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Advantages of bispecific hybridomas in one-step immunocytochemistry and immunoassays

(bispecific monoclonal antibodies/substance P)

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ABSTRACT **A chemical selection procedure has been used to prepare a hybrid hybridoma cell line (P4C1) following fusion of two previously established hybridomas secreting antiperoxidase and antisubstance P, respectively. P4C1 secretes bispecific monoclonal antibody alongside the two parental antibodies, with no visible inactive heterologous heavy-light chain pairs. The bispecific monoclonal antibody is thus easy to purify in excellent yields. The advantage of its monovalency for one antigen and simultaneous binding of a marker enzyme has been explored for its potential use in competitive immunoassays. Its use in immunocytochemistry led to major improvements in sensitivity, signal-to-noise ratio, simplification of staining procedures, and ultrastructural preservation of subcellular elements. Particularly remarkable was that, unlike conventional procedures, the immunoreaction with the bispecific monoclonal antibody was homogeneously distributed across the entire thickness of a 50-μm section.**

Hybrid hybridomas are derived by fusing two (or more) antibody-producing cells. The resulting cells secrete hybrid immunoglobulins exhibiting the binding characteristics of the two individual parental hybridomas in a single molecule (1, 2). These bispecific monoclonal antibodies (bs mAb) are structurally bivalent but functionally univalent for each combining site.

The potential of such hybrid molecules has been studied with the antisomatostatin-antiperoxidase hybrid hybridoma P4C7 (1, 2). This was derived by fusing spleen cells from immunized rats and a hypoxanthine/aminopterin/thymidine-sensitive antiperoxidase rat hybridoma. Although this approach offers several advantages, it is apparent that quality and affinity of the derived bs mAb remains a matter of chance and depends on the contribution of the spleen partner. It was desirable to derive hybrid hybridomas derived from two well-characterized hybridomas to compare their performance relative to that of the parental lines. This paper describes the use of a chemical selection procedure to generate a hybrid hybridoma derived from the fusion of a well-characterized antisubstance P (anti-SP) hybridoma (NC1/34) (3)¶ and an antiperoxidase hybridoma (YP4) (1). The derived antiperoxidase-anti-SP bs mAb (P4C1) was found to be better for the histochemical detection of antigenic sites in a single-step incubation than the mAb NC1/34 developed by peroxidase-antiperoxidase (PAP) methods (4).

MATERIALS AND METHODS

Materials. SP, horseradish peroxidase (HRP), 4-chloro-1-naphthol, emetine, and actinomycin D were from Sigma. Nitrocellulose sheets were from Schleicher & Schuell. SP was conjugated to bovine serum albumin (BSA) using glutaraldehyde (5).

Fusion Protocol. Fusion was between hybridomas YP4 (1) [a rat antiperoxidase hybridoma (IgG1)] and NC1/34 anti-SP (a rat–mouse hybrid secreting rat IgG2a). The sensitivity of NC1/34 to emetine—an irreversible inhibitor of protein synthesis—was determined. Typically, 10^6 cells were incubated with doubling dilutions of the drug for 1 hr at 37°C in 10 ml of Dulbecco's modified Eagle's medium (DMEM). The cells were collected by centrifugation, washed, resuspended in 10 ml of DMEM at 37°C for 30 min to allow efflux of any residual free drug in the cell, and plated. Critical drug concentration was defined as <1% survival of the cells, beyond which no cell growth was observed. For NC1/34 and YP4, this was 200 μM emetine and 100 ng of actinomycin D per ml, respectively. For fusion, 5×10^7 cells in 50 ml of DMEM were thus treated for 1 hr, and following the efflux phase, the cells were mixed in a 1:1 ratio and fused with 1 ml of 45% PEG (M_r 1400, Merck)/10% dimethyl sulfoxide (Me_2SO) in DMEM as described (6, 7). The washed pellet was incubated in 50 ml of 20% DMEM for 4–6 hr, and an aliquot was cloned on the same day on soft agarose at four dilutions. About 100 clones were expanded in 24-well plates with 10% DMEM. Supernatants were assayed and the best were recloned at least twice.

bs mAb Assay. The principle of the assay is described in Fig. 1. About 2–5 μl of antigen (100 μg/ml, SP-BSA) was spotted on a nitrocellulose sheet at the center of 1 × 1 cm squares and dried for 5 min. The sheet was blocked with 5% BSA in phosphate-buffered saline (PBS) for at least 10 min and gently pressed between two filter papers, and 2–5 μl of the hybrid hybridoma supernatants was spotted on top of the antigen. After 2–5 min, the sheet was washed three times with PBS, incubated for 15 min with HRP (20 μg/ml in PBS/5% BSA), washed three times with PBS, and developed with 0.4 mg of 4-chloro-1-naphthol per ml/0.03% H_2O_2.

Molecular Analysis of Hybrid Hybridoma Immunoglobulins. The selected clones were expanded for the large-scale production of serum-free supernatants (7). The proteins were precipitated with 100% saturated $(NH_4)_2SO_4$, dialyzed against 10 mM Na_2HPO_4 (pH 7), loaded on a DE-52 column, and eluted with a linear gradient of 10–100 mM Na_2HPO_4 (pH 7.5). Three major peaks were identified—the middle peak exhibiting the bs mAb activity, the two others exhibiting the

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Abbreviations: bs mAb, bispecific monoclonal antibody(ies); HRP, horseradish peroxidase; H, heavy; L, light; BSA, bovine serum albumin; PAP, peroxidase-antiperoxidase; Me_2SO, dimethyl sulfoxide; CNS, central nervous system; SP, substance P.

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¶Since NC1/34 recognizes the C-terminal portion of SP (3), SP immunoreactivity may include other neurokinins having the same C-terminal structure.

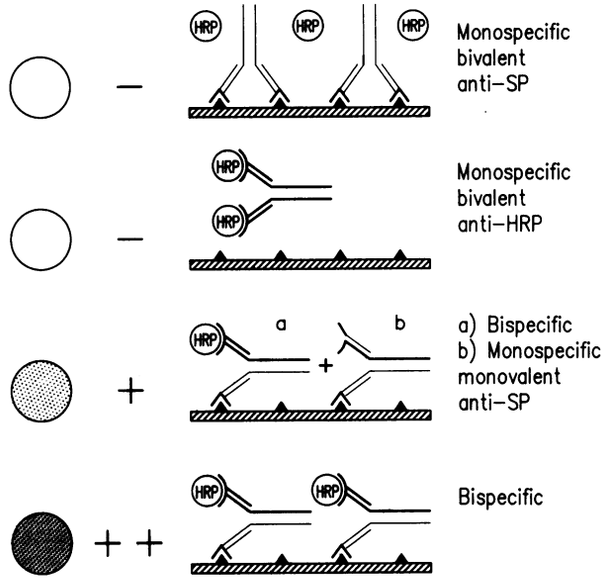

FIG. 1. bs mAb assay. Only anti-SP antibodies bind to immobilized SP. The bs mAb binds with one arm and the other is available for peroxidase binding. This is revealed by 4-chloro-1-naphthol and H_2O_2. Heterologous L–H-chain associations give rise to inactive species that are competitive inhibitors of the bispecific reaction. Specific examples of the use of these reactions are shown in Fig. 2.

two parental immunoglobulin activities. Other procedures were as described (2, 6).

Immunohistochemical Procedure. For screening, the substantia gelatinosa of the spinal trigeminal nucleus of the rat was chosen. Tissues were treated as described (3). Tissue samples were cut at 10 μm in a cryostat (−20°C) and mounted on coated glass slides. Staining was as follows. (*i*) Rinse with 0.1 M PBS (pH 7.2) containing 0.02% Triton X-100 for 10 min at room temperature and incubate overnight with hybridoma supernatants (or NC1/34). Supernatants were diluted 1:1 in 0.1 M phosphate buffer/Triton X-100 containing 5 μg of HRP per ml (grade VI, Sigma). Incubations were carried out in humid chambers at ≈4°C. (*ii*) After rinsing in 0.1 M phosphate buffer/Triton X-100 for 30 min, sections were incubated for 15 min in 0.05 M Tris·HCl (pH 7.5) containing 0.06% 3,3′-diaminobenzidine (Sigma) and incubated an additional 15 min following the addition of H_2O_2 to a final concentration of 0.01%. (*iii*) After rinsing in Tris·HCl buffer or PBS, sections were dehydrated, transferred to xylene, mounted on De Pex, and observed with a bright-field microscope. NC1/34 was developed by the PAP reaction (4). For this, tissue samples were incubated with 1:100 dilutions in PBS of NC1/34, as in ref. 2, followed by incubations with 1:10 dilutions of anti-rat IgG (Miles), and developed with a monoclonal rat PAP reagent, as described (6, 8). For electron microscopy, 50-μm-thick vibratome sections of the spinal cord fixed by perfusion with 4% paraformaldehyde/0.1–0.5% glutaraldehyde were incubated with P4C1-HRP or incubated following the PAP procedure and processed as described (9). Blocks of spinal cord tissue were also cut perpendicularly to the main plane of sections, and semithin and thin sections were prepared covering the total tissue thickness. Semithin and thin sections were observed and photographed using an automated Leitz photomicroscope and a Phillips 410 electron microscope, respectively.

RESULTS AND DISCUSSION

In this work we fused two established hybridomas, treating one cell line with a critical concentration of emetine and the other with actinomycin D. Emetine, an inhibitor of ribosome translocation, and actinomycin D, an inhibitor of RNA synthesis, have been used together to inhibit the proliferation of CEM cells (10). Sensitivities of hybridoma lines NC1/34 and YP4 to emetine and actinomycin D were determined. For the fusion, drug concentrations used were such that <1% of the cells survived the 1-hr treatment (6). Up to 40% of all of the primary clones obtained after fusion were positive in the hybrid monoclonal assay [although the intensity of the signal differed (Figs. 1 and 2)]. The remaining 60% of the primary clones may include hybrids that either have not retained the two parental heavy (H)- and light (L)-chain genes due to chromosome losses or are hybrids of the same lineage whose residual survival capacity was amplified by homofusions. As for the differential intensity of the positive clones, they may represent clones with different concentrations of the bispecific species or they may contain different proportions of inactive components (Fig. 1).

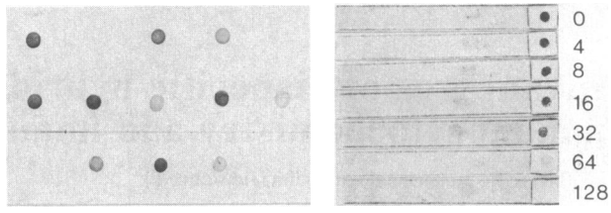

FIG. 2. Bispecific binding assay and dipstick competitive immunoassay with a bs mAb. (*Left*) Assays of supernatants of P4C1 clones. The SP-BSA was spotted on a nitrocellulose sheet marked with squares and blocked with BSA, and supernatants were added to cover each spot. After rinsing, color was developed with 4-chloro-1-naphthol and H_2O_2. (*Right*) The competitive assay is based on the inhibition of the binding of the bs mAb by soluble antigen. Coated nitrocellulose squares containing a 2-μl spot of SP-BSA were prepared as described in the legend to Fig. 1 and glued to the right end of a plastic stick. Standard solutions containing the indicated concentrations of SP (nanomolar) were also prepared. For the assay, purified bispecific fraction II of P4C1/11 to a final concentration of 2 μg/ml and HRP to 300 μg/ml were added to the standard SP solution. After immersing the antigen-coated sticks for 15 min, they were removed and rinsed and the color was developed.

A hybrid hybridoma codominantly expresses the immunoglobulin H (H_1 and H_2) and L (L_1 and L_2) chains of both of its parents. Ten different IgG molecular species are theoretically predicted to assemble. However, a number of quanti-

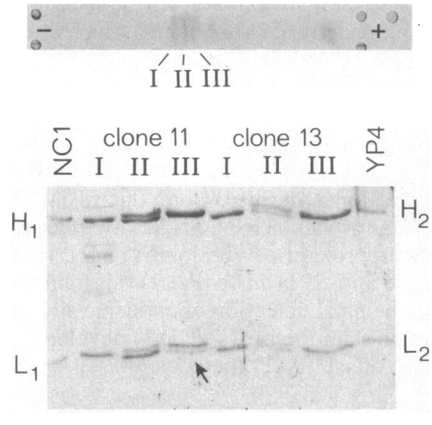

FIG. 3. Molecular analysis of hybrid hybridoma clones P4C1/11 and P4C1/13. (*Upper*) Cellulose acetate electrophoretic pattern of the ascitic fluid of a P4C1/11 tumor-bearing nude mouse showing the intensities of the three bands approximately as 1:2:1. The three immunoglobulin bands were resolved by DE-52 and chains were analyzed by $NaDodSO_4$/PAGE (*Lower*). (I) Anti-SP activity. (II) Bispecific hybrid activity. (III) Anti-HRP. The parental antibodies are also run alongside. Note the additional L chain in peak III of P4C1/13 (arrow).

tative factors influence the proportion of each species assembled. These include association of homologous vis-à-vis heterologous chains, which may be preferential or even 100% restricted, different rates of synthesis of chains, and subclass characteristics of the H chains. Three basic types of assembly can be schematically considered. *Type 1*: Association of all chains generating 10 different molecular species. *Type 2*: H–L-chain association restricted to the original hybridoma pairs. This would generate the two parental mAb molecules and a single heterodimer (L_1H_1–H_2L_2) of the two H–L-chain pairs. This ideal pattern of association provides the best yields of bs mAb. *Type 3*: Restricted association of one H–L-chain pair but not the other. For example, L_1 can associate with H_2 (in addition to H_1) but L_2 does not associate with H_1.

In type 1 and type 3, molecular species are generated containing inactive heterologous HL combinations that compete for antigen but, as they do not contribute to the final reaction, they become competitive inhibitors in the hybrid assay (Fig. 1). The more strongly positive P4C1 primary clones were evaluated by the one-step immunohistochemical assay. A few were recloned twice, a stage at which 95% of the reclones were positive in the hybrid assay. Large amounts of antibody were obtained from spinner cultures grown in serum-free medium (7) or as ascitic fluid in the peritoneal cavity of nude mice.

Cellulose acetate electrophoresis of ascitic fluid disclosed three immunoglobulin bands (Fig. 3 *Upper*), as observed in other hybrid hybridomas (1, 2, 6). These three components were resolved on a DE-52 column with a linear gradient of

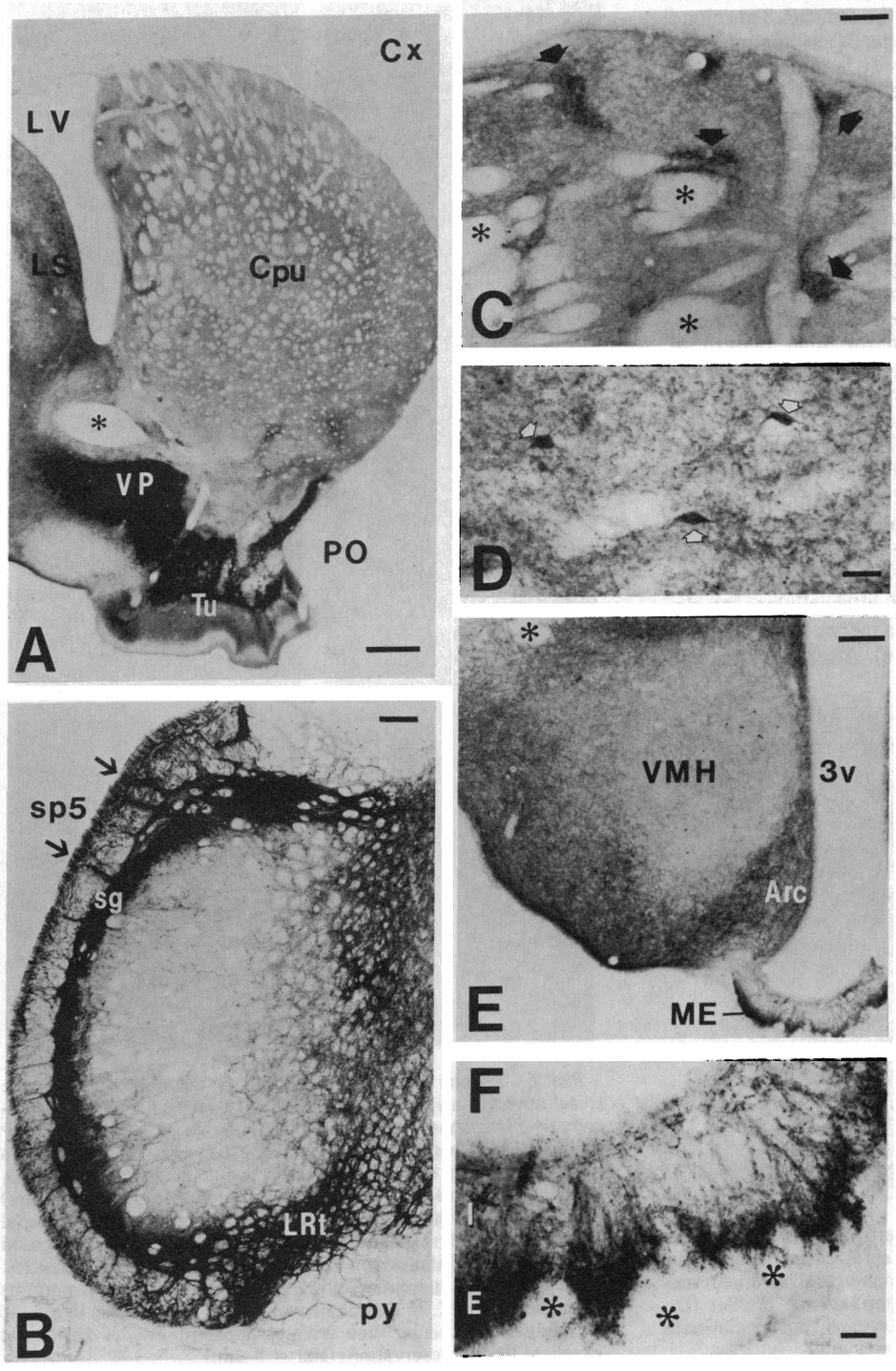

FIG. 4. (*A*) Lower-magnification micrograph of a frontal section of the rat brain immunostained with P4C1. Note the clear-cut differential localization and reaction intensities across various nuclear regions. LS, lateral septum; Cpu, caudate putamen; Cx, neocortex; PO, primary olfactory cortex; Tu, olfactory tubercle; VP, ventral pallidum; LV, lateral ventricle. Asterisk denotes total absence of background staining in myelinated bundle of the anterior commissure. (Bar = 600 μm.) (*B*) Lower-magnification micrograph of the lower medulla oblongata showing the dense network of SP-immunoreactive fibers terminating in the substantia gelatinosa of the spinal trigeminal nerve (sg). Arrows indicate bundles of sensory, immunoreactive fibers, leaving the tractus spinalis of the trigeminal nerve (sp5) toward the corresponding nucleus. LRt, lateral reticular formation; py, pyramid. (Bar = 225 μm.) (*C*) Patchy distribution of P4C1-immunoreactive fibers in the rat neostriatum (arrows). Asterisks denote corticostriatal fiber tracts. (Bar = 150 μm.) (*D*) Higher-magnification micrograph revealing SP-immunoreactive cell bodies (arrows) in the rat neostriatum without colchicine treatment. (Bar = 50 μm.) (*E*) Lower magnification of the rat hypothalamus at the level of the ventral medial nucleus (VMH). The center of this nucleus displays little immunoreactivity, whereas abundant fibers and terminals are found in the surrounding neuropile. Intense immunoreaction can be observed in the median eminence (ME) and arcuate nucleus (Arc). 3v, Third ventricle. Asterisk denotes fiber bundle of the fornix. (Bar = 250 μm.) (*F*) Higher-magnification micrograph from *E*, displaying intense immunoreactive fibers in the median eminence of the rat, particularly intense in the external layer (E). I, internal layer. Asterisks denote location of primary plexus of portal capillary vessels. (Bar = 50 μm.)

10–100 mM Na_2HPO_4 (pH 7.5). Analysis of the L- and H-chain composition of the immunoglobulins present in the three peak fractions from clone 11 (Fig. 3) shows that peaks I and III represent the parental antiperoxidase and anti-SP, respectively, with no detectable cross-contamination of L chains. The middle peak shows the H and L chains expected for a hybrid molecule. This pattern corresponds to the uniquely restricted H–L-chain combination type 2 associations described above. This favorable combination to form fully functional arms is further optimized by their random association through the H chain to generate the anti-SP:antiperoxidase bs mAb in the ratio of 1:2:1 (Fig. 3 *Upper*).

Four of five clones analyzed gave such a pattern—namely, P4C1/5, P4C1/6, P4C1/11, and P4C1/12. However, at least one clone P4C1/13 was anomalous, showing two L chains in the antiperoxidase peak III (Fig. 3, arrow). The second L chain has the same mobility as that of the anti-SP. This clone therefore exhibits the pattern of type 3 association (see above). Predictably, the bs mAb activity is inhibited by the presence of an inactive antiperoxidase arm, as shown in Fig. 1. This was particularly borne out by immunohistochemical assays (data not shown). Although all of the P4C1 clones were derived from the same two parents in a single fusion experiment, the distinctive chain association pattern of P4C1/13 vis-à-vis the other P4C1 clones presents an interesting paradox. It is pertinent to note that the various hybrid hybridoma clones are not necessarily derived by fusion of only one YP4 and one NC1 cell. Three or more cells could fuse and retain a variable number of H- and/or L-chain genes. It is possible that the difference in the final pattern of preferential association is a reflection of the altered ratios of the two competing L chains to produce alternative H/L-chain pairs.

Inhibition of Binding of bs mAb to a Solid Support. bs mAb are functionally monovalent. It follows that binding of the bs mAb to antigen immobilized on a solid support can be inhibited in a linear fashion by soluble antigen. Therefore, they could be valuable to develop competitive "dipstick" immunoassays. To test the validity of this approach, we coated with antigen small pieces of nitrocellulose as described for the bispecific assay. These pieces were mounted on "sticks" (semiflexible plastic strips). After blocking with BSA, the sticks were submerged in solutions containing the bs mAb, HRP, and various concentrations of SP. After 10 min of incubation, they were washed and immersed in the developer. The color intensity was inversely proportional to the amount of SP in the solution (Fig. 2 *Right*). An interesting

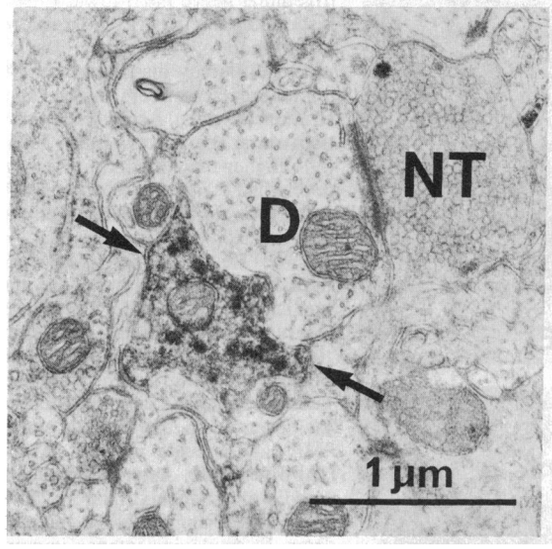

FIG. 5. Electron micrograph of the superficial layers of the dorsal horn of the rat spinal cord. Arrows indicate a P4C1-immunoreactive nerve terminal and NT indicates a nonimmunoreactive nerve terminal, both in contrast with two non-immunodendritic profiles (D). Note clarity of ultrastructural detail of postsynaptic differentiation in D, microtubules, and mitochondria. (Bar = 1 μm.)

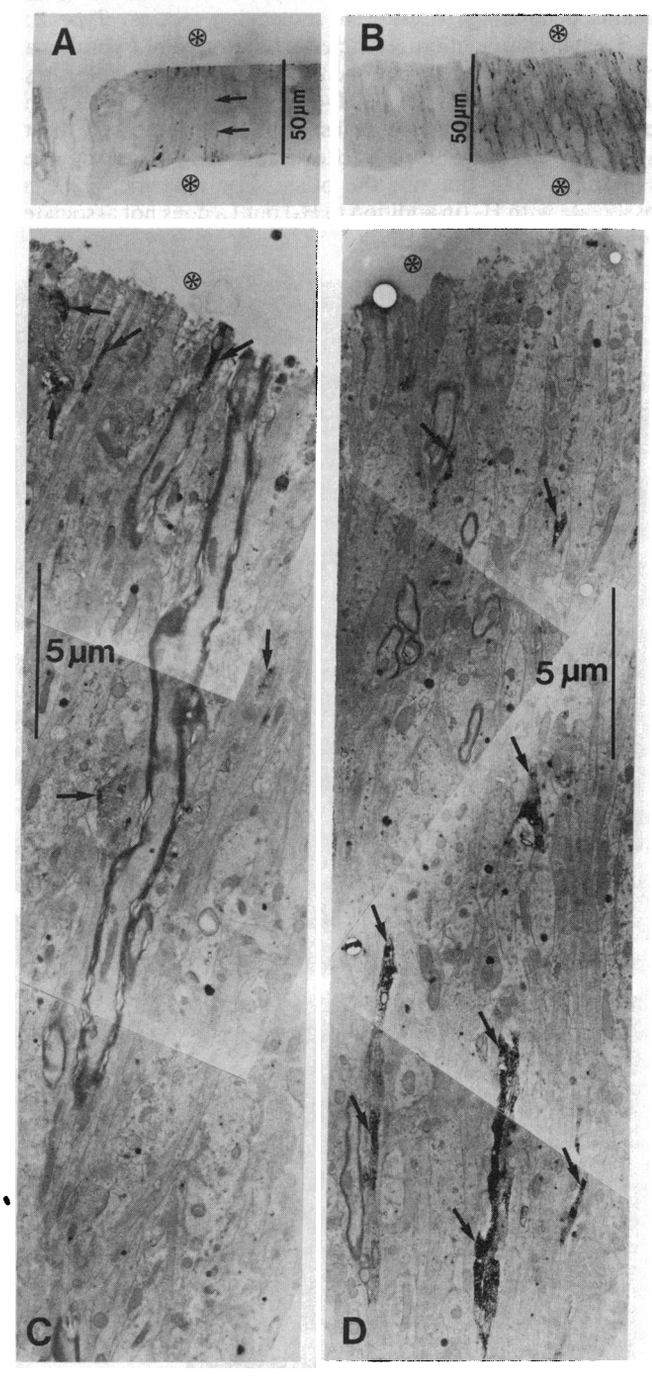

FIG. 6. (*A* and *B*) Semithin sections of the dorsal horn of the spinal cord cut across a 50-μm-thick slice viewed by light microscopy. (*A*) Immunostained with NC1/34 and developed by the PAP procedure. (*B*) Immunostained with the bs mAb P4C1. Asterisks indicate surfaces exposed to incubation medium. Arrows in *A* indicate the reduced extent of the immunoreaction. (Bar = 50 μm.) (*C* and *D*) Low-magnification electron micrographs of material shown in *A* and *B*, respectively. Asterisks indicate surfaces exposed to incubation medium. Arrows indicate immunoreactive varicosities. Note the gradual diminution of immunoreactivity in deeper portions of the section in *C*. In *D*, immunoreactive profiles to the bispecific antibody P4C1 can be seen without apparent loss of signal and excellent tissue preservation. (Bar = 5 μm.)

aspect of this approach is that it can be adapted to multiple assays within a single experiment. This implies coating a single stick with several spots, each containing a different antigen. This stick would then be immersed in the unknown fluid after the addition of a mixture containing several bs mAb, one arm specific for each antigen, and the other in all cases specific for the same marker molecule (HRP in our case, but it could be radioactive, fluorescent, etc.). Such methods would have the advantage of being a simple dipstick immunoassay for multiple antigens.

Improved Immunocytochemistry with the Bispecific Hybridoma in Light and Electron Microscopy. The application of the bs mAb (P4C1) in immunocytochemistry for the detection of SP-immunoreactive sites in the central nervous system (CNS) revealed positive elements in all areas known to contain the peptide. This was attained by a single-step incubation of a mixture of the bispecific P4C1 and HRP. The procedure implies a drastic simplification of current techniques in which one or several developing antibodies are used. The fact that the bispecific antibody molecule is monovalent did not seem to affect the apparent avidity compared to the parental antibody (NC1/34). On the other hand, though the pattern and intensity of the signal were consistent with previous reports (11, 12), the procedure offered clear advantages. The two most obvious were the total absence of background and the higher sensitivity as compared with previous and parallel experiments using the PAP approach. The absence of background was such that identification of nuclear landmarks obvious with conventional techniques was very faint to the point that, occasionally, phase-contrast microscopy or counterstaining was required. The absence of background allowed very fine morphological and unambiguous definition of immunoreactive sites. This feature can best be appreciated in the entirely negative reaction of CNS myelinated tracts, such as corpus callosum, anterior commissure, fornix, etc. (Fig. 4 *A* and *E*), making possible the detection of isolated positive immunoreactive axons in fiber tracts. The lack of background together with greater sensitivity enhanced the contrast between immunopositive and lesser immunopositive or negative areas. Sharp differences in peptide immunoreactivity were found throughout the CNS (see Fig. 4 *A–F*). Thus, extremely intense immunoreaction was found in the ventral pallidum (Fig. 4*A*), medial amygdala, substantia nigra, and spinal nucleus of the trigeminal nerve (Fig. 4*B*). The combined advantages of increased sensitivity and lower background allowed the demonstration of SP immunoreactivity otherwise not readily evident in the rodent CNS—for example, the bundling of positive fibers in the caudate putamen, responding to the mosaic organization of this structure (Fig. 4*C*) (13), fibers in the external layer of the median eminence (Fig. 4 *E* and *F*), and immunoreactive cell bodies. This should improve the experimental use of antibodies for the detection of biological substances and also contribute to eliminating the problem of "false positive" reactions in immunodiagnostic tests (histopathology, ELISA, etc.). The improvement in signal-to-noise ratio is probably due, among other factors, to the elimination of "link" antibodies, which can create considerable noise in conventional immunohistochemical procedures. These are usually polyclonal, commercial antibodies with considerable batch variation. In addition, second and third layers of developing antibodies might occlude immunoreactive sites, as described for the PAP technique at high antigen and primary antibody concentrations (14).

Another indication of the enhanced sensitivity achieved using the bs mAb is the relative ease at which peptide immunoreactivity could be demonstrated in well-documented CNS SP containing neuronal cell bodies (Fig. 4*D*). In these cell bodies, peptides are normally stored at relative low concentration and their demonstration usually requires the application of colchicine or other agents that inhibit axonal transport. This was not necessary in the experiments described here. It is generally believed that sensitivity in immunocytochemistry is greatly enhanced by the "amplification" offered by multiple layers of developing antibodies. Bispecific antibodies bind a single enzyme molecule per antibody bound to the antigen, whereas, in the PAP procedure, two or more PAP complexes, each containing three enzyme molecules, are expected for each antibody bound.

The present observations suggest that stereochemical considerations are at least as important as amplification. This is highlighted by the remarkable results obtained at the electron microscopic level. Clear results at the electron microscopic level were obtained using P4C1 bs mAb in a one-step procedure. Unusual resolution of ultrastructural detail was achieved in those experiments (Fig. 5). These improvements are probably due to a number of factors, an important one being the use of glutaraldehyde (up to 0.5%) without noticeable loss of immunoreactivity. This may again be related to higher sensitivity and better penetration. Other factors are likely to be the reduction of incubation steps and lesser tissue manipulation to allow antibody penetration. Indeed, the most remarkable feature was the improvement in tissue penetration, demonstrable by the fact that the immunoreaction was homogeneously present across the entire thickness of the section. This is emphasized by comparing the results with those obtained when applying conventional procedures, in which only the superficial layers display adequate immunoreaction (Fig. 6). In this context, it is perhaps relevant that preformed HRP complexes have a molecular size of ≈90,000 daltons compared to >500,000 daltons for regular PAP complexes.

This work is dedicated to L. F. Leloir on the occasion of his 80th birthday. M.R.S. and C.M. are grateful to Richard Pannell for his skilled assistance throughout this work and A.C.C. is grateful for the technical assistance of Mrs. N. Robitaille-Giguere. The electron microscopical material was processed by Dr. A. Ribeiro da Silva and Miss K. Hewitt. A.C.C. gratefully acknowledges the support of the Medical Research Council (Canada), the office of the Dean, Faculty of Medicine, McGill University, and Medicorp.

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