In Vivo Downregulation of T Helper Cell 1 Immune Responses Reduces Atherogenesis in Apolipoprotein E–Knockout Mice

E. Laurat, MD; B. Poirier, PhD; E. Tupin, MSc; G. Caligiuri, MD, PhD; G.K. Hansson, MD, PhD; J. Bariéty, MD, PhD; A. Nicoletti, PhD

Background—A chronic immune response involving proinflammatory T helper cell 1 (Th1) lymphocyte activation occurs in the atherosclerotic lesion, but whether this activation is protective or deleterious remains unclear.

Methods and Results—We modulated the immune response of the atherosclerosis-prone apolipoprotein E–deficient (apoE−/−) mouse. Eight-week-old apoE−/− mice were treated daily with pentoxifylline (PTX), a known inhibitor of the Th1 differentiation pathway, or PBS (control) for 4 weeks or 12 weeks. Twelve-week PTX treatment reduced atherosclerotic lesion size by 60% (P<0.01). PTX-treated mice developed lesions that were limited to the degree of fatty streaks. In contrast, control mice developed mature fibrofatty atherosclerotic lesions. In parallel, the proportion of interferon (IFN)–γ–producing Th1 splenic lymphocytes was significantly reduced by PTX, and lesion size was correlated to the proportion of IFN-γ+ T cells. In vitro addition of PTX to cultured spleen cells did not modify the production of IFN-γ but increased the production of IL-10 by T cells, indicating that PTX does not suppress IFN-γ production but rather blocks Th1 polarization while promoting Th2 polarization.

Conclusions—Thus, PTX protected mice from atherosclerosis by reducing the Th1 polarization of T helper lymphocytes. This study demonstrates that the Th1 immune response associated with atherosclerosis is deleterious and that a modulation of the Th1 differentiation pathway may provide a new pharmacological tool to treat this disease.

Key Words: atherosclerosis • lymphocytes • inflammation • interleukins • inhibitors

Atherosclerosis is associated with immune system activation.1 Atherosclerotic plaques are infiltrated by T helper (Th) lymphocytes (CD4+), which suggests that a specific immune response may be involved in the pathogenesis of the disease. Nevertheless, it is still debated whether the immune response plays a deleterious or protective role. To test this hypothesis, we performed an immunomodulation in apolipoprotein E–deficient (apoE−/−) mice, which is an adequate model to study the role of cellular immunity in atherosclerosis, because their atherosclerotic lesions are infiltrated with macrophages and CD4+ and CD8+ T cells.

Substantial evidence suggests the existence of functionally polarized responses by the CD4+ T helper cells. Proinflammatory Th1 cells produce interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and interleukin (IL)-2, which activate macrophages and are involved in delayed-type hypersensitivity reactions.2 In contrast, Th2 cells produce IL-4, IL-5, IL-10, and IL-13, which promote antibody responses and inhibit several macrophage functions.3 Furthermore, Th1 and Th2 cytokines exert negative cross-regulatory effects. Interestingly, IL-124,5 and IFN-γ4,6 are found in atherosclerotic plaques both in humans and in atherosclerotic mice, demonstrating that atherosclerotic lesions contain Th1-promoting cytokines, which would contribute to a proinflammatory deleterious environment in the plaque similar to the one involved in rheumatoid arthritis.7

Pentoxifylline (PTX) is a phosphodiesterase inhibitor that has been used in various types of peripheral and cerebrovascular diseases.8,9 PTX inhibits the Th1 differentiation pathway of T cells10,11 and has dramatic protective effects in experimental diseases mediated by Th1 autoimmune responses, such as experimental autoimmune encephalomyelitis11 and experimental rheumatoid arthritis.12 Therefore, we examined whether a short-term (4 weeks) or a long-term (12 weeks) PTX treatment could modulate the Th1 immune response and affect atherogenesis in apoE−/− mice.

Twelve-week PTX treatment reduced the Th1 polarization of T helper lymphocytes and the size of the atherosclerotic lesions by 60%. Our data support the hypothesis that Th1 immune responses associated with atherosclerosis are deleterious and that a modulation of the Th1/Th2 balance may provide a new pharmacological tool to treat this disease.

Methods

Experimental Protocol

Eight-week-old apoE−/− male mice (IFFA-CREDO, France) were randomly separated into control and PTX groups (see Table for...
numbers of mice). In the 4-week experiment, PTX (Sigma; 120 mg · kg⁻¹ · d⁻¹ IP) was administered by 2 injections in a fixed volume of 0.1 mL. In the 12-week experiment, PTX was administered at a dose of 120 mg · kg⁻¹ · d⁻¹ for 4 weeks, and 60 mg · kg⁻¹ · d⁻¹ was used for the last 8 weeks. The dose was decreased to alleviate the side effects (trembling, prostration). Control mice received a 0.1-mL IP injection of PBS twice a day. Mice were maintained on a normal chow diet and kept in standard conditions. Mice were euthanized under anesthesia. Blood was collected by cardiac puncture and allowed to clot. After vascular perfusion with sterile PBS, the heart and ascending aorta and the spleen were dissected. All experiments were approved by our institutional ethical committee for animal welfare.

Serum Measurements and Quantification of Atherosclerotic Lesions

Serum was separated by centrifugation and stored at −80°C. Serum total cholesterol (TC) level was measured with a Boehringer-Mannheim kit (CHOD-PAP), and serum HDL cholesterol (HDL-C) and triglyceride (TG) levels were measured with Konelab kits (Finland) according to the manufacturer’s instructions.

The aortic root was dissected and lesions were quantified as previously described.13

Immunohistochemistry

Aortic root cryosections were also used for immunohistochemistry to visualize the presence and localization of vascular cell adhesion molecule (VCAM)-1- and I-Aα (class II MHC)—positive cells. Cryosections were air-dried and fixed in acetone. Binding of VCAM-1 and I-Aα (Pharmingen) biotinylated primary antibodies (1/100) was revealed with avidin–alkaline phosphatase (Vectastain ABC kit, Vectorlab) and fast red substrate (Dakopatts). Sections were counterstained with hematoxylin, mounted under glass coverslips, and photographed.

Cell Culture

Proliferation

Spleen cells were prepared as described.11 We cultured 500,000 mononuclear cells/well at 37°C in 0.5% CO₂ in 96-well flat-bottom plates (triplicates), with or without concanavalin A (0.5 to 2.5 μg/mL, Sigma) and with or without PTX at 10, 100, or 1000 μg/mL. After 48 hours, 1 μCi of [³H]thymidine was added to each well. After 1 more day of incubation, incorporated [³H]thymidine was counted in a Microbeta counter. Results are expressed as counts per minute.

FACS Analysis

Cell populations: Spleen T and B cells were assessed with CD45-CD3-CD19 triple staining, and T-cell subsets with CD3-CD4-CD8 triple staining with a FACSCalibur (Becton Dickinson) flow cytometer as described.13

Intracellular staining of cytokines: The intracellular staining of cytokines was adapted from the method described by Openshaw et al.14 We cultured 1 500 000 spleen cells per mouse overnight in the presence of 2 U/mL IL-2 (Genzyme). Then, cells were either kept untouched or stimulated with 50 ng/mL phorbol myristate acetate and 500 ng/mL ionomycin for 4 hours at 37°C. Protein secretion was blocked by addition of 10 μg/mL brefeldin A during the last 2 hours. The in vitro effect of PTX was assessed by duplicating these experiments in the presence of PTX at the indicated concentrations. Th cells were stained with an anti–CD4-Cy antibody (Pharmingen), fixed with 4% formaldehyde in PBS, and permeabilized with PBS/BSA 0.2%/saponin 0.5% containing a mixture of IFN-γ–FITC and IL-10–PE antibodies (Pharmingen). Cells were analyzed with a FACSCalibur (Becton Dickinson).

Statistical Analysis

Results are expressed as mean±SEM. Simple regression and ANOVA were performed with Statview 5.0 software (SAS Institute Inc). Differences between groups were considered significant at a value of P<0.05.

Results

Effect of PTX on Physiological Parameters

The PTX treatment did not modify the serum TC or HDL-C levels (Table). The ratio HDL-C/TC was also similar in all groups of mice. Conversely, PTX induced a 9% to 15% reduction of body weight (Table). After 12 weeks of PTX treatment, the serum TG content was significantly reduced, an effect not observed in the short-term experiment (Table).

Effect of PTX on Plaque Formation

In the 4-week experiment, mice exhibited only fatty streak lesions independently of treatment (Figure 1A). In the 12-week experiment, the PTX group still showed fatty streak lesions, whereas lesions in control mice progressed to the degree of complex fibrofatty plaques (Figure 1A). In addition, the immunoinflammatory plaque infiltrate was less abundant in the PTX group than in controls. MHC class II⁺ cells could be detected both at the luminal side and in the adventitia at lesion sites (Figure 1B). Such cells could barely be detected in the PTX group (Figure 1B) or in the aorta of mice from the 4-week experiment (data not shown).

The surface area covered by lesions was quantified on oil red O–stained sections, and this surface area was divided by the aorta surface area to obtain the fractional area of lesion.13 As mentioned, a 4-week administration of PTX did not modify early lesion formation (Figure 2A). Twelve weeks of PTX treatment, however, reduced the fractional area of lesion dramatically, by 60% (1.99±0.70% versus 5.35±0.64% in controls, P<0.01; Figure 2A). A longitudinal analysis of

### Table: Serum TC, HDL-C, and TG Levels and Body Weights

<table>
<thead>
<tr>
<th></th>
<th>TC (mmol/L)</th>
<th>HDL-C (mmol/L)</th>
<th>HDL-C/TC ratio</th>
<th>TG (mmol/L)</th>
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<td>4 Weeks</td>
<td></td>
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<td>Control (n=9)</td>
<td>8.8±0.6</td>
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<td>0.46±0.08</td>
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<td>PTX (n=9)</td>
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<td>3.3±0.5</td>
<td>0.31±0.05</td>
<td>1.58±0.23</td>
<td>24.9±0.6*</td>
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<tr>
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<td>12.6±0.8</td>
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<td>1.76±0.29</td>
<td>29.3±0.5</td>
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<tr>
<td>PTX (n=7)</td>
<td>12.9±1.0</td>
<td>4.6±0.3</td>
<td>0.39±0.02</td>
<td>0.84±0.15*</td>
<td>26.7±1.1*</td>
</tr>
</tbody>
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Values are mean±SEM. *P<0.05 vs aged-matched controls.

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1. Openshaw et al. 2017
2. Mannheim kit (CHOD-PAP) and with or without PTX at 10, 100, or 1000 μg/mL.
lesions confirmed the antiatherogenic effect of PTX and demonstrated that measurements were performed accurately at the same levels of the root in both groups, with the largest lesion extension located at 600 μm (Figure 2B).

Phosphodiesterase inhibitor–induced increase in the intracellular content of cAMP downregulates the expression of adhesion molecules, especially VCAM-1 and E-selectin, in cultured endothelial cells. Because downregulation of adhesion molecules is likely to be atheroprotective, we assessed whether PTX could have protected apoE−/− mice by modulating the expression of VCAM-1. Immunohistochemistry in the aortic root revealed that in the 4-week experiment, intimal cells in both groups of mice weakly expressed VCAM-1 (data not shown). Conversely, in the 12-week experiment, expression of VCAM-1 was reduced in PTX-treated mice (Figure 3).

**Effect of PTX on Immune Responses**

**Spleen Cell Proportion and Proliferation**

To determine whether PTX could affect the proportion of different immune cells and inhibit the proliferative capacity of T cells, we examined the spleen cell population and the ex vivo proliferation of T cells in response to a polyclonal mitogen. Four-week or 12-week PTX treatments did not affect the relative proportions of CD3⁺, CD4⁺, or CD8⁺ T cells or the proportion of CD19⁺ B cells (data not shown).

PTX-treated mouse spleen cells exhibited an identical basal proliferation rate (data not shown). Concanavalin A stimulation resulted in an equivalent proliferation in both groups of mice. In vitro addition of high doses of PTX

**Figure 1.** PTX limits lesion development and decreases inflammatory infiltrate. A, Serial sections of aortic root in PTX and control mice (magnification ×40). Insets, Magnification ×200 showing fatty streaks in 12-week PTX group and complex plaques with fibrous cap (arrowheads) in control group; areas of inflammatory infiltrates can be detected both in intima and in adventitia of controls (arrows). B, Immunohistochemistry for I-A^b^ cells in 12-week experiment. Lesions from control mice were rich in I-A^b^ inflammatory cells in adventitia (arrows) and fibrous cap (arrowheads). PTX-treated group showed fewer I-A^b^ cells (arrows). L indicates lumen; magnification ×200.

**Figure 2.** PTX reduces plaque formation. A, Fractional area of lesion (lesion/vessel surface area ×100). B, Lesion distribution according to distance from appearance of first cusp. ○ indicates 12-week control; ●, 12-week PTX; □, 4-week control; and ■, 4-week PTX. Values are means±SEM. †P<0.01, PTX vs control.
systematically inhibited the basal and concanavalin A–induced proliferation. Inhibition of proliferation by PTX was dose-dependent, 1 mmol/L PTX completely blocking T-cell proliferation (data not shown).

Spleen Lymphocyte Th1/Th2 Differentiation

The pool of Th1 and Th2 cells was estimated by quantifying the proportion of spleen CD4+ cells able to produce IFN-γ and IL-10, respectively, on stimulation. The Th1 and Th2 pools estimated with this setting are directly representative of the in vivo Th1/Th2 balance.

In the control mice, the pool of Th1 cells increased by 24% (Figure 4A; \( P < 0.05 \)) during the course of the disease, and the pool of Th2 cells decreased by 45% (Figure 4B; \( P < 0.05 \)), demonstrating a shift of the Th balance toward Th1-dominant immune responses.

In vivo PTX treatment significantly reduced the pool of Th1-polarized T cells, as indicated by the smaller proportion of IFN-γ1 Th cells compared with control mice (Figure 4A). In contrast, addition of PTX in vitro did not further modify the proportion of IFN-γ1 Th cells or the mean fluorescence intensity of IFN-γ1 Th cells (357 ± 14 in the control group versus 392 ± 16 in the PTX group, \( P = NS \)), indicating that PTX is probably acting on Th1 polarization rather than directly on the capacity of T cells to produce Th1 cytokines.

In vivo treatment with PTX did not influence the proportion of Th2 spleen cells (Figure 4B) or the production of IL-10 as assessed by the mean fluorescence intensity of IL-101 Th cells (125 ± 3 in the control group versus 119 ± 8 in the PTX group, \( P = NS \)). In contrast, high doses of PTX increased the proportion of IL-101 Th cells in vitro (Figure 4B).

Linear regression analysis revealed that the number of IFN-γ1 spleen Th cells was correlated to lesion size (Figure 5).

Discussion

The Th1/Th2 balance controls inflammation and may therefore be important in atherosclerosis. To assess whether a modulation of this balance could affect the course of the disease, atherosclerosis-prone apoE−/− mice were treated for either 4 or 12 weeks with PTX, a phosphodiesterase inhibitor known to block the Th1 polarization. PTX treatment for 12 weeks decreased the size of atherosclerotic lesions by 60% compared with control mice. Of note, the degree of protection was of the same magnitude as the one obtained by gene targeting of the IFN-γ receptor in apoE−/− mice. Furthermore, lesions observed in PTX-treated mice were limited to the fatty streak stage, whereas control mice developed mature fibrofatty plaques with significant inflammatory infiltrates. Our data indicate that the protective effect of PTX was...
achieved through an in vivo modulation of the Th1 differentiation pathway.

The proposition that Th1-promoting cytokines would contribute to a proinflammatory deleterious environment in the plaque is supported by recent studies based on genetic modulation of the Th1/Th2 balance\(^6\)–\(^8\) or on Th1 cytokine administration\(^9\) in atherosclerosis-prone mice. One should be cautious, however, when extrapolating data obtained in mice to humans: Murine Th1 clones do not produce IL-10. Conversely, human Th1 and Th2 clones can produce IL-10, which can inhibit the proliferation of both Th1 and Th2 cells.\(^{10}\) IL-10 is thus modulating the function of both cell populations in humans, whereas its regulatory role in mice is oriented toward Th1 responses.

To assess whether PTX did modulate the Th1 pathway in apoE\(^{-/-}\) mice, spleen T cells were studied in detail. First, we found that in parallel with disease progression, the Th balance shifts toward Th1-dominant immune responses in apoE\(^{-/-}\) mice, without changes in the proportion of CD4\(^+\), CD8\(^+\), and CD19\(^+\) spleen cell populations. Our data show that PTX treatment blocked the in vivo proinflammatory Th1 polarization, as detected by a decrease of 25% to 60% in the proportion of Th1 spleen cells. Inflammatory infiltrates in the lesions and in the adventitia, commonly observed in apoE\(^{-/-}\) mice,\(^{20}\) were reduced by PTX treatment, indicating that PTX anti-Th1 effects had beneficial effects on the inflammatory process at the lesion sites.

To dissect the protective mechanism of PTX, we performed in vitro experiments. PTX did not alter the proportion of Th1 cells or the IFN-\(\gamma\) T-cell production in spleen-cell cultures. Indeed, it has been suggested that phosphodiesterase inhibitors block the Th1 differentiation pathway without altering the synthesis of IFN-\(\gamma\). They rather act by blocking either the production or the signaling of IL-12\(^{21}\) and/or TNF-\(\alpha\).\(^{22}\) In our in vitro study, PTX increased the number of IL-10-producing cells by 30%, in agreement with previous reports.\(^{23}\) Thus, PTX may act by both blocking the Th1 polarization and promoting the Th2 polarization. Our data suggest that the latter effect requires high doses of PTX. Remarkably, the proportion of Th1 cells in vivo was correlated to lesion size. These data strongly suggest that PTX achieved atheroprotection through its anti-Th1 effect.

In vivo PTX treatment did not affect concanavalin A–induced proliferation of spleen cells, whereas addition of PTX in vitro blocked it. This latter effect was not due to PTX cytotoxicity. Indeed, absence of PTX cytotoxicity was assessed by trypan blue exclusion and propidium iodide stainings (data not shown). Given that concanavalin A exerts its proliferative stimulation through mitogen-activated protein kinase (MAPK) activation, the antiangiogenic effect of PTX may rather be due to inhibition of the MAPK cascade through the increase in cAMP and activation of protein kinase A, as described in many systems.\(^{24}\) Such an antiproliferative effect did not occur in vivo, most likely because the PTX dose to which cells were submitted in vivo was lower than the 100 \(\mu\)mol/L that is necessary to inhibit proliferation. Indeed, the usual plasma level of PTX after a 400-mg capsule is \(\approx 1 \mu\)g/mL.\(^{25}\) In our experiments, this would correspond to plasma levels of PTX ranging from \(\approx 10 \) to 50 \(\mu\)mol/L. Thus, these results indicate that PTX did not exert its action by a systemic downregulation of the cellular immune responses.

Given the much faster metabolism of mice than of humans, the dose of 160 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\) was previously used experimentally, for instance in nonobese diabetic mice.\(^{26}\) Therapeutic effects were achieved at a dose of 300 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\) in a rat model of experimental autoimmune encephalomyelitis.\(^{11}\) Initially, we used a lower dose than in these studies, and to alleviate the side effects observed with the 120-mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\) injections, the dose was further decreased to 60 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\). This dose is closer to the dose of PTX commonly used in humans (10 to 20 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\)).

Because downregulation of adhesion molecules is likely to be atheroprotective,\(^{27}\) PTX could have exerted protection through its ability to downregulate their expression.\(^{15}\) Indeed, VCAM-1 expression was reduced in the aorta of PTX-treated mice. This effect was seen only in the 12-week experiment, however. We can expect that if this were the main atheroprotective effect of PTX, a difference in the expression of VCAM-1 would also have been detected in the 4-week experiment and would have bestowed atheroprotection to treated mice. Actually, it is reasonable to speculate that the decrease in the expression of this inflammatory marker is probably a result of the anti-Th1 effect of PTX.

On one hand, TNF-\(\alpha\) promotes hypertriglyceridemia by inhibiting the lipoprotein lipase synthesis in adipocytes.\(^{28}\) On the other hand, PTX is known to inhibit the production of TNF-\(\alpha\),\(^{22}\) and a long-term PTX treatment is thus expected to decrease the serum TG concentration. This is precisely what was observed in the group of mice treated during 12 weeks with PTX. Importantly, TG serum levels were not correlated with lesion size (data not shown). Furthermore, the serum levels of TC and HDL-C and the HDL-C/TC ratio were not modified by the PTX treatment. Thus, the atheroprotective effects of PTX cannot be explained by its effects on the lipid metabolism.

Our data show that in vivo inhibition of the Th1 pathway dramatically reduced atherosclerosis in apoE\(^{-/-}\) mice. To the best of our knowledge, this is the first study showing the potential benefit of in vivo Th1/Th2 modulation in experimental atherosclerosis. Can these data be extrapolated to the human disease? Indeed, several kinds of evidence suggest that human T cells are less easily polarized into Th1 or Th2 phenotypes than are mouse cells.\(^{29}\) Th0 cells that promote an inflammatory process, however, use “Th1” cytokines, such as IFN-\(\gamma\) and TNF-\(\alpha\). Consequently, at least in short-term analyses, antagonizing the Th1 polarization or the Th1 proinflammatory effectors is expected to yield similar results. Thus, PTX may exert the same atheroprotective effect in humans as in mice, because it achieves similar Th1 cytokine production inhibition on human and murine cells.\(^{21,30}\) Through such anti-Th1 effects, PTX was shown to weaken the consequences of rejection on graft survival in humans (a Th1-dependent disorder)\(^{31}\) or to induce a Th2 immune deviation in patients with multiple sclerosis.\(^{32}\) It will now be interesting to design clinical regression trials to examine the antiatherogenic efficiency of Th1 immunomodulation.
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References
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