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The CCR5 receptor acts as an alloantigen in CCR5Δ32 homozygous individuals: Identification of chemokine- and HIV-1-blocking human antibodies

(chemokine receptor/RANTES/allograft rejection/HIV-1 neutralization)

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ABSTRACT **The chemokine receptor CCR5 is the major coreceptor for infection by macrophage-tropic R5 HIV-1. A 32-bp deletion in the gene coding for CCR5 (CCR5Δ32) occurs with a frequency of 10% in the Caucasian population and results in a receptor protein that is truncated and not expressed at the cell surface. CCR5Δ32 homozygous individuals are apparently normal but resistant to infection with R5 HIV-1. In two individuals homozygous for CCR5Δ32, who had been repeatedly exposed to CCR5-expressing blood cells through sexual activity, we have identified antibodies to CCR5 that bound specifically to the surface of CCR5-expressing cell lines. Serum from these individuals, in contrast to serum from $CCR5^{+/+}$ individuals, competed with radiolabeled RANTES for binding to the CCR5 receptor and inhibited infection of peripheral blood mononuclear cells with R5, but not X4, primary isolates of HIV-1. The identified human antibodies to CCR5 define an alloantigen that may cause allograft rejection in a mismatch situation even in individuals with no history of blood transfusions or i.v. drug abuse.**

CCR5, the receptor for the CC-chemokines RANTES, MIP-α, and MIP-β is expressed on macrophages and on a subset of both $CD4^+$ and $CD8^+$ T cells. High expression of CCR5 is found on activated T and memory T cells, characterized as $CD45RO^+$, and exhibiting high expression of CD26 and CD95 (1). CCR5 recently was identified as the major coreceptor for R5 HIV-1 strains, which led to the discovery of a CCR5 allele with a 32-bp deletion (CCR5Δ32 allele) that in homozygous individuals, conferred almost complete protection against HIV-1 infection (2). The mutation has a surprisingly high allele frequency in the Caucasian population, especially in northern Europe, and results in the production of a receptor that is truncated and not expressed on the cell surface (2–4). More recently, a single point mutation at position 303 on one CCR5 allele, in combination with the CCR5Δ32 allele, has been described to have similar effect (5). Over 1% of the population of northern Europe is homozygous for the CCR5Δ32 allele, and they are accordingly natural gene knockouts for the CCR5 receptor protein. These individuals could be expected to develop an immune response against CCR5 if exposed to the CCR5 alloantigen. This is the case for another seven-transmembrane chemokine receptor called DARC (the Duffy antigen) found on erythrocytes and used by *Plasmodium vivax* for cell entry (6, 7). Some individuals lack the Duffy blood group antigen either because of mRNA down-regulation or a 14-bp deletion. In these patients, a strong antibody response to the Duffy antigen, i.e., the DARC chemokine receptor, can be observed at blood transfusion (8).

A Danish study of the frequency of the CCR5Δ32 allele and its effect on the clinical outcome of HIV infection included a cohort of high-risk HIV-1 seronegative individuals for comparison (9). Two of these individuals, both with a history of sexually transmitted diseases with erosions of the genital and rectal epithelia, were found to be homozygous for the Δ32 allele. Their medical history rendered them particularly vulnerable to immunization through multiple exposures to CCR5-expressing cells, and herein we report the identification and characterization of antibodies to CCR5 in these two individuals. The major part of the antibody response seemed to be directed against the ligand-binding site, although the serum also inhibited infection of peripheral blood mononuclear cells (PBMCs) with R5 primary isolates of HIV-1. The identified human antibodies to CCR5 define an alloantigen that may cause allograft rejection in a mismatch situation. Further, the human anti-CCR5 antibodies may form the basis for development of immunotherapeutic reagents for HIV-1 and other CCR5-associated diseases.

MATERIALS AND METHODS

Receptor and Ligand. Wild-type CCR5 (accession no. X91492) was cloned by PCR technologies from cDNA extracted from human blood. RANTES, expressed in *Escherichia coli* and HPLC-purified, was kindly provided by Tim Wells (Glaxo Biomedical Research Institute, Plan Les Quates, Switzerland).

Transfection and Tissue Culture. cDNA coding for wild-type CCR5 was cloned into the pTEJ8 eukaryotic expression vector, and COS-7 cells were transiently transfected by the calcium phosphate precipitation method, as described (10). HEK-293 cells were stably transfected by a calcium phosphate precipitation method, and clones were selected by G-418 (1 mg/ml). Stably transfected Chinese hamster ovary (CHO) cells were kindly provided by Tim Wells (11).

Confocal Laser Scanning Microscopy. CCR5-expressing CHO and HEK-293 cells were grown in RPMI medium 1640 containing 10% fetal calf serum and allowed to adhere to chambered coverslips (Nunc) for 48 h at 37°C, 5% CO_2 to form monolayers. Live cells were incubated with the human sera and

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Abbreviations: PBMC, peripheral blood mononuclear cell; CHO, Chinese hamster ovary.

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a murine mAb against CCR5 (MAB181, R & D Systems) for 1.5 h at room temperature, washed three times with cold culture medium, and fixed with 2% paraformaldehyde. Cells were blocked with normal goat serum and incubated with fluorescein isothiocyanate-labeled goat anti-human Fab antibody (Pierce) or Texas Red-labeled goat anti-mouse IgG (Pierce) diluted 1:200, in PBS for 1 h at room temperature. After secondary antibody incubations, the cells were washed twice in PBS for 15 min at room temperature and mounted in antifading reagent (30 mM DTT/PBS/glycerol, 2:9:1). Cell staining was evaluated by confocal laser scanning microscopy. As a control, all experiments were duplicated with omission of the primary antibody.

SDS/PAGE and Western Blotting. CCR5-expressing or nontransfected CHO and HEK293 cells were resuspended in lysis buffer [1% Nonidet P-40 (Sigma), 20 μg/ml of phenylmethylsulfonylfluoride in 50 mM Tris-buffered saline] and mixed with equal volume of 2× sample buffer (4% SDS, 0.2% bromophenol blue, 20% glycerol in 100 mM Tris-buffered saline) and boiled for 5 min. The samples were electrophoresed on a 7.5% solving gel (Bio-Rad), and the proteins were electroblotted onto Immobilon P (Millipore). The Immobilon sheet was cut into strips of 5 mm, blocked in 0.1% Tween-20 in Tris-buffered saline for 15 min, and incubated with the sera for 3 h at room temperature. Bound antibody was detected with horseradish peroxidase-labeled goat anti-human Fab antibody (Pierce) and visualized by chemiluminescent substrate (Supersignal Substrate, Pierce) and autoradiographic film (Life Science). The monoclonal anti-CCR5 antibody (MAB181) did not recognize the SDS-denatured CCR5.

Inhibition of RANTES Binding. Monoiodinated RANTES (catalogue no. IM288) was obtained from Amersham. One day after transfection and 1 day before the binding experiments, the transfected COS-7 cells were transferred to 24-well culture plates with 1×10^5 cells per well aiming at 15% binding of ^{125}I-RANTES in 0.5 ml of binding buffer consisting of 25 mM Hepes, supplemented with 5 mM $MgCl_2$ and 1 mM $CaCl_2$, pH 7.2, supplemented with 0.5% (wt/vol) BSA (Sigma). To remove salt and smaller molecules, serum samples were buffer-exchanged to binding buffer using Bio-Spin 6 Chromatography Columns (exclusion limit 6 kDa, Bio-Rad) immediately before the binding assays. The buffer-exchanged sera were studied in various dilutions in competition binding for 4 h at 4°C with 15–18 pM iodinated RANTES ($1.5–2 \times 10^4$ cpm/well). The reaction was stopped by washing the wells four times in 0.5 ml ice-cold binding buffer supplemented with 0.5 M NaCl, followed by addition of 1 ml lysis buffer containing 8 M urea, 2% (vol/vol) Nonidet P-40, and 3 M acetic acid (12). Specific binding constituted around 80% of the total. Binding data were analyzed, and IC_{50} value for the homologues competition binding curve was determined by computerized nonlinear regression analysis using the INPLOT program (GraphPad, San Diego), giving a B_{max} was 4.7 ± 0.6 fmol receptor/10^5 cell, and IC_{50} of $1.6 \pm 0.2 \times 10^{-10}$ M ($n = 6$) with a Hill coefficient of -0.8 ± 0.03 (data not shown) (13). Similar experiments ($n = 2$) also were performed using stable CCR5 transfected CHO cells. Further, to remove any chemokines, serum samples were also buffer-exchanged to binding buffer using Bio-Spin 30 Chromatography Columns (exclusion limit 30 kDa, Bio-Rad) immediately before the binding assays.

Neutralization of HIV-1 Using a p24 Production Assay. Virus neutralization of primary HIV-1 isolate and laboratory-adapted HIV-1 strains were assessed by using phytohemagglutinin-stimulated PBMCs and H9 cells, respectively, as indicator cells, and with determination of p24 antigen production as the end point. The virus isolates were: the two R5 primary isolates BaL and 93US073 (pediatric), the X4 using primary isolate 92UG029, and the X4 laboratory-adapted strain HxB2 obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. The viruses 93US073, BaL, 92UG029, and HxB2 were 90% neutralized by the potent neutralizing human antibody IgG1b12 at concentrations of 3.1 μg/ml, 10 μg/ml, 10 μg/ml, and 0.04 μg/ml, respectively (14) (P. W. Parren, M.W., A. Trkola, J. M. Binley, M. Purtscher, H. Katinger, J. P. Moore, and D.R.B., unpublished results). Briefly, the neutralization assays were performed as follows: PBMCs were stimulated with 5 μg/ml phytohemagglutinin for 48 h, washed and incubated with 40 units/ml of interleukin 2 in RPMI medium 1640 containing 10% heat-inactivated fetal calf serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM L-glutamine for 72 h. Six 2-fold serial dilutions of each sera were prepared and a 500-μl aliquot of each was transferred to three replicate wells of a 96-well flat-bottom culture plate. Fifty microliters containing 100 $TCID_{50}$ of primary isolate stock or laboratory strain was added and incubated for 1 h at 37°C. The calculated neutralization titers refer to the serum dilution during this preincubation step. The serum/virus mixture then was diluted 2-fold by addition 5×10^4 stimulated PBMCs or 2×10^4 H9 cells in 100 μl. After 4-h incubation, the cells were washed twice and cultured for 7 days. The cultures were collected, treated with 1% vol/vol Empigen detergent, and tested in triplicate for p24 antigen content using an ELISA. Virus production in the absence of serum was measured from three wells containing 50 μl of virus, 50 μl of medium, and 100 μl of PBMCs or H9 cells. The ratios of p24 antigen production in serum-containing cultures to p24 antigen production in control cultures were estimated, and the serum dilution causing 50% inhibition were determined. No binding to recombinant HIV-1 gp120 (JRFL strain) with all CCR5Δ32 and $CCR5^{+/+}$ sera in ELISA was observed.

RESULTS AND DISCUSSION

From a cohort of 35 HIV-1 seronegative homosexual men with previous high-risk behavior, defined as having unprotected sexual intercourse with more than 10 partners per year in the period 1983–1985 and having a history of at least one episode of a sexually transmitted disease, two individuals were found to be homozygous for the Δ32 allele (9). Because of the history of sexually transmitted diseases with erosions of the genital and rectal epithelia, these individuals were particularly vulnerable to immunization through multiple exposures to CCR5-expressing cells. Neither of the individuals had a medical history indicating blood transfusions or intravenous drug abuse. Sera from these two individuals were examined for the presence of anti-CCR5 antibodies.

Initially, the sera were tested for binding to CHO and HEK-293 cells, both stably transfected with the CCR5 receptor, by using confocal laser scanning microscopy. Strong staining of the cell surface of the CCR5-expressing CHO cells was observed with serum from both CCR5Δ32 individuals (Fig. 1*A*). Similar surface staining was obtained with a murine mAb against CCR5 (Fig. 1*B*). A subpopulation of CHO cells were found to be stained neither by the human sera nor by the murine antibodies, conceivably because of the lack of CCR5 expression (Fig. 1 *A* and *B*, arrowheads). Serum from both CCR5Δ32 donors, as well as the murine anti-CCR5 antibody, showed staining of the surface of the CCR5-expressing HEK-293 cells (data not shown). In contrast, no staining of CCR5-transfected CHO cells (Fig. 1*C*) or HEK-293 cells was observed with serum from eight homozygous $CCR5^{+/+}$ sera, including four from the cohort of the 35 exposed HIV-1 seronegative homosexual men. No staining was observed with the sera from the two individuals homozygous for the Δ 32 allele or the four $CCR5^{+/+}$ individuals on nontransfected CHO or HEK-293 cells grown under identical conditions. Staining for CCR5 was observed with sera from the two Δ32

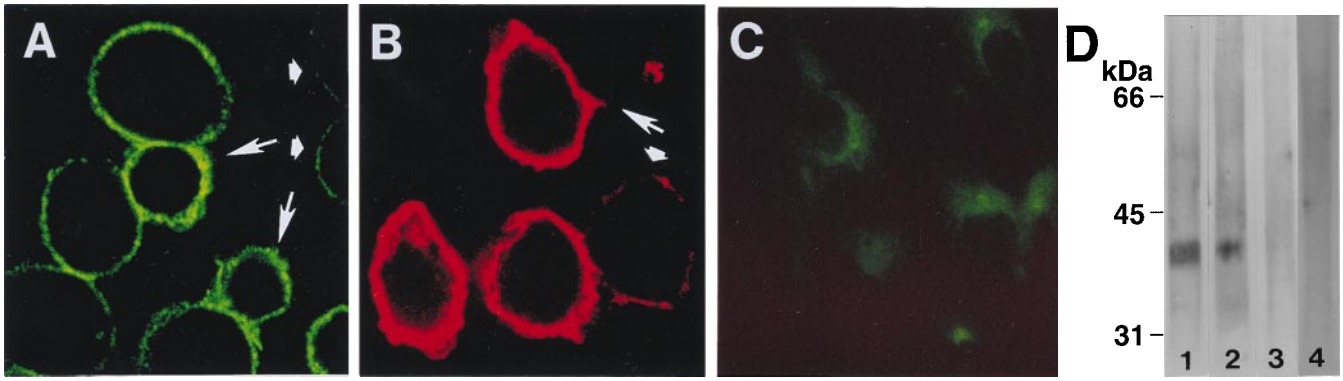

FIG. 1. Analysis of the reactivity of serum antibodies from CCR5Δ32 donors using confocal laser scanning microscopy and Western blotting. Surface binding of serum from CCR5Δ32 donor 1 (1:50) (*A*, arrows), murine anti-CCR5 mAb (5 μg/ml) (*B*, arrows), and serum from a $CCR5^{+/+}$ individual (1:50) (*C*) to live CCR5-expressing CHO cells. Similar staining of the cell surface was observed with the CCR5Δ32 sera and the murine anti-CCR5 mAb whereas no surface staining was obtained with $CCR5^{+/+}$ sera. A subpopulation of CHO cells show no binding of CCR5Δ32 sera or the anti-CCR5 mAb (arrowheads) and neither was staining observed with the two CCR5Δ32 and the four $CCR5^{+/+}$ sera on nontransfected CHO cells grow n under identical conditions. Western blot analysis (*D*) of CCR5-expressing CHO cell extract using serum antibodies from CCR5Δ32 donors 1 (lane 1) and 2 (lane 2), and a $CCR5^{+/+}$ individual (lane 3) or nontransfected CHO cells using serum antibodies from CCR5Δ32 donor 1 (lane 4).

allele individuals up to a dilution of 1:200 and 1:50, respectively.

The sera also were tested for binding to Western blots of SDS/PAGE-separated CCR5-expressing CHO and HEK293 cell lysate. Both CCR5Δ32 sera, at a dilution of 1:10, stained a band with a molecular mass of approximately 40 kDa (Fig. 1*D*, lanes 1 and 2) whereas no staining was observed with six $CCR5^{+/+}$ sera, including four of the 35 exposed HIV-1 seronegative individuals. No staining of SDS/PAGE-separated nontransfected CHO cells with the two CCR5Δ32 sera was observed.

We then tested serum from the two CCR5Δ32 and four $CCR5^{+/+}$ homozygous individuals in a competition binding assay using iodinated RANTES and CCR5-expressing, transiently transfected, COS-7 cells. Sera were gel-filtered to remove salt and smaller molecules, and incubated in different dilutions with ^{125}I-labeled RANTES. As shown in Fig. 2, the sera from the two CCR5Δ32 homozygous individuals displaced RANTES significantly more effectively than control sera from the CCR5-expressing cells. The 1:20 dilution of the two homozygous CCR5Δ32 sera displaced all specifically bound, radioactive RANTES, compared with 39% displacement with the homozygous $CCR5^{+/+}$ sera at this dilution. The 1:100 and 1:500 dilutions of the two homozygous CCR5Δ32 sera displaced 65% and 40% specifically bound RANTES, respectively, whereas no displacement was seen at these dilutions for the homozygous $CCR5^{+/+}$ sera. Subsequently, we repeated the competition experiments by using CCR5-expressing CHO cells to verify that the cell type had no influence on the binding kinetics. Similar affinities of the two homozygous CCR5Δ32 sera for CCR5-expressing CHO cells and CCR5-expressing COS-7 cells was observed.

Phytohemagglutinin-activated $CD4^+$ lymphocytes from exposed-noninfected individuals has been shown to produce more RANTES, MIP-α, and MIP-β than phytohemagglutinin-activated $CD4^+$ lymphocytes from unexposed noninfected individuals (15). To verify that the observed inhibition of iodinated RANTES was not caused by increased chemokine levels (RANTES, MIP-α, MIP-β, and MDC) in the two CCR5Δ32 sera compared with the homozygous $CCR5^{+/+}$ sera,

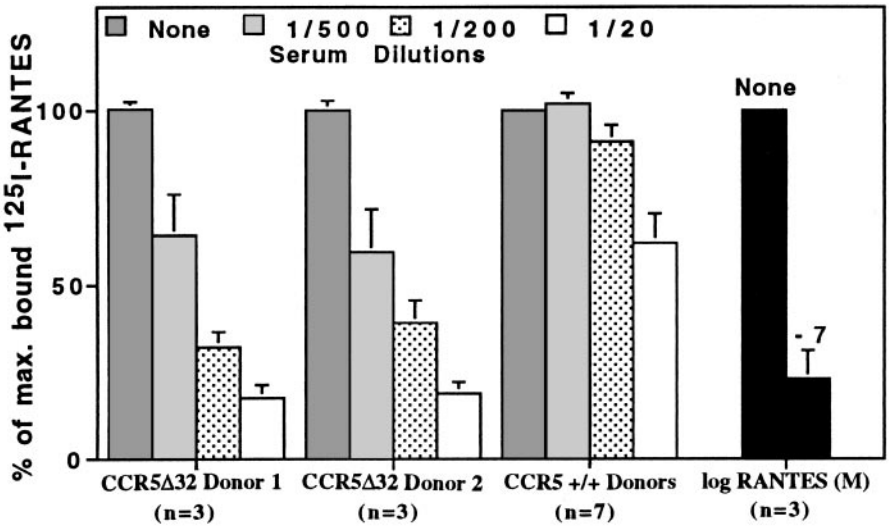

FIG. 2. Displacement of iodinated RANTES by CCR5Δ32 sera, $CCR5^{+/+}$ sera and RANTES from transiently transfected COS-7 cells expressing CCR5. As shown, the sera diluted (1:20, 1:50, and 1:500) from the two Δ32 individuals displaced binding of RANTES, in contrast to a panel of $CCR5^{+/+}$ sera. Maximally bound iodinated RANTES corresponded to approximately 1,500 cpm. No specific binding was observed with COS-7 transiently transfected with the pTEJ8 eukaryotic expression vector. Nonspecific binding of ^{125}I-RANTES was determined by homologues displacement by using 10 μM RANTES (black columns).

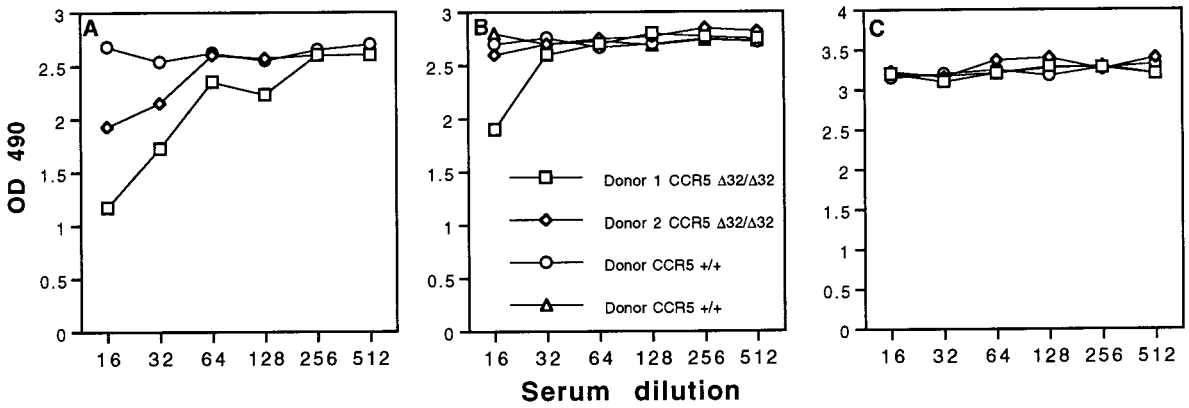

FIG. 3. Neutralization of the macrophage-tropic (CCR5, R5) primary isolate 93US073 (*A*), BaL (*B*) and the CXCR4 (X4) laboratory isolate HxB2 (*C*) by sera from two CCR5Δ32 individuals, and sera from exposed HIV-1 seronegative $CCR5^{+/+}$ individuals, as measured by a p24 production assay. The data in *A* and *B* is the average of two independent neutralization experiments.

the sera were passaged through a gel filtration column that removes all molecules under 30 kDa. As a control, RANTES was completely depleted from a sample containing 200 μM RANTES after passage through the column. The flow-through from the CCR5Δ32 sera was tested in the competition experiments and the results were identical to those shown in Fig. 2, demonstrating that the mentioned chemokines had no influence on the results obtained by competition binding.

The presence of antibodies against CCR5 in certain individuals has immediate practical implications for blood serology, because transfusion with leukocyte-containing blood and transplantation with CCR5-positive organs to a CCR5-negative individual may lead to adverse reactions. Although antibodies are clearly involved in graft rejection in situations other than the hyperacute rejection (because of preformed antibodies to major histocompatibility complex antigens), their precise role in acute and chronic rejection remains unclear. Depending on whether CCR5 also can be recognized by the cellular arm of the immune system, bone marrow transplantation between a CCR5Δ32 homozygous donor and a $CCR5^{+/+}$ recipient may lead to graft versus host disease (GVHD) and, in that case, CCR5 must be recognized as a minor histocompatibility antigen. Studies over the years of other minor histocompatibility antigens (16) have shown that disparity of some of these antigens between HLA-matched bone marrow donors and recipients is a major risk factor for GVHD. With assays readily available for typing, it will be important to re-evaluate larger populations of GVHD from northern Europe to determine whether the CCR5 mismatch can explain some of these cases.

CCR5 generally has been considered to be a rather poor immunogen. However, the present study suggests that multiple exposure to CCR5 encountered through damaged mucosal surfaces can elicit a quite efficient antibody response. Further studies will determine whether this also occurs in individuals receiving leukocyte-containing blood transfusions and organ transplants. Moreover, whether a mismatch situation in feto-maternal incompatibility situations gives rise to an anti-CCR5 response remains to be elucidated.

We finally tested the ability of the sera from the CCR5Δ32 individuals to block infection by CCR5 by using R5 primary isolates of HIV-1 in a p24 assay (Fig. 3). With one of the two sera (donor 1), two repeated experiments resulted in more than 50% neutralization of the pediatric 93US073 isolate at serum dilutions of 1:32 and 1:16, respectively (Fig. 3*A*). At the same dilutions, homozygous +/+ serum, including two of the 35 exposed HIV-1 seronegative individuals, exhibited no neutralization. The sera also were tested against the BaL isolate and, again, the sera of one of the two individuals homozygous for the Δ32 allele showed some neutralization capability, but did not reach 50% at the 1:16 dilution (Fig. 3*B*). The sera also were tested against two CXCR4 by using X4 HIV-1 viruses: the laboratory stain HxB2 (Fig. 3*C*) and the primary isolate 92UG029. As expected, these two viruses were not neutralized by the sera from the CCR5Δ32 individuals.

Characterization of murine mAbs against CCR5 and mutational analysis of this receptor indicate that the binding site for the gp120 HIV envelope protein and the chemokine binding sites are only partially overlapping (17–20). Both of the human antisera identified in the present study blocked chemokine binding, but only one showed HIV neutralization activity. Whether this reflects a quantitative or a qualitative phenomenon remains to be clarified. In any case, the major part of the antibody response in the two individuals homozygous for the Δ32 allele seemed to be directed against the ligand-binding site, conceivably located mainly in the second extracellular loop, rather than against the site interacting with gp120 (17). Technologies are now available to clone of each part of the immune response from such Δ32 allele homozygotes (21), thus allowing production of antibodies with defined functions that, for example, might be used for therapy.

Most immunotherapy strategies have focused on targeting HIV virus itself. In contrast, blocking the CCR5 receptor by antibodies or other reagents aims at protecting cells, particularly activated and memory T cells from infection, which may be of major importance to the clinical outcome. A central issue for CCR5 antagonist immunotherapy is whether CCR5 receptors are essential for other immune functions. Initial studies have indicated that individuals homozygous for the Δ32 allele may have no clinical phenotype. However, recent studies have indicated that rheumatoid arthritis patients carrying the Δ32 allele may exhibit less inflammation than their wild-type counterparts, suggesting beneficial roles other than that involved in HIV pathogenesis of this allele and pointing to anti-CCR5 as a possible part of anti-inflammatory therapy (26). This is supported by observations that target disruption of the β chemokine receptor CCR1 in mice, which has overlapping function with CCR5, appear to result in a diminished inflammatory response that protects against tissue injury. (22). Blocking the CCR5 receptor by ligands (RANTES, MIP-α, or MIP-β) (23, 24) or modified ligand (aminooxypentane-RANTES) (13) has been shown to inhibit infection by non-syncytia-inducing, R5 HIV-1 strains with varying efficiency. Additionally, murine mAbs recognizing the second extracellular loop or the NH_2-terminal region of CCR5 have been generated that neutralize R5 viruses (1, 17). Such antibodies may have therapeutic potential, although for *in vivo* use human antibodies are more attractive. These antibodies may be cloned by Epstein–Barr virus transformation or phage display technology from immune donors.

In conclusion, we have demonstrated the occurrence of an antibody response to CCR5 in individuals homozygous for the Δ32 allele who had been repeatedly exposed to CCR5-expressing blood cells through sexual contact, rather than blood transfusions. This observation strongly suggests that CCR5 is a unique alloantigen that should be evaluated as potentially causing allograft rejection in mismatch situations. Furthermore, our observations on antichemokine receptor antibodies also may have broader implications, because polymorphism of another chemokine receptor recently has been reported (25). Although this CCR2b amino acid substitution is relatively neutral others might cause changes in the structure of chemokine receptors and at alloimmunization elicit an antibody response. Finally, human anti-CCR5 antibodies may form the basis for the development of immunotherapeutic reagents for HIV and other CCR5-associated diseases.

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