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Identification of a hexapeptide that mimics a conformation-dependent binding site of acetylcholine receptor by use of a phage-epitope library

(anti-acetylcholine receptor monoclonal antibody 5.5/conformation-dependent epitope/"mimotope")

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ABSTRACT **Monoclonal antibody (mAb) 5.5 is directed against the ligand-binding site of the nicotinic acetylcholine receptor. The epitope for this antibody is conformation-dependent, and the antibody does not react with synthetic peptides derived from the receptor sequence. We have identified a ligand peptide that mimics this conformation-dependent epitope from a phage-epitope library composed of filamentous phage displaying random hexapeptides. Among 38 positive phage clones, individually selected from the library, 34 positive clones carried the sequence Asp-Leu-Val-Trp-Leu-Leu (DLVWLL), 1 positive clone had the sequence Asp-Ile-Val-Trp-Leu-Leu (DIVWLL), and 3 positive clones expressed the sequence Leu-Ile-Glu-Trp-Leu-Leu (LIEWLL), none of which are significantly homologous with the nicotinic acetylcholine receptor α subunit sequence. All of these phages bind specifically to mAb 5.5. The synthetic peptide DLVWLL inhibits binding of mAb 5.5 to the related peptide-presenting phage and to the nicotinic acetylcholine receptor in a concentration-dependent manner; the IC_{50} value is of the order of 10^{-4} M. Bioactivity of the peptide "mimotope" DLVWLL was demonstrated *in vivo* in hatched chickens by inhibition of the mAb 5.5 effect by the peptide. The neuromuscular block and myasthenia gravis-like symptoms that are induced in chicken by passive transfer of mAb 5.5 were specifically abolished by DLVWLL. This study shows the potential of a random peptide phage-epitope library for selecting a mimotope for an antibody that recognizes a folded form of the protein, where peptides from the linear amino acid sequence of the protein are not applicable.**

The muscle nicotinic acetylcholine receptor (AcChoR) is a well-characterized ligand-gated ion channel located in the postsynaptic folds of the neuromuscular junction (1, 2). This receptor, which is also recognized as the major autoantigen in the neuromuscular autoimmune disease myasthenia gravis, is a transmembrane glycoprotein composed of four subunits present in a molar stoichiometry of $\alpha_2\beta\gamma\delta$. The cholinergic binding site of AcChoR is in the α subunit (3), within a small fragment containing the two tandem cysteines at positions 192 and 193. Workers from our laboratory (4, 5) have previously demonstrated that a synthetic dodecapeptide corresponding to aa 185–196 of the AcChoR α subunit contains the essential elements of the ligand-binding site. Nevertheless, some additional amino acid residues in other regions of the α subunit, and even in other subunits (6), were reported in close proximity to the ligand upon binding and possibly to participate in the binding site. Thus, the native binding site of AcChoR is probably obtained by a certain spatial conformation of the receptor and, indeed, a denatured form of the receptor does not bind cholinergic ligands nor does it bind the receptor-specific neurotoxins—e.g., α-bungarotoxin (7).

In earlier studies from our laboratory (8), Mochly-Rosen and Fuchs described an anti-AcChoR monoclonal antibody (designated mAb 5.5 G.12 and here termed mAb 5.5) that is specific to the ligand-binding site of the receptor. This antibody blocks the binding of α-bungarotoxin to AcChoR, and its binding to AcChoR is inhibited by α-neurotoxins and by other cholinergic ligands in accordance with their affinities to the receptor. Immunochemical characterization of mAb 5.5 showed that it reacts with native intact AcChoR but does not react with denatured AcChoR (8), indicating that it does not recognize a sequential peptide fragment in the receptor but rather recognizes a structural antigenic determinant resulting from a specific spatial conformation of the receptor protein. mAb 5.5 was also shown to affect AcChoR function *in vivo*; it inhibits carbamoylcholine-induced sodium transport in muscle cells (9) and modifies single channel properties of the receptor (10). Moreover, passive transfer of mAb 5.5 in chickens results in the induction of a neuromuscular block similar to that seen in experimental autoimmune myasthenia gravis (11).

To search for a possible ligand for this antibody, we have used the phage-epitope library (12) and isolated a hexapeptide epitope that specifically recognizes mAb 5.5 and mimics its natural conformational structured epitope, both *in vitro* and *in vivo*. The construction and application of several epitope libraries in which different peptides are expressed on the surface of filamentous phage (fd) has been described (12). Such libraries were used recently by several groups to isolate peptide ligands that react specifically with mAbs (12, 13), lectins (14, 15), or streptavidin (16). The epitopes for mAbs identified so far are compatible with peptide sequences present in the original immunogen (which induced the corresponding antibody). In contrast, the epitope for mAb 5.5 isolated from the hexapeptide library in this study has no homology in the sequence of the immunogen AcChoR and is thus defined as a "mimotope" (17, 18). The availability of simple synthetic ligands (e.g., peptides) that mimic natural complex epitopes might have an important application in the particular case of mAb 5.5, in view of its specific biological activity, and may contribute, in general, to the elucidation of the molecular basis of biological recognition.

MATERIALS AND METHODS

Epitope Library. The hexapeptide epitope library used in this study was provided by George P. Smith (University of

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Abbreviations: AcChoR, nicotinic acetylcholine receptor; mAb, monoclonal antibody. Peptide sequences are given in single-letter amino acid codes.

Missouri, Columbia, MO) and was constructed by use of the phage fd-derived vector fUSE5 as described (12). This library consists of 2×10^8 original phage clones and was amplified ≈500 times. The phage clones each contain a hexapeptide fused to the minor coat protein PIII. Theoretically, the library can represent, at most, 69% of the 6.4×10^7 possible hexapeptides (12).

Preparation, Purification, and Biotinylation of Antibodies. The anti-AcChoR mAb 5.5 was prepared as described (8). Two milliliters of ascitic fluid containing mAb 5.5 was diluted with 10 ml of loading buffer (3 M NaCl/1.5 M glycine, pH 8.9) and passed through a column (1×0.5 ml) of immobilized protein A and protein G (Schleicher & Schuell). The column was washed with 10 ml of the loading buffer and eluted with 0.1 M glycine·HCl buffer, pH 2.8. Fractions of 0.7 ml were collected into tubes containing 70 μl of 1 M Tris·HCl buffer, pH 8.0. For biotinylation, 100 μg of the immunoglobulin fraction of mAb 5.5 in 100 μl of 0.1 M $NaHCO_3$, pH 8.6, were incubated for 2 hr at room temperature with 5 μg of biotinamidocoproate *N*-hydroxysuccinimide ester (Sigma, B 2643) from a stock solution of 1 mg/ml in dimethylformamide and dialyzed at 4°C against phosphate-buffered saline (PBS; 0.14 M NaCl/0.01 M phosphate buffer, pH 7.4).

Isolation of Epitope-Presenting Phage from the Hexapeptide Epitope Library. A library sample containing 3.8×10^9 infectious phage particles was subjected to three rounds of selection (panning) and amplification. For each selection cycle a biotinylated mAb 5.5 (60 μg/ml) in a total volume of 50 μl was used. The phages were preincubated with the biotinylated antibody overnight at 4°C, and the reaction mixtures were then layered in 1 ml of PBS/0.5% Tween 20 on streptavidin-coated 60-mm polystyrene Petri dishes and incubated for 30 min at room temperature. Unbound phages were removed by extensive washings (10 times for 10 min each) in PBS/0.5% Tween 20, and the remaining phages were eluted with 0.8 ml of 0.1 M HCl titrated to pH 2.2 with glycine. The eluate was neutralized and used to infect *Escherichia coli* K91 cells. After three rounds of panning, individual bacterial colonies containing amplified phage clones were grown in a microtiter plate, and the phages were tested by ELISA for their ability to specifically bind mAb 5.5.

Antibody Binding to Isolated Phages. Binding of antibodies to phages was analyzed by ELISA. Wells of microtiter plates (Maxisorb, Nunc) were coated with 100 μl of a 1:1000 dilution (in 0.1 M $NaHCO_3$, pH 8.6) of rabbit antiphage M13 serum by incubation overnight at 4°C. Coated plates were washed three times with PBS/0.05% Tween 20, and 100 μl of enriched phage clones, containing 10^9 phage particles, were then added to the wells and incubated for 1 hr at 37°C. Wells were blocked with 1% bovine serum albumin (BSA) (in PBS) for 1 hr at room temperature, washed, and incubated with the antibody (1 μg/ml or as otherwise specified) overnight at 4°C. For inhibition experiments, peptides or proteins were preincubated with the antibody for 30 min at 37°C, before their addition to phage-coated wells. After washing, bound antibody was detected by incubation with alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulins (BioMakor, Rehovot, Israel), diluted 1:2000, for 1 hr at room temperature. The bound antibody was monitored by the enzymatic activity of alkaline phosphatase, with *n*-paranitrophenylphosphate used as a substrate, and the color developed after 50 min was determined by a microtiter plate reader at 405 nm. Positive phage clones were propagated, and their DNA was sequenced in the epitope region by using a Sequenase version 2.0 kit (United States Biochemical) according to the manufacturer's instructions.

For experiments on the inhibition of mAb 5.5 binding to AcChoR, ELISA plates were coated with purified *Torpedo californica* AcChoR (30 μg/ml) at 4°C overnight, and the experiments were done as described above for the inhibition of mAb 5.5 binding to phages.

AcChoR and Peptide Preparations. AcChoR was solubilized with Triton X-100 from the electric organ of *T. californica* and purified as described (19). Denatured AcChoR preparation (reduced carboxymethylated-AcChoR) was prepared by reduction and carboxymethylation of AcChoR in 6 M guanidine hydrochloride, as described by Bartfeld and Fuchs (7). Recombinant peptides corresponding to aa 122–205 of *Torpedo* and mouse AcChoR α subunit were prepared as described (20) and were obtained from Sylvia Katchalsky (The Weizmann Institute of Science, Rehovot, Israel). Hexapeptides were synthesized in the Unit for Chemical Services at the Weizmann Institute by the solid-phase method of Merrifield (21).

Passive Transfer Experiments. A neuromuscular block was induced in chickens by passive transfer of mAb 5.5, essentially as described by Souroujon *et al.* (11). Male hatched chickens, 3–5 days old, each weighing ≈50 g, were injected i.p. with mAb 5.5 (200 μg of purified immunoglobulins in 1 ml of PBS). To examine the effect of the synthetic peptide DLVWLL, chickens were injected with a mixture (1 ml in PBS) of mAb 5.5 (200 μg; minimal effective dose) and the competing peptide, after preincubation of the mixture for 1 hr at 37°C with gentle shaking. The animals were followed for myasthenia-like symptoms, including muscle weakness and, at later stages, breathing difficulties and paralysis.

RESULTS

Isolation of Peptide-Presenting Phages by mAb 5.5. To identify a peptide epitope for mAb 5.5, which may block the biological activity of this antibody, we screened the hexapeptide phage-epitope library with biotinylated mAb 5.5. After three cycles of panning and phage amplification, 90 individually isolated bacterial colonies were grown in microtiter plates, and their phages were assayed for antibody binding. ELISA analysis revealed that 87 of these clones bound specifically to mAb 5.5. DNA from 38 of the positive clones was sequenced, and the deduced peptide sequences are shown in Table 1. The sequence DLVWLL appeared in 34 clones; one additional clone had a peptide sequence DIVWLL, with just one replacement of leucine for isoleucine. In three additional clones the inserted peptide sequence was LIEWLL, in which the three carboxyl-terminal residues (WLL) are identical to those in the other clones, whereas the other three differ. It should be noted that these three latter clones gave consistently lower binding values to mAb 5.5. Most of the following analysis was done with the DLVWLL-phage—i.e., the sequence most often found.

Binding of mAb to the DLVWLL-phage was concentration dependent; half-maximal binding was obtained at an antibody

Table 1. Binding of mAb 5.5 to phages isolated from an epitope library

Epitope sequence*	Binding	Phages identified, no.
DLVWLL	779 ± 15	34
DIVWLL	789 ± 47	1
LIEWLL	442 ± 15	3

Binding data were obtained by ELISA. Enriched phage clones containing 10^9 phage particles in 100 μl were added to the anti-phage M13-coated wells and incubated later with mAb 5.5 (1 μg/ml), as described. Binding values are expressed by the absorbance at 405 nm $\times 10^3$ (mean ± SD) after subtraction of background readings obtained with an unrelated phage (background readings were, in all cases, <15% of sample readings).

*Amino acid sequences (deduced from DNA) of the inserted hexapeptide.

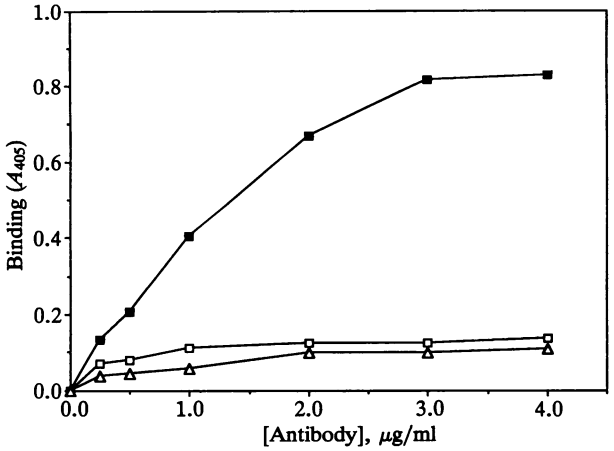

FIG. 1. Binding of mAb 5.5 to DLVWLL-phage. Antibodies were added to the phage-coated wells, and binding was analyzed by ELISA as described. Binding of mAb 5.5 (■) and mAb 5.14 (□) to DLVWLL-phage. Binding of mAb 5.5 to an unrelated HGPAWK-phage (△).

concentration of 1 μg/ml, corresponding to 4×10^{-9} M (Fig. 1). An unrelated anti-AcChoR mAb (mAb 5.14; ref. 9) did not bind significantly to the DLVWLL-phage. Likewise, mAb 5.5 did not bind to an unrelated phage, bearing the sequence HGPAWK (Fig. 1). These two controls demonstrate the specificity of mAb 5.5 binding to DLVWLL-phage.

Specificity of Antibody–Phage Recognition. Specificity of the interaction of mAb 5.5 with DLVWLL-phage was further assessed by inhibition experiments. Fig. 2 shows that the synthetic peptide DLVWLL, and not other peptides tested, inhibited mAb 5.5 binding to the phage with an IC_{50} value of 2×10^{-4} M. Interestingly, a synthetic peptide with an inverse amino–carboxy orientation to DLVWLL (i.e., LLWVLD) showed no significant inhibitory activity. Although the DLVWLL sequence does not exist in AcChoR, AcChoR inhibited the binding of mAb 5.5 to DLVWLL-phage with an IC_{50} value of 1 nM (Fig. 3). This result suggests that the natural epitope for this antibody in the receptor is not represented by a sequential peptide but rather is represented by some structured epitope formed by appropriate folding of the receptor molecule and, thus, the synthetic hexapeptide could be defined as a mimotope for this natural structured epitope. Indeed, as depicted in Fig. 3, a denatured form of the

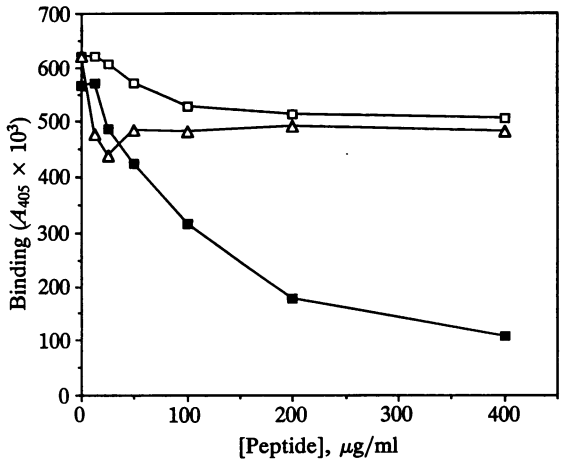

FIG. 2. Inhibition of mAb 5.5 binding to DLVWLL by synthetic peptides. The assay was done as described with 0.1 μg of mAb 5.5 per phage-coated well and increased concentrations of DLVWLL (■), LLWVLD (□), and FRHSVV (△).

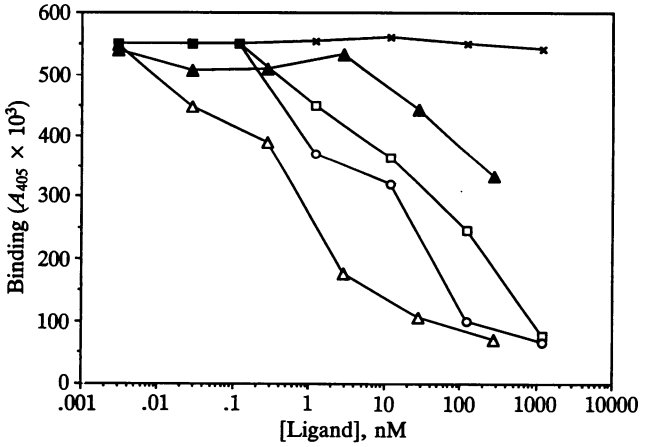

FIG. 3. Inhibition of mAb 5.5 binding to DLVWLL-phage by AcChoR and derivatives. The assay was done as described with 0.1 μg of mAb 5.5 per phage-coated well and increased amounts of purified *Torpedo* AcChoR (△), denatured (reduced and carboxymethylated) AcChoR (▲), recombinant AcChoR fragment corresponding to aa 122–205 of *Torpedo* (○) and mouse (□) α subunit and of the cloning vector (X) as a control.

receptor (reduced carboxymethylated AcChoR) was at least three orders of magnitude weaker as an inhibitor of the interaction of the antibody with the phage. Recombinant peptide fragments corresponding to aa 122–205 of *Torpedo* and mouse AcChoR also inhibited the binding of mAb 5.5 to the DLVWLL-phage, although at a higher molar concentration than that of the intact AcChoR (Fig. 3). These two fragments contain the ligand binding-site domain of AcChoR and were shown to bind both α-bungarotoxin and mAb 5.5 (20).

Specificity of the interaction of DLVWLL with mAb 5.5 was also tested by its capacity to inhibit binding of the antibody to AcChoR (Fig. 4). The peptide DLVWLL inhibits the binding of mAb 5.5 to AcChoR in a concentration-dependent manner with an IC_{50} value of ≈150 μg/ml (2×10^{-4} M), which is similar to the IC_{50} value from inhibition of mAb 5.5 binding to the peptide-presenting phage (see Fig. 2). On a molar basis this concentration is significantly higher than the concentration of intact AcChoR (2×10^{-7} M) required for a similar inhibition. Nevertheless, inhibition by DLVWLL is specific because the peptide with identical composition but opposite orientation (LLWVLD) caused no inhibition.

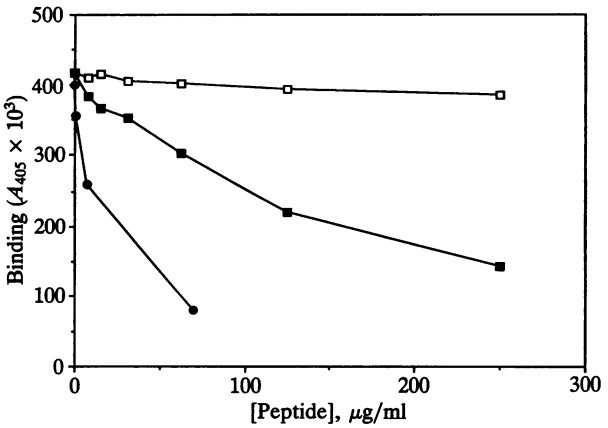

FIG. 4. Inhibition of mAb 5.5 binding to AcChoR by synthetic DLVWLL. The assay was done as described with 0.1 μg of mAb 5.5 per AcChoR-coated well and increased amounts of DLVWLL (■), LLWVLD (□), or *Torpedo* AcChoR (●).

Table 2. Effect of peptide DLVWLL on the neuromuscular block induced in chickens by mAb 5.5

Peptide sequence	Peptide, mg	mAb 5.5, μg	MG-like signs*	Animals tested, no.
None		200	+	6
DLVWLL	0.5	200	+	3
DLVWLL	1	200	+	3
DLVWLL	2	200	±†	3
DLVWLL	4	200	−	6
FRHSVV‡	4	200	+	2
DLVWLL	4		−	2

Chicken were injected i.p. with a solution (1 ml in PBS) of mAb 5.5 and synthetic peptide (after their preincubation for 1 hr at 37°C).
*Myasthenia gravis (MG) signs are characterized by muscle weakness, breathing difficulties, and paralysis, as determined 20 hr after passive transfer; animals in each group exhibited similar signs.
†Moderate expression of muscle weakness was detected 20 hr after passive transfer (Fig. 5), and the animals had completely recovered 2 days later.
‡Unrelated control peptide.

Peptide Epitope Inhibits the mAb Effect *in Vivo*. Finally, the synthetic hexapeptide DLVWLL was shown to inhibit the effect of mAb 5.5 on AcChoR *in vivo*. As reported earlier by workers from our laboratory (M. C. Souroujon, A. R. Pachner, and S.F.) (11), passive transfer of mAb 5.5 to chickens resulted in an induction of experimental autoimmune myasthenia gravis (Table 2 and Fig. 5). This neuromuscular block of AcChoR by mAb 5.5 could be abolished by preincubation of the antibody with the hexapeptide before the passive transfer. Administration into chickens of 4 mg of DLVWLL with mAb 5.5 (200 μg) completely prevented the induction of experimental autoimmune myasthenia gravis symptoms by the antibody. Administration of a lower amount of peptide (2 mg per chicken) led to amelioration of the clinical symptoms, and the chickens had completely recovered 2 days later (Fig. 5).

DISCUSSION

Using a hexapeptide phage-epitope library (12), we have identified a hexapeptide, DLVWLL, that interacts specifically with an anti-AcChoR mAb (mAb 5.5). This particular antibody was shown to be directed to the receptor ligand-binding domain and to compete with the binding of α-bungarotoxin and other cholinergic ligands (8). Thus, the epitope for this mAb should be at or near the ligand-binding site of AcChoR. Also, mAb 5.5 was shown to interact with a conformation-dependent epitope that is not formed by a continuous amino acid sequence in the protein. It was, therefore, not unexpected that the sequence of the isolated mimotope, DLVWLL, does not exist in AcChoR. It is not known (*i*) whether in the intact AcChoR the natural epitope for mAb 5.5 is formed by these amino acid residues, which are not present contiguously but are brought together by proper folding of the protein, or (*ii*) whether the peptide in this sequence forms a structure that resembles the natural epitope and is not necessarily homologous in sequence and/or in amino acid composition to the original epitope.

Sequence analysis of 38 independent peptide-phage clones that bind to mAb 5.5 indicates that they all have a tryptophan residue at the fourth position in the hexapeptide, supporting the assumption that the presence of tryptophan, or of aromatic residues in general, is required for cholinergic ligand binding. Trp-187 of the AcChoR α subunit was shown to play an essential role in the binding of α-bungarotoxin to AcChoR (5, 22). Moreover, this tryptophan, as well as additional aromatic residues (tryptophans and tyrosines) in the α subunit, interact with the ligand upon its binding to AcChoR (6). Additional support for the participation of an aromatic residue in cholinergic ligand binding comes from the identification of an "aromatic gorge" as part of the ligand-binding domain of the enzyme acetylcholinesterase (23).

The random epitope libraries were originally designed and used to map peptide epitopes on proteins. There have also been some reports on the isolation of peptide mimotopes for epitopes of a nonpeptidic nature, such as biotin (16) and α-D-mannopyranoside (14, 15). The present study, however, is an example of the application of a phage-epitope library to obtain a protein-derived epitope that is recognized by a *folded* form of the protein (AcChoR) and not by a continuous amino acid sequence. In this respect protein-derived epitopes that are conformation-dependent may belong to the category of nonpeptide epitopes. For such protein-derived epitopes,

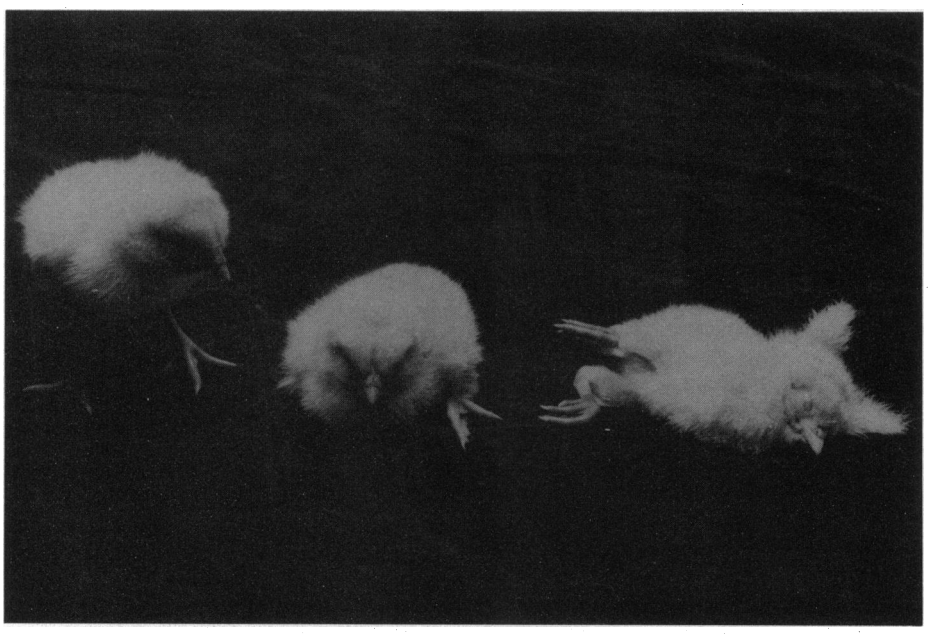

FIG. 5. Inhibition of the *in vivo* effect of mAb 5.5 in hatched chickens by synthetic DLVWLL. Chickens were injected i.p. with 200 μg of mAb 5.5 alone (right) or with a mixture of 200 μg of mAb 5.5 with 2 mg (middle) or 4 mg (left) of DLVWLL (preincubated for 1 hr at 37°C). Photographs were taken 20 hr after antibody (or antibody and peptide) administration.

synthetic peptides corresponding to sequences of the original protein are not suitable for isolation of an epitope or a mimotope. On the other hand, random peptide libraries constructed by either molecular biological (12, 16, 24) or synthetic chemical techniques (25, 26) should prove to have a potential application. By using such random libraries, it is possible to find a ligand mimotope for a biologically active molecule (be it a mAb, receptor, lectin, etc.) that differs in structure from the binding site of the "natural" ligand but mimics its specificity. Indeed, the peptide DLVWLL, which bears no sequence homology to AcChoR, binds specifically to mAb 5.5 and blocks its biological activity both *in vitro* and *in vivo*.

Although the interaction of DLVWLL with mAb 5.5 is specific, the affinity of the peptide is rather moderate. The synthetic DLVWLL inhibits competitively the binding of mAb 5.5 to the related cloned phage or to AcChoR with IC_{50} values of $\approx 10^{-4}$ M. The apparent binding of the cloned phage as well as of AcChoR to mAb 5.5 is about three orders of magnitude higher. That the phage appears to bind better than the free peptide may reflect a multivalent binding interaction of the phage, which carries several copies of the minor coat protein PIII-fused hexapeptide. The preferential interaction of the peptide-linked phage may also result from the restricted conformation of the peptide as a consequence of its immobilization within the phage proteins. It is possible that, by using longer synthetic peptides derived from phage libraries with longer inserted peptides or by extending the hexapeptide at both ends with amino acid residues flanking the hexapeptide in the phage, one could obtain mimotopes with higher affinity to the receptor. Nevertheless, it should be noted that the moderate affinity obtained with the peptide mimotope might be an intrinsic limitation and may represent the best fit one can get in this system for a conformation-dependent epitope formed by a noncontinuous sequence in a protein or for epitopes that are of a nonprotein nature.

The inhibitory effect of DLVWLL on the neuromuscular block induced by administration of mAb 5.5 is particularly encouraging with respect to the potential application of peptide mimotopes for *in vivo* targeting of molecules with biomedical importance. Epitope libraries might be used to identify peptide epitopes or mimotopes capable of interacting specifically with immunopathological antibodies—e.g., autoantibodies in myasthenia gravis or in other immunological disorders. Such specific peptide ligands may form the basis for the development of therapeutic agents designed by appropriate modification and derivatization of such selective peptide mimotopes.

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