JOURNAL OF VIROLOGY, Feb. 1993, p. 961–968
0022-538X/93/020961-08$02.00/0

Vol. 67, No. 2

An Antibody- and Synthetic Peptide-Defined Rubella Virus E1 Glycoprotein Neutralization Domain

JERRY S. WOLINSKY,[1]* ELENA SUKHOLUTSKY,[1] WILLIAM T. MOORE,[2] AMY LOVETT,[1] MICHELINE McCARTHY,[1] AND BARBARA ADAME[1]

Department of Neurology[1] and Analytical Chemistry Center,[2] The University of Texas Health Science Center at Houston, P.O. Box 20708, Houston, Texas 77225

Received 31 August 1992/Accepted 9 November 1992

We previously described a monoclonal antibody (MAb) library generated by infecting BALB/c mice with rubella virus (RV) and selected by an enzyme-linked immunosorbent assay (ELISA) using purified virion targets. Plasmid pARV02-01, which expresses the fusion protein $RecA_{1-35}$-GIGDLGSP-$E1_{202}$-$E1_{283}$-GDP-$LacZ_{9-1015}$ in *Escherichia coli*, was shown to be a ligand for MAbs E1-18 and E1-20 (J. S. Wolinsky, M. McCarthy, O. Allen-Cannady, W. T. Moore, R. Jin, S. N. Cao, A. Lovett, and D. Simmons, J. Virol. 65:3986–3994, 1991). Both of these MAbs neutralize RV infectivity. A series of five overlapping synthetic peptides was made to further explore the requirements of this MAb binding domain. One of these peptides (SP15; $E1_{208}$ to $E1_{239}$) proved an effective ligand for both MAbs in the ELISA. Stepwise synthesis of SP15 defined the minimal amino-terminal requirement for binding MAb E1-18 as $E1_{221}$ and that of MAb E1-20 as $E1_{223}$; the minimal carboxyl-terminal requirement is uncertain but does not exceed $E1_{239}$. Immunization of mice and rabbits with SP15 induced polyvalent antibody reactive with SP15, with other overlapped and related but not unrelated synthetic peptides, and with RV. The rabbit anti-SP15 antibody showed neutralization activity to RV similar to that of MAbs E1-18 and E1-20 but lacked hemagglutination inhibition activity. These data define a neutralization domain on E1 and suggest that the RV epitopes conserved by SP15 may be critical for protective host humoral immune responses.

Rubella virus (RV) is the sole *Rubivirus* subgroup representative of the togaviruses. In humans, its only natural hosts, RV usually causes only a mild or subclinical infection, although more serious manifestations of primary infection and the devastating consequences of early gestational infection on the developing fetus are well recognized (reviewed in reference 48). The nucleotide sequence and organization of the RV gene have been resolved (9). The virion contains three structural proteins, designated E1, E2, and C (reviewed in reference 50). It is the E1 glycoprotein that bears the majority of the monoclonal antibody (MAb)-defined erythrocyte binding and neutralization sites (12, 13, 17, 18, 41, 43, 44).

We previously constructed a map of selected conformation-independent epitopes of the structural proteins of RV recognized by murine humoral responses during the course of an experimental infection (51). A subset of MAbs generated following RV infection of BALB/c mice was used to define these epitope-containing domains when they were expressed as fusion proteins in a prokaryotic system. Several of these domains were confirmed and further refined by the use of synthetic peptides (29, 51). One rather large domain of E1, $E1_{202}$ to $E1_{283}$, was defined by MAb E1-18, which neutralizes RV infectivity, and MAb E1-20, which neutralizes infectivity and modestly inhibits hemagglutination (49, 51). The possible importance of this region of E1 has also been noted by others (4, 23, 27, 39). In this study, we show that the epitope defined by MAbs E1-18 and E1-20 can be simulated by a synthetic peptide (SP15; $E1_{208}$ to $E1_{239}$) and is contained within a minimum domain bounded by $E1_{221}$ and $E1_{239}$. Further, SP15 can induce antibodies in mice and rabbits, and the latter are capable of neutralizing RV infectivity in vitro.

MATERIALS AND METHODS

Antibodies. The generation and characterization of the murine MAbs have previously been reported (43, 44, 51). MAbs E1-18 (immunoglobulin G2a [IgG2a]) and E1-20 (IgG1) are reactive with the E1 glycoprotein of RV. MAb C-1 (IgG2a) is reactive with the capsid (C) protein of RV and was used in this study as a control reagent. To determine whether selected synthetic peptides were immunogenic, 2- to 4-week-old male BALB/c mice were immunized by repeated intrascapular subcutaneous injections of 100 μg of synthetic peptide emulsified in complete (initial immunization) or incomplete (subsequent booster immunization) Freund's adjuvant. The mice were bled by retro-orbital puncture, using fine capillary tubes. Blood was separated by centrifugation in the capillary tubes, and the 10 to 25 μl of serum recovered was diluted in buffered saline for determination of antibody reactivity by enzyme-linked immunosorbent assay (ELISA) under standard conditions (29). Rabbits were bled by ear vein and then immunized with repeated intrascapular subcutaneous injections of 100 μg of synthetic peptide emulsified in complete (initial immunization) or incomplete (subsequent booster immunization) Freund's adjuvant. The capacity of the MAbs and rabbit sera to inhibit hemagglutination was determined as previously detailed (44) with and without pretreatment with heparin-manganese chloride (46).

Plaque reduction assay. A predetermined amount of RV containing 50 PFU was added to 10-fold dilutions of crude MAb containing ascites fluid or rabbit sera in Hanks balanced salt solution (HBSS) containing 0.1% bovine serum albumin and incubated for 1 h at room temperature. A 1-ml aliquot of each sample was inoculated into each of three

* Corresponding author.

TABLE 1. Synthetic peptides

Peptide	Location	Sequence
SP11	$E1_{202}$–$E1_{225}$	DLVEYIMNYTGNQQSRWGLGSPNC
SP15	$E1_{208}$–$E1_{239}$	MNYTGNQQSRWGLGSPNCHGPDWASPVCQRHS
SP27	$E1_{221}$–$E1_{239}$	GSPNCHGPDWASPVCQRHS
SP12	$E1_{226}$–$E1_{247}$	HGPDWASPVCQRHSPDCSRLVG
SP7	$E1_{233}$–$E1_{256}$	SRLVGATPERPRLRLVDADDPLLR
SP13	$E1_{264}$–$E1_{283}$	DPLLRTAPGPGEVWVTPVIGSQ
SP10	C_{64}–C_{97}	GNRGRGQRRDWSRAPPPPEERQETRSQTPAPKPS
SP18	C_{64}–C_{89}	GNRGRGQRRDWSRAPPPPEERQETRS
SP17	C_{76}–C_{89}	RAPPPPEERQETRS
SP9	C_{78}–C_{97}	PPPPEERQETRSQTPAPKPS

monolayers of Vero cells grown in 35-mm wells of a six-well flat bottom culture plate (Corning Glass Works, Corning, N.Y.) that had been washed once with HBSS. The monolayers were then incubated at 37°C for 2 h. The inoculum was aspirated, the monolayers were washed once with HBSS, and 2.5 ml of Eagle minimal essential medium containing 2.0% fetal bovine serum and 0.5% agarose (SeaKem HGT; FMC BioProducts, Rockland, Maine) was added to each plate. The agarose was hardened at room temperature, and the plates were incubated at 37°C for 5 to 6 days. The number of plaques was determined following application of the vital dye neutral red (51).

Peptide synthesis. Peptides were synthesized by solid-phase methods based on standard *tert*-butyloxycarbonyl amino acid addition protocols, using an automated peptide synthesizer (model 430A; Applied Biosystems Inc., Foster City, Calif.) controlled by standard Applied Biosystems system software version 1.40 for 0.5 mM scale synthesis as previously detailed (51). Peptides were deprotected and cleaved from resin by treatment with anhydrous hydrogen fluoride (HF) in the presence of either anisole alone or anisole with dimethylsulfide and ethanedithiol when methionine was present. The cleaved peptide and resin were then washed with peroxide-free ethyl ether, and the peptide was extracted from the resin with 15% acetic acid. If methionine was present, 2% β-mercaptoethanol was included. After an additional ethyl ether extraction, the peptides were lyophilized. Preparative HF cleavages were performed commercially (Immuno-Dynamics, Inc., La Jolla, Calif.).

To eliminate the possibility of false-negative solid-phase ELISA results due to incorrect assembly of synthetic peptides or failure to completely remove side chain blocking groups used during the synthesis of the peptides, stringent assessment of the structural status of each peptide was performed by fast atom bombardment mass spectrometry (FABMS) on the final HF-derived product (3). For SP15, structural assessment was performed stepwise by FABMS on analyte peptide released by micro-trifluoromethanesulfonic acid (TFMSA) cleavage from selected peptide-resin aliquots automatically removed after each coupling cycle, using previously detailed methods (28, 29).

RESULTS

MAb binding to synthetic peptides. We had previously shown that plasmids p9 ($RecA_{1-35}$-GIGDLGSP-$E1_{162}$-$E1_{332}$-DP-$LacZ_{9-1015}$) and pARV02-01 ($RecA_{1-35}$-GIGDLGSP-$E1_{202}$-$E1_{283}$-GDP-$LacZ_{9-1015}$) express trihybrid proteins in *Escherichia coli* that should contain an N-terminal 43-amino-acid segment of the bacterial protein RecA fused to either 171 or 82 residues of the mid-portion of the RV E1 glycoprotein backbone followed in short order by a minimally truncated but enzymatically active β-galactosidase (LacZ) gene product (51). Proteins harvested from bacteria expressing these plasmids were shown to contain the epitopes defined by E1-18 and E1-20 when probed by these MAbs in both ELISA and Western immunoblot assays (49, 51). To better delineate the domain containing these MAbs, we fabricated a set of five overlapping peptides to span the common RV sequence expressed by the two plasmids.

The synthetic peptides in this set represent the amino acid residues deduced from the nucleotide sequence derived from the Therien strain of RV (9). These peptides include amino acids $E1_{202}$ to $E1_{225}$ (SP11), $E1_{208}$ to $E1_{239}$ (SP15), $E1_{226}$ to $E1_{247}$ (SP12), $E1_{233}$ to $E1_{256}$ (SP7), and $E1_{264}$ to $E1_{283}$ (SP13) and are shown in Table 1 along with the five other peptides from a region of the RV capsid protein that were used as control reagents in this study. When this overlapped set of synthetic peptides was used as target antigens in the solid-phase ELISA, only SP15 proved to be an adequate ligand for E1-18 or E1-20 binding (Fig. 1). Both of these MAbs showed excellent binding to SP15 when the peptides were coated on the 96-well polycarbonate ELISA plates at peptide concentrations of 4 to 40 pM per well (Fig. 1). None of the other anti-RV MAbs from our library of E1, E2, and C-specific reagents bound to SP15 or the other overlapped synthetic peptides of this region of E1 (data not shown).

To better define the limits of the protein sequence required to bind these MAbs, the micro-TFMSA cleavage method

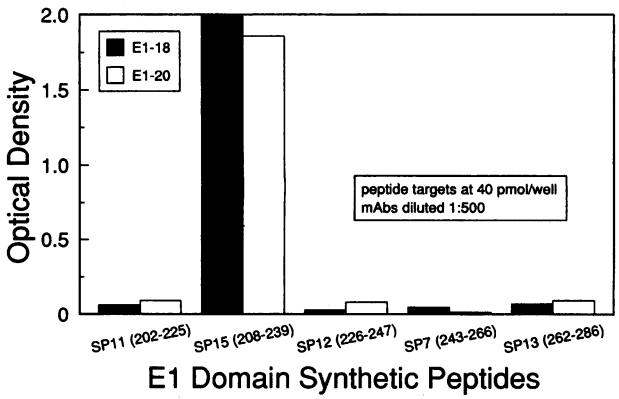

FIG. 1. Specificity of MAbs E1-18 and E1-20 for binding to the synthetic peptides. Peptides were allowed to adsorb overnight at 4°C to individual wells of 96-well microtiter plates diluted at a concentration of 40 pmol per well in bicarbonate buffer. MAb-containing ascites fluids diluted 5×10^{-2} were then added, and the reaction was carried out under otherwise standard ELISA conditions.

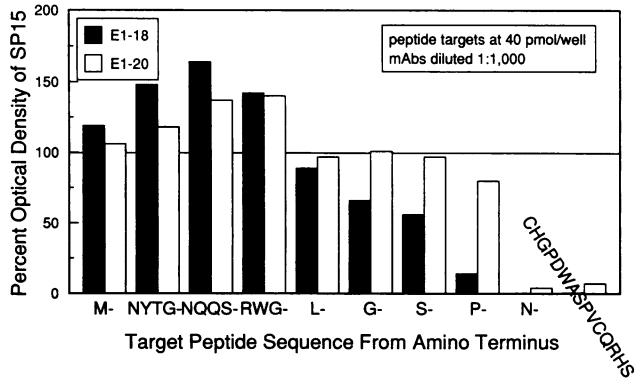

FIG. 2. Minimal amino-terminal residue requirements for binding MAbs E1-18 and E1-20 to SP15. Micro-TFMSA cleavage products of the stepwise synthesis of SP15 were bound to the wells of microtiter plates at a concentration of 40 pmol per well. In this representative experiment, the MAb was diluted 10^{-3} and then added to an otherwise standard ELISA reaction. Data were normalized to the percent of maximal binding (as measured by optical density) of the MAb to the micro-TFMSA cleavage product divided by the binding observed on the same microtiter plate of that MAb to the full-length SP15 molecule following micro-TFMSA cleavage. The monitored peptide synthesis is represented by the single-letter amino acid code from the far right of the x axis beginning with the peptide $E1_{225}$–$E1_{239}$ and extended at its amino terminus in variable steps to the full-length SP15.

was applied to 1- to 10-mg amounts of each of the assembled peptide-resin derivatives available from the stepwise synthesis of SP15. In this fabrication of SP15, care was taken to use mesitylene-sulfonyl-Arg in the synthesis since the blocking group used to protect the side chain of tosyl-Arg is incompletely removed by micro-TFMSA chemical cleavage (29). As seen in Fig. 2, the stepwise addition of amino acids to the amino terminus of the growing peptide chain was associated with the binding of E1-20 from and beyond the addition of the proline at $E1_{223}$, as determined in the solid-phase ELISA. E1-18 required the addition of several more amino-terminal residues to the peptide chain before binding comparable to that seen with the intact SP15 occurred (Fig. 2).

To further confirm these findings, a truncated form of SP15, SP27, was fabricated (Table 1). When first synthesized and studied following deprotection and release from the Pam-copoly(styrene–1% divinylbenzene) resin with the micro-TFMSA cleavage method, the new peptide bound only E1-20 well, and then only when SP27 was applied to the wells of the microtiter plates at very high concentrations. Analysis of the peptide by FABMS showed that it was composed of a mixture of species including the desired product, as well as molecules with protonated masses consistent with the presence of unremoved tosyl groups on the arginine at $E1_{237}$ and methylbenzyl groups on the cysteines at $E1_{225}$ and/or $E1_{235}$. The desired molecule was purified by high-pressure liquid chromatography and restudied. It was able to serve as an adequate ligand for binding both E1-18 and E1-20 when used at low concentrations in the solid-phase ELISA (data not shown).

Immunogenicity of SP15 in mice and rabbits. To determine whether the synthetic peptides of the $E1_{202}$–$E1_{283}$ domain could induce a polyvalent antibody response, BALB/c mice were immunized with synthetic peptide emulsified first in complete Freund's adjuvant and then two or more times in incomplete Freund's adjuvant. The synthetic peptides were not otherwise chemically modified or coupled to carrier molecules for their use in the immunization protocols. Immunization with SP11 induced a brisk antibody response, with antibody measurable in the ELISA that reacted well with both SP11 and SP15, to a limited extent with SP12, SP13, and RV, but not with SP7 as target antigens (Fig. 3a). Immunization with SP12 produced a delayed but strong response to SP12 and SP15, with little measurable response to the other peptides or RV (Fig. 3b). In contrast, immunization including three booster injections of mice with SP7 produced no detectable humoral immune response in any of seven animals, as measured in the solid-phase peptide-specific ELISA (data not shown). An identical immunization schedule with SP13 produced a delayed response which was restricted to SP13, seen only after the second booster injection and then found in only three of six mice even after a third challenge (data not shown). Most notably, immunization with SP15 induced a humoral immune response that was somewhat delayed compared with that seen with SP11, but one that became more broadly reactive to include measurable responses to SP15, SP11, and SP12 and to RV antigens in the solid-phase ELISA (Fig. 3c).

The delayed development of a measurable antibody titer to SP15 until after the second immunization (first booster injection with SP15 in incomplete Freund's adjuvant) was consistently observed in three independent immunization series. None of 19 mice immunized with SP15 showed an immune response after primary immunization, 15 of 19 developed a readily measurable response at a serum dilution of 10^{-3} to the peptide after the second immunization, and all had measurable antibody after the second challenge with SP15. Attempts to develop a stable hybridoma cell line from spleen cells of SP15-immunized mice have not yet been successful. However, we have observed hybridoma cell lines which transiently produce antibody with characteristics similar to those of E1-18 and E1-20 (data not shown).

To obtain sufficient quantities of sera for functional testing and to determine whether SP15 could induce an immune response in a species other than the mouse, rabbits were immunized with either SP15 or SP18, a peptide of the capsid protein (C_{64} to C_{89}). SP18 was selected as a control for this series of studies since it is similar in size to SP15. Further, like SP15, SP18 induces a delayed humoral immune response in BALB/c mice (data not shown). Finally, since SP18 represents a subsequence from the RV capsid (Table 1), it should not induce antibodies with functional activity in either the neutralization or hemagglutination inhibition assay.

The immunization protocol and reactivity profiles of the anti-SP15 rabbit sera to a panel of peptide antigens and RV are shown in Fig. 4. Sera from the SP15-immunized rabbit showed a rising titer to SP15 in the assay, with a broadening pattern of reactivity over time that encompassed SP11, SP12, and RV but not SP7 or SP13. No significant response to the peptides of the C domain could be found by ELISA in sera from the SP15-immunized rabbit. The cross-reactivity of the rabbit anti-SP15 sera with SP11 and SP12 is readily explained by the considerable shared sequences between these molecules; SP11 has 18 amino acids common to SP15, and SP12 shares 14 residues with SP15 (Table 1). A similar though more delayed response was seen in the SP18-immunized rabbit (data not shown). The antipeptide antibody titer was always highest to the immunizing peptide. No significant response to any of the peptides of the E1 domain could be found by ELISA in sera from the SP18-immunized rabbit. The pattern of cross-reactivity of the rabbit anti-SP18 sera

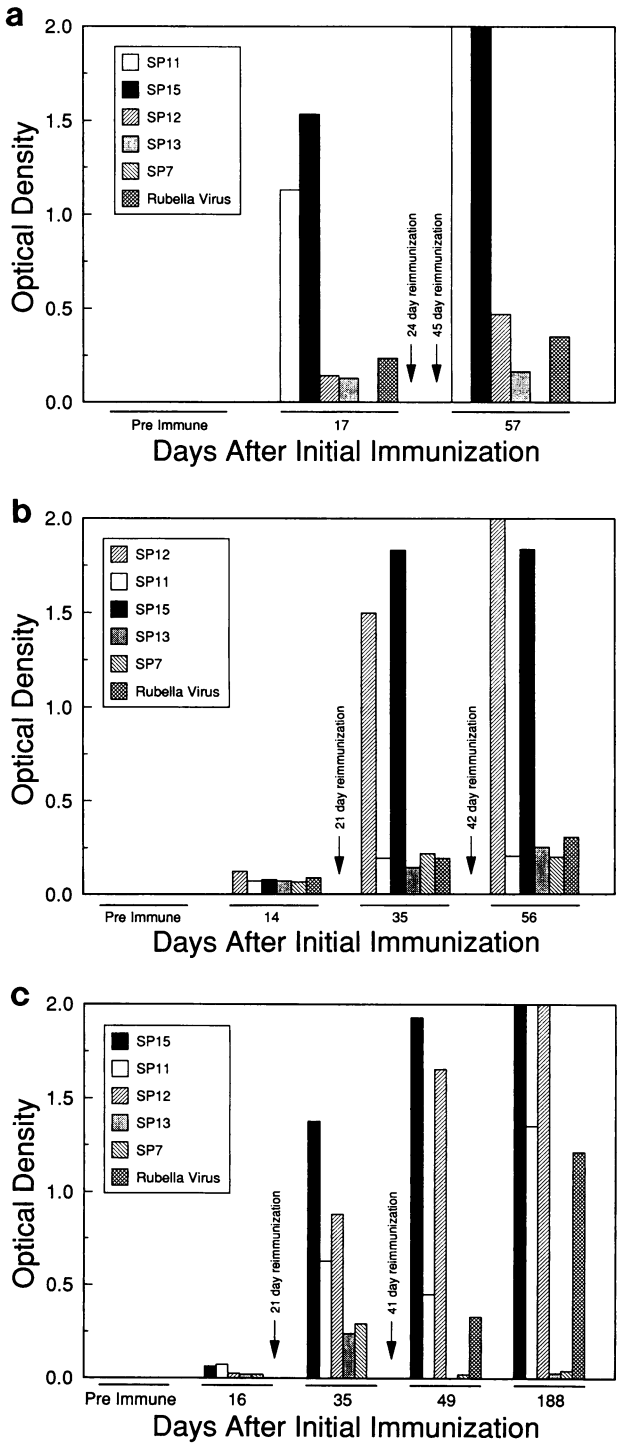

FIG. 3. Immune response of BALB/c mice following immunization with SP11 (a), SP12 (b), and SP15 (c). Mice were immunized with 100 μg of SP11, SP12, or SP15 in complete or incomplete Freund's adjuvant at the times indicated and bled by retro-orbital puncture, and the sera were analyzed for antibody against the synthetic peptides or RV by ELISA. Individual sera were diluted 10^{-3}, and peptide targets were used at a concentration of 40 pmol per well. Each datum point reflects the mean optical density of sera obtained from each of four to nine mice sampled at the indicated times.

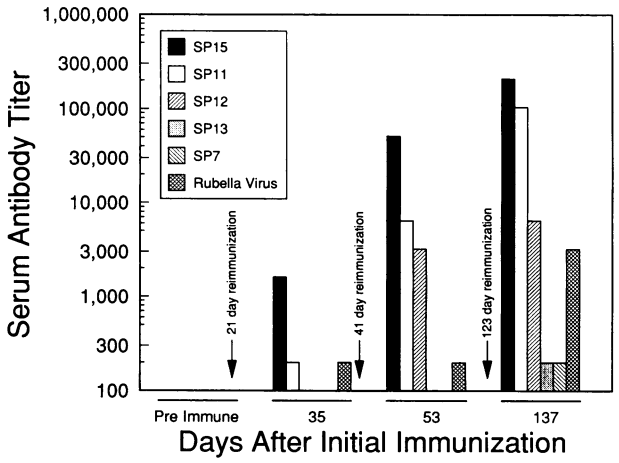

FIG. 4. Rabbit immune response following immunization with SP15. The rabbit was immunized with 1 mg of SP15 in complete or incomplete Freund's adjuvant at the times indicated and bled by ear vein puncture, and the sera were analyzed for antibody against the synthetic peptides or RV by ELISA. Sera were evaluated at an initial concentration of 10^{-2} and then serially diluted 1:1 to determine their end dilution titer to each antigen. Peptide targets were used at a concentration of 40 pmol per well.

among overlapped capsid peptides SP18 and SP10 but limited, if any, cross-reactivity to SP9, SP17, and RV suggest that the polyvalent response in this serum is directed primarily at residues at the amino-terminal portion of this C domain (Table 1) and that this antigenic site is not very accessible on RV under the conditions of the ELISA.

Functional properties of selected murine monoclonal and the rabbit polyvalent antibodies. MAbs E1-18 and E1-20 were tested in parallel with the two hyperimmune rabbit antipeptide antisera (anti-SP15 and anti-SP18) for functional activity in neutralization and hemagglutination inhibition assays. MAb C-1 and preimmune rabbit serum served as additional control reagents in these studies. As seen in Fig. 5, ascites fluids containing MAbs E1-18 and E1-20 produced an estimated 50% reduction of RV infectivity at dilutions of 5,000 and 8,000/ml, respectively. MAb C-1 failed to significantly inhibit virus infectivity at the dilutions tested. The rabbit anti-SP15 serum had an estimated 50% neutralization titer of 300 U/ml. Comparable neutralizing activity of the preimmune rabbit serum was estimated at ≤10 U/ml. The anti-SP18 serum failed to show neutralizing activity at the dilutions tested.

We previously reported that MAbs E1-20 and E1-18 had little or no activity in the hemagglutination inhibition assay (51). However, when reanalyzed in this study, the MAb-containing ascites fluids inhibited the agglutination of chicken erythrocytes at dilutions of 1:8,192 and 1:32,768, respectively. Ascites fluid containing MAb C-1 failed to inhibit hemagglutination at dilutions greater than 1:2. Pretreatment of the samples with heparin-manganese chloride did not appreciably change the results. We are uncertain as to the apparent change in the activity of MAbs E1-18 and E1-20 in this assay. However, we suspect that it may reflect differences in the particular harvest of ascites fluid containing antibody used, the purity of current RV antigen preparations, or more likely the sensitivity to aggregation of the erythrocytes, which are derived from a different source than that used in previous studies. Both hyperimmune rabbit sera weakly inhibited hemagglutination in this assay at dilutions

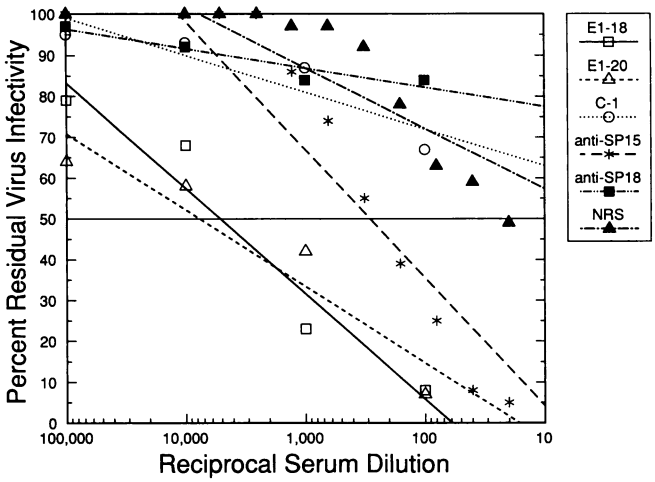

FIG. 5. Neutralization capacity of the monoclonal and polyvalent antibodies. Rabbits were bled by ear vein to provide preimmune normal serum (NRS) and then immunized with repeated injections of SP15 or SP18 as detailed in Materials and Methods. The rabbit polyvalent hyperimmune sera (anti-SP15 and anti-SP18) used in this experiment were harvested after the third booster injection as detailed in the legend to Fig. 4. The neutralization assay was performed as detailed in the text. The percent reduction in virus activity was determined by dividing the number of plaques in the antibody treated monolayers by those in untreated infected monolayers. Individual datum points are shown with symbols, and logarithmic regression lines for each sample tested are indicated. None of the control sera (preimmune normal serum, anti-SP18, or MAb C-1) showed significant inhibition of infectivity. The neutralization titers of the sera expressed as the reciprocal of the dilution that produced a 50% reduction of infectivity as estimated from the regression curves were as follows: preimmune normal serum, <10; anti-SP18, ≤10; MAb C-1, <10; anti-SP15, 300; MAb E1-18, 5,000; and MAb E1-20, 8,000.

of 1:64. Again, pretreatment of the rabbit sera had no effect on the results of this assay. We interpret these data to suggest that SP15 is not capable of inducing a measurable specific hemagglutination inhibition response in rabbits.

DISCUSSION

As often as not, rubella passes as a subclinical infection in humans with no evidence of rash or other clinical symptoms (48). Mice infected with RV also undergo subclinical infection with no discernible effects of the infection. However, the infected mice do develop cellular and humoral rubella-specific immune responses that seem to parallel those seen in humans. We therefore chose to study the repertoire of MAbs produced by the mouse as a first approximation of the fine specificity of the immune response that might be anticipated in humans. The preponderance of murine MAbs that are active in functional assays which measure neutralization and hemagglutination inhibition activity react with the E1 glycoprotein of the virus (12, 13, 17, 41, 43, 44, 51). Further, the overwhelming majority of the E1-specific MAbs that we have recovered from BALB/c mice interact with epitopes that do not withstand denaturation with sodium dodecyl sulfate and reduction of this molecule (51). MAbs E1-18 and E1-20 are exceptions in that they bind to denatured E1. The neutralizing epitope that these MAbs define would appear to be of considerable importance in that it must be exposed on native virions, and when occupied by antibody, either the epitope itself or structures in its surrounding domain are blocked from critical early interactions with the host cell such that infection cannot proceed. Alternatively, more distant changes in the E1 molecule or postulated E1-E2 heterodimer spike are induced by MAb binding to the epitope that in turn account for this important neutralization effect. Therefore, overlapping synthetic peptides, based on the sequence of the RV E1 protein segment expressed as recombinant fusion proteins that bind MAbs E1-18 and E1-20 (49, 51), were used to deduce the minimum epitope binding requirements of these antibodies.

Of the initial set of five peptides fabricated, only SP15 proved to be an adequate ligand for MAbs E1-18 and E1-20 when evaluated as targets in the solid-phase ELISA. Analysis of stepwise synthesis products of SP15 extending from the carboxyl-terminal equivalent residue of $E1_{239}$ showed that the minimum amino-terminal requirements for binding of the two MAbs differed slightly, with MAb E1-20 showing good binding following the addition of the proline at $E1_{223}$ and E1-18 requiring the extension of the peptide in the amino-terminal direction for an additional two or three residues. Independent synthesis of a truncated form of SP15, SP27, confirmed these binding requirements for E1-18 and E1-20. Thus, the neutralizing epitope(s) defined by these MAbs is bounded by $E1_{221}$ and $E1_{239}$.

Peptides usually elicit adequate antibody responses in animals. However, the antibodies produced, while capable of binding to the immunizing peptide, are not often reactive with the protein from which the peptide was derived when it is presented in the intact and native state (1, 14, 19). Further, derivatization of the peptide may be required to evoke a measurable or maximal humoral response (6). SP15 proved competent to evoke a measurable antibody response in both mice and rabbits when emulsified in complete Freund's adjuvant. More important, the antibody generated was capable of binding to both the immunizing peptide, peptides containing related but not those with unrelated RV sequences, and purified rubella virions when tested by ELISA. However, the integrity of RV when used as a target antigen under the conditions of the ELISA is uncertain. Therefore, the E1 glycoprotein presented in this manner cannot be assumed to be in its native conformation. Nonetheless, the ability of the hyperimmune rabbit anti-SP15 sera to neutralize the infectivity of RV confirmed the capacity of the peptide to induce a population of antibodies reactive with epitopes present and exposed on the surfaces of intact and functional virions.

Several investigators have reported the ability of selected synthetic peptides to induce neutralizing antibody responses to a limited number of animal viruses (6, 7, 26, 32, 34). Unfortunately, in some instances the critical sequences required for antibody induction appear to lie in regions of the virus that are quite variable between different virus isolates and strains, limiting the potential usefulness of these peptides as synthetic antigens or as candidate vaccines (8, 20, 21, 54). Sequence data available for several wild-type and vaccine strains of RV suggest that the region of RV represented by SP15 is well conserved. Identical amino acid sequences are predicted for the Therien (9, 11, 42), Judith (39), M_{33} (5), and HPV77 (53) strains, and the RA27/3 vaccine strain is reported to differ only by a histidine→tyrosine change at $E1_{210}$ (30). The latter is well outside the critical minimal epitope-containing domain of SP15.

SP15 and its truncated form, SP27, are composed of an interesting sequence with two cysteines separated by nine

intervening amino acid residues. Failure to completely remove side chain blocking groups on either cysteine of SP27 with micro-TFMSA cleavage resulted in peptides which were not adequate ligands for E1-18 and E1-20. Furthermore, replacement of either cysteine with α-aminobutyric acid, a molecule quite homologous to cysteine but unable to form disulfide bonds (10, 36, 47), abrogates binding of MAbs E1-18 and E1-20 (51). These observations and the ELISA data from the stepwise synthesis products of SP27 suggest that the amino acids involved in the epitopes recognized by these MAbs are distributed over a region of at least 13 amino acids for E1-20 and as many as 16 to 17 residues for maximal MAb binding in the case of E1-18. It is of interest that both the RV E1 and E2 glycoproteins are rich in cysteine and readily form homo- and heterodimeric species (2, 45). The formation of disulfide bonds is a common feature of the alphaviruses, to which RV is related. These may stabilize critical domains of the native molecules in such a manner that most neutralizing MAbs to E1 or E2 of the togaviruses are directed at highly conformation-restricted epitopes (34, 38, 51). The length specifications of peptides SP15 and SP27 and the requirements for two noncontiguous cysteines suggest that these peptides may express conformation-dependent epitopes (22, 37). It is also of interest that a number of other synthetic peptides that mimic antibody-defined neutralization sites contain spaced cysteine residues that may ensure the stabilization of the peptide and preserve critical conformations present in the native protein (21, 24, 40).

Several laboratories have also used peptides (4, 23, 27, 31) or proteolytic peptide fragments (18, 39) to attempt to define functional sites on the E1 glycoprotein of RV. Ho-Terry et al. (18) localized MAb-defined hemagglutination inhibition and neutralization sites to $E1_{245}$ to $E1_{285}$ and further deduced three critical regions for MAb binding ($E1_{245}$ to $E1_{251}$, $E1_{260}$ to $E1_{266}$, and $E1_{225}$ to $E1_{234}$). The first two subsequences are wholly contained within SP7, as are the putative tripeptide ($E1_{250}$ to $E1_{252}$) and tetrapeptide ($E1_{260}$ to $E1_{263}$) epitopes defined by Lozzi and colleagues (23) and their related extended peptides optimized for binding human sera (31). However, SP7 is nonimmunogenic in mice. Similarly, SP13 contains most of the residues of the latter two sequences defined by Terry et al. (39) and further studied by Neri et al. (31), but it is also nonimmunogenic in mice. Further, peptides in this region ($E1_{245}$ to $E1_{285}$) show rather poor binding of immunoglobulin fractionated from pooled human high-titer antirubella sera (31).

Chayne and coworkers (4) have recently shown that a MAb that neutralizes RV infectivity but does not block erythrocyte agglutination binds to an E1 synthetic peptide ($E1_{219}$ to $E1_{233}$) and shows better binding characteristics when additional amino acid residues are included in a related and larger synthetic molecule ($E1_{199}$ to $E1_{233}$). Reactivity of this group's MAbs with a panel of fusion proteins and deletion mutants expressing various portions of the E1 segment in vitro and in vivo suggested binding domains for two other MAbs, one with neutralization activity and one with hemagglutination inhibition activity within $E1_{214}$ to $E1_{233}$ and $E1_{214}$ to $E1_{240}$, respectively (4). Taken together, the data from the aforementioned groups and from our own laboratory support the concept of the mid-portion of the E1 glycoprotein of RV being exposed and critically involved in early steps in the infectious process. Our data, as discussed above, also suggest that cysteines in this region may be crucial in maintaining optimal configurations of E1 for such interactions. The importance of this region is further underscored by the observations of Mitchell and associates; using a synthetic peptide ($E1_{213}$ to $E1_{239}$), they were able to show strong binding of nine distinct MAbs and various human immune sera by ELISA (27).

The ability of nonderivatized SP15 to induce an immune response in BALB/c mice and New Zealand White rabbits suggests that SP15 may contain sequences necessary for providing the T-cell help required for efficient B-cell induction and antibody production in vivo. However, it does not appear that SP15 has T-cell epitopes that can be recognized by a significant proportion of human seropositive donors, as measured by their in vitro peptide-specific immunoproliferative responses (25). Whether chimeric synthetic peptides designed from sequences of SP15 and more distant regions of E1 or even RV capsid protein domains will prove to be more effective immunogens in laboratory animals than is SP15 alone is currently under investigation. Accumulated data for other viral systems (15, 16, 33, 35) suggest that such chimeric molecules might provide a prototype RV vaccine.

ACKNOWLEDGMENT

This study was supported in large part by grant AI26943 from the National Institutes of Health to J.S.W.

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