

## Review Article

# Trichomoniasis and Lactoferrin: Future Prospects

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Received 22 March 2012; Revised 5 July 2012; Accepted 18 August 2012

Academic Editor: Bryan Larsen

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*Trichomonas vaginalis* is a parasitic protozoan which infects the urogenital tract and requires iron as an essential nutrient. Iron is known to upregulate various adhesins required for cytoadherence and other factors involved in pathogenesis. At mucosal surfaces, iron is chelated by lactoferrin resulting in low levels of free iron. However, pathogens have evolved mechanisms for an increased uptake of iron. The present review highlights the role of iron in survival of *Trichomonas* during fluctuating concentrations of iron at mucosal surfaces during the menstrual cycle. Future prospects in terms of new drug and vaccine targets related to iron and its receptors have also been described.

## 1. Introduction

*Trichomonas vaginalis* is a flagellated, parasitic protozoan which causes trichomoniasis by infecting urogenital tract. Trichomoniasis is one of the most common causes of non-viral genitourinary sexually transmitted infection (STI) in humans with a worldwide prevalence of 170 million cases annually. According to WHO estimates, it accounts for almost half of all curable STIs [1]. Despite high prevalence, it is one of the poorly studied parasites with respect to virulence properties, pathogenesis, and immunopathogenesis. The present paper highlights the importance of various targets in pathogenesis where iron and its related transporting proteins play an important role. The role of lactoferrin (Lf) has been studied in various infections including parasitic infections, and in the present paper we will discuss the role of iron and lactoferrin in trichomoniasis.

## 2. Brief Description of the Parasite

*T. vaginalis* was discovered by Alfred Donné in 1836 and is an amitochondrial, microaerotolerant flagellate. *Trichomonas vaginalis* varies in shape and size. In a pure culture, typical shape of *T. vaginalis* is pyriform but amoeboid shapes have also been documented in parasites adhering to vaginal tissue. The average size of *T. vaginalis* is about 9 by 7  $\mu\text{m}$ . It has

five flagella; 4 are present anteriorly and the other flagellum is incorporated within the undulating membrane. It lacks a cystic stage and exists as a trophozoite. The trophozoite divides by binary fission and gives rise to progeny in the lumen or on the mucosal surfaces of urogenital tract of humans. Internal organelles include nucleus and axostyle which spans through the cell from anterior end to posterior end. Hydrogenosomes are the energy producing organelles, which appear as chromatic granules under light microscope [2].

## 3. Transmission and Clinical Features

Trophozoites of *Trichomonas* are transmitted from person to person through sexual contact. *T. vaginalis* trophozoites colonize the epithelial surface of the human urogenital tract in which they obtain nutrients, multiply, and face a constant challenge from host immune surveillance. The trophozoites divide by longitudinal binary fission after attaching themselves to mucosal surfaces of the urogenital tract. Incubation period varies from 4 to 28 days [3]. It can survive for long term in the acidic environment of vagina and the disease itself may be chronic. The clinical spectrum in women ranges from asymptomatic carrier state in approximately 50% of infected women, while symptomatic patients may suffer from mild-to-severe inflammation with a foul-smelling

discharge and severe irritation. Infection during pregnancy may be associated with premature rupture of membranes, preterm delivery, and low-birth-weight babies. In men, the clinical spectrum varies from asymptomatic carrier state to acute state characterised by purulent urethritis, dysuria, or mild pruritus [4].

#### 4. Iron an Essential Nutrient

Iron is an essential nutrient required for the survival of both humans and pathogenic protozoa. Iron is essential for wide range of biological process, including oxygen transport, DNA synthesis, and electron transport [5]. Some protozoa such as amitochondriate protists (e.g., *Trichomonas*, *Giardia*, and *Entamoeba*) require a high extracellular iron concentration (50–200  $\mu\text{M}$ ) for their growth as compared to other prokaryotic or eukaryotic cells (0.4–4  $\mu\text{M}$ ) [6]. Such a high concentration of iron is required for functional energy metabolism system which relies heavily on Fe-S proteins [7]. To protect against invading pathogens mammals have evolved the scavenging mechanisms to limit the availability of iron near the vicinity of the pathogen. Ferric iron is chelated by lactoferrin which is an extracellular glycoprotein of the host immune system. Pathogens have also developed several mechanisms to obtain iron from the host holo-lactoferrin (holo-Lf) [6, 8]. Thus, it is the delicate balance between the invading pathogen and host immune response which ultimately determine the clinical outcome.

#### 5. Iron as Ferric or Ferrous Ions

Iron in the earth exists as ferric ( $\text{Fe}^{3+}$ ) or ferrous ( $\text{Fe}^{2+}$ ) state. It can accept or donate the electrons and catalyzes the important biochemical reactions such as the Fenton reaction: (a)  $\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}\cdot + \text{OH}^-$ , (b)  $\text{Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{2+} + \text{OOH}\cdot + \text{H}^+$ . It catalyses the conversion of hydrogen peroxide to toxic free radicals [9, 10]. Thus, it cannot be available in the free state in tissues or cells: it is available only bound to proteins, limiting its ability to cause the damage. Intracellular iron is bound to ferritin or it is present within hemoglobin as a major iron content of the body. Extracellular iron is bound to transferrin which helps in transferring the iron to all cells and at mucosal surfaces it is bound to lactoferrin (Lf) [6, 8].

#### 6. Lactoferrin (Lf)

Lactoferrin (Lf) is a mammalian non-heme iron-binding glycoprotein which can bind two ferric ions with very high affinity even at an acidic pH of 2.5–4 [11, 12]. A glycan moiety provides the protection from proteolysis and also helps in decreasing its immunogenicity [13]. Lf exists in two different conformations which are governed by the iron binding status and these are apo-Lf (without iron) and holo-Lf (with iron) [14]. Apo-Lf has an open conformation which can bind to iron, and holo-Lf exhibits a closed conformation which is saturated with iron. Lf is a highly conserved glycoprotein having a homology of approximately 70% between humans

and mice and greater than 70% between humans and cattle with respect to amino acid sequence [15].

Lf is found in various mucosal secretions, including saliva, tears, vaginal fluids, semen, nasal and bronchial secretions, gastrointestinal fluids, urine, and most abundantly in milk and colostrum [16, 17]. It is also found in blood, amniotic fluid, and in secondary neutrophils granules, where it plays an important physiological role [18].

#### 7. Functions of Lactoferrin (Lf)

Lf is a multifunctional protein with a wide range of biological activities including regulation of iron absorption, immune response, antioxidant, anticarcinogenic, anti-inflammatory properties, and antimicrobial activity [12, 19–21]. Recently, Lf is emerged as a pivotal component of iron and inflammatory homeostasis capable of overcoming pregnancy-associated anemia, while decreasing serum IL-6 levels [22–24].

#### 8. Anti-Inflammatory Properties of Lf

*In vivo* and *in vitro* evidence characterizes Lf as a potent anti-inflammatory compound able to reverse/attenuate inflammatory response triggered by Toll-like receptor (TLR) engagement in antigen-presenting cells. Recently, Puddu et al. [25] have shown that monocyte-derived dendritic cells (MD-DCs) generated in the presence of bovine Lf (bLf) failed to undergo activation by upmodulating CD83, costimulatory and major histocompatibility complex molecules. Consistent with an impaired maturation, bLf-MD-DC primed T lymphocytes exhibit a unresponsiveness characterized by impaired expression of IFN- $\gamma$  and IL-2. These immunosuppressive effects correlate with an increased expression of molecules with negative regulatory functions. Berlutti et al. [26] have shown that Lf downregulates proinflammatory cytokines in intestinal epithelial cells infected with invasive and noninvasive *Escherichia coli* strains. Valenti et al. [27] have also shown similar anti-inflammatory activity of Lf in cystic fibrosis bronchial cells invaded by *Burkholderia cenocepacia*. Thus, Lf may be used as a therapeutic agent as it prevents the inflammation-related damage. Little information is available about the molecular details of interaction of Lf with the cells to mediate its effects. Recently, a study by Suzuki et al. [28] has shown that the N1 domain of human Lf is required for internalization by caco-2 cells and targeting to the nucleus to mediate its effects.

#### 9. Microbiostatic and Microbicidal Effects

Apo-Lf is secreted as microbicidal product by secondary granules of neutrophils. Thus, apo-Lf sequesters the iron at the site of infection, resulting in deprivation of iron to microbes. Hence, microbes must compete with host Lf to meet their iron requirement. Due to its iron-chelating property, it is known as microbiostatic [29]. Apo-Lf is a highly cationic in nature and thus, readily binds to anionic

charges on bacterial surfaces, provided by lipopolysaccharides, porins, and teichoic acids. Such binding results in destabilizing the bacterial membrane [30]. Apo-Lf also exhibits a synergistic effect with IgA, lysozyme, antibiotics, and drugs which helps in eradication of microorganisms [20, 31].

## 10. Lactoferrin Friend or Foe

When apo-Lf acquires iron, it becomes saturated with iron and forms holo-Lf which is an important source of iron for microbes. Thus, iron not only abolishes Lf's innate immune effect but also acts a source of nutrition to microbes. Microbes have developed the various strategies to acquire the iron from holo-Lf which helps them in replication causing acute or chronic infections and damage to host. Thus, for microbes iron-loaded Lf is a friend but becomes a foe when iron is not present [29, 32].

## 11. Mechanisms Adopted by Parasites to Acquire Iron from Holo-Lf

Basically there are four different mechanisms by which parasites acquire iron as a source of nutrient from holo-Lf and these are as follows [33].

(1) *Lf Binding Receptor*. Expression of lactoferrin binding receptors or proteins (Lbps) which directly bind to holo-Lf (*Trichomonas vaginalis*).

(2) *Enzymatic Degradation*. Secretion of proteases to cleave holo-Lf to release iron as a source of nutrient (*Trichomonas fetus* and *Entamoeba histolytica*).

(3) *Reducing Enzymes*. Reductases help in reducing the ferric state to ferrous state which is more useful to the parasite (*Leishmania* spp.).

(4) *Xenosiderophores*. This mechanism is used by bacteria and fungi to obtain iron by producing and secreting siderophores, which have high affinity for iron. They can remove iron from holo-Lf, which in turn is captured by specific receptors, and after providing iron to the pathogen, siderophore again becomes available to carry fresh iron from the environment [34]. *Trichomonas fetus* is able to use a wide range of foreign siderophores *in vitro* (ferrioxamine B, coprogen, ferrichrome, enterobactin, and pyoverdine) [35].

## 12. Iron Acquisition by *Trichomonas* Is Receptor Mediated

*T. vaginalis* acquire iron either from holo-Lf or hemoglobin [36, 37]. It recognizes human holo-Lf by two surface proteins which are 178 and 75 kDa. Approximately 90,000 receptors have been documented to be present on each trichomonad. *T. vaginalis* only recognizes holo-Lf, as it is not able to recognize apo-LF or holotransferrin (holo-Tf). A study

by Lehker and Alderete [38] has shown the dynamics of lactoferrin-binding proteins and receptors to changing iron concentration. Lf-binding activity has been shown to be increased by 1.6-fold, under iron-depleted conditions as compared to iron rich conditions. Increase in Lf-binding activity has also been documented when trichomonads with depleted intracellular iron pools were assayed. It has also been observed that a number of holo-Lf-binding receptors increase 2.5 times when trichomonads were cultivated in iron-depleted conditions compared to iron rich-conditions. The number and affinity of holo-Lf receptors in response to changing external and internal iron concentrations clearly shows relevance to *in vivo* situations. Lf concentration in human vaginal mucosa constantly fluctuates throughout the menstrual cycle, varying from 9 µg/mL during mid cycle to 200 µg/mL [39]. Thus, increased affinity during decreased Lf concentration may be a successful mechanism to obtain iron during such conditions.

## 13. Role of Iron on Growth of *T. vaginalis* In Vitro

Yuan and Xue [40] have shown the effect of varying concentration of iron in the TYM (trypticase-yeast extract-maltose) medium for trophozoites of *T. vaginalis*. The generation times were found to be shorter when trophozoites were grown in iron-rich conditions (100–400 µmol/L iron ion). Minimal lethal concentration (MLC) of metronidazole, tested by serial dilution method, was found to be significantly lower in iron-rich media as compared to control group.

## 14. Role of Iron in Cytoadherence

Levels of cytoadherence to HeLa cells have been found to be modulated by different iron levels. Trichomonads isolated from iron-rich media have been shown to mediate higher level of cytoadherence. The extent and expression of adherence property is directly proportional to the concentration of iron added to the medium. This increase in cytoadherence is mediated by increase in expression of genes coding for adhesins. Under iron-rich conditions, increased synthesis of adhesins mediate better cytoadherence. Actinomycin D and alpha-amanitin have been shown to prevent the expression of adhesin molecules resulting in decreased cytoadherence. Thus, iron upregulates the expression of adhesins and the level of cytoadherence, representing an important initial step in pathogenesis. The mucus layer of the vaginal tract is the first barrier encountered by the *T. vaginalis*. *Trichomonas* interacts specifically with the mucin, the predominant component of the mucus. Then the organism comes in contact with the vaginal epithelium cells (VECs) which are under the influence of various hormonal changes induced by the menstrual cycle. So, to colonize the vaginal epithelium *T. vaginalis* has evolved multiple mechanisms. Lipophosphoglycan (LPG) is a major adherence factor but other proteins which help in cytoadherence are adhesion proteins (AP), fibronectin (FN)-binding protein, laminin-binding protein,  $\alpha$ -actinin, enolase, phosphoglucomutase,

GTP-binding protein (GTP-BP) [41–43]. There occurs an upregulation of four major iron-regulated adhesion proteins (AP65, AP51, AP33, and AP23), GAPDH, and several hypothetical proteins in a specific receptor-ligand fashion. These APs have sequence homology to metabolic enzymes and the majority are positively regulated by iron at the level of transcription and translation. The ap65-1 gene encodes a 65 kDa malic enzyme involved in cytoadherence [43]. The transcription of this gene is critically regulated by its promoter region where presence of multiple closely spaced DNA regulatory elements regulate iron-induced transcription [44].

Another 120 kDa adhesion protein (AP120) is also induced under iron-rich conditions and has sequence homology with pyruvate: ferredoxin oxidoreductase A (PFO A), a hydrogenosomal enzyme that is absent in humans [7, 45]. The main function of the hydrogenosome, an organelle typical of trichomonads, and converting malate or pyruvate to H<sub>2</sub>, CO<sub>2</sub>, and acetate by a pathway associated with ATP synthesis. This pathway relies on activity of iron-sulphur proteins such as pyruvate: ferredoxin oxidoreductase (PFOR), hydrogenase, and ferredoxin. Studies have shown that like AP120, PFO is localized to the parasite surface and participates in cytoadherence [46]. Thus, *T. vaginalis* PFO is an example of a surface-associated cell-binding protein which lacks enzyme activity and is involved in cytoadherence. Additionally, PFO behaves like AP120 in parasites, when grown under iron-rich conditions.

These adhesion proteins help parasite attachment and their role in pathogenesis has been confirmed by coculture experiments. It has been shown in these experiments that antibodies to adhesion proteins (APs) reduce the parasite adhesion and subsequent cytopathic effects (CE) on host cells. FN-binding proteins bind to multiple FN domains including the cell-binding domain (CBD), N-terminal domain (NTD), and gelatine-binding domain (GBD). During these processes, iron along with calcium and phosphatase is essentially required for differential gene expression which helps in survival, growth, and colonization of parasite in the vaginal hostile environment [42, 47].

*T. vaginalis* glutaraldehyde-3-phosphate dehydrogenase (GAPDH) has been identified as a fibronectin-binding protein that is localized at the parasite surface. The expression and surface localization of GAPDH are positively regulated by iron [41, 48]. Thus, *T. vaginalis* is part of a growing list of microbial pathogens that contain surface-associated enzymes that have alternate, nonenzymic functions. However, the mechanisms by which these enzymes are localized at the *Trichomonas* surface and the pathways in which they act are poorly understood.

The responses of *T. vaginalis* to iron limitation or iron excess have been well established. There is ~80% lower rates of protein synthesis and ≥3-fold decrease in cell densities when organisms are grown in iron-limited culture system as compared to iron-rich conditions. These parasites also exhibit generation times of approximately 10 hours, 2.5-fold longer than organisms grown in the usual complex medium [49].

## 15. Effect of Iron on the Virulence of *T. vaginalis*

Iron is an essential element which has a significant effect on the virulence of *T. vaginalis*. Ryu et al. [50] evaluated the role of iron in relation to the virulence of *T. vaginalis* in mice. Trophozoites cultivated from normal Diamond's trypticase-yeast extract-maltose (TYM) media and iron-supplemented TYM media produced subcutaneous abscesses, whereas trophozoites cultivated in iron-deficient media failed to produce any pathology. Iron also affects the level of adherence and the cytotoxicity of trichomonads to HeLa cells, which are significantly reduced when trophozoites were cultivated in iron-deficient media.

## 16. Iron Upregulates Proteinases Involved in Complement Resistance

It has been seen that *T. vaginalis* is readily lysed by the activation of alternative complement pathway. However, parasites become resistant to complement when grown in iron-rich media. The resistance to complement has been shown to be dependent on iron concentration, and other divalent ions other than iron do not modulate the complement activity. Lactoferrin is known to provide the iron source to the trophozoites and it also renders the low-iron parasites resistant to complement lysis. Pretreatment of high-iron, complement-resistant parasites with proteinase inhibitors prevented the degradation of C3 on the trichomonad surface and results in degradation of parasite by complement activity. Thus, proteinases are the enzymes which provide resistance to complement degradation when iron sources are available [51].

*T. vaginalis* faces the profound change in the environment during menstrual cycle. The vaginal microenvironment undergoes dramatic change during menstruation as it is flooded with serum proteins, erythrocytes, and other macromolecules. During this stage, *T. vaginalis* encounters the complement which is an important effector system against *T. vaginalis* infection. Activation of alternative complement system does not require antibodies and it can be activated by magnesium ions, factors B and D, properdin, and C3 for activation. It has been observed that menstrual blood complement is trichomonacidal, mediated by activation of alternative pathway. However, trichomonads are known to undergo adaptive changes *in vivo*, which enable the parasites to avoid lysis by complement as a survival strategy [51]. Most important being the iron which regulates the gene expression of surface immunogens and adhesions as described above.

Even after menstruation, infection persists as some parasites evade the immune mechanism of alternative complement pathway by secreting various proteinases. These proteinases not only help the parasites acquire the nutrients through lysis of erythrocytes but also help in recognition and binding to host cells. Thus, trichomonad proteinases have dual role in host pathology and parasite survival.



## 17. Iron Modulates Major Surface Immunogen P270

P270 is an immunogenic protein which causes phenotypic variation of *T. vaginalis* infected with double-stranded RNA virus (designated as TVV: *T. vaginalis* virus) [52, 53] on the basis of its differential cytoplasmic versus surface expression. Two types of isolates have been documented during infection with *T. vaginalis*. Type I isolates comprise homogeneous nonfluorescent (negative phenotype) trichomonads which synthesize and express P270 in the cytoplasm. In contrast, type II isolates comprise both fluorescent and nonfluorescent subpopulations (positive and negative phenotypes). Growth of virus-positive organisms in high-iron medium induces expression of trichomonad adhesins but yields parasites without surface P270. P270 has been found to be highly phosphorylated in high-iron parasites and modulating the low surface expression of P270. Thus, iron plays a role in modulating surface localization of P270 in virus-harboring parasites [54].

## 18. Irony about Iron Regulation of CP65 Cysteine Proteinase

It has been seen that iron is essential for the virulence of the parasite and *Trichomonas* has evolved different strategies for acquiring iron. In *T. vaginalis*, iron up-regulates amounts of adhesins and levels of cytoadherence [55, 56]. Iron also up-regulates the cysteine proteinases (CPs) involved in complement resistance and several parasite functions as described above. However, a study by Alvarez-Sánchez et al. [57] has shown that iron specifically down-regulates proteolytic activity, expression, and transcription of CP65, negatively affecting trichomonal cytotoxicity *in vitro*. Several CPs participate in the virulence of *Trichomonas vaginalis* and 65 kDa CP, CP65, is one involved in cytotoxicity. A similar effect has also been observed with CP39, involved in cytotoxicity [58]. Similarly, iron also downregulates the other genes such as *tcp12* and *flp-1,2* which encodes for papain-like CP and fibronectin-like proteins, respectively [59, 60]. Thus, it is very surprising that similar iron concentrations exert opposite effects on expression of adhesions and some CPs. This irony has been explained by the fact that iron concentration fluctuates in the vagina during menstrual cycle. Lactoferrin concentration is also known to vary during the different phases of menstrual cycle; that is, it is present in highest concentration after the menstruation and decreases progressively during the cycle with minimum levels present immediately before menstruation. *Trichomonas* has evolved the two different mechanisms to maintain its pathogenicity at different phases of menstrual cycle which helps in its survival. It has been postulated that just before the menstruation when iron concentration is lowest, *T. vaginalis* may enhance the level of cytotoxicity by increasing the CP65 activity. Simultaneously, low iron levels may reduce the expression of adhesions leading to decreased level of cytoadherence, allowing the parasite to move around in search for new iron sources. The opposite effect might be occurring during

the highest level of iron concentrations (during and after menstruation); there occurs an up-regulation of adhesin expression, providing an opportunity for the parasites to adhere firmly to prevent the flushing by menstrual blood flow [57].

## 19. Future Prospects

Drug resistance among the protozoa is increasing at an alarming rate, with relatively few options left for the treatment. In 1962, resistance among *T. vaginalis* was first reported [61] and is at risk of developing increased resistance to recommended therapeutics due to empirical use of drugs. The 5-nitroimidazole family of drugs (metronidazole and tinidazole) are the only class of drugs approved for the treatment of trichomoniasis [62] and P. Upcroft and J. A. Upcroft [63] have reported up to 10% of infections which are not responding to treatment in the United States. Resistant organisms are cosmopolitan in distribution and are of considerable concern as *Trichomonas* infections are linked to human immunodeficiency virus (HIV) transmission. Further scientific research is required to fill the gaps in knowledge about understanding the pathogenesis, so that newer drug targets can be evaluated in order to overcome the increasing burden of drug resistance.

## 20. *T. vaginalis* Receptor as a Vaccine Candidate

Lehker and Alderete [38] examined the sera of patients by immunoblotting to detect the proteins from parasites grown in low- and high-iron media, recognized by antibodies. Twelve trichomonad immunogens from parasites grown in iron-rich medium and 7 from those grown in low-iron medium were found. Sera from patients with vaginitis recognized the purified Lf receptor protein. Thus, *T. vaginalis* receptor could be tested as a vaccine candidate.

## 21. Drug Delivery

Lf may be used as a carrier for drug delivery as it specifically binds to receptor at the surface of *Trichomonas*. Moreover, it is concentrated at places where infection is present at the mucosal surface.

## 22. Inhibition of Lf and Receptor Binding

Inhibition of Lf binding sites at *Trichomonas* surface may deprive the parasites from iron availability and can be used as an attractive therapeutic strategy.

Thus, search for newer drug targets and vaccine candidates in relation to lactoferrin, its receptors, and *Trichomonas* is required in coming years to win the fight against this parasite.

## 23. Conclusion

Iron is an essential nutrient required by *T. vaginalis* to maintain its virulence in changing vaginal environment due to

cyclic menstruation. *Trichomonas* has evolved the receptor-mediated uptake of iron through holo-Lf and its concentration and affinity vary according to the availability of iron in the external environment. Usually, iron is known to upregulate various adhesins and other factors which increase which help in maintaining the virulence. However, it has been seen that cysteine proteinases (CP65 and CP39) are downregulated when the external iron concentration is high. It is important for the survival of parasite as when abundant iron is available, that is, during menstrual flow, there is an increase in cytoadherence, preventing flushing of parasites during blood flow of menstruation. On the other hand, just before menstruation, iron concentration is low; thus cytoadhesion is also decreased and CP65 and CP39 are activated which help moving the parasite in search of iron and maintain its virulence. Furthermore, drugs can be formulated which can target the holo-Lf receptor or various immunogens can be tested which can provide immunity or holo-Lf can be used a carrier for drug delivery. However, studies are required in animal models to check the efficacy and adverse effects of new drugs, targeting Lf or its receptor. At present there is no drug available which targets the Lf or its receptor for the treatment of trichomoniasis. Finally to conclude apo-Lf is lethal to parasite, but when it acquires iron (holo-Lf), it is used as a iron source by the parasite.

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