

Detection of substance P in the central nervous system by a monoclonal antibody

(neuropeptides/immunocytochemistry/radioimmunoassay)

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ABSTRACT Peptides with transmitter-like characteristics are being found in many brain areas. The application of immunocytochemical and radioimmunoassay methods has contributed much to the clarification of these neuronal systems. Here we report the development of a rat monoclonal antibody produced by a hybrid myeloma and its application to the study of one of these peptides, substance P. The hybrid clone, isolated after fusion of mouse myeloma cells with hyperimmune rat spleen cells, allowed us to obtain a standardized and permanent source of monoclonal substance P antibodies in a culture cell system. This antibody recognizes the COOH-terminal part of substance P in radioimmunoassay down to 10–20 fmol. It does not crossreact with other known mammalian brain peptides tested. By immunofluorescence the antibody was shown to bind specifically and with a remarkably low background to nerve terminals and cell bodies located in clearly defined nuclear organizations of the central nervous system.

There is among neurobiologists an increasing interest in the functional significance of naturally occurring peptides that resemble the known neurotransmitters in their distribution, cellular localization, and release characteristics (see reviews 1–3). Among these peptides, substance P and the endorphins have received considerable attention for their possible implication in pain mechanisms and behavior. Substance P has been clearly associated with the transmission of primary sensory information in the spinal cord (4–6). This peptide also seems to be related to a variety of other neuronal pathways (7, 8) and it has been reported to be located in synaptic vesicles (9, 10) and larger membrane-bound cellular elements (11). The peptide seems to coexist in the same neuron with another presumptive neurotransmitter, 5-hydroxytryptamine (12, 13). Much of the present knowledge about the occurrence and function of these peptides derives from the application of immunological techniques. But conventional antibodies have serious limitations. They constitute a heterogeneous population of antibody molecules that vary from preparation to preparation, making the standardization of reagents an impossible task. The application of monoclonal antibodies produced by hybrid myelomas overcomes these limitations (14). This technique permits the derivation of permanent cell lines capable of continuous production of highly specific antibodies. The derivation and use of a monoclonal antibody to substance P are described in this paper.

MATERIALS AND METHODS

Immunization Procedure. Substance P (Peninsula Laboratories, San Carlos, CA) was conjugated to bovine serum albumin with carbodiimide as coupling agent (Sigma). Six intraperitoneal injections of 0.3 or 3 μ g of the immunogen dis-

solved in complete Freund's adjuvant were given over a period of three months to male adult Wistar rats. Periodical bleedings were tested by a radioimmunoassay. An antibody response was detected only in rats receiving 3- μ g doses, and one of these was injected intravenously with 3 μ g of the conjugate in saline and sacrificed 72 hr later. The spleen was immediately used for cell fusion.

Cell Fusion. Spleen cells were fused with NS1/1-Ag 4-1 (NS1), a cell line derived from the mouse myeloma MOPC 21, which is resistant to 20 μ g of 8-azaguanine per ml and does not secrete IgG chains but contains intracellular chains (15). The fusion protocol and the growth of hybrids in selection medium were as described (16). Immediately after fusion the cells were distributed in 96 wells (Linbro BCL-50h). Culture supernatants were periodically assayed for the presence of anti-substance P antibodies as detailed below. Selected cultures were cloned in soft agar as described (17).

Analysis of Products Secreted by Myeloma Hybrids. Cells from the cloned line were incubated for 24 hr with [14 C]lysine medium, and the supernatants were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis as described (15).

Concentration and Partial Purification of Antibody. The hybrid clone NCl/34 HL was adapted to grow in Dulbecco's modified Eagle's medium (DMM) containing only 2.5% fetal calf serum. Cells were allowed to grow to stationary phase and the supernatant fluid was precipitated by addition of solid $(\text{NH}_4)_2\text{SO}_4$ to 50% saturation. The precipitate was dissolved in about 1/50th of the volume of original supernatant with phosphate buffered saline, pH 7.4 (P_i/NaCl), and dialyzed three times against 50 vol of P_i/NaCl . This is referred to as Ig fraction.

Radioimmunoassay. [Tyr^8]Substance P (Peninsula Laboratories) was iodinated (^{125}I , The Radiochemical Centre, Amersham, England) by the chloramine-T method (18). The assay (19) was carried out under nonequilibrium conditions at 4°C in 0.05 M, pH 8.6, barbitone/acetate buffer containing 0.1% gelatin. Synthetic substance P or tissue extracts were preincubated for 4 hr with 1:1000 dilutions of the Ig fraction and further incubated with ^{125}I -labeled [Tyr^8]substance P for 18 hr at 4°C. The bound and unbound labeled substance P were separated by the addition of a 200- μ l suspension of 5.0% charcoal and 5.0% dextran T-70 in barbitone/acetate buffer (0.05 M, pH 8.6). After 30 min at 4°C the charcoal/dextran absorbed peptide (free) was separated from the supernatant (bound) by centrifugation at 1500 \times g for 7 min and the radioactivity was measured in a gamma counter.

Immunocytochemistry. Male adult Wistar rats weighing approximately 250 g were anaesthetized with equithesin and perfused through the heart with cold 4% (wt/vol) paraform-

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Abbreviation: P_i/NaCl , phosphate-buffered saline.

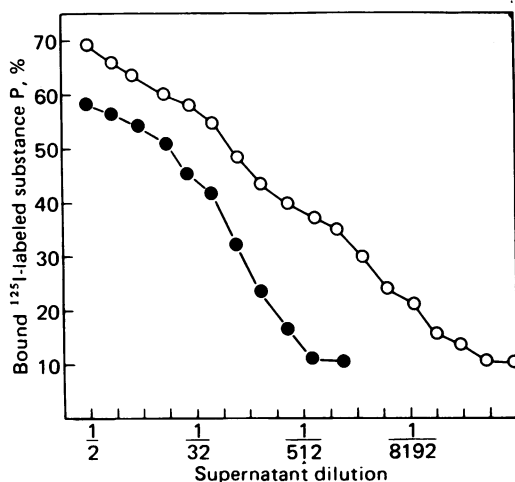


FIG. 1. Binding of substance P to NCl/34. ●, Cell culture medium (NCl/34 antibody). ○, Concentrated partially purified preparation from the same culture medium (Ig fraction). For this experiment the radioimmunoassay procedure conditions were used, omitting incubations prior to the addition of radiolabeled substance P.

aldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The brains were removed, kept in fixative for 2–4 hr, left overnight in the same buffer containing 5% sucrose, and cut into 10- μ m-thick sections on a cryostat at -20°C . Sections were immersed in P_i/NaCl containing 0.2% Triton X-100 for 15 min at room temperature and then for 30 min at 37°C , either in 1:40 dilutions of the Ig fraction of NCl/34 in $\text{P}_i/\text{NaCl}/\text{Triton X-100}$ or, as controls, in the same antibody preparation previously adsorbed with 200 μg of substance P per ml. Assays were also performed with crude culture media from positive and negative clones. Sections were washed several times in $\text{P}_i/\text{NaCl}/\text{Triton X-100}$ and further incubated with fluorescein isothiocyanate-conjugated rabbit immunoglobulin specific for rat IgG (Miles–Yeda) diluted in $\text{P}_i/\text{NaCl}/\text{Triton X-100}$ for 40 min at 37°C . The sections were mounted in 3:1 glycerol/ P_i/NaCl (vol/vol) after several washes and examined under a Leitz Sm-Lux or a Leitz Dialux 20 microscope equipped with a 50-W high pressure mercury lamp and epifluorescence optics. The following (Leitz) filter combination was used in a single block: excitatories KP 590 \times 2 plus GG 455, suppressor K 515, and a dichroic beam splitting mirror TK 510. Photographs were taken

with a Leitz Orthomat camera with Kodak 400 ASA Tri-X panchromatic film.

RESULTS

Two weeks after fusion of myeloma and spleen cells, vigorous growth of hybrids was observed in 86 of the 94 wells, and 8 were shown to possess anti-substance P antibodies. Seven of these cultures rapidly lost their activity, but it was possible to freeze active cells from two of them. Clones from one of these, NCl/34, were grown 4 weeks after fusion and of the 17 tested 8 were positive and 9 were negative. Three of the antibody-producing clones were selected and two, NCl/34.11 and NCl/34.15, were recloned.

The secreted products of four subclones (NCl/34.11.1, NCl/34.11.4, NCl/34.15.2, and NCl/34.15.3) were analyzed to check for clonal homogeneity and antibody secretion. All subclones appeared identical and all secreted two antibody-specific immunoglobulin chains, a γ heavy chain (not expressed in the myeloma NSI parent) had a light chain different from that of the parental myeloma, indicating that the selected clones did not express the parental myeloma light chains. Clone NCl/34.11.1 was used in all the subsequent studies and was denominated NCl/34.HL. “Antibody NCl/34” refers in all cases to spent medium. Partially purified concentrates of it are described as “Ig fraction.”

The binding of substance P to antibody NCl/34 and to the Ig fraction is shown in Fig. 1. With 1:1000 dilutions of the IgG fraction, [Leu]enkephalin, [Met]enkephalin, somatostatin, or β -endorphin did not compete with substance P at any of the concentrations investigated, but approximately 5% crossreactivity was found with the related peptide eleidoisin (Fig. 2). The five-, six-, and eight-amino-acid COOH-terminal fragments of substance P and intact substance P were shown to be about equally able to displace labeled substance P (Fig. 2).

In ordinary radioimmunoassay conditions the monoclonal antibody allowed the accurate measurement of nonlabeled substance P to 10–20 fmol. Tissue extracts, as obtained for substance P radioimmunoassay, do not interfere with the assay (results not shown). A linear relationship was observed between the amount of tissue extract and the detectable substance P (Fig. 3).

Sections of rat brain stained by indirect immunofluorescence with NCl/34 antibody (crude supernatants) gave a strong fluorescent reaction in the substantia gelatinosa of the spinal cord,

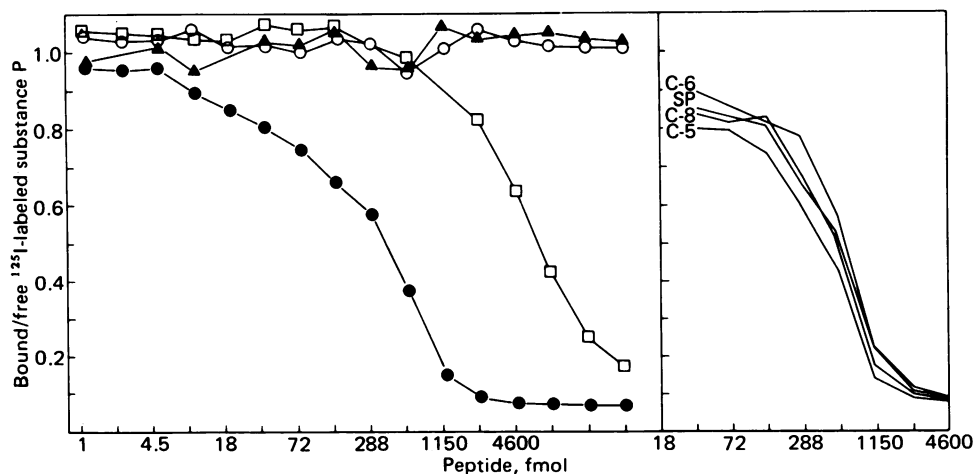


FIG. 2. (Left) Radioimmunoassay for substance P and crossreactivity with other brain peptides. ●, Substance P; ▲, β -endorphin; and ○, somatostatin. [Leu]enkephalin and [Met]enkephalin, also negative, are not included in the figure. A partial crossreactivity is found with the related peptide eleidoisin (□), which contains the same tripeptide sequence in its COOH-terminal region. (Right) Radioimmunoassays for substance P (SP) and identity of the binding of the COOH-terminal fragments of the peptide (C-5, C-6, and C-8) with substance P.

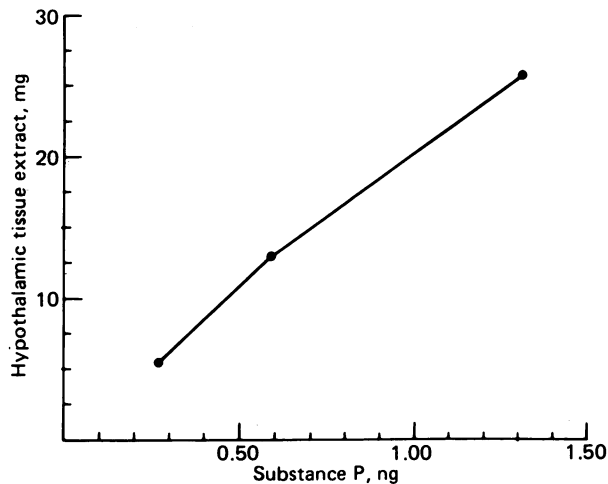


FIG. 3. Application of the monoclonal antibody to the estimation of tissue content of substance P. Linearity of the displacement of the radiolabeled substance P by increasing amounts of tissue extracts.

but this was obscured by an exaggerated fluorescent background. On the other hand, the partially purified Ig fraction resulted in a very intense and remarkably clean immunofluorescent reaction, primarily located in the substantia gelatinosa of the dorsal horn (Fig. 4A). The fibrillar immunofluorescence corresponded well to the terminal field of primary sensory neurones, which has been claimed to contain substance P (4–6). The fluorescence in the neuronal elements was not observed in controls in which the antibody was preincubated with substance P (Fig. 4B). A similar intense reaction was found in many other brain areas such as the substantia nigra of the mesencephalon (Fig. 4C), and the distribution of the fluorescence was largely as described in previous reports (7, 8). However, the fluorescence was more circumscribed and there was less background than that shown by conventional anti-substance P guinea pig sera. In addition, cell bodies were revealed in a variety of regions of the central nervous system after the inhibition of axonal transport by colchicine (Fig. 4D).

DISCUSSION

Monoclonal antibodies produced by hybrid myelomas (14) are potentially superior to conventional antisera in a variety of ways (20). Many hybrid myelomas (21) recognizing cell surface antigens and other complex antigenic structures, such as viruses, have now been prepared. But the potential of the technique for the production of standard laboratory reagents for radioimmunoassay of, for instance, hormones and peptides of pharmacological interest, has not been documented. Furthermore, the usefulness of monoclonal antibodies in the characterization and localization of neurotransmitters or neurotransmitter candidates has not been properly tested. The work described in this paper was intended to explore these aspects. The clone derived is a mouse–rat hybrid and no attempt was made to grow it in immunosuppressed or immunodeficient animals. All the antibody was prepared from culture fluid; this does not contain carrier rat immunoglobulin, which would have been present in the serum of tumor-bearing rats. Indirect immunofluorescence is improved when no carrier rat immunoglobulin is present. This may be one of the reasons why the immunofluorescence was so free from background.

The results demonstrate that the NCl/34 is suitable for radioimmunoassays, but no precise kinetic studies were performed to establish the affinity of the NCl/34 antibody. The method of screening for the presence of antibody-producing clones, involving radioimmunoassay, ensured adequate af-

finity because low-affinity antibodies would have been scored negative. The crossreactivity experiments indicate that the antibody recognizes a determinant located in the COOH-terminal portion of substance P. Complete crossreactivity was obtained with three COOH-terminal fragments of substance P; 5% crossreactivity was obtained with the nonmammalian related peptide eleidoisin, which shares with substance P the three COOH-terminal residues as well as proline in position 2 and phenylalanine in position 7.

The antibody performed well in a nonequilibrium competitive radioimmunoassay and femtomole amounts of the synthetic peptide could be detected. Under the conditions used, tissue extracts did not interfere with the assay. Different groups have reported quite different concentrations of substance P in the central nervous system. For the hypothalamus they were (ng/g): 884–659 (22), 280 (23), and 120 (19). The amount of “substance P” detected in the hypothalamus was therefore $\frac{1}{3}$ or $\frac{1}{10}$ the amount of endogenous “substance P” reported (and also obtained by us, unpublished results) by using conventional sera. A similar discrepancy was also found in preliminary studies with other tissues. Although we do not have a satisfactory explanation for the discrepancies, it seems possible that the material recognized as “substance P” with conventional multivalent sera is not a single molecular entity. Such antisera vary from batch to batch and may recognize other molecules that share with substance P antigenic determinants other than the one recognized by NCl/34. By using NCl/34 as an immunoadsorbant, it should be possible to test the hypothesis and to establish if substance P-like components with pharmacological activity crossreact with conventional antisera.

In immunocytochemistry, NCl/34 was much better than antisera obtained directly from immunized animals. A very clean fluorescent reaction was observed in the areas tested. The reason for such improvement must be at least partly the absence of inactive rat immunoglobulin, but it may also reflect lack of crossreactivity with other components. It will be of interest to compare in detail the distribution of immunoreactive elements in the central nervous system as revealed by NCl/34 and conventional antisera. Crossreactive substances are suggested by both the immunofluorescence and radioimmunoassay results.

NCl/34 has also been used successfully to show depletion of substance P immunoreactivity in the spinal nucleus of the trigeminal nerve after surgical lesions interrupting sensory input[†] and to show building up of axonally transported substance P after colchicine administration (unpublished data) or nerve ligatures (24). It has also been used to demonstrate by immunofluorescence and immunoenzyme methods the presence of neurons with substance P-like immunoreactivity in the pigeon brain (H. J. Karten, personal communication) at dilutions of up to 1:1000 of the Ig fraction.

The hybrid myeloma strategy is applicable to substances that have not been fully characterized, such as neurotransmitter receptor molecules. Antibodies to these substances are generally raised by injecting purified or semipurified preparations that contain impurities of equal or higher immunogenicity. Hybrid myelomas circumvent this problem and may facilitate the preparation of specific antibodies against receptor molecules. The same is true of neuronal surface molecules and “recognition” molecules as well as neurotransmitter biosynthetic enzymes, for which a compromise has to be found between the degree of purification and the preservation of the biological activity. The fact that the monoclonal antibody can be pro-

[†] Del Fiaco, M. & Cuello, A. C. (1978) in *Proceedings of the 7th International Congress of Pharmacology*, Abstr. 2551.

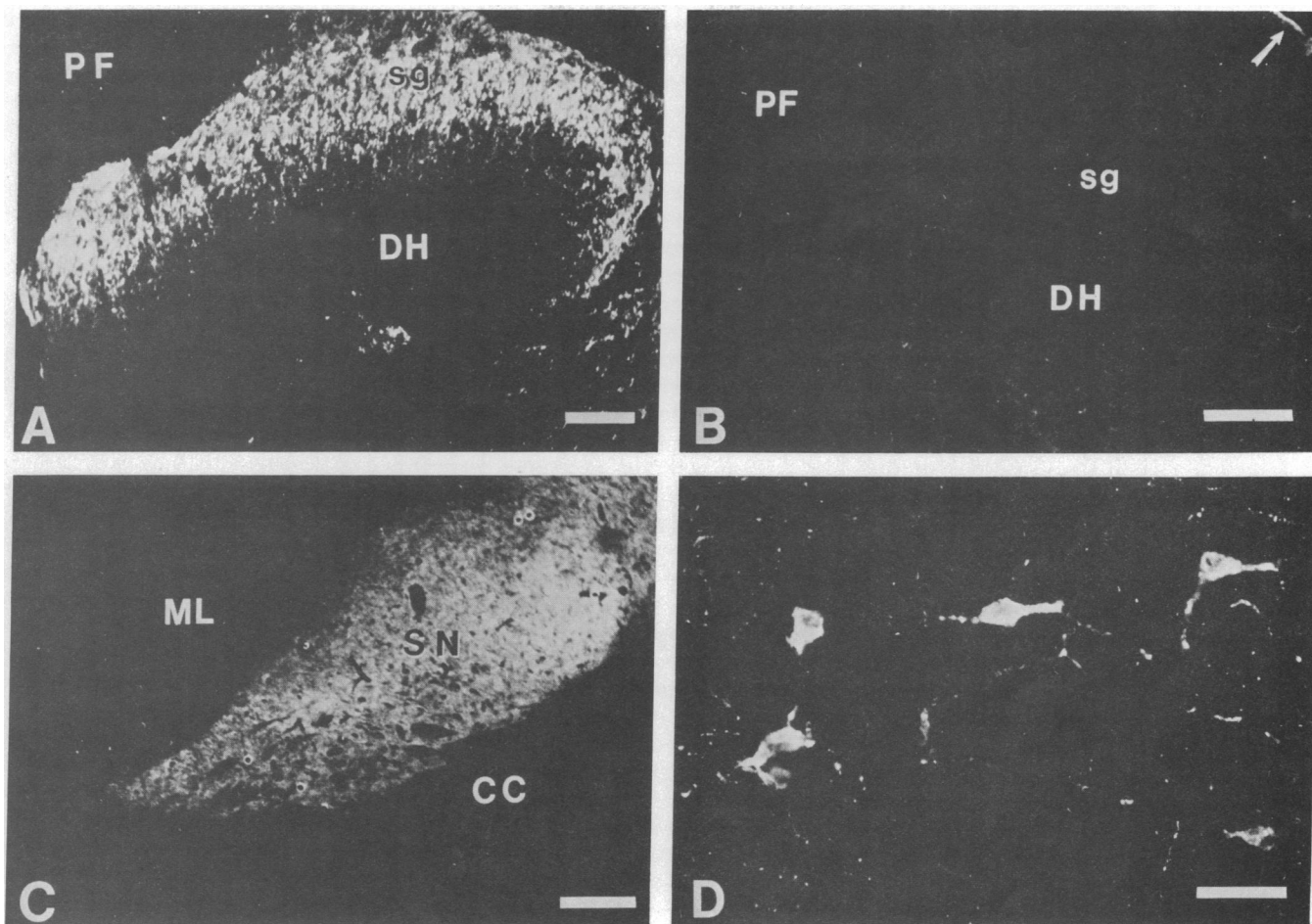


FIG. 4. (A) Section of the rat spinal cord (cervical level). NCI/34, revealed by indirect immunofluorescence, bound to fibrillar elements restricted to the superficial layers of the dorsal horn (DH). PF, posterior funiculus; sg, substantia gelatinosa. Bar = 100 μ m. (B) Section consecutive to A, incubated with NCI/34 Ig fraction preabsorbed with synthetic substance P (220 μ g/ml). Note lack of immunofluorescence in the area of the substantia gelatinosa. Arrow points to some unspecific fluorescence due to excess free second antibody in the pial surface of the spinal cord. Bar = 100 μ m. The unspecific binding is not observed in A. (C) Strong diffuse immunofluorescence of rat substantia nigra (SN) obtained with NCI/34. CC, crus cerebri; ML, medial lemmiscus. Bar = 100 μ m. (D) Neuronal cell bodies and processes after colchicine treatment (50 μ g/2 μ l intraventricularly) and immunocytochemistry with NCI/34, Ig fraction. The section is through the lateral reticular formation of the rat medulla oblongata. (From M. Del Fiocco.) Bar = 50 μ m.

duced perpetually is of great value for a standard reagent, particularly when it may be difficult to reproduce immunological results. NCI/34 and other monoclonal antibodies could eventually be adapted to electron microscopical studies or to techniques such as radioimmunoassay with labeled antibody or immunoradioautography using internally labeled antibody prepared in culture.

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