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Brief Report

A Study of the Immunology, Virology, and Safety of Prednisone in HIV-1–Infected Subjects with CD4 Cell Counts of 200 to 700 mm^{-3}

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Summary: Adult Clinical Trials Group Study 349 examined the immunology, virology, and safety of 40 mg/d prednisone as an adjunct to antiretroviral therapy in 24 HIV-infected subjects with >200 $CD4^+$ T cells/mm^3 in a randomized placebo-controlled trial. After 8 weeks, median lymphocyte and $CD4^+$ cell numbers increased >40% above baseline values ($p = .08$). No effect was observed on markers of cell activation or apoptosis, although the proportion of $CD28^+$ $CD8^+$ T cells increased ($p = .006$). Prednisone inhibited monocyte TNFα production without affecting T-cell responses to antigens or mitogens. Two subjects assigned to prednisone were subsequently found to have asymptomatic osteonecrosis of the hip. Many questions remain regarding the role of activation-induced sequestration and apoptosis as causes of progressive $CD4^+$ T-cell loss in AIDS. The potential role of corticosteroids as tools to examine this question will be limited by concerns regarding their toxicity; however, further studies of other agents to limit cellular activation in AIDS are warranted. **Key Words:** Activation—AIDS pathogenesis—Clinical trial—Corticosteroids—Apoptosis.

Many questions remain regarding the mechanisms causing progressive T-cell loss in AIDS, which may include direct viral cytotoxicity, programmed cell death (apoptosis), and trapping of activated T cells in lymphoid tissue (1). Corticosteroids may exert potentially beneficial effects on all these mechanisms (2–5). One uncontrolled clinical study published in 1995 indicated that prolonged administration of corticosteroids resulted in sustained increases in CD4 counts (5). This potential benefit must be balanced, however, by the recognized detrimental effects of corticosteroids on cellular immune function, glucose homeostasis, and bone metabolism. The primary objectives of the Adult Clinical Trials Group (ACTG) Study 349 therefore were to determine the effects of 40 mg/d prednisone on CD4 cell count and plasma HIV RNA in HIV-infected individuals on stable antiretroviral therapy. Secondary end points included other measures of immune activation and function, metabolism, and clinical outcome.

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METHODS

Study Design

The ACTG Study 349 was a multicenter, randomized, double-blind, placebo-controlled study of 40 mg/d prednisone for 8 weeks followed by 20 mg/d for 4 weeks in HIV-1–infected subjects with $CD4^+$ T-cell counts of 200 to 700 cells/mm^3 on stable antiretroviral therapy with at least two agents for >12 weeks before entry. Institutional review board approval and written informed consent were obtained according to the U.S. Department of Health and Human Services guidelines. Pregnant women; persons with a prior history of malignancy, diabetes, hypertension, pancreatitis, heart failure, peptic ulcer disease, osteoporosis, or autoimmune diseases; and recipients of any immunomodulatory therapies within 4 weeks of study entry were also excluded. Additional exclusionary criteria were anemia (hemoglobin <9.1 g/dL for men and <8.9 g/dL for women), platelet count <50,000 cells/mm^3, amylase >1.5 times the upper limit of normal, alanine and aspartate aminotransferase levels more than >5.0 times the upper limit of normal, creatinine >2.0 times the upper limit of normal, and glucose >160 mg/dL.

Immunologic Studies

Lymphocyte subsets were enumerated in heparinized blood with directly labeled monoclonal antibodies against CD3, CD4, CD8, CD28, CD38, CD95, CD62L, CD45RA, and HLA-DR (PharmMingen, San Diego, CA) (6). Naive cells were defined as $CD45RA^+/CD62L^+$. Plasma TNFα, sTNFrII, sIL-2r, neopterin, and β_2 microglobulin were measured according to ACTG consensus methods (7). Induction of TNFα (using *Escherichia coli* lipopolysaccharide) and interferon (IFN)-γ (using phytohemagglutinin A and *Mycobacterium avium* culture filtrate) were done by the whole-blood method (7). Lymphoproliferation induced by *Candida albicans* extract, streptokinase, or pokeweed mitogen was measured using mononuclear cells (7).

Virologic Studies

Blood samples were drawn into vacuum tubes containing EDTA anticoagulant and were processed on the same day. HIV-1 RNA was measured in plasma using either the Amplicor HIV Monitor or Ultra-Sensitive Monitor assay (Roche Molecular Systems, Branchburg, NJ), according to ACTG consensus methods (8–10). The lower quantitative sensitivity of the viral RNA assay was 400 copies/mL of plasma for some subjects and 50 copies/mL for others. DNA was extracted from 10^6 frozen or cryopreserved peripheral blood mononuclear cells (PBMCs) using QIAamp Blood DNA Mini Kits (Qiagen, Valencia, CA). PCR for HIV *gag* was performed using forward and reverse primers of GAG ACC ATC AAT GAG CAA GC and SK431, (11) respectively, and a probe consisting of 6FAM-AAA GAG ACC ATC AAT GAG GAA GCT GCA GAA-TAMRA. PCR was performed using an ABI 7700 with 500 ng of cellular DNA per tube. Potential contamination with PCR product was minimized using uracil N-glycosidase. β-Globin DNA was simultaneously amplified. Controls included DNA extracted from uninfected PBMCs, in vitro HIV-infected PBMCs, and HIV-1_{LAV}–infected A3.01 cells. Assay results were considered valid only if β-globin DNA was detected in both duplicate specimen reactions, the correlation coefficient (R^2) of standard curves was ≥0.95, negative control reactions resulted in C_T values of ≥40, and positive control reactions resulted in calculated input copy numbers of between 50 and 200 copies per reaction. The number of HIV DNA copies per 10^6 CD4 cells was calculated assuming 1 μg extracted DNA per 150,000 cells.

Metabolic and Anthropometric Studies and Screening for Osteonecrosis

Anthropometric measurements were performed according to ACTG procedures. Fasting blood specimens for insulin and lipids were collected in EDTA, and they were collected for glucose in sodium fluoride/potassium oxalate. Blood was processed within 6 hours of collection. Plasma triglyceride and total and HDL cholesterol were measured by β-estimation; LDL cholesterol was calculated using the Friedewald equation. Plasma insulin, proinsulin, and C-peptide concentrations were determined by radioimmunoassay (Linco Research, St. Louis, MO). Insulin sensitivity by homeostasis model assessment (HOMA) was calculated as glucose (mmol/L) × insulin (μU/mL) / 22.5 using 1 mmol/L = 0.05551 mg/dL. Screening for avascular necrosis of the hips was performed by conventional radiographs (using anterior/posterior and "frog-leg" views of both hips) and by MRI.

Statistical Analysis

The original sample size ($n = 118$) was estimated to yield 80% power to detect changes of +40% and −29% in $CD4^+$ T-cell counts and 1 $\log_{10}$ changes in HIV-1 RNA, with $\alpha = 5\%$ and Bonferroni adjustment for multiple testing of the two primary objectives. Analysis of adverse events other than osteonecrosis was restricted to those occurring while on treatment/placebo or within 56 days after discontinuation. Treatment arms were compared using exact stratified Wilcoxon tests, stratifying only on HIV-1 RNA. A three-level ordinal classification (increase, no change or undetectable on both determinations, or decrease) was used to examine changes in HIV-1 RNA, HIV-1 DNA, TNFα, and LPA. CIs on median changes were determined by inverting the Wilcoxon signed-rank test. All p values represent two-tailed tests, without adjustment for multiple comparisons.

RESULTS

Accrual to this study began in March 1999, with a targeted enrollment of 118 subjects. Later that year, preliminary findings from a retrospective analysis were released by researchers at the Warren Grant Magnuson Clinical Center at the National Institutes of Health indicating that prednisone treatment of individuals with HIV infection might be associated with an unacceptably high rate of osteonecrosis of the hip (12). Accordingly, ACTG Study 349 was prematurely closed to accrual in December 1999. Subjects included in the study were rapidly tapered off study drug. Beginning in April 2000, those who had been assigned to prednisone were offered screening evaluations for osteonecrosis by questionnaire, conventional radiography, and MRI. The questionnaire, examination, and plain films were repeated 6 months and 1 year later. To make the best use of the limited sample size, the analysis plan of the study as a whole was revised to an as-treated design, including only those subjects who had received at least 8 weeks of their originally

planned treatment. One subject assigned to prednisone was excluded because he had received a daily dose of 20 mg rather than 40 mg. Another assigned to placebo was excluded because he had discontinued antiretroviral therapy shortly after randomization. The treatment assignments of the remaining 24 subjects were well balanced with respect to baseline characteristics, including age, gender, race/ethnicity, injection drug use, Karnofsky score, CD4 cell count, plasma HIV RNA, and antiretroviral therapy (all $p > .18$).

Cell Counts

The median $CD4^+$ T-cell count on entry was 387 cells/μL. After 8 weeks, counts in prednisone recipients increased by a median of 172 cells (45%, range: 6–391 cells), whereas only a 4-cell increase (1%, range: −18–139) occurred in placebo recipients; this difference approached statistical significance ($p = .08$). Responses in prednisone recipients correlated with baseline CD4 counts ($r = 0.55$, $p = .067$). Responses reflected a generalized increase in total lymphocyte count (42%, $p = .08$); as a result, there was no change in the proportion of CD4 cells. The median $CD8^+$ T-cell count increased by 26% in the prednisone arm, although the difference between arms did not reach statistical significance ($p = .17$). The effect of prednisone on CD4 cell number was lost by week 12, 4 weeks after a dose reduction to 20 mg; however, a true effect cannot be excluded because of the low power of the study at that time point (<25%).

Advanced Flow Cytometry

Compared with placebo, prednisone was associated with a significant increase in the proportionate expression of CD28 on $CD8^+$ cells. Before treatment, this marker was expressed on 43% of CD8 cells. Treatment with prednisone increased this value by 9.8%, whereas the corresponding change in the placebo arm was −1% ($p = .006$). CD28 expression on $CD4^+$ cells was 89.5% at baseline and was not affected by treatment. There were no significant differences between the prednisone and placebo recipients in other markers on either $CD4^+$ or $CD8^+$ cells, including those indicating naivety ($CD45RA^+$ and $D62L^+$), activation ($HLA\text{-}DR^+$ and $CD38^+$), apoptosis ($Tunel^+$), or Fas expression ($CD95^+$).

Monocyte and T-Cell Function

The effects of treatment on monocytes and T cells were studied by stimulation in vitro and by examining soluble activation markers in serum. Prednisone treatment resulted in a reduction in LPS-induced TNFα by monocytes to 61% of pretreatment values (range: 26%–91%, $p = .046$ compared with placebo). This was reflected in vivo by a change in serum of soluble TNF type II receptor of −363 pg/mL (range: −880 to −21, $p = .03$ compared with placebo). No significant change was detected in plasma IL-6, neopterin, β_2-microglobulin, or TNFα (which was below the lower limit of the assay on both determinations in 16 of the 21 subjects). No effects were observed on antigen- or mitogen-induced IFNγ production or lymphoproliferation, whether analyzed according to response category (positive or negative) or numeric values (not shown).

Virology

Plasma HIV-1 RNA and cellular proviral DNA were below the level of detection at baseline in 67% and 29% of subjects. A categoric scoring system was used to evaluate changes during treatment; none was detected for either parameter ($p = .95$ and $p = .84$, respectively).

Body Composition and Metabolic Assays

The median body mass index at baseline was 26 kg/m^2. There was no significant treatment effect, although a trend toward greater weight gain was noted in prednisone-treated subjects (+0.58 [CI: −0.37, 1.1] vs. +0.14 in controls [CI: −0.71, 0.56], $p = .20$). Circumference measured at the umbilicus increased after prednisone treatment (1 cm [CI: −2.1, 3.3] vs. −2.7 in controls [CI: −6, −0.43], $p = .026$); similar trends were noted in other indices, but these did not reach statistical significance. Total and HDL cholesterol increased significantly in the prednisone arm compared with placebo (total cholesterol: 39 mg/dL [CI: 12, 52] vs. 1 [CI: −13, 19], $p = .022$; HDL: 23.5 mg/dL [CI: 15, 28] vs. 1 [CI: 0, 7], $p < .001$). No significant differences were detected in LDL cholesterol, triglycerides, insulin, proinsulin, c-peptide, glucose, or insulin sensitivity by HOMA; however, assessments of insulin resistance in this study may have been limited by small sample size. A significant positive correlation was found between changes in $CD4^+$ T-cell number and change in HDL cholesterol (rank correlation = 0.62, $p = .031$).

Adverse Events

No subjects discontinued study medication because of adverse effects. One subject assigned to placebo developed a grade 3 elevation in serum alanine aminotransferase. One subject assigned to prednisone developed

bacterial pneumonia due to penicillin-resistant *Streptococcus pneumoniae*; treatment did not require a change in study medication. There were no other grade 3 or 4 adverse events and no defined opportunistic infections or deaths.

Thirteen of 17 subjects who received prednisone consented to evaluation for osteonecrosis. Twelve underwent conventional hip radiography; all these studies were normal. Eleven underwent MRI evaluation, of which 2 (18%) showed evidence of osteonecrosis. Both had been treated with protease inhibitors; 1 had also received treatment with lipid-lowering agents and testosterone. Both individuals remained asymptomatic during the year of follow-up.

DISCUSSION

The main finding of this study is that prednisone treatment resulted in an increase of >40% in circulating $CD4^+$ T cells and total lymphocytes and by a similar trend in $CD8^+$ T cells. The mechanisms responsible for these increases are uncertain. T cells ordinarily respond to antigen and cytokine stimulation by undergoing transient clonal expansion (1). Only a small proportion of the expanded cell population enters (or re-enters) the pool of memory T cells; most instead undergo activation-induced programmed cell death (apoptosis). Elegant studies in which replicating cells were labeled in vivo have documented increased turnover rates of both $CD4^+$ and $CD8^+$ T lymphocytes in subjects with HIV infection (13). In that study, interruption of HIV replication by highly active antiretroviral therapy was accompanied by reduced lymphocyte proliferation without affecting the rate of cell death. These findings are consistent with a model in which the lymphocyte proliferation and apoptosis are driven by sustained expression of HIV antigens. In vitro data suggest that in HIV infection, glucocorticoids reduce T-cell death without significantly affecting proliferation (14,15). Rescue from activation-induced programmed cell death may therefore account for the increase we observed.

Binding of HIV to resting $CD4^+$ cells results in upregulation of L-selectin (CD62L), CD44, CD11a, and Fas (16). It is believed that these cells preferentially migrate to lymphoid tissues, where they undergo apoptosis. The death of these cells would pass undetected in the blood, possibly explaining the lack of effect on apoptosis in the current study. Analysis of apoptotic events in lymphoid tissues and of T-cell receptor gene excision circle DNA (TREC) (17) and Ki67 expression may assist future studies to determine the relative contributions of regeneration, redistribution, and antiapoptosis to the responses we observed.

Two previous clinical trials have examined the effects of corticosteroids in HIV-1–infected individuals. In an uncontrolled study, Andrieu et al. (5) found that prednisolone treatment resulted in increased $CD4^+$ T-cell counts and reduced levels of apoptosis. Antiretroviral therapy in that study consisted of, at most, zidovudine monotherapy. Although plasma HIV RNA was not measured, it likely was higher than in the current study. The effects that Andrieu and his colleagues reported on apoptosis may reflect higher levels of HIV expression. A second study by McComsey et al. (18) found no effect of prednisone on $CD4^+$ cell counts. The lack of effect in that study may be due to the lower baseline $CD4^+$ T-cell counts of its subjects (<200 cells/μL), given the relationship we observed between baseline count and magnitude of response.

The present study also found that corticosteroid treatment increased expression of CD28 by $CD8^+$ T cells. CD28 is the major costimulatory molecule of T cells (19). Its engagement by B7-1 (CD80) or B7-2 (CD86) on antigen-presenting cells results in T-cell activation and proliferation accompanied by upregulation of antiapoptotic $BCL\text{-}X_L$ pathways (20,21). In the absence of CD28 ligation, T-cell receptor binding results in apoptosis. CD28 expression by $CD4^+$ and $CD8^+$ cells is reduced in subjects with HIV-1 infection in association with defects in lymphocyte proliferation and IL-2 secretion; these are most pronounced in patients with advanced disease stage (22,23). It is not clear whether the increased proportion of circulating $CD28^+$ cells caused by prednisone treatment represents de novo expression or redistribution of cells from lymphoid tissue.

Weight loss, decreased HDL cholesterol, and increased triglyceride levels (due to an increase in very LDL) characteristically occur in HIV infection (24–26). Other investigators have noted a negative correlation between HDL cholesterol and TNFα or sTNFRII levels in HIV infection (25–27). These metabolic changes are similar to those seen in other chronic infections and are thought to be caused by the inflammatory response. It is therefore not surprising that we observed truncal weight gain, increased total cholesterol, and increased HDL cholesterol due to prednisone. Although these have not previously been reported in HIV-1 infection, similar glucocorticoid-induced increases in HDL cholesterol have been reported in patients with other chronic inflammatory diseases, including systemic lupus erythematosus, sarcoidosis, and organ transplantation (28,29). In these instances, increased HDL cholesterol occurs predominantly in the HDL_2 fraction and is associated with in-

creased risk of atherosclerosis rather than protection (30). The prognostic significance of the increases we observed is unknown. The period of corticosteroid administration in this study was brief; it is likely that other adverse metabolic consequences would become apparent with prolonged administration. Because body composition was not assessed, some prednisone-treated subjects may have lost muscle despite increases in body mass.

Two of 11 prednisone-treated subjects (18%) who agreed to MRI screening had asymptomatic lesions consistent with osteonecrosis. The retrospective survey by Miller et al. (12) that prompted the premature closure of this study found the incidence of asymptomatic osteonecrosis to be 4.4%. The duration of corticosteroid exposure in that report ranged from several days to several weeks; the intensity of exposure could not be accurately estimated. Miller and his colleagues identified several other factors also associated with osteonecrosis, including low testosterone, treatment with lipid-lowering agents or testosterone, and routine use of body-building devices. One of the 2 subjects with osteonecrosis in the present report also had two of these additional risk factors. Two other recent studies have also linked corticosteroid use and osteonecrosis in persons with HIV infection (31,32). Symptomatic osteonecrosis is a potentially debilitating condition that can require hip replacement surgery. Many gaps remain in our understanding of osteonecrosis in HIV infection, including the natural history of asymptomatic lesions. Until such data are collected, it would be prudent to limit corticosteroid use in HIV-infected individuals.

In summary, treatment of HIV-infected individuals with prednisone resulted in increased numbers of circulating $CD4^+$ T cells and reduced TNFα production by monocytes but did not affect antigen- or mitogen-stimulated T-cell proliferation or IFNγ expression. The potential role of corticosteroids in AIDS as a tool to examine pathogenesis or as adjunctive treatment will be limited by concerns regarding their toxicity, but further studies of other agents to inhibit apoptosis or limit T-cell activation are warranted.

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