ELSEVIER

Available online at www.sciencedirect.com
SCIENCE DIRECT®

Microbial Pathogenesis 36 (2004) 263–271

MICROBIAL PATHOGENESIS

www.elsevier.com/locate/micpath

Heme-iron increases levels of AP65-mediated adherence by *Trichomonas vaginalis*

J.F. Alderete[a,*], J. Nguyen[a], V. Mundodi[a], M.W. Lehker[b]

[a]*Department of Microbiology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., MC 7758, San Antonio TX 78284-7758, USA*
[b]*Department of Biology, University of Texas at Brownsville and Southmost College, Brownsville, TX 78250, USA*

Received 5 October 2003; accepted 22 December 2003

Abstract

Trichomonas vaginalis is a protozoan responsible for the number one, non-viral sexually transmitted disease. Surface proteins (AP65, AP51, AP33 and AP23) mediate adherence to vaginal epithelial cells (VECs). Iron increases growth of trichomonads and synthesis and surface placement of adhesins. We observed by immunofluorescence using monoclonal antibody (mAb) 12G4 the placement of AP65 on surfaces of trichomonads supplemented with hemoglobin or hemin as a source of iron. We, therefore, tested the hypothesis that heme-bound iron is an alternative source of iron important to trichomonal growth and regulation of expression of the adhesin genes. Here we show that the inhibition of parasite growth by the iron chelator 2,2-dipyridal is rescued by hemoglobin or hemin, but not protoporphyrin IX. Importantly, trichomonads grown in iron-limiting medium supplemented with free iron, hemoglobin and hemin had elevated levels of *ap65* transcript that were 12.6-, 12.3- and 9.2-fold higher, respectively, than low-iron organisms, as determined by RT-PCR. Similarly, the amounts of AP65 were 8.9-, 11.2-, and 4.8-fold higher in parasites grown in free iron, hemoglobin and hemin, respectively, than organisms in low-iron medium. The heme-iron-regulated AP65 increased adherence of parasites to immortalized VECs. Not surprisingly, parasites pretreated with anti-AP65 serum IgG had decreased adherence compared to organisms incubated with prebleed serum IgG. These data illustrate that heme-bound iron is a source of iron similar to lactoferrin. This work extends our findings about the multiple sources of iron for regulating virulence genes of *T. vaginalis*.
© 2004 Elsevier Ltd. All rights reserved.

Keywords: Hemoglobin; Hemin; Host–parasite; Iron; Regulation; *Trichomonas vaginalis*; Vaginal epithelial cells; Virulence

1. Introduction

The flagellated protozoan, *Trichomonas vaginalis*, causes the most prevalent non-viral sexually transmitted disease (STD) worldwide [1]. This non-self limiting disease, trichomonosis, causes symptoms in women ranging from mild to severe inflammation with a foul-smelling discharge and severe irritation [2–4]. Trichomonosis has been associated with increased risk for adverse pregnancy outcome [2–8], cervical cancer [9], and HIV seroconversion [10,11]. It is important to study aspects of the host–parasite interrelationship that lead to understanding host infection and pathogenesis. Parasitism of the vaginal tract by *T. vaginalis* is a multi-step process involving distinct mechanisms of interaction with macromolecules, cells and tissues. Trichomonads interact in a specific manner with mucin [12] followed by contact with the VECs where four iron-regulated surface proteins of *T. vaginalis* mediate cytoadherence [13–15]. Penetration of the epithelium likely leads to specific associations with the extracellular matrix-basement membrane glycoproteins fibronectin [16] and laminin [17].

In the constantly changing environment of the vagina, a number of host and parasite factors may contribute to growth, multiplication, and cytoadherence. *T. vaginalis* has an absolute high iron requirement for growth and multiplication [15,18]. Previous reports indicate increased virulence of trichomonads following iron injection in mice [19] as well as a relationship of iron levels to the activity of hydrogenosomal enzymes that are critical to the energy needs of this organism [18,20]. Supplementation of medium with numerous alternative sources to free iron, including iron-binding proteins and iron-containing proteins, reversed the growth-inhibitory effect induced by the iron-chelator

* Corresponding author. Tel.: +1-210-567-3940; fax: +1-210-567-6612.
E-mail address: alderete@uthscsa.edu (J.F. Alderete).

0882-4010/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.micpath.2003.12.007

2,2-dipyridal [15]. Lactoferrin, ferritin, hemoglobin, and cytochrome c, but not transferrin, apolactoferrin and trace metals without iron, promoted growth in a qualitative growth assay in batch culture. The uptake of iron results immediately from the binding by trichomonads of lactoferrin [15,21]. Similar to that seen for free iron, iron from lactoferrin regulates the property of adherence [22]. Further, an additional nutrient acquisition system for *T. vaginalis* is the specific erythrocyte associations with parasites followed by hemolysis [23]. In addition to providing lipids, the erythrocytes' hemoglobin bound parasites in a highly specific, receptor-mediated fashion. In light of the importance of iron to the biology of this organism, it would not be surprising if the distinct iron sources also were involved in modulating virulence properties, such as adherence [13,14,20]. We hypothesized that heme-iron was an alternative source of iron for trichomonal growth and for modulating adherence to VECs. This report shows that indeed, heme-iron can rescue organisms in an iron-limiting medium and increase transcription and surface placement of the AP65 adhesin, which results in higher levels of adherence. We discuss the significance of our results.

2. Results

2.1. AP65 is surface associated in T. vaginalis grown in iron-depleted medium supplemented with various bound iron sources

In vivo trichomonads encounter various iron-containing proteins, and these proteins stimulate growth and multiplication in qualitative assays [15,18,23]. Since free iron regulates the expression and surface placement of adhesins [20,22], we wanted to determine whether bound iron sources also regulate similar expression of the prominent adhesin AP65. As done in an earlier study [20], we visualized the surface expression of AP65 in trichomonads by immunofluorescence with monoclonal antibody (mAb) 12G4. As expected, trichomonads grown in iron-replete (Fig. 1(a1)), but not in iron-restricted (Fig. 1(4a)), medium upregulated surface expression of AP65, as seen by strong fluorescence of non-permeabilized parasites. Surprisingly, similar strong fluorescence was evident among parasites grown in low-iron medium supplemented with hemoglobin (Fig. 1(2a)) and hemin (Fig. 1(3a)) as a source of iron. An mAb called L64 to a small molecular weight trichomonad cytoplasmic protein [24] gave no fluorescence like that seen in Fig. 1(4a). The brightfield pictures show the integrity of trichomonads used for fluorescence.

2.2. Growth kinetics of T. vaginalis organisms with hemoglobin and hemin as a source of iron

In order to test whether heme-iron regulates transcription and surface placement of AP65, we first measured the kinetics of growth of trichomonads with varying concentrations of hemoglobin added to iron-limiting medium. Fig. 2 illustrates a representative growth experiment and compares the parasite density in batch culture in iron-replete (closed circles) versus iron-limiting (open circles) medium. Growth for 24 h gave a density approaching $3 \times 10^6\ ml^{-1}$ for high-iron organisms. Low-iron parasites underwent one doubling in the same time. Low-iron parasites supplemented with 5.0–25.0 μM hemoglobin after 30 h incubation gave cell densities equal to trichomonads in high-iron medium. A concentration of 2.5 μM hemoglobin eventually gave an equivalent high density,

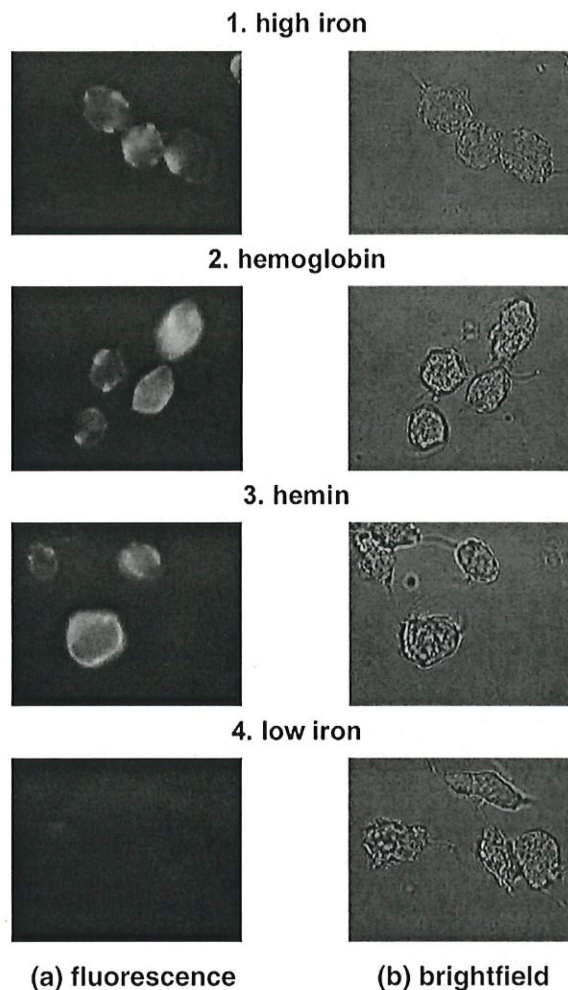

Fig. 1. Immunofluorescence (part a) and corresponding darkfield microscopy (part b) showing surface expression of AP65 on *T. vaginalis* organisms grown on various iron sources. Trichomonads grown in low-iron medium (Section 4) (panel 4) were added to medium supplemented with free iron (high iron, panel 1), 25 μM hemoglobin (panel 2) and 25 μM hemin (panel 3). AP65 was detected with mAb 12G4 that recognizes an epitope accessible on the parasite surface [20]. The mAb L64 to a cytoplasmic trichomonad protein [24] or secondary fluoresceine-conjugated goat anti-mouse IgG gave no fluorescence, as seen for low-iron trichomonads in panel 4.

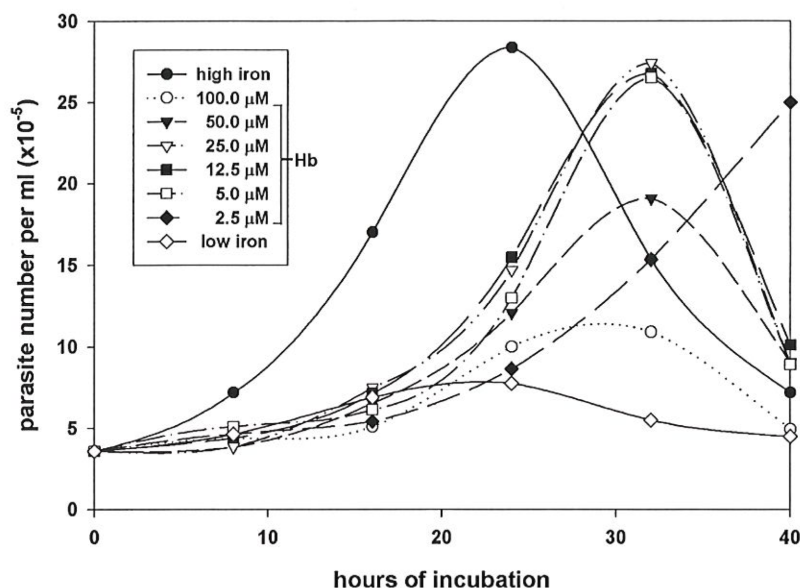

Fig. 2. Growth kinetics of a representative experiment for trichomonads grown in high-iron (solid circles) and low-iron (open diamonds) medium and compared with medium supplemented with varying amounts of hemoglobin (Hb), as indicated by the symbols in the inset. Growth experiments were performed on four different occasions with similar results. Numbers of parasites were determined by a Neubauer counting chamber.

albeit at a slower rate of growth. A decrease in growth was apparent at concentrations of hemoglobin >25 μM presumably due to toxicity.

In the representative growth experiment shown in Fig. 3, addition to iron-limiting medium of 25.0 μM purified hemin (closed squares) as a source of iron gave after 30 h correspondingly equivalent densities $\sim 2.5\times 10^6\ \mathrm{ml}^{-1}$ as that achieved by trichomonads in high-iron medium (closed circles) by 24 h. Amounts $\geqq 50.0$ μM hemin resulted in decreased densities. Prolonged growth greater than 40 h with ≤ 12.5 μM hemin never achieved parasite densities equal to 25.0 μM hemin. Protoporphyrin IX did not support growth and gave results equal to those of low-iron-grown trichomonads (open diamonds), showing the role of heme-bound iron in modulating growth and multiplication of parasites.

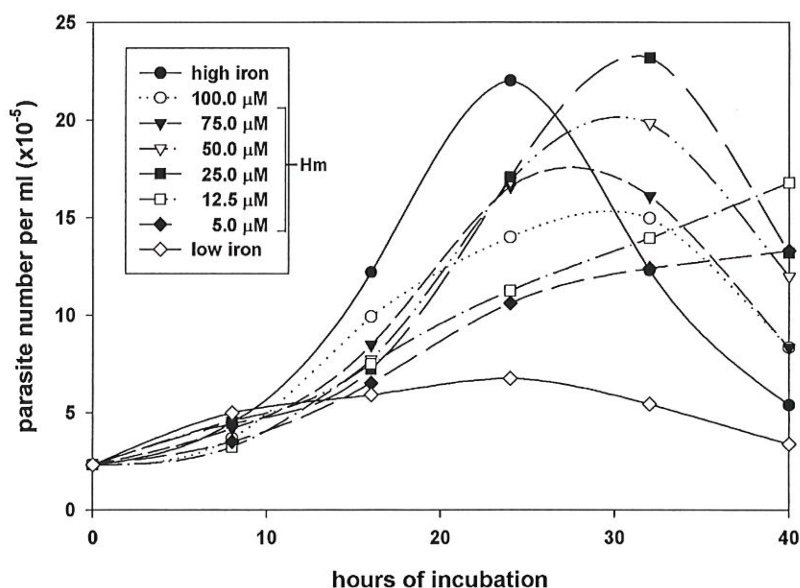

Fig. 3. Growth kinetics of a representative experiment as in Fig. 2 comparing high- and low-iron-grown trichomonads with medium supplemented with varying amounts of hemin (Hm), as indicated by the symbols in the inset. Growth experiments were performed on four different occasions with similar results.

2.3. Levels of *ap65* mRNA and amounts of AP65 increase with hemoglobin and hemin

We were now in a position to determine by RT-PCR the effect of various iron sources on transcription of *ap65* encoding the prominent adhesin of *T. vaginalis* [20]. Fig. 4a presents the results of a representative experiment. Lane 1 shows the 631-pb PCR product (arrow) of the positive control (Section 4) to confirm the specificity of primers and size of product. Greater amounts of the mRNA for *ap65* were obtained for low-iron-grown trichomonads supplemented with high-iron (lane 3), hemoglobin (lane 4), and hemin (lane 5) compared to organisms grown in low-iron (lane 6) medium. Quantitation of the PCR product bands was done using the Scion Image for Windows program version Beta 4.0.2, and the fold increases in amounts of transcript were 12.6, 12.3, and 9.2 for high-iron-, hemoglobin- and hemin-grown parasites, respectively, when compared to low-iron-grown parasites where the amount of product was normalized to 1.0 for comparison. Protoporphyrin IX gave low amounts of transcript consistent with low-iron parasites (lane 7). The faint PCR product (~ 1 kbp) seen in all the reactions except in the positive control (lane 1) is a non-specific amplification that is likely due to the low annealing temperature of the PCR reaction. Finally, as an internal control to validate the amounts of *ap65* in the different iron sources (Fig. 4a), we performed identical experiments on the *p270* gene (Section 4). As shown in Fig. 4b, similar, if not identical, amounts were detected for the expected PCR product regardless of the iron source or levels.

We next determined if the elevated levels of *ap65* transcript corresponded to increased amounts of AP65. We employed the ligand assay using immortalized MS-74 VECs to measure relative amounts of host cell-binding AP65 [20]. Eluted bound protein was immunoblotted after SDS-PAGE with anti-AP65 mAb 12G4. In the representative data presented in Fig. 5, elevated amounts of AP65 were detected

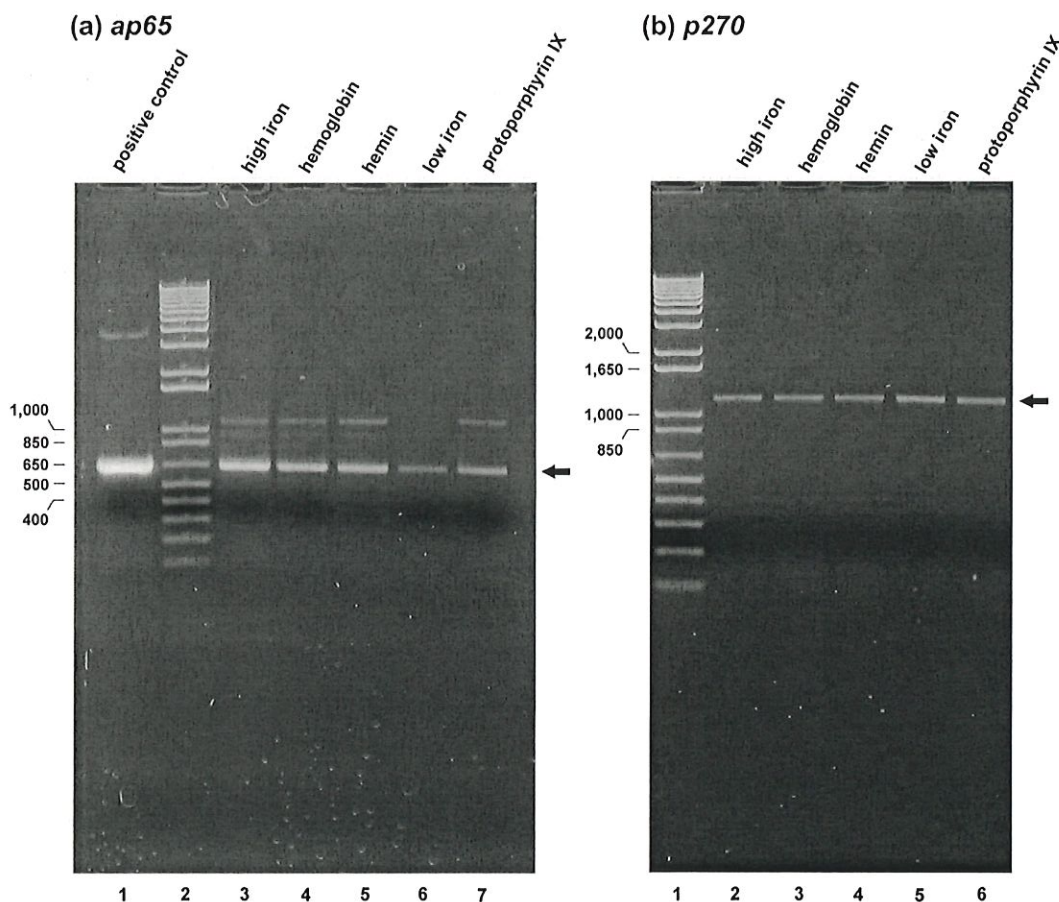

Fig. 4. RT-PCR demonstrating transcription of the *ap65* gene (part a) compared to the *p270* gene (part b). The RT-PCR products for both the *ap65* and *p270* genes (arrows) represent the correct estimated size based on the position of the primers (Section 4). Positive control for the *ap65* gene (lane 1) refers to the reaction using plasmid with the *ap65-3* gene as described in Section 4. Trichomonads grown in iron-limiting medium (low iron) were transferred to iron-replete medium (high iron) or medium supplemented with 25 μM hemoglobin, 25 μM hemin, and 25 μM protoporphyrin IX. After the RT-PCR reaction, the products were electrophoresed in 1% agarose gel followed by staining with ethidium bromide. The size of the reaction product was estimated from the DNA size markers in lane 2 labeled MW, and numbers on the left indicate the sizes for some of the DNA bands of the size markers.

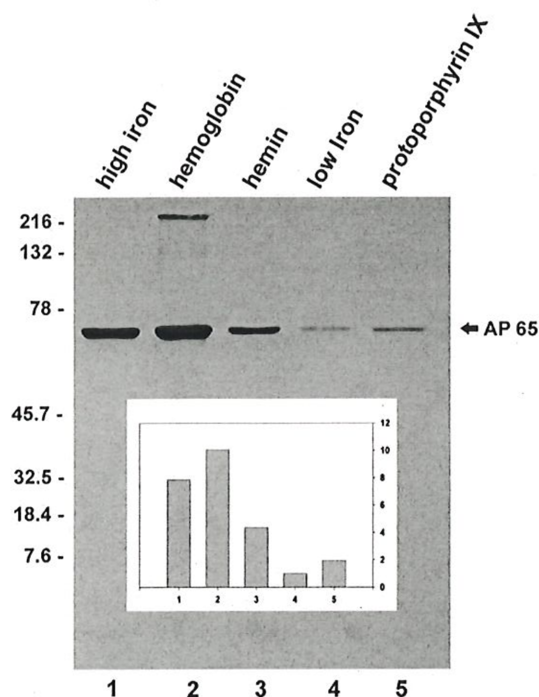

Fig. 5. The amounts of AP65 (arrow) synthesized by *T. vaginalis* organisms grown in medium supplemented with different sources of iron. Trichomonads were grown in iron-limiting medium (lane 4) and used to inoculate medium supplemented with high iron (lane 1), 25 μM hemoglobin, 25 μM hemin (lane 3), and 25 μM protoporphyrin IX for use in a ligand assay. In this experiment, extracts of organisms were added to immortalized MS-74 VECs as described in Section 4, and VEC-binding AP65 was eluted for electrophoresis and immunoblotting onto nitrocellulose (NC). NC blots were then probed with mAb 12G4. Intensity of the AP65 band corresponds to levels of transcription as examined by RT-PCR in Fig. 4, and the baseline amount of AP65 obtained with trichomonads grown in low-iron medium is as previously reported [20, 22]. The inset shows the quantitation of amounts of AP65 as fold increase (ordinate) in the corresponding numbered immunoblots. The intensity of band for low-iron-grown organisms is arbitrarily given the number one for comparison. Numbers at left are size markers (×1000).

in the organisms grown in iron-limiting medium supplemented with free iron (lane 1), hemoglobin (lane 2), and hemin (lane 3). Only low amounts of AP65 were evident for the low-iron- (lane 4) and protoporphyrin IX-treated (lane 5) trichomonads. The inset shows the fold-increase (ordinate axis) in amounts of mAb-reactive AP65 band (arrow) when compared to AP65 of low-iron-grown organisms normalized to a value of 1.0. High-iron- (bar 1), hemoglobin- (bar 2) and hemin-grown (bar 3) trichomonads had 8.9-, 11.2-, and 4.8-fold greater amounts of AP65, respectively, than did low-iron- (bar 4) and protoporphyrin IX-grown (bar 5) organisms. Quantitation was with the SCION IMAGE program as above. Finally, two-dimensional analysis of the AP65 revealed six spots (data not shown), suggesting synthesis of all members of the six-gene family, as before [20]. These results reinforce the notion that heme-iron is a regulator of both transcription and translation of *ap65*.

2.4. Adherence is increased with hemoglobin and hemin

Next, we felt it important to determine if the increased transcription, translation, and surface placement of AP65 led to corresponding higher levels of adherence, as previously shown for free iron [20]. Fig. 6 presents results from four separate experiments with quadruplicate samples measuring the level of adherence to immortalized MS-74 VECs among *T. vaginalis* organisms grown in iron-restricted (bar graph labeled low) medium versus supplementation with free iron (high), hemoglobin (Hb) and hemin (Hm). As can be seen, trichomonads with 25 μM hemoglobin and 25 μM hemin as a source of iron gave levels of adherence equivalent to parasites given free-iron. The level of adherence achieved in the presence of hemoglobin and hemin were similar to those using lactoferrin as the source of iron (not shown) [22]. Finally, Fig. 7 illustrates two representative experiments on the inhibition of adherence when trichomonads grown in 25 μM hemoglobin or 25 μM hemin were first pretreated with anti-AP65 IgG. These results reaffirm the role of AP65 regulated by heme-iron on adherence to immortalized VECs.

3. Discussion

The menstrual cycle plays a key role in modulating the vaginal environment. The hormones estrogen and progesterone regulate in part the fluctuations in iron sources and other nutrients [25,26]. Amounts of lactoferrin, a source of iron for *T. vaginalis* organisms, are elevated after the postmenstrual phase and steadily decline until menstruation [26]. Trichomonads grown in low-iron medium supplemented with lactoferrin have elevated levels of adherence mediated by increased amounts of the adhesins [22]. The evidence points to the existence of unique receptors on *T. vaginalis* for binding of distinct iron-binding and iron-containing proteins. While these distinct sources of iron support growth and multiplication of trichomonads (Figs. 2 and 3), what remains unknown is whether the iron-containing proteins, in particular heme-bound iron, play a role in modulating virulence properties, such as adherence. This report shows, for the first time, the regulation of transcription, translation and surface expression of adhesins through heme-iron.

Heme iron utilization is a common theme among pathogenic and non-pathogenic bacteria and fungi [27–33]. Indeed, receptors in microorganisms for specific hemin and heme binding have been described [28,34], and uptake of intact heme has been established in bacteria [35]. In the case of *T. vaginalis*, host hemoglobin and heme-bound iron may represent an abundant pool of iron during menses and/or during cytolysis of epithelial cells [15]. We previously suggested that iron from the heme moiety enters the iron utilization pathway, as does iron derived from

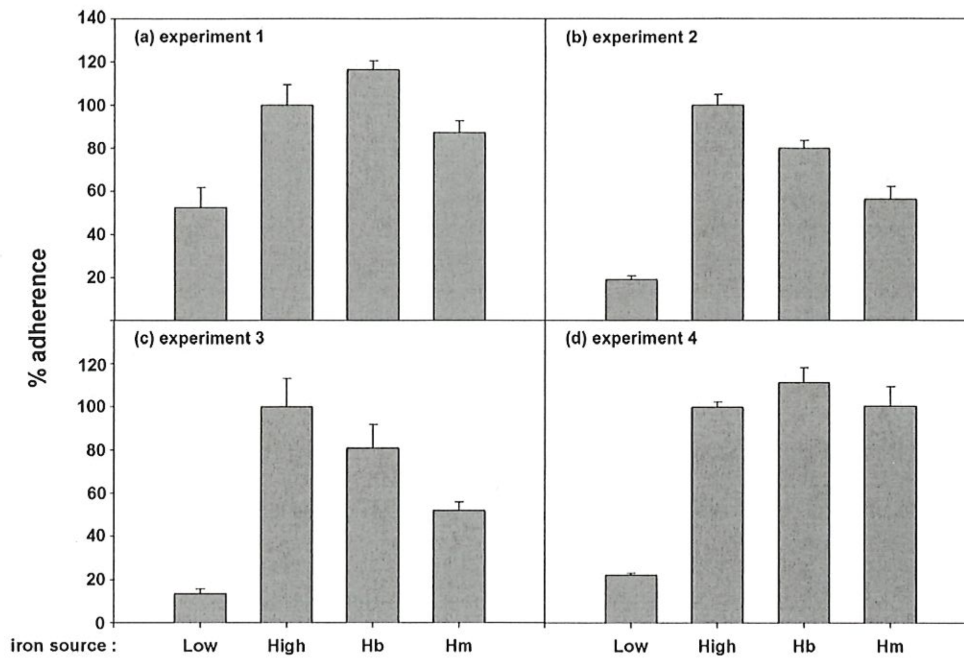

Fig. 6. The elevated levels of adherence to MS-74 immortalized VECs by *T. vaginalis* organisms grown overnight in iron-replete medium (high) or medium supplemented with 25 μM hemoglobin (Hb) or 25μM hemin (Hm) compared to iron-limiting medium (low). The extent of adherence by parasites grown in high-iron medium was normalized to 100% in each experiment for comparative purposes, as before [14,20,22]. Each experiment was performed with quadruplicate samples.

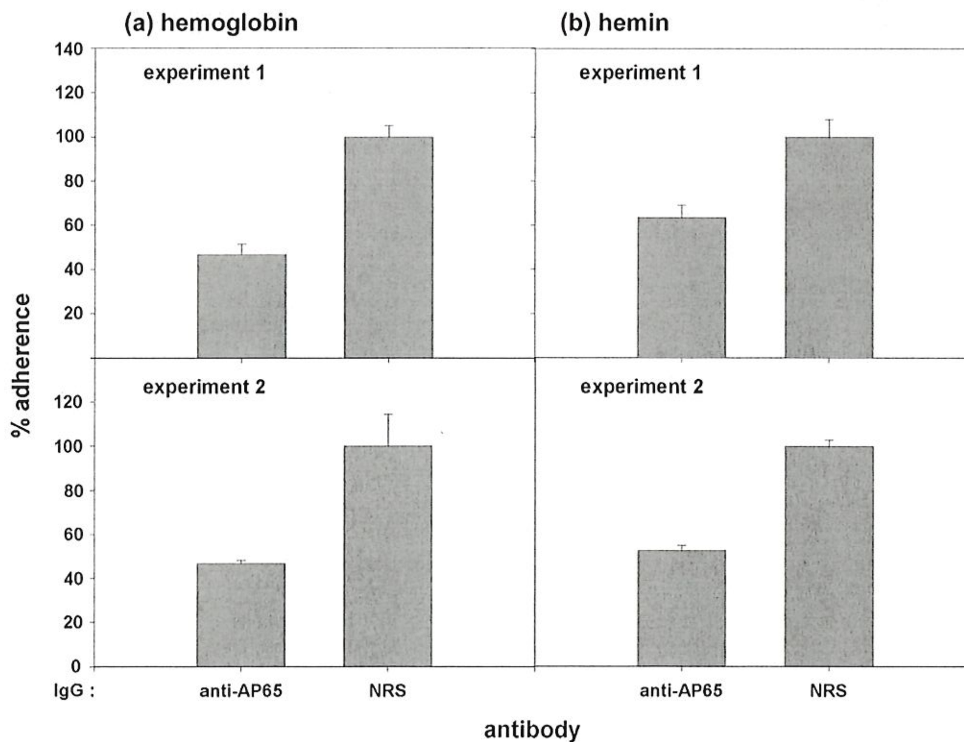

Fig. 7. Rabbit anti-AP65 serum IgG treatment of *T. vaginalis* organisms inhibits adherence to MS-74 immortalized VECs compared to control prebleed normal rabbit serum IgG (NRS). The trichomonads were grown overnight in medium supplemented with hemoglobin (a) or hemin (b). The level of adherence obtained for control treated trichomonads was normalized to 100% to compare with anti-AP65 antibody-treated parasites. Each experiment was performed with quadruplicate samples.

lactoferrin [15,21]. Since lactoferrin-iron regulates gene transcription of virulence genes, we hypothesized that heme-iron may have the same effect. Unquestionably, we now show that heme-bound iron, similar to lactoferrin, up-regulates transcription of the *ap65* gene, as evidenced by data from RT-PCR (Fig. 4) and the ligand assay (Fig. 5). As AP65 and the adhesins are also enzymes [20], this report underscores the significance of multiple iron sources in up-regulating expression of enzymes for the hydrogenosome organelle. It is important to point out that to our knowledge, it has not yet been demonstrated that trichomonads degrade the heme ring. Thus, the possibility that heme itself could be a modulator of transcription cannot be excluded at this time. Heme is a known regulator of gene transcription in eukaryotic cells, where binding of heme activates a signaling cascade leading to gene transcription or by directly interacting with transcription factors [36–38]. Similarly, in *Bordetella* sp. heme was found to regulate gene transcription of the heme-uptake system by directly interacting with a transcriptional activator distinct from the FUR transcriptional regulator [39,40]. In *Candida albicans* pathways regulated by heme are involved in morphogenic change [33], and hemoglobin induces fibronectin associations [41]. It is conceivable, therefore, that the heme moiety itself may regulate trichomonal transcription and possibly other signaling pathways. As the iron-regulatory sequences have been characterized for one of the *ap65* genes [42], the question of whether the heme moiety utilizes common or distinct sequences would be of interest. Nonetheless, these data now direct our attention toward clarifying the true mechanisms by which heme-bound iron regulates expression of trichomonad genes and proteins.

It is noteworthy that trichomonads grown in iron-limited medium supplemented with hemoglobin or hemin displayed a growth delay when compared to organisms supplemented with free iron. The significance of this observation is not entirely clear yet, but may indicate that either heme iron does not enter into the biologically active iron pool immediately or that acquisition of heme-iron requires the induction of a heme acquisition system. The latter possibility may confer an advantage to *T. vaginalis* in the different stages of infection. During the colonization of intact mucosal surfaces, lactoferrin iron, but not heme iron, may be the major source of iron. Once established, the elaboration of parasite cytolytic factors that damage the vaginal epithelium and/or availability of erythrocytes readily hemolysed by trichomonads [23] may now provide sufficient heme for growth and virulence gene regulation. This differential expression of various iron acquisition systems depending on available iron sources will allow for rapid adaptation to the changing host environment.

We have proposed that this urogenital parasite is highly evolved, being capable of surviving in a dynamic and constantly-changing host environments. What is clear is the ability of *T. vaginalis* to colonize effectively the urogenital region through the existence on trichomonads of distinct functional proteins, such as adhesins for host epithelial cytoadherence [14,20,22] and receptors for extracellular matrix-basement proteins [16,17]. Further, that these properties are under the control of host iron suggests a need for multiple receptor-mediated acquisition systems for binding of the iron-containing and iron-binding proteins [15]. Data strongly indicate the presence of receptors for lactoferrin [15,21] and hemoglobin [23]. The existence on the parasite surface of such receptors involved in iron-acquisition would be highly advantageous to survival at sites inside the host that have different nutritional compositions. As amounts of lactoferrin and the availability of hemoglobin through erythrocyte hemolysis fluctuate during the menstrual cycle [23,26], any regulation of transcription, translation and surface placement of functional molecules, like adhesins, would be a survival force for the parasite. This may explain past observations on analysis of parasites from fresh clinical isolates for properties modulated by iron, such as cytoadherence [13] and phenotypic variation [43], and findings that a large percentage of trichomonads from vaginal secretions were high-iron-organisms.

4. Materials and methods

4.1. Culture and growth of T. vaginalis

The fresh clinical *T. vaginalis* isolate T016 [14,16,20] was grown no longer than 4 weeks by daily passage in Trypticase–yeast extract–maltose medium supplemented with 5% heat-inactivated horse serum (TYM-serum) [44]. Low-iron-grown trichomonads were obtained as previously detailed [15,16,22]. Briefly, washed parasites at a starting density of 10^5 organisms per ml were cultured overnight in TYM-serum containing 50 μM of the iron chelator 2,2-dipyridil (2,2-DP; Sigma Chemical Co., St Louis, MO) for 24 h at 37 °C [15,22]. These organisms were then suspended in TYM-serum supplemented with 50 μM 2,2-DP for an additional 24 h incubation before growth kinetic experiments. To obtain high-iron organisms, low-iron parasites were washed once and suspended in TYM-serum medium containing 200 μM ferrous ammonium sulfate (Sigma) and incubated as for the low-iron-grown organisms for 24 h at 37 °C. Deferrated hemoglobin (Sigma) and hemin (Sigma) stock solutions were prepared in addition to iron-restricted medium at concentrations indicated in the individual experiments. For adherence assays, trichomonads grown overnight in T25 flasks in the different iron sources were radiolabeled with [^{3}H]-thymidine, as before [14,20]. The specific activity of labeled washed parasites was determined prior to the adherence assay in order to compare the extent of adherence under the different growth conditions. All experiments involving organisms grown under different iron conditions and iron sources were performed on at least four separate occasions unless otherwise indicated. In each

growth experiment, the growth trend was similar when comparing the different iron conditions and iron sources.

4.2. *Immunofluorescence of parasites*

Fluorescence of non-permeabilized trichomonads grown in iron-restricted medium alone or supplemented with the various iron sources as indicated in Fig. 1 was performed using standardized conditions recently detailed [20]. Parasites were treated with control, irrelevant mAb or mAb 12 G4 reactive to surface AP65 [20]. After washing, parasites were suspended in fluorescein isothiocyanate-conjugated secondary goat anti-mouse IgG (Sigma). As a negative control, mAb L64 to a low molecular weight cytoplasmic protein [24] was used, as before [20].

4.3. *RT-PCR*

The endogenous levels of *ap65* mRNA were measured by quantitative RT-PCR. The TRIZOL™ method (Invitrogen Life Technologies, Carlsbad, CA) extracted total RNA from 5×10^6 trichomonads. RNase-free DNaseI removed contaminating DNA prior to quantitation of purified total RNA. The cDNA was prepared from 2 μg of total RNA using Superscript II Reverse transcriptase (Invitrogen) as per the manufacturer's protocol. PCR amplification was performed with 1 μl of the cDNA as template by using the gene-specific oligonucleotides. The sense primer was 5′-CAGTCAGTCGACCAGTTAGATATGGGTACAGAC-3′, and the antisense primer was 5′-GTGACAG-GATCCCGCTCGCAGTTAGCGCATGTAG-3′. The primers amplified a 631-bp product of the *ap65* internal coding region. The *ap65-3* gene [45] cloned in pBlue-Script™ (Strategene, La Jolla, CA) plasmid was a template in a standard PCR reaction and served as a positive control to show the specificity of primers and for comparison with RT-PCR reaction products. As a control for these experiments, we performed RT-PCR for the *p270* gene [24,43]. The sense primer was 5′-GTTGATAGAGAAGGTAGG-GATAAC-3′, and the antisense primer was 5′-TATATT-TATAATAAATTAGACTTCAACTCC-3′. All RT-PCR experiments involving organisms grown in the different iron conditions and iron sources were performed on four separate occasions. In each case, the trend for the amount of PCR product was similar when comparing the different iron conditions and iron sources.

4.4. *Host cells, ligand assay, adherence, and antibody inhibition of adherence*

The adherence of *T. vaginalis* and the binding of the adhesins, in particular AP65 [20] was determined with immortalized MS-74 VECs. The cells were processed as described for both the adherence and ligand assays. Adherence assays and antibody inhibition experiments were performed using quadruplicate samples. Affinity purified IgG from prebleed NRS and from rabbit anti-AP65 serum as described [20] was used in the inhibition experiments.

Acknowledgements

This study was supported by Public Health Service grant AI-43940 from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health. The laboratory would like to thank Mr Julio Ramirez for initiating these experiments while participating in a Minority Scientist Development Program supported by NIGMS (R25 GM55286) directed by Dr Damon Herbert of the Department of Cellular and Structural Biology of the UTHSCSA.

References

[1] World Health Organization. An overview of selected curable sexually transmitted diseases. WHO Global Programme on AIDS Report 1995.
[2] Krieger JN, Wolner-Hanssen P, Stevens C, Holmes KK. Characteristics of *Trichomonas* vaginalis isolates from women with and without colpitis macularis. J Infect Dis 1990;161:307–11.
[3] Soper DE, Bump RC, Hurt WG. Bacterial vaginosis and trichomoniasis vaginitis are risk factors for cuff cellulitis after abdominal hysterectomy. Am J Obstet Gynecol 1990;163:1016–21.
[4] Wölner-Hanssen P, Krieger JN, Stevens CE, Kiviat NB, Koutsky L, Critchlow C, DeRouen T, Hillier S, Holmes KK. Clinical manifestations of vaginal trichomoniasis. JAMA 1989;261:571–6.
[5] Cotch MF, Pastork JGD, Nugent RP, Yerg DE, Martin DH, Eschenbach DA. Demographic and behavioral predictors of *Trichomonas* vaginalis infection among pregnant women. The vaginal Infections and Prematurity Study Group. Obstet Gynecol 1991;78: 1087–92.
[6] Hardy PH, Hardy JB, Nell EE, Graham DA, Spence MR, Rosenbaum RC. Prevalence of six sexually transmitted disease agents among pregnant inner-city adolescents and pregnancy outcome. Lancet 1984; 2:333–7.
[7] Minkoff H, Grunebaum AN, Schwarz RH, Feldman J, Cummings M, Crombleholme W, Clark L, Pringle G, McCormack WM. Risk factors for prematurity and premature rupture of membranes: a prospective study of the vaginal flora in pregnancy. Am J Obstet Gynecol 1984; 150:965–72.
[8] Read JS, Klebanoff MA. Sexual intercourse during pregnancy and preterm delivery: effects of vaginal microorganisms. The Vaginal Infections and Prematurity Study Group. Am J Obstet Gynecol 1993; 168:514–9.
[9] Zhang SF, Begg CB. Is *Trichomonas vaginalis* a cause of cervical neoplasia? Results from a combined analysis of 24 studies. Int J Epidemiol 1994;23:682–90.
[10] Laga M, Manoka A, Kiravu M, Malele B, Tuliza M, Nzila N, Goeman J, Behets F, Batter V, Alary M, Heyward WL, Ryder RW, Piot P. Non-ulcerative sexually transmitted diseases as risk factors for HIV-1 transmission in women: results from a cohort study. AIDS 1993;7: 95–102.
[11] Wasserheit JN. Epidemiological synergy. Interrelationships between human immunodeficiency virus infection and other sexually transmitted diseases. Sex Transm Dis 1992;19:61–77.
[12] Lehker MW, Sweeney D. Trichomonad invasion of the mucous layer requires adhesins, mucinases, and motility. Sex Transm Infect 1999; 75:231–8.

[13] Alderete JF, Demeš P, Gombošova A, Valent M, Fabušova M, Janoská A, Štefanovic J, Arroyo R. A specific parasitism of purified vaginal epithelial cells by *Trichomonas vaginalis*. Infect Immun 1988; 56:2558–62.

[14] Arroyo R, Engbring J, Alderete JF. Molecular basis of host epithelial cell recognition by *Trichomonas vaginalis*. Mol Microbiol 1992;6: 853–62.

[15] Lehker MW, Alderete JF. Iron regulates growth of *Trichomonas vaginalis* and the expression of immunogenic trichomonad proteins. Mol Microbiol 1992;6:123–32.

[16] Crouch ML, Alderete JF. *Trichomonas vaginalis* interactions with fibronectin and laminin. Microbiology 1999;145:2835–43.

[17] Costa e Silva Filho F, de Souza W, Lopes JD. Presence of laminin-binding proteins in trichomonads and their role in adhesion. Proc Natl Acad Sci USA 1988;85:8042–6.

[18] Gorrell TE. Effect of culture medium content on the biochemical composition and metabolism of *Trichomonas vaginalis*. J Bacteriol 1985;161:1228–30.

[19] Budilova M, Kulda J. Further studies on the effect of ferric ammonium citrate on the virulence of *Tritrichomonas foetus* to mice. J Protozool 1977;24:49.

[20] Garcia AF, Chang T-H, Benchimol M, Klumpp DJ, Lehker MW, Alderete JF. Iron and contact with host cells induce expression of adhesins on surface of *Trichomonas vaginalis*. Mol Microbiol 2003; 47:1207–24.

[21] Peterson KM, Alderete JF. Iron uptake and increased intracellular enzyme activity follow host lactoferrin binding by *Trichomonas vaginalis* receptors. J Exp Med 1984;160:398–410.

[22] Lehker MW, Arroyo R, Alderete JF. The regulation by iron of the synthesis of adhesins and cytoadherence levels in the protozoan *Trichomonas vaginalis*. J Exp Med 1991;174:311–8.

[23] Lehker MW, Chang T-H, Dailey DC, Alderete JF. Specific erythrocyte binding is an additional nutrient acquisition system for *Trichomonas vaginalis*. J Exp Med 1990;171:2165–70.

[24] Alderete JF, Demes P, Gombosova A, Valent M, Janoska A, Fabusova H, Kasmala L, Garza GE, Metcalfe EC. Phenotypes and protein-epitope phenotypic variation among fresh isolates of *Trichomonas vaginalis*. Infect Immun 1987;55:1037–1041.

[25] Reith E, Ross M. Atlas of descriptive histology, 2nd ed. New York, NY, USA: Harper and Row; 1965.

[26] Cohen MS, Britigan BE, French M, Bean K. Preliminary observations on lactoferrin secretion in human vaginal mucus: variation during menstrual cycle, evidence of hormonal regulation and implications for infection with *Neisseria gonorrhoeae*. Am J Obstet Gynecol 1987; 157:1122–5.

[27] Noya F, Arias A, Fabiano E. Heme compounds as iron sources for nonpathogenic *Rhizobium* bacteria. J Bacteriol 1997;179:3076–8.

[28] Carroll JA, Coleman SA, Smitherman LS, Minnick MF. Hemin-binding surface protein from *Bartonella Quintana*. Infect Immun 2000;68:6750–7.

[29] Murphy ER, Sacco RE, Dickenson A, Metzger DJ, Hu Y, Orndorff PE, Connell TD. BhuR, a virulence-associated outer membrane protein of *Bordetella avium*, is required for the acquisition of iron from heme and hemoproteins. Infect Immun 2002;70:5390–403.

[30] Coulton JW, Pang JCS. Transport of haemin by *Haemophilus influenzae* type b. Curr Microbiol 1983;9:93–8.

[31] Lewis LA, Sung MH, Gipson M, Hartman K, Dyer DW. Transport of intact porphyrin by HpuAB, the hemoglobin–haptoglobin utilization system of *Neisseria meningitidis*. J Bacteriol 1998;180:6043–7.

[32] Stojilijkovic I, Hantke K. Transport of haemin across the cytoplasmic membrane through a haemin-specific periplasmic binding-protein-dependent transport system in *Yersenia enterocolitica*. Mol Microbiol 1994;13:719–32.

[33] Casanova M, Cervera AM, Gozalbo D, Martinez JP. Hemin induces germ tube formation in *Candida albicans*. Infect Immun 1997;65: 4360–4.

[34] Lei B, Liu M, Voyich JM, Prater CI, Kala SV, Deleo FR, Musser JM. Identification and characterization of HtsA, a second heme-binding protein made by *Streptococcus pyogenes*. Infect Immun 2003;71: 5962–9.

[35] Mazmanian SK, Skaar EP, Gaspar AH, Humayun M, Gornicki P, Jelenska J, Joachmiak A, Missiakas M, Schneewind O. Passage of heme-iron across the envelope of *Staphylococcus aureus*. Science 2003;299:906–9.

[36] Chang L, Hach A. Molecular mechanism of heme signaling in yeast: the transcriptional activator Hap1 serves as the key mediator. Cell Mol Life Sci 1999;56:415–26.

[37] Killeen AJE, Gong P, Naquin R, Hu B, Steward D, Ingelfinger JR, Nath KA. Heme activates the heme oxygenase-1 gene in renal epithelial cells by stabilizing Nrf2. 2002. Am J Physiol Renal Physiol 2003;284:F743–52.

[38] Nirodi SS, Nirodi CS, Ram N, Prabhu L, Padmanaban G. A 65-kda protein mediates the positive role of heme in regulating the CYP2B1/B2 gene in rat liver. J Biol Chem 1997;272:8895–900.

[39] Vanderpool CK, Armstrong SK. Heme-responsive transcriptional activation of Bordetella *bhu* gene. J. Bacteriol. 2003;185:909–17.

[40] Kirby AE, Metzger DJ, Murphy ER, Connell TD. Heme utilization in Bordetella avium is regulated by RhuI a heme-responsive extra-cytoplasmic function sigma factor. Infect Immun 2001;69:6951–61.

[41] Yan S, Rodrigues RG, Roberts DD. Hemoglobin-induced binding of *Candida albicans* to the cell-binding domain of fibronectin is independent of the Arg-Gly-Asp sequence. Infect Immun 1998;66: 1904–9.

[42] Tsai CD, Liu HW, Tai JH. Characterization of an iron-responsive promoter in the protozoan pathogen *Trichomonas vaginalis*. J Biol Chem 2002;277:5153–62.

[43] Alderete JF, Kasmala L, Metcalfe EC, Garza GE. Phenotypic variation and diversity among *Trichomonas vaginalis* and correlation of phenotype with contact-dependent host cell cytotoxicity. Infect Immun 1986;53:285–93.

[44] Diamond LS. The establishment of various Trichomonas of animals and man in axenic cultures. J Parasitol 1957;43:488–90.

[45] O'Brien JL, Lauriano CM, Alderete JF. Molecular characterization of a third malic enzyme-like ap65 adhesin gene of *Trichomonas vaginalis*. Microb Pathogen1996;20:335–349.