

## Varicella Zoster Virus–Specific Immune Response after Treatment with Sodium Stibogluconate for Cutaneous Leishmaniasis

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**Abstract.** Sodium stibogluconate has been associated with the reactivation of varicella zoster virus (VZV) in otherwise healthy adults who receive the drug as treatment for cutaneous leishmaniasis. Ten patients receiving daily sodium stibogluconate underwent phlebotomy at baseline and at day 10. Flow cytometry–based immunophenotyping, VZV-specific IgG levels, and lymphocyte proliferative responses and intracellular cytokine secretion to VZV, cytomegalovirus, tetanus toxoid, superantigen, and mitogens were performed at both time points. The absolute number of total leukocytes, total lymphocytes, and lymphocyte subsets decreased overall without predilection for any particular subset of lymphocytes, such that the percentage of the total lymphocyte population for each lymphocyte subset did not change significantly (except for a marginal increase in percentage of cytotoxic T cells). Antibodies to VZV were measured in seven patients before and after treatment, and did not change. Lymphocyte proliferative responses to VZV and other antigens and mitogens did not change from baseline. The mechanism for the increased rate of VZV reactivation after treatment with sodium stibogluconate remains undefined.

### INTRODUCTION

Leishmaniasis is a parasitic disease transmitted by the bite of a phlebotomine sandfly, with manifestations of infection ranging from asymptomatic disease to self-healing skin sores to progressive visceral infection and death, depending on the strain of the infecting organism and the immune status of the host.<sup>1</sup> For decades, the standard therapy for most forms of leishmaniasis has been sodium stibogluconate, which is available in the United States as Pentostam® (GlaxoSmithKline, London, United Kingdom).

Sodium stibogluconate, although efficacious, has been associated with numerous toxicities including pancreatitis, hepatitis, myalgias/arthralgias and electrocardiographic changes.<sup>2,3</sup> Varicella zoster virus (VZV) infection can occur in patients treated with sodium stibogluconate and various other antimonial compounds.<sup>4–6</sup> In a review of patients treated with sodium stibogluconate at one center, incidence of VZV reactivation (shingles) was 3.6%, which was greater than the reported incidence of 0.2% in healthy persons 20–30 years of age.<sup>7</sup> More recently, a patient with concomitant shingles and VZV meningitis after sodium stibogluconate treatment has been reported.<sup>8</sup>

The mechanism for the reactivation of VZV is not completely understood. Presumably, latent VZV can reactivate and produce symptoms when VZV-specific cell-mediated immunity decreases below a critical threshold level.<sup>9</sup> For example, the decrease in VZV-specific cell-mediated immunity that occurs with age is associated with an increased frequency of VZV infection.<sup>10</sup> In a prospective study of eight patients with cutaneous leishmaniasis receiving sodium stibogluconate, a decrease in helper T cells of 306 cells/mm<sup>3</sup> or 67% of baseline was reported by day seven of treatment. The investigators surmised that the decrease in T cells occurring with treatment with sodium stibogluconate may have predisposed

to VZV reactivation.<sup>7</sup> The current study seeks to expand the findings of this report by examining the antibody- and cell-mediated immune response to VZV that occurs during treatment with sodium stibogluconate.

### METHODS

The study was performed under an approved human use protocol, and all study volunteers provided written informed consent. Patients with a confirmed diagnosis of cutaneous leishmaniasis who were scheduled to receive sodium stibogluconate for treatment of leishmaniasis were offered participation in the study. Phlebotomy was performed at baseline and then after 10 daily doses ( $\pm$  one dose) of sodium stibogluconate (20 mg/kg/day, with no upper limit on dose). A total of 32 mL of blood was collected at each time point in serum-separator tubes, heparin tubes, and cell-preparation tubes. Peripheral blood mononuclear cells (PBMCs) were separated from heparinized blood by Ficoll-hypaque gradient centrifugation and cryopreserved.

Quantitative levels of IgG to VZV were measured by enzyme-linked immunosorbent assay according to the manufacturer's instructions (Wampole Laboratories, Princeton, NJ). Phenotypes of T cell subsets and natural killer (NK) cells were determined by flow cytometry with fluorochrome-labeled monoclonal antibodies against the cell surface markers CD3, CD4, CD8, CD45RO, CD25, CD16, and CD56 (all from BD Bioscience, San Jose, CA). Briefly, 100  $\mu$ L of whole blood was stained with appropriate antibodies at room temperature for 30 minutes. After incubation, erythrocytes were lysed with a fluorescent-activated cell sorter (FACS) lysing solution (BD Bioscience) and T cell subsets and NK cells were analyzed using a FACSCalibur flow cytometer and CellQuest Analysis Software (BD Bioscience).

A lymphocyte proliferation assay (LPA) was used for pre-stibogluconate and day 10 samples by incubating  $1 \times 10^5$  PBMCs with VZV lysate at dilutions of 1:100 and 1:200 or control cell lysate prepared in the same way as the VZV lysate at a dilution of 1:100 (University of Colorado Hospital Clinic, Denver, CO). In addition, PBMCs were incubated

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with cytomegalovirus (CMV) lysate at concentrations of 5 and 2.5  $\mu\text{g/ml}$  (Advanced Biotechnologies Inc., Columbia, MD), tetanus toxoid at concentrations of 5 and 2.5  $\mu\text{g/ml}$  (Staten Serum Institute, Copenhagen, Denmark), and the mitogens (Sigma-Aldrich, St. Louis, MO) phytohemagglutinin (PHA, 2  $\mu\text{g/ml}$ ), concanavalin A (ConA, 20  $\mu\text{g/ml}$ ), and pokeweed mitogen (PWM, 1.25  $\mu\text{g/ml}$ ). After three days of incubation with the mitogen and six days with the tested antigens, cells were pulsed with 1  $\mu\text{Ci/well}$  of  $^3\text{H}$ -thymidine for six hours, harvested using the Tomtec, Mach3M (EG&G Wallac, Gaithersburg, MD), and counted in a 1450 microbeta tritium (EG&E Wallac). The data were expressed as a lymphocyte stimulation index (LSI) = PBMC counts per minute (cpm) + antigen/mitogen/PBMC cpm + medium to define antigen specificity. Positivity was defined as an LSI  $\geq 5$ .

Fresh heparinized blood was used in a whole blood intracellular cytokine secretion (ICS) assay as described previously.<sup>11</sup> Briefly, test samples were stimulated with VZV and CMV lysates (ABI, Columbia, MD), the negative control sample was stimulated with CD28/CD49d alone, and the positive control sample was stimulated with *Staphylococcus* enterotoxin B (SEB, Sigma, Milwaukee, WI) and CD28/CD49d. Samples were incubated at 37°C for 6 hours, and subsequently placed at 4°C overnight. After overnight incubation, 50  $\mu\text{L}$  of EDTA was added to each sample, and the samples were incubated for 15 minutes at room temperature. Five milliliters of FACS Lysing Solution (Becton Dickinson Immunocytometry Systems, San Jose, CA) was added to each sample. The samples were incubated for 10 minutes at room temperature and subsequently stored at -80°C. For staining, pre-treatment and post-treatment samples were thawed at 37°C and 7 mL of wash buffer was added to each sample. Samples were centrifuged at 1,800 rpm for 8 minutes and the supernatant was decanted. Two milliliters of wash buffer was added and each sample was divided between two staining tubes and centrifuged. The PBMCs were permeabilized with 500  $\mu\text{L}$  of Perm 2 (Becton Dickinson Immunocytometry Systems) for 10 minutes at room temperature. Samples were washed and stained by adding cocktails of antibodies consisting of either CD3/APC/CD8 fluorescein isothiocyanate/interferon- $\gamma$  (IFN- $\gamma$ ) phycoerythrin (PE)/interleukin-2 (IL-2)

PE or CD3/APC/CD4PerCpCy5.5/IFN- $\gamma$  PE/IL-2 PE. Cells were analyzed in a FacScan (Becton Dickinson, Mountain View, CA) and with FLOWJO software (Tree Star, Palo Alto, CA). Criteria for any antigen-specific response was that the net % CD8 + or CD4 + positive (unstimulated control sample background subtracted) must be  $\geq 0.05\%$  and  $3 \times$  background.

## RESULTS

A total of 11 adult patients with a diagnosis of cutaneous leishmaniasis requiring treatment with sodium stibogluconate were initially enrolled in the study. One patient was withdrawn from the study for reasons unrelated to the study, leaving 10 subjects for whom data was available. The mean dose of sodium stibogluconate administered to each patient was 1,865 mg (range = 1,336–1,955 mg). No patient developed reactivation of VZV during the study or during six months of follow-up. Compared with baseline, at day 10 the absolute number of total leukocytes decreased by a median of 2,400/mL ( $P = 0.004$ ), total lymphocytes by 800/mL ( $P = 0.002$ ), helper T cells by 265/mL ( $P = 0.002$ ), cytotoxic T cells by 159/mL ( $P = 0.002$ ), memory T cells by 321/mL ( $P = 0.002$ ), CD4CD25 + regulatory T cells by 29/mL ( $P = 0.006$ ), and NK cells by 54/mL ( $P = 0.004$ ). The percentage of the total lymphocyte population for each lymphocyte subset did not change significantly except for a marginal increase in percentage of cytotoxic T cells by 0.77% ( $P = 0.049$ ). Therefore, lymphocyte subset numbers decreased overall without predilection for any particular subset. Antibodies to VZV were measured and detected in seven patients before and after treatment, and did not change ( $P = 0.69$ ).

Four persons had positive IFN- $\gamma$ /IL-2 CD4 + T cell responses to VZV as detected by the whole blood ICS assay with a positive cut-off defined as the net IFN- $\gamma$ /IL-2 response 3 times the VZV control cell lysate and  $\geq 0.05\%$ . Only one person (LZ01) had a positive VZV response before and after treatment with sodium stibogluconate, two had responses before treatment but not after treatment (LZ05 and LZ06), and one had a response only after treatment (LZ11) (Table 1).

TABLE 1

Varicella zoster virus (VZV) T cell responses pre-treatment and post-treatment with sodium stibogluconate in *Leishmania*-infected persons

Subject	Time	Lymphocyte stimulation index (LSI)*			% CD4 interferon- $\gamma$ /interleukin-2-positive T cells†
		VZV 1:100	VZV 1:200	Control lysate	
LZ01	Pre-treatment	<b>34</b>	<b>12</b>	1	<b>0.08</b>
	Post-treatment	<b>144</b>	<b>1131</b>	1	<b>0.07</b>
LZ02	Pre-treatment	4	6	1	0.04
	Post-treatment	7	5	1	0.02
LZ04	Pre-treatment	18	10	1	0.04
	Post-treatment	1	1	1	0.02
LZ05	Pre-treatment	12	20	1	<b>0.06</b>
	Post-treatment	10	4	ND	0.03
LZ06	Pre-treatment	9	5	1	<b>0.51</b>
	Post-treatment	6	7	1	0.08
LZ07	Pre-treatment	8	6	1	0.04
	Post-treatment	40	25	1	0.04
LZ10	Pre-treatment	8	4	1	0.00
	Post-treatment	2	2	1	0.00
LZ11	Pre-treatment	9	8	1	0.03
	Post-treatment	6	4	1	<b>0.07</b>

\* Positive responses (LSI  $\geq 5$ ) are in **bold**. ND = not done.

† Results are shown with background (control lysate) subtracted. Positive cytokine responses (see text) are in **bold**.

The PBMCs from two persons secreted IFN- $\gamma$ /IL-2 after stimulation with CMV lysate, but the net % IFN- $\gamma$ /IL-2 positive CD4 + T cells did not change before and after treatment. None of the persons had detectable CD8 + T cell responses to VZV. Responses to the control antigen (SEB) were robust in all persons and ranged from 1% to 8% IFN- $\gamma$ /IL-2-positive T cells. The average CD4 + and CD8 + SEB response was  $3 \pm 1.1\%$  and  $3.1 \pm 1.8\%$  respectively.

For the thawed PBMCs used in the LPA, the mean  $\pm$  SD viability of the PBMCs was  $82\% \pm 6\%$ . Eight persons had LPA responses to VZV with an LSI up to 44; six of these persons had responses to VZV detected before and after treatment with sodium stibogluconate, and two persons had a positive LPA response to VZV before treatment but not after treatment (Table 1). All persons had robust responses to the three mitogens (PHA, PWM, and ConA). In five of the eight persons, pre-treatment and post-treatment mitogen responses were assessed, and the average LSI was 171 pre-treatment and 166 post-treatment. The LSI pre-treatment and post-treatment were highly correlated, and there was no significant difference between the values ( $P = 0.79$ , by paired  $t$  test). In three of eight patients, the pre-treatment LSI to the three mitogens was robust, but there were insufficient PBMC available after treatment for testing. In parallel PBMCs from two normal donors were assessed, and the LSI to VZV was  $< 3$ . None of the controls or patient samples responded to the control viral lysate preparation; all LSI were  $< 1$ . Responses to tetanus and CMV antigen were seen in five of eight persons, and the responses were similar pre-treatment and post-treatment.

## DISCUSSION

Despite several decades of use, the exact mechanism of action of sodium stibogluconate is unknown, as are the reasons for its side effects. The reactivation of VZV during or shortly after the administration of sodium stibogluconate is well-described, and is presumably caused by suppression of cellular immunity. The current study reports a decrease in CD4 + T cells of 265/mL at day 10 of therapy, which is similar to the decrease of 306/mL reported in a previous study.<sup>7</sup> The current study found no decrease in antibody titers against VZV, which is perhaps not surprising given the kinetics of antibody production and the relatively narrow time collection points.

The LPA and ICS analysis of cellular immune responsiveness before and after 10 daily doses of sodium stibogluconate found no changes in lymphocyte proliferation in response to stimulation with VZV. The LPA appeared to be more sensitive in detecting VZV-specific responses than the ICS assay. The range of LPA responses are in agreement with those of previous studies.<sup>12</sup>

The lack of a demonstrable decrement in functional cellular immune response to VZV during sodium stibogluconate therapy in this study may have several explanations. Reactivation of VZV, although more likely to occur after sodium stibogluconate therapy, is a relatively rare occurrence, and no patient in this study cohort experienced that adverse event. It is possible that patients who do end up developing VZV reactivation experience more marked impairment in their immune control of VZV than did patients in the current study.

Alternatively, the immunologic defect which sodium stibogluconate inflicts may not be measurable with the assays used in this study, and more precise and sensitive tools may be required. The time frame for measuring VZV-specific cellular immune responses post stibogluconate may not have been ideal because the interval between assays was only ten days. Demonstrable changes in cellular immune function may take longer to be observed. Finally, this was an observational study and the number of persons may have been too small to be able to detect significant changes in cellular responses.

Nevertheless, the findings of this study confirm that the administration of sodium stibogluconate results in a broad decrease in lymphocyte subsets, without a demonstrable effect on functional cellular immune response to VZV or a range of other antigens and mitogens. The general decrease in lymphocyte subsets does, however, translate to a decrease in absolute numbers of VZV-specific lymphocytes, possibly accounting for the increased risk for VZV reactivation. It remains unclear how long this period of decreased VZV-specific lymphocytes lasts because we were not able to measure the lymphocyte recovery period after discontinuation of sodium stibogluconate treatment. Potentially, VZV-specific T cell numbers may take longer to recover than other antigen-specific T cells or total lymphocytes, leading to increased VZV risk. Alternatively, VZV may be more likely to take advantage of specific cellular immune suppression than other potential infectious agents, resulting in the cases of VZV reactivation seen after sodium stibogluconate treatment.

Future studies could examine the kinetics of total and antigen-specific lymphocyte recovery, as well as target those patients who actually experience VZV reactivation after receiving sodium stibogluconate. For now, the exact relationship between VZV reactivation and sodium stibogluconate still remains undefined, and future investigation is required to explain this relationship.

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