BRIEF METHOD

Improved Sensitivity for Cell Mapping of Hepatitis C Virus RNA Sequences and Cellular Surface Antigens in Blood Cells

Jaime Gosálvez, Nuria Ortiz-Movilla, Altea Gosálbez, Elena Rodríguez-Iñigo, Javier Bartolomé, and Vicente Carreño

Genetic Unit (JG), Department of Biology, Science Faculty, Universidad Autónoma de Madrid, and Fundación para el Estudio de las Hepatitis Virales (NO-M, AG, ER-I, JB, VC), Madrid, Spain

Simultaneous morphologic localization of specific RNA sequences and proteins in a given cell lineage may be of critical relevance in basic and applied biomedical research. When analyzing blood cells, erythroblast-specific markers may be used for flow sorting and then, isolated cells may be studied for the presence of a specific RNA by fluorescent *in situ* hybridization (FISH). An alternative routine is the simultaneous detection of both target molecules on cells fixed on the same slide (Choolani et al, 2001; Gosálvez et al, 2002; Speel et al, 1994). However, covisualization of both types of molecules is not an easy task because the methods that are used most (paraformaldehyde fixation or frozen tissue sections) to stabilize proteins within cells or tissue sections abolish or reduce RNA molecule detection and vice versa (Bisucci et al, 2000). To solve these problems we have developed an alternative method that allows for the simultaneous detection of nucleic acids by FISH and cell-specific proteins by conventional immunocytochemistry in unfixed agarose gel trapped cells (AGTC). In this report we have applied this new method for the simultaneous detection of hepatitis C virus (HCV) RNA sequences and the T-cell marker CD3 in peripheral blood mononuclear cells (PBMC) from patients with chronic hepatitis C.

PBMC from 10 patients with histologically proven chronic hepatitis C and with HCV-RNA detectable in serum and in PBMC by RT-PCR were included in this study. PBMC from 10 healthy controls without markers of HCV infection were used as negative controls. PBMC were isolated from heparinized blood by centrifugation on Ficoll-Hypaque gradients. Isolated PBMC were divided into two aliquots. In the first one, the cells were resuspended in freshly prepared 4% paraformaldehyde in PBS, fixed during 10 minutes at

4° C, and pipetted onto a glass slide. The cells were air-dried, washed three times in PBS, and dehydrated through a graded series of ethanol dilutions. The second aliquot of cells was processed for the AGTC-FISH protocol. For that purpose, cells were resuspended in KCl (0.075 M) for 10 minutes at 37° C, pelleted at 500 ×g for 5 minutes, and resuspended in 15 µl of PBS. Resuspended cells were mixed with 35 µl of low melting point agarose maintained at 37° C, deposited onto a glass slide (that was previously precoated with 0.65% standard agarose and dried at 80° C), covered with a coverslip, and allowed to solidify at 4° C. Finally, coverslips were gently removed by immersing the slides in PBS at room temperature. Both samples (paraformaldehyde fixed and AGTC) were hybridized with the cDNA corresponding to the complete 5' noncoding region of the HCV genome isolated from the plasmid p5'NC (Rodríguez-Iñigo et al, 2000). The probe was nick-translated with digoxigenin-11 dUTP, denatured for 5 minutes at 90° C, and quenched on ice; 20 ng of the labeled cDNA was applied to each slide. The hybridization was performed at 42° C for 16 hours in a humid chamber. After hybridization the samples were washed at 42° C in 2× SSC, 0.5× SSC, and 0.1× SSC (15 minutes each) (20× SSC: 3 M NaCl, 3 mM trisodium citrate). Digoxigenin-labeled hybrids were detected with an anti-digoxigenin fluorescein isothiocyanate conjugate antibody (Roche Molecular Biochemicals, Indianapolis, Indiana). The intensity of the signals was amplified using the Fluorescent Antibody Enhancer set for DIG detection kit (Roche Molecular Biochemicals). Cells were counterstained using 4,6-diamidino-2-phenylindole. The fluorescent signals were visualized using single, double, and triple band-pass filters and mounted on a LEICA DMR epifluorescence microscope. Images were captured with a high-sensitivity CCD camera (Photometrics, Roper Scientific, Phoenix, Arizona) and 24-bit RGB color depth and stored as .tiff files. Images for fluorescence quantification were visualized under the single band-pass filter and analyzed using a macro designed on Visilog 5.1 soft-

DOI: 10.1097/01.LAB.0000077009.54320.6B

Received November 21, 2002.

Address reprint requests to: Dr. V. Carreño, Fundación para el Estudio de las Hepatitis Virales, C/ Guzmán el Bueno 72, 28015 Madrid, Spain.
E-mail: fevhpa@fehv.org

ware. Two parameters were obtained and correlated: area of hybridization and whole fluorescence intensity, both in arbitrary units. The specificity of the FISH was assessed by digestion of the cell preparations with RNase A (0.2 mg/ml) (Sigma, St. Louis, Missouri) for 2 hours at 37° C before hybridization and by hybridization with an unrelated probe—a 360-bp fragment of the bacterial chloramphenicol acetyl transferase gene generated by PCR and cloned in the pCRII-TOPO vector (Invitrogen, Carlsbad, California).

For surface antigen detection, slides containing AGTC were incubated with 20 μ l of biotinylated anti-CD3 (Sigma) for 30 minutes at 4° C in a moist chamber and covered with coverslips cut from a parafilm strip. After that, the slides were washed in PBS for 5 minutes. The bound antibody was detected with streptavidin-Cy3 (Sigma) in 4 $\times$ SSC, 1% BSA, and 0.1% Triton X-100, for 25 minutes at 37° C. After CD3 detection, the slides were washed three times (2 minutes each) in 4T buffer (4 $\times$ SSC, 0.1% Triton X-100). After the successful detection of the targeted cells was confirmed by fluorescence microscopy, the slides were processed for AGTC-FISH, as described above.

Fluorescent hybridization signals were detected in the PBMC of the 10 patients with chronic hepatitis C either in cells fixed in paraformaldehyde or in cells embedded in agarose. The presence of fluorescent signals in the PBMC of the HCV chronic carriers does not imply that HCV replication takes place in these cells, because the probe used for the detection of the viral RNA (a double-stranded DNA) does not discriminate between the HCV-RNA of positive and negative polarity. No hybridization signals were detected in the PBMC from the 10 healthy controls nor in the specificity controls. Figure 1 shows the images obtained in the HCV-RNA detection in paraformaldehyde fixed cells (Fig. 1a) and in AGTC (Fig. 1b). The cell volume retained after paraformaldehyde fixation does not allow for the precise localization of the RNA molecule, and in most of the cells, fluorescent signal appears to be in the nuclei because of cytoplasm retraction. However, the AGTC-FISH shows that the virus invariably maps on the cell cytoplasm. The area and the fluorescence intensity rendered by AGTC-FISH was 50 times higher than in cells fixed in paraformaldehyde (data not shown). The percentage of cells showing fluorescent signals was 0.62% $\pm$ 0.37% (range, 0.1–

Figure 1.

Fluorescent in situ hybridization (FISH) mapping of hepatitis C virus (HCV)-RNA in peripheral blood mononuclear cells (PBMC) under two experimental conditions. a, paraformaldehyde fixation; b, unfixed cells using the agarose gel trapped cells (AGTC)-FISH protocol; c, Colocalization of CD3 antigens and HCV-RNA using the AGTC-FISH after antigen detection. Green corresponds to HCV-RNA, red to anti-CD3, and blue to the cell nuclei.

1.2%) in paraformaldehyde fixed cells and $2.5\% \pm 1.4\%$ (range, 0.9–5%) in AGTC. This indicates that HCV-RNA detection in AGTC is between four and five times more sensitive than in paraformaldehyde fixed cells and suggests that in paraformaldehyde fixed cells FISH detects HCV-RNA only in those cells supporting the highest HCV-RNA levels. This fact may be of relevance when the targeted RNA molecule shows a low number of copies per cell. Additionally, unfixed cells immersed in agarose retain the antigenicity of the cell surface markers. In this experiment, surface antibodies targeting the CD3-positive cells were used (Fig. 1c). Although both fluorescent signals colocalize out of the nuclei, the use of two fluorochrome emissions allows for an easy discrimination of signal corresponding to the RNA molecules and that of surface proteins (green and red, respectively, in Fig. 1c). This opens the possibility of the detection of a target RNA in different cells types within the same slide by using a panel of specific antibodies against different cell markers labeled with distinct fluorochromes.

In summary, the method presented makes it possible to enhance the sensitivity to detect RNA molecules when compared with other existing systems and to simultaneously characterize cell subpopulations expressing a given RNA.

References

- Bisucci T, Hewiston TD, and Darby IA (2000). cRNA Probes: Comparison of isotopic and non-isotopic detection methods. In: Darby IA, editor. *Methods in molecular biology: In situ hybridization protocols*, vol 123. Totowa: Humana Press, 291–303.
- Choolani M, O'Donnell H, Campagnoli C, Kumar S, Roberts I, Bennett PR, and Fisk NM (2001). Simultaneous fetal cell identification and diagnosis by epsilon-globin chain immunophenotyping and chromosomal fluorescence in situ hybridization. *Blood* 98:554–557.
- Gosálvez J, de la Torre J, Pita M, Martínez-Ramírez A, López-Fernández C, Goyanes V, and Fernández JL (2002). FISHing in the microwave: the easy way to preserve proteins. I. Colocalization of DNA probes and surface antigens in human leukocytes. *Chromosome Res* 10:137–143.
- Rodríguez-Iñigo E, Casqueiro M, Navas S, Bartolome J, Pardo M, and Carreño V (2000). Fluorescent in situ hybridization of hepatitis C virus RNA in peripheral blood mononuclear cells from patients with chronic hepatitis C. *J Med Virol* 60:269–274.
- Speel EJ, Herbergs J, Remaekers FC, and Hopman AH (1994). Combined immunocytochemistry and fluorescence in situ hybridization for simultaneous tricolor detection of cell cycle, genomic, and phenotypic parameters of tumor cells. *J Histochem Cytochem* 7:961–966.