JOURNAL OF VIROLOGY, Dec. 1988, p. 4745–4751
0022-538X/88/124745-07$02.00/0

Vol. 62, No. 12

Minimum Requirements for Immunogenic and Antigenic Activities of Homologs of a Synthetic Peptide of Influenza Virus Hemagglutinin

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Received 31 May 1988/Accepted 30 August 1988

Synthetic peptides of increasing length and corresponding in sequence to the C-terminal end of the HA_1 molecule of influenza virus were constructed and examined for their immunogenic and antigenic properties. Peptides containing at least the four C-terminal amino acids, when coupled to keyhole limpet hemocyanin, were capable of eliciting antibody in BALB/c mice that bound to the 24-residue parent peptide H3 HA_1 (305 to 328). In the absence of a carrier, the C-terminal decapeptide was the shortest peptide capable of eliciting antibody. The specificity of this antibody was indistinguishable from that of a monoclonal antibody to the parent peptide which recognizes an epitope encompassed by the C-terminal seven residues. All peptides containing at least the C-terminal four residues were able to inhibit completely the binding of this monoclonal antibody to the parent peptide. Taken together, these results indicate that (i) the tetrapeptide is capable of eliciting specific antibody when coupled to a carrier, (ii) this tetrapeptide possesses all of the antigenic information necessary to occupy the paratope of a monoclonal antibody elicited by the longer parent peptide, and (iii) the decapeptide contains all of the information necessary to elicit a specific immune response and therefore carries an epitope recognized by T cells as well as one recognized by B cells.

The synthetic peptide H3 HA_1 (305 to 328), which represents the C-terminal 24 amino acid residues of the heavy chain (HA_1) of influenza virus hemagglutinin (HA) of the H3 subtype, contains three antigenic sites which are recognized by antibody elicited in BALB/c mice (11). Two of these sites are also recognized by monoclonal antibodies (MAb) which are capable of binding simultaneously to the same peptide molecule with high affinity (4). Antibodies to the peptide are subtype specific (5, 8), and the immune response to the peptide is *I-E*^{d} restricted (2). One of the three antigenic sites is encompassed by the sequence Met-320-Arg-Asn-Val-Pro-Glu-Lys-Gln-Thr-328 and contains at least one epitope recognized by polyclonal antisera elicited by the parent peptide. An epitope recognized by MAb 1/1, also elicited by the parent peptide, is encompassed by the seven amino acids Asn-322-Val-Pro-Glu-Lys-Gln-Thr-328, of which residues Asn-322, Glu-325, and Gln-327 are necessary for binding to the antibody (11).

The purpose of the study reported here was to determine the minimum structural requirements for immunogenic and antigenic activity of this model peptide. The C-terminal antigenic site was chosen because it allows progressively longer peptides (homologs) to be synthesized from the C terminus, which is a known boundary of that site. Knowledge of the contact residues recognized within this site by a MAb to the 24-residue C-terminal peptide also allows comparative specificity studies to be performed on antisera resulting from immunization with the family of homologs. Furthermore, because the parent peptide elicits antibody in mice in the absence of a covalently coupled carrier protein (2, 5, 8), it must contain at least one epitope recognized by helper T (T_h) cells. Progressive elongation of peptides from the C terminus should allow delineation of the boundaries of such a T_h cell epitope, together with the minimum structure associated with the B-cell epitope. If homologs are covalently coupled to a carrier that provides the necessary T-cell help, it should also be possible to determine whether any sequence shorter than the complete B-cell epitope displays immunogenicity in vivo and whether the antibody produced differs in specificity from that elicited by the parent peptide. Such subepitopic sequences could then be assayed for antigenicity in vitro and their specificities for various antibodies could be compared with those of the native epitope.

MATERIALS AND METHODS

Synthetic peptides. The synthetic peptides H3 HA_1 (305 to 328) and H2 HA_1 (305 to 328), representing the C-terminal 24 amino acid residues of the HA_1 polypeptides from the viruses A/Memphis/1/71 (H3) and A/Japan/305/57 (H2), respectively, and also analog A and analog B (see Fig. 1) were assembled with an Applied Biosystems synthesizer, model 430A, as previously described (5). The panel of tri- to decapeptides representing different-length homologs (see Fig. 1) of the parent peptide H3 HA_1 (305 to 328) were synthesized semimanually with a Cambridge Research Biochemical Pepsynthesizer. Briefly, syntheses were performed by the solid-phase procedure with Pepsyn K resin (0.1 mmol/g) and fluorenylmethoxycarbonyl amino acid derivatives (3). Peptides were assembled in a stepwise manner, and after addition of the third amino acid from the C terminus, samples of the resin were removed sequentially after each amino acid coupling cycle. In this way, peptides representing 3 (C3) to 10 (C10) residues of the amino acid sequence of peptide H3 HA_1 (305 to 328) were assembled. These peptides were cleaved from the resin with 95% trifluoroacetic acid–5% water, taken up into 30% aqueous acetic acid, and extracted with ether, and the aqueous phase was lyophilized to yield free peptide. The crude peptides were purified by preparative high-pressure liquid chromatography on an RP-300 C18 column with acetonitrile-water gradient elution. The

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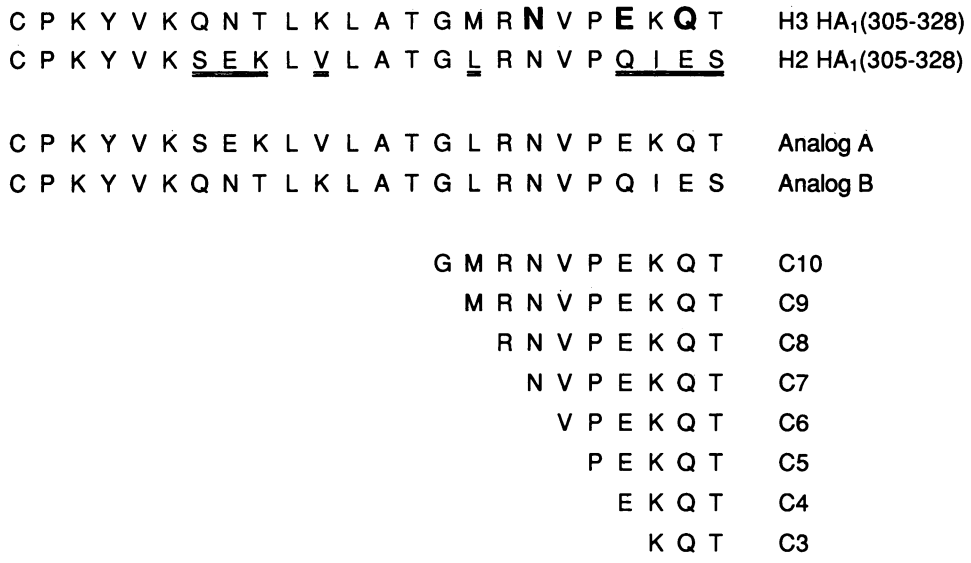

FIG. 1. Amino acid sequences of the peptides used in this study. The sequence of the parent peptide H3 HA_1 (305 to 328), the corresponding sequence from the H2 subtype, H2 HA_1 (305 to 328), and analogs and homologs of the parent sequence are shown in single-letter code. The numbering refers to the positions within the sequences of the HA_1 polypeptides from which the synthetic peptides were derived. The residues in boldface within the sequence of peptide H3 HA_1 (305 to 328) have been shown (11) to be contact residues for MAb 1/1. Underscored residues indicate the differences in sequence between the H3 and H2 subtypes in this region of the HA_1 molecule.

purified peptides were found to be homogeneous as assayed by amino acid analysis and analytical high-pressure liquid chromatography.

Conjugation of synthetic peptides to a carrier. Synthetic peptides were coupled to keyhole limpet hemocyanin (KLH) with glutaraldehyde. Equal volumes of each peptide and KLH, each at 2 mg/ml in phosphate-buffered saline (PBS), were added together, and an equal volume of glutaraldehyde (0.25% in water) was added dropwise over a period of 10 min. The resulting solution was kept at room temperature for 6 h in the dark with occasional mixing. After dialysis against a solution of PBS containing 50 mM glycine, the sample was exhaustively dialyzed against PBS.

Inoculation of animals. Groups of five female BALB/c mice 6 to 8 weeks old were immunized intraperitoneally with either the free peptide or the peptide conjugated to KLH. In each case, animals received 100 μg of a peptide emulsified in complete Freund adjuvant. After 4 weeks, animals received a similar dose of peptide or peptide conjugate in complete Freund adjuvant and were bled after a further 3 weeks. The sera from individual animals were pooled and stored frozen at −20°C until used.

ELISA. Enzyme-linked immunosorbent assays (ELISA) were performed in flat-bottom wells of flexible polyvinyl chloride microtiter trays which had been coated with 50 μl of antigen solution (5 μg/ml) for 18 h. After the antigen solution was aspirated, unoccupied sites on the wells were blocked by addition of 100 μl of a solution (10 mg/ml) of bovine serum albumin in PBS. After 1 h, the wells were washed thoroughly with PBS containing 0.05% Tween 20. Antibody preparations diluted in PBS–0.05% Tween 20 containing bovine serum albumin (5 mg/ml) were then added and kept at ambient temperature. After 18 h, trays were washed and a solution (50 μl at a 1/400 dilution) of horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G (Dako, Glostrup, Denmark) was added. After 1 h, the plates were washed, and the bound antibody was detected by addition of 100 μl of the substrate 2,2′-azino-di-3-ethylbenzthiazoline sulfonic acid at a concentration of 0.2 mM in 50 mM citrate buffer (pH 4) containing 0.004% H_2O_2. The optical density of the resulting solutions was determined in a Titertek Multiscan apparatus, and the values were normalized by expression as a percentage of the maximum value obtained within the assay. Inhibition assays were performed by adding dilutions of the peptide to a constant amount of the antibody which gave less than maximum binding to antigen-coated wells in the absence of an inhibitor.

MAb 1/1. MAb 1/1, raised in a BALB/c mouse inoculated with the parent synthetic peptide H3 HA_1 (305 to 328), was derived and characterized as previously described (5). This MAb recognizes an epitope encompassed by residues Asn-322-Val-Pro-Glu-Lys-Gln-Thr-328, of which residues Asn-322, Glu-325, and Gln-327 are necessary for binding of the antibody (11).

RESULTS

Immunogenicity of peptide homologs with and without a carrier. Sera obtained from BALB/c mice immunized with individual peptides ranging in length from 3 to 10 amino acid residues (Fig. 1), either free or covalently coupled to KLH, were examined for their ability to bind to the parent peptide H3 HA_1 (305 to 328). The results show that, when coupled to KLH as a carrier (Fig. 2), the minimum-length peptide capable of eliciting antibody that binds to the parent peptide is the tetrapeptide Glu-325-Lys-Gln-Thr-328 (C4). Production of antibody requires addition of residue Glu-325 to the immunosilent peptide Lys-326-Gln-Thr-328. KLH-coupled peptides C5 and C6 induced antibody of similar titer. In contrast, peptides C7, C8, C9, and C10 induced antibody of much higher titer, comparable to that of MAb 1/1. This quantal difference between the two classes of immunogens is associated with the addition of residue Asn-322 to peptide Val-323-Pro-Glu-Lys-Gln-Thr-328.

A different result was obtained when the immunogenic activity of carrier-free peptides was examined (Fig. 3).

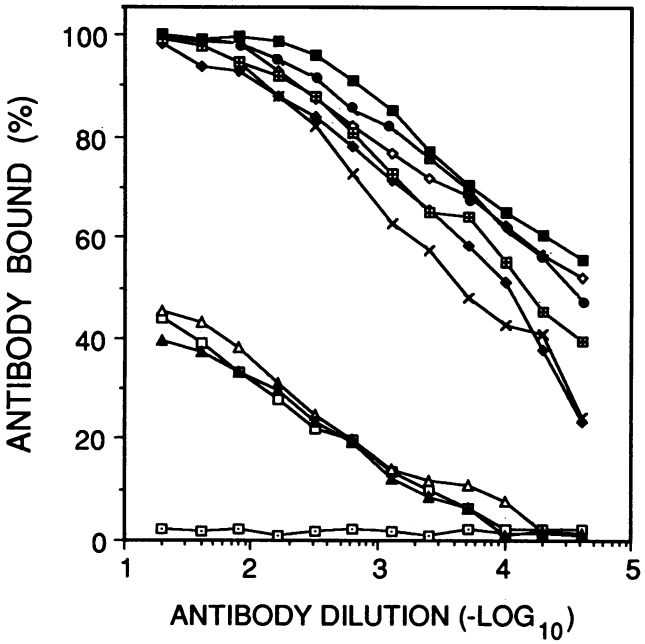

FIG. 2. Titration of anti-(peptide-KLH) sera tested against peptide H3 HA_1 (305 to 328). Serial dilutions of antisera obtained from mice inoculated with Lys-326-Gln-Thr-328–KLH (⊡), Glu-325-Lys-Gln-Thr-328–KLH (□), Pro-324-Glu-Lys-Gln-Thr-328–KLH (△), Val-323-Pro-Glu-Lys-Gln-Thr-328–KLH (▲), Asn-322-Val-Pro-Glu-Lys-Gln-Thr-328–KLH (◇), Arg-321-Asn-Val-Pro-Glu-Lys-Gln-Thr-328–KLH (◆), Met-320-Arg-Asn-Val-Pro-Glu-Lys-Gln-Thr-328–KLH (×), Gly-319-Met-Arg-Asn-Val-Pro-Glu-Lys-Gln-Thr-328–KLH (●), and Ala-317-Thr-Gly-Met-Arg-Asn-Val-Pro-Glu-Lys-Gln-Thr-328–KLH (⊞) were assayed against the parent peptide in a direct-binding ELISA. MAb 1/1 (■) was also incorporated in the assay for comparison. The antibody bound is expressed as a percentage of the maximum level of binding observed for any particular antibody preparation.

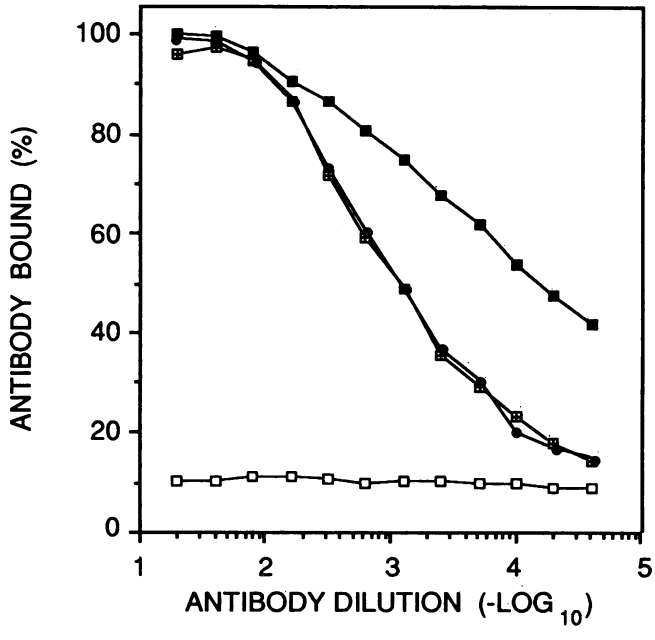

FIG. 3. Titration of anti-peptide sera tested against peptide H3 HA_1 (305 to 328). Serial dilutions of antisera obtained from mice inoculated with peptides without a carrier were assayed against the parent peptide in a direct-binding ELISA. Symbols: ●, anti-peptide Gly-319-Met-Arg-Asn-Val-Pro-Glu-Lys-Gln-Thr-328; ⊞, anti-peptide Ala-317-Thr-Gly-Met-Arg-Asn-Val-Pro-Glu-Lys-Gln-Thr-328. Anti-peptides Lys-326-Gln-Thr-328, Glu-325-Lys-Gln-Thr-328, Pro-324-Glu-Lys-Gln-Thr-328, Val-323-Pro-Glu-Lys-Gln-Thr-328, Asn-322-Val-Pro-Glu-Lys-Gln-Thr-328, Arg-321-Asn-Val-Pro-Glu-Lys-Gln-Thr-328, and Met-320-Arg-Asn-Val-Pro-Glu-Lys-Gln-Thr-328 all showed superimposable titration curves and are represented here as a single profile (□) for clarity. MAb 1/1 (■) was also incorporated in the assay for comparison.

Under these circumstances, the shortest peptide capable of eliciting antibodies that bind to the parent peptide was Gly-319-Met-Arg-Asn-Val-Pro-Glu-Lys-Gln-Thr-328 (C10). The titers of the antibodies elicited by the two immunogenic homologs C10 and C12 were, however, lower than that of MAb 1/1. These results contrast with those obtained when C10 and C12 were conjugated to KLH; in that case, the titers of the antibodies induced were comparable to that of MAb 1/1. The finding that the decapeptide and dodecapeptide induce antibody in the absence of a carrier implies that, in addition to a B-cell epitope(s), they also possess a T_h-cell epitope(s). The lower titer of antisera produced in response to these two homologs when they were injected in the absence of a carrier than that obtained when coupled to KLH indicates that the T_h epitopes present on the KLH carrier molecule provide more help than does the T_h epitope(s) present within the sequence Gly-Met-Arg-Asn-Val-Pro-Glu-Lys-Gln-Thr.

Specificity of antibodies induced by peptide homologs. The specificity of the antibodies elicited by the various free peptides and peptide conjugates was examined by using an inhibition ELISA. This assay has been shown to provide information on the fine specificity of antibody-antigen reactions (2) and also avoids the possibility of adventitious cross-reactions which can arise from conjugation of a peptide with a carrier (1). Each antipeptide serum was examined, but because the antibodies fell into discrete patterns of behavior, the results of only three representatives are presented for clarity. Antibodies elicited by the C4-KLH conjugate were representative of antibodies produced in response to C4-KLH, C5-KLH, and C6-KLH conjugates, whereas antibodies produced in response to the C7-KLH conjugate were typical of those elicited by C7-KLH, C8-KLH, C9-KLH, and C10-KLH conjugates. The properties of antibodies induced by the carrier-free peptide Gly-319-Met-Arg-Asn-Val-Pro-Glu-Lys-Gln-Thr-328 form a third group. Parallel inhibition experiments were performed with MAb 1/1, which has known binding specificity (11), and are presented as a reference for the inhibition assays performed with antisera.

Two analogs, A and B, of the parent peptide H3 HA_1 (305 to 328) were used to inhibit binding between the parent peptide and antisera to homologs of the parent peptide. Each analog contains one of the distinctive H3 amino acid sequences incorporated into a sequence which is otherwise identical to the HA_1 C-terminal 24 residues of the H2 subtype (Fig. 1). In every case, only the parent peptide and analog A were capable of inhibition (Fig. 4). Both of these peptides include the sequence Arg-321-Asn-Val-Pro-Glu-Lys-Gln-Thr-328 and therefore contain the relevant amino acid residues which bind antibodies elicited by the peptide homologs. With these inhibitors, the antisera elicited by the various immunogens demonstrated similar specificities to MAb 1/1.

The fine specificities of antibodies induced by the various peptide homologs were investigated by using the family of

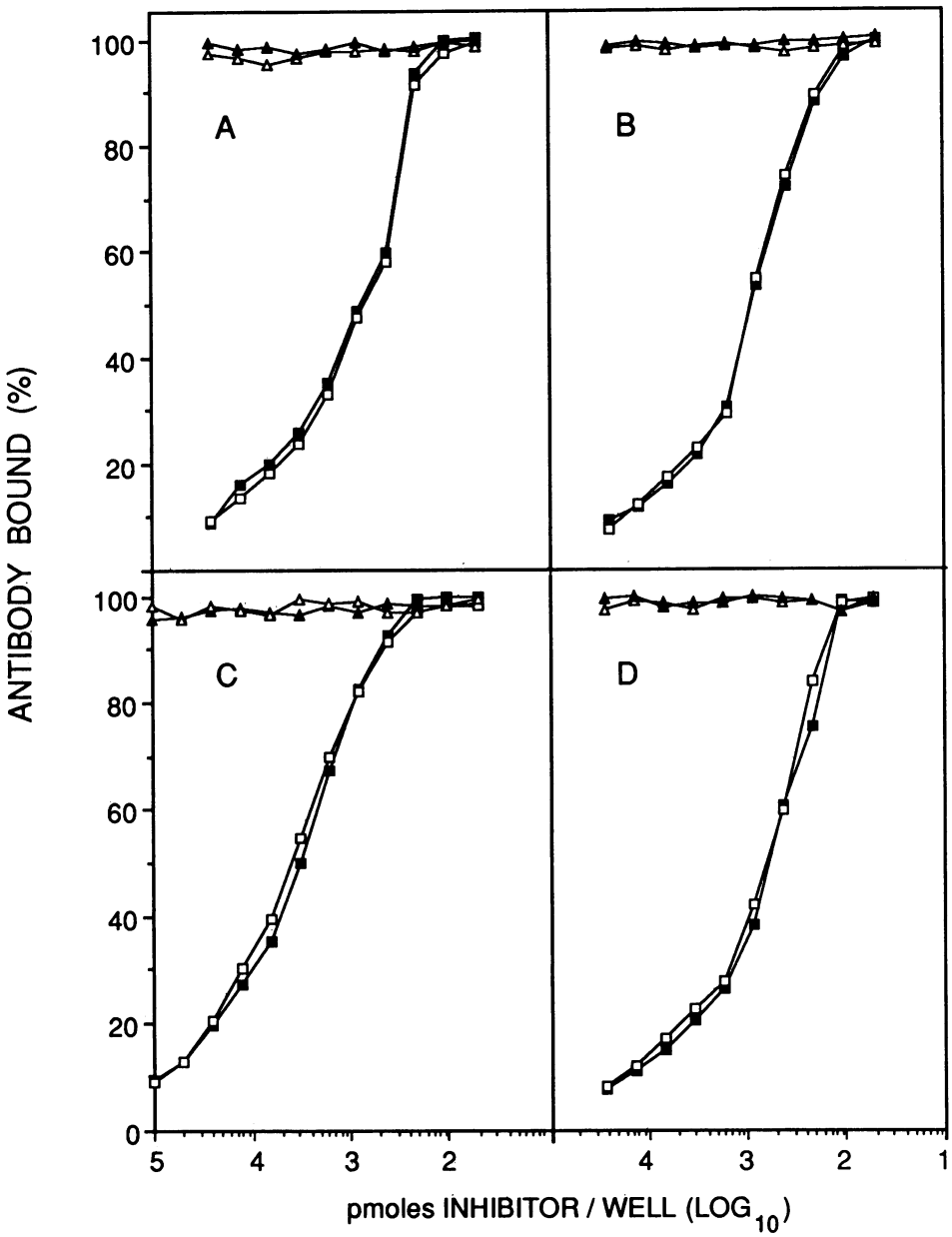

FIG. 4. Inhibition by peptides and peptide analogs of binding to peptide H3 HA_1 (305 to 328) of antisera to homologs. Antisera elicited by the conjugates Glu-325-Lys-Gln-Thr-328–KLH (panel A) and Asn-322-Val-Pro-Glu-Lys-Gln-Thr-328–KLH (panel B) or by the free peptide Gly-319-Met-Arg-Asn-Val-Pro-Glu-Lys-Gln-Thr-328 (panel C) and MAb 1/1 (panel D) were tested by ELISA for their ability to bind to peptide H3 HA_1 (305 to 328) in the presence of dilutions of peptide H3 HA_1 (305 to 328) (■), peptide H2 HA_1 (305 to 328) (▲), analog A (□), or analog B (△).

short peptides themselves as inhibitors (Fig. 5). Antibodies induced by the conjugates C4-KLH, C5-KLH, and C6-KLH were inhibited by peptides C4, C5, C6, C7, and C8 and the parent peptide to similar extents and at equimolar concentrations (Fig. 5A). Antibodies elicited by the conjugates C7-KLH, C8-KLH, C9-KLH, and C10-KLH showed a different pattern of inhibition; i.e., peptides C4, C5, and C6 demonstrated comparable but relatively inefficient inhibition, whereas peptides C7, C8, C9, and C10 and the parent peptide formed a second distinct group of more efficient inhibitors (Fig. 5B). A similar dual pattern of inhibition by the two distinct groups of homologs was observed with antibodies elicited by carrier-free homolog C10 (Fig. 5C) and MAb 1/1 (Fig. 5D). These results indicate that antibodies elicited by conjugates C7-KLH, C8-KLH, C9-KLH, and C10-KLH, and also carrier-free C10, possess specificities similar to that of MAb 1/1 and that these specificities are different from those of the antibodies elicited by the conjugates C4-KLH, C5-KLH, and C6-KLH.

Antigenic activity of peptide homologs. In addition to providing information on the fine specificities of the various antisera, the inhibition experiments also illustrate the antigenic properties of the peptide homologs. As with immunogenic activity, antigenic activity, i.e., the ability to bind to any of the anti-peptide antibodies, was first detected when residue Glu-324 was added to the antigenically silent tri-

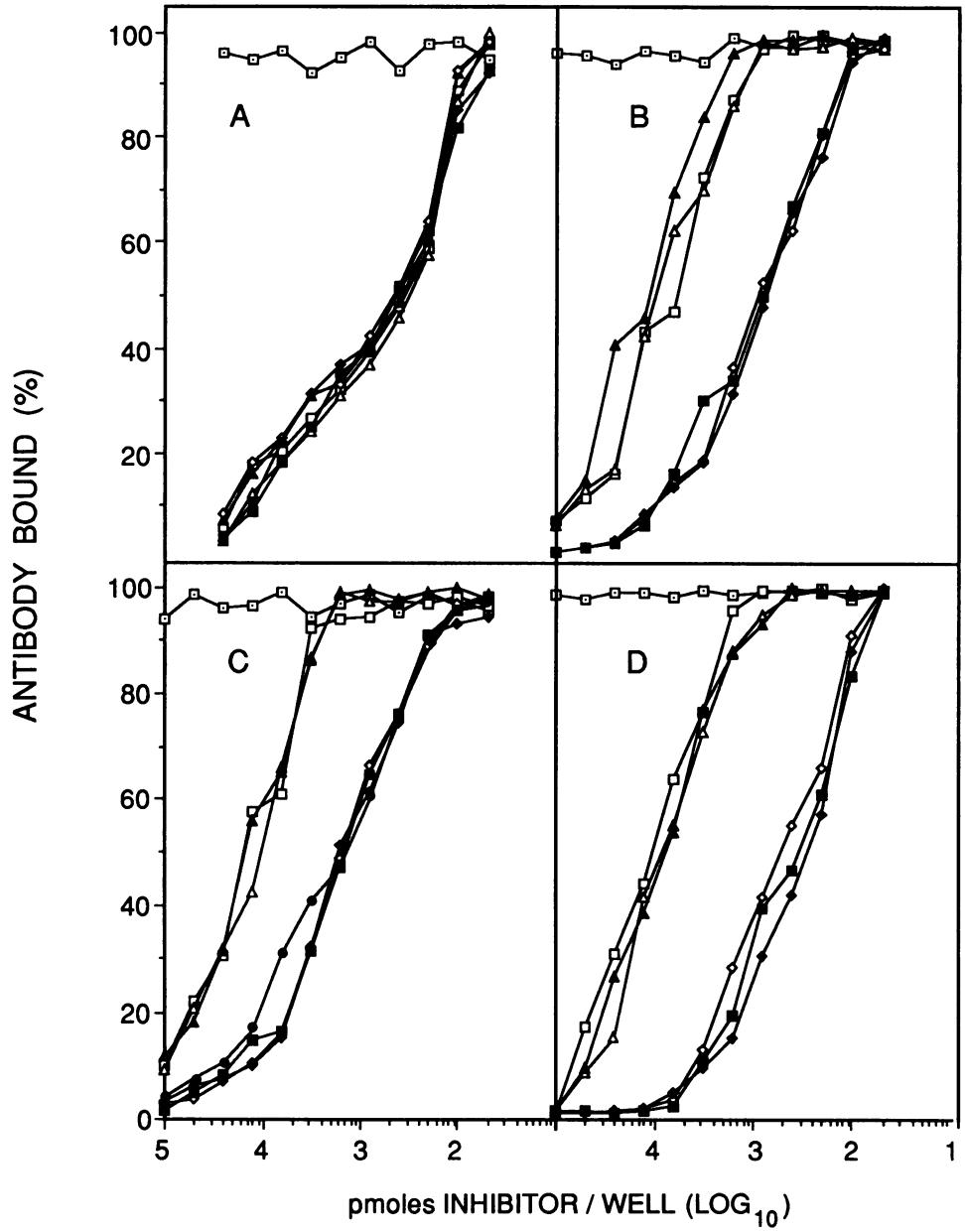

FIG. 5. Inhibition by peptide homologs of the binding of antisera to parent peptide H3 HA_1 (305 to 328). Antisera elicited by the conjugates Glu-325-Lys-Gln-Thr-328–KLH (panel A) and Asn-322-Val-Pro-Glu-Lys-Gln-Thr-328–KLH (panel B), the free peptide Gly-319-Met-Arg-Asn-Val-Pro-Glu-Lys-Gln-Thr-328 (panel C), and MAb 1/1 (panel D) were tested by ELISA for their ability to bind to peptide H3 HA_1 (305 to 328) in the presence of dilutions of peptides Lys-326-Gln-Thr-328 (⊡), Glu-325-Lys-Gln-Thr-328 (□), Pro-324-Glu-Lys-Gln-Thr-328 (△), Val-323-Pro-Glu-Lys-Gln-Thr-328 (▲), Asn-322-Val-Pro-Glu-Lys-Gln-Thr-328 (◇), Arg-321-Asn-Val-Pro-Glu-Lys-Gln-Thr-328 (◆), Gly-319-Met-Arg-Asn-Val-Pro-Glu-Lys-Gln-Thr-328 (●), and H3 HA_1 (305 to 328) (■).

peptide Lys-Gln-Thr, whereas addition of residue Asn-322 to peptide Val-Pro-Glu-Lys-Gln-Thr resulted in a second abrupt increase in activity. These experiments also show that all peptide homologs were antigenically similar when examined by using antibodies elicited by peptide C4, C5, or C6 (Fig. 5A), whereas the homologs fell into two distinct classes of antigen when examined by using antisera induced by C7, C8, C9, and C10 (Fig. 5B and C). The lack of antigenic distinction made between any of the homologs when they were examined with antisera to peptides C4, C5, and C6 and the clear antigenic differences seen when examined with antisera to C7, C8, C9, and C10 indicate that different residues are involved in binding to these two groups of antibodies.

The similarity in inhibitory activity of the parent peptide H3 HA_1 (305 to 328), analog A, and the homologs C7, C8, and C10 indicates not only that each of these contains the residues that make up the antigenic epitope but also that the conformation assumed by the constellation of essential residues is similar. This is not surprising considering the relatively small size of the peptide homologs and may merely reflect their assumption of the correct conformation by a mechanism of induced fit when the peptide occupies the antibody paratope.

DISCUSSION

The results of the experiments described here indicate that the shortest immunologically active peptide homolog of the antigenic site which occurs at the C-terminal end of the parent peptide H3 HA_1 (305 to 328) is the tetrapeptide Glu-325-Lys-Gln-Thr-328 (C4). This homolog satisfies the minimum requirements for antigenic activity and also, when coupled to a carrier, those for immunogenic activity. To elicit antibodies without coupling to a carrier, the C4 homolog has to be extended by an additional six residues. This extension to C10 produces the minimum structure incorporating T_h- and B-cell epitopes. Inspection of the primary structure of this homolog (Gly-Met-Arg-Asn-Val-Pro-Glu-Lys-Gln-Thr) reveals that it contains a sequence, Asn-Val-Pro-Glu-Lys, that fulfils the requirements for T_h-cell epitopes postulated by Rothbard (9) and Rothbard and Taylor (10), namely, a charged residue followed by two hydrophobic residues and then two polar residues. In humans, the parent peptide has been reported to possess the immunodominant T-cell epitope(s) of the hemagglutinin molecule (7), but in that case the epitopes were found to be located at the N-terminal end of the sequence (6).

The pattern C3 = 0, C4 = C5 = C6 $\ll$ C7 = C8 = C9 = C10 is seen in expression of both the antigenic and the immunogenic properties of this family of homologs. Antibodies produced by peptides C4 through C6 are both quantitatively (titer) and qualitatively (specificity) different from those elicited by peptides C7 through C10. Addition of residues Val-323 and Pro-324 to the C-terminal tetrapeptide had little or no effect on the titer or specificity of the antibody induced, but with addition of residue 7, Asn-322, the titer of the antisera jumped some 2,000-fold. This C-terminal heptapeptide also matches the parent peptide H3 HA_1 (305 to 328) in its antigenic activity, since C7 is able to inhibit the binding of antibodies elicited by any of the shorter peptide homologs and also that of MAb 1/1. Although Glu-Lys-Gln-Thr blocked the binding to the parent peptide of all of the antibodies examined and therefore contained sufficient information to bind specifically to the antibody paratope, its inhibitory titer was about 30-fold lower than that of Asn-322-Val-Pro-Glu-Lys-Gln-Thr-328. This presumably reflects the contribution of residue Asn-322 to the total energy of binding of the epitope to the paratope. Although analog B and peptide H2 HA_1 (305 to 328) each contain the sequence Arg-321-Asn-Val-Pro-324, in common with analog A and peptide H3 HA_1 (305 to 328), neither of these analogs was capable of inhibiting the binding to the native H3 sequence of any of the antibody preparations. Only peptides containing the sequence Glu-325-Lys-Gln-Thr-328 inhibited the reaction.

The epitope defined by MAb 1/1 is encompassed by the sequence Asn-322-Val-Pro-Glu-Lys-Gln-Thr-328, in which the three residues Asn-322, Glu-325, and Gln-327 each make a substantial contribution to antibody binding (11). The results of the present study indicate that, for both immunogenic and antigenic activities of peptide homologs, two of these residues are sufficient but addition of the third leads to a substantial increase in activity. These results are consistent with the hypothesis that the antibodies elicited by the series of homologs each bind to the same residues recognized by MAb 1/1, but the possibility that antibodies elicited by the peptide homologs bind to different contact residues within the sequence Glu-Lys-Gln-Thr cannot be formally excluded. Similarly, the acquisition of activity by addition of Glu-325 to the inactive peptide Lys-Gln-Thr may not reflect addition of an essential contact residue but could conceivably be due to the introduction of some other feature, such as charge, which permits the correct orientation of the molecule in the antibody paratope without that residue necessarily being directly involved in binding. In the same way, we cannot exclude the possible end effect of the free alpha amino group of Asn-322, which may affect the conformation of the short peptide sequence Asn-Val-Pro-Glu-Lys-Gln-Thr by, for example, interaction with the side-chain carboxyl and amino groups of the glutamic acid and lysine residues. It is, however, difficult to predict what effect, if any, these sorts of charge interactions and end effects will have. Nevertheless, because addition of residue Asn-322 to peptide Val-Pro-Glu-Lys-Gln-Thr leads to such a dramatic increase in activity, it is clear that this residue is important for optimal antigenic and immunogenic activity of the peptide homologs C7, C8, C9, C10, and C12.

The fact that Glu-Lys-Gln-Thr is the smallest peptide able to inhibit the antibody-antigen-binding reactions indicates that it constitutes the core of the epitope recognized by all of the antibodies elicited by the various peptide homologs, as well as MAb 1/1. Extension of the core sequence to five or six residues did not change the specificity of the resulting antibodies. Similarly, though addition of the seventh residue greatly enhanced activity, there was no discernible difference in specificity among antibodies elicited by C7, C8, C9, and C10. It seems, therefore, that induction of an antibody of the correct specificity requires particular residues with appropriate spatial separation but that the intervening residues do not affect that specificity.

ACKNOWLEDGMENTS

We thank Lorena E. Brown and E. Margot Anders for constructive comments made during the course of this work and during preparation of the manuscript. We also thank Mary Jones for excellent technical assistance.

This work was supported by project grant 840626 from the National Health and Medical Research Council of Australia.

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