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A Second Envelope Glycoprotein Mediates Neutralization of a Pestivirus, Hog Cholera Virus

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Several monoclonal antibodies (MAbs) raised against hog cholera virus (HCV) reacted with the HCV structural glycoprotein gp44/48 and neutralized the virus. The presence of HCV gp44/48 on the viral surface was directly demonstrated by immunogold electron microscopy. Eight anti-HCV gp44/48 MAbs were tested by immunoperoxidase assay against a panel of pestivirus strains. Each MAb showed a distinct pattern of reactivity with HCV strains. It is suggested that the MAbs are well suited for epidemiological investigations of HCV outbreaks.

Members of the genus *Pestivirus* are causative agents of animal diseases that occur worldwide, namely, hog cholera, bovine viral diarrhea, and border disease of sheep (12). Molecular characterization of two members of the genus, bovine viral diarrhea virus (BVDV) and hog cholera virus (HCV), demonstrated that the pestivirus genome is represented by a single-stranded RNA of about 12.5 kb which is of positive polarity and comprises a single large open reading frame (ORF) (2, 3, 15, 17). For HCV, this ORF is translated into a hypothetical polyprotein of 3,898 amino acids. The mature proteins are released from this polyprotein by virus-encoded and host cell proteases.

The 5′-terminal part of the HCV ORF encodes one nonstructural protein (the HCV autoprotease p23) and four structural proteins (the nucleocapsid protein p14 and three glycoproteins, gp44/48, gp33, and gp55). HCV gp44/48 and gp55 each form disulfide-linked homodimers, while gp55 also dimerizes to a heterodimer with the putative transmembrane glycoprotein gp33; these dimers are present in HCV-infected cells as well as in virions (22). It was previously shown that one of the pestiviral glycoproteins, HCV gp55/BVDV gp53, mediated neutralization (4, 25, 27); this feature is an indirect indication of the presence of HCV gp55/BVDV gp53 at the surface of the respective virions. We show here that the envelope glycoprotein HCV gp44/48 also induces neutralizing monoclonal antibodies (MAbs). For the first time, the localization of a pestiviral glycoprotein at the surface of virions is directly demonstrated.

MATERIALS AND METHODS

Cells and viruses. The pig lymphoma cell line $38A_1D$ was kindly provided by W. Schäfer (Max-Planck-Institut für Virusforschung, Tübingen, Germany) (21). Cells were grown in Dulbecco's modified Eagle medium with 10% fetal calf serum. Swine testis epitheloid (STE) cells were derived from a cell clone of McClurkin's ST cell line which had been received from J. B. Derbyshire (Guelph, Ontario, Canada) (13). The cells were grown as monolayers in a medium consisting of an amino acid mixture of Eagle's minimum essential medium including nonessential amino acids and Leibovitz's L-15 medium, supplemented with 10% tryptose phosphate broth and 10% horse serum. HCV Alfort was obtained from B. Liess (Veterinary School, Hannover, Germany). Additional pestivirus isolates were obtained from Central Veterinary Institute, Lelystad, Netherlands (HCV strains Brescia, Henken, and Bergen), Behringwerke AG, Marburg, Germany (HCV strain Behring), and Philips-Duvar (HCV strain Duvaxin); HCV Krefeld I 1990, Weilburg 1990, Rhön 1989, and the BVDV strain are isolates obtained from the field.

Preparation of MAbs. Preparation of anti-HCV MAbs was performed essentially as previously described (25). Spleen cells of two mice (no. 1 and no. 4) that had been immunized with HCV strain Alfort were fused with SP2/0 myeloma cells in the presence of polyethylene glycol. The fusion products of one-third of each spleen were plated into four 96-well plates and two 24-well plates. Growth of hybridoma cultures was observed in 215 (mouse no. 1) and 211 (mouse no. 4) of the wells. By indirect immunofluorescence of HCV-infected cells, 66 hybridomas secreting anti-HCV antibodies were identified.

ELISA with bacterial fusion proteins. The positions of HCV clones expressed as fusion proteins in bacteria have been described elsewhere (20). One fusion protein contains part of HCV gp44/48, whereas the second fusion protein contains part of HCV gp55. Coating of enzyme-linked immunosorbent assay (ELISA) plates and further processing of the plates were performed as described previously (25).

Infection of cells. $38A_1D$ cells were infected in suspension in a volume of 20 to 30 ml at a cell concentration of 5×10^7 cells per ml at 37°C for 90 min at a multiplicity of infection of 0.01 to 0.001. Thereafter, the cells were incubated in bottles with gentle stirring (Tecnomara, Fernwald, Switzerland).

Metabolic labeling of cells. $38A_1D$ cells at 10^7 cells per ml were labeled for 24 h with 0.5 mCi of [^{3}H]glucosamine (Amersham Buchler, Braunschweig, Germany) per ml. The labeling medium contained 20 mM fructose instead of glucose. After the labeling period, the cells were stored at −70°C. Labeling started 48 h after infection.

Preparation of virus. Supernatant from infected $38A_1D$ cells was first centrifuged at 5,400 × *g* for 20 min to remove cells, nuclei, and large cell debris. After the virions had been pelleted according to a sedimentation coefficient described for pestiviruses ($s_{20,w}$ of about 100) (16), the pellet was resuspended in a buffer suitable for Western blot (immunoblot) analysis and stored at −70°C.

Western immunoblot. Virus extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide) (11) under reducing or nonreducing conditions and transferred to nitrocellulose filters. Prestained molecular weight markers were from

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Bethesda Research Laboratories. The filters were blocked with 1% bovine serum albumin–0.05% Tween 20 dissolved in phosphate-buffered saline (PBS) for 1 h, followed by overnight incubation with undiluted tissue culture supernatant of MAbs. After several washes and incubation with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (Ig) antibodies (Dianova, Hamburg, Germany), the substrates 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (both from Fluka, Neu-Ulm, Germany) were added. The reaction was stopped with 20 mM Tris (pH 8.0)–5 mM EDTA.

Radioimmunoprecipitation and SDS-PAGE. Cell extracts were prepared for immunoprecipitation as described earlier (18). For precipitation under denaturing conditions, the infected cells were disrupted by boiling with 2% SDS (7). The extracts were incubated with 100 μl of hybridoma supernatant. Precipitates were formed with cross-linked *Staphylococcus aureus* (8), analyzed by SDS-PAGE (12% polyacrylamide) (5) under reducing conditions, and processed for fluorography by using En^{3}Hance (New England Nuclear). The dried gels were exposed to Kodak XAR5 X-ray films at −70°C. The ^{14}C-labeled molecular weight standards were from Amersham Buchler.

Immunogold electron microscopy. STE cells infected in suspension with HCV were grown as monolayers on multitest slides. At 24 h after infection, they were incubated without previous fixation sequentially with MAb 24/16, rabbit anti-mouse IgG (Dianova), and protein A-colloidal gold particles (8 nm in diameter) (26). Cell monolayers were then fixed with 2.5% glutaraldehyde and 1% osmium tetroxide, dehydrated, and in situ embedded in Araldite. Ultrathin sections were stained with uranyl acetate and lead citrate.

Neutralization assay. Neutralization experiments were all performed with STE cells grown in microtiter plates. HCV-infected monolayers were immunostained with the indirect immunoperoxidase assay by using a mixture of anti-HCV gp55 MAbs as the first antibodies and a goat anti-mouse IgG horseradish peroxidase conjugate (Dianova) as the second antibody. To study the neutralizing capacity of anti-HCV gp44/48 MAbs, undiluted culture supernatant and approximately 10^2 50% tissue culture infective doses ($TCID_{50}$) of the HCV strain Alfort were inoculated with STE cells in suspension. The cultures were grown for 3 days in 5% CO_2 before immunostaining. The synergism of neutralization was assayed by incubating individual MAbs and mixtures of MAbs with approximately 10^1, 10^2, 10^3, and 10^4 $TCID_{50}$ of HCV Alfort. Each MAb was tested in duplicate alone and in combination with each of the other MAbs.

Immunoperoxidase assay. STE cells were seeded simultaneously with different HCV isolates in microtiter plates at a multiplicity of infection such that all cells in a plate were uniformly infected after 3 days. After incubation for 3 or 4 days at 37°C, the supernatants were discarded. The cells were washed once with PBS diluted 1/3 in water, dried, fixed with a mixture of acetone and methanol for 10 min, and dried again. Each well then received 50 μl of a 1:10 dilution of different MAb hybridoma supernatants. The plates were incubated for 2 h at 37°C. After discarding of the supernatants and thorough washing with PBS plus 0.05% Tween-20, 50 μl of a rabbit anti-mouse IgG peroxidase conjugate was added, and the plates were again incubated for 1 h, followed by washing three times with PBS-Tween and once with water. Aminoethyl carbazole and H_2O_2 in sodium acetate buffer (pH 5.0) served as substrate in the staining reaction, which lasted up to 30 min. Excess substrate was removed, and 50 μl of water per well was added.

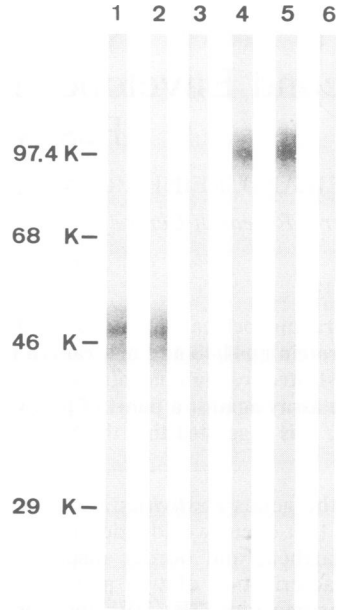

FIG. 1. Western blot analysis with MAbs against HCV gp44/48. Pelleted virions were separated by SDS-PAGE (10% polyacrylamide) under reducing (lanes 1, 2, and 3) and nonreducing (lanes 4, 5, and 6) conditions, transferred to nitrocellulose, and incubated with anti-HCV MAb 24/16 (lanes 1 and 4), anti-HCV MAb 1d43 (lanes 2 and 5), and control MAb anti-foot-and-mouth disease virus (lanes 3 and 6). K, kilodaltons.

RESULTS

Preparation and characterization of MAbs against HCV gp44/48. After fusion of spleen cells from immunized mice with myeloma cells, the first screening was performed by immunofluorescence on noninfected and HCV-infected STE cells; 66 positive hybridomas which secreted antibodies reacting only with HCV-infected cells were identified. In order to identify the proteins recognized by the hybridoma supernatants, they were first analyzed in an ELISA for reactivity with different bacterial fusion proteins encompassing parts of HCV-encoded structural proteins (20). Several of the hybridomas reacted with the HCV gp55-derived fusion protein, but only two hybridomas recognized the HCV gp44/48-derived polypeptide. It was assumed that the latter represented antibodies able to recognize denatured gp44/48. These two hybridoma supernatants were tested in a Western blot. Pelleted virions were separated under reducing and nonreducing conditions (Fig. 1); both conditions were applied because HCV gp44/48 is found in virions in a monomeric and a disulfide-linked homodimeric form (size, about 100 kDa) (22). The two hybridoma supernatants recognized the HCV gp44/48 monomer (Fig. 1, lanes 1 and 2) as well as the disulfide-linked gp44/48 homodimer (Fig. 1, lanes 4 and 5).

In a previous article, preparation and characterization of MAbs against HCV gp55 have been described (25). Interestingly, the immunofluorescence with all of the anti-HCV gp55 MAbs was considerably stronger and less diffuse than that observed with the two anti-HCV gp44/48 MAbs (Fig. 2). We assumed that the kind of fluorescence may be specific for either glycoprotein. According to this criterion, additional MAbs were isolated, representing promising candidates for recognition of HCV gp44/48. In order to identify the anti-

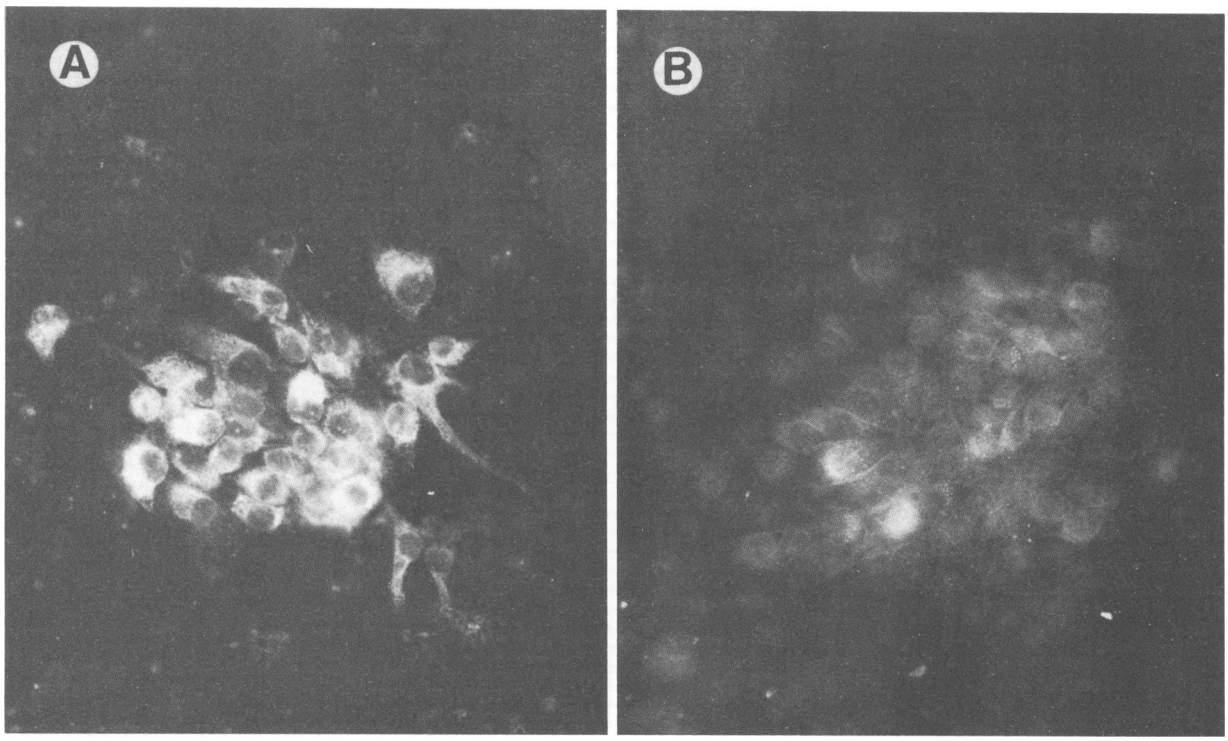

FIG. 2. Immunofluorescence patterns with HCV-infected cells. HCV-infected STE cells were incubated after fixation with anti-HCV gp55 MAb f48 (A) or anti-HCV gp44/48 MAb 24/16 (B).

gen(s) reactive with these MAbs, immunoprecipitation experiments with extracts from metabolically labeled HCV-infected cells were performed. To differentiate MAbs that recognize native and/or denatured protein(s), different precipitation conditions were applied. Figure 3 shows the separation of immunoprecipitated proteins obtained under native (Fig. 3A) and denaturing (Fig. 3B) conditions. All eight MAbs recognized native HCV gp44/48, including the two MAbs reactive with denatured gp44/48 in the Western blot analysis. It was expected that the latter two MAbs should also precipitate HCV gp44/48 under denaturing conditions. Surprisingly, only one of these two MAbs was able to precipitate HCV gp44/48 under denaturing conditions.

The data obtained by immunoprecipitation show that distinct patterns of immunofluorescence could be used reliably to identify MAbs against HCV gp44/48. By the different approaches outlined above, a panel of 15 anti-HCV gp44/48 antibody-producing hybridomas was identified; 8 of these hybridomas were subjected to cloning and further analysis.

Demonstration of HCV gp44/48 on the surface of virions. Virions from HCV are composed of four structural proteins—the nucleocapsid protein p14 and three envelope glycoproteins, HCV gp44/48, gp33, and gp55 (22). HCV gp55 is expected to be located on the surface of virions, because neutralizing MAbs are directed against this glycoprotein. It has been postulated that gp33 represents a transmembrane glycoprotein to which the envelope glycoprotein gp55 is covalently linked by disulfide bridges (22, 25). Except for its presence in preparations from complete virions (22), nothing is known about the localization of HCV gp44/48.

In order to investigate the localization, immunogold electron microscopy studies with anti-gp44/48 MAb and HCV-infected cells were performed. As shown in Fig. 4, extracellular viral particles were immunostained by the applied anti-gp44/48 MAb, directly demonstrating the presence of gp44/48 on the surface of virions. HCV gp44/48 is therefore not only part of the viral envelope but also accessible on the surface of HCV particles.

Neutralization of HCV by anti-HCV gp44/48 MAbs. Pseudorabies virus recombinants that expressed HCV gp55 have been shown to induce a protective immune response in pigs (23). Experiments with vaccinia virus/HCV recombinants indicated, however, that a protein(s) in addition to HCV gp55 is involved in protection of the natural host (19). The localization of HCV gp44/48 at the viral surface makes this glycoprotein another promising candidate to be involved in the induction of a protective immune response.

One obvious question is whether MAbs against HCV gp44/48 can neutralize the virus. With undiluted cell culture supernatant, seven of eight anti-HCV gp44/48 MAbs led to detectable neutralization of the HCV strain used (Fig. 5A). Neutralization observed with an anti-HCV gp55 MAb was, however, much stronger (Fig. 5, control). In order to investigate whether neutralization via gp44/48 could be enhanced, different combinations of MAbs were applied. Incubation of certain pairs of anti-HCV gp44/48 MAbs with 10^2 $TCID_{50}$ of HCV Alfort led to complete prevention of HCV multiplication (Fig. 5B). In contrast to the individual MAbs, the paired MAbs were able to partially neutralize 10^4 $TCID_{50}$ of HCV Alfort. Use of all possible 27 pairs showed that anti-HCV gp44/48 MAbs (Fig. 5A, a to g) act synergistically in certain combinations. The neutralizing effect of these pairs was detectable at a dilution of the hybridoma supernatants of 1:8 to 1:16 (data not shown).

Variability of HCV gp44/48. Comparison of the deduced amino acid sequences of HCV gp55/BVDV gp53 indicated

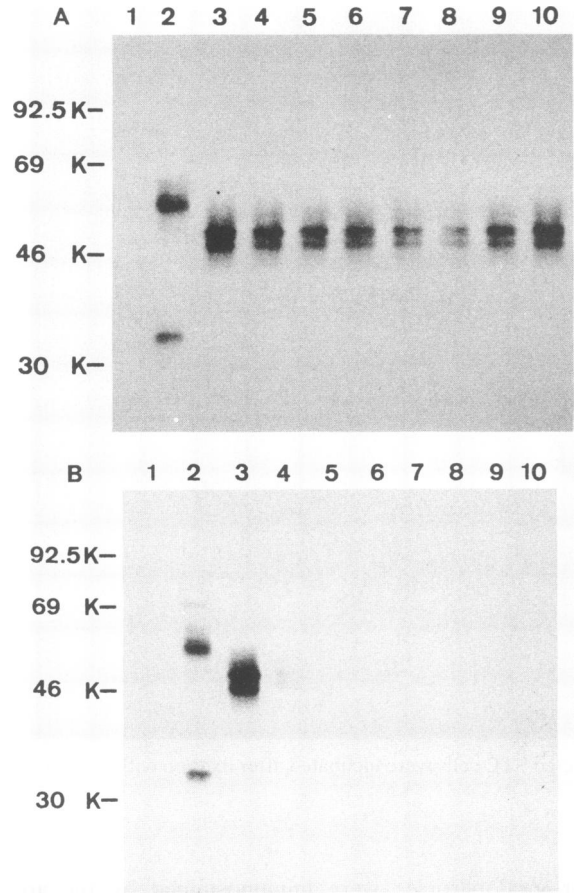

FIG. 3. Immunoprecipitation of HCV glycoproteins with MAbs. $38A_1D$ cells were infected with HCV and metabolically labeled with [^{3}H]glucosamine. Extracts from cells (under native [A] and denaturing [B] conditions) were incubated with control MAb anti-foot-and-mouth disease virus (lane 1), anti-HCV gp55 MAb a18 (lane 2), and anti-HCV gp44/48 MAbs 24/16 (lane 3), 1B5 (lane 4), 1B11 (lane 5), 1c6 (lane 6), 1d43 (lane 7), 4d20 (lane 8), 1d29 (lane 9), and 4b6 (lane 10). The precipitates were separated under reducing conditions by SDS-PAGE (12% polyacrylamide). K, kilodaltons.

that this structural glycoprotein shows a higher variability than HCV gp44/48/BVDV gp48 or HCV gp33/BVDV gp25 (25). It therefore seemed appropriate to use the respective MAbs (against HCV gp55 and BVDV gp53, respectively) to discriminate HCV and BVDV as well as pestivirus strains. Such investigations are important for diagnostic and epidemiological studies. While BVDV and HCV are easily distinguishable by this approach, the identification of HCV strains was less satisfactory, at least with the MAbs against HCV gp55 available to us (data not shown).

To elucidate the reactivity pattern of anti-HCV gp44/48 MAbs, indirect immunoperoxidase assays were performed after infection of STE cells with different HCV strains and one BVDV isolate. Table 1 summarizes the results obtained with the eight selected anti-HCV gp44/48 MAbs. As expected, all MAbs reacted with the HCV strain Alfort, which was used for the preparation of the MAbs. Interestingly, each MAb showed a distinct pattern with the other selected HCV strains. No reactivity was observed with the selected BVDV isolate. Seven of the eight anti-HCV gp44/48 MAbs reacted with HCV Rhön 1989. In contrast, HCV strain

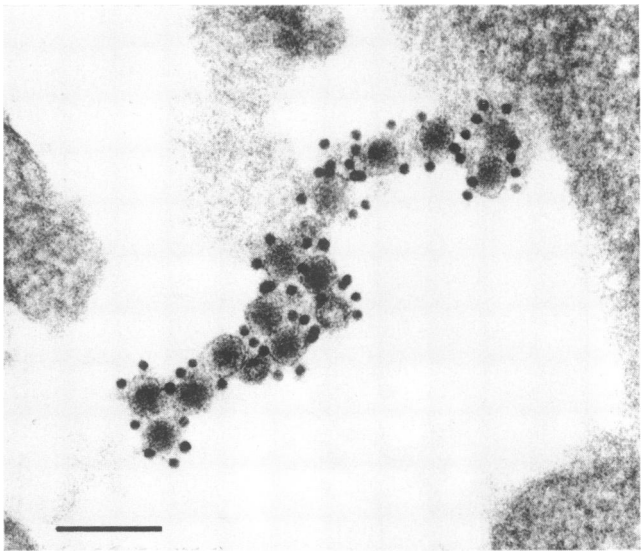

FIG. 4. Immunogold electron microscopy of HCV-infected STE cells with anti-HCV gp44/48 MAb 24/16. Bar, 100 nm.

Behring showed no binding with any of the anti-HCV gp44/48 MAbs but reacted with certain anti-HCV gp55 MAbs (data not shown). These results suggest that the eight anti-HCV gp44/48 MAbs are directed against different epitopes. We conclude from these studies that MAbs against HCV gp44/48 represent a promising diagnostic tool for the identification of HCV isolates.

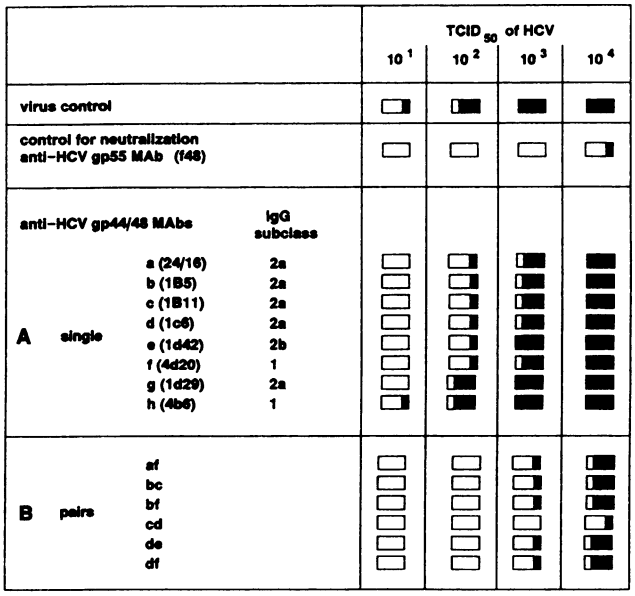

FIG. 5. Neutralization of HCV by anti-HCV gp44/48 MAbs. ■, all cells of monolayer infected (no neutralization); ◨, fewer than half of monolayer cells infected (partial neutralization); ◧, more than half of monolayer cells infected (partial neutralization); □, cells not infected (full neutralization).

TABLE 1. Reactivity pattern of anti-HCV gp44/48 MAbs against HCV and BVDV strains in immunostaining

Strain or isolate	Reactivity of MAb against HCV gp44/48[a]							
	24/16	1B5	1B11	1c6	1d43	4d20	1d29	4b6
HCV								
Alfort	+	+	+	+	+	+	+	+
Behring	−	−	−	−	−	−	−	−
Duvaxin	−	+	−	−	−	−	−	−
Brescia	+	+	−	+	−	−	−	−
Henken	+	+	−	+	−	−	−	+
331 USA	−	−	+	−	+	−	+	+
Krefeld I 1989	+	−	+	−	+	−	+	+
Bergen	+	+	−	+	+	−	+	+
Weilburg 1990	+	−	+	−	+	+	+	+
Rhön 1989	+	+	+	+	+	−	+	+
BVDV								
Unclassified isolate	−	−	−	−	−	−	−	−

[a] +, positive reaction in immunoperoxidase assay; −, no reaction in immunoperoxidase assay.

DISCUSSION

MAbs against HCV gp44/48 were obtained by using an immunization protocol which had been applied earlier for the preparation of anti-HCV gp55 MAbs (25). In order to obtain a panel of anti-HCV gp44/48 MAbs, different assays had to be applied. After identification of anti-HCV-producing hybridomas by indirect immunofluorescence, an ELISA against a bacterial fusion protein which encompassed part of the HCV gp44/48 region was employed. Two positive supernatants from hybridoma cells were isolated in this way. A Western blot analysis confirmed reactivity with HCV gp44/48, because these two hybridoma supernatants recognized monomeric as well as disulfide-linked dimeric gp44/48. The observation that immunofluorescence patterns on HCV-infected cells with previously characterized anti-HCV gp55 MAbs and these two anti-HCV gp44/48 MAbs were quite different facilitated the identification of additional anti-HCV gp44/48 MAbs. The reason for the different fluorescence patterns observed with MAbs against these two HCV envelope glycoproteins is not known. It apparently does not reflect a lower degree of avidity of the anti-HCV gp44/48 MAbs, since in immunoprecipitation experiments from cell extracts no difference among the MAbs against both glycoproteins has been found. In addition, the distribution of antibody subclasses is similar for both groups of MAbs. HCV gp44/48 is supposed to start with amino acid 268 of the predicted ORF just behind a typical signal sequence with Ala and Val in positions −1 and −3, respectively (22, 24). The C terminus has been predicted to be amino acid 485 (20). According to these assumptions, stretches of hydrophobic amino acids necessary for membrane anchoring are missing within gp44/48. A partial loss of HCV gp44/48 from the membranes of infected cells may therefore result in the specific fluorescence pattern. N-terminal protein sequencing of purified pestivirus glycoproteins HCV gp44/48 and gp33 will be performed to determine directly whether gp44/48 lacks an effective membrane anchor.

The demonstration of gp44/48 at the surface of virions and the successful neutralization experiments with anti-HCV gp44/48 MAbs indicate that this pestiviral glycoprotein is a candidate to contribute to protection in the natural host. In a previous study, we investigated the role of different proteins for induction of a protective immune response using the vaccinia virus expression system (19). The experiments showed that expression of the four structural proteins—HCV p14, gp44/48, gp33, and gp55—by a recombinant vaccinia virus induced protective immunity in pigs against a lethal dose of HCV. Interestingly, pigs immunized with a vaccinia virus/HCV recombinant lacking most of the HCV gp55 coding sequences were also protected against a lethal challenge (19). Our failure to detect neutralizing antibodies in animals immunized with the respective vaccinia virus/HCV recombinant could be explained by low titers of the induced antibodies which were not detected by a standard neutralization assay. The development of effective vaccines against hog cholera should not be restricted to the structural glycoprotein HCV gp55 (23). As a direct consequence of the data presented, protection experiments that use a vaccinia virus/HCV gp44/48 recombinant will be performed.

Antibodies can neutralize virus by acting during certain steps of a viral infection, and the binding to different sites on a viral protein can cause different mechanisms of neutralization (14). However, the mechanisms of pestivirus neutralization have yet to be defined. We do not know which step in HCV infection (e.g., binding to cell surface receptors, virus penetration, or virus uncoating) is affected by MAbs directed against either HCV gp55 or HCV gp44/48. The observation that the neutralizing activity of anti-HCV gp44/48 MAbs is quite different from that of anti-HCV gp55 MAbs probably refers to different mechanisms of neutralization. Synergistic neutralization of viruses by pairs of MAbs directed against viral glycoproteins has been described for several viruses, such as La Crosse virus (9), bovine herpesvirus 4 (6), and respiratory syncytial virus (1). Pairs of MAbs specific for a glycoprotein of La Crosse virus (a member of the California serogroup of bunyaviruses) are able to enhance neutralization of this virus. The same MAbs are also reactive with other members of the serogroup but do not increase neutralization of these viruses (10). It will be interesting to determine whether pairs of anti-HCV gp44/48 MAbs reactive with HCV strains other than Alfort are also able to neutralize these other strains.

None of the eight established anti-HCV gp44/48 MAbs detected an epitope common among different HCV strains. Determination of the amino acid sequences from gp44/48 proteins of other HCV strains will be of interest; this is especially true for the HCV strain Behring, because none of the MAbs available is reactive with this strain. BVDV gp48 of one selected BVDV isolate was not recognized by any of the anti-HCV gp44/48 MAbs, in contrast to gp53 of different BVDV strains, which was recognized by certain anti-HCV gp55 MAbs. This is surprising, because the HCV gp44/48 and BVDV gp48 sequences are more conserved than the HCV gp55 and BVDV gp53 sequences (25). Additional studies with more MAbs and pestivirus strains are needed before definitive conclusions can be drawn.

The panel of available neutralizing MAbs directed against HCV gp55 and gp44/48 will be useful for epitope mapping that uses bacterial fusion proteins and synthetic peptides (for the MAbs reactive with denatured proteins) and different vaccinia virus/HCV recombinants expressing truncated HCV glycoproteins. This panel of MAbs will also help to identify different steps in pestivirus infection which may be blocked by the neutralizing MAbs. The knowledge of neutralization epitopes and mechanisms of neutralization are of basic interest and are also important for the development of effective recombinant vaccines.

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