Production of Monoclonal Antibodies against Parainfluenza 3 Virus and Their Use in Diagnosis by Immunofluorescence

JOSEPH L. WANER,1,2* NORMA J. WHITEHURST,1 TAMYRA DOWNS,2 AND DONALD G. GRAVES2

Department of Pediatrics, Division of Infectious Diseases,1,2* and Department of Microbiology and Immunology,2
Oklahoma University Health Sciences Center, Oklahoma City, Oklahoma 73190

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Monoclonal antibodies were produced against parainfluenza virus type 3 (PI-3) and used to identify PI-3 clinical isolates in cell culture and PI-3 antigen in cells obtained from nasopharyngeal (NP) washes of patients. Two (2E9 and 4G5) of the three monoclonal antibodies characterized reacted by immunoblotting with a 67,000-dalton PI-3 protein, and one antibody (4E5) reacted with two viral proteins in the range of 29,000 to 31,000 daltons. The three monoclonal antibodies did not cross-react by indirect immunofluorescence (IFA) with PI-1 or PI-2 and identified by IFA 18 isolates of PI-3 in cell culture. The 2E9 antibody reacted with PI-3 antigen in cells of 8 NP wash specimens that also yielded PI-3 in cell culture. Cells from 12 specimens reactive by IFA for respiratory syncytial virus, 1 specimen yielding adenovirus in cell culture, and 5 specimens yielding influenza virus were not reactive.

Parainfluenza virus type 3 (PI-3) is an important cause of lower respiratory tract disease in infants (4, 15). Infection with PI-3 may produce symptoms similar to those of respiratory syncytial virus (RSV), although the clinical sequelae may be less severe (5, 7). Laboratory diagnosis is routinely accomplished by isolating the virus in cell culture and identifying the isolate by hemagglutination inhibition tests or fluorescent-antibody procedures (2). Immunofluorescent-antibody tests have been used to identify viral antigen in infected cell cultures or in cells from nasopharyngeal (NP) washes obtained from infected patients (3, 8, 11, 18). The inconsistency in the quality and availability of reagents, however, has hindered development of rapid and sensitive methods for diagnosis. The success of the indirect fluorescent-antibody (IFA) technique, in which commercial or monoclonal antibodies to RSV are used for rapid detection of RSV antigen in cell cultures and cells from NP washes (1, 9, 12, 13), prompted us to make monoclonal antibodies against PI-3 and to test the efficacy of their use in diagnostic procedures.

MATERIALS AND METHODS

Preparation of immunogen. The NW strain of PI-3, isolated from a 6-month-old patient with bronchiolitis, was adapted to grow with the appearance of cytopathic effect (CPE) in HEp-2 cells. Cell cultures were maintained on minimal essential medium supplemented with 2% fetal bovine serum, 2 mM glutamine, and 50 μg of gentamicin per ml. HEp-2 cultures growing in culture flasks (75 cm²) were inoculated with cell-free virus at a multiplicity of infection of 1 and incubated at 34°C until greater than 90% of the cells showed CPE, usually 24 to 48 h after inoculation. Cells of infected cultures were dislodged with a rubber policeman, collected by centrifugation at 500 × g for 10 min, and washed twice with Tris-buffered saline (0.01 M; pH 7.2). The cell pellet obtained from three 75-cm² flasks was suspended in 1 ml of glycerine-buffered saline (0.01 M; pH 8.0) containing 0.1% Triton X-100 and 0.1% deoxycholate. The preparation was frozen and thawed once and centrifuged at 750 × g for 10 min.

The resulting supernatant fluid was used as the soluble immunogen.

Viability HEp-2 cells infected for 48 h with virus were also used as an immunogen. Infected cells were scraped from the flask, washed twice in Hanks balanced salt solution, and suspended in Hanks balanced salt solution.

Production of monoclonal antibodies. Female BALB/c mice (6 weeks old) were inoculated intraperitoneally (i.p.) with 0.2 ml of the soluble immunogen and then injected subcutaneously 2 weeks later with 5 × 10⁵ infected cells. Ten days later, the mice were reinoculated i.p. with soluble immunogen, and 3 days later, the spleens were removed. Suspensions of spleen cells were depleted of erythrocytes by osmotic lysis in H₂O. The remaining splenocytes were then fused with NS-1 myeloma cells at a ratio of 4:1 by using 50% polyethylene glycol. After fusion, a suspension of 5 × 10⁴ cells per 0.1 ml of RPMI-1640 (RPMI medium supplemented with 10% NCTC-109, hypoxanthine-thymidine, 15% fetal bovine serum, and 10% spent medium from NS-1 cell cultures) was distributed in 96-well cell culture plates. Twenty-four hours later and for 2 weeks thereafter, the cultures were refed with RPMI-1640 supplemented with aminopterin. Culture fluids were screened for antibody to PI-3 by an IFA test. Hybridomas producing antibody were cloned twice by limiting dilution. The immunoglobulin fraction of culture fluid was precipitated with saturated ammonium sulfate, the precipitate was dissolved in distilled H₂O and dialyzed against phosphate-buffered saline (PBS; 0.01 M, pH 7.2) for 24 h with four changes of buffer, and culture fluid was concentrated 20-fold.

Isotyping of the monoclonal antibodies was done by using the Ouchterlony double diffusion test. Concentrated monoclonal immunoglobulins were placed in the outside wells of an Ouchterlony plate. The center wells contained anti-mouse antibody specific to either immunoglobulin G1 (IgG1), IgG2a, IgG2b, or IgM obtained from Meloy Laboratories, Inc., Springfield, Va. 

IFA tests. PI-1, PI-2, and PI-3, influenza A virus (H3N2), and influenza B virus (Singapore) were grown in primary rhesus monkey kidney cells (PRMK); RSV was grown in HEp-2 cells. Infected or uninfected cells were scraped from the surface of the culture vessel and placed on circumscribed...
areas of glass slides, dried, and fixed in acetone for 10 min at 4°C; cells from cultures inoculated with clinical specimens were similarly treated. Fluids from hybridoma cultures were diluted 1:2 with PBS, and monoclonal antibodies obtained after (NH₄)₂SO₄ precipitation were diluted 1:20 with PBS and then incubated on cells for 30 min at 37°C. Slides were washed twice in PBS and twice in distilled water, and then fluorescein-conjugated goat anti-mouse IgM and IgG (Tago) were applied for 30 min at 37°C. Slides were washed three times in PBS and once in distilled water; the last wash of PBS contained 0.02% Evans blue as a counterstain. Each hybridoma culture fluid was tested on infected and uninfected cells. The IFA procedure for detecting PI-3 or RSV antigen in cells obtained from NP washes was performed as described above; reagents for the RSV test were purchased from Burroughs Wellcome Co., Research Triangle Park, N.C.

Immunoblotting procedure. Antigen for immunoblots was prepared as described for production of immunogen except that infected or uninfected HEP-2 cells were suspended to 20% by volume in Tris-buffered saline (0.01 M; pH 7.2) containing 1% Triton X-100 and 1% deoxycholate. After one freeze-thaw, the preparations were centrifuged at 1,000 x g for 15 min, and the supernatant phase was used as antigen. Antigen preparations of PI-3 and extracts of uninfected HEP-2 cells were treated with a sample buffer (0.5% sodium dodecyl sulfate, 1.25% β-mercaptoethanol, 0.02 M Tris-HCl, 7% glycerol, 0.02% bromophenol blue [pH 6.8]) for 3 min at 100°C. Antigen samples were electrophoresed through a 5 to 15% polyacrylamide gradient gel by using a discontinuous buffer system described by Laemmli (10). Electrophoresis was performed at 15 mA per gel for 5.5 h or until the anode front reached the bottom of the gel. Molecular weight protein standards were included in each run. After electrophoresis, the gels were used for immunoblotting by a method similar to that described by Towbin et al. (17). Briefly, the proteins were transferred electrophoretically to nitrocellulose paper (pore size, 0.45 μm) at 150 mA for at least 16 h. A portion of the membrane containing one lane with the molecular weight standards, one lane of PI-3, and one lane of uninfected cell extract was stained with amido black stain (0.2 g in a mixture containing 90 ml of methanol, 20 ml of acetic acid, and 80 ml of distilled water). The other portion of the membrane was incubated with 0.5% Tween 20 in PBS for 1 h at 37°C. The sheets were subsequently cut into strips, and each strip was incubated for 2 h at room temperature with a 1:15 to 1:25 dilution of an (NH₄)₂SO₄-concentrated monoclonal antibody and then washed four times in PBS (containing 0.05% Tween 20) over a period of 30 min. The membrane strips were then incubated for 1 h with peroxidase-conjugated goat anti-mouse IgG (1:1,000 dilution in PBS [pH 7.6] containing 0.05% Tween 20) and washed four times in PBS. Finally, the antigenic bands were observed after the addition of substrate, 4-chloro-1-naphthol. Substrate solution was made by adding 25 mg of 4-chloro-1-naphthol to 0.5 ml of 95% ethanol and then adding this mixture to 100 ml of 0.05 M Tris buffer (pH 7.4). The substrate solution was stirred in the dark for 30 min, filtered, and used immediately after the addition of 17 μl of 30% H₂O₂. Antigenic reactive bands stained a deep purple color in 3 to 5 min. The blots were rinsed in distilled water, dried, and stored in the dark until photographed.

Clinical specimens. NP wash specimens received in the virology laboratory of the Oklahoma Children’s Memorial Hospital for isolation of respiratory viruses were inoculated into duplicate cultures of PRM cell, diploid human fibroblasts, and HEP-2 cells. Cultures were viewed daily for CPE; PRM cultures were tested for hemadsorption with guinea pig erythrocytes at 4 and 22°C every 2 to 3 days.

Cells from NP washes were prepared for direct fluorescent-antibody tests for PI-3 or RSV antigen as previously described (3, 12).

RESULTS

Characterization of monoclonal antibodies. Eight hybridomas producing antibodies against PI-3 antigens were identified by IFA testing. Three of the antibodies (2E9, 4G5, and 4E5) were selected for study because of their strong fluorescent reactions. These three monoclonal antibodies did not react in IFA tests with antigens of PI-1, PI-2, influenza A virus (H3N2), influenza B virus (Singapore), adenovirus type 1, or RSV. Antibodies 4E5 and 4G5 were of the IgG2a isotype and 2E9 was an IgG1. The fluorescent reactions of the 2E9 and 4G5 monoclonal antibodies were characterized by a speckling pattern in the cytoplasm of infected cells; 4E5 produced a smooth fluorescing pattern in the cytoplasm.

PI-3 proteins recognized by the monoclonal antibodies. The specificity of the monoclonal antibodies was investigated by

FIG. 1. Western immunoblots of electrophoretically separated proteins from PI-3-infected HEP-2 cells and uninfected HEP-2 cells reacted with murine monoclonal antibodies 2E9, 4E5, and 4G5. Nitrocellulose strips containing peptides of uninfected HEP-2 cells (lane g) and PI-3-infected HEP-2 cells (lane h) along with one lane containing molecular weight protein standards (lane i) were transferred and then stained with amido black protein stain. Polyptides from uninfected HEP-2 cells (lanes a, c, and e) and PI-3-infected cells (lanes b, d, and f) were incubated with antibodies 2E9 (lanes a and b), 4G5 (lanes c and d) and 4E5 (lanes e and f). Molecular weight markers: myosin, 200,000; β-galactosidase, 116,250; phosphorylase b, 92,500; bovine serum albumin, 66,000; ovalbumin, 45,000; dehydrogenase, 36,000; carbonic anhydrase, 29,000; trypsinogen, 24,000; trypsin inhibitor, 20,000; and alpha-lactalbumin, 14,000.
the immunoblot technique. Nitrocellulose strips containing separated proteins from PI-3-infected HEp-2 cells and uninfected HEp-2 cells were either incubated with the individual monoclonal antibodies or stained with amido black. Antibodies 2E9 and 4G5 were specific for a viral protein of approximately 67,000 daltons, whereas antibody 4E5 reacted with two proteins in the virus-infected cells in the molecular weight regions of 31,000 and 29,000 daltons (Fig. 1). Occasionally, a second reactive band was seen with 2E9 in the molecular weight region of approximately 140,000 daltons.

The PI-3 monoclonal antibodies did not react with separated proteins from uninfected HEp-2 cells. Concentrated culture fluid from hybridomas to other viral proteins (i.e., bovine leukemia virus) failed to react with PI-3 proteins under the same conditions (data not shown).

Identification of PI-3 isolates in cell cultures. HEp-2 or PRMK cell cultures showing CPE or hemadsorption of guinea pig erythrocytes after inoculation with 40 different NP specimens obtained from October 1984 through May 1985 were tested for reactivity with the three PI-3 monoclonal antibodies. Twelve isolates of PI-3 were identified by the PI-3 monoclonal antibodies (Fig. 2A); 19 isolates of RSV, 4 isolates of influenza A virus (H3N2), 3 isolates of PI-1, and 2 isolates of adenovirus were not reactive. Additionally, the PI-3 monoclonal antibodies reacted with two isolates of PI-3 obtained in February 1983 and 4 isolates obtained between April and June of 1984 that were recovered from infected cell cultures frozen at -70°C.

A pool of the three PI-3 monoclonal antibodies was prepared by mixing equal volumes of the individual preparations. The pool was then used to stain cells from the original cultures of PI-3 isolates that had been frozen at -70°C. The degree and pattern of fluorescence observed did not indicate an improved sensitivity over the individual use of the three PI-3 monoclonal antibodies.

Detection of PI-3 antigen in cells obtained from NP washes. Cells collected from the NP washes of eight patients with culture-proven PI-3 infection were reactive with the 2E9 monoclonal antibody (Fig. 2B). Similar preparations from 12 patients that grew RSV and were reactive for RSV antigen by IFA did not react with 2E9, nor did 5 specimens yielding influenza virus and 1 specimen yielding adenovirus; 28 additional specimens were negative by IFA testing for PI-3 and RSV and did not produce a virus isolate.

The fluorescing pattern of PI-3 antigen in infected cell cultures and in cells from NP washes was restricted to the cytoplasm and was speckling in appearance. The speckling pattern presented a smoother appearance, however, in NP cells that appeared to be derived from columnar epithelium.

DISCUSSION

The three monoclonal antibodies studied were specific and sensitive for identifying PI-3 isolates in cell cultures. In preliminary tests with laboratory strains and with a limited number of clinical isolates obtained from NP washes, the monoclonal antibodies identified PI-3 isolates and did not cross-react with other common respiratory viruses or uninfected cells. Pooling of the three antibodies did not increase the sensitivity of the IFA reaction. The use of a single antibody, 2E9, was satisfactory for use in identifying cell culture isolates and for detecting PI-3 antigen directly in cells obtained from NP washes. A change or an absence of the target epitope on other PI-3 isolates, however, is possible. Additional isolates from other geographic locations should be tested, and a monoclonal antibody pool should be used, therefore, to confirm and maintain the diagnostic reliability of the reagents.

The 2E9 and 4G5 antibodies reacted by immunoblotting with a viral protein of approximately 67,000 daltons. A protein of comparable molecular weight was reported to be associated with the nucleocapsids of purified virions and was the most abundant protein in the virion (16). Although antigen derived from infected cells was used in the immuno blotting procedure, the effectiveness of 2E9 may be explained by its reaction with this most abundant PI-3 protein. The speckling fluorescent patterns of 2E9 and 4G5 were identical, while that of 4E5 was smooth; 4E5 reacted by immunoblotting with 29,000- and 31,000-dalton proteins.

FIG. 2. IFA reactions of the 2E9 monoclonal antibody on PRMK cells infected with a clinical isolate of PI-3 (A) and cells from an NP wash of a patient with PI-3 infection (B).
35,000-dalton protein was associated with the envelope of purified virions (16). The occasional reactivity of 2E9 with a 140,000-dalton protein may be due to the nature of the antigen preparation and could represent a reaction with aggregates or a precursor of the 67,000-dalton protein.

The effectiveness of identifying PI-3 antigen in cells obtained from NP washes may be particularly useful in making a rapid diagnosis. Application of the IFA technique for PI-3 and RSV to cells obtained from an NP wash would result in the rapid diagnosis of the two most common causes of bronchiolitis and pneumonia in infants (6, 14). Additionally, preliminary experiments have indicated that a pool of three monoclonal antibodies against PI-1, PI-2, and PI-3 can identify isolates of the three viruses (unpublished data). Thus, four of the most common viral causes of respiratory disease may be rapidly diagnosed by the conventional procedure of direct immunofluorescence of cells from NP washes. The availability of specific and sensitive reagents should also stimulate the application of other immune test constructions to the rapid diagnosis of the parainfluenza viruses.

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