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Detection of Adenovirus in Nasopharyngeal Specimens by Radioactive and Nonradioactive DNA Probes

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The presence of adenovirus DNA in clinical specimens was analyzed by nucleic acid hybridization assays by both radioactive and enzymatic detection systems. The sensitivity of the hybridization tests was in the range of 10 to 100 pg of homologous adenovirus DNA. Minimal background was noticed with unrelated viral and nonviral DNA. Twenty-four nasopharyngeal mucus aspirate specimens, collected from children with acute respiratory infection, were assayed in the hybridization tests and also by an enzyme immunoassay for adenovirus hexon antigen which was used as a reference test. Sixteen specimens positive by the enzyme immunoassay also were positive in the two nucleic acid hybridization tests, and the remaining eight specimens were negative in all of the tests. The results indicate that nucleid acid hybridization tests with both radioactive and nonradioactive probes can be used for diagnosis of microbial infections.

Nucleic acid hybridization techniques have recently been applied successfully in the detection of several microorganisms (1–3, 5, 7, 8, 10, 18–20). In the test, nucleic acids bound onto a solid phase are detected by labeled DNA probes. The method is specific, sensitive, and relatively rapid, and it also enables the detection of latent infections (9). The use of recombinant DNA technology has made it possible to produce probes in almost unlimited quantities.

The disadvantage of the hybridization assays for routine diagnostic use is the need for radioisotopes which have short half-lives and are difficult to handle. Recently a method based on the use of biotinylated probes and the interaction of these with enzyme-conjugated streptavidin has been introduced (12, 13). In the present study this principle was used to detect viral DNA in clinical specimens.

MATERIALS AND METHODS

Specimens. Nasopharyngeal mucus aspirates were collected from children with acute respiratory infection with disposable mucus collectors as described previously (16). The specimens were diluted (1:5) with a buffer containing 20% inactivated fetal bovine serum, 2% Tween 20, and 0.02% merthiolate in phosphate-buffered saline solution (PBS; pH 7.3). The specimens were sonicated to break up the mucus and stored at −20°C until tested. Sixteen positive and eight negative specimens, based on the hexon antigen enzyme immunoassay (EIA) (16), were selected for further analysis.

Preparation of probes. Adenovirus type 2 was propagated in HeLa cells, and the virus was purified in two successive CsCl gradients. DNA was isolated from virions by treatment with proteinase K and phenol extraction (14).

Adenovirus type 2 DNA and pBR322 DNA were digested with the restriction endonuclease *Hin*dIII (Boehringer GmbH, Mannheim, Federal Republic of Germany) and ligated together with T4-DNA ligase (Boehringer GmbH). Recombinant plasmid DNA was purified from Amp^r Tet^s colonies, digested with *Hin*dIII, and analyzed by Southern blotting (17) with the adenovirus 2 DNA probe. The recombinant plasmid containing the *Hin*dIII A fragment was used in the hybridization tests.

Spot hybridization with radioactive probes. HeLa cells were infected with adenovirus type 2 at a multiplicity of 10. When a cytopathic effect was observed in at least 80% of cells, they were collected and stored at −20°C until assayed. For the hybridization test, either 200 μl of cells in PBS or 200 μl of sonicated nasopharyngeal mucus specimens were digested with proteinase K (E. Merck AG, Darmstadt, Federal Republic of Germany) at a concentration of 0.1 mg/ml for 1 h at 37°C. The specimens then were extracted with phenol-chloroform, and the nucleic acids were concentrated by ethanol precipitation. The pellet was dissolved in 200 μl of 0.3 M NaOH and boiled for 5 min to denature the DNA. The specimens immediately were chilled on ice, neutralized by adding 3 M HCl, and adjusted to contain 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with a stock solution of 20× SSC.

The treated samples were spotted onto nitrocellulose filters (BA 85; Schleicher & Schuell Co., Dassel, Federal Republic of Germany) with a filter manifold. The filters were air dried, baked at 80°C for 1 h, and prehybridized for 1 h at 65°C in 6× SSC–5× Denhardt solution (4) supplemented with 0.1 mg of herring sperm DNA per ml, denatured by boiling at an alkaline pH. A 1-μg amount of the DNA probe was labeled with ^{32}P by nick translation (15) to a specific activity of 10^7 to 10^8 cpm/μg and added to the hybridization bag in 6× SSC–5× Denhardt solution–0.5% sodium dodecyl sulfate. The filter was hybridized overnight at 65°C and washed three times for 20 min each in 2× SSC–0.5% sodium dodecyl sulfate at 65°C. The radioactivity bound to the filter was detected either by autoradiography for 4 to 16 h with X-ray film (Trimax XD; 3M, Milano, Italy) or by scintillation counting, in a 1217 Rackbeta Liquid Scintillation Counter (Wallac, Turku, Finland).

Spot hybridization with enzymatic probe system. The treatment of the specimens and the hybridization reaction were carried out as described above, with the exception that the probe was labeled with biotinylated dUTP (ENZO Biochem, New York, N.Y.; 12). After the filters were washed, they were incubated for 1 h in PBS supplemented with 2% bovine serum albumin and 0.1% Triton X-100 to minimize the nonspecific binding of the streptavidin conjugate onto the filter.

Streptavidin (Bethesda Research Laboratories, Gaithersburg, Md.) was conjugated with alkaline phosphatase (Type VII, Sigma Chemical Co., St. Louis, Mo.) as earlier de-

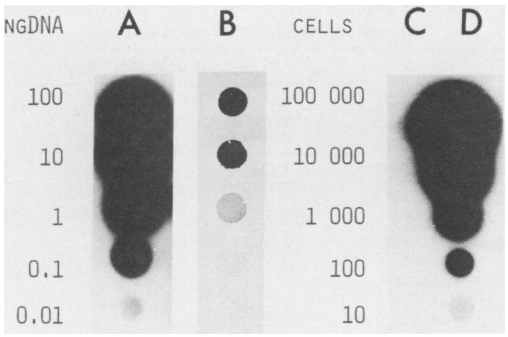

FIG. 1. Detection of adenovirus DNA by spot hybridization with radioactive (A, C, and D) and enzymatic (B) probe systems. Various amounts of adenovirus DNA (A and B) and infected (D) and control (C) cell DNA were spotted onto nitrocellulose and tested in the hybridization assay with ^{32}P-labeled (A, C, and D) or biotinylated (B) adenovirus DNA probe. The results are shown as autoradiographs (A, C, and D) or by enzymatic color reaction on the filter caused by incorporated streptavidin-alkaline phosphatase complex (B).

scribed (6). The filter was incubated for 30 min at 20°C with the conjugate diluted in PBS and washed twice for 10 min in PBS and once in the staining buffer (1 M Tris [pH 9.7], 0.3 mM $MgCl_2$, 0.02 mM $ZnSO_4$). The color was developed for 1 to 5 min with 1 mg of both α-naphthyl phosphate (Sigma) and 4-aminodiphenylamine diazonium sulfate (Sigma) per ml in the staining buffer (11). For the measurement of A_{405}, the spots were cut out and incubated in test tubes in diethanolamine-$MgCl_2$ buffer (pH 10; Orion Diagnostiga, Espoo, Finland) supplemented with 1 mg of paranitrophenyl phosphate (Orion Diagnostica) per ml. The optical density was measured with a Multiskan MC spectrophotometer (Eflab Oy, Helsinki, Finland).

Adenovirus antigen detection assay. The details of the antigen detection assay have been described earlier (16). Briefly, the specimens were incubated for 1 h at 37°C on microtiter plates coated with an immunoglobulin fraction from guinea pigs immunized with highly purified adenovirus hexon antigen. The antigen bound onto the solid phase was detected by rabbit anti-hexon immunoglobulin and horseradish peroxidase-conjugated anti-rabbit immunoglobulins.

RESULTS

The sensitivity of the hybridization assays was evaluated by testing various amounts of homologous DNA and cells infected with adenovirus type 2. The radioactive whole adenovirus type 2 DNA probe gave a positive signal with 10 pg of homologous DNA (Fig. 1), whereas the cloned *Hin*dIII restriction fragment probe detected 100 pg of DNA (data not shown). The sensitivity of the alkaline phosphatase detection system was at the level of 100 pg of DNA. No background was noticed when herpes simplex virus DNA was tested. The sensitivity of the test for the detection of adenovirus DNA in infected cells was between 10 and 100 cells (Fig. 1). A slight background appeared when large amounts of uninfected HeLa cells with whole adenovirus DNA probe were evaluated (Fig. 1).

To compare the two hybridization tests with an antigen detection assay the results were also analyzed both as counts per minute after scintillation counting and as absorbance values (Fig. 2). The sensitivity was 100 and 1,000 pg of DNA with the radioactive and enzymatic probe systems, respectively, whereas the antigen detection assay had a sensitivity of 1,000 pg.

Twenty-four nasopharyngeal mucus aspirate specimens were tested by the nucleic acid hybridization assays and by the EIA for hexon antigen. Of the 24 specimens, 16 were positive in the antigen assay, and all these 16 were positive in the hybridization assays when the results were interpreted visually (Fig. 3). When analyzed quantitatively, one of the EIA-positive specimens was negative in the hybridization test with radioactive probes and one of the EIA-negative specimens appeared positive (Table 1). When the enzyme reaction in test tubes was used to measure hybridization, 10 positive specimens were found, all of which were also positive by the antigen assay.

DISCUSSION

A test for the detection of adenovirus DNA by radioactive and nonradioactive probes was developed. To evaluate the sensitivity of the test, different probes and assay systems

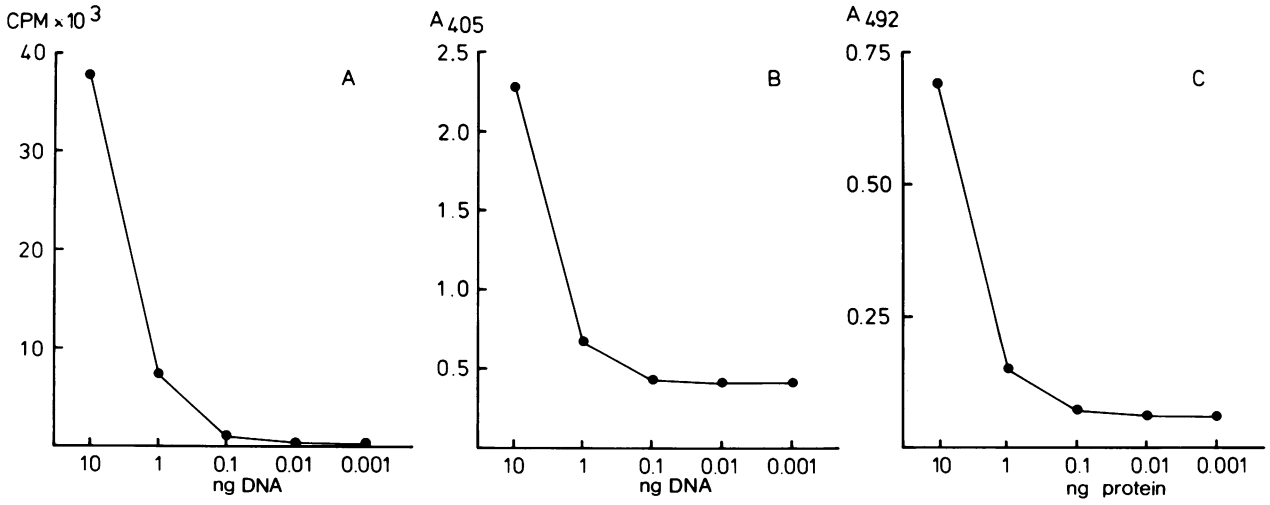

FIG. 2. Detection of adenovirus DNA and hexon antigen. Various amounts of purified adenovirus DNA (A and B) and hexon antigen (C) were tested in the nucleic acid hybridization assays by radioactive (A) or enzymatic (B) detection systems and in the antigen detection test (C). The results are expressed as counts per minute (A) after scintillation counting or as absorbance values (B and C).

were compared. DNA probes can be labeled to a very high specific activity (10^8 cpm/μg) by using one or more radioactive precursors. The sensitivity of the test can be further increased with long exposure times. Although the enzymatic probe system is less sensitive, it can be successfully used to detect viral DNA sequences in clinical specimens, and it has several advantages. Since the probe is stable, it can be prepared in large quantities and stored for long periods of time. No special facilities are required when this detection system is used, and no exposure on X-ray films is needed to visualize the results.

The size of the probe is critical for the specificity of the test. In this study both the total adenovirus DNA genome and a restriction fragment cloned in a plasmid were used. The sensitivity of the hybridization assay with the cloned probe was lower; this can be partly explained by the smaller portion of viral DNA represented. The specificity was better, as expected, and no background with uninfected control cells was noticed. Both probes were equally sensitive in the detection of adenovirus DNA in clinical specimens.

The developed test was used for the detection of adenovirus DNA in 24 mucus aspirate specimens. An antigen detection system for adenovirus hexon antigen was used as a reference test. The results of the tests were in complete agreement when the interpretation of the hybridization assays was based on the spots in the autoradiogram or on the filter. The values in counts per minute disagreed in two specimens and the absorbance values disagreed in six specimens when compared with the EIA results. The reason for these discrepancies needs to be further analyzed if such interpretations are to be used in routine diagnosis. No direct correlation was noticed between the levels of adenovirus DNA and hexon antigen in the clinical specimens.

The results were in agreement with those of earlier reports (20; P. Stålhandske, T. Hyypiä, A. Allard, P. Halonen, and U. Pettersson, submitted for publication), which have shown the presence of adenovirus DNA in stool and mucus specimens. Although some of the nasopharyngeal specimens contained color, their color differed distinctly from the dark-brown color of the precipitate in the enzyme reaction (Fig. 1). Stool specimens cannot be directly tested in the spot hybridization assay with the enzymatic probe system because the color of the samples causes difficulties in the interpretation of the test results. To avoid this problem a sandwich-type of assay (20) may be favorable.

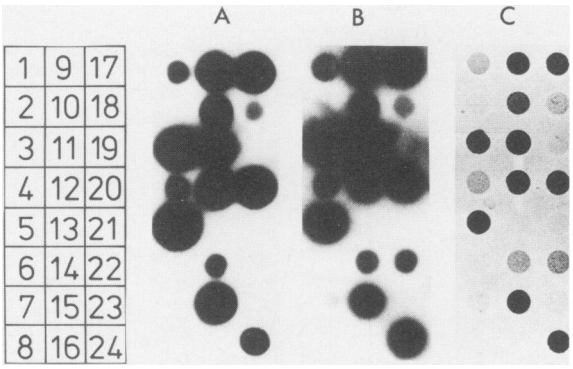

FIG. 3. Detection of adenovirus DNA in nasopharyngeal mucus aspirates. The specimens were treated with proteinase K, phenol extracted, denatured, and applied onto nitrocellulose. Adenovirus DNA was detected by nucleic acid hybridization with radioactively labeled whole adenovirus DNA (A) and a cloned *Hin*dIII A fragment in pBR322 (B) as probes and by the enzymatic detection system with whole adenovirus DNA probe (C). Positive signals were observed in specimens 1, 3, 4, 5, 7, 9, 10, 11, 12, 14, 15, 17, 18, 20, 22, and 24 in the original autoradiographs (A and B) and filters (C).

TABLE 1. Detection[a] of adenovirus DNA and hexon antigen in nasopharyngeal mucus aspirates

Specimen no.	EIA[b]	Nucleic acid hybridization with:		
		Adenovirus DNA probe		*Hin*dIII-A (cpm)[e]
		cpm[c]	A_{405c}[d]	
1	2.326	3,208*	0.243*	2,707*
2	0.095	372	0.150	589
3	2.395	22,538*	0.532*	28,008*
4	0.682	2,992*	0.144*	2,763*
5	2.352	55,734*	0.476*	30,209*
6	0.143	140	0.109	202
7	0.646	1,207*	0.131*	549*
8	0.158	233	0.126	96
9	1.908	16,650*	0.821*	29,152*
10	1.774	8,900*	0.425*	5,626*
11	2.019	21,792*	0.798*	26,222*
12	2.069	33,688*	0.655*	25,113*
13	0.101	400	0.133	395
14	1.820	5,568*	0.145*	2,185*
15	1.231	25,492*	0.566*	15,048*
16	0.139	500	0.150	173
17	2.050	15,647*	0.797*	28,588*
18	1.429	2,099*	0.224*	1,001*
19	0.139	1,805	0.189	1,300
20	2.442	31,530*	0.694*	26,372*
21	0.118	194	0.169	380
22	1.373	641*	0.206*	2,493*
23	0.138	122	0.156	522
24	2.435	7,919*	0.543*	24,258*

[a] The specimens considered positive in the test are underlined. The positive assay results based on visual interpretation (Fig. 3) of the hybridization tests are marked with an asterisk. The specimens with values exceeding 2 × the mean of the negatives were considered positive.

[b] A_{492} values of the EIA for adenovirus hexon antigen.

[c] Values in counts per minute by the hybridization test with ^{32}P-labeled adenovirus DNA probe.

[d] A_{405} values of the hybridization test with biotin streptavidin-alkaline phosphatase detection system.

[e] Values in counts per minute by the hybridization test with ^{32}P-labeled cloned *Hin*dIII A DNA fragment probe.

In routine diagnostic assays the time required for completion of the test is critical. We have not yet optimized the test. Our preliminary results indicated that the baking of the filters for 1 to 2 h at 80°C could be replaced by 2 min in a microwave oven and that the prehybridization step, if necessary at all, could be reduced to 15 min. Although optimal hybridization occurs in 16 h, shorter hybridization times (1 to 4 h) can also be used for purposes in which the sensitivity is not critical. The washing of the filters can be done in 15 to 30 min, and even tap water can be used. The fastest way to obtain results is either by an enzyme reaction on the filter or by scintillation counting. A critical step involves the treatment of the specimens. Phenol extraction is a time-consuming procedure, and it may not be optimal. It possibly could be replaced by denaturing the specimens directly on the filter (19).

By using stable probes the hybridization assay provides an alternative method for microbial diagnosis. In infections in which the production of viral antigens is low or the microorganisms are difficult to isolate, the system described here offers new possibilities for definitive diagnosis.

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