A RANTES-Antibody Fusion Protein Retains Antigen Specificity and Chemokine Function[1]

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The successful eradication of cancer cells in the setting of minimal residual disease may require targeting of metastatic tumor deposits that evade the immune system. We combined the targeting flexibility and specificity of mAbs with the immune effector function of the chemokine RANTES to target established tumor deposits. We describe the construction of an Ab fusion molecule with variable domains directed against the tumor-associated Ag HER2/*neu*, linked to sequences encoding the chemokine RANTES (RANTES.her2.IgG3). RANTES is a potent chemoattractant of T cells, NK cells, monocytes, and dendritic cells, and expression of RANTES has been shown to enhance immune responses against tumors in murine models. RANTES.her2.IgG3 fusion protein bound specifically to HER2/*neu* Ag expressed on EL4 cells and on SKBR3 breast cancer cells as assayed by flow cytometry. RANTES.her2.IgG3 could elicit actin polymerization of THP-1 cells and transendothelial migration of primary T lymphocytes. RANTES.her2.IgG3 prebound to SKBR3 cells also facilitated migration of T cells. RANTES.her2.IgG3 bound specifically to the CCR5 chemokine receptor, as demonstrated by flow cytometry, and inhibited HIV-1 infection via the CCR5 coreceptor. RANTES.her2.IgG3, alone or in combination with other chemokine or cytokine fusion Abs, may be a suitable reagent for recruitment and activation of an expanded repertoire of effector cells to tumor deposits. ***The Journal of Immunology*****, 1998, 161: 3729–3736.**

Stimulation of an antitumor immune response is a stepwise process requiring the accumulation and activation of immune effector cells in the vicinity of tumor cells. Monocytes and lymphocytes initially interact with adhesion molecules on endothelial cells, followed by migration of immune effector cells in response to chemotactic gradients in tissues. Effector cells in the tumor vicinity are then available for activation and subsequent stimulation of an antitumor immune response. Chemokines are low m.w. proteins that act as potent chemoattractants and are involved in the migration of inflammatory cells. They are divided according to the configuration of the first cysteine residues at the amino terminus of the proteins. Different subfamilies of chemokines have been shown to attract different classes of inflammatory cells. C-C chemokines predominantly attract monocytes and lymphocytes, while C-X-C chemokines attract neutrophils in addition to lymphocytes (1). RANTES is a member of the C-C chemokine family and is a potent chemoattractant of T cells, NK cells, monocytes, eosinophils, basophils, and dendritic cells (2). RANTES present at high concentrations (1 μM) has been shown to stimulate T cell activation and proliferation (3, 4). RANTES-mediated T cell activation can also lead to the generation of an antitumor immune response and tumor rejection as shown in gene transfer studies performed in syngeneic murine EL4 (our manuscript in preparation) and MCA205 tumor models (5). Therefore, direct delivery of RANTES to tumor deposits may assist in the recruitment and/or activation of immune effector cells for the purpose of cancer immunotherapy.

Successful eradication of cancer cells in the setting of minimal residual disease may require targeting of widely scattered metastatic tumor deposits that are not accessible to direct gene transfer. We have developed a novel protein for the targeting of a specific immune response against micrometastatic disease by linking a tumor-specific Ab to a chemokine. In this paper we describe an Ab fusion protein with variable domains specific for HER2/*neu*, linked to the chemokine RANTES. HER2/*neu* is a tumor-associated Ag highly expressed in ovarian, breast, lung, and other cancers (6–9). The Ab domain of the fusion molecule should allow tumor targeting, while the fused chemokine would act to recruit and activate antitumor effector cells. We demonstrate that fusion protein binds specifically to the HER2/*neu* Ag expressed on breast cancer cells. Biologic activity of the RANTES domain in the fusion protein was confirmed using assays for F-actin[3] polymerization of monocytic cells and for transendothelial migration of primary T lymphocytes. Our results demonstrate that a chemokine can be functionally linked to Abs directed against tumor Ags. Such fusion proteins may be useful for the generation of an antitumor immune response.

Materials and Methods

Cell lines and reagents

SKBR3, THP-1, EL4, Sp2/0, and P3X63-Ag.653 cells were obtained from American Type Culture Collection (Manassas, VA). Sp2/0, P3X63Ag8.563

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[3] Abbreviations used in this paper: F-actin, filamentous actin; ECD, extracellular domain; NBD-phallacidin, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phallacidin.

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and EL4 cells were cultured in Iscove's medium supplemented with 5% FBS, L-glutamine, penicillin, and streptomycin. SKBR3 and THP-1 cells were maintained in RPMI 1640 medium containing 10% FBS, L-glutamine, penicillin, and streptomycin. Recombinant human RANTES (rRANTES) was obtained from R&D Systems (Minneapolis, MN).

Ab expression vectors

For the construction of a humanized anti-HER2/*neu* Ab (referred to as her2-IgG3), the variable light and heavy chain sequences were obtained from the humanized humAb4D5-8 Ab (provided by Dr. P. Carter, Genentech, South San Francisco, CA) (10, 11) and cloned into previously described mammalian expression vectors for human κ light chain and IgG3 heavy chain, respectively (12). To construct anti-HER2/*neu* Ab fusion protein with the chemokine RANTES (referred to as RANTES.her2.IgG3), human RANTES sequences were amplified from the plasmid pBS- RANTES (a gift from T. Schall, ChemoCentryx, Mountain View, CA) using the sense primer 5′-GGCATAA GCTTGATATCTGAAGCCATGGGC-3′ and the antisense primer 5′-GCG CGGTTAACCGTTATCAGGAAAATGC-3′, and the PCR product was subcloned as a *Hin*dIII/*Hpa*I fragment at the 5′ end of a cassette encoding the $(Ser\text{-}Gly^4)^3$ linker sequences fused to the anti-HER2/*neu* V_H sequences. The resulting RANTES-linker-V_H coding sequences were isolated as an *Eco*RV/*Nhe*I fragment and cloned into an expression vector for human IgG3 heavy chain (13).

Recombinant Ab expression, immunoprecipitation, and purification

Transfection, expression, and purification of the recombinant Abs were performed as previously described to obtain both her2.IgG3 and RANTES.her2.IgG3 fusion protein (12). Briefly, Sp2/0 or P3X63-Ag.653 myeloma cells were transfected with 10 μg of each of the anti-HER2/*neu* light chain and heavy chain expression vectors by electroporation. Transfected cells were plated at 10^4 cells/well in 96-well U-bottom tissue culture plates and selected in 0.5 mM histidinol (Sigma, St. Louis, MO). Wells were screened for Ab secretion using a human IgG-specific ELISA, and positive wells were expanded.

To determine the sizes of the secreted recombinant her2.IgG3 and RANTES.her2.IgG3 Abs, supernatants from Sp2/0 cells grown overnight in medium containing [^{35}S]methionine (Amersham, Arlington Heights, IL) were immunoprecipitated with goat anti-human IgG (Zymed, San Francisco, CA) and staphylococcal protein A (IgGSorb, The Enzyme Center, Malden, MA). Precipitated Abs were analyzed on SDS-PAGE gels in the presence or the absence of the reducing agent β-ME. For purification of her2.IgG3 and RANTES.her2.IgG3, high producing clones were expanded in roller bottles in Hybridoma Serum-Free Medium (Life Technologies, Grand Island, NY), and 2 to 4 liters of cell-free medium was collected. Culture supernatants were passed through a GammaBind protein G column (Pharmacia, Piscataway, NJ), and the column was washed with 10 ml of PBS. The proteins were successively eluted with a total of 10 ml of 0.1 M glycine at pH 4.0, pH 2.5, and pH 2.0, and the eluate was neutralized immediately with 2 M Tris-HCl, pH 8.0. Eluted fractions were dialyzed against PBS and concentrated using Centricon filters with a m.w. cut-off of 50,000 Da (Amicon, Beverly, MA).

Flow cytometry studies

SKBR3 cells were detached by treatment with 0.5 mM EDTA. Cells to be stained were washed in PBS, incubated with 10 μg/ml her2.IgG3 or RANTES.her2.IgG3 Abs for 1 to 2 h at 4°C, washed and stained with FITC-conjugated anti-human IgG (Sigma) or, alternatively, with biotin-conjugated anti-human RANTES (R&D Systems) followed by streptavidin-phycoerythrin (Sigma), and analyzed by flow cytometry.

Affinity analysis

The affinity of RANTES.her2.IgG3 for its HER2/*neu* Ag was compared with that of the parental her2.IgG3 Ab using an IAsys Optical Biosensor (Fisons Applied Sensor Technology, Paramus, NJ). Soluble HER2/*neu* Ag (extracellular domain (ECD), provided by Genentech) was immobilized on a sensitized microcuvette according to the manufacturer's instructions. Abs at a 1×10^{-7}-M concentration diluted in PBS with 0.05% Tween 20 were added to the cuvette, and association and dissociation rates were measured. Rate constants were calculated using the FASTfit software (supplied with the IAsys System).

HIV inhibition experiments

Pseudotyped HIV-1 virions containing HIV-1 JR-FL envelope were produced by electroporation of COS cells with the plasmids HIV-luc-Δ*env* and pLET-JR-FL as described previously (14). These virions allow detection of infection through assay of luciferase activity. Virus stocks were harvested at 48 h after electroporation and were frozen at −80°C. HOS-CD4-CCR5 target cells (at 10^6 cells) were seeded into 10-cm culture plates. The next day, the cells were preincubated with medium alone or medium containing 125 nM rRANTES, RANTES.her2.IgG3, or her2.IgG3 for 15 min at 37°C, followed by infection with HIV-luc-JR-FL, at an estimated 50 ng of viral p24 protein, in the presence of 10 μg/ml polybrene (Sigma). Forty-eight hours postinfection, cells were washed with medium and lysed in 100 μl of luciferase lysis buffer (Promega, Madison WI). Lysates (20 μl) were then analyzed for luciferase activity following the manufacturers's instructions (Promega).

F-actin polymerization studies

THP-1 cells, at 1×10^6 cells/ml, were stimulated with cAMP at 1 μM for 72 h. Stimulated cells were washed and incubated with recombinant RANTES (rRANTES), RANTES.her2.IgG3, or control her2.IgG3. Reactions were stopped at 0, 0.5, 1, 3, 5, and 10 min by fixing the cells in paraformaldehyde for >48 h as previously described (15). Fixed cells were stained with NBD-phallacidin (Molecular Probes, Eugene, OR) and analyzed by flow cytometry. The relative increase in fluorescence over that at control time zero was plotted.

Transendothelial migration assays

HUVECs were obtained from term umbilical cords through the courtesy of Dr. Lee Ann Sporn (University of Rochester, Rochester, NY). Umbilical cords were flushed with lactated Ringer's solution injected with pronase (Calbiochem, San Diego, CA) and incubated for 20 min, after which the endothelial cells were flushed from the vein. First-passage HUVECs were cultured in McCoy's 5A medium (Life Technologies) supplemented with 20% FBS, 50 μg/ml endothelial mitogen (Biomedical Technologies, Stoughton, MA), and 100 μg/ml heparin (Sigma) in flasks precoated with 1% porcine gelatin (Sigma). At confluence, cultures were detached with trypsin/EDTA (Life Technologies), washed, and plated in Iscove's medium supplemented with 15% FBS, 15% horse serum, 180 ng/ml hydrocortisone (Sigma), 100 μg/ml endothelial growth factor (BioSource, Camarillo, CA), 50 μg/ml heparin, 1% L-glutamine, and 1% penicillin-streptomycin on a 3-μm porous membrane insert of a Transwell plate (Costar, Cambridge, MA). All HUVECs used in these studies are early passage cells (p3–p5). Transendothelial migration experiments were performed when HUVECs reached confluence following plating (~2–3 days) using methods adapted from Mohle et al. (16). Primary T cells were purified from Ficoll-Hypaque-separated PBMC from normal donors using T cell enrichment columns (R&D Systems) and were plated over the HUVEC monolayer in the upper well of a Transwell plate in X-Vivo 10 serum-free medium (BioWhittaker, Walkersville, MD). Recombinant RANTES, RANTES.her2.IgG3, or her2.IgG3 control were diluted in X-Vivo 10 medium as indicated and added to the lower wells. The plates were incubated at 37°C for 24 h, and cells that migrated to the lower well were counted using a hemocytometer. Neutralization experiments were performed by preincubating chemokine or control preparations with a neutralizing anti-RANTES Ab (R&D Systems) at 5 μg/ml for 30 min in the lower well of the Transwell plate before plating the T cells in the upper well. Alternatively, T cells were preincubated with pertussis toxin (Calbiochem-Novabiochem, La Jolla, CA) at 100 ng/ml for 36 h before performing the transendothelial migration assay. In another set of experiments, SKBR3 cells were preincubated with 10 μg/ml of either her2.IgG3 or RANTES.her2.IgG3 for 2 h at 4°C. The cells were then washed three times, resuspended in X-Vivo 10 medium, and plated in the lower well of the Transwell plate at 2 to 4×10^4 cells/well, and transendothelial migration was assayed 24 h later as described above.

Results

Ab fusion protein design and expression

We designed and constructed the Ab fusion protein RANTES.her2.IgG3 in which the chemokine RANTES was linked to the amino terminus of the heavy chain of the humanized anti-HER2/*neu* heavy chain Ab via a $(Ser\text{-}Gly^4)^3$ flexible linker (Fig. 1). Expression vectors encoding the anti-HER2/*neu* light chain and the RANTES.her2.IgG3 heavy chain were transfected into Sp2/0 myeloma cells, and stable transfectants were identified and expanded. Recombinant protein was purified using a protein G affinity column. Assembly and secretion of the H_2L_2 form of the recombinant fusion protein were verified by SDS-PAGE. A complete H_2L_2 form (~185 kDa) of the RANTES.her2.IgG3 fusion

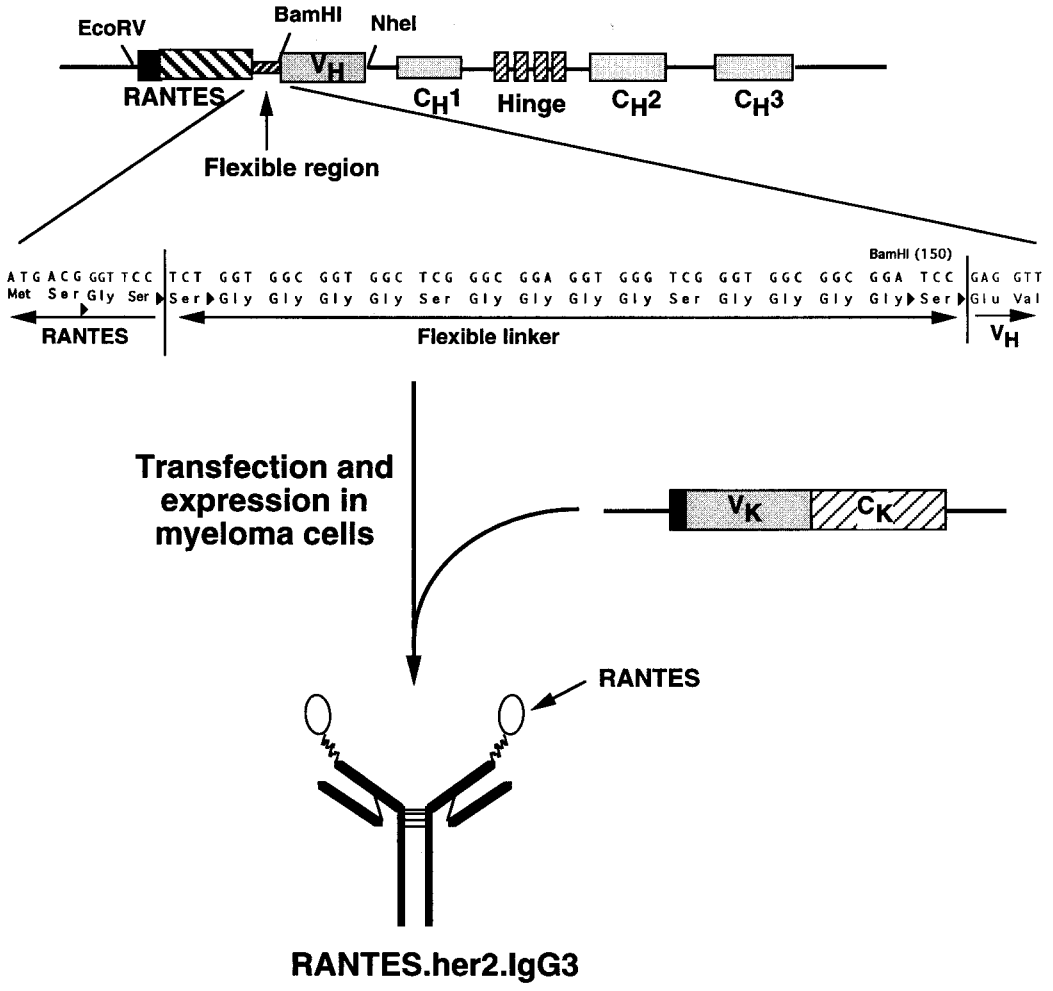


FIGURE 1. Construction of vector for the expression of anti-HER2/*neu* RANTES.her2.IgG3. RANTES was cloned at the 5′ terminus of human her2.IgG3 heavy chain through a flexible linker maintaining the open reading frame of the fusion protein. After transfection of both anti-HER2/*neu* light chain and RANTES heavy chain fusion genes into myeloma cells, an H_2L_2 form of the Ab was assembled and secreted.

protein is secreted by the myeloma cells (Fig. 2*A*, *lane 2*). Following reduction with 2-ME, both RANTES.her2.IgG3 heavy chain (Fig. 2*A*, *lane 4*), which has higher apparent m.w. than the her2.IgG3 heavy chain (Fig. 2*A*, *lane 3*), and intact anti-HER2/*neu* light chain (~25 kDa) were detected. Both her2.IgG3 and RANTES.her2.IgG3 recombinant Abs were detected with an anti-human IgG Ab (Fig. 2*B*), whereas only RANTES.her2.IgG3 was specifically detected with an anti-RANTES Ab (Fig. 2*C*).

Characterization of the binding domains of RANTES.her2.IgG3 fusion protein

To test the ability of recombinant RANTES.her2.IgG3 to bind to the HER2/*neu* Ag, SKBR3 cells, a breast cancer cell line known to express high levels of HER2/*neu*, were incubated with an isotype control human IgG3 (anti-dansyl IgG3), her2.IgG3, or RANTES.her2.IgG3. Cells were then stained with either FITC-conjugated anti-human IgG or biotin-conjugated anti-RANTES Ab followed by phycoerythrin-conjugated streptavidin and were analyzed by flow cytometry. Both her2.IgG3 (Fig. 3*B*) and RANTES.her2.IgG3 (Fig. 3*C*) bound specifically to SKBR3 cells as detected using an anti-human IgG Ab. Therefore, fusion of the extracellular domain of RANTES to the amino terminus of her2.IgG3 did not interfere with recognition of the HER2/*neu* Ag by the Ab domain. SKBR3 cells incubated with RANTES.her2.IgG3, but not with her2.IgG3, also stained positively with anti-human RANTES, indicating that after binding of RANTES.her2.IgG3 to Ag, the RANTES domain was still accessible to Ab (Fig. 3, *E* and *F*). The same experiment was repeated using EL4 cells engineered to stably express the human HER2/*neu* Ag by gene transfer. Binding to cell surface HER2/*neu* Ag was detected by flow cytometry on EL4/Her2 cells (Fig. 3*H*), while no binding was detected on parental cells that did not express the HER2/*neu* antigen (Fig. 3*G*).

The affinities of RANTES.her2.IgG3 and her2.IgG3 for Ag were directly compared using an IAsys Biosensor (Fig. 4). The soluble ECD of HER2/*neu* was immobilized on a microcuvette as described in *Materials and Methods*. Her2.IgG3 or RANTES.her2.IgG3 was added to the ECD-coated cuvette, and the association and dissociation rate constants were determined. The affinity (K_D) of RANTES.her2.IgG3 was 5.3×10^{-8} M, similar to the affinity of 7.0×10^{-8} M determined for the parental her2.IgG3. These studies indicate that fusion of the low m.w. RANTES molecule at the amino terminus of the her2.IgG3 heavy chain did not appreciably alter the affinity of the anti-HER2/*neu* Ab for its Ag.

Binding of RANTES.her2.IgG3 to the CCR5 chemokine receptor

To assess the ability of RANTES.her2.IgG3 to bind to its CCR5 receptor, we obtained HOS/CD4-CCR5 and HOS/CD4 cells from the National Institutes of Health/AIDS repository. RANTES has been shown to bind to CCR5 but not to the CXCR4 (or fusin)

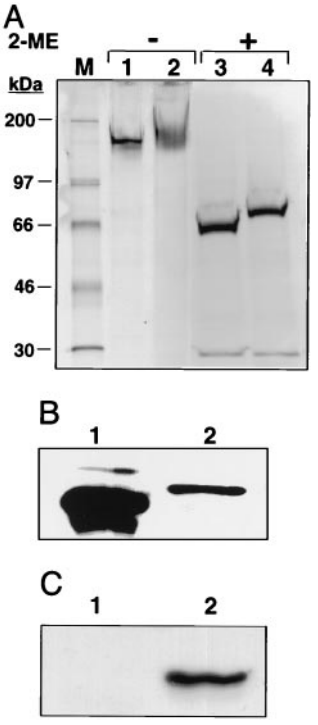


FIGURE 2. SDS-PAGE analysis of the secreted recombinant Abs. *A*, Myeloma cells secreting her2.IgG3 (*lanes 1* and *3*) or RANTES.her2.IgG3 (*lanes 2* and *4*) were metabolically labeled with [^{35}S]methionine, the supernatant was precipitated with goat anti-human IgG followed by staphylococcus A, electrophoresed on an SDS-PAGE gel in the presence (*lanes 3* and *4*) or the absence (*lanes 1* and *2*) of 2-ME, and analyzed by autoradiography. The her2.IgG3 (*lane 1*) and RANTES.her2.IgG3 (*lane 2*) purified from culture supernatants were run on an SDS-PAGE gel, blotted onto nitrocellulose membrane, and analyzed using horseradish peroxidase-conjugated anti-human Ig (*B*) or mouse anti-RANTES followed by horseradish peroxidase-conjugated anti-mouse Ab (*C*). The Western blots were developed using a chemiluminescent substrate and analyzed by exposure to x-ray film.

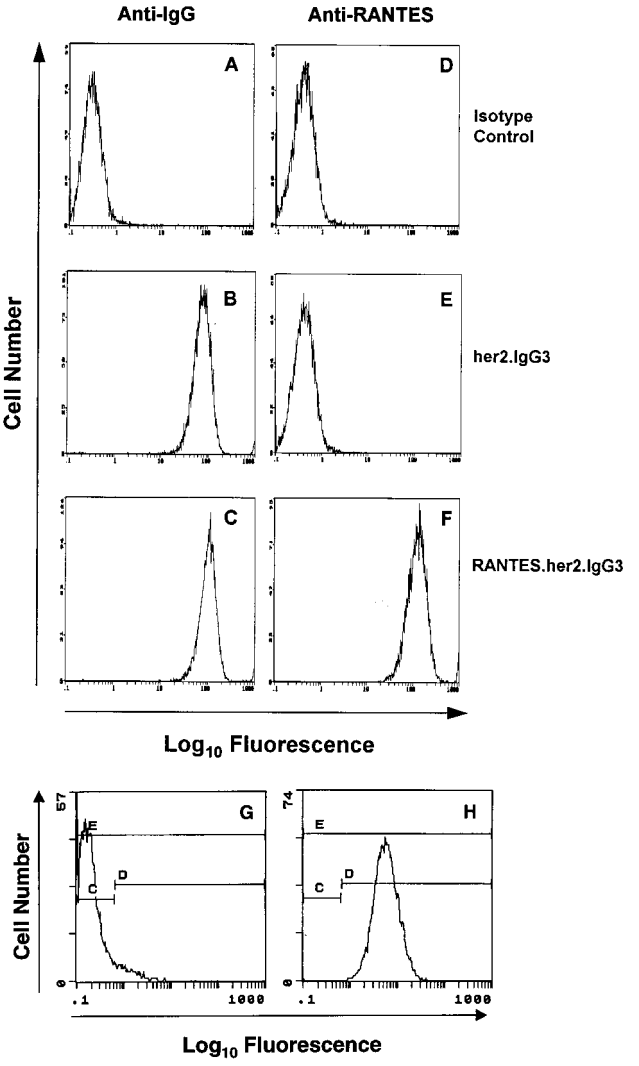


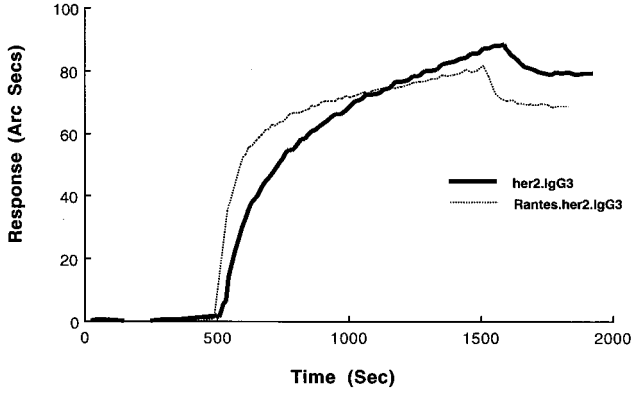
FIGURE 3. Flow cytometric analysis of the recombinant Abs. SKBR3 cells were incubated with an isotype control Ab (*A* and *D*), her2.IgG3 (*B* and *E*), or RANTES.her2.IgG3 (*C* and *F*) as described in *Materials and Methods*, washed, and stained with either FITC-conjugated anti-human IgG (*A–C*) or biotin-conjugated anti-RANTES Ab followed by PE-conjugated streptavidin (*D–F*). EL4 (*G*) or EL4/HER2 (*H*) cells were incubated with RANTES.her2.IgG3, washed, and stained with FITC-conjugated anti-human IgG. The samples were then analyzed by flow cytometry.

chemokine receptor. The specific binding of RANTES.her2.IgG3 to CCR5 was first determined by flow cytometry (Fig. 5*A*). Increased fluorescence was observed when RANTES.her2.IgG3 was incubated with HOS/CD4-CCR5 compared with HOS/CD4-CXCR4 cells (Fig. 5*A*) and HOS/CD4 (data not shown). CCR5 expression on HOS/CD4-CCR5 was first confirmed using anti-CCR5 Ab (2D7, National Institutes of Health/AIDS repository) as shown in Figure 5*B*.

The CCR5 receptor of RANTES has been described as a coreceptor for the macrophage-tropic HIV-1 and is required for the infection of monocytes by macrophage-tropic isolates, an important event in the initial stages of HIV-1 infection. Several studies have successively used the natural ligand of CCR5, rRANTES, as an inhibitor of HIV infection in vitro (17, 18). We, therefore, tested the ability of RANTES.her2.IgG3 to inhibit HIV-1 infection via the CCR5 receptor using HOS/CD4-CCR5 cells as infection targets. A recombinant macrophage-tropic vector, HIV-luc-JR-FL, in which *nef* sequences have been replaced by the luciferase gene was used to infect HOS/CD4-CCR5 cells. We show that RANTES.her2.IgG3 fusion protein, but not her2.IgG3, inhibited HIV-1 replication with efficacy similar to that of rRANTES (Fig. 5*C*). No inhibition by RANTES.her2.IgG3 was observed when the T cell-tropic HIV-1 isolate (LAI) was tested (data not shown).

RANTES.her2.IgG3 transmits a chemotactic signal

The chemotactic effect of RANTES is accompanied by a change in the configuration of intracellular actin in the cytoskeleton. We used an F-actin polymerization assay to study the biologic effect of RANTES.her2.IgG3 fusion protein (19). In this assay, cAMP-differentiated THP-1 monocytic cells were treated with parental her2.IgG3 Ab, RANTES.her2.IgG3 fusion protein, or rRANTES (Fig. 6). Aliquots of the treated cells were harvested at 0.5, 1, 3, 5, and 10 min; fixed; and stained with NBD-phallacidin, which detects polymerized actin. RANTES.her2.IgG3 induced F-actin polymerization within 0.5 min of treatment, and the polymerization response was maintained for about 3 min, while her2.IgG3 did not increase the F-actin content. The polymerization curve obtained with RANTES.her2.IgG3 was similar to that observed with rRANTES. The actin response obtained with RANTES.her2.IgG3 is therefore mediated by the RANTES domain of the fusion protein and not the IgG3 domain.



	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D(M)
her2.IgG3	147867	0.0070	7.0×10^{-8}
RANTES.her2.IgG3	153538	0.0082	5.3×10^{-8}

FIGURE 4. Affinity studies of her2.IgG3 and RANTES.her2.IgG3 proteins to their Ags. Binding of her2.IgG3 or RANTES.her2.IgG3 to a ECD-coated microcuvette was assayed using an IAsys Optical Biosensor system as described in *Materials and Methods*, and the association (k_a) and dissociation (k_d) constants were calculated using the Fastfit program. The affinity constant K_D was calculated as k_d/k_a. Binding following the addition of both proteins at 1×10^{-7} M is shown.

RANTES.her2.IgG3 mediates transendothelial migration of effector cells

To determine whether the RANTES.her2.IgG3 fusion protein could facilitate the transendothelial chemotaxis required for recruitment of effector cells to the site of the tumor, we used a modified Boyden chamber chemotaxis assay. HUVEC monolayers were grown to confluence on the culture insert of a Transwell culture plate. The migration of primary peripheral blood T cells plated in the upper well was studied in response to different concentrations of RANTES.her2.IgG3 or rRANTES added to the lower well. Table I summarizes the data from four different experiments, and the average migration index of all experiments is plotted in Figure 7*A*. The chemotactic response of purified peripheral blood T cells to RANTES.her2.IgG3 was similar to that to rRANTES. Control her2.IgG3 did not elicit significant T cell chemotaxis. Therefore, the chemotactic response is mediated by the RANTES domain of the RANTES.her2.IgG3 fusion protein. When RANTES.her2.IgG3 was compared with her2.IgG3 Ab, significant migration of T cells was observed in response to RANTES.her2.IgG3 at 1.0 and 10.0 ng/ml ($p = 0.0133$ and 0.0062, respectively). The migration of T cells observed with RANTES.her2.IgG3 was specifically neutralized with anti-RANTES Ab at 5 μg/ml. The number of migrating T cells observed in response to RANTES.her2.IgG3 was reduced to the background number observed in the control wells (Fig. 7*B*). Since the RANTES chemokine signal is known to be mediated via a G protein effector, we also tested the effects of pertussis toxin on RANTES.her2.IgG3-mediated chemotaxis activity. Pertussis toxin treatment of T cells inhibited migration induced by rRANTES as well as by RANTES.her2.IgG3 (Fig. 7*C*). This demonstrates that RANTES.her2.IgG3 fusion protein acts via a pertussis toxin-sensitive G protein effector similarly to rRANTES.

We next tested the ability of RANTES.her2.IgG3 to induce migration following binding to the surface of tumor cells through its Ab domain. SKBR3 cells, which express high levels of HER2/*neu*, were preincubated with her2.IgG3 or RANTES.her2.IgG3, unbound protein was removed by washing, and the cells were placed in the lower well of a chemotaxis Transwell plate. Migration of peripheral blood T cells through a confluent HUVEC layer was measured 24 h later as described above. Anti-HER2/*neu* RANTES.her2.IgG3, but not her2.IgG3, prebound to the cells was capable of inducing the migration of T cells (Fig. 8). Levels of migration with RANTES.her2.IgG3 actually exceeded those seen

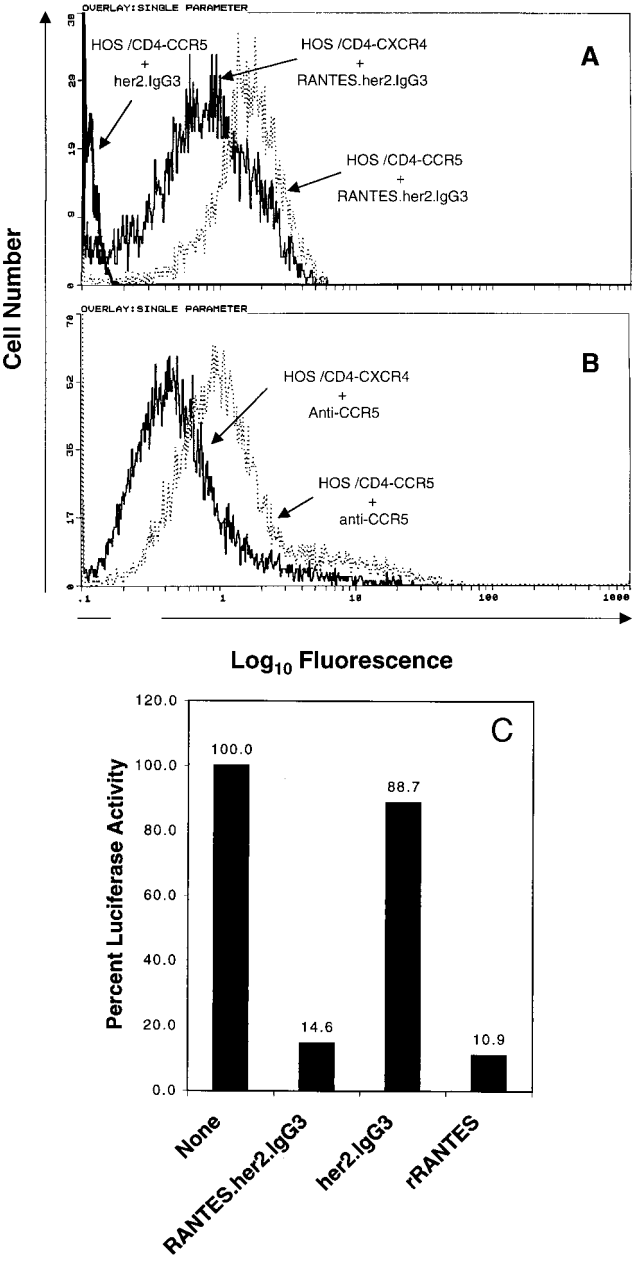


FIGURE 5. Interaction of RANTES.her2.IgG3 with CCR5 receptor. *A*, HOS/CD4-CXCR4 (black line) or HOS/CD4-CCR5 (dotted line) cells were incubated with 1 μg of RANTES.her2.IgG3 (black and dotted lines) or her2.IgG3 (dark black line) at 4°C for 30 min. Cells were then washed and incubated with FITC-conjugated anti-human Ab and analyzed by flow cytometry. *B*, HOS/CD4-CXCR4 (black line) or HOS/CD4-CCR5 (dotted line) cells were incubated with 1 μg of mouse anti-CCR5 Ab at 4°C for 30 min, washed, incubated with FITC-conjugated anti-mouse Ab for another 30 min, and analyzed by flow cytometry. *C*, HOS/CD4-CCR5 cells were incubated with 125 nM her2.IgG3, RANTES.her2. IgG3, rRANTES, or medium alone for 15 min at 37 C. The cells were then infected with HIV-luc-JR-FL macrophage-tropic HIV-1 recombinant virus. The cells were lysed 48 h later, and assayed for luciferase activity. The experiment was repeated four times with similar inhibition results.

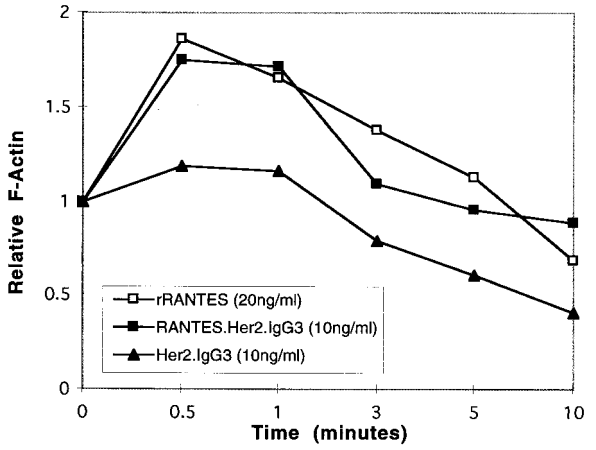


FIGURE 6. F-actin polymerization of differentiated THP-1 cells. THP-1 were prestimulated with cAMP, washed, and incubated with rRANTES, RANTES.her2.IgG3, or her2.IgG3. At different time intervals, an aliquot of the cells was fixed with paraformaldehyde and stained with NBD-phallacidin. The samples were analyzed by flow cytometry, and relative F-actin was calculated as the mean fluorescence relative to time zero. This experiment was repeated three times with similar inhibition results.

in response to soluble rRANTES at 0.1 and 1 ng/ml. These experiments demonstrate that the RANTES.her2.IgG3 fusion protein bound to the tumor cell surface was capable of creating a gradient necessary for its chemotactic activity in the vicinity of targeted tumor cells.

Discussion

We describe the construction and in vitro characterization of an Ab-chemokine fusion protein in which the chemokine RANTES was linked by genetic engineering to an Ab specific for the tumor-associated Ag HER2/*neu*. We hypothesize that RANTES.her2.IgG3 will localize to the tumor vicinity through the Ab domain of the fusion protein. The accumulation of the fusion protein at the tumor site should then create a local chemokine gradient that would enhance the transendothelial migration of effector cells such as T lymphocytes, NK cells, monocytes, and dendritic cells. An increase in local immune effectors should then enhance the development of an active cellular immune response at the site of the tumor.

The anti-HER2/*neu* Ab used in this study is based on the humanized humAb4D5–8 Ab currently in phase III clinical trials (10, 20). The variable sequences of the Ab were cloned into a human IgG3 backbone to provide greater flexibility in folding of the fusion protein mediated by the long hinge region of IgG3. Our results indicate that RANTES can be effectively linked to the amino terminus of the heavy chain of the Ab, with retention of both Ab specificity and RANTES activity. We also demonstrate that anti-HER2/*neu* affinity of the RANTES.her2.IgG3 fusion protein for its antigenic target is similar to that of the her2.IgG3 parental Ab. In an assay of biologic activity, RANTES.her2.IgG3 was capable of inducing F-actin polymerization of monocytic cells. We consistently observed that the activity of RANTES in the fusion protein is higher than that of rRANTES on a molar basis. This may be due to the fact that the larger m.w. RANTES.her2.IgG3 fusion protein (185 vs 8 kDa for rRANTES) is providing greater stability of the fusion protein and thereby greater activity. Alternatively, the bivalency of RANTES in RANTES.her2.IgG3 may increase its potency. In assays for transendothelial migration in vitro, we demonstrated that both peripheral blood T cells and monocytes migrated in response to RANTES.her2.IgG3 fusion protein, while limited migration was observed using the her2.IgG3 Ab. This suggests that the Ab fusion protein in soluble form is capable of effectively stimulating transendothelial migration of inflammatory cells.

The chemotactic effect of chemokines appears to be mediated by the generation of a chemokine gradient in the tumor vicinity. To test for the ability of RANTES Ab fusion protein to elicit a gradient when bound to Ag-expressing cells, we measured the effect of cell surface chemotactic effects exhibited by cell surface immobilized RANTES.her2.IgG3. Anti-HER2/*neu* RANTES.her2.IgG3 bound to SKBR3 cells was capable of inducing transendothelial migration of T cells in a Transwell migration assay. Ab affinity, avidity, as well as equilibrium binding (association and dissociation) may all contribute to the generation of a local RANTES gradient by the fusion protein. Shedding of the HER2/*neu* Ag fusion protein complex may also contribute to the formation of a gradient. Such shedding of HER2/*neu* Ag alone or following binding of Ab has been observed in vitro, and soluble HER2/*neu* (ECD) can be measured in vivo in breast cancer patients (21). Recently, a member of a new class of chemokines, a CX_3C chemokine, expressed by endothelial cells has been described (22). The CX_3C molecule exists in a secreted and a membrane-bound

Table I. *Transendothelial migration of peripheral blood T cells in response to RANTES.her2.IgG3*[a]

Condition	Concentration (ng/ml)	% Migration (migration index)[b]				Average Migration Index ± SEM
		Expt. 1	Expt. 2	Expt. 3	Expt. 4	
None		12.9 (1.0)	16.4 (1.0)	5.2 (1.0)	5.9 (1.0)	1.0 ± 0.00
rRANTES	0.1	24.9 (1.9)	23.0 (1.4)	9.2 (1.8)	8.4 (1.4)	1.6 ± 0.13
	1.0	34.4 (2.7)	27.5 (1.7)	14.3 (2.8)	9.9 (1.7)	2.2 ± 0.30
	10.0	ND	ND	7.6 (1.5)	6.8 (1.2)	1.3 ± 0.11
RANTES.her2.IgG3	0.1	28.2 (2.2)	26.7 (1.6)	6.7 (1.3)	8.1 (1.4)	1.6 ± 0.20 ($p = 0.2665$)[c]
	1.0	38.3 (3.0)	34.4 (2.1)	15.5 (3.0)	13.3 (2.2)	2.6 ± 0.24 ($p = 0.0133$)[c]
	10.0	44.9 (3.5)	6.2 (1.6)	15.1 (2.9)	14.3 (2.4)	2.6 ± 0.40 ($p = 0.0062$)[c]
her2.IgG3	0.1	18.5 (1.4)	6.7 (0.4)	6.6 (1.3)	9.6 (1.6)	1.2 ± 0.27
	1.0	18.1 (1.4)	10.0 (0.6)	9.6 (1.9)	9.6 (1.6)	1.4 ± 0.27
	10.0	18.1 (1.4)	6.7 (0.4)	8.9 (1.7)	5.9 (1.0)	1.1 ± 0.28

[a] A HUVEC monolayer was grown to confluence on the porous membrane of a transwell plate (see *Materials and Methods*). Peripheral blood T cells were purified from blood obtained from normal donors and plated in the upper well of the transwell plate. rRANTES, RANTES.her2.IgG3, or her2.IgG3 were added to the lower wells at the indicated concentrations. Migration was allowed to proceed at 37°C for 24 h, and the number of migrated cells in the lower well was counted and recorded as percent migration.

[b] Values correspond to percent of T cells placed in the upper well (1×10^5 in expt. No. 1, 4.7×10^4 in expt. No. 2, 2×10^5 in expts. No. 3 and No. 4) that migrate to the lower well in response to the described conditions. In parentheses, we show migration index for each experiment calculated as percent migration for a specific condition divided by control percent migration in medium only.

[c] The *p* value was calculated from paired *t* test comparing RANTES.her2.IgG3 to her2.IgG3 in all four experiments at the same concentration.

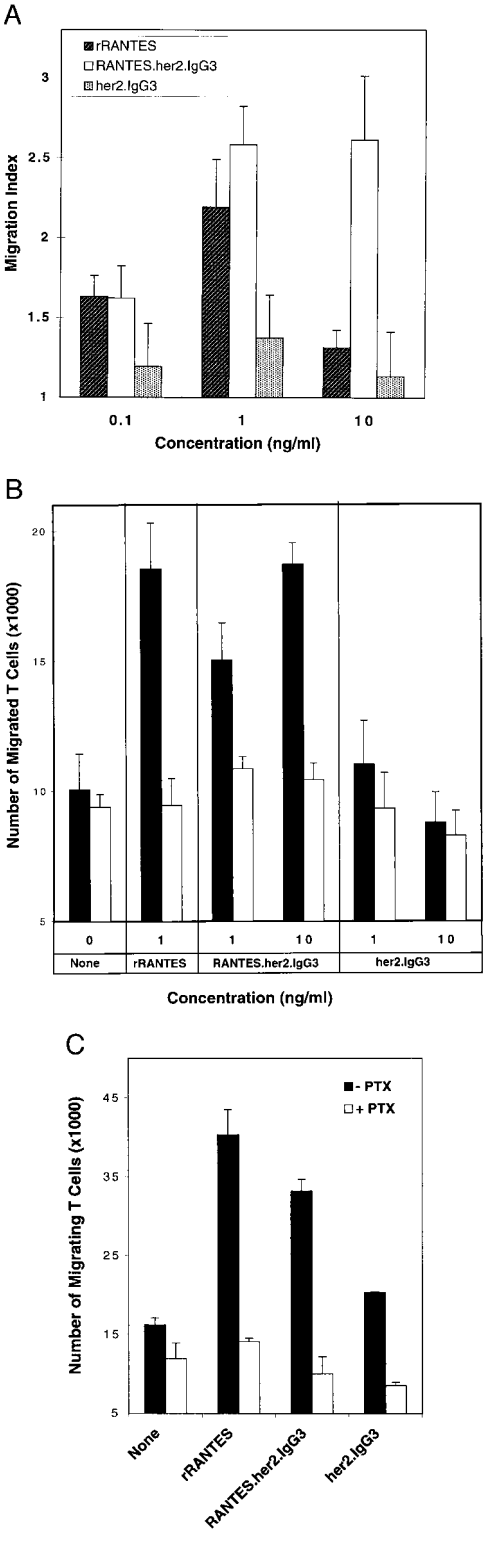


FIGURE 7. Transendothelial migration of peripheral blood T cells in response to soluble RANTES.her2.IgG3. *A*, The average of the migration indexes for all four experiments described in Table I is plotted. *B*, Chemokines or Abs at the indicated concentrations were preincubated with medium alone (black bars) or with anti-RANTES Ab at 5 μg/ml for 30 min before T cell addition (white bars), and the assay was allowed to proceed as described in *A*. *C*, T cells were pretreated with pertussis toxin (white bars) or with medium alone (black bars) for 36 h, washed, and then placed in the insert well on an endothelial monolayer. Medium, rRANTES (1 μg/ml), her2.IgG3 (10 μg/ml), or RANTES.her2.IgG3 (10 μg/ml) was placed in the lower well. Transendothelial migration was measured 24 h later as described in *A*. The error bars represent the SEM.

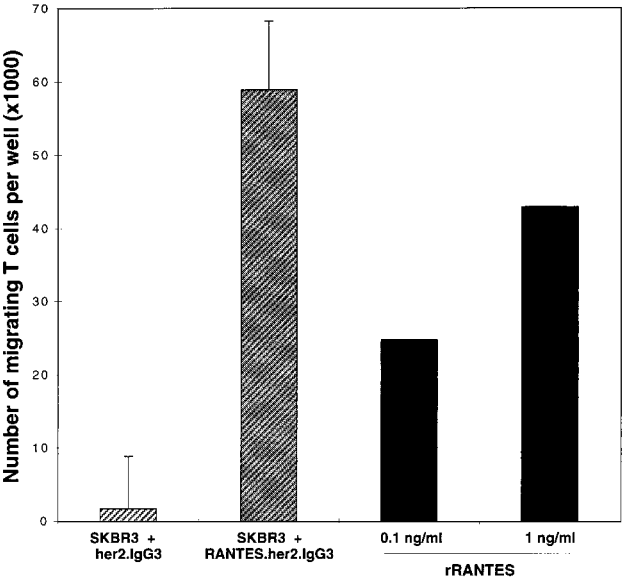


FIGURE 8. Transendothelial migration of primary peripheral blood T cells in response to cell surface Ag-bound RANTES.her2.IgG3. SKBR3 cells were preincubated with her2.IgG3 or RANTES.her2.IgG3 for 2 h at 4°C. The SKBR3 cells were then washed and placed in the lower well of a Transwell plate in which a confluent HUVEC monolayer was grown on the porous membrane. In separate wells, rRANTES was added at the indicated concentrations instead of preincubated SKBR3 cells. Purified peripheral blood T cells, at 3×10^5 cells/well, were added to the upper well, and the Transwell plates were incubated at 37°C overnight. Migration was measured by counting the number of T cells in the lower well. Background migration in presence of medium only was subtracted from the sample cell number.

form as further evidence that a membrane-bound chemokine can promote effector cell migration.

RANTES has been reported to induce two calcium influx signals in T cells. The first is of short duration and characteristic of chemokines, whereas the second is similar to the TCR activation signal leading to Ag-independent T cell proliferation (3). Taub et al. (4) has shown that RANTES can also potentiate B7.1-mediated T cell costimulation. Studies ongoing in the laboratory are testing potential synergy between the RANTES.her2.IgG3 fusion protein and another fusion protein developed in our laboratory in which the extracellular domain of the B7.1 costimulatory molecule was fused to an antitumor Ab (23). RANTES was recently shown to generate an antitumor immune response when MCA-205 sarcoma cells engineered to express RANTES were injected in vivo into syngeneic immunocompetent mice (5). Similar results were seen in our laboratory using the murine EL4 lymphoma. We have observed that RANTES provides protection from tumor growth whether introduced stably ex vivo through retroviral vectors (unpublished data) or introduced transiently through herpes simplex-derived amplicon vector in vivo in established tumors (manuscript in preparation). Protection is associated with an increase in CTL activity and development of systemic immunity capable of rejecting parental RANTES-nonexpressing tumor cells upon rechallenge. Therefore, local delivery of RANTES may be a suitable strategy for the recruitment and activation of a tumor-specific immune response.

One potential limitation to the bioavailability of RANTES-Ab fusion protein is the presence of a promiscuous receptor for C-C and C-X-C chemokines on the surface of RBC that may serve as

a "sink" for free chemokines (24). While chemokine receptor/ligand interactions on target inflammatory cells appear to be specifically regulated, erythrocytes have been observed to possess a multispecific receptor that binds chemokines of both C-C and C-X-C classes. This receptor has been cloned and shown to be identical with the Duffy Ag (Dfy) (24, 25). Preliminary experiments suggest that RANTES.her2.IgG3 does not bind to Dfy^a and Dfy^b Ag by flow cytometry (data not shown). Further characterization of Dfy Ag binding is currently being performed in the laboratory. It is not yet conclusively known whether fusion decreases the affinity of RANTES for the erythrocyte chemokine receptor. It also may be possible to mutate RANTES so that it no longer binds the RBC receptor, but retains its ability to recruit immune effector cells. Alternatively, the RANTES.her2.IgG3 fusion protein could be delivered intratumorally or in settings in which red cell binding is less likely to present a problem, such as for i.p. or intrapleural disease.

In conclusion, we describe the construction and characterization of a chemokine Ab fusion protein with specificity for a tumor-associated Ag. While several Ab cytokine fusion proteins have been described (26–30), this is the first report of an Ab chemokine fusion protein. In theory, such a fusion protein may have the ability to recruit a large repertoire of T cells and other inflammatory cells to the tumor vicinity and thereby enhance the antitumor immune response. Recruitment of a large cohort of effector cells may augment the likelihood of activating tumor-specific memory cells or may allow activation of naive T cells through provision of additional costimulatory signals as well as processed tumor Ags. Chemokine-Ab fusion proteins might be useful, alone or in combination with other previously described fusion proteins, such as fusions with IL-2 (28, 29) and/or B7.1 (23), in eliciting an enhanced antitumor immune response.

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