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RANTES-Induced T Cell Activation Correlates with CD3 Expression

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The chemokine RANTES induces a unique biphasic cytoplasmic Ca2+ signal in T cells. The first phase of this signal, similar to that of other chemokines, is G-protein mediated and chemotaxis associated. The second phase of this signal, unique to RANTES and evident at concentrations greater than 100 nM, is tyrosine kinase linked and results in a spectrum of responses similar to those seen with antigentic stimulation of T cells. We show here that certain Jurkat T cells responded to RANTES solely through this latter pathway. A direct correlation between the RANTES-induced second phase response and CD3 expression was demonstrated in these cells. Sorting the Jurkat cells into CD3high and CD3low populations revealed that only the CD3high cells were responsive to RANTES. Furthermore, stimulation of these Jurkat cells with anti-CD3 mAb significantly depresses their subsequent response to RANTES. While a RANTES-specific chemokine receptor is expressed at a low level on these Jurkat cells, the RANTES-induced activation is dependent on the presence of the TCR. Thus, stimulation through TCR may partially account for RANTES' unique pattern of signaling in T cells.

The chemokine RANTES mediates chemotaxis in a variety of leukocytes, including subpopulations of lymphocytes, basophils, eosinophils, and monocytes (1–5). Furthermore, exposure of T cell clones to concentrations of RANTES greater than 100 nM leads to a unique biphase pattern of cytoplasmic Ca2+ mobilization representing two distinct signaling pathways that can be dissected pharmacologically (6). One pathway generates signals associated with chemotaxis, while the other generates signals associated with cellular activation, including increased secretion of the cytokines IL-2 and IL-5, up-regulation of IL-2 receptors, and enhanced cellular proliferation (6).

The first phase of this biphase signal, which is transient in nature and associated with chemotaxis, can be inhibited by the heterotrimeric Gai protein inhibitor pertussis toxin (PTX).6 This is consistent with signaling through other C-C chemokine receptors, such as the CCR-1 (7). By contrast, the second phase of this signal, which comprises a sustained Ca2+ influx, is insensitive to pertussis toxin PTX, but sensitive to the tyrosine kinase inhibitor herbimycin A (HA). The second phase signal is associated with cellular activation and has been shown by whole cell patch clamp analyses to be similar to TCR-mediated early activation events (6), a feature that suggests signaling apart from the seven-transmembrane, G protein-linked chemokine receptors.

To identify and characterize the RANTES-responsive elements mediating the second phase signal, we have identified two cell lines that manifest only one of the two RANTES-induced signaling pathways. The monocytic cell line THP-1 responded to RANTES through a G protein-linked pathway, while the T cell line Jurkat responded through a tyrosine kinase-mediated pathway. Furthermore, we showed a direct correlation between CD3 expression and the RANTES response in the Jurkat cells, raising the possibility that RANTES may engage the TCR complex as a way of effecting cellular activation.

Materials and Methods

Cell culture

THP-1 cells were obtained from the American Type Culture Collection (ATCC TIB-202) and the Jurkat cell lines were developed at DNAX. Both cells were grown in RPMI 1640 medium (JRH Biosciences) containing 10% heat-inactivated FBS, 25 mM HEpes (pH 7.5), 5 μM penicillin, and 5 μg/ml streptomycin.

Cytoplasmic Ca2+ mobilization assays

Cells were labeled with 3 μM indo-1 acetoxymethyl ester (Molecular Probes) in complete growth medium at a density of 107 cells/ml for 45 min at 20°C with gentle mixing. Cells were washed, resuspended at 107 cells/ml in HBSS (138 mM NaCl, 5 mM KCl, 5.6 mM D-glucose, 4 mM sodium bicarbonate) (Life Technologies) containing 1% FBS, and maintained at 20°C for up to 2 h. RANTES (R&D Systems) or mAb to human CD3 (UCHT1, Immunotech) was added to 107 cells in 2 ml of flush buffer (HBSS with 1.6 mM CaCl2 and 10 mM HEpes (pH 7.5)) and maintained at 37°C in an acrylic cuvette with constant stirring. Fluorescence measurements to determine the increases in cytoplasmic free Ca2+ concentration ([Ca2+]i) were performed with a Photon Technologies spectrofluorometer at an excitation wavelength of 350 nm (4 nm bandwidth) and simultaneous emission measurements at 400 and 490 nm (10 nm bandwidth). The ratio of 400 nm/490 nm was recorded at a rate of 2 Hz.

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6 Abbreviations used in this paper: PTX, pertussis toxin; MIP-1α,β, macrophage inflammatory protein -1α,β; MCP-1,2,3, macrophage chemotactic protein -1, 2, 3; PF-4, platelet factor 4; HA, herbimycin A.
Pharmacologic inhibition of the chemokine response

Cells were incubated at a concentration of 10^6 cells/ml for 16 h in complete growth medium with either 10 mM HA (Calbiochem) or 100 ng/ml PTX (Calbiochem).

FACS analysis and Jurkat cell sorting

FACS analyses were performed using standard protocols. Briefly, cells were washed in PBS containing 1% BSA, resuspended at 2 x 10^5 cells per well in 96-well V-bottom plates (Costar) and incubated with FITC-conjugated human anti-CD3 mAb (UCHT1, Immunotech) for 30 min. They were washed three times and analyzed on a FACScan (Becton Dickinson). Acquisitions were based on the forward and side-scatter characteristics and 10,000 events were acquired. For FACS sorting, two independent acquisition gates were applied to the CD3-positive and CD3-negative populations respectively based on their FL1 fluorescence. 10^6 cells were collected for each population.

Overnight TCR stimulation of Jurkat T cells

Jurkat cells were incubated for 16 h at a density of 10^6 cells/ml in complete growth medium with the addition of 2.5 μg/ml human anti-CD3 mAb (UCHT1, Immunotech) for 30 min. They were washed three times and analyzed on a FACScan (Becton Dickinson). Acquisitions were based on the forward and side-scatter characteristics and 10,000 events were acquired. For FACS sorting, two independent acquisition gates were applied to the CD3-positive and CD3-negative populations respectively based on their FL1 fluorescence. 10^6 cells were collected for each population.

RANTES equilibrium binding

A standard filtration binding protocol was employed (8). Briefly, 10^6 cells were incubated with approximately 0.1 nM ^125I-RANTES (Dupont NEN) in the presence of increasing amounts of unlabeled RANTES competitor using the following buffer: 25 mM HEPES, 80 mM NaCl, 1 mM CaCl_2, 5 mM MgCl_2, adjusted to pH 7.4. Scintillant (MicroScint 10, Packard) was added to the filters, and the retained radioactivity measured using a TopCount scintillation counter (Packard). The data were then analyzed using IgorPro software (WaveMetrics).

Results

Dissection of the RANTES-induced biphasic Ca^{2+} response

A biphasic Ca^{2+} response was observed upon the addition of RANTES to a final concentration of 1 μM (Fig. 1A) in both the SPB21 CD4 T cell clone and in PBL (6). This response comprised a short transient spike of increased cytoplasmic Ca^{2+} followed by a more vigorous and sustained response accompanying the opening of extracellular Ca^{2+} channels.

To segregate the two phases of the RANTES-induced response seen in the SPB21 T cell clone, we surveyed a number of cell types and assessed their signaling characteristics in response to RANTES. THP-1 cells exhibited a heterotrimeric G protein-dependent pathway, consistent with what is commonly known of chemokine receptor signal transduction. By contrast, Jurkat cells showed a signaling pathway dependent on tyrosine phosphorylation, a feature consistent with the second phase RANTES response in the SPB21 T cell clone. Superimposing the responses of THP-1 and Jurkat cells mirrored the biphasic Ca^{2+} flux profile seen with the SPB21 T cell clone (compare Fig. 1B with 1A). This also highlighted a difference in the kinetics of the two responses:
the initiation of the THP-1 response was more rapid that of the Jurkat response.

RANTES dose-response studies highlighted a second major difference between the two cellular responses (Fig. 1, C and D). Transient increases of cytoplasmic Ca$^{2+}$ were seen in THP-1 cells with as little as 0.1 nM RANTES, which is consistent with known THP-1 in vitro chemotactic and signal transduction characteristics and chemokine binding affinities (K$_D$ values around 1 nM) (8–10). By contrast, the Jurkat cells did not exhibit significant Ca$^{2+}$ mobilization at less than 100 nM RANTES; maximal stimulation was observed at 1 µM, while only a minimal response was seen at 100 nM, with no response observed at or below 10 nM RANTES. This closely mirrored the dose-response of the RANTES-induced second phase signal in the SPB21 T cell clone.

Pharmacologic analysis of the RANTES-induced response

The pharmacologic sensitivities of the RANTES-induced cellular responses in THP-1 and Jurkat cells were examined. PTX, which inhibits heterotrimeric G$_i$ protein signal transduction (11), and HA, a selective inhibitor of the Src family of protein tyrosine kinases (12), were used. HA treatment of THP-1 cells had no effect on the RANTES-induced signal, while PTX inhibited the response (Fig. 2A). This was consistent with G protein-linked signaling in these cells. By contrast, the RANTES response in Jurkat cells was not affected by the addition of PTX, while treatment with HA resulted in a marked abrogation of signal (Fig. 2B). Thus the RANTES response in Jurkat cells seemed transduced through a tyrosine kinase pathway and not through a PTX-sensitive G protein pathway; suggesting that RANTES is capable of signaling through two distinct and separable pathways. The THP-1 cells exhibited a rapid transient response mediated via G proteins; the Jurkat cells showed a delayed and sustained response via tyrosine kinases. The Jurkat cells provided us with a means to investigate this tyrosine kinase-linked RANTES response.

Selective activation of Jurkat by RANTES

To ascertain whether the RANTES-induced tyrosine kinase pathway could be engaged by other chemokines, we tested chemokines of both C-X-C and C-C classes on the Jurkat cells. Figure 3 shows the Jurkat cell Ca$^{2+}$ response to final concentrations of 1 µM MIP-1α, MIP-1β, MCP-3, and PF-4. The C-C chemokines MIP-1α and MIP-1β, which are functionally and structurally closely related to RANTES, had no effect on the Jurkat cells. MCP-3 and PF-4 resulted in little, if any, resultant Ca$^{2+}$ flux as compared with RANTES. Furthermore, the chemokines MCP-1 and IL-8 also failed to induce a Ca$^{2+}$ flux in these Jurkat cells (data not shown). These data were consistent with results observed with the SPB21 T cell clone (6), indicating that RANTES is unusual in its ability to activate this tyrosine kinase signaling pathway.

Ca$^{2+}$ response profiles after RANTES or anti-CD3 stimulation

We next compared RANTES- and CD3-mediated signaling in Jurkat cells. The addition of 1 µM RANTES or 2.5 µg/ml anti-CD3 mAb (saturating concentration) resulted in Ca$^{2+}$ fluxes of similar
FIGURE 4. Comparison of RANTES and anti-CD3 induced cytoplasmic Ca\(^{2+}\) levels in Jurkat cells. RANTES and anti-CD3 stimulation both cause Ca\(^{2+}\) release from intracellular stores and import of extracellular Ca\(^{2+}\) into the cytoplasm. A, Jurkat cells responding to the addition of 1 \(\mu\)M RANTES. The top trace is representative of intracellular Ca\(^{2+}\) levels in cells stimulated with RANTES at time 0 as in the previous experiments. The bottom trace (EGTA) depicts cytoplasmic Ca\(^{2+}\) levels in cells from a representative experiment where EGTA has been added to the cell suspension buffer at 10 mM final concentration (sufficient to chelate five fold the level of extracellular Ca\(^{2+}\)) before stimulation of the cells with RANTES. The middle trace (Ca\(^{2+}\)) is representative of experiments where the extracellular Ca\(^{2+}\) level has been increased from 2 mM to 10 mM approximately 70 s after the addition of RANTES. B, Experiments conducted as in A except that the stimulation is provided by addition of anti-CD3 to 2.5 \(\mu\)g/ml final concentration.

FIGURE 5. Signal response of different Jurkat cell lines. Analysis of the Jurkat cell line and the NR (nonresponder) Jurkat cell line. Shown are Ca\(^{2+}\) mobilization assays upon addition of 1 \(\mu\)M RANTES (A), Ca\(^{2+}\) mobilization assays upon addition of 2.5 \(\mu\)g/ml anti-CD3 mAbs (B), and a FACS analysis using anti-CD3 mAbs (C, D).
magnitude in these Jurkat cells (Fig. 4). When a fivefold excess of the extracellular Ca\(^{2+}\) chelator EGTA was added, the Ca\(^{2+}\) release from intracellular stores was clearly visible as a transient spike. This indicated that the sustained Ca\(^{2+}\) fluxes seen in response to RANTES or anti-CD3 Ab were attributable to Ca\(^{2+}\) entry from the extracellular source. To test this hypothesis, we increased the extracellular Ca\(^{2+}\) concentration from 1.6 mM to 10 mM approximately 70 s following stimulation. This increase in extracellular Ca\(^{2+}\) concentration caused a temporary rise in the Ca\(^{2+}\) flux followed by a rapid correction, presumably through the closing of extracellular Ca\(^{2+}\) channels. This phenomenon was seen with both RANTES and anti-CD3 mAb.

**Segregation of RANTES response by CD3 phenotype**

We noted that not all Jurkat cell lines responded equally to RANTES stimulation. We isolated a non-responding (NR) Jurkat cell line that responded to RANTES in the Ca\(^{2+}\) mobilization assay with less than 20% of the magnitude seen with the responding Jurkats (Fig. 5A). Strikingly, these NR Jurkat cells also had a marked attenuation of their anti-CD3 response (Fig. 5B). FACS analyses of CD3 expression
on these two Jurkat populations revealed that 95% of the responding Jurkats expressed CD3, while only 5% of the NR Jurkats were CD3 positive (Fig. 5, C and D, respectively). This suggested an association between CD3 expression and RANTES responsiveness.

To dissect the molecular nature of this association, we FACS-sorted the NR Jurkat population into two subpopulations: CD3<sup>high</sup> and CD3<sup>low</sup>. Subsequent analyses of these two NR Jurkat subpopulations indicated that the segregation had been greater than 99% successful (Fig. 6). While CD3 expression was dramatically different in these two populations, FACS analyses of these two subpopulations using a variety of other cellular markers (i.e., CD2, CD28, CD44) did not discern any other differences (data not shown).

The CD3<sup>high</sup> NR Jurkats responded strongly to anti-CD3 stimulation, while CD3<sup>low</sup> NR Jurkats showed no discernible response (Fig. 6). The responses to RANTES mirrored that of anti-CD3 responses: CD3<sup>high</sup> Jurkats responded strongly to RANTES, the CD3<sup>low</sup> Jurkats responded very weakly (Fig. 6). Thus RANTES appeared to require the presence of CD3 to induce a Ca<sup>2+</sup> signal in these Jurkat cells.

Cross-modulation of RANTES and anti-CD3 induced signals
We examined whether anti-CD3 stimulation could down-regulate the subsequent RANTES response. A mixed population of Jurkat cells, incubated overnight with anti-CD3 mAb, were assayed for cytoplasmic Ca<sup>2+</sup> mobilization in response to RANTES or anti-CD3 stimulation. Following the overnight anti-CD3 mAb incubation, the surface expression of CD3 (as well as TCR) was down-regulated as analyzed by FACS (using three different anti-CD3 mAbs and an anti-TCR α/β mAb for FACS analysis). Subsequently, the cellular responses of these cells to both anti-CD3 mAb and RANTES stimulation were significantly decreased (Fig. 7). Overnight incubation with an IgG1 isotype control did not down-regulate CD3 expression and had no effect on subsequent cellular responses to RANTES and anti-CD3 mAb stimulation (Fig. 7). These data strengthened the connection between CD3 expression and RANTES responsiveness.

RANTES binding to Jurkat cells
To address the question of whether a RANTES-binding receptor was expressed on these Jurkat cells, we employed equilibrium binding (Fig. 8). Displaceable binding with a $K_d$ of 0.1 nM was observed on these cells; however, the calculated receptor expression level was low, approximately 600 sites per cell. These data then indicate that at least one chemokine receptor is expressed upon these cells, but it is not at a level that could easily explain the magnitude and kinetics of the resulting RANTES-induced calcium flux.
that receptor being coexpressed with CD3. A third possibility is that RANTES could bind to other cell surface moieties (such as extracellular matrix components), crosslinking the TCR through chemokine aggregation.

Ligand binding studies shed some light on these different possibilities. First, the data suggest that a high affinity receptor for RANTES is expressed on Jurkat cells, although the expression level is low. While Jurkat cells do have signaling responses to RANTES in a manner consistent with a pathway through this high affinity receptor (15), neither the binding affinity nor the receptor expression level are consistent with the magnitude and kinetics of the responses reported here. In addition, in our experience low expression levels (less than a thousand sites per cell) of chemokine receptors on cells of this type normally do not give rise to significant cytoplasmic calcium flux in response to chemokine stimulation.

RANTES aggregation appears to be a dynamic process with protein aggregating and dissociating rapidly at equilibrium, and is dependent upon protein concentration and buffer conditions (data not shown). It is possible that glycosaminoglycans binding sites for RANTES exist on the surface of Jurkat cells, allowing the chemokine to attach to the cell surface via relatively low affinity interactions, but promoting subsequent aggregation of RANTES into higher order aggregates. This could explain the effect observed in the experiment depicted in Figure 8 upon addition of unlabeled RANTES at concentrations greater than 10 nM. Also, we have noted that monoclonal antibodies specific for RANTES will react with the cell surface of Jurkat cells which have been pre-incubated with the chemokine, and that the intensity of antibody staining increases with increasing amounts of RANTES added to the cells (data not shown). A cell surface aggregation process involving tethered RANTES could result in the cross-linking of CD3/TCR complexes in a type of molecular latticework, not dissimilar to what has been hypothesized for CD4 (16), resulting in the activation of tyrosine kinase signaling downstream of the TCR.

A wide variety of cell types express chemokines, either constitutively or under appropriate stimulation (e.g., TNF-α, IFN-γ, or IL-1β). RANTES, for example, is expressed by leukocyte subpopulations, fibroblasts, and endothelial cells (4). During an inflammatory reaction, a significant chemokine release is thought to occur within the immunologic microenvironment. It is possible that chemokines from a transient release could be sequestered within local environment and presented to responding target cells. In this way, the local chemokine concentration of RANTES might well exceed 100 nM in vivo. Chemokine concentration gradients, where the concentration is directly proportional to the distance from the site of inflammatory reaction, may exist to serve two distinct purposes. At a distance away from the inflammatory site, the lower concentrations of chemokine could serve a chemoattractant role for leukocytes populations. Closer to the inflammatory site, however, higher chemokine concentrations (at least of RANTES) may serve to activate specific populations of responding cell. Consistent with a T cell priming role, RANTES activation would leave the CD3/TCR surface expression intact, perhaps facilitating specific Ag recognition subsequently.

In summary, we have shown that chemokine activation might be mediated through the action of molecules other than seven-transmembrane G protein-coupled receptors. Such knowledge may be useful in evaluating the roles of chemokines in inflammatory pathologies and in the design of therapeutic agents.
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