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differed between NMDA and KA (Fig. 3c, d), and in individual spinal cord neurones KA-evoked increases in [Ca\(^{2+}\)] were always much smaller than those evoked by NMDA. These experiments suggest that Na\(^{+}\) is a poor trigger for inducing an increase in [Ca\(^{2+}\)], since in several neurones the inward (Na\(^{+}\)) current activated by KA produced no detectable arsenazo III. These currents activated by KA produced no detectable arsenazo III signals reported here. This reflects interactions between permeant ions within the channel with Ca\(^{2+}\) acting as both a permeant ion and as a blocker of monovalent cation flux.

The experiments reported here provide evidence for an agonist-triggered increase in [Ca\(^{2+}\)] in mammalian spinal cord neurones. Previously, ion-sensitive microelectrodes were used to measure changes in intracellular ionic activity triggered by excitatory amino acids in frog motoneurones. The latter experiments suggested an increase in both [Na\(^{+}\)] and [Ca\(^{2+}\)] during perfusion with L-glutamate but the results were difficult to interpret clearly as (1) neurones were not voltage-clamped and thus it is difficult to separate the relative contributions of Ca\(^{2+}\) influx via voltage-dependent calcium channels and agonist-activated channels, and (2) L-glutamate is a mixed agonist that acts at multiple subtypes of excitatory amino acid receptors.

The response to NMDA-receptor activation thus provides a second source of calcium flux, distinct from that resulting from conventional voltage-dependent calcium channels, which may have important long-term effects on excitability. Our finding that the ion channels linked to the NMDA receptor subtype are more permeable to Ca\(^{2+}\) than those linked to KA receptors, has implications for the role of excitatory amino acid receptors in CNS function. It is possible that Ca\(^{2+}\) influx activated by NMDA receptors underlies the synaptic plasticity generating long-term potentiation, as the latter is prevented by intracellular injection of EGTA to chelate Ca\(^{2+}\) (ref. 29), or by blocking NMDA receptors with selective antagonists. For example, Ca\(^{2+}\) influx localized at transmitter-operated ion channels could have a role in organizing and regulating postsynaptic structures in an appropriate spatial relation to transmitter-releasing presynaptic terminal boutons, and it is important to consider that Ca\(^{2+}\) influx occurring at NMDA receptors located on dendritic spines might produce an especially large but localized elevation in intracellular Ca\(^{2+}\) concentration, due to restriction of Ca\(^{2+}\) diffusion along the narrow shaft of the spine. In addition, our results have some bearing on the mechanisms of desensitization of NMDA receptors, as the link that has been demonstrated between [Ca\(^{2+}\)] and desensitization of nicotinic receptors at the neuromuscular junction may occur also for other receptor-ionophore complexes. Thus our results may help to explain the similar desensitization evoked by either large doses of NMDA or depolarizing voltage jumps, which trigger Ca\(^{2+}\) entry through NMDA channels and voltage-dependent calcium channels, respectively.

Received 3 January; accepted 1 April 1986.


Replacing the complementarily-determining regions in a human antibody with those from a mouse

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The variable domains of an antibody consist of a β-sheet framework with hypervariable regions (or complementarity-determining regions—CDRs) which fashion the antigen-binding site. Here we attempted to determine whether the antigen-binding site could be transplanted from one framework to another by grafting the CDRs. We substituted the CDRs from the heavy-chain variable region of mouse antibody B1-8, which binds the hapten NP-cap (4-hydroxy-3-nitrophenacetyl caproic acid; \(\text{K}_{\text{NP-cap}} = 1.2 \times 10^{9} \text{M}^{-1}\)), for the corresponding CDRs of a human myeloma protein. We report that in combination with the B1-8 mouse light chain, the new antibody has acquired the hapten affinity of the B1-8 antibody (\(\text{K}_{\text{NP-cap}} = 1.9 \times 10^{9} \text{M}^{-1}\)). Such 'CDR replacement' may offer a means of constructing human monoclonal antibodies from the corresponding mouse monoclonal antibodies.

The three-dimensional structures of several immunoglobulins show that the variable domains consist of two β-sheets pinned together by a disulfide bridge, with their hydrophobic faces packed together. The individual β-strands are linked by loops which at one tip of the β-sheet may fashion a binding pocket for small haptenes. Sequence comparisons among heavy- and light-chain variable domains (\(\text{V}_{\text{H}}\) and \(\text{V}_{\text{L}}\), respectively) reveal that each domain has three CDRs flanked by four relatively conserved regions (framework regions—FRs). As seen in the structure of the human myeloma protein NEWM (Fig. 1), the CDRs include each of the three main loops. Often the CDRs also include the ends of the β-strands, suggesting that side chains at the ends of the β-strands may help to fix the conformation or orientation of the loops. The framework regions form the bulk of the β-sheet, although for example in the \(\text{V}_{\text{H}}\) domain of NEWM, FR1 includes part of the loop between the two β-sheets and CDR2 not only forms a loop but a complete β-strand (Fig. 1). The structure of the β-sheet framework is similar in different antibodies, as the packing of different side chains is accommodated by slight shifts between the two β-strands. Furthermore, the packing together of \(\text{V}_{\text{H}}\) and \(\text{V}_{\text{L}}\) FRs is conserved, therefore the orientation of \(\text{V}_{\text{L}}\) with respect to \(\text{V}_{\text{H}}\) is fixed. We wondered whether the FRs represent a simple β-sheet scaffold on which new binding sites may be built, and

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whether the structure of the CDRs (and antigen binding) is therefore independent of the FR context. To answer these questions experimentally, we have grafted the CDRs from one antibody to another, to determine whether antigen binding transfers with the CDRs.

We grafted the CDRs from the VH domain of the mouse monoclonal antibody B1-8 (ref. 7) into the VH domain of the human myeloma protein NEWM, whose crystallographic structure is known1,3. The VH domain of the CDR donor (B1-8) is attached to a μ constant region and associated with a mouse A1 light chain, and the antibody is directed against the hapten NP-cap. Both the VH and VL domains seem to have a role in determining the affinity of the antibody for NP-cap as the substitution of either domain by other, highly related variable domains can destroy hapten binding (refs 7, 9 and M.S.N., unpublished results). In the VH domain, each of the CDRs has been implicated in NP-cap binding1,2, but the class of constant domains attached to VH does not seem to affect binding of hapten1,11. The CDRs from the VH domain of antibody B1-8 (ref. 13) are longer than the CDRs which they replace in NEWM1,3 and this may give rise to a deeper binding pocket.

Most of the residues conserved between the VH domains of B1-8 and NEWM are located in FR2, FR4 and the carboxy-terminal third of FR3 (Fig. 2a) and largely form the region of β-sheet which is packed against the light chain. Therefore, it might be expected that the VH domain of B1-8 (hereafter abbreviated to MVNP) and the hybrid B1-8/NEWM domain (HuVNP) would dock in a similar manner with the mouse VL domain to form the antigen-combining site4. The more variable FR1 and N-terminal two-thirds of FR3 form the other β-sheet which is exposed to solvent (Fig. 1c).

The gene encoding the HuVNp domain was constructed by gene synthesis (Fig. 2b). We then constructed a plasmid, pSV-HuVNpHe, in which the HuVNp domain was linked to a human e constant region, and cloned into a pSV2-gpt-derived vector14. The plasmid DNA was introduced into cells of the J558L mouse myeloma by spheroplast fusion. J558L secretes λ1 light chains which have been shown to associate with heavy chains containing a MVNP variable domain, to create a binding site for NP-cap or the related hapten NIP-cap (3-iodo-4-hydroxy-5-nitrophenylacetyl caproic acid)3. As the plasmid pSV-HuVNpHe contains the gpt marker (encoding guanine phosphoribosyltransferase), stably transfected myeloma cells could be selected in medium containing mycophenolic acid14; transfectants would be expected to secrete an antibody (HuVNp-IgE) with a heavy chain composed of a HuVNp variable domain and human e constant regions, and the λ1 light chain of the J558L myeloma. The culture supernatants of several gpt' clones were assayed by radioimmunoassay and found to contain NIP-cap-binding antibody. The antibody secreted by one such clone was purified from the culture supernatant by affinity chromatography on NIP-cap-Sepharose, and by SDS-polyacrylamide gel electrophoresis the protein was indistinguishable from the mouse chimeraic MVNP-IgE (ref. 12) (results not shown). The HuVNp-IgE antibody competes effectively with MVNP-IgE for binding to both anti-human e (Fig. 3a) and NIP-cap coupled to bovine serum albumin (NIP-BSA) (Fig. 3b).

The affinities of HuVNp-IgE for NP-cap and NIP-cap were
The maximum quench was varied from 0% to 100% and divided into FRs and CDRs according to Kabat et al. and nucleotide sequence of the residues are marked with a line above and below the residue. VH domain encoding the coding sequence is derived from the synthetic oligonucleotides therefore differ from the method. The synthetic gene for using the computer program ANAL blocks (A-D and A' - D') containing oligonucleotides 1, 3, 5 and 7 (block A), 2, 4, 6 and 8 (block A'), 9, 11, 13a and 13b (block B), 10a, 10b and 12/14 (block B'), 15 and 17 (block C), 16 and 18 (block C'), 19, 21, 23 and 25 (block D), and 26, 22, 24, 26a and 26b (block D'). In a typical assembly, for example, by sequence of block A, 50 pmol of oligonucleotides 1, 3, 5 and 7 were phosphorylated at the 5' end with T4 polynucleotide kinase and mixed with 5 pmol of the terminal oligonucleotide which had been phosphorylated with 5 µCi of [γ-32P]ATP (Amersham; 3000 Ci mmol⁻¹). These oligonucleotides were annealed by heating to 80°C and cooling to room temperature over 30 min with unkinased oligonucleotides 2, 4 and 6 as splints in 150 µl of 50 mM Tris-HCl pH 7.5, 10 mM MgCl₂. For the ligation, ATP (1 mM) and dithiothreitol (10 mM) were added, together with 0.1 µg of T4 DNA ligase (Anglian Biotechnology Ltd), and the mixture was incubated for 30 min at room temperature. EDTA was added to 10 mM, the sample extracted with phenol, precipitated from ethanol, dissolved in 20 µl of water and boiled for 1 min with an equal volume of formamide dye. Then the sample was loaded onto a thin (0.3 mm) 8 M urea/10% polyacrylamide gel and a band of the expected size detected by autoradiography and eluted by soaking. The two full-length single strands were assembled from A-D and A'-D' using splint oligonucleotides; thus, A-D were annealed and ligated in 30 µl as above with 100 pmol each of oligonucleotides 10a, 16 and 20 as splint, then incubated overnight (A'-D' were constructed with oligonucleotides 7, 13b and 17 as splints). After phenol/ether extraction blocks A-D were annealed with blocks A'-D', small amounts were cloned in the vector M13mp8 (ref. 28) then cut with PstI and HindIII and the gene sequenced by the double-strand technique. The MVNP gene was transferred as a HindIII–BamHI fragment from the vector pSV-NRP (ref. 12) to the vector M13mp8 (ref. 30). To facilitate the replacement of MVNP coding sequences by the synthetic HuVNp sequence, then HindIII sites were removed from the 5' noncoding sequence by site-directed mutagenesis, and a new HindIII site was subsequently introduced near the end of FR4. By cutting the vector with PstI and HindIII, most of the coding sequence falls out and the fragment could be introduced as a PstI–HindIII fragment. The sequence at the HindIII site was corrected to give NEWM FR4 by site-directed mutagenesis. The HindIII–BamHI fragment, now carrying the HuVNp gene, was excised from M13 and cloned back into pSV-NRP to replace the MVNP gene (and yield the vector pSV-HuVNp). Finally, the heavy-chain constant domains of human IgE (ref. 26) were introduced as a BamHI fragment to yield the vector pSV-HuVNp, which was transfected into the myeloma line J558L by electroporation. The sequence of the HuVNp gene in pSV-HuVNp was checked by re-cloning the HindIII–BamHI fragment back into M13mp8 (ref. 30).

**Table 1** Affinity of HuVNp-IgE and MVNP-IgE for the hapten (NCAP and NPCAP)

<table>
<thead>
<tr>
<th>Hapten</th>
<th>K_{NCAP} (µM)</th>
<th>K_{NPCAP} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MVNP-IgE</td>
<td>1.2 ± 0.1</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>HuVNp-IgE</td>
<td>1.9 ± 0.2</td>
<td>0.07 ± 0.02</td>
</tr>
</tbody>
</table>

The affinity of HuVNp-IgE and MVNP-IgE for NPCAP was determined by fluorescence quenching with excitation at 295 nm and emission observed at 340 nm (ref. 22). Antibody solutions were diluted to 100 nM in phosphate-buffered saline, filtered (0.45 µm-pore cellulose acetate) and titrated with NPCAP in the range 0.2–20 µM. As a control, the mouse D1-3 antibody, which does not bind hapten, was titrated in parallel. A decrease in the ratio of the fluorescence of HuVNp-IgE or MVNP-IgE (as appropriate) to that of the D1-3 antibody was taken as being proportional to NPCAP occupancy of the antigen-binding sites. The maximum quench was 40% for both HuVNp-IgE and MVNP-IgE, and hapten dissociation constants were determined from least-squares fits of triplicate data sets to a hyperbola. The concentration of NPCAP was varied from 10 to 300 nM, and ~50% quenching of fluorescence was observed at saturation. As the antibody concentrations were comparable to the values of the dissociation constants, data were fitted by least-squares to an equation describing tight binding inhibition.

then measured directly using the fluorescence quench technique, and compared with those of MVNP-IgE (Table 1). The antibodies HuVNp-IgE and MVNP-IgE have similar affinities for either hapten (NPCAP or NPCAP), and although the affinity of HuVNp-IgE for both hapten is slightly lower than that of MVNP-IgE (2–3-fold, 0.3–0.6 kcal mol⁻¹), the difference in affinity is less than expected for loss of either a hydrogen bond or van der Waals' contact from the active site of an enzyme. It seems that binding affinity and specificity for hapten can be conferred on a human antibody by grafting in the CDRs from an appropriate mouse antibody.

Is this result likely to be general? This would assume (1) that antigen usually binds to the CDRs, and any contacts to the FRs are made to the polypeptide backbone or to conserved side chains, and (2) that substitutions in the FRs do not usually affect the conformation of the CDR loops. These assumptions seem reasonable: thus, in the structure of a complex of the D1-3 antibody with lysozyme (R. A. Mariuzza, S. Phillips and R. J. Poljak, personal communication) most contacts to the lysozyme are made by the CDRs but there is also a hydrogen bond in FR1 of the Vκ domain from the β-OH of Thr 30 (often conserved or replaced by Ser). Similarly, the conformation of CDR loops
between β-strands depends on loop size and specific interactions of the loop back to the β-sheet. However, in the same class of variable domains (VH or VL) these interactions are usually conserved (ref. 5 and A. M. Lesk and C. Chothia, personal communication).

While human monoclonal antibodies have therapeutic potential in human disease, they can be difficult to prepare and treatment of patients with mouse monoclonal antibodies often increases the titre of circulating antibody against the mouse immunoglobulin. As chimeric antibodies containing human constant domains (Cm) and variable domains made by grafting mouse CDRs into human FRs, could have therapeutic potential, we wondered whether the HuVNp-lgE antibody loses antigenic determinants associated with the MVnp variable region (idiotypes). The binding of HuVNp-lgE and MVnp-lgE to both monoclonal and polyclonal anti-idiotypic antibodies directed against the MVnp domain was examined by using inhibition assays. As shown in Fig. 3d, the HuVNp-lgE antibody has lost the MVnp idiotype determinant recognized by antibody Ac38 (ref. 21). Furthermore, HuVNp-lgE also binds the antibody Ac38 (ref. 21) less well (Fig. 3c), therefore it is not surprising that HuVNp-lgE has lost many of the determinants recognized by a polyclonal rabbit anti-idiotypic antiserum (Fig. 3e). While the loss of idiotypic determinants that accompanies ‘humanizing’ of the VH region is reassuring in view of potential therapeutic applications, it does suggest that the recognition of the hapten and of anti-idiotypic antibodies is not equivalent. Thus the HuVNp-lgE antibody retains hapten binding but has lost idiotypic determinants, indicating that the immunoglobulin uses different sites to bind hapten and anti-idiotypic antibodies. It appears, therefore, that both FR and CDR side chains form the binding site for these anti-idiotopes, but mainly CDR side chains interact with hapten.

We thank C. Milstein for suggesting this project, K. Rajewsky and M. Reth for the anti-idiotypic antibodies Ac38 and Ac146, and A. M. Lesk, C. Chothia, R. J. Leatherbarrow and C. Milstein for helpful discussions. J.F. is a Fellow of the Jane Coffin Childs Memorial Fund for Medical Research.

Received 17 February; accepted 17 March 1986.