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Microbicidal effect of the lactoferrin peptides Lactoferricin17–30, Lactoferrampin265–284, and Lactoferrin chimera on the parasite *Entamoeba histolytica*

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Abstract *Entamoeba histolytica* is a parasitic protozoan that produces amoebiasis, an intestinal disease characterized by ulcerative colitis and dysentery. In some cases, trophozoites can travel to the liver leading to hepatic abscesses and death. Recently, lactoferrin and lactoferricin B have been shown to be amoebicidal in axenic cultures. The aim of this work was to determine whether the lactoferrin-peptides lactoferricin amino acids 17–30, lactoferrampin amino acids 265–284, and lactoferrin chimera which is a fusion product of the two peptides, are capable of producing a microbicidal effect to trophozoites of *E. histolytica*. We evaluated the killing effect of these peptides in growth kinetics carried out in axenic culture medium to which different concentrations of peptides were added. At 50 μM of peptide concentration, lactoferricin and lactoferrampin had a moderate amoebicidal effect, since a 45–50% of trophozoites remained viable at 24 h culture. However, at 50 μM of the lactoferrin chimera 75% amoeba were killed whereas at 100 μM all cells died. These data indicate that of lactoferrin-peptides mainly the chimera have amoebicidal activity in a time- and concentration-dependent manner. The lactoferrin-peptides might be useful as therapeutic agents against amoebiasis and thereby diminish the use of metronidazole, which is extremely toxic for the host.

Keywords *Entamoeba histolytica* · Amoebicidal effect · LFcin · LFampin · LFchimera

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Introduction

Amoebiasis is the third cause of death by parasites worldwide. A current estimate of this disease is that approximately 50 million people are symptomatically infected and 100,000 people die annually, essentially in developing countries. Amoebiasis is caused by the extracellular enteric protozoan *Entamoeba histolytica*, which cysts are transmitted through the fecal-oral route from contaminated water or food. Trophozoites of this primitive parasite are able to invade the intestinal

mucosa causing dysentery, fever and abdominal pain. By unknown reasons, they often spread to other organs, especially the liver, brain and lung; severe cases can lead to ulcerative colitis, liver abscesses and death (Espinosa-Cantellano and Martinez-Palomo 2000).

Lactoferrin (LF) is an iron-binding glycoprotein of the mammalian innate immune system. LF without iron (apo-LF) is more rigid and stable, with a high isoelectric point (pI >9) than iron-charged LF (holo-LF), and thus, it can bind to a variety of cells; this explains its additional remarkable biological activities as a potent antitumoral, microbicidal and antioxidant agent (Baker and Baker 2009; Brock 2002; Weinberg 2003; Tomita et al. 2009). LF is found as a secreted product of exocrine glands located at the gateways of the digestive, reproductive, and respiratory tracts (Arnold et al. 1982; Lönnerdal and Iyer 1995; Masson et al. 1966); LF is present in e.g., colostrum, milk, tears, saliva and semen. Additionally, apo-LF is an acute-phase reactant that chelates iron in the hyposiderhaemia of infection, during which it is released at infection sites by the secondary granules of neutrophils to deprive pathogens of the iron required for growth. Neutrophil LF is mainly unsaturated (up to 86%), making it an efficient iron scavenger (Arnold et al. 1982; Valenti et al. 2004; Van Snick et al. 1974; Wilson and Britigan 1998; Wooldridge and Williams 1993).

The digestion of bovine lactoferrin generates lactoferricins (LFcin), having more potent activity against bacteria than the native protein (Bellamy et al. 1992; Yamauchi et al. 1993). Another LF peptide named LFampin was discovered (Van der Kraan et al. 2004), which also presents bactericidal (Van der Kraan et al. 2006) and candidacidal (Van der Kraan et al. 2005) activity.

Both, LF and LF-peptides are able to kill a wide variety of pathogenic microorganisms such as bacteria, fungi, parasites, and also enveloped viruses, most probably due to their membrane disturbing activities (Bellamy et al. 1992; van der Kraan et al. 2004, 2006; Wakabayashi et al. 1996; Haney et al. 2007, 2009; León-Sicairos et al. 2006a, b, 2009; Bolscher et al. 2009; Marr et al. 2009). Therefore, LF and LF-peptides are a promising class of antimicrobial compounds to fight pathogenic microbes. Linking LFcin and LFampin into one molecule named LFchimera resulted in a strikingly stronger bactericidal activity than that of its constituent peptides (Bolscher et al. 2009).

Previously, we reported that apo-LF displayed microbicidal effect versus *E. histolytica* trophozoites when cultured in low-iron medium. Bovine LFcin from Sigma (aa RRWQWRMKKLG) was also studied as amoebicidal (León-Sicairos et al. 2006a, b). Interestingly, in medium with iron, in which apo-LF is converted in holo-LF, the amoeba used this protein as an iron source, suggesting that iron plays a double role in the interaction between amoeba and lactoferrin (León-Sicairos et al. 2005).

In the present study we have investigated the amoebicidal effect of the lactoferrin-peptides LFcin17–30, LFampin265–284, and LFchimera in axenic in vitro amoeba cultures. LFchimera displayed the strongest amoebicidal effect against *E. histolytica* trophozoites and may be used as an effective antiamoebic.

Materials and methods

Materials

Peptides, LFcin17–30, LFampin265–284, and LFchimera were synthesized at the department of Oral Biochemistry, Academic Center for Dentistry Amsterdam (ACTA), Amsterdam, The Netherlands (Bolscher et al. 2009). Chelex-100 resin, propidium iodide (PI), and trypan blue (TB) dyes were purchased to Sigma Chemical Co. (St. Louis MO.). All solutions used in the experiments were treated with Chelex-100 resin in order to remove iron traces from reagents. Glassware used was previously treated with 6 N HCl for 16 h to remove residual iron.

Cultures and media used

Trophozoites of the *E. histolytica* strain HM-1:IMSS were axenically grown in BI-S-33 medium (Dibico, Mexico; Diamond et al. 1978) supplemented with 16% (v/v) bovine serum (Microlab, Mexico); it contained approximately 100 μM iron. The low-iron medium used was BI-S-33 without ammonium ferric citrate (AFC), which was treated with the Chelex-100 resin (5%, w/v) and sterilized after removal of the resin by filtration. This low-iron medium contained approximately 6.5 μM iron. Iron concentration was measured by the method of ferrozine using an automatic analyzer (Hitachi 747, Japan). Media and iron concentration are shown in Table 1.

Table 1 Media used in this study

Medium	Iron concentration (μM)[a]	Iron source
BI-S-33	100	AFC, serum and iron traces of reagents[b]
Low-iron	6.5	Serum[c]
BI-S-33 or Low-iron plus LF peptides	100 or 6.5	

AFC ammonium ferric citrate

[a] Iron concentration was measured using ferrozine reagent and an automatic analizar (Hitachi 747, Japan)

[b] Diamond et al. 1978

[c] Serrano-Luna et al. 1998

Viability assays

Trophozoites from 48 h culture in BI-S-33 medium at 37°C were used for the experiments. They were incubated in an ice bath for 5 min in order to remove the cells from the tube wall and centrifuged at 500 g for 5 min. The pellet was washed with PBS and suspended (1×10^4 cells) in BI-S-33 medium or in low-iron medium containing LFcin17–30, LFampin265–284, or the chimera of both peptides, to obtain a final concentration of 25, 50, 75 and 100 μM of each compound. Amoebae were incubated at 37°C for different periods of time. After every period, amoeba samples were obtained and washed by centrifugation. To examine the exclusion of TB dye as a criterion of amoeba viability, a cell suspension was mixed with an equal volume of PBS containing 0.5% TB and observed under a light microscope in a Neubauer's chamber. All of the experiments were preformed in three independent assays, each in triplicate.

As an alternative method to estimate viability, amoebas were incubated with the peptides as above mentioned. The samples were stained with 10 μg ml^{-1} PI for 10 min in an ice bath and washed twice in PBS. Exclusion of PI by 1×10^4 cells was determined by flow cytometry (FACScan, Beckton Dickinson, St. Jose, CA). Positive and negative controls of viability were amoebas grown in BI-S-33 or in low iron medium, respectively. All of the experiments were performed in three independent assays, each in triplicate.

Results

LFcin17–30 and LFampin265–284 have a moderate amoebicidal effect

Since we found that iron avoided the amoebicidal effect of LFcin B (aa RRWQWRMKKLG; Leon-Sicairos et al. 2006b), we tested whether iron is also able to avoid the amoebicidal effect of LFcin17–30 and of LFampin265–284. To determine whether LFcin and LFampin cause a microbicidal effect against the *E. histolytica* trophozoites, two viability assays were performed with amoebas incubated with these peptides at different times and concentrations. Moreover, the effect of iron in the protection of this parasite was determined. The results using BI-S-33 medium (with ammonium ferric citrate) indicated that 50 μM of these peptides caused approximately 50% of trophozoite mortality in 24 h (Fig. 1A, C). At 100 μM of each compound approximately 18–20% of trophozoites were viable in that time. This result suggests a moderate amoebicidal effect dependent on concentration. The result of the trophozoites incubated with these peptides in low iron medium (Fig. 1B, D, respectively) was similar to that in medium BI-S-33 with iron. Thus iron does not protect the amoeba against these peptides. Similar results were found when the interaction between LFcin17–30 and LFampin265–284 with amoeba was determined by flow cytometry (not shown).

LFchimera displayed a higher amoebicidal effect than its constituent peptides

Using a similar viability assay we found that LFchimera possessed a higher microbicidal activity against the *E. histolytica* trophozoites than its constituent peptides LFcin17–30 and LFampin265–284, and that this effect was independent on iron (Fig. 1E, F). This peptide also showed a concentration-dependent amoebicidal action, but at 50 μM only 25–30% of trophozoites remained viable after 24 h of interaction; at 100 μM of chimera the culture died (5–6% viability), and the effect was drastic since only 18% of trophozoites were viable at 3 h of interaction. These data indicate a strong killing activity of LF chimera against the amoeba cell.

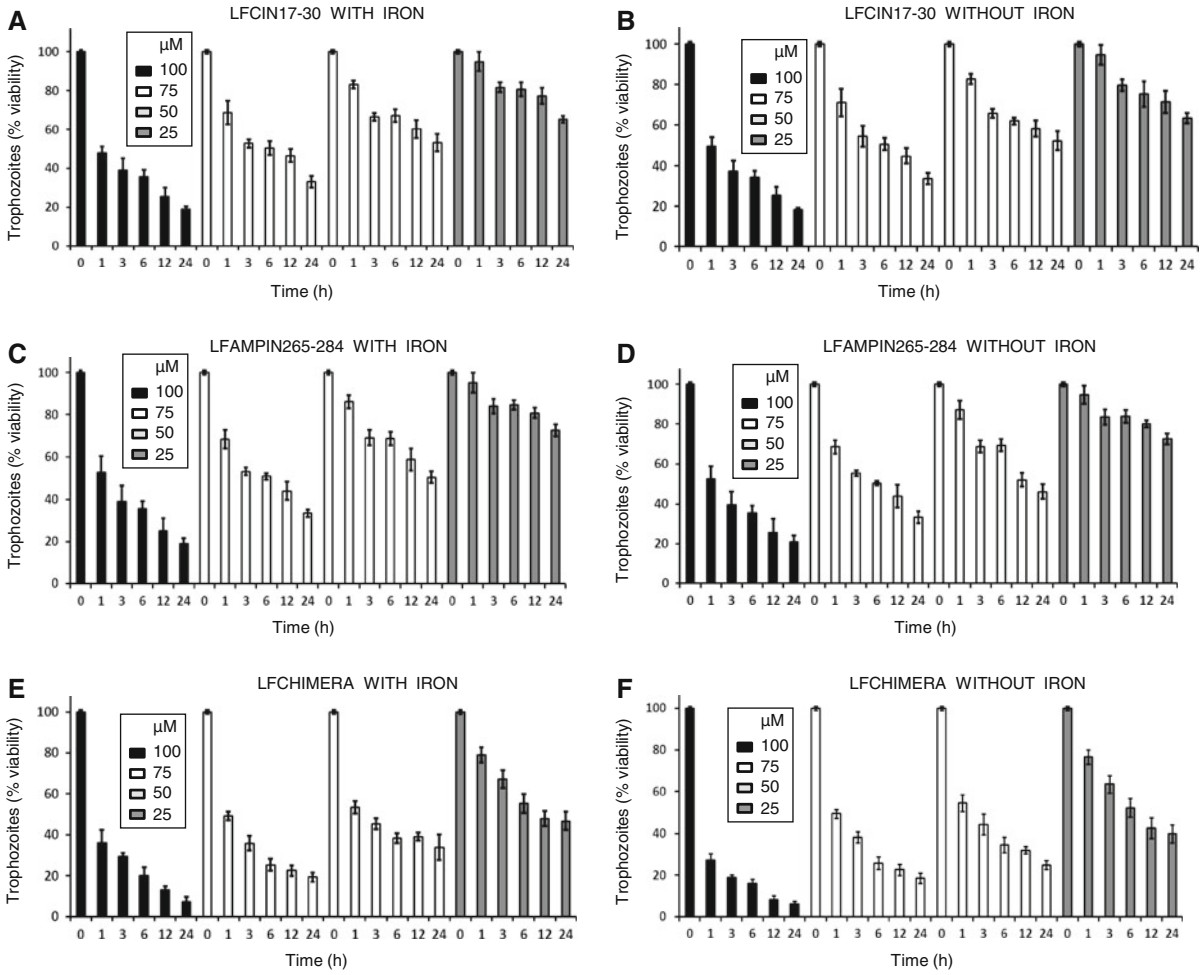

Fig. 1 Amoebicidal effect of Lactoferricin, Lactoferrampin and Lactoferrin chimera. Trophozoites (10^4) were incubated in BI-S-33 medium (100 µM iron; *panel a*, *c* and *e*) or in low-iron medium (6.5 µM iron; *panel b*, *d* and *f*) containing: **A** and **B**, LFcin17–30, **C** and **D**, LFampin265–284, **E** and **F**, LFchimera, at the concentrations and period of time indicated. Cell viability was estimated using trypan blue to evaluate cell permeability. In all of the cases, data are the means of three independent experiments performed in triplicate ± SD

Discussion

Globally, amoebiasis is the third leading cause of death due to parasites, after malaria and schistosomiasis. According to the World Health Organization (WHO), amoebiasis causes 100,000 deaths annually. This disease mainly affects countries with poor hygienic conditions and nowadays it is a great public health problem (Espinosa-Cantellano and Martinez-Palomo 2000).

Metronidazole is the gold choice for the treatment of amoebiasis, in particular for the extra-intestinal disease. However, this drug is at risk for the patient since it originates DNA adducts (Roe 1983; Dobias et al. 1994; Harder et al. 2001). In addition, it has been reported that *E. histolytica* laboratory strains may become resistant to metronidazole (Wassmann et al. 1999). Therefore, it is especially necessary to search constantly for new molecules for the treatment of amoebiasis, and to diminish and/or eliminate the use of metronidazole and other toxic drugs.

Resistance of microorganisms to drugs has leaded to the development of alternative therapies. A new option is the use of cationic antimicrobial peptides

derived from LF. These compounds are microbicidal by themselves and they could enhance the activity of certain antibiotics and drugs. In bacteria, the mechanism of action is inducing permeability of the bacterial membrane. Moreover, they may act in synergy with antibiotics (Sánchez-Gómez et al. 2008). Lactoferrin-peptides provide a rapidly expanding source of new antimicrobial compounds, which could be used in the treatment of parasitic diseases. We have previously described human and bovine LF and the bovine peptide Lactoferricin B (aa RRWQWRMKKLG) as amoebicidal agents. In addition, LF as well as LFcin potentiated the activity of metronidazole, leading to the possibility of using low doses of the drug and thus diminish its toxicity (León-Sicairos et al. 2006b).

In the present study we show that the pathogenic *E. histolytica* strain HM1:IMSS was susceptible to the LFchimera within short incubation times (3 h), whereas the parasite showed a moderate sensibility to LFcin17–30 and LFampin265–284, achieving amoeba death only after prolonged incubation times. Interestingly, the activities were not affected in medium containing iron, suggesting that this element does not participate in the amoebicidal effect of LFcin17–30, LFampin265–284 and LFchimera. Probably diet iron will not influence the antiamoebic activity. Thus, LFchimera may be an excellent candidate as antiamoebic. Of course it is necessary to test the LFchimera's lethality to amoeba in animal models in order to confirm its amoebicidal effect in vivo.

This work is the first step in the study of the amoebicidal action of LFchimera, which could be considered as an excellent candidate to combat the parasite and help to the cure of amoebiasis.

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