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Depletion of CCR5-Expressing Cells with Bispecific Antibodies and Chemokine Toxins: A New Strategy in the Treatment of Chronic Inflammatory Diseases and HIV

Hilke Brühl,* Josef Cihak,† Manfred Stangassinger,† Detlef Schlöndorff,* and Matthias Mack[1]*

The chemokine receptor CCR5 is expressed on the majority of T cells and monocytes in the inflammatory infiltrate of diseases such as rheumatoid arthritis, renal diseases, and multiple sclerosis. In contrast, little expression of CCR5 is found on peripheral blood leukocytes. A specific depletion of CCR5$^+$ cells could therefore be a useful strategy to reduce the cellular infiltrate in chronic inflammations. Moreover, CCR5 is the major coreceptor for M-tropic HIV-1 strains. Depletion of CCR5$^+$ leukocytes may help to eliminate cells latently infected with HIV-1. We designed two constructs that specifically destroy chemokine receptor-positive cells. The first construct, a bispecific Ab, binds simultaneously to CCR5 and CD3. Thereby it redirects CD3$^+$ T cells against CCR5$^+$ target cells. The Ab specifically depletes CCR5$^+$ T cells and monocytes, but is inactive against cells that do not express CCR5. Furthermore, ex vivo the bispecific Ab eliminated >95% of CCR5$^+$ monocytes and T cells from the synovial fluid of patients with arthritis. Also, we designed a fusion protein of the chemokine RANTES and a truncated version of *Pseudomonas* exotoxin A. The fusion protein binds to CCR5 and down-modulates the receptor from the cell surface. The chemokine toxin completely destroyed CCR5$^+$ Chinese hamster ovary cells at a concentration of 10 nM, whereas no cytotoxic effect was detectable against CCR5$^-$ Chinese hamster ovary cells. Both constructs efficiently deplete CCR5-positive cells, appear as useful agents in the treatment of chronic inflammatory diseases, and may help to eradicate HIV-1 by increasing the turnover of latently infected cells. ***The Journal of Immunology,*** **2001, 166: 2420–2426.**

The chemokine receptor CCR5 is a member of a large family of G protein-coupled seven-transmembrane domain receptors that binds the proinflammatory chemokines RANTES, macrophage-inflammatory protein 1α (1), macrophage-inflammatory protein 1β, and monocyte chemotactic protein 2 (1, 2). Chemokines act in concert with adhesion molecules to induce the extravasation of leukocytes and to direct their migration to sites of tissue injury (3). In a variety of chronic inflammatory diseases, an impressive accumulation of CCR5-positive T cells and macrophages is found at the site of inflammation. An accumulation of CCR5$^+$ cells has been demonstrated in several types of arthritis (4, 5), inflammatory renal diseases including transplant rejection (6, 7), multiple sclerosis (8, 9), and inflammatory bowel diseases (10). In contrast, in the peripheral blood of these patients only a minority of T cells and monocytes express CCR5. Therefore, CCR5 appears to be an excellent marker to identify leukocytes that are involved in chronic inflammation. The occurrence of a 32-bp deletion in the CCR5 gene that prevents expression of CCR5 (11) allows study of the pathophysiological role of CCR5 in chronic inflammatory diseases. In patients with rheumatoid arthritis, the frequency of CCR5-deficient (CCR5-Δ32/Δ32)[2] (5) individuals is significantly reduced (12). Moreover, the mean survival of kidney transplants is significantly longer in CCR5-Δ32/Δ32 patients (D. Schlöndorff, personal communication). These results make CCR5 look like a promising target for therapeutic intervention. Furthermore, the predominance of CCR5-positive leukocytes in the diseased tissue in contrast to its rare expression on peripheral blood leukocytes suggests that a specific elimination of CCR5-positive leukocytes may be therapeutically useful by reducing the number of infiltrating cells in chronic inflammation without significantly depleting peripheral blood leukocytes. Eliminating CCR5-positive leukocytes from the inflammatory infiltrate should be of greater therapeutic benefit than simply blocking chemokine receptors of these cells, as they have already infiltrated the tissue.

Besides its role in inflammation, CCR5 is the primary coreceptor for M-tropic HIV-1 strains that predominate early in the course of an infection (13, 14). Transmission of HIV-1 depends on the presence of CCR5, as individuals with a homozygous Δ32 deletion of the CCR5 allele are highly resistant against infection with HIV-1 (11). Although antiretroviral therapy can efficiently suppress replication of HIV-1, complete eradication of HIV has not been achieved to date. The main obstacle appears to be the inactivity of antiretroviral therapy against latently infected cells that can survive for several years and function as an endogenous source for HIV-1 (15). Many of these cells fail to express viral proteins and can evade the immune response. However, the majority of latently infected cells may still express CCR5, as this receptor was necessary for their initial infection. We therefore propose that depletion of CCR5-positive cells should significantly reduce the number of latently infected cells in HIV-1 infection. Other strategies to eliminate HIV-1-infected cells that depend on a specific recognition of viral proteins, e.g., surface-expressed gp120 (16), would be less effective against latently infected cells.

To test these possibilities, we designed two strategies to destroy CCR5-positive cells. First, we constructed a bispecific single-chain Ab that binds with one arm to CCR5 and with the other arm to

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[2] Abbreviations used this paper: CCR5-Δ32/Δ32, homozygous 32-bp deletion in the CCR5 alleles; CHO, Chinese hamster ovary; PE38, truncated version of *Pseudomonas* exotoxin A; CXCR, CXC chemokine receptor.

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CD3 expressed on T lymphocytes. Thereby the bispecific Ab induces a close contact between CCR5-positive target cells and CD3-positive T cells. Cross-linkage of CD3 by the Ab triggers the cytotoxic activity of T lymphocytes and leads to lysis of CCR5-positive cells (Fig. 1). Bispecific Abs were originally developed for oncological therapy to redirect cytotoxic T cells against malignant cells (17). Various types of bispecific Abs have been described so far. Although the chemical cross-linkage of two mAbs and the hybrid-hybridoma technology (18) is hampered by ill-defined protein aggregates and low yields, diabodies and bispecific single-chain Abs have overcome these problems (19–23). In 1995, we described functional expression of bispecific single-chain Abs in Chinese hamster ovary (CHO) (3) cells (20, 24). These Abs are composed of two single-chain Fv fragments that are joined by a flexible linker consisting of glycine and serine molecules. We now propose a new application for bispecific Abs, namely, elimination of infiltrating leukocytes in chronic inflammation. The CCR5-CD3-bispecific Ab would specifically deplete CCR5-positive cells and reduce considerably the number of cells that infiltrate inflamed tissue. T cells and monocytes that are not involved in the inflammation would largely remain unaffected because they rarely express CCR5.

In a second approach to destroy chemokine receptor-positive cells, we designed a fusion protein of the chemokine RANTES and a truncated version of the *Pseudomonas* exotoxin A. Several fusion proteins with a truncated version of *Pseudomonas* exotoxin A have been designed so far. Most of them have been used to target and destroy malignant cells (25). A truncated version of the toxin (PE38) was used for the construct (26), as the full-length protein binds with its first domain to the ubiquitous α_2-macroglobulin receptor and is therefore toxic to most eukaryotic cells (27). To overcome this problem, the first domain of *Pseudomonas* exotoxin A can be replaced by a specific sequence to alter the binding specificity of the toxin (26). We investigated whether a chemokine can serve as ligand sequence on the toxin, direct it to specific targets by binding to the specific chemokine receptor, and induce uptake of the toxin and lysis of chemokine receptor-positive cells. We considered chemokines to efficiently mediate the uptake of the toxin as chemokine receptors are rapidly internalized upon binding of chemokines (28, 29). In this study, we show that in fact the chemokine RANTES fused to PE38 binds to CCR5 and efficiently destroys CCR5-positive target cells. Thus, both the bifunctional CD3-CCR5 Abs and the RANTES-*Pseudomonas* A toxin constructs are promising candidates as therapeutic agents.

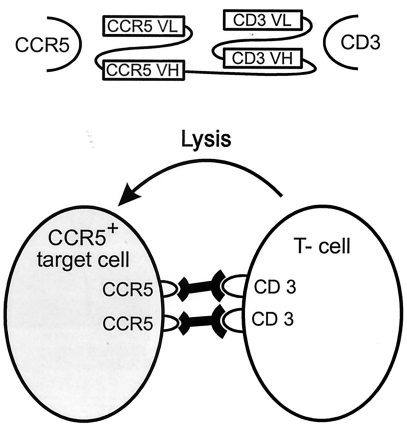

FIGURE 1. Scheme of the bispecific single-chain Ab. The anti-CCR5 single-chain fragment (CCR5 V_L/CCR5 V_H) derived from the hybridoma MC-1 is fused to the N terminus of a single-chain fragment directed against CD3 (CD3 V_H/CD3 V_L). Binding of the bispecific Ab to $CD3^+$ T cells and CCR5-positive target cells results in cross-linkage of CD3, activation of effector T cells, and lysis of CCR5-positive target cells.

Materials and Methods

PBMC, synovial fluid, and cell lines

PBMC were isolated from buffy coats or full blood of healthy donors by Ficoll density gradient centrifugation. Where indicated, PBMC were used from donors with a homozygous 32-bp deletion in the CCR5 allele (CCR5-Δ32/Δ32) preventing surface expression of CCR5. Synovial fluid of patients with arthritis was obtained from diagnostic or therapeutic arthrocentesis and used for the experiments without further preparation. Informed consent was obtained from all patients.

CHO cells were stably transfected with CCR5 or CXC chemokine receptor (CXCR) 4 as described previously (28).

Construction and expression of the bispecific single-chain Ab anti-CCR5-anti-CD3

The light (V_L) and heavy (V_H) variable domains from the anti-CCR5 hybridoma MC-1 were cloned using PCR amplification (30). Reverse transcription was conducted with random hexamer nucleotides and SuperScript reverse transcriptase (Life Technologies, Grand Island, NY). The variable domains were amplified by PCR with Pfu-polymerase, subcloned into the vector PCR-script Amp SK^+ (Stratagene, La Jolla, CA), and sequenced. As described previously, the light and heavy variable domains were joined to a single-chain fragment using a $(Gly_4Ser_1)_3$ linker and expressed in the periplasmic space of *Escherichia coli* to test binding of the recombinant protein to CCR5. Subsequently, the DNA sequence of the anti-CCR5 single-chain fragment was subcloned with BsrG1 and BspE1 into an eukaryotic expression vector (pEF-DHFR) that contained a single-chain fragment directed against CD3 with a C-terminally attached tail of six histidine residues (20). The anti-CCR5 and anti-CD3 single-chain fragments were joined by a linker coding for Gly_4Ser_1. The bispecific Ab was expressed in DHFR-deficient CHO cells and purified from the culture supernatant by affinity chromatography on immobilized Ni^{2+} ions (Ni-NTA; Qiagen, Chatsworth, CA).

Construction and expression of RANTES-PE38

A PCR fragment of RANTES, generated with the primers P1 and P2, was subcloned with *Stu*I and *Sal*I into a vector for periplasmic expression in *E. coli* (20). The restriction site *Stu*I had previously been introduced at the 3′ terminus of the OmpA signal sequence. The DNA of a truncated version of Pseudomonas exotoxin A (PE38), kindly provided by I. Pastan (26), was amplified by PCR with Pfu-polymerase using the primers P3 and P4 and subcloned with *Bsp*E1 and *Hin*dIII into the vector that already contained the cDNA of RANTES. Primer P4 also added a tail of 6 histidine residues at the 3′ terminus of PE38. During the periplasmic expression, the OmpA signal sequence is cleaved off such that the recombinant protein starts with the first amino acid of RANTES. The C-terminally attached tail of six histidine residues allowed purification by affinity chromatography on Ni-NTA (Qiagen). List of primers is as follows: P1, 5′-AAAGGCCTC CCCATATTCCTCGGA; P2, 5′- AAAGTCGACTCCGGACATCTCCAAAGA GTTGATGTAC; P3, 5′-AATCCGGAGGCGGCAGCCTGGCCGC; and P4, 5′-GGGAAGCTTAGTGATGGTGATGGTGATGCTTCAGGTCCTCGCGCGG.

FACS analysis and Western blot

Binding of the bispecific single-chain Ab to CHO cells or PBMC was determined by FACS analysis. The cells were incubated with the bispecific Ab for 60 min on ice followed by an Ab against 6xHis (Dianova, Hamburg, Germany) and a PE-conjugated polyclonal rabbit anti-mouse $F(ab')_2$ fragment (R439; Dako, Hamburg, Germany).

Western blots to detect the bispecific Ab or RANTES-PE38 were stained with the mAb against 6xHis (Dako) and a peroxidase-labeled polyclonal rabbit anti-mouse Ab (P260; Dako). Western blots to detect CCR5 in the cell-free supernatant were performed as previously described (31).

Down-modulation of chemokine receptors

PBMC were incubated for 30 min at 37°C with various concentrations of RANTES or RANTES-PE38 diluted in RPMI 1640 with 10% FCS in a volume of 100 μl. Medium alone was used as control. The cells were then stained on ice for surface CCR5 expression using the mAb MC-1 or medium as negative control followed by the PE-conjugated anti-mouse Ab R439. As shown previously, the presence of RANTES bound to CCR5 does not block the binding of MC-1 to CCR5 (28). The same could be

demonstrated for RANTES-PE38. The FACS analysis was performed on a FACSCalibur (Becton Dickinson, Mountain View, CA) and CellQuest software. Lymphocytes and monocytes were distinguished by their forward and sideward light scatter properties and expression of CD14, CD4, and CD8. Relative surface CCR5 expression was calculated as [mean channel fluorescence (experimental) − mean channel fluorescence (negative control)]/[mean channel fluorescence (medium) − mean channel fluorescence (negative control)].

Depletion of cells with the bispecific anti-CCR5-anti-CD3 Ab and RANTES-PE38

PBMC from CCR5 wild-type or CCR5-deficient (Δ32/Δ32) donors were incubated overnight to induce expression of CCR5 on monocytes. Cultured PBMC or freshly drawn synovial fluid of patients with arthritis were incubated with different concentrations of purified anti-CCR5-anti-CD3-bispecific Abs or medium as control for 20 h. No preactivation of effector T cells was performed. Surviving cells were analyzed on a FACSCalibur and counted.

CHO cells expressing CCR5 or CXCR4 were grown to subconfluence on 24-well culture plates and incubated with different concentrations of purified RANTES-PE38 or medium as control. After 40 h, the adherent and nonadherent cells were recovered and analyzed by FACS to measure the percentage of dead cells. We have previously established that dead (propidium iodide-positive) CHO cells can be identified by their light scatter properties.

Results

Production of a bispecific single-chain Ab directed against CCR5 and CD3

As described in *Materials and Methods*, we amplified the DNA sequences coding for the variable domains of the light (V_L) and heavy chain (V_H) of the CCR5-specific hybridoma MC-1 by RT-PCR. Subsequently, we constructed a single-chain fragment by joining the V_L and V_H sequences with a linker coding for $(Gly_4Ser_1)_3$ and expressed it in the periplasmic space of *E. coli* to test the binding activity of the construct. To obtain the bispecific single-chain Ab, we joined the DNA sequence of the CCR5 Ab fragment to an Ab fragment directed against CD3 using a linker coding for Gly_4Ser_1. The bispecific single-chain Ab was expressed in CHO cells and purified via a C-terminally attached histidine tail with an overall purification yield of ~900 μg/L culture supernatant. SDS-PAGE showed a single band of ~60 kDa under reducing and nonreducing conditions without any detectable proteolysis or degradation of the protein (Fig. 2).

Binding of the bispecific Ab to CCR5 and CD3

Binding of the bispecific Ab to CD3-positive T cells was demonstrated by FACS analysis (Fig. 3). As the bispecific Ab would also bind to CCR5, we performed the analysis with PBMC that lack expression of CCR5 due to a homozygous 32-bp deletion in the CCR5 alleles. The Ab showed good binding to T cells as identified by costaining with Abs against CD4 and CD8 (Fig. 3). In addition,

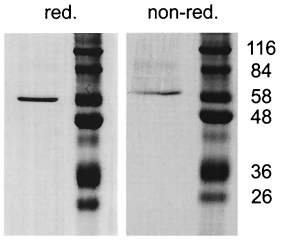

FIGURE 2. SDS-PAGE of the purified bispecific single-chain Ab anti-CCR5-anti-CD3. A single band of ~60 kDa is visible under reducing (*left*) and nonreducing (*right*) conditions. No degradation or proteolysis of the bispecific Ab is detectable.

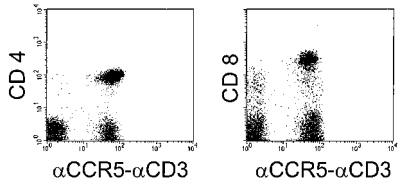

FIGURE 3. Binding of the anti-CCR5-anti-CD3-bispecific Ab to CD3 on CCR5-Δ32/Δ32 lymphocytes. Costaining with CD4 and CD8 demonstrated that the bispecific Ab binds to the subpopulation of $CD4^+/CD8^+$ T cells.

the bispecific Ab competed with the monoclonal CD3 Ab OKT-3 for binding to T cells (data not shown).

Binding of the bispecific Ab to CCR5 was demonstrated on CCR5-overexpressing CHO cells and human monocytes (Fig. 4). The Ab showed excellent binding to CCR5-transfected CHO cells (Fig. 4*A*) and cultured monocytes (Fig. 4*B*), while no binding was detectable on CHO cells transfected with CXCR4 or on cultured monocytes from a donor with a homozygous CCR5-Δ32/Δ32 deletion. Overnight cultivation of monocytes induces expression of CCR5 on wild-type monocytes, while monocytes from donors with a homozygous CCR5-Δ32/Δ32 deletion fail to express CCR5. Moreover, the CCR5 signal detectable with the bispecific Ab on cultured monocytes could be reduced to values below 15% by preincubation of monocytes for 30 min at 37°C with AOP-RANTES, a CCR5 ligand that is known to efficiently induce internalization of CCR5 (data not shown) (28). Preabsorption of the bispecific Ab on $CCR5^+$ CHO cells prevented subsequent binding to CD3 and preabsorption on CCR5-deficient T cells almost completely abolished subsequent binding to CCR5 (data not shown). This further indicates that both specificities of the bispecific Ab are contained in the same molecule.

CCR5-specific depletion of monocytes from cultured PBMC

To test the ability of the anti-CCR5-anti-CD3-bispecific single-chain Ab to deplete CCR5-positive primary cells, we incubated human PBMC with the bispecific Ab (Fig. 5). Before incubation the PBMC were cultured overnight to up-regulate CCR5 expression on monocytes (28, 32). By retargeting cytotoxic T cells, the bispecific Ab depleted the majority of monocytes within 20 h in a concentration-dependent manner (Fig. 5). In contrast to the bispecific Ab, the monovalent single-chain Fv fragments directed

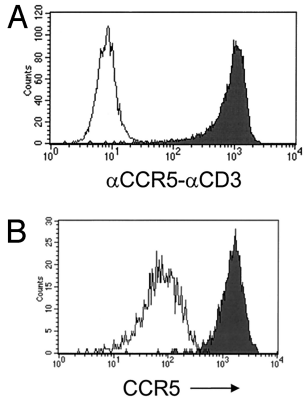

FIGURE 4. Binding of the anti-CCR5-anti-CD3-bispecific Ab to CCR5 on transfected CHO cells (*A*) and cultured monocytes (*B*). CHO cells transfected with CCR5 and monocytes from a CCR5-positive donor are shown in black, while CXCR4-positive CHO cells and monocytes from a CCR5-deficient donor (Δ32/Δ32) served as negative control and are shown in white.

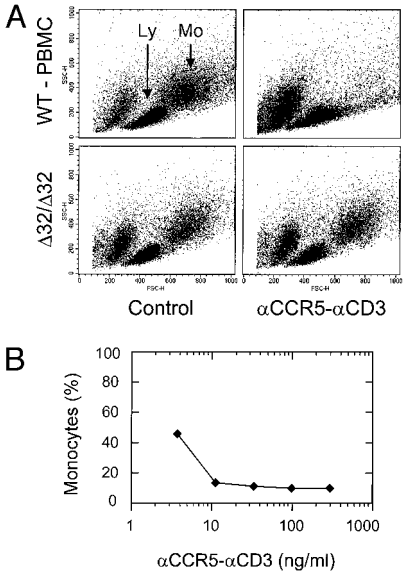

FIGURE 5. Depletion of CCR5-positive monocytes by the bispecific Ab. *A,* CCR5-deficient PBMC (Δ32/Δ32) or wild-type PBMC (WT-PBMC) were cultured overnight and incubated with the bispecific Ab (100 ng/ml) or medium as control for 20 h. Remaining monocytes (Mo) and lymphocytes (Ly) were identified by their light scatter properties in FACS. The CCR5-positive wild-type monocytes were completely depleted by the bispecific Ab, whereas the CCR5-deficient monocytes survived. *B,* Dose response showing depletion of cultured monocytes with various concentrations of the anti-CCR5-anti-CD3-bispecific Ab. More than 90% of the monocytes were depleted at a concentration of 33 ng/ml.

against CCR5 and CD3 used either alone or in combination were not able to deplete CCR5-positive monocytes (data not shown). To verify that the depletion of monocytes with the bispecific Ab was due to their expression of CCR5, we performed the same experiment with PBMC from a donor with a homozygous 32-bp deletion in the CCR5 allele that prevents surface expression of CCR5. No depletion of CCR5-deficient monocytes occurred after 20 h, indicating that the depletion of cells with the bispecific Ab is restricted to monocytes that express CCR5 (Fig. 5*A*). As further control, we preincubated PBMC with an excess of the parental mAb MC-1 or a control Ab and then added the bispecific Ab. Preincubation with MC-1 considerably reduced the depletion of monocytes while the control Ab had no effect (data not shown).

Depletion of monocytes and T lymphocytes from the synovial fluid of patients with arthritis

The bispecific single-chain Ab could potentially be applied to deplete CCR5-positive T cells and monocytes from the inflamed joints of patients with arthritis. We therefore determined the depletion of CCR5-positive cells from the synovial fluid of patients with various types of arthritis. Before each depletion experiment, we confirmed by FACS analysis that the majority of lymphocytes and monocytes in the synovial samples express CCR5, whereas no expression of CCR5 was detectable on granulocytes (data not shown). The synovial fluid was incubated ex vivo with different concentrations of the bispecific Ab immediately after arthrocentesis without prior preparation or washing steps. Using freshly drawn synovial fluid, we aimed at testing the efficacy and stability of the bispecific Ab under conditions resembling most closely the situation in vivo. Within 20 h, the bispecific Ab induced depletion of the majority of lymphocytes and monocytes from the synovial fluid, while granulocytes that do not express CCR5 remained unaffected (Fig. 6).

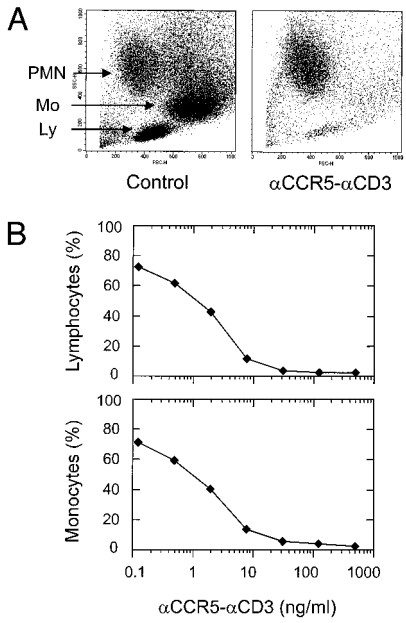

FIGURE 6. The bispecific anti-CCR5-anti-CD3 Ab depletes lymphocytes and monocytes from the synovial fluid of a patient with chronic arthritis. *A,* Freshly draw synovial fluid was incubated with the bispecific Ab (500 ng/ml) or medium as control for 20 h and analyzed by FACS. The bispecific Ab completely depleted the CCR5-positive monocytes and lymphocytes, whereas the CCR5-negative granulocytes (PMN) survived. Consistent with our previous data, all monocytes and lymphocytes in this synovial fluid expressed CCR5, whereas no expression of CCR5 was found on granulocytes (data not shown). *B,* Dose response for the depletion of monocytes and lymphocytes from the synovial fluid with the anti-CCR5-anti-CD3-bispecific Ab. More than 95% of both cell types were depleted at a concentration of 31 ng/ml.

Construction of the chemokine toxin RANTES-PE38

As described in *Materials and Methods*, the DNA sequence of RANTES was fused with the sequence of a truncated version of the *Pseudomonas* exotoxin A (PE38) kindly provided by I. Pastan (26). In a first version of the construct, a Gly-Ser linker was spaced between RANTES and PE38. However, this resulted in a considerable proteolytic degradation of the fusion protein during expression in *E. coli* (data not shown). To stabilize the construct, we removed the linker and the first three amino acids of PE38. The new fusion protein showed no proteolysis during expression in the periplasmic space of *E. coli* as demonstrated by SDS-PAGE and Western blot (Fig. 7).

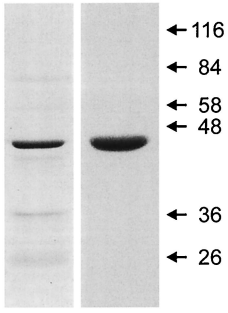

FIGURE 7. SDS-PAGE (*left*) and Western blot (*right*) of the purified protein RANTES-PE38. A distinct band with the expected size of ~46 kDa is visible in the Coomassie-stained SDS-PAGE and Western blot. Most likely our affinity purification is not 100%, which explains the faint bands in the Coomassie stain.

Biologic activity of the RANTES-PE38 construct

The fusion of RANTES to the N terminus of a truncated version of the *Pseudomonas* exotoxin A is supposed to result in specific binding of the construct to cells expressing RANTES receptors such as CCR5, CCR1, and CCR3. Internalization of the chemokine receptors upon binding of the modified toxin would enhance the cellular uptake and cytotoxic activity of the construct. We therefore analyzed whether RANTES-PE38 is able to internalize CCR5 from the surface of primary monocytes and T cells (Fig. 8). Internalization of CCR5 would indicate that the construct is able to bind to CCR5 and that RANTES remains functionally active after fusion to PE38. As shown in Fig. 8, the construct is able to internalize CCR5 from the surface of monocytes and lymphocytes. Compared with unmodified RANTES, the chemokine toxin was somewhat less effective in down-modulating CCR5. To exclude the possibility that RANTES-PE38 increases the shedding of CCR5 from the cell surface and thereby reduces surface expression of CCR5, we quantified by Western blot the amount of CCR5 in the cell-free supernatant after incubation of CCR5-positive CHO cells for 30 min at 37°C with unmodified RANTES, RANTES-PE38, or medium alone. Compared with the medium control, no increased shedding of CCR5 was detectable with either RANTES or RANTES-PE38, indicating that the reduced surface expression is due to internalization of CCR5.

We then analyzed the cytotoxic activity of RANTES-PE38. For that purpose, we incubated CHO cells expressing human CCR5, murine CCR5, and human CXCR4 with various concentrations of the chemokine toxin or medium. No surviving (i.e., adherent) human or murine CCR5-positive CHO cells were detectable by light microscopy after a 40-h incubation with as little as 10 nM RANTES-PE38. In contrast, regular growth and survival was observed when the CCR5-positive cells were incubated with medium or when CXCR4-positive CHO cells were incubated with equal concentrations of the chemokine toxin (data not shown). To quantify the percentage of dead cells, we analyzed the adherent and nonadherent cells by FACS. We have previously established that living and dead CHO cells can be identified by their light scatter properties. As shown in Fig. 9*A*, no cytotoxic effect of RANTES-PE38 was seen on CHO cells expressing CXCR4, whereas CHO cells expressing human CCR5 were completely killed by 10 nM RANTES-PE38. As further control, we preincubated $CCR5^+$ CHO cells with 10 μg/ml unconjugated RANTES and then added the chemokine toxin (Fig. 9*B*). Preincubation of the $CCR5^+$ cells with unconjugated RANTES completely prevented their destruction by RANTES-PE38. RANTES alone did not influence the viability of the cells.

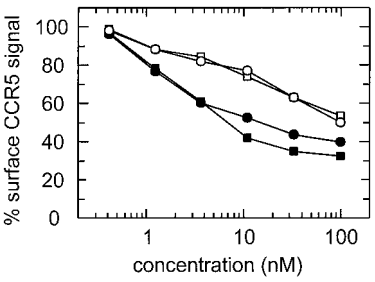

FIGURE 8. Down-modulation of CCR5 from the surface of PBMC by incubation with RANTES-PE38 (○, □) and RANTES (●, ■) for 30 min at 37°C. Surface expression of CCR5 was determined on lymphocytes (□, ■) and monocytes (○,●) and is given as percentage of the medium control. The fusion protein RANTES-PE38 is able to down-modulate CCR5 from the cell surface with a somewhat lower efficiency than unmodified RANTES.

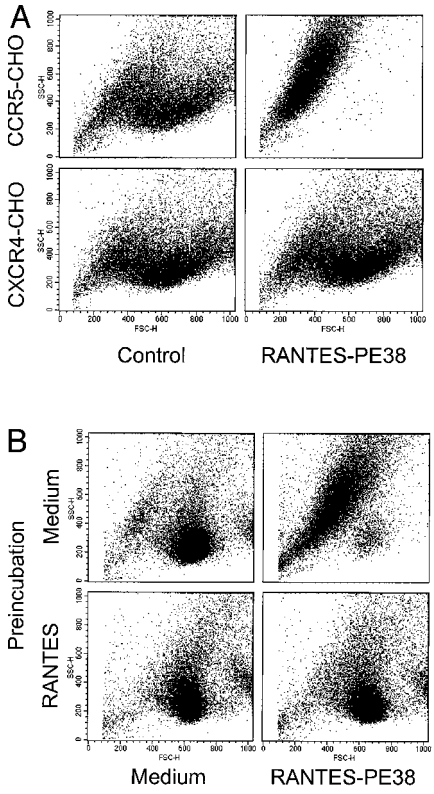

FIGURE 9. Destruction of CCR5-positive CHO cells with the chemokine toxin RANTES-PE38. *A*, CCR5-positive CHO cells and CXCR4-positive CHO cells were incubated for 40 h with the chemokine toxin (10 nM) and analyzed by FACS. Dead cells appear in the *left upper region* of the forward and sideward light scatter plot. RANTES-PE38 completely destroyed the CCR5-positive CHO cells, whereas it had no effect on the CXCR4-positive CHO cells. *B*, The cytotoxic activity of RANTES-PE38 could be completely blocked by addition of unmodified RANTES (10 μg/ml) 60 min before incubation with RANTES-PE38. Preincubation with medium served as control and did not prevent the killing of cells.

These experiments indicate that RANTES-PE 38 is able to internalize CCR5 from the surface of cells and induces depletion of cells expressing the RANTES receptors human CCR5 or murine CCR5. The inactivity of the construct against CXCR4-positive CHO cells demonstrates that the cytotoxic activity of the construct is restricted to cells that express specific chemokine receptors.

Discussion

We propose that a specific depletion of CCR5-positive cells may be therapeutically useful in chronic inflammatory diseases and HIV-1 infection. In chronic inflammation such as rheumatoid arthritis (4, 5), inflammatory renal diseases including transplant rejection (6), multiple sclerosis (8, 9), and inflammatory bowel disease (10), a clear predominance of T cells and monocytes expressing the chemokine receptor CCR5 is found within the affected tissues. In contrast, in the peripheral blood only a minority of T cells and monocytes express CCR5 (4). A depletion of CCR5-positive cells may therefore considerably reduce the infiltrate in the inflamed tissue while other leukocytes would remain largely unaffected. Also, identification of latently HIV-infected cells by CCR5 and their specific depletion could be a clinical application of the bispecific Abs and chemokine toxins.

We present two possibilities to deplete CCR5-positive cells. First, we describe a bispecific single-chain Ab that simultaneously binds to CCR5 and CD3 and thereby redirects T effector cells

against CCR5-positive target cells (Fig. 1). A precondition for the efficacy of this bispecific Ab is the presence of T effector cells. In rheumatoid arthritis, T cells are enriched in the synovial fluid and tissue and their percentage further increases with disease duration. Since most of these T cells are CCR5 positive, they would deplete each other as shown for the synovial fluids of patients with arthritis (Fig. 6). Many of the infiltrating T cells potentially recognize autoantigens and therefore perpetuate the disease process. In chronic glomerulonephritis, interstitial nephritis, and transplant rejection, CCR5-positive T cells are abundantly present in areas of interstitial infiltration and their number correlates with the degree of renal insufficiency (6). Apart from T cells, CCR5 is expressed by the majority of monocytes/macrophages within the inflamed tissue. Monocytes/macrophages are responsible for destruction of inflamed tissue. The depletion of infiltrating monocytes/macrophages may therefore considerably reduce the tissue damage, e.g., joint destruction in rheumatoid arthritis. Moreover, CCR5 is highly expressed on dendritic cells (33) that are found in the synovial fluid and synovial tissue in rheumatoid arthritis and are thought to play a major role for initiation and perpetuation of the inflammatory process (34).

A specific depletion of CCR5-positive cells appears to have several advantages over conventional immunosuppressive or immunomodulatory therapy: 1) Depletion of infiltrating cells would only take a couple of hours, while immunomodulatory agents usually need several weeks for their onset of action. 2) Conventional treatments only suppress the activity of infiltrating cells which leads to a rapid relapse after termination of the therapy. In contrast the bispecific Ab would eliminate the infiltrating cells and may therefore exert a prolonged benefit. 3) Elimination of CCR5-positive leukocytes is supposed to be well tolerated as CCR5-positive cells are enriched in the inflamed tissue and only rarely encountered in the peripheral blood. A local application of bispecific Abs, e.g., intra-articular injection in arthritis, would further reduce potential side effects.

Chemokine toxins significantly differ from bispecific Abs in their mechanism of depletion. Although bispecific Abs depend on T effector cells to deplete other cells, the mere binding and internalization of chemokine toxins is sufficient to induce cell death. Binding and internalization of chemokine toxins depends on the expression of appropriate chemokine receptors. In many cases, chemokines bind to more than one receptor, which would result in a more extensive destruction of cells. The chemokine toxin RANTES-PE38 binds to CCR5 and eliminates CCR5-positive CHO cells. However, we assume that the construct would also recognize and destroy CCR1- or CCR3-positive cells since RANTES also binds to these receptors (35). The main advantage of chemokine toxins is their ease of production, as the chemokine moiety can be rapidly exchanged by one cloning step. For most receptors, one would also be able to select a chemokine that only binds to one receptor. Chemokine toxins would therefore be an ideal tool to study depletion of selected subtypes of cells in various animal models of inflammation.

The expression of chemokine receptors on leukocytes is associated with certain types of an immune response. Recently, it was described that the receptors CCR5 and CXCR3 are primarily expressed on TH-1 cells, while CCR3, CCR4, and CCR8 are mainly found on TH-2 cells (36–39). Depletion of chemokine receptor-positive cells may therefore induce an immune deviation from TH-2 to TH-1 or vice versa. Depletion of CCR3-positive cells may be of benefit in allergic diseases, as CCR3 is not only expressed on TH-2 cells but also on eosinophils and basophils (40, 41).

Apart from chronic inflammatory diseases, the depletion of CCR5-positive cells could also be useful for the treatment of HIV-1 infection to reduce the number of latently infected cells. Early in the course of an HIV infection, preferentially CCR5-positive cells are infected with HIV-1 (14). A small fraction of these cells, mainly $CD4^+$ T cells, macrophages, and potentially dendritic cells, become latently infected and function as an endogenous source for HIV-1 (15). Latently infected cells can survive for several years and are thought to be responsible for the failure to completely eradicate HIV-1 despite prolonged highly active antiretroviral therapy. We therefore propose that depletion of $CCR5^+$ cells would shorten the half-life of latently infected cells and could help to eradicate HIV-1 in combination with highly active antiretroviral therapy. Latently infected cells cannot be identified by surface expression of viral proteins, as little viral genes are expressed when the virus is dormant. CCR5 however would still be expressed on latently infected cells as it was necessary for initial infection.

Specific depletion of chemokine receptor-positive cells can be achieved with bispecific Abs and chemokine toxins and may represent a new strategy in the treatment of chronic inflammatory diseases and HIV-1.

References

1. Samson, M., O. Labbe, C. Mollereau, G. Vassart, and M. Parmentier. 1996. Molecular cloning and functional expression of a new human CC-chemokine receptor gene. *Biochemistry 35:3362*.
2. Gong, W., O. M. Howard, J. A. Turpin, M. C. Grimm, H. Ueda, P. W. Gray, C. J. Raport, J. J. Oppenheim, and J. M. Wang. 1998. Monocyte chemotactic protein-2 activates CCR5 and blocks CD4/CCR5-mediated HIV-1 entry/replication. *J. Biol. Chem. 273:4289*.
3. Butcher, E. C., and L. J. Picker. 1996. Lymphocyte homing and homeostasis. *Science 272:60*.
4. Mack, M., H. Bruhl, R. Gruber, C. Jaeger, J. Cihak, V. Eiter, J. Plachy, M. Stangassinger, K. Uhlig, M. Schattenkirchner, and D. Schlondorff. 1999. Predominance of mononuclear cells expressing the chemokine receptor CCR5 in synovial effusions of patients with different forms of arthritis. *Arthritis Rheum. 42:981*.
5. Qin, S., J. B. Rottman, P. Myers, N. Kassam, M. Weinblatt, M. Loetscher, A. E. Koch, B. Moser, and C. R. Mackay. 1998. The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. *J. Clin. Invest. 101:746*.
6. Segerer, S., M. Mack, H. Regele, D. Kerjaschki, and D. Schlondorff. 1999. Expression of the C-C chemokine receptor 5 in human kidney diseases. *Kidney Int. 56:52*.
7. Eitner, F., Y. Cui, K. L. Hudkins, D. M. Anderson, A. Schmidt, W. R. Morton, and C. E. Alpers. 1998. Chemokine receptor (CCR5) expression in human kidneys and in the HIV infected macaque. *Kidney Int. 54:1945*.
8. Balashov, K. E., J. B. Rottman, H. L. Weiner, and W. W. Hancock. 1999. $CCR5^+$ and $CXCR3^+$ T cells are increased in multiple sclerosis and their ligands MIP-1α and IP-10 are expressed in demyelinating brain lesions. *Proc. Natl. Acad. Sci. USA 96:6873*.
9. Sorensen, T. L., M. Tani, J. Jensen, V. Pierce, C. Lucchinetti, V. A. Folcik, S. Qin, J. Rottman, F. Sellebjerg, R. M. Strieter, et al. 1999. Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients. *J. Clin. Invest. 103:807*.
10. Agace, W. W., A. I. Roberts, L. Wu, C. Greineder, E. C. Ebert, and C. M. Parker. 2000. Human intestinal lamina propria and intraepithelial lymphocytes express receptors specific for chemokines induced by inflammation. *Eur. J. Immunol. 30:819*.
11. Samson, M., F. Libert, B. J. Doranz, J. Rucker, C. Liesnard, C. M. Farber, S. Saragosti, C. Lapoumeroulie, J. Cognaux, C. Forceille, et al. 1996. Resistance to HIV-1 infection in Caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature 382:722*.
12. Gomez-Reino, J. J., J. L. Pablos, P. E. Carreira, B. Santiago, L. Serrano, J. L. Vicario, A. Balsa, M. Figueroa, and M. D. de Juan. 1999. Association of rheumatoid arthritis with a functional chemokine receptor, CCR5. *Arthritis Rheum. 42:989*.
13. Berger, E. A., P. M. Murphy, and J. M. Farber. 1999. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. *Annu. Rev. Immunol. 17:657*.
14. Connor, R. I., K. E. Sheridan, D. Ceradini, S. Choe, and N. R. Landau. 1997. Change in coreceptor use coreceptor use correlates with disease progression in HIV-1-infected individuals. *J. Exp. Med. 185:621*.
15. Chun, T. W., and A. S. Fauci. 1999. Latent reservoirs of HIV: obstacles to the eradication of virus. *Proc. Natl. Acad. Sci. USA 96:10958*.
16. Bera, T. K., P. E. Kennedy, E. A. Berger, C. F. Barbas III, and I. Pastan. 1998. Specific killing of HIV-infected lymphocytes by a recombinant immunotoxin directed against the HIV-1 envelope glycoprotein. *Mol. Med. 4:384*.
17. Staerz, U. D., O. Kanagawa, and M. J. Bevan. 1985. Hybrid antibodies can target sites for attack by T cells. *Nature 314:628*.
18. Staerz, U. D., and M. J. Bevan. 1986. Hybrid hybridoma producing a bispecific mAb that can focus effector T cell activity. *Proc. Natl. Acad. Sci. USA 83:1453*.

19. Holliger, P., T. Prospero, and G. Winter. 1993. "Diabodies": small bivalent and bispecific Ab fragments. *Proc. Natl. Acad. Sci. USA 90:6444.*
20. Mack, M., G. Riethmuller, and P. Kufer. 1995. A small bispecific Ab construct expressed as a functional single-chain molecule with high tumor cell cytotoxicity. *Proc. Natl. Acad. Sci. USA 92:7021*.
21. Kurucz, I., J. A. Titus, C. R. Jost, C. M. Jacobus, and D. M. Segal. 1995. Retargeting of CTL by an efficiently refolded bispecific single-chain Fv dimer produced in bacteria. *J. Immunol. 154:4576*.
22. Mallender, W. D., and E. W. Voss, Jr. 1994. Construction, expression, and activity of a bivalent bispecific single-chain antibody. *J. Biol. Chem. 269:199.*
23. Gruber, M., B. A. Schodin, E. R. Wilson, and D. M. Kranz. 1994. Efficient tumor cell lysis mediated by a bispecific single chain antibody expressed in *Escherichia coli*. *J. Immunol. 152:5368*.
24. Mack, M., R. Gruber, S. Schmidt, G. Riethmuller, and P. Kufer. 1997. Biologic properties of a bispecific single-chain Ab directed against 17-1A (EpCAM) and CD3: tumor cell-dependent T cell stimulation and cytotoxic activity. *J. Immunol. 158:3965*.
25. Kreitman, R. J. 1999. Immunotoxins in cancer therapy. *Curr. Opin. Immunol. 11:570.*
26. Theuer, C. P., R. J. Kreitman, D. J. FitzGerald, and I. Pastan. 1993. Immunotoxins made with a recombinant form of *Pseudomonas* exotoxin A that do not require proteolysis for activity. *Cancer Res. 53:340*.
27. Kounnas, M. Z., R. E. Morris, M. R. Thompson, D. J. FitzGerald, D. K. Strickland, and C. B. Saelinger. 1992. The α_2-macroglobulin receptor/low density lipoprotein receptor-related protein binds and internalizes *Pseudomonas* exotoxin A. *J. Biol. Chem. 267:12420*.
28. Mack, M., B. Luckow, P. J. Nelson, J. Cihak, G. Simmons, P. R. Clapham, N. Signoret, M. Marsh, M. Stangassinger, F. Borlat, et al. 1998. Aminooxypentane-RANTES induces CCR5 internalization but inhibits recycling: a novel inhibitory mechanism of HIV infectivity. *J. Exp. Med. 187:1215*.
29. Mack, M., and D. Schlondorff. 2000. Down-modulation and recycling of chemokine receptors. *Methods Mol. Biol. 138:191.*
30. Orlandi, R., D. H. Gussow, P. T. Jones, and G. Winter. 1989. Cloning Ig variable domains for expression by the PCR. *Proc. Natl. Acad. Sci. USA 86:3833*.
31. Mack, M., A. Kleinschmidt, H. Brühl, C. Klier, P. J. Nelson, J. Cihak, J. Plachy, M. Stangassinger, V. Erfle, and D. Schlöndorff. 2000. Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles: a mechanism for cellular HIV 1 infection. *Nat. Med. 6:769*.
32. Sozzani, S., S. Ghezzi, G. Iannolo, W. Luini, A. Borsatti, N. Polentarutti, A. Sica, M. Locati, C. Mackay, T. N. Wells, et al. 1998. IL-10 increases CCR5 expression and HIV infection in human monocytes. *J. Exp. Med. 187:439*.
33. Lee, B., M. Sharron, L. J. Montaner, D. Weissman, and R. W. Doms. 1999. Quantification of CD4, CCR5, and CXCR4 levels on lymphocyte subsets, dendritic cells, and differentially conditioned monocyte-derived macrophages. *Proc. Natl. Acad. Sci. USA 96:5215*.
34. Thomas, R., L. S. Davis, and P. E. Lipsky. 1994. Rheumatoid synovium is enriched in mature antigen-presenting dendritic cells. *J. Immunol. 152:2613.*
35. Baggiolini, M., B. Denwald, and B. Moser. 1997. Human chemokines: an update. *Annu. Rev. Immunol. 15:675*.
36. Sallusto, F., D. Lenig, C. R. Mackay, and A. Lanzavecchia. 1998. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J. Exp. Med. 187:875.*
37. Bonecchi, R., G. Bianchi, P. P. Bordignon, D. D'Ambrosio, R. Lang, A. Borsatti, S. Sozzani, P. Allavena, P. A. Gray, A. Mantovani, and F. Sinigaglia. 1998. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J. Exp. Med. 187:129*.
38. Sallusto, F., C. R. Mackay, and A. Lanzavecchia. 1997. Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. *Science 277:2005.*
39. Zingoni, A., H. Soto, J. A. Hedrick, A. Stoppacciaro, C. T. Storlazzi, F. Sinigaglia, D. D'Ambrosio, A. O'Garra, D. Robinson, M. Rocchi, et al. 1998. The chemokine receptor CCR8 is preferentially expressed in Th2 but not Th1 cells. *J. Immunol. 161:547*.
40. Heath, H., S. Qin, P. Rao, L. Wu, G. LaRosa, N. Kassam, P. D. Ponath, and C. R. Mackay. 1997. Chemokine receptor usage by human eosinophils: the importance of CCR3 demonstrated using an antagonistic monoclonal antibody. *J. Clin. Invest. 99:178*.
41. Uguccioni, M., C. R. Mackay, B. Ochensberger, P. Loetscher, S. Rhis, G. J. LaRosa, P. Rao, P. D. Ponath, M. Baggiolini, and C. A. Dahinden. 1997. High expression of the chemokine receptor CCR3 in human blood basophils: role in activation by eotaxin, MCP-4, and other chemokines. *J. Clin. Invest. 100:1137*.