The biology of RANTES (regulated on activation normal T cell expressed) aggregation has been investigated using RANTES and disaggregated variants, enabling comparison of aggregated, tetrameric, and dimeric RANTES forms. Disaggregated variants retain their Gi-type G protein-coupled receptor-mediated biological activities. A correlation between RANTES aggregation and cellular activation has been demonstrated. Aggregated RANTES, but not disaggregated RANTES, activates human T cells, monocytes, and neutrophils. Dimeric RANTES has lost its cellular activating activity, rendering it noninflammatory. Macrophage inflammatory protein 1α, macrophage inflammatory protein-1β, and erythrocytes are potent natural antagonists of aggregated RANTES-induced cellular activation.

There is a clear difference in the signaling properties of aggregated and disaggregated RANTES forms, separating the dual signaling pathways of RANTES and the enhancing and suppressive effects of RANTES on human immunodeficiency virus infection. Disaggregated RANTES will be a valuable tool to explore the biology of RANTES action in human immunodeficiency virus infection and in inflammatory disease.

Human RANTES1 is a proinflammatory chemokine that promotes cell accumulation and activation in chronic inflammatory conditions (1–3). RANTES expression has been associated with transplant rejection, atherosclerosis, arthritis, atopic dermatitis, airway inflammatory disorders, delayed-type hypersensitivity reactions, glomerular nephritis, asthma, endometriosis, and cancers (4–8). In addition, RANTES may be a key regulator of HIV-1 infection. It is the most potent natural chemokine inhibitor of M-tropic HIV-1 infection (9), but at high concentration can also act as a stimulator, enhancing viral infection (10, 11).

Numerous elements of the RANTES signaling cascade have been characterized, but the whole process remains poorly understood (12). RANTES has been shown to act via two different signal transduction pathways in T cells (13). The high affinity Gi-type G protein-coupled receptor (GPCR) signaling pathway acts at relatively low concentrations of RANTES (≤50 nM) and mediates chemotaxis, transient calcium mobilization, and suppression of HIV infection, although the last may require only receptor binding and not signaling. The low affinity signaling pathway acts via protein tyrosine kinases (PTKs) and is observed only at higher concentrations of RANTES (≥1 μM). RANTES-induced stimulation of the low affinity PTK pathway leads to T cell activation, including proliferation of T cells, induction of interleukin-2 (IL-2) expression, homotypic aggregation and increases in expression of cell surface molecules such as the IL-2 receptor (CD25), CD49, CD28, CD11b, and CD11c (13–15). Both the Gi-type GPCR- and PTK-mediated responses to RANTES stimulation can be studied independently by assessing calcium mobilization in THP-1 cells, which respond only via the Gi-type GPCR signaling pathway, or in CD3+ Jurkat cells, which respond only via the PTK pathway (16). These studies lead us to conclude that at high concentration, RANTES is a potent immune modulator, distinct from antigen, which activates T cells, and may be an important factor in immune pathologies lacking obvious antigenic stimulation.

There is interest in potential clinical uses for chemokines, but the proinflammatory nature of RANTES may inhibit its clinical evaluation. A single intradermal injection of RANTES into dog skin resulted in a dose-dependent, eosinophil- and macrophage-rich inflammatory lesion within 4 h, leading to full dermal thickening after 24 h, indicative of significant proinflammatory activity (3). In a clinical setting, such inflammatory responses could amount to serious undesirable side effects. There is a need, therefore, for noninflammatory RANTES analogues, and particularly those that retain the native agonist properties, to enable preclinical evaluation.

MIP-1α, MIP-1β, and RANTES are chemokines that share the unusual tendency to self-associate, forming high molecular weight aggregates in a concentration-dependent manner. It is our belief that their aggregation must be fully characterized and its in vivo relevance determined if the immunomodulatory properties of these chemokines are to be fully understood. Systematic mutagenesis has identified key residues in MIP-1α, MIP-1β, and RANTES that are critical for aggregation and has enabled the production of fully active disaggregated proteins (11). All of the receptor binding, Gi-type GPCR-mediated signal transduction and chemotactic activities associated with the aggregating chemokines were maintained in the disaggregated mutants. However, a significant biological difference between aggregating and nonaggregating forms of RANTES was iden-
Characterization of Noninflammatory RANTES Mutants

Materials and Methods

Chemokine Proteins—Synthetic chemokine gene construction, generation of mutants, yeast expression of chemokine protein, and its purification have been described (11). Briefly, they were expressed in Saccharomyces cerevisiae and were purified after secretion from the culture supernatant. Ion-exchange and reverse-phase HPLC (high performance liquid chromatography) produced proteins >95% purity with correct molecular mass by mass spectrometry. Monocyte chemoattractant protein-1, interleukin (IL)-8, and stromal-derived factor-1 were purchased from R&D Systems (Abingdon, United Kingdom).

Cells—The human mononuclear THP-1 and T cell-like CD4+ Jurkat cell lines were obtained from the European Collection of Animal Cell Cultures. They were cultured in RPMI 1640 medium, 2 mM glutamine, 10% (v/v) fetal calf serum at 37 °C in 5% CO2. Blood was obtained from healthy volunteers by venepuncture. Peripheral blood mononuclear cells (PBMCs) were purified from heparin-anticoagulated blood by Ficoll-Hyapaque (Sigma) separation. Primary human T cells were expanded from human PBMCs (106/ml) in RPMI 1640 medium, 2 mM glutamine, 10% (v/v) fetal calf serum by phytohemagglutinin (PHA) (Sigma; 5 μg/ml) and lymphocult-T-HP (1% (v/v), Boehr1) stimulation. After 2 days, the cells were washed, resuspended at 10⁶/ml in the same medium without PHA, and kept in culture for a few weeks. Human neutrophils were isolated from EDTA-anticoagulated blood by dextran sedimentation and Ficoll-Hyapaque separation. After extensive washing in PBS, the erythrocytes were resuspended in RPMI 1640 medium at 10⁶ cells/ml.

Calcium mobilization assay—This assay was performed as described in Czaplewski et al. (11). Briefly, cells (2 × 10⁶/ml) in growth medium were incubated with Fura-2/AM (1 μM) for 45 min and washed and resuspended in Tyrodes buffer at 2 × 10⁶ cells/ml. A Perkin-Elmer LS-50B fluorometer was used to measure Fura-2 fluorescence emission excited at 340 nm with a 10-nm bandwidth, and the emission was continuously recorded at 500 nm with a 5-nm bandwidth. Chemokines were added (20 μM, 100-fold final concentration), and the increase in intracellular calcium was noted. To achieve the very high chemokine concentrations used in some experiments, 1 ml of chemokine (final concentration, 2× in Tyrodes buffer) was added to 1 ml of cells at 4 × 10⁶/ml. The effect of pertussis toxin (Calbiochem) was assessed by preincubation (1 μg/ml) for 30 min prior to assay.

Flow cytometry—Cells (0.5 × 10⁶/ml) in fetal calf serum-free RPMI 1640 medium were incubated with chemokines, PHA (Sigma), or IL-8 or left untreated (addition of PBS as a control) and incubated for 4 h at 37 °C. The cells were harvested, washed in PBS, and incubated with appropriate monoclonal antibodies for 20 min at room temperature. Monoclonal antibodies used in this study were anti-human CD3 (PerCP), CD4 (FITC), CD6 (PE), CD69 (FITC or PE), CD11b (Mac-1-PE), CD11c (gp-150, 95-PE), and CD14 (PE) from Becton Dickinson. Cells were washed and analyzed by flow cytometry on a Becton Dickinson FACSCalibur. When whole blood was used, it was diluted with an equal volume of RPMI 1640 medium and used as above. Erythrocytes were lysed using a Coulter Q-prep (EPICS, Immunology). The effect of erythrocyte addition was assessed by mixing purified erythrocytes (10⁹/ml) with purified neutrophils (10⁶/ml) or Jurkat cells (10⁶/ml) to approximate blood cell concentration. After incubation with chemokines, the cell mixtures were harvested, and the cells were stained with antibodies as described above. Erythrocytes were lysed as above prior to fluorescence-activated cell sorter analysis.

Results

Characterization of the Self-association and G1-type GPCR Biology of RANTES and Disaggregated RANTES Mutants—The concentration-dependent self-association of RANTES, RANTES E26A, RANTES E66S, and RANTES E26A/E66S was analyzed by sedimentation equilibrium analytical ultracentrifugation at 0.1 and 0.5 mg/ml (Table I). Substitution of acidic residues at position 26 or 66 disaggregated RANTES resulting in the generation of protein solutions with weight average molecular weights consistent with tetramers and dimers respectively. Substitutions at both of these positions did not further disaggregate the variants. The self-association of the variants was insensitive to protein concentration over the range of concentrations suitable for analytical ultracentrifugation analysis. Use of RANTES, RANTES E26S, and RANTES E66S in experiments allows the biology of aggregating RANTES, “tetrameric” RANTES, and “dimeric” RANTES to be compared. It must be appreciated that at higher protein concentrations (up to 4 mg/ml), even the disaggregated variants will self-associate, although their tendency to do so is greatly reduced. We have focused on the comparison of RANTES and RANTES E66S.

The maintenance of GPCR binding and signal transduction activities by disaggregated RANTES variants has been described using recombinant cell lines expressing chemokine receptors (11). We have confirmed the G1-type GPCR potency of RANTES E66S using THP-1 cells that naturally express RANTES receptors (Fig. 1A). RANTES and RANTES E66S induced comparable transient calcium flux, indicative of a GPCR receptor. The results obtained with the supraoptimal 1 μM dose of chemokines are presented to enable direct comparison at that dose with the results obtained using Jurkat cells (Fig. 2A). To ensure that RANTES E66S retained potency through the G1-type GPCR-mediated signal transduction pathway, the chemotactic activities of RANTES and RANTES E66S for mononuclear cells were evaluated and were comparable (Fig. 1B).

Aggregation of RANTES Is Responsible for Induction of the Protein Tyrosine Kinase Pathway—We have used Jurkat cells,
Characterization of Noninflammatory RANTES Mutants

Disaggregated RANTES E66S Does Not Activate Human T Lymphocytes—T cell activation induced by RANTES or disaggregated RANTES was assessed using the early activation marker CD69. We also looked at the expression of the adhesion molecules CD11b and CD11c, members of the integrin family. Unlike wild-type RANTES, RANTES E66S was unable to induce any marker up-regulation on cultured human (50% CD4+/50% CD8+) T cells (Fig. 3) and therefore did not activate the T cells. The effect of RANTES was concentration-dependent, and it was necessary to use a higher concentration of RANTES (5 μM) to reproducibly observe activation in the cultured human T cells. RANTES-mediated stimulation of CD69, CD11b, and CD11c expression was insensitive to pertussis toxin (data not shown). Similar results were obtained using Jurkat cells and T cells fluorescence-activated cell sorter-gated from freshly purified PBMCs (data not shown). RANTES-induced homotypic adhesion of human T cells described by Bacon et al. (14) was not observed in the presence of RANTES E66S (not shown).

RANTES but Not Disaggregated RANTES E66S Activates Purified Human Neutrophils and Monocytes—The integrin CD11b can be used as a cell surface marker of neutrophil activation. Although RANTES effects on neutrophils have seldom been described in literature, Conklyn et al. (17) showed that RANTES could act on neutrophils in EDTA-anticoagulated whole blood, elevating CD11b expression in a concentration-dependent manner. We reproduced these results and showed that neutrophils in whole blood responded to both RANTES and RANTES E66S with relatively modest increases in CD11b expression compared with that obtained by treatment with IL-8 (Fig. 4A). However, on purified neutrophils, the response was quite different with substantial elevation of CD11b at 5 μM RANTES and no stimulation by 5 μM RANTES E66S (Fig. 4B).
This increase in CD11b expression on purified neutrophils was not inhibited by pertussis toxin, but the effects of IL-8 (which acts through the G\text{i}-type GPCR pathway) were pertussis toxin-sensitive (data not shown). These data led to the conclusion that two signaling pathways were involved in the response. As in T cells, RANTES appears to act on neutrophils via two independent mechanisms: an aggregation-independent mechanism (Gi-type GPCR-mediated because RANTES E66S is active) that acts at a relatively low concentration to modestly elevate CD11b expression, and an aggregation-dependent mechanism (non-Gi-type GPCR or non-GPCR-linked) that is observed only in purified neutrophils. We assume that a RANTES-induced GPCR response was not observed in purified neutrophils and that the IL-8-induced response decreased compared with whole blood assay, because this pathway may have been affected by cell purification.

We have also demonstrated the presence of the aggregation-dependent RANTES signaling pathway in monocytes using cell surface expression of CD69 to estimate cellular activation by RANTES (5 \( \mu \text{M} \)) and RANTES E66S (5 \( \mu \text{M} \)) (Fig. 4C). Lower
concentrations of chemokine did not induce significant up-regulation of CD69 on monocytes (not shown). Because the RANTES Gi-type GPCR signaling pathway has already been described in monocytes (1), this observation implies that dual signaling pathways induced by RANTES exists in these cells as well as in T cells and neutrophils.

**Erythrocytes Inhibit Cellular Activation by RANTES**—The aggregation-dependent RANTES signaling pathway was observed in purified neutrophils but not neutrophils in whole blood (Fig. 4, A and B). Here, we show that purified T cells respond to aggregated RANTES (Fig. 3), but we have been unable to demonstrate activation of T cells in whole blood by high concentrations of aggregated RANTES (data not shown). To explore why RANTES activated purified cells but not cells in whole blood, reconstruction experiments were used, focusing on neutrophils. We first observed that RANTES-induced activation was reduced by addition of serum to the medium, although this could not account for the whole effect (data not shown). Addition of different purified white cell-types did not inhibit purified neutrophil activation by RANTES (data not shown). However, addition of purified erythrocytes (within the concentration range found in blood) to purified neutrophils abolished RANTES-induced activation of CD11b expression (Fig. 5A). Neutrophil stimulation by IL-8 was largely unaffected by erythrocyte addition. A general increase in background fluorescence due to erythrocyte lysis was noted. Erythrocytes express the Duffy antigen, which is a relatively nonspecific chemokine receptor that binds RANTES (18); therefore, the effect of Duffy-negative (FYa, FYb) erythrocyte addition was evaluated (erythrocytes were a kind gift from Dr. Geoff Poole). Erythrocytes inhibited RANTES-induced neutrophil activation regardless of their Duffy status. This appears to be a general observation because the effects of erythrocytes on RANTES-induced CD11c expression by Jurkat cells were similar (Fig. 5B).

**Disaggregated RANTES E66S, MIP-1α, and MIP-1β Antagonize RANTES-induced Leukocyte Activation**—To characterize the mechanism of RANTES-induced cellular activation, we explored the ability of chemokines to antagonize the up-regulation of CD11c on Jurkat cells (Fig. 6). Addition of RANTES (5 μM) increased CD11c expression. None of the other chemokines (5 μM) on their own had any effect on CD11c expression (data not shown). Co-incubation with a 2-fold excess (10 μM) of disaggregated RANTES E66S, MIP-1α, or MIP-1β substantially inhibited CD11c up-regulation. Although RANTES E66S was an effective antagonist, neither of the other disaggregated chemokines tested, MIP-1α E66S or MIP-1β E67S, antagonized. IL-8 and monocyte chemoattractant protein-1 were also ineffective antagonists. Similar results were obtained with neutrophils (data not shown).

**DISCUSSION**

The discovery that aggregation is responsible for the ability of RANTES to stimulate HIV infection in vitro (11) led us to investigate whether there were other activities associated with RANTES aggregation. We have previously shown that amino acid substitution of residues E26 and E66 in RANTES inhibits aggregation (11). Here, we show that the RANTES E26A/E66S double mutant does not offer any advantage over the variants with single substitutions. We have compared the biological activities of wild-type aggregating RANTES with RANTES E26A, which is essentially tetrameric, and RANTES E66S, which is essentially dimeric at 0.1–0.5 mg/ml. Wild-type and dimeric RANTES have equivalent Gi-type GPCR-mediated activities on THP-1 and human mononuclear cells. We have used
Jurkat cells to demonstrate that there is a correlation between RANTES aggregation and stimulation of the protein tyrosine kinase signal transduction pathway associated with T cell activation. At high concentrations (up to 10 μM) disaggregated RANTES is essentially inactive on Jurkat cells. When challenged with 0.5 mM RANTES E66S, Jurkat cells respond, and we estimate that RANTES is approximately 200-fold more potent than RANTES E66S in this assay. RANTES, but not RANTES E66S, stimulates the cell surface expression of the activation marker CD69 and integrins CD11b and CD11c on human T cells and Jurkat cells. The RANTES dual signaling pathway has already been described for T cells (13). Here, we extend this observation, showing that both monocytes and neutrophils possess dual signaling pathways and that in each cell type, a RANTES aggregation-dependent signaling pathway exists. Evaluating the significance of monocyte and neutrophil activation by RANTES requires further investigation.

These observations suggest that RANTES aggregation may be responsible for a proportion of its proinflammatory activity. The relative inability of RANTES E66S to stimulate the protein tyrosine kinase pathway and to activate the leukocytes suggests that dimeric RANTES is noninflammatory.

RANTES acts on T lymphocytes via two independent signal transduction pathways. Both RANTES and disaggregated RANTES bind to RANTES G protein-coupled receptors and signal, leading to migration of the T cells. However, only RANTES can trigger the PTK pathway, leading to cell activation and many associated events, such as proliferation or apoptosis, adhesion molecule expression, and release of cytokines described in this study and in the literature (13–15). This study indicates that hematopoietic cells generally possess two distinct RANTES-induced signal transduction pathways. One is mediated by Gq-type G proteins, resulting in a transient mobilization of calcium, and the other, involving protein tyrosine kinase, results in a prolonged mobilization of calcium. Chemokine receptors have been shown to couple to alternative G proteins, such as Go, Gs, and Gz (19, 20). It is possible that these PTX-insensitive G proteins mediate a signal leading to activation of PTKs. Several studies have characterized chemokine receptor (GPCR)-mediated signal transduction pathways, which lead to activation of protein tyrosine kinases (21–23). These observations apparently provide a mechanism to support the two signaling pathways used by RANTES. However, the pharmacology of these signal transduction systems does not currently match that of the system we have observed. We see it as a RANTES-specific, concentration-dependent phenomenon. MIP-1α and MIP-1β do not induce cellular activation. The protein tyrosine kinases Pyk2 and Pyk2-H have been implicated in GPCR-mediated responses (22, 24). RANTES-mediated activation of Pyk2 is sensitive to PTX, indicating that it is directed through a Gq-type GPCR and is inconsistent with the observed pharmacology (21). Although the PTX sensitivity of Pyk2-H has not been reported, both RANTES and MIP-1β have been shown to induce its activation (22). These observations indicate that RANTES-mediated cellular activation is probably not directed through Pyk2 or Pyk2-H. RANTES has also been shown to induce the tyrosine kinase activity of the zeta-associated protein (ZAP)-70 and the focal adhesion kinase pp125FAK (14). The detailed pharmacology of this signal transduction route is not yet fully resolved, and it is not clear whether there is a direct role for GPCRs or whether another interaction induces signaling by aggregated RANTES.

Aggregation of RANTES on the T cell surface, perhaps bound to cells via GPCRs or glycosaminoglycan interactions (25, 26), may trap cell surface molecules, such as the T cell receptor complex, in a relatively nonspecific way to increase their local concentration and trigger signaling. This hypothesis is supported by the work of Dairaghi et al. (16), who have shown that the presence of CD3 is essential for RANTES-induced T cell activation. Neutrophils or monocytes can be activated by cross-linking of surface molecules, such as L-selectins, integrins, or Fcγ receptors II (27–29). In these cells RANTES may cross-link these molecules through nonspecific aggregation, inducing signal transduction leading to cellular activation.

Recently, Gordon et al. (10) described an enhancement of HIV infection in the presence of high concentrations of RANTES in vitro, and they believe that this may be related to cellular activation by RANTES. Our study supports this theory because disaggregated RANTES, which does not promote cell activation, does not enhance HIV infection (11).

Although RANTES activates purified neutrophils in an aggregation-dependent manner, it does not activate neutrophils in whole blood. Reconstruction experiments adding serum, leukocytes, or erythrocytes to purified neutrophils show that erythrocytes were responsible for most of the inhibition of RANTES-mediated neutrophil activation. Erythrocytes also inhibit the RANTES-mediated activation of Jurkat cells, suggesting that their inhibitory activity is not restricted to primary cells. The Duffy chemokine receptor expressed on the erythrocytes of some individuals does not appear to be responsible for this activity. The identification of the inhibitory activity of erythrocytes leads to some interesting biological questions. There may be an erythrocyte concentration-dependent mechanism to modulate RANTES-induced cellular activation in different cellular compartments. Thus, in the absence of erythrocytes, RANTES may induce cellular activation in intercellular spaces, lymph, and secreted fluids (such as mucus) but may be relatively inactive in blood. There are special circumstances that may be interesting to investigate, such as hemorrhage and
injury, in which erythrocytes enter other cellular compartments and may modulate inflammation via their ability to inhibit the proinflammatory activities of RANTES.

An additional level of control of RANTES-mediated cellular activation has been identified. Disaggregated RANTES E66S, MIP-1α and MIP-1β are effective antagonists; thus the level of co-expression of MIP-1α and MIP-1β may be crucial to the overall outcome when investigating the biological consequence of RANTES expression. The observation that disaggregated RANTES, MIP-1α, and MIP-1β, but not disaggregated MIP-1α or MIP-1β, antagonize RANTES is interesting, and further investigation of these differences is likely to lead to a better understanding of the biology of aggregating chemokines. It is not clear how these chemokines inhibit RANTES activation of cells. It is possible that they bind to the RANTES binding sites on the cell and block interactions with RANTES. Alternatively, they may bind to RANTES, forming heteromultimers, and thus inhibit the formation of the high molecular weight inflammatory RANTES aggregates. Heteromultimers of RANTES with MIP-1α or MIP-1β have not been described, but their formation may be feasible given the high degree of homology between these chemokines and the observation that their self-association is controlled by homologous residues (11).

Our speculative study of RANTES aggregation has led to interesting biology, such as the in vitro stimulation of HIV infection by aggregated RANTES, the role of aggregation in RANTES-mediated cellular activation, the identification of erythrocytes as anti-inflammatory cells and the discovery of MIP-1α and MIP-1β as potent natural antagonists of RANTES-mediated cellular activation. The in vivo relevance of these studies, which have used RANTES at extremely high concentrations, is open to debate. High local concentrations of RANTES may be expected at infection or inflammation sites. In addition, the identification of natural antagonists of RANTES-mediated cellular activation indicates that control mechanisms may be in place and suggests that these in vitro phenomena may have in vivo relevance. In addition to investigating the role of RANTES aggregation in chronic inflammatory diseases,
perhaps by using RANTES E66S or MIP-1β as antagonists, we are also interested in the generation of transgenic mice expressing disaggregated chemokines to evaluate the role of aggregation in vivo.

It is clear that aggregating RANTES may be unsuitable for clinical evaluation and that disaggregated variants that are noninflammatory may be preferred for future evaluation of the clinical potential of RANTES. RANTES-mediated activation of cells by high concentrations of RANTES may not be directed via GPCRs, and alternative modes of interaction via, for example, glycosaminoglycans are feasible. The search for therapeutic chemokine receptor antagonists to treat inflammatory diseases is advanced but may be partially misdirected if non-GPCR-mediated proinflammatory activities of chemokines are not inhibited.

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