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Trichomonas vaginalis NYH286 Phenotypic Variation May Be Coordinated for a Repertoire of Trichomonad Surface Immunogens

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Trichomonas vaginalis **isolate NYH286 was fractionated with immunoglobulin G of sera from patients with trichomoniasis. Subpopulations of trichomonads with phenotypes of either patient serum-immunoglobulin G reactive (PS^+) or nonreactive (PS^-) were obtained. Flow cytofluorometry of PS^+ and PS^- subpopulations with a monoclonal antibody called C20A3 which reacts with a high-molecular-weight immunogen of *T. vaginalis* gave corresponding fluorescent (positive) and nonfluorescent (negative) phenotypes. No relationship was seen between PS^+ and PS^- phenotypes and binding of soybean agglutinin, wheat germ agglutinin, and concanavalin A, showing that PS^- organisms still possessed carbohydrate moieties on their surfaces based on lectin binding. Phenotypic variation among the PS^+ and PS^- trichomonads was observed during in vitro growth. A positive-to-negative phenotype shift was also recorded for parasites obtained from lesions of mice subcutaneously infected with PS^+ trichomonads. The involvement of surface proteins in the differential PS^+ and PS^- reactions was supported by soluble antigen and whole cell radioimmunoprecipitation assays. Finally, enhanced parasitism and killing of HeLa cells in monolayer cultures were observed for PS^- subpopulations as compared with PS^+ counterparts. The data support the idea that phenotypic variation for *T. vaginalis* may be coordinated for a repertoire of trichomonad immunogens and that such membrane dynamics influence expression of virulence determinants for these sexually transmitted disease agents.**

Trichomonas vaginalis is capable of undergoing phenotypic variation (6). This phenomenon was characterized by using a monoclonal antibody (MAb) which recognized a prominent immunogenic molecule (6, 8), and this high-molecular-weight immunogen was one of several which appeared to be either present on or absent from trichomonal membranes (4, 8, 9). Because earlier studies indicated that several parasite proteins were responsible for antigenic heterogeneity among trichomonal isolates and subpopulations of isolates (9), it became necessary to test whether *T. vaginalis* phenotypic variation (6) might occur simultaneously for a repertoire of immunogens, which were detected previously with sera from patients with trichomoniasis (9).

MATERIALS AND METHODS

Microorganisms. The *T. vaginalis* long-term-grown isolate NYH286 has been recently described (3, 4, 6, 8, 9, 14). NYH286 trichomonads were passaged daily for up to 2 years. Parasites were cultivated in Diamond's Trypticase (BBL Microbiology Systems, Cockeysville, Md.) yeast extract-maltose medium (10) without agar and supplemented with 10% heat-inactivated horse serum. Only trichomonads in the logarithmic phase of growth were used for experiments (13).

Antibody reagents. Reactive sera (class IA) (9) from patients with trichomoniasis (IHS) were recently examined for antibody to *T. vaginalis* surface proteins and compared with pooled sera from normal, uninfected humans used as a control. The sera from mice infected subcutaneously with live organisms (IMS) have also been described (8, 9). This mouse antiserum contained high-titered antibody to most, if not all, trichomonad surface proteins as determined by various assays, including enzyme-linked immunosorbent assay (2), soluble antigen radioimmunoprecipitation (Sol-RIP), and whole cell (WC-RIP) (1, 8, 9). Preinfection sera from the same mice used for infection served as a control. Finally, an MAb designated C20A3 which recognized a high-molecular-weight protein of *T. vaginalis* (8) was also used. This MAb was employed to study phenotypic variation among trichomonal isolates (6) and reacts with live parasites similar to the pooled class IA IHS (9). An irrelevant MAb of the same immunoglobulin G (IgG) isotype but nonreactive with *T. vaginalis* was used as a control (6, 8).

Indirect immunofluorescence and flow cytofluorometry. The procedures detailing the handling of parasites and incubations with serum, serum IgG, and MAb reagents were published recently (1, 8, 9). Fluorescein isothiocyanate-labeled anti-mouse or anti-human immunoglobulins (Cooper Biomedical, Inc., West Chester, Pa.) were used as required for individual experiments. Indirect immunofluorescence of parasite subpopulations was conducted in duplicate, and experiments were performed at least three times.

Flow cytofluorometry for fluorescein isothiocyanate-conjugated lectins, such as soybean agglutinin, wheat germ agglutinin, and concanavalin A (all purchased from Sigma Chemical Co., St. Louis, Mo.), was performed with 1 to 10 μg of lectin. Reactions with lectins were always examined microscopically before flow cytofluorometry to determine the minimal concentration which gave fluorescence with no agglutination of trichomonads. Asparagus pea lectin (Sigma) was used as a negative control because it reacted little, if any, with trichomonads under the same conditions. Cytofluorometric analysis of parasites with MAb was performed as recently described (6). All assays were done no less than three times.

Parasites recovered from lesions of at least two BALB/c mice subcutaneously infected with subpopulations of trichomonads as described below were also evaluated by flow cytofluorometry. The numbers of organisms and conditions used for infection of mice were as described by others (11). At various days after inoculation, infected sites were opened and washed several times with phosphate-buffered saline until no additional trichomonads were recovered.

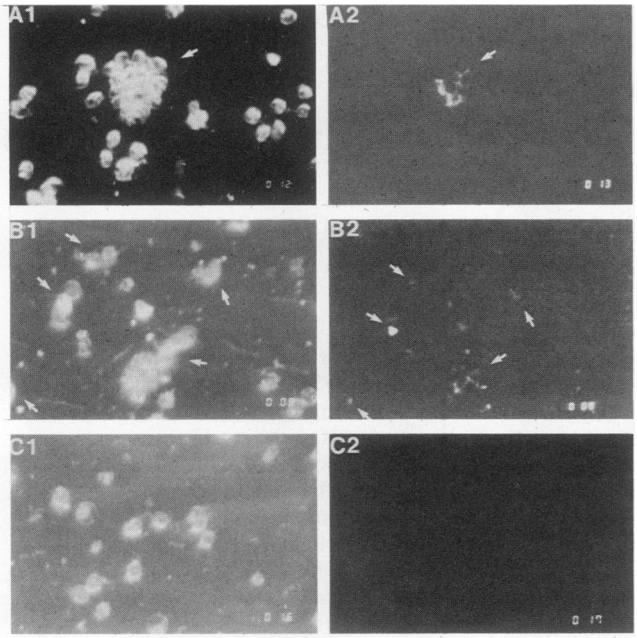

FIG. 1. Dark field (A1, B1, and C1) and corresponding indirect immunofluorescence (A2, B2, and C2) of *T. vaginalis* NYH286 subpopulations fractionated with magnetic particles possessing IgG of sera from patients with trichomoniasis. The original heterogeneous isolate NYH286 was evaluated with MAb C20A3 before fractionation (A). PS^+ (B) and PS^- parasites (C) were incubated with IgG of IHS. Note the absence of fluorescence in the PS^- trichomonads; identical results were obtained with MAb C20A3. Arrows point to corresponding representative fluorescent trichomonads.

Parasites too few in number for analysis were detectable in washings from mouse lesions after 8 days postinfection. Washings were pooled and centrifuged at 2,000 × *g* for 10 min, and pellets consisting of live trichomonads, host cells, and debris were suspended to 0.5 ml. Motile organisms were enumerated and were always examined by indirect immunofluorescence and dark-field microscopy simultaneously (8). Flow cytofluorometry was then immediately performed. At no time was nonspecific fluorescence of host cells or debris seen when using the MAb C20A3 or irrelevant MAb. Cytofluorometric analysis of *T. vaginalis* obtained from lesions of no less than three mice was conducted on four separate occasions.

Fractionation of trichomonal subpopulations. Trichomonads reactive (positive [Pos] phenotype) and nonreactive (negative [Neg] phenotype) with IgG of sera from patients with trichomoniasis (IHS) as determined by indirect immunofluorescence were separated by using protein A-bearing magnetic particles (Boehringer Mannheim, La Jolla, Calif.). After being washed twice with phosphate-buffered saline, the particles were added to *T. vaginalis* organisms pretreated with a 1:5 dilution of IHS. After incubation for 2 h at room temperature, parasites which were patient serum IgG positive (PS^+) were immobilized by a magnet on the side of the test tube. Nonreactive, patient serum-IgG-negative (PS^-) parasites were transferred to another tube containing medium. After incubation of PS^+ and PS^- subpopulations in medium for 1 to 2 days, trichomonads grew to sufficient densities for passage and analysis by indirect immunofluorescence.

RIP and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sol-RIP and WC-RIP were performed as described recently (1, 8, 9). In both assays, trichomonads were iodinated (1) immediately after flow cytofluorometric analysis of the parasite populations with MAb C20A3 or after indirect immunofluorescence with IgG of IHS. A detergent extract of iodinated trichomonad proteins or surface-labeled, motile organisms (1) was then used for Sol-RIP and WC-RIP, respectively.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of iodinated parasite proteins precipitated by RIP was done on 7.5% acrylamide gels as detailed previously (1, 9, 13).

Cytadherence and cytotoxicity of HeLa cell monolayers. The ability of *T. vaginalis* to parasitize HeLa cells and the quantitative colorimetric assay for determining the extent of parasite-mediated, contact-dependent killing of HeLa cells were performed as described recently (3, 7).

RESULTS

Reaction of IHS with parasite surface proteins and fractionation of trichomonal subpopulations. IHS which contained antibody reactive to numerous trichomonad proteins as determined by the WC-RIP assay (1, 9) was used to separate subpopulations of *T. vaginalis* NYH286. Fractionation was accomplished with IgG of IHS bound to protein A-bearing

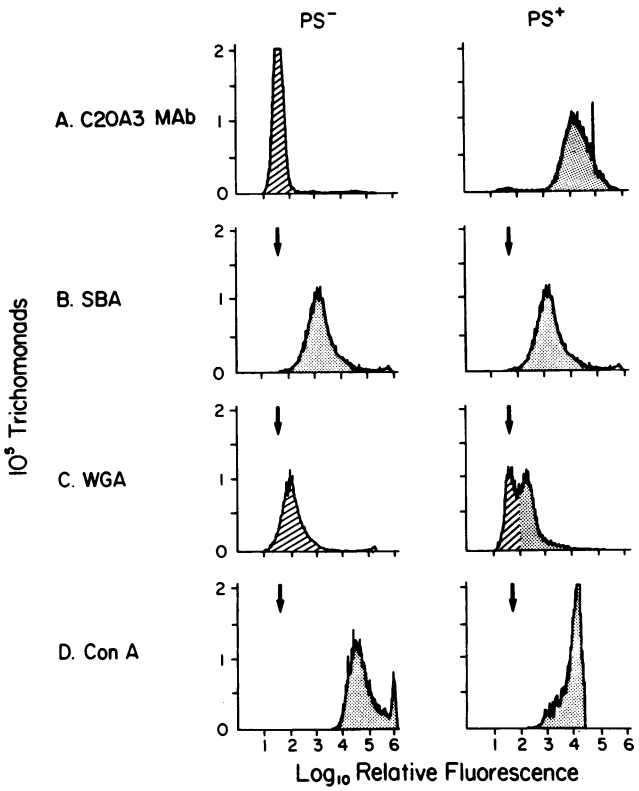

FIG. 2. Representative flow cytofluorometry analysis of *T. vaginalis* NYH286 subfractionated by IgG of sera from patients with trichomoniasis. PS^+ and PS^- trichomonads were incubated with hybridoma supernatant containing MAb C20A3 (6) (A) and 7 μg of lectins (B through D) as described in Materials and Methods. Lines areas and arrows indicate the fluorescence intensity of Neg phenotype parasites incubated with an MAb of the same isotype but nonreactive with *T. vaginalis* NYH286. SBA, Soybean agglutinin; WGA, wheat germ agglutinin; Con A, concanavalin A.

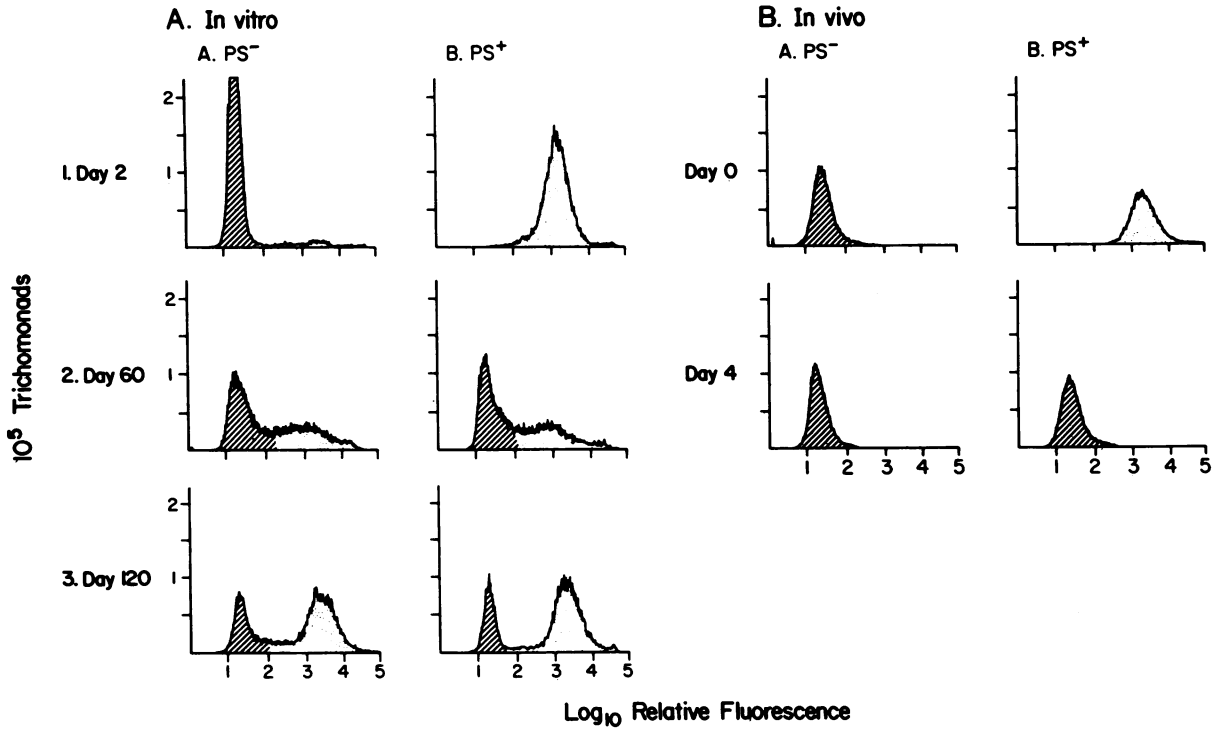

FIG. 3. Representative flow cytofluorometric patterns showing phenotypic variation of PS^- and PS^+ subpopulations during extended in vitro cultivation (A) or during subcutaneous infection of mice (B). Patterns represent the fluorescence distribution of parasites with MAb C20A3. Lined areas indicate absence of fluorescence or Neg phenotype determined with a control, irrelevant MAb of the same isotype. (B) Recovery of trichomonads from lesions of three mice infected subcutaneously with PS^- and PS^+. Parasites were pooled and represented 18 and 24% of the initial total inoculum for the three mice given PS^- and PS^+ organisms, respectively.

magnetic particles. Figure 1 shows dark field and corresponding fluorescence of the same fields of NYH286 organisms which were heterogeneous (panels A) and of the fractionated patient serum-reactive (panels B) and nonreactive (panels C) subpopulations. MAb C20A3 detected only parasites expressing the high-molecular-weight immunogen, and a similar heterogeneity of the population was seen with IHS (panel A). Also, fluorescence was only seen for the purified PS^+ (panels B) but not PS^- organisms (panels C) with the IgG of IHS.

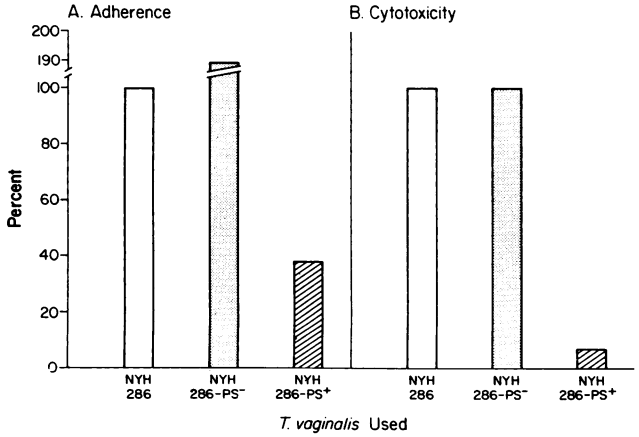

FIG. 4. Comparative adherence (A) and cytotoxicity (B) of PS^-, PS^+, and parent NYH286 parasites. Trichomonads were incubated with HeLa cell monolayers. Details describing the adherence and cytotoxicity parameters are described in Materials and Methods and published elsewhere (3, 6, 7).

Reaction of PS^- and PS^+ subpopulations with MAb and lectins. Flow cytofluorometry with MAb C20A3 was performed, and consistent with indirect immunofluorescence (Fig. 1B and C), PS^- and PS^+ trichomonal subpopulations gave corresponding Neg and Pos cytofluorometric phenotypes (Fig. 2A). No similar differentiation was seen with three lectins, soybean agglutinin, wheat germ agglutinin, and concanavalin A (Fig. 2B through D), which have been used by others in attempts to correlate surface carbohydrate moieties and virulence (15, 16). For example, homogeneous Pos phenotype reactions were recorded for both subpopulations with soybean agglutinin and concanavalin A. Wheat germ agglutinin yielded a homogeneous Neg phenotype for PS^- trichomonads but gave heterogeneity among PS^+ organisms. These data show the absence or presence of immunogens, such as those seen by C20A3 (6, 8, 9), from surfaces of the PS^- and PS^+ subpopulations, respectively. No relationship was apparent under these experimental conditions between membrane carbohydrates as measured with these lectins (15, 16) and serum IgG reactions.

Phenotypic variation of PS^- and PS^+ subpopulations. It was important to determine whether the subpopulations of *T. vaginalis* fractionated with IgG of IHS undergo phenotypic variation (6). PS^- and PS^+ organisms cultivated in vitro for prolonged periods were evaluated by flow cytofluorometry with C20A3. Figure 3A shows the emergence of a population of opposite phenotype from respective PS^- and PS^+ starting phenotypes. Indirect immunofluorescence

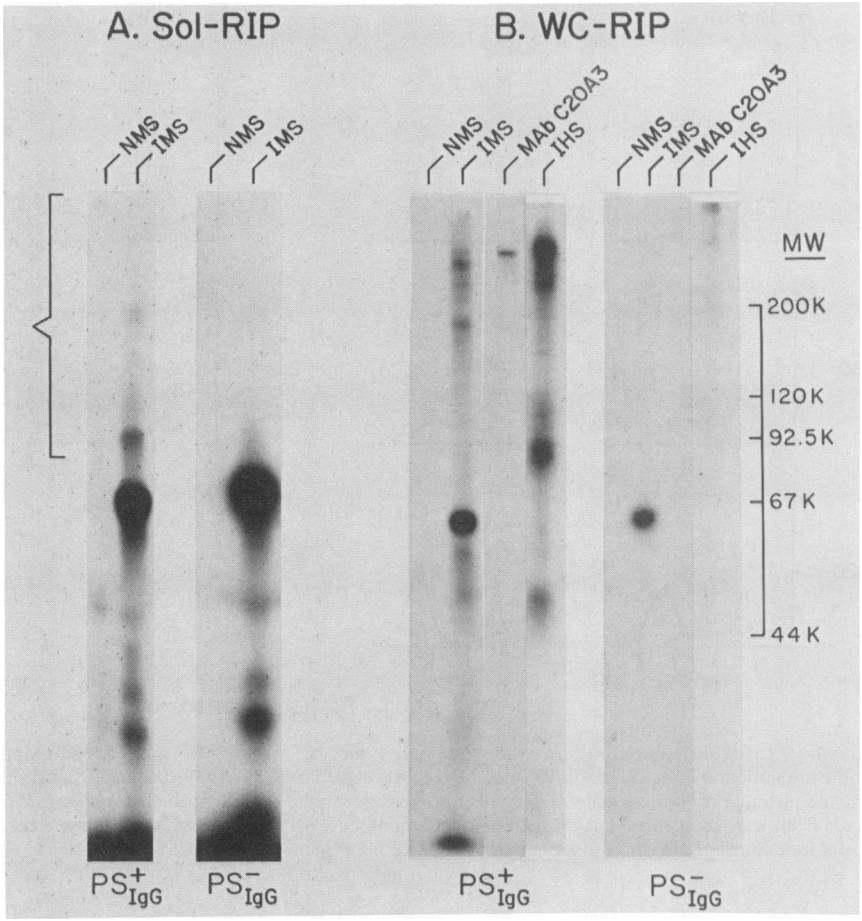

FIG. 5. Autoradiograms after sodium dodecyl sulfate-polyacrylamide gel electrophoresis of iodinated trichomonad proteins used for Sol-RIP (A) and WC-RIP (B) assays performed as described in the text. Iodinated detergent extracts or live, motile parasites were incubated with pooled normal mouse sera (NMS), pooled sera from infected mice (IMS), pooled highly reactive sera from patients with trichomoniasis (IHS), and MAb C20A3. Pooled sera from normal, uninfected women and an irrelevant MAb were used as controls and gave patterns similar to normal mouse serum. The bracketed area emphasizes the region of high-molecular-weight proteins believed to be involved in the coordinated phenotypic reactions. MW, Molecular weight; K, $\times 10^3$.

with the IgG of IHS also gave staining and nonstaining parasites as seen with C20A3 (Fig. 1), showing phenotypic variation (6) for the immunogens reactive with IgG of patient sera.

It was of interest to examine whether phenotypic variation might occur among PS^- and PS^+ *T. vaginalis* organisms during subcutaneous infection into the hindquarters of BALB/c mice. This was important because of the use of this procedure for assessing the pathogenicity among isolates of *T. vaginalis* (11). Figure 3B illustrates cytofluorometric patterns which show the recovery of only Neg phenotype trichomonads from mouse lesions regardless of the original infecting phenotype.

Enhanced cytadherence and cytotoxicity of HeLa cell monolayers by PS^- trichomonads. It was necessary to examine the ability of PS^- and PS^+ trichomonads to parasitize and kill HeLa cells (3, 7, 12), since parasites without C20A3-reactive immunogen on their surface display higher levels of contact-dependent cytotoxicity of HeLa cells (6). Both cytadherence and cytotoxicity levels were greater with the parent NYH286 isolate and the PS^- subpopulation when compared with PS^+ organisms evaluated simultaneously (Fig. 4). In fact, in this and other experiments, the level of PS^- cytadherence was up to twice that seen for the heterogeneous parent NYH286 population of trichomonads. These data show the relationship between the phenotype of these microorganisms and host cytadherence and cytotoxicity virulence properties (6).

Evidence for a repertoire of trichomonad proteins responsible for PS^- and PS^+ phenotypes. Finally, I attempted to demonstrate the role of immunogenic proteins in the patient serum IgG phenotypic distinctions of *T. vaginalis* subpopulations. This was important because of the putative role of high-molecular-weight proteins in antigenic heterogeneity among trichomonal isolates (6, 8, 9). Figure 5 shows results of Sol-RIP and WC-RIP with iodinated detergent extracts and intact cells of *T. vaginalis* NYH286, respectively, incubated with IMS, IHS, and MAb C20A3. In both Sol-RIP and WC-RIP assays, PS^- parasites failed to yield the same number and intensity of precipitable high-molecular-weight bands (Fig. 5, bracketed area) compared with PS^+ organisms, even after prolonged exposure of X-ray film or use of more concentrated IMS. The patterns of precipitated iodinated protein bands by IHS in the WC-RIP assay for PS^+ parasites were consistent with previously published results (9). No similar WC-RIP recognition of proteins by IHS was

evident for the PS^- parasites. As expected, C20A3 precipitated the iodinated immunogen only in PS^+ trichomonads. It is noteworthy that iodinated proteins of ≤65,000 daltons were not readily detected by WC-RIP, also as has been previously reported (9). Few, if any, protein bands were seen with normal mouse serum or normal human serum used in duplicate samples of the same iodinated preparations (data not shown) (9).

DISCUSSION

Preliminary experiments implicated a group of trichomonad immunogens as candidates responsible for the antigenic heterogeneity of *T. vaginalis* (4, 9). A key feature was that only some, but not all, isolates displaced the repertoire of immunogens on their surface (4, 8, 9). Evidence for this phenomenon was also provided with an MAb (C20A3) (8), and more recently, the immunogen recognized by C20A3 was found to undergo phenotypic variation (6).

Data provided in this report reinforce the view that phenotypic variation may be coordinated for a family of surface components. For example, with MAb C20A3 as a probe, PS^- and PS^+ subpopulations from isolate NYH286 were cultivated in vitro and yielded trichomonads of opposite phenotype. It was possible to reisolate the respective Pos and Neg phenotypes by using magnetic particles incubated with IHS from emerging heterogeneous populations (Fig. 3A). The newly fractionated PS^- and PS^+ subpopulations gave indirect immunofluorescence and RIP reactions consistent with those seen in Fig. 2 and 5. Importantly, similar results have been obtained using a fresh isolate, JHH (6, 8, 9), grown in vitro for no longer than 5 days, showing that phenotypic variation of a repertoire of immunogens occurs for other isolates besides the common laboratory isolate, NYH286.

Also interesting was the binding of lectins to both PS^- and PS^+ parasites. These data indicate that although glycoproteins are members of the repertoire of molecules involved in the antigenic distinctions (5, 8), they are not the only determinants of *T. vaginalis* heterogeneity. Otherwise, it would have been expected that more well-defined differentiation of trichomonal isolates on the basis of sugar specificities would have been obtained (15, 16).

The IHS used for my present studies possessed very little, if any, antibody to NYH286 iodinated surface proteins of ≤70,000 molecular weight (Fig. 5) (9). This suggests that antibody to high-molecular-weight immunogens was indeed responsible for fractionation of the parent population with the IgG of IHS. In addition, lower-molecular-weight antigens (≤70,000), which are common and stable molecules of *T. vaginalis* isolates (4), appear inaccessible to antibody recognition as seen by their lack of precipitation by WC-RIP with IMS (Fig. 4B). Thus, these antibody-inaccessible immunogens would not play a major role in the antigenic heterogeneity of *T. vaginalis*.

Only Neg phenotype parasites, albeit 10% of the starting inoculum, were recovered from lesions of mice injected subcutaneously with PS^+ organisms. Although little is known about the factors responsible for the killing of parasites within the lesions, an early antibody response has been demonstrated (8) toward members of the high-molecular-weight repertoire of immunogens, including the C20A3-reactive molecule studied here. Since Pos phenotype parasites can be killed by antibody (5), Neg phenotype organisms which emerge during an infection might survive host antibody targeted to these high-molecular-weight immunogens.

Neg phenotype trichomonads (PS^-) promote higher levels of parasitism and killing of HeLa cells, as reported earlier (6). These data (6) (Fig. 5) show an inverse relationship between the expression of the repertoire of major immunogens and the ability to attach to host cells, possibly through protein adhesins. Candidate adhesins of *T. vaginalis* (unpublished observations) have recently been identified, and initial studies confirm the alternating expression of groups of proteins on trichomonal membranes. These and future studies may clarify the relevance of our observations with respect to virulence and disease pathogenesis for this model system.

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