Iron Modulates Phenotypic Variation and Phosphorylation of P270 in Double-Stranded RNA Virus-Infected *Trichomonas vaginalis*

J. F. ALDERETE*

Department of Microbiology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas

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*Trichomonas vaginalis* infected with a double-stranded RNA virus undergoes phenotypic variation on the basis of surface versus cytoplasmic expression of the immunogenic protein P270. Examination of batch cultures by flow cytometry with monoclonal antibody (MAb) to P270 yields both fluorescent and nonfluorescent trichomonads. Greater numbers and intensity of fluorescent organisms with surface P270 reactive with MAb were evident in parasites grown in medium depleted of iron. Placement of iron-limited organisms in medium supplemented with iron gave increased numbers of nonfluorescent trichomonads. Purified subpopulations of trichomonads with and without surface P270 obtained by fluorescence-activated cell sorting reverted to nonfluorescent and fluorescent phenotypes when placed in high- and low-iron media, respectively. No similar regulation by iron of P270 was evident among virus-negative *T. vaginalis* isolates or virus-negative progeny trichomonads derived from virus-infected isolates. Equal amounts of P270 were detectable by MAb on immunoblots of total proteins from identical numbers of parasites grown in low- and high-iron media. Finally, P270 was found to be highly phosphorylated in high-iron parasites. Iron, therefore, plays a role in modulating surface localization of P270 in virus-harboring parasites.

Trichomonosis (14, 17) is the most common nonviral sexually transmitted disease (vaginitis) and is caused by infection with the protist *Trichomonas vaginalis* (28). Trichomonosis has major health consequences for women, as it is associated with adverse pregnancy outcomes (10), enhanced susceptibility to human immunodeficiency virus (20, 27) and possibly cervical neoplasia (29).

Experiments aimed at understanding the reported extensive antigenic heterogeneity among *T. vaginalis* isolates (9, 13, 18, 24) led to the discovery of the property of phenotypic variation (6). This was defined on the basis of surface versus cytoplasmatic expression of a repertoire of high-M<sub>m</sub> immunogens (1, 2, 4, 5). The monoclonal antibody (MAb) C20A3 recognized an epitope tandemly repeated within the highly immunogenic surface protein termed P270 (6–8). Analyses by flow cytometry and fluorescence-activated cell sorting (FACS) of fresh clinical isolates revealed heterogeneous immunoreactivity, such as fluorescent and nonfluorescent subpopulations by indirect immunofluorescence with MAb (4, 6). These reactivities with MAB were similar to those reported for isolates with MAb, with polyclonal experimental sera, or with sera from patients with trichomonosis (8, 9, 13, 18, 24). Based on flow cytometry with MAb, it became evident that two types of isolates occur naturally during infections with *T. vaginalis* (4). Type I isolates were homogeneous nonfluorescent (negative phenotype) trichomonads that synthesize and express P270 in the cytoplasm. In contrast, type II isolates were heterogeneous and comprised both fluorescent and nonfluorescent subpopulations (positive and negative phenotypes) that were then purified by FACS (6). Each purified subpopulation reverted to the opposite phenotype but only upon long-term daily passage in batch culture (6). It was further demonstrated that both MAb and polyclonal Ab from patients reactive with P270 were lytic for trichomonads with surface P270 in a complement-independent fashion (5, 11). In vivo, among type II isolates from patients, the percent of trichomonads with surface P270 ranged from 0 to 10% (4), suggesting that the host environment either eliminates parasites with surface P270 or favors cytoplasmic expression. Finally, the identification of the double-stranded RNA (dsRNA) virus within *T. vaginalis* organisms established a relationship between virus infection and phenotypic variation (26). The virus is multisegmented (15), and loss of virus from parental type I isolate organisms by batch culture (16, 26) produced virus-negative progeny such as type I isolate parasites that were incapable of surface placement of P270.

The complete sequence of a p270 gene of a fresh clinical isolate was recently reported (23). Furthermore, it has been shown that, except for the number of tandemly repeated units (23), the gene was highly conserved among type I and type II isolates (3). The repeated domain was flanked by 69 bp (23 amino acids) of upstream and 1,185 bp (395 amino acids) of downstream nonrepeat, coding regions (23). The sequences of the repeats within the p270 gene were identical (23). Furthermore, recent analyses revealed that the amino- and carboxy-terminal, nonrepeated regions were identical for P270s of different isolates (4), showing that protein sequences were not responsible for surface versus nonsurface placement of P270 during phenotypic variation and among isolates.

A relationship was established between iron and levels of cytoadherence and amounts of adhesins synthesized by *T. vaginalis* (21). Insofar as the cytoadherent type II trichomonads synthesizing adhesins were known to lack surface P270 (6, 21), our group hypothesized that iron directly modulated surface placement of P270. In this report I show that growth in low-iron medium promotes surface placement of P270 for virus-infected but not virus-negative parasites. Conversely, growth of virus-positive organisms in high-iron medium, which induces expression of trichomonad adhesins (21), yields parasites without surface P270. It is also shown that high-iron trichomonads highly phosphorylate P270 compared to organisms grown in low-iron medium.
TABLE 1. Relationship between iron and surface expression of P270 on T. vaginalis type I and type II isolates

<table>
<thead>
<tr>
<th>Isolate, isolate type, and level of iron in medium used</th>
<th>% of organisms with surface P270 in indicated experiment</th>
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</thead>
<tbody>
<tr>
<td>T076, I Low</td>
<td>0</td>
</tr>
<tr>
<td>High</td>
<td>0</td>
</tr>
<tr>
<td>T068-II, II Low</td>
<td>95</td>
</tr>
<tr>
<td>High</td>
<td>92</td>
</tr>
<tr>
<td>T066, II Low</td>
<td>38</td>
</tr>
<tr>
<td>High</td>
<td>30</td>
</tr>
<tr>
<td>AL8, II Low</td>
<td>79</td>
</tr>
<tr>
<td>High</td>
<td>83</td>
</tr>
<tr>
<td>Replete</td>
<td>10</td>
</tr>
<tr>
<td>AL8-N, virus-negative from AL8d</td>
<td>0</td>
</tr>
<tr>
<td>Low</td>
<td>0</td>
</tr>
<tr>
<td>High</td>
<td>0</td>
</tr>
</tbody>
</table>

* a Isolates were as defined on the basis of infection by the dsRNA virus (16, 26). Presence or absence of virus was determined using various criteria, as described previously (16). Briefly, dsRNA was detected in agarose gels after electrophoresis of total nucleic acids from T. vaginalis isolates and/or Northern analysis by using either the radiolabeled dsRNA segments or cDNAs corresponding to one of the dsRNA segments of the virus as probes (15). The normal complex medium of Trypticase–yeast extract–maltose supplemented with 5% heat-inactivated horse serum was used for batch culture of trichomonads (12). Low- and high-iron media were prepared as described before (21), and parasites were grown under each condition for no less than 24 h prior to examination by indirect immunofluorescence. Briefly, low-iron medium was prepared by the addition of 2,2-dipyridyl (Sigma Chemical Co., Saint Louis, Mo.) (0.15 mM final concentration) to growth medium. High-iron medium was made by the addition to growth medium of ferrous ammonium sulfate-hexahydrate (Sigma) (0.2 mM final concentration) from a 100-fold stock solution made in 50 mM sulfosalicylic acid.

* b The percentage of organisms with surface P270 was determined by counting no fewer than 10 fields or 100 organisms three separate times upon visualization by fluorescence microscopy, as before (6, 8).

* c ALS-N represents virus-negative progeny trichomonads derived from an agar clone of a single trichomonad from the parental T. vaginalis isolate AL8 (16, 26). Repeated daily passage in normal medium resulted in the spontaneous abortion of the dsRNA virus from parasites (6, 16, 26).

Relationship between iron levels in medium and P270 surface expression among type II isolate trichomonads. Indirect immunofluorescence with live trichomonads was performed by using established conditions with the MAb C20A3. As seen for two representative experiments, whose results are given in Table 1, the type II clinical isolates T068-II, T066, and AL8 were heterogeneous for surface reactivity with MAb. The numbers of fluorescent trichomonads were always lower when parasites were grown overnight in the complex medium supplemented with iron. In medium depleted of iron, increased numbers of organisms with surface P270 were evident. This dramatic change in the percentage of the opposite phenotype in just three to four generations was unusual; earlier studies involving batch culture required weeks to produce a similar change in fluorescence patterns (6). Importantly, the dsRNA virus is lost among some isolates by daily passage in batch culture requiring weeks to produce a similar change in the percentage of the opposite phenotype. Importantly, other divalent cations were added to iron-depleted medium, as was done previously by this laboratory (21). No similar change from surface to cytoplasmic expression of P270 as seen in Fig. 1B1 was observed, even after batch cultures were maintained over a period of several days (data not shown). No fluorescence was detected with an irrelevant MAb.

It has been established that iron induces synthesis of trichomonad adhesins and enhances levels of cytoadherence (21). As another control, comparative experiments monitoring the extent of cytoadherence in relation to expression of surface P270 were performed. As shown for two representative experiments (Table 2), T. vaginalis organisms grown in medium depleted of iron gave levels of cytoadherence lower than those seen for organisms grown in iron-replete medium. It was also noted that the overall extent and intensity of fluorescence was greater for the live parasites grown in low- versus high-iron medium. The established ligand assay was also performed to identify the four adhesins (21). Increased amounts of adhesins mediating cytoadherence were evident in high-iron organisms, and no synthesis was detectable in low-iron parasites. These results reaffirm the alternating expression of at least two groups of proteins on the surface of T. vaginalis (2). Phosphorylation of P270 and cytoplasmic expression occurs in high iron. Figure 2A shows P270 bands from autoradiograms after SDS-PAGE (8, 19) of immunoprecipitated P270 from a RIP assay. Detergent extracts were prepared from 107 T. vaginalis T068-II organisms labeled overnight with 1 mCi of [32P]orthophosphate in 15 ml of high- versus low-iron medium. P270 is readily phosphorylated in parasites grown overnight in high- compared to low-iron medium. Similarly, greater intensities of phosphorylated proteins were seen in autoradiograms (Fig. 2B) of the total trichomonad protein gels (Fig. 2D) after SDS-PAGE. Gel lanes contained proteins from equal numbers of parasites. Interestingly, as seen in Fig. 2C, immunoblotting
Definitive evidence that iron directly modulates surface expression of P270 among virus-harboring *T. vaginalis* organisms was lacking in earlier data. This study establishes that iron mediates surface placement of P270 and, not unexpectedly, reaffirms that iron regulates the synthesis and surface expression of the adhesin proteins (21). Furthermore, the relationship between levels of iron in the growth medium and the phosphorylation of P270 is demonstrated (Fig. 2), which may begin to provide a biochemical basis to further elucidate the contribution of the dsRNA virus to the property of phenotypic variation.

The *p270* gene of isolate T068-II was recently sequenced (23). This *p270* gene has a 333-bp unit which contains the epitope recognized by the MAb, and this domain is tandemly repeated at least 18 times. The nonrepeat coding regions for the *5*′ and *3*′ ends were 69 nucleotides (23 amino acids) and 1,185 nucleotides (395 amino acids), respectively. More re-
FIG. 2. Higher levels of phosphorylation of P270 in T. vaginalis organisms grown in high-iron (H) versus low-iron (L) medium. (A) Autoradiograms from RIP assay performed as detailed before (4, 6, 8) by using detergent extracts of [32P]orthophosphate-labeled trichomonads incubated with MAb C20A3 or irrelevant control MAb. Immune complexes were precipitated by using protein A-bearing Staphylococcus aureus (6, 8). All extracts contained N-α-p-tosyl-L-lysine chloromethyl ketone to inhibit cysteine proteinase released upon solubilization of parasites. Immunoprecipitated 32P-labeled proteins were then solubilized by boiling S. aureus for 3 min. After bacteria were pelleted, the supernatant was subjected to SDS-PAGE, and gels were dried for autoradiography, as before (6, 8). (B) SDS-PAGE-autoradiography of total proteins as in panel B was then blotted onto nitrocellulose for probing with MAb C20A3 by using established protocols (8, 25). Control irrelevant MAbs of the same isotype were used as controls and did not give any reactivity with trichomonad proteins on the nitrocellulose blots. (D) Coomassie brilliant blue-stained gels of total proteins after SDS-PAGE show the complex patterns for both high- and low-iron parasites, respectively. These serve as internal controls to monitor the iron status of trichomonads.


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