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Pretreatment with a 55-kDa Tumor Necrosis Factor Receptor–Immunoglobulin Fusion Protein Attenuates Activation of Coagulation, but not of Fibrinolysis, during Lethal Bacteremia in Baboons

Tom van der Poll, Patty M. Jansen, Kimberly J. Van Zee, C. Erik Hack, Hester A. Oldenburg, Hansruedi Loetscher, Werner Lesslauer, Stephen F. Lowry, and Lyle L. Moldawer

Cornell University Medical College, Laboratory of Surgical Metabolism, New York, New York; Academic Medical Center, Department of Internal Medicine, University of Amsterdam, and Department of Pathophysiology of Plasma Proteins, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, Netherlands; F. Hoffmann-La Roche, Basel, Switzerland; University of Florida College of Medicine, Department of Surgery, Gainesville

Baboons *(Papio anubis)* receiving a lethal intravenous infusion with live *Escherichia coli* were pretreated with either a 55-kDa tumor necrosis factor (TNF) receptor–IgG fusion protein (TNFR55:IgG) ($n = 4$, 4.6 mg/kg) or placebo ($n = 4$). Neutralization of TNF activity in TNFR55:IgG-treated animals was associated with a complete prevention of mortality and a strong attenuation of coagulation activation as reflected by the plasma concentrations of thrombin–antithrombin III complexes ($P < .05$). Activation of fibrinolysis was not influenced by TNFR55:IgG (plasma tissue-type plasminogen activator and plasmin–α_2-antiplasmin complexes), whereas TNFR55:IgG did inhibit the release of plasminogen activator inhibitor type I ($P < .05$). Furthermore, TNFR55:IgG inhibited neutrophil degranulation (plasma levels of elastase–α_1-antitrypsin complexes, $P < .05$) and modestly reduced release of secretory phospholipase A_2. These data suggest that endogenous TNF contributes to activation of coagulation, but not to stimulation of fibrinolysis, during severe bacteremia.

Tumor necrosis factor-α (TNF) is considered to be a key mediator in the pathogenesis of sepsis syndrome. TNF is released into the circulation early after intravenous bacterial challenges in animals, and neutralization of endogenous TNF prevents lethality in these acute models [1]. The role of TNF in disturbances of the hemostatic mechanism during systemic infection is less clear. Sepsis- or endotoxin-induced activation of the coagulation system is driven by the tissue factor–mediated extrinsic pathway [2]. TNF potently up-regulates tissue factor expression on endothelial and mononuclear cells, suggesting a role for this cytokine in coagulation activation [1, 2]. In accord, intravenous injection of recombinant TNF into humans or baboons induced activation of the common pathway of the coagulation system [3, 4]. However, treatment of baboons with severe bacteremia or endotoxemia with a neutralizing anti-TNF monoclonal antibody did not result in a noticeable effect on activation of the coagulation system, in spite of the fact that such treatment afforded significant protection against

lethality [5–7]. Similarly, anti-TNF did not influence coagulation activation during nonlethal endotoxemia in chimpanzees [8]. By contrast, anti-TNF completely prevented the fibrinolytic response to nonlethal endotoxemia [8], whereas in the lethal models fibrinolysis was not investigated.

Recently, the capacity of a 55-kDa TNF receptor–IgG fusion protein (TNFR55:IgG) to effectively neutralize TNF activity and to strongly reduce lethality in baboons infused with a lethal dose of live *Escherichia coli* was reported [9]. Interestingly, treatment with TNFR55:IgG attenuated fibrinogen consumption and the increases in prothrombin and partial thromboplastin times caused by the bacteremia. These findings prompted us to study in more detail the effect of TNFR:IgG on the activation of coagulation and fibrinolysis.

Methods

Study design. The present study was performed simultaneously with a previously reported investigation [9]. Details of the animal study have been reported elsewhere [9]. Briefly, baboons *(Papio anubis)* (10–14 kg) were challenged with *E. coli* with or without pretreatment with TNFR55:IgG at the Research Animal Resource Center of Cornell University Medical College. At time zero, all animals received 10^{10}–10^{11} cfu/kg live *E. coli* (O86:B7) through a femoral venous catheter over 30 min. Baboons were randomized to receive either TNFR55:IgG (Ro 45-2081; 4.6 mg/kg; $n = 4$) or placebo ($n = 4$) as a 15-min intravenous infusion directly prior to infusion of bacteria. Endotoxin concentration of the TNFR55:IgG preparation was <0.4 EU/mg of protein. Arterial blood was obtained at -0.5, 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, and 8 h relative to the infusion of *E. coli*.

Assays. All assays were performed in heparinized plasma samples and were described in detail previously [4, 10]. Coagulation activation was determined by measuring thrombin−antithrombin III (TAT) complexes by ELISA. Fibrinolytic activation was monitored by measurements of tissue type plasminogen activator (tPA) by ELISA, plasminogen activator inhibitor type I (PAI-1) by ELISA, and plasmin−α_2-antiplasmin (PAP) complexes by RIA. Levels of PAP complexes are expressed as percentage of the level present in normal baboon plasma in which a maximal amount of PAP complexes was generated by a 1-h incubation with an equal volume of urokinase (50 μg/mL) in the presence of 0.4 M methylamine. Neutrophil degranulation was determined by measurement of the plasma concentrations of elastase−α_1-antitrypsin complexes by RIA. Secretory phospholipase A_2 (sPLA$_2$) was measured with an ELISA.

Statistical analysis. All values are expressed as mean ± SE. Differences within groups were tested by repeated-measures analysis of variance. Differences between groups were tested by repeated-measures analysis of variance (interaction between treatment and time). *P* values are therefore derived from analyses in which data from all time points were included. $P < .05$ was considered to represent a significant difference.

Results

In the previously reported study, treatment with TNFR55:IgG had been found to completely neutralize TNF activity throughout the observation period and to significantly attenuate the severe hypotensive response and lethality observed in the animals infused with *E. coli* [9]. Three of four baboons pretreated with placebo had died, after 6, 30, and 36 h, respectively, while all baboons treated with TNFR55:IgG survived.

The infusion of *E. coli* was associated with a strong activation of the common pathway of the coagulation system, as reflected by a sustained rise in the plasma concentrations of TAT complexes, peaking after 6 h (959 ± 296 ng/mL; $P < .05$ vs. time) (figure 1). This coagulation response was significantly blunted by treatment with TNFR55:IgG. Peak levels of TAT complexes in TNFR55:IgG-treated animals were 322 ± 90 ng/mL ($P < .05$ vs. placebo). Bacteremia also induced a marked activation of the fibrinolytic system, as indicated by increases in the plasma concentrations of tPA (peak of 12.9 ± 2.2 ng/mL after 8 h; $P < .05$ vs. time), PAI-1 (peak of 3669 ± 1298 ng/mL after 8 h; $P < .05$), and PAP complexes (peak of 0.68% ± 0.32% after 4 h; $P < .05$) (figure 1). Treatment with TNFR55:IgG had no influence on the rise in tPA levels but significantly attenuated the release of PAI-1 during bacteremia. Peak PAI-1 levels in TNFR55:IgG-infused baboons were 1095 ± 314 ng/mL ($P < .05$ vs. placebo). The plasma concentrations of PAP complexes tended to be higher in TNFR55:IgG-treated animals (peak of 0.85% ± 0.32% after 4 h), but the difference compared with control animals did not reach statistical significance.

As reported earlier [9], bacteremia was associated with a sustained neutrocytopenia, which was partially reversed by
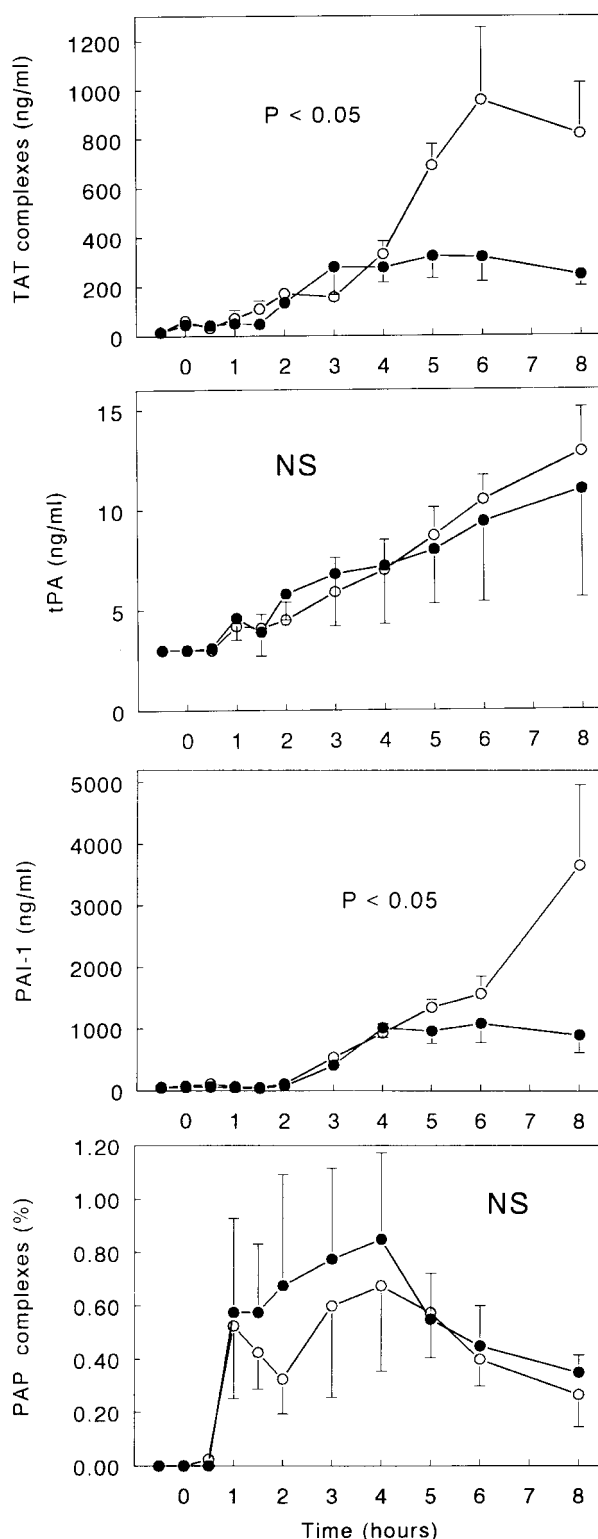
Figure 1. Effect of pretreatment with 55-kDa TNF receptor−IgG fusion protein (TNFR55:IgG) on activation of coagulation and fibrinolysis. Data are mean (±SE) plasma concentrations of thrombin-antithrombin III (TAT) complexes, tissue-type plasminogen activator (tPA), plasminogen activator inhibitor type I (PAI-1), and plasmin−α_2-antiplasmin (PAP) complexes after infusion of *Escherichia coli* in baboons pretreated with either TNFR55:IgG ($n = 4$, ●) or placebo ($n = 4$, ○). *P* indicates difference between groups by analysis of variance. NS = nonsignificant.
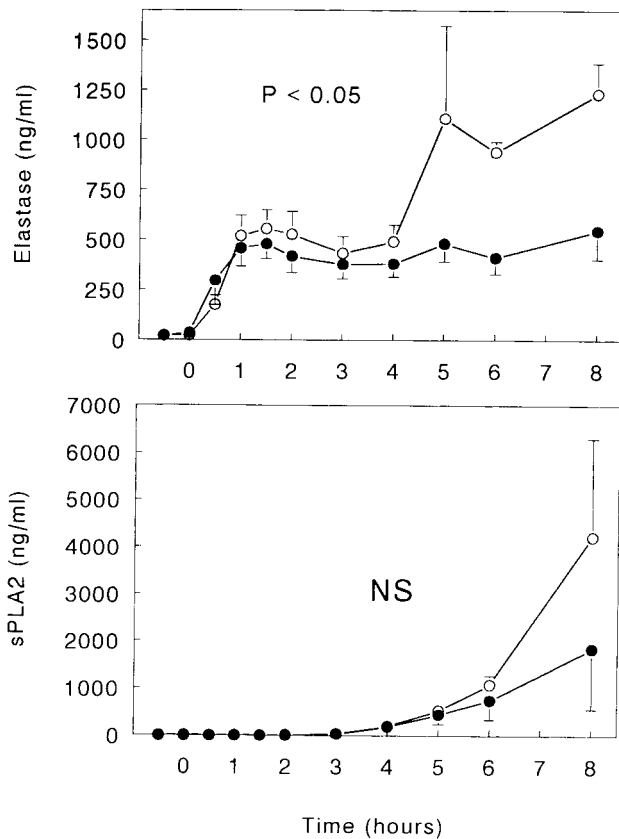
Figure 2. Effect of 55-kDa TNF receptor–IgG fusion protein (TNFR55:IgG) on neutrophil degranulation and secretory phospholipase A2 (sPLA2) release. Data are mean ($\pm$SE) plasma concentrations of elastase–α_1-antitrypsin complexes and sPLA2 after infusion of *Escherichia coli* in baboons pretreated with either TNFR55:IgG ($n = 4$, ●) or placebo ($n = 4$, ○). *P* indicates difference between groups by analysis of variance. NS = nonsignificant.

TNFR55:IgG. Infusion of *E. coli* also resulted in degranulation of neutrophilic granulocytes, as reflected by an increase in the plasma levels of elastase–α_1-antitrypsin complexes, maximal levels being measured at the end of the study period at 8 h (1234 $\pm$ 153 ng/mL; *P* < .05 vs. time) (figure 2). TNFR55:IgG significantly inhibited this response, with elastase–α_1-antitrypsin complexes reaching an approximately constant level at ~500 ng/mL from 1 to 8 h (*P* < .05 vs. placebo). *E. coli* bacteremia further caused a rise in the plasma concentrations of sPLA$_2$, the highest levels being measured at the end of the observation period (8 h: 4217 $\pm$ 2081 ng/mL; *P* < .05 vs. time) (figure 2). Although TNFR55:IgG reduced sPLA$_2$ release (8 h: 1845 $\pm$ 1284 ng/mL), the difference with control baboons did not reach statistical significance because of a large interindividual variation.

Discussion

The main finding of this study is that pretreatment of baboons with severe *E. coli* bacteremia with a TNF receptor–IgG fusion protein results in an attenuated procoagulant response, as reflected by inhibition of release of TAT complexes, in the absence of an effect on activation of fibrinolysis. This result is in line with the previously reported inhibition of more rough measures of coagulation activation, such as fibrinogen consumption and prolongation of prothrombin and partial thromboplastin times [9]. However, it should be noted that, thus far, studies in which endogenous TNF was neutralized in primates with either lethal or sublethal bacteremia or endotoxemia did not reveal any effect on activation of the coagulation system [5–8], while anti-TNF did abrogate the fibrinolytic response to low-dose endotoxin in chimpanzees [8]. We do not have an explanation for the apparent discrepancy of the present results with those from earlier studies, other than that the compound used to neutralize TNF activity, a dimeric 55-kDa TNF receptor–IgG fusion protein (which also has affinity for lymphotoxin), was different.

We consider it unlikely that our results are influenced by less-than-complete neutralization of TNF. Plasma TNF activity remained completely neutralized (as determined by the highly sensitive WEHI cytotoxicity assay) in animals treated with TNFR55:IgG throughout the entire observation period [9]. In fact, even lower doses of TNFR55:IgG than used in this study were able to completely neutralize TNF activity [9]. Further, TNFR55:IgG has been found to form stable complexes with TNF, in which aspect it differs from a similar IgG fusion protein containing a dimeric p75 TNF receptor [11]. Further, we consider it unlikely that the large spread in the levels of PAP complexes had an important influence on our main conclusion regarding an unaltered fibrinolytic response in animals treated with TNFR55:IgG. Indeed, in our previous study of low-grade endotoxemia in chimpanzees, anti-TNF completely prevented any rise in tPA and PAP complexes [8, 12], while in the present study, the levels of PAP complexes even tended to be higher in animals in which TNF was effectively neutralized.

Excessive activation of neutrophils may contribute in an important way to tissue injury in sepsis [13]. The plasma concentrations of elastase–α_1-antitrypsin complexes have been used as indicators of neutrophil degranulation in vivo and correlate with mortality rates in patients with sepsis [13]. TNF has been found to trigger neutrophil degranulation in vitro [1] and in humans and baboons in vivo [4, 14]. The present results that TNFR55:IgG reduced the release of elastase in bacteremic baboons are in line with a similar inhibition of elastase release by an anti-TNF antibody in endotoxemic chimpanzees [8] and therefore extend the role of TNF in neutrophil degranulation to lethal bacteremia.

sPLA$_2$ is a regulatory enzyme controlling the synthesis of eicosanoids and platelet-activating factor, which has been implicated in the pathogenesis of tissue injury associated with sepsis. Injection of TNF into baboons elicited a rapid release of sPLA$_2$ [4]. Pretreatment with TNFR55:IgG was associated with a modest, nonsignificant inhibition of sPLA$_2$ release, suggesting that TNF is not a critical mediator of this response in sepsis. Similarly, infusion of an anti-TNF antibody also mod-

estly reduced sPLA$_2$ secretion in an earlier study with bacter-emic baboons [15].

One control animal had died at the time the last blood sample was taken (after 8 h). The lack of this sample is unlikely to influence the results, since plasma concentrations of PAI-1, elastase$-\alpha_1$-antitrypsin complexes, and sPLA2 were higher in more ill animals, and thus the availability of an 8-h sample from the baboon that died early would likely have made differences between control animals and animals treated with TNFR55:IgG more pronounced.

Severe *E. coli* bacteremia in baboons may be a useful model to study pathogenetic mechanisms underlying inflammatory re-sponses during fulminant septic shock. We herein demonstrate that neutralization of endogenous TNF by pretreatment with a 55-kDa TNF receptor−IgG fusion protein not only prevents lethality but also significantly attenuates coagulation activation while not influencing fibrinolysis. Compared with earlier find-ings in primates with mild endotoxemia, revealing unaltered coagulation activation and inhibited fibrinolytic activation in animals treated with an anti-TNF antibody [8], these data illus-trate the divergence of various sepsis models, which depend on the severity and the time course of toxicity evoked by the bacterial insult, and also point to the difference in the effects exerted by the various anti-TNF agents used in such studies.

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Proteases from *Aspergillus fumigatus* Induce Release of Proinflammatory Cytokines and Cell Detachment in Airway Epithelial Cell Lines

J. F. Chris Tomee, Albertus T. J. Wierenga, Pieter S. Hiemstra, and Henk F. Kauffman

Department of Allergology, University Hospital Groningen, and Department of Pulmonology, Leiden University Hospital, Netherlands

Aspergillus fumigatus is a pathogen causing diverse respiratory disorders. Several studies have suggested that fungal proteases may play a role in the pathogenicity of fungi. Since the airways are the most common route for entry of *A. fumigatus,* this study focused on the ability of fungal proteases to induce the release of proinflammatory cytokines and to cause cell detachment in human pulmonary epithelial cell lines. It was shown that fungal serine protease activity induced the production of interleukin (IL)-8 and IL-6 and monocyte chemotactic protein-1 and caused cell detachment in a dose-dependent fashion. Chymostatin, antipain, phenylmethylsulfonyl fluoride, and heat treatment completely inhibited fungal protease activity, cytokine production and cell detachment; antileukoprotease partially inhibited these activities. By causing cell detachment, fungal proteases may decrease the physical barrier function of the epithelium; however, by eliciting a cytokine response, the epithelium may signal the mucosal inflammatory response against *A. fumigatus.*

Aspergillus fumigatus is a fungal pathogen of major clinical importance that predominantly affects the respiratory system and may infiltrate the bronchial mucosa [1, 2]. The virulence of *A. fumigatus* is due to specific properties by which it is able to bypass the cellular and humoral defenses of the host, and the production of extracellular proteases has been implicated as an important factor in its pathogenicity [3–5]. Although *A. fumigatus* mostly enters the human host by the pulmonary route, information regarding the interactions between fungal proteases and the human airways is limited.

The airway epithelium and mucus layer function as a physical barrier for the exclusion and removal of particulate matter, including microorganisms. In addition, the epithelium may play a paramount role in the orchestration of the local immune response by releasing inflammatory products and expressing various types of cell adhesion molecules (reviewed in [6]). The present study focuses on the production of proinflammatory cytokines and the induction of cell detachment in two pulmonary epithelial cell lines following exposure to *A. fumigatus* protease activity.

Methods

A. fumigatus culture filtrates. Preparation of culture filtrates from early-phase *A. fumigatus* cultures in collagen–yeast carbon base medium (early-phase collagen culture filtrate; E-ColCF) was performed as described elsewhere [7]. Heat treatment of E-ColCF was performed at 65°C for 30 min.

Quantification of fungal protease activity and inhibition of enzyme activity. Elastase activity was quantified using the elastase-specific substrate *N*-succinyl-alanyl-alanyl-prolyl-leucine *p*-nitroanilide (Sigma, St. Louis). Substrate (final concentration, 2.5 m*M*) was added to E-ColCF (final concentration, 0.33 mg protein/mL) in buffer (100 m*M* TRIS-HCl, 200 m*M* NaCl, 0.05% Triton X-100, pH 7.8) in a 96-well flat-bottomed microtiterplate (Greiner Labortechnik, Alphen a/d Rijn, Netherlands) at 37°C. The A_{405} was read every 30 s for 5 min with a computerized plate reader (Thermomax; Sopar-biochem, Nieuwegein, Netherlands). One unit of elastase activity is defined as the amount of enzyme that results in an increase of 1 optical density unit (A_{405}) per minute.

Total protease and gelatinase activities were measured according to the methods described elsewhere [7] using casein (Merck, Darmstadt, Germany) and orange gelatin (BIOK, Vilnius, Lithuania) as substrates, respectively. One unit of enzyme activity is the amount of enzyme that hydrolyses 1 mg of casein (total protease activity) or 1 mg of orange gelatin (gelatinase activity) in 30 min. When protease inhibitors were used, E-ColCF was incubated with the inhibitor for 15 min at 37°C before being added to the protease substrate. The inhibitors chymostatin (10 μg/mL), antipain (10 μg/mL), phenylmethylsulfonyl fluoride (PMSF, 100 μ*M*; Sigma), and human recombinant antileukoprotease (rALP, 40 μ*M*; gift from Synergen, Boulder, CO) were used.

Cell cultures. A549 cells, a human alveolar type II epithelium-like cell line, and NCI-H292 cells, a human epithelial cell line with characteristics of bronchial epithelial cells, were obtained from American Type Culture Collection (Rockville, MD). Cells were cultured at 37°C in RPMI 1640 (Life Technologies GIBCO BRL, Breda, Netherlands) with 10% heat-inactivated fetal calf serum (BioWhittaker, Verviers, Belgium) and 50 μg/mL gentamicin sulfate (Centrafarm Services, Etten-Leur, Netherlands).

Incubation of epithelial cell cultures with fungal culture filtrates. Epithelial cells were cultured in sterile 24-well culture dishes (Costar, Cambridge, MA) until 80%–90% confluence was reached. Following overnight incubation in serum-free RPMI

1640, epithelial cell cultures were washed and incubated for 48 h with various concentrations of E-ColCF (range, 5×10^{-4} to 200 μg/mL) in serum-free RPMI 1640 at 37°C and 5% CO_2. Epithelial cells incubated with interleukin (IL)-1α (20 U/mL; Boehringer Mannheim, Mannheim, Germany) (A549 cells) or phorbol myristate acetate (PMA, 80 n*M;* Sigma) (NCI-H292 cells) for 48 h served as positive controls. Epithelial cells incubated with serum-free RPMI 1640 served as the negative control. After 48 h of culture, supernatants were collected and stored at −20°C.

Quantification of cytokine and growth factor release, epithelial cell detachment, cell viability, and metabolic activity. Cytokine production was quantified using commercially available EIAs. IL-1β, IL-6, and IL-8 immunoassays were obtained from the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (Amsterdam). Granulocyte-macrophage colony-stimulating factor (GM-CSF) and monocyte chemotactic protein (MCP)-1 immunoassays were obtained from R&D Systems (Minneapolis). After 48 h of incubation with E-ColCF, cell detachment was scored using an inverted microscope and quantified on a 3-point scale (i.e., no, partial, or complete detachment). Cell viability was quantified microscopically by incubating epithelial cells with trypan blue (0.2% in 0.9% NaCl). A colorimetric assay was used for the quantification of cell viability, activation, and proliferation using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) [8].

Statistics. All data are presented as mean ± SE. Comparisons were made using the two-tailed Student's *t* test after recalculating cytokine productions to percentages of increase compared with that of the negative control. Differences were considered significant if *P* was < .05. Values are from at least four different experiments performed in duplicate.

Results

Cytokine production by bronchial and alveolar epithelial cells after incubation with fungal culture filtrates. E-ColCF caused a dose-dependent increase in IL-8 production by A549 cells that was significant at concentrations of ≥0.32 μg/mL E-ColCF (figure 1B). At 1.6 μg/mL E-ColCF, the IL-8 levels reached maximum, and cell detachment became evident (figure 1B). With E-ColCF concentrations >1.6 μg/mL, IL-8 production steadily decreased but remained elevated over that of the negative control (figure 1). Comparable results were found for the production of IL-6 by A549 cells; however, IL-6 production was optimal at 8 μg/mL E-ColCF (data not shown). MCP-1 production by A549 cells (figure 1B) paralleled the dose-response curve for the production of IL-8 in this cell line. MCP-1 levels were significantly increased between 0.32 and 8.0 μg/mL E-ColCF (*P* < .004), with a maximum at 1.6 μg/mL E-ColCF. In NCI-H292 cells, IL-6 production was significantly increased at 0.32 μg/mL E-ColCF (*P* < .008), with an optimum at 1.6 μg/mL E-ColCF (figure 1A). At 200 μg/mL E-ColCF, the IL-6 response was significantly lower than that of the negative control (*P* < .004). The dose-response curve for IL-8 was markedly different from that for IL-6 in this cell line; an increase in IL-8 production was found at 1.6 μg/mL E-ColCF (*P* < .04; figure 1A), reaching high

levels (>30 ng/mL IL-8) at 200 μg/mL E-ColCF. Despite cell detachment, IL-8 production did not decrease with higher E-ColCF concentrations. Partial cell detachment in NCI-H292 cell cultures was found at E-ColCF concentrations of 8.0–40.0 μg/mL (figure 1A); complete detachment was found with >40.0 μg/mL (figure 1A). No MCP-1 was found in supernatants from NCI-H292 cells. IL-1ß and GM-CSF could not be detected in supernatants from either cell line. Control experiments showed that the collagen–yeast carbon base medium up to 200 μg/mL did not increase cytokine production or cause cell detachment.

To exclude the possibility of enzymatic breakdown of secreted cytokines, thereby reducing their immunoreactivity in our detection procedures, IL-1β, IL-6, IL-8, GM-CSF, and MCP-1 were incubated with various E-ColCF concentrations for 24 h at 37°C. No decrease in immunoreactivity was found for any of the cytokines except MCP-1. At 200 μg/mL E-ColCF, MCP-1 immunoreactivity was decreased by 58%. At lower concentrations of E-ColCF, the decrease in immunoreactivity of MCP-1 was negligible (<8%, data not shown). In A549 cells, the metabolic activity was not affected by higher E-ColCF concentrations. In NCI-H292 cells, however, there was a 65% decrease in MTT reduction with 200 μg/mL E-ColCF. E-ColCF concentrations up to 200 μg/mL did not affect cell viability (trypan blue exclusion assay) in A549 or NCI-H292 cells; viability was always >95% (data not shown). Furthermore, the addition of E-ColCF did not affect the measurements of IL-8 and IL-6 in cell supernatants.

Inhibition of fungal protease activity by the serine protease inhibitors chymostatin, antipain, PMSF, and rALP. Quantification of the protease activities in E-ColCF revealed 7.4, 11.2, and 3.3 U/mg protein for elastase, gelatinase, and total protease activities, respectively (data not shown). Chymostatin (an inhibitor of chymotrypsin-like serine protease activity) completely abolished elastase and gelatinase activities and largely inhibited total protease activity (75.8%). Comparable results were obtained with the serine protease inhibitors antipain and PMSF (data not shown). With 40 μM rALP (an endogenous serine protease inhibitor), the activities for elastase, gelatinase, and total protease were inhibited by 68.9%, 31.3%, and 15.2%, respectively. Heat treatment of E-ColCF completely abolished all enzyme activities (data not shown).

Inhibition of cytokine production by protease inhibitors and heat treatment of E-ColCF. Heat treatment or addition of chymostatin, antipain, or PMSF to E-ColCF blocked its ability to induce IL-8 production in A549 cells at 1 μg/mL (table 1). Identical results were found for the production of MCP-1 and IL-6 (data not shown). In NCI-H292 cultures, the increase in IL-8 production with 40 μg/mL E-ColCF was partially inhibited by chymostatin (69.0%), antipain (71.9%), and PMSF (74.3%) and largely inhibited by heat treatment of E-ColCF (85.4%; table 1). Cell detachment was completely inhibited by the serine protease inhibitors and heat treatment of the E-ColCF (table 1). rALP partially inhibited the E-ColCF–induced IL-8 production (64.7%) and cell detachment in NCI-H292 cells
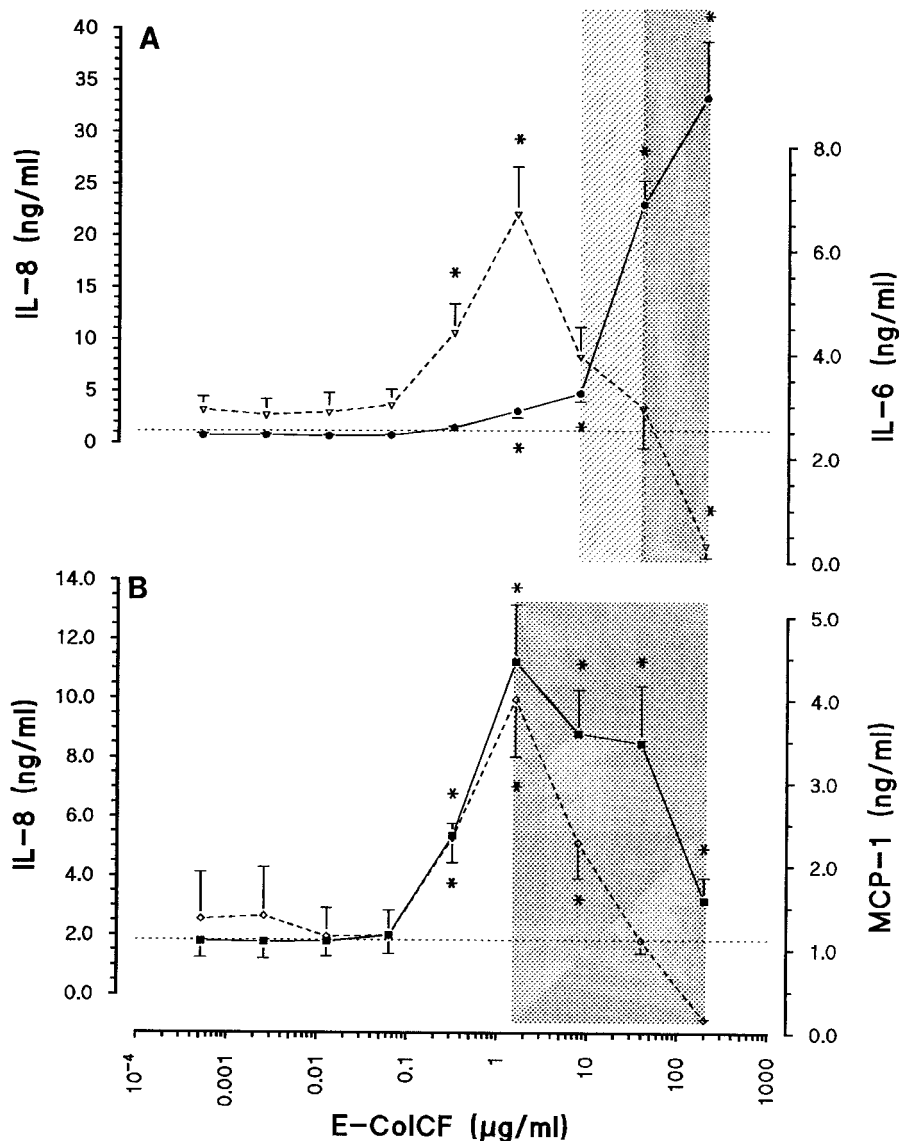
Figure 1. Quantification of immunoreactive cytokines produced by pulmonary epithelial cell lines after incubation with various concentrations of early-phase collagen culture filtrate (E-ColCF) (ordinate). **A**, interleukin (IL)-8 (solid line) and IL-6 (dashed line) in NCI-H292 culture supernatants. Light stippled area = partial cell detachment; dark stippled area = complete cell detachment. **B**, IL-8 (solid line) and monocyte chemotactic protein (MCP)-1 (dashed line) in A549 culture supernatants. Stippled area = complete cell detachment. Comparisons were made using 2-tailed Student's t test after recalculating cytokine production to % increase compared with that of negative control. $P < .05$ was considered significant. Values are from at least 4 experiments performed in duplicate. * Significantly increased or decreased cytokine production compared with that of negative control. Horizontal stippled lines = negative controls.

(data not shown). Control experiments showed that the inhibitors had no effect on spontaneous and IL-1α− or PMA-induced cytokine production or on viability and metabolic activity of cells from either cell line. Viability was always >95% (data not shown).

Discussion

In this study, we showed that culture filtrates from *A. fumigatus* induced the production of proinflammatory cytokines and caused cell detachment in pulmonary epithelial cell lines. Both phenomena were inhibited by the inhibition of fungal serine protease activity, suggesting that serine proteases released from *A. fumigatus* mediate these effects.

The production of MCP-1 and IL-8 by the epithelial cells may important for inflammatory processes. MCP-1 and IL-

8 are prototype members of the C-C and C-X-C families of chemokines and act as potent chemoattractants and activators of monocytes, lymphocytes, and neutrophils, respectively [9, 10]. Production of these chemokines in vivo may, therefore, provide a set of signals responsible for activating a broad spectrum of cellular host defense involving monocytes, lymphocytes, and neutrophils.

The observed decrease in cytokine production at higher E-ColCF concentrations is remarkable. The decrease in cytokine production by the A549 cells coincided with cell detachment without affecting cell viability or metabolic activity. In NCI-H292 cells, however, cell detachment coincided with a 65% reduction in metabolic activity, but in these cells the IL-8 production (in contrast to the IL-6 production) continued to increase with higher E-ColCF concentrations. Since the immunoreactivities of IL-6 and IL-8 were not affected at higher fungal

Table 1. The effect of inhibition of fungal protease activity on interleukin (IL)-8 production in A549 or NCI-H292 cells.

	IL-8 production (ng/mL)		
	A549	NCI-H292	Cell detachment
Positive control	73.3 ± 4.7	19.8 ± 2.4	−
E-ColCF alone	4.7 ± 0.4	17.1 ± 5.0	+
+ chymostatin	0.1 ± 0.0	5.3 ± 1.6	−
+ antipain	0.0 ± 0.2	4.8 ± 1.1	−
+ PMSF	0.0 ± 0.1	4.4 ± 1.6	−
Heat treatment	0.1 ± 0.2	2.5 ± 0.8	−

NOTE.   Cell cultures were incubated with 1 (A549) or 40 (NCI-H292) $\mu g/$ mL early-phase collagen culture filtrate (E-ColCF) with or without chymostatin (10 $\mu g/mL$), antipain (10 $\mu g/mL$), or phenylmethylsulfonyl fluoride (PMSF; 100 μM). Maximal production values found for positive controls (A549 cells: 20 U/mL of IL-1α; NCI-H292 cells: 80 nM of phorbol myristate acetate) are indicated. Microscopic observations of cell detachment in NCI-H292 cell cultures are indicated as: (+) complete, (±) partial, or (−) no detachment. Mean ± SE of individual readings minus spontaneous cytokine release of at least 4 independent experiments is given. Spontaneous IL-8 release in A549 and NCI-H292 cell cultures was 1.7 ± 0.2 and 0.9 ± 0.3 ng/mL, respectively.

protease concentrations, cytokine production appears to be decreased.

It is difficult to relate the fungal protease concentrations used in our experiments to the concentrations found in the airways following environmental exposures or exposures under pathologic conditions. The rapid release and high concentrations of fungal proteases found in *A. fumigatus* culture filtrates [7], however, could support the assumption that high focal concentrations on the apical surface of the epithelium may be reached. Furthermore, high IgG antibody responses, specific for fungal serine proteases, can be found in sera from patients with aspergillosis [7, 11], suggesting that fungal proteases may be produced in vivo during transient and chronic *Aspergillus* infections.

Several lines of evidence suggest a role for fungal proteases in the pathogenicity of *A. fumigatus* [3–5, 11, 12]. Although the production of extracellular proteases is obviously not the only factor that explains the marked pathogenicity of *A. fumigatus*, results from the present study indicate that fungal protease activity may lead to epithelial cell detachment. The ability to cause cell detachment may be an important feature of pathogenic fungi because weakening of the epithelium as a physical barrier may expose subepithelial structures and facilitate fungal adhesion [13–15]. Following epithelial cell detachment and adhesion, the fungus may gain ready access to the lung parenchyma and subsequently cause infection. However, by eliciting a cytokine response, the epithelium may signal the mucosal inflammatory response against *A. fumigatus,* resulting in a broad spectrum of immunologic response, possibly involving both polymorphonuclear and mononuclear cells. Endogenous protease inhibitors such as ALP may partially inhibit fungal serine protease activity, thereby inhibiting the production of proinflammatory cytokines by airway epithelial cells.

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Oxamniquine Cures *Schistosoma mansoni* Infection in a Focus in Which Cure Rates with Praziquantel Are Unusually Low

Foekje F. Stelma, Souleymane Sall, Bocar Daff, Seydou Sow, Malick Niang, and Bruno Gryseels

Department of Parasitology, University of Leiden, Leiden, Netherlands; Département de Santé Publique, Université de Cheikh Anta Diop, Dakar, and Projet de Lutte contre la Bilharziose dans la Région du Fleuve, Région Médical, St. Louis, Senegal; Prins Leopold Institute for Tropical Medicine, Antwerp, Belgium

An outbreak of *Schistosoma mansoni* in northern Senegal was observed in 1988, and chemotherapy with praziquantel in this recently established focus resulted in very low parasitologic cure rates. Among other explanations, the emergence of a praziquantel-tolerant parasite strain was feared. To study this hypothesis further, 138 persons with endemic *S. mansoni* infection were randomly allocated to treatment with either 20 mg/kg oxamniquine or 40 mg/kg praziquantel. Parasitologic cure rates at 6 weeks were significantly higher in the oxamniquine group (79%) compared with those in the praziquantel group (36%; $P = .0043$). The reduction in egg counts was generally good, but 12% less reduced in the praziquantel group. These results confirm that cure rates with praziquantel were abnormally low, whereas oxamniquine performed satisfactorily, as in other areas in which *S. mansoni* is endemic. The possibility of a praziquantel-tolerant *S. mansoni* strain must therefore be studied carefully.

During the last decade, praziquantel has been the drug of choice for the treatment of intestinal schistosomiasis in Africa [1]. This drug is considered to be safe and efficacious. However, in a *Schistosoma mansoni* focus in northern Senegal, cure rates after praziquantel treatment were low compared with those in other areas in which *S. mansoni* is endemic [1, 2]. This focus has only recently emerged and is characterized by extremely high intensities of infection [3]. Lack of immunity may also play a role, considering the synergistic action of praziquantel with host immune responses [4]. However, the spread of a genetically homogeneous schistosome strain with low susceptibility to praziquantel cannot be excluded [2, 5]. The aim of the present study was to compare the efficacy of praziquantel in this focus with that of another schistosomicidal drug, oxamniquine, in a controlled field trial.

Population and Methods

The study village, Ndombo, consists of 3000–4000 inhabitants and is situated along a main irrigation canal, not far from the town of Richard-Toll in northern Senegal. The area has been described in detail elsewhere [3]. All study subjects ($n = 138$) were residents from this village, and after extensive information about the research and its aims, they volunteered to participate. Each person submitted to a parasitologic examination: 2 stool samples were collected at 2 different days before treatment, and duplicate 25-mg Kato slides were prepared from each sample [6]. The slides were examined 24–72 h after preparation by two microscopists unaware of the given treatment. Participants were prestratified by age, intensity of infection, and history of previous praziquantel treatment and then randomly allocated to treatment with either 40 mg/kg praziquantel (Distocide; Shin Poong, Seoul, Korea) or 20 mg/kg oxamniquine (Vansil; Pfizer, Groton, CT) by lottery. Pregnant women and children <5 years old were excluded from the study. Parasitologic examination was repeated at 3 and 6 weeks after treatment.

Egg counts per gram of feces (epg) were calculated as the arithmetic mean of individual egg counts multiplied by 40. As egg counts approximate a log-normal distribution, geometric means were derived from $\log^{10}$-transformed epg from positive egg counts (epg >0) only. χ^2 test and t test statistics and 95% confidence intervals of means were used to compare groups. Parasitologic cure was defined as not excreting eggs in stools at 6 weeks after treatment. Egg count reduction was calculated as $[1 - (\text{geometric mean epg after treatment/geometric mean epg before treatment})] \times 100$.

Results

The study population is described in detail in table 1. No significant differences were found between treatment groups

Table 1. Characteristics of treatment groups; no significant differences were found regarding age, sex, history of previous treatment, and intensity of infection.

	Praziquantel	Oxamniquine	Significance
5–19 years	34 (51%)	39 (54%)	$\chi^2 = 0.020$
≥20 years	32 (49%)	33 (46%)	$P = .888$
Age range (years)	5–75	5–71	
Sex ratio (no. male/no. female)	22/44	30/42	$\chi^2 = 0.694$
			$P = .405$
Previous praziquantel treatment	27 (41%)	34 (47%)	$P = .1530$
Geometric mean egg count (eggs/g of stool)	260	393	$P = .167$
95% confidence interval	174–389	258–598	
n	66	72	

with respect to age, sex, intensity of infection, and history of previous treatment. According to the parasitologic data, subjects treated with oxamniquine showed significantly better cure rates at 6 weeks after treatment than did those who received praziquantel: 79% versus 36% ($P = .0043$; figure 1). The reduction in egg counts 6 weeks after treatment was higher in the oxamniquine group than in the praziquantel group: 94% versus 82% (figure 2).

Discussion

Treatment with praziquantel normally results in cure rates between 60% and 95% [2]. However, in this focus in northern Senegal, treatment with the standard drug regimen of 40 mg/kg resulted in a parasitologic cure of only 18%–36%, depending on the follow-up period after treatment [2, 7]. Various explanations could be forwarded, such as the very high intensities of infection and the recent nature of this focus; however, after several in-depth studies, these possibilities could be rejected (unpublished data). The most important component of

Figure 1. Parasitologic cure (% positive) after treatment with praziquantel (▲, 40 mg/kg) and oxamniquine (●, 20 mg/kg). $\chi^2 = 18.92$; $P = .0043$.

the chemotherapeutic field trials was a comparison between praziquantel and oxamniquine. Normally, both drugs are equally effective against *S. mansoni;* however, if important differences in cure between the two drugs were to be found, they might be caused by regional parasite strain characteristics.

The potential emergence of a parasite strain less susceptible to praziquantel treatment has been extensively debated [8]. Although praziquantel resistance has never been reported in other areas in which schistosomiasis is endemic, the epidemic nature of this focus may have caused a homogeneous and praziquantel-tolerant strain to become apparent. The existence of strain-related variations in tolerance was described for different antimonial drugs [9]; however, this phenomenon was only recently reported for praziquantel by Fallon and Doenhoff [10]. In a laboratory setting, they were able to select a schistosome strain not susceptible to praziquantel, but this characteristic did not remain constant when the drug pressure was removed. Additionally, in the same setting, 1 isolate from the Senegal focus appeared to be more tolerant to praziquantel treatment [5]. We therefore compared praziquantel with oxamniquine in a field study, as oxamniquine is considered as effective as praziquantel for community treatment [11].

The best follow-up period for monitoring parasitologic cure has been a topic of discussion [8]. Six weeks is considered the optimal follow-up period for the evaluation of schistosomiasis treatment, as this is the ''parasitologic window'' in which most eggs from previous infections would be excreted and eggs from prepatent infections would not yet have appeared. At the 6-week follow-up in this focus, the parasitologic cure rate obtained by oxamniquine treatment was significantly better than that of praziquantel and, in contrast to praziquantel, highly comparable with what has been observed in other areas [1, 12].

Prepatent infections may explain in part the difference in cure, as such infections become susceptible to praziquantel treatment 1 week later than they become susceptible to oxamniquine treatment [13]. However, after the randomization procedure, the oxamniquine group still possessed higher intensities of infection and therefore probably maintained even more prepatent infections than the praziquantel group. One would therefore expect that the former group would exhibit lower cure
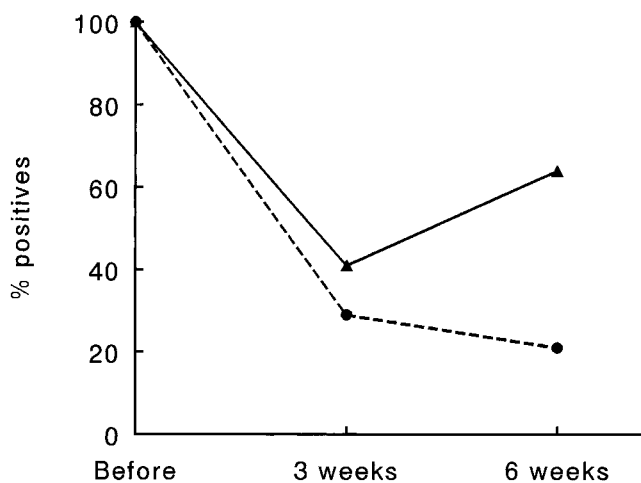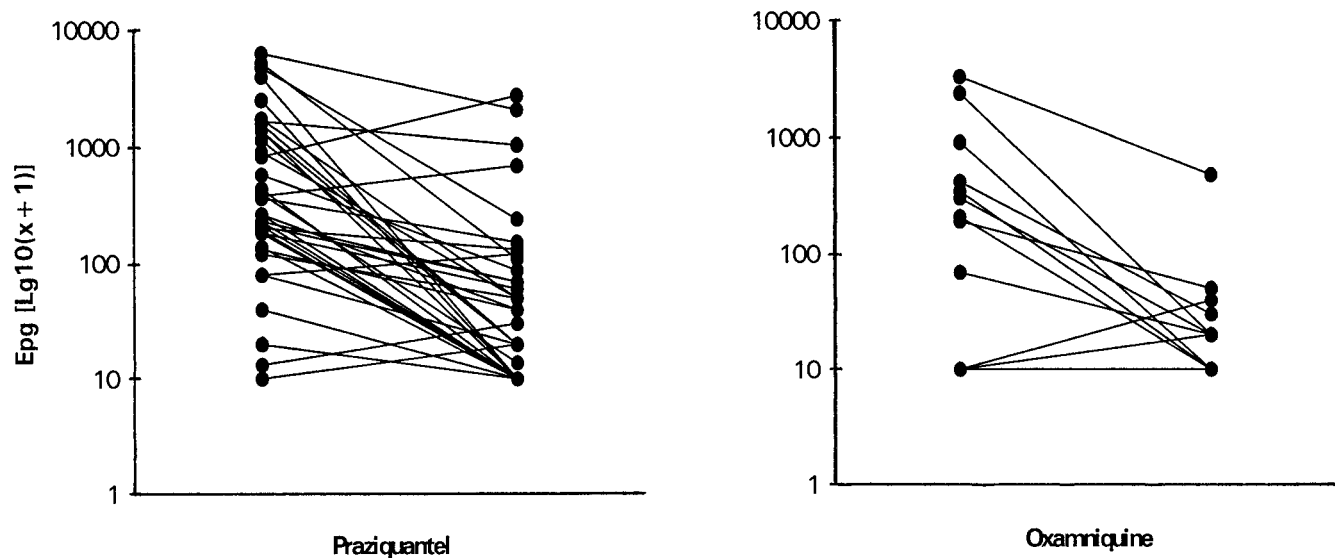
Figure 2. Egg count reduction in subjects who did not achieve cure after treatment with either praziquantel ($n = 42$) or oxamniquine ($n = 15$), before and 6 weeks after treatment.

rates or at least cure rates similar to those of the praziquantel group. Furthermore, in the focus of Maniema, Zaire, intensities of infection equally high as those in this Senegalese focus were encountered [12]. However, in the Zairian focus, cure rates after oxamniquine treatment were inferior to those obtained by praziquantel treatment [12]. An abundance of prepatent infections alone can thus not sufficiently explain the low parasitologic cure rates observed after praziquantel treatment in this Senegalese schistosomiasis focus.

The different working mechanisms of praziquantel and oxamniquine may justify the divergence in cure rate observed; oxamniquine is thought to bind with the parasite DNA, which then leads to cellular damage and death over a prolonged period [9]. Our observations correspond with this theory, as the curve of cure in the oxamniquine group continued to decline after 3 weeks (figure 1). Praziquantel, however, binds to the adult worm tegument, causing acute damage, tetanoid immobilization, and lysis of the worm tegument through host-dependent immunologic responses [4]. During in vitro experiments, it has been shown that worms can recover from these damages when challenged with a subcurative dose [14], and we probably observed this phenomenon, as cure rates appeared to decline after 3 weeks in the praziquantel group.

Immune responses are necessary for an optimal result of praziquantel treatment, as they act synergistically with this drug [4]. It is possible that these responses still were immature in this recently emerged focus. However, if insufficient immune responses were to explain the low cure rate after praziquantel treatment, cure rates would improve in subjects receiving subsequent treatments, and this was not the case (unpublished data).

In conclusion, we have demonstrated that parasitologic cure rates after treatment with 20 mg/kg oxamniquine were signifi-

cantly better than after 40 mg/kg praziquantel. This observation supports the hypothesis about a Senegalese schistosome strain with an increased tolerance to the latter drug. To still be able to design adequate control strategies in the future, further studies should be undertaken to verify this hypothesis, and efforts should be made to understand the underlying mechanisms of this phenomenon.

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