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Monoclonal antipeptide antibodies recognize epitopes upon VP4 and VP7 of simian rotavirus SA11 in infected MA104 cells

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Summary. To study morphogenetic events of rotavirus SA11-infected MA104 cells with strictly defined reagents we produced monoclonal antibodies against synthetic peptides from both outer capsid proteins VP4 (aa residues 228–241: QNTRNIVPVSIVSR) and VP7 (aa residues 319–326: SAAFYYRV) of simian rotavirus SA11. Two of the selected monoclonal antibodies proved to be reactive with determinants of SA11-infected MA104 rhesus monkey kidney cells, with purified SA11 as well as with the particular peptides used for immunization. The anti-VP4 antibody had a demonstrable neutralizing titer of 200 (50% focus reduction) whereas the anti-VP7 MuMAb revealed no detectable neutralizing activity. In peptide-inhibition experiments, the corresponding peptide inhibited its MuMAb whereas the noncorresponding peptide had no effect on antibody binding to intracellular viral antigen. Localization of VP7 was preceded by VP4 as shown by immunofluorescence microscopy.

Introduction

Rotaviruses have been shown to be an important cause of gastroenteritis in human infants and in young animals [9]. Detailed knowledge of the virion structure and virus receptor may provide targets for antiviral agents [13]. This may also be valid for the assembly of subviral particles and the intracellular morphogenesis of infectious particles due to interference of substances in the course of protein–protein interaction.

Because of the ubiquity of rotaviruses, members of many species come into contact with these viruses very early during the postnatal period, and their sera contain antibodies against rotaviruses. Furthermore, the extensive heterotypic reactivity among rotaviruses results in some unpredictabilities in the production of polyclonal sera which often show crossreactivity against many polypeptides of different rotavirus serotypes, sometimes interfering with an unequivocal

polypeptide determination of biologically active antigens. Therefore, hybridoma antibodies free of any contaminating animal serum antibodies (e.g., free of nonevaluated or IgG-containing horse or bovine sera, mouse ascites, etc.) elicited against defined determinants of particular viral polypeptides, e.g., determined by synthetic peptides should be a promising tool for studying antigenic determinants of rotaviruses and their subviral particles. As shown for other systems synthetic peptides have been proven to be a valuable tool in studying the repertoire and the morphology of determinants [16, 25].

Here we report on the combination of both techniques, namely the monoclonal antibody and the synthetic peptide technique. We produced monoclonal antibodies against synthetic peptides which are prospective epitopes that meet the requirements of hydrophilicity and chain flexibility on both outer capsid proteins of simian rotavirus SA11 although the peptides do not represent typical determinants according to the Kyte-Doolittle model. These epitopes have been probed previously with polyclonal antipeptide sera [27, 28]. Here we show a more rigorous approach to the problem. The antibodies were characterized, tested for their capacity to neutralize SA11 and probed topographically in SA11-infected acetone-fixed MA104 cells. The intracellular location of the studied VP4 and VP7 determinants were demonstrated at different times after infection.

Materials and methods

Peptide synthesis and induction of monoclonal antipeptide antibodies

Peptides were synthesized via the Fmoc-strategy and purified by reversed phase chromatography as previously reported [27, 28]. BALB/c mice were immunized with peptide BSA conjugate prepared by the glutaraldehyd method [27, 29] (0.1 mg of SA11 VP7 aa 319–326 and 0.6 mg of SA11 (fm) VP4 aa 228–241 respectively). The antigen was dissolved in 50 µl PBS and emulsified with 50 µl complete Freund's adjuvant and injected subcutaneously. On day 21, the procedure was repeated with incomplete Freund's adjuvant. 10 days before fusion, a final boost of glutardialdehyde-crosslinked peptide was given (0.1 mg and 0.3 mg respectively). Fusion of splenic lymphocytes with mouse myeloma cells (NSO) was performed by the standard procedure of Köhler and Milstein [10] as described previously for the preparation of virus induced monoclonal antibodies [6]. Selection of antibody secreting cell clones was done by ELISA in wells coated with peptide [29]. Briefly, 2–5 µg of the appropriate peptide OVA-conjugate were applied to each well of an ELISA-plate (Maxisorp; NUNC, Wiesbaden, Federal Republic of Germany) in 100 µl of 0.05 M CO_3^{2-} buffer (pH 9.6). All washing and dilution steps were performed with Dulbecco's phosphate buffered saline (PBS; pH 7.5) containing 0.05% Tween 20 and 0.1% OVA (Sigma, Deisenhofen, Federal Republic of Germany). Supernatants from antibody secreting cells were applied in a dilution of 1 : 5 in PBS. Enzyme-labeling was performed by a peroxidase-conjugated anti-mouse polyvalent immunoglobulin antibody (Sigma). O-phenylenediamin (Dako Diagnostika, Hamburg, Federal Republic of Germany) was used for substrate reaction.

Estimation of antibody concentration by ELISA

The concentration of antipeptide antibodies was determined in an ELISA by comparison with the isotypically identical antibody of known concentration. Microtiter wells (Dynatech, Plochingen, Federal Republic of Germany) were coated with isotype-specific antibodies

(Sigma) in a dilution of 1 : 3,000 in 0.5 M CO_3^{2-} buffer (pH 9.6). After overnight incubation at 5 °C the wells were washed 3 times with PBS-0.05% Tween 20, and 0.5% defatted milk powder. Unspecific adsorption to the plastic surface was blocked with 5% milk powder in PBS-0.05% Tween 20 for 1 h at room temperature. The monoclonal antibodies and a reference antibody with a known concentration of 1 mg/ml were successively diluted in logarithmic steps and added to the wells. Following a 1 h incubation at 37 °C unbound antibody was washed off, and a biotinylated anti-mouse antibody (Sigma) was added in a dilution of 1 : 5,000 for another hour. After a further washing step, extravidin-peroxidase conjugate (Sigma) was added in a dilution of 1 : 10,000 and incubated for one more hour. The wells were washed again and incubated with 0.04% O-phenylenediamine, 0.012% H_2O_2 in phosphate-citrate buffer (pH 5.0) for 15 min in the dark. The reaction was stopped with 2 N sulfuric acid. The absorbance of the wells was measured in an Anthos reader 2001 photometer at 492 nm. On the assumption that the reference antibody and the antipeptide antibody are of the same isotype, we estimated the concentration of the antibodies by comparing the different dilutions of the antibodies at half of their maximal ELISA-absorbance.

Microneutralization assay

Virus was activated with 200 U trypsin (from bovine pancreas, Sigma, T.8253)/ml of virus suspension for 30 min at 37 °C. Incubation of 100 µl activated virus (approximately 200 focus-forming units per 0.1 ml) with 100 µl of the appropriate antibody concentration was performed for 90 min at 37 °C. 100 µl of this suspension was mixed with 10^4 MA104 cells in 100 µl medium containing 50 U trypsin/ml and seeded into the wells of a 96 well tissue culture microtiter plate. 48 h p.i. cell monolayers were washed with PBS and fixed with ethanol for 5 min. Viral proteins were enzymatically stained. For this purpose fixed cells were overlaid with 50 µl of a 1 : 1,000 serum dilution of a rabbit anti-rotavirus hyperimmune serum in PBS for 1 h at 37 °C. Unbound antibody was washed off with PBS, and a biotinylated antirabbit antibody (Dakopatts, Glostrup, Denmark) diluted 1 : 3,000 was added for 1 h. After a further washing step, extravidin-peroxidase conjugate (Sigma) followed for another hour. Subsequent to a last washing step substrate solution was put onto the cells (10 ml 0.05 M Tris-HCl, pH 7.8; 1 ml H_2O_2 1 : 6,000 diluted in H_2O; and 200 µl of a stock solution containing 0.1 g of 3-amino-9-ethylcarbazole in 10 ml ethanol). Enzyme substrate reaction was stopped after approximately 30 min by washing with water. Eight wells were seeded with a 1 : 2 dilution of the virus-working dilution for reference cut off (approx. 50 p.f.u.). Foci were counted and the mean (n = 8) was used as the 50% reduction level. Antibody dilutions reducing the foci count to or below the cut off value were regarded to be positive.

Immunofluorescence microscopy

Indirect immunofluorescence was carried out on subconfluent monolayers of MA104 cells grown on eight-chambered tissue culture slides. Cells were infected with our plaque-purified stock of SA11 (m.o.i. = 0.1) originally obtained from T. H. Flewett and cultivated in our laboratory since 1979. At 0 h, 9 h, 12 h, and 18 h p.i. post infection cells were washed in Dulbecco's phosphate buffered saline (PBS; pH 7.5), and fixed twice in ice-cold acetone for 5 min each. The air dried slides were first treated with the mouse monoclonal antipeptide antibodies, diluted 1 : 10 in PBS for 45 min at 37 °C. After a washing step in PBS, the slides were incubated with a fluorescein-conjugated anti-mouse antibody (Sigma) diluted 1 : 100 in PBS for 30 min at 37 °C. Specimens were examined using a Leitz photomicroscope and photographed using a 400 ISO Fujichrome film.

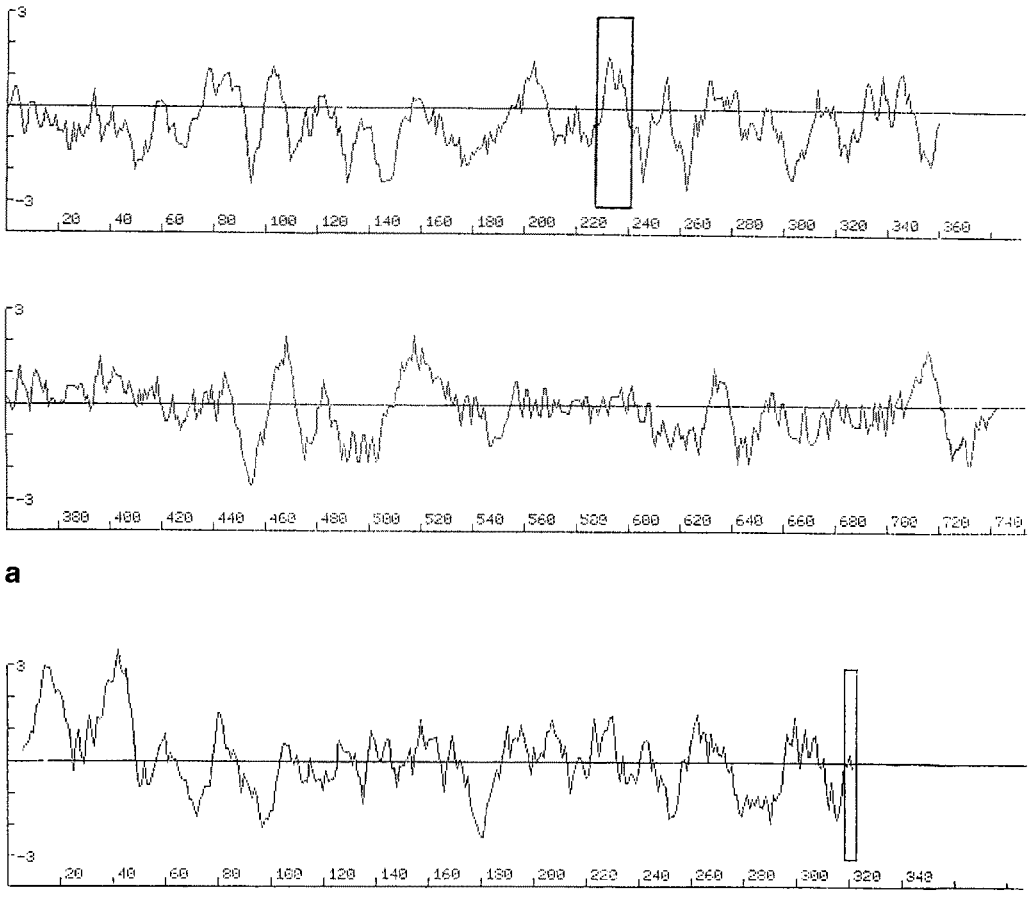

Fig. 1. Kyte-Doolittle plot of the relevant epitopes. Hydropathy plots of **a** SA11 (4 fm) VP4 (sequence see [8, 18]) and **b** VP7 [2] outer capsid polypeptides from SA11 (4 fm) (serotype 3). Windows indicate the areas of synthesized peptides. Positive values at the ordinate correspond to hydrophobicity

Results

Prediction of B cell epitopes on SA11 structural proteins VP4 and VP7

As a prerequisite for B cell epitopes accessibility for antibodies is postulated. A common model for prediction of B cell epitopes is the Kyte-Doolittle plot [11], which displays the hydropathic character of a protein. Both the carboxy terminus of VP7 and the proposed carboxy terminus of VP8*, the minor cleavage product of VP4 [14] do not meet the requirements of this model. Nevertheless, the carboxy terminus is hydrophilic by its charge and has the option of chain-end flexibility [17, 19]. The importance of proteolytic cleavage for viral infectivity has been demonstrated for rotaviruses [4, 5]. Several examples have been shown to influence viral infectivity by manipulating the cleavage site [22–24]. For these reasons the octapeptide SAAFYYRV corresponding to aa 319–326

Table 1. Epitopes of the outer capsid structural proteins of rotavirus SA11. Monoclonal antibodies directed against synthetic peptides from SA11 (4fm) VP4 and SA11 VP7

Peptide 1	SA11 (4fm) VP4 aa 228–241[a]
Amino acid sequence	QNTRNIVPVSIVSR
Monoclonal antibody	16B04
Isotype	IgM
Ig-concentration	0.16 mg/ml
Neutralizing titer	$FR_{50} = 200$
Peptide 2	SA11 (crWa) VP7 aa 319–326[b]
Amino acid sequence	SAAFYYRV
Monoclonal antibody	15C01
Isotype	IgM
Ig-concentration	0.13 mg/ml
Neutralizting titer	$FR_{50} < 50$

The synthesis of these peptides, their purification and their preparation as an immunogen to produce polyclonal antipeptide sera in rabbits have been described previously

[a] [27]

[b] [28]

crWa Crossreactive to Wa (human serotype 1)

of SA11 VP7 and the tetradecapeptide QNTRNIVPVSIVSR derived from aa 228–241 of SA11 (4 fm) VP4 were selected for synthesis. The relevant positions in the amino acid sequence are indicated in the Kyte-Doolittle plots of rotavirus SA11 VP4 and VP7 (Fig. 1).

Monoclonal antibodies directed against rotavirus SA11 induced by synthetic peptides

Both synthetic peptides corresponding to simian rotavirus SA11 (4 fm) VP4 aa 228–241 and SA11 VP7 aa 319–326 were immunogenic in mice, demonstrating the recognition of these predicted B cell epitopes. Prefusion sera of mice immunized with SA11 (4 fm) VP4 aa 228–241 revealed neutralizing antibodies with titers up to 400 regarding a 50% focus-reduction in our microneutralization assay. MuMAb of hybridoma 16B04 corresponding to the same peptide revealed weak neutralizing activity against SA11 (data summarized in Table 1).

The viral antigen is recognized in infected cells by monoclonal antipeptide antibodies

The monoclonal antibodies 16B04 and 15C01 (Table 1) proved to be helpful for the localization of the appropriate viral protein in infected cells. In an indirect immunofluorescence experiment discrete staining patterns were observed for both antibodies. In a kinetic study with 16B04 staining gradually

increased from approximately 8 h p.i. to 20 h p.i. (Fig. 2 a, c, and d). Specificity of the obtained immunofluorescence staining patterns was demonstrated in an inhibition experiment (Fig. 3). The reactivity of the appropriate antibody could be completely inhibited by preincubation of the antibody with 20 µg of the corresponding peptide-OVA-conjugate per ml of antibody fluid. Crossincubation with the same amount of the noncorresponding peptide-OVA-conjugate did not influence the reactivity of monoclonal antibodies 16B04 and 15C01, respectively. This clearly demonstrates that only the homologous peptide has the appropriate specificity for antibody binding.

Interestingly, the staining pattern observed for 15C01 followed with a certain time lag. In our experiments characteristic circular staining patterns could be monitored with 16B04 around 9 h p.i. Homologous patterns are stained with 15C01 around 12 h p.i. indicating a time lag of about 3 h. This effect is not due to the antibody concentration, because both antibodies were used in similar concentrations and are of the same isotype. Evaluation of photographs taken at 9 h p.i. and 12 h p.i. from simultaneously infected (one culture dish) and handled slides demonstrates this finding clearly.

Discussion

In this study, we show the reactivity of monoclonal antibodies elicited by immunization with synthetic peptides. The extent of reactivity and the intracellular localization of particular epitopes of rotavirus SA11 were shown in infected MA104 cells by immunofluorescence microscopy. Staining with anti-VP4 produced discrete fluorescent patterns approximately 8 h p.i. gradually increasing up to 20 h p.i. Interestingly, the reactivity of MuMAb SA11VP7-15C01 against VP7 compared with the reactivity of MuMAb SA11VP4-16B04 against VP4 appeared with a lag period of approx. 3 h. Although a cytoplasmically located protein, and, from a morphogenetic point of view, located between VP6 of the single-shelled particle and the membrane-bound VP7, VP4 seems to be located at the outermost edge of the mature virus particle from a topological point of view [21, 31]. However, little is known about the different forms of subviral and intermediate particles and the sequence of apposition of VP4 and VP7 onto the single-shelled particles. The single-shelled particle seems to be transported to or comes in close association with the endoplasmic reticulum

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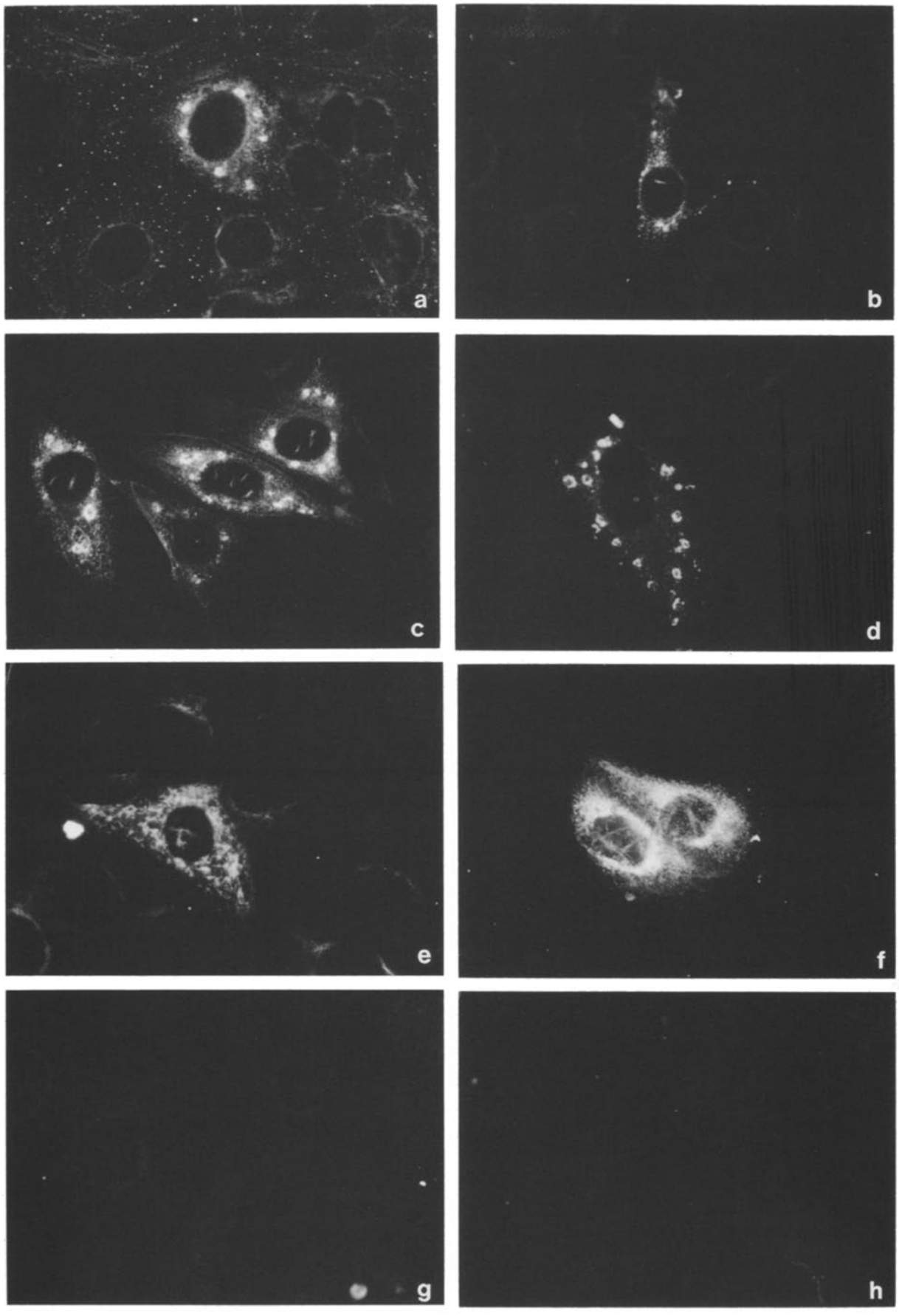
Fig. 2. Kinetic of the antipeptide antibodies in rotavirus SA11-infected MA104 cells. MA104 cells were infected with trypsin-activated simian rotavirus SA11 (m.o.i. 0.1–0.5). At 9 h (**a** and **b**), 12 h (**c** and **d**), and 18 h p.i. (**e** and **f**), cells were washed in PBS, fixed in acetone and decorated with MuMab SA11VP4-16B04 (anti-VP4; **a**, **c**, **e**, and **g**) or SA11VP7-15C01 (anti-VP7; **b**, **d**, **f**, and **h**). Cells were extensively washed and MuMabs detected with an FITC-conjugated goat-IgG directed against Igs (AMG) of the mouse. Noninfected MA104 cells were included as control (**g** and **h**)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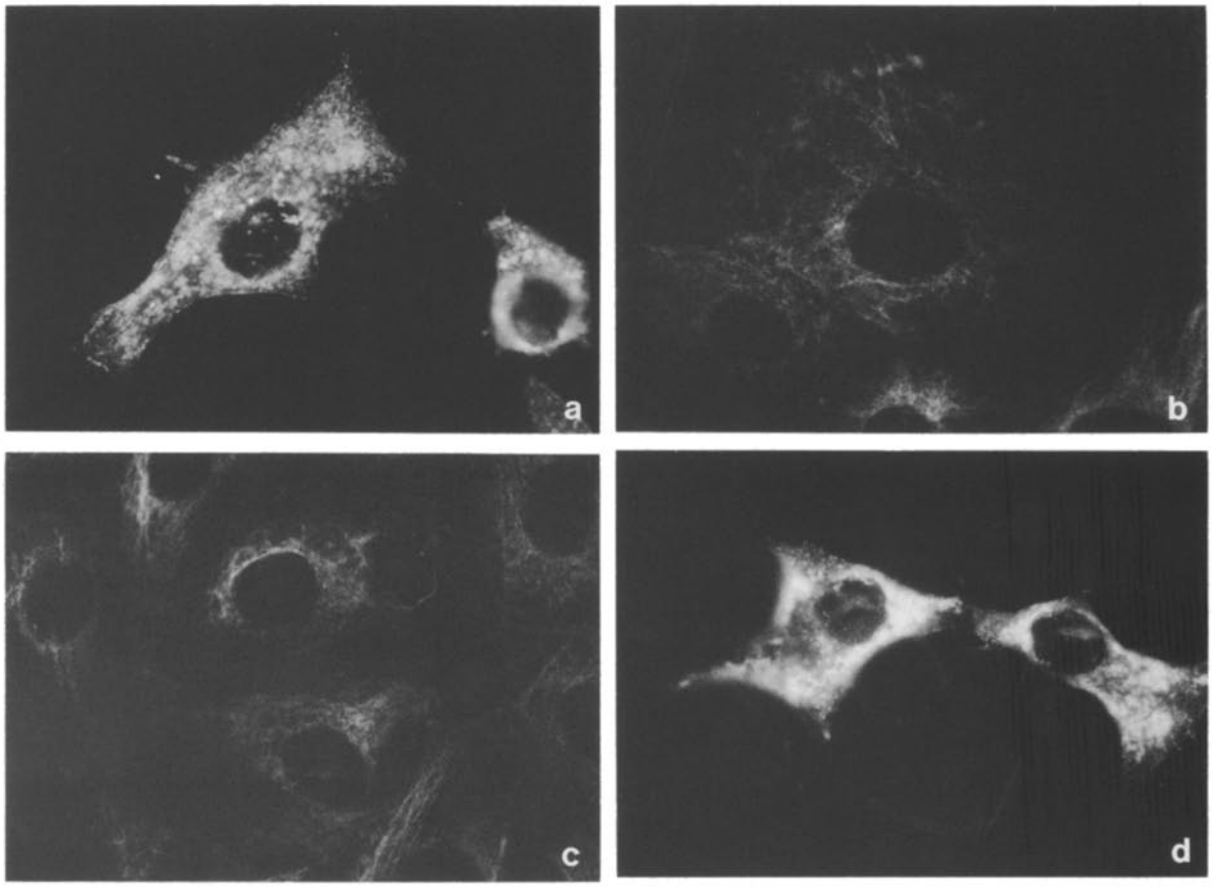

Fig. 3. Inhibition of MuMAb antirotavirus reactivity by homologous but not by heterologous peptide. Anti-VP4 (MuMAb SA11VP4-16B04; **a** and **b**) and anti-VP7 (MuMAb SA11VP7-15C01; **c** and **d**) were incubated with the synthetic peptide SA11 VP7-peptide (**a** and **c**) or SA11 (4 fm) VP4-peptide (**b** and **d**) at a peptide concentration of 20 µg/ml. Conditions of immunofluorescence microscopy are the same as in Fig. 2. The relative reduction of specific immunofluorescence shows the degree of specific inhibition by corresponding and noncorresponding peptide respectively

(RER) [26] where it triggers the endogenous budding process. The nonstructural glycosylated membrane protein NS28 seems to guide both the mature single-shelled particles and the cytoplasmic VP4 to the endoplasmic reticulum membrane [15] where NS28 and VP7 lodges [1, 3, 20, 30]. During the budding process, VP7 is added to the intermediate particle. Kabcenell et al. [7] report on a rapid assembly of the inner capsid particle and apposition of VP4 and VP7 occurring after a lag period of 15 min. This, however, is in contrast to our findings where immunostaining of VP4 precedes immunostaining of VP7 for a longer period. Whereas Kabcenell et al. come to their conclusion through pulse chase experiments and kinetic studies our data are deduced by immunological means. This discrepancy might possibly be explained by the exposure of individual determinants on intermediate particles and fully assembled virions. There-

fore, if reliable predictive measures are at hand, the chosen approach might lead to highly discriminating reagents. The drawback of contaminating antibodies even in monospecific polyclonal sera and the time consuming determination of a particular epitope for a conventionally produced monoclonal antibody could thus be avoided. This is of special interest in rotavirus research. For discontinuously composed epitopes, however, this approach will be less suitable until reliable predictive methods have been developed which display the three-dimensional structure of the antigen.

The low neutralizing activity of the anti-VP4 antibody may be a result of our chosen approach. We expected that antibodies directed against the proteolytic cleavage site of the VP4 protein might influence the infectivity of the virus. As demonstrated previously [27] this assumption was proved to be correct for the induction of polyclonal antipeptide sera in rabbits by synthetic peptide corresponding to SA11 (fm 4). The murine antipeptide sera reacted with rotavirus analogously (data not shown) [27, 28].

However, it is not clear whether the viral epitope is able to induce neutralizing antibodies in vivo in high concentrations. On the other hand binding of the peptide-induced antibodies to the infectious virus may be restricted by conformational requirements. Aware of the conceptual difficulties in defining an epitope [11], reagents produced in the above mentioned manner might be ideal for tailor-made class-, subclass-, serotype-, and even monotype-specific serological assays.

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