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Detection of Parvovirus B19 DNA in Bone Marrow Cells by Chemiluminescence In Situ Hybridization

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A chemiluminescence in situ hybridization method was developed for the search of B19 parvovirus DNA in bone marrow cells, employing digoxigenin-labeled B19 DNA probes, immunoenzymatically detected with a highly sensitive 1,2-dioxetane phosphate as chemiluminescent substrate. The light emitted from the in situ-hybridized probe was analyzed and measured by a high-performance luminograph connected to an optical microscope and to a personal computer for the quantification of the photon fluxes from the single cells and for image analysis. The chemiluminescence in situ hybridization was applied to bone marrow cell smears of patients with aplastic crisis or hypoplastic anemia, who had been previously tested by in situ hybridization with colorimetric detection, dot blot hybridization, and nested PCR. The chemiluminescent assay provided an objective estimation of the data, proved specific, and showed an increased sensitivity in detecting B19 DNA compared with in situ hybridization with colorimetric detection.

Human parvovirus B19 is the etiologic agent of a wide range of clinical syndromes such as erythema infectiosum, fetal hydrops, postinfectious arthropaty, transient aplastic crises in patients with haemolytic disorders, and chronic bone marrow failure in immunocompromised patients (1, 4, 26). As parvovirus B19 cannot be efficiently grown in established cell cultures, the diagnosis of B19 infection relies mainly on the detection of specific immune response to B19 and/or detection of B19 genomes by hybridization assays or by PCR (11, 18, 20, 29, 31). In situ hybridization is a successful method for the localization of specific viral nucleic acids inside individual cells, with the preservation of cellular morphology also permitting retrospective studies on archival specimens. In situ hybridization techniques have been successfully used for the detection of B19 nucleic acids in bone marrow cells and in fetal tissues using either isotopic or nonisotopic probes such as biotinylated or digoxigenin-labeled probes (11, 17, 25, 27, 28). Digoxigenin-labeled probes have proved very sensitive and specific for the detection of B19 and other virus genomes in cells or tissue specimens, giving levels of positive signal similar to those obtained with radioactive probes and proving more sensitive and specific than biotinylated probes (9, 19, 24).

In in situ hybridization assay, digoxigenin-labeled probes are immunoenzymatically revealed by using antidigoxigenin Fab fragments labeled with alkaline phosphatase or with peroxidase followed by colorimetric substrates giving a final colored product at the site of the enzyme (11). In recent years, chemiluminescent substrates have been proposed as a more sensitive alternative to colorimetric substrates in various analytical techniques in which small amounts of analytes or enzymes have to be detected (7, 14–16, 22), and they have also been successfully employed for the search of B19 DNA in dot blot hybridization assays (13, 23, 30). Moreover, using newly synthesized dioxetane derivates as chemiluminescent substrates for alkaline phosphatase, we were able to detect as little as 10 fg of B19 DNA in a dot blot hybridization format, with the chemiluminescent substrates proving about 10- to 50-fold more sensitive than the colorimetric ones (12). Continuing improvements in chemiluminescent substrates have been matched by new developments in photon imaging instrumentation such as high-performance luminographs based on a charge-coupled device video camera or high-dynamic-range pickup tube (Saticon) combined with a video amplifier. These instruments not only allow a quantification of emitted light at the single-photon level but also permit localization of the chemiluminescent emission on a target surface. Moreover, by connecting the luminograph to an optical microscope, it is possible to localize the light emission inside tissues or cells. All these data prompted us to explore the use of highly sensitive chemiluminescent substrates and a high-performance luminograph connected to a light microscope for the in situ detection of B19 DNA inside bone marrow cells of patients with aplastic crisis or hypoplastic anemias using digoxigenin-labeled B19 DNA probes constructed in our laboratory.

Samples. Bone marrow aspirates from 15 patients with aplastic crisis or hypoplastic anemias caused by B19, diagnosed by in situ hybridization with colorimetric detection and dot blot hybridization or nested PCR in peripheral blood serum samples were analyzed in the study (2, 11, 20, 21, 31). Dot blot hybridization was performed with digoxigenin-labeled probes as previously described (2), and the sensitivity of the assay with colorimetric detection ranged between 1.5×10^5 and 3×10^4 genome copies. Nested PCR was performed as previously described (20) on peripheral blood samples, treated with a lysis solution, and the sensitivity of the assay was between 1 and 20 genomes.

Of the 15 patients, 6 were bone marrow transplant recipients, 6 were patients with lymphoproliferative disorders under immunosuppressive therapy, 2 were thalassemic patients, and 1 was an AIDS patient with haemophilia. Moreover, samples from a bone marrow recipient with hypoplastic anemia who had proved positive for B19 DNA by in situ hybridization but negative by dot blot hybridization and nested PCR in peripheral blood were also analyzed.

As controls, bone marrow aspirates from 24 patients with

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hematologic disorders who had proved negative for B19 DNA by in situ hybridization with colorimetric detection, dot blot hybridization, and nested PCR in peripheral blood were studied.

Bone marrow aspirates from each patient were processed for chemiluminescent and colorimetric in situ hybridization with the same probes and in the same run. Samples were analyzed in duplicate for both chemiluminescence detection and colorimetric detection.

As negative controls cells from the interleukin 3-dependent leukemic cell line TF-1 were used.

B19 parvovirus DNA probe. The B19 probe was prepared from the molecular clone of a 5.0-kbp insert (nucleotides 282 to 5310), which represents the complete coding sequence of B19 DNA, cloned in vector pUC18 (10). Routine methods for large-scale preparation of plasmids were used. Digoxigenin-labeling of the probe was performed as previously described (2) from the excised insert, by the randomly primed DNA labeling method. The digoxigenin-labeled probe was hybridized with serially diluted unlabeled parvovirus B19 DNA bound to nylon membranes, and between 0.5 and 0.1 pg of B19 DNA (corresponding approximately to 1.5×10^5 and 3×10^4 genome copies) could be visualized by colorimetric detection, and 10 fg (corresponding approximately to 3×10^3 genome copies) could be visualized by chemiluminescence detection. The B19 DNA probe could be stored at −20°C for at least 1 year with no decrease in its activity.

Bone marrow smear preparation. Bone marrow aspirates were smeared on slides pretreated as previously described (11) and were air dried and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) (0.15 M, pH 7.4) for 10 min. After fixation, smears were washed three times in PBS for 10 min each and then dehydrated with ethanol washes (30, 60, 80, 95, and 100%) for 5 min each. Smears were then air dried and stored at 4°C until use.

In situ hybridization reaction. Cell smears were hydrated in PBS and placed in 0.02 N HCl for 10 min. After three washes with PBS, cells were treated with 0.01% Triton X-100 in PBS for 2 min. After three further washes with PBS, cell smears were treated with pronase (Boehringer, Mannheim, Germany) (0.5 mg/ml in 0.05 M Tris-HCl [pH 7.6], 5 mM EDTA) for 5 min. Smear preparations were then washed twice with PBS containing 2 mg of glycine per ml. After these treatments cell smears were postfixed with 4% paraformaldehyde in PBS and washed twice with PBS containing 2 mg of glycine per ml. Smears were then dehydrated by ethanol washes (30, 60, 80, 95, and 100%). Dehydrated monolayers were overlaid with 10 μl of the hybridization mixture. The hybridization mixture consisted of 50% deionized formamide, 10% dextran sulfate, 250 ng of carrier calf thymus DNA per μl, and 2 ng of digoxigenin-labeled probe DNA per μl in 2× SSC buffer (0.3 M NaCl, 0.03 M sodium citrate [pH 7.0]). A clean glass coverslip was then applied, and the edges were sealed with rubber cement to prevent loss of the mixture during denaturation and hybridization. Cell smears and hybridization mixture were denatured together by heating in an 85°C water bath for 5 min and were then incubated at 37°C overnight. After hybridization, the coverslips were carefully removed, and cell smears were washed in stringent conditions (11).

Chemiluminescence detection. For the detection of hybridized probes, slides were briefly washed in a 100 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl. Smears were then incubated for 30 min with sheep polyclonal antidigoxigenin Fab fragments, conjugated to alkaline phosphatase, and diluted 1/500 in blocking reagent (Boehringer). After incubation, bone marrow smears were washed by two 15-min washes with Tris-HCl buffer and equilibrated for 2 min with equilibration buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$ [pH 9.5]). The chemiluminescence detection of alkaline phosphatase was performed by treating the cells with undiluted adamantil-1,2-dioxetane phenyl phosphate substrate (CDP-star) (Tropix, Inc., Bedford, Mass.). After an optimized incubation of 30 min at room temperature, the solution was removed, and the luminescent signal from the hybrid formation was detected and analyzed with a system which consisted of luminograph LB-980 (EG&G Berthold, Bad Wilbad, Germany), which is a high-performance, low-light-level imaging apparatus with a high-dynamic-range pickup tube (Saticon) combined with a video amplifier, connected to a model BH-2 light microscope (Olympus Optical, Tokyo, Japan) and to a personal computer with a commercially available program for image analysis. The microscope was enclosed in a dark box to prevent contact with the external light. The system operated in consecutive steps. Firstly, bone marrow cells were recorded in transmitted light; then, the luminescent signal from the hybrid formation was measured; and then, after a computer elaboration of the luminescent signal with pseudocolors corresponding to light intensity, an overlay of the two images on the screen provided by the transmitted light and by the luminescent signal allowed the spatial distribution of the target analyte to be localized and evaluated. Digital images of the light emission from bone marrow cells were optimized with 2-s integration intervals for 1 min of total accumulation time. The light emission from each cell was quantified by defining a fixed area and summing the total number of photon fluxes per second from within this area.

As negative controls TF-1 cells were analyzed as described above, providing threshold background levels. A mean of 50 negative cells were analyzed for each run, and the average value of photon fluxes per second plus fivefold the standard deviation was considered the positive threshold for chemiluminescence in situ detection of B19 DNA. Corrections for instrumental background and flat-field variations were automatically performed by the LB-980 apparatus.

To assess the specificity of the chemiluminescence assay, a series of controls were performed: (i) B19-positive bone marrow cells were hybridized with the unlabeled B19 DNA probe; (ii) B19-positive bone marrow cells were hybridized with the unlabeled B19 DNA probe; (iii) B19-positive bone marrow cells were hybridized with the digoxigenin-labeled B19 DNA probe, but the incubation with anti-digoxigenin antibody was replaced with an incubation with nonimmune sheep serum; and (iv) B19-positive bone marrow cells were hybridized with the digoxigenin-labeled B19 DNA probe, but the incubation with antidigoxigenin antibody was omitted. The light emission from control experiments was analyzed and evaluated as described above.

Colorimetric detection. Hybridized bone marrow cells were treated with the colorimetric alkaline phosphatase substrate as previously described (11), and the development of a dark blue precipitate at the enzyme site in positive cells was monitored by microscopic examination.

Of the 15 bone marrow aspirates from patients who had been proved positive for B19 DNA both by in situ hybridization and by dot blot hybridization and/or nested PCR, all 15 samples proved positive by chemiluminescence in situ hybridization (Fig. 1). When a comparison between the numbers of cells found positive by chemiluminescence in situ hybridization and by colorimetric in situ hybridization was made, after the samples from the same patient had been processed in the same run and with the same batch of probe, the two methods showed a high correlation ($P = 0.0005$; Spearman rank correlation).

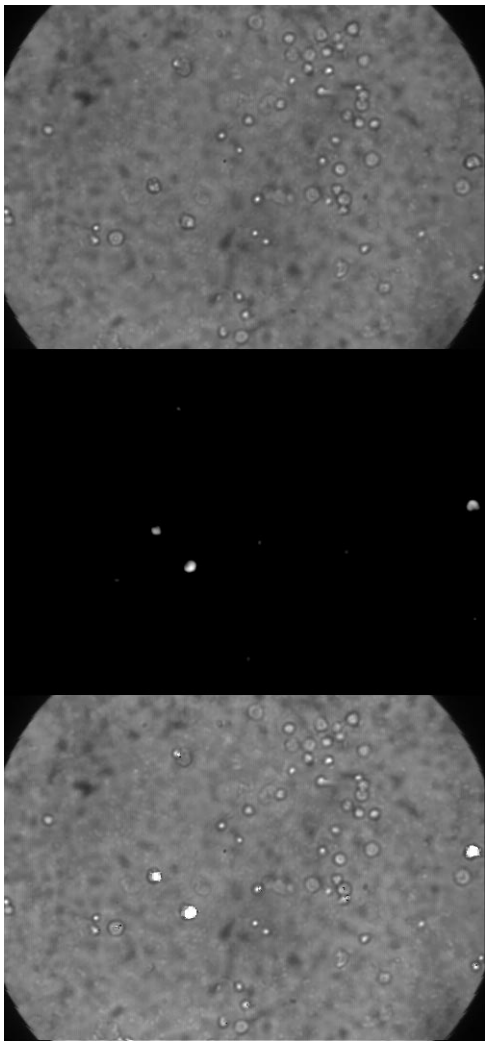

FIG. 1. Chemiluminescence in situ hybridization revealing B19 parvovirus in bone marrow cells. From top to bottom: live image, luminescent signal, and overlay of the live image and luminescent signal.

With the chemiluminescence method a mean of 19.8 positive cells per 100 counted cells could be found versus the mean of 4.6 positive cells per 100 counted cells obtained with the colorimetric method. This difference was highly significant ($P = 0.0007$; Wilcoxon signed test for paired data).

The bone marrow samples from the patient with hypoplastic anemia who had been proved positive by in situ hybridization with colorimetric detection but negative by dot blot hybridization and nested PCR in peripheral blood was confirmed positive by chemiluminescence in situ hybridization.

Of the 24 bone marrow aspirates from patients who had previously proved negative for B19 DNA by in situ hybridization with colorimetric detection, by dot blot hybridization, and by nested PCR in peripheral blood, 23 samples proved negative by both chemiluminescence in situ hybridization and colorimetric in situ hybridization. One sample, however, proved positive by chemiluminescence in situ hybridization but negative by colorimetric in situ hybridization. The sample was from a patient with a chronic anemia after bone marrow transplantation. To establish whether this sample was a false positive, we tested the sample with another B19 DNA probe of 700 bp that we had used in previous studies (2, 11, 23, 31), and the positive result obtained by the chemiluminescence in situ hybridization and the negative result obtained by the colorimetric reaction were confirmed. As the peripheral blood from this patient when retested with nested PCR was confirmed negative for B19, the nested PCR was performed directly on bone marrow cells, and a clear positive result was achieved.

A series of control experiments definitely proved that the chemiluminescence hybridization reaction was detecting parvovirus B19 sequences specifically. In fact, no chemiluminescent signal was observed when B19-positive bone marrow cells were hybridized with the plasmid pUC18 control DNA labeled probe and treated with antidigoxigenin Fab fragment conjugated with alkaline phosphatase and with the chemiluminescent substrate. Similarly, no luminescent signal was detectable after hybridization with unlabeled probes followed by the immunoenzymatic chemiluminescence treatment. Moreover, B19-positive bone marrow cells were completely negative after hybridization with the B19 labeled probes when the primary incubation with antidigoxigenin antibody was either omitted or replaced with incubation with nonimmune sheep serum.

In recent years there has been a growing interest in the application of in situ hybridization for the diagnosis of viral diseases, especially for those viruses, like parvovirus B19, that cannot be diagnosed by isolation procedures. The possibility of using a nonradioactive label, such as digoxigenin, which has a sensitivity similar to that of radioactive labels and a better resolution, has made this technique more attractive for diagnostic laboratories trying to avoid problems related to the short life of radioactive compounds, disposal, and personnel safety. In this study we have developed a chemiluminescence in situ hybridization assay for the search of B19 DNA which could combine the specificity of digoxigenin-labeled probes, the sensitivity of alkaline phosphatase chemiluminescent substrates, and the spatial morphological resolution of in situ hybridization, using a high-performance, low-light-level imaging luminograph connected to a light microscope and to a computer for image analysis. Although the technological equipment employed in this work is mainly a research tool at this time, in our study we tried to explore its potentiality for diagnostic purposes. In our assay we used digoxigenin-labeled probes which were immunoenzymatically revealed by using antidigoxigenin Fab fragments conjugated with alkaline phosphatase. The use of Fab fragments in the detection of hybridized probes can be highly specific without any background due to the presence of cells expressing Fc receptors, and the use of alkaline phosphatase avoids many of the disadvantages of horseradish peroxidase, such as interference from endogenous peroxidase activity in cells and the need to pretreat cells to inhibit the enzyme. The chemiluminescence detection of the alkaline phosphatase-labeled antibody was performed with the 1,2-dioxetane substrate CDP-star, which represents one of the most sensitive detection systems, being able to reveal as few as 1.6 zeptomoles of the enzyme (3); moreover, CDP-star has glowing kinetics with a steady-state emission which permits easier handling of the specimens, and, since the signal intensity is proportional to enzyme activity or concentration, it also allows accurate analysis of the samples. In our chemiluminescence in situ hybridization assay, signal acquisition was done after the liquid film of the substrate over the section was removed. This permitted a sensitive and sharp topographical localization of the signal, as the mobility of the luminescent product within the liquid film was avoided and the path and scatter of the photons were as limited as possible. Moreover, processing the images by using an image superposition function avoided the problems which may arise from the sequential analysis of the

tissue structure and the chemiluminescent signal obtained on the screen in turn (15).

A sharp topographic distribution of the probe within the cell was achieved, as the LB-980 instrumentation with the conventional setup has a spatial resolution of 240 μm, i.e., one pixel corresponds to this value, but once the instrument is connected to the microscope and with the use of the 40× and 100× lenses, pixel size is reduced to 1.1 and 0.41 μm, respectively.

Our chemiluminescence in situ hybridization to detect B19 DNA in bone marrow cells proved very specific and very sensitive, as all the positive samples from patients with a diagnosed B19 infection also proved positive with our assay, with a larger number of positive cells per sample thus permitting an easier evaluation of the sample. Moreover, the sample which had proved positive by in situ hybridization with colorimetric detection, from a patient who had tested negative for the presence of B19 DNA by dot blot hybridization and nested PCR in peripheral blood, was confirmed positive by chemiluminescence in situ hybridization.

Moreover, among 24 samples from patients with hematological disorders who had proved negative for B19 DNA by in situ hybridization with colorimetric detection, dot blot hybridization, and nested PCR in peripheral blood, chemiluminescence in situ hybridization confirmed the negative result for 23 samples and for 1 sample gave a positive result, proving as sensitive as nested PCR performed on bone marrow cells. Our chemiluminescence in situ hybridization thus offers the possibility of diagnosing B19 infection in a small percentage of the total number of cells without extraction of viral nucleic acids and obtaining information on the molecular aspects of B19 infection, with wide applications for both research and diagnostic purposes. As the ability of B19 to persist in bone marrow cells over long periods of time in the absence of detectable viremia has been demonstrated by PCR directly on bone marrow aspirates (4, 6, 8), our chemiluminescence in situ hybridization could also be useful for monitoring persistent replication of B19 in bone marrow cells in chronic infections.

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