HIV Target Cells in Schistosoma haematobium-Infected Female Genital Mucosa

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Abstract. The parasite Schistosoma haematobium frequently causes genital lesions in women and could increase the risk of human immunodeficiency virus (HIV) transmission. This study quantifies the HIV target cells in schistosome-infected female genital mucosa. Cervicovaginal biopsies with and without schistosomiasis were immunostained for quantification of CD4+ T lymphocytes (CD3, CD8), macrophages (CD68), and dendritic Langerhans cells (S100 protein). We found significantly higher densities of genital mucosal CD4+ T lymphocytes and macrophages surrounding schistosome ova compared with cervicovaginal mucosa without ova (P = 0.034 and P = 0.018, respectively). We found no increased density of Langerhans cells (P = 0.25). This study indicates that S. haematobium may significantly increase the density of HIV target cells (CD4+ T lymphocytes and macrophages) in the female genitals, creating a beneficial setting for HIV transmission. Further studies are needed to confirm these findings and to evaluate the effect of anti-schistosomal treatment on female genital schistosomiasis.

INTRODUCTION

Schistosomiasis is a major cause of morbidity and mortality in sub-Saharan Africa.1 The waterborne parasite is co-endemic with human immunodeficiency virus (HIV) infection and several studies have suggested an association between the two diseases.2–6

The lower female reproductive tract is the main site of HIV-1 transmission, and is also the predilection site for female genital Schistosoma haematobium ova deposition.7–9 Similar to sexually transmitted infections (STIs), it has been suggested that genital lesions caused by S. haematobium ova may provide points of entry for HIV.3,10 The schistosome-infected cervix appears inflamed with abnormal mucosal blood vessels, contact bleeding, and damage to the mucosal surfaces.9

The CD4+ T lymphocytes located in the female genital mucosa are fundamental to sexual transmission of HIV-1 to women.11–13 Cervicovaginal CD68+ macrophages and Langerhans cells are also thought to play a key role.14,15 Studies have shown that CD4+ T lymphocytes and macrophages are present in the local immune response to Schistosoma mansoni ova.16

The aim of this study was to quantify the density of periovular CD4+ T lymphocytes, macrophages, and Langerhans cells in schistosome-infected female genital mucosa.

MATERIAL AND METHODS

Study subjects. The study populations have previously been described in detail.17,18 In short, following informed consent, a medical history was taken, a gynaecological examination performed, and biopsies from the cervix and/or vagina sampled from 61 sexually active Malawian women 15 to 49 years of age with urinary S. haematobium infection. Female genital schistosomiasis was defined as S. haematobium ova found in cervicovaginal biopsies. Malawian women with urinary schistosomiasis but without genital S. haematobium ova served as endemic negative controls. Serological testing was not performed. Non-endemic control specimens were drawn from the diagnostic tissue biobank at Oslo University Hospital. Biopsies with normal morphology of the ectocervix and biopsies with chronic non-specific cervicitis were included as non-endemic negative and positive controls, respectively.

Histopathology and immunohistochemistry. The biopsies were fixed in formalin, routinely processed, embedded in paraffin, serial sectioned, and stained. The results of the histological examination of hematoxylin and eosin (HE) stains have been published previously.19

The immunohistochemical stains were performed using Benchmark XT, antibody diluent (251-018), and Detection Kit Ventana ultraView Universal DAB (760-500) (Ventana Medical Systems, Inc., Tucson, AZ). Similar to findings in previous immunohistochemical studies, antibodies to CD4 cross-reacted non-specifically and could not be interpreted.20 Therefore, the density of CD4+ T lymphocytes was quantified by subtracting the density of CD8+ cells (cytotoxic T cells) from the density of CD3+ cells (mature T lymphocytes) on two consecutive 3.5 μm thick sections of each biopsy. The T cells were identified by applying mouse monoclonal antibodies to CD8 (C8/144B, Dako Denmark AS, Glostrup, Denmark) and rabbit monoclonal antibodies to CD3 (clone 2GV6, Ventana), respectively. Macrophages were identified using mouse monoclonal antibodies to CD68 (clone KP1, Dako Denmark) and Langerhans cells were identified using rabbit polyclonal antibodies to S100 protein (Ventana). Negative and positive controls were applied and the immunohistochemical stains were controlled by morphological identification. Unfortunately, the limited quantity of formalin-fixed biopsies in this study proved insufficient for further analyses of receptor expression or activity.

To study the histopathology directly adjacent to the ova, only biopsies with ova present were included in the analyses. Ova negative slides were either included as negative endemic controls or excluded if ova had been found in a previous section of the same biopsy.21 The maximum number of excluded specimens caused by undetectable ova was 18, whereas six endemic cases and 10 endemic controls were excluded because of insufficient tissue.

Microscopy and image analysis. The sections were examined using a Leica DM3000 microscope (Leica Microsystems
The morphological and immunohistochemical analyses were performed in a standardized manner. First, the HE-stained sections were evaluated. Schistosome ova were defined as “viable” if miracidia with eosinophilic glands or germinal cells were seen, whereas ova containing dark purple stain identified histologically as calcification were defined as “calcified.” For the quantification of T lymphocytes and macrophages, the HE-stained sections were photographed with a Leica DFC420 digital camera. To avoid the influence of adjacent but undetected ova, the biopsies were photographed only in areas containing ova. In biopsies with more than one cluster of ova, the most representative area for the respective biopsy’s tissue reaction was included. Likewise, because ova are usually located subepithelially, the control biopsies were also photographed in a subepithelial area representative for the main tissue reaction in the respective biopsy. Thereafter, the near-exact same areas of the consecutive immunostained sections, identified by histological anatomical structures, were photographed. The investigators were blinded for the immunostains until the HE-stained sections had been chosen and photographed. All sections were examined microscopically and all photographs controlled by an experienced pathologist (BR).

The principles for the computer-assisted image analyses of immunostained target cells are outlined in Figure 1. Stromal cell structures stained with antibodies to CD3, CD8, and CD68 were quantified using ImageJ, a Java image processing application downloaded free of charge from the website of the National Institutes of Health (NIH). The computer algorithms for the image analyses were developed according to previously described techniques. An outline of the measured structures was superimposed on the original photograph to permit manual validation. For the quantification of T lymphocytes, intact lymphocytic cells were counted separately from non-intact cell structures histopathologically identified as apoptotic cell fragments. All photographs and cell counts were controlled by an experienced pathologist and if necessary a second senior pathologist was consulted.

The statistical analyses and sample size estimation were performed using SPSS version 16.0 (SPSS, Inc., Chicago, IL) and PS Power and sample size calculations version 2.1.31. Most variables were not normally distributed and non-parametric tests were therefore used when studying associations. Medians and interquartile ranges (IQR) were used to describe the results. The Mann-Whitney U and
Kruskal-Wallis H tests were applied where appropriate. Spearman’s rank correlation coefficient was used when studying associations between continuous variables. Intra- and inter-observer reliabilities were determined by calculating the intra-class correlation coefficients after log-transformation of the data. A 5% significance level was used throughout.

**Ethical considerations.** Permissions for the histopathological and immunohistochemical investigations of anonymized archival Malawian and Norwegian biopsies, without additional consent from the study subjects, were granted by the National Health Science and Research Committee of Malawi (2009/ NHSRC #620) and the Norwegian Regional Ethics Committee (2009/1250a). The permissions were based on the fact that the proposed analyses did not require identifiable information or history, did not have any direct relevance to the physical, mental or social well-being, and did not have any direct diagnostic or therapeutic implications for the study women.

Study information was provided to the original study population in the local languages Yao and Chichewa and free informed oral consent was obtained. Following consent, all women who had urinary schistosomiasis were offered gynaecological examination. Consent was also reascertained by the physician before each step of the investigations and sampling. Treatment and follow-up for schistosomiasis, STIs, cancer, and other conditions were given in collaboration with the physicians in Mangochi District Hospital. The participating women were not asked for HIV testing. All women, including those who declined further investigations, were offered treatment with praziquantel. All non-endemic controls were followed up by the referring clinician in Norway.

**RESULTS**

The median age of the endemic women with genital *S. haematobium* ova was 20.5 years of age (IQR = 18.0–28.0), of the endemic controls without ova 26.0 years (IQR = 22.0–32.0), and of the non-endemic controls 44.0 years (IQR = 30.5–55.5). Of the endemic cases included in this study, schistosome ova were found in 19 of 42 (45%). The median number of ova per high-power field was 4 (IQR = 2–8), of which 84% were calcified and viable ova were found together in the subepithelial tissue. Calcified and viable ova were found in 19 of 42 (45%). As shown in Figure 3, the highest densities of *S. haematobium* ova compared with endemic and non-endemic tissue without ova (asterisk, *P* < 0.05). The circles represent outliers (values >1.5 times IQR). The most representative area for each biopsy was carefully selected by an experienced pathologist. In biopsies containing ova, the most representative high-power field surrounding the ova was analyzed. (1) Non-endemic = Norwegian (*N* = 45). (2) Endemic = Malawian (*N* = 29). (3) There were no significant differences between specimens with viable schistosome ova (*N* = 2) and the other study groups.

**Figure 3.** The density of CD4 + T lymphocytes in each study group. The density of CD4 + T lymphocytes/mm² cervicovaginal stroma was significantly higher surrounding calcified *Schistosoma haematobium* ova compared with endemic and non-endemic tissue without ova (*P* < 0.001, respectively). There was no significant difference in the density of CD4 + T lymphocytes between tissue with viable ova (*N* = 2) and tissue with calcified ova (*N* = 11) or endemic controls without ova (*P* = 0.69 and *P* = 0.89, respectively). As shown in Figure 3, the highest densities of genital mucosal CD4 + T lymphocytes were found in tissue with calcified *S. haematobium* ova and in non-endemic chronic cervicitis, between which there was no significant difference (*P* = 0.082). Furthermore, the density of intact CD4 + T lymphocytes was significantly associated with the density of apoptotic CD4 + cells (*P* = 0.026). There was no significant association between the density of CD8 + T cells and schistosome ova in female genital mucosa (*P* = 0.23).

**Macrophages.** The density of CD68 + macrophages was significantly higher in cervicovaginal mucosa with viable *S. haematobium* ova compared with endemic women with calcified ova (*P* = 0.044), endemic women without genital schistosomiasis (*P* = 0.018), and non-endemic women with healthy cervical tissue (*P* = 0.006). The highest densities of macrophages were found in cervicovaginal mucosa with viable ova and in non-endemic chronic cervicitis, and between them there was no significant difference (*P* = 0.32). The lowest densities of macrophages were seen in cervicovaginal tissue with calcified ova and endemic and non-endemic controls without ova, and between them there was no significant difference (*P* = 0.45).

**Langerhans cells.** We found no difference in the density of genital mucosal Langerhans cells between women with genital *S. haematobium* ova and endemic or non-endemic controls without ova (*P* = 0.25 and *P* = 0.43, respectively). Furthermore, we found no association between the densities of genital mucosal Langerhans cells and schistosome ova in female genital mucosa (*P* = 0.23).

**CD4 + T lymphocytes.** As shown in Figure 3, the density of genital mucosal CD4 + T lymphocytes surrounding calcified schistosome ova was significantly higher compared with endemic and non-endemic controls without ova (*P* = 0.034 and *P* < 0.001, respectively). There was no significant difference in the density of CD4 + T lymphocytes between tissue with viable ova (*N* = 2) and tissue with calcified ova (*N* = 11) or endemic controls without ova (*P* = 0.69 and *P* = 0.89, respectively). As shown in Figure 3, the highest densities of genital mucosal CD4 + T lymphocytes were found in tissue with calcified *S. haematobium* ova and in non-endemic chronic cervicitis, between which there was no significant difference (*P* = 0.082). Furthermore, the density of intact CD4 + T lymphocytes was significantly associated with the density of apoptotic CD4 + cells (*P* = 0.026). There was no significant association between the density of CD8 + T cells and schistosome ova in female genital mucosa (*P* = 0.23).
of CD4+ T lymphocytes and epithelial Langerhans cells in the genital mucosa of endemic women with and without ova ($P = 0.88$).

**Medical history.** The density of mucosal CD4+ T lymphocytes was not associated with the different menstrual phases (follicular $P = 0.15$ and luteal $P = 0.73$, respectively). Our sample was too small to perform statistical analyses correcting for the frequency of sexual intercourse, prior treatment of STIs, or prior anti-schistosomal treatment.

**DISCUSSION**

*Schistosoma haematobium* ova frequently cause lesions in the female genital mucosa and studies suggest that female genital schistosomiasis may increase the risk of HIV transmission.2,4-26 In this study from Malawi, cervicovaginal mucosa with *S. haematobium* ova contained more CD4+ T lymphocytes and macrophages than genital mucosa without ova. The CD4+ T lymphocytes were associated with calcified ova and CD4+ cell apoptosis. Calcified ova are typical of chronic *S. haematobium* infection, although cell apoptosis may indicate an active, ongoing immune reaction in this chronically infected tissue.27 Viable *S. haematobium* ova on the other hand, were associated with an increased density of macrophages. These findings suggest that both recent and chronic *S. haematobium* infection stimulate tissue responses in the cervicovaginal mucosa that could influence HIV susceptibility.

In this study, the density of epithelial Langerhans cells in the cervicovaginal mucosa was not associated with schistosome ova in the underlying stroma. A recent study indicates that the function of dendritic cells might be impaired in human *S. haematobium* infection.28 Results from experimental studies indicate that *S. mansoni* infection may activate dendritic cells by a modulated pathway.29 It was not possible to explore dendritic cell function or activation in these formalin-fixed specimens because of their limited sizes.

Previous histopathological studies of human *S. haematobium* infection in the urinary and female genital tracts have shown a range of immune cells surrounding parasite ova.19,21,22,27,30-35 These studies did not include control populations. However, the findings are in line with results from experimental studies on hepato-intestinal schistosomiasis.26-38 To our knowledge, this study from Malawi is the first to quantify the periovular density of HIV target cells in schistosome infected cervicovaginal mucosa.

The sample size is small and the findings are prone to type 1 and type 2 errors. This makes the interpretation of the results related to viable ova difficult, particularly when they are non-significant. Furthermore, in presumed negative cases, schistosome ova might have been located just outside the studied sections. The density of CD4+ T lymphocytes was estimated from the number of CD3+ and CD8+ cells on two consecutive 3.5 μm thick sections. Although the thickness of the sections is less than half the average diameter of a lymphocyte, the reported CD4+ cell number may to some extent differ from its true count.25 Our calculation of CD4+ T lymphocytes may be slightly underestimated because antibodies to CD8 also recognize some γδ T cells and natural killer cells (Dako Denmark).

Some shortcomings, if resolved, would probably increase the analyzed differences between cases and controls. First, it is probable that some endemic controls have a missed diagnosis of genital schistosomiasis.9 Second, the HIV prevalence has subsequently been estimated to be approximately 10% of the population in 1994,39 but individual HIV diagnosis was not a part of the original project and could therefore not be done. The HIV-1 infection would likely reduce the CD4+ cell density.13

In a clinical cross-sectional study in Zimbabwe, the odds for being infected with HIV was nearly three times higher in women with genital schistosomiasis than in women without.4 The results from our study suggest that periovular CD4+ T lymphocytes could play an important role in the interaction between the two infections.40 Clinical data indicate that female schistosomiasis causes chronic damage to the cervicovaginal mucosa and urinary tract if not prevented by anti-schistosomal treatment in childhood.1,14 Mass treatment is deemed necessary to prevent morbidity and reinfection and has been implemented on a large scale in several African countries.40

In conclusion, this study shows that *S. haematobium* ova in female genital mucosa are surrounded by an increased density of CD4+ T lymphocytes and macrophages. The CD4+ T lymphocytes are concentrated around calcified ova, indicating that *S. haematobium* ova may sustain an increased long-lasting HIV susceptibility in the cervicovaginal mucosa. Further studies are needed to validate these findings, to further explore the immunopathology, and to evaluate the effect of preventive anti-schistosomal mass treatment on female genital schistosomiasis.

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