Immunoblot Analysis of the Human Antibody Response to Respiratory Syncytial Virus Infection

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SUMMARY

The protein specificities of the antibodies induced during natural respiratory syncytial (RS) virus infection in humans were investigated using immunoblotting of human sera against virus proteins resolved by gel electrophoresis. Sera from 33 patients, who had complement-fixing antibodies against RS virus, were analysed. All the patients showed a response against the virus nucleoprotein (VPN41) and glycoprotein (VGP48). Antibodies against the virus proteins GP90, VPP32, VPM27 and two proteins of molecular weights 24800 and 23700 were also demonstrated. The spectrum of protein specificities of the antibodies varied among patients. There was an increase in the level of antibody for these virus proteins between the acute and convalescent serum samples from five patients. The protein specificities of the IgG and IgM antibodies were determined for five patients; VPN41, VPP32 and VPM27 induced IgG and IgM in all of these sera. Two of the five patients had detectable IgG but not IgM antibodies against VGP48, whereas the remaining three sera had detectable VGP48-specific IgG and IgM antibodies.

INTRODUCTION

Human respiratory syncytial (RS) virus is a major cause of respiratory infections with a peak incidence in children of 2 months of age, although infections in adults have been recorded (Hall et al., 1976; Parrott et al., 1973). The virus has a single-stranded RNA genome coding for at least 10 polypeptides ranging in mol. wt. from 9500 to 200000 (Cash et al., 1977; Pringle et al., 1981; Huang et al., 1985).

The immune response to RS virus infection in humans has been investigated by a number of workers. Mills et al. (1971) reported that respiratory tract secretory antibody rather than serum neutralizing antibody is important in limiting virus infection. The data on the protection of infants against RS virus by maternally derived antibodies are conflicting (Hall et al., 1976; Parrott et al., 1973). The virus has a single-stranded RNA genome coding for at least 10 polypeptides ranging in mol. wt. from 9500 to 200000 (Cash et al., 1977; Pringle et al., 1981; Huang et al., 1985).

The immune response to RS virus infection in humans has been investigated by a number of workers. Mills et al. (1971) reported that respiratory tract secretory antibody rather than serum neutralizing antibody is important in limiting virus infection. The data on the protection of infants against RS virus by maternally derived antibodies are conflicting. Parrott et al. (1973) demonstrated RS virus-neutralizing antibodies, derived from the mother, during the acute phase of infection suggesting that they are not protective. However, several studies show that maternally acquired immunity may be protective against pulmonary disease induced by RS virus and that the level of the antibody might be critical for protection. Babies born to mothers with high levels of IgG antibodies against RS virus are protected during the first month of life when the risk of severe disease is greatest (Ogilvie et al., 1981; Glezen et al., 1981). Ward et al. (1983) used immunoprecipitation of radioiodinated virion nucleocapsids and surface proteins to determine the protein specificities of the antibodies in convalescent sera from adults and children with recent RS virus infection. Sera from infants immunoprecipitated the virus nucleoprotein (VPN41), two virus glycoproteins (VGP48 and VGP20) and a 68000 M_r protein. In addition to these proteins, the virus glycoprotein GP90 was precipitated by normal adult sera. In the same study an association between high levels of antibody to VPN41 in serum from mothers and protection of their offspring against RS virus infection was described. In contrast, Vainionpaa et al. (1985) examined 17 human sera by immunoprecipitation of in vivo radiolabelled virus proteins. They reported that in primary RS virus infection the antibody...
response is predominantly directed against VGP48 and the virus matrix protein (VPM27), with only a very weak response to VPN41.

To extend and clarify these data we have used immunoblotting to determine the protein specificities of antibodies induced during RS virus infections in humans. We observed that antibodies against VPN41 and VGP48 were present in all sera analysed; the amounts of antibodies to the remaining virus proteins were variable among different sera.

METHODS

Sources of virus, cells and monoclonal antibodies. The source of the RS virus isolates (RS Long, RS A2, RSN-2, RSF-20, 35, 38, 41 and 44) and details of their growth in either BS-C-1 or HEp-2 cells have been documented (Gimenez et al., 1986). The monoclonal antibodies against VPM27 and against the virus phosphoprotein VPP32 have been described elsewhere (Gimenez et al., 1984). The monoclonal antibody to VPN41 was prepared in this laboratory against RSN-2 (H. B. Gimenez, unpublished data). The monoclonal antibody to the 23K protein was kindly provided by Dr G. Toms (University of Newcastle, Newcastle upon Tyne, U.K.).

Source of human sera. Human sera, from patients aged between 3 and 82 years, were kindly provided by Dr M. A. J. Moffat (Virus Diagnostic Laboratory, Department of Bacteriology, University of Aberdeen). Antibody titres against RS virus were determined by members of staff in the Virus Diagnostic Laboratory using the complement fixation (CF) test. Apart from the acute sera, all of the sera had CF titres to RS virus of at least 1:128; the titres of the acute sera are specified in the text.

Serum samples used for the detection of IgM antibodies were screened for rheumatoid factor by a latex agglutination assay ('Rheuma-Wellcotest', Wellcome). In addition, the sera were extracted with Staphylococcus aureus (Cowan I strain) to reduce the level of IgG antibodies. Briefly, the bacteria, which had been washed three times in phosphate-buffered saline (PBS), were added to 100 μl of human serum and incubated for 30 min at 37 °C followed by an additional 30 min incubation at 18 °C. The bacteria were removed by centrifugation in a microfuge and the supernatant serum was used in the immunoblot assay. The sera were assayed for IgM and IgG antibodies at a dilution of 1:20.

Immunoblot analysis of human serum. Unless stated otherwise, the antigen was prepared from purified RS virus. The proteins were resolved by discontinuous SDS–PAGE and electroblotted onto nitrocellulose membranes which were then blocked to prevent non-specific binding of the antibodies (Gimenez et al., 1984, 1986). The nitrocellulose membranes were incubated for 60 min with human sera at the dilutions specified. After extensive washing with PBS containing 0.2% Triton X-100 and 10% foetal calf serum, the membranes were incubated for 60 min with horseradish peroxidase-linked second antibody. For the screening of all immunoglobulin classes the second antibody was goat anti-human immunoglobulin (Nordic Immunological Laboratories, Maidenhead, U.K.) used at a dilution of 1:1000. For the assay of IgM and IgG antibody classes the second antibodies were murine monoclonal antibodies to human lgM and IgG (Miles Laboratories) used at a dilution of 1:1000. The monoclonal antibody to human IgM was of subclass IgG2b and that to human IgG was of subclass IgG1. Peroxidase-conjugated, affinity-purified rabbit anti-mouse IgG2b and IgG1 antibodies were used as third antibodies. In all cases diaminobenzidine tetrahydrochloride was used as the enzyme substrate (Gimenez et al., 1984). To control for non-specific binding, strips of nitrocellulose were processed either without the human serum for the analysis of total immunoglobulins, or without the first or second antibody for the IgM and IgG assays.

Protein molecular weights were determined by co-electrophoresis with the following standard protein markers: ovalbumin (43000), α-chymotrypsinogen (25700) and β-lactoglobulin (18400) (Bethesda Research Laboratories); trypsinogen (24000) and trypsin inhibitor (20100) were obtained from Sigma.

RESULTS

Determination of the screening conditions for human sera

Two variables which may have influenced our results were (i) the virus subtype used as the antigen in the immunoblot assay and (ii) the concentration of the human serum assayed. It is possible that the virus which infected the patient was antigenically distinct from the virus isolate used in the screening assay. Consequently, we screened three human sera, which showed different antibody patterns (see below) at a dilution of 1:200, against three RS virus isolates as the antigens. The viruses had been isolated over a period of 26 years from widely distinct geographical locations and included the two antigenic subtypes of RS virus (Gimenez et al., 1984, 1986). The pattern of antibody specificities to the major virus proteins (VGP48, VPN41 and VPP32) was independent of the RS virus isolate used as the antigen (Fig. 1a). However, for patient W no antibodies were detected for VPM27 when the serum was screened against the
Human antibody response to RS virus

Fig. 1. Effect of the antigenic virus type on the antibody protein specificities. (a) The proteins of partially purified virus of three RS virus isolates [RSN-2 (N2), A2 and Long (L)], prepared as described by Gimenez et al. (1984), were used as antigens in the immunoblot assay against three human serum samples (S, W and G; at dilutions of 1:200). (b) Intracellular proteins of five RS virus isolates, collected during 1984, were prepared as described by Gimenez et al. (1986) and used as the antigens in the immunoblot assay against a single human serum (dilution 1:200).

proteins of the RSN-2 strain whereas antibodies against VPM27 were detected using the RS A2 and RS Long strains. When five RS viruses, isolated from patients in north-east Scotland during 1984, were used as antigens with a single human serum sample (at a dilution of 1:200) the pattern was the same for all five (Fig. 1b). As a result of these data the RSN-2 strain was used routinely as the antigen for the following immunoblot assays. The data shown in Fig. 1(b) revealed differences in the mobility of the virus proteins which were probably related to the subtype of the RS virus; RSF-44 is subtype I and the other isolates are subtype II (Gimenez et al., 1986).

Since the results of the immunoblot assay may depend on the human serum concentration used, we screened sera over a range of concentrations. Two sera were assayed at dilutions of 1:50, 1:100 and 1:200; a similar pattern was observed irrespective of the serum concentration although the intensity of the bands increased with increasing serum concentration (data not shown). Therefore, we screened all sera at a routine dilution of 1:200 which was a suitable dilution for all of the sera characterized.

The virus proteins VPN41, VPP32 and VPM27 were identified by immunoblotting with the corresponding monoclonal antibodies (Fig. 2a). A monoclonal antibody prepared against the 23K protein of the RS A2 strain reacted with a protein migrating in the position of VPM27 of
RSN-2 (Fig. 2b). However, when the same antibody was reacted with antigen from RS A2 two bands were observed co-migrating with VPM27 and a protein of Mr 24 800 (data not shown). It was likely that at least two proteins migrated in the position of VPM27. On occasion (see Fig. 1, 3 and 4) a protein was detected migrating between VPP32 and VPM27; this protein was also detected when uninfected cell proteins were used as the antigen.

Screening of human sera for RS virus antibodies

Fig. 3 shows the analysis of paired acute and convalescent sera from five patients (W, S, H, P and O). All five patients had a greater than fourfold rise in CF antibody titre to RS virus between the collection of the serum samples. The sixth patient (D) had CF titres against RS virus of > 256 for both sera. Patients W, S, H and D (aged 56, 76, 82 and 4 years respectively) had severe respiratory infections, including pneumonia, whereas patients P and O (aged 3 and 4 years respectively) had milder respiratory infections. These two patients had low CF titres (< 16) in the acute sera and showed very low levels of antibody at a serum dilution of 1:200 by immunoblotting. Acute sera (CF titres <16) from patients S and H had antibodies against VPN41, VGP48 and GP90; patient H also had trace levels of antibodies to VPP32, VPM27 and a protein of Mr 24 800 (designated 25K). Patient W had antibodies against GP90, VGP48,
Human antibody response to RS virus

VPN41, VPP32, VPM27 and 25K; the broader antibody spectrum was probably related to the higher CF titre (1:32) of this acute serum compared to the acute sera from the other patients. For patients W, S, H, P and O there was a change in the antibody spectrum between the collection of the acute and convalescent sera. All the convalescent sera had antibodies against GP90, VGP48, VPN41, VPP32, VPM27 and 25K. In addition, antibodies against a virus protein of Mr 23700 (designated 24K) were found for all patients except P and O. A broadening of the antibody response for patients P, O and S was also observed when just the IgG antibodies were assayed (data not shown).

Single serum samples from a further 27 patients were screened at a dilution of 1:200; representative data are shown in Fig. 4. All of the data in Fig. 3 and 4 were obtained using a single preparation of virus antigen. The sera were divided into six groups according to the protein specificities of the antibodies. All of the sera had antibodies against VPN41 and VGP48 whereas the presence of antibodies against the other virus proteins was variable among the groups.

Comparison of IgG and IgM protein specificities

In the experiments presented above, the second antibody used for the immunoblot assay recognized all human immunoglobulin classes. To extend these data we also determined the protein specificities for the IgM and IgG antibodies in the sera from eight patients. All of these sera had RS virus-specific IgM antibodies as shown by ELISA (data not shown). None of the serum samples we analysed contained rheumatoid factor detectable by the 'Rheuma-Wellcotest'.

Fig. 3. Analysis of paired human sera. Paired acute (A) and convalescent (C) sera from six patients (W, S, H, D, P and O) were analysed at a dilution of 1:200 by immunoblotting.
assay. However, low levels of rheumatoid factor, undetected by this test, may have been present which could have given false positive results in the IgM assays. To avoid this, the sera were extracted with *S. aureus* in order to reduce the level of IgG antibodies and so eliminate possible interference by IgM anti-IgG rheumatoid factor. As seen from the data in Fig. 5, IgG still remained after extraction, but a 10-fold higher serum concentration had to be used for its detection compared to the unextracted sera. Three sera had IgM and IgG antibodies against four of the major virus proteins (VGP48, VPN41, VPP32 and VPM27); representative data for one patient are shown in Fig. 5(a). These sera reacted with some proteins of slightly higher $M_r$ than VGP48. We were not able to correlate these bands with previously described RS virus proteins. Three sera had no detectable IgM antibodies when screened at a dilution of 1:20 but had IgG antibodies against VGP48, VPN41, VPP32 and VPM27 (data not shown). The two remaining patients (P and D) showed different protein specificities for IgM and IgG. Both had IgM and IgG antibodies against VPN41, VPP32 and VPM27 but neither had detectable IgM antibodies against VGP48, a subunit of the virus fusion protein VP70. Both sera had IgG antibodies against VGP48; the data for patient P are shown in Fig. 5(b). It was possible that IgM antibodies were present which reacted with VP70 but not with VGP48. Therefore, we determined whether VP70-specific IgM was present in these two sera. Virus proteins were

Fig. 4. RS virus protein specificities of human serum antibodies. Representative data for the different patterns of protein specificities of the antibodies present in human sera are presented. Sera were screened at a dilution of 1:200 by immunoblotting. The figures below the lanes are the numbers of sera showing each pattern.
Human antibody response to RS virus

Fig. 5. Determination of RS virus protein specificities of IgG and IgM antibodies. Sera were analysed either before (−) or after (+) extraction with S. aureus at dilutions of 1:200 and 1:20 respectively. The IgG and IgM antibodies were identified as described in the text. (a) Convalescent (C) serum sample screened for IgG and IgM antibodies; (b) paired acute (A) and convalescent (C) sera from patient P (Fig. 3); (c) convalescent (C) sera from patient P; for this assay the virus proteins were resolved by SDS–PAGE in the absence of 2-mercaptoethanol as described in the text. Tot., all immunoglobulin classes detected.

resolved under non-reducing conditions and used as the antigen in the immunoblot assay (Gimenez et al., 1986). Under these conditions IgM antibodies were detected which reacted with the unreduced VP70 (Fig. 5c).

DISCUSSION

The data we have presented complement those of Ward et al. (1983) and Vainionpaa et al. (1985) in establishing the protein specificities of the antibodies induced as a result of RS virus infection. Some differences in the results were apparent which may in part be due to the methods used. Although different virus isolates were used by each group, our data indicate that this had little influence on the resulting pattern of antibodies. Apart from one experiment, the data of Ward et al. (1983) were obtained by immunoprecipitation of a virus antigen prepared by in vitro radioiodination of the virus nucleocapsid and glycoproteins. Consequently, it is likely that antibodies to only a limited number of the virus proteins were detected by this approach.
Vainionpaa et al. (1985) also immunoprecipitated virus proteins but in this case they used intracellular virus proteins labelled \textit{in vivo} with \([35S]\)methionine. Nevertheless, even with this approach there could be problems in the detection of antibodies against, for example, GP90 which incorporates only small amounts of methionine (Pringle et al., 1981). This method may thus lead to a possible under-representation of antibodies against GP90. We observed a similar situation for 25K and 24K which were detected by immunoblotting but not by radiolabelling with \([35S]\)methionine (unpublished data). Immunoblotting circumvents these problems since antibodies against all of the virus proteins may in principle be identified. In contrast to the data of Ward et al. (1983) and ourselves, Vainionpaa et al. (1985) detected only a very weak immune response to VPN41 in patients with primary RS virus infections. We invariably observed antibodies to VPN41 in single serum samples with high CF titres (> 128) to RS virus as well as showing a rise in titre to VPN41 between paired acute and convalescent sera from patients.

Immunoblot analysis of 33 human sera clearly demonstrated differences between sera in the antibody-binding patterns. Two possible explanations for the different patterns were: (i) some patients had an immune response to only a subset of the virus proteins, and (ii) the single serum samples analysed represented different stages in the development of an immune response to all the virus proteins. From the analysis of paired sera from five patients, it appeared that the major antibody response was initially against VPN41 and VGP48 with a broadening of the immune response to the remaining virus proteins later in the course of the infection. Following the peak of the antibody titres it is possible that antibodies with different protein specificities decayed at different rates which also could have accounted for the varying antibody-binding patterns.

We analysed the protein specificities of the IgG and IgM antibodies. Two patients had different protein specificities for IgM and IgG; IgM to VP70 was detected but not to VGP48, whereas VP70- and VGP48-specific IgG antibodies were detectable. Since we were able to demonstrate IgM antibodies against VGP48 for three other sera, the difference observed for the IgM and IgG specificities was unlikely to be due to technical difficulties in assaying for IgM but may have represented a true biological phenomenon. A possible explanation is that at some stage of the immune response the level of IgM was too low for its detection whereas the equivalent IgG antibodies were high enough to be detected. Alternatively, the difference may be accounted for by either the unreduced or reduced forms of VP70 being exposed during the different stages of the development of the immune response. Differences between the IgM and IgG protein specificities have been documented during \textit{Toxoplasma gondii} infection (Partanen et al., 1984). Partanen et al. (1984) suggested that in some instances an IgG response may not always be preceded by a corresponding IgM response.

A detailed serological follow-up of infected individuals will be required to determine the importance of the different antibody binding patterns.

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Human antibody response to RS virus


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