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Technical Advance

Sensitive *in Situ* Hybridization with Catalyzed Reporter Deposition, Streptavidin-Nanogold, and Silver Acetate Autometallography

Detection of Single-Copy Human Papillomavirus

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The usefulness of standard* in situ *hybridization for viral nucleic acid detection is occasionally limited by its sensitivity limit of 10 to 50 copies per cell. A modified version of the recently described signal amplification method, catalyzed reporter deposition (CARD), and its application to formalin-fixed cells and tissue sections is presented. Deposition of the reporter is facilitated by using horseradish peroxidase catalyzing the deposition of biotinylated tyramide on the location of the probe target. The biotin accumulation created is usually detected with streptavidin-labeled enzymes or fluorochromes. In the present investigation, this step was replaced by streptavidin-Nanogold and combined with silver acetate autometallography. This resulted in deep-black precipitation at positive* in situ *hybridized reaction sites. The sensitivity of this new approach was tested with a biotinylated, genomic probe specific for human papillomavirus (HPV)-16/18. SiHa cells, a cervical carcinoma-derived cell line with one to two HPV16 copies per cell, and 10 histologically confirmed cervical carcinomas were used for the study. All samples were previously HPV16 positive with solution polymerase chain reaction, but only two of the cervical carcinomas were positive with standard* in situ *hybridization with barely visible signals. When employing CARD-Nanogold, SiHa cells and 9 of 10 biopsies proved positive with marked signals. It is concluded that this nonisotopic method can detect single viral copies* in situ *in routinely fixed material and may have the potential to replace* in situ *polymerase chain reaction in many applications. (Am J Pathol 1997, 150:1553–1561)

Since its introduction in 1969 by Gall and Pardue,[1] *in situ* hybridization (ISH) techniques have become important tools to detect nucleic acid target sequences. Regarding the detection of viral nucleic acids, it was demonstrated that radioisotopic labels can be replaced by nonradioisotopic reporter molecules such as biotin, digoxigenin, or diverse fluorochromes without loss in sensitivity or specificity.[2,3] Currently, commercially available, genomic, viral DNA or RNA probes can be confidently applied to most routine diagnostic material. However, the detection sensitivity of both radioisotopic and nonradio-

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isotopic labels in genomic probes is 10 to 50 viral copies per cell in formalin-fixed samples.[2–4] During the last few years, several attempts have been described to amplify ISH signals. Some of these include the silver intensification of diaminobenzidine precipitates or immunogold silver staining.[5] The possibility of exploring the amplification of nucleic acids by *in situ* polymerase chain reaction (ISPCR) has been extensively reported.[6–11] We and others could detect single viral copies only when ISPCR was used.[8,9,11] Although equipment dedicated to ISPCR exists, this technique is not entirely problem-free. Two groups have, for instance, reported back-diffusion artifacts during the amplification process and how this may be dealt with.[6,9] Amplicon leakage may jeopardize ISPCR results and is frequently not satisfactorily addressed in most publications. An important and valid strategy is the pursuit of signal amplification as an alternative that avoids problems with target (nucleic acid) amplification.

Such an amplification principle, designated catalyzed reporter deposition (CARD) and based on horseradish-peroxidase-mediated deposition of biotin, was recently introduced.[12–16] After hybridization, CARD uses as a first detection layer an antihapten immunoglobulin- or streptavidin-labeled horseradish peroxidase. Signal amplification is achieved by deposition of biotinylated tyramide (a biotin-phenolic compound) at the location of the probe label through the enzymatic action of horseradish peroxidase. The accumulated biotin source is usually detected with enzyme- or fluorochrome-labeled streptavidin. The principle was originally developed for enzyme-linked immunosorbent assay and Western blot by Bobrow et al[12,13] and has been applied immunohistochemically by Adams et al,[14] who demonstrated its unprecedented sensitivity. Its use has been successfully extended to ISH of endogenous DNA sequences with the possibility of single-gene detection using probes that vary in length ranging from several kilobases to some hundred base pairs.[15,16] So far, no published *in situ* CARD application has dealt with foreign nucleic acid detection, eg, viruses or other infectious agents.

The objective of this study was to determine whether single viral copy detection was possible by employing ISH with CARD in formalin-fixed samples using a biotinylated, genomic human papillomavirus (HPV) DNA 16/18 probe of ~8 kb. CARD was modified such that the amplified biotin molecules were detected with streptavidin-Nanogold and silver acetate autometallography[17] rather than with enzymes or fluorochromes (Figure 1). Gold particles coupled with silver intensification (autometallography) can help to achieve extremely sensitive ISH and immunohistochemistry, offering improved detection over both fluorescent and radioisotopic probes.[18] Although colloidal gold had been used earlier, it has several disadvantages; its size, charge, and aggregation limits penetration, and the adsorption of streptavidin or antibodies is not fully stable. A recent improvement in gold technology has been the synthesis of a 1.4-nm gold cluster, Nanogold, that may be covalently attached to streptavidin or antibodies.[19] We have found that use of streptavidin-Nanogold overcomes many problems associated with colloidal gold and makes it the visualization method of choice over fluorescent, enzymatic, and radioisotopic methods. It has a combination of properties not achieved by other molecules: 1) its small size gives excellent tissue and nucleus penetration, 2) it has stable covalent gold attachment, 3) silver acetate autometallography gives ultrahigh sensitivity, 4) it gives rapid and permanent color development and does not require lengthy film exposure or processing, 5) its black color is compatible with full strength standard cell and nuclear histological stains, which greatly improves morphological assessment, 6) it is observable in bright field, and therefore no expensive fluorescent optics is required and no problems with bleaching and autofluorescence arise, 7) resolution is high enough even for ultrastructural electron microscopic localization, and 8) it is nonradioisotopic. Single viral copy detection was consistently achieved in routinely fixed material by ISH with CARD-Nanogold.

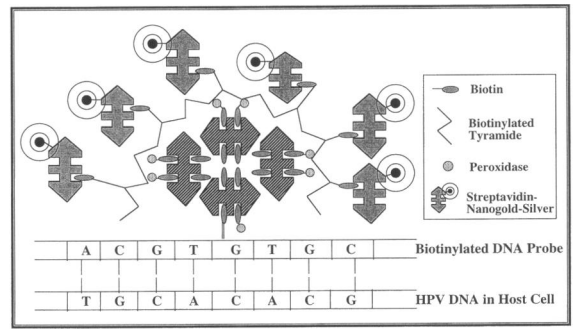


Figure 1. *Diagram of ISH with CARD-Nanogold. The viral DNA is demonstrated with a biotinylated nucleic acid probe. Every single biotin molecule acting as a DNA-coupled reporter can be detected with one molecule of a streptavidin-biotin-peroxidase complex. For simplicity, only four such complexes are shown. The following biotinyl tyramide layer is used to accumulate biotin molecules at peroxidase-labeled sites. These are then further detected by streptavidin-Nanogold and visualized by silver precipitation (autometallography). Using this combination, a multiplication of the original label is reached, allowing the detection of single viral copies.*

Materials and Methods

Cell and Tissue Samples

For monitoring the detection sensitivity of ISH with CARD-Nanogold, formalin-fixed SiHa cells[20] were used as a model system. This cell line contains one to two HPV16 copies per cell integrated in region q21-q31 of chromosome 13.[21] To check ISH, formalin-fixed CaSki cells[21] with 200 to 800 HPV16 copies per cell integrated in at least 11 chromosomal sites[22] were used. Both cell lines are derived from cervical carcinoma and were obtained from American Type Culture Collection (Rockville, MD). Previously, SiHa was reported to prove negative with standard ISH[4,8,11] but proved positive with ISPCR.[8,11] To assess the utility of ISH with CARD-Nanogold in clinical samples, 10 formalin-fixed and histologically confirmed cervical carcinoma biopsies were tested. All samples included in the study were previously HPV16 positive with solution PCR,[23] but only two of the cervical carcinoma biopsies tested positive when performing standard ISH[4,5] with only barely visible signals.

Preparation of Samples

SiHa and CaSki cells were grown until confluency at 37°C in a humidified incubator with 5% CO_2 atmosphere in a medium consisting of 90% Eagle's medium and 10% fetal calf serum, with glutamine and antibiotics (100 U of penicillin and 50 mg/ml streptomycin) added. The cells were harvested with 0.25% trypsin and centrifuged in medium in a Beckman TJ-6 at 2000 rpm for 5 minutes at room temperature. Thereafter, they were washed in Dulbecco's Mg^{2+}- and Ca^{2+}-free phosphate-buffered saline (PBS; a 10X solution consists of 9.16 g of Na_2HPO_4, 2.0 g of KCl, 2.0 g of KH_2PO_4, and 80.0 g of NaCl in 800 ml of ultrapure water. The pH was adjusted to 7.2 with concentrated NaOH, and ultrapure water was added to a final volume of 1 L). The solution was centrifuged again, the PBS was decanted, and it was fixed in 4% neutral-buffered formaldehyde solution (4.5 g of $NaH_2PO_4 \cdot H_2O$ and 8.3 g of $NaHPO_4 \cdot H_2O$ in 110 ml of concentrated formaldehyde solution and distilled water to a final volume of 1 L), pH 7.2, at room temperature for 15 hours. The fixative was decanted, the cells were resuspended in PBS, and approximately 10^4 cells were spotted onto organo-silane-treated slides[24] by gravitation. Superfluous fluid was removed with a tissue after 10 minutes. The spots were air dried under a laminar hood and dehydrated with 70%, 95%, and absolute ethanol and air dried again. This treatment resulted in a well preserved morphology. To mimic routine biopsy procedures, paraffin blocks of 4% neutral-buffered formaldehyde-fixed SiHa cells were also prepared. Before fixation, these cells were resuspended in 500 ml of normal AB serum and clotted with approximately 25 U of thrombin (Gentrac, Middletown, WI). The biopsies deriving from cervical carcinoma tissue were fixed in 4% neutral-buffered formaldehyde for 15 hours and thereafter processed in upgrading ethanols as above and xylene and embedded in paraffin. The biopsies and the SiHa cell block were cut as 4- to 6-μm sections onto organo-silane-treated slides, baked for 1 hour at 60°C, brought to room temperature, and deparaffinized in xylene twice for 15 minutes each, rinsed in absolute ethanol, and air dried. Specimens were then ready for unmasking nucleic acids and used within a few days.

ISH with CARD

For good accessibility of target DNA to the biotinylated, genomic HPV16/18 DNA probe of ~8 kb (ENZO Diagnostics, New York, NY), the formalin-fixed specimens (cell spots and cut biopsy and cell sections alike) were permeabilized with 0.01% proteinase K (Boehringer Mannheim, Mannheim, Germany) dissolved in PBS at 37°C for 8 to 30 minutes as described earlier.[4] Protease action was inactivated by three 3-minute washings in distilled water. This was followed by quenching of the endogenous peroxidase with 3% H_2O_2 at room temperature for 30 minutes. Denaturation of the target DNA in the presence of the probe was performed at 92 to 94°C for 10 minutes followed by hybridization in a moist chamber containing 2X standard saline citrate (SSC; a 20X SSC solution consists of 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of ultrapure water). The pH was adjusted to 7.0 with concentrated NaOH, and ultrapure water added to a final volume of 1 L at 37°C for 2 hours. Post-hybridization washes were done in 2X SSC at room temperature for 5 minutes, 0.2X SSC at 37°C two times for 5 minutes each, and 2X SSC at room temperature for 5 minutes. Samples were incubated in Lugol's iodine (Merck, Darmstadt, Germany) at room temperature for 5 minutes, then washed in tap water and ultrapure water followed by 2.5% sodium thiosulfate until they became colorless, and washed again in tap water for 3 minutes and ultrapure water twice 3 minutes each. For standard ISH, the procedure was continued as published previously[4,5] or carried out with streptavidin-Nanogold

(Nanoprobes, Stony Brook, NY) directly as described below. For ISH with CARD, samples were blocked with 4X SSC containing 0.5% blocking reagent (supplied in the tyramide signal amplification kit, TSA, DuPont-NEN, Boston, MA) at 37°C for 30 minutes followed by a brief rinse in 4X SSC containing 0.05% Tween-20 (Boehringer Mannheim). Immunochemical detection was performed with streptavidin-biotinylated horseradish peroxidase complex (Duett streptavidin, Dako, Glostrup, Denmark) at a concentration of 1:200 in SSC with blocking reagent at room temperature for 30 minutes. Extensive washing steps consisted of 3 changes SSC with Tween-20 for 5 minutes each, followed by PBS for 5 minutes and 50 mmol/L Tris/HCl, pH 7.5 (1 mol/L Tris/HCl consists of 121.1 g of Tris base dissolved in 800 ml of ultrapure water. The pH was adjusted to 7.0 with concentrated HCl, and ultrapure water was added to a final volume of 1 L for 5 minutes. Subsequently, hybridized reaction sites were amplified at room temperature for 10 minutes with biotinyl tyramide at a concentration of 0.001 to 0.002 mg/ml borate buffer/H_2O_2 diluent (TSA) followed by four consecutive washes in PBS containing 0.05% Tween-20 and 20% dimethylsulfoxide at room temperature for 5 minutes each. Dimethylsulfoxide facilitates penetration by rendering the plasma membrane permeable.

Streptavidin-Nanogold and Silver Acetate Autometallography Detection

Samples were immersed in Tris-buffered saline (TBS; 8.8 g of NaCl and 12.1 g of Tris base in 800 ml of ultrapure water). The pH was adjusted to 7.6 with concentrated HCl, and ultrapure water was added to a final volume of 1 L containing 0.1% fish gelatin (Aurion, Wageningen, The Netherlands) for 5 minutes and incubated with streptavidin-Nanogold (Nanoprobes; Stony Brook, NY) diluted 1:1500 in TBS containing 1% bovine serum albumin (Boehringer Mannheim) at room temperature for 60 minutes. This was followed by two 5-minute washes in TBS and one 5-minute wash in TBS with fish gelatin and then briefly in PBS. Post-fixation was performed in 2% glutardialdehyde, electron microscopy grade (Merck) in PBS for 2 minutes followed by repeated washes in deionized water for at least 15 minutes and the last two rinses in ultrapure water. One hundred milligrams of silver acetate (Fluka Chemie, Buchs, Switzerland) was dissolved in 50 ml of ultrapure water by continuous stirring (solution A). The autometallography process was performed in low pH citrate buffer (23.5 g of trisodium citrate dihydrate and 25.5 g of citric acid monohydrate in 850 ml of ultrapure water adjusted to pH 3.8 with citric acid). Two hundred and fifty milligrams of hydroquinone (BDH Chemicals, Poole, UK) was dissolved in citrate buffer (solution B). Just before the process of autometallography, solution A was mixed with solution B. Development was checked under a light microscope, and when optimal staining was reached (after 4 to 10 minutes), the process was stopped by repeated washes in tap water. Slides were rinsed in tap water for at least 3 minutes and sections briefly counterstained with 1% eosin and nuclear fast red, dehydrated in ethanols, cleared in xylene, and mounted in DPX (BDH Chemicals).

Controls

To ensure that the observed ISH signals were specific, negative control experiments included an irrelevant probe, ie, the biotin-labeled vector pBR322 without target sequences (Digene, Silver Springs, MD), which was additionally applied to all HPV-tested samples. Target-negative tissue, ie, an epidermal biopsy, served as another negative control. CaSki cells served as the positive ISH control for the HPV16/18 probe.

Results

SiHa and Cervical Carcinoma Biopsies

When performing standard ISH with the HPV16/18 probe, SiHa was negative, regardless of whether it was prepared as cell spots or embedded in paraffin, and only 2 of 10 biopsies tested positive. ISH with the HPV16/18 probe and CARD-Nanogold demonstrated HPV presence both in SiHa cell spots and cut sections as well as in 9 of 10 cervical carcinoma biopsies. Positive *in situ* hybridized reaction sites were seen as deep-black precipitation in HPV-infected nuclei (Figure 2, A and C, and Figure 3, A, C, and D). This, in combination with 1% eosin and nuclear fast red counterstaining, facilitates evaluation, especially in low-magnification bright-field microscopy.

Controls

SiHa (Figure 2, B and D, and Figure 3B) and the 10 cervical carcinoma biopsies tested negative with the irrelevant probe pBR322 and ISH with CARD-Nanogold. The target-negative epidermal biopsy proved negative with the HPV16/18 probe and ISH with

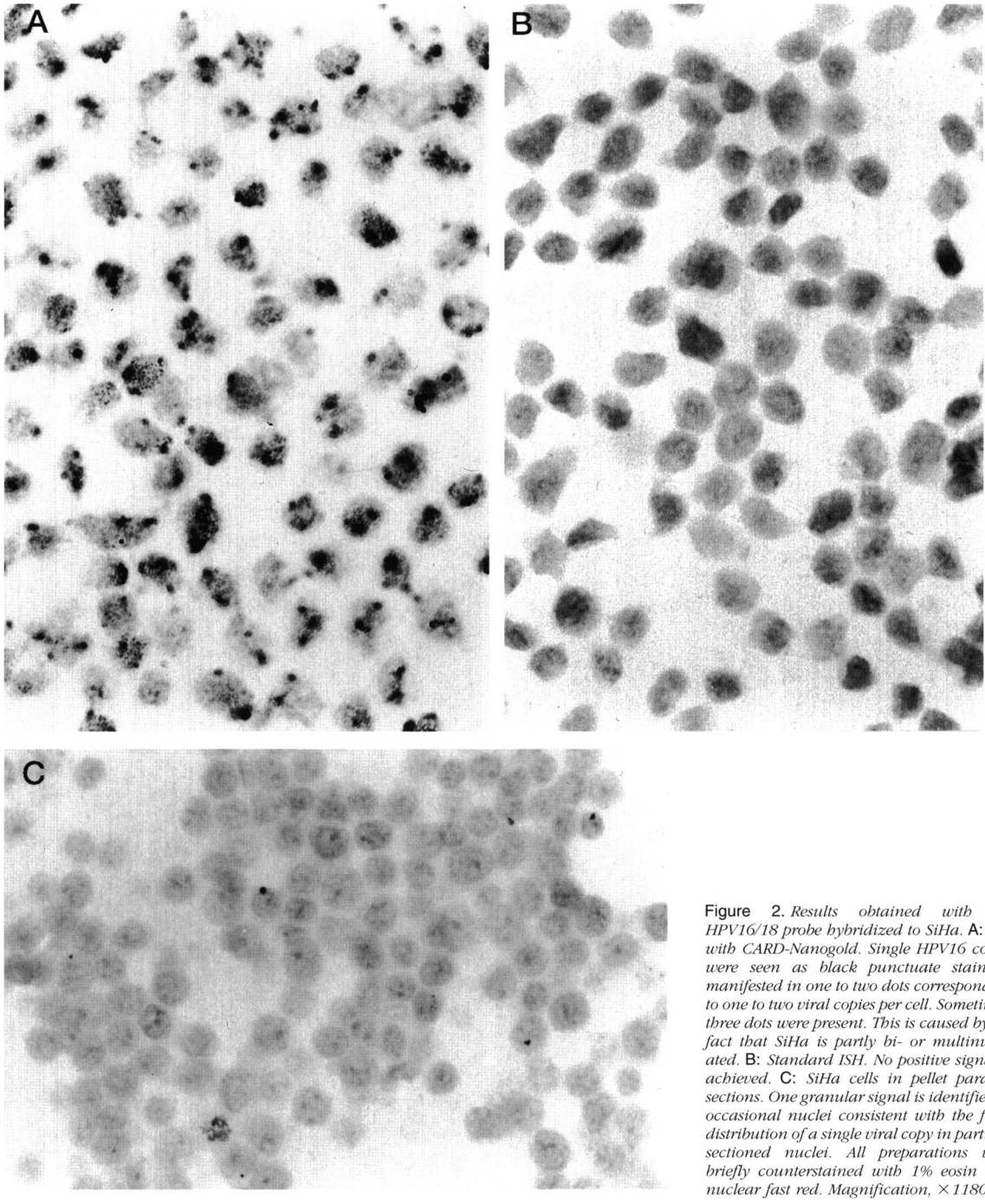
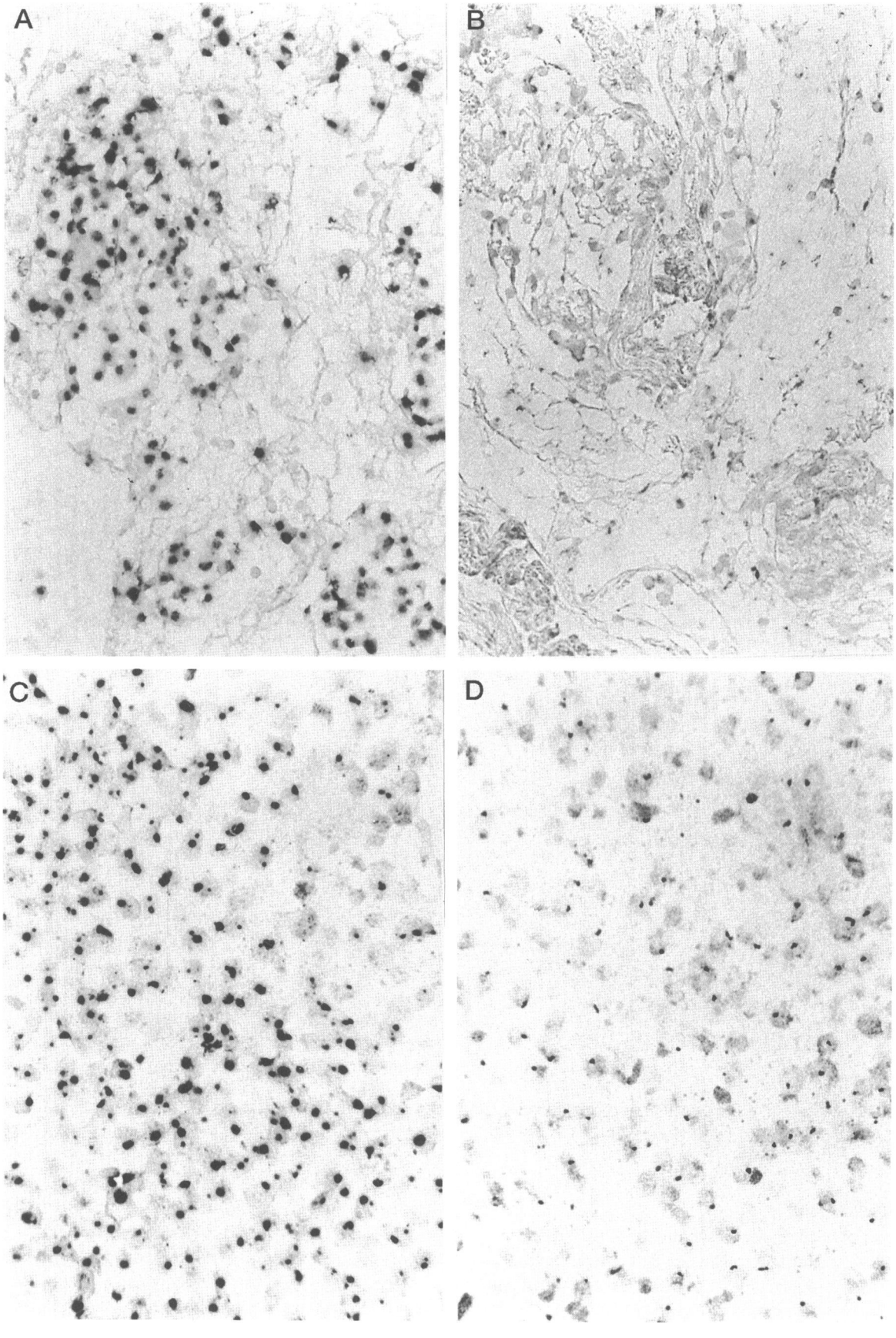


Figure 2. *Results obtained with the HPV16/18 probe hybridized to SiHa.* A: *ISH with CARD-Nanogold. Single HPV16 copies were seen as black punctuate staining, manifested in one to two dots corresponding to one to two viral copies per cell. Sometimes, three dots were present. This is caused by the fact that SiHa is partly bi- or multinucleated.* B: *Standard ISH. No positive signal is achieved.* C: *SiHa cells in pellet paraffin sections. One granular signal is identified in occasional nuclei consistent with the focal distribution of a single viral copy in partially sectioned nuclei. All preparations were briefly counterstained with 1% eosin and nuclear fast red. Magnification, ×1180.*

CARD-Nanogold. CaSki cells tested positive with the HPV16/18 probe and standard ISH.

Discussion

The aim of this study was to present CARD modified for ISH with streptavidin-Nanogold to detect single viral copies in routinely fixed samples by applying biotinylated genomic probes of several kilobases. CARD is based on amplification of the detection signal rather than amplification of nucleic acids as in ISPCR. Both methods allow the detection of single-copy genes or viruses with probes ranging from a few hundred base pairs to several kilobases.[8,9,11,15–16] ISPCR is even successful when using oligoprobes of ~30 to 50 bp.[6,10] So far, this has not been convincingly demonstrated with CARD. In applications in which, for instance, point mutations of nucleic acid sequences are


A
B
C
D

to be tested, oligoprobes seem the adequate alternative to confirm such alterations. It remains to be seen whether CARD is a sensitive enough tool for this issue. However, in most cases, genomic or subgenomic probes suffice, and in such applications the benefits of CARD compared with ISPCR are profound. Leakage of amplicons to target-negative sites, still the principal drawback of ISPCR and inadequately addressed in much of the published literature to date, is no longer a problem. Costs are low because no expensive equipment or reagents are needed, and the reproducibility of CARD fulfills the requirements for research and clinical work. Moreover, signal amplification is applicable both to rare nucleic acid sequences and antigen (gene product) detection. Finally, the whole procedure, including hybridization, can be completed within one working day and is on the whole less onerous than ISPCR.

Commercially available, ready-to-use CARD reagents and biotin-labeled probes, eg, those used in this study as well as others, even with reporter molecules such as diverse fluorochromes and digoxigenin, make applications easy. Additionally, self-made PCR probes with diverse labels have been successfully used.[16] That the positive signals were specific and not due to artifacts, eg, to silver acetate nonspecifically bound to nucleolar organizing regions, was proven by our rigorous negative control system.

The specific application of ISH with CARD-Nanogold was found superior over an initially performed version with enzymatic detection, eg, with peroxidase because signals appeared more distinct (data not shown). We therefore optimized the current protocol accordingly, with emphasis on duration and concentration of streptavidin and biotinyl tyramide as well as protein blocking and extensive washing steps. Protease treatment was carried out as described earlier, ie, 0.01% for at least 15 minutes, which is necessary to achieve the desired detection sensitivity of single viral copies. Additional adjustments were made during detection with streptavidin-Nanogold, which eventually could be used in a dilution ~15 times higher than is advised for standard ISH by the supplier.

Lugol's iodine followed by decolorization with sodium thiosulfate is used in nearly all immunogold silver-staining procedures described in the literature. This step has empirically been found to increase the autometallographic staining efficiency in most applications, including immunohistochemistry and ISH.[5] If iodine treatment is avoided, the autometallography amplification process needs to be drastically prolonged. The chemistry of Lugol's iodine followed by sodium thiosulfate has been discussed controversially and has not yet been explained convincingly. Other types of oxidation solutions, eg, potassium permanganate or hydrogen peroxide with and without the combination of a chemical reduction step, and also complete avoidance of oxidation steps were all systematically tried (data not shown). However, no other type of pretreatment has resulted in the same detection sensitivity comparable to Lugol's iodine in combination with autometallography. Its effect seems to be connected with silver amplification. No deleterious effect was seen on DNA or RNA detectablility by hybridization (G. W. Hacker et al, unpublished observations).

To accomplish the highest possible contrast between viral DNA-positive nuclei and negative nuclei, only weak counterstaining was performed, which reduces assessability of morphological details to some degree. For improved morphological resolution, ie, to make target-negative nuclei appear more distinct, counterstaining would have to be increased accordingly.

Taken together, we are confident that single viral copy detection can be achieved using ISH with CARD-Nanogold in formalin-fixed samples. Our present protocol worked always satisfactorily on cultured cells because conditions could be well controlled. However, one should bear in mind that the quality of clinical material may vary depending, eg, on fixation time/temperature and how fast a specimen was put into fixative. This is a general and recognized dilemma and not confined to CARD alone when performing immunohistochemistry or immunohistochemical detection after ISH on routinely fixed material. One case was rated negative simply because safe evaluation was difficult due to background noise, even in the negative control but less marked there, possibly masking positive, specific signal. As the sensitivity of CARD can be manipulated, components such as fixation, protease treatment, streptavidin, and biotinyl tyramide have to be

Figure 3. *Clinical samples tested with the HPV16/18 probe. Two cases of nonkeratinizing squamous cell carcinoma of cervix uteri are shown.* **A:** *ISH with CARD-Nanogold. Signals are marked in the malignant keratinocytes. The connective tissue is entirely negative. This case was negative with standard ISH.* **B:** *Same case as* **A**; *negative control in which the specific HPV16/18 probe was replaced by an irrelevant probe (pBR322). No signal is seen.* **C:** *Different case of nonkeratinizing squamous cell carcinoma. ISH with CARD-Nanogold again shows relatively large spots of positive signal.* **D:** *Same case as* **C**; *control experiment using ISH with Nanogold without CARD amplification. Specific signal is present, although it appears as much smaller, dot-like spots compared with* **C**. *All sections are semi-consecutive and were briefly counterstained with 1% eosin and nuclear fast red. Magnification, ×375.*

balanced very carefully. This requires that all negative controls be used as indispensable references in every staining performance. Positive CARD results are not valid unless the negative controls are free from artifacts. If these precautions are observed, CARD may well become the tool of choice for low-copy nucleic acid and antigen *in situ* detection in many applications.

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