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Immunogenicity of Peptides Simulating a Neutralization Epitope of Transmissible Gastroenteritis Virus

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Previously, an epitope recognized by a set of neutralizing monoclonal antibodies directed against the S protein of transmissible gastroenteritis has been identified. This neutralization epitope can be simulated by a single peptide combining residues 380 to 387 and 1176 to 1184 of the S protein; this combination peptide (SFFSYGEI–QLAKDKVNE) was more antigenic than its single constituents. Here we describe the immunogenicity of this combination peptide, in comparison with monomer and tandem peptides of both constituents, and with a cyclic peptide consisting of residues 373 to 398. All antisera, raised in rabbits, bound to the peptide used as immunogen. Only sera that recognized the residues 380 to 387 bound to whole virus. Three of the four antisera with the highest binding titers to whole virus also had neutralization activity. Analysis of the fine-specificity of the antisera with PEPSCAN peptides indicated that the spectrum of antibodies induced by the 380 to 387 sequence depended on the presentation of this sequence in a peptide to the immune system. The nonbinding and nonneutralizing anti-(380 to 387)-sera appeared to contain a limited spectrum of antipeptide antibodies. Furthermore, the lack of neutralization of the antiserum against the combination peptide could be explained by the immunodominance in rabbits of the 1176 to 1184 sequence over the 380 to 387 sequence. These findings demonstrate a few fundamental problems of simulating discontinuous epitopes by single synthetic peptides.

Transmissible gastroenteritis virus (TGEV) is a member of the Coronaviridae family. It causes enteric disease in pigs of all ages with high mortality for newborn piglets (*1, 2*). Neutralizing antibodies are elicited by the spike protein S (1447 amino acids), which is located on the viral surface (*3, 4*). Mutual competition of neutralizing monoclonal antibodies (MAbs) indicated that five antigenic sites of the S protein are involved in neutralization (Refs. (*5–7*); L. Enjuanes, personal communication). The binding of MAbs specific for one of these sites (group IV) to short overlapping peptides derived from the S amino acid sequence revealed an epitope that consisted of the residues 380 to 387 with contributions of residues from the region 1176 to 1184 (*7*). The combination peptide SFFSYGEI–QLAKDKVNE, which contained the residues of both regions was a better antigen than each of its constituents. One MAb of this group IV, CVI-TGEV-57.57 (MAb 57.57), strongly binds to whole virus, has a high neutralizing activity, and recognizes peptides from the S protein residue regions 378 to 389 and 1173 to 1187 and the combination peptide (7). In this study we describe the biological properties of antipeptide sera, raised in rabbits, against the combination peptide SFFSYGEI–QLAKDKVNE, against the monomer and tandem peptides of both constituents, and against a cyclic peptide consisting of residues 373 to 398. The binding of the antipeptide sera to viral antigen was determined in an ELISA and in a virus neutralization assay (7). PEPSCAN analysis was used to determine the fine-specificity of the binding of the antipeptide sera to the peptides (7). This fine-specificity was then correlated with the ability of the antipeptide sera to bind to whole virus and to neutralize virus infectivity.

Peptide synthesis and immunization of rabbits were as described (7). Table 1 lists the sequences of the peptides, designated A, B, C, D, E, and F. A cysteine residue was added at the C terminus of the peptides B, C, D, E, and F to couple the peptide to keyhole limpet hemocyanin. Peptide A was oxidized to form cyclic monomers via a disulfide bridge between the N- and C-terminal cysteine; this peptide was used to immunize two rabbits without coupling to a carrier protein. The antipeptide sera, designated α-$A_{1,2}$, α-$B_{1,2}$, α-$C_{1,2}$, α-$D_{1,2}$, α-$E_{1,2}$, and α-$F_{1,2}$, were collected 77 days after immunization (Table 2).

The binding of the antipeptide antibodies with the peptides used for immunization was tested by an ELISA with coated peptides (Table 2) and with peptides covalently bound to a solid support; the PEP-

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TABLE 1

SEQUENCES OF THE PEPTIDES USED FOR IMMUNIZATION AND PEPSCAN

Peptide	Sequence[a]	Designation of antipeptide serum	Designation of PEPSCAN peptides[b]
A	CYTVSDSSFFSYGEIPEGVTDGPRYC	α-A	—
B	SFFSYGEI-C	α-B	b
C	SFFSYGEI-SFFSYGEI-C	α-C	c
D	QLAKDKVNE-C	α-D	d
E	QLAKDKVNE-QLAKDKVNE-C	α-E	e
F	SFFSYGEI-QLAKDKVNE-C	α-F	f
—	QLAKDKVNE-SFFSYGEI	—	f′

[a] -C is a nonsequential cysteine residue at the C terminus of the peptides, used to couple the peptides to keyhole limpet hemocyanin via the coupling agent *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester.

[b] The PEPSCAN peptides were synthesized without an additional cysteine residue.

SCAN analysis (Fig. 1). Antibodies raised against peptides A, B, and C bound to peptides that contained the SFFSYGEI sequence (peptides A, B, C, b, c, F, and f′) and not to peptides that contained only the QLAKDKVNE sequence (peptides D, E, d, and e). Conversely, antibodies raised against peptides D and E bound to peptides D, E, F, d, e, f, and f′ and not to peptides A, B, C, b, and c. In the peptide ELISA all antisera had similar titers with the peptides used as immunogen. However, with PEPSCAN peptides the α-$D_{1,2}$ sera and α-$E_{1,2}$ sera have been diluted 10 times more than the α-$A_{1,2}$, α-$B_{1,2}$, α-$C_{1,2}$, and α-$F_{1,2}$ sera to obtain an equal degree of binding. Furthermore, the α-$F_{1,2}$ sera against the combination peptide bound better to the peptides D, E, d, and e than to B, C, b, and c (Table 2; Fig. 1). Apparently, the QLAKDKVNE sequence alone or in combination with the SFFSYGEI sequence is in rabbits more immunogenic than the SFFSYGEI sequence. Changing the order of the constituents of the combination peptide SFFSYGEI-QLAKDKVNE (f) into the peptide QLAKDKVNE-SFFSYGEI (f′) did not significantly influence the binding pattern of the antipeptide sera (Fig. 1). Essentially the same results were obtained when the constituents of the tandem and combination peptides were separated by a spacer of one, two, or three glycine residues (data not shown).

TABLE 2

ANTIVIRAL AND ANTIPEPTIDE TITERS OF 12 ANTIPEPTIDE SERA[a]

Antipeptide serum	Virus ELISA[b]	Virus neutralization[c]	Peptide ELISA[d] A	B	C	D	E	F
α-A_1	4.3	2.4	4.4	3.6	3.6	<	<	3.7
α-A_2	3.7	1.2	3.8	3.0	3.1	<	<	3.2
α-B_1	4.2	<	3.9	5.1	5.0	<	<	4.2
α-B_2	3.3	<	3.8	4.9	4.8	<	<	3.8
α-C_1	<	<	3.4	4.5	4.6	<	<	3.4
α-C_2	4.1	2.6	4.3	5.0	5.0	<	<	4.3
α-D_1	<	<	<	<	<	4.9	4.9	5.0
α-D_2	<	<	<	<	<	4.3	4.6	4.4
α-E_1	<	<	<	<	<	4.2	4.3	4.1
α-E_2	<	<	<	<	<	4.1	4.3	4.3
α-F_1	2.3	<	3.1	2.9	2.5	3.7	3.8	4.0
α-F_2	2.9	<	3.5	3.5	3.3	4.3	4.4	4.4
MAb 57.57	6.8	3.7	6.5	6.2	6.2	4.8	4.6	6.4

[a] Titers are expressed as the $-\log_{10}$ of the serum dilution. The antipeptide sera were collected 77 days after immunization. Each peptide (see Table 1) was used to immunize two rabbits.

[b] <, virus ELISA titer is ≤2.0.

[c] <, virus neutralization titer ≤0.9. The neutralization was assayed in a microdilution system with SK6 cells, the Purdue strain of TGEV, and serial dilutions of the antisera starting at a dilution of 1:8 (7).

[d] Peptides A, B, C, D, E, and F were coated onto the wall of a well of a microtiter plate (1 μg). The binding of the antipeptide sera were tested in an ELISA, starting at a serial dilution of $1:10^3$ (7). <, peptide ELISA titer is ≤2.0.

The binding and neutralization properties of the antipeptide sera to whole TGEV are shown in Table 2. Only antisera that recognized the peptides A, B, C, and F (sera α-$A_{1,2}$, α-$B_{1,2}$, α-C_2, and α-$F_{1,2}$) showed binding to the virus. The α-C_1 serum is exceptional because it bound to peptides A, B, C, and F but not to whole virus. The highest titers of binding to whole virus were observed with the α-A_1, α-A_2, α-B_1, and α-C_2 sera. Three of these sera (α-A_1, α-A_2, and α-C_2) had neutralization activities that correlated with their binding titers to whole virus. The α-$D_{1,2}$ and α-$E_{1,2}$ sera did not recognize whole virus nor did they have any neutralization activity. Therefore, we conclude that only antibodies specific for the S protein residues 380 to 387 (SFFSYGEI) bind to virus and may have neutralization activity. This is in agreement with the observation that antipep-

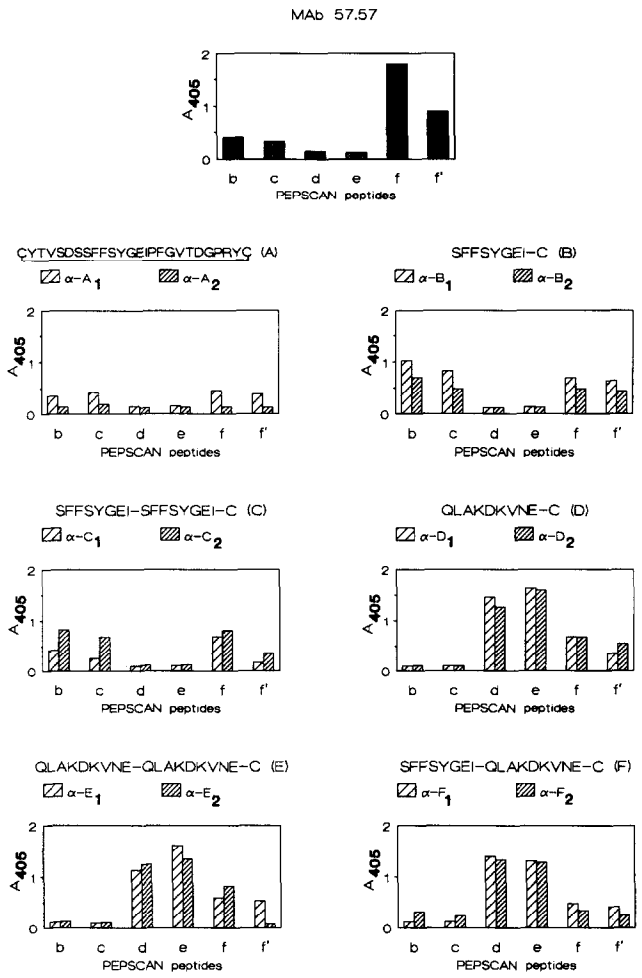

FIG. 1. The sequence of the peptides b, c, d, e, f, and f′ are given in Table 1. The first plot shows the binding of MAb CVI-TGEV-57.57, diluted $1:10^6$. On top of each next plot the peptides used as immunogen and the antisera from two rabbits are indicated. The antibody binding, measured as the extinction at 405 nm by an ELISA (A_{405}), is plotted vertically. The antipeptide sera α-$A_{1,2}$, α-$B_{1,2}$, α-$C_{1,2}$, and α-$F_{1,2}$ were diluted $1:10^2$ and the sera α-$D_{1,2}$ and α-$E_{1,2}$ were diluted $1:10^3$.

tide sera against the residues 377 to 391 had neutralization activity while antipeptide sera against residues 1171 to 1185 did not bind to whole virus and had no neutralization activity (*7*).

To explain most of the biological properties of the antipeptide sera α-A to α-F, we determined their fine-specificities using PEPSCAN peptides. Overlapping peptides that vary in length from three to nine amino acids derived from the regions 372 to 389 and 1166 to 1201 (Fig. 2) were synthesized on polyethylene rods and tested as described previously (*7, 8*). Each first peptide of length N consisted of the first N amino acids of the specified regions. Subsequent peptides contained the last N − 1 amino acids of the preceding peptide and the next amino acid of the specified region. Antibody binding was measured as the extinction at 405 nm by an ELISA and plotted vertically. The binding patterns of the antipeptide sera were compared with the binding patterns of MAb 57.57. Our experience indicates that the differences in specific binding of the antipeptide sera are both reproducible and significant.

The recognition patterns obtained with the tri- to nonapeptides of the 372 to 389 region (Fig. 2A) indicate that α-$A_{1,2}$, α-$B_{1,2}$, α-$C_{1,2}$, and α-$F_{1,2}$ antipeptide sera contain a broad spectrum of antibodies. Apparently, this depends more on the presentation of the 380 to 387 sequence—as a cyclic (A), a coupled monomer (B), a coupled tandem (C), or a coupled combination (F) peptide—than on the individual rabbit (from an outbred population) used for immunization. Most antipeptide sera recognized shorter peptides than the antiviral MAb 57.57.

A most significant observation may be that some of the binding patterns seem to reflect a limited paratope specificity (a limited spectrum of antibodies). This was observed previously with antipeptide sera that can neutralize foot-and-mouth disease (FMD) virus infectivity (*8*). Thus, apart from the high background, the α-B_1 serum appears to contain mainly antibodies that bind specifically to the heptapeptide FFSYGEI (residues 381 to 387) and also to the shorter derivatives FFSYGE, FSYHE, YGEI, and GEI. Likewise, α-B_2 binds to peptides that start with the residues FFS and shows again an optimal binding to FFSYGEI. The recognition of peptides starting with the sequence VSDS (376 to 379) must be aspecific since these residues are not part of the peptide immunogen (Fig. 2A). The α-C_1 serum shows a binding pattern that is typical for a monoclonal antibody [cf. (*7, 9*)], while the pattern of the α-F_2 serum is remarkably similar to the pattern of MAb 57.57. It is of interest that these monospecific antipeptide sera are nonneutralizing or, as α-C_1, do not even bind to whole virus. In contrast, the neutralizing antipeptide sera α-$A_{1,2}$ and α-C_2 have clearly a more complex recognition pattern. This suggests an explanation for the different biological properties of the set of antipeptide sera: a heterogeneous immune response raised against a peptide is more likely to contain neutralizing antibodies than a monospecific response.

Apparently, binding of antibodies to whole virus does not necessarily imply neutralization. This is also indicated by our previous finding that not all MAbs that bind to the 376 to 389 region of the S protein have neutralization ability and that the neutralizing and nonneutralizing MAbs have different fine-specificities (*7*). The α-F_2 antiserum raised against the combination peptide has a specificity that is remarkably similar to

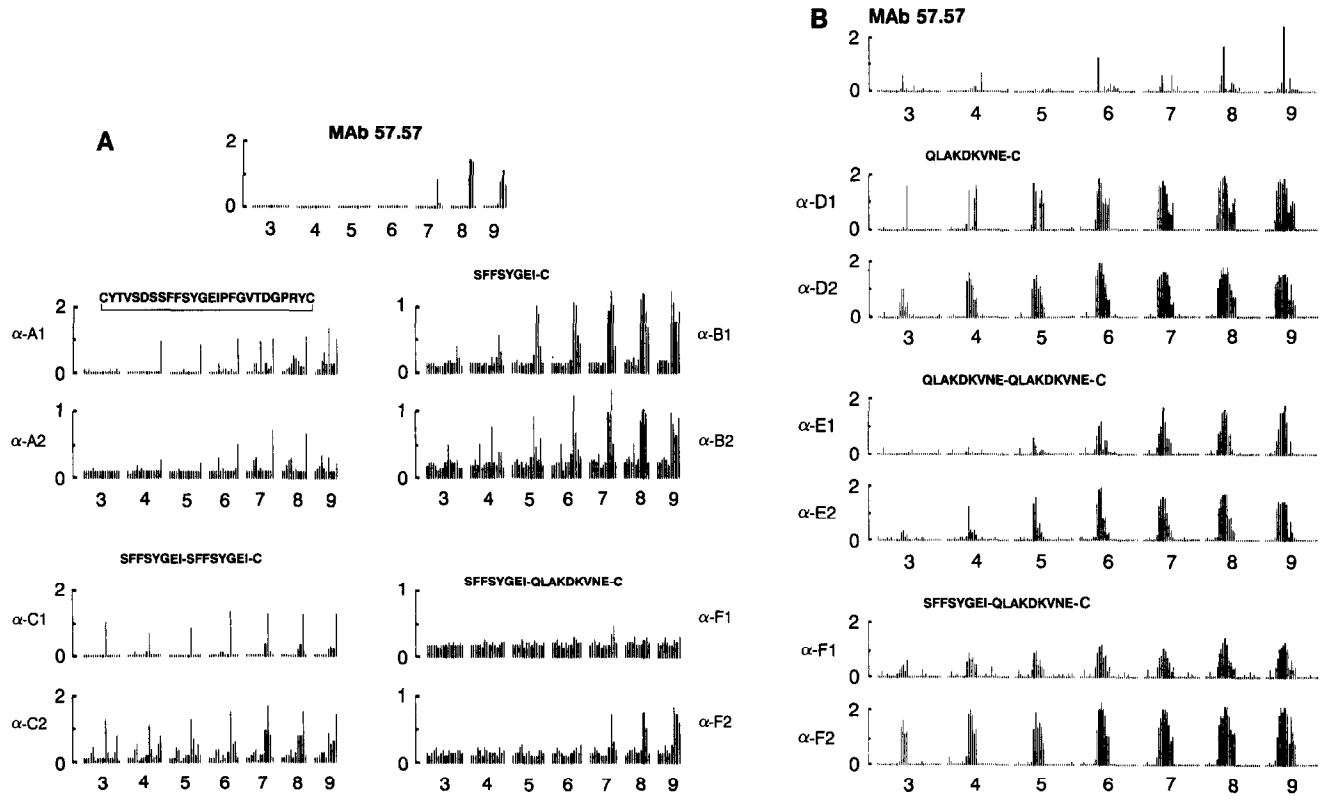

FIG. 2. Binding of antipeptide antibodies with overlapping PEPSCAN peptides that vary in length from three to nine amino acids. The antibody binding, expressed as the extinction at 405 nm by ELISA, is plotted vertically and the numbers below the horizontal axis correspond to the number of amino acids present in the peptides. The sequence of the peptide immunogen is shown above each pair of scans. (A) The PEPSCAN peptide sequences are derived from the S protein sequence region 372 to 389. Boldface lines indicate the peptides with N-terminal amino acid 380. At the top the recognition pattern of MAb CVI-TGEV-57.57 (diluted $1:3 \times 10^4$) is given (*7*). The other recognition patterns, in pairs, are of the antipeptide sera α-$A_{1,2}$, α-$B_{1,2}$, α-$C_{1,2}$, and α-$F_{1,2}$ respectively (each diluted $1:10^3$). (B) The PEPSCAN peptide sequences are derived from the S protein sequence region 1166 to 1201. Boldface lines indicate the peptides with N-terminal amino acid 1176. At the top the recognition pattern of MAb 57.57 (diluted $1:5 \times 10^3$) is given (7). The other recognition patterns, in pairs, are of the antipeptide sera α-$D_{1,2}$, α-$E_{1,2}$, and α-$F_{1,2}$ respectively (each diluted $1:10^3$).

the pattern of the neutralizing MAb 57.57 (Fig. 2A). However, the binding titer of α-F_2 is about 10^4 times lower than the titer of MAb 57.57. Therefore, the low virus titers and the lack of neutralization (Table 2) can be explained by the low concentration of antibodies with a high affinity for the SFFSYGEI sequence. As suggested by the data in Table 2 and Fig. 1, this is most likely caused by the immunodominance of the 1173 to 1184 sequence in rabbits.

The immunodominance of this 1173 to 1184 sequence is also indicated by the PEPSCAN patterns of the antipeptide sera α-$D_{1,2}$, α-$E_{1,2}$, and α-$F_{1,2}$ with the tri- to nonapeptides of the 1166 to 1201 sequence (Fig. 2B). The recognition of several overlapping tripeptides by the α-$D_{1,2}$ and α-$F_{1,2}$ sera clearly reflects the presence of a broad antibody spectrum. The α-$F_{1,2}$ responses appear to contain a limited antibody spectrum, but none of the patterns resembles the pattern of MAb 57.57.

The use of synthetic peptides that combine linear antigenic sites to mimic a discontinuous epitope is a logical approach to induce antibodies that bind to virus and have neutralization activity. Various attempts were made to combine antigenic peptides that are separated on the primary sequence but are in close range in the tertiary structure. A combination peptide of the two epitopes of FMD virus that contains the residues of the regions 140 to 160 and the C terminus (residues 200 to 213) of VP_1 induced high neutralizing antibodies in guinea pigs (*10*). Recently, Francis and co-workers have shown that combination peptides of the sequence of the major epitope of two different FMD virus strains can elicit weak to moderate neutralizing antibodies in guinea pigs (*11*).

Our findings, however, reveal some of the problems with the design of complex peptide antigens that mimic a discontinuous epitope. Notably, the induction of antipeptide antibodies with a virus neutralization activity may depend on the presentation of the peptide sequence. We have demonstrated that a detailed in-

vestigation of the fine-specificity of antipeptide sera by PEPSCAN analysis can contribute to an explanation of their biological properties. This may be most relevant for the investigation of the effect of peptide vaccination in the natural host.

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REFERENCES

1. SIDDELL, S., WEGE, H., and TER MEULEN, V., *J. Gen. Virol.* **64,** 761–776 (1983).
2. STURMAN, L. S., and HOLMES, K., *Adv. Virus Res.* **28,** 35–112 (1983).
3. JIMÉNEZ, G., CORREA, I., MELGOSA, M. P., BULLIDO, M. J., and ENJUANES, L., *J. Virol.* **60,** 131–139 (1986).
4. LAUDE, H., CHAPSAL, J. M., GELFI, J., LABIAU, S., and GROSCLAUDE, J., *J. Gen. Virol.* **67,** 119–130 (1986).
5. CORREA, I., JIMÉNEZ, G., SUÑÉ, C., BULLIDO, M. J., and ENJUANES, L., *Virus Res.* **10,** 77–94 (1988).
6. DELMAS, B., GELFI, J., and LAUDE, H., *J. Gen. Virol.* **67,** 1405–1418 (1986).
7. POSTHUMUS, W. P. A., LENSTRA, J. A., SCHAAPER, W. M. M., VAN NIEUWSTADT, A. P., and MELOEN, R. H., *J. Virol.* **64,** 3304–3309 (1990).
8. GEYSEN, H. M., BARTELING, S. J., and MELOEN, R. H., *Proc. Natl. Acad. Sci. USA* **82,** 178–182 (1985).
9. KUSTERS, J. G., JAGER, E. J., LENSTRA, J. A., KOCH, G., POSTHUMUS, W. P. A., MELOEN, R. H., and VAN DER ZEIJST, B. A. M., *J. Immunol.* **143,** 2692–2698 (1989).
10. DIMARCHI, R., BROOKE, G., GALE, C., CRACKNEL, V., DOEL, T., and MOWAT, N., *Science* **232,** 639–641 (1986).
11. FRANCIS, M. J., HASTINGS, G. Z., CLARKE, B. E., BROWN, A. L., BEDDELL, C. R., and ROWLANDS, D. J., *Immunology* **69,** 171–176 (1990).