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Plasma cytokines and oxidative damage in HIV-positive and HIV-negative adolescents and young adults: A protective role for IL-10?*

CHARLES B. STEPHENSEN[1], GRACE S. MARQUIS[2,†], STEVEN D. DOUGLAS[3,‡], & CRAIG M. WILSON[4,¶]

[1]*USDA-ARS Western Human Nutrition Research Center at the University of California, Davis and Department of Nutrition, University of California, Davis, CA 95616, USA,* [2]*Department of Food Science and Human Nutrition, Iowa State University, 1127 HNSB, Ames, IA 50011, USA,* [3]*Division of Allergy and Immunology, Department of Pediatrics, University of Pennsylvania School of Medicine, Joseph Stokes, Jr. Research Institute at The Children's Hospital of Pennsylvania, Philadelphia, PA, USA, and* [4]*Department of Pediatrics and Medicine, University of Alabama at Birmingham, Birmingham, AL 35294-2170, USA*

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Abstract
HIV infection causes immune activation that leads to oxidative damage. Proinflammatory cytokines may promote such damage and the regulatory cytokine IL-10 may protect against such damage. To examine the relation of these cytokines to oxidative damage, 67 cases of oxidative damage and 67 matched controls were selected from the reaching for excellence in adolescent health (REACH) study. Subjects were young (15–23 years), largely female (76%), HIV-positive (73%) and black (69%). Proinflammatory cytokines were not significantly associated with oxidative damage but plasma IL-10 had a significant, negative association with oxidative damage. This finding is consistent with a protective role for IL-10 in diminishing oxidative damage during immune activation.

Keywords: *Oxidative damage, HIV, malondialdehyde, protein carbonyl, IL-10*

Introduction

Subjects with HIV infection are at increased risk of oxidative stress due to immune system activation [6]. This stress may hasten the progression of disease by increasing the rate of HIV replication and impairing immune function due to oxidative damage [8]. Age, advanced disease and low intake of antioxidant nutrients may exacerbate oxidative damage. In a recent study we found that adolescents and young adults with HIV infection from the multi-center reaching for excellence in adolescent health (REACH) study did not have higher plasma concentrations of two markers of oxidative damage, protein carbonyls and malondialdehyde, than did HIV-negative subjects [10]. This may have been due to the relative youth of

Correspondence: C. B. Stephensen, USDA-ARS Western Human Nutrition Research Center at the University of California, Davis and Department of Nutrition, University of California, Davis, CA 95616, USA. Tel: 530 754 9266. Fax: 530 752 8966.
E-mail: cstephensen@ucdavis.edu

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†Tel: 515 294 9231. Fax: 515 294 5390. E-mail: gmarquis@iastate.edu
‡Tel: 215 590 1978. Fax: 215 590 3044. E-mail: douglas@email.chop.edu
¶Tel: 205 975 7608. Fax: 205 934 7138. E-mail: cwilson@uab.edu

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these subjects, adequate intake of some antioxidant nutrients (e.g. vitamin C) [7] and the fact that few subjects had advanced disease. However, markers of immune activation, including the number of activated CD8 + T-lymphocytes and plasma C-reactive protein (CRP) concentration, were associated with greater oxidative damage.

Proinflammatory cytokines such as IL-1β, TNF-α IL-6, IL-8 and IL-12 may increase oxidative damage by inducing production of reactive oxygen species. On the other hand, regulatory cytokines such as IL-10 may have direct antioxidant properties as well as the ability to down-regulate immune activation [5]. We measured the plasma levels of these cytokines in a subset of REACH subjects with oxidative damage matched to control subjects without oxidative damage.

Materials and methods

The REACH study, conducted at 15 US clinical sites, was a prospective, observational, cohort study on the progression of HIV infection in adolescents 12–18 years of age who had become infected through sexual activity or intravenous drug use [9,13]. HIV-negative adolescents were recruited from the same sites using selection criteria to make the HIV-negative and HIV-positive groups comparable with regard to risk-behavior profiles and demographic characteristics (including age, gender, race and ethnicity). For the present analysis, a supplemental cross-sectional study was conducted at 14 of these sites during one study visit in 2000. Among the 436 active participants at these sites, 391 agreed to participate in this sub-study (264 HIV-positive and 127 HIV-negative). The study received approval by the human subjects review boards at the University of California, Davis, Iowa State University, University of Alabama at Birmingham, and each clinic site. All participants provided informed written consent.

Cases of oxidative damage were defined as subjects with a plasma concentration for either protein carbonyls or malondialdehyde above the 90th percentile for all REACH subjects, as described [10]. Controls below this cut-off of the same gender and HIV status as the case were selected from the same clinical site using a chronological list of enrollment. Controls were selected with an enrollment date as close as possible to the case. From among 73 total cases of oxidative damage 67 were identified with appropriate controls and adequate available plasma for cytokine analysis. Most laboratory procedures have been described elsewhere [10].

Plasma cytokine concentrations were determined using Cytometric Bead Array reagents and a FacsCalibur flow cytometer (BD-Pharmingen, San Jose, CA). Plasma was frozen at −70°C and most samples were thawed and refrozen once or twice before analysis. The limit of detection for all cytokines was approximately 1 pg/ml. Approximately 10% of samples were analyzed in duplicate to assess within day and between day variation. The mean within-day variation (mean difference/mean) for all cytokines was 13%, while the variations for IL-12, TNF-α, IL-10, IL-6, IL-1 β and IL-8 individually were 17, 11, 6, 24, 11 and 9% respectively ($n = 10$ samples measured in duplicate). The mean between-day variation for all cytokines was 24%, while the individual variations were 23, 22, 19, 37, 15 and 30% respectively ($n = 16$ samples measured in duplicate). Samples were analyzed on five separate days. Fifty-two of the 67 case-control pairs (78%) were analyzed together on one of these days.

Cytokine and other immune activation markers were $\log_{10}$ transformed to normalize their distributions before statistical analysis. Since some samples had undetectable cytokine levels we adjusted all data by adding a value of one before $\log_{10}$ transformation. Mean values presented in Figure 1 were calculated on the untransformed data (including undetectable values as zeroes). Categorical variables were compared by Chi-squared test. Cytokine concentrations and other continuous variables were compared between cases and controls by paired Student's *t*-test. Multiple logistic regression analysis was used to adjust the cytokine analysis for other immune activation markers known to be associated with oxidative damage in these subjects [10]. The logistic regression models shown in Table I were also examined using methods to adjust for pairing, and the same variables were identified. Variables with *p*-values < 0.10 were retained in the model. Unless otherwise indicated, data are presented as mean ± standard error (SE). These analyses were performed using SigmaStat for Windows version 2.03 (Jandel Scientific, San Rafael, CA) and SAS (SAS Institute, Carey, NC).

Results

Seventy six percent (51/67) of the case-control pairs were female and 73% (49/67) were HIV-positive. Seventy three percent of the controls (48/66) and 69% of the cases (46/67) reported their race as black ($p = 0.74$). Fifteen percent of the controls (10/66) and 22% of the cases (15/67) reported their ethnicity as Hispanic ($p = 0.40$). Forty five percent of controls (30/67) and 43% of cases (29/67) reported smoking ($p = 0.10$). The mean age of the controls was 19.7 ± 0.2 years while that of the cases was 20.0 ± 0.2 years ($p = 0.19$).

Five markers of immune activation were measured. Neutrophil counts did not differ between cases and controls ($p = 0.76$, data not shown), nor did the concentrations of the acute phase protein ceruloplasmin ($p = 0.48$), or the concentration of neopterin ($p = 0.76$), a marker for activated macrophages. The number of activated CD8 + T-lymphocytes

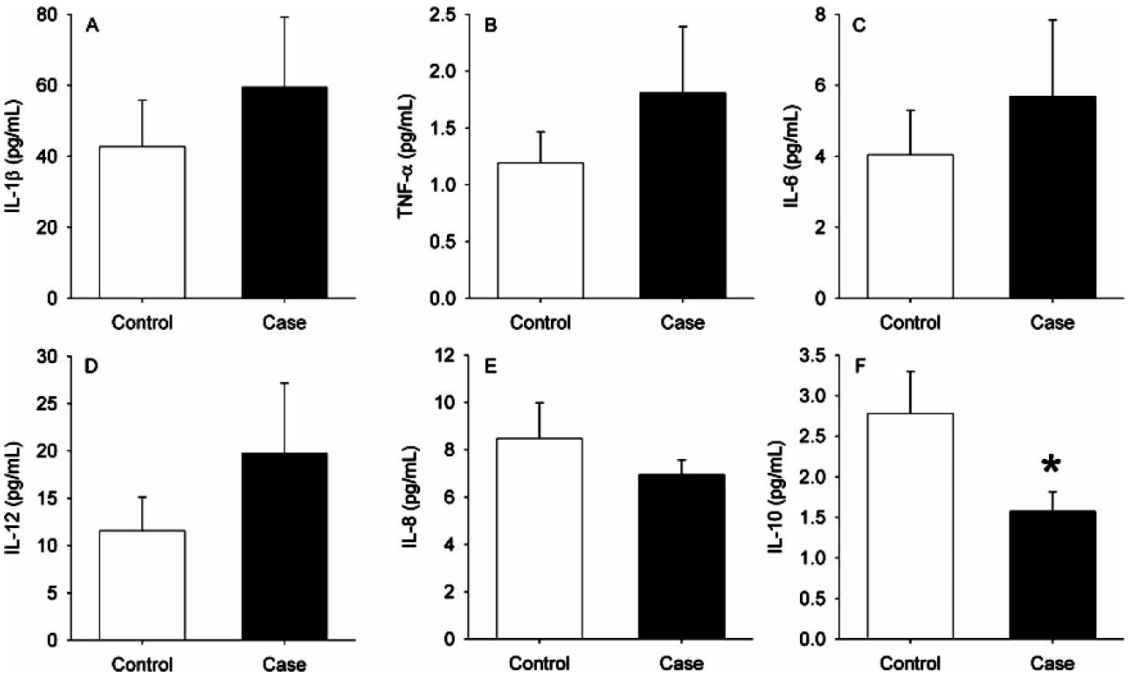

Figure 1. Mean ± SE plasma cytokine concentrations in 67 cases of oxidative damage and 67 matched controls from the multi-center reaching for excellence in adolescent health (REACH) study of adolescents and young adults with and without HIV infection. Cases had elevated levels of either plasma malondialdehyde or plasma protein carbonyls, as defined in the text. Controls were matched by gender, HIV status and study site. An asterisk (*) indicates a significant difference ($p < 0.05$) between cases and controls when compared by paired Student's *t*-test on $\log_{10}$ transformed data. Panels A through F present results for IL-1, TNF, IL-6, IL-12, IL-8 and IL-10, respectively.

($p = 0.097$) and the plasma CRP concentration ($p = 0.10$) tended to be higher in the cases than controls but these differences were not statistically significant. Among the 49 HIV-positive case-control pairs the mean CD4 + T-lymphocyte count of the controls, $578 \pm 51\,\text{mm}^{-3}$ ($n = 49$), did not differ from that of the cases, $485 \pm 39\,\text{mm}^{-3}$ ($n = 49$; $p = 0.18$). The percentage of subjects using antiretroviral therapy (ART) did not differ between controls (59%, 29/49) and cases (49%, 24/49; $p = 0.42$). Plasma virus load data were available for only 63% (31/49) of HIV-positive case-control pairs but the mean for the controls ($4.20 \pm 0.18\,\log_{10}$ genome copies/ml) was higher than for cases (3.81 ± 0.14, $p = 0.049$).

The percentage of subjects positive for plasma cytokines did not differ significantly between cases and controls. For controls ($n = 67$), the percent positive for IL-1β, TNF-α, IL-6, IL-12, IL-8 and IL-10 were 58, 61, 60, 64, 99 and 69% ($n = 39$, 41, 40, 43, 66 and 46, respectively). The corresponding percentages for cases were 54, 64, 64, 58, 100 and 54% ($n = 36$, 43, 43, 39, 67 and 36; *p*-values = 0.73, 0.86, 0.72, 0.60, 1.0 and 0.11, respectively).

Mean cytokine concentrations for controls and cases are shown in Figure 1. Concentrations of the proinflammatory cytokines, TNF-α IL-6, IL-12 and IL-8 did not differ significantly between the controls and cases ($p = 0.95$, 0.82, 0.97, 0.91 and 0.79, respectively) although the cases had higher mean concentrations for four of these five cytokines (139, 152, 141, 171 and 82%, respectively). In contrast, the mean concentration for the regulatory cytokine IL-10 was significantly greater ($p = 0.040$) in controls than cases. The mean for controls was 176% that of cases. When multiple, stepwise regression analysis was used to identify predictors of IL-10 concentration from among measures of immune activation, only plasma neopterin concentration was a significant, positive predictor ($R^2 = 0.182$, $p < 0.001$, $n = 134$).

Elevated IL-10 concentrations remained associated with a lower risk of oxidative damage when immune activation variables previously shown to be associated with oxidative damage in these subjects [10] were accounted for using multiple logistic regression analysis (Table I). In this analysis the regression equation uses multiple independent variables to predict the likelihood that the dependent variable will have a value of 1, indicating a case of oxidative damage, or will have a value of 0, indicating a control subject without such damage. Positive coefficients thus indicate a greater risk of oxidative damage for a particular variable while negative coefficients indicate a lesser risk. When all case-control pairs were examined in a single regression model, plasma CRP concentration and the count of activated CD8 + T-lymphocytes were both positively associated with oxidative damage (as indicated by the positive coefficients in Table I), while IL-10 was negatively associated (as indicated by the

Table I. Association of plasma IL-10 with risk of oxidative damage.

Variable	All case-control pairs (n=58 pairs)				HIV+ case-control pairs (n=41 pairs)			
	Coefficients*	SE	p-value	Odds ratio	Coefficients*	SE	p-value	Odds ratio
Constant	−0.633	0.557	0.0256	0.531	−4.474	8.988	0.619	0.014
CD4 + T-cells (mm^{-3})					−0.00153	0.000879	0.082	0.998
Log_{10} act. CD8 + T-cells (mm^{-3})	0.730	0.342	0.033	2.08	1.352	0.515	0.009	3.87
Log_{10} CRP (μg/l)	0.522	0.318	0.100	1.68	1.152	0.497	0.020	3.17
Log_{10} IL-10 (ng/l)	−1.512	0.640	0.018	0.220	−2.744	0.913	0.003	0.064
Log_{10} ceruloplasmin (mg/dl)					2.773	5.800	0.633	16.00
Antiretroviral therapy (ART)					22.092	11.186	0.048	3.92×10^{9}
ART × log_{10} ceruloplasmin †					−14.445	7.227	0.046	5.33×10^{-7}

Coefficients, standard errors (SE) and p-values are from a multiple logistic regression equation predicting status as a "case" with oxidative damage (dependent variable = 1) vs. a "control" without such damage (dependent variable = 0). The Odds ratio indicates the increase (or decrease) in odds of being a "case" for every one unit increase (or 10-fold increase, in the case of log_{10} transformed variables) in each independent variable.

*A positive coefficient indicates increased risk of oxidative damage, while a negative coefficient indicates decreased risk. †Interaction term. All terms (ceruloplasmin, ART and the interaction term) must be considered together when considering risk.

negative coefficient). This finding is consistent with the lower plasma concentrations of IL-10 seen in subjects with oxidative damage compared to those without such damage (as shown in Figure 1). In addition, the odds ratios reported for IL-10 was 0.220, indicating that the odds of being identified as a case of oxidative damage decrease by a factor of 0.220 with every unit increase of IL-10. Conversely, the odds of being a case increase by a factor of 2.08 for every unit increase of activated CD8 + T-cells.

When HIV-positive case-control pairs were examined alone, IL-10 was again negatively associated with oxidative damage. The association was somewhat stronger in HIV-positive subjects as indicated by the odds ratio of 0.064, compared to 0.220 for the model with all subjects. Ceruloplasmin concentration was also associated with a lower risk of oxidative damage. This association is only seen in subjects not taking ART [10], as represented by the interaction term in Table I. Plasma virus load was marginally ($p = 0.100$) associated with a lower risk of oxidative damage when included in this model (using the 28 case control pairs with plasma virus load data for both subjects). When the 17 HIV-negative case-control pairs were examined separately none of the variables shown in Table I was a significant predictor of oxidative damage.

Discussion

To our knowledge this is the first report that plasma IL-10 concentrations are associated with a lower risk of oxidative damage during HIV infection. This observation is not entirely unexpected, however, as it was recently proposed that IL-10 may be an antioxidant cytokine [5]. A principal basis for this suggestion is that IL-10 treatment *in vitro* decreases the production of reactive oxygen and nitrogen species by activated phagocytes. This decrease, in turn, could decrease the production of markers of oxidative damage such as malondialdehyde and protein carbonyls, which were used in this study to identify "cases" of oxidative damage. Thus IL-10 may act directly on "activated" cells, including phagocytes, to decrease oxidative stress and resulting oxidative damage.

It is also likely that IL-10 acts indirectly to decrease oxidative damage by down-regulating immune activation. For example, IL-10 can decrease the production of the pro- inflammatory cytokines IL-1 β, TNF-α IL-6 and IL-12, inhibit Th1 lymphocyte development [3] and IFN-γ secretion, and decrease proliferation and survival of T lymphocytes [2,5]. These regulatory properties of IL-10 could decrease immune activation that is associated with oxidative damage in the REACH subjects. Specifically, the number of activated cytotoxic T-lymphocytes was positively associated with oxidative damage in these subjects. Since a cytotoxic T- lymphocyte response is induced by Th1 cytokines such as IFN-γ, IL-10 may

directly down-regulate this response. In addition, plasma CRP concentration was positively associated with oxidative damage. Since CRP is synthesized by hepatocytes in response to increased systemic levels of IL-1 β, TNF-α and IL-6, it is reasonable to speculate that IL-10-mediated decreases in phagocyte activation could lead to decreases in oxidative damage as well as subsequent decreases in hepatic CRP synthesis.

Another possible explanation of the negative association of IL-10 with oxidative damage is that some aspect of oxidative stress could decrease IL-10 production. *In vitro* studies have shown that 4-hydroxynonenal, a product of lipid peroxidation, can decrease IL-10 production by lipopolysaccharide-stimulated peripheral blood mononuclear cells [4]. This explanation is plausible but the weight of evidence currently available favors a protective role for IL-10 in decreasing oxidative damage.

Interestingly, plasma levels of TNF-α and IL-6 were not significantly associated with oxidative damage, although higher levels were seen in cases than in controls. This observation suggests that CRP concentration is a better systemic indicator of the pro-oxidant potential of phagocyte-mediated inflammation than are these individual proinflammatory cytokines, since CRP was significantly associated with oxidative damage in the same analysis. This may not be true in all pathologic conditions, however. In patients with chronic heart failure plasma IL-6 correlated positively with oxidative damage while IL-10 concentrations did not show a significant association [14], although CRP was not measured in that study.

The role of IL-10 in HIV pathogenesis is controversial [2]. Elevated IL-10 levels have been associated with more advanced HIV disease [1] and progression of disease within individuals [11]. These data are consistent with the suggestion that IL-10 may down-regulate protective immunity and hasten progression of disease. On the other hand, IL-10 may decrease the production of proinflammatory cytokines that can increase HIV replication [2] and thus could slow disease progression. Our findings are more consistent with a protective role for IL-10 in HIV pathogenesis. An IL-10-mediated decrease in oxidative stress could decrease HIV replication by decreasing NF-κ B activity [8]. In addition, elevated counts of activated CD8 + T-lymphocytes are a strong predictor of disease progression in the REACH cohort [12] and are associated with oxidative damage in this study. These observations tend to support the view that elevated oxidative damage markers are associated with underlying immune-mediated pathology, and that IL-10 mediated decreases would be beneficial rather than detrimental.

Three other variables—CD4 T-lymphocyte count, ceruloplasmin concentration and ART use—were also associated with oxidative damage in the present study. The negative association of CD4 T-lymphocyte count with oxidative damage was of marginal statistical significance but suggests that oxidative damage is a greater risk with more advanced disease. In addition, we have previously reported [10] that ceruloplasmin concentration is negatively associated with oxidative damage in HIV-positive subjects, but this apparent protective association is seen only in those not taking ART.

In summary, this study suggests that IL-10 may play an antioxidant role during HIV infection. This intriguing possibility needs confirmation, and clarification as to whether the regulatory aspects of IL-10 provide a net benefit or detriment with regard to progression of HIV disease.

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