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TECHNICAL PROGRESS REPORT - 1991

UNIDO/ICGEB Collaborative Research Programme 1988 (CRP/GRE88-05)
Contract No. 90/017 dated 6 February 1990

PROJECT TITLE:
STRUCTURE AND PATHOGENICITY OF THE ACETYLCHOLINE RECEPTOR

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SUMMARY

Antibodies against the muscle acetylcholine receptor (AChR) induce the disease myasthenia gravis (MG). We found in the past that the majority of the monoclonal antibodies (mAbs) bind to the main immunogenic region (MIR) which is located within residues 67-76 of the AChR α-subunit. We are studying in depth the structure and pathogenicity of the AChR with main emphasis on the MIR. In 1991 the following were performed:

Linear and cyclic analogues of the MIR decapeptide were produced in large amounts. Their binding capacity to the corresponding mAbs was tested by radioimmunoassays whereas their conformation, while free or bound to the mAbs, was studied by 2D-NMR and molecular dynamics approaches.

The cDNAs of VH and VL domains of four anti-MIR mAbs were cloned and sequenced revealing extensive similarities among them. Production of single-chain Fv fragments is in process. The final aim is to construct a mutated Fv with high binding affinity for the AChR in order to be used as protector of the AChR in MG. To achieve this aim we need to know the exact binding sites and conformation of the mAbs. Thus, small synthetic peptides mimicking certain CDRs of the mAbs were produced. Some of these peptides exhibited significant AChR-binding activity, thus probably uncovering the most critical parts of the antigen combining sites of these mAbs. In parallel, aiming at the detailed crystallographic analysis of the anti-MIR mAbs, the conditions were established for the production of small crystals of the Fab fragments.

The epitopes for 17 mAbs to the δ-subunit of *Torpedo* and human AChR were precisely mapped by the use of overlapping synthetic peptides. A very immunogenic epitope was identified on the cytoplasmic side of the subunit.

Bivalent $F(ab)_2$ fragments of an anti-MIR mAb injected into rats caused AChR loss and induced myasthenic symptoms. Because these fragments cannot act through complement it is concluded that antigenic modulation of AChR is capable of inducing MG.

DETAILED REPORT

Myasthenia gravis (MG) is caused by autoantibody-mediated loss and perhaps functional blockage of the nicotinic acetylcholine receptor (AChR) at the neuromuscular junction. We have found in the past that the majority of the anti-AChR monoclonal antibodies (mAbs) bind to a region on the α-subunit, named main immunogenic region (MIR).

Monoclonal antibodies against the MIR cause AChR loss in cell cultures and experimental MG in rats. The indirectly measured anti-MIR antibody fraction in MG sera is primarily responsible for the ability of the sera to cause AChR loss in cell cultures. Thus the MIR seems to play a critical role in MG. By the use of AChR fragments, binding of several anti-MIR mAbs has been localized between residues α67-76 (residues WNPADYGGIK).

Our goal is to gain information about the pathological mechanisms underlying MG and to suggest a targeted immunosuppressive treatment of this disease. This is approached by determining the antigenic specificities, binding characteristics and functional effects of anti-AChR mAbs and myasthenic patients' antibodies. Special emphasis is devoted to the study of the MIR due to its apparently critical role in MG. At the same time valuable information is obtained for the AChR molecule itself.

During 1991 we performed the following:

1. Investigation of the antigenic role of each amino acid within the MIR decapeptide α67-76.

The main aim of this part is to eventually construct a peptide analogue having conformation and antigenicity similar with that of the MIR on the intact AChR. Such a molecule would be valuable for the study of the anti-AChR antibodies in MG sera and for planning therapeutic strategies.

In 1990 by the use of the PEPSCAN technique of Geysen (PNAS 81:3998-4002, 1984) we produced about 250 peptides attached to polyethylene rods and tested their binding to the anti-MIR mAbs. This year some of the MIR analogues which exhibited improved binding activity were produced in large quantities in soluble form, by our collaborator Prof. C. Sakarellos, The so far synthesized peptides have not proved of high affinity for the AChR. In order to recover the high affinity which was detected by the PEPSCAN peptides, acetylated soluble peptides will be further produced and tested. In addition to linear peptides, two cyclic MIR decapeptides were synthesized in an effort to mimic the conformation of the MIR on the intact AChR. These peptides, however, apparently acquired unfavorable conformation and thus they did not exhibit significant binding activity. (Submitted).

2. Molecular studies on the anti-MIR monoclonal antibodies

Despite our considerable knowledge on the structure of the MIR (and of other mAb epitopes on the AChR) we know practically nothing about the structure of the anti-MIR antibodies and especially about their binding sites. We have thus initiated molecular studies towards the characterization of the anti-MIR mAbs. Total RNAs were isolated from 5 hybridomas (4 anti-MIR mAbs and one non-MIR). cDNAs containing the variable regions of the mAbs were synthesized using the M-MLV reverse transcriptase and anti-sense primers from the constant CH_1 and Ck regions. The cDNAs were amplified enzymatically by the polymerase chain reaction (PCR) using Taq polymerase and two primers, one sense from the 5' end of the VH or Vk regions, and one anti-sense from the 3' end of these regions. The

amplified regions were cloned into the SmaI site of the Bluescript vector and their sequences were determined. The cDNA sequences of the VL domains from all four anti-MIR mAbs tested were very similar to each other (on average 90% homology within each pair) but very different from those of non-MIR mAbs (on average 55% homology). Similar homologies were observed among the VH domains. We are now in the process of constructing and expressing single-chain Fv fragments containing both VL and VH domains.

In order to identify critical antibody binding points on the antigen binding sites of the above mAbs, we synthesized peptides corresponding to various complementarity-determining regions (CDRs) of VL and VH domains of the sequenced mAbs. Intact, fragmented and hybrid CDRs were synthesized on polyethylene rods. The peptides were tested for binding ^{125}I-α-bungarotoxin labeled AChR. CDR_2 of VH and a hybrid of VL CDR_1 plus VL CDR_2 bound very well the AChR. Anti-MIR Fab inhibited AChR binding. Active CDR segments could be as small as decapeptides (for VL CDR_{1+2}) and tetrapeptides (for VH CDR_2). Single residue substitutions revealed the binding role of each residue. One substitution enhanced AChR binding.

We expect that these studies, in addition to helping us to understand the properties of the anti-MIR antibodies, will form the basis for the subsequent construction of improved affinity and humanized mAbs for potential therapeutic use.

3. 2D-NMR and crystallographic studies of MIR peptide - antibody interactions. (In collaboration with Dr. M.T. Cung, Dr. M. Marraud, Prof. C. Sakarellos and Dr. N. Oikonomakos).

2D-NMR studies and molecular dynamics simulations on the conformation of the α67-76 peptide and its analogues continued this year. It was found that the affinity of the anti-MIR mAb (no. 6) is related to the three-dimensional structure of the N-terminal hexapeptide, and is favored when flexibility of the C-terminal fragment is sufficient to allow the maximum number of close contacts with the antibody. The most probable conformation of the MIR decapeptide in contact with the anti-MIR mAb was determined and was suggested that this structure is probably very similar to that in the intact AChR (publications 3,5).

A detailed understanding of antigen - antibody interactions requires elucidation of the three-dimensional structure of the antibody combining sites, both in the free state and as complexes with the antigen. The principal method for arriving at this picture is high-resolution X-ray diffraction analysis of crystals of antibodies and co-crystals of antigen-antibody complexes. Thus, we aim to the 3-D structural determination of anti-MIR Fab and Fv crystals and their cocrystals with the MIR decapeptide α67-76.

We have established conditions for reproducible production of crystals of Fabs from two anti-MIR mAbs, though still of small size. Low resolution X-ray diffraction photographs have been already obtained. In the course of crystallizing these Fabs it was found that success in obtaining larger crystals was determined primarily by the purity of the proteins and that the microheterogeneity was very detrimental to crystallization. Work is in progress aiming to improve the size and diffraction properties of the crystals as well as to co-crystallize the Fabs together with the synthetic MIR peptide. These studies will be instructive in designing the above mAb manipulations by molecular biology approaches.

4. Precise mapping of the epitopes for mAbs to the cytoplasmic side of the AChR δ-subunit.

By the use of the Pepscan technique we synthesized many continuously overlapping peptides corresponding to the sequence 322-469 of the Torpedo and human AChR δ-subunit. The precise limits of the epitopes for 17 mAbs were thus determined. 11 anti-δ-subunit mAbs bound to the same amphipathic octapeptide of the *Torpedo* and human α-subunit (δ352-359). Synthesis of many single-residue analogues of this peptide uncovered small but characteristic differences in the binding requirements of each of the 11 mAbs (Tzartos and Valkana, in preparation).

5. Pathogenicity of the MIR and its protection against myasthenic sera. In vivo experiments.

The mechanisms involved in the pathogenicity of the anti-MIR mAbs were investigated. Experimental animals were injected with intact mAbs, Fab and $F(ab)_2$ fragment of anti-MIR mAbs. Intact mAbs and bivalent $F(ab)_2$ fragment efficiently induced MG symptoms. Univalent Fab did not induce MG. The fact that $F(ab)_2$, which do not bind complement but induce antigenic modulation of the AChR are also capable of inducing experimental MG suggests that antigenic modulation by the anti-MIR mAbs is sufficient to induce MG. Nevertheless, the much lower efficiency of these mAbs, as compared with that of the intact mAbs, confirms that complement also plays a critical role in the pathogenicity of the anti-MIR mAbs. (Loutrari and Tzartos, submitted).

In another set of experiments we tried to protect the animals against the activity of injected human MG sera or of mAbs by shielding the MIR of their AChR by non-pathogenic Fab of anti-MIR mAbs. Apparently, due to the relatively low affinity of the Fab for AChR, they did not succeed in protecting the animals against the disease. We shall repeat the protection experiments when we construct recombinant mutant Fv proteins of high affinity for the AChR.

PUBLICATIONS

1. Tzartos, S.J. and Remoundos, M.S. (1990). Fine localization of the major α-bungarotoxin binding site to residues α189-195 of the Torpedo acetylcholine receptor. Residues 189, 190 and 195 are indispensable for binding. J. Biol. Chem. 265, 21462-7.
2. Tzartos, S.J., T., Cung, M.T., I. Kordossi, A. Loutrari, E., Marraud, M., Papadouli, I., Sakarellos, C., Sophianos, D. and V. Tsikaris (1990). Myasthenia gravis. Studies with monoclonal antibodies against the acetylcholine receptor. Rev. Clin. Pharmacol. and Pharmacokinetics. 4, 148-163.
3. Cung, M.T., Demange, P., Marraud, M., Tsikaris, V., Sakarellos, C., Papadouli, I., Kokla, A., Tzartos, S.J. (1991). Two-dimensional ^{1}H-NMR study of antigen-antibody interactions: binding of synthetic decapeptides to an anti-acetylcholine receptor monoclonal antibody. Biopolymers. 31, 769-776.
4. Tzartos, S.J., Cung, M.T., Loutrari, H., Mamalaki, A., Marraud, M., Papadouli, I. and Sakarellos, C. (1991) The main immunogenic region (MIR) of the nicotinic acetylcholine receptor and the anti-MIR antibodies. Molecular Neurobiology, Vol. 5, in press.

5. Cung, M.T., Tsikaris, V., Demange, P., Papadouli, I., Tzartos, S.J., Sakarellos, C., and Marraud, M. (1992) 2D-NMR and molecular dynamics analysis of the *Torpedo* acetylcholine receptor α67-76 fragment and of its Ala^{76}-analogue. Peptide Research in press.

Athens, March 5, 1992

The Principal Investigator

Socrates Tzartos

The General Director of the Hellenic Pasteur Institute

Demetris Rapakoulias

FINANCIAL REPORT - 1991

UNIDO/ICGEB Collaborative Research Programme 1988 (CRP/GRE88-05)
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PROJECT TITLE:
STRUCTURE AND PATHOGENICITY OF THE ACETYLCHOLINE RECEPTOR
UNIDO/ Project No. GE/GLO/89001 GRE88-05

Principal Investigator:
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	1991 (US$)	
	Original budget	Used funds
SMALL EQUIPMENT (pipetmen, vortex and magnetic stirrer)	5,600	2,550
CONSUMABLES & CHEMICALS	6,200	8,530
EDUCATION & TRAINING	1,700	2,320
LITERATURE	1,200	1,180
OTHER EXPENSES	1,800	1,920
TOTAL	16,500	16,500

Athens, March 5, 1992

Principal Investigator:

Socrates Tzartos

Financial Officer

ΕΛΛΗΝΙΚΟΝ ΙΝΣΤΙΤΟΥΤΟΝ ΠΑΣΤΕΡ ΑΘΗΝΑΙ

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