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Evaluation of Monoclonal Antibodies for Subtyping of Currently Circulating Human Type A Influenza Viruses

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The hemagglutinin subtype specificities of six monoclonal antibodies (MAbs) to influenza type A viruses were evaluated in a rapid culture assay by immunoperoxidase staining. Confluent monolayers of MDCK cells in multiwell plates were inoculated with (i) 23 reference viruses, (ii) 200 isolates collected during the influenza season 1995 to 1996, and (iii) 28 clinical specimens previously found to be influenza virus positive. After overnight incubation, the cells were fixed and stained with MAbs IVA1/B10, IIF4/D3, 12L/5, 13L/6, 18L/1, or 18L/4. Type-specific MAbs were included as controls. All antibodies gave intensive cytoplasmic staining with infected cells in the absence of any reaction with uninfected cells. MAbs 12L/5, 13L/6, 18L/1, and 18L/4 exclusively reacted with viruses of the subtype H1, and the antibodies IVA1/B10 and IIF4/D3 exclusively reacted with viruses of the subtype H3. None of these MAbs reacted with viruses of the H2 subtype or with influenza type B viruses. Of the 200 recent isolates, 63 were identified as influenza virus type A, subtype H1, 95 were identified as type A, subtype H3, and 41 were identified as type B. One isolate contained a mixture of a type A (H3) and a type B influenza virus. Of the 28 previously positive clinical specimens, 15 contained an influenza virus A, subtype H3, 1 contained an influenza virus A, subtype H1, and 9 contained an influenza B virus. The subtype of a very weakly positive specimen could not be determined, and two specimens remained negative. The MAbs described here allow for a rapid typing and subtyping of influenza virus isolates and for the type- and subtype-specific detection of influenza viruses in clinical specimens.

Global influenza virus activity is monitored by laboratories participating in a surveillance network coordinated by the World Health Organization. These laboratories serve a variety of important functions. Primarily, they produce diagnostic data that help physicians to take appropriate therapeutic measures and to prevent nosocomial spread of influenza viruses in institutions such as nursing homes (5). Further, antigenic and genetic analyses of clinical isolates provide information about the evolution and geographic spread of newly emerging epidemic strains and ultimately help identify suitable virus strains to be included in the trivalent influenza vaccine (2).

Most laboratories are able to identify the type of an influenza virus isolate. Monoclonal antibodies (MAbs) and polyclonal sera for this purpose are widely available. For further analysis of influenza A viruses, the isolate is often forwarded to a reference laboratory, where the subtype is commonly identified by the hemagglutination inhibition test with specific antisera raised in sheep, ferrets, or chickens. This is a time-consuming procedure which often delays the reporting of accurate epidemiologic information by weeks or even months. To expedite the processing of influenza virus isolates in diagnostic laboratories during an influenza season, simple methods based on well-characterized reagents for the identification of type and subtype are needed.

We have recently developed a rapid culture assay for the type- and subtype-specific identification of currently circulating human influenza viruses in clinical specimens (12). In this assay we used pools of MAbs against influenza type A and type B viruses to identify the virus type (10). Subtypes were determined with one MAb specific for H3 and one MAb cross-reacting among H1, H3, and several other hemagglutinin subtypes (7). In this study we evaluated a new panel of MAbs for their suitability to identify the subtypes H1N1 and H3N2 and we used them in a test format which allows for the rapid processing of substantial numbers of clinical isolates.

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MATERIALS AND METHODS

Cells and reference viruses. All experiments were done with MDCK cells (ATCC CCL34; American Type Culture Collection, Rockville, Md.). They were grown in Dulbecco's modified Eagle medium supplemented with 1 to 5% fetal bovine serum (FBS), 0.2% bovine serum albumin, 25 mM HEPES, and gentamicin. Cultures in multiwell dishes were incubated in a 5% CO_2 atmosphere. Reference viruses included in this study are listed in Table 1. The viruses A/Texas/36/91(H1N1), A/Beijing/353/89(H3N2), and B/Panama/45/90 served as controls in all experiments.

MAbs. MAbs were prepared by standard procedures by using the viruses A/Singapore/6/86(H1N1) (9) and A/Dunedin/4/73(H3N2) (6) as immunizing antigens. The viruses were grown in embryonated hens' eggs and purified on sucrose gradients. Immunoglobulins were purified from mouse ascitic fluids by affinity chromatography on protein A-Sepharose. The subtype specificities of antibodies were initially determined by solid-phase radioimmunoassay as described elsewhere (9). Based on their reaction pattern, MAbs IVA1/B10, IIF4/D3, 12L/5, 13L/6, 18L/1, and 18L/4 were selected for all further experiments. The following MAbs were used as reference reagents: (i) MAb 1L3, with specificity to the nucleoprotein of influenza virus type A (9); (ii) a pool containing two MAbs against the nucleoprotein of influenza A virus (pool A) (10); (iii) a pool containing one MAb against the nucleoprotein and one against the hemagglutinin of influenza B virus (pool B) (10); (iv) MAb HA1-71, an antibody specific to viruses of the subtype H3 (7, 12); and (v) MAb HA2-76, an antibody which reacts with hemagglutinins of the subtypes H1 and H3 (7, 12).

Characterization of MAbs by rapid culture assay. Rapid culture assay was performed as described previously (12). Briefly, wells of 96-well cell culture plates (Costar, Cambridge, Mass.) were seeded with approximately 2.5×10^4 MDCK cells in 100 μl of medium containing 1% FBS. After incubation at 36°C

TABLE 1. Reaction patterns of MAbs with influenza viruses of different hemagglutinin subtypes

Virus (subtype)	Reaction pattern of MAb						
	IVA1/B10	IIF4/D3	12L/5	13L/6	18L/1	18L/4	1L3
A/Wisconsin/33(H1N1)	−	−	+	+	+	+	+
A/Puerto Rico/8/34(H1N1)	−	−	+	+	+	+	+
A/Belgium/47(H1N1)	−	−	+	+	+	+	+
A/FM/1/47(H1N1)	−	−	+	+	+	+	+
A/Switzerland/74/85(H1N1)	−	−	+	+	+	+	+
A/Singapore/6/86(H1N1)	−	−	+	+	+	+	+
A/Texas/36/91(H1N1)	−	−	+	+	+	+	+
A/Bratislava/4/57(H2N2)	−	−	−	−	−	−	+
A/Singapore/1/57(H2N2)	−	−	−	−	−	−	+
A/Hong Kong/1/68(H3N2)	+	+	−	−	−	−	+
A/England/42/72(H3N2)	+	+	−	−	−	−	+
A/Dunedin/4/73(H3N2)	+	+	−	−	−	−	+
A/Victoria/3/75(H3N2)	+	+	−	−	−	−	+
A/Bratislava/101/76(H3N2)	+	+	−	−	−	−	+
A/Bangkok/1/79(H3N2)	+	+	−	−	−	−	+
A/Belgium/2/81(H3N2)	+	+	−	−	−	−	+
A/Philippines/2/82(H3N2)	+	+	−	−	−	−	+
A/Praha/2/83(H3N2)	+	+	−	−	−	−	+
A/Caen/1/84(H3N2)	+	+	−	−	−	−	+
A/Mississippi/1/85(H3N2)	+	+	−	−	−	−	+
A/Beijing/352/89(H3N2)	+	+	−	−	−	−	+
A/Hong Kong/23/92(H3N2)	+	+	−	−	−	−	+
B/Panama/45/90	−	−	−	−	−	−	−

overnight, 95 μl of medium containing 4 μg of tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Sigma Chemical Co., St. Louis, Mo.) per ml was added to each well. The 23 viruses listed in Table 1 were used in these experiments. Each virus was inoculated into 12 wells. The volume of the inoculum was 5 μl per well. The plates were again incubated at 36°C overnight. The medium was then aspirated, and the cells were washed twice with phosphate-buffered saline (PBS) and fixed with absolute methanol at room temperature for 10 min. After the cells were washed further with PBS, 80 μl of MAbs diluted in PBS containing 5% nonfat dry milk was added. In preliminary experiments the optimal dilutions of the MAbs were determined and found to be 1.17 μg/ml for IVA1/B10, 0.16 μg/ml for IIF4/D3, 0.37 μg/ml for 12L/5, 0.36 μg/ml for 13L/6, 0.35 μg/ml for 18L/1, 0.51 μg/ml for 18L/4, and 1.0 μg/ml for 1L3, respectively. After a 60-min incubation at 36°C, the plates were washed four times with PBS. Peroxidase-conjugated rabbit antibodies to mouse immunoglobulins (Dako, Glostrup, Denmark) at a dilution of 1:200 in PBS containing 5% nonfat dry milk were added to each well, and the plates were again incubated at 36°C for 60 min. After washing of cells as described above, substrate solution was added and the color reaction was developed at room temperature for approximately 30 min. For the substrate solution, 20 mg of 3-amino-9-ethylcarbazole (Sigma) was dissolved in 5 ml of dimethylformamide and then diluted to a final concentration of 200 μg/ml in sodium acetate buffer (pH 5.0). Before use, 1 μl of 30% H_2O_2 per ml was added. One hundred microliters of this solution was added to each well. After a 30-min incubation at room temperature, the plates were read at a ×100 magnification with an inverted microscope. The criterion for a positive reaction was distinct intracellular red staining in the absence of any staining in uninfected control cells.

Influenza virus isolates and clinical specimens. A total of 200 influenza virus isolates submitted to the World Health Organization Collaborating Center for Influenza at the Centers for Disease Control and Prevention (Atlanta, Ga.) during the influenza season 1995 to 1996 were typed and subtyped in this study. Of those, 140 were from the Americas, 19 were from Europe, 38 were from Asian countries, and 3 were from Australia. All isolates were passaged either once or twice in MDCK cells and stored frozen at −70°C for several months before being tested. Further, 28 influenza virus-positive throat swabs, nasal swabs, and nasopharyngeal aspirates that had been stored frozen at −70°C for between 3 months and 3 years were also available for testing. These specimens had initially been tested by standard virus isolation and by a rapid culture assay for the detection of influenza type A and B viruses (11) at the diagnostic laboratory of the Department of Virology, University of Turku.

Typing and subtyping of influenza virus isolates by rapid culture assay. The 200 isolates were typed and subtyped by the rapid culture assay described above. Isolates were inoculated into eight wells at a final dilution of 1:100. After an overnight incubation and fixation, one well of each isolate was stained with one of the six MAbs: IVA1/B10, IIF4/D3, 12L/5, 13L/6, 18L/1, and 18L/4. The two remaining wells were stained with pool A (diluted 1:1,000) or pool B (diluted 1:1,000). All isolates were also typed and subtyped by the standard method (12).

Type- and subtype-specific identification of influenza viruses in clinical specimens. Approximately 10^5 cells in 500 μl of Dulbecco's modified Eagle medium containing 5% FBS were added to each well of 24-well cell culture plates. At confluency, the growth medium was replaced with 450 μl of maintenance medium containing TPCK-trypsin. Fifty to 100 μl of a clinical sample was inoculated into each of four wells of a plate and then centrifuged at 700 × *g* at ambient temperature for 45 min. After an incubation at 36°C for 18 h, the cells were washed and fixed as described above. One well of each specimen was stained with pool A, one was stained with pool B, one was stained with a mixture of the MAbs IVA1/B10 and IIF4/D3, and one was stained with a mixture of MAbs 12L/5, 13L/6, 18L/1, and 18L/4, diluted as mentioned above.

RESULTS

The cytoplasms and the membranes of infected cells were intensely stained with the MAbs IVA1/B10, IIF4/D3, 12L/5, 13L/6, 18L/1, and 18L/4. Infected cells could be easily distinguished from uninfected cells. Preliminary experiments with reference viruses indicated that the antibodies 12L/5, 13L/6, 18L/1, and 18L/4 exclusively reacted with viruses of type A, subtype H1N1, and the antibodies IVA1/B10 and IIF4/D3 were specific to type A, subtype H3N2 influenza viruses (Table 1). None of these antibodies reacted with influenza virus type B, herpes simplex virus, cytomegalovirus, poliovirus type 3, echovirus type 30, adenovirus, parainfluenza viruses, or respiratory syncytial virus.

Two hundred influenza virus isolates were typed and subtyped by the rapid culture assays described here and by the reference assay (12). With the reference antibodies pool A, pool B, HA1-71, and HA2-76, 158 isolates were distinguished as influenza virus type A, of which 63 were of subtype H1 and 95 were of subtype H3. Forty-one viruses were typed as influenza virus B. A mixture of a type A (H3N2) and a type B influenza virus was identified in one isolate. The MAbs 12L/5, 13L/6, 18L/1, and 18L/4 correctly identified all 63 viruses of subtype H1. MAb IIF4/D3 reacted with all of the 95 isolates of subtype H3, and MAb IVA1/B10 reacted with all except 1 of these isolates. One isolate that contained a mixture of an influenza virus type A H3 and an influenza virus type B showed a positive reaction with both H3-specific MAbs.

Twenty-eight influenza virus-positive specimens were analyzed by rapid culture assay with pool A, pool B, a pool of MAbs 12L/5, 13L/6, 18L/1, and 18L/4 for the detection of A, subtype H1N1 viruses, and a mixture of MAbs IVA1/B10 and IIF4/D3 for the identification of A, subtype H3N2 viruses. Twenty-six of these specimens were found to be positive in this experiment. Fifteen were identified as influenza virus type A, subtype H3N2, one was identified as influenza virus type A, subtype H1N1, and nine were identified as influenza virus type B, respectively. One specimen showed only two positive cells in the well stained with pool A but no positive cells in any of the other wells. Two other specimens were negative in all wells.

DISCUSSION

Six MAbs were evaluated and found suitable for subtyping currently circulating human influenza type A viruses in a rapid culture assay system. Reference type A influenza viruses of the subtypes H1N1, H2N2, and H3N2 and a large number of recent influenza virus isolates were used to determine the subtype specificities and the intrasubtype cross-reactivities of the antibodies. Four antibodies reacted exclusively with viruses of the subtype H1, and two reacted exclusively with those of subtype H3. Although some of the reference viruses were isolated several decades ago, all were recognized by the MAbs of the corresponding subtype specificity. This indicates that the antibodies identify epitopes on the hemagglutinin molecule which are not directly exposed to the selection pressure caused by

neutralizing antibodies and have been well conserved over this period. In fact, in a radioimmunoassay with native, purified viruses at various pHs, the antibodies preferentially reacted with the low-pH form of the hemagglutinin (4, 8a). With these specificities, it is likely that the antibodies described will also react with the homologous viruses of future influenza epidemics. However, as it has been shown by others, subtype-specific MAbs may react reliably with isolates collected during one season but may fail to identify viruses from a subsequent epidemic (8). With the continuous molecular evolution of type A influenza viruses (1), any diagnostic reagent for influenza virus has to be carefully evaluated against new epidemic strains every year.

In the assay system described, none of the six antibodies reacted with the viruses A/Singapore/1/57 and A/Bratislava/4/57. These viruses are of subtype H2N2, a subtype which disappeared in 1969 after the H3N2 viruses emerged. It is possible, however, that H2N2 viruses will someday reemerge in the human population and cause a pandemic. The inclusion of a reagent that broadly reacts with all hemagglutinin subtypes, such as pool A or MAb 1L3, allows for the detection of type A influenza viruses of all hemagglutinin subtypes. Any isolate that is recognized by these type-specific antibodies but does not react with any of the subtype-specific MAbs has to be further analyzed by the hemagglutination inhibition test with antisera raised against viruses of other hemagglutinin subtypes (3).

The inclusion of H1-specific antibodies is a significant improvement of the assay used as a reference test in this study (12), which is able to discriminate only between H3 and non-H3, e.g., H1 viruses. The panel of antibodies used in this study also allows for the identification of mixtures between viruses of the subtypes H1 and H3. In fact, one of the 200 isolates contained a type B influenza virus and a small fraction of a type A influenza virus of the H3 subtype. It could not be determined whether this specimen derived from an individual who was simultaneously infected with two viruses or whether the mixture reflected a laboratory contamination.

The antibodies have also been used for the subtype-specific detection of influenza viruses in clinical specimens. After storage at −70°C for several months to several years, 26 of the 28 specimens still contained enough infectious virus to allow for the identification of the type and the subtype after an incubation of only 18 h. There is, at present, no clinical benefit if the subtypes of influenza A viruses can be identified by a rapid diagnostic test. However, simultaneous detection, typing, and subtyping of influenza viruses in one step obviate the need for additional passages of the virus for further identification by a hemagglutination inhibition test and can therefore diminish the risk of laboratory contaminations and may reduce costs. In addition, the reporting of accurate epidemic data can be done in a more timely manner.

Rapid culture assays for the detection of many different viruses, including respiratory viruses, have found wide application in clinical diagnostic laboratories. Because such assays are easy to perform, read, and interpret and because their sensitivities favorably compare with that of standard virus isolation, they may replace standard procedures in some diagnostic laboratories. However, for additional antigenic and genetic characterization of influenza viruses after the type and subtype have been determined, it is important to obtain an isolate of the virus. The result from the rapid culture assay is usually available within less than 24 h after the specimen has been received by the laboratory. In an optimal transport medium, virus infectivity in the original specimen should be well preserved during this period. Once a positive result is obtained, standard cell culture tubes or eggs can be inoculated with an aliquot of the specimen to obtain an isolate.

The assay described here serves the diagnostic and epidemiologic aspects of influenza virus surveillance. The antibodies will be carefully evaluated with isolates collected in different geographic areas during coming seasons, and they will be investigated for further applications.

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