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Protocol

EliCell assay for the detection of released cytokines from eosinophils

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Abstract

Eosinophils contain several preformed cytokines within their specific granules. Therefore, without requiring them in de novo synthesis of cytokines, eosinophils can release quantities of granule-derived cytokines by highly regulated mechanisms. However, eosinophil "degranulation" is poorly understood, in part, because available methodologies did not appear appropriate for analyzing vesicular mobilization and transport of eosinophil granular contents. The EliCell assay is a microscopic methodology substantially modified from other techniques employed to detect cytokine release (i.e., ELISPOT). The method is a dual antibody capture/detection system in which viable eosinophils are incubated in a solid streptavidin-conjugated agarose matrix, which contains a biotinylated capture antibody against the cytokine of interest. Released cytokine is detected around non-permeabilized eosinophils with a separate fluorochrome-labeled detection antibody. Thus, the EliCell system captures and detects extracellular cytokines at the site of their release from eosinophils. As examples, we have used EliCell essays to detect the selective release of either IL-4 or IL-12 cytokines found preformed in eosinophils—from eotaxin- or anti-CD9-stimulated eosinophils, respectively. With appropriate pairs of antibodies, any preformed cytokine found into eosinophil granules could be studied and the mechanisms of their secretion evaluated by using the EliCell assay.

Keywords: EliCell; ELISA; Eosinophils; Cytokines; Degranulation; Cytokine release

1. Background

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 by Gleich et al., 1992; Kita et al., 1998). 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 dozens of cytokines or chemokines with diverse proinflammatory

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and immunoregulatory properties (reviewed by Lacy and Moqbel, 1997; Kita et al., 1998). Notably, many, if not all of these cytokines (e.g., RANTES, IL-4 and IL-5), are preformed within eosinophils and stored within specific granules.

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. How are these cytokines released? 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. 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).

Available methodologies did not appear appropriate for analyzing the mechanisms of eosinophil degranulation based on vesicular mobilization and transport of granule contents. First, eosinophil degranulation assays are conventionally preformed with eosinophils in suspension or adherent to derivatized beads, with degranulation quantified by assay of released proteins present in supernatant fluids by ELISA assays. 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 by ELISA, especially early in the release process, 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). Second, tissue eosinophils are adherent cells that interact with elements of the extracellular matrix. To recapitulate responses of tissue eosinophils, studying eosinophils in an appropriate gel-phase matrix would be advantageous. Third, 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. Altogether, the study of eosinophil vesicular transport as a means to mobilize preformed eosinophil granule cytokines and other proteins requires new experimental assays.

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). The method 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 around non-permeabilized eosinophils with a separate fluorochrome-labeled detection antibody. Initially, we developed the EliCell assay to study the release of RANTES from eosinophils (Bandeira-Melo et al., 2000), but, currently, we have adapted the technique to evaluate the mechanisms of release of several other cytokines, including IL-4 (Bandeira-Melo et al., 2001), IL-12, IL-13 and IL-5.

2. Type of research

(i) Extracellular detection of different cytokines released by individual eosinophils under stimulation.
(ii) Study of immune functions of human eosinophils.

3. Time required

3.1. Ahead of time

Chemical coupling of streptavidin to agarose: 2–3 days (stock solution good for at least 6 months).

3.2. Stepwise

Preparation of agarose/cell mixture: 20 min.
Preparation of stimuli: 20 min.

Preparation of chambered slides containing agarose/cell mixtures: 20 min.
Incubation of EliCell preparations: ranging from 10 min to 3 h.
Fixation and washing of EliCell preparations: 5 and 10 min.
Labeling of released cytokines: 45 min.
Washing the unbound detecting antibody: 3 ×, 10 min each.
Drying slides: at least 20 min or overnight in the fume hood.
Mounting slides: 10 min.
Analysis of cells by microscopy: slides can be analyzed until 7 days after mounting.

4. Materials

4.1. Streptavidin-conjugated agarose

Agarose with a low melting point (65.5 °C), which remains liquid at 37 °C until solidified by cooling (24 °C gelling point) after which it remains solid when re-warmed to 37 °C, is from Promega (catalog no. V2111, Madison, WI). Streptavidin hydrazide is from Pierce Chemical (catalog no. 21120, Rockford, IL).

4.2. Medium for cell incubation

(a) RPMI-1640 medium supplemented with 0.1% ovalbumin (grade VI, catalog no. A-2512; Sigma, St. Louis, MO). In addition, the capturing antibodies and all stimuli are also diluted with such medium.
(b) 10 × concentrated RPMI-1640 liquid (Sigma) containing 1% ovalbumin is added in one-tenth volumes to the agarose matrix.

4.3. Pairs of capturing and detection anti-cytokine antibodies

Biotinylated capturing antibodies and fluorochrome-labeled detection antibodies against a specific targeted cytokine:

(a) RANTES: biotinylated capture goat polyclonal antibody to RANTES (catalog no. BAF278) plus Alexa546-labeled detection monoclonal antibody to RANTES (clone 21418.211; mouse IgG1) (both from R&D Systems, Minneapolis, MN).
(b) IL-4: biotinylated capture goat polyclonal antibody to IL-4 (catalog no. BAF204) plus Alexa546- or Alexa488-labeled detection monoclonal antibody to IL-4 (clone 3010.211; mouse IgG1) (both from R&D Systems).
(c) IL-12: biotinylated capture goat polyclonal antibody to IL-12 (catalog no. BAF219) plus Alexa546-labeled detection monoclonal antibody to IL-12 (clone 24910.1; mouse IgG1) (both from R&D Systems).
(d) IL-5: biotinylated capture goat polyclonal antibody to IL-5 (catalog no. BAF205; R&D Systems) plus PE-labeled detection monoclonal antibody to IL-5 (clone JES1-39D10; rat IgG2a; BD Pharmingen).
(e) IL-13: biotinylated capture mouse monoclonal antibody to IL-13 (catalog no. 555054; BD Pharmigen) plus Alexa-labeled detection monoclonal antibody to IL-13 (catalog no. MAB213; R&D Systems).

Alexa546- and Alexa448-labeled matching isotype mouse IgG1 (clone 11711.11; R&D Systems) and PE-labeled non-immune rat IgG2a (catalog no. IC006P; R&D Systems) are used as irrelevant controls. Alexa546- and Alexa488-labeled antibodies were prepared using the Alexa™546 (red) or Alexa™488 (green) protein labeling kits, respectively, according to the manufacturer's instructions (Molecular Probes, Eugene, OR).

4.4. Fixative

Solution in HBSS (Gibco, Rockville, MD) of 2% paraformaldehyde (catalog no. 15710; Electron Microscopy Sciences, Washington, PA) is methanol-free, to avoid any potential unwanted cell loss of cellular membrane selectivity, and should be used in the same day.

4.5. Mounting medium for slides

Aqueous mounting medium (Polysciences, Warrington, PA).

4.6. Potential stimuli

(1) A classical physiological stimulation with the recombinant chemokine eotaxin (R&D Systems); (2) a cytolytic/exocytic non-physiological challenge with the calcium ionophore A23187 (Sigma); and (3) an activating anti-CD9 antibody (mouse IgG1; clone ALB6; Immunotech, Jackson ImmunoResearch, West Grove, PA).

4.7. Special equipment

(a) Ultra-microtips (elongated shape) (catalog no. 1111-4000; USA Scientific, Ocala, FL).
(b) Microscope slides (frosted) from Fisherfinest (catalog no. 12-544-2).
(c) CoverWell™ perfusion chambers (catalog no. PC1L-0.5) from Grace Bio-Labs (Bend, OR).
(d) Hydrophobic barrier Pap-pen (catalog no. 71310) from Electron Microscopy Sciences (optional).
(e) Two water baths (at 70 and at 37 °C each).
(f) Cell incubator at 37 °C with a humidified 5% CO_2 atmosphere.
(g) Fluorescence inverted microscope (Eclipse TE300, Nikon, Tokyo, Japan) equipped with a Plan Apo 100 × 1.4 Ph3 objective (Nikon) and a Cooled Color Digital camera (Spot 1.3.0, Diagnostic Instruments, Sterling Heights, MI) in conjunction with an image editing software (Adobe Photoshop 5.5, Adobe Systems, San Jose, CA).
(h) Image analysis program IPLab (version 3.2.4; Scanalytics, Fairfax, VA).

5. Detailed procedure

5.1. Chemical coupling of streptavidin to agarose

The attachment of streptavidin to agarose is a two-step procedure. First, 5 ml of a low-melting temperature agarose, 2.5% w/v in sterile dH_2O, is solubilized at 70 °C, poured into thin layers in flasks ~ 3 in. in diameter (250-ml Erlenmeyer glass) and then solidified at 4 °C. To generate aldehyde functions in the agarose molecules, pour 5 ml of 10 mM $NaIO_4$ in pH 5.5 100 mM sodium acetate buffer on top of the solidified agarose and incubate at 4 °C overnight. The oxidized gel is then extensively washed with 1 l of sterile dH_2O (e.g., 50 washes with 20 ml for about 10 min each) before the coupling step. To chemically couple streptavidin, incubate overnight the reactive agarose with 5 ml of a molar excess of streptavidin hydrazide (0.2 mg/ml; Pierce Chemical) at room temperature, allowing the metaperiodate-oxidized agarose to react with the hydrazide group of the streptavidin. After extensive washes with 1 l of sterile dH_2O (e.g., 50 washes with 20 ml for about 10 min each), store aliquots of the streptavidin–agarose conjugate at 4 °C and use them for 3–6 months. The extent of streptavidin conjugation of agarose can be assayed spectrophotometrically (500 nm) with 2-(4′-hydroxyazobenzene)-benzoic acid (catalog no. 28010ZZ; Pierce Chemical) (Janolino, 1996). The concentration of streptavidin in five different agarose preparations ranged from 0.45 to 0.7 μg/ml.

5.2. Preparation of agarose/cell mixture

To prepare the cytokine-capturing agarose/cell matrix, first melt the stock solution of streptavidin-conjugated agarose at 70 °C and then place it in a water bath at 37 °C. While still liquid at 37 °C, mix nine volumes of agarose matrix with one volume of 10 × concentrated RPMI-1640 medium containing 1% ovalbumin. Still at 37 °C, mix one volume of this medium-supplemented streptavidin–agarose 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 against the targeted cytokine (stock solution: 100 μg/ml in RPMI-1640 medium containing 0.1% ovalbumin).

5.3. Addition of stimuli

Potential agonists are added in one-tenth volumes to agarose/eosinophil mixtures. Prepare small test tubes containing 2 μl of each agonist at concentrations 10 × higher than the desired ones. Then, add 20 μl of agarose/cell matrix and gently pipette the mixture once up and down to mix the cells with stimulus.

5.4. Preparation of chambered slides

Immediately thereafter, gently ‘‘spread’’ (with special elongated tips) or simply put a ‘‘drop’’ containing

20 μl of mixture samples (with or without stimulus) onto microscope slides. In the first case, avoid touching the slides with the tip (use surface tension to move agarose mixture throughout the slide). Cover gently this thin layer of agarose/cell matrix with the perfusion chambers. Overlay the agarose/cell mixture (which is already inside of a chamber) by pipetting 400 μl RPMI-1640 medium containing 0.1% ovalbumin plus an identical concentration of the agonist present in the agarose/eosinophil mixture through access ports found in the chamber surface. The slides should be briefly (5 min) cooled at 4 °C before incubation.

5.5. Cell incubation and fixation

The slides are incubated at 37 °C in a humidified 5% CO_2 atmosphere. After 5, 30, 60 or 180 min, stop the incubations by removing the chambers carefully from the slides and immediately fix the cells by immersing them into a 2% paraformaldehyde in HBSS for 5 min. Avoid higher concentrations of paraformaldehyde or longer incubations as this may lead to cell membrane permeabilization.

5.6. Staining of released cytokines

After a 10-min wash with HBSS, place the slides flat in a humidified environment. Use a hydrophobic barrier pen or simply dry the slide border to define the area within the slides containing cells. In the first case, do not touch the agarose with barrier pen to avoid cell damage. Centrifuge the fluorochrome-labeled anti-cytokine detection antibody (15 000 × *g*, 30 min) and add the diluted (1:1000) supernatant (400 μl; concentration of stock solution will vary) to those areas for 45 min. Wash the slides three times with fresh HBSS (10 min each). Let the slides dry at room temperature and apply the aqueous mounting medium (Polysciences) to each slide before coverslip attachment.

5.7. Data analysis and presentation

Slides are viewed with a 100× 1.4 Plan Apo Ph3 objective (Nikon) by both phase-contrast and fluorescence microscopy. To document cell morphology and fluorescent pattern of immunoreactivity of the targeted cytokines, digital photography is performed with a Spot Cooled Color Digital camera (model 1.3.0, Diagnostic Instruments) in conjunction with the image-editing program Adobe Photoshop 5.5 (Adobe Systems). A total of 200 eosinophils are scored, and the percentage of those exhibiting fluorescent staining for extracellular cytokine release is then calculated. Any damaged cells showing morphological alterations are not scored. In addition, the amount of immunoreactive cytokine released by individual eosinophils can be evaluated by quantifying fluorescence intensity around each cell using the image analyzes software IPLab. To this end, 50 consecutive eosinophils are evaluated per experimental condition. Fluorescence intensity at each pixel is quantified in arbitrary units ranging from 0 to 255. We have arbitrarily fixed the background fluorescent value, evaluated with the non-immune fluorochrome-labeled antibody, as 35 units. The cumulative fluorescence intensities in excess of this background threshold are summed for all pixels overlying each analyzed eosinophil. None of the images have saturated pixels.

5.8. Essential controls

A fluorochrome-labeled matching antibody needs to be routinely included as a non-immune isotype control for the anti-targeted cytokine detection antibody. In addition, three other conditions, (i) employing agarose lacking streptavidin, (ii) omitting the biotinylated anti-targeted cytokine capture antibody and (iii) using an irrelevant biotinylated isotype control capture antibody in combination with the fluorochrome-labeled anti-targeted cytokine detection antibody, can be evaluated.

6. Results

Human blood eosinophils were stimulated in order to release cytokines to be detected extracellularly by the EliCell strategy. EliCell results can be analyzed by the following two complementary ways.

6.1. Evaluating cell morphology and immunofluorescent patterns of a released cytokine

This kind of analysis is an elegant and powerful way to identify the secretory process involved in a

given stimulatory condition. As an example, Fig. 1 illustrates that punctate immunoreactivity for IL-4 and IL-12 detected at a few discrete loci proximate to cell surface is the prevalent fluorescent pattern around eotaxin- and anti-CD9-stimulated eosinophils. The form, size, number and surrounding extracellular localization of the immunoreactive cytokines under such physiological stimulation are compatible with release occurring by means of vesicular transport-mediated piecemeal degranulation. With increasing concentrations of eotaxin (0.6 and 6.0 nM) or anti-CD9 mAb (0.25 and 2.5 μg/ml), there was a progressive increase in the percentage of eosinophils showing immunofluorescent staining for released IL-4 or IL-12 (Fig. 2).

A different pattern, featuring intense, uniformly broad and even intracellular immunostaining for both IL-4 (Fig. 1) and IL-12, was elicited by A23187 stimulation, indicating the involvement of release processes resulting from cytolysis and/or exocytosis. Moreover, by assessing morphological parameters in EliCell preparations, eosinophil features of either

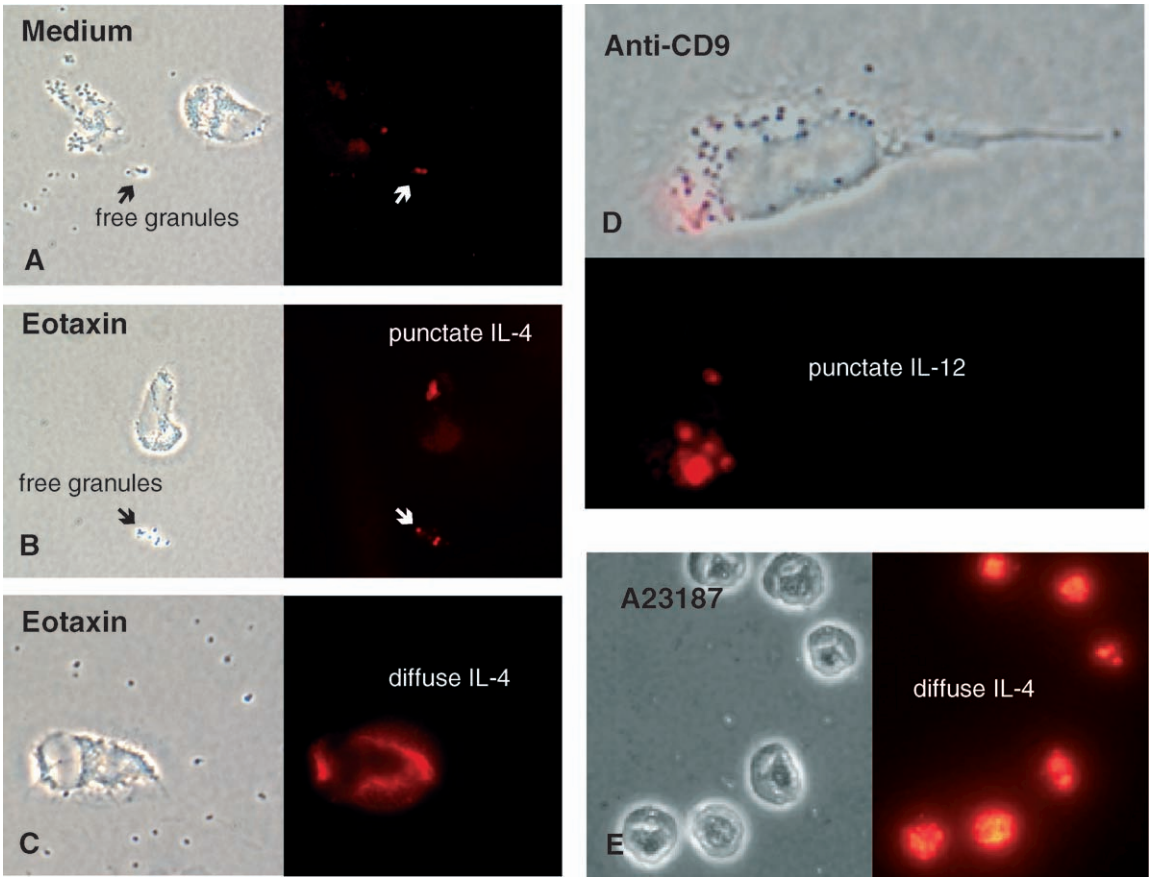

Fig. 1. Phase-contrast and fluorescence microscopy (red stainings) of identical fields of eosinophils incubated in IL-4 (A, B, C and E)- or IL-12 (D)-capturing EliCell preparations. The streptavidin-linked gel matrix containing biotinylated anti-IL-4 or anti-IL-12 capture antibody was stained with Alexa546-labeled anti-IL-4 or anti-IL-12 detection antibody, respectively. In A, non-stimulated eosinophils do not show fluorescent immunoreactive IL-4. B and C show representative eosinophils stimulated with 6 nM of eotaxin exhibiting punctate (B) or diffuse (C) immunoreactive staining. D shows a representative eosinophil stimulated with 2.5 μg/ml of anti-CD9 exhibiting punctate IL-12 red fluorescence and in E, several eosinophils, stimulated by A23187 (0.5 μM), exhibit diffuse IL-4 immunoreactivity. Arrows indicate free granules from cytolytic cells showing fluorescence staining. Digital pictures were taken in a Spot camera on a Nikon or Olympus microscope using 100 × magnification oil objective.

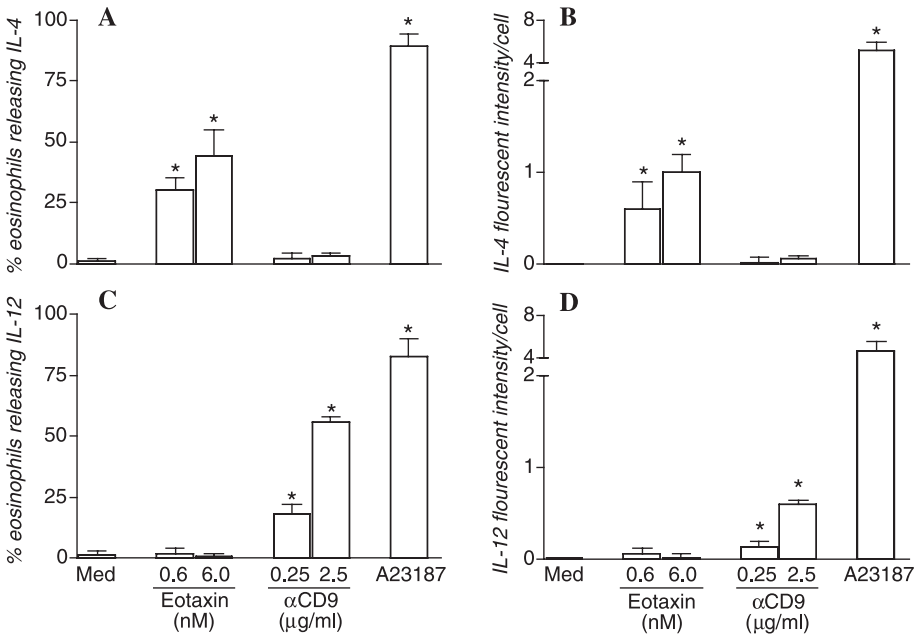

Fig. 2. Extracellular release of IL-4 (A and B) and IL-12 (C and D) by human eosinophils in EliCell assays. In A and C, the percentages of eosinophils with fluorescent staining for each cytokine are presented; and in B and D, the fluorescent intensities were electronically measured to evaluate cytokine release. Extracellular release of IL-4 and IL-12 was detected after stimulation with eotaxin (0.6 or 6 nM) or anti-CD9 antibody (0.25 μg/ml or 2.5 g/ml). The cells were kept in agarose–streptavidin matrix containing biotinylated anti-IL-4 or anti-IL-12 for 1 h. The detection of released protein was performed by adding the Alexa546-labeled-anti-IL-4 or anti-IL-12 antibodies. Stimulation with the cytolytic/exocytotic A23187 (0.5 μM) was used as a positive control. Analysis of eosinophils was performed by phase-contrast and fluorescence microscopy. Results are expressed as the means ± S.D. of different donors. *Means $P < 0.05$ compared with non-stimulated eosinophils.

activation (increase in cell size, presence of vacuoles and voluminous nucleus with high segmentation) or damage (condensed nucleus and/or cytoplasm, loss of nuclear segmentation, disrupted cell, free granules) can be easily detected.

6.2. By indirect semi-quantification of the amounts of a released cytokine

In EliCell preparations, such analysis can be performed by electronic measurements of the fluorescence intensity around each eosinophil. Fluorescence intensity values are derived from color pixels in acquired images and expressed in arbitrary numbers that, in turn, denote the amounts of released cytokine. The capacity of the EliCell assay to capture released cytokine at the site of its release on the surface of individual eosinophils has a high level of sensitivity for detecting even low amounts of extracellular cytokine. As shown on Fig. 2, IL-4 and IL-12 release were detectable by EliCell system with concentrations of eotaxin and anti-CD9 that were not able to trigger extracellular levels of released cytokine detectable by conventional ELISA assay of cell supernatants (not shown) (Bandeira-Melo et al., 2000, 2001).

7. Discussion

The EliCell assay captures extracellularly released proteins at the site of their release on the surface of individual eosinophils. Inasmuch as eosinophil-derived cytokines are stored preformed in the eosinophil granules, the EliCell system was developed to enable a specific and sensitive detection of low levels of cytokines released gradually, especially by vesicular transport, from eosinophils. For the EliCell examples shown here, we were able to detect either IL-4 or IL-12-cytokines known to exist preformed in eosinophils. As physiological agonists, we used the chemo-

kine eotaxin and an activating antibody against the surface molecule CD9. In addition, the calcium ionophore A23187 was used as a cytolytic/exocytic stimulus.

Valid EliCell analyses require that cells should remain viable and non-permeabilized during the process. The kinetics of cell death was analyzed during all the EliCell steps by the ethidium bromide exclusion test and by cell morphological evaluation both in phase-contrast and bright-field (staining with Hema 3®, catalog no. 122-911; Fisher Scientific) microscopy. Most cells (95%) remain viable during all the procedures, even at the end of EliCell (Fig. 3), but a small population (1–5%) undergo degeneration and die by apoptosis (Fig. 3). Any cells showing apoptotic signs (nuclear and cytoplasmic condensation and loss of nuclear segmentation amongst other features) can be identified at the end of the EliCell procedures.

7.1. Trouble shooting

The following are the common problems and non-obvious features found in EliCell preparations with their possible explanations and potential solutions.

7.1.1. No cytokine detection

When few or no cytokine specific immunostaining is observed (but expected), the problem usually lies in the selection of the anti-cytokine antibodies (most commonly the capturing antibody). To solve such problem, it is possible to increase the concentration of capture antibody or the time of exposure of the detection antibody. Alternatively, the chosen pair of capture/detection antibody may be incompatible and may need to be changed. Capture and detection antibodies should not recognize the same antigenic determinant of a cytokine, therefore, it is advisable to use a monoclonal antibody in association with a polyclonal

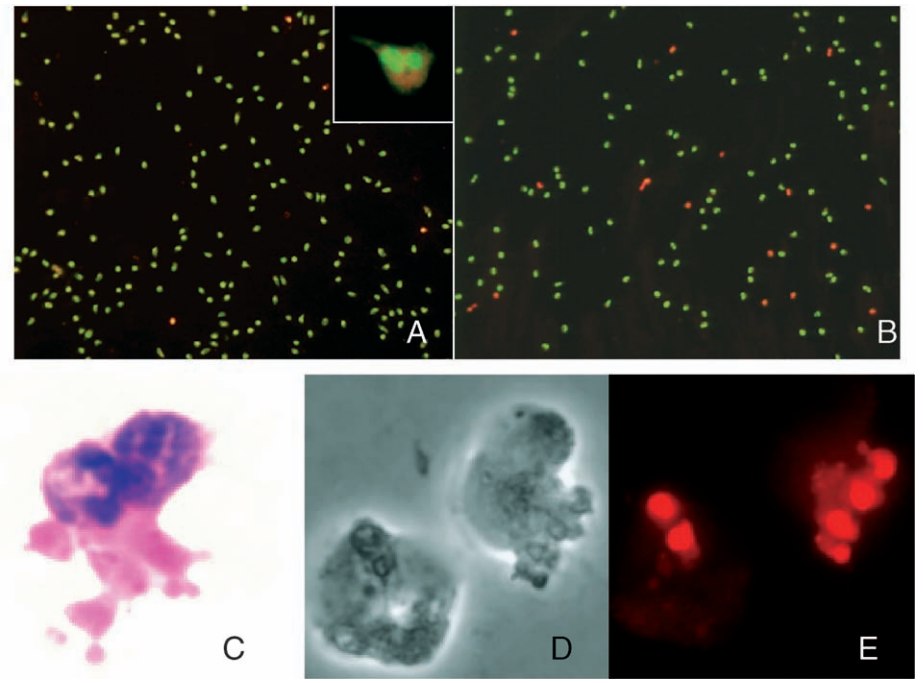

Fig. 3. Fluorescence (A, B and E), phase-contrast (D) or bright-field (C) microscopy of representative non-stimulated eosinophils. (A and B) Most cells show green fluorescent nucleus (live cells) after staining with ethidium bromide in the beginning—10 min (A)—or at the end—3 h (B)—of EliCell procedures. In C, an apoptotic cell observed at the end of the EliCell assay is shown. D and E exhibit identical fields of dying eosinophils incubated in IL-4-capturing EliCell preparations showing large fluorescent spots. The streptavidin-linked gel-matrix containing biotinylated anti-IL-4 capture antibody was stained with Alexa546-labeled anti-IL-4 detection antibody. Bright-field observations were performed after staining with Hema 3. Digital pictures were taken in a Spot camera on a Nikon or Olympus microscope using 20× (A and B) or 100× (C–E) magnification objective.

antibody. In addition, the lack of cytokine release can be due to inefficient stimulation; a positive control with a known agonist should be always included in the experiments.

7.1.2. Cytokine detection around non-stimulated cells

As an important negative control, non-stimulated eosinophils should not show any immunostaining for the targeted cytokine. However, eosinophil activation during the procedures of cell isolation or EliCell preparation can lead to a spontaneous, stimulus-independent release of granule-derived cytokines. Throughout cell purification and preparation, care is needed to ensure that eosinophils are not mechanically, chemically or immunologically stimulated. To assess such problems, EliCell preparations containing both capturing and detection antibodies for a specific cytokine but without a stimulus need to be included. Unexpected cytokine detection onto eosinophils can also result from non-specific detection (discussed below).

7.1.3. Non-specific detection of extracellular cytokines

Fluorescent detection antibodies may non-specifically bind to released proteins deposited around stimulated eosinophils or bind to the eosinophils themselves. To investigate in EliCell preparations, non-specific binding—the tendency for cells to be sticky and bind antibodies through low affinity non-antigen biding site-mediated interactions—a proper control using host/isotype-matched irrelevant antibodies, must be included. If non-specific staining is too high (>10% positive), there are several possible remedies. The detecting antibody may be diluted further, or a different one from a different host may be tried. Also, it is possible to try an adsorbing reagent that effectively blocks out non-specific sites, such as a normal serum (same host of the detecting antibody). Non-specific fluorescence can also be detected when the solution of detecting antibody contains a high degree of aggregated antibody. It is important to centrifuge the detecting antibody, as emphasized in Section 5.6. In addition, apoptotic cells, mainly in advanced stage of disintegration, can exhibit large and sharp fluorescent spots and should be excluded by morphological evaluation in phase-contrast (Fig. 3). Non-specific fluorescence found during EliCell for disintegrating apoptotic cells was also described by Janatpour et al. (2002) in leucocyte debris during flow cytometry-base method.

7.1.4. Cytokine detection inside non-stimulated cells

The EliCell assay is designed to study released cytokines found extracellularly around stimulated eosinophils. The unwanted intracellular immunostaining of granular stocks of preformed cytokines is a potential problem with the EliCell system. The trained observer can easily discriminate between real secreted cytokine and this artifact. Analysis under confocal microscopy can confirm such problem. Loss of cell membrane impermeability enables antibody to enter the cell and results in fluorescent immunostaining of cytoplasmic granules without labeling of the nucleus. The penetration of the detecting antibody inside eosinophils can be avoided by care during both agarose embedding and fixation steps. In addition, extra care is important so as not to dry the slides during all the procedures. The slides are dried only at the end of EliCell. Paraformaldehyde fixation cross-links proteins are often sufficient to protect cytoplasmic antigens from being washed out of the cells since such fixative adequately preserve cell membrane integrity. However, high concentrations of and/or long incubations with paraformaldehyde can damage eosinophil plasma membranes, therefore enabling antibody entrance. The presence of methanol in paraformaldehyde solutions could worsen the problem by dissolving membrane lipids and enhancing permeability to detecting antibodies. Therefore, the use of methanol-free paraformaldehyde solution and a re-evaluation of fixative concentration and duration may resolve such problem.

7.1.5. Cytolysis

There are 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 (Erjefält and Persson, 2000). Throughout the EliCell preparations, it is possible to observe signs of eosinophil lysis, indicated by the presence of free eosinophil granules. Occasionally, these granules showed fluorescent staining (Fig. 1). Although the evaluation of the extend of cytolysis is difficult, we have tried to study it by counting the proportion of cytolytic eosinophils (presence of surrounding free granules) per EliCell preparation. Of note, the cytolytic population can be variable depending upon cell

donor, but it usually does not represent more than 15% of whole eosinophil population. Such proportion may or not be present in the beginning of incubation and does not increase, even, after 3 h of incubation. Moreover, we have not identified any agonist (tested physiological stimuli include eotaxin, RANTES, IL-16, IL-5, IFN-γ, anti-CD9 and others) capable of inducing further eosinophil cytolysis. Mechanical cytolysis of eosinophils can be also caused by inappropriate manipulation of the cells. Agarose spreading is a critical step and extra care should be taken when long tips are used. Alternatively, a simple drop can be placed onto slides and the agarose/cell matrix will spread by adding the subsequent medium.

7.1.6. Pericellular cytokine immunostaining

In EliCell preparations, the punctate pattern that characterizes the extracellular cytokine immunostaining—closely proximate to the eosinophil surface—is ultimately controlled by the concentration of the biotinylated capturing antibody needed to fix the cytokine at the immediate sites of its release. This kind of immunostaining pattern is an advantageous feature of EliCell parameters, because it reveals the involvement of vesicular transport in cytokine release. Intentional or accidental reductions in the concentrations of capturing antibody levels in the gel matrix will let the released cytokine diffuse from its site of release. As shown in Fig. 1, for eotaxin-induced IL-4 release in a gel matrix containing half of the usual concentration of anti-IL-4 capturing antibody, the immunostaining pattern appears as a fluorescent rim surrounding the eosinophil perimeter without defining the spots of vesicular release. Interestingly, this provides documentation that the focal release of preformed cytokine by eosinophils may enable the cytokine to diffuse locally to stimulate adjacent cells, such as in sites of allergic inflammation.

8. Quick procedure

8.1. Agarose preparation

(i) Weigh 0.125 g agarose and suspend in 5 ml of sterile distilled water in a 250-ml Erlenmeyer glass flask. Do not swirl to avoid agarose binding onto the wall.
(ii) Melt the agarose in a 70 °C water bath for 15 min.
(iii) Solidify at 4 °C for 20 min.
(iv) Add 5 ml of periodate solution.
(v) Place at 4 °C overnight with protection from light.
(vi) Discard the periodate solution.
(vii) Wash by adding 20 ml of distilled sterile water at 4 °C (50 ×, 10 min each).
(viii) Add 5 ml of 0.2 mg/ml streptavidin–hydrazide solution.
(ix) Incubate overnight at room temperature.
(x) Wash by adding 20 ml of distilled sterile water at room temperature (50 ×, 10 min each).
(xi) Store streptavidin–agarose at 4 °C.

9. EliCell itself

(i) Melt streptavidin-conjugated agarose at 70 °C.
(ii) Place in water bath at 37 °C until use.
(iii) Prepare the agarose/cell mixture in one small test tube in the following proportion:

  (a) RPMI 10 × plus OVA 1% + agarose → 1 volume
  (b) Biotinylated capture antibody → 1 volume
  (c) Purified cells → 3 volumes.

(iv) Place 20 μl agarose/cell mixture into agonist or medium tube (containing in advance 2 μl agonist—10 × concentrated—or 2 μl medium).
(v) Gently transfer 20 μl above the agarose–cell mixture onto a slide.
(vi) Cover gently with the chamber.
(vii) Overlay 400 μl RPMI-1640 medium (containing 0.1% ovalbumin plus the desired agonist concentration) through access ports in the chamber surface.
(viii) Briefly cool the agarose matrix to solidify.
(ix) Incubate at 37 °C (5% CO_2) for different times.
(x) Carefully remove the chamber.
(xi) Fix the slides by immersing in 2% parafomaldehyde for 5 min.
(xii) Wash the slides by immersing in HBSS for 10 min.
(xiii) Define the slide area containing cells by Pappen or drying.

(xiv) Add 400 μl of Alexa-fluorochome labeled detector antibody (1:1000) diluted in HBSS.
(xv) Incubate at room temperature for 45 min.
(xvi) Wash the slides by immersing HBSS for 10 min, 3 × each.
(xvii) Leave the slides to dry at room temperature and mount with aqueous mounting medium.
(xviii) Analyze the slides by phase-contrast and fluorescence microscopy.

10. Essential literature references

Bandeira-Melo et al. (2000, 2001).

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