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EliCell: a gel-phase dual antibody capture and detection assay to measure cytokine release from eosinophils

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Abstract

Eosinophils contain many preformed cytokines and chemokines, which are stored in specific granules along with cationic granule proteins. Mobilization and release of these granule contents can be selective and mediated by vesicular transport. We have developed a sensitive method to detect and quantitate eosinophil vesicular transport-mediated release of specific eosinophil proteins. Our EliCell assay is based on microscopic observations of individual viable eosinophils embedded in an agarose matrix that contains immobilized antibody to the protein of interest. Following stimulation of eosinophils, released protein is bound by the capture antibody at its site of release and is detected by a fluorochrome-conjugated detection antibody. We have validated this assay by evaluating interferon-γ-induced release of RANTES from eosinophils. Extracellularly released RANTES was visualized as focal immunoflourescent staining and was quantitated by scoring the numbers of eosinophils releasing RANTES and by measuring the fluorescent intensity over individual eosinophils. In comparison with ELISA assays of RANTES released into supernatant fluids by interferon-γ-stimulated eosinophils, EliCell assays were more sensitive enabling detection of RANTES release at earlier times and at lower levels of interferon-γ stimulation. The EliCell assay provides a sensitive method to study the regulated release of eosinophil-derived cytokines, chemokines and other granule proteins.

Keywords: Eosinophils; ELISA; ELISPOT; EliCell; cytokines; degranulation

1. Introduction

The specific granules of eosinophils are notable for their content of four distinct cationic proteins, major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein and eosinophil-derived neurotoxin, whose extracellular release may cause dysfunction and destruction of other cells (reviewed in Gleich et al., 1992; Kita et al., 1998). Thus, the ill understood process of 'degranulation' constitutes an important mechanism by which eosin-

Abbreviations: IL, Interleukin; RANTES, Regulated upon Activation Normal T-cell Expressed and Secreted; MBP, Major Basic Protein; EPO, Eosinophil Peroxidase; Ig, Immunoglobulin; rh, recombinant human; IFN, Interferon; HBSS, Hanks' Balanced Salt Solution; ELISA, Enzyme-Linked Immunosorbent Assay; SD, Standard Deviation; ANOVA, Analysis of Variance; ELISPOT, Enzyme-Linked ImmunoSpot.

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ophils release their preformed stores of deleterious cationic granule proteins.

In addition, a major advance in the understanding of the functional capabilities of eosinophils has been the recognition that eosinophils are sources of cytokines. Human eosinophils synthesize at least two dozen cytokines or chemokines with diverse proinflammatory and immunoregulatory properties (reviewed in Lacy and Moqbel, 1997; Kita et al., 1998). Notably, many, if not all, of these cytokines are preformed within eosinophils and stored within specific granules. Cytokines, including granulocyte-macrophage colony-stimulating factor (GM-CSF) (Levi-Schaffer et al., 1995; Desreumaux et al., 1998), tumor necrosis factor (TNF)-α (Beil et al., 1993; Beil et al., 1995), IL-16 (Lim et al., 1996), RANTES (Lim et al., 1996), IL-6 (Lacy et al., 1998), IL-3 (Desreumaux et al., 1998), IL-5 (Dubucquoi et al., 1994; Moller et al., 1996a; Desreumaux et al., 1998), IL-8 (Braun et al., 1993), IL-4 (Moller et al., 1996b), vascular endothelial growth factor (VEGF) (Horiuchi and Weller, 1997) and transforming growth factor (TGF)-α (Egesten et al., 1996), have been localized to eosinophil specific granules by techniques including subcellular fractionation, immunogold electron microscopy and/or light microscopy. Within specific granules, the subcompartmental localizations of cytokines have not been fully established, although immunogold electron microscopy has indicated distinctly differing localizations for some cytokines. IL-4 (Moller et al., 1996b) and IL-5 (Moller et al., 1996a) have been localized to the MBP-rich crystalloid core, whereas TNF-α (Beil et al., 1993; Beil et al., 1995) and TGF-α (Egesten et al., 1996) are principally in the granule matrix. IL-3, IL-5 and GM-CSF have been localized by immunogold electron microscopy to the matrix of specific granules (Desreumaux et al., 1998), and RANTES has been localized by confocal fluorescence microscopy to the granule matrix (Lacy et al., 1999).

The packaging of preformed cytokines within eosinophil specific granules enables eosinophils to release quantities of cytokines without the need to engage in their *de novo* synthesis. An example is the rapid release of IL-4 from murine eosinophils to initiate a Th2 response to parasite antigens (Sabin and Pearce, 1995; Sabin et al., 1996). How are these cytokines released? With an armamentarium in the granule of four cationic proteins and over two dozen cytokines with diverse biologic activities, it is probable that not all granule proteins are concomitantly released from eosinophils. Rather it is highly likely that there are regulated mechanisms to release specific granule proteins. Indeed, while the mechanisms of eosinophil 'degranulation' are poorly understood, ultrastructural observations strongly support the existence of mechanisms for selective mobilization of specific eosinophil granule contents.

Unlike mast cells or basophils that undergo acute exocytotic degranulation in response to cross-linking of their high affinity Fcε receptors, a physiologic mechanism to elicit comparable acute degranulation of eosinophils has never been identified (Weller and Dvorak, 1995). In contrast to the acute exocytotic eosinophil 'degranulation' elicitable in vitro by cross-linking Fcγ or Fcα receptors or by agents such as phorbol esters, multiple electron microscopic observations of tissue samples demonstrate that specific eosinophil granule contents are mobilized and released by mechanisms not involving the wholesale exocytosis of specific granules (Dvorak et al., 1994). The ultrastructure of lesional eosinophils provides compelling evidence that eosinophil specific granule contents are mobilized in vivo by selective incorporation into small vesicles that traffic to the cell surface and release these granule contents by a process of 'piecemeal' degranulation based on vesicular transport (Dvorak et al., 1994). In addition, there are some reports showing that, in human diseases, intact membrane-bound eosinophil granules are released into tissues (Gleich et al., 1984; Davis et al., 1998), indicating that besides vesicular transport of granules contents, eosinophils can undergo cytolysis.

Eosinophil degranulation assays are conventionally performed with eosinophils in suspension or adherent to derivatized beads or a solid surface, with degranulation quantitated by assay of released proteins present in supernatant fluids. These assays are not well suited to analyzing the mechanisms of eosinophil degranulation based on vesicular mobilization and transport of granule contents (Weller and Dvorak, 2000). Vesicular transport will mobilize selected granule proteins and release them progressively in small packets in the microenvironment around the eosinophil. Consequently, the levels of

released proteins may not be readily detectable in fluid phase assays of supernatants, especially early in the release process, either because overall levels in the supernatant fluids are too low for assay and/or because some proteins remain bound to eosinophils (as shown for EPO in Henderson and Chi, 1985). Tissue eosinophils are adherent and interact with elements of the extracellular matrix. Integrin-mediated (both $\alpha 4$ and $\beta 2$) interactions are known to augment eosinophil degranulation (Anwar et al., 1993; Anwar et al., 1994; Horie and Kita, 1994; Kaneko et al., 1995). To recapitulate responses of tissue eosinophils, studying eosinophils in an appropriate solid-phase matrix would be advantageous. Further, since the process of vesicular transport of granule-derived proteins has the potential to selectively mobilize some and not all granule-derived proteins, an assay of eosinophil piecemeal degranulation should have the capacity to evaluate the release of more than a single granule component concomitantly. Thus, the study of eosinophil vesicular transport as a means to mobilize preformed eosinophil granule cytokines and other proteins requires new experimental assays.

In this study, we describe a novel method to sensitively evaluate the extracellular release of eosinophil-granule derived cytokines or chemokines. The method, we term the EliCell assay, is a dual antibody detection system in which viable eosinophils are incubated in a solid streptavidin-conjugated agarose matrix which contains a biotinylated capture antibody to the cytokine of interest. Released cytokine is detected with a separate fluorochrome-labeled detection antibody. Since human eosinophils are known to store and release RANTES (Lim et al., 1996; Ying et al., 1996; Lacy et al., 1999), we have utilized the EliCell assay to study the release of this chemokine from eosinophils.

2. Material and methods

2.1. Materials

Agarose with a low-melting point (65.5°C) that remains liquid at 37°C until solidified by cooling (24°C gelling point) after which it remains solid when rewarmed to 37°C was from Promega (catalog # V2111, Madison, WI). Biotinylated capture polyclonal antibodies to RANTES and IL-4 were from R&D Systems (Minneapolis, MN). Detection monoclonal antibody to RANTES (clone 21418.211; R&D Systems) and isotype control mouse IgG_1 (clone 11711.11; R&D Systems) were fluorochrome-labeled using the Alexa™546 protein-labeling kit according to the manufacturer's instructions (Molecular Probes, Eugene, OR). rhIFN-γ, was purchased from Biosource (Camarillo, CA) and A23187 from Sigma Chemical Co. (St. Louis, MO). rhIL-5 and rhGM-CSF were from R&D Systems.

2.2. Purification of human eosinophils

Peripheral blood was obtained with informed consent from a total of 12 normal donors (subjects with no history of atopy), used in different sets of experiments. Blood was mixed with sodium citrate (130 mM, pH 5.2) anticoagulant and 6% dextran-saline (MacGaw, Irvine, CA) in a ratio of 4:1:1 for 30–45 min to facilitate erythrocyte sedimentation. The leukocyte-enriched plasma was harvested, overlaid onto Ficoll-Paque (Amersham Pharmacia, Uppsala, Sweden) and centrifuged at room temperature at 250*g* for 20 min. The granulocyte-enriched cell pellets were collected, washed at 4°C with calcium- and magnesium-free HBSS, and then depleted of erythrocytes by hypotonic saline lysis. Eosinophils were negatively selected using the MACS system (Miltenyi Biotec, Auburn, CA) with anti-CD16 and anti-CD3 immunomagnetic beads to remove neutrophils and remaining T lymphocytes, respectively (Lim et al., 1996). The viability of freshly isolated eosinophils was >95% (by trypan blue exclusion) and the purity was >99% (by HEMA3® staining; Fisher Scientific Co., Pittsburgh, PA). Purified eosinophil suspensions were adjusted to 2×10^6 or 15×10^6 cells/ml in RPMI-1640 medium containing 0.1% ovalbumin (Sigma Chemical Co.) for use in ELISA or EliCell assays, respectively.

2.3. Chemical coupling of streptavidin to agarose

The attachment of streptavidin to agarose was made in a two step procedure. First, 5 ml of a molten low-melting temperature agarose, 2.5% w/v in sterile dH_2O, was poured into thin layers in flasks

(250 ml glass Erlenmeyer), solidified and then activated by overnight incubation at 4°C with 5 ml of 10 mM $NaIO_4$ in pH 5.5 100 mM sodium acetate buffer to generate aldehyde functions. The oxidized gel was washed extensively (e.g. 50 washes for 10 min each) with 20 ml of sterile dH_2O before the coupling step. Reactive agarose was incubated overnight with a molar excess of streptavidin hydrazide (0.2 mg/ml; Pierce Chemical Co., Rockford, IL) at room temperature, allowing the metaperiodate-oxidized agarose to react with the hydrazide group. After extensive washes (e.g. 50 washes for 10 min each) with 10 ml of sterile dH_2O, the streptavidin-agarose conjugate was stored at 4°C and used for at least 3 months. The extent of streptavidin conjugation of agarose assayed spectrophotometrically (500 nm) with 2-(4′-hydroxyazobenzene)-benzoic acid (catalog #28010ZZ, Pierce Chemical Co.) (Janolino, 1996) was ~0.5 μg/ml.

2.4. EliCell assay for the detection of eosinophil-released RANTES

To prepare the agarose matrix, streptavidin-conjugated agarose was melted at 70°C; and then while liquid at 37°C, nine volumes of agarose were mixed with one volume of 10× concentrated RPMI-1640 medium containing 1% ovalbumin at 37°C. Then, one volume of this medium supplemented streptavidin-agarose was mixed at 37°C with three volumes of eosinophils at 15×10^6 cells/ml in RPMI-1640 medium containing 0.1% ovalbumin and one volume of biotinylated antibody to RANTES (100 μg/ml in RPMI-1640 medium containing 0.1% ovalbumin). As indicated, potential agonists, either rhIFN-γ (5 – 500 U/ml) or A23187 (0.5 μM), were added in one tenth volumes to agarose/eosinophil mixtures. Immediately thereafter, 20 μl samples were gently spread onto microscope slides and then covered with CoverWell™ chambers (catalog # PC1L-0.5; Grace Bio-Labs, Bend, OR, USA). The slides were briefly cooled to solidify the agarose matrix and then overlaid with RPMI-1640 medium containing 0.1% ovalbumin and an identical concentration of the stimulus present in the agarose/eosinophil mixture. The slides were incubated at 37°C in a humidified 5% CO_2 atmosphere. After 5, 30, 60 or 180 mins, incubations were stopped by removing the chambers and fixing the cells with 2% paraformaldehyde in HBSS for 5 min. After a quick wash with HBSS, the Alexa546-labeled anti-RANTES detection antibody was added (400 μl of 20 μg/ml) for 45 mins. Slides were washed with HBSS, and aqueous mounting medium (Polysciences Inc., Warrington, PA) was applied to each slide before coverslip attachment. Slides were viewed with a Plan Apo 100× 1:4 Ph3 objective (Nikon; Tokyo, Japan) by both phase-contrast and fluorescence microscopy using a Eclipse TE300 Nikon fluorescence microscope. Electronic photography was performed with a Spot Cooled Color Digital camera (model: 1.3.0-Diagnostic Instruments Inc., Sterling Heights, MI) in conjunction with the image editing program Adobe Photoshop 5.5 (Adobe Systems Inc., San Jose, CA). Two hundred eosinophils were scored and the percentage of those exhibiting red staining for extracellular RANTES release was calculated. In addition, the amount of immunoreactive RANTES released by individual eosinophils was evaluated by quantitating fluorescence intensity around each cell using the software program IPLab (version 3.2.4; Scanalytics Inc., Fairfax, VA). Fifty consecutive eosinophils were evaluated per experimental condition. Fluorescence intensity at each pixel was quantitated in arbitrary units ranging from 0 to 255. Background fluorescence, evaluated with non-immune antibody, was 35 units. The cumulative fluorescence intensities in excess of the background threshold were summed for all pixels overlying each analyzed eosinophil.

An Alexa546-labeled mouse IgG_1 was routinely included as a nonimmune isotype control for the anti-RANTES detection antibody. In addition, three other conditions, (i) employing agarose lacking-streptavidin; (ii) omitting the biotinylated anti-RANTES capture antibody; or (iii) using an irrelevant biotinylated anti-IL4 capture antibody in combination with the Alexa546-labeled anti-RANTES detection antibody, were evaluated.

2.5. Eosinophil viability assay

At the end of incubations, slides containing eosinophils were fixed with 2% paraformaldehyde in HBSS for 5 min, exactly as in EliCell preparations for detection of RANTES release. After a wash with

HBSS, eosinophils were stained in darkness for 15 min at room temperature, following the manufacturer's instructions, with a mixture of the LIVE/DEAD fluorescent dyes (Molecular Probes), consisting of the membrane-permeant SYTO® 10 Green plus the viable cell-impermeant DEAD Red™-fluorescent nucleic acid stain. Percentages of viable cells (green stained cells) were calculated.

2.6. ELISA for detection of eosinophil-derived RANTES

Eosinophils at 2×10^6 cells/ml were incubated (37°C; 5% CO_2) with different concentrations of rhIFN-γ (5–500 U/ml) or A23187 (0.5 μM) in RPMI-1640 medium containing 0.1% ovalbumin. After varying time points, the rhIFN-γ- or ionophore-induced degranulation was stopped on ice and collected supernatants were stored at −20°C until assayed. Soluble RANTES was measured with a dual antibody sandwich Quantikine ELISA kit according to the manufacturer's instructions (R&D Systems). With this immunoassay, the sensitivity was less than 8 pg/ml and the dynamic range was from 31.2 to 2000 pg/ml.

2.7. Statistical analysis

Released-RANTES detected by ELISA assay was quantitatively represented as pg/ml of eosinophil supernatants and by EliCell, as average total cellular fluorescence intensity per eosinophil or as percentage of eosinophils showing immunoreactivity for extracellular RANTES. Data were expressed as mean±SD. Statistical comparisons were done by ANOVA followed by Newman-Keuls Student's test. Differences were considered significant when $P<0.05$.

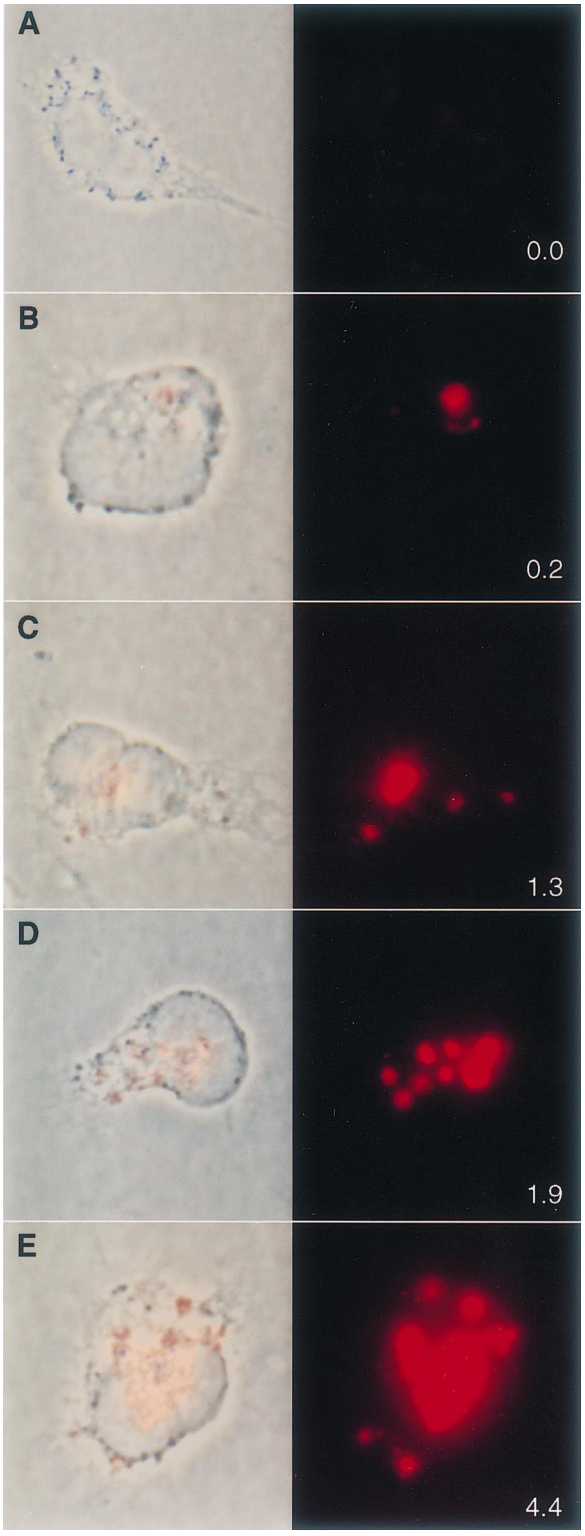
3. Results

3.1. EliCell assay of eosinophil extracellular release of RANTES

The EliCell assay system was developed to enable the sensitive detection and specific quantitation of proteins released, especially by vesicular transport, from eosinophils. These eosinophil-derived proteins include preformed cytokines, chemokines and other granule-stored proteins. To this end, the EliCell system was evaluated by investigating the release of the chemokine RANTES, known to exist preformed in eosinophils (Lim et al., 1996; Ying et al., 1996). Viable eosinophils maintained in tissue culture media without serum or growth factor cytokines were admixed and then incubated in a solidified streptavidin-conjugated agarose matrix. The agarose-matrix bound a biotinylated anti-RANTES antibody to capture and localize any cell released RANTES. After incubations with and without stimuli for 'degranulation', eosinophils were fixed without being permeabilized; and a fluorochrome-labeled anti-RANTES detection antibody was added.

In the EliCell assay, eosinophils incubated for 3 h in the absence of 'degranulating' stimuli exhibited no immunoreactive RANTES fluoresence (Fig. 1A), indicating that intracellular stores of preformed RANTES were neither being detected in the dual antibody capture/detection system nor being released by cytolytic or other mechanisms. In contrast, the degranulation elicited by the calcium ionophore A23187 (0.5 μM) yielded very prominent immunoflourescent staining for RANTES (Fig. 1E). With increasing concentrations of rhIFN-γ (5–500 U/ml), a cytokine known to promote the release of RANTES from eosinophils (Ying et al., 1996; Lacy et al., 1999), there was a progressive increase in the intensity of immunofluorescent staining for released RANTES (Fig. 1B–D).

To validate this dual antibody capture/detection system for the measurement of released RANTES, each element of the system was tested. As noted in Table 1, no RANTES staining was found when streptavidin was not conjugated to the agarose or when either the biotinylated anti-RANTES capture antibody was omitted or was substituted with an irrelevant biotinylated antibody, indicating that the capacity to capture released RANTES locally around the cells was critical to its subsequent detection. In addition, the use of an irrelevant fluorochrome-labeled non-immune control (Alexa546-labeled mouse IgG_1) instead of the Alexa546-labeled anti-RANTES detection antibody yielded no staining (data not shown), supporting the specificity of the immunoreactive RANTES detection. Thus, the



EliCell assay detects extracellularly released RANTES which must be captured and retained in place by a specific capture antibody in order to be subsequently detected by the anti-RANTES detection antibody.

The viability of eosinophils in the 3 h EliCell assays was also monitored. With dual fluorescent dyes to measure both dead and viable eosinophils, 88±5% (mean±SD; $n=4$) of eosinophils were viable in the absence of any serum or growth factors. Addition of either rhGM-CSF (100 ng/ml) or rhIL-5 (100 ng/ml), two cytokines which enhance eosinophil viability, did not further improve their viability (90±4 and 92±4%, respectively; $n=3$) and were not needed to sustain eosinophil survival in EliCell preparations. Even when eosinophils were stimulated with a high dose of rhIFN-γ (500 U/ml), which is also known to enhance eosinophil survival (Valerius et al., 1990), viability remained high (87±8%; $n=3$), indicating that maximal RANTES release was not due to cytotoxic effects on eosinophils of the high dose rhIFN-γ stimulation. In contrast, eosinophils stimulated with 0.5 μM A23187 were only 35±7% ($n=3$) viable. Moreover, A23187 clearly induced eosinophil cytolysis, inasmuch as a large number of cells in the gel-matrix lacked plasma membrane integrity and were surrounded by free granules (data not shown).

Fig. 1. Phase-contrast (left panels) and fluorescent (right panels) microscopy of identical fields of eosinophils incubated for 3 h in RANTES-capturing EliCell preparations. The streptavidin-linked gel-matrix contained biotinylated anti-RANTES capture antibody and was stained with Alexa546-labeled anti-RANTES detection antibody. RANTES immunoreactivity sites (red stainings) are overlaid on phase-contrast images, in order to facilitate their localization around cells. In **A**, non-stimulated eosinophil do not display any fluorescent anti-RANTES staining. **B**, **C** and **D** show representative eosinophils stimulated with 5, 50 or 500 U/ml of rhIFN-γ exhibiting punctate RANTES immunoreactive staining. **E** shows a representative eosinophil stimulated with A23187 (0.5 μM) exhibiting diffuse RANTES immunoreactivity. Digital pictures were taken by a Spot camera on a Nikon microscope using 100× magnification oil objective. The displayed numerical values are the fluorescent intensities of immunoreactive RANTES released by each of the shown eosinophils, as electronically quantified by the software program IPLab.

Table 1
Requirement and specificity of EliCell capture conditions to detect stimulated extracellular release of RANTES from eosinophils[a]

Stimuli	RANTES capture condition		% Eosinophils Releasing RANTES
	SA-matrix	Biotinylated-capture Ab	
Medium	+	anti-RANTES	1.7±1.2
rhIFN-γ	+	anti-RANTES	60.3±14.5**
rhIFN-γ	−	anti-RANTES	Not detected
rhIFN-γ	+	–	Not detected
rhIFN-γ	+	anti-IL4	Not detected

[a] Cells were incubated for 3 h with 500 U/ml of IFN-γ. All different conditions were stained with Alexa546-labelled anti-RANTES detection antibody. Values represent the percentage of total eosinophils surrounded by secreted extracellular RANTES immunoreactivity (red staining). Results are expressed as mean±SD of five donors at non-stimulated and positive conditions and three donors to other groups. **$P<0.01$ compared with non-stimulated condition. SA, streptavidin; Ab, antibody.

3.2. EliCell assay of rhIFN-γ-induced eosinophil RANTES release

By utilizing both phase-contrast and fluorescence microscopy of EliCell preparations, we detected a punctate pattern of RANTES immunoreactivity at a few discrete loci proximate to the surface of 3 h rhIFN-γ-stimulated eosinophils (Fig. 1B–D). The size, number and surface localization of the released RANTES would be compatible with release occurring by means of vesicular transport from viable eosinophils. In contrast, A23187 evoked a different pattern of immunostaining, featuring intense and homogenous pattern of diffuse RANTES reactivity (Fig. 1E) within 3 h, but already detectable as fast as 5 min in 78±9% (mean±SD, $n=5$) of eosinophils and clearly due to disruption of granule/plasma membranes. Therefore, in contrast to rhIFN-γ-induced extracellular RANTES secretion, part of the A23184-induced RANTES immunoreactivity may reflect detection of non-released RANTES remaining within permeabilized and damaged cells. In fact, confocal images revealed that in A23187-stimulated eosinophils the immunoreactive RANTES extensively colocalized within the cell, while RANTES was localized outside of IFN-γ-stimulated cells (data not shown).

With a low concentration of stimulating rhIFN-γ (5 U/ml), just a few small areas of punctate RANTES release (Fig. 1B) were observed and these increased gradually in number, size and intensity with increasing rhIFN-γ concentrations (Fig. 1C–D). Computerized quantitation of fluorescence proximate to stimulated eosinophils confirmed that rhIFN-γ (5–500 U/ml) induced RANTES release in a concentration-dependent fashion from eosinophils (Fig. 2B). Of note, the number of releasing cells also increased with increasing rhIFN-γ (Fig. 2C). For instance, at 5 U/ml of rhIFN-γ small amounts of RANTES were released by ~25% of eosinophil population. On the other hand, at 50 or 500 U/ml, greater proportions of rhIFN-γ-stimulated eosinophils were surrounded by high levels of extracellular RANTES. A comparable high proportion of eosinophils was positive after ionophore challenge (A23187; 0.5 μM), but as expected the amounts of RANTES around these degranulating and damaged eosinophils were significantly higher than with the highest dose of rhIFN-γ (500 U/ml).

Kinetic studies evaluated the time course of RANTES release and the proportion of eosinophils releasing RANTES in response to rhIFN-γ (500 U/ml; Fig. 3). As early as 5 min after stimulation, 15% of rhIFN-γ-stimulated eosinophils were surrounded by immunoreactive RANTES (Fig. 3C). With increasing time, the proportions of eosinophils releasing RANTES progressively increased up to ~75% at 1 h. Parallel quantitative analyses of fluorescence intensity of released immunoreactive RANTES revealed that RANTES was detectable within 5 min, increased further at 30 min and achieved high levels after 1 h (Fig. 3B). Fig. 4 displays a series of images depicting eosinophils releasing RANTES at different time points after

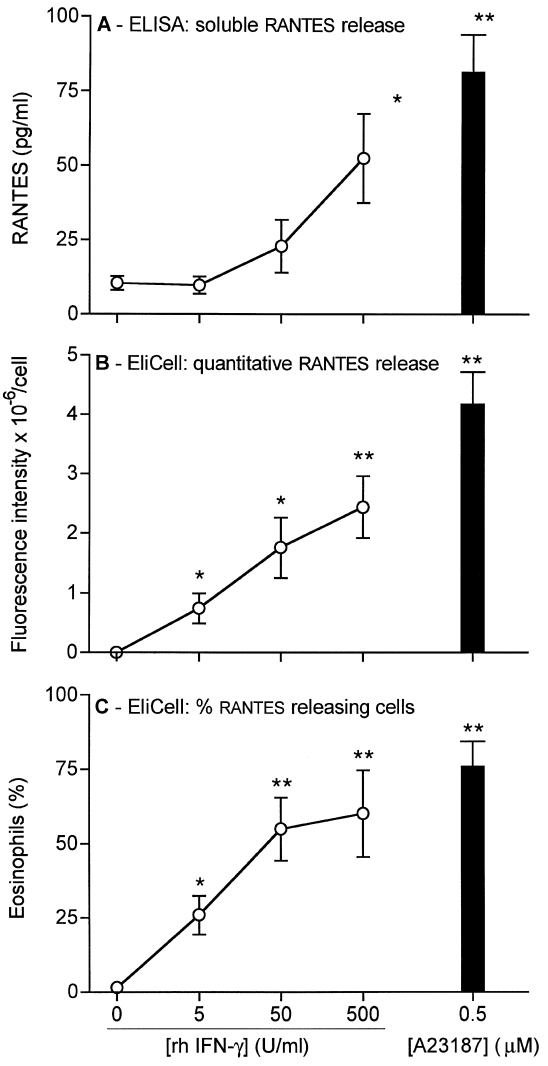


Fig. 2. Dose dependence of rhIFN-γ-induced (5–500 U/ml) RANTES release by eosinophils within 3 h. For comparison, the effect of A23187 (0.5 μM) is also shown (closed bars). **A** shows results obtained using a dual antibody ELISA assay of fluid-phase samples and expressed as pg/ml. **B** and **C** show results from EliCell analysis of gel-phase samples incubated with streptavidin-linked gel-matrix with biotinylated anti-RANTES capture antibody, and stained with Alexa546-labeled anti-RANTES detection antibody. For EliCell assays, RANTES secretion is expressed in two distinct ways: (**B**) average total cellular fluorescence intensity for immunoreactive RANTES around individual eosinophils and (**C**) the percentage of eosinophils surrounded by extracellularly released RANTES. Results are expressed as the mean±SD of five donors at 500 U/ml and three donors at other rhIFN-γ concentrations. * and ** represent $P<0.05$ and $P<0.01$ compared with non-stimulated conditions, respectively.

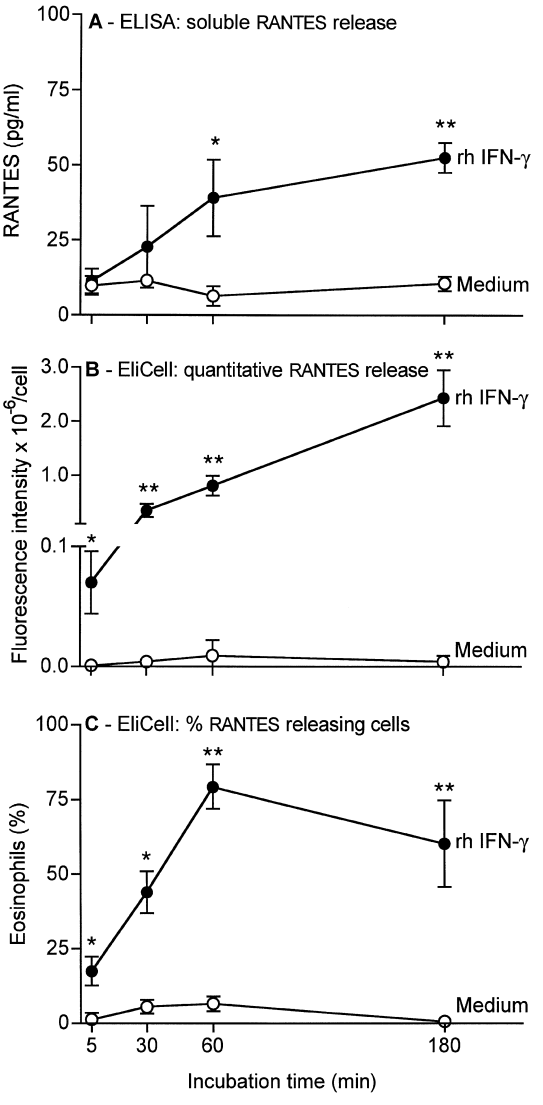


Fig. 3. Time course of RANTES release by eosinophils stimulated with rhIFN-γ (500 U/ml), evaluated by ELISA of fluid-phase samples (**A**) or EliCell of gel-phase samples (**B–C**). ELISA was performed in eosinophils supernatants using a dual antibody assay and the amount of released RANTES is expressed as pg/ml. EliCell preparations included streptavidin-linked gel-matrix with biotinylated anti-RANTES and were stained with Alexa546-labeled anti-RANTES. For EliCell assay, RANTES secretion is expressed in two distinct ways: (**B**) average total cellular fluorescence intensity for immunoreactive RANTES around individual eosinophils and (**C**) the percentage of eosinophils surrounded by extracellularly released RANTES. Results are expressed as the mean±SD of five donors at 3 h and three donors at the other time points. * and ** represent $P<0.05$ and $P<0.01$ compared with non-stimulated conditions at same time points, respectively.

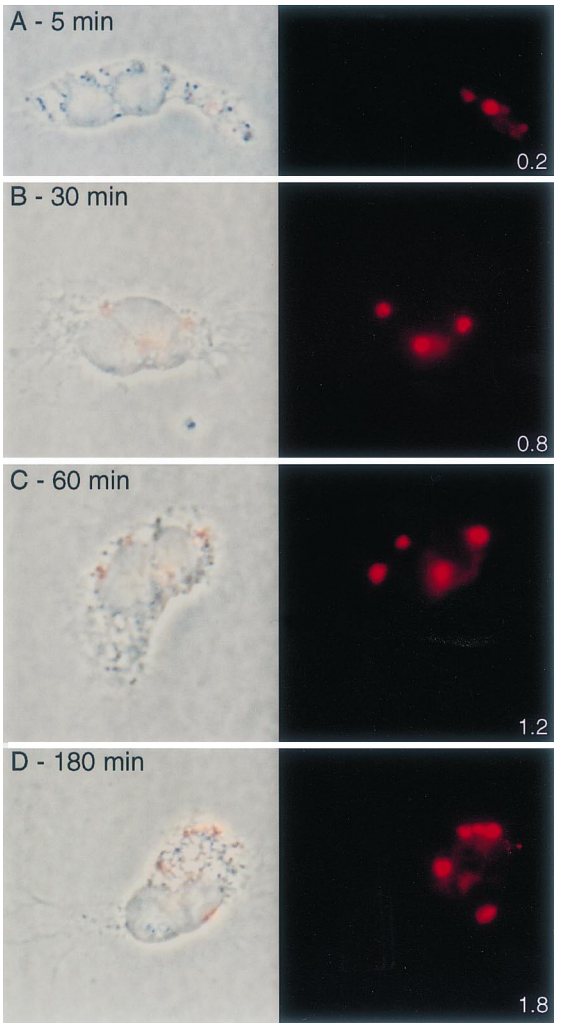


Fig. 4. Phase-contrast (left panels) and fluorescent (right panels) microscopy of identical fields of eosinophils incubated for 5 min (**A**), 30 min (**B**), 1 h (**C**) or 3 h (**D**) in RANTES-capturing EliCell preparations. The streptavidin-linked gel-matrix contained biotinylated anti-RANTES capture antibody and was stained with Alexa546-labeled anti-RANTES detection antibody. RANTES immunoreactivity sites (red stainings) are overlaid on phase-contrast images, in order to facilitate their localization around cells. The images show representative eosinophils stimulated with 500 U/ml of rhIFN-γ exhibiting punctate RANTES immunoreactive staining. Digital pictures were taken by a Spot camera on a Nikon microscope using 100× magnification oil objective. The displayed numerical values are the fluorescent intensities of immunoreactive RANTES released by each of the shown eosinophils, as electronically quantified by the software program IPLab.

IFN-γ stimulation. Although the amount of released RANTES was very low within 5 min, it was significant, punctate and likely due to secretion of preformed stores (Fig. 4A). These findings are in agreement with the rapid depletion of intracellular RANTES stores of IFN-γ-stimulated eosinophils evaluated by cellular fractionation (Lacy et al., 1999). The Elicell assay was highly reproducible, as the same general profile of rhIFN-γ- or A23187-induced RANTES release by human eosinophils was observed in EliCell preparations from 6 different normal subjects.

3.3. Comparison of EliCell and ELISA assays for eosinophil RANTES release

To compare the sensitivities of ELISA versus EliCell assays, both the dose response of RANTES release induced by increasing concentrations of rhIFN-γ (5–500 U/ml; Fig. 2) and the time course of rhIFN-γ-induced RANTES release (Fig. 3) were evaluated in supernatants of fluid phase eosinophils by ELISA and in the EliCell assays with gel-embedded eosinophils.

At lower doses of rhIFN-γ (5–50 U/ml), the EliCell assays detected RANTES release (Fig. 2B–C), whereas by ELISA assays eosinophil supernatants contained no detectable RANTES (Fig. 2A). Analogously, EliCell assays, unlike the ELISA assays, had the sensitivity to detect early rhIFN-γ-induced RANTES release after 30 and even 5 min of stimulation (Fig. 3B–C). By ELISA, statistically significant secretion of RANTES was detected only as late as 1 h post rhIFN-γ (Fig. 3A).

4. Discussion

Conventional ELISPOT assays routinely permeabilize cultured cells, such as lymphocytes, to detect newly formed cytokines or immunoglobulins (King et al., 1990). These assays are not suitable for eosinophils, which contain preformed cytokines and other granule proteins. Thus, to detect only extracellularly released proteins by immunostaining, eosinophils must remain viable and impermeant to detecting antibodies. Our EliCell assay is based on substantial modifications of techniques employed to

capture and detect cytokines released by other cell types (i.e. the sandwich ELISPOT method) (Favre et al., 1997; Beech et al., 1997). Low-gelling point agarose is used since after it is melted it can can be kept liquid at 37°C which enables eosinophils to be incorporated within it without thermal damage to the cells. Eosinophils in the medium-enriched agarose remained viable, responded to a known stimulus, IFN-γ, by releasing RANTES, and remained impermeant to anti-RANTES detection antibody. Conjugation of the agarose with streptavidin enabled a biotinylated capture antibody to bind and localize extracellularly released RANTES. As shown in Table 1, without these capture conditions, released RANTES was not retained and detected. Thus the assay was not confounded by the intracellular stores of preformed RANTES, but specifically detected only RANTES released extracellularly from the IFN-γ-stimulated eosinophils.

The capacity of the EliCell assay to capture released RANTES (or other proteins) at the site of its release on the surface of individual eosinophils has several benefits. First, the assay has high sensitivity. In comparison with conventional ELISA assays of RANTES released into supernatant fluids by eosinophils, the EliCell assay proved to be very appreciably more sensitive. RANTES release was detectable by EliCell with 100 fold lower concentrations of IFN-γ stimulus than needed in ELISA assays. Second, fewer eosinophils are needed in EliCell assays than in conventional fluid phase degranulation assays. Third, the heightened sensitivity enabled release of RANTES to be detected as early as 5 minutes after stimulation, at levels not detectable by ELISA. As noted in the Introduction, it is likely that vesicular transport of granule-derived proteins is an active means by which eosinophils release packets of their preformed cytokines and other granule proteins. Such release may yield high local concentrations of eosinophil-derived cytokines that would be pertinent in vivo to cell–cell interactions in tissue sites, but these local levels are not detectable by conventional ELISA assays. EliCell assays have both the inherent sensitivity and the capacity to monitor the morphologic features of eosinophil ‘degranulation’ that will be needed to study the regulation of eosinophil vesicular transport mediated degranulation.

While we have validated the EliCell assay by evaluating the IFN-γ stimulated release of RANTES from eosinophils, these assay methods are amenable to multiple modifications. EliCell can be used to assay other cytokines released by eosinophils, including IL-4 (data not shown). Further the assay can be used to study differential response of eosinophil from normal and atopic donors. Fibronectin or other extracellular matrix components can be added to the agarose matrix to facilitate studies of integrin-mediated augmentation of degranulation responses. EliCell assays can utilize the co-stimulatory effects of extracellular matrix components to study immobilized eosinophils, in contrast to studying eosinophils artificially suspended in fluid phase media. Moreover, EliCell assays have the sensitivity to detect differential release from single eosinophils of one or more granule-derived proteins. An additional biotinylated capture antibody can be included concomitantly to evaluate differential release of two proteins or release of two or more proteins can be evaluated concomitantly in parallel assays. Thus, our EliCell technique with viable eosinophils immobilized in an agarose matrix provides a novel and robust method to investigate vesicular transport and piecemeal degranulation from eosinophils.

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