ANTIGENIC VARIATION OF A CYSTEINE-RICH PROTEIN IN *GIARDIA LAMBLIA*

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Extensive differences have been noted among different isolates of *Giardia lamblia* with respect to surface-labeled proteins (1, 2), antigenic profile (1–4), restriction endonuclease patterns (5), and isoenzyme patterns (4, 6). Antigenic variation has been shown for the WB isolate of *G. lamblia* and may explain some of the differences in surface labeling and antigenic profile (Nash, T. E., A. Aggarwal, and R. D. Adam, submitted for publication).

An mAb (6E7) specific for a 170-kD antigen expressed on the surface of some isolates, including WB, is cytotoxic for organisms expressing this antigen (7). *G. lamblia* WB trophozoites were cloned four times in succession. These cloned lines (referred to as parents) were then incubated with mAb 6E7 and most organisms were killed within minutes. Survivors (referred to as progeny) were resistant to killing by mAb 6E7, and the 170-kD antigen was no longer detectable on the surface or in whole cell extract. When these resistant organisms were surface labeled with ^{125}I, the 170-kD band was replaced by multiple bands ranging in size from ~50 to 170 kD. These bands appeared to be derived from several subpopulations of antigenic variants, since some clones of these antigenic variants only expressed one major surface-labeled band (Nash, T. E., A. Aggarwal, and R. D. Adam, submitted for publication).

By using mAb 6E7, we have isolated a portion of the gene encoding the 170-kD antigen (or a closely related member of the gene family) from a λgt11 expression library and have used this cloned gene fragment as a probe to examine the mechanism of antigenic variation in *Giardia*. We have also demonstrated that the 170-kD antigen has a high cysteine content by sequence analysis of the cloned gene fragment and by metabolic labeling with [^{35}S]cysteine.

Materials and Methods

Giardia Isolates. Clones of the WB isolate of *G. lamblia* are described in detail elsewhere (Nash, T. E., et al., submitted for publication). A doubly cloned WB line (WB2X) (8) was further cloned twice in succession by limiting dilution to yield "parent lines." The parent lines (WB A6-6E7S, WB C9-6E7S, WB E11-6E7S, WB A10-6E7S, WB C1-6E7S) were all sensitive to mAb 6E7 by cytotoxicity studies (7), and immunoblotting of whole cell lysate revealed a 170-kD antigen reacting with mAb 6E7. These parents (6E7S lines) were each incubated with mAb 6E7 and survivors (progeny lines) were isolated that no longer expressed the 170-kD antigen (6E7R lines) by immunoblotting (7). The progeny lines are called WB A6-6E7R, WB C9-6E7R, WB E11a-6E7R, WB E11b-6E7R, WB A10-6E7R, and WB C1-6E7R. The WB A6-6E7R line was cloned and one of these clones was used

for the cysteine labeling. WB DK (a clone of WB) and WB MU are WB isolates from our laboratory that spontaneously lost expression of the 170-kD antigen.

Construction of DNA Library and Immunoscreening. A λgt11 expression library of an uncloned WB line expressing the 170-kD antigen was constructed by digesting the DNA with mung bean nuclease (9). The DNA fragments between 0.5 and 4 kb were packaged into λgt11 using Eco RI linkers (9). The insert DNA was not methylated. The library was screened with polyclonal rabbit anti-*Giardia* serum raised against the Isr *Giardia* isolate (2) that expresses the 170-kD antigen (10, 11). Horseradish peroxidase-labeled anti-rabbit IgG was used as the second antibody. The three positive plaques (M2, M3, M16) were purified and rescreened with mAb 6E7, and one positive clone (M2) was identified. Fusion proteins were produced in λ lysogen *Escherichia coli* $1089R^-$, and whole cell lysates were separated by SDS-PAGE under reducing conditions, transferred to nitrocellulose, and probed with mAb 6E7 (10).

DNA Isolation and Subcloning. Purified λM2 DNA was digested with Eco RI and found to contain two inserts. The inserts were isolated by electroelution from an agarose gel, and each was subcloned back into λgt11. The two separately cloned fragments are referred to as M2-1 (1 kb) and M2-2 (500 bp).

Sequencing. Sequencing was performed as described (12, 13) using restriction enzyme fragments of M2-1 subcloned into M13mp18 (14) (Fig. 4*A*). Deaza-dGTP (15) and restriction enzyme mapping were used to clarify the sequence in GC-rich regions.

Cysteine Labeling. 2×10^7 *Giardia* trophozoites in 2 ml of TYI-S-33 medium (1) without cysteine were incubated with 250 μCi of L-[^{35}S]cysteine HCl (Amersham Corp., Arlington Heights, IL) for 4-5 h at 37°C. The trophozoites were washed extensively with PBS, pH 7.2, and aliquots were frozen at −70°C. For antibody precipitation, pellets were dissolved in PBS, pH 7.2, with 1% Triton, incubated overnight with mAb 6E7, and immune complexes were precipitated with Staphylococcal protein A (Behring Diagnostics, La Jolla, CA) (1). Pellets were boiled in sample buffer, and SDS-PAGE was performed as before (1).

Blots and Probes. *Giardia* DNA was isolated and Southern blotting was performed as previously described (5). Washing was performed at 52°C in 0.2× SSC, 0.1% SDS.

Total RNA was extracted (16) using guanidinium isothiocyanate and hot phenol, electrophoresed on a formaldehyde gel with RNA markers (Bethesda Research Laboratories, Gaithersburg, MD), and transferred to nitrocellulose. Hybridization was performed at 37°C (50% formamide, 5× SSPE, 5× Denhardt's 0.1% SDS; T_m −37°C) and blots were washed at low (25°C, 2× SSC, 0.1% SDS; T_m −86°C) and high (65°C, 0.1× SSC, 0.1% SDS; T_m −12°C) stringency. Therefore, T_m −37°C and T_m −12°C were the effective stringencies for washing.

dsDNA was labeled with ^{32}P by nick translation (16) or random priming (17), and ssDNA was labeled with ^{32}P using the Klenow fragment (18). Unincorporated nucleotides were separated from labeled probes by column chromatography using Sephadex G50.

Results

Immunoscreening of Genomic Library. A mung bean expression library of DNA from a WB *Giardia* isolate that expresses the 170-kD antigen was screened with polyclonal rabbit serum raised against a *Giardia* isolate expressing the 170-kD antigen. Three clones were identified (M2, M3, M16). An immunoblot of the fusion proteins of the three recombinant clones was probed with mAb 6E7 and revealed a protein of ~145 kD produced by the M2 clone (Fig. 1*A*). Electrophoretic analysis of the m2 clone revealed two Eco RI fragments, 1.0 and 0.5 kb in size. These were subcloned into λgt11 and only the 1-kb fragment (M2-1) produced a fusion protein reacting with mAb 6E7.

RNA Blotting. To determine whether the gene for the 170-kD antigen was transcribed in the antigenic variants, total RNA was separated by formaldehyde gel electrophoresis, transferred to nitrocellulose, and probed with M2-1 (Fig. 2).

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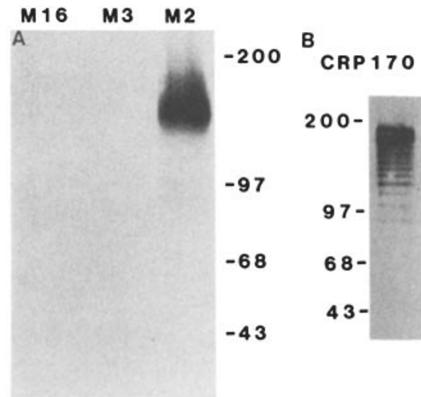

FIGURE 1. (*A*) mAb 6E7 was used to probe a Western blot of the fusion proteins produced by the three clones in λgt 11 that were positive with polyclonal antiserum. Molecular weights (× 10^3) are on the right. The size of the fusion protein is ~145 × 10^{-3}. (*B*) Western blot of whole cell lysate from a CRP170-expressing (6E7S) *Giardia* isolate probed with mAb 6E7. The sample was boiled with 2-ME. Positions of molecular weight markers (× 10^{-3}) are on the left.

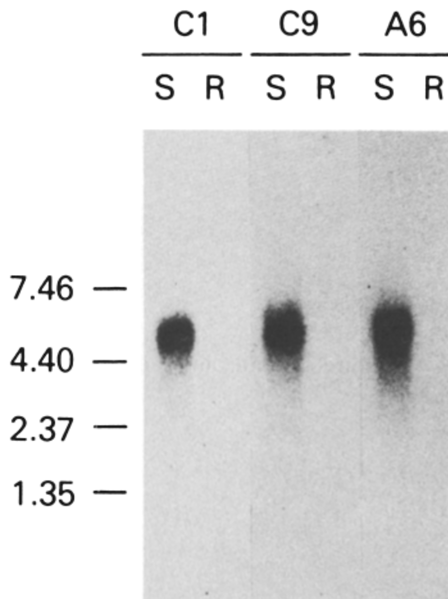

FIGURE 2. Northern blot of total RNA from WB C1, WB C9, and WB A6 probed with M2-1 and washed at 65°C in 0.1× SSC, 0.1% SDS. In adjacent lanes are the 6E7S clones (*S*) and their 6E7R (*R*) antigenically variant progeny. Positions of RNA size markers (Bethesda Research Laboratories) are indicated on the left. The transcripts identified are ~5.4 kb.

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The probe hybridized to a 5.4-kb transcript from each of three 6E7S clones and did not hybridize to RNA from their antigenically variant progeny, even at low-stringency (T_m −37°C) washing. Since the WB C1-6E7R line expresses a surface protein that is similar in size to its 6E7S parent (Nash, T. E., et al., submitted for publication), this rules out simple truncation of the gene as a possible mechanism of antigenic variation, at least in the WBC1 line.

DNA Blotting. Southern blots of parent/progeny sets were probed with M2-1 to compare lines expressing the 170-kD antigen (6E7S) with their immediate 6E7R progeny. In these 6E7R antigenic variants, the 170-kD antigen was replaced by surface-labeled bands ranging in size from ~50 to 170 kD (Nash, T. E., et al., submitted for publication). There were ~7–10 DNA bands ranging in size from 6.2 to ~28 kb with varying intensities of hybridization, suggesting that this is a multigene family (Fig. 3*A*).

Differences were readily apparent among the different lines (Fig. 3*A*); how-

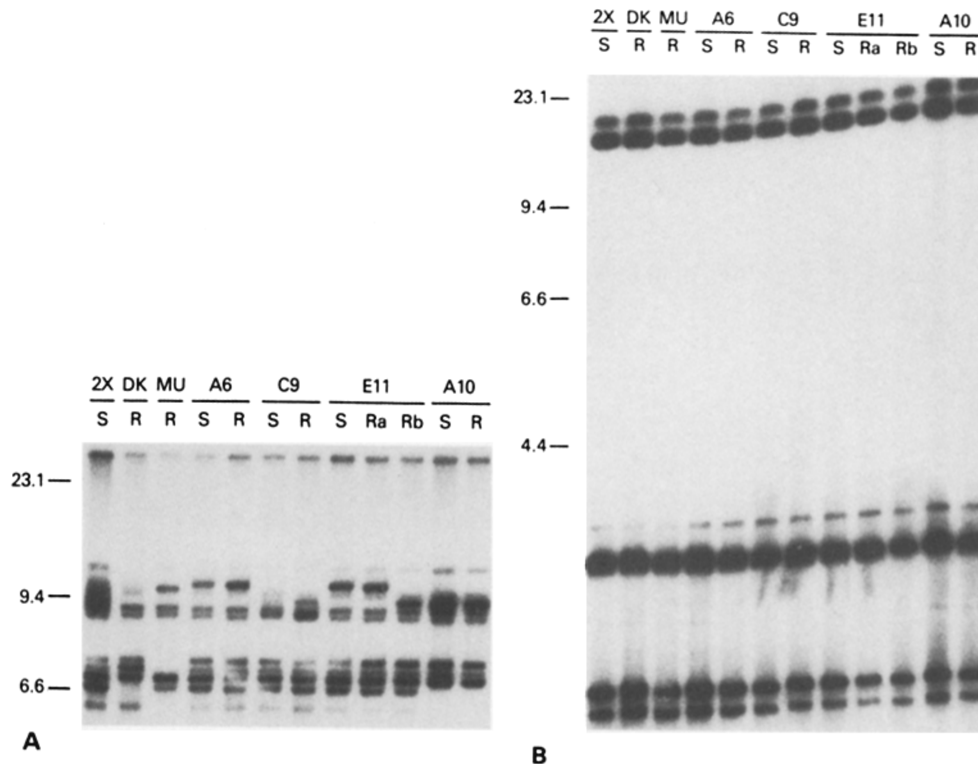

FIGURE 3. Southern blots of different WB Giardia isolates; WB 2X, WB DK, WB MU, and four parent (*S*)/progeny (*R*) sets; WB A6, WB C9, WB E11, WB A10. DNA marker sizes (kb) are on the left. Blots were probed with (*A*) M2-1, (*B*) G6A10 (used previously to distinguish classes of *Giardia* isolates) (5).

ever, there was less difference between immediate parent and progeny. The major exception is one of the two progeny of the WB E11-6E7S clone, (WB E11b-6E7R), in which a 10-kb band is replaced by a 9.3-kb band. DNA from the same isolates was also probed with G6A10, a random cloned fragment of WB *Giardia* DNA, which has previously been shown to differentiate among classes of *Giardia* (5) and all isolates showed the WB banding pattern (Fig. 3*B*). Therefore, genomic rearrangements involving the 170-kD antigen gene family are frequent, occurring within 50–100 generations, but are not clearly associated with antigenic variation.

Sequencing. Sequence analysis of the M2-1 fragment (Fig. 4*B*) revealed that the one open reading frame was also in frame with the β-galactosidase gene of λgt11. Northern blots of RNA from WB A6-6E7S were probed with strand-specific DNA from M13mp18 subclones of M2-1. Only one strand hybridized with the RNA, consistent with the orientation of the observed open reading frame (data not shown).

The nucleotide and putative amino acid sequence of M2-1 (Fig. 4*B*) reveals two tandem repeats and part of a third. The repeats are 195 bp (65 amino acids) in length and identical with the exception of a silent substitution in the last codon of the second full repeat. The repeating as well as the nonrepeating portions of

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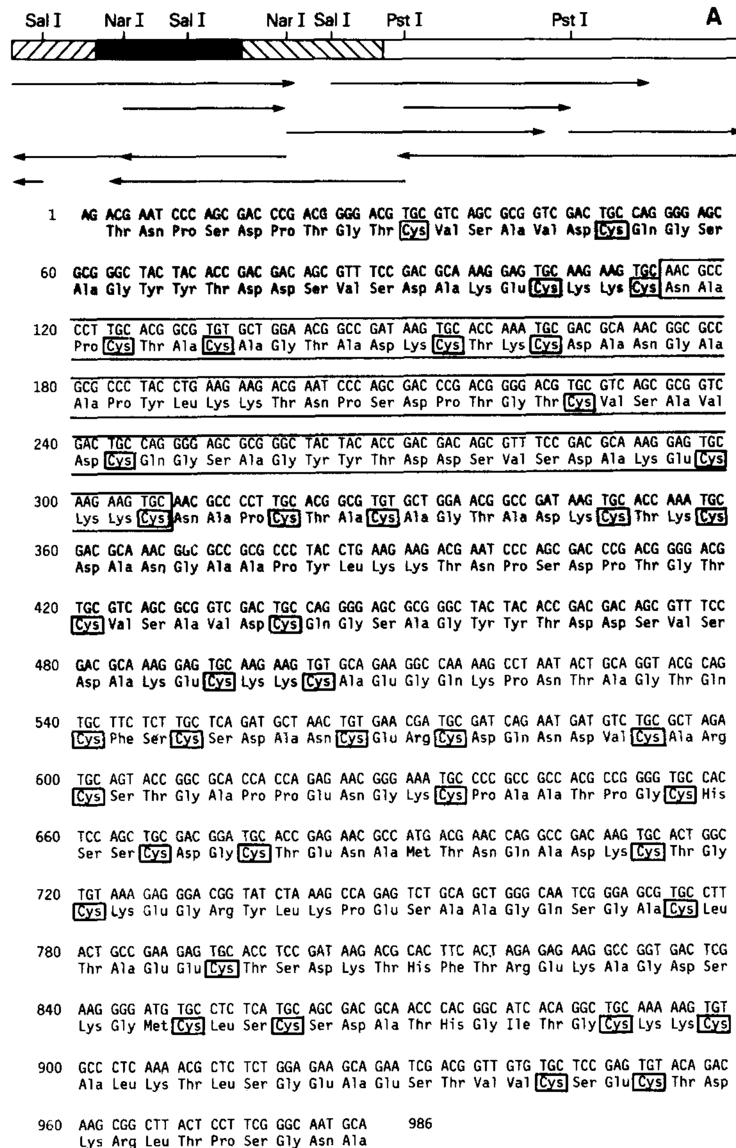

FIGURE 4. (*A*) Sequencing strategy for M2-1. Subclones for sequencing were generated by restriction enzymes. Tandem repeats are shown by alternating crosshatched and shaded areas. (*B*) DNA and amino acid sequence of M2-1 (not including the Eco RI linkers). The polypeptide encoded by M2-1 is 33,436 M_r with 12% cysteine. Two tandem repeats 65 amino acids in length and part of a third (37 amino acids) comprise base 1 through 503 of the sequence (shown by alternating shaded and lined segments).

M2-1 are cysteine-rich (12%). The molecular weight predicted from the amino acid sequence is 33,436, in close agreement with the 29,000 M_r predicted by the β-galactosidase fusion protein (145 kD minus the 116 kD accounted for by β-galactosidase). Because of the cysteine-rich region within the 170-kD antigen, we have designated this protein CRP170.

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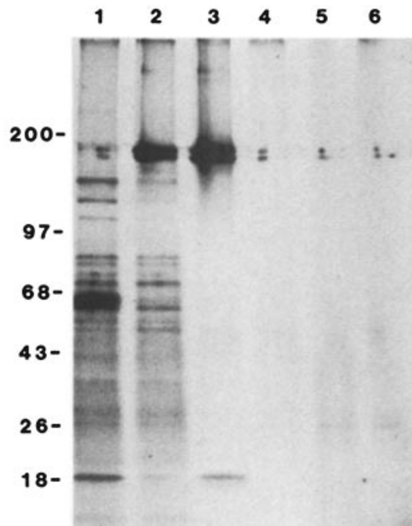

FIGURE 5. SDS-PAGE of *Giardia* metabolically labeled with [^{35}S]cysteine. (*1*) clone of WB A6-6E7R (antigenic variant), (*2*) WB A6-6E7S (CRP170-expressing); (*3*) WB A6-6E7S immunoprecipitated with mAb 6E7; (*4*) WB A6-6E7S immunoprecipitated with control ascites; (*5*) WB A6-6E7R immunoprecipitated with mAb 6E7; (*6*) WB A6-6E7R immunoprecipitated with control ascites. Molecular weight markers (× 10^{-3}) are on the left. Note that artifact is present at 170 × 10^{-3} mol wt marker on lanes *1* and *4–6* due to spillover from lanes *2* and *3*.

Metabolic Labeling with Cysteine. As further confirmation that the M2-1 probe is from the CRP170 gene, *Giardia* trophozoites were metabolically labeled with [^{35}S]cysteine (Fig. 5). A cysteine-labeled doublet of ~170 kD from the WB A6 (parent) clone was selectively precipitated by mAb 6E7, whereas a clone of WB A6-6E7R expressed a 64-kD cysteine-labeled protein that was not precipitated by mAb 6E7. The high cysteine content may explain the observation that a major surface antigen of *Giardia* is polydisperse (1, 2, 19) or forms a stair-step pattern (Fig. 1*B*) on SDS-PAGE. This effect is much less pronounced when the sample is prepared without a reducing agent (2), and is most likely due to differential breakage of disulfide bonds (20). At this time, we are unable to explain why the cysteine-labeled protein migrates as a doublet, although it may be related to processing of the protein before it is secreted. This would also explain why only a single band is seen with ^{125}I surface labeling.

Discussion

We have cloned a portion of the gene for an antigenically variant surface protein (CRP170) in *Giardia* and shown that it has a high cysteine content. Since the CRP170 gene is from a multigene family, the possibility still exists that the M2-1 probe is from a pseudogene rather than the actual CRP170 gene. Regardless, it is a useful tool for analysis of antigenic variation in *Giardia* because of the following characteristics. The cloned gene fragment (M2-1) produces a fusion protein in λgt11 that reacts with mAb 6E7. It hybridizes at high stringency (T_m −12°C) to 5.4-kb transcripts from 6E7S isolates and does not hybridize to RNA from the 6E7R antigenic variants. As predicted by the amino acid sequence of M2-1, CRP170 is readily labeled in vivo with cysteine, and is replaced by a cysteine-labeled protein of different molecular weight in the antigenic variant.

It is known that *Giardia* has a high nutritional requirement for cysteine, independent of its reducing properties (21), and that thiol groups are present on the surface of *Giardia* (22). Therefore, the finding of a cysteine-rich protein(s) in *Giardia* is not unexpected.

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Antigenic variation has been studied in a number of other protozoa and bacteria. *Paramecium*, a ciliated protozoan, also has a cysteine-rich (11%) surface protein that undergoes antigenic variation (23–25). Change from one immobilization (i) antigen to another may be induced by antiserum against the expressed antigen or by changes in environmental conditions, and a cytoplasmic factor has a major effect on which i-antigen is expressed (23).

The variant surface antigens of *Giardia* show stable expression for a limited number of generations. The entire population does not shift from expression of one antigen to another; rather, a small subpopulation already expresses a new antigen and is selected by antiserum (Nash, T. E., et al., submitted for publication). The gene for CRP170 of *Giardia* appears to be part of a multigene family, and DNA rearrangements involving this gene family are frequent. In contrast, the i-antigen genes of *Paramecium* are single copy genes and have not been observed to undergo rearrangements (26).

Certain isolates of *Trichomonas vaginalis* are able to alternate between two different phenotypes (27), an ability that is associated with the presence of a dsRNA virus (28). This phenomenon is clearly different from antigenic variation in *G. lamblia* in a number of ways: (*a*) the repertoire of surface antigens in *Giardia* appears extensive and reversion back to expression of CRP170 has not been observed (Nash, T. E., et al., submitted for publication); (*b*) the WB isolate used for these studies does not possess the dsRNA virus (Wang, C. C., personal communication) that is present in some isolates of *Giardia* (29); (*c*) in phenotypic variants of *T. vaginalis*, the previous surface antigen, while not present on the surface, is present in whole cell lysate (27).

Antigenic variation in *Neisseria gonorrhea* (30–32), *Borrelia hermsii* (33), and *Trypanosoma brucei* (34) is associated with DNA rearrangement. The most common mechanism of DNA rearrangement leading to antigenic variation is duplicative transposition in *N. gonorrhea* and in *T. brucei*. Each organism has a repertoire of silent genes for the variant antigen, Variant Surface Glycoprotein (VSG) in *T. brucei* and the pilus and opacity proteins in *N. gonorrhea*. Antigenic variation occurs when a silent gene is duplicated and the extra copy is placed into an expression site, displacing the previously expressed gene. *B. hermsi* has a repertoire of silent genes and one expression site for the Variable Major Protein (VMP), but in this case, the mechanism of antigenic change may involve a mechanism other than duplicative transposition (33).

Although our data do not show a clear correlation of antigenic change in *Giardia* with DNA rearrangement, there is extensive rearrangement involving the CRP170 gene family, and it may be that a correlation will be seen when a different portion of the CRP170 gene is used as a probe. Indeed, the rapid rate of change (10^{-3}) (Nash, T. E., et al., submitted for publication) is consistent with programmed DNA rearrangement as a potential mechanism of antigenic change in *Giardia* (35). Further investigation using the entire CRP170 gene as well as CRP genes from antigenic variants will be required. Examination of the 5′ or 3′ flanking areas may explain whether changes in control regions result in selective expression of any particular gene.

In addition to the mechanism of antigenic variation, a number of important questions remain. Does antigenic variation also occur in vivo, or is it an in vitro

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phenomenon? Do other *Giardia* isolates also undergo antigenic variation? Since *Giardia* may cause a chronic infection, it is conceivable that antigenic variation may play a role in the chronicity as it does in infections caused by African trypanosomes and *Borrelia*. It is hoped that human volunteer and animal model studies will shed further light on these important questions.

Summary

The WB isolate of *Giardia lamblia* expresses a cysteine-rich 170-kD surface antigen (CRP170) that undergoes antigenic variation. An (6E7), cytotoxic for isolates expressing CRP170, was used in another study to select antigenic variants from clones of the WB isolate of *Giardia*. CRP170 was replaced by surface-labeled bands ranging in size from ~50 to 170 kD.

In this study, mAb 6E7 was used to isolate a 1-kb portion of the CRP 170 gene (M2-1) from a λgt11 expression library. The M2-1 clone hybridized to a 5.4-kb transcript from isolates expressing CRP170 but did not hybridize to RNA from antigenic variants. Evidence was found for frequent rearrangements at the CRP170 gene locus.

DNA sequencing of the M2-1 clone revealed the presence of long tandem repeats. The putative amino acid sequence of M2-1 reveals a 12% cysteine content, and CRP170 is readily labeled in vivo with cysteine.

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