Effects of 6-Chloro-2', 3'-Dideoxyguanosine (6-Cl-ddG) in Surface Lymph Nodes of Rhesus Monkeys (Macaca mulatta) Chronically Infected with Simian Immunodeficiency Virus (SIVmac239)

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ABSTRACT. We studied the effects of 6-chloro-2', 3'-dideoxyguanosine (6-Cl-ddG), an antiretroviral drug, in surface lymph nodes of rhesus monkeys (Macaca mulatta) chronically infected with simian immunodeficiency virus (SIV). The rhesus monkeys were treated with 25 mg/kg of 6-Cl-ddG every 8 hr for 2 weeks. We performed sequential biopsies of the surface lymph nodes three times: before, during, and after the drug treatment. The 6-Cl-ddG dramatically decreased the number of infectious virus (measured by limiting dilution assay) in lymph node mononuclear cells. This decrease was consistent with the decrease in the number of viral RNA-positive cells in lymph nodes (analyzed by in situ hybridization). Histopathological analysis revealed that hyperplastic lymphoid follicles were reduced in size, especially, enlarged areas of centroblasts in lymphoid follicles (the so-called dark areas of germinal centers) were declined. Our results demonstrated that 6-Cl-ddG decreased the viral burden concomitantly with reduced hyper-activation of germinal centers in lymphoid follicles of SIV-infected rhesus monkeys. — KEY WORDS: antiretroviral drug, 6-chloro-2', 3'-dideoxyguanosine, histopathological change, lymph node, simian immunodeficiency virus.

Lymph nodes are known to be major reservoirs of human immunodeficiency virus (HIV) and main sites for HIV replication [4, 5, 8, 10, 16]. Many researchers have clearly demonstrated that a large amount of HIV persistently replicate in lymph nodes even during the asymptomatic stage of infection (indicators of this stage are a CD4+ T cell count exceeding 300/µl in peripheral blood and a low viral burden in plasma) [8, 16]. Moreover, the high viral burden in lymph nodes may result in lymphoid histological disorders (e.g., follicular hyperplasia, follicular involution and lymphoid depletion) [18]. Therefore, in antiretroviral therapy, reducing the viral burden in lymph nodes and relieving the lymphoid structural disorders are essential. However, many studies that evaluated antiretroviral drugs focused on the kinetics of CD4+ T cells and on the viral burden in peripheral blood [2, 6, 20, 24]. Little is known about the effect of antiretroviral drugs in lymph nodes. In this study, we examined direct effect of an antiretroviral drug in lymph nodes.

The 6-chloro-2', 3'-dideoxyguanosine (6-Cl-ddG) is a highly lipophilic antiretroviral drug [9, 15, 22], whose antiretroviral activity is as high as that of ddG or that of AZT [22]. Manouilov et al. demonstrated that lipophilicity is a key element in the targeting of antiretroviral drug to lymph nodes [14]. They showed that, in mice, administered AZT rapidly disappear from lymph nodes compared with the lipophilic prodrug of AZT, dipalmitylphosphatidyl-AZT [14]. The unique structure of 6-Cl-ddG (i.e., the chlorine at portion 6 of 2', 3'-dideoxyguanosine) increases lipophilicity [22], and may promote efficacy of ddG in lymph nodes.

For evaluating the effects of antiretroviral drugs in vivo, rhesus monkeys infected with simian immunodeficiency virus (SIV) are most suitable at present [23]. During SIV infection in rhesus monkeys, histological disorders observed in lymph nodes are comparable to disorders during HIV infection in humans [1, 13, 17, 21, 25]. We recently reported significant effects of 6-Cl-ddG on the viral burden in plasma and in peripheral blood mononuclear cells of a rhesus monkey in the ARC/AIDS stage [9]. In this study, we investigated the effects of 6-Cl-ddG on viral burden and virus-induced histopathological abnormalities in surface lymph nodes of rhesus monkeys chronically infected with SIV.

MATERIALS AND METHODS

Animals: We used two juvenile rhesus monkeys (Mm9408 and Mm9421) that were intravenously inoculated with 100 TCID50 of SIVmac239 [19] 12 months before being used in our experiments. Although these monkeys manifested lymphoid adenopathy, p27 gag antigen in their plasma was undetectable before the experiments. For control studies, lymph nodes were biopsied from five SIV-negative and clinically healthy rhesus monkeys (Mm9406, Mm9416, Mm9419, Mm9422, Mm9427). All monkeys were
serologically negative for B virus, simian T-cell leukemia virus type I and simian type D retrovirus. This experiment was approved by the Animal Care and Use Committee of National Institute of Infectious Diseases, Japan.

Administration of 6-Cl-ddG: Two SIV-infected monkeys were subcutaneously administrated with 25 mg/kg of 6-Cl-ddG every eight hours for two weeks as previously described [9].

Sampling of lymph nodes: Axillary and inguinal lymph nodes were surgically biopsied under general anesthesia with intramuscular administration of 15 mg/kg of ketamine hydrochloride (Sankyo). The operations were done using a safety cabinet at the P3 facility in Tsukuba Primate Center. Each biopsied lymph node was immediately divided into two pieces at its hilum lymphonodi. One piece was used for histopathological analysis and the other was minced for making single cell suspension of lymph node mononuclear cells (LNMCs). The lymph nodes were biopsied three times: 1 week before the treatment of 6-Cl-ddG, the last day of the treatment, and 4 weeks after the end of the treatment.

Titration of infectious virus in LNMCs (limiting dilution assay): LNMCs were serially diluted in duplicate at concentrations of 10⁰, 10⁴, 10⁵, 10⁶, 10⁷, and 10⁸ cells/ml/well. Then, 2×10⁴ cells of CEMx174, an SIVmac-susceptible human cell line, were added to the wells. The cell mixtures were cultured for 4 weeks in RPMI1640 medium (Gibco BRL) supplemented with 10% fetal bovine serum, 50mM of 2-mercaptoethanol, L-glutamine, and antibiotics. Virus replication was confirmed by detecting the formation of syncytia (using an inverted microscope) and by p27 gag antigen production in the supernatant with an SIV Enzyme-Linked Immno Assay Kit (Coulter).

Fixation and making tissue section: The tissues of biopsied lymph nodes were fixed with 4% paraformaldehyde-0.1 M phosphate buffer pH 7.4 for 24 hr at 4°C, dehydrated through gradient ethyl-alcohol (from 70% to 100%), and then kept in a dry chamber at 4°C until staining.

Preparation of digoxigenin-labeled RNA probes: Portions of gag, pol, and nef genes of SIVmac239 [19] were inserted into pSPT19 and pSPT18 (Boehringer Mannheim), and each linearized product was used as a template for antisense and sense probes, respectively. All probes were generated from T7 promoter in the presence of digoxigenin-labeled UTP with a DIG RNA Labeling Kit (Boehringer Mannheim) according to the manufacturer’s instruction. To obtain a majority of fragments that contained 200 to 300 nucleotides for enhancing the penetration into tissue sections, the digoxigenin-labeled RNA was subjected to mild alkaline hydrolysis.

In situ hybridization: We used a hybridization technique described elsewhere [11]. In brief, the tissue slides were deparaffinized and rehydrated. The specimens here were digested with 20 µg/ml of proteinase K (Sigma) to enhance the permeability of the probes, and then acetylated in 0.25% acetic anhydride - 0.1 M triethanolamine to minimize the background noise before hybridization. The hybridization mixture contained the digoxigenin-labeled antisense or sense SIV probes at 2 µg/ml, 50% formamide, 10% (w/v) dextran sulfate, 600 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1mM EDTA (pH 8.0), 1xDenhardt’s solution (Sigma), sodium dodecil sulfate (0.25%), and yeast tRNA (200 µg/ml). Hybridization was carried out at 50°C for 16 hr in humid chambers. After hybridization, the slides were rinsed in 50% formamide-5x saline-sodium citrate buffer (SSC) at 50°C for 60 min. Excess unhybridized probes on the slides were digested with 20 µg/ml RNase A (Sigma), and then removed by rinsing with 2x SSC at 50°C for 60min and 0.2x SSC at 50°C for 40 min. The hybridized probes were reacted with alkaline phosphatase conjugated sheep anti-digoxigenin antibody (Boehringer Mannheim). Alkaline phosphatase was colored by dipping in the substrate (257 µM nitro-blue-tetrazolium-chloride, 415 µM 5-bromo-4-chloro-3-indolylphosphate, 50 mM Tris-HCl (pH 9.5), 100 mM NaCl and 50 mM MgCl₂) for 8 hr at 25°C in the dark. The specimens were counterstained with Kernechtrot before coverslipping with a layer of Glycergel (Dako).

Immunohistochemistry: We used mouse monoclonal and rabbit polyclonal antibodies against the following human antigens: CD68 (KP-1; Dako), CD20 (L20; Dako), Ki-67 (MIB1; Zymed Laboratories), HLA-DR (CR3/43; Dako), S100 (Z311; Dako), and CD3 (A452; Dako). The streptavidin-biotin-peroxidase complex technique (LSAB-2 peroxidase kit; Dako) was used for detecting the antibodies. We used 3, 3'-diaminobenzidine-tetrahydrochloride (DAB) as a chromogen. All specimens were counterstained with hematoxylin solution. Endogenous peroxidase was inactivated with 0.3% hydrogen peroxide in methanol. For enhancing the immunoreaction to CD68 and CD3, the sections were pre-treated with 0.05% pronase (Dako). For staining Ki-67, the sections were pre-heated in a microwave oven.

RESULTS

Virus titration with LNMCs: Before 6-Cl-ddG administration, Mm9408 had 500 SIV-infected cells in 10⁶ LNMCs and Mm9421 had more than 3162 (Fig. 1A). After 2 weeks of 6-Cl-ddG treatment, these numbers were dramatically reduced, to 1.6 and 3.2, respectively (Fig. 1B). Therefore, 6-Cl-ddG treatment decreased the number of SIV-infected cells by a factor of 300 and by over 1000, respectively. However, at 4 weeks after the end of the drug treatment, SIV-infected cells rapidly increased, to 1581 and 21.5 respectively. The hybridization mixture contained the digoxigenin-labeled antisense or sense SIV probes at 2 µg/ml, 50% formamide, 10% (w/v) dextran sulfate, 600 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1mM EDTA (pH 8.0), 1xDenhardt’s solution (Sigma), sodium dodecil sulfate (0.25%), and yeast tRNA (200 µg/ml). Hybridization was carried out at 50°C for 16 hr in humid chambers. After hybridization, the slides were rinsed in 50% formamide-5x saline-sodium citrate buffer (SSC) at 50°C for 60 min. Excess unhybridized probes on the slides were digested with 20 µg/ml RNase A (Sigma), and then removed by rinsing with 2x SSC at 50°C for 60min and 0.2x SSC at 50°C for 40 min. The hybridized probes were reacted with alkaline phosphatase conjugated sheep anti-digoxigenin antibody (Boehringer Mannheim). Alkaline phosphatase was colored by dipping in the substrate (257 µM nitro-blue-tetrazolium-chloride, 415 µM 5-bromo-4-chloro-3-indolylphosphate, 50 mM Tris-HCl (pH 9.5), 100 mM NaCl and 50 mM MgCl₂) for 8 hr at 25°C in the dark. The specimens were counterstained with Kernechtrot before coverslipping with a layer of Glycergel (Dako).

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In situ hybridization: Fifteen lymph nodes in total were analyzed for expression of SIV-RNA by in situ hybridization (Fig. 2). Before 6-Cl-ddG administration, the average numbers of SIV-RNA positive cells in a section were 29.3 ± 2.7 (n=3) in Mm9408 and 21.5 ± 8.5 (n=4) in Mm9421. SIV-RNA positive cells were mainly in paracortical areas.
EFFECT OF 6-CL-DDG ON SIV IN LYMPH NODES

The majority of SIV-RNA positive cells were identified as small lymphocytes (Fig. 3B). Positive signal in a reticular pattern at germinal center was too faint to distinguish from the background noise. After 2 weeks of 6-Cl-ddG treatment, one lymph node from Mm9408 and three from Mm9421 were biopsied. SIV-RNA positive cells were detected only in one lymph node from Mm9421 (i.e., 2 positive cells in a section). There was no detectable SIV-RNA positive cell in the other specimens. Four weeks after the end of 6-Cl-ddG treatment, the number of SIV-RNA positive cells was high, 39.3 ± 17.7 (n=3) and 42 (n=1) in Mm9408 and Mm9421, respectively.

No positive signal was detected in any section hybridized with sense probes (Fig. 3C) or in sections from any SIV-seronegative animal hybridized with antisense probes.

Immunohistochemical analysis: The most remarkable changes observed in the lymph nodes during 6-Cl-ddG treatment occurred in the lymphoid follicles. Before the drug treatment, follicular hyperplasia with large irregular germinal centers was seen in all lymph nodes of both animals (Figs. 4A, 5A). In some follicles, these germinal centers were fused together (Fig. 4A). Generally, the dark zones of germinal centers in the hyperplastic follicles were fairly expanded (Figs. 4A, 5A, 5D). Almost all part of the dark zones of lymphoid follicles were occupied by CD20+Ki67+ proliferative B lymphocytes, so-called centroblasts (Figs. 5A, 5D). On the other hand, light zones of germinal centers, where CD20+Ki67− B lymphocytes (so-called centrocytes), CD3+ T lymphocytes, and S100+ follicular dendritic cells were predominant in stead of centroblasts, were compressed by the expanded dark zones (Figs. 4A, 5A, 5D). Also seen were involuted follicles in which CD3+ T lymphocytes had invaded the mantle zone (data not shown).

In both animals two weeks after the onset of the drug treatment, the hyperplastic lymphoid follicles, especially, the expanded dark zones of germinal centers, had dramatically decreased in size (Figs. 4B, 5B, 5E). However, involuted follicles were not influenced (data not shown).

On the contrary, at 4 weeks after the end of the treatment, the lymphoid follicles had grown and the germinal centers with enlarged dark zone had reappeared (Figs. 4C, 5C, 5F). The histological structure was indistinguishable from the basal levels (i.e., before treatment).
Despite remarkable changes in lymphoid follicles, structural alteration in sinus and in paracortical area was not obviously detected during the administration of 6-Cl-ddG (data not shown). The relative number and the location of CD68+ macrophages and HLA-DR+ interdigitating dendritic cells were not influenced by 6-Cl-ddG treatment (data not shown).

**DISCUSSION**

We previously demonstrated the antiretroviral activity of 6-Cl-ddG in non-human primate models by monitoring viral burden and several biological markers in peripheral blood [9]. However, what remained unclear were the direct antiretroviral effects of 6-Cl-ddG in the lymph nodes, which
are major sites of HIV or SIV replication. The present study showed that administration of 6-Cl-ddG for 2 weeks to two rhesus monkeys chronically infected with SIVmac239 dramatically decreased the number of SIV-infected LNMcs (Fig. 1) and SIV-RNA positive cells in sections of lymph nodes (Fig. 2). Furthermore, hyper-activated lymphoid follicles were decreased in size by 6-Cl-ddG treatment (Fig. 4). In the lymphoid follicles, dark zones of germinal centers were also significantly reduced in size (Fig. 5).

Cohen et al. recently reported that AZT monotherapy in the earlier stages of HIV-infected persons (CD4+ T cells were 659 cells/µl) did not decrease the viral amount in lymph nodes [4]. They also reported that administration of AZT and ddi as combination therapy to moderately advanced carriers of the disease (CD4+ T cells were less than 400 cells/µl) effectively decreased viral replication in lymph nodes. However, changes in histopathological structure during the examination were not clarified [5].

This is the first report that an antiretroviral drug significantly improves histopathological lymphoid disorders by decreasing the viral burden in lymph nodes in primates that have a chronic infection of immunodeficiency virus.

Manouilov et al. demonstrated that lipophilicity is a key factor for retention of antiretroviral drugs in lymph nodes [14]. The 6-Cl-ddG is a highly lipophilic antiretroviral prodrug [22]. In a study now being done, we found that the values of area under the concentration time curve (AUC1/2) in inguinal lymph nodes or mesenteric lymph nodes after intrasubcutaneous inoculation of 6-Cl-ddG to rats were 1.3 to 2 times higher than that of ddG (manuscript in preparation). Therefore, the remarkable antiretroviral effects of 6-Cl-ddG in lymph nodes seen in the current study was probably due to the drug’s lipophilicity, resulting in its high concentration in the lymph nodes. However, we could not definitively determine if 6-Cl-ddG is more effective in lymph node than other antiretroviral drugs due to the lack of published reports on the others in non-human primates.

The large irregular dark zone of germinal center in hyperplastic lymphoid follicle is a feature unique to the chronic SIV and HIV infections [17, 18]. The dark zone of germinal center is the site of B cell replication [12, 17]. Therefore, our histopathological results indicate that 6-Cl-ddG eventually suppressed inappropriate B cell proliferation in lymph nodes.

Although the exact mechanism of forming unique hyper-activated lymphoid follicles in chronic HIV and SIV infections is still unclear, abnormal production of cytokine is considered as the causative factor. Clerici and Shearer. reported that during the progression toward AIDS, the cells that produce TH2-type cytokines (e.g., interleukin (IL)-4 and IL-10) dominate the cells that produce TH1-type cytokines (e.g., interferon-gamma and IL-2) [3]. Because IL-4 has been known to induce B cell proliferation [7], overproduction of IL-4 may be involved in the expanding dark zone of germinal centers in SIV and HIV infection.

Many studies report that follicular dendritic cells (FDC) trap HIV or SIV particles on their cytoplasmic membrane [1, 4, 8, 16, 21, 25]. However, all sections in our study had only a scant amount of SIV-RNA on FDC (Fig. 3). Wyand et al. reported that the detection of SIV on FDC was more difficult by in situ hybridization than by immunohistochemistry [21, 25]. It is possible that our method was less sensitive in detecting small amount of non-replicative SIV-RNA included in SIV particles trapped by FDC. Therefore, the kinetics of SIV on FDC during 6-Cl-ddG administration remains unclear.

At 4 weeks after the end of 6-Cl-ddG administration, the viral amount in lymph nodes (Figs. 1, 2) and histological abnormality of lymphoid follicles (Figs. 4, 5) rebounded to their basal levels. For enhancing the effects of 6-Cl-ddG, it may available to extend the period of the treatment or to use with other antiretroviral drugs as a combination therapy [2, 6, 20].

During administration of 6-Cl-ddG, the number of red blood cells in peripheral blood slightly decreased (data not shown). This probably was a side-effect of 6-Cl-ddG as described previously [9].

In conclusion, we emphasize the availability of 6-Cl-ddG in lymph nodes, and the importance of investigating the viral burden and the histological structure in lymph nodes in evaluation of antiretroviral drugs.

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