Localization and Synthesis of an Antigenic Determinant of Herpes Simplex Virus Glycoprotein D That Stimulates the Production of Neutralizing Antibody

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An antigenic determinant capable of inducing type-common herpes simplex virus (HSV)-neutralizing antibodies has been located on glycoprotein D (gD) of HSV type 1 (HSV-1). A peptide of 16 amino acids corresponding to residues 8 to 23 of the mature glycoprotein (residues 33 to 48 of the predicted gD-1 sequence) was synthesized. This peptide reacted with an anti-gD monoclonal antibody (group VII) previously shown to neutralize the infectivity of HSV-1 and HSV-2. The peptide was also recognized by polyclonal antibodies prepared against purified gD-1 but was less reactive with anti-gD-2 sera. Sera from animals immunized with the synthetic peptide reacted with native gD and neutralized both HSV-1 and HSV-2.

Herpes simplex viruses (HSVs) cause a number of human diseases, including cold sores, eye and genital infections, and encephalitis (21). HSV glycoproteins are structural components of the virion envelope and have been implicated in virus-induced alterations of mammalian cells (22, 27). These glycoproteins are expressed on infected cell plasma membranes and act as major antigenic stimuli for the cellular and humoral responses of the host (22, 27). Four virion glycoproteins, designated gB, gC, gD, and gE have been described (26). One of these, glycoprotein D (gD), apparently plays a major role in the immune response to HSV. Evidence to support this suggestion is as follows. (i) Purified gD of HSV type 1 (oral; HSV-1) (gD-1) or HSV-2 (genital) (gD-2) stimulates high titers of type-common virus-neutralizing antibody (6, 30); (ii) passive immunization with monoclonal antibodies directed against gD protects mice from challenge by a lethal dose of HSV (3, 11, 18); (iii) gD mediates antibody-dependent, complement-mediated cytotoxicity and antibody-dependent, cell-mediated cytotoxicity (3, 23, 25); (iv) purified gD is able to protect mice against lethal challenge with either HSV-1 or HSV-2 (D. Long, T. J. Madara, M. Ponce de Leon, G. H. Cohen, P. C. Montgomery, and R. J. Eisenberg, *Infect. Immun.*, in press).

Recently, we mapped the domains of gD involved in its biological and immunological activities by the use of a panel of gD-specific monoclonal antibodies which recognize eight separate antigenic determinants on the molecule (12). Some of these determinants are type specific and others are cross-reactive or type common. Two groups of monoclonal antibodies, groups V and VII, recognized the reduced and alkylated form of gD-1, suggesting that the determinants recognized by these antibodies were sequential or continuous in nature (2; M. Ponce de Leon, D. Long, R. J. Eisenberg, J. T. Matthews, and G. H. Cohen, unpublished data). In addition, group VII antibodies recognized both gD-1 and gD-2 and neutralized the virus infectivity of both HSV-1 and HSV-2. The continuous nature of the determinant

recognized by group VII antibodies made it feasible to consider the synthesis of a peptide that might stimulate production of type-common neutralizing antibody. Such a synthetic peptide might have potential as a vaccine (1).

For this report, peptides were synthesized corresponding to the most probable amino acid sequence of the group VII determinant of gD. Analysis of these peptides with antibodies directed against gD-1 and gD-2 indicated that the group VII epitope was contained within one of these peptides (16-mer). This peptide was coupled to protein carriers and was used to immunize rabbits and mice. Anti-16-mer sera reacted with the synthetic peptides and with native gD and neutralized HSV in a type-common fashion.

MATERIALS AND METHODS

Cells and virus. Conditions for the growth and maintenance of BHK and KB cells and the propagation of virus have been described previously (7). For infection, an input multiplicity of 20 PFU of HSV-1 (strain HF) and 10 PFU of HSV-2 (strain SAVAGE) per cell was used.

Labeling procedures. HSV-1- and HSV-2-infected cells were labeled by the methods described previously (6, 12, 13). Monolayers (75-cm² flask) were pulse-labeled for 15 min at 6 h postinfection in Hanks salts with [³⁵S]methionine (specific activity, 600 Ci/mmol, 750 µCi), and cytoplasmic extracts were prepared (6, 12, 13). Purification of gD from HSV-1-infected KB cells and HSV-2-infected BHK cells was carried out as previously described (13). The isotope was purchased from New England Nuclear Corp.

Preparation of antisera and monoclonal antibodies. Anti-gD-1 and anti-gD-2 sera were prepared in rabbits and mice against purified preparations of gD-1 and gD-2 (6, 13). Preparation of group VII monoclonal antibodies (represented by antibody 170) with strain G of HSV-2 was previously described (12, 24). Antisera to the synthetic peptides were prepared as follows. BALB/c mice were immunized four times at biweekly intervals by intraperitoneal injection of 15 µg of 16-mer alone or 16-mer covalently bound to ovalbumin (OVA) or keyhole limpet hemocyanin (KLH) and emulsified

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in 50% complete Freund adjuvant. Female New Zealand rabbits were immunized with the same preparations of 16-mer OVA or 16-mer KLH at five weekly intervals by intramuscular injection (6). A total of 350 μ g of 16-mer was used.

Immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. gD was immunoprecipitated from infected cell extracts by the use of anti-gD sera (6, 13) or anti-peptide sera and *Staphylococcus aureus* protein A (IgG Sorb, New England Nuclear Corp.). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out in slabs of 10% polyacrylamide cross-linked with 0.4% *N,N'*-diallyltartardiamide (26). After electrophoresis, the gels were stained with Coomassie brilliant blue. For fluorography (5), the gels were impregnated with 2,5-diphenyloxazole, dried on filter paper, placed in contact with Kodak X-Omat AR5 film, and stored at -70°C .

Peptide synthesis. All *N*-tert-butoxycarbonyl amino acids were purchased from Sigma Chemical Co.; BOC-Cys-*o*-Resin, L-*t*-Amyloxycarbonyl-*N*-Tosyl-L-Arginine, and L-BOC-*O*-Benzyl-L-Serine were purchased from Peninsula Laboratories, Inc. Peptides were manually synthesized using Merrifield solid-phase methods (28) with the following modifications. (i) A series of three washes, each of methylene chloride-absolute ethanol-methylene chloride, was used instead of dioxane and chloroform before and after deprotection of *N*-*t*-BOC amino acid groups; (ii) *N*-*t*-BOC amino acids were deprotected with 25% trifluoroacetic acid in methylene chloride; (iii) completeness of the deprotection and coupling reactions was monitored using the color tests described by Kaiser et al. (17). After synthesis, the resin was dried, and 50 equivalents of thioanisole was added. The side protection groups were removed, and the peptide was then cleaved from the resin with anhydrous hydrogen fluoride. After removal of anhydrous hydrogen fluoride, the peptide resin mixture was extensively washed with ethyl acetate to remove the thioanisole. The cleaved peptide was extracted with 1.5% NH_4HCO_3 and lyophilized. To couple peptides to protein carriers, the maleimide group was incorporated into either KLH or OVA with *M*-malimidobenzol-*N*-hydroxysuccinimide ester (MBS) as described by Liu et al. (20), and the MBS-modified proteins were allowed to react with a 20 M excess of the peptide (20). Because the coupling reaction requires the presence of a thiol group, and the putative determinant does not contain cysteine residues, cysteine was included in all the synthetic peptides as the first amino acid at the carboxyl terminal. Peptides were labeled with ^3H -*N*-hydroxysuccinimide propionate by the method described by A. H. Ross (personal communication). Tracer amounts of labeled peptides were added to the unlabeled peptide to determine the extent of coupling. The coupling level for MBS-modified OVA (mole of peptide per mole of MBS-OVA) was eight. Sequencing was performed as described by Hunkapillar and Hood (16).

Dot-blot analysis of synthetic peptides. A modification of the antigen spot test was used (14). Nitrocellulose strips (BA-85; 0.45 μm ; Schleicher & Schuell Co.) were spotted with 3 μl of purified gD-1 (28 ng), gD-2 (32 ng), and uninfected KB cell extract (128 ng). The buffer for each of these was 0.01 M Tris (pH 7.3), containing 0.15 M NaCl and 0.1% Nonidet P-40 (TNS). The synthetic peptides were dissolved in TNS, and 500 ng was spotted. The strips were air dried and immersed in modified 5 $\times$ Denhardt solution (DS) (10) consisting of DS with 0.1% Nonidet P-40, 1.5% bovine serum albumin, 1 $\times$ borate-buffered saline (pH 8.2), 0.05% gelatin, and 0.04% NaN_3 (N. Fraser, personal com-

munication and reference 29). Dilutions of polyclonal (1:200) or monoclonal antibodies (1:50) were prepared in DS. The strips were transferred to tubes containing 1 ml of diluted antibodies and shaken for 1 h. All washes and incubations were done at 4°C . The strips were washed three times with DS and transferred to tubes containing ^{125}I -Pro A (New England Nuclear Corp.) (5×10^5 cpm) in 1 ml of DS and incubated for 1 h. The strips were removed and washed five times in DS, dried thoroughly, and placed on Kodak XAR-5 film with a Dupont Cronex screen (Lightning-Plus). The dot-blot assay was linear in the range of 60 to 1,000 ng of antigen, although we were able to detect as little as 10 to 15 ng visually. Binding of 1,000 ng of 16-mer was equivalent to the binding of 60 ng of gD-1 to group VII antibody 170.

RESULTS

Prediction of the location of the group VII determinant of gD-1. Previously we used *S. aureus* protease V8 to isolate a 12,000-dalton fragment of gD-1 bound by group VII antibody (12). Tryptic peptide analysis indicated the presence of a type-common, arginine-containing peptide called "f" in the 12,000-dalton fragment. To identify the f peptide, we carried out tryptic peptide analyses of gD labeled with one of several different radioactive amino acids. Peptide f contained proline and alanine (G. H. Cohen, D. Long, and R. J. Eisenberg, unpublished data) in addition to methionine and arginine (12). In addition, we isolated the f peptide and determined its size on Bio-Gel P4 to be ca. 700 daltons (G. H. Cohen, D. Long, and R. J. Eisenberg, unpublished data). These data suggested that the f peptide consisted of residues 36 to 41 of the deduced amino acid sequence of gD-1 (30). Together, these data suggested that the group VII determinant was near the amino terminus of gD-1.

Figure 1 shows a computer representation of the secondary structure of gD-1, by the use of the predicted amino acid sequence of Watson et al. (30) and rules established by Chou and Fasman (8, 9). For these calculations, we excluded the first 25 amino acids of the predicted sequence (30) from consideration, since direct N-terminal sequence analysis showed that lysine residue 26 of the deduced sequence was the amino terminus of mature gD-1 (11a). By the use of stringent criteria (probability of a turn $[\text{Pt}] \geq 7.5 \times 10^{-5}$), 17 β -turns are predicted for this sequence. Also shown in Fig. 1 is a second empirical analysis which assumes that hydrophilic regions of protein structures have a greater immunological potential (15). The predictive value of this method for positioning the group VII determinant is partly based on the assumption that the epitope is continuous in nature (2). The two types of analyses show that the first β -turn in the N-terminal region of the molecule overlaps with a highly hydrophilic region (open circles) and therefore is a likely candidate for an antigenic site. The sequence of the 23 amino acids encompassing this turn is shown in the insert (Fig. 1) and includes the f peptide (met-ala-asp-pro-asn-arg).

Recognition of the synthetic peptide by group VII monoclonal antibody and by polyclonal anti-gD-sera. To test the prediction that the group VII epitope was in the first β -turn, we synthesized the series of peptides shown in Fig. 2. Cysteine was added to the carboxy terminus of each peptide to facilitate coupling to a protein carrier.

We used a modification of the antigen spot test (14) to detect and measure quantities in nanograms of purified gD (13) and the synthetic peptides. Figure 3, row 1 shows that group VII antibody 170 reacted with purified native gD-1

FIG. 1. Predicted secondary structure and hydrophilicity of gD-1. The secondary structure of gD-1 was predicted by a computer program by the rules devised by Chou and Fasman (8, 9). Probabilities for the occurrence of helix (Pa), pleated sheet (Pb), and β -turns (Pt) were evaluated using stringent conditions ($P_t > 1.0 \times 10^{-4}$ or $P_t > 7.5 \times 10^{-5}$; $P_t > P_a$ and $P_t > P_b$). Shaded circles indicate hydrophobic regions, and open circles indicate hydrophilic areas (15). The radius of a circle over a residue position is proportional to the average hydrophilicity as calculated for that residue and the next five residues. The value is therefore distorted at the C-terminal end. The insert shows the amino acid sequence of the N-terminal region (1 to 23) of gD-1.

(lane B), gD-2 (lane C), and the 16-mer (lane F), but not with the 11-mer (lane E), 7-mer (lane D), or uninfected KB cell extract (lane A). Thus, the 16-mer contains the epitope recognized by group VII monoclonal antibodies.

Polyclonal anti-gD-1 serum obtained from rabbit 1 (row 2) recognized gD-1 (lane B), gD-2 (lane C), the 16-mer (lane F), and the 11-mer (lane E) but not the 7-mer (lane D). A second antiserum (rabbit 2), prepared against gD-1, also recognized the 7-mer (lane D, row 3). These results suggest that the first β -turn of gD-1 is highly immunogenic and consists of several overlapping epitopes which comprise an antigenic determinant.

Polyclonal antiserum prepared to purified gD-2 (rabbit 3) did not react with the 16-mer (lane F, row 4), and serum from another gD-2 immunized animal (rabbit 4) showed only slight reactivity with this peptide (lane F, row 5). Similarly, pooled mouse sera prepared against purified gD-2 did not react with the 16-mer (data not shown). The pooled mouse anti-gD-1 serum (lane F, row 6), however, did react with the 16-mer. These results suggest that the antigenic determinant of gD-1 which is present in the 16-mer is different from the corresponding determinant in gD-2.

Specificity of the reaction between monoclonal antibody and the 16-mer peptide. Representatives of six of the eight groups of monoclonal antibodies to gD (12) were tested in the dot-blot assay against gD-1, gD-2, and the 16-mer (Fig. 4). Each antibody (rows 1 to 6) reacted with either gD-1 (lane B), gD-2 (lane C), or both. However, only group VII antibody 170

reacted with the 16-mer (lane D, row 2), thus demonstrating the specificity of the reaction.

Immune response to the 16-mer. (i) Dot-blot assay. Antisera were prepared in both mice and rabbits to the 16-mer peptide covalently bound to KLH or OVA. The reactivity of these sera with gD-1 and gD-2, as well as with the 7-, 11-, and 16-mer peptides, is shown in Fig. 5. Rabbit antisera prepared to the 16-mer KLH (row 5) and the 16-mer OVA (rows 6 and 7)

FIG. 2. Amino acid sequence of the synthetic peptides that overlap the first β -turn of gD-1. Three peptides were synthesized, and in each case, cysteine was added to the carboxy terminus to facilitate coupling to a protein carrier. The numbers in parentheses refer to the residue numbers of the predicted sequence of gD-1 (30). The numbers outside parentheses refer to the residue numbers of the mature glycoprotein (no signal peptide).

FIG. 3. Dot-blot analysis of the synthetic peptides with polyclonal anti-gD antibodies. The antibodies used were as follows: row 1, monoclonal antibody 170; row 2, anti-gD-1 (rabbit 1); row 3, anti-gD-1 (rabbit 2); row 4, anti-gD-2 (rabbit 3); row 5, anti-gD-2 (rabbit 4); row 6, anti-gD-1 (mouse pooled sera); row 7, pooled sera from mice sham-immunized with complete Freund adjuvant; row 8, preimmune rabbit serum. Antigens: lane A, KB cell extract; lane B, gD-1; lane C, gD-2; lane D, 7-mer; lane E, 11-mer; lane F, 16-mer. Exposure time of the film was 3 h.

recognized gD-1 (lane B) but reacted poorly with gD-2 (lane C). However, these three rabbit sera reacted quite well with the 16-mer peptide (lane F, rows 5 to 7). A diversity in this response, however, is suggested by the reaction of these antisera with the 7-mer and the 11-mer. Antisera prepared in mice against the uncoupled 16-mer (row 11) or the 16-mer OVA (row 9) gave a poor response, reacting only slightly with the 16-mer or with gD-1 or gD-2. However, mouse anti-16-mer-KLH serum (row 10) reacted strongly with gD-1 (lane B) and the 16-mer (lane F). This antiserum reacted poorly with gD-2 (lane C) and not at all with the other synthetic peptides (lanes D and E).

(ii) Radioimmunoprecipitation of gD by antisera prepared against the 16-mer. Figure 6 shows the electrophoretic pattern of radioactive proteins which were immunoprecipitated from cytoplasmic extracts of HSV-1- or HSV-2-infect-

FIG. 4. Dot-blot analysis of the 16-mer synthetic peptide with different monoclonal antibodies. The antibodies used (grouped as described in reference 12) were as follows: row 1, group I, HD-1; row 2, group VII, 170; row 3, group V, 57S; row 4, group IV, 1S; row 5, group III, 11S; row 6, group VI, 45S; row 7, pooled sera from sham-immunized mice; row 8, anti-gD-1 (rabbit 1); row 9, anti-gD-2 (rabbit 3); rows 10 and 11, preimmune rabbit serum. The strips in rows 1 to 7 were exposed to X-ray film for 7 days; rows 8 to 10 were exposed for 3 days. Antigens: lane A, KB cell extract (128 ng); lane B, gD-1 (28 ng); lane C, gD-2 (32 ng); lane D, 16-mer (500 ng).

ed cells that were pulse-labeled with [35 S]methionine (6). Control reactions with polyclonal anti-gD-1 and anti-gD-2 sera (Fig. 6A, lanes 1 and 2 and Fig. 6B, lane 1) show the positions of the precursors pgD-1 and pgD-2 (6). Each serum prepared against the 16-mer (Table 1) was tested against these cell extracts in a similar fashion. Mouse anti-16-mer-KLH serum reacted with pgD-1 (Fig. 6A, lane 4) and pgD-2 (Fig. 6B, lane 3). None of the other mouse (Fig. 6A, lanes 5 and 6 and Fig. 6B, lanes 4 and 5) or rabbit sera prepared against the 16-mer immunoprecipitated pgD (data not shown for rabbit sera).

(iii) Virus neutralization. Previously we showed that antisera prepared against purified gD-1 or gD-2 neutralized both HSV-1 and HSV-2 (6, 13). In addition, group VII monoclonal antibody exhibited low titers of type-common neutralizing activity (12, 24). Each serum prepared against the 16-mer was tested for virus neutralization (Table 1). As controls, each of the polyclonal sera and antibody 170 was titrated in the same assay. Rabbit sera prepared against the 16-mer OVA and the 16-mer KLH and mouse anti-16-mer-KLH serum neutralized both HSV-1 and HSV-2. The titers were comparable to those obtained with antibody 170. When serum from rabbit 7 (anti-16-mer OVA) was depleted of complement by heat inactivation, the titers were significantly lower. In contrast, inactivation of complement had little or no effect on the titers of hyperimmune anti-gD sera, mouse anti-16-mer-KLH serum, or antibody 170.

DISCUSSION

This report describes the localization and synthesis of an antigenic determinant of gD of HSV-1. Experimental evidence (12; G. H. Cohen, D. Long, and R. J. Eisenberg, unpublished data) suggested that the N-terminal region of gD-1 contained the type-common group VII antigenic epitope. Predictions of secondary structure (8, 9) and hydro-

FIG. 5. Dot-blot analysis of the antisera from rabbits or mice immunized with the 16-mer covalently bound to KLH or OVA. The antibodies used were as follows: row 1, anti-gD-1 (rabbit 1); row 2, anti-gD-1 (rabbit 2); row 3, anti-gD-2 (rabbit 3); row 4, anti-gD-2 (rabbit 4); row 5, anti-16-mer KLH (rabbit 5); row 6, anti-16-mer OVA (rabbit 6); row 7, anti-16-mer OVA (rabbit 7); row 8, preimmune rabbit serum; row 9, anti-16-mer OVA (pooled mouse sera); row 10, anti-16-mer KLH (pooled mouse sera); row 11, anti-16-mer (pooled mouse sera). Complete Freund adjuvant was used in all immunization. Antigens: lane A, KB cell extract (128 ng); lane B, gD-1 (28 ng); lane C, gD-2 (32 ng); lane D, 7-mer (500 ng); lane E, 11-mer (500 ng); lane F, 16-mer (500 ng).

FIG. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of radioactively labeled pgD-1 and pgD-2 immunoprecipitated by mouse antisera prepared to the purified glycoproteins and to the 16-mer. Fluorograms of 10% acrylamide gels cross-linked with *N,N'*-diallyltartardiamide. For metabolic labeling of gD, monolayers (KB for HSV-1 and BHK for HSV-2) were infected with HSV-1 (strain HF) or HSV-2 (strain SAVAGE). At 2 h postinfection, the monolayers were overlaid with Eagle minimal medium. At 6 h postinfection, the cells were incubated for 15 min in Hanks salts containing 750 μ Ci of [35 S]methionine. The cells were washed with iced saline and lysed, and cytoplasmic extracts were prepared and immunoprecipitated. (A) An extract of HSV-1-infected KB cells was immunoprecipitated by anti-gD-1 serum (lanes 1 and 2); anti-gD-2 serum (lane 3); anti-16-mer-KLH serum (lane 4); anti-16-mer serum (lane 5); and sham-immunized mouse sera (lane 6). (B) An extract of HSV-2-infected BHK cells was immunoprecipitated by anti-gD-1 serum (lane 1); anti-gD-2 serum (lane 2); anti-16-mer-KLH serum (lane 3); anti-16-mer serum (lane 4); and sham-immunized mouse sera (lane 5).

philic regions (15) suggested that the determinant was in the first β -turn near the amino terminus of gD-1. We were able to localize this determinant between residues 8 and 23 of gD-1, based on the specificity of group VII monoclonal antibody 170 for the 16-mer peptide.

The reactivity of anti-gD-1 sera with the 7-mer, 11-mer, and 16-mer suggests the existence of several epitopes in the N-terminal region of gD-1. In contrast, the almost complete lack of reactivity of anti-gD-2 sera with any of the peptides suggests that gD-2 is structurally different in this region from gD-1. The amino acid sequences of gD-1 and gD-2 appear to be quite similar at the amino terminus (11a). However, it is possible that a difference in even one amino acid could impose drastic changes in structure and antigenicity. Knowledge of the full sequence of gD-2 should help to explain the observed differences.

In this study, we assessed the immunogenicity of the 16-mer by injecting mice and rabbits with preparations of this peptide either uncoupled or coupled to two different carriers, KLH and OVA. We observed that the coupled 16-mer induced a diverse antibody response. The antisera recognized the synthetic peptides, as well as gD-1 and gD-2. Of interest was the finding that at least some of these antisera neutralized HSV-1 and HSV-2. In one case, the titer appeared to be partly complement dependent, whereas the

neutralizing activity of monoclonal antibody 170 appears to be largely independent of complement. This may be a reflection of the diversity of antibodies raised to the synthetic peptide. It was also noted that the neutralization titers of the anti-peptide sera were considerably lower than those of the anti-gD sera but were comparable to that of antibody 170. We showed previously that several groups of gD monoclonal antibodies exhibit high titers of neutralizing activity (12). Thus, the high neutralizing activity of polyclonal anti-gD serum represents a composite of antibodies to several determinants, of which the group VII determinant is only one. Future experiments will assess the protective effect induced by immunization with the synthetic peptide. For foot-and-mouth disease virus, a synthetic peptide corresponding to a portion of VP1 capsid protein stimulated production of neutralizing antibody and protected guinea pigs against subsequent virus challenge (4). A similar philosophy has been utilized in manufacturing potential synthetic peptide vaccines for a number of viruses (reviewed in reference 19). Though it is possible that the production of neutralizing antibody may not be a prerequisite for protection against HSV infection, the synthetic peptide nevertheless does stimulate an immune response to gD which includes virus neutralization and thus has potential practical application.

TABLE 1. Serum neutralization of HSV infectivity

Antiserum ^a	Neutralization titer ^b	
	HSV-1 (strain HF)	HSV-2 (strain SAVAGE)
gD-1 (rabbit 2)	1,024 (1,024)	800
gD-2 (rabbit 4)	640	1,600
Preimmune (rabbit)	None	None
gD-1 (mouse)	1,024	640
gD-2 (mouse)	256	512
Sham-immunized (mouse)	None	None
Group VII monoclonal antibody 170	64 (40)	32
16-mer KLH (rabbit 5)	32	16
16-mer OVA (rabbit 6)	32	32
16-mer OVA (rabbit 7)	64 (16)	32 (16)
16-mer KLH (mouse)	64 (64)	8
16-mer OVA (mouse)	8	8
16-mer (mouse)	None	None
KLH (mouse)	None	None

^a Each mouse serum was pooled from five mice.

^b Highest serum dilution that reduced infectivity by 50% (6, 13). The titer of sera after heating at 56°C for 30 min to inactivate complement is indicated in parentheses.

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