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Detection of Epstein-Barr Virus by *In Situ* Hybridization

Progress Toward Development of a Nonisotopic Diagnostic Test

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This work presents some initial quantitation of an* in situ *hybridization method for detection of Epstein-Barr (EB) virus nucleic acids. The purpose is to develop evaluative criteria for diagnosis of viral presence in clinical tissue specimens. In this work simultaneous denaturation of probe and target DNA and an alkaline phosphatase conjugate to detect biotinated probe were used as described by Unger et al.[28] For evaluation of the hybridization, a variety of cell lines, both productively and latently infected, that were hybridized* in situ *using nick translated ^{32}P-labeled viral probe sequences and counted by scintillation after the method of Lawrence and Singer were used.[23] Producer cells (B95-8) showed intense foci of staining in approximately 5% of cells, with most of the other cells showing varying staining intensity. Raji cells showed varying amounts of signal from cell to cell. Namalwa cells exhibited one spot in most cells that was decreased after cells were treated with Actinomycin D (dactinomycin, Merck Sharp & Dohme, West Point, PA). Signal was identified in only a third of these same cells after sectioning. EB virus-negative Ramos cells showed no signal. The nuclear punctate nature of the signal generated is diagnostic of infected cells, and may be a useful test for cultured cells or pathologic specimens. (Am J Pathol 1989, 135:1035–1044)

Epstein-Barr (EB) virus, a DNA gamma herpes virus, was discovered in 1964 and by 1984 was completely sequenced.[1–4] As is the case with other members of the gamma herpes family of viruses, EBV possesses the property of persistent infection in host cells.[5,6] It also has a unique tropism for mature B cells and immortalizes them for tissue culture.[7] Clinically, the virus produces a flu-like illness or the syndrome of infectious mononucleosis.[8,9] The virus produces lymphoproliferative disorders in certain experimental animals and may be related to certain malignancies in humans.[10–12] Developing sensitive methods for detecting an active or latent infection, particularly in intact human tissue, will promote a better understanding of the relationship of this virus to a variety of disease etiologies.

Diagnosis of EBV infections currently relies on serology demonstrating antibodies to nuclear and membrane antigens.[13–17] However, antibody detection relies on the production of viral proteins, and the maintenance of their antigenicity through the tissue preparation techniques. Modern molecular techniques to detect viral nucleic acids have recently become available. Filter hybridization can detect EBV sequences in DNA extracted from affected tissue.[16,17] However, this technique does not permit the histologic localization of the viral nucleic acids within tissue sections. By contrast, the technique of *in situ* hybridization allows the detection of viral specific DNA or RNA sequences within cells compatible with microscopic examination and, furthermore, requires only a small amount of material.[18–22]

Our laboratory used a quantitative method, involving ^{32}P-labeled probes, to optimize conditions for *in situ* hybridization to chicken muscle cells in culture.[20,22,23] Applying this approach to the detection of nuclear sequences used EBV integrated into human genomic DNA to de-

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velop *in situ* hybridization technology for single copy sensitivity.[24] This approach used the lymphoblastoid cell line, Namalwa, followed by high resolution fluorescence detection. In the work presented here, we began using an approach originally reported as useful for routine pathologic investigations by Brigati et al[19] and Unger et al.[25] The center of this work is the denaturation of the target sequences (another herpesvirus, CMV) in the presence of the probe. Because both target and probe are double-stranded DNA, self-reannealing of either is a significant consideration. In routine tissue sections in which preparations vary, penetration of the probe to the target may be delayed sufficiently to allow reannealing. Hence, a period in denaturing conditions would allow this equilibration. We wished to explore this approach for EBV on cultured cells with known copy numbers to evaluate whether it is capable of detection sensitive enough to employ on tissue sections.

For these studies, we used as a model system established cell lines in which the virus infection is either productive (B95-8 cells established from a marmoset[26]) or latent (Namalwa: two copies of viral DNA per cell[24,27]; or Raji, estimated to have 50 copies per cell[28]). Control cells were EBV negative Ramos.[29] Analysis of the hybridization and detection indicated that this was a useful diagnostic approach for tissue sections, and standard procedures were used to prepare sections from cultured Namalwa cells. These sections were compared with slides containing the whole cultured cells to evaluate the effect of sectioning positive cells.

Materials and Methods

B95-8 cells, Raji cells, and EB virus-negative cells (gift from Dr. J. Sullivan) were maintained in minimum essential medium (MEM) and 10% fetal calf serum (FCS, GIBCO Laboratories, Grand Island, NY). Fifteen percent FCS was used for Ramos cells. EB virus Bam W (3.1 Kb) and Bam A (12 Kb) fragments (gift of Dr. J. Skare) were grown in pBR 322 vector. Because of the repetitive nature of Bam W, the mixture corresponds to a target of approximately 30 kb of viral genome.[27] An equal mix of Bam W and Bam A probe 0.1 mg/ml or pBR 322 probe alone was radiolabeled by nick translation to a specific activity of 3.5×10^8 cpm/μg of probe using ^{32}P-dCTP (3000 Ci/mM, Amersham).

Acid cleaned glass coverslips (Clay Adams, Lincoln Park, NJ) were dipped in 0.5% poly-L-lysine (Sigma Chemical Co., St. Louis, MO) and dried overnight at 37 C. Cultured cells were labeled overnight with tritiated thymidine (Dupont/NEN, Billerica, MA, 10 μCi/ml). Subsequently, the cells were washed twice in phosphate-buffered saline (PBS) with 5 mM $MgCl_2$ and resuspended in the same solution at a cell concentration of 10^6 cells per ml. One hundred μl of the cell suspension were mixed with 100 μl of fetal calf serum and deposited by cytocentrifuge (1800*g* for 1 minute) onto polylysine-coated glass coverslips. The cells were allowed to dry for 3 minutes and fixed in 4% paraformaldehyde (pH 7.4) for 10 minutes. The cells can be stored in 70% ethanol until used. Before use, the cells were washed twice in phosphate-buffered saline (PBS) plus 5 mM $MgCl_2$, and then treated in a solution of 0.5% saponin, 0.5% triton X-100 (Boehringer, Indianapolis, IN) in PBS for 10 minutes and washed again in PBS-$MgCl_2$. The cells were next placed in a solution of tris-glycine, pH 7.4, for an additional 10 minutes. Before hybridization, the cells were placed in 50% deionized formamide (Sigma) in 2× SSC (0.3M sodium chloride, 0.03M sodium citrate, pH 7.3) for 5 to 10 minutes.

In Situ *Hybridization*

Ten to 20 ng of probe were added to 4 μl of carrier DNA-RNA, ie, an equal mix of E. coli tRNA 10 mg/ml (Boehringer) and sheared herring sperm DNA 10 mg/ml (Sigma). The mixture was lyophilized in a vacuum under centrifugation (Savant). The probes were resuspended in 11 μl of deionized formamide and heated to 95 C for 2 minutes just before hybridization. Eleven μl of hybridization mix (10% dextran sulfate, 4× SSC, 2% bovine serum albumin, and 0.1% sodium pyrophosphate) were then added to the heated probe, mixed well, and pipetted onto parafilm spread over a glass plate. The blotted coverslips incubating in 50% formamide, 2× SSC, were then carefully placed on the probe to prevent the trapping of air bubbles. The coverslips were covered with a second parafilm layer, placed in an oven at the desired temperature for 10 minutes, and transferred to a 37 C incubator for the desired period.

Washing Conditions

The parafilm covering the glass coverslips was cut with a sharp razor blade. The moist coverslips were removed by floating in 2× SSC formamide (50%) and placed first in a solution of 2× SSC in 50% formamide for a half hour at 37 C and then in 2× SSC at 37 C for another half hour. The coverslips were then washed twice in 1× SSC for 10 minutes each at room temperature with shaking.

Detection

Scintillation

The coverslips were transferred to 1× PBS-$MgCl_2$ cut in half with a diamond pencil, and counted for 2 minutes in a scintillation counter (Beckman, Palo Alto, CA) in PBS using Cerenkov radiation. The coverslips were then dried through a graded ethanol series, air-dried overnight, and counted again in scintillation fluid to detect tritium as well as ^{32}P. The scintillation counts were normalized so that probe hybridized could be expressed per 100,000 cells.

Autoradiography

After drying through ethanol, those coverslips not used for scintillation were mounted cell side up onto microscope slides using permount, dipped in Kodak NTB3 emulsion, and stored at 4 C in the dark for the desired period. The cells were developed in D_{19} (Kodak, Rochester, NY) for 5 minutes at 14 C, in 0.1% acetic acid for 1 minute, and in Kodak fixer for 5 minutes. After several washes in deionized water, the slides were counterstained with 0.5% methyl green and 0.5% pyronin. Cells were viewed using water and stored dry at 4 C.

Nonisotopic Detection

Bam H1 fragments (A and W) of EBV cloned into pBR 322, as well as the vector alone, were nick translated using biotinylated dUTP (BRL, Gaithersburg, MD) according to standard protocols. For some experiments, biotinylated Bam W and pBR 322 probes were purchased from Enzo Biochemical, New York, NY. These probes were found to give high levels of nonspecific background. Because this introduced variation, these probes were not found to be useful for *in situ* hybridization. Identical conditions were used as detailed previously. However, after the last wash, the coverslips were exposed to 4× SSC in 0.01% triton (2 × 10 minutes). Streptavidin-alkaline-phosphatase complex[31] (a gift of BRL) was diluted 1:1,000 in 4× SSC in 1% BSA, and 40 μl of this solution was added to each coverslip and incubated for 20 minutes. We also evaluated the use of a streptavidin-alkaline-phosphatase conjugate purchased from DAKO (Santa Barbara, CA) and found it to be acceptable under the same conditions, but diluted 1:200. The coverslips were washed (4× SSC, 0.01% triton) and placed in alkaline buffer (0.1M tris in 50 mM $MgCl_2$, pH 9.5) for 2 minutes. For color development, 1 ml of this buffer containing Bromochloroindolyl phosphate (BCIP) and Nitroblue tetrazolium (NBT) (BRL) (50 μl of BCIP at 50 mg/ml and 65 μl of NBT at 75 mg/ml in 15 ml) was incubated in the dark with continuous shaking.

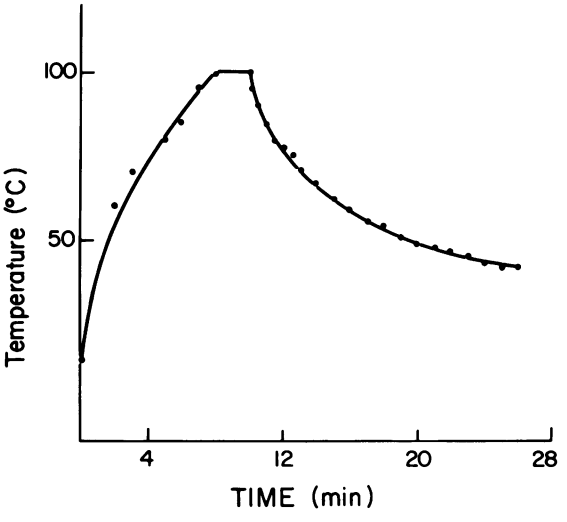

Figure 1. *Sample temperature as a function of time. Glass plate surface temperature was measured in degrees centigrade over time. The plates were placed in a 100 C oven at time zero and removed to a 37 C incubator at 10 minutes (the point at which the curve drops).*

Color development of the slides was monitored microscopically. Times varied from 5 minutes to overnight depending on the cell samples and desired sensitivity of detection. The reaction was stopped by dipping the slides in 4% paraformaldehyde and counterstaining with methyl green. The slides were mounted in crystal mount (Biomedia Corp., Foster City, CA) or stored dry.

Results

Use of the cytocentrifuge to deposit washed cells on poly-L-lysine-coated glass coverslips gave the most consistent cell retention. Use of tritiated thymidine to label the cells allowed correction of the results for cell number after counting several cell samples by microscopy. Coverslips could then be stored in ethanol until use. The hybridized probe, labeled by ^{32}P, could be counted by scintillation and then expressed as nanograms hybridized per tritium counts corresponding to 10^5 cells, the approximate amount on each coverslip. Using this approach, we sampled some of the parameters which may be important for their effect on signal.

Denaturation Conditions

We denatured the probe simultaneously with the cells and tissues, a method originally employed by Brigati et al[19] and Unger et al.[25] Different temperatures were evaluated by monitoring a thermometer located under the parafilm

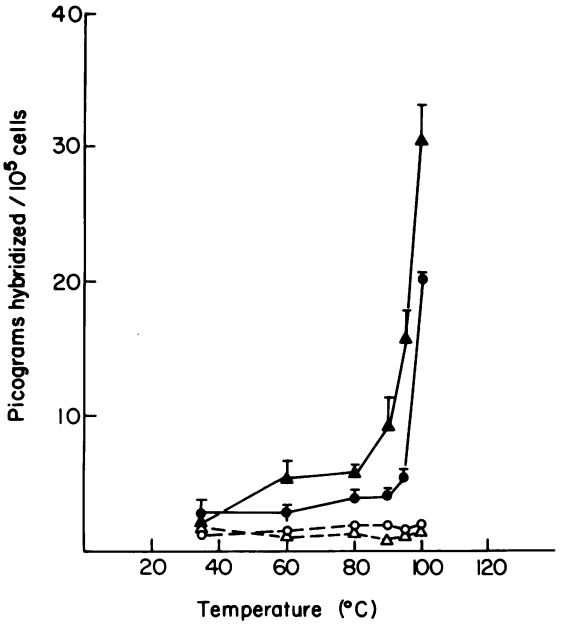

Figure 2. *Hybridization signal in B95-8 and Raji cells as a function of temperature. Cells were exposed to varying temperatures for 10 minutes before hybridization. Scintillation counts on triplicate coverslips were expressed as picograms of ^{32}P-Bam W/A probe hybridized per 10^5 tritiated B95-8 cells (closed triangles) or Raji cells (closed circles) with increasing temperature. Open triangles and circles represent hybridization of ^{32}P pBR 322 control probe to the same cells under the same conditions.*

next to the coverslips. Temperature reached 100 C in 8 minutes, and the samples were heated for an additional 2 minutes (Figure 1). For lower temperatures, cells were maintained for 10 minutes in the oven at the appropriate temperature. Figure 2 shows signal increased dramatically after the temperature reached 80 C for B95-8 cells and 90 C for Raji cells. It should be noted here that the increase in signal represents probe denaturation as well as target denaturation.

Probe Concentration

Increase of labeled probe concentration gave increasing signal, which saturated at approximately 40 pg per 10^5 B95-8 cells (Figure 3) and 20 pg per 10^5 Raji cells (Figure 4). This corresponds to approximately 1000 kilobases/cell for B95-8 or 500 kilobases per cell for Raji. If the composite of Bam W and Bam A equals approximately 30 kilobases, this corresponds to an average of 33 copies per cell for B95-8 and 16 copies per cell for Raji. This is fewer than the 50 DNA copies per cell estimated for Raji and may indicate a lower efficiency for detecting episomes. Because a small percentage of B95-8 has high

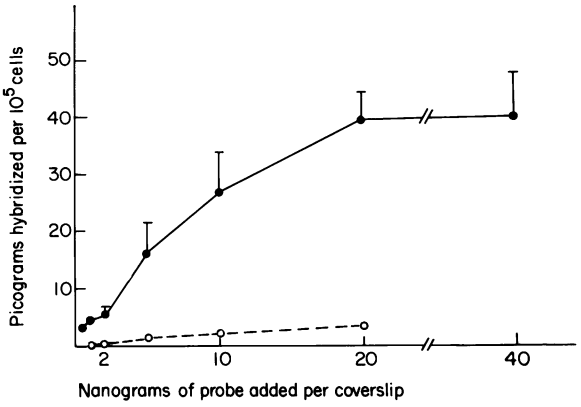

Figure 3. *Saturation curve of B95-8 cells with increasing probe concentration. The dark circles represent picograms of ^{32}P Bam W/A probe hybridizing to 10^5 B95-8 cells, with increasing probe concentration, after duplicate samples were heated to 100 C and hybridized for 3 hours at 37 C. The open circles represent hybridization of ^{32}P-pBR 322 control probe to the same cells under the same conditions.*

copy number, these productively infected cells can be estimated to contain as much as 4×10^4 kb or 1200 copies of EBV.

Hybridization Time

After denaturation, coverslips containing cytospun cells were hybridized at 37 C for increasing periods (Figure 5).

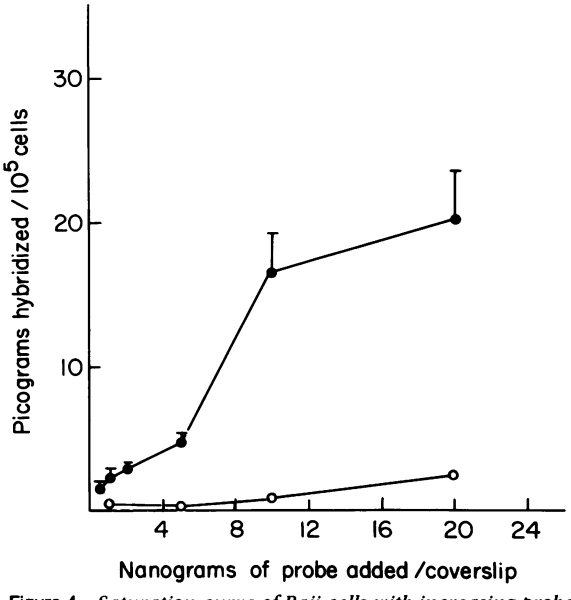

Figure 4. *Saturation curve of Raji cells with increasing probe concentration. The dark circles represent picograms of ^{32}P Bam W/A probe hybridizing to Raji cells with increasing probe concentration, as in Figure 3. The open circles represent hybridization of ^{32}P pBR 322 control probe to the same cells.*

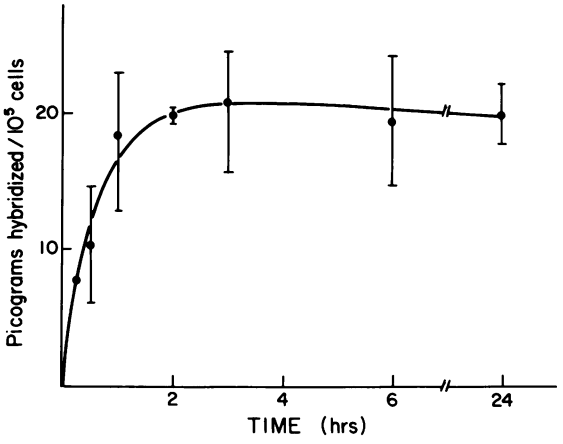

Figure 5. *Hybridization kinetics. Ten ng of ^{32}P Bam W/A probe and B95-8 cells were simultaneously denatured at 100 C for 10 minutes and then left to hybridize at 37 C for different periods. These were duplicate samples except for the first point.*

The signal plateau was reached in 1 to 2 hours, and did not increase significantly after 24 hours of hybridization. Subsequent hybridizations were carried out for 3 hours.

Autoradiographic Development

Development time was overnight for B95-8 cells after hybridization with ^{32}P probe (Figure 6A). When the slides were allowed to develop for 5 days, the positive foci extended beyond the confines of the cell (Figure 6B). Intense foci of ^{32}P activity were seen in only a small percentage of the cells. The control probe showed no foci of hybridization.

Raji cells hybridized with the viral probe showed that the silver grain distribution over the cells was not uniform (Figure 6C). Figure 7A represents grain counts over a number of Raji cells to quantitate the distribution compared with a control probe (Figure 7B). More than 94% of Raji cells probed with pBR 322 had fewer than five grains per cell (Figure 6D). In contrast, 37% of the Raji cells probed with the EBV probe were indistinguishable from the control, 23% had weak but detectable signal, 35% had strong signal, (ie, significantly increased grain counts), and a small number (5%) had extremely high signal (ie, very large numbers of grains). Because all cells in this line are believed to be carrying EBV genomes, either this *in situ* hybridization protocol induces an artifactual heterogeneity or the cell line is intrinsically heterogeneous, either in accessibility of the targets or in actual copy number of sequences (see Discussion).

Nonisotopic Detection

Streptavidin-alkaline-phosphatase detection after hybridization to B95-8 cells with biotinylated viral probe showed foci of intense nuclear signal in a small percentage of the cells, as was seen with autoradiography (Figure 6E). Other cells showed varying degrees of signal intensity within the nucleus, usually punctate in nature and possibly representing sites of viral replication or transcription. Raji cells showed a varying degree of nuclear signal from cell to cell as was indicated with grain counts from autoradiography (Figure 6F). Hybridization with biotinylated pBR 322 probe showed occasional diffuse cytoplasmic background around the nucleus but no nuclear signal was noted (not shown). Hybridization of the probe to Namalwa cells, which contain two EBV DNA copies per cell in two discrete foci in G_2 cells and one in G_1 cells,[24] were found to contain either one or two foci of signal per cell (Figure 6G). No signal was found with the control probe. When Namalwa cells were treated with 4 μg/ml actinomycin for 4 hours, the nuclear foci were very much diminished and barely detectable in most cells. The signal in most part represents, therefore, RNA transcripts within the nucleus that have been shown to be equivalent to 900 kb of RNA.[30] Hybridization to Ramos, EBV-negative cells showed no nuclear signal (Figure 6H).

Detection of EBV in Sectioned Material

Namalwa cells centrifuged into a pellet were treated according to a standard regimen used at the pathology lab at Massachusetts General Hospital (courtesy of Dr. Nancy Harris). The sectioned material, received on glass slides, was hybridized as described above with the following exceptions. After dewaxing of the paraffin sections, the slides were rehydrated in 70% ethanol and treated with 0.2N HCl for 10 minutes. Proteinase K was used (4 μg/ml, Sigma) at 37 C for 10 minutes. They were then incubated in 0.1M Tris-HCl, 0.2M glycine at pH 7.4 for 10 minutes, in 0.1M triethanolamine for 10 minutes, and finally stored in 2× SSC 50% formamide until hybridization. Single or double spots representing the aforementioned RNA transcripts were seen in 30% to 40% of the cells (Figure 8). Ramos cells processed identically showed no signal. Of note, however, was an increase in background after the sectioning protocol that made it somewhat more difficult to analyze the signal.

Discussion

Although this study employs simultaneous denaturation of viral targets and the probe within the tissue, certain strengths and weaknesses of this approach became evident during the course of this work. For instance, the heat required for DNA denaturation caused significant morpho-

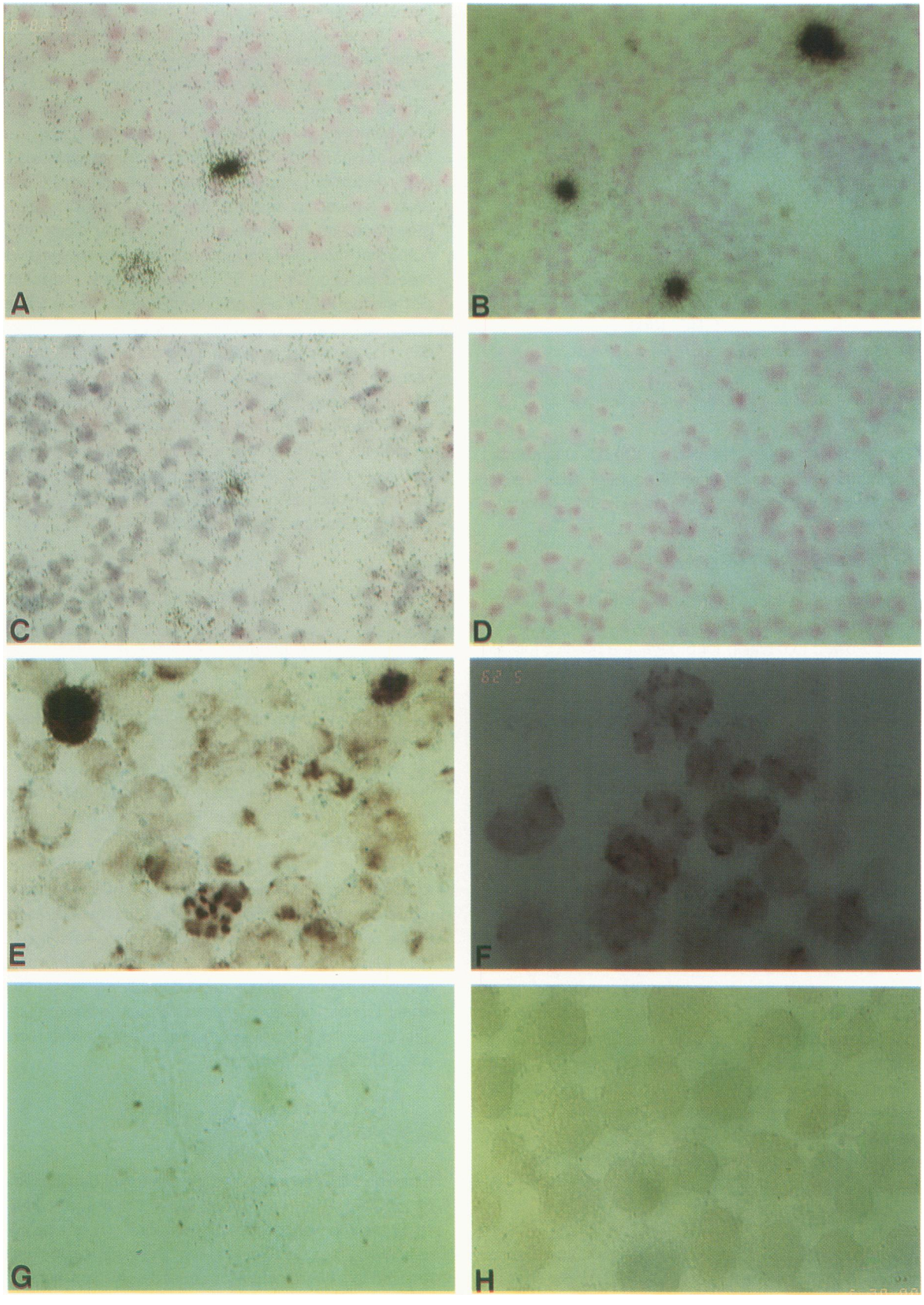
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Figure 6. *Microscopy of various cell lines infected with EBV detected by isotopically and nonisotopically labeled probes.* **A, B:** *Autoradiograms of hybridized B95-8 cells. Probe as described above was hybridized to the cells (original magnification ×100).* **A:** *Overnight development.* **B:** *Five days of development.* **C, D:** *Autoradiographs of hybridized Raji cells.* **C:** *The probe was hybridized as described and developed for 10 days (×100).* **D:** *Hybridization of ^{32}P pBR 322 probe to the same cells and developed as above (original magnification ×100).* **E:** *EBV nucleic acids in productively infected cells detected nonisotopically after* in situ *hybridization. B95-8 cells hybridized with biotinylated Bam W probe and developed with streptavidin-alkaline-phosphatase (original magnification ×500).* **F, G, H:** *EBV nucleic acids in latently infected cell lines and control cells detected by nonisotopic methods after* in situ *hybridization (original magnification ×500).* **F:** *Raji cells (50 copies EBV per cell) hybridized with and detected as in E.* **G:** *Namalwa (two copies EBV per cell) cells hybridized and detected as in E.* **H:** *Ramos (no EBV) cells hybridized and detected as in E.*

logic disruption and was very destructive for chromosomes prepared by standard methods. Tissue sections fared considerably better, perhaps due to more structural integrity. RNA was damaged by the heat as well; better morphology and RNA preservation were obtained using a more gentle protocol.[24,30] This is an important consideration because, as indicated by the Namalwa results, most of the signal is from RNA. A prediction from this is that single-stranded probes, or completely melted double-stranded probes applied to dehydrated sections, could possibly be used in the absence of heat. These possibilities are being investigated. Nonetheless, the technique was sensitive enough to detect a single copy of EBV and its associated transcripts (approximately 900 kb of EBV total target), as evidenced by the Namalwa cells. Although this is not currently the most sensitive method for detecting EBV in Namalwa cells (see References 24 and 30), it appears sufficient for the EBV-infected cells tested. Further developments using alkaline phosphatase detection will be directed toward improvement of sensitivity. Because of the stability of the color detection, which provides a permanent record, and because it is possible to use standard bright field microscopy, these efforts are worthwhile.

In a transformed B cell line from cotton-top marmosets[32] (B95-8), a small percentage of the cells are productively infected by EB virus. A significant proportion of the virus in this line exists in a linear form.[26] A human Burkitt lymphoma cell line, Raji, contains approximately fifty viral genomes per cell as determined by filter hybridization.[28] Approximately 8 to 12 copies of the virus are believed to be in linear form and associated with host DNA; the remaining copies exist as circular supercoiled episomes.[33,34] The difference in hybridization temperatures between these two cell types (greater than 80 C for B95-8 cells and greater than 90 C for Raji cells) may reflect a difference between these two cell types, or the conformation or concentration of their respective viral DNA or RNA.

Denaturation of DNA viral sequences at 100 C was employed by Brigati et al[19] and Unger et al.[25] Their suggestion that double-stranded viral DNA may reanneal after denaturation served as the rationale for our approach. However, it may be important in cells that contain large concentrations of linear DNA, as is the case in cells associated with productive infections, because previous work showed it was not necessary in Namalwa cells in which viral DNA is integrated and in which reannealing is not a factor.[24] Furthermore, in cases in which most of the signal is RNA, as is the case with Namalwa cells, denaturation of the target nucleic acids was unnecessary.[30] It should also be noted that exposing cultured cells to 100 C was destructive to cellular morphology, chromosomes, and RNA retention, and the minimal amount of heat necessary should be used (in our case, only 2 minutes at 100 C). In paraffin sections of the same cell line the heat treatment was less destructive, possibly because the hardening

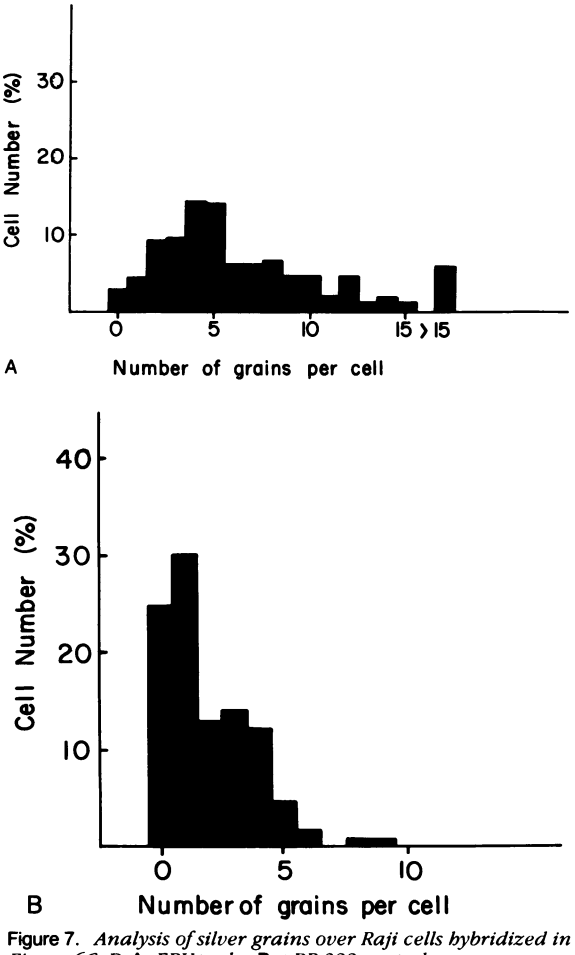

Figure 7. *Analysis of silver grains over Raji cells hybridized in Figure 6C, D.* **A:** *EBV probe.* **B:** *pBR 322 control.*

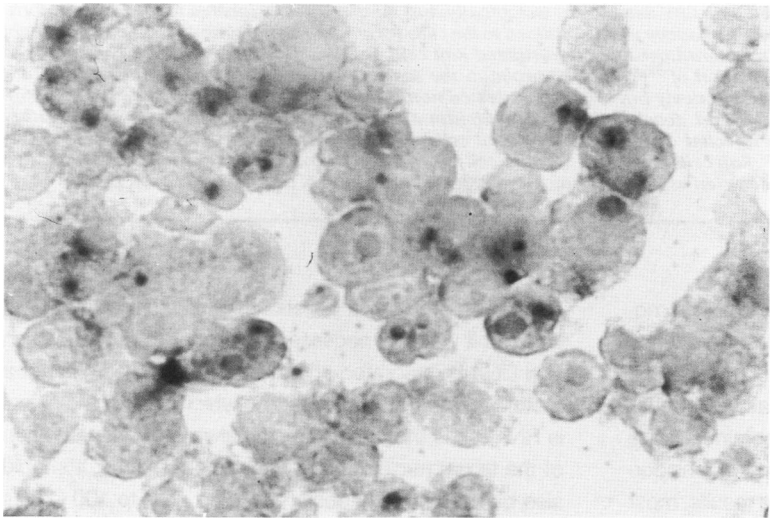

Figure 8. *The Namalwa cells in Figure 6G were pelleted in serum and fixed, embedded, and sectioned in a pathology laboratory. Formaldehyde fixation was overnight. Sections were deparaffinated, acid-treated, proteinased, and rehydrated in 50% formamide, 2× SSC, and hybridized as described in Materials and Methods. Alkaline phosphatase development was for 30 minutes (original magnification ×500; no counterstain).*

and embedment protects the morphology, and may have been more directly analogous to the findings described by Unger et al.[25]

Northern blot analysis of B95-8 cells indicated a significant EB virus-translated RNA content that was markedly enhanced by phorbol esters.[35] The experiments described in this work on B95-8 cells were repeated using fluorescent detection[30] rather than alkaline phosphatase and more cells were detected as positive (as many as 34%, Marselle L, Lawrence J, personal communication). The probe appeared to be detecting mostly RNA, as most of the signal disappeared with RNase treatment. This single-cell data obtained by microscopy confirmed the alkaline phosphatase detection in that most positive signal was found in a small percentage of B95-8 cells, which were lytically infected with EB virus; the rest contained latent virus.[24,32] Further quantitative work using fluorescence was directed toward clarifying the exact amounts of RNA and DNA on the cellular level.[30] Similarly, hybridization to Raji cells under normal (nondenaturing) conditions showed that most signal was due to hybridization to RNA, a finding in agreement with the result of Northern blot analysis.[35–38]

Estimates of 50 EB virus copies per Raji cell were derived from Southern blot analysis of DNA extracted from these cells.[28] Our quantitation detected fewer copies per cell (RNA and DNA) on the average, perhaps due to lesser accessibility of episomal targets to the probe compared with linear targets. The variation in distribution of P^{32} grains over these cells in our study indicated that there may be variation in virus content or, more likely, RNA expression from cell to cell. This variation was also evident when using nonisotopic detection of hybridization. That the variation is not an artifact of hybridization was suggested by results using Namalwa cells, for which more than 85% of the cells were seen to have signal comprising a single or double spot of hybridization per cell. Other work[21,39] used *in situ* hybridization of biotinated EBV probes followed by antibody detection. We found results similar to theirs, including variation in EBV signal in individual Raji cells. These researchers[21] suggested that mitotic cells may have increased EBV signal.

Sectioned Namalwa cells give an indication of the reduction in sensitivity of detection of EBV that occurs when samples are subjected to routine preparation procedures found in most pathology laboratories. Because sectioning selects cellular material, the EBV signal may not appear in some sections. The reduction of the signal in sectioned cells (30% to 40%) compared with that in cultured cells (85%) is a combination effect resulting from the sectioning and the use of histologic procedures that have not been optimized for *in situ* hybridization. The application of this approach to clinical diagnosis will be presented elsewhere.[40]

These results give an upper limit estimate of the maximum amount of positive signal that would be apparent in sectioned material if 100% of the cells were positive for EBV, but each contained a single site of EBV expression. The striking punctate nature of the signal is diagnostic of EBV expression or replication and increases both the sensitivity and the certainty of identifying the viral infection, productive or latent. Future developments and clinical applications will be important to verify the diagnostic usefulness of this technique.

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