Profile of Leukocyte-Endothelial Cell Interactions Induced in Venules and Arterioles by Nucleoside Reverse-Transcriptase Inhibitors In Vivo

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Background. There is controversy regarding cardiovascular (CV) toxicity of the nucleoside reverse-transcriptase inhibitors used to treat human immunodeficiency virus infection.

Methods. We evaluated the effects of nucleoside reverse-transcriptase inhibitors on leukocyte-endothelium interactions, a hallmark of CV diseases, in rat mesenteric vessels using intravital microscopy and in human arterial cells using a flow chamber system.

Results. Abacavir and didanosine increased rolling, adhesion and emigration in rat vessels. These effects were reversed with antibodies against Macrophage-1 antigen (Mac-1) or intercellular adhesion molecule 1 and were reproduced in human cells. Lamivudine, zidovudine, emtricitabine, and tenofovir had no effects.

Conclusions. Our results support the association of abacavir and didanosine with CV diseases.

Keywords. Abacavir; didanosine; tenofovir; lamivudine; zidovudine; emtricitabine; leukocyte-endothelium interactions; cardiovascular diseases.

Combined antiretroviral therapy has reduced the mortality rates associated with human immunodeficiency virus (HIV) infection. However, with the increased longevity of patients there is growing concern about the adverse effects generated by this life-long treatment. The association with cardiovascular (CV) diseases is especially worrying given that HIV-infected subjects are already at risk of developing such conditions because of the virus [1]. Combined antiretroviral therapy is a combination of at least 3 drugs: a nonnucleoside reverse-transcriptase inhibitor and/or a protease inhibitor plus 2 nucleoside reverse-transcriptase inhibitors (NRTIs), the latter of which always forms a part of this therapy. Despite the controversy surrounding the CV toxicity of abacavir (ABC) [2], recent guidelines continue to recommend an initial regimen of this NRTI with lamivudine (3TC) [3].

Evidence supports the implication of vascular inflammatory mechanisms in the association between ABC and CV, including enhanced platelet reactivity, T-cell activation, arterial stiffness, and endothelial dysfunction [1]. In addition, we have recently demonstrated in vitro the specific capacity of ABC and didanosine (ddI) to elicit leukocyte accumulation in venular endothelium [4, 5], a hallmark of vascular diseases characterized by inflammation such as atherosclerosis [2]. The endothelium is the principal controller of white cell traffic between the blood stream and extravascular space. This process involves a cascade of adhesive interactions between leukocytes and endothelial cells manifested in leukocyte rolling, leading to firm adhesion and subsequent endothelial transmigration. Although leukocyte recruitment via postcapillary venules is particularly important in many inflammatory diseases, despite being less common, the interaction with arteries is crucial to the mononuclear infiltration appearing in the genesis of atherosclerotic processes [6].

We have performed in vivo experiments, in venules and arterioles, to evaluate the relevance of and mechanisms implicated in the leukocyte-endothelium interactions induced by ABC and ddI. In addition, we have compared their actions with those of other NRTIs and assessed their effects on the interactions of leukocytes with human arterial cells in vitro.

METHODS

Intravital Microscopy

Leukocyte-endothelial cell interactions were evaluated in anesthetized Sprague-Dawley rats (200–250 g; anesthetized with sodium pentobarbital [65 mg/kg, administered intraperitoneally]) according to a standard technique [6]. A segment of the midjejunum was placed over an optically clear viewing pedestal at 37°C for tissue transillumination. The exposed mesentery was superfused with bicarbonate buffer saline (BBS; pH, 7.4; 37°C; 2 mL/min) and visualized using an orthostatic...
microscope (Nikon Optiphot-2, SMZ1; Nikon) coupled to a video camera (Sony SSC-C350P; Sony) to allow image capturing on videotape (Sony SVT-5300P) for playback analysis. A video caliper (Microcirculation Research Institute, Texas A&M University) allowed the diameter of arterioles (15–30 µm) and single unbranched mesenteric venules (25–40 µm) to be measured online (during the acquisition of the images). Hemodynamic parameters were calculated as described elsewhere [6].

Experimental Protocol
Animals were injected (2.5 mL, administered intraperitoneally) with saline or 1 of the following NRTI solutions: ABC (1–10 µmol/L; equivalent to 8–80 µg/kg), ddI (5 µmol/L; 15 µg/kg), 3TC (10 µmol/L; 30 µg/kg), ZDV (5 µmol/L; 17 µg/kg), emtricitabine (FTC; 10 µmol/L; 30 µg/kg), or tenofovir (TDF; 1–5 µmol/L; 4–18 µg/kg). All doses were representative of plasma concentrations in patients [7–9] and administered intraperitoneally, as is usual in intravital microscopy [6, 10]; ABC was also orally (100 mg/kg) administered [11]. Images were recorded 4 hours later (in some cases 24 or 48 hours later) during a 5-minute period and leukocyte parameters were evaluated offline (during a separate time period) by a blind observer.

Adhesion molecules were determined by pretreatment (30 minutes before NRTI) with blocking or with the corresponding control antibodies (monoclonal antibodies) injected through the tail vein in doses of 2 mg/kg for WT-5 (anti-rat macrophage-1 antigen [Mac-1]; CD11b) and 1 mg/kg for WT-3 (anti-rat β2-integrins; CD18) and 1A29 (anti-rat intercellular adhesion molecule 1 [ICAM-1]; CD54), which have been shown to block the in vivo function of the adhesion molecules analyzed [6, 12].

Flow Cytometry
Portal blood samples were incubated (40 µL; 20 minutes; on ice and in darkness) with saturating amounts of the corresponding conjugated monoclonal antibody anti-rat fluorescein isothiocyanate and then lysed and fixed [6]. Expression of adhesion molecules in granulocytes, monocytes and lymphocytes was analyzed with a FACSCalibur flow cytometer (BD).

Cell Culture
Human umbilical arterial endothelial cells (HUAECs; passage 1) and peripheral blood mononuclear cells (PBMCs) from healthy volunteers were from Hospital Clínico Universitario, Valencia, Spain. The PBMCs and HUAECs were treated independently (4 hours; 37°C) with ABC (1–10 µmol/L), ddI (5 µmol/L), 3TC (10 µmol/L), ZDV (5 µmol/L), FTC (10 µmol/L), TDF (1 µmol/L), or control [4, 5].

Dynamic Adhesion Assay
The parallel plate flow chamber model used has been described elsewhere [5]. In brief, cover slips containing confluent HUAEC monolayers were inserted in a chamber (37°C), and a portion (5 x 25 mm) was exposed to flow. The chamber was mounted on an inverted microscope (Nikon Eclipse TE 2000-S; ×40) with a video-camera (Sony Eyeware HAD). The PBMCs were resuspended in buffer (Dulbecco phosphate-buffered saline [DPBS] with Ca2+ and Mg2+ containing 20 mmol/L HEPES and 0.1% human serum albumine [HSA]) at 0.5 x 10^6 cells/mL and drawn across the monolayer (flow rate, 0.36 mL/min; shear stress, 0.7 dyn/cm^2). Images of a single field were recorded (5 minutes), and parameters were determined.

Materials
Materials included Sprague-Dawley rats (Charles River Laboratories); pentobarbital (Guinama); antibodies and lysis solution (BD Bioscience); Dulbecco PBS, EGM-2 culture media and fetal bovine serum (Lonza); HSA (albuminulate 25%), Roswell Park Memorial Institute 1640 medium supplemented with 20 mmol/L HEPES, Hank’s balanced salt solution (HBSS), fibronectin, and dextran (Sigma Chemical); Ficoll-Paque Plus (GE Healthcare); cover slips (Nunc; Thermo Fisher Scientific); PBS, collagenase, and trypsin (Gibco; Invitrogen); and antiretrovirals (Sequoia Research Products).

Ethics and Statistics
Human (all donors provided informed consent) and animal procedures followed the Spanish laws and were approved by the respective hospital or faculty ethic committee. One-way analysis of variance with a Newman-Keuls correction was used (mean ± standard error of the mean; n ≥ 4; P < .05).

RESULTS
Leukocyte-Endothelial Interactions in Venules and Arterioles
ABC induced a significant and dose-dependent decrease in leukocyte rolling velocity (Figure 1A), while increasing rolling flux (Figure 1B), adhesion (Figure 1C), and emigration (Figure 1D) in rat venules and adhesion in rat arterioles (Figure 1E). The effects of ABC (5 µmol/L, administered intraperitoneally) were reproduced after oral administration (100 mg/kg; data not shown). The effects of ABC (10 µmol/L, administered intraperitoneally) were acute and reversible, with values returning to levels similar to control levels 48 hours later (data not shown). The in vivo results obtained in rat arterioles were reproduced in vitro in human cells. ABC also induced a significant decrease in PBMC rolling velocity and an increase in rolling flux and adhesion (Supplementary Figure 1). ddI (Figure 1 and Supplementary Figure 1), but not ZDV, 3TC, FTC, or TDF (data not shown), triggered a response similar to that caused by ABC, both in vivo and in vitro.

Role of Mac-1 and ICAM-1
Flow cytometry revealed that both ABC and ddI induced a significant increase in CD11b and CD18 in rat neutrophils and monocytes, while having no effect on CD11a, CD49d, or...
CD62L (Supplementary Table 1). In animals treated with ddI, CD11b/c expression was enhanced in monocytes but not in neutrophils, whereas ABC had no bearing on either of these leukocyte populations. Neither ABC nor ddI exerted any influence on the adhesion molecules of lymphocytes (data not shown). The effects of ABC and ddI were reversed by pretreatment with antibodies against Mac-1 (CD11b/CD18) or its ligand ICAM-1 (CD54; Figure 2 and Supplementary Figure 2).

**DISCUSSION**

To our knowledge, this is the first study to analyze in vivo (in venules and arterioles) and in vitro (in arteries) the effects of the NRTIs most widely used to treat HIV infection (ABC, ddI, 3TC, ZDV, FTC, and TDF) on the trafficking of leukocytes from blood to tissue. Our in vivo results show that ABC and ddI induce inflammatory events through the interaction of the...
leukocyte’s Mac-1 with its endothelial ligand ICAM-1. These results expand preliminary in vitro data, which suggested that ABC and ddI elicit leukocyte accumulation in venular endothelial cells [4]. We have now performed an in-depth investigation with intravital microscopy, a technique that has been instrumental in understanding the inflammatory cascade associated with vascular diseases and its pharmacological modulation [10, 13], to analyze the nature and characteristics of the leukocyte-endothelial cell interplay induced by NRTIs.

In this setting, concentrations of ABC (1–10 µmol/L) and ddI (5 µmol/L) that mimicked those present in patients (1–8 and 3–10 µmol/L respectively) [7–9] not only induced

Figure 2. Role of Mac-1 and intercellular adhesion molecule 1 (ICAM-1) in abacavir (ABC)–induced leukocyte-endothelium interactions in rat mesenteric postcapillary vessels. Rats were treated intraperitoneally with saline (vehicle) or ABC (10 µmol/L). Some animals were treated intravenously with anti-CD11b monoclonal antibody (mAb), anti-CD18 mAb, anti–ICAM-1, or the corresponding control mAb 30 minutes before administration of ABC. Responses of leukocyte rolling velocity (A), rolling flux (B), adhesion (C), and emigration (D) in venules and adhesion (E) in arterioles were quantified 4 hours later. Rolling flux was assessed offline by counting the number of leukocytes passing a reference point in the vessel per minute. Leukocyte rolling velocity was calculated by measuring the time required for these cells to travel along 100 µm of the venule, expressed as micrometers per second. A leukocyte was considered to have adhered to the endothelium if it remained stationary for ≥30 seconds (cells per 100 µm of vessel), and emigration was expressed as the number of interstitial leukocytes per field. Results represent means ± standard errors of the mean (n = 4–5). **P < .01 for comparison with corresponding value in vehicle-treated group; +++P < .01 for comparison with corresponding value in ABC-treated group (analysis of variance followed by Newman-Keuls test). Abbreviations: IgA, immunoglobulin A; IgG1, immunoglobulin G1.
leukocyte rolling and adhesion in postcapillary venules—signs of which we have observed previously [4]—but also caused significant leukocyte emigration. The fact that similar results were obtained with oral and intraperitoneal ABC rules out a potential limitation of our intravital approach, that is, that the pharmacokinetics of intraperitoneal administration of the drug do not replicate the clinical reality. Furthermore, the effects of ABC were reversible, and although we did not measure the intracellular concentrations of its active metabolite, carbovir-triphosphate, the finding that these effects had a duration matching the longer intracellular half-life of the metabolite (12–21 hours) [9], but not that of plasmatic ABC (around 2 hours), suggests that carbovir-triphosphate was implicated.

The migratory effect evidences the high activation elicited by ABC and ddI, because white cells moving toward inflamed tissue represent the last and critical step of leukocyte-endothelial interactions. Indeed, emigration is the point of no return in inflammatory responses, because preceding phases—rolling and adhesion—are reversible, and most leukocytes that attach to postcapillary venules at the site of inflammation eventually reenter the circulation. In contrast, leukocytes that commit to diapedesis do not return, or at least not as the same cell type [14]. Importantly, we have appreciated that these 2 NRTIs also provoke leukocyte adhesion in arterioles. Although the level of adhesion was lower than in venules, and no rolling or emigration was apparent, these arterial effects are surprising, because few stimuli are capable of modifying the arteriolar-leukocyte parameters revealed by intravital macroscopy, including perivascular laser injury, cigarette smoke, tumor necrosis factor α or angiotensin II [6]. Indeed, interaction with arteries is essential for mononuclear infiltration during genesis of the atherosclerotic process. Therefore, it is highly relevant that both drugs induced mononuclear (PBMC) rolling and adhesion in human arterial cells. This effect was evaluated in a dynamic flow adhesion assay, a well-established in vitro system used, for example, to analyze leukocyte recruitment in patients with CV diseases or the vascular pro- or anti-inflammatory effects of drugs [5].

Flow cytometry revealed that ABC and ddI induced selective up-regulation of Mac-1 (CD11b/CD18) on the surface of rat neutrophils and monocytes without influencing the expression of CD11a, CD49d, or CD62L. The only disparity between the 2 drugs was the augmentation of CD11b/c on rat monocytes by ddI. Furthermore, the in vivo interactions induced by ABC or ddI were prevented by antibodies against CD11b, CD18, or its endothelial ligand ICAM-1 (CD54). As a whole, these data clearly implicate Mac-1 (CD11b/CD18) and ICAM-1 (CD54) in the leukocyte-endothelial interactions induced by these 2 antiretrovirals. Interestingly, Mac-1 has been proposed as the link between cellular adhesion and thrombosis [15]. It is present in neutrophils and monocytes, where it interacts with ICAM-1, causing them to adhere to the endothelium [4]. It also interacts with platelet ligands [4] to mediate the leukocyte engagement of platelets, a fundamental step in the onset of the thrombotic process [15].

The effects of ABC and ddI in venules and arterioles were not reproduced (in vivo or in vitro) by the other NRTIs evaluated (data not shown [4, 5]). Remarkably, if the chemical structure of the compounds is taken into account, a pattern of activity emerges; changes were induced by cyclic purine analogues (ABC or ddI), but not by pyrimidine analogues (3TC, ZDV, and FTC) or the acyclic nucleotide TDF.

We must be cautious when extrapolating experimental data to the clinical human situation, particularly when complex pathological situations are involved. One potential limitation of this study could be that we have not used cells from HIV-infected patients to evaluate the effects of the drugs on their vascular responses. However, even if this is taken into account, the differences we observed in CV toxicity profiles support the recent clinical association of ABC and ddI with CV diseases.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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