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Lesions of Primary and Secondary Syphilis Contain Activated Cytolytic T Cells†

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This study demonstrates that $CD8^+$ cytotoxic lymphocytes (CTL) are found in both primary and secondary syphilis lesions. $CD8^+$ T cells were detected by immunohistology, and mRNAs for granzyme B and perforin were detected by reverse transcription and PCR, suggesting that $CD8^+$ cytotoxic lymphocytes are activated.

Syphilis has primary and secondary clinical stages, with large numbers of *Treponema pallidum* organisms found in mucous membrane and skin lesions. The majority of the organisms are cleared from lesions by the immune response, although some spirochetes persist in the host and can lead to severe late manifestations of infection. Several observations support the hypothesis that cellular immunity is more important than humoral immunity in the clearance of *T. pallidum* from early syphilis lesions (9). First, secondary syphilis lesions, containing high numbers of bacteria, develop in the presence of high titers of antibody against treponemal antigens. Second, T lymphocytes and macrophages are the major infiltrating cells in primary and secondary syphilis and are associated temporally with *T. pallidum* clearance (10, 12). Finally, *T. pallidum* antigens can be seen within macrophages in healing lesions (11), and macrophages can ingest and kill *T. pallidum* in vitro (1). Our earlier studies (15) have demonstrated a Th1 cytokine response in primary and secondary lesions, consistent with a role for activated macrophages in *T. pallidum* clearance. This cytokine milieu is also conducive to cytotoxic T lymphocyte (CTL) development (2, 15).

Although $CD8^+$ cells have been demonstrated in syphilis lesions (3, 14), it is unclear whether these cells function as CTLs or perform some other function. Functional activity of CTLs can be implied by the presence of mRNA for perforin and granzyme B, two proteins believed to be responsible for cytolysis of target cells (5, 8). In this study, primary and secondary syphilis lesions were examined for the presence of activated $CD8^+$ CTLs by immunohistology and testing for the presence of perforin and granzyme B mRNA.

Biopsy specimens were obtained from five patients with primary syphilis chancres and eight patients with secondary syphilis lesions at a sexually transmitted disease clinic in Nairobi, Kenya (Table 1). Informed consent was obtained under a protocol approved by the human subjects committees at the University of Nairobi and University of Washington. Human immunodeficiency virus (HIV) serologic testing was performed on each subject, but $CD4^+$ T-cell counts were not obtained. Biopsies of healthy skin and a lesion of recurrent herpes simplex virus type 2 (HSV-2) (gift of David Koelle, University of Washington) were used as controls. Punch biopsy specimens (diameter, 4 mm) were obtained, embedded in tissue-freezing medium (Miles, Elkhart, Ind.), and stored at −70°C. These specimens were previously used to investigate the presence of cytokines in syphilitic lesions (15).

The clinical characteristics of the patients are listed in Table 1. *T. pallidum* was identified in all lesions by dark-field microscopy. The serum samples of four of five patients with primary syphilis were nonreactive in the rapid plasma reagin (RPR) test, consistent with very early infection. All patients except two with secondary syphilis had reactive RPR test results; these two had reactive results on follow-up. Five of the thirteen syphilis patients were infected with HIV-1.

Cryostat sections of syphilis lesions were stained by using mouse monoclonal antibodies, a biotin-coupled horse anti-mouse antibody, and avidin-biotin-peroxidase complexes (Vector Laboratories, Burlingame, Calif.). The sections were developed with diaminobenzidine-NiCl and counterstained with methyl green. Controls with the use of an isotype-matched monoclonal antibody directed to irrelevant antigens were negative in matched specimens (data not shown). The monoclonal antibodies used were anti-CD4 (T4; Coulter Corp, Hialeah, Fla.), anti-CD8 (T8; Coulter), and anti-CD56 (NKH-1; Coulter). The cellular infiltrates of syphilis contained many $CD4^+$ and $CD8^+$ T cells (Fig. 1) in almost even proportions. Virtually no $CD56^+$ cells were found (data not shown), demonstrating that these lesions contain few natural killer (NK) cells.

To investigate whether the $CD8^+$ T cells were likely to have cytolytic activity, the lesions were studied by reverse transcription and PCR for granzyme B and perforin mRNA (5, 8). Total RNA was isolated from half of a 4-mm punch biopsy excised from the frozen block and homogenized with a disposable pestle (Kontes, Vineland, N.J.) in RNAzol B solution (Biotecx Laboratory, Friendswood, Tex.). cDNA was synthesized from total RNA as previously described (15). Hypoxanthine phosphoribosyltransferase (HPRT) cDNA was quantitated by competitive PCR, and each sample was adjusted for equivalent concentrations of HPRT cDNA (15). Two microliters of each HPRT-equivalent cDNA was used in a 50-μL PCR reaction mixture containing 50 mM Tris (pH 8.3), 10 mM MgCl, 200 μM deoxynucleoside triphosphate, 1 μM (each) specific 5′ and 3′ oligonucleotide primer, and 4 U of *Taq* polymerase. The PCR cycling conditions were 94°C for 1 min and 65°C for 2 min for 40 cycles with the oligonucleotides GGGGAAGCTCCA TAAATGTCACCT (sense) and TACACACAAGAGGGCCT CCAGAGT (antisense) (7) for granzyme B and 94°C for 1 min

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† This paper is dedicated to the memory of our friend and colleague James M. Nasio.

TABLE 1. Clinical findings and CTL mRNA for 13 subjects with primary or secondary syphilis

Diagnosis and patient no.	HIV status	Result for:			
		RPR test	Dark-field microscopy	Granzyme B	Perforin
Primary syphilis					
1	−	−	+	+	+
2	−	+	+	+	+
3	+	−	+	+	+
4	+	−	+	+	+
5	+	−	+	+	+
Secondary syphilis					
6	−	+	+	+	+
7	−	−[a]	+	+	+
8	−	−[a]	+	+	+
9	−	+	+	+	+
10	−	+	+	+	+
11	−	+	+	+	+
12	+	+	+	+	+
13	+	+	+	+	+
Herpes simplex virus	−			+	+
Healthy skin	−			−	−

[a] Undiluted serum sample was RPR negative at initial visit but was RPR positive at follow-up. Additional serum samples from the initial visit were unavailable to determine whether a prozone effect was responsible for the initial negative RPR test result.

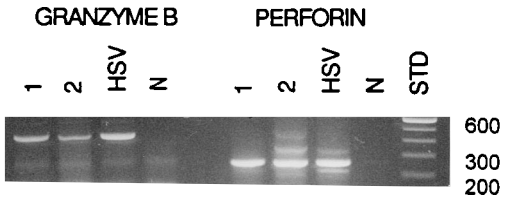

FIG. 2. Ethidium bromide-stained PCR products. PCR products representing mRNA for granzyme B (432 bp) and perforin (250 bp) are found in specimens from patients with primary (patient no. 4) and secondary (patient no. 10) syphilis and recurrent herpes simplex virus but not in healthy skin specimens.

and 58°C for 2 min for 40 cycles with the oligonucleotides GCAATGTGCATGTGTCTGTG (sense) and GAAGTGG GTGCCGTAGTTGG (antisense) for perforin. Ten microliters of the PCR product was electrophoresed on a 2% agarose gel and stained with ethidium bromide. Negative and positive controls, including no cDNA, cDNA from healthy skin, cDNA from herpetic skin, and cDNA from phytohemagglutinin-stimulated peripheral blood mononuclear cells, were included in each run. The PCR products for granzyme B, perforin, and HPRT were confirmed to be derived from the expected cDNA by restriction endonuclease cleavage with *Ava*II, *Ava*II, and *Hin*cII, respectively (data not shown). All syphilis specimens and the HSV and phytohemagglutinin control specimens had mRNA for perforin and granzyme B, but the healthy skin biopsies lacked mRNA for perforin and granzyme B (Fig. 2

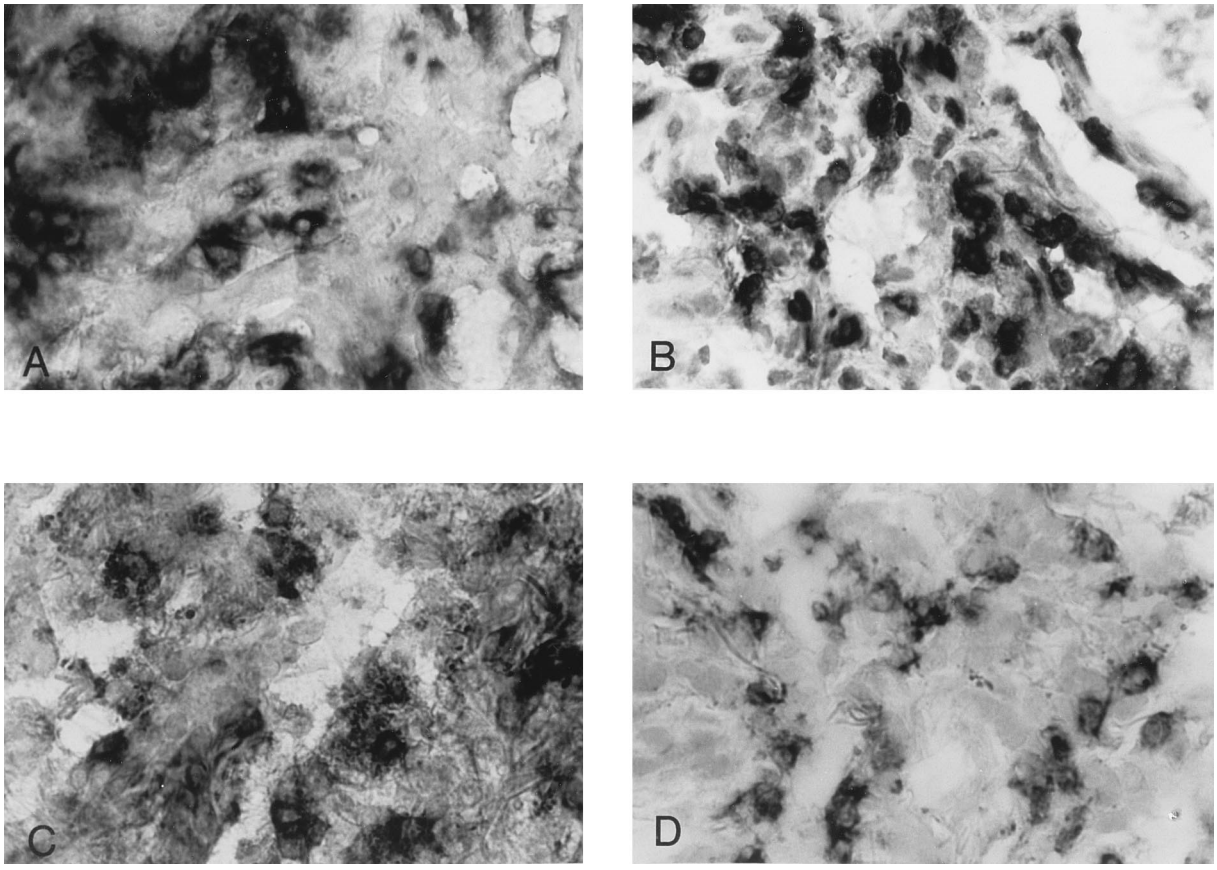

FIG. 1. Photomicrographs of immunohistochemically stained frozen sections. $CD4^+$ (A and C) and $CD8^+$ (B and D) cells are found in the infiltrates of a representative case of primary (patient no. 5) (A and B) or secondary (patient no. 13) (C and D) syphilis. Few, if any, $CD56^+$ (NK) cells were seen in any of the lesions (data not shown). Magnification, ×400.

and Table 1). This finding indicates that cytotoxic lymphocytes are present in the syphilis infiltrates.

The findings of an abundance of $CD8^+$ T cells, the paucity of NK cells, and the presence of mRNA for both granzyme B and perforin support a role for activated $CD8^+$ CTLs in the immune response to syphilis. While it is remotely possible that the granzyme B and perforin mRNAs originate from $CD4^+$ CTLs or from the small numbers of NK cells in the lesions, the more likely conclusion is that the abundantly present $CD8^+$ cells have cytolytic function. This suggests that the levels of interleukin-2, interleukin-12, and gamma interferon found in syphilis lesions (15) are biologically relevant, as these cytokines are major stimulators of $CD8^+$ CTL proliferation and activation (2), as well as stimulators of macrophage activation. The $CD8^+$ T cells are likely responding to *T. pallidum* antigens presented via the major histocompatibility complex class I pathway. *T. pallidum* has been found free in the cytoplasm of nonphagocytic cells (13), and *T. pallidum* antigens could enter the class I pathway from this reservoir. Alternatively, macrophages can present ingested exogenous antigens via the class I pathway (4, 6). The role of $CD8^+$ cells in the immune response to *T. pallidum* is not clear but could include lysis of cells harboring intracellular *T. pallidum* or production of gamma interferon to enhance phagocytosis and killing of treponemes by macrophages.

No qualitative differences were seen in perforin or granzyme B mRNA in primary versus secondary syphilis, or in HIV-infected versus HIV-uninfected patients. The pathologic and microbiologic changes are similar in primary and secondary syphilis, and it is not surprising that the cytokine and enzyme milieus are similar. Although not addressed in our study, quantitative analysis might reveal subtle differences in the amounts of cytokines or enzymes, which could account for the perceived delay in healing of syphilis lesions in HIV-infected patients.

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