Resistance to HIV-1 infection among African sex workers is associated with global hyporesponsiveness in interleukin-4 production


*Departments of Medical Microbiology; †Pediatrics and Child Health; ‡Immunology, University of Manitoba, Winnipeg, Canada. R3E 0W3; and §Departments of Medical Microbiology, and ‖Community Health, University of Nairobi, Kenya

Corresponding author: Dr. Kent HayGlass, Department of Immunology, University of Manitoba, 626-730 William Ave. Winnipeg, MB R3E 0W3. E-mail: hayglass@ms.umanitoba.ca

ABSTRACT

We previously identified HIV-1 resistant prostitutes who remain persistently HIV-1 PCR- and antibody-negative despite continued heavy exposure to HIV-1 through sex work. We hypothesized that differences in virus-specific cytokine responses are associated with resistance vs. susceptibility to infection. Although polyclonal activation failed to reveal such differences, antigen-mediated activation of peripheral blood mononuclear cells (PBMC) in primary culture by using intact HIVIIIB demonstrates that resistance is associated with enhanced virus-driven interferon γ and markedly reduced IL-4 responses relative to those seen in HIV-1 seropositive prostitutes (CDC stage A1, CD4>500/ml). No changes were detectable in HIV-stimulated interleukin (IL) 10 and IL-13 production, but IL-5 responses were somewhat increased in resistant sex workers. Moreover, the IL-4 responses of HIV-1 resistant women to a panel of unrelated recall antigens were more than 20-fold reduced relative to HIV-infected prostitutes or those of healthy Kenyan women not involved in sex work. Thus, resistant women differ from seropositive-infected women and healthy controls by exhibiting a profound global hyporesponsiveness in their capacity to generate IL-4 responses.

Key words: HIV-1 resistance • type 1/type 2 immune responses • immunity

HIV exposure can lead to a broad spectrum of outcomes ranging from rapid progression to AIDS to survival for many years (1, 2). A small percentage of individuals exhibit resistance to HIV-1 infection, remaining seronegative, PCR-negative, and healthy despite multiple exposures. The mechanisms underlying resistance in these exposed/uninfected individuals appear heterogeneous and may include defective expression of the CCR5 co-receptor for HIV-1 internalization, elevated production of CC chemokines, RANTES, MIP-1a and MIP-1b, HIV-specific T helper and CTL responses, or secretory IgA responses in the genital tract (3–11).

The Nairobi prostitute cohort exhibits convincing epidemiological evidence of resistance to HIV infection in the face of intense sexual exposure (12). These individuals average more than 60
unprotected sexual exposures to HIV-1 per year. They have been followed for up to 15 years, and they remain HIV-1 antibody and PCR negative. Resistance in these women is not associated with enhanced levels of CC chemokines or mutations in the CCR5 gene, and their PBMC are readily infected with a wide range of HIV-1 variants of differing cellular tropism (13). Many of these women exhibit CTL responses to HIV env, gag, and pol gene products and interleukin (IL)2 production to HIV peptides (14), which suggests that immunologic factors may be involved in this type of HIV-1 resistance.

We hypothesized that differential immune responses may underlie resistance vs. susceptibility to HIV infection among Nairobi sex workers. Although exposed/uninfected individuals clearly possess HIV-1 specific, MHC class I and II restricted responses (3, 15–17), why these responses develop is unclear. In particular the nature of the immunoregulatory cytokine response in resistant (seronegative) vs. susceptible populations is largely unknown. A number of years ago, studies addressed the hypothesis of a Th1 to Th2 shift in immune responsiveness during HIV progression and its potential role in the pathogenesis of AIDS (18–20). They examined seropositive nonprogressors vs. seropositive progressors at different stages of disease. Many used mitogens or chemical stimuli rather than antigen-mediated activation. Many also relied on analysis of cytokines such as IL-2 and IL-10 that are now recognized as poor indicators of human type 1 or type 2 dominance. Quantitative analyses of cytokine responses by fresh PBMC stimulated in primary culture by intact HIV-1, exhibiting a spectrum of potential HIV epitopes, have not been carried out to date.

MATERIALS AND METHODS

Using an Institutional Protocol Review board-approved protocol, we examined HIV-1 resistant Kenyan prostitutes (n=14, chronically HIV-1 exposed, negative serology 8–56 times over 4–13 years, consistently HIV-1 PCR-negative (12); HIV-1 infected Kenyan prostitutes (n=11, PCR and HIV-1 antibody positive, CD4>500/ul), and healthy, low-risk female Kenyan controls (seronegative and PCR-negative, n=11). All subjects had received BCG immunization at birth. In initial experiments each culture parameter—including the optimal cell concentration, antigen or polyclonal activator concentration and culture period—was evaluated independently to identify conditions yielding optimal cytokine responses. Unselected PBMC populations, obtained by standard techniques using Histopaque-1077 (Sigma, St Louis, MO) (21), were used directly ex vivo in preference to highly purified CD4 T cells so as to not exclude a priori, antigen-driven cytokine production by non-CD4 cells. We cultured cells at 3×10^5/well (200 ul) in RPMI-10% fetal calf serum medium by using U-bottom 96-well plates (Corning Inc., Corning, NY). These primary cultures were stimulated with intact, psoralen/UV-inactivated HIV-1IIIB (Advanced Biotechnologies Inc., Columbia, MD) at 1.25×10^3 and 1.25×10^2 virus particles/ml (stock 10^{10.5} TCID_{so} /ml in H9 cells before inactivation); soluble anti-CD3 mAb (100 ng/ml OKT3 purified from hybridomas obtained from the American Type Culture Collection, (Rockville, MD); phytohemagglutinin (1%, Difco, Detroit, MI); streptokinase (5000 U/ml, Streptase^R, a gift from Aventis, Montreal, Canada); purified protein derivative (5 TU/ml, Tubersol^R from Connaught/Pasteur Merieux, Toronto, Canada); Candida albicans extract (50 ug/ml, Greer Laboratories, Lenoir, NC); and culture medium alone as negative control. Mitogen and antigen (streptokinase) controls yielded equivalent responses ± psoralen (at 10 pg/ml), which indicated that this reagent did not interfere with cytokine responses
under the conditions tested. Culture supernatants were harvested for analysis at days 1, 2, and 4 following polyclonal activation and d. 4, 6, 8 following antigen activation with peak cytokine responses typically observed at days 2 and 6, respectively. Cytokine responses were quantitated by ELISA (21, 22). IFN-γ was calibrated against WHO Gg23-901-530 (assay detection limit 0.3 U/ml); IL-4 against WHO standard 88/656 (detection limit ~0.45 pg/ml); IL-5, IL-13, and IL-10 against recombinant standards from Pharmingen (San Diego, CA) (assay detection limits of 5, 1, and 5 pg/ml). All samples were evaluated in at least duplicate assays, with the concentration of cytokine in each supernatant calculated from a minimum of three points falling on the linear portion of titration curves calibrated against recombinant cytokine standards serially diluted on each plate. Standard errors typically ranged from 3% to 10%. Differences between groups were analyzed by Mann-Whitney U test and Fisher’s Exact test (two-tailed), with median values indicated.

RESULTS

HIV-1 specific cytokine synthesis in primary culture

We examined antigen-specific cytokine responses in HIV-1 resistant and infected CDC stage A1 Kenyan sex workers. All participants were asymptomatic, exhibited CD4 > 500/ul, and had not received anti-retroviral therapy. Following short-term primary culture of PBMC with purified whole inactivated HIV-1IIIB or, for comparison, stimulation with polyclonal activators or unrelated recall antigens, cytokine production in culture supernatants was evaluated by ELISA.

All resistant and HIV-1 susceptible (i.e., infected) subjects exhibited HIV-dependent responses in primary culture, with detailed kinetic studies revealing peak cytokine levels at days 4 to 6 of culture in both groups (data not shown). Concomitant analyses of low-risk, seronegative, presumably unexposed, Kenyan women (n=11) did not yield detectable responses on identical stimulation (data not shown).

The nature of the cytokine response was markedly different in resistant and susceptible women. The frequency of resistant women exhibiting detectable IFN-γ responses on culture with HIV-1 was 86% (12 of 14 subjects) compared with 18% of susceptible women (2/11, Fisher’s two-tailed, P<0.001). Resistant women also exhibited more intense IFN-γ production than did HIV-1 infected individuals in response to virus-mediated stimulation (Fig. 1, Mann-Whitney P<0.005). IL-2 was not examined, as it is an unreliable indicator of type 1 cytokine synthesis. In marked contrast to IFN-γ, HIV-1 stimulated IL-4 responses in primary culture were substantially less frequent in the resistant population, with 2 of 14 resistant women (14%) generating detectable IL-4 responses vs. 10 of 11 susceptible women (91%, Fisher’s P<0.0002 ). The median intensity of this HIV-stimulated IL-4 production was at least 20-fold lower among resistant compared with susceptible women (<0.45 vs. 9.3 pg/ml; P<0.005). When these data are examined as the ratio of virus-stimulated IL-4:IFN-γ production, a commonly used strategy for evaluation of type 1 vs. type 2 dominance of immune responses (23, 24), resistant subjects exhibited patterns commonly termed “type 1” dominance, whereas healthy HIV-infected subjects displayed “type 2” dominated responses (IL-4: IFN-γ ratios of 0.27 vs. 46, P<0.0002, Mann-Whitney, Fig. 2).

A limited number of cytokines have often been studied as surrogates for Th1- vs. Th2-like
activation. Given that most immune responses and individual T cells exhibit a much more complex spectrum of cytokine responses than the bimodal pattern initially observed with T cell clones (23–27), we evaluated HIV-1 stimulated responses of a number of other type 2 cytokines. Notwithstanding the ~20-fold lower median IL-4 production seen in the resistant population, virus-stimulated IL-13 production was intense and similar in both groups (P=0.09, Fig. 1). HIV-driven IL-5 responses were significantly higher in the resistant population, with a median response of 71 pg/ml vs. undetectable (<3.9 pg/ml) among susceptible prostitutes. Thus, IL-5:IFN-γ or IL-13:IFN-γ ratios were indistinguishable between resistant and infected groups (P>0.05, Fig. 2), which supports the argument against a classical Th2 association with resistance vs. susceptibility to HIV-1 infection. IL-10 production, not consistently associated with either human Th1- or Th2-like T cell clones in vitro nor type 1 or type 2 responses in vivo (23), also did not differ significantly between resistant and susceptible populations (P=0.34, Fig. 1).

**Polyclonal stimuli fail to reveal alterations in type 1 vs. type 2 cytokine balance**

Use of polyclonal activators as surrogate antigens elicits intense, hence readily quantified, cytokine responses, which led to their widespread usage. However, polyclonal stimuli initiate distinct intracellular signaling events and frequently elicit cytokine responses that differ from those seen following MHC-dependant T cell activation (17, 22). Comparisons seeking to identify associations between differential cytokine responses and HIV-1 disease progression (i.e., comparing seropositive nonprogressors with other seropositive individuals at various stages of disease) largely relied on various polyclonal activators or HIV peptides (generally <25 amino acids) to evaluate the net HIV-specific immune response. Thus, for purposes of comparison, primary cultures were established by using immobilized anti-CD3 monoclonal antibody or phytohemagglutinin (PHA) as polyclonal stimuli (22). As anticipated, median cytokine responses by all resistant and HIV-1 infected subjects were 5- to 50-fold more intense than those seen in that individual’s HIV-specific response. However, IFN-γ, IL-4, IL-5, or IL-10 production in the study groups did not differ significantly under any of the conditions tested (P>0.05, data not shown). Median IL-13 production was marginally (P=0.04) higher following anti-CD3, but not PHA stimulation.

**Recall antigen-mediated stimulation reveals a global deficiency in the capacity of HIV-1 resistant women to mount IL-4 responses**

Because resistant women exhibit a strongly IFN-γ-dominated cytokine response to HIV-1 and because resistance to HIV has been associated with enhanced type 1 responses (IFN-γ synthesis, CTL activity), we considered the hypothesis that these women exhibit a generalized tendency for enhanced IFN-γ production relative to the general population. We therefore examined their capacity to respond to a panel of common recall antigens compared with low-risk, also seronegative, presumably unexposed Kenyan women.

Short-term culture with polyclonal stimuli (PHA, anti-CD3) or immunologically unrelated antigens (streptokinase, purified protein derivative, or Candida) revealed that the increased IFN-γ response to HIV seen among HIV-1 resistant prostitutes (Fig. 1) is not paralleled by general hyper-responsiveness in IFN-γ production (Fig. 3). Similarly, production of IL-5, IL-10, and IL-13 to these
environmental antigens was comparable with the HIV-1 resistant, HIV-infected, and HIV-1 unexposed groups (data not shown).

In marked contrast, IL-4 responses generated by HIV-1 resistant women to these recall antigens were dramatically lower than those of either healthy presumably unexposed Kenyan women or HIV-infected women examined above (Fig. 4). This decreased capacity of HIV-1 exposed/uninfected women to mount IL-4 responses was evident whether we examined the frequency of individuals in each group capable of mounting Ag-specific IL-4 responses (Fisher’s, \( P = 0.02 \) to 0.005 for the five different stimuli examined) or the median intensity of the IL-4 responses generated (Mann-Whitney \( P = 0.01 \) to 0.0015). In contrast, IL-4 responses to streptokinase, purified protein derivative (PPD), or Candida were indistinguishable in HIV-1 infected vs. HIV-1 unexposed populations (Mann-Whitney \( P = 0.43 \) to 0.54). Thus, HIV-1 resistant women exhibit a global hyporesponsiveness in their capacity to generate IL-4 responses to HIV or to common recall antigens when compared with either HIV-1 infected or unexposed groups.

DISCUSSION

Despite recognition that HIV-1 specific Th activity is likely to be important in host resistance, rate of disease progression, and immunotherapeutic intervention (30–34), the nature of this response is not well understood. In part, this may be due to a widespread experimental focus on T cell proliferation or IL-2 production, which, although they provide evidence of helper T cell activation, provide little information on the nature of that response. Previous studies of cytokine production, and of Th activity, have tended to use synthetic peptides of 15-20 amino acids, individual gene products (i.e., baculovirus expressed p24) or, in analysis of cytokines such as IL-4, IL-5 or IL-13, polyclonal activators as stimuli. Given the often low frequency of individuals demonstrating proliferative responses to single protein Ags (i.e., 3 of 41 subjects to baculovirus expressed p24, 4 of 41 responding to gp160(34)) and the exquisite selectivity of HIV-1 specific responses in some subjects (35), it is extremely difficult for us to predict with confidence the array of relevant epitopes that will be recognized in a population. Consequently, we chose to evaluate the cytokine response by short-term culture in response to intact, inactivated HIV-1 IIIB to provide a more compete cross section of antigens.

The data in this report, obtained from individuals who provide one of the most clear-cut examples of resistance to HIV-1 infection in the face of ongoing HIV-1 exposure, indicate that these resistant subjects exhibit profoundly different cytokine responses to HIV-1 and to common environmental antigens. Specifically, resistance vs. susceptibility to HIV-1 infection in these women is distinguished by reciprocal differences in the intensity of HIV-1 driven IFN-\( \gamma \) and IL-4 cytokine production, which is apparently independent of substantial alterations in IL-5, IL-10, and IL-13 responses. Such changes are not readily interpretable in terms of classical type 1 vs. type 2 skewing of the HIV-specific immune response. Whether the global deficiency in the capacity of this population to generate IL-4 responses is associated with other clinical consequences, such as a decreased incidence of atopic diseases or asthma, remains to be determined.

The events occurring during the earliest stages of viral infections, in particular the nature of the immunoregulatory response that develops, are pivotal in determining the type and intensity of
immune response that develops, as well as the eventual outcome of the disease (35–39). Patients with self-limited viral disease maintain a high number of anti-viral CD4 T cell responses with a strong inverse correlation between the intensity of such responses and viral RNA levels, supporting the hypothesis of viral control dependent on CD4 T cells (30, 36, 40, 41). Conversely, an absence of detectable virus-specific CD4 T cell responses is associated with lack of immune control and progressive infection. The presence of such Th activity is required for maintenance of effective CTL activity and other immune effector responses throughout the chronic phase of infection. The importance of such Th activity is underlined by the recent findings of Rosenberg et al, who report the reappearance of gag-specific T cell responses (measured as proliferation) in HIV-1 infected subjects at levels comparable with those of long-term nonprogressors in those instances where HAART (highly active anti-retroviral therapy) led to complete (<50 copies/ml) suppression of plasma viremia (32). These data underline the importance of Th activity and emphasize the importance of defining more precisely the nature of the immunoregulatory response, as central steps in determining clinical outcome of HIV-1 exposure.

It is not yet possible to say whether resistance to HIV-1 is the cause or result of the globally reduced IL-4 responses seen among resistant women. If resistance reflects acquired immunity, individuals with reduced capacity to mount IL-4 responses to HIV-1 may preferentially develop resistance. Alternatively, the reduced IL-4 responsiveness evident in these women may be secondary to the induction of resistance. HIV-1 has been reported to replicate more efficiently in IL-4 producing T cell clones (Th2/Th0) than in Th1 clones (42, 43). Certainly, IL-4 is a pivotal cytokine in promoting commitment of naive T cells to IL-4 dominated response patterns (44–47) and is capable in vitro of overriding potent IFN-γ/Th1 promoting stimuli such as IL-12. We also note that rIL-4 substantially up-regulates in vitro expression of CXCR4, a coreceptor for T cell tropic HIV-1, making those cells more susceptible to HIV-1 infection (48, 49) and resulting in increased HIV-1 production (50), whereas IFN-γ down-regulates expression of this receptor (43, 51, 52). Collectively, these observations raise the possibility that resistant individuals who exhibit markedly lower HIV- and environmental Ag-driven IL-4 responses in vitro may be characterized by lower levels of infectious HIV production at initial exposure, combined with enhanced clearance of HIV-1, which is attributable in part to intense IFN-γ dominated responses and CTL generation.

An ideal HIV-1 vaccine would elicit sterilizing immunity so that, after exposure, the virus would never be detected in the body (53). The finding that these women, with functional immunity and undetectable levels of virus despite chronic exposure to infectious virus, exhibit global hyporesponsiveness in their capacity to mount IL-4 responses may be of particular relevance to HIV-1 vaccine development. Also of interest in this regard is the fact that antigen-specific activation was accomplished here by using HIV-1IB, a widely available clade B virus. Currently dominant Kenyan isolates are from clades A, C, D (54, 55), although B clade viruses do occur. The finding that HIV-1IB readily evokes cytokine responses among PBMC from subjects with minimal exposure to this clade is consistent with the epidemiologic finding that these women who have exposure to a broad variety of HIV variants within and between clades are resistant to a broad range of HIV-1 subtypes. Thus, resistance to HIV-1 in these women appears broadly cross-protective. Similar cross-reactivity in CTL activity (56) and β chemokine production (57) between HIV-1 and HIV-2 responses has also been observed. This observation may also have significant implications for design of HIV vaccines.
ACKNOWLEDGMENTS

This work was supported by grants from the National Health Research and Development Program and the Medical Research Council of Canada to F.A.P., J.E.E., and K.T. H.H.T. was supported by the Canadian Commonwealth Scholarship Plan. K.T.H. and F.A.P. hold Canada Research Chairs.

REFERENCES


30. Matloubian, M., Concepcion, R. J., and Ahmed, R. (1994) CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. *J. Virol.* **68**, 8056-8063


42. Maggi, E., Mazzetti, M., Ravina A, et al. (1994) Ability of HIV to promote a TH1 to TH0 shift and to replicate preferentially in TH2 and TH0 cells. *Science* **265**, 244-248


Received December 14, 2000; revised March 22, 2001.
Figure 1. HIV-1 resistant sex workers exhibit significantly enhanced IFN-γ (Mann-Whitney P < 0.005) and lower IL-4 responses (P < 0.005) on in vitro re-exposure to intact HIV-1. Short-term primary cultures were set up with unselected PBMC from 14 HIV-1 resistant (●) and 11 HIV-1 susceptible (PCR and seropositive, CD4>500 per ul) sex workers (□). Cells were cultured for 2–8 days with intact inactivated HIV IIIB, and in parallel wells (not shown), polyclonal stimuli anti-CD3 mAb or PHA. The intensity of peak cytokine responses in culture supernatants was determined by ELISA against WHO or commercial standards as described at Methods and Materials.
Figure 2. Comparison of HIV-1 driven, type 1/type 2 cytokine dominance in resistant (●) and susceptible (□) women. The ratios of the indicated cytokines were determined from the primary data of Figure 1, reflecting peak HIV-driven cytokine responses.
Figure 3. HIV-1 resistant women do not exhibit generalized hyper-responsiveness in their capacity to mount IFN-γ responses to unrelated recall antigens. PBMC from seronegative resistant (●) and seronegative presumably unexposed (▲) women were cultured with streptokinase (5000 U/ml), PPD (5 TU/ml), Candida extract (50 µg/ml), PHA (1%) or anti-CD3 (100 ng/ml, not shown) with the intensity of the maximum IFN-γ response determined at days 2–8. Peak cytokine responses are shown. Some groups contain fewer subjects due to the limited number of cells available. Mann-Whitney $P > 0.05$ in all cases.
Figure 4. Resistant women are hyporesponsive in their capacity to mount recall antigen driven IL-4 responses. PBMC were cultured as for Figure 3, and peak streptokinase (a) and PPD-specific (b) responses in resistant (●) and susceptible (□) prostitutes, as well as low-risk controls (▲), following primary culture were determined by ELISA.