocyte cell cultures of the pyoverdin Pf, a bacterial iron chelating agent isolated from *Pseudomonas fluorescens* CCM 2798.

This substance is a chromopeptide having a chromophore derived from 2,3-diamino-6,7-dihydroxyquinoline. Pyoverdin Pf possesses a linear peptide chain, SerCTHPMD-Gly-(L)-Ser-(D)-*threo*-OHAsp-(L)-Ala-Gly-(D)-Ala-Gly-(L)-OHOrn (cyclic), which contains an unusual natural amino acid, a cyclic amidine derived due to the condensation of one mole of serine and one mole of 2,4-diaminobutyric acid ([7, 8]; Structure 1). Pyoverdin Pf possesses three bidentate groups which bind iron (III) firmly giving very stable octahedral complexes ($K_{Assoc.} = 10^{32}$) [9, 10].

Since the hepatocytes are a major target of iron overload toxicity, we have employed cultured hepatocytes for assessing pyoverdin Pf effects.

In this paper we clearly demonstrate that pyoverdin Pf could effectively prevent the iron toxicity produced by iron overload in hepatocyte cultures.

MATERIALS AND METHODS

Ferric nitrilotriacetate solution

A ferric nitrilotriacetate solution was prepared according to the method of White and Jacobs [11]. To 47 mg nitrilotriacetic acid disodium salt (Sigma) dissolved in 10 mL sterile water, was added ferriammonium citrate (20 mg) (Merck). The final concentration of ferric ion was 10 mM and the molar ratio nitrilotriacetic acid/ferric ion was 2:1. The solution was sterilized before use by filtration on a 0.22 μm filter.

Cell culture

Adult rat hepatocytes were isolated from 2-month-old Sprague–Dawley male rats by cannulating the portal vein and perfusing the liver with a collagenase solution, as previously described [12]. Cells were collected in Leibovitz medium containing 2 mg bovine serum albumin/mL. Cell suspensions were filtered on gauze and allowed to sediment for 20 min in order to remove cell debris, blood and sinusoidal cells. The cells were washed three times by centrifugation at 50 *g*, tested for viability and

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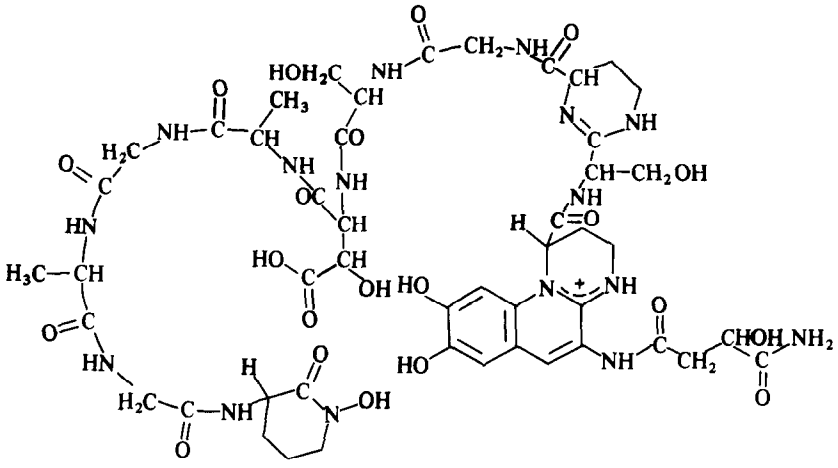
¶ Abbreviations: DFO, desferrioxamine B or Desferal; AST, aspartate amino transferase; LDH, lactate dehydrogenase; SerCTHPMD for 2-Seryl-4(6)-carboxy-3,4,5,6-tetrahydropyrimidine; MDA, malondialdehyde.



Structure 1.

counted. The hepatocytes were suspended in a mixture of 75% Eagle's minimum essential medium and 25% medium 199 with Hank's salts, supplemented with 10% fetal calf serum and containing (per mL): streptomycin (50 μg), penicillin (7.5 IU), bovine insulin (5 μg), bovine serum albumin (1 mg). Usually, 2×10^5 hepatocytes were suspended in 1 mL of medium in Multiwell tissue culture plates. The medium was changed 3–4 hr later. The effects of iron and iron chelators were studied in cultures maintained in bovine serum albumin and fetal calf serum free medium. For experimental purposes, the cultures were maintained for 24 hr in the control conditions (neither iron nor chelators), in the presence of iron alone or iron plus chelators (desferrioxamine B or pyoverdin Pf). Iron or chelators were added on day 1 of culture; the culture medium and the hepatocytes were collected on day 2.

Iron chelators

DFO was purchased from Ciba-Geigy. Pyoverdin Pf was isolated from *Pseudomonas fluorescens* [7].

Enzyme assays

Lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) activities were measured in the culture medium and intracellularly as an index of cytotoxicity, employing LDH and AST kits (Roche or Boehringer), adapted to the Cobasbio analyzer.

The enzyme activity was expressed in mIU/well. Inter- and intra-assay variations did not exceed 10%.

Protein assay

In order to determine the total protein per well, the media were decanted and the cell cultures were rinsed with a phosphate-buffered saline. Cells were sonicated for 3 × 5 sec. The protein content was determined according to the method of Bradford [13] employing bovine serum albumin as standard.

Albumin assay

Standard rat albumin and the antiserum were obtained from Cappel Laboratories (Cochranville, PA, U.S.A.).

The media from the culture dishes at the end of the treatment were withdrawn and stored at −80°. Albumin was quantified by immunonephelemetry [14]. Standard albumin solution and incubation media were mixed with an appropriate dilution of antiserum against albumin in 0.9% NaCl containing 4% polyethylene glycol and incubated at room temperature for 2 hr. Absorbance was determined at 700 nm. The limit of sensitivity of the assay was 2 μg/mL.

Free malondialdehyde (MDA) evaluation

HPLC procedure. MDA quantification was performed according to a HPLC method described previously [15]. The HPLC system (LDC Milton Roy) was equipped with a Spherogel-TSK G1000 PW size exclusion column 7.5 mm i.d./30 cm (Cluzeau, France). The eluant was composed of 0.1 M disodium phosphate buffer, pH 8 at a flow rate of 1 mL/min. The absorbance was monitored at 267 nm. The injections (250 μL) were performed by an autosampling injector (Promis, LDC) and the data were recorded and treated using a chromatography software (Thermochrom, LDC).

Preparation of free MDA standard. Five microlitres of 1,1,3,3-tetramethoxypropane (Sigma) were hydrolysed in 5 mL of 0.1 N HCl for 5 min in boiling water. This solution was then diluted 1000 times in 0.01 M NaH_2PO_4 buffer pH 7.45, corresponding to a 6 μM MDA solution. The concentration of MDA in samples was calculated using a standard curve of free MDA.

Preparation of the samples for HPLC analysis. After 24 hr of incubation, culture media were collected and hepatocytes were washed twice with 0.01 M phosphate buffer, pH 7.45 before being resuspended in 1 mL of the same buffer. Cells were sonicated for 3 × 5 sec. The protein content of the

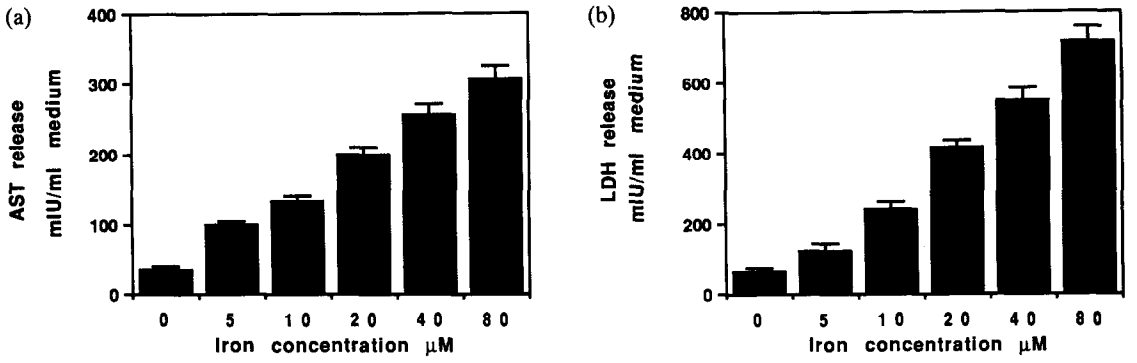


Fig. 1. Releases of AST (a) and LDH (b) in rat hepatocyte cultures maintained for 24 hr in the presence of increasing concentrations of iron. Each point is the mean ± SEM of quadruplicate cultures.

cell lysate was determined by the method of Bradford [13]. The samples (culture media and cell homogenates) were filtered through 500 Da membrane ultrafilter (Amicon, U.S.A.) in a 10 mL Amicon cell pressurized at 4 bars with nitrogen gas. The filtrate was used for HPLC procedure. Free MDA was quantified separately in culture media and in cell homogenates and then was expressed as total MDA present in each culture sample (MDA in the cells + MDA in the culture medium).

Fe^{55} mobilization from hepatocyte cultures

Rat labelled hepatocytes were obtained by maintaining the cultures during 1 day in the presence of 1 μM Fe^{55} ferric chloride (sp. act. 1.5 mCi/mg Fe; Radiochemical Center, Amersham). In order to demonstrate that the chelators were able to remove iron from the cells, 100 μM of desferrioxamine B or pyoverdin Pf were added on 1 day to the culture medium. The iron chelator effects were compared with that of a culture control which consists of culture medium without addition of chelators.

Statistics

Experimental values are means ±SEM. Significance was assessed using Student's *t*-test at the level of 0.05.

RESULTS

Evaluation of iron toxicity in rat hepatocyte cultures

In the cultures containing iron ranging from 5 to 80 μM, a significant increase in AST and LDH release was observed (Fig. 1a and b). These enzyme releases could be seen with 5 μM iron and a highly significant level ($P < 0.001$) was obtained with 80 μM iron. The enzyme activity determined in the culture medium was accountable for the disappearance of the corresponding activity from the cells (Fig. 2a and b). The cellular decline in activity was statistically significant ($P < 0.001$).

Under similar conditions secretion of albumin was decreased as compared to the control ($P < 0.001$, Fig. 3).

It appeared clearly that iron overload, particularly at concentrations as high as 80 μM, was highly toxic for rat hepatocyte cultures. This toxic effect was also confirmed by the increase observed in total free MDA production of the cultures treated with 80 μM of iron ($P < 0.001$; Fig. 4).

Protective effect of desferrioxamine B and pyoverdin Pf

In the cultures treated with 80 μM of iron, a highly significant release ($P < 0.001$) of AST and LDH was observed (Fig. 5a and b).

To circumvent this enzyme release due to iron overload, DFO (100 μM) and pyoverdin Pf (100 μM) were effective ($P < 0.001$; Fig. 5a and b). This protective effect of DFO and pyoverdin Pf was concomitantly seen on the decrease in the albumin secretion ($P < 0.001$; Fig. 6) as well as on the total free MDA production ($P < 0.001$; Fig. 4).

Effect of desferrioxamine B and pyoverdin Pf on Fe^{55} mobilization from rat hepatocyte cultures

After the cultures have been maintained for 1 day in a culture medium containing 1 μM of Fe^{55}, the hepatocytes were treated for 1 more day with either control medium or medium plus 100 μM of DFO or pyoverdin Pf. A significant increase of the intracellular iron concentration was observed ($P < 0.001$; Fig. 7a) in the cultures incubated with Fe^{55}. The addition of the chelators was followed by a decrease in the intracellular iron level ($P < 0.001$; Fig. 7a) which was well correlated to an increase of the iron concentration in the culture medium accountable to a release from the hepatocytes ($P < 0.001$; Fig. 7b).

DISCUSSION

In the present study we have demonstrated that the iron overload was toxic for rat hepatocyte cultures. This toxic effect was inhibited by desferrioxamine B and by pyoverdin Pf. It appeared that the bacterial siderophore isolated from *Pseudomonas fluorescens* proved to be as effective as desferrioxamine in protecting toxic effect of iron overload as well as removing iron from the loaded hepatocytes.

In this work iron overload in rat hepatocyte

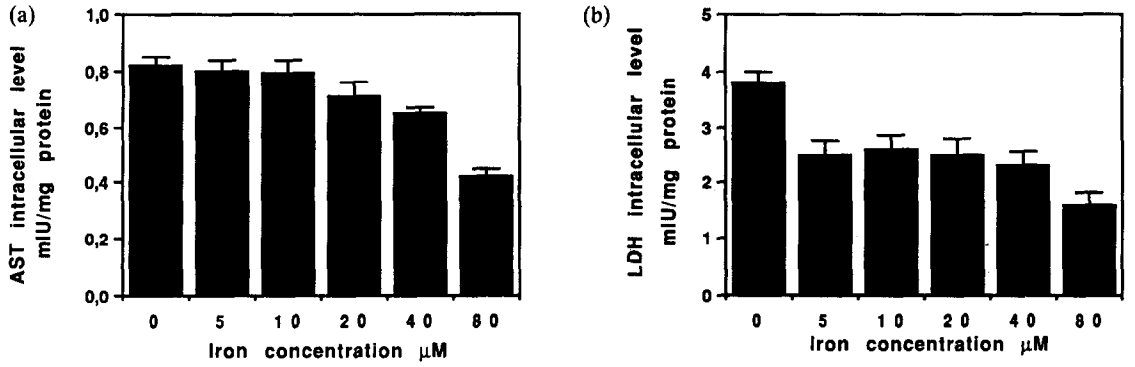


Fig. 2. Intracellular levels of AST (a) and LDH (b) in rat hepatocyte cultures maintained for 24 hr in the presence of increasing concentrations of iron. Each point is the mean ± SEM of quadruplicate cultures.

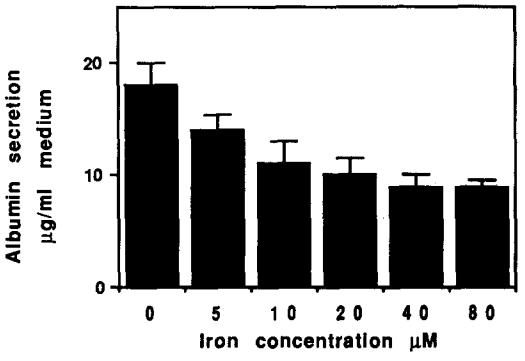


Fig. 3. Albumin secretion in rat hepatocyte cultures maintained for 24 hr in the presence of increasing concentrations of iron. Each point is the mean ± SEM of quadruplicate cultures.

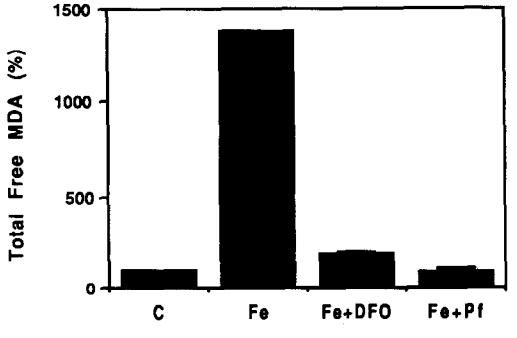


Fig. 4. Total free MDA production in rat hepatocyte cultures maintained for 24 hr in the control conditions (C), in the presence of 80 μM of iron (Fe) or 80 μM of iron plus 100 μM of desferrioxamine B or pyoverdin Pf (Fe + DFO or Pf). Each point is the mean ± SEM of triplicate cultures and is expressed in per cent of total free MDA measured in the control cultures.

cultures was produced due to iron uptake in a non-transferrin bound form. Indeed, in genetic or secondary hemochromatosis, this form of iron is increased in the serum, possibly reflecting the saturation of transferrin binding capacity [16, 17]. Moreover, the hepatic process for this form of iron is efficient and is regulated either by a membrane carrier or by some rate-limiting metabolic step [18, 19]. Therefore, high plasma levels of non-transferrin iron may play an important role in hepatic toxicity during primary or secondary hemochromatosis. In the present study, ferric nitrilotriacetate was used to obtain iron overload. Indeed, we have demonstrated previously that iron under its complexed form with nitrilotriacetic acid was able to penetrate into the rat hepatocytes in culture. Labelled iron entered the hepatocytes within the first hour of its treatment. Progressive increase of iron in the cells was obtained, almost doubling its concentration in 3 hr, and was about four times higher after 24 hr [15]. These findings were in agreement with those obtained previously on cell pellets where iron concentration was measured by flameless atomic spectrophotometry [20]. However, it is well established that ferric nitrilotriacetate, due to its favorable configuration, reacts rapidly with transferrin to fill all the sites [21]. Therefore, in these experimental conditions it is possible that a part of the iron entering hepatocytes may be in a transferrin-bound form.

The present study provides evidence that iron overload was toxic for cultured rat hepatocytes since the release of LDH or AST in the culture medium and the production of total free MDA were increased. This toxicity was also confirmed by a decrease in the albumin secretion.

We have demonstrated that the toxic effect of iron overload was inhibited by desferrioxamine B since this chelator was able to block the increase observed in enzyme leakage and in the production of total free MDA. Moreover, in our experimental model, desferrioxamine B appeared also to be able to remove iron from the hepatocytes. It is well known that iron induces lipid peroxidation [22] by a mechanism involving formation of oxygen free

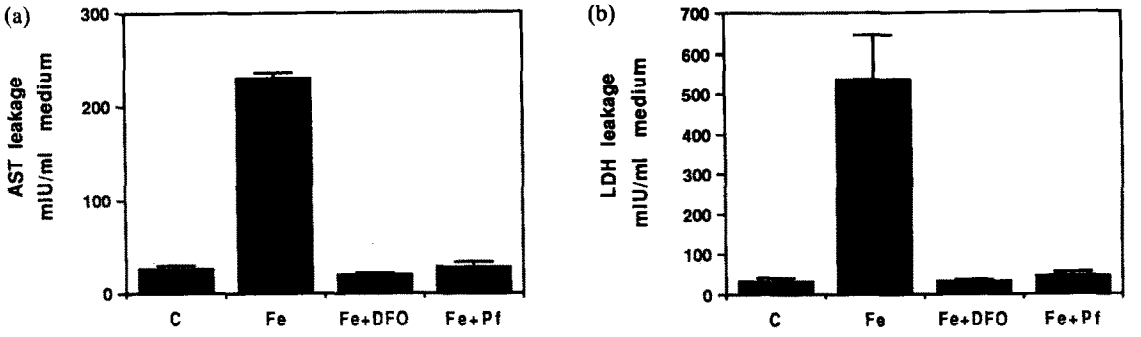


Fig. 5. Releases of AST (a) and LDH (b) in rat hepatocyte cultures maintained for 24 hr in the control conditions (C), in the presence of 80 μM of iron (Fe) or 80 μM of iron plus 100 μM of desferrioxamine B or pyoverdin Pf (Fe + DFO or Pf). Each point is the mean ± SEM of quadruplicate cultures.

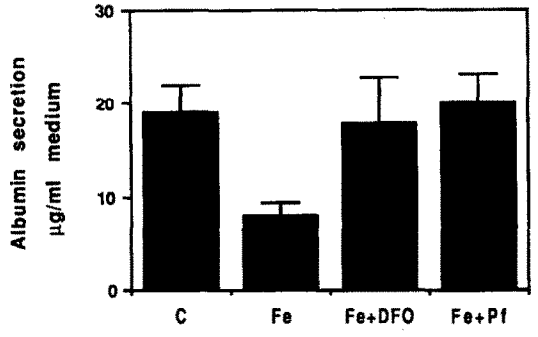


Fig. 6. Albumin secretion in rat hepatocyte cultures maintained for 24 hr in the control conditions (C), in the presence of 80 μM of iron (Fe) or 80 μM of iron plus 100 μM of desferrioxamine B or pyoverdin Pf (Fe + DFO or Pf). Each point is the mean ± SEM of quadruplicate cultures.

radical species such as hydroxyl radical $OH^{\cdot}$ or superoxide anion $O_2^{\cdot-}$ [23]. Desferrioxamine B, the powerful chelator of Fe(III), inhibits iron-dependent lipid peroxidation by blocking the generation of highly reactive oxygen species involved in mediating tissue injury observed in human diseases [24]. *In vitro* studies have shown that oxygen radical generation promoted by low levels of iron (5 μM) can be inhibited by desferrioxamine B at concentrations equimolar to those of iron [25]. More recently, Schwarz [26] has also demonstrated that desferrioxamine B markedly inhibited malondialdehyde production as well as the rise of LDH in a human hepatoblastoma cell line ($HepG_2$) maintained in the presence of various concentrations of iron ascorbate.

The present data show also that pyoverdin Pf was as effective as desferrioxamine B in the protection of hepatocyte cultures against the toxic effect of iron overload by decreasing the enzyme leakage and the MDA production and removing the iron from the cells. There was no significant difference between the efficiency of the two types of chelators. Indeed, in the cultures maintained in the presence of iron plus desferrioxamine B or pyoverdin Pf, the enzyme

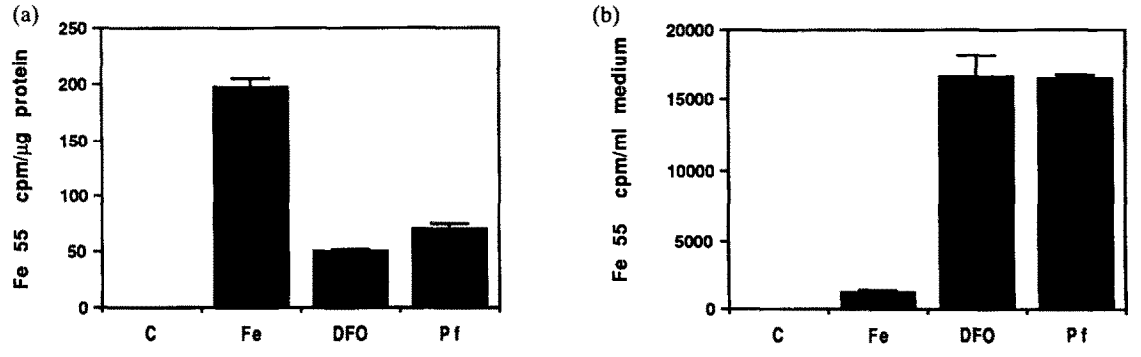


Fig. 7. Intracellular level (a) and release (b) of Fe^{55} in rat hepatocyte cultures maintained for 24 hr in a culture medium containing 1 μM Fe^{55} and treated 1 day more with either the control medium (Fe) or medium plus 100 μM DFO or pyoverdin Pf (DFO or Pf). C: cultures maintained in the control medium during the experimental procedure. Each point is the mean ± SEM of quadruplicate cultures.

release as well as the MDA production and the albumin secretion were at the same levels as in the controls. This indicates that pyoverdin Pf is a valuable substance for preventing iron toxicity in hepatocyte cultures. The putative mechanism by which pyoverdin Pf may act is very clearly related to its powerful ability to chelate iron.

Thus, in the present study a cellular model has been used which may prove useful in the study of the effect of drugs similar to those employed here. The use of such a peptidic siderophore seems to have advantages over compounds like DFO since the latter is subject to protease degradation unlike pyoverdin [27].

In conclusion, our work highlights the iron chelating properties of pyoverdin Pf in a biological model.

REFERENCES

1. Bassett ML, Halliday JW and Powell LW, Value of hepatic iron measurements in early hemochromatosis and determination of critical iron level associated with fibrosis. *Hepatology* **6**: 24–29, 1986.
2. Grace ND and Powell LW, Iron storage disorders of the liver. *Gastroenterology* **64**: 1257–1283, 1974.
3. Brissot P and Deugnier Y, Genetic hemochromatosis. In: *Oxford Textbook of Clinical Hepatology* (Eds. McIntyre N, Benhamou JP, Bircher J, Rizzetto M and Rodés J), Vol. 2, pp. 948–958. Oxford University Press, Oxford. 1991.
4. Bradbear RA, Bain C, Siskind V, Schofield FD, Webb S, Axelsen EM, Halliday JW, Bassett ML and Powell LW, Cohort study of internal malignancy in genetic hemochromatosis and other chronic non-alcoholic liver diseases. *J Natl Cancer Inst* **75**: 81–84, 1985.
5. Pippard MJ, Letsky GA, Callender SJ and Weatherall DJ, Prevention of iron loading in transfusion dependent thalassaemia. *Lancet* **1**: 1178–1181, 1978.
6. Hunt J, Richards RJ, Harwood R and Jacobs A, The effect of desferrioxamine on fibroblasts and collagen formation in cell cultures. *Br J Haematol* **41**: 69–76, 1979.
7. Demange P, Bateman A, Mertz C, Dell A, Piemont Y and Abdallah MA, Bacterial siderophores: the structure of Pyoverdins Pt siderophores of *Pseudomonas tolaasii* NCPPB 2192 and pyoverdins Pf, siderophores of *Pseudomonas fluorescens* CCM 2798. Identification of an unusual natural amino acid. *Biochemistry* **46**: 11057–11067, 1990.
8. Demange P, Bateman A, Macleod JK, Dell A and Abdallah MA, Bacterial siderophores; unusual 3,4,5,6-tetrahydropyrimidine-based amino acids in pyoverdins from *Pseudomonas fluorescens*. *Tetrahedron Lett* **31**: 7611–7614, 1990.
9. Meyer JM and Abdallah MA, The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification and physicochemical properties. *J Gen Microbiol* **107**: 319–328, 1978.
10. Demange P, Wendenbaum S, Linget C, Bateman A, Macleod JK, Dell A, Albrecht AM and Abdallah MA, *Pseudomonas* siderophores: structure and physico-chemical properties of pyoverdins and related peptides. In: *Second Forum on Peptides* (Eds. Aubry A, Marraud M and Vitoux B), Vol. 174, pp. 95–98. John Libbey Eurotext Ltd, Montrouge/INSERM Paris, 1989.
11. White GP and Jacobs A, Iron uptake by Chang cells from transferrin, NTA and citrate complexes. The effects of iron-loading and chelation with desferrioxamine. *Biochim Biophys Acta* **543**: 217–225, 1978.
12. Guguen-Guillouzo C and Guillouzo A, Methods for preparation of adult and fetal hepatocytes. In: *Isolated and Cultured Hepatocytes* (Eds. Guillouzo A and Guguen-Guillouzo C), pp. 1–12. John Libbey Eurotext Ltd, Montrouge/INSERM, Paris, 1986.
13. Bradford MM, A rapid and sensitive method for quantification of microgram quantities of proteins utilizing the principle of protein dye binding. *Anal Biochem* **72**: 248–254, 1976.
14. Ritchie RF, Automated immunoprecipitation analysis of serum proteins. In: *The Plasma Proteins: Structure, Functions and Genetic Control* (Ed. Putman PW), Vol. 2, pp. 375–425. Academic Press, New York, 1975.
15. Morel I, Lescoat G, Cillard J, Pasdeloup N, Brissot P and Cillard P, Kinetic evaluation of free malondialdehyde and enzyme leakage as indices of iron damage in rat hepatocyte cultures. *Biochem Pharmacol* **39**: 1647–1655, 1990.
16. Hershko C, Graham C, Bates GW and Rachmilewitz EA, Non specific serum iron in thalassaemia: an abnormal serum iron fraction of potential toxicity. *Br J Haematol* **40**: 255–263, 1978.
17. Batey FG, Lai Chung Fong P, Shamir S and Sherlock S, A non transferrin bound serum iron in idiopathic hemochromatosis. *Dig Dis Sci* **25**: 340–346, 1980.
18. Brissot P, Wright TL, Ma WL and Weisiger RA, Efficient clearance of non-transferrin bound iron by rat liver. Implications for hepatic iron loading in iron overload states. *J Clin Invest* **76**: 1463–1470, 1985.
19. Wright TL, Brissot P, Ma WL and Weisiger RA, Characterization of non-transferrin-bound iron clearance by rat liver. *J Biol Chem* **261**: 10909–10914, 1986.
20. Desvergne B, Baffet G, Loyer P, Rissel M, Lescoat G, Guguen-Guillouzo C and Brissot P, Chronic iron overload inhibits protein secretion by adult rat hepatocytes maintained in long-term primary culture. *Eur J Cell Biol* **49**: 162–170, 1989.
21. Bates GW, Billups C and Saltman P, The kinetics and mechanism of iron (III) exchange between chelators and transferrin. *J Biol Chem* **242**: 2810–2815, 1967.
22. Beckman JK, Borowitz SM and Burr IM, The role of phospholipase A activity in rat liver microsomal lipid peroxidation. *J Biol Chem* **262**: 1479–1481, 1987.
23. Dianzani MU, The role of free radicals in liver damage. *Proc Nutr Soc* **46**: 43–52, 1987.
24. Halliwell B, Protection against tissue damage *in vivo* by desferrioxamine: what is its mechanism of action. *Free Radical Biol Med* **7**: 645–651, 1989.
25. Aruoma OI, Grootveld M and Halliwell B, The role of iron in ascorbate-dependent deoxyribose degradation. Evidence consistent with a site-specific hydroxyl radical generation caused by ions bound to the deoxyribose molecule. *J Inorg Biochem* **29**: 289–299, 1987.
26. Schwarz B, Effect of iron and desferrioxamine on malondialdehyde production in HepG2 cells. *Hepatology* **8**: abstract 639, 1378, 1988.
27. Döring G, Pfestorff M, Botzenhart K and Abdallah MA, Impact of proteases on iron uptake of *Pseudomonas aeruginosa* pyoverdin from transferrin and lactoferrin. *Infect Immunity* **56**: 291–293, 1988.