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Iron regulates growth of *Trichomonas vaginalis* and the expression of immunogenic trichomonad proteins

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Summary

Iron is an essential nutrient for *Trichomonas vaginalis* and is acquired via highly specific receptor-mediated mechanisms from the host. Responses of *T. vaginalis* to conditions of iron limitation or iron excess were analysed in order to determine whether iron levels in the growth medium regulate certain properties of the parasite. When compared with organisms grown in excess iron, iron limitation resulted in ≥80% lower rates of protein synthesis and ≥3-fold decreases in cell densities. These parasites also exhibited generation times of ≈10 hours, 2.5-fold longer than organisms grown in the usual complex medium. Iron-restricted growth also resulted in increased binding of lactoferrin by trichomonads, which paralleled elevated expression of the lactoferrin-binding receptor protein having a relative molecular mass of 136000 daltons (136kDa). A M_r 126kDa protein was concomitantly repressed in low-iron-grown parasites. The greater amounts of lactoferrin bound by iron-depleted *T. vaginalis* organisms corresponded with both the expression of additional receptors onto trichomonal surfaces and increased affinity of the receptor for the lactoferrin molecule. Finally, immunoblot analysis of parasites grown under high- and low-iron conditions using sera from patients with trichomoniasis further revealed the synthesis by *T. vaginalis* of at least 19 iron-regulated immunogens, and patients' sera also detected the lactoferrin receptor. These data not only show the overall importance of iron to the biology of this protozoan, but illustrate the *in vivo* iron modulation of gene expression of the biofunctional lactoferrin receptor and other immunogens.

Introduction

Trichomonas vaginalis, a mucosal parasite of the urogenital tract, is responsible for one of the most common clinically recognized sexually transmitted diseases in humans (Krieger, 1981). Trichomoniasis in women ranges from an asymptomatic carrier state to intense inflammation of the vaginal epithelium, which can be accompanied by itching, a purulent white discharge, and severe abdominal pain. The reasons for the tremendous variation in symptomatology (Krieger *et al.*, 1990) are unknown but may be related to parasite as well as host factors. Successful parasitism of the vaginal epithelium by this microorganism relates, in part, to the ability of trichomonads to acquire essential nutrients, such as iron (Peterson and Alderete, 1984a; Lehker *et al.*, 1990) and lipids (Lehker *et al.*, 1990; Peterson and Alderete, 1984b) following specific cytadherence to vaginal epithelial cells (Alderete *et al.*, 1988).

Iron, not readily available *in vivo*, is an essential element for the growth of many pathogens (Griffith, 1985). The multiplicity of mechanisms used by microorganisms to obtain iron from their host (Griffith, 1985) underscores its importance for microbial survival. Thus, it is not surprising that microorganisms confronted by the iron-restricted environment of the host are able to acquire iron from host iron-binding proteins (Griffith, 1985).

Although poorly understood, the role and importance of iron in modulating pathogenicity of *T. vaginalis* is receiving our increased attention because of the diverse and highly specific mechanisms of iron acquisition used by microorganisms (Griffith, 1985). For example, acquisition of iron by this parasite by binding of host lactoferrin and haemoglobin by distinct receptors has been demonstrated (Peterson and Alderete, 1984a; Lehker *et al.*, 1990), and the evidence points to the existence of other receptors on *T. vaginalis* for binding of distinct iron-binding and iron-containing proteins. Additionally, a previous report indicated increased virulence of trichomonads following iron injection in mice (Budilova and Kulda, 1977). The importance of iron for *T. vaginalis* is further demonstrated by the relationship of iron levels to the activity of hydrogenosomal enzymes which are critical to the energy needs of this organism (Gorrell, 1985). Maximal enzyme activity in these organelles is achieved only at ≥200 μM iron, an extremely high concentration of iron for microorganisms. This amount of iron contrasts greatly with the 0.4 – 4 μM iron required by bacterial pathogens (Griffith, 1985; Robins Browne and Prpic, 1985). It would not be surprising, therefore, that iron-restriction may dramatically modify

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Table 1. Growth of *T. vaginalis* NYH 286 in TYM medium differing in iron content.

Sample number	Chelator concentration (mM)	Medium supplement	Density[a] ($\times 10^5$ parasites ml^{-1}
1	0.00	None	36.0
2	0.01	None	28.0
3	0.05	None	25.0
4	0.10	None	9.8
5	0.20	None	4.1
6	0.30	None	≤0.01
7	0.30	Fe^{2+} (0.3 mM)	35.0
8	0.30	Trace metals[b]	≤0.01
9	0.30	Ltf[c] (200 μg ml^{-1})	18.6
10	0.30	aLtf[c] (200 μg ml^{-1})	≤0.01
11	0.30	Trf[c] (200 μg ml^{-1})	≤0.01

a. Test tubes containing 5 ml of medium under the various conditions (*Experimental procedures*) were inoculated with 2×10^5 trichomonads. Cell density was determined at 6 h intervals during 24 h of growth until maximal cell density was achieved. Numbers of parasites are the average of three determinations by haemacyometer counting.
b. Trace metal solution was prepared as described by Linstead (1981), except that iron was omitted.
c. Abreviations: Ltf, iron-saturated human lactoferrin; aLtf, apolactoferrin; Trf, iron-saturated human transferrin.

properties of *T. vaginalis*, either through expression of novel mediators of virulence, which ensures the parasite's acquisition of iron *in vivo*, or through modification of general physiological properties, which allows the parasite to survive in the nutrient-limiting environment of the vagina (Lehker and Alderete, 1990). This hypothesis is attractive in view of the possible iron limitation in the vagina relative to the high iron levels required by the parasite (Gorrell, 1985).

In this report, data are presented that show for the first time that iron levels influence important properties of this urogenital pathogen (*T. vaginalis*). These properties include, but are not limited to, growth rate, overall protein synthesis and protein–immunogen profiles of the parasite. The trichomonad lactoferrin receptor identified in earlier studies (Peterson and Alderete, 1984a) was found to be one of several proteins regulated by iron. At least 18 other trichomonad immunogens were identified as being responsive to changes of iron levels in the growth medium. Our results indicate the existence of *in vivo* environmental niches for this sexually transmitted disease parasite that allow for gene expression of both low- and high-iron-regulated proteins and immunogens.

Results

Effects of iron limitation on T. vaginalis *growth properties*

Table 1 (samples 1 to 6) shows the progressive reduction in cell density of isolate NYH 286 trichomonads in the presence of increasing amounts of 2,2-dipyridal, which limits the availability of iron. Parasites were killed at ≥300 μM 2,2 dipyridal (sample 6). The non-toxicity of the chelator was shown by recovery of growth after addition of excess ferrous ammonium sulphate to medium with 300 μM chelator (sample 7). Finally, the addition of trace metals (Linstead, 1981) without iron, but with iron chelator, (sample 8) also did not support growth.

The growth-inhibitory effect of 2,2-dipyridal was reversed by human ferrolactoferrin (sample 9), but not apolactoferrin or transferrin (samples 10 and 11, respectively), consistent with the reported iron uptake following specific lactoferrin binding by *T. vaginalis* (Peterson and Alderete, 1984a). These experiments were performed at least three times. Identical results were also obtained with five other isolates examined.

Table 2 shows the reduction in maximal cell densities of trichomonal isolates in the presence of 100 μM 2,2-dipyridal (low iron). The generation time of trichomonads grown under low-iron conditions was two times that seen for parasites grown in high-iron medium, which gave doubling times of 4–6 h as previously reported (Peterson and Alderete, 1982). At the same time, low-iron-grown organisms had decreased protein synthesis relative to high-iron-grown parasites. Identical results were obtained with trichomonads grown in the trypticase/yeast extract/maltose (TYM)-serum medium treated with Chelex 100 and

Table 2. Growth characteristics of two *T. vaginalis* isolates grown in low- and high-iron medium.

	Generation Time[a]		Range of Maximal Density[b] (h)[c]		Rate of ^{35}S Incorporation[d]	
Isolate	H[e]	L[e]	H	L	H	L
NYH 286	4.6±0.1	7.2±0.3	2.8–3.5 (32)[c]	0.8–1.5 (32)[c]	650±21	132±14
IR 78	5.3±0.4	10.6±1.1	4.3–5.9 (42)	0.7–1.3 (36)	568±32	205±17

a,e. As described in the *Experimental procedures*. The difference between H (high-iron medium containing 250 μM iron) and L (low-iron medium containing 100 μM 2,2-dipyridal) trichomonads was significant ($P \leq 0.01$) based on the Student's *t* test. Generation time was determined during the exponential phase of growth.
b. Maximal densities from five independent experiments were determined by haemacytometer counting of 4 h intervals after inoculation with 1×10^5 parasites per ml. Numbers are given as 10^6 organisms per ml.
c. Numbers in parenthesis refer to the hours of growth needed to achieve maximal cell densities.
d. Rate is given as c.p.m. [^{35}C]-methionine incorporated per hour, measured over a 4 h period.

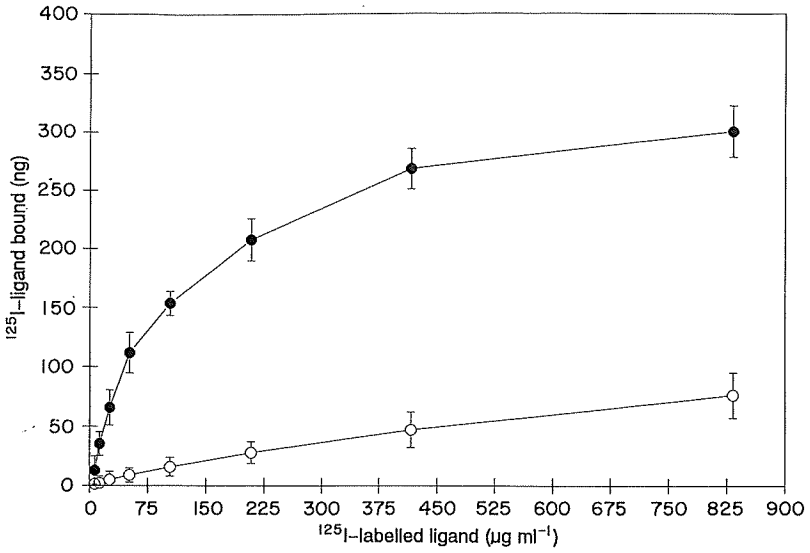

Fig. 1. Concentration-dependent binding of human lactoferrin to *T. vaginalis.* Parasites were totally depleted of intracellular iron pools (●) (*Experimental procedures)* or grown in high-iron medium (○). Approximately 1 × 10^7 parasites in a 0.5 ml final volume were incubated with various amounts of ^{125}I-labelled lactoferrin at 4°C. Specific activity of lactoferrin was 1.3 × 10^5 c.p.m. μg^{-1}. Results are the averages of duplicate determinations of two experiments.

supplemented with trace metals. These data strongly indicate that various growth and metabolic processes of *T. vaginalis* are specifically affected by levels of iron in the growth medium

Iron regulation of lactoferrin binding levels

To clarify the role of lactoferrin in reversing the effects of iron limitation, we compared the binding of lactoferrin to live trichomonads grown under iron-replete (Table 3, sample 1) and iron-limiting conditions. The level of lactoferrin-binding activity increased by ≈1.6-fold when trichomonads were placed in low-iron conditions (samples 2–4). Trichomonads with depleted intracellular pools of iron (as described in the *Experimental procedures*) had an approximately fivefold increase in lactoferrin binding (sample 5). The increased lactoferrin bound was due to reduced iron levels, since replenishment of medium with excess iron in the presence of chelator reduced lactoferrin association to baseline (sample 6).

Trichomonads were also grown in low-iron medium supplemented with iron-saturated lactoferrin (samples 7 and 8). Levels of lactoferrin bound were like those seen for low-iron-grown organisms and remained unchanged, even in the presence of what is considered a high concentration (20 μM) of lactoferrin. This indicates that downregulation of possible receptors does not occur under these conditions, which nonetheless provides sufficient iron for some growth and multiplication.

Estimation of lactoferrin receptor number and affinity.

Earlier calculations of the lactoferrin receptor number for this parasite were performed with organisms grown in normal complex medium (Peterson and Alderete, 1984a). Thus, it was now necessary to obtain a more accurate determination of receptor numbers of trichomonads depleted of intracellular pools of iron.

Saturation binding kinetics of lactoferrin acquisition by iron-depleted parasites confirmed the responsiveness of lactoferrin receptors to iron (Fig. 1). Scatchard plot analysis (Fig. 2) indicated the existence of 2.5 × 10^5 receptors for iron-depleted trichomonads, and this was 2.5 times greater than that seen for iron-rich parasites (Fig. 2, inset).

Table 3. Expression of lactoferrin-binding activity of *T. vaginalis* NYH 286 grown in media differing in iron content.

Sample number	Medium supplement[a]		Cell density[b]	Binding activity[c]
1	250 μM	Fe	32.0	64.3 ± 15.4
2	25 μM	2,2-DP[d]	21.0	95.6 ± 12.6
3	75 μM	2,2-DP	15.0	103.3 ± 15.8
4	100 μM	2,2-DP	8.8	105.7 ± 17.3
5[e]	100 μM	2,2-DP	11.0	309.2 ±10.6
	100 μM	2,2-DP+		
6	250 μM	+Fe	19.0	65.8 ± 8.7
7	1 μM	+Ltf	11.0	102.0 ± 14.8
8	20 μM	+Ltf	15.0	93.9 ± 5.4

a. TYM medium with serum supplemented as indicated was inoculated with trichomonads from an overnight-grown culture to an initial density of 2 × 10^5 parasites per ml.

b. Density was determined by haemocytometer counting, and numbers are given as 10^5 parasites per ml.

c. Binding activity was determined as described in the *Experimental procedures*, and numbers ± standard deviation are given as ng lactoferrin bound per 10^7 parasites.

d. Abbreviations: Fe, ferrous ammonium sulphate; Ltf, iron-saturated human lactoferrin; 2,2-DP, 2,2-dipyridal.

e. Trichomonads underwent two cycles of iron-limitation for iron depletion of intracellular pools (*Experimental procedures).* After 36 h of growth in the presence of iron chelator, organisms were subcultured into new low-iron medium for another 24 h.

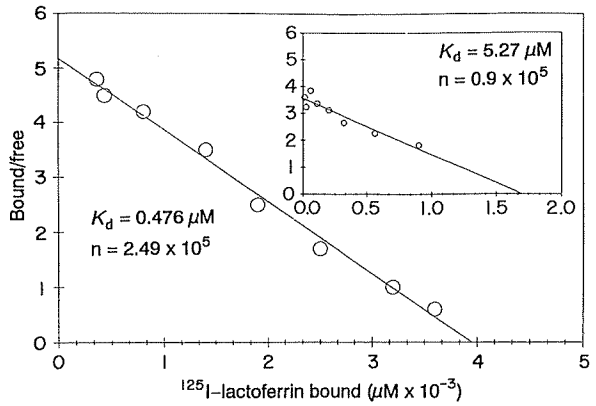

Fig. 2. Estimation of lactoferrin receptor number by Scatchard plot analysis. Data presented in Fig. 1 of lactoferrin binding to trichomonads grown in high-iron medium (inset) were compared with totally iron-depleted parasites and used to calculate number of receptors (N) and affinity (K_d).

The increased number of receptors was confirmed by a dot-blot assay (Schryvers and Morris, 1988) (*Experimental procedures*). The $\approx 10^5$ receptor number of high-iron-grown trichomonads was identical to that previously reported by us (Peterson and Alderete, 1984a).

Finally, the Scatchard data indicated that the K_d values for receptors of organisms grown under iron-replete and iron-limited conditions were 5.27 and 0.47 μM, respectively. Next, we investigated the idea that the lactoferrin receptor of iron-limited organisms had a higher affinity for the lactoferrin ligand. In this experiment, binding of lactoferrin to low-iron-grown parasites was still evident in the presence of NaCl added to the binding buffer (Table 4); no similar binding was seen in an identical experiment with high-iron-grown parasites. To show that the lactoferrin-binding proteins with the different K_d values were identical and that the iron state of the cell influenced the affinity, the receptors were affinity purified as before (Peterson and Alderete, 1984a) from the high-iron- and low-iron-grown organisms. Both receptors had identical electrophoretic mobilities, suggesting that affinity for lactoferrin, and not expression of different-sized receptors, was affected by the level of iron.

Time period for lactoferrin-receptor induction and repression

Trichomonads grown in high-iron medium were shifted to iron-depleted medium, and lactoferrin binding was monitored. Levels of lactoferrin bound increased ≈10-fold within 6 h (Fig.3), and part of the overall increase could be inhibited by cycloheximide. This indicated that some pre-formed lactoferrin receptors were mobilized initially during iron limitation, and this was followed by the synthesis of new receptor molecules at the later time-points.

The inset in Fig. 3 illustrates the return to baseline levels for the lactoferrin receptors upon addition of iron to iron-depleted parasites. As controls, trace metals without iron added to Chelex 100- or 2,2-dipyridal-treated medium failed to show a similar decrease in the number of lactoferrin receptors.

Do iron sources other than lactoferrin overcome the growth-inhibitory effect of iron-limitation?

Addition of iron through supplementation of medium with numerous alternative sources, including free iron, iron-binding proteins, and iron-containing proteins, reversed the growth inhibitory effect induced by 200 μM 2,2-dipyridal (Table 5, samples 1 to 7). The absence of parasite growth and multiplication when apolactoferrin (aLtf), iron-saturated transferrin (Trf), or trace metal solution (TMS) without iron (samples 8 to 10) were added to the medium illustrates the specificity of iron acquisition by this parasite.

Specific iron regulation of expression of T. vaginalis *lactoferrin receptor*

Figure 4 (Panel A) shows stained gels of total proteins of two isolates (NYH 286 and IR 78) grown in low- and high-iron media. Proteins of 136 and 72 kDa were readily visible in stained gels only during iron-restricted growth

Table 4. Binding of ^{125}I-labelled lactoferrin to *T. vaginalis* at various ionic strengths.[a]

	Per Cent Bound ^{125}I-Labelled Lactoferrin[b]	
Binding buffer	high iron[c]	low-iron[c]
PBS	100.0	100.0
PBS ± NaCl (mM)		
25	90.0 ± 3.0	100.0
50	86.1 ± 1.3	100.0
75	75.7 ± 2.5	100.0
100	55.2 ± 1.4	84.3 ± 1.8
125	40.3 ± 1.7	60.7 ± 2.3
150	30.3 ± 2.4	39.4 ± 3.9
200	21.9 ± 3.2	26.6 ± 3.1

a. 2 × 10^5 parasites in 50 μl of PBS were added to individual wells of microtitre plates and air-dried at 37°C. Parasites were then fixed with 50 μl of ethanol at 37°C. Plates were used immediately or stored at −20°C until needed.

b. Lactoferrin binding was determined by addition of 50 μl of 1 mg ml^{-1} ^{125}I-labelled lactoferrin to the microtitre wells. After incubation for 30 min at 37°C, wells were washed 5× with 200 μl of PBS before counting was done by scintillation spectroscopy. Non-specific binding as c.p.m. recovered from wells containing a 20-fold excess of unlabelled lactoferrin was substracted from all counts.

c. High- and low-iron media were prepared as described in the *Experimental procedures.* Per cent binding is the average of five experiments performed in duplicate.

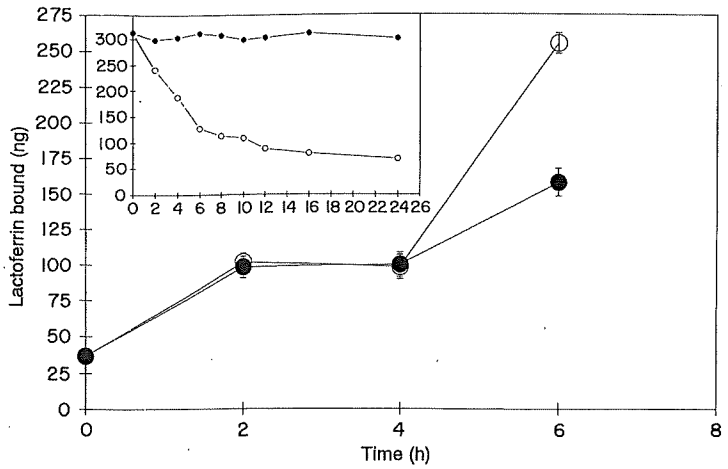

Fig. 3. Time period for lactoferrin receptor induction and repression. Parasites were grown in high-iron medium and pretreated for 1 h with (●) or without (○) 100 μM cycloheximide, which did not result in lysis of organism and did not affect trichomonal motility. Trichomonads were then incubated for up to 6 h in TYM-serum medium containing 200 μM 2,2-dipyridal. Lactoferrin binding was measured as described in the *Experimental procedures*. Protein synthesis of cycloheximide-treated organisms was inhibited as has been described (Alderete and Garza, 1988). The inset demonstrates the return to baseline levels of lactoferrin binding after addition of iron (○) but not trace metal solution (●) (Linstead, 1981) of parasites grown under stringent iron-limitation.

(lanes 1 and 3). A 128 kDa protein was seen only in organisms grown in high-iron medium (lanes 2 and 4). Also, these proteins resided on the parasite surface, as evidenced by their ability to be extrinsically labelled (Panel B, lanes 1 and 2) and the absence of the iodinated bands with trypsin treatment of iodinated trichomonads prior to electrophoresis (lanes 3 and 4). The parasite origin of the three proteins was confirmed using [^{35}S]-methionine-labelled trichomonads (Peterson and Alderete, 1984a). In data not shown, the 136 and 72 kDa proteins were found to be expressed only at ≤50 μM iron levels, whereas the 128 kDa protein was only detectable after growth in ≥50 μM iron. Finally, TYM-serum medium containing reduced amounts of either serum, maltose or nucleotides, which also gave decreased cell densities and increased generation times, had no effect on the expression of any of these proteins. It is noteworthy that identical results were obtained for four other isolates, and these data were indicative of iron regulation of expression of specific trichomonad proteins.

Confirmation that the lactoferrin receptor is the 136 kDa protein

Electrophoresis was performed on the 136 kDa iron-repressible protein purified from acrylamide gels and on the trichomonad lactoferrin receptor obtained from affinity chromatography (Peterson and Alderete, 1984a). Both of these proteins had the same electrophoretic mobility (Fig. 5, lanes 2 and 3, respectively). Immunoblots were then performed on these comigrating proteins. Purified antibody immunoreactive with the lactoferrin receptor (*Experimental procedures*) detected both the iron-repressible protein and the affinity-purified lactoferrin receptor. These data confirm the identity of the 136 kDa iron-regulated protein as the lactoferrin receptor, as previously suggested (Peterson and Alderete, 1984a).

Does iron regulate expression of immunogenic proteins in vivo*?*

Finally, we investigated whether the lactoferrin receptor and possibly other iron-regulated proteins not readily detectable in stained gels or fluorograms (Fig. 4) were expressed *in vivo*. Sera of patients were examined by immunoblotting for detection of proteins of parasites grown in low- and high-iron medium. To our surprise, antibody in patients' sera recognized numerous iron-regulated proteins (Fig. 6, Panels A–E). Representative immunoblots of total proteins from low-iron-grown trichomonads (lanes labelled L) revealed seven immunogens detected by patients' serum antibody. Likewise, 12 proteins were induced in high-iron-grown parasites. In all, 24 sera from patients were analysed and, as presented and summarized in Fig. 6, no two sera gave identical reactions with low- or high-iron-regulated immunogens. Importantly, expression of these immunogens was not

Table 5. Various iron sources added to medium for growth of *T. vaginalis*.[a]

Isolate	Sample Number									
	1	2	3	4	5	6	7	8	9	10
	Fe^{3+b}	Ltf[b]	Frt	Hgl	Cyt	HeLa[c]	RBC[c]	Trf	TMS	aLtf
NYH 286	+[d]	+	+	+	+	+	+	−[c]	−	−
IR 78	+	+	+	+	+	+	+	−	−	−

a. Trichomonads of isolates NYH 286 and IR 78 were inoculated into TYM-serum medium with sufficient 2,2-dipyridal to completely inhibit growth in the absence of iron (Table 1). The ability to reverse growth inhibition was evaluated by counting organisms at 24 and 36 h post-inoculation.

b. Abbreviations of additions to medium: Ltf, iron-saturated lactoferrin; aLtf apolactoferrin; Frt, ferritin; Hgl, haemoglobin; Cyt, cytochrome *c*, Trf, transferrin; TMS, trace metal solution without iron; RBC, human red blood cells; Fe^{3+} was added to 250 μM.

c. HeLa and RBC were added to a final density of 2×10^6 cells per ml. Iron-binding and iron-containing proteins were added at 1 mg ml^{-1}.

d. +, Growth; −, no growth.

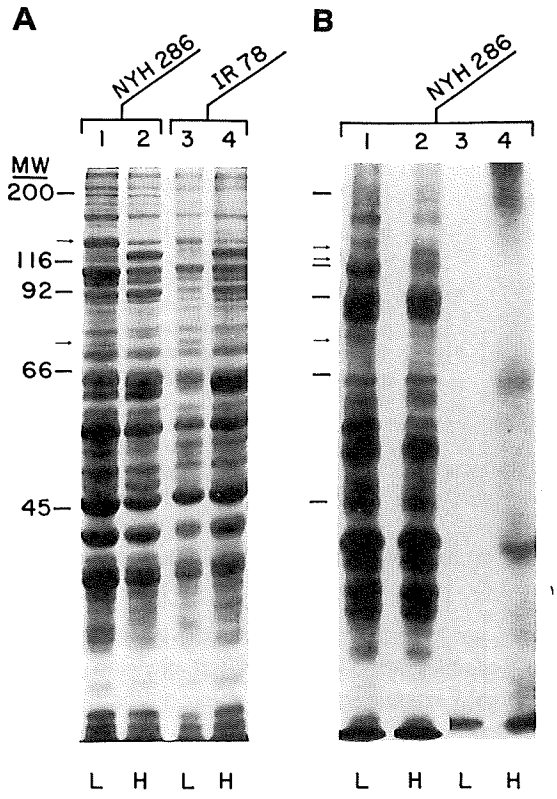

Fig. 4. Induction of surface proteins of *T. vaginalis* NYH 286 and IR 78 in response to iron-limited and iron-replete growth conditions.
A. SDS–PAGE patterns of NYH 286 (lanes 1 and 2) and IR 78 (lanes 3 and 4) grown in low-iron (L) and high-iron (H) medium.
B. Autoradiogram of ^{125}I-labelled trichomonad proteins of 1×10^6 parasites grown in low-iron (lane 1) and high-iron medium (lane 2). Lanes 3 and 4 show the protein pattern of the same number of live, iodinated trichomonads first treated with 10 mg of trypsin for 30 min at 37°C before TCA precipitation of proteins (Peterson and Alderete, 1982).

dependent on the growth rate of the parasite. The immunogens were never detected by the patients' sera on *T. vaginalis* grown in TYM-serum medium containing reduced amounts of either serum, maltose or nucleotides, or the calcium chelator, ethyleneglycol-bis(β-amino-ethylether)-*N, N, N′, N′*-tetraacetic acid (EGTA). These latter modifications of the growth medium also affected the growth rate and cell densities. Eighty per cent (19/24) of sera from patients with trichomoniasis reacted with purified lactoferrin receptor in an immuno-dot-blot assay (data not shown). No similar protein reactivity to the lactoferrin receptor was ever detected with normal human serum as a control. These data dramatize both the *in vivo* expression of iron-regulated immunogens and the existence of low- and high-iron conditions for this parasite in the human vagina.

Discussion

We now show that iron specifically affects a variety of properties of the sexually transmitted disease parasite, *T. vaginalis*. For example, withdrawal of iron is manifested by inhibition of growth and multiplication, which is reflected in an overall decreased rate of protein synthesis (Tables 1 and 2). It is not surprising that the interrelationship between host and parasite would translate into the evolution of mechanisms by which this protozoan can obtain iron through specific associations with iron-binding (lactoferrin and ferritin) and iron-containing (haemoglobin and cytochrome) proteins (Table 5). This is especially relevant in the vagina, which is an iron-limiting environment because of the absence of free iron.

The differential expression of lactoferrin receptors in response to different iron levels provided evidence that iron regulates at least one receptor important to *T. vaginalis* iron-acquisition. We show a protein synthesis-dependent and -independent increase in lactoferrin receptors (Fig. 3), illustrating that this microorganism responds quickly to changing environmental iron conditions. The mobilization of pre-formed lactoferrin receptors to the surface, coupled with the gene expression and protein synthesis of lactoferrin receptors as a result of

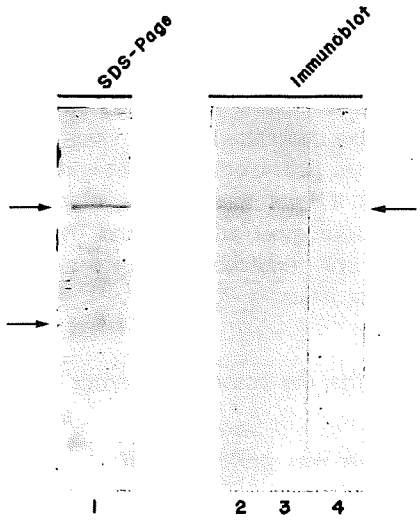

Fig. 5. Confirmation that the 136 kDa iron-regulated protein is the lactoferrin receptor. The lactoferrin receptor was purified by lactoferrin-affinity column chromatography (lane 1) as described in the *Experimental procedures*. The purified lactoferrin-binding protein (lane 1) and the 136 kDa iron-repressible protein excised from a gel of total proteins of low-iron-grown trichomonads were then re-electrophoresed, blotted onto nitrocellulose and reacted with monospecific, rabbit anti-lactoferrin receptor antibody (lanes 2 and 3, respectively) or normal rabbit antiserum (lane 4). This anti-lactoferrin receptor antibody was purified by elution (Garvey *et al.*, 1977) from the preparative blots of the receptor reacted with total anti-*T. vaginalis* serum (Alderete, 1983a). The reaction of the antibody with the lactoferrin receptor and the co-migrating 136 kDa iron-repressible proteins is evident.

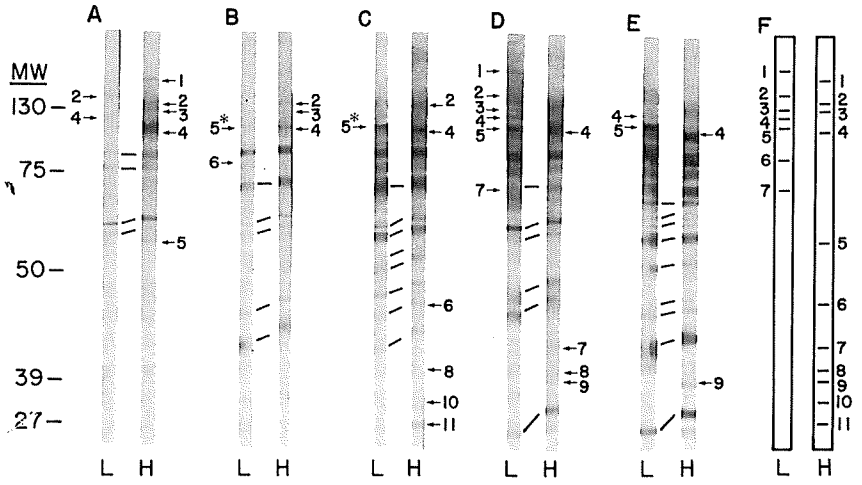

Fig. 6. Representative reaction patterns of five different patient's sera (Panels A to E) with proteins of trichomonads grown in low- (lane L) or high-iron (lane H) medium. Panel F shows the summary of differential immunogens detected for low- (L) or high-iron (H) grown parasites from 24 total sera from patients with confirmed trichomoniasis. The protein labelled 5 of low-iron parasites is the 136 kDa lactoferrin-binding protein.

falling internal levels of iron, shows that multiple mechanisms are available to the parasite for dealing with this critical aspect of nutrient acquisition.

The ability of *T. vaginalis* to modulate number and affinity of lactoferrin receptors (Figs 2 and 3) in response to changing external and internal iron concentrations clearly allows for regulation of iron acquisition at multiple levels. This may be relevant to the *in vivo* situation, in which lactoferrin concentrations are constantly fluctuating (from 200 $\mu g\,ml^{-1}$ after menses to a low of 9 $\mu g\ ml^{-1}$ during the mid-cycle; Cohen *et al.*, 1987). Although an increased affinity for lactoferrin may be a mechanism that ensures removal of iron from the limiting and finite pool of lactoferrin molecules in vaginal secretions (Cohen *et al.*, 1987), it is intriguing that the lactoferrin receptor may exist in two different conformational states leading to different affinities for the same ligand. An attractive hypothesis is that increased receptor density favours co-operative interactions, leading to higher affinity. The possibility that the receptor may possess two distinct binding sites, one for lactoferrin and another for iron, may be an alternative explanation. Binding of an iron atom either from lactoferrin or from the medium could then change the affinity of the lactoferrin-binding site, as in models of allosteric inhibition (Freifelder, 1982). No similar model has been proposed for iron acquistion in other microorganisms, but this possibility may be consistent with the release of intact apolactoferrin from *T. vaginalis* (Peterson and Alderete, 1984a).

The absolute necessity of iron for growth and multiplication of *T. vaginalis* and the high iron requirement of this parasite (Gorrell, 1985) may make it impossible to overcome the nutritional immunity imposed by the host, if trichomonads relied only on the interaction of lactoferrin with its trichomonal receptor. The ability of trichomonads to lyse human erythrocytes (Krieger *et al.*, 1983; Dailey *et al.*, 1990) and HeLa cells and epithelial cells (Alderete and Pearlman, 1984; Krieger *et al.*, 1985) has been established. That red blood cells and epithelial cell extracts stimulate growth in iron-limited medium (Table 5) strongly suggests that intracellular iron sources, such as cytochromes, ferritin, and haemoglobin, are also likely iron sources for the parasite during infection. These multiple iron-acquisition systems may be beneficial to this microbial pathogen by providing co-operative or alternative mechanisms for iron sequestration during establishment and maintenance of infection.

It is noteworthy that the lactoferrin receptor (Fig. 4) is a minor component on the cell surface of trichomonads grown in normal growth medium. Thus, earlier immunochemical characterization studies by us (Alderete, 1983a,b; Alderete and Garza, 1986) and others (Su-Lin and Honigberg, 1983; Teras, 1966; Torian *et al.*, 1984) may not have identified this immunogenic biofunctional molecule. In contrast, organisms depleted of intracellular iron pools may have the lactoferrin receptor as a major component of the cell surface (Fig. 4, panels A and B). In most patients, the presence of antibody to the trichomonad lactoferrin receptor further confirms the *in vivo* expression of this gene. Although it may be envisaged that antibodies to this receptor control infection, through interruption of iron uptake from lactoferrin, the alternative iron-acquisition systems ensure the survival of this parasite. In addition, any immuno-cross reactivity between the trichomonad and mammalian cell lactoferrin receptors (a possibility requiring investigation) may be detrimental to host cells and contribute to host cytopathology.

The *in vivo* differential expression of proteins was also revealed (Fig. 6). A total of 19 iron-regulated proteins were identified (Fig. 6). That human antibody to proteins synthesized by organisms under either high- or low-iron conditions are expressed *in vivo* highlights the incredibly complex nature of this parasite–host interaction. For

example, this suggests that this microorganism experiences low- and high-iron environments during infection in a single host. One possibility is that subpopulations of *T. vaginalis* express high- or low-iron-regulated proteins depending on the iron status of different micro-environments within the vagina. It is equally possible to envisage that a high-iron micro-environment might be depleted of iron by vigorous parasite growth and multiplication, thereby creating an iron-limiting region. Also, lactoferrin concentrations vary dramatically during the menstrual cycle (Cohen *et al.*, 1987), and erythrocytes as a major source of haem iron (Lehker *et al.*, 1990) for trichomonads are present only during menstruation or may exist only in defined foci of infection. Finally, it was intriguing to find that individual patients responded only to subsets of iron-regulated proteins (Fig. 6), since none of the sera of 24 patients recognized all of the iron-regulated proteins. This differential expression of only a few in the total repertoire of iron-regulated proteins might possibly be indicative of subsets of iron-responsive proteins being induced according to the levels of iron.

An extensive literature exists which describes pronounced antigenic heterogeneity among *T. vaginalis* isolates (Su-Lin and Honigberg, 1983; Teras, 1966; Teras *et al.*, 1966). Our discovery of the general property of phenotypic variation, defined on the basis of surface expression and/or synthesis of several repertoires of trichomonad proteins (Alderete *et al.*, 1985; 1986; Alderete, 1987; Neale and Alderete, 1990) and including the iron-regulated proteins shown here, continues to define and clarify the molecular basis of antigenic heterogeneity. The data in this report and an earlier study by us, which demonstrates the ability of *T. vaginalis* to respond to specific environmental signals with concomitant induction of protein immunogens (Lehker and Alderete, 1990), and the human serum antibody response depending on a multiplicity of variables, such as *in vivo* expression of the phenotypically varying trichomonad proteins, which include prominent immunogens (Alderete, 1987), proteinases (Neale and Alderete, 1990), erythrocyte-binding proteins (Lehker *et al.*, 1990), and now the iron-regulated proteins (Fig. 6), contribute to this parasite's overall antigenic diversity and complexity. Our work reaffirms the idea that antibody responses among patients with trichomoniasis is toward proteins that are differentially expressed depending on the specific host micro-environment(s). All of these data and earlier work suggest strongly that organisms of freshly obtained isolates of *T. vaginalis* comprise a heterogeneous population, in which no two parasites may be identical when examined in the context of the several phenotypes, as described above. This heterogeneity in the parasite population may itself ensure survival in a constantly changing host vaginal environment.

Experimental procedures

Media and growth

T. vaginalis isolates NYH 286 and IR 78 (Alderete, 1983a) were used in this study. Parasites were grown as described previously (Peterson and Alderete, 1982), in trypticase-yeast extract-maltose (TYM) medium supplemented with 10% heat-inactivated (HI) horse serum. Low-iron medium was prepared by addition of 2,2-dipyridal (Sigma Chemical Co.) at 100 μM final concentration, except where otherwise indicated. High-iron medium was made by addition of ferrous ammonium sulphate hexahydrate (Sigma) at 250 μM final concentration, and a 100-fold stock solution of ferrous ammonium sulphate was made in 50 mM sulphosalicylic acid (Gorrell, 1985). Modified TYM-serum medium was also used in order to demonstrate that iron, and not other factors and conditions, was responsible for the observed results. Trichomonads were grown in TYM medium either with only 1% HI-serum or in the absence of maltose. Organisms were also grown in a semi-defined medium described before, except without nucleotides (Peterson and Alderete, 1984b). For some experiments, TYM-serum medium was treated with Chelex 100 (BioRad Laboratories) to remove iron. This medium was supplemented with trace metals without iron (Linstead, 1981) in order to replace other metals possibly removed by Chelex 100.

Maximal lactoferrin receptor numbers were determined with trichomonads totally depleted of intracellular iron pools. In this case, TYM-serum medium was supplemented with 100 μM 2,2-dipyridal and seeded at an initial density of 1×10^5 organisms per ml. After 36 h of growth, parasites were subcultured into new medium containing 100 μM 2,2-dipyridal and incubated for another 24 h.

Cell densities were determined by enumeration of organisms with a haemocytometer (Reichert). Generation time (G) was estimated by the formula $G = (t\log 2)/(\log b - \log a)$, where t is the time interval between two measurements. The variables a and b represent the parasite densities at the two time-points, and generation time was always the average of three experiments, each done in triplicate.

Rate of protein synthesis

Trichomonads grown in low- or high-iron medium were harvested during the late-logarithmic growth phase, and after washing, cells were suspended in phosphate buffered saline (PBS)–30 mM maltose to a density of 1×10^6 organisms per ml. Then, 250 μM iron or 100 μM 2,2-dipyridal was added to the parasite suspension. Parasites were radiolabelled by the addition of 100 μCi per ml of [^{35}S]-methionine (specific activity 1500 Cimmol^{-1}; Amersham Corp.) and incubated for up to 4 h at 37°C. Neither increased cell numbers nor cell death was observed under these conditions. At 1-h intervals, 50 μl (5×10^4 parasites) of the parasite suspension was removed and washed four times with ice-cold PBS before precipitation of trichomonad proteins by trichloroacetic acid (TCA) (Alderete, 1983b). The extent of radioactivity incorporated into proteins was determined by scintillation counting.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE), immunoblotting and lactoferrin ligand blotting

SDS–PAGE of total TCA-precipitated proteins and electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose

(NC) have been described (Towbin *et al.*, 1979; Alderete *et al.*, 1985). Molecular weight markers were from BioRad.

NC blots were blocked with 5% non-fat dry milk (NFDM) in Tris-buffered saline (TBS) (Tris, 20 mM; NaCl, 500 mM; pH 7.4) for 2 h at room temperature (RT). Patients' sera diluted 1:10 in NFDM/TBS/0.05% Tween 20 was then added and incubated overnight at RT. The blots were then washed three times with TBS/0.05% Tween 20 at RT. Goat anti-human immunoglobulin conjugated to horseradish peroxidase (BioRad) was then added at a final dilution of 1:1500 in NFDM/TBS/0.05%. The blots were incubated for 6 h at RT. After washing of blots three times for 10 min with TBS 0.05% Tween 20, blots were developed with 4-chloro-1-naphthol (2 mg ml^{-1}) prepared in TBS containing 20% methanol and 0.015% H_2O_2.

For dot-blot (Schryvers and Morris, 1988) analysis, 5 μl of serial dilutions of trichomonal detergent extracts (200 μg ml^{-1} protein) (Peterson and Alderete, 1984a) were spotted onto NC and air-dried. The NC was blocked for 1 h with NFDM in PBS before placing NC in TBS containing 120 mM NaCl, 0.01% NP-40, and 500 ng ml^{-1} iron-saturated human lactoferrin. After incubation at 37°C for 2 h, the blot was washed three times with PBS and incubated with rabbit anti-human lactoferrin antibody (Peterson and Alderete, 1984a) for 2 h at 37°C. Blots were finally washed and incubated for 1 h at RT with mouse anti-rabbit immunoglobulin horseradish peroxidase-conjugated antibody (BioRad) at a 1:2000 dilution. The blots were washed and developed as above.

Surface labelling

Radioiodination of membrane proteins was accomplished by the Chloramine-T labelling technique (Alderete, 1983b), using 0.5 mCi of $Na^{125}I$ for radioiodination of 2×10^7 parasites.

Preparation of iron-binding proteins

Lactoferrin and transferrin (1 mg ml^{-1}) were dissolved in 100 mM sodium citrate/100 mM sodium carbonate buffer, pH 8.6, and saturated with iron by the addition of a 50-fold molar excess of $FeCl_3$. Free iron was removed by chromatography using a 1.0 × 15 cm column of Sephadex G-25 (Pharmacia). Apotransferrin and apolactoferrin were prepared by dissolving the proteins in a solution of 100 mM sodium acetate/100 mM sodium phosphate/25 mM ethylenediaminetetracetic acid (EDTA), pH 5.2, and incubated at 4°C overnight before chromatography through Sephadex G-25 (Peterson and Alderet, 1984a). The degree of saturation of the preparation was measured spectrophotometrically at an absorbance of 465 nm (Mazurier and Spik, 1980). Lactoferrin was radioiodinated by lactoperoxidase as described previously (Peterson and Alderete, 1984a) and chromatographed on Sephadex G-25 to remove free radioiodine. Efficiency of labelling was determined by TCA precipitation of proteins (Garvey *et al.*, 1977).

Lactoferrin cell-binding assay

Binding of lactoferrin to live *T. vaginalis* was described previously (Peterson and Alderete, 1984a). Briefly, washed trichomonads were suspended to a density of 1×10^7 parasites in a 0.5 ml final volume of PBS. Iodinated lactoferrin (200 μg ml^{-1} final concentration) prepared in PBS was then added, and parasites were incubated at 4°C for 45 min. Unbound protein was then removed by centrifugation at 500 × *g*, and organisms were washed twice with ice-cold PBS. Finally, pelleted trichomonads were resuspended and transferred to another tube before determination of lactoferrin avidly-bound to parasites by scintillation counting.

Affinity purification of trichomonad lactoferrin-binding proteins

Affinity purification of the lactoferrin-receptor was accomplished as previously described (Peterson and Alderete, 1984a). A trichomonal detergent extract was chromatographed on a 1 × 10 cm lactoferrin-Sepharose affinity column, followed by washing in PBS. The bound material was eluted with 3 M KSCN, pH 7.2 and dialysed against PBS before SDS–PAGE.

Antibody reagents

Patient's sera were obtained from women with confirmed trichomoniasis. Control normal sera were from women with no previous history of this or any other sexually transmitted disease. All sera were non-reactive with medium components, and the control sera did not detect any trichomonad proteins by immunoblot. High-titred rabbit antisera to *T. vaginalis* NYH 286 and to human lactoferrin have been described before (Alderete, 1983a). Purified antibody to the trichomonad lactoferrin receptor was obtained by elution of antibody bound to NC blots containing affinity-purified lactoferrin receptor. In this case, NC blots with bound antibody were treated with a 50 mM glycine/150 mM NaCl buffer, pH 2.3. Eluted antibody was dialysed and concentrated by ammonium sulphate precipitation (Freifelder, 1982).

Acrylamide gel purification of proteins

Total TCA-precipitated trichomonad proteins or lactoferrin affinity-purified proteins were separated by SDS–PAGE using 5% separating acrylamide gels. After staining and destaining (Alderete, 1983a), bands of interest were cut from the gel and re-electrophoresed, using 7.5% acrylamide in the separating gel.

Growth stimulation assay

The ability of trichomonads to utilize different complexed iron sources was determined by a growth stimulation assay recently described (Lehker *et al.*, 1990). Briefly, parasites (2×10^5 organisms per ml) were suspended in TYM-serum medium supplemented with 200 μM 2,2-dipyridal. This medium does not support growth and multiplication of *T. vaginalis*. Various iron sources were then added to the medium, and cell densities monitored throughout a 24–36 h incubation period.

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