Monoclonal Antibodies to Human Respiratory Syncytial Virus and Their Use in Comparison of Different Virus Isolates

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SUMMARY

Monoclonal antibodies to the RSN-2 isolate of human respiratory syncytial (RS) virus have been characterized with regard to their specificity for viral polypeptides and for different RS virus isolates. Five hybridoma antibodies recognized the phosphoprotein VPP32 and the other recognized the matrix protein VPM27. Evidence was obtained to support the view that VPP32 was associated with the nucleoprotein VPN41. Three of the antibodies to VPP32 showed cytoplasmic immunofluorescent staining while the other two showed only surface staining of virus-infected cells. The immunoblot technique was used to determine the immunoreactivity of four of the hybridoma antibodies against human RS isolates other than RSN-2. One of these antibodies reacted exclusively with RSN-2 virus isolate whereas the others detected determinants shared by all human RS isolates tested. Extension of this approach may offer the possibility of typing RS isolates using monoclonal antibodies.

INTRODUCTION

Human respiratory syncytial (RS) virus, classified in the genus Pneumovirus, is a major cause of lower respiratory tract illness in young infants (Holzel et al., 1963). It is morphologically similar to the other members of the group: bovine respiratory syncytial (BRS) virus and pneumonia virus of mice (PVM). BRS virus is antigenically related to human RS virus (Paccaud & Jacquier, 1970) and the polypeptide profile is similar to that of human RS virus (Cash et al., 1977) whereas PVM is antigenically unrelated to human RS virus (Berthiaume et al., 1974) and the polypeptide pattern is distinct from those of both BRS and human RS viruses (Cash et al., 1977).

The human RS virus particle contains three glycoproteins designated GP90, VGP48 and GP26; the latter two glycoproteins are linked by disulphide bonds. The other polypeptides in the virus particle include a nucleoprotein VPN41, a phosphoprotein VPP32, a matrix protein VPM27, and minor proteins VP200, VP25 and VP10 (Pringle et al., 1981; Fernie & Gerin, 1982; Gruber & Levine, 1983).

Human convalescent serum failed to detect serological differences between RS virus isolates, although minimal differences were found using antisera raised in ferrets (Coates et al., 1966). However, it is possible that the increased specificity afforded by analysis using monoclonal antibodies could demonstrate the existence of different types of human RS virus. Monoclonal antibodies to the Long isolate of human RS virus have previously been produced and used in the immunological analysis of viral polypeptides and in the study of the biological function of envelope proteins (Cote et al., 1981; Fernie et al., 1982). More recently, Walsh & Hruska (1983) have described the production of six monoclonal antibodies, three reacting with the polypeptides associated with the envelope, one reacting with the nucleocapsid protein and two reacting with a 37000 mol. wt. protein.
We report here the production of monoclonal antibodies specific to the polypeptides VPP32 and VPM27 of the RSN-2 strain of human RS virus and their cross-reactivity with other human RS isolates. The antigenic relationship between human RS virus and the other members of the genus is also discussed.

METHODS

Virus and cells. HEp-2 cells were obtained from Flow Laboratories. BSC-1 cells, human RS virus isolates (RSN-2, A2 and Long), BRS virus and PVM were kindly supplied by Professor C. R. Pringle (University of Warwick, Coventry, U.K.). The RSF-4 virus was isolated during 1982 from a baby suffering from severe bronchiolitis in the Royal Aberdeen Children's Hospital (H. B. Gimenez & P. Saul, unpublished results). The cell lines were propagated in Eagle's medium (Glasgow modification) supplemented with 10% newborn calf serum. The mouse myeloma cell line X63. Ag8. 653 (Flow Laboratories) was propagated in Dulbecco's medium containing 10% horse serum. Hybridomas were maintained in Dulbecco's medium with 10% foetal calf serum (FCS) since the horse serum was found to contain antibodies reacting with human RS virus polypeptides.

The conditions of infection and labelling with radioactive isotopes were as described by Cash et al. (1977) except that actinomycin D was not included in our experiments and that the radioactive isotope used was L-[35S]methionine (1500 Ci/mmol) (Amersham International) at a final concentration of 20 μCi/ml.

Gradient-purified and pelleted virus. The method of Ueba (1978) was used for virus purification except that the final linear sucrose gradient was omitted. On occasion, pelleted virus was used. This was obtained as follows. The cells were scraped into the medium and clarified by centrifugation at 1800 g for 30 min at 4 °C. The virus in the supernatant was pelleted through 30% sucrose (prepared in 0.15 M-NaCl, 0.05 M-Tris–HCl, 1 mM-EDTA pH 7.5) at 51 000 g for 60 min. The virus protein were prepared for polyacrylamide gel electrophoresis as described by Marsden et al. (1976).

Polyacrylamide gel electrophoresis (PAGE). The cell extracts were treated with lysis buffer (0.15 M-NaCl, 0.05 M-Tris–HCl pH 7.5, 1% Triton X-100, 1% sodium deoxycholate) for 5 min on ice, and clarified for 5 min at 15 000 g. Clarified lysates were incubated with antisera or hybridoma antibodies as required and antigen–antibody complexes were precipitated with Staphylococcus aureus (Gimenez & Compans, 1980). PAGE analysis was carried out using a discontinuous buffer system with a 10%o resolving gel as described by Marsden et al. (1976). The gels were fluorographed using En3Hance (New England Nuclear) and exposed against pre-flashed X-ray film. Densitometry of fluorographs was carried out using an LKB Laser densitometer with associated computer software.

Bovine serum negative control and anti-RS virus serum, obtained from Wellcome Laboratories were included in the analysis to locate the virus polypeptides.

Immunization of mice and production of monoclonal antibodies. Two groups of BALB/c mice were immunized. One group was injected with gradient-purified RSN-2 virus treated with 0.1% SDS at 100 °C for 2 min, and the other injected with gradient-purified RSN-2 virus inactivated with short-wavelength u.v. radiation for 1 h at 20 °C. Each mouse was inoculated intraperitoneally with 0.2 ml of virus (10^5 to 10^6 p.f.u.) mixed with 0.2 ml Freund's complete adjuvant. After 3 to 4 weeks, each group of mice received a second immunization with their respective inoculum mixed with Freund's incomplete adjuvant. Three days before removal of the spleen all of the mice received a boost of live gradient-purified RSN-2 virus (10^6 p.f.u./mouse). The spleen and myeloma cells were fused as described by Köhler & Milstein (1975). Briefly, 10^6 spleen cells and 2 × 10^7 myeloma cells were treated with 50% polyethylene glycol (PEG) 1500. After dilution of the PEG with Dulbecco's medium, the cells were pelleted and resuspended in the same medium supplemented with 10% FCS and distributed in 96-well plates. The following day, an equal volume of medium containing 2 × HAT was added to all wells (Littlefield, 1964). Five to 7 days later the medium was replaced with fresh Dulbecco's medium containing 10% FCS and HAT. After a further incubation of 5 to 7 days the tissue culture fluid (TCF) from wells showing cell growth was screened for RS virus antibodies.

The screening of TCF was carried out using the enzyme-linked immunosorbent assay (ELISA) method. Plates (96-well) were coated with either RSN-2 virus-infected or uninfected BSC-1 cells or gradient-purified RSN-2 virus. The cell antigens were prepared as follows. BSC-1 cells were grown in 96-well plates and alternate rows were infected with RSN-2 virus leaving the remaining rows in the same plate with uninfected BSC-1 cells. When cytopathic effect was evident, the cells were washed, fixed with ethanol–acetic acid and stored at −20 °C. Gradient-purified RSN-2 virus was added to 96-well plates at approximately 1 μg/well and dried overnight at 37 °C. Before the ELISA assay all wells were coated with 5% bovine serum albumin in phosphate-buffered saline (PBS). Negative controls included in each plate were pre-immune serum and TCF from X63. Ag8. 653 cells. Anti-RS virus serum raised in mice acted as the positive control. TCF from hybridomas was added to the plates (50 μl/well) and incubated overnight at 4 °C. The plates were washed and sheep F(ab')2 anti-mouse immuno-globulin conjugated to horseradish peroxidase (New England Nuclear) added at the recommended dilution of 1/500. The plates were incubated for 1 h at 37 °C and washed. The substrate o-phenylenediamine was added and
the plates incubated for 30 min at 37 °C. After termination of the assay with 4·5 M-H_2SO_4 the wells were scored using a Multiskan plate reader (Flow Laboratories). Hybridomas that were positive with both the gradient-purified RSN-2 virus and the RSN-2 virus-infected BSC-1 cells but negative with uninfected cells were propagated, cloned and used for production of ascites tumours as described by McKearn (1980). The hybridoma cell lines 3-5, 3-4 and 4-14 were cloned by a dilution method. Briefly, 96-well plates containing 100, 500 and 5000 hybrid cells were prepared. Thymocyte cells were used as feeder cells. The plates that showed 30% of the wells with growing cells were examined by microscopy. Wells containing only one colony of cells were screened and positive cell clones propagated further. The data presented for 3-1, 3-3 and 3-7 were obtained using hybridoma lines from 96-well plates where the frequency of wells showing growth was less than 30%.

**Immunofluorescence staining.** Indirect immunofluorescence staining was performed as described by Faulkner et al. (1976).

**Immunoblot technique.** For this analysis the amount of protein applied to each lane for PAGE was 3 to 5 μg for gradient-purified virus and 10^5 cells for the cell lysates. After PAGE, polypeptides were transferred to nitrocellulose as described by Towbin et al. (1979). The nitrocellulose was washed in PBS containing 10% FCS and 0·2% Triton X-100 (PBS–FCS–T) for 30 min with continuous movement. All steps were carried out at room temperature. The membrane was cut into strips and incubated for 1 h with either serum or ascites fluid diluted in PBS–FCS–T by 50-fold and 10-fold respectively; TCF was used undiluted. The membrane was washed four times with PBS–FCS–T and incubated with goat anti-mouse immunoglobulin conjugated to horseradish peroxidase for 1 h with continuous movement. After washing with PBS–FCS–T the substrate (50 mg dianaminobenzidine tetrahydrochloride in 100 ml 0·1 M-Tris–HCl pH 7·6, 0·01% H_2O_2) was added and the reaction continued for up to 5 min. Strips were washed in PBS and air-dried.

**RESULTS**

Lymphocytes from immunized mice were fused with mouse myeloma cells and the tissue culture fluids from the hybrid cells were tested by ELISA as described above. TCF from 30 hybridomas reacted with both gradient-purified RSN-2 virus and RSN-2 virus-infected cells but did not react with uninfected BSC-1 cells. Here we describe the characterization of six of these hybridomas. The hybridomas designated 3-1, 3-3, 3-4, 3-5 and 3-7 were obtained from a group of mice immunized with SDS-treated RSN-2 virus and the hybridoma 4-14 was obtained from mice immunized with u.v.-inactivated RSN-2 virus.

**Immunofluorescence staining**

Antibodies present in TCF or ascites fluids (AF) were tested by indirect immunofluorescence staining with both fixed and unfixed RSN-2 virus-infected BSC-1 cells. With the fixed cells, two different patterns of staining were observed, consistent with cytoplasmic and peripheral antigens. The cytoplasmic staining was exhibited by the 3-5 hybridoma antibody (Fig. 1 a) as well as the 3-4 and 4-14 hybridoma antibodies (not shown). Late in infection, when the cytopathic effect was extensive, monoclonal antibody 3-5 also produced fluorescent staining on the peripheries of fixed infected cells. Peripheral fluorescent staining of fixed infected cells was exhibited by the 3-3 and 3-1 hybridomas (not shown). The 3-7 hybridoma antibodies gave no detectable immunofluorescent staining.

When the experiments were carried out on unfixed cells, hybridoma antibodies 3-3 (Fig. 1 b) and 3-1 recognized antigen on the surface of the cells but 4-14, 3-4, 3-5 and 3-7 were negative. All hybridoma antibodies gave no detectable immunofluorescence staining with uninfected BSC-1 cells.

**Identification of proteins recognized by the monoclonal antibodies**

The specificity of the antibodies was investigated using the immunoblot technique. A nitrocellulose strip incubated with RSN-2 virus antiserum raised in mice was included in each analysis to locate the virus polypeptides (Fig. 2 b, f, i). Fig. 2 (a, c, e, g, h) shows that the antibodies produced by hybridomas 3-5, 3-4, 4-14, 3-3 and 3-1 respectively were specific to the polypeptide VPP32. Hybridoma 3-7 produced antibodies to the polypeptide VPM27 (Fig. 2 d). Non-immune mouse serum and TCF from a number of hybridoma cell lines failed to react with RS virus polypeptides under these conditions (data not shown).
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Fig. 1. Indirect immunofluorescence staining of RSN-2-infected BSC-1 cells. (a) Ethanol-acetic acid-fixed cells incubated with 3-5 hybridoma antibodies. (b) Live cells incubated with 3-3 hybridoma antibodies.

Fig. 2. Immunoblot analysis. Polypeptides of RSN-2 gradient-purified virus were separated by PAGE and transferred to nitrocellulose. The nitrocellulose strips were incubated with RSN-2 virus mouse antiserum (b, f, i), or hybridoma antibodies 3-5 (a), 3-4 (c), 3-7 (d), 4-14 (e), 3-3 (g) and 3-1 (h).

Analysis of the specificity of the antibodies for viral proteins was extended using the immunoprecipitation of proteins for RS virus-infected cells labelled in vivo with $[^{35}S]$methionine. By this procedure the antibody produced by hybridoma 3-4 seemed to react with both VPN41 and VPP32 (Fig. 3d). The band of radioactivity located above VGP48 was probably due to non-specific precipitation of a cellular polypeptide, because it was also precipitated by bovine serum negative control (Fig. 3b). Similar results were obtained with the antibodies produced by hybridomas 3-5 and 4-14. The polypeptides VGP48 and VPM27 were not detected in the
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Fig. 3. Electrophoretic analysis of polypeptides precipitated by bovine and mouse antibodies. [35S]-Methionine-labelled RSN-2-infected BSC-1 cells were treated as described in the text and divided into two aliquots. One aliquot was immunoprecipitated using bovine anti-RS virus serum (a), bovine serum negative control (b), mouse anti-RS virus serum (c) or hybridoma antibody 3-4 (d). The other aliquot was centrifuged for 1 h at 100000 g and immunoprecipitated with bovine anti-RSN-2 virus serum (e), hybridoma antibody 3-4 (f) or hybridoma antibody 3-5 (g).

immunoprecipitates although they were readily immunoprecipitated using anti-RS virus mouse serum (Fig. 3c). No polypeptides were immunoprecipitated by antibodies from the hybridomas 3-3, 3-1 or 3-7.

It was considered unlikely that VPN41 and VPP32 share common epitopes recognized by the antibodies 3-4, 3-5 and 4-14 since only VPP32 was recognized by the immunoblot procedure (Fig. 2). Therefore, we investigated the possibility that the cell extract contained the VNP41 and VPP32 both associated in a macromolecular complex and also as free polypeptides. The putative macromolecular complex was pelleted from the cell lysate by centrifugation for 1 h at 100000 g and the supernatants were subjected to immunoprecipitation. Again, the antibodies produced by the hybridomas 3-4 and 3-5 immunoprecipitated VPN41 and VPP32 but this time the VPP32 band was more intense than that of VPN41 (Fig. 3f, g). The amount of VPP32 and VPN41 polypeptides immunoprecipitated by 3-4 antibodies from cell lysates before and after ultracentrifugation (Fig. 3d and f respectively) were determined by densitometry of the fluorogram. The ratios VPP32 : VPN41 were 0.36 and 2.99 for lanes (d) and (f) respectively. This suggested that these antibodies were specific to VPP32 and immunoprecipitated a macromolecular complex with which the polypeptide VPN41 was associated.

Cross-reactivity with different human RS virus isolates

RS virus isolates from different locations were analysed using the hybridoma TCF and AF. Cell extracts of RSN-2, RS A2, RS Long and RSF-4 virus-infected cells were subjected to PAGE
and then analysed by the immunoblot technique. RSF-4 virus was grown in HEp-2 cells whereas the other RS virus isolates were grown in BSC-1 cells. Both uninfected HEp-2 and BSC-1 cells were included as controls (Fig. 4a, g, i, o). Antibodies produced by hybridoma 3-5 reacted with all of the RS virus isolates used (Fig. 4j to n). In the A2 isolate three bands in the region of VPP32 reacted with 3-5 antibodies but only two bands with RSN-2, Long and RSF-4 virus isolates. These bands were always detectable by the immunoblot technique and occasionally by immunoprecipitation of [3H]leucine-labelled RS virus-infected cell extracts (not shown). Antibody produced by hybridoma 3-1 also cross-reacted with all the RS virus isolates analysed (not shown). However, the antibodies produced by hybridoma 4-14 reacted only with the RSN-2 virus isolate (Fig. 4c, d). The immunoblot of gradient-purified RSN-2 virus with mouse antiserum was included for comparison (Fig. 4h). The origin of the band present in lanes (j), (l), (m) and (n) just above the BSC-1 cellular band (lane o) is unknown.

The 3-7 hybridoma antibody did not react with VPM27 of pelleted RS Long virus, although this polypeptide was detectable using the mouse antisera (not shown). Results for 3-7 hybridoma antibody with pelleted RS A2 and RSF-4 viruses were equivocal because of the small amounts of VPM27 in these preparations.

Using the immunoprecipitation technique, 3-5 and 3-4 antibodies precipitated both VPP32 and VPN41 polypeptides for all isolates tested (data not shown). The precipitation of VPN41 polypeptide was again considered to be non-specific for the reasons discussed above.

**Cross-reactivity with other members of the genus Pneumovirus**

At least two BRS virus polypeptides (approximate mol. wt. 41000 and 32000) reacted by immunoblot with mouse antiserum to human RS virus (Fig. 5c). Four human RS virus polypeptides (VGP48, VPN41, VPP32 and VPM27) were immunoprecipitated by bovine anti-RS virus serum (Fig. 3a). The antibody produced by hybridoma 3-5 cross-reacted with the 32000
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Fig. 5. Immunoblot analysis of PVM, BRS and human RS viruses. The polypeptides transferred to nitrocellulose were obtained from uninfected BSC-1 cells (a), pelleted PVM virus (b, e, h), pelleted BRS virus (c, f, i) and gradient-purified RSN-2 virus (d, g, j). The nitrocellulose strips were incubated with the following antibodies: mouse anti-RSN-2 virus serum (a to d), hybridoma antibody 4-14 (e to g) and hybridoma antibody 3-5 (h to j).

The mol. wt. polypeptide of BRS virus (Fig. 5f) previously identified by Cash et al. (1977) as the counterpart of the polypeptide VPP32 of human RS virus. These results support the antigenic relationship of BRS and human RS viruses reported by Paccaud & Jacquier (1970) on the basis of neutralization tests and the immunoprecipitation results of Pringle & Cross (1978). The 4-14 antibody did not react with any BRS polypeptides (Fig. 5f).

There was no reaction between the antibodies from either the 3-5 or 4-14 hybridomas and PVM polypeptides (Fig. 5 e, h). However, the nucleoprotein of PVM, BRS and human RS viruses cross-reacted when RSN-2 virus mouse antiserum was used (fig. 5b, c, d). There was no reaction between the polypeptides of any of the three viruses and sera from non-immunized mice. Berthiaume et al. (1974) reported that PVM is antigenically unrelated to human RS virus by complement fixation and neutralization tests. The difference between our results and those of Berthiaume et al. (1974) may be due to the different techniques used.

DISCUSSION

We have described the production, characterization, and use of hybridoma antibodies specific to two human RS virus polypeptides: VPP32 and VPM27. Our results show that the epitope on VPP32 of the RSN-2 isolate recognized by the 4-14 hybridoma antibody is absent in the other RS virus isolates used: A2, Long and RSF-4. RSN-2 virus differs from the A2 and Long isolates in the electrophoretic mobility of the VPP32 polypeptide (Cash et al., 1977); our own unpublished data indicate that RSF-4 virus is grouped with the A2 and Long isolates by this criterion. This preliminary correlation between the antigenic difference determined by mono-
clonal antibody 4-14 and the groups described by Cash et al. (1977) has to be confirmed using more virus isolates and with a greater range of monoclonal antibodies. In contrast, the epitope detected by monoclonal antibody 3-5 was conserved amongst all the human RS virus isolates studied and BRS virus. Neither 3-5 nor 4-14 hybridoma antibodies reacted with PVM polypeptides.

Either one, two or three VPP32 bands could be identified in different RS virus isolates using the hybridoma 3-5 antibody (Fig. 4 and 5). These polypeptides could have been generated by post-translational processing of a common precursor that possessed the epitope recognized by 3-5 antibody. Alternatively, VPP32 has been shown to be phosphorylated and the multiplicity of bands could be accounted for by different levels of phosphorylation of the polypeptide. A double band in the region of VPP32 has previously been reported by other workers (Huang & Wertz, 1983). In addition, Cash et al. (1979) reported that the unphosphorylated VPP32 migrated slightly more rapidly than the phosphorylated form.

The 3-4 hybridoma antibody immunoprecipitated two polypeptides, VPN41 and VPP32 (Fig. 3), but when tested by immunoblotting reacted with only VPP32. A possible interpretation of these results is that VPP32 exists as a macromolecular complex with VPN41, possibly the nucleocapsid, which is immunoprecipitated by 3-4 antibodies due to their specificity for VPP32. The cytoplasmic location of VPP32 determined by immunofluorescent staining with 3-4 and its probable association with the nucleoprotein VPN41 is consistent with the suggestion by Peeples & Levine (1979) that VPP32 is a nucleocapsid protein.

The antibodies to VPP32 exhibited either cytoplasmic or surface staining by immunofluorescence microscopy of infected cells. There are two possible explanations for this observation. First, if the same VPP32 polypeptide is responsible for both cytoplasmic and surface staining, the antigenic determinants recognized by the surface-staining antibodies may be generated by post-translational processing of cytoplasmic VPP32 during assembly of the virus and only exposed after budding. The determinant recognized by 3-5 must be lost or otherwise obscured during the transport of the protein to the cell surface. This would be a late event, since peripheral immunofluorescence staining by 3-5 antibodies was demonstrated late in infection. The hypothetical processing step would simultaneously expose the determinant recognized by the 3-1 antibody. It would follow that VPP32, probably a nucleocapsid protein (see above), would be located on the surface of virus-infected cells some time during virus replication. In support of this, Fernie et al. (1981) demonstrated the presence of a nucleocapsid protein at the surface of mouse cells persistently infected with RS virus and Walsh & Hruska (1983) detected the presence of VPN41 on the surface of syncytia when cytopathic effect was extensive. Similarly, expression of influenza A virus internal antigens on the surface of infected cells has been reported by Yewdell et al. (1981). A second explanation for the immunofluorescent staining pattern is that there may be two polypeptides of 32000 mol. wt., one detectable by cytoplasmic staining and associated with the nucleocapsid, and the other, of unknown location within the virion, detectable by surface fluorescence. High-resolution gel electrophoresis and two-dimensional gel electrophoresis may help to identify these polypeptides.

The hybridoma antibody 3-7 was specific to VPM27 but it was not possible to locate the polypeptide by immunofluorescent microscopy probably because of the low level of protein VPM27 present in infected cells.

One of the main purposes of this work was the investigation of antigenic variation between isolates of human RS virus. This is important for future vaccine development. Previously, comparison of different RS isolates was carried out by serological analysis using conventional antisera and by electrophoresis of the viral polypeptides. The availability of monoclonal antibodies and the use of the immunoblot technique have allowed us to extend these studies. The monoclonal antibody 4-14 was capable of distinguishing between different human RS virus isolates and it is likely that a larger panel of monoclonal antibodies of VPP32 produced from different RS virus isolates could provide the basis for a method of typing human RS virus.

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