Benchmarks

amount of tryptase that is spontaneously released from cells.

Figure 3 shows dose responses of tryptase release by anti-IgE at various IgE concentrations. Maximal activation of the cells occurred with 0.1 $\mu\text{g/mL}$ IgE with no detectable activation at 0.0001 $\mu\text{g/mL}$ IgE (Figure 3). The EC_{50} values for anti-IgE were 51 ± 15 , 56 ± 7.3 , 145 ± 60 , and 167 ± 43 ng/mL, respectively, for 1, 0.1, 0.01, and 0.001 $\mu\text{g/mL}$ IgE (Figure 3). When the data were analyzed in a reciprocal manner, the EC_{50} values for IgE were 1.7 ± 0.3 , 2.3 ± 0.4 , 3.4 ± 0.3 , 5.1 ± 0.8 , 7.9 ± 1.8 , 17.6 ± 3.2 , and 26 ± 7.8 ng/mL, respectively, for 10, 3, 1, 0.3, 0.1, 0.03, and 0.01 $\mu\text{g/mL}$ anti-IgE.

A real-time tryptase release assay has been developed. The profile of inhibition of the proteolytic activity released by these cells is consistent with tryptase activity. Tryptase is released by activation of Fc ϵ RI in a time- and dose-dependent manner (Figures 2 and 3). This homogeneous real-time assay requires small numbers of cells, few processing steps, and offers the possibility of high-throughput screening because it is formatted in 96 wells. In addition, this assay allows for the simultaneous identification of both inhibitors and activators of mast cells without alteration of the assay format. The initial lag in activity (Figure 2) allows for the identification of activators when compounds are pre-incubated with cells. Using this real-time assay, the EC_{50} values for both IgE and anti-IgE for release of tryptase from CBMCs were determined. Additionally, the maximal amount of tryptase released through Fc ϵ RI was compared with that released by calcium ionophore (data not shown). These amounts were equivalent, which is consistent with previous studies (12) in which the percent histamine release through Fc ϵ RI was found to be 100% of that released by ionophore from cells cultured in IL-4. The amount of tryptase released in our studies was typically 10%–20% of total tryptase consistent with 10%–20% histamine release from the same cultures. In addition, this assay has been used to examine tryptase release mediated by endothelin-1, platelet activating factor, phorbol ester, and ATP (data not shown). This methodology should also

be applicable to mast cells isolated from other species; however, the protease profile of these mast cells will have to be characterized. This assay will be useful to screen for modulators of tryptase release from CBMCs and will allow the identification of both inducers and inhibitors of release.

REFERENCES

1. **Yong, L.C.J.** 1997. The mast cell: origin, morphology, distribution, and function. *Exp. Toxic. Pathol.* 49:409-424.
2. **Lichtenstein, L.M. and A.G. Osler.** 1964. Studies on the mechanisms of hypersensitivity phenomena IX. Histamine release from human leukocytes by ragweed pollen antigen. *J. Exp. Med.* 120:507-530.
3. **Morel, A.M. and M.A. Delaage.** 1988. Immunoanalysis of histamine through a novel chemical derivatization. *J. Allergy Clin. Immunol.* 82:646-654.
4. **Bruce, C., R. Weatherstone, A. Seaton, and W.H. Taylor.** 1976. Histamine levels in plasma, blood, and urine in severe asthma, and the effect of corticosteroid treatment. *Thorax* 31:724-729.
5. **Bhat, K.N., C.M. Arroyave, S.R. Marney, D.D. Stevenson, and E.M. Tan.** 1976. Plasma histamine changes during provoked bronchospasm in asthmatic patients. *J. Allergy Clin. Immunol.* 58:647-656.
6. **Erger, R.A. and T.B. Casale.** 1992. Comparison of methodologies to measure human lung histamine. *J. Immunol. Methods* 31:115-121.
7. **Lavens, S.E., D. Proud, and J.A. Warner.** 1993. A sensitive colorimetric assay for the release of tryptase from human lung mast cells in vitro. *J. Immunol. Methods* 166:93-102.
8. **Schwartz, L.B., T.R. Bradford, C. Rouse, A.M. Irani, G. Rasp, J.K. Van der Zwan, and P.W. Van der Linden.** 1994. Development of a new, more sensitive immunoassay for human tryptase: use in systemic anaphylaxis. *J. Clin. Immunol.* 14:190-204.
9. **Nilsson, G. and K. Nilsson.** 1995. Effects of interleukin (IL)-13 on immediate-early response gene expression, phenotype and differentiation of human mast cells. Comparison with IL-4. *Eur. J. Immunol.* 25:870-873.
10. **Caughey, G.H., W.W. Raymond, E. Bacci, R.J. Lombardy, and R.R. Tidwell.** 1993. Bis(5-amidino-2-benzimidazolyl)methane and related amidines are potent, reversible inhibitors of mast cell tryptase. *J. Pharm. Exp. Ther.* 264:676-682.
11. **Elrod, K.C., W.R. Moore, W.M. Abraham, and R.D. Tanaka.** 1997. Lactoferrin, a potent tryptase inhibitor, abolishes late-phase airway responses in allergic sheep. *Am. J. Respir. Crit. Care Med.* 156:375-381.
12. **Toru, H., R. Pawankar, C. Ra, J. Yata, and T. Nakahata.** 1998. Human mast cells produce IL-13 by high-affinity IgE receptor cross-linking: enhanced IL-13 production by IL-4-primed human mast cells. *J. Allergy Clin. Immunol.* 102:491-502.

Address correspondence to Dr. Scott Greenfeder, Schering-Plough Research Institute, 2015 Galloping Hill Rd. MS-1600, Kenilworth, NJ 07033, USA. e-mail: scott.greenfeder@spcorp.com

Received 18 November 2002; accepted 14 February 2003.

S. Greenfeder, H. Gilchrest, B. Cheewatrakoolpong, S. Eckel, M. Billah, R.W. Egan, and J.C. Anthes
*Schering-Plough Research Institute
Kenilworth, NJ, USA*

Double-Color Fluorescence In Situ Hybridization with RNA Probes

BioTechniques 34:914-918 (May 2003)

In situ hybridization is a useful tool for localization and partial quantification of gene expression. However, the method is costly and laborious, especially when used to measure the expression of multiple genes. Most previously reported protocols for double-labeled fluorescence probes involve DNA probe labeling (1,2). The disadvantages of using DNA probes are lower sensitivity and higher background signals. Some methods for double-labeled RNA probes have also been reported, but most involve radioactive probes (3,4). Here we report the development of a unique method of double-color fluorescence in situ hybridization (FISH) using RNA probes.

Previous studies have indicated the expression of specific isoforms of sulfonylurea receptors in subpopulations of dopamine-containing cells of the substantia nigra (5). We took advantage of this information to demonstrate the

Benchmarks

feasibility and utility of double-color FISH for the simultaneous identification of two distinct species of mRNA in individual cells. To our knowledge, this is the first report showing co-localization of sulfonylurea receptor 1 (SUR1) and dopamine transporter (DAT) mRNA in substantia nigra pars compacta by double-color FISH. We labeled an RNA probe (probe 1) for SUR1 with digoxigenin and a second RNA probe (probe 2) for the DAT with fluorescein. After hybridization with target gene transcripts, the signal from the digoxigenin-labeled SUR1 probe was amplified by anti-digoxigenin-HRP and visualized following the reaction of HRP with cyanine 3 tyramide (red color). Subsequently, anti-fluorescein-HRP was used to amplify the signal from the fluorescein-labeled DAT probe, followed by the reaction of HRP with fluorescein tyramide (green color).

Both the red color from cyanine 3 tyramide and the green color from fluorescein tyramide were clearly visible under a fluorescence microscope. A yellow signal obtained by using a dual-

pass filter could be seen in the cells expressing both target genes (Figure 1C). The results indicate that this method can be successfully employed to detect gene co-expression with reasonably high sensitivity when coupled with signal amplification.

For probe preparation, SUR1 probes (sense and antisense) were labeled by incorporation of digoxigenin-UTP (Roche Applied Science, Indianapolis, IN, USA). DAT probes were labeled by incorporation of fluorescein-UTP (Roche Applied Science). The labeling procedure, using 40 μ L total reaction volume, is detailed in the instruction manual in the MAXIscripTM SP6/T7 kit (Ambion, Austin, TX, USA). After transcription, the template DNA was removed by incubation at 37°C for 15 min with 4 U DNase I, followed by 2 μ L 0.5 M EDTA with a final concentration of 25 mM to stop the reaction. Each labeled RNA probe was precipitated with 0.1 volumes of 4 M LiCl and 3 volumes of 100% ethanol, washed with 70% ethanol, and resuspended in 40 μ L RNase-free water. The quality

and quantity of probes were determined by electrophoresis and spectrophotometry at an absorbance of 260 nm. The concentration of labeled probe was approximately 0.5 μ g/ μ L.

For in situ hybridization, Sprague-Dawley rat brains were rapidly removed from decapitated animals, frozen immediately on dry ice, and stored at -80°C until use. Frozen sections (16 μ m thick) were collected on slides. The mounted sections were fixed in 4% paraformaldehyde in DEPC-treated PBS, pH 7.4, for 15 min. Slides were rinsed twice with PBS, acetylated with 0.25% acetic anhydride in TEAS buffer (0.1 M triethanolamine, 0.9% NaCl, pH 8.0), and washed in an ordered series (70%, 95%, 100% ethanol, 5 min each, 15 min chloroform, and 100%, 95% ethanol, 5 min each). The sections were air-dried immediately before hybridization. Two antisense probes or two sense probes were mixed with yeast tRNA and DEPC water to reach 1 volume of probe mixture. After the probe mixture was denatured at 90°C for 3 min, 4 volumes of the

Figure 1. SUR1 and DAT mRNA expression in substantia nigra using in situ hybridization. Panel A shows the broad expression of SUR1 mRNA visualized by red filter (cyanine 3 tyramide signal). Panel B shows DAT mRNA expressed in a subpopulation of substantia nigra cells visualized by green filter (fluorescein tyramide signal). Panel C shows the population of cells co-expressing both SUR1 and DAT, as indicated by the yellow color. Panels D and E show the background with sense probes visualized by red or green filter.

Benchmarks

hybridization cocktail (Amresco, Solon, OH, USA) were added. The final probe concentration was 0.5 ng/ μ L, and the final tRNA concentration was 800 ng/ μ L. The final volume of the probe mixture was determined by the number of slides (100 μ L probe mixture/slide). Sections were hybridized with two antisense probes, two sense probes, or no probe at 60°C for 15 h in a chamber humidified with 2 $\times$ SSC. After hybridization and rinsing with 2 $\times$ SSC, slides were treated with 20 μ g/mL RNase A in RNase buffer (10 mM Tris, pH 8.0, 0.5 M NaCl, 0.75 mM EDTA) at 37°C for 30 min to digest the unhybridized RNA, followed by incubation for 30 min at 37°C with only RNase buffer. A serial high-stringency wash was applied after RNase digestion (1 $\times$ SSC for 15 min at room temperature, 0.1 $\times$ SSC for 30 min at 60°C, and 0.1 $\times$ SSC for 15 min at room temperature).

For signal amplification and visualization, after a 30-min incubation in blocking solution (2% normal sheep serum in TNT buffer) at 37°C, the slides were incubated with anti-digoxigenin-HRP at 1:200 dilution with TNT buffer for 30 min at 37°C. The TNT

buffer contained 0.1 M Tris, pH 7.5, 0.15 M NaCl, plus 0.3% Triton[®] X-100 or 0.05% Tween[®] 20. Slides were then washed three times (5 min each time) with TNT at room temperature to remove the unbound anti-digoxigenin-HRP. The bound HRP reacted with cyanine 3 tyramide at 1:50 dilution in 1 $\times$ amplification solution (TSA system; Perkin Elmer Life Sciences, Boston, MA, USA). The sections were incubated at room temperature for 3–10 min. The excess cyanine 3 tyramide was removed by three 5-min washes with TNT buffer at room temperature. Any remaining HRP was deactivated with PBS containing 1% H₂O₂, followed by the washes as described above for the removal of excess cyanine 3 tyramide. The signal from the second probe, labeled with fluorescein, was amplified with anti-fluorescein-HRP (Perkin Elmer Life Sciences). The slides were incubated with anti-fluorescein-HRP at 1:100 dilution with TNT buffer plus 1% normal sheep serum for 30 min at room temperature or 37°C, and then washed three times (5 min each) with TNT buffer at room temperature. The visualization of the signal from anti-fluorescein-HRP was achieved by the reaction of HRP with fluorescein tyramide (TSA system). The slides were incubated with fluorescein tyramide (1:50 dilution in 1 $\times$ amplification buffer) for 3–10 min at room temperature, followed by the same three TNT buffer washes described above. Finally, all slides were dehydrated through a serial wash (70%, 90%, 100% ethanol, 5 min each), embedded in Vectashield[®] (Vector Laboratories, Burlingame, CA, USA) and viewed by fluorescence microscopy using appropriate filters. The slides were stored at 4°C in the dark to optimize the life of the signal.

The results (Figure 1) show that SUR1 mRNA and DAT mRNA are co-expressed in the dopaminergic substantia nigra (SN) neurons, as previously reported in a study using combined electrophysiological analysis with single-cell RT-multiplex PCR protocols (5). The development of this procedure provides a sensitive, nonradioactive method for the detection of simultaneously expressed genes across a fairly broad anatomical region and should be useful in wide range of future studies.

REFERENCES

1. Nederlof, P.M., S. van der Flier, J. Wiegant, A.K. Raap, H.J. Tanke, J.S. Ploem, and M. van der Ploeg. 1990. Multiple fluorescence in situ hybridization. *Cytometry* 11:126-131.
2. Wiegant, J., C.C. Wiesmeijer, J.M. Hoovers, E. Schuurin, A. d'Azzo, J. Vrolijk, H.J. Tanke, and A.K. Raap. 1993. Multiple and sensitive fluorescence in situ hybridization with rhodamine-, fluorescein-, and coumarin-labeled DNAs. *Cytogenet. Cell Genet.* 63:73-76.
3. Esclapez, M. and C.R. Houser. 1995. Somatostatin neurons are a subpopulation of GABA neurons in the rat dentate gyrus: evidence from colocalization of pre-prosomatostatin and glutamate decarboxylase messenger RNAs. *Neuroscience* 64:339-355.
4. Ozden, S., C. Aubert, D. Gonzalez-Dunia, and M. Brahic. 1990. Simultaneous in situ detection of two mRNAs in the same cell using riboprobes labeled with biotin and 35S. *J. Histochem. Cytochem.* 38:917-922.
5. Liss, B., R. Bruns, and J. Roeper. 1999. Alternative sulfonyleurea receptor expression defines metabolic sensitivity of K-ATP channels in dopaminergic midbrain neurons. *EMBO J.* 18:833-846.

This work was support by a US Army Grant no. DAMD 17-97-2-7013 to D.H.R. and R.J.M. Address correspondence to Dr. Roy J. Martin, Pennington Biomedical Research Center, 6400 Perkins Road, Baton Rouge, LA 70808, USA. e-mail: martinrj@pbrc.edu

Received 16 November 2001; accepted 7 March 2003.

X. Xi, D.S. Roane¹, J. Zhou, D.H. Ryan, and R.J. Martin
*Pennington Biomedical Research Center
Baton Rouge, LA
¹University of Louisiana at Monroe
Monroe, LA, USA*