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CCR5-Reactive Antibodies in Seronegative Partners of HIV-Seropositive Individuals Down-Modulate Surface CCR5 In Vivo and Neutralize the Infectivity of R5 Strains of HIV-1 In Vitro

Lucia Lopalco,* Claudia Barassi,* Claudia Pastori,* Renato Longhi,† Samuele E. Burastero,* Giuseppe Tambussi,* Francesco Mazzotta,‡ Adriano Lazzarin,* Mario Clerici,§ and Antonio G. Siccardi¶

Exposure to HIV does not necessarily result in infection. Because primary HIV infection is associated with CCR5-tropic HIV variants (R5), CCR5-specific Abs in the sera of HIV-seronegative, HIV-exposed individuals (ESN) might be associated with protection against infection. We analyzed sera from ESN, their HIV-infected sexual partners (HIV+), and healthy controls (USN) searching for CCR5-specific Abs, studying whether incubation of PBMC with sera could prevent macrophage inflammatory protein 1β (Mip1β) (natural ligand of CCR5) binding to CCR5. Results showed that Mip1β binding to CCR5 was not modified by sera of either 40 HIV+ or 45 USN but was greatly reduced by sera of 6/48 ESN. Binding inhibition was due to Abs reactive with CCR5. The CCR5-specific Abs neutralized the infectivity of primary HIV isolates obtained from the corresponding HIV+ partners and of R5-primary HIV strains, but not that of CXCR4-tropic or amphitropic HIV strains. Immunoassorption on CCR5-transfected, but not on CXCR4-transfected, cells removed CCR5-specific and virus-neutralizing Abs. Epitope mapping on purified CCR5-specific Abs showed that these Abs recognize a conformational epitope in the first cysteine loop of CCR5 (aa 89–102). Affinity-purified anti-CCR5-peptide neutralized the infectivity of R5 strains of HIV-1. Anti-CCR5 Abs inhibited Mip1β-induced chemotaxis of PBMC from healthy donors. PBMC from two ESN (with anti-CCR5 Abs) were CCR5-negative and could not be stimulated by Mip1β in chemotaxis assays. These results contribute to clarifying the phenomenon of immunologic resistance to HIV and may have implications for the development of a protective vaccine.


During the last few years, documentary evidence of the existence of individuals who remain HIV-seronegative and are apparently uninfected despite multiple exposures to HIV-1 (exposed seronegative, ESN) has been produced by a number of groups, suggesting that it is possible to achieve some degree of resistance to the virus. Some ESN show signs of unconventional HIV-related immunity, including 1) Abs to cellular Ags, including HLA class I and CD4 (1–3), 2) IgA-mediated and mucosally confined HIV humoral immune responses (4, 5), 3) HIV-specific systemic cell-mediated immunity (6–8) as well as production of type 1 cytokines (9–11) and β-chemokines (12) by HIV peptide-stimulated PBMC, and 4) HIV-specific CD8+ T lymphocyte-mediated cytotoxicity directed toward early HIV proteins (13–15).

Anti-cell Abs and T cell-mediated immunity have been associated with protection in primate models of lentivirus infection (16–18), but the role of these immune responses in humans is still unclear. More recently, it was shown that the cells of some ESN couldn’t be infected in vitro by M-tropic (R5) strains of HIV because they lack the essential CCR5 coreceptor (19, 20). In the CCR5 gene, at least two mutations (20, 21) have been associated with total or partial resistance to infection by M-tropic R5 strains of HIV, and one mutation was associated with slowing progression of the disease (22). R5 strains (23) account for most of the transmission of HIV infections (particularly sexually transmitted infections) and are associated with the earlier phases of the disease (24). Because CCR5-defective individuals were described to have normal inflammatory and immune reactions, CCR5 has been interpreted as a redundant molecule in adults, and has thus become an important potential target for blocking drugs (25) and immune modulation (26–28). Therefore, we searched for anti-CCR5 autoantibodies in the sera of ESN individuals on the assumption that, if such Abs exist, they are unlikely to affect immune function but may interfere with the HIV coreceptor function of CCR5.

Materials and Methods

Study population

Two different cohorts of monogamous couples discordant for HIV serostatus were selected for the study. A first cohort was enrolled in Milan (3 homosexual and 37 heterosexual couples); a second group was enrolled in Florence (2 homosexual and 6 heterosexual couples). The 48 ESN subjects included in the study were 26 heterosexual females, 17 heterosexual males,
and 5 homosexual males (passive recipients). Written informed consent was obtained from all the participants. The inclusion criterion was a history of penetrative sexual intercourse without condom, at least twice per week and for at least 2 years with no other known risk factors during that period or afterward. HIV-1 infection was excluded because of the absence of anti-HIV-1 Abs (ELISA and Western blot) and viral DNA (PCR). Seronegativity was confirmed 6 mo after enrollment. Forty-five serum samples from unexposed, sexually active, seronegative individuals (USN) (29 females, 16 males) were used as negative controls. Forty serum samples from HIV-seropositive patients of ES were also analyzed. After enrollment in the protocol, counseling was offered to all of the couples and many (but not all) switched to safe-sex practices. The ESN were characterized for the presence of CCR5-A32 alleles (courtesy of Drs. V. Nardese and P. Lusso), anti-CD4 Abs, and HIV-specific IgA (3, 5).

Purification of CD4+ cells

PBMC from healthy blood donors were isolated by Ficoll-Hypaque centrifugation and stimulated for 3 days with PHA (3 μg/ml) (Sigma-Aldrich, Steinheim, Germany) and rIL-2 (100 U/ml) (Amersham, Buckinghamshire, U.K.). CD4+ cells were purified from activated PBMC by immunoadsorption to anti-CD4 magnetic beads (Oxford, Hampshire, U.K.). Purified CD4+ cells were stimulated for 3 days with IL-2 before the macrophage inflammatory protein 1β (Mip1β) binding assay. PBMC were characterized on the basis of the presence of CCR5-A32 alleles; only PBMC from donors not carrying the CCR5-A32 allele were used.

Mip1β binding assay

The assay was performed as described (29). Briefly, 10^6 purified CD4+ cells in 200 μl of RPMI 1640 (Life Technologies, Milan, Italy) (containing 0.05 M Na2EDTA, 1% BSA, and 25 mM HEPES) were incubated with appropriate dilutions of sera and/or Ig-enriched fractions; after 45 min of incubation, 125I-Mip1β (DuPont-NEN, Mechelem, Belgium) was added (final concentration, 0.1 nM, 0.2 μCi), and the cells were further incubated for 2 h on ice. Unbound radioactivity was separated by centrifugation on a two-step gradient (30) in 0.3-m1 tubes (Nunc, Roskilde, Denmark) as follows: the lower layer consisted of FCS containing 10% sucrose; the upper layer consisted of 80% silicone (Sigma-Aldrich) and 20% mineral oil (Sigma-Aldrich). The bound radioactivity in the cell pellets was measured in a gamma counter. Serum samples were diluted 1:10 (five replicates for each sample). A specificity control consisting of a 100-fold excess of unlabeled Mip1β was included in all experiments. The binding of the 125I-Mip1β to activated CD4+ cells ranged between 1000 and 6000 cpm. The cut-off value was set at 12% (three SD above the mean value of the 45 USN serum samples).

Anti-CCR5 Ab assay

Anti-CCR5 Abs bound to CCR5-positive cells were revealed by 0.5 μCi/ml of 125I-sheep anti human Ig F(ab’)2 Ab fragments (Amersham). Activated CD4+ T cells were treated with human sera diluted 1/10 and then processed as described for the Mip1β binding assay. The cut-off value (1065 cpm) was established as 3 SD above the mean of 10 USN serum samples. Cellular suspensions from CCR5-transfected U87 cells monolayers pretreated with human sera diluted 1/10 were processed as described above. A pool of 10 USN sera was used as negative control, while 0.1 nM 125I-Mip1β and the anti-CCR5 mouse mAb 2D7 (31) were used as positive controls. The mAb was used at 0.3 μg/ml and revealed by 0.5 μCi/ml 125I-sheep anti-mouse IgG F(ab’)2 Ab fragments (Amersham).

Chemotaxis assay

PBMC from one healthy donor (selected for high expression of CCR5) were activated with PHA and IL-2 for 3 days (see above) in the presence of two concentrations of purified Igs (250 and 62 μg/ml) from ESN34, ESN55, and USN5. Then, 5 x 10^6 activated PBMC in 50 μl of RPMI 1640 medium containing 0.3% human serum albumin were placed in the upper chamber of transwells (Costar, Europe, Amsterdam, The Netherlands). Chemotaxis was conducted in the presence of 1.5 μg/ml of Mip1β (placed in the lower chamber). The transwells were incubated for 2 h at 37°C; cells that migrated from the upper to the lower chamber were then quantified by FACs analysis. PBMC from ESN34 and ESN55 were also used in chemotaxis assays to evaluate the capacity of ESN PBMC to migrate in the presence of Mip1β. The results were expressed as chemotaxis index, which represents the fold increase in the number of migrated cells in response to Mip1β over the spontaneous cell migration in control medium.

C-C chemokines assay

Serum chemokines concentrations (RANTES, Mip1α, and Mip1β) were determined with commercial ELISA kits (R&D Systems, Minneapolis, MN).

Ig purification

Anti-human polyclonal Ig-coupled Agarose (Sigma-Aldrich) was used to purify total Ig from the sera of ESN and USN. Briefly, 100 μl of serum were incubated overnight at 4°C in columns containing 5 ml of anti-human Ig-agarose. After recovering the column washout (Ig-depleted fraction), the columns were washed six times in phosphate buffer (0.01 M with 0.5 M NaCl). Bound Igs were eluted with glycine/NaCl 0.2 M, and the eluted fractions neutralized with 1 M Tris (Ig-enriched fraction). Ig-enriched and Ig-depleted fractions were concentrated on Ultrafree-15 Biomax 30 membranes (Millipore, Bedford, MA) with a cut-off of 30 kDa and dialyzed against RPMI 1640. Ig concentration was determined by ELISA using commercial Igs as standard and adjusted to 2.5 mg/ml (corresponding to a serum dilution of 1:10). The Ig-depleted fractions were diluted by the same factor.

Virus isolation and titration

HIV was isolated from the PBMC of HIV-seropositive partners by cocultivation with PHA-stimulated PBMC of two USN. Cultures were maintained until increasing levels of HIV-p24 Ag were detected in two consecutive determinations. The infectivity (ID50) of each virus isolate was determined on PBMC from one single donor as follows: six replicates (150 μl) of 5-fold serial dilutions (from 1:5 to 1:125) of virus were added to two wells of a round-bottom Microtiter plate (Nunc) containing 10^5 resting PBMC in 75 μl of medium, incubated for 2 h, washed, and resuspended in RPMI 1640 medium containing PHA (3 μg/ml) and 10 U/ml rIL-2. HIV-1 p24 Ag was titrated after 5, 7, and 9 days of culture by standard assays (32). ID50 titers were defined as the reciprocal of the virus dilution yielding 50% positive wells (Reed-Muench calculation).

Phenotypic characterization of viral coreceptor usage

Each virus isolate was used to infect U87 human glioma cell lines (provided by Dr. P. Lusso) expressing one of the following chemokine receptors: CCR1, CCR2B, CCR3, CCR5, or CXCR4. Then, 1 ml of virus-containing culture supernatant was incubated with 8 x 10^5 U87 cells in 12-wells plates for 4 h at 37°C. Cells were washed twice and incubated in RPMI 1640 medium supplemented with 10% FCS. Cells were observed daily for cytopathic effect. Culture supernatants, collected from each well 2, 5, and 7 days after infection, were analyzed for HIV-1 p24 Ag.

Virus neutralization assays

The “resting cell assay” was performed according to Zolla-Pazner (33). Briefly, 2 x 10^5 resting PBMC were added to 75 μl of serial dilutions of Ig-enriched fractions from ESN or USN; after 1 h incubation, 75 μl of a virus dilution (ID50 adjusted to 20) was added. The cultures were incubated for another 2 h, washed, and resuspended in PHA- and IL-2-containing medium. HIV-1 p24 Ag in the supernatants was determined on days 7 and 9. Percent neutralization was calculated relatively to a nontreated control.

Adsorption of purified Igs on U87 cell lines

Ig-enriched fractions from ESN and USN were adsorbed on monolayers of CCR5- or CCR3-transfected U87 cell lines obtained by seeding 8 x 10^5 cells in Microtiter wells; 100 μl of Ig-enriched fractions (2.5 mg Ig/ml) were immunoadsorbed on the cell monolayers (10 min at 37°C); the liquid was then collected and passed to a second cell monolayer (10 min at 37°C). The procedure was repeated 10 times.
Synthesis of peptides and preparation of peptide/beads

Peptides were synthesized by the solid phase F-moc method (34) using an Applied Biosystem model 433 A peptide synthetizer (Foster City, CA). After the peptide assembly, the side chain-protected peptidyl resin was deblocked as previously described (35) and purified to apparent homogeneity by reverse-phase chromatography. An extra-sequence cysteine was added to peptides 1, 3, and 4 to obtain conformationally cyclic peptides. These peptides were treated overnight with a 5-fold excess of oxidized glutathione and purified by reverse-phase chromatography.

Coupling of CCR5 peptides to tosyl-activated Dynabeads M280 (Dynal, Oslo, Norway) was obtained following standard procedures. Briefly, $3 \times 10^7$ beads were incubated with 9 mg of CCR5 peptides in 50 mM borate buffer pH 9.5 (16 h at 37°C). After four washes in PBS, peptide/beads were ready for use.

Affinity-purification of Abs on peptide/beads

Binding of anti-CCR5 specific Igs to peptide/beads was obtained by incubating 9 µg Igs to 9 µg peptide/beads for 1 h at 4°C. Igs were eluted in 0.5 M acetic acid, dialyzed against RPMI 1640 medium, then tested in Mip1β binding assays and/or in HIV neutralization assays. To establish if the region recognized by anti-CCR5 Abs corresponds to a conformational epitope, the specific peptide/beads were incubated with 10 mM of 2-ME, and subsequently with 30 mM of N-ethyl maleimide (final concentration, 30 mM) for 60 min before Ab binding.

Results

Inhibition of Mip1β binding to surface CCR5 by sera of ESN

Unlike RANTES and Mip1α (the other two CCR5-binding β-chemokines that inhibit infection by R5 strains of HIV), Mip1β binds exclusively to CCR5. For this reason, a Mip1β radio-binding assay to measure the presence and function of CCR5 on cell surfaces was recently introduced (29). We used a similar radio-binding inhibition assay to search for anti-CCR5 Abs in sera of ESN and USN individuals as well as in sera of HIV-infected patients. A total of 133 serum samples from 45 USN, 40 HIV-seropositives, and 48 ESN were assayed (at a 1:10 dilution; five replicas for each serum) to investigate whether they could inhibit Mip1β binding to purified CD4+ cells. The ability of ESN sera to inhibit Mip1β binding to purified CD4+ cells was higher than that observed with sera from either USN or HIV-positives, and the difference was statistically significant ($t = 1.939; p < 0.05$). The arithmetic means ± SE of the inhibition values were 1.42 ± 0.7 (USN), 1.23 ± 0.06 (HIV-seropositives), and 5.2 ± 2.2 (ESN). The sera of six ESN (ESN9, 31, 32, 34, 53, 55) had values well above the cut-off (mean ± 3 SE of USN controls) (Fig. 1).

To exclude the possibility that the observed inhibition may have been due to high serum concentrations of β-chemokines, we measured the concentration of RANTES, Mip1α, and Mip1β in the six ESN sera in which Mip1β-inhibiting activity was detected and in the CD4+ sera of 10 USN control. The concentrations of all three chemokines were comparable between ESN and USN sera (data not shown).

Inhibition of Mip1β binding to CCR5 by sera of ESN is mediated by Abs

The sera of the six ESN that inhibited Mip1β binding to CCR5 and of three USN were purified by affinity chromatography (on agarose-bound anti-human Ig Abs) to obtain Ig-enriched and Ig-depleted fractions. Both fractions were then tested in the radio-binding assay. Fig. 2A shows that Mip1β-binding inhibition was associated with the Ig-enriched fraction in all ESN samples, whereas no activity was observed in the Ig-depleted fractions. Both the Ig-enriched and Ig-depleted fractions from USN sera were devoid of inhibitory activity. The data suggest the Mip1β-binding...
inhibition to be due to Abs. The dose-dependence of the binding inhibition is shown in Fig. 2B. In all but one case (ESN55), significant inhibition was observed at a concentration of 62.5 μg (corresponding to a serum dilution of 1:40); all fractions lost their inhibitory activity at a concentration of 15.6 μg (corresponding to a serum dilution of 1:160).

**Anti-CCR5 and not anti-Mip1β Abs are responsible for the inhibitory activity**

To exclude the possibility that the inhibitory Abs could be directed against Mip1β (rather than against CCR5), we set up an ELISA to detect anti-Mip1β Abs. Mip1β-binding Abs could not be detected in either the whole sera or Ig-enriched fractions of either ESN or 10 USN (data not shown), although the ELISA was sensitive enough to detect concentrations as little as 100 pg/ml of anti-Mip1β Abs. Therefore, it is likely that the observed inhibition is due to Abs interacting with CCR5 and blocking Mip1β binding to its receptor, possibly by steric hindrance.

Anti-CCR5 Abs were demonstrated in five of five of the Mip1β-inhibiting sera by a similar radio-binding assay, which employs CD4 T cells or CCR5-transfected U87 cells and radiolabeled anti-human Ig secondary Abs. A strong increase in binding was observed when CCR5-transfected U87 cells were compared with CD4 T cells, as shown in Fig. 3, A and B.

**Anti-CCR5 Abs inhibit the chemotactic response to Mip1β**

To evaluate whether the cells of ESN in whom anti-CCR5 Abs are detected are functionally CCR5-negative, purified Ig fractions from ESN34, ESN55, and from one control (USN5) were used to inhibit the chemotactic response induced by Mip1β on PBMC from healthy controls. The results show that anti-CCR5 Abs from ESN34 and ESN55 reduced chemotaxis in a dose-dependent manner (Fig. 3C). Additionally, whereas control PBMCs migrated in response to Mip1β, PBMCs from ESN34 and ESN55 were not responsive to Mip1β (Fig. 3C). These results indicate that anti-CCR5 Abs are functionally active as they suppress chemotaxis and suggest that, in vivo, these Abs down-regulate CCR5 expression.

**Lack of correlation between anti-CCR5 Abs and other immune correlates of protection**

No correlation was found between the presence of anti-CCR5 Abs and other immune correlates of protection previously associated with the ESN status. ESN were tested for the following correlates and resulted negative (data not shown): 1) the presence of a homozygous CCR5 deletion (CCR5-D32), 2) anti-CD4 Abs (anti-CD4 Abs were only detected in ESN53), or 3) serum HIV-specific IgA. Moreover, no correlation was found between anti-CCR5 Abs and gender: ESN55, 32, and 53 were males from heterosexual couples, the other three were females. Interestingly, the anti-CCR5 response is persistent in time: in the four cases tested (ESN34, 32, 53, and 55), anti-CCR5 titers persisted in sera obtained after 2 years of safe sex (data not shown).

**Anti-CCR5 Abs inhibit the infectivity of R5 strains of HIV**

The data reported above demonstrate that anti-CCR5 Abs can be found in the sera of ESN, and that these Abs prevent binding of Mip1β to CCR5.
Mip1β to CCR5. Because CCR5 is the main coreceptor for R5 strains of HIV, we verified whether anti-CCR5 Abs could also inhibit HIV-1 binding to CCR5. Thus, we assayed the activity of Ig fractions from five of the ESN in whom anti-CCR5 Abs were observed (ESN31, 32, 34, 53, and 55) in HIV-1 neutralization assays using primary virus isolates derived from seropositive patients. A number of primary isolates were analyzed for coreceptor usage; three isolates were chosen: HIV36 (R5 strain that uses CCR5 as its coreceptor), HIV26 (X4 strain that uses CXCR4 as its coreceptor), and HIV45 (amphitropic R3, R5, X4 strain that uses CCR3, CCR5, and CXCR4 as coreceptors). Additionally, the isolates from four seropositive partners (all using CCR5 as coreceptor) were used in the neutralization assay. Ig-enriched fractions from all five ESN inhibited infection by the R5 strain in a dose-dependent manner (Fig. 4A); these fractions had no inhibitory effect on the other two HIV-1 strains (data not shown). The purified Ig fractions of four ESN (ESN34, 53, 31, and 55) inhibit the replication of the primary isolate derived from the corresponding four HIV+ partners is shown in B–E.

These data are consistent with the activity of Abs reactive with CCR5 and blocking the infectivity of CCR5-restricted viruses rather than with the activity of Abs reacting with HIV-1 epitopes involved in the interaction with CCR5, because in the latter case the amphitropic virus would also have been inhibited.

Evidence that anti-CCR5 Abs are also responsible for the neutralizing activity on the R5 strains was sought by immunoadsorption: Ig-enriched fractions from sera of three ESN and three USN were adsorbed on either CCR5- or CXCR4-transfected U87 cells. Adsorption on CCR5-positive cells (but not on CXCR4-positive cells) reduced both the virus-neutralizing activity (Fig. 5A) and the Mip1β-binding inhibitory activity (Fig. 5B). In particular, 1) in the neutralization assay, the concentration of unabsorbed fractions giving 50% inhibition (IC50), as well as that of CXCR4-adsorbed fractions, was ~30 µg/assay, whereas the IC50 of CCR5-adsorbed fractions was >200 µg/assay; 2) Mip1β-binding inhibition was ~82% in both unabsorbed and CXCR4-adsorbed fractions, but was 57% in CCR5-adsorbed fractions. ESN inhibiting sera could also neutralize another primary R5 isolate (HIV53) in both resting and activated PBMC neutralization assays (data not shown). These data are consistent with the hypothesis that the Abs removed by adsorption on CCR5 are the same responsible for the neutralization of HIV-1 R5 strains.

CCR5 expression on PBMC from Ab-positive ESN individuals

The issue of in vivo coexistence of anti-CCR5 Abs and CCR5 Ag on the surface of cells was addressed by studying the CCR5 phenotype of purified CD4+ cells from two patients (ESN55 and ESN34) and from four USN controls using the Mip1β binding assay. No binding could be detected for the two patients, while all the USN exhibited normal binding values (range, 2000–3500 cpm; data not shown). To evaluate CCR5 expression on the cell surface, purified, preactivated CD4+ cells from ESN55 and ESN34 and one
USN control were stained with a CCR5-specific Ab (mAb 2D7) and tested in a flow cytometry assay, which employs FITC-labeled anti-mouse Ig secondary Abs. Results (Fig. 6) showed that, whereas CCR5 was normally detected on lymphocytes of the USN, no CCR5 expression was observed on the cells of the two ESN individuals.

**Epitope mapping of anti-CCR5 Abs**

Anti-CCR5 Abs were tested on a panel of synthetic peptides covering the complete sequence of the extra membrane region of CCR5 (Table I). Specific Ab binding to peptide 3 (aa 89–102), corresponding to the second external domain (first cystein loop) of CCR5, was observed. As shown in Fig. 7A, the Abs are highly selective for this epitope as no binding was observed on a panel of other peptides (peptides 1, 2, 4, and 5). Binding of the anti-CCR5 Abs from ESN sera to peptide 3 was abolished by addition of 2-ME and N-ethyl maleimide, which cause reduction and alkylation of the cystein loop (Fig. 7A), suggesting that Ab recognition requires the maintenance of the epitope tertiary structure.

**Affinity-purified anti-CCR5 Abs inhibit HIV-1 replication**

Abs to peptide 3 were affinity-purified from ESN55 and ESN32 sera and tested in HIV neutralization assays. The neutralizing titers obtained with Igs eluted from peptide 3 (Fig. 7B) were higher (IC\textsubscript{50} of 1 \(\mu\)g/ml for ESN55 and 5 \(\mu\)g/ml for ESN32) than those of total serum Igs (IC\textsubscript{50} of 13 \(\mu\)g/ml for ESN55 and 82.7 \(\mu\)g/ml for ESN32) (Fig. 4A).

**Discussion**

Exposure to HIV does not inevitably lead to infection. Thus, individuals who do not seroconvert despite being repeatedly exposed to HIV have been described in different cohorts of at-risk individuals including: sexually exposed gay men, neonates of HIV-seropositive mothers, accidentally exposed health care workers, prostitutes, and heterosexual partners of HIV-seropositive individuals. ESN individuals are clearly a heterogeneous group because a number of different immunological features have been associated with the ESN status at variable frequencies (1–7, 12). That resistance to HIV-infection can also have a genetic basis was suggested by the observation that a mutant form of one of the most important coreceptors for HIV on the surface of CD4\textsuperscript{+} cells, CCR5, is present in a subset of such individuals. Mutations in CCR5 lead to a defective coreceptor function for those HIV-1 virus strains that use CCR5 as their coreceptor (R5 strains), thereby impeding the penetration of HIV into the target cell and the onset of disease (19, 21).

Given that 1) the specific ligand for CCR5 is Mip1\(\beta\), 2) a functional CCR5 coreceptor is a requirement for infection by HIV-1 R5 strains (36, 37), 3) R5 strains are responsible for the establishment of most new infections (24), and 4) the occupancy of CCR5 receptor by its ligands (38, 39) or by Abs (31, 40) prevents infection by HIV, we investigated whether binding of Mip1\(\beta\) to CCR5 could

| Peptides covering the sequence of the extramembrane region of CCR5 |
|------------------------|------------------|
| Peptides               | Sequence         |
| 1                      | cMDQVQSSPFIYDYYIYTSEPC |
| 2                      | YYTSEPCOKINVKIAARLLP |
| 3                      | cYAAQWDFGNTMRCQ |
| 4                      | CSSHPFYSQYFWKCPQNLKc |
| 5                      | FOEFFGLINCCSSSNR |

\*Amino acids not in capital letters were introduced to obtain conformationally peptides.
be inhibited by sera of ESN. Sera from neither controls nor HIV-infected individuals could interfere with the binding of Mip1β to CCR5; in contrast, inhibitory activity was detected in sera of a number of ESN: binding of Mip1β to CCR5 was inhibited by sera of 6/48 (12.5%) ESN, but not by sera of either 45 USN or 40 HIV+ seropositive individuals \( (p < 0.001) \). Characterization of this inhibitory activity indicated that anti-CCR5 Abs 1) are present in the sera of some ESN, 2) recognize a conformational epitope in the first cysteine loop of CCR5, 3) inhibit Mip1β binding to CCR5, 4) down-modulate CCR5 expression on PBMC in vivo, 5) inhibit Mip1β-induced chemotaxis of control PBMC, and 6) block the HIV coreceptor function of CCR5, neutralizing the infectivity of R5 strains of HIV-1.

Although the potentially protective role of such Abs is evident, their origin is a matter of speculation: anti-CCR5 Abs 1) may be “naturally” present in some rare individuals \(< 1/85, \text{which is the sum of the two control populations})\), 2) may be elicited by exposure to HIV or by particular and still unidentified factor(s) involved in HIV exposure, or 3) may be elicited by some HIV-unrelated antigenic stimulation. In this context, it is interesting that a recent report showed that autoantibodies to CCR5 could be induced in C57BL/6 mice by inoculation with a papilloma virus modified to express CCR5 peptides \((41)\). Such Abs could inhibit binding of β-chemokines to CCR5, as well as block infection with HIV-1 \((41)\). Interestingly, the CCR5 epitope used to immunize mice and the epitope recognized by the CCR5-specific IgGs detected in ESN sera show >90% homology.

The autoantibodies produced during the course of viral infections have been attributed to virus-induced self-alterations that become autoimmunogenic \((42)\). Therefore, the infrequent anti-CCR5 response might be due to a particular antigenic stimulation or to the particular reactivity of rare individuals. Lehner et al. \((43)\) have shown that xenogenic immunization of macaques with SIV grown in human cells induces protective immunity and the generation of Abs reactive with simian CCR5 that are capable of neutralizing SIV in in vitro assays. Allogenic, rather than xenogenic, immunization might have produced the same result in our ESN individuals. The recent report that CCR5 can act as an alloantigen in CCR5-Δ32 homozygous individuals elicitng Abs that compete with RANTES and inhibit infection by R5, but not X4, primary isolates of HIV-1 supports this hypothesis \((44)\). Along the same lines, therapeutic strategies aimed at preventing HIV-1 infection by means of Abs to CCR5 elicited via immunization with a modified CCR5 gene are currently being developed \((45)\). In this context, it is interesting that, whereas interruption of at-risk sex in ESN is followed by a sharp drop in the titers of HIV-specific IgA and in the loss of HIV-specific cell-mediated immunity \((5)\), no modifications were observed in the concentration of anti-CCR5 Abs. These observations indicate that, whereas HIV-specific immunity in ESN is dependent on a continuous exposure to the virus, anti-CCR5 Abs could be maintained independently of exposure to HIV. These results also suggest that 1) immune protection against HIV infection may be secondary to the activation of multiple mechanisms that, beside potent HIV-specific cell-mediated immunity in peripheral blood and mucosal immunity in the genital tract, include anti-CCR5-specific Abs; 2) anti-CCR5 Ab-mediated protection against HIV infection could be maintained even in the absence of HIV-specific immune responses in ESN; 3) vaccine strategies aimed at elicitation of anti-CCR5 Abs could be less dependent on the need for repeated vaccine boost, compared with vaccine strategies based on the induction of HIV-specific immune responses.

In conclusion, our results provide further information on the heterogeneous mechanisms underlying resistance to HIV infection in ESN individuals and reinforce the concept that immune protection can be mediated by multiple mechanisms, one of which could be anti-CCR5 Abs. Because other, still unclear mechanisms, including complement, chemokines, and other antiviral factors, are likely to be involved in the phenomenon of resistance to HIV infection, more research is necessary to shed further light on the immunologic correlates of this fortunate condition.

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