Antigen-driven C–C Chemokine-mediated HIV-1 Suppression by CD4+ T Cells from Exposed Uninfected Individuals Expressing the Wild-type CCR-5 Allele

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Summary

Despite repeated exposure to HIV-1, certain individuals remain persistently uninfected. Such exposed uninfected (EU) people show evidence of HIV-1–specific T cell immunity and, in rare cases, selective resistance to infection by macrophage-tropic strains of HIV-1. The latter has been associated with a 32–base pair deletion in the C–C chemokine receptor gene CCR-5, the major coreceptor of macrophage-tropic strains of HIV-1. We have undertaken an analysis of the HIV-specific T cell responses in 12 EU individuals who were either homozygous for the wild-type CCR-5 allele or heterozygous for the deletion allele (CCR-5Δ32). We have found evidence of an oligoclonal T cell response mediated by helper T cells specific for a conserved region of the HIV-1 envelope. These cells produce very high levels of C–C chemokines when stimulated by the specific antigen and suppress selectively the replication of macrophage-tropic, but not T cell–tropic, strains of HIV-1. These chemokine-producing helper cells may be part of a protective immune response that could be potentially exploited for vaccine development.

Many individuals who remain persistently uninfected despite repeated exposure to HIV display evidence of HIV-specific immunity, including antigen-driven T helper cell–mediated cytokine production (1–3) and cytotoxicity induced by HIV early proteins (4–6). This implies that in some cases chronic exposure to HIV may lead to protective immunity rather than infection. A well-characterized pathway of HIV suppression involves CD8 T cells producing C–C chemokines (7). Evidence for the in vivo relevance of such a pathway comes from the observation that rare individuals homozygous for a deletion within the C–C chemokine receptor gene CCR-5 (CCR-5Δ32) are resistant to HIV-1 infection (8, 9), although infection in a CCR-5Δ32 homozygous individual has also been reported (10). CCR-5 is the major coreceptor of macrophage-tropic nonsyncytium-inducing (NSI) strains of HIV-1 (11–14), which are considered to be preferentially involved in sexual transmission and constitute the predominant phenotype in newly infected individuals. T cell–tropic syncytium-inducing (SI) strains appear later in infection (15). It is estimated that CCR-5Δ32 homozygosity is present in 1% of the Caucasian population (8). In high risk groups, the frequency is only slightly elevated (2.8%) (16); thus, the CCR-5Δ32 deletion

does not fully account for HIV resistance. Other mechanisms of resistance to infection may involve other mutations in the CCR-5 gene or in genes coding for alternative coreceptors. On the other hand, specific immune responses induced by exposure to HIV antigens may prevent infection by interfering with the same pathway of HIV entry. Such immune responses may play a role in CCR-5 wild-type homozygous exposed uninfected (EU) individuals whose cells are fully competent to support the growth of macrophage-tropic strains of HIV-1 (12). Specific immunity may be driven by cytotoxic as well as helper T cells producing C–C chemokines. Indeed, CD4+ T cells from EU individuals have been shown to produce high levels of C–C chemokines upon polyclonal activation (12). It is not known whether the production of C–C chemokines by EU helper T cells is part of an antigen-driven immune response or is under the control of other factors.

Here, we present findings from a cohort of heterosexual couples in which one partner was HIV infected and the other remained persistently uninfected despite having engaged in unprotected sexual intercourse (EU partner). The aim of the study was to identify immune mechanisms of resistance to infection, possibly involving the C–C chemo-

kine pathway, in those EU partners expressing the wild-type CCR-5 allele. We identified HIV gp120-specific CD4^{+} T cell clones, which were highly represented in the helper cell population of EU partners, and assessed their ability to produce C–C chemokines and suppress HIV replication when stimulated with the specific antigen.

Materials and Methods

Study Population. 12 long-term sexually active heterosexual couples with discordant HIV serological status, i.e., one partner was infected and seropositive and the other seronegative and uninfected (EU), were enrolled in the study. At the time of entry into the study, and regularly thereafter, the infected partners were evaluated for laboratory (serum p24 antigen and CD4 cell count) and clinical parameters of HIV infection, and were assigned to a Centers for Disease Control (CDC) classification of disease stage. All CDC disease stages were represented with no preponderance of any one group. The EU partners were tested for HIV-1/2 antibodies, serum p24 antigen, and plasma HIV DNA (by PCR) at the time of entry into the study. Thereafter, they were monitored clinically and tested for HIV-1/2 antibodies, p24 antigen, and HIV DNA PCR (NASBA, Organon Teknika, Veedijk, Belgium) every 3 mo. Couples were followed for at least 24 mo, and during that time, none of the EU seronegative partners seroconverted or showed any clinical or laboratory evidence of HIV infection.

CCR-5 Genotyping. A portion of the CCR5 gene was amplified by PCR from PBMC genomic DNA. The following primers were used: primer 1, 5' GTC TTC ATT ACA CCT GCA GCT C 3'; primer 2, 5' GTG AAG ATA AGC CTC ACA GCC 3'. PCR was conducted with 1 μg of genomic DNA using 0.2 mM dNTPs, 0.2 μM primers, and 1.25 U of AmpliTAq Gold polymerase (PE Applied Biosystems, Branchburg, NJ) for 35 cycles (94°C for 40 s; 60°C for 40 s; 72°C for 40 s) after an initial 10-min denaturation at 94°C. The resulting PCR products were separated on a 2% nusieve agarose gel.

Limiting Dilution Assays. Serial dilutions of PBMC from EU and control uninfected unexposed individuals were plated in 48 replicate microwells in the presence of 5×10^4 γ-irradiated autologous PBMC pulsed with 50 μg/ml of the C5 peptide. Cell proliferation was tested 8 d later by [³H]thymidine incorporation. The precursor frequency, i.e., the average number of cells needed to generate a single clone, was determined by plotting the number of cells plated per well against the percent-negative wells (17).

Generation and Characterization of EU T Cell Clones. T cell lines were generated from freshly isolated PBMC after a single round of in vitro stimulation with the peptide and then cloned by limiting dilution in the presence of allogeneic γ-irradiated PBMC, PHA, and hrIL-2 (Proleukin, Chiron B.V., Amsterdam, Netherlands). Clones exhibiting peptide-specific proliferative activity were maintained in culture by monthly restimulation with PHA and irradiated allogeneic PBMC. Fine mapping of epitopes recognized by the T cell clones was performed using autologous EBV-transformed B cell lines (EBV-B) incubated with a panel of synthetic peptides overlapping by one amino acid. For cytofluorimetric analysis, T cell clones (2×10^5/sample) were stained with Vβ-specific monoclonal antibodies (PharMingen, San Diego, CA; Immunotech, Marseille, France) for 30 min at 4°C, washed twice, and suspended in PBS. The cells were analyzed in a FACScan® (Becton Dickinson, Mountain View, CA).

PCR Amplification and Sequencing of TCR cDNA. Total RNA was extracted from T cell clones and used for first-strand cDNA synthesis using an oligo (dT) primer and MMLV reverse transcriptase (GIBCO BRL, Gaithersburg, MD). 1/60 of each cDNA sample was amplified using the Vα–Cα- or Vβ–Cβ-specific oligonucleotides previously described (18). To characterize the N-region sequence of the Vβ chain, the remaining PCR amplified product was subsequently run on a 12% native PAGE. DNA bands with similar migration properties were excised and eluted from the gel. DNA was subsequently precipitated and sequenced.

Virus Isolation and Characterization. Viruses were isolated from PBMC of HIV infected patients by co-cultivation with PHA-stimulated PBMC from two healthy donors as previously described (19). In brief, 2×10^6 patient PBMC were cultivated with 15×10^6 donor PBMC in RPMI-1640 (GIBCO BRL) containing 10 U/ml rhIL-2, 10% FCS, glutamine, and antibiotics (IL-2 medium). Cultures were followed until three consecutive positive determinations of HIV-1 p24 antigen were obtained. To prepare virus stock, 30×10^6 donor PBMC were infected with p24 Ag-positive supernatant. After 7 d, supernatant was collected, centrifuged, aliquoted, and frozen at −80°C. Primary viruses were characterized as SI or NSI by differential growth on MT2 or Jurkat–tat cells.

Virus Inhibition Assays with Supernatants from EU Clones. Supernatants (75 μl) of CD4 clones collected after 48-h stimulation with PHA in the presence of autologous B-LCL cells were added to triplicate wells of PHA-activated healthy donor PBMC (10^5 cells/well in 75 μl IL-2 medium) that had been inoculated with 75 μl of serially diluted virus. After incubation overnight at 37°C, plates were washed by centrifugation and fresh IL-2 medium was added, with or without a 1:3 dilution of supernatant. Washing and readdition of medium was repeated after 2 d. At days 7 and 11, p24 Ag was measured by ELISA. Virus ID_{50} was newly determined for each experiment and virus inhibition measured using the virus dilution giving a $TCID_{50} >10$. Neutralization of the EU clone suppressive activity by antichemokine antibodies was obtained by preincubating clone supernatants with a mixture of anti–RANTES (50 μg/ml), anti–MIP-1α (30 μg/ml), and anti–MIP-1β (30 μg/ml) goat IgG (R&D Sys. Inc., Mineapolis, MN) for 30 min at room temperature and adding the supernatants to cultures of control PBMC inoculated with a NSI primary isolate (HIV-1₄₃). The purified IgG fraction from a nonimmune goat serum was used as control (110 μg/ml). Virus growth was monitored after 7–10 d by p24 Ag release.

Results

CCR-5 Genotypes. PCR amplification of genomic DNA corresponding to the 32–base pair deletion of CCR-5 was used to determine the CCR-5 genotype of the 12 EU partners. Eleven were homozygous for the wild-type CCR-5 allele, one was CCR-5Δ32 heterozygous, and none were CCR-5Δ32 homozygous (data not shown).

HIV Envelope-specific T Cells. HIV-specific T cell responses in the EU partners were detected using three HIV gp120 envelope peptides containing known T cell epitopes (20) plus an additional peptide corresponding to the fifth conserved region of gp120 (peptide C5). Proliferative responses to the peptides were measured after a single round of stimulation of fresh PBMC. Eighteen unexposed uninfected (UU) subjects were used as controls. The C5 peptide stimulated 9 of 11 EU PBMC tested but none of the UU
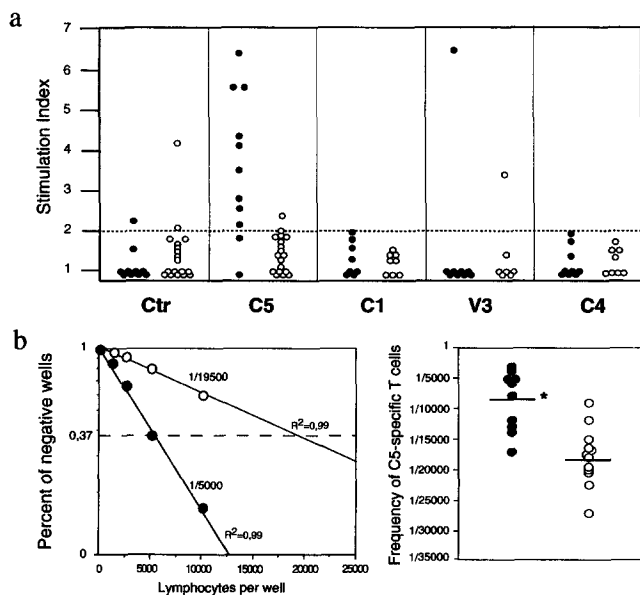
a

b

Figure 1. (*a*) Reactivity of EU T cells to different HIV-1 gp120 peptides. PBMC from exposed uninfected (EU) partners (*closed symbols*) and control unexposed uninfected (UU) individuals (*open symbols*) were cultured in RPMI medium supplemented with 5% human serum in the presence of 30 μg/ml of peptide. On day 7, cells were washed and then incubated for a further 5 d in fresh medium containing rIL-2 (20 U/ml). On day 12, 3×10^4 T cells were cultivated together with 10^5 autologous irradiated PBMC in the presence of the specific peptide and the proliferative responses measured after 3 d by [³H]thymidine incorporation. The data are expressed as stimulation index (*S.I.*). An individual was considered positive when the S.I. was >2. The peptides used were derived from the following HIV-1IIIb sequence: peptide C1, HEDIISLWDQSLK-PCVKLT; peptide V3, RIHIGPGRAFYTTKN; peptide C4, KQFINM-WQEWGKAMYA; peptide C5, SELYKYKVVKIEPLGVAPTKAKRR. The sequence of the control peptide was TPSLLEQEVKPSTELEYLGP-DEND. (*b*) Frequency of gp120–C5 specific T cells in EU partners. (*Left*) A representative experiment with PBMC from one EU and one control UU individual. (*Right*) Individual frequencies of C5-specific T cells from EU and UU PBMC.

Table 1. *TCR Genes of GP120–C5-specific CD4 Clones from Exposed Uninfected Individuals*

Donor	Clone	Vβ	N-D-N	Jβ	Vα
EU 23	EU23-08	Vβ5.2	TTGAGGGGTGTAGAC	Jβ1.5	Vα9
	EU23-09	Vβ5.2	TTGAGGGGTGTAGAC	Jβ1.5	Vα9
	EU23-20	Vβ5.2	TTGAGGGGTGTAGAC	Jβ1.5	Vα9
EU 25	EU25-01	Vβ6	CCGGGTGGG	Jβ2.1	
	EU25-02	Vβ3	TCGAAGGTCACG	Jβ2.1	
	EU25-03	Vβ3	TCGAAGGTCACG	Jβ2.1	
	EU25-04	Vβ3	TCGAAGGTCACG	Jβ2.1	
	EU25-06	Vβ3	TCGAAGGTCACG	Jβ2.1	
	EU25-13	Vβ3	TCGAAGGTCACG	Jβ2.1	
EU 26	EU26-02	Vβ17	ATAGGGCTAGCGGGT	Jβ2.2	Vα17
	EU26-03	Vβ17	ATAGGGCTAGCGGGT	Jβ2.2	
	EU26-05	Vβ17	ATAGGGCTAGCGGGT	Jβ2.2	
	EU26-11	Vβ17	ATAGGGCTAGCGGGT	Jβ2.2	Vα17
	EU26-18	Vβ17	ATAGGGCTAGCGGGT	Jβ2.2	Vα17
	EU26-45	Vβ17	ATAGGGCTAGCGGGT	Jβ2.2	Vα17
	EU26-50	Vβ3	ND		ND	ND
EU 28	EU28-01	Vβ7.1	ACCAGGGGACCTTGGT	Jβ2.7	
	EU28-04	Vβ7.1	ACCAGGGGACCTTGGT	Jβ2.7	

PBMC, whereas the other three peptides had no activity except for the V3 peptide, which stimulated one EU PBMC and one UU PBMC (Fig. 1 *a*). These results indicated that the T cell repertoire of EU but not UU individuals contains a high number of gp120-C5–specific T cells. To quantify more precisely the differences between EU and UU individuals we performed limiting dilution analysis of EU and UU peripheral T lymphocytes upon stimulation with the C5 peptide. As shown in Fig. 1 *b*, the mean frequency of C5-specific EU T cells was significantly higher ($P < 0.001$) than that of UU T cells (1 in 7,700 $\pm$ 4,700 for EU versus 1 in 18,000 $\pm$ 6,500 for UU). The C5 peptide was therefore selected for further analysis.

Generation and Characterization of CD4 T Cell Clones. C5-specific T cells from four EU partners, including three CCR-5 homozygous (EU23, EU26, EU28) and one CCR-5Δ32 heterozygous (EU25), were cloned under limiting dilution conditions, and each of the clones characterized. All clones generated were CD4+/CD8−. The TCR Vβ genes usage was determined by flow cytometry using

specific monoclonal antibodies and by oligonucleotide-directed PCR amplification of cDNA of the Vβ-D-Jβ junctional region and Vα genes. Table 1 lists the TCR genes expressed by each of the clones. This analysis revealed that the majority of clones derived independently from each EU partner expressed the same TCR as if they were derived from the expansion of a single precursor. The presence of an oligoclonal response in the four EU partners from which the clones were derived, together with the high number of C5-specific T cells detected in the majority of EU partners tested, suggested that the C5 peptide contains one or more dominant epitopes recognized by EU T cells. All EU clones were then tested on a panel of 12-residue synthetic peptides representing the C5 region. Three epitopes, YKVVKIEPLGVAPT, LGVAPTKAKRRV, and IEPLGVAPTKAK, were identified (data not shown).

Antigen-driven C–C Chemokine Production by EU Clones. The availability of CD4 clones specific for an HIV peptide allowed us to ask the question of whether such clones, upon recognition of the specific antigen presented by self–antigen-presenting cells, were capable of producing HIV-suppressive C–C chemokines. Stimulation of EU clones with the specific peptide or with PHA, but not with the control peptide, induced the release of high amounts of all three C–C chemokines (16 $\pm$ 5.6 ng/ml RANTES, 67 $\pm$
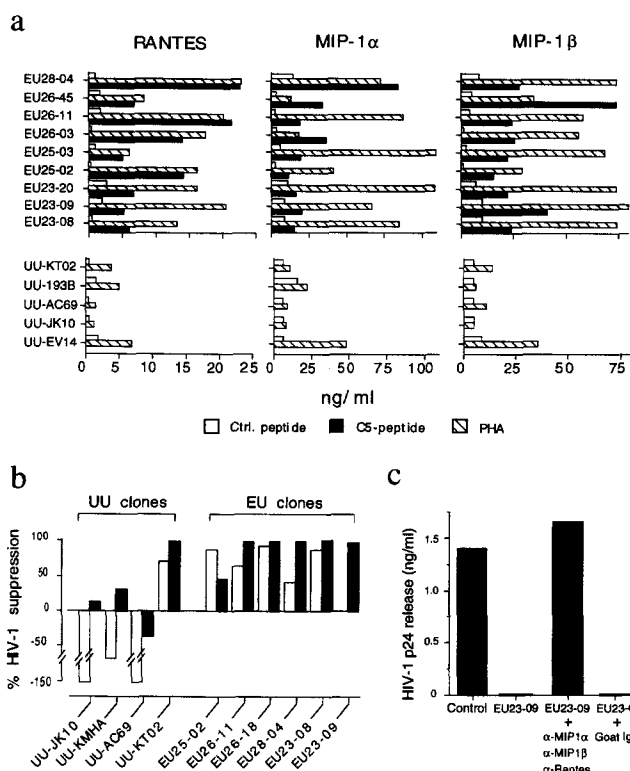
Figure 2. (*a*) Antigen driven release of C–C chemokines by EU clones. Peptide C5-specific clones from exposed uninfected (EU) partners (10^6 cells/ml) were cultivated with autologous B-LCL cells (5×10^5/ml) in the presence of 30 μg/ml of the C5 peptide (*closed bars*), PHA (*dashed bars*), or an unrelated control peptide (*open bars*). Supernatants were collected after 48 h and tested for the presence of RANTES, MIP-1α, and MIP-1β using commercially available ELISA (R&D Systems). Clones from unexposed uninfected individuals (UU) were stimulated under the same conditions in the presence of PHA or control peptide only. (*b*) EU clones suppress HIV-1 NSI primary isolates. Supernatants of EU clones collected after 48-h stimulation with PHA in the presence of autologous B-LCL cells were added to cultures of PBMC from UU subjects that had been inoculated with two HIV-1 NSI primary isolates, HIV-1$_{40}$ (*open bars*), HIV-1$_{43}$ (*closed bars*). Results are expressed as percent suppression of p24 Ag production. (*c*) Anti-C–C chemokine antibodies abrogate the suppressive activity of EU clones. Growth of a NSI primary isolate (HIV-1$_{43}$) on control PBMCs in the presence of supernatant from clone EU23.9, which was pretreated with a mixture of α-RANTES, α-MIP-1α, and α-MIP-1β antibodies or control goat IgG.

36 ng/ml MIP-1α, and 61 ± 18 ng/ml MIP-1β). In contrast, PHA stimulation of the UU clones failed to induce production of the three chemokines as efficiently (4 ± 2.3 ng/ml RANTES, 19 ± 17 ng/ml MIP-1α, and 14 ± 12 ng/ml MIP-1β) (Fig. 2 *a*). Comparison of the levels of the three C–C chemokines produced under polyclonal versus antigen-specific activation revealed that induction of RANTES production by antigen-specific and PHA stimulation was similar in the majority of clones tested, whereas, for MIP-1α and MIP-1β, PHA stimulation generally induced higher levels. In all cases, however, the levels of the three chemokines were substantially higher than those previously reported (10) for CD4 clones.

HIV-1 Inhibitory Activity of EU Clones. Next, we investigated the ability of the C5-specific EU clones to suppress HIV infection in vitro. Supernatants from PHA-stimulated EU and UU clones were added to cultures of control PBMC inoculated with two HIV-1 NSI (HIV-1$_{40}$, HIV-1$_{43}$) and two HIV-1 SI (HIV-1$_{45}$, HIV-1$_{48}$) primary isolates. All six EU clones tested potently suppressed the two NSI isolates (Fig. 2 *b*), but showed no activity on the two SI isolates (data not shown). In contrast, three of the four UU clones failed to suppress all isolates tested but instead significantly enhanced replication of the two NSI isolates (Fig. 2 *b*). One UU clone (UU-KT02) displayed a level of suppressive activity toward the two NSI isolates similar to that of EU clones.

The selectivity of the suppressive activity of the EU clones for the NSI isolates suggested that in this experimental system, C–C chemokines were the major suppressive factor. To exclude the role of other suppressive factors, the cell-free culture supernatants of the EU clones were pretreated with a mixture of neutralizing antibodies against RANTES, MIP-1α, and MIP-1β before measuring the suppressive activity against the NSI isolate HIV-1$_{43}$. As shown in Fig. 2 *c*, the anti-chemokine antibodies completely abrogated the suppressive activity, whereas no effect was observed with the control antibodies, indicating that C–C chemokines are the major, if not the only, HIV suppressive factor released by EU CD4 clones.

Discussion

These data are evidence that, upon antigen stimulation, HIV-specific helper T cells can produce C–C chemokines that suppress infection by CCR-5–dependent HIV-1 viruses. In vitro, such HIV-suppressive activity requires the production of relatively high levels of C–C chemokines, because clones that produce low levels of C–C chemokines have the opposite effect on HIV replication, i.e., enhancement. The enhancing effect may be due to the production of other factors, including cytokines, which upregulate HIV replication. The previous observation that CD4 clones from EU individuals produce higher levels of C–C chemokines than clones from control unexposed individuals (12) was confirmed by our study. This was also associated with the expression of potent HIV-suppressive activity specific for NSI isolates. However, suppressive activity could also be detected in one UU clone (UU-KT02) that produced only limited quantities of C–C chemokines. Thus, lack of enhancing cytokine production may have resulted in potentiation of the HIV-suppressive activity of C–C chemokines.

One could predict that protection against sexual transmission depends on the balance between enhancing and suppressive activity resulting from either the number of HIV-specific T cells producing suppressive chemokines or extra production at the single cell level. It is not clear which factors determine an increase in the number of clones producing higher levels of C–C chemokines seen in EU individuals. A high number of C–C chemokine-producing cells may be the result of an immune response maintained

in vivo by repeated exposures to HIV antigens. The finding in each EU partner of a large number of clones bearing identical T cell receptors specific for the C5 env region suggests a critical role of this region in inducing protective responses to HIV. On the other hand, numerous reports of robust Th1 type responses in EU individuals (1) suggest that Th1 cytokines might upregulate C–C chemokine production. Thus, some mechanism of control of C–C chemokine production by other cells of the immune system may be upregulated in EU individuals.

Significant differences in the levels of C–C chemokines produced by EU versus UU clones were also detected after polyclonal activation. EU individuals may therefore represent a population with a genetic predisposition to high production of C–C chemokines, which may help in generating HIV-suppressive responses upon the first encounter with HIV antigens. An immune-mediated mechanism and genetic predisposition to high C–C chemokine production are not necessarily mutually exclusive, and indeed might be synergistic. For vaccine design, it will be important to understand to what extent genetic predisposition favors the generation of C–C chemokine-mediated protective responses and whether an immune manipulation targeted against particular HIV antigens could enhance C–C chemokine production to a level sufficient to prevent sexual transmission.

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