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Site-directed serology with synthetic peptides representing the large glycoprotein G of respiratory syncytial virus

(immunogenic epitopes/antibody response/surface antigen)

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ABSTRACT **A set of 23 nested 15-amino-acid-long peptides with overlaps of 5 amino acids, representing the complete extramembranous part of the large glycoprotein G of respiratory syncytial (RS) virus, was analyzed in ELISA against different sera containing virus-specific antibodies. Seven of the peptides reacted with rabbit hyperimmune sera against purified virions. In contrast, only one of these seven peptides reacted with murine monoclonal antibodies specific for G. In connection with RS virus infections in humans, increase of antibody titers against three peptides was found in about one-third of the cases. These three peptides were included among those identified by both murine and rabbit antibodies. The present findings may open possibilities for site-directed clinical serology in the case of RS virus infections.**

Respiratory syncytial (RS) virus is a unique human respiratory pathogen. It causes the largest fraction of pneumonia and bronchiolitis among infants and children, especially among those between 2 and 6 months of age (1, 2). Many of these infections occur in the presence of passively transferred neutralizing antibodies. Although high titers of antibodies appear to ameliorate the disease process in infants less than 9 months of age (3, 4), it has been speculated that immunopathological events may potentiate the damage caused by virus replication in the respiratory tract. This concept was reinforced by the observation of aggravated disease in children who had received a parenteral immunization with a formalin-inactivated and concentrated RS virus vaccine (5–7). The particular features of the immunopathology of RS virus infections need to be elucidated by expansion of our knowledge of the immunobiology of RS virus structural components.

RS virus has two envelope-associated glycoproteins. These are the large glycoprotein G (molecular mass ≈90 kDa), which may be responsible for viral attachment to cells (8, 9), and the putative RS virus fusion protein F. The latter protein shows many similarities to the homologous protein of other paramyxoviruses. Proteolytic cleavage gives a disulfide-linked complex of a 48-kDa F1 and a 20-kDa F2 protein (10). In contrast, the G protein shows a number of features that are unique to RS virus.

The structure of G has been deduced from nucleotide sequence data on cDNA clones (11, 12). The protein is 298 amino acids long, which gives a molecular mass of 32,588 Da. A single 23-amino-acid-long hydrophobic domain has been interpreted to allow membrane insertion at 41 residues from the NH_2 terminus. Heavy glycosylation with both N- and O-linked sugars (9, 11, 12) is the cause of the apparent molecular mass of 90 kDa of the G protein in NaDodSO$_4$/polyacrylamide gels.

Some data pertaining to the relative role of glycoproteins G and F in immunoprotection have been presented. Monoclonal antibodies against both proteins give passive immunity in cotton rats and mice (13, 14). However, effective neutralization *in vitro* is seen primarily with monoclonal antibodies against F (15, 16). Polyclonal rabbit hyperimmune sera against both immunoaffinity chromatography-purified G and F have neutralizing activity (8, 17). Still a relatively more immunodominant role of F is indicated by results of *in vivo* protection experiments in cotton rats and mice with vaccinia vector-borne RS virus genes for G and F.

Recently it has been found that isolates of RS virus can be classified into two subgroups on the basis of their reactivity with monoclonal antibodies (16, 18). Members of these subgroups differ in immunological properties of at least four structural proteins, and some homologous proteins also show distinct molecular mass characteristics (19). These differences involve both envelope glycoproteins. Five of six overlapping epitopes in glycoprotein G were found to be unique for subgroup A virus by characterization with monoclonal antibodies against a representative of this subgroup. Similarly, one of two epitopes on glycoprotein F differed, and in addition, subgroup-specific size differences of the F cleavage products F1 and F2 were identified.

In this study a nested set of overlapping peptides representing the extramembranous part of G were prepared by the method of simultaneous multiple peptide synthesis (20) and cleaved with a multiple vessel apparatus with liquid hydrogen fluoride (21). These peptides were used to gain further understanding of structural–functional relationships in this protein. The reactions between different peptides and rabbit hyperimmune sera against purified virions, murine monoclonal antibodies against the G protein, and paired acute and convalescent sera from individuals infected with RS virus were examined in enzyme-linked immunosorbent assay (ELISA). The capacity to react with peptides varied with the different kinds of sera. Possibilities for site-directed serology are highlighted by the findings of this study.

MATERIALS AND METHODS

Synthesis of Peptides Representing the Extramembranous Part of the G Protein. The amino acid sequence deduced from nucleotide sequence data on the G gene in ref. 11 was used. Fifteen-amino-acid-long peptides with an overlap of 5 amino acids, representing the whole extramembranous part of the G protein, were synthesized (Fig. 1). A cysteine residue was

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Abbreviations: RS, respiratory syncytial; F, fusion protein; ELISA, enzyme-linked immunosorbent assay; RIPA, radioimmunoprecipitation assay.

added at the end of each peptide. The total number of peptides was 23, and they were numbered consecutively from the COOH terminus. The peptides were synthesized in 2 weeks in 50- to 75-mg quantities by the method of simultaneous multiple-peptide synthesis (20) and were cleaved with liquid hydrogen fluoride in a 24-vessel apparatus (21).

Sera. Rabbit hyperimmune antisera were prepared by immunization with virus purified by centrifugation in discontinuous sucrose gradients as described (22). The sera had high titers of antibodies in ELISA and reacted with all major viral structural proteins in radioimmunoprecipitation assays (RIPA) but only to a limited extent with control antigen from uninfected cells (E.N., unpublished data). The collection of nine murine monoclonal antibodies against the G protein has been described (16). Antibody-containing ascites fluid was used. Sera from both children and adults with RS virus infections were examined. The etiology of the respiratory infections was defined by virus isolation and/or demonstration of antibody increases in complement fixation tests or ELISA. Some sets of sera were taken from a study of subgroup characteristics of RS virus strains from children with two consecutive symptomatic infections (M.A.M., unpublished data).

ELISA. Two different variants of ELISA were used and referred to as dry and wet tests. Each peptide (100 pmol) was adsorbed to separate wells of 96-well microtiter soft-plastic plates (Titertek Immuno Assay-Plate, Flow Laboratories). This amount of peptide was distributed in 50 μl of either phosphate-buffered saline (pH 7.2) or in 10 mM sodium carbonate buffer (pH 9.5). In the former case, plates were left open at 37°C overnight to dry, whereas in the latter case, the plates were sealed to prevent evaporation during the overnight incubation at 37°C. The plates with peptide antigen in the dried state were fixed with 50 μl of methanol per well for 5 min at room temperature. In whole-virus ELISA tests, infected cells (50,000 per well) were dried onto plates and used as antigen. Hereafter the plates for dry and wet tests were treated identically. In parts of the study 1% (wt/vol) bovine serum albumin was substituted for 0.25% bovine nonfat dry milk as blocking reagent, carrier, and wash solution. The previously described method for ELISA was used (23). Peroxidase conjugates were swine anti-rabbit, rabbit anti-mouse, and rabbit anti-human IgG (Dakopats). Extinction values greater than 0.2 were considered positive.

RESULTS

Reactions of Peptides with Rabbit Hyperimmune Sera Against Purified Virions. A collection of nine rabbit sera against four strains of subgroup A and five strains of subgroup B of RS virus (cf. refs. 16 and 19) was tested against all 23 peptides, and representative data are given in Table 1. Some sera reacted distinctly (ELISA titer in excess of 160) with a fraction of the peptides. This fraction included 7 of the 23 peptides. The results with peptides 11 and 18 were ambiguous because of high background values. In three regions of the protein, reactions were seen with overlapping peptides: peptides 10–12 (reaction of peptide 11 with rabbit antisera was uncertain, but a distinct reaction was seen with human convalescent sera; see below), 15 and 16, and 20 and 21. Peptides 15 and 16 were from a region of the protein that was highly hydrophilic (Fig. 2), but the other regions with primary structures active in antibody binding showed variable hydrophilicity.

There was no correlation between either antibody titers in ELISA with virus-infected cells or the G-specific reactivity identified in RIPA tests (data not shown) of the rabbit sera and their capacity to react with diffrent peptides. Rather, sera showing high activity against one peptide gave high titers also with a range of certain other peptides. Since at least one antigenic site in the G protein is subgroup specific (16), it was anticipated that also reactions with one or more of the peptides might show such a restricted specificity. However, generally this was not the experience. Only in the case of peptides 12 and 15 was there an increased tendency for peptide antibody-containing sera of subgroup A specificity to show comparatively higher titers than sera of subgroup B specificity (Table 1).

Reactions of Peptides with G Protein-Specific Murine Monoclonal Antibodies. Nine different monoclonal antibodies

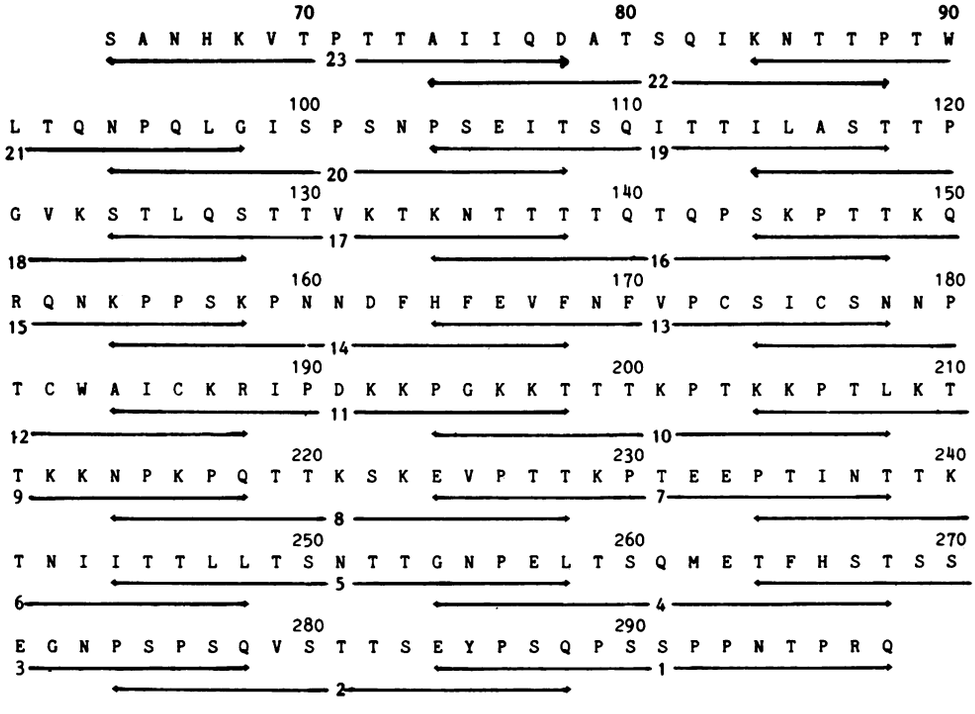

FIG. 1. Fifteen-amino-acid-long overlapping peptides representing the extramembranous part of the G protein of RS virus. The peptides were numbered 1 through 23 starting from the COOH-terminal end of the protein.

Table 1. Reaction of rabbit hyperimmune sera against purified virions of RS virus strains representing subgroups A and B with different G-specific peptides in wet ELISA tests

Strain used to raise rabbit antiserum		Virus-infected cells	ELISA titer with peptide						
Strain	Subgroup		7	10	12	15	16	20	21
WV12138 (1086)	A	80,000	1280	1280	5120	2560	1280	5120	2560
A2 (1088)	A	80,000	1280	2560	2560	1280	1280	5120	2560
WV9894 (1094)	A	320,000	80	80	80	160	<80	160	160
CH287 (1096)	A	640,000	40	80	80	<80	<80	160	80
WV3212 (1090)	B	160,000	1280	1280	320	640	640	2560	1280
WV4843 (1092)	B	160,000	1280	640	320	320	1280	640	1280
WV1293 (1098)	B	160,000	320	80	80	<80	80	80	80
WV6873 (1100)	B	160,000	80	<80	<80	80	160	<80	80
CH18537 (1104)	B	80,000	320	<80	160	320	320	320	160

against the G protein were tested with the different peptides. The antibodies were previously classified into six different categories (epitopes) based on results of competition ELISA (16). There was a considerable overlap in these tests, showing that all antibodies bound at the same antigenic site. The only two monoclonal antibodies that were mutually exclusive in the competition ELISA were C793 (epitope 1) and B119 (epitope 6).

Only 1, peptide 12, of all 23 peptides had a capacity to react with the monoclonal antibodies (Table 2). This peptide reacted in wet ELISA with seven of nine monoclonal antibodies. Generally the difference between the whole virus and peptide ELISA titers was about 100-fold. Antibody B18 showed a comparatively low peptide ELISA titer. The monoclonal antibodies B119 and C793 did not show any demonstrable peptide ELISA titer. Since the whole-virus ELISA titer of B119 was low, it may be that this limited the possibilities for detection of any antibody activity with peptide 12. Antibody C793 had an ELISA titer with virus-infected cells of 20,000 but did not react in a 1:10 dilution with any peptide. Thus, the behavior of this antibody is clearly different from that of the other monoclonal antibodies, possibly excepting B119. Interestingly, C793 is the only one among the nine G-specific monoclonal antibodies that shows intersubgroup reactivity (16).

Reactions of Peptides with Antibodies Appearing in Connection with RS Virus Infection in Humans. Significant increases in antibody titers with G-specific peptides were seen in some human RS virus infections (Table 3). These reactions were seen with 3 of the 23 peptides. Among these peptides were peptide 12, which was seen by antibodies of both rabbit and mouse origin, and peptides 11 and 15, of which 15 also was identified with rabbit anti-virion sera, whereas results with peptide 11 were ambiguous (Fig. 2). The differences observed between dry and wet ELISA titers are exemplified with peptide 15 in Table 3. The differences seen in these two variations of the test were moderate with this peptide, but with peptides 11 and 12, the reactions in wet ELISA were much more pronounced. In fact, no significant increase in antibody titers was seen in the matched sera with these peptides in dry ELISA.

The occurrence of antibodies reacting with G peptides in connection with RS virus infections was unpredictable. Besides the paired sera from patients 1 and 2 showing antibody increases, four other pairs of sera from a corresponding selection of samples from adults with RS virus infections did not show antibody increases with peptides, although increases were seen with virus-infected cells as antigen. Originally this was interpreted to reflect the possibility that peptides might allow a preferable identification of

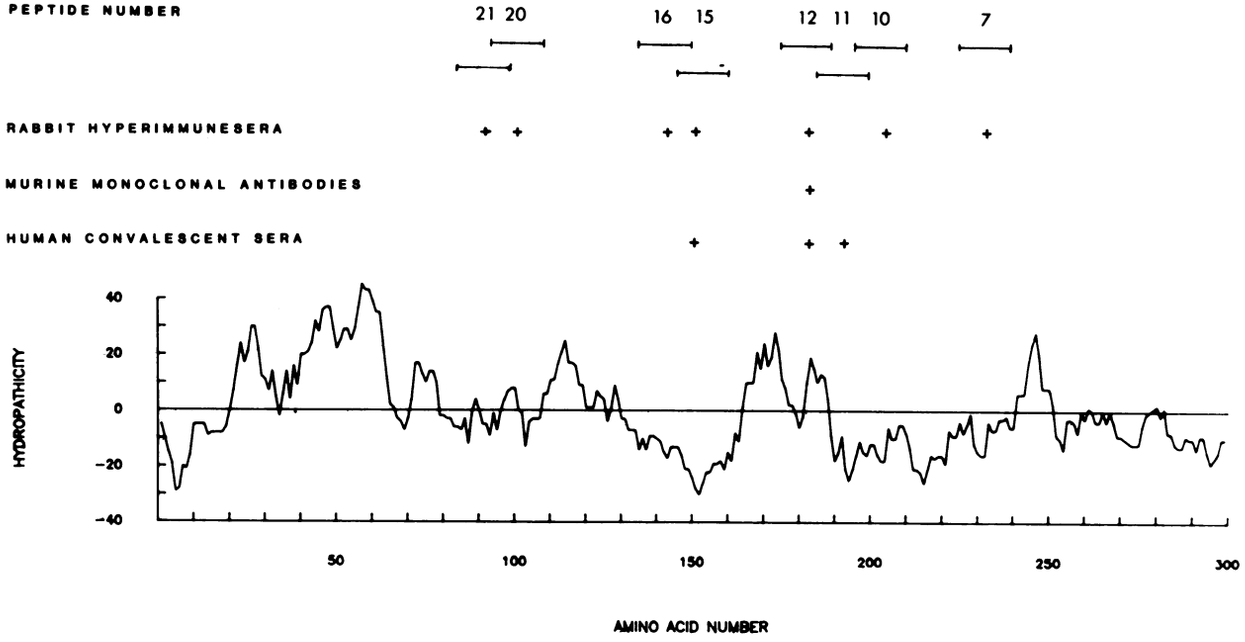

FIG. 2. Depiction of positions of peptides reacting with RS virus-specific antibodies in rabbit anti-virion hyperimmune sera, murine hybridoma ascites fluid, and human convalescent sera in relation to the hydropathy diagram of RS virus glycoprotein G (reproduced from ref. 11 with permission, copyright IRL Press Ltd.).

Table 2. Reactions of RS virus subgroup A G-specific murine monoclonal antibodies (mAb) in ELISA with virus-infected cells and G peptide 12

mAb clone	Epitope* specificity	ELISA titer: Virus-infected cells	ELISA titer: Peptide 12 Dry	ELISA titer: Peptide 12 Wet
C793	G1	20,000	<10	<10
B14	G2	5,000	<10	80
B18	G2	20,000	<10	10
B23	G2(?)	80,000	80	1280
B109	G3	160,000	320	640
B158	G3	80,000	<10	640
B17	G4	40,000	<10	1280
B25	G5	80,000	<10	2560
B119	G6	2,500	<10	<10

*Based on competition ELISA (16). A considerable overlap in competition reactions was seen with all mAbs.

subgroup-specific reactions, a concept reinforced by the observation made with sera from patient 3 (Table 3). In this child a significant increase of peptide antibody titers was seen after the initial infection with a subgroup A virus strain, whereas the antibody titers remained low after the subsequent infection with a subgroup B virus strain. However, another child (patient 4) with an RS virus subgroup A infection followed by subgroup B infection showed a different pattern of appearance of antibodies (Table 3). In this case significant antibody increases were seen only as an accumulated effect of the two consecutive infections. In five other cases from the same study of consecutive symptomatic infections (M.A.M., unpublished data), no increase in antibody titers with peptide antigens was found in spite of the presence of significant antibody increases in ELISA with virus-infected cells as antigen.

DISCUSSION

Synthetic antigens allow detection of antibody responses to linear epitopes and possibly some discontinuous epitopes in which one of the participating polypeptide strands may have a dominance (24, 25). In addition to this restriction, a limiting factor in the use of peptide antigens is that the epitope they represent may be covered by carbohydrate chains associated with the mature native protein (26). In addition, discontinuous epitopes involving both carbohydrate and polypeptide chains may occur. The G protein of RS virus is heavily glycosylated with both N- and O-linked sugars (9, 11, 12). This extensive glycosylation may restrict the number of immunogenic sites available in the protein. It is noticeable that peptides representing a long comparatively hydrophilic stretch at the COOH-terminal part of G did not appear to be immunogenic. In fact, a survey of the hydrophobicity/hydrophilicity profile of G does not give good guidance for the identification of immunodominant linear epitopes in this protein (Fig. 2). Although the immunogenic site identified by peptides 15 and 16 occurs in a comparatively hydrophilic region, other immunodominant sites demonstrated by the positive ELISA reactions with peptides 10–12, 20, and 21 partly have a hydrophobic nature.

There was a difference between the immunogenicity of the various peptides in different species and under the various conditions of hyperimmunization and immunization in connection with virus replication. Peptide 12 was uniquely immunogenic in rabbits, mice, and humans. The highest selectivity of reaction was seen with the set of nine G-specific monoclonal antibodies. Studies with other monoclonal and polyclonal murine G-specific reagents are required to further define the overall immunogenicity of the G protein in mice. The broadest reactivity with the synthetic peptides was seen with rabbit hyperimmune sera. This may have both quantitative and qualitative explanations. Because the rabbit sera were derived from hyperimmunization, including primary immunization with Freund's complete adjuvant and a later intravenous booster with aqueous antigen, the ELISA titers of the reagents were high. Further, it is possible that the immunization procedure may have broadened the antibody response to include also certain "nonnative" sites.

Human convalescent sera from RS virus infection in some cases contained significantly increased titers of antibodies to G peptides. These sera identified the important immunogenic site represented by peptide 12 and also a second site covered by peptide 15. Immunogenic peptides also have been identified in other virus systems. Thus, by use of peptides, it has been possible to detect antibodies to the Epstein–Barr nuclear antigen 1 in Epstein–Barr virus infections (27), to the pre-S antigen in hepatitis B virus infections (28), and to the transmembranous glycoprotein of human immunodeficiency virus (29).

The use of site-directed serology offers a number of attractions. The antigen used in the test is biochemically defined and, therefore, can be standardized readily. Unlimited amounts may be synthesized and, compared with tissue

Table 3. Reaction of sera from individuals with RS virus infections in ELISA with virus-infected cells and different G peptides

Patient	Serum: Infection stage	Serum: No.	Virus-infected cells	ELISA titer with peptide: 11*	12*	15 Dry	15 Wet	20*
1†	Acute	22	2,500	<40	<40	<40	40	<40
	Conv.	23	40,000	80	80	40	320	<40
2†	Acute	25	5,000	<40	<40	80	160	<40
	Conv.	26	80,000	160	320	640	1280	<40
3	Acute A	6,745	2,500	80	80	<80	80	<80
	Conv. A	8,485	40,000	320	1280	1280	640	<80
	Acute B	10,190	2,500	80	80	<80	80	<80
	Conv. B	10,851	10,000	80	80	<80	80	<80
4	Acute A	756	640	80	<80	80	80	<80
	Conv. A	857	10,000	160	80	160	160	<80
	Acute B	2,201	5,000	160	80	320	320	<80
	Conv. B	2,900	80,000	640	160	320	320	<80

Conv., convalescent.
*Wet ELISA titer.
†The RS virus subgroup characteristics of this infection were not determined.

culture-prepared materials, synthetic peptides will be inexpensive. The application of this technology to different virus systems will have to be evaluated individually. At present no predictions can be made because available knowledge on protein structure does not allow a definition of amino acid sequences that may represent highly immunogenic epitopes. Therefore, an empirical approach, preferably including evaluation of a nested set of peptides as performed in this study, is to be recommended. The method of simultaneous multiple-peptide synthesis (20) allows the preparation of a large number of peptides within a short time.

The end-point titers of both murine monoclonal antibodies and human sera were frequently much higher with the wet than with the dry ELISA. This is interpreted to signify that the wet form of ELISA allows a presentation of peptides in a configuration more closely related to the homologous epitope in the native protein.

It is not known why only about one-third of human convalescent antibodies reacted with peptides representing the RS virus G protein. Antibodies to discontinuous or combined carbohydrate–protein epitopes might play a dominating role in sera from patients not showing any reactivity. In other systems (e.g., concerning antibodies to human immunodeficiency virus), essentially all postinfection sera showed a reaction (29). The most dominant linear epitope in the RS virus G protein appeared to be located in the region of the protein represented by peptide 12. Antibodies in polyclonal postinfection and hyperimmune sera as well as monoclonal antibodies reacted with this peptide. The antibody titers of monoclonal antibody reagents were about 100 times higher with whole-virus antigen than with the synthetic peptide. Thus, the efficacy of interaction within a single epitope was higher with the native antigen. It is possible that alternate peptides from the part of G that includes peptide 12 or a combination of peptides may both show an improved capacity to react with the epitope-specific monoclonal antibodies and with antibodies of corresponding specificity in convalescent sera.

In further work towards the practical application of site-specific serology focusing on the G protein of RS virus, the occurrence of both intrasubgroup- and intersubgroup-specific epitopes also needs to be considered. This study is based on the amino acid characteristics of the G protein of a subgroup A virus deduced from nucleotide sequencing data. The corresponding data will soon be available for the G gene of a subgroup B RS virus. The availability of these data will allow a comparison of primary protein structure of the G component of subgroup A and B RS viruses. Based on this comparison, a selection of potential subgroup-unique and subgroup-shared linear epitopes can be made. Potentially, a peptide-dependent serological test for RS virus could be designed to identify antibodies against epitopes of either of these specificities.

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