

Immune activation in Africa is environmentally-driven and is associated with upregulation of CCR5

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Background: HIV infection in Africa is associated with immune activation and a cytokine profile that stimulates CCR5 expression. We investigated whether this immune activation is environmentally driven; if a dominant expression of CCR5 could indeed be detected in African individuals; and if R5 HIV strains would be prevalent in this population.

Methods: Freshly drawn peripheral blood mononuclear cells from HIV-uninfected African and Italian individuals living in rural Africa, from HIV-uninfected Africans and Italians living in Italy, and from HIV-infected African and Italian patients were analysed. Determinations of HIV coreceptor-specific mRNAs and immunophenotype analyses were performed in all samples. Virological analyses included virus isolation and characterization of plasma neutralizing activity.

Findings: Results showed that: immune activation is detected both in Italian and African HIV-uninfected individuals living in Africa but not in African subjects living in Italy; CCR5-specific mRNA is augmented and the surface expression of CCR5 is increased in African compared with Italian residents (CXCR4-specific mRNA is comparable); R5-HIV strains are isolated prevalently from lymphocytes of African HIV-infected patients; and plasma neutralizing activity in HIV-infected African patients is mostly specific for R5 strains.

Conclusions: Immune activation in African residents is environmentally driven and not genetically predetermined. This immune activation results in a skewing of the CCR5 : CXCR4 ratio which is associated with a prevalent isolation of R5 viruses. These data suggest that the selection of the predominant virus strain within the population could be influenced by an immunologically driven pattern of HIV co receptor expression.

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Introduction

An abnormal activation of the immune system has repeatedly been postulated to be involved in the pathogenesis of African HIV infection and recent data confirm that lymphocytes from African HIV-infected and uninfected individuals show functional and phenotypic signs of activation [1–3]. Thus, interleukin (IL)-2, interferon gamma (IFN γ), and IL-10 production is increased when cytokine production by antigen-stimulated peripheral blood mononuclear cells (PBMC) of African HIV-infected and -uninfected individuals is compared to that of HIV-infected and -uninfected European subjects [1–3]. Additionally, CD4 and HLA class II-expressing lymphocytes and the CD4CD45RO : CD4CD45RA ratio are augmented in African compared with European individuals [3]. Immune activation is thus suggested to characterize the AIDS epidemic in Africa together with a series of other differences that include a mainly heterosexual transmission, faster progression to AIDS, and shorter survival time after AIDS diagnosis [4–9]. The observation that immune activation is detected both in African HIV-infected individuals and healthy controls could be explained by genetic or by environmental factors including parasitic and non-parasitic infections, critical hygienic conditions, and nutritional deficiencies. Immune activation is likely to result in profound modifications of the interaction between the immune system and HIV as supported by the observation that different cytokines preferentially modulate the surface expression of the two main HIV coreceptors: IL-2, IFN γ , and IL-10 upregulate CCR5 on the cell surface [10–12] and CXCR4 is stimulated by IL-4 [13]. These coreceptors are also preferentially expressed by different cell subtypes: CXCR4 is predominant on naive T cells (CD45RA) and CCR5 is highly expressed on effector/memory cells (CD45RO) [12], a T cell subpopulation that is augmented in African individuals [3].

Based on the following observations, R5 HIV strains might be favoured in African HIV infection at least partially because of a cytokine-induced predominance of CCR5 expression: (i) different cytokines stimulate the preferential expression of CCR5 and CXCR4 on the surface of cells [10–13]; (ii) the coreceptor usage of non-syncytium- and syncytium-inducing (SI) viruses is different (CCR5 and CXCR4, respectively) [14]; (iii) the predominant cytokine profile observed in Africa (secretion of IL-2, IFN γ , and IL-10) is associated with a more frequent *in vitro* isolation of non-SI (R5) strains of HIV [15]; and (iv) HIV-1 SI variants are isolated rarely from patients infected with HIV subtypes A, C, and D [16–19] (the prevalent clades in Uganda) [20].

To investigate if, (i) in African HIV infection, immune activation is secondary to environmental factors, (ii) the prevalent cytokine profile would stimulate a preferential

expression of CCR5 on cell surface, (iii) and mainly CCR5-dependent HIV strains would be isolated, we examined immunological and virological parameters in HIV-infected and -uninfected individuals from Milano, Italy, and Gulu, Uganda. The same parameters were examined in HIV-seronegative Italian individuals who had been living in Gulu for a long period of time (> 10 years) and in HIV-uninfected subjects from rural Uganda who had been living in Italy for > 10 years.

Material and methods

The Ugandan–Italian AIDS Project for HIV/AIDS

In 1994 a research project on HIV/AIDS was initiated by Ugandan and Italian institutions. This project supports virological, immunological, and epidemiological research in HIV infection in Uganda and is linked to a public health programme addressing diagnosis, treatment, and health education. The two components are integrated in the ‘Global Support to the National Plan for HIV/AIDS Control in Uganda Program’ which is run on the Italian side by a co-ordinated effort of the Italian Istituto Superiore di Sanita, the Ministry of Foreign Affairs, the First Infectious Disease Unit, L. Sacco Hospital, Milan, and the Chair of Immunology of the University of Milan, and on the Ugandan side by the Lacor St. Mary’s Hospital Lacor, Gulu, the Ministry of Health, and the Ugandan Virus Research Institute.

Blood collection, shipping and patient selection

Whole blood was collected by venepuncture in to EDTA-containing vacutainer tubes (Becton Dickinson, Rutherford, New Jersey, USA). An aliquot of blood was processed to obtain plasma samples by two-step centrifugation at 400 and 800 **g** for 20 min at 4°C. The samples were aliquoted, frozen and kept in liquid nitrogen. A second aliquot of blood was hand-carried on an overnight flight from Gulu, Uganda to Milan, Italy. Upon arrival, PBMC were separated on lymphocyte separation medium (Organon Teknika Corp., Durham, North Carolina, USA), washed twice in phosphate-buffered saline, and the number of viable leukocytes was determined by Trypan blue exclusion. The entire procedure was completed within 30 h of blood collection; all specimens were coded and laboratory personnel were blinded throughout the experimental procedures. Frozen plasma aliquots were shipped to Italy in temperature-controlled (liquid nitrogen) thermal containers. Blood specimens were obtained from 14 Ugandan HIV-infected patients followed at St. Mary’s Lacor Hospital Lacor, Gulu, Uganda, and from 13 Italian HIV-infected patients followed by the First Infectious Disease Unit, L. Sacco Hospital in Milan, Italy. All patients were diagnosed to be affected by advanced HIV disease (to minimize differences between

the two groups, blood from the Milan patients was processed after a minimum of 24 h from collection). Fifteen healthy individuals from Uganda, 15 healthy Italian individuals, 15 healthy Italian individuals who had been living in Uganda (Gulu region) for at least 10 years; and eight Ugandan individuals from the Gulu region who had been living in Italy for at least 10 years were also included in the study. All individuals were age- and sex-matched and were screened to exclude viral or parasitic infections. This protocol was supported by the Research Ethics Committee, Ospedale Luigi Sacco, Milano, Italy. Written informed consent was obtained from all patients before enrolment.

Immunophenotypic analyses

Lymphocyte subsets were evaluated using an Epics XL flow-cytometer (Coulter Electronics Inc., Miami Lakes, Florida, USA) using 100 μ l EDTA-treated peripheral blood incubated for 30 min at 4°C with fluorochrome-labelled monoclonal antibodies (MAb). Erythrocyte lysis was obtained after incubation with the Immuno-Prep Epics Kit (Coulter Electronics) and Q-prep Work Station (Coulter Electronics). Lymphocytes were analysed selectively using forward and side scatter properties. For each sample, multiparametric data were acquired for 5000 events.

CCR5 and CXCR4 expression on the cell membrane was evaluated by incubating 2×10^5 PBMC with 10 μ l mouse antibodies to CCR5 and CXCR4 (183 and 173, respectively) (R & D Systems, Minneapolis, Minnesota, USA) for 1 h at 4°C. Cells were then washed in PBS and incubated with fluoresceine isothiocyanate-conjugated anti-mouse IgG (Fab2 fragment) (Sigma, St. Louis, Missouri, USA) diluted 1 : 250 in phosphate-buffered saline containing 1% bovine serum albumin for 30 min at 4°C.

RNA extraction

Total RNA was extracted from lymphocytes according to the acid guanidium thiocyanate-phenol-chloroform method. The purity of the extracted RNA was determined by spectrophotometry. Finally, the extracted RNA was treated with RNase-free DNase (RQ1 DNase, Promega, Madison, Wisconsin, USA) to remove contaminating of genomic DNA.

Reverse transcription

One μ g total RNA from lymphocytes was reverse transcribed into first-strand cDNA in a 20 μ l final volume containing 1 μ M random hexanucleotide primers, 1 μ M oligo dT and 200 U Molony murine leukaemia virus reverse transcriptase.

Normalization of sample β -actin cDNA content by competitive PCR

To compare cytokine mRNA expression in the different samples, it was essential to use equivalent amounts

of substrate cDNA. All samples were therefore normalized for β -actin cDNA content by competitive PCR. Briefly, one set of primers (5'-ATCTGGCACCA-CACCTTCTACAATGAGCTGCG-3' and 5'-CGT CATACTCCTGCTTGCTGATCCACATCTGC-3') amplified both β -actin cDNA in samples and competitor cDNA. The PCR products of both target and competitor were separated by electrophoresis. The competitor cDNA generated a shorter PCR product (619) than the target cDNA (838 bp). Densitometry (INTAS) was used to quantify the density of the bands of samples and competitor PCR products. The concentration of substrate sample cDNA was calculated from the concentration of competitor cDNA that generated an equal amount of competitor and sample products. Therefore, by plotting the ratio of sample density to competitor PCR product against the known amount of competitor substrate cDNA, the amount of substrate β -actin cDNA in each sample was calculated. To normalize the cDNA sample concentration all samples were diluted to the same concentration as the sample with the lowest cDNA concentration.

Quantification of CCR5 and CXCR4 by competitive PCR

CCR5 and CXCR4 were quantified using an exogenous competitor in competitive PCR. The competitor and the template have similar length and the same primer recognition sequences, thus ensuring identical thermodynamics and amplification efficiency for both template species. Following amplification, products of both templates are distinguishable by gel electrophoresis to allow densitometric evaluation of the relative intensities of the bands. The ratio of amplification products reflects the ratio between the initial amount of the template, thus allowing the precise evaluation of amount of CCR5 and CXCR4 cDNA.

Virus isolation

PBMC from HIV-seronegative individuals (AFR_{neg}, ITA_{neg} and ITA/AFR_{neg}) were stimulated for 24 h with phytohemagglutinin (PHA; Sigma Aldrich, Milan, Italy). Cell (5×10^6) were co-cultured with 5×10^6 cells from HIV-seropositive patients (AFR_{pos} and ITA_{pos}) for 3 weeks in RPMI medium supplemented with 20% foetal bovine serum (FBS; Irvine Scientific, Irvine, California, USA) and 10% human recombinant IL-2 (Boehringer Mannheim, Monza, Italy). Supernatants were sampled twice a week and tested for p24 antigen (p24Ag) production using a commercial ELISA kit (Coulter Corporation, Miami, Florida, USA). Once a week; fresh 1 day PHA pre-stimulated HIV-seronegative cells were added to the cultures, maintaining a 1 : 1 ratio. A culture was considered positive if two serial supernatant samples were positive, and showed a 10-fold increase in p24Ag.

Infection of MT2 cell line to establish biological phenotype of primary viruses

The phenotype of primary HIV-1 isolates was determined by syncytium induction and replication in MT-2 cell lines as described previously [21]. Briefly, cryo-preserved cell-free supernatants from primary PBMC co-cultures were used to infect 5×10^6 MT-2 cells. After overnight incubation, MT-2 cells were washed twice, incubated in culture flasks at 37°C, 5% CO₂, 100% humidity, in RPMI 1640 medium supplemented with 10% FBS and antibiotics. Cultures were screened daily for cytopathic effect and twice a week for p24Ag determination (Immunotest Innogenetics N.V., Antwerp, Belgium). MT-2 cultures were carried for 3 weeks. MT-2 characterization was performed both on 'early' (taken from primary PBMC co-cultures as soon as p24Ag became detectable) as well as from 'late' (taken at the end of the culture period) supernatants.

Infection of U87 cell line to define coreceptor usage of primary viruses

Virus isolates from Italian seropositive subjects and the African primary viruses obtained from different co-culture (AFRpos \times AFRneg, AFRpos \times ITA neg, ITApos \times AFRneg, ITApos \times ITAneg, AFRpos \times ITA/AFR neg and ITApos \times ITA/AFR neg) were used to infect U87 human glioma cell lines (provided by D. Littman, courtesy of P. Lusso) expressing one of the following chemokine receptors: CCR5; CXCR4, CCR3; CCR2B. One ml of virus-containing culture supernatant was incubated with 8×10^4 U87 cells in 12-well plates for 4 h at 37°C. Cells were then washed twice and incubated in RPMI medium supplemented with 10% FBS. Cell cultures were observed daily for cytopathic effect. Culture supernatants were collected 2, 5 and 7 days after infection.

Virus titration

The infectivity (50% tissue culture infective dose; TCID₅₀) of each virus isolate was determined on PBMC from a single donor. Virus was diluted (fivefold dilution starting with 1 : 5). Each dilution (150 μ l) was seeded into six parallel wells of a round-bottomed microtiter plate (Nunc, Roskilde, Denmark) containing 1×10^5 resting PBMC in 75 μ l medium, incubated for 2 h, washed, and resuspended in medium containing PHA and 10 U/ml recombinant (r)IL-2 (Amersham, Little Chalfont, UK). Nine and 11 days later HIV-1 p24Ag was quantified in samples from each well [17]. TCID₅₀ were defined as the reciprocal of the virus dilution resulting in 50% positive wells (Reed-Muench calculation).

Virus neutralization assays

Neutralization activity of plasma of 20 Ugandan HIV-infected individuals was measured using the method of Zolla-Pazner, with minor modifications. Resting PBMC (2×10^5) from a healthy donor were added to

96 microplate wells containing 13 and 14.5 TCID₅₀ HIV-1#26 and HIV-1#36 (primary isolates) in a total volume of 150 μ l. After 2 h, the cultures were incubated with 75 μ l of different dilutions of plasma (range: 1 : 50 to 1 : 3200) from Ugandan HIV-infected subjects; 60 min later the PBMC were washed and resuspended in PHA and IL-2-containing medium. Supernatant p24Ag levels were determined 9 and 11 days after infection. Each set of assays was correlated with one negative (plasma from five Italian healthy donors) and two different positive controls: a neutralizing anti-CD4 MAb (500, 125, 31.2, and 7.8 ng/ml final dilutions), and serum from one Italian HIV-seropositive individual used at the same dilution of the Ugandan plasma. The mean value obtained with a specific plasma dilution was compared to the mean values from the six corresponding replicates in the absence of plasma. The optical density obtained in the absence of plasma ranged from 2.0 to 2.2 for HIV-1#26 (X4 strain) and from 1.8 to 2.0 for HIV-1#36 (R5 strain).

Statistical analyses

The mean values of the immunophenotypic markers with their 95% confidence intervals are reported in Table 1 for Italian and African healthy individuals. Differences in the expression of different immunophenotypic markers were evaluated using the Mann-Whitney non-parametric test.

Results

Immune activation is environmentally-driven

Immunophenotypic analyses were performed in PBMC of healthy HIV-seronegative Italian individuals residing in either Italy or Uganda as well as in PBMC of healthy Ugandan subjects living either in Uganda or in Italy. Results showed that a similar pattern of immune activation is present in healthy African residents independent of their ethnic background (Italian or Ugandan). Immune activation disappears in Ugandan individuals who live in Italy. Thus, CD3 and CD3/CD4 T lymphocytes were diminished whereas CD16 cells were augmented both in Italian and Ugandan individuals residing in Africa compared with Italians and Ugandans living in Italy. The percentage of activated CD4 T cells (CD4/DRII) was augmented significantly in both groups of African residents compared with Italians and Ugandans living in Italy, whereas no differences were observed in the percentage of CD8/CD38 lymphocytes. The percentage of CD4- and CD8-naïve cells (CD45RACD62L) was reduced and that of CD4 and CD8 memory lymphocytes (CD45RO) was augmented in both groups of African residents compared with Italians and Ugandans living in Italy. Finally, the percentage of CD4CD7CD57 cells

was reduced and that of CD4+CD7-CD57+ lymphocytes was augmented in African compared with Italian residents. CD4+CD7+CD57+ cells were suggested to preferentially produce type 1 cytokines whereas CD4+CD7-CD57+ cells produce increased amounts of IL-4 and IL-10 [22,23]. These results are shown in Table 1.

A lower CXCR4 : CCR5 ratio is observed in African residents independent of their ethnic background

Because a robust production of IL-2, IFN- γ , and IL-10 is detected in PBMC of both Italian and Ugandan African residents (previously published data), and these cytokines are known to be associated with the expression of CCR5, CCR5 and CXCR4 mRNA was analysed in PBMC of healthy African residents (Italians or Ugandans) and of healthy Italians and Ugandans living in Italy. The same parameters were also investigated in PBMC of HIV-infected Italian and Ugandan individuals. Results obtained in eight individuals per group show that a six- to sevenfold increase in the amount of CCR5-specific mRNA is detected in African residents (Italian and Ugandan) compared with Italians and Ugandans living in Italy. The mean CCR5 mRNA was 2100 copies/ml in Ugandan individuals, 1858 copies/ml in Italians living in Africa, 342 copies/ml in Italians, and 309 copies/ml in Ugandans living in Italy (*P* values reaching statistical significance: Italian versus Ugandans, 0.001; Italians versus Italians living in Africa, 0.012; Ugandans versus Ugandans living in Italy, 0.001). In contrast, no significant differences are evident when the amount of CXCR4 is compared in cells of healthy, HIV-seronegative African or Italian residents. The median CXCR4 : CCR5 ratio was 107 : 1 in the Ugandans living in Africa, 123 : 1 in the Italians living in Africa, 274 : 1 in the Italians living in

Italy, and 187 : 1 in Ugandans living in Italy. The increase detected in CCR5 is thus independent of the ethnic background of the individuals enrolled in the study.

When Ugandan and Italian HIV-infected individuals were compared (*n* = 10 in each group) a similar pattern emerged. Thus, a similar amount of mRNA for CXCR4 was detected in cells of both groups of individuals whereas a sixfold augmentation of CCR5 was present in cells of the Ugandans compared with the Italian patients (mean CCR5 mRNA: Africans, 2512 copies/ml; Italians, 418 copies/ml; *P* = 0.001). This resulted in a skewing of the CXCR4 : CCR5 ratio which was 33.8 : 1 in African and 167 : 1 (median values) in Italian patients. Fig. 1 shows results obtained in representative individuals from each group.

These results are based on quantification of HIV coreceptors mRNA by PCR techniques. The intracellular amount of mRNA might not be correlated directly with surface protein expression. Thus, the amount of these coreceptors on the cell surface of the same eight healthy African residents (Italians or Ugandans) and Italians living in Italy in whom PCR analyses were performed were examined (these analyses could not be performed in cells of Africans living in Italy because of technical problems). Immunofluorescence analyses were performed using a panel of CCR5- and CXCR4-specific MAbs. Results confirmed that the CXCR4 : CCR5 ratio is skewed in African compared with Italian residents. The mean (range) CXCR4 : CCR5; ratio was 0.7 (0.5–1.2 : 1) in Ugandan individuals; 1.5 (1–1.8 : 1) in Italian individuals; and 1 (0.6–1.6 :) in Italians living in Africa. Results of three representative subjects are shown in Fig. 2.

Table 1. Phenotypic analyses in HIV-seronegative individuals. Four groups are considered: Italians living in Italy, Africans living in Uganda, Italians who have been living in Uganda for at least 10 years, and Ugandans who have been living in Italy for at least 10 years Arithmetic means are shown.

Marker	Italians (n = 15) Mean (95%CI)	Ugandans (n = 15) Mean (95%CI)	Italians living in Uganda (n = 15) Mean (95%CI)	Ugandans living in Italy (n = 8) Mean (95%CI)
CD3	80.9 (77.8–83.9)	73.0 ^a (69.5–76.5)	66.1 ^b (60.6–71.5)	77.5 (71.6–79.4)
CD19	15.4 (13.6–17.2)	13.7 (12.1–15.3)	9.4 ^b (7.5–11.4)	14.3 (8.9–15.4)
CD16	9.9 (8.2–11.5)	12.7 (9.9–15.4)	14.6 (11.7–17.6)	8.6 (6.5–12.2) ^c
CD3/CD4	52.9 (47.7–54.1)	41.2 ^a (38.8–43.7)	39.8 ^b (37.8–41.7)	44.8 (39.8–55.2) ^c
CD4/DRII	1.5 (1.1–1.9)	3.2 ^a (2.2–4.2)	3.1 ^b (2.4–3.8)	2.2 (1.9–3.2)
CD4/45RO	26.3 (21.8–30.8)	30.9 (28.6–33.1)	30.1 (27.9–32.3)	28.1 (26.8–33.1)
CD4/45RA/62L	15.1 (12.2–18.0)	12.6 ^a (10.7–14.4)	10.0 ^b (7.8–12.2)	13.2 (10.2–16.8)
CD4/7+/57+	31.8 (27.6–36.0)	28.7 (25.7–31.6)	24.8 ^b (22.0–27.5)	35.4 (27.2–33.5)
CD4/7-/57+	8.4 (6.1–10.7)	14.7 ^a (13.4–15.9)	15.1 ^b (12.0–18.2)	NT
CD3/CD8	25.9 (23.1–28.7)	23.6 (21.2–25.9)	22.8 (19.9–25.7)	24.0 (21.2–28.4)
CD8/45RO	6.1 (5.2–7.0)	9.5 ^a (7.6–11.4)	11.0 ^b (8.9–13.2)	8.9 (7.1–12.2)
CD8/45RA/62L	8.2 (7.0–9.4)	4.9 ^a (3.7–6.0)	4.0 ^b (3.4–4.7)	NT
CD8/38	12.4 (4.9–19.9)	8.8 (6.2–9.5)	11.5 (7.8–15.3)	NT

^aStatistically significant differences between Italians and Ugandans (*P* < 0.05). ^bStatistically significant differences between Italians and Italians living in Uganda (*P* < 0.05). ^cStatistically significant differences between Ugandans and Ugandans living in Italy (*P* < 0.05). CI, Confidence interval; NT, not tested.

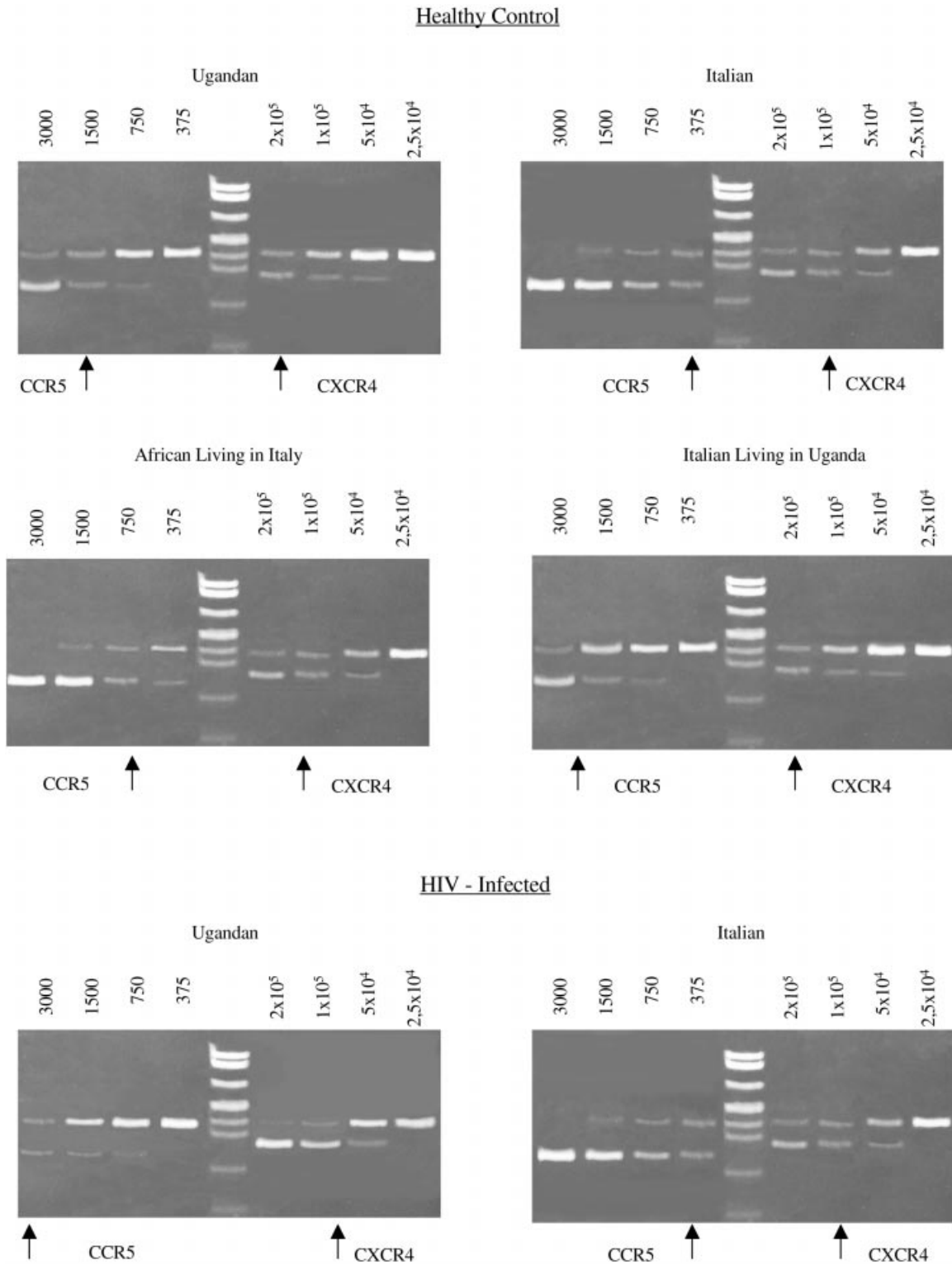


Fig. 1. CCR5- and CXCR4-specific mRNA in resting PBMC of representative HIV-infected and -uninfected individuals. Upper panels: PBMC of one Ugandan (left) and one Italian (right) HIV-seronegative individual. Middle panels: PBMC of a healthy Ugandan living in Italy (left) and of a healthy Italian living in Uganda (right). Lower panels: PBMC of one Ugandan (left) and one Italian (right) HIV-seropositive individuals. CCR5-specific mRNA is shown on the left side of each panel, CXCR4-specific mRNA is shown on the right. The upper bands show chemokine receptor specific cDNA; the lower bands show different dilutions of the competitors. The numbers above the panels indicate the number of copies of the chemokine receptors specific competitor used; arrows show the dilution at which equivalence is observed between cDNA and the competitor.

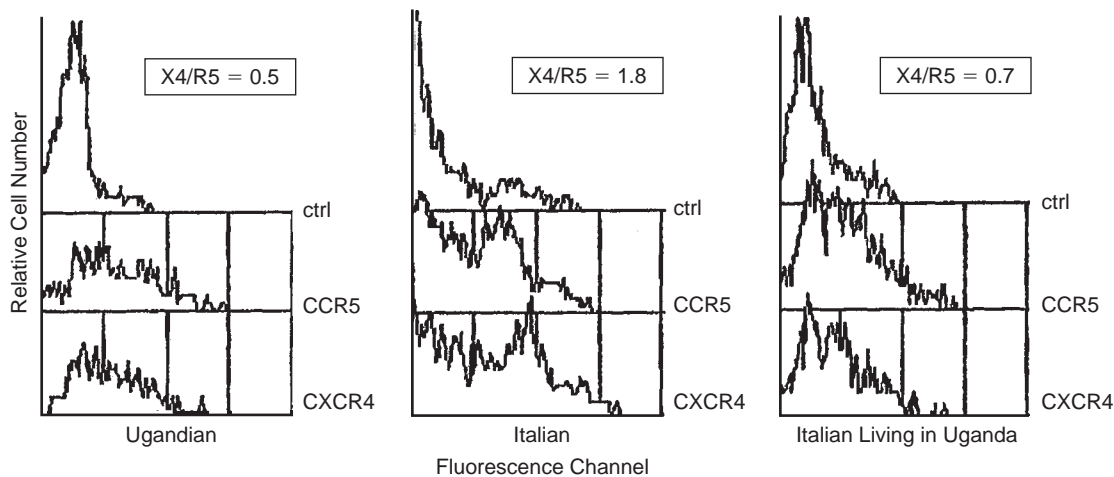


Fig. 2. FACS analysis (mean fluorescence channel) of CCR5 and CXCR4 expression on the surface of resting PBMC from representative HIV-infected and -uninfected Ugandan and Italian healthy controls. Left panel: PBMC of an Ugandan individual; middle panel: PBMC of an Italian individual; right panel: PBMC of an HIV-seronegative Italian individuals living in Uganda.

Overall these data show that the expression of the two main HIV co receptors is not influenced by the genetic background but rather is a product of the immunological environment.

C5-tropic strains of HIV are predominantly isolated in Ugandan HIV-infected individuals

PBMC from Ugandan and Italian HIV-seropositive individuals were cocultured with PBMC of HIV-seronegative Italians living in Italy, Italians living in Uganda, or Ugandans living in Uganda, to verify if the phenotype of the virus isolated in these conditions would be influenced by the immune activation status. The supernatant of each co-culture was collected at early (as soon as p24 was detectable in the supernatant) and late (after three consecutive positive results in the p24 assay) times. Supernatants were used to determine the biological tropism of HIV (MT-2 assay) and to evaluate the coreceptor usage (the U87 CD4 glioma cell lines transfected with and expressing different chemokine receptors was used). Results showed that HIV non-SI strains were predominantly observed when PBMC of Italian or Ugandan HIV-infected individuals were co-cultured those of HIV-seronegative individuals residing in Africa (irrespective of ethnic background), and that HIV SI strains were predominantly obtained when the same PBMC were co-cultured with PBMC of HIV-seronegative Italians living in Italy.

Similar results were obtained when HIV isolated from the co-cultures was used to infect the U87 CD4 cell line expressing either CCR5, CXCR4, CCR3, or CCR2b. Thus: (i) viruses obtained from co-culture of PBMC from Italian or African HIV-seropositive individuals with PBMC of HIV-seronegative African residents (Ugandan or Italian) used mainly CCR5 (R5); (ii) viruses obtained from co-culture of the same HIV-positive PBMC with PBMC of HIV-seronegative

Italians living in Italy used mainly CXCR4 (X4). No viruses used CCR3 or CCR2b as coreceptors (data not shown). These findings confirm that virus growth is influenced by the cell used in the co-culture system and suggest that immunological activation may be involved in selecting the dominant biological phenotype within the quasispecies.

HIV-1 R5-specific neutralizing activity is detected mainly in plasma from African HIV-infected individuals

Neutralizing activity was analysed in plasma of 20 African HIV-seropositive individuals to verify if the observed prevalent isolation of R5 strains would be associated with an R5-specific humoral immune response. Infectivity reduction was evaluated using two different primary viruses: HIV-1#36 (R5 strain) and HIV-1#26 (X4 strain) obtained from HIV-seropositive individuals and characterized on the HIV coreceptor-expressing U87 glioma cell line. Plasma of 17 out of the 20 (70%) African individuals down-modulated the infectivity of the R5 virus; when the X4 virus was used infectivity reduction was observed only in two out of 20 (10%) cases. The 50% and 90% inhibitory concentrations are shown in Table 2. To exclude the possibility that inhibition may have been caused by elevated concentrations of plasma beta chemokines, RANTES, Mip1 α and Mip1 β were measured in plasma of the 20 African HIV-infected individuals and of 20 HIV-seronegative controls. Plasma concentrations of the three beta chemokines were comparable between patients and controls (data not shown) suggesting that the neutralizing activity is due to the immunoglobulin fraction. These results suggest that in African HIV-infected individuals isolation of R5 viruses is associated with the development of an R5-specific immune response.

Table 2. Neutralizing activity in plasma from HIV-seropositive Ugandan individuals.

Sample	HIV#36 (-R5 strain)		HIV#26 (-X4 strain)	
	IC ₅₀	IC ₉₀	IC ₅₀	IC ₉₀
546	> 3200	> 3200	–	–
959	390	220	–	–
1296	440	230	–	–
1316	> 3200	> 3200	–	–
1324	110	60	–	–
1474	< 50	< 50	–	–
1493	1.125	270	380	230
1514	100	< 50	–	–
1649	< 50	< 50	–	–
1654	3.2	380	–	–
1665	100	60	–	–
1790	> 3200	> 3200	1.105	100
1798	< 50	< 50	–	–
1804	75	< 50	–	–
1811	150	60	–	–
1845	95	< 50	–	–
1849	< 50	< 50	–	–
1879	–	–	–	–
1884	–	–	–	–
1882	–	–	–	–
SIM4 (anti-CD4 MAb)	340	90	320	80
Serum HIV ⁺ ^a	440	220	460	250
Negative control ^b	–	–	–	–

^aPlasma of a representative HIV seropositive Italian patient. ^bMean value of plasma from five seronegative healthy donors. IC₅₀, Dilution giving 50% neutralization; IC₉₀, dilution giving 90% neutralization.

Discussion

Immune activation is observed in African HIV-infected and -uninfected individuals; this activation is characterized by an abnormal pattern of cytokine secretion (robust production of IL-2, IFN γ and IL-10) [3] and an increase in activated lymphocytes and memory cells [1–3]. We investigated if immune activation in Africa is genetically determined or whether it is secondary to environmental conditions by comparing immune parameters in Ugandan individuals who live in either Uganda or Italy and in Italian subjects who live in Italy or have been long-term residents of Uganda. Results showed that an immune activation similar to the one detected in Ugandan individuals is observed in Italians who reside in Africa and that this activation disappears in Ugandans living in Italy. We thus conclude that environmental factors influence the peculiar pattern of immune response seen in Africa.

Because CCR5, the coreceptor for R5 (M-tropic or non-SI) viruses, is prevalently expressed on memory T cells [12] and its expression is stimulated by the same cytokines whose production is augmented in African individuals [10–12], we analysed whether immune activation in Africa would correlate with an increased expression of CCR5. The results confirmed that, whereas CXCR4 expression is comparable in all groups of individuals examined, CCR5 is augmented in cells

of HIV-infected and -uninfected African residents. Immune activation therefore is associated with an imbalance in the CXCR4 : CCR5 ratio that can be detected even in HIV-uninfected individuals. We next performed virological analyses on HIV-infected Italian and African individuals to confirm previous reports suggesting that X4 strains of HIV are isolated rarely in African HIV-infected patients. Results showed that R5 HIV strains are isolated mainly from lymphocytes of Ugandan HIV-infected subjects, and that neutralizing activity in plasma of HIV-infected African patients is specific mainly for R5 strains.

HIV infection in Uganda is caused mostly by viruses of clades A, C and D [20]; these same clades are present in countries such as Ethiopia, Ivory Coast, and Cameroon [24,25] where the prevalent isolation of R5 viruses has been described [16–19]. Thus, whereas progression of HIV infection in European and American HIV-infected individuals is often correlated with the isolation of X4 strains of HIV [26–30] and with virus adaptation to a promiscuous use of multiple coreceptors [31], R5 viruses are prevalently isolated throughout the course of HIV infection in Africa [16–19]. This phenomenon could be an intrinsic property of non-B clades of HIV; nevertheless, it could be speculated that the augmented expression of CCR5 seen in cells of Ugandan individuals could be a possible selective factor within the viral quaspecies. Thus, X4 viruses could be

at a selective disadvantage and the appearance of X4 variants would not be favoured in African HIV infection secondarily to an alteration of the CXCR4:CCR5 ratio. In this regard it is interesting that longitudinal studies showed that the expression of CCR5 declines even in the progression of B clade-supported HIV infection, and that this decline is associated with the emergence of X4 viruses [32]. Thus our results allow us to speculate that the prevalence of R5 HIV strains seen in Ugandan HIV-seropositive individuals might be secondary to a higher amount of CCR5 expression on cells of Ugandan residents. These results also confirm the recent findings by Kalinkovich and colleagues who reported that CCR5 expression is indeed augmented on cells of Ethiopian HIV-seronegative individuals [33].

The observation that the CXCR4:CCR5 ratio is diminished even in both Ugandan and Italian HIV-seronegative individuals living in Africa suggests that the skewing in the expression of the two major HIV coreceptors is secondary to environmentally driven immune activation. In this light it is interesting to observe that once African HIV-infected individuals move to industrialized countries, immune activation gradually disappears (this data, [34]), and the rate of isolation of R5 and X4 viruses becomes comparable to the one described for European patients HIV [34]. Another observation stemming from these results, and reinforcing the hypothesis that the state of immune activation and of coreceptor expression might exert a selective pressure on HIV, is that the main HIV isolate was different according to the immune characteristics of the cell on which virus was grown. Thus, cocultivation of cells from the same HIV-infected individuals led to different results when the only variable introduced to the system was the immune status of the cocultured cells (Italian cells: X4 strains; African cells: R5 strains). Although it has long been known that virus growth is influenced by the cell used in the culture system, it has to be noticed that CXCR4 expression was comparable and dominant in all groups of individual studied. These data thus suggest that the CXCR4:CCR5 ratio, rather than the absolute amount of coreceptors expressed on cell surface, might be associated with the prevalent isolation of either R5 or X4 strains of HIV.

In vitro virus isolation is not unequivocally correlated with the situation *in vivo*. To approach the *in vivo* scenario HIV-specific humoral immune responses were analysed in Ugandan and Italian HIV-infected individuals. Results showed this response to be qualitatively different in the two groups of patients. Therefore, whereas both R5- and X4-specific neutralizing activity was detected in Italian patients, R5-restricted neutralizing activity was present in the vast majority (82%) of Ugandan HIV-seropositive individuals. These results

suggest that neutralization sensitivity of HIV primary isolates in Africa is determined by coreceptor usage, and are in apparent contrast with data reported previously indicating an independence between neutralizing activity and coreceptor usage [35–39]. The discrepancy can be explained by the observation that, whereas other investigators measured neutralizing activity by using sera of American and European HIV-infected individuals, we used freshly drawn sera from therapy-naïve African patients. Selective pressure, use of antiretroviral therapy, and a different immune status can therefore explain these differences and suggest that the present observations resemble the *in vivo* situation more closely.

Short-lived immune activation is classically observed during primary HIV infection in which both R5 and X4 strains of HIV are transmitted [40]. If immune activation would result in a time-limited alteration of the CXCR4:CCR5 ratio this could help to explain the disappearance of X4 strains of HIV, which is usually observed shortly thereafter. Finally, because an alteration of the CXCR4:CCR5 ratio is detected in the female genital tract [41], the higher prevalence of R5 strains of HIV within the population in sub-Saharan Africa could be at least partially responsible for the prevalent heterosexual transmission of HIV observed in these regions of the world.

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Appendix

The Italian-Ugandan AIDS Project

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