Histochemistry (1990) 95:155–163

Histochemistry

Production of a bi-specific monoclonal antibody recognizing mouse kappa light chains and horseradish peroxidase

Applications in immunoassays

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Accepted September 17, 1990

Summary. The production of a bi-specific monoclonal antibody that simultaneously recognizes mouse kappa light chains and horseradish peroxidase (HRP) for use as a general developing reagent in a wide variety of immunobased techniques is described. This antibody, named McC10, was produced by the fusion of an aminopterin-sensitive interspecies hybridoma which secretes rat monoclonal antibodies against HRP ($RAP_2 \cdot Ag$) and splenocytes from a rat immunized with whole mouse immunoglobulin (Ig)G. The hybrid-hybridoma generated from this fusion expresses and secretes rat Igs of the IgG_1 and IgG_{2a} subclasses, as determined by radial immunodiffusion. In competitive binding solid-phase enzymatic assays, McC10 was found to cross-react with all four mouse IgG subclasses as well as mouse kappa light chains. In contrast, in this type of assay, McC10 did not appear to recognize mouse IgA, IgM or lambda light chains. However, IgM-bearing kappa light chains were recognized by immunocytochemistry. Epitope specificity of this bi-specific antibody was more clearly determined on immunoblots where McC10 was found to exclusively recognize mouse kappa light chains and display no cross-reactivity with mouse Ig heavy chains nor with kappa light chains from rat or rabbit. In addition, McC10 was used successfully in two-step immunocytochemistry (ICC) for the localization of enkephalin, nerve growth factor (NGF) receptor and paired helical filament-immunoreactive sites in rat brain, rat skin and human brain, respectively, using mouse IgG's and IgM's as primary antibodies. McC10 compared favourably with peroxidase-anti-peroxidase (PAP) ICC with respect to sensitivity but was markedly superior with respect to specificity when used in fixed human brain or rat skin. This study demonstrates some of the potential advantages of using an epitope specific monoclonal bi-specific developing reagent like McC10 in an immunobased technique like ICC. Its potential use in a variety of other immunobased procedures is discussed.

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Introduction

Hybridoma technology has made a considerable impact in immunocytochemistry (ICC) and a variety of other immunobased techniques. This technology has provided an unlimited supply of standard, well characterized and highly specific antibodies from pure or impure immunogens. Consequently, it is not surprising that monoclonal antibodies are increasingly used as primary antibodies in a large number of studies. When using primary monoclonal antibodies, immunostaining can be obtained with the use of direct, indirect or unlabelled staining procedures (Sternberger et al. 1970). Techniques which depend upon external chemical conjugation protocols may suffer from a number of shortcomings like loss of immunoreactivity. Thus, they have been favoured by the unlabelled immunobased staining procedure. Hybridoma technology has also contributed to developments in this latter technique with the production of monoclonal anti-immunoglobulin (Ig) (MacMillan et al. 1984) and anti-peroxidase (Cuello et al. 1984b; Mason et al. 1982; Semenenko et al. 1985) antibodies. More recently, with the development of two new techniques [internal radiolabelling of monoclonal antibodies (Cuello and Milstein 1981; Cuello et al. 1982) and the production of bi-specific monoclonal antibodies (Milstein and Cuello 1983; 1984)], hybridoma technology has renewed interest in direct immunostaining procedures. Bi-specific monoclonal antibodies which possess binding sites for the antigen under investigation and an immunohistochemical marker like horseradish peroxidase (HRP) on the same Ig molecule have been produced and used successfully in a variety of studies (Suresh et al. 1986; Semenenko et al. 1988; Kenigsberg et al. 1990). Bi-specific monoclonal antibodies used in one- or two-step immunocytochemistry (ICC) have been found to be remarkably sensitive and highly specific, dramatically enhancing the signal-to-noise ratio. Furthermore, the introduction of these bi-specific monoclonal antibodies has considerably shortened standard staining procedures. These shortened protocols may inevitably help to preserve tissue

and antigen integrity which is especially important for ultrastructural studies at the electron microscopic level. Despite these observations, it will undoubtedly become an overwhelming task to develop bi-specific monoclonal antibodies for every antigen under investigation. Consequently, we have attempted to overcome this step and further the use of the bi-specific antibodies by producing a bi-specific developing antibody capable of the simultaneous recognition of mouse Igs and HRP to be used as general developing reagents. The generation of this bi-specific antibody, its characterization and its use in two-step immunostaining procedures are described.

Materials and methods

Immunizations

Adult female Sprague Dawley (Charles River) rats were immunized with 500, 50 or 5 µg/injection of either HRP (Sigma Grade VI) or whole mouse IgG (Schripps). Both immunogens were first dissolved in phosphate buffered saline (PBS) pH 7.4 and then emulsified in an equal volume of Freund's adjuvant (Difco). Each rat received a total of 200 µl of the emulsified immunogen per injection which was administered in multiple sites intracutaneously and intraperitoneally (Vaitukaitis et al. 1971). The animals received a total of four such injections, the first three administered at weekly intervals and the final after a 3-week rest period. Five to 10 days after the final immunization, blood samples were obtained and the presence of anti-HRP antibodies was determined in conventional peroxidase antiperoxidase (PAP) ICC. The presence of anti-mouse IgG antibodies in animal sera was assessed in conventional PAP ICC and the direct enzyme-linked immunosorbent assay (ELISA), using whole mouse IgG as the coating antigen. Three to 4 days before fusion, the appropriate host animal received an intrasplenic boost (Spitz et al. 1984) of either immunogen dissolved in PBS at the dose used in the previous immunizations.

Cell fusion

Both fusions were carried out as described previously (Kohler and Milstein 1975; Kohler et al. 1976) with several modifications. Splenocytes from a hyperimmune rat immunized with HRP were mixed with non-producing mouse myeloma cells $Sp_2/O \cdot Ag$ at a ratio of 10:1, respectively. The mixed cells were pelleted by centrifugation at 100 *g* for 5 min and then exposed to the fusagen, 45% polyethylene glycol (PEG) (BDH) and 5% dimethyl sulfoxide for a total of 2 min. Dimethyl sulfoxide was included since it has been found to significantly enhance PEG-induced fusion (Norwood et al. 1976). Five millilitres of glucose potassium (GKN) buffer, containing 8.0 g NaCl, 0.4 g KCl, 3.56 g Na_2PO_4, 0.78 g NaH_2PO_4, 5.0 g glucose and 0.01 g phenol red/1 H_2O (MRC Cellular Immunology Unit, Oxford, UK), was than added over 1 min and the volume was increased to 45 ml with GKN buffer. The cells were then collected by centrifugation at 100 *g*, resuspended to a final volume of 48 ml in RPMI 1640 (Gibco) supplemented with 20% fetal bovine serum (FBS) (Gibco) and then equally distributed in 4 24-multiwell Corning plates (Fisher Scientific), already containing a feeder layer of 1×10^6 rat spleen cells per well. The following day, 1 ml of RPMI, 20% FBS plus double strength hypoxanthine aminopterin thymidine (Sigma) was added to each well. Hybrid cell growth was monitored and media was replenished when necessary. Ten to 30 days after fusion, spent media was collected from wells with conspicuous hybrid cell growth and tested for presence of antiperoxidase antibodies by PAP ICC. Positive hybrids were cloned twice by limiting dilution, expanded and then frozen in liquid N_2. One final hybridoma, secreting monoclonal antibodies against HRP coded RAP_2, was rendered resistant to 8-azaguanine (ICN) in a step-wise manner by growing the hybridoma in successively higher doses of 8-azaguanine. Cells surviving in 15 µg/ml 8-azaguanine were then cloned by limiting dilution and retested for antiperoxidase activity as well as aminopterin sensitivity. The final aminopterin-sensitive anti-HRP hybridoma was coded $RAP_2 \cdot Ag$.

For the production of the hybrid-hybridoma, splenocytes from a rat immunized with whole mouse IgG were once again mixed with $RAP_2 \cdot Ag$ cells at a ratio of 10:1, respectively. Fusion was carried out essentially as described for the production of rat anti-peroxidase antibodies. The presence of bi-specific anti-mouse IgG antiperoxidase activity in fusion wells and subsequent clones was determined by the direct ELISA, using whole mouse IgG as the coating antigen. Positive supernatants were retested by direct ELISA with the 4 heavy chain subclasses of mouse IgG and in ICC with appropriate mouse primary antibodies. One clone, named McC10, was eventually isolated and used for final characterization.

Direct ELISA

Incubations were carried out at room temperature and the volume of reagents added per well for all steps except blocking were kept constant at 50 µl. In addition, assay wells were washed 3 to 4 times with PBS, following each of the incubation steps. Microassay (Immunlon I, Dynatech) plates were coated with either 50 µg/ml whole mouse IgG (Schripps), 10 µg/ml purified mouse IgG subclasses (ICN) or PBS alone (control wells) for 2 h. Unoccupied binding sites were subsequently blocked by incubating for 1 h with 200 µl/well of a 1% ovalbumin solution (Sigma) prepared in PBS. Following this step, mouse anti-HRP monoclonal antibodies (MAP.A6, Semenenko et al. 1985) were preincubated with 5 µg HRP/ml for 1 h at room temperature and then applied to the microassay wells for a 45 min incubation. Finally, peroxidase activity was determined colourimetrically with 0.4% 2,2-azino-bis (3-ethylbenzthiazoline sulfonic acid) (ABTS, Sigma) and 0.01% H_2O_2 prepared in phosphate citrate buffer pH 4.0. The peroxidase reaction was allowed to proceed over a 30-min period and was then stopped with the addition of 1% oxalic acid. The change in optical density was determined at 414 nm with the use of an EIA-Reader (Bio-Rad Model 2550).

For the determination of bi-specific antibody activity, the microassay plates were coated and blocked as described above. Following the blocking step, hybrid-hybridoma supernatant from fusion wells or clones were preincubated with HRP (5 µg/ml supernatant) for 1 h before application to the assay wells. Incubation with culture supernatant was allowed to proceed over a 1-h period and peroxidase activity was revealed as previously described.

Dilution curve for McC10

Optimal coating efficiency for whole mouse IgG was determined by the peroxidase saturation technique and by the checker-board titration technique (Munoz et al. 1986), using undiluted McC10 supernatant supplemented with HRP (5 µg/ml). The optimum solid-phase antigen coating concentration determined by the two different methods were very similar and were found to be approximately 2 µg mouse IgG/ml PBS. Consequently, this coating concentration was used in all subsequent assays.

Microassay wells were coated with 2 µg/ml whole mouse IgG and blocked as previously described. McC10 hybridoma supernatant was serially diluted in PBS and supplemented with HRP (final concentration, 5 µg HRP/ml). Peroxidase activity was determined as detailed earlier and a dilution curve for McC10 was constructed. The dilution of the hybridoma supernatant, which fell on the linear portion of the curve, was used in the competitive binding assays.

Competitive binding assay

Competitive binding assays, with purified mouse IgG subclasses, IgA, IgM, lambda and kappa light chains, were performed using McC10 supernatant diluted 1:150 in PBS and preincubated with various amounts of the mouse immunoglobulins. The final concentration of HRP added to these supernatants was kept constant at 5 μg/ml. Mouse IgG_1, IgG_{2a}, IgG_{2b} and IgG_3 are myeloma IgGs isolated from the tumour lines MOPC 21 (IgG_{1k}), UPC 10 and RPC 5 (IgG_{2ak}) or J606 and FLOPC 21 (IgG_{3k}), respectively. Mouse IgA and IgM are myeloma Igs isolated from the tumour lines MOPC 315 ($IgA_{\lambda 2}$) or TEPC (IgM_k), respectively, while mouse kappa and lambda light chains were isolated from MOPC 41 or RPC 20 tumour lines. All mouse tumours were of BALB/c origin. Microassay wells were coated with 2 μg/ml whole mouse IgG and blocked as previously described. Incubations with diluted McC10 supernatants with or without competing immunoglobulins, were allowed to proceed over 1 h. Peroxidase activity was assessed as described above.

Radial immunodiffusion

To determine the classes and subclasses of the rat antiperoxidase (RAP_2) and bi-specific (McC10) monoclonal antibodies, hybridoma supernantants were concentrated 10-fold with a Minicon concentrator (Amicon Corp.). Seventy-five microlitres of the concentrated supernatants were added to the central wells of a 1% agarose gel, and 10 μl of highly specific rabbit antisera against each of the 4 heavy chain subtypes of mouse IgG as well as IgA and IgM (Serotec) were applied to the surrounding wells. The development of sharp precipitin lines could be visualized within 2 days.

Polyacrylamide gel electrophoresis, Western blots and immunoblots

Whole mouse, rat and rabbit IgG, as well as mouse IgA, IgM, the four heavy chain subclasses of mouse IgG, mouse and rat kappa light chains and mouse lambda light chains, were electrophoretically separated on 10% polyacrylamide slab gels under denaturing conditions using β-mercaptoethanol as a reducing agent. In addition, IgM was also separated after reduction with dithiothreitol. After separation, the proteins were transferred electrophoretically onto nitrocellulose membranes at 20 V for 8 h with the use of a trans-blot apparatus (Bio-Rad). A portion of the membrane containing the molecular weight standards was cut, and the bands were revealed with 1% Amido black. The remaining part of the membrane was blocked with 5% bovine serum albumin for 1 h at 37° C and then incubated for 1 h at room temperature with undiluted McC10 hybridoma supernatant which had been preincubated with HRP (5 μg/ml). The blots were then washed 3 to 4 times with PBS and finally exposed to the peroxidase substrates, 0.06% diaminobenzidine (DAB, Sigma) and 0.01% H_2O_2 in PBS. The peroxidase reaction was stopped after 10–15 s by removal of the substrates, washed extensively with PBS and then dried and photographed.

Detection of antiperoxidase activity for the production of a rat anti-HRP monoclonal antibody

The following immunocytochemical procedure was used for the detection of antiperoxidase activity. Forty μm sledge microtome sections of 4% paraformaldehyde fixed rat spinal cord were incubated overnight at 4° C with 1:100 (v/v) anti-substance P rat monoclonal antibody (NCl/34, Cuello et al. 1979), diluted in PBS/0.2% Triton X-100 (PBS+T). Sections were then washed in PBS+T and incubated for 1 h at room temperature with dialyzed fractionated rabbit antisera containing anti-rat IgG antibodies (Sigma). This antisera was reconstituted according to directions and then further diluted 1:20 in PBS+T before use. Following this, sections were incubated for 1 h at room temperature in diluted sera collected from rats which had been immunized with HRP or with undiluted supernatant from fusion wells or clones in the generation of RAP_2. The diluted rat sera or hybridoma supernatants were preincubated with HRP (5 μg/ml) for at least 1 h at room temperature before being applied to the tissue sections. After washing with PBS+T, sections were exposed to 0.06% DAB for 15 min and then H_2O_2 (final concentration 0.01%) was added. Sections were then washed free of substrates, dehydrated, cleared and mounted for visualization. All microtome sections were processed free-floating.

Immunocytochemical localization of NGF receptor, enkephalin and paired helical filament immunoreactivity

Adult male Wistar rats were perfused transcardially with a mixture of 4% paraformaldehyde, 0.1% glutaraldehyde and 15% saturated picric acid solution in 0.1 *M* phosphate buffer, pH 7.4, followed by the same fixative mixture devoid of glutaraldehyde. After perfusion, the lower lips were excised and further fixed by immersion in the latter fixative mixture for 1 h at 4° C. Parasagittal sections were obtained with a cryostat and collected on glass slides. Sections were subsequently immersed in 0.5% H_2O_2 in PBS for 15 min, washed in PBS+T and incubated overnight in the undiluted hybridoma supernatant from the 192 IgG cell line (Chandler et al. 1984) at 4° C. All the subsequent incubations were carried out at room temperature. After two washes in PBS+T, the tissue was incubated for 90 min in the undiluted hybridoma supernatant from the McC10 cell line. After one wash in PBS+T, the sections were incubated in 5 μg/ml HRP dissolved in PBS+T, rinsed three times in PBS+T and reacted with DAB (0.06%) and H_2O_2 (0.01%). The tissue was then dehydrated, clarified, mounted and then examined under bright field and interference-contrast optics.

For the detection of enkephalin immunoreactivity, a similar fixation procedure was applied except that the fixative used was simply 4% paraformaldehyde. Thirty-five μm sections of the spinal cord were generated using a sledge microtome and were incubated overnight with mouse anti-enkephalin monoclonal antibody coded NOCl (Cuello et al. 1984a). The reaction was developed, using McC10 as described above.

For electron microscopy, rat spinal cord tissue, prepared in accordance with Connaughton et al. (1986), was quickly frozen by immersion in liquid nitrogen and thawed in 0.1 *M* phosphate buffer at 25° C. Fifty μm-thick sections were obtained with a Vibratome, treated for 30 min in 1% sodium borohydride in PBS and then rinsed several times in PBS (Kosaka et al. 1986). The sections were then incubated free-floating with mouse anti-enkephalin antibody, and the DAB reaction was carried out with double intensification as described by Adams (1981). Triton was not added to the tissue sections. After the DAB reaction, the tissue was rinsed twice with phosphate buffer, osmicated for 1 h in 1% osmium tetroxide in 0.1 *M* phosphate buffer at 4° C, dehydrated and flat-embedded in Epon between thick acetate foil and a plastic coverslip. After polymerization of the Epon, the sections were examined by light microscopy, and the selected fields were trimmed and re-embedded for electron microscopic examination. The ultrathin sections were observed either uncontrasted or after double-contrast staining with uranyl and lead.

Fifty μm sections of human post-mortem samples from the frontal cortex of Alzheimer's patients were obtained using a sledge-microtome, subsequently rinsed in PBS and incubated in 0.5% H_2O_2 in PBS for 15 min at room temperature. Immediately thereafter, the sections were rinsed twice in PBS and once in PBS+T and then incubated overnight with the 6.423 mAB (Wischik et al. 1988) (diluted 1:100) in PBS+T at 4° C. After two buffer rinses, the sections were then developed, using either McC10 as earlier

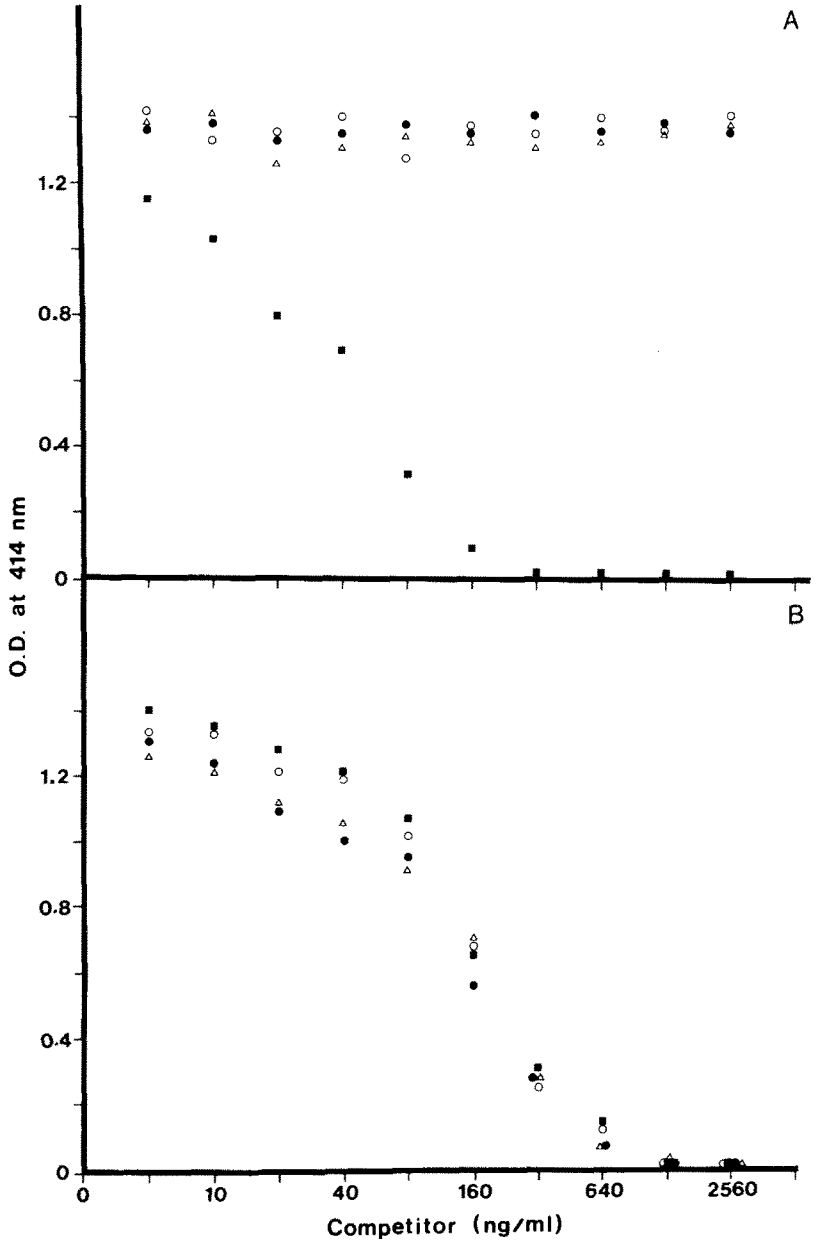

Fig. 1A, B. Competitive binding assays for McC10 expressed as optical density (O.D.) (*ordinates*) at 414 nm versus amount of competitor (*abscissae*). Displacement of binding of McC10 to immobilized whole mouse IgG coated on microassay dishes was determined for mouse lambda (*closed circles*) and kappa light chains (*closed squares*) as well as IgM_k (open circles) and IgA_l (open triangles) in **A**; mouse IgG_{1k} (closed circles), IgG_{2ak} (open triangles), IgG_{2bk} (open circles) and IgG_{3k} (closed squares) in **B**. Values represent means obtained from quadruplicate assay wells

described or the PAP procedure which employs rabbit antisera against mouse Ig (1:50), followed by mouse anti-HRP (1:30) (Semenenko et al. 1985) supplemented with 5 μg HRP/ml.

Results

Five of the 6 rats which had been immunized with HRP were found to produce antiperoxidase antibodies as determined by PAP ICC for substance P with the use of a rat anti-substance P monoclonal primary antibody (NC1-34) and rabbit anti-rat IgG antisera as the bridge antibody. The intensity of the immunostaining was very similar for sera from those animals immunized with 50 and 500 μg of HRP and weakest for that from one rat immunized with a 5-μg dose of peroxidase. One rat immunized with an intermediate dose was selected for cell fusion.

All of the six animals immunized with mouse IgG displayed some degree of antibody activity against whole mouse IgG as determined by the direct ELISA. One rat which received 50 μg of the IgG per injection possessed the highest titre sera and was therefore selected for cell fusion.

Fusion efficiencies of the rat splenocytes with the mouse myeloma $Sp_2/O \cdot Ag$ or the interspecies antiperoxidase hybridoma $RAP_2 \cdot Ag$ were relatively high. Hybrid cell growth were detected in about 70% of the fusion wells of the former fusion and about 94% of the latter. The presence of antiperoxidase activity was evident in about 50% of the fusion wells tested. Wells containing hybrids with the highest antiperoxidase activity were cloned twice by limiting dilution, and the final clone was rendered resistant to 8-azaguanine. Aminopterin sensitivity was not accompanied by loss in specific anti-

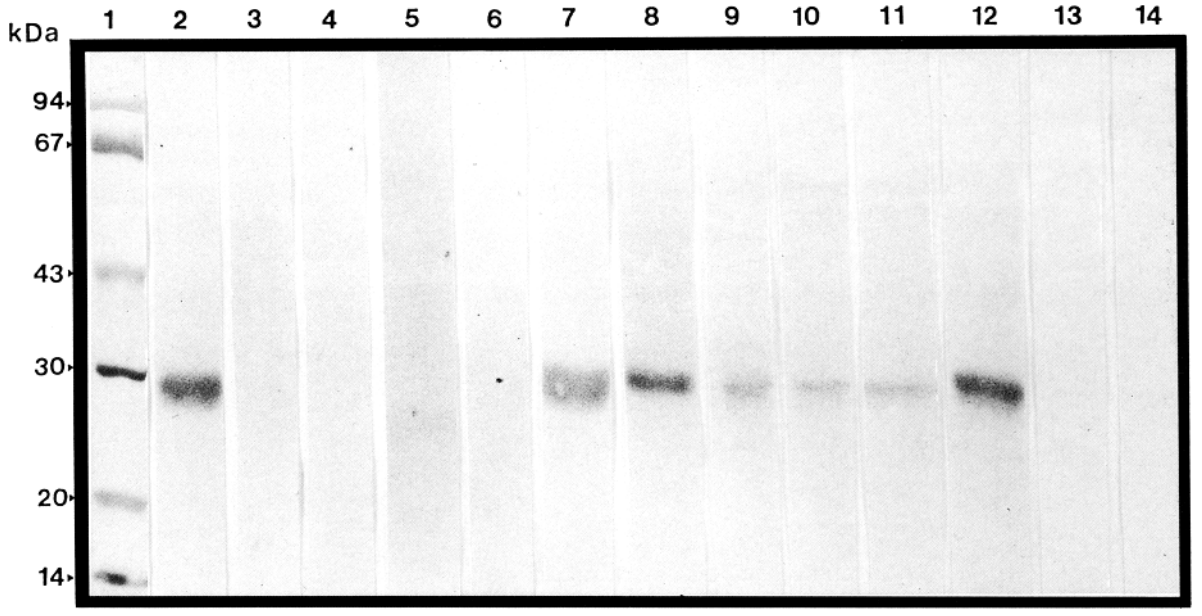

Fig. 2. Immunodetection of mouse kappa light chains on Western blots with the use of McC10. Whole mouse (*lane 2*), rat (*lane 3*) and rabbit (*lane 4*) IgG, mouse IgA_1 (*lane 5*), IgM_k (*lanes 6 and 7*), IgG_{1k} (*lane 8*), IgG_{2ak} (*lane 9*), IgG_{2bk} (*lane 10*), IgG_{3k} (*lane 11*), mouse kappa (*lane 12*), rat kappa (*lane 13*) and mouse lambda (*lane 14*) light chains were separated electrophoretically and then transferred to nitrocellulose membranes as described in the text. β-Mercaptoethanol was used as reducing agent for all samples except IgM (*lane 7*) where dithiothreitol was employed. Molecular weight standards were revealed with amido black (*lane 1*). Immunoreactivity was detected using DAB and H_2O_2 as substrates

body production as revealed in the final cloning of $RAP_2 \cdot Ag$.

Although fusion efficiency was somewhat higher with rat splenocytes from rats immunized with mouse IgG and the hybridoma $RAP_2 \cdot Ag$, the presence of hybrid-hybridomas secreting the desired bi-specific antibodies was detected in only 30% of the fusion wells tested. Six fusion wells displaying the highest bi-specific antibody activity were retested by direct ELISA using the four mouse IgG subclasses as coating antigens. Two of the fusion wells contained hybrid-hybridomas which secreted bi-specific antibodies that recognized the IgG_1 subclass only, one recognized IgG_{2a} exclusively and three recognized both IgG_{2a} and IgG_{2b}, while only two recognized all four subclasses. There appeared to be no wells containing clones which displayed exclusive recognition of either IgG_{2b} or IgG_3 alone. One fusion well, secreting bi-specific antibodies capable of binding all four IgG subclasses, was used for final cloning. One final clone, coded McC10, was isolated and further characterized.

By radial immunodiffusion, the monoclonal antiperoxidase antibodies (RAP_2) were found to be of the IgG_1 rat subclass, while the bi-specific antibodies (McC10) secreted by the hybrid-hybridoma were of the IgG_1 and IgG_{2a} rat subclasses. Consequently, the IgG_{2a} portion of McC10 was undoubtedly inherited from its lymphocyte parent and contains the recognition sites for mouse Igs.

McC10 binding sites on mouse Ig were determined both in competitive binding assays and more directly on Western blots. In the competitive binding assays, McC10 was found to recognize all of the four mouse IgG subclasses. Each of the subclass-specific IgGs were equally effective in displacing the binding of McC10 to immobilized whole mouse IgG coated on the microassay plate (Fig. 1 B). Binding was competely displaced with 1.28 μg of the IgG subclasses. In contrast, mouse IgA or IgM were not effective competitors (Fig. 1 A). Since McC10 appeared to cross-react only with the kappa light chains (Fig. 1 A), its lack of cross-reactivity with IgM was surprising as mouse IgM isolated from the TEPC tumour line contains kappa light chains.

This confusing finding from the competition assays was resolved on Western blots. Equimolar amounts of whole mouse, rat or rabbit IgG, mouse IgM, IgA, the four gamma subclasses of mouse IgG as well as lambda and kappa light chains of the mouse and rat kappa light chains, were electrophoretically separated on 10% polyacrylamide slab gels. The reactivity of McC10 revealed on immunoblots showed that McC10 exclusively recognized the mouse kappa light chains (Fig. 2). No cross-reactivity with the three heavy chain classes, subclasses or lambda light chains was demonstrated. Furthermore, when IgM_k was reduced with β-mercaptoethanol prior to separation and transfer, McC10 did not cross-react with this Ig (Fig. 2, lane 6). In contrast, when dithiothreitol was used as a reducing agent, a distinct band appeared at approximately 25 kDa, corresponding to the molecular weight of the light chain (Fig. 2, lane 7). Amido black staining of IgM after separation showed that reduction was incomplete with the use of β-mercaptoethanol.

The application of McC10 as a general developing reagent for the immunocytochemical detection of a variety of mouse primary antibodies of various classes and

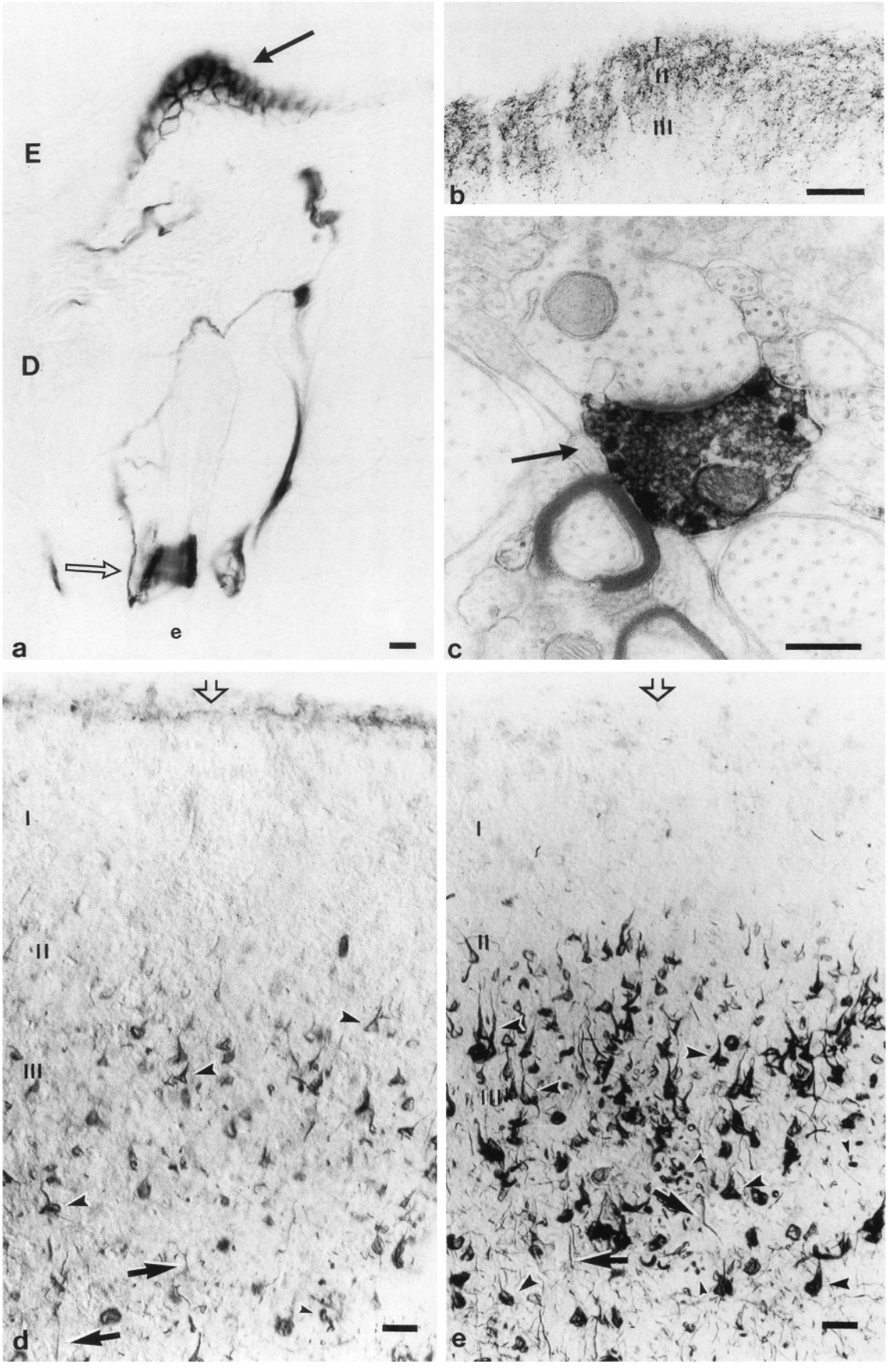
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Table 1. Mouse Ig binding sites detected with McC10

Specificity	Reference	Code	Ig Class and Subclass	Tissue
Tyrosine hydroxylase	Semenenko et al. (1986)	TOH-A1.1	IgG_1	Rat substantia nigra
Leu-enkephalin Met-enkephalin	Cuello et al. (1984a)	NOC_1	IgG_3	Rat spinal cord*
NGF-receptor	Chandler et al. (1984)	192 IgG	IgG_1	Rat CNS, skin*
Core of paired helical filaments (tau related epitope)	Wischik et al. (1988)	6.423	IgG_{2b}	Human post-mortem brain* (Alzheimer's Disease)
Alzheimer's protein A68 (tau related epitope)	Wolozin et al. (1986)	AL50	IgM	Human post-mortem brain

* Indicates results illustrated in Fig. 3

Fig. 3. a Photomicrograph of the skin of the rat lower lip, stained for the localization of nerve growth factor receptor-immunoreactive sites with the use of mouse IgG 192 followed by the bi-specific McC10 antibody. Note the complete absence of non-specific staining of collagen which is usually seen in samples rich in connective tissue. *Arrowheads* point to an area of epidermal staining, *small arrow* to nerves and the *open arrow* to a hair follicle receptor. *E* – epidermis; *D* – dermis. *Scale bar* = 20 μm.

b–c Staining of the dorsal horn of rat spinal cord with the use of an anti-enkephalin monoclonal antibody and the bi-specific McC10. **b** Light microscopic photograph demonstrates enkephalin immunoreactivities in laminae I to III. Note absence of background staining, making the tissue landmarks difficult to recognize. *Scale bar* = 20 μm. **c** Electron micrograph from lamina I, demonstrating a strongly anti-enkephalin immunoreactive axonal varicosity (*arrow*) with a few large granular and many agranular synaptic vesicles, presynaptic to a dendrite. Note the excellent preservation of the membranes and the virtual absence of non-specific staining. *Scale bar* = 0.5 μm.

d–e Frontal cortex from patient with Alzheimer's Disease, displaying immunoreactivity to the monoclonal antibody 6.423 directed against the pronase-resistant core of the paired helical filaments. The immunoreactivity was developed either with the PAP complex **d** or McC10 **e**. McC10 hybridoma supernatant immunoreactive neurofibrillary tangles (NFT, *big arrowheads*), enlarged neurites (*small arrowheads*) and thread-like elements (*small arrows*) appeared to be numerous and densely stained in cortical layers II and III. However, in adjacent section **d**, fewer immunoreactive elements were visualized when the PAP procedure was applied and a non-specific reaction in the piamater (*open arrow*) was apparent. Interference contrast microscopy, *bar* = 30 μm

subclasses is shown in Table 1 and Fig. 3. McC10 was used successfully in a number of different tissue preparations, revealing specific and distinct antigenic sites. Some of these findings are illustrated in Fig. 3a where, in the skin of the rat lower lip, a tissue prone to high background staining in keratinous and collagen-rich structures, McC10 detected specific NGF-receptor-immunoreactive epidermis for the first time. These light microscopic observations revealed the finer details of the relationship that exists between NGF-receptor-bearing peripheral nerve fibers with blood vessels, epithelial patches and hair follicle structures. These observations are also being extensively reported elsewhere (A. Ribeiro-da-Silva, R.L. Kenigsberg and A.C. Cuello, submitted). McC10 was equally effective in revealing enkephalin-immunoreactive sites in nerve fiber networks of the superficial layers of the rat spinal cord (Fig. 3b). In addition, this bi-specific reagent was used in a shortened protocol for the localization of specific enkephalin immunoreactivity at the electron microscopic level which helped preserve ultrastructural detail (Fig. 3c).

Finally, the ultimate demonstration of the highly specific nature of McC10 is illustrated when difficult tissues like human brain pathological archive samples are employed. In this regard, specific paired helical filament immunoreactive sites could be revealed more readily with McC10 with little, if any, background staining (Fig. 3e) when compared to the PAP procedure (Fig. 3d). A full report of this observation will be published elsewhere (Mena et al., submitted).

Discussion

In this study, we report the development of a bi-specific monoclonal antibody that is capable of simultaneously recognizing mouse kappa light chains and the histochemical marker, HRP. This bi-specific antibody proves to be a truly universal developing reagent for the detection of mouse Igs. Although whole mouse IgG was used as the immunogen, we managed, by careful screening, to isolate a hybrid-hybridoma that produces bi-specific antibodies that recognize all four mouse gamma subclasses. In contrast to the human gamma subclasses which are remarkably similar, with 90–95% homology in the domain regions, the mouse gamma subclasses are only 60–70% homologous (Ellison and Hood 1982; Wang et al. 1980). Therefore, the likelihood that McC10 was binding to a conserved portion of the mouse gamma globulin chain, based on its cross-reactivity with all four gamma subclasses, and represented a heavy chain specific antibody, seemed unlikely. Upon further characterization of McC10, this proved not to be the case. The exclusive cross-reactivity McC10 displays with the mouse kappa light chains has many advantages. For example, although the percentage of light chains represented by kappa or lambda classes varies among species, the kappa:lambda ratio is as high as 95:5 in the mouse (Nisonoff et al. 1975). In addition, as kappa or lambda light chains can pair equally well with any heavy chain class or subclass, one can assume that the majority of mouse

Igs contain kappa light chains. Furthermore, as in the mouse, like the human, there is only one kappa constant region gene in the haploid genome and there exists only one kappa isotype form (Potter and Lieberman 1967). Finally, it has been shown that there exists only 37% homology between the kappa light chain constant regions from mouse, rat, human and rabbit (Novotny and Franck 1975), while heavy chain homology among these divergent species may be as high as 60–70% (Jeske and Capra 1984). Consequently, it appears that we have, with the development of a kappa chain specific bi-specific monoclonal antibody, produced a highly selective developing reagent that should theoretically, recognize most of mouse Igs irrespective of heavy chain class or subclass. The potential merits of its high specificity renders this antibody very valuable in double-immunostaining procedures, where, for example, primary antibodies from other species are employed. Nevertheless, despite the theoretical assumptions made above, it appears that McC10 does not cross-react with IgM_k in a competitive binding assay. However, upon reduction of IgM with dithiothreitol, kappa light chain recognition was resumed. As circulating IgM normally exists as a pentamer of molecular weight 950 kDa, its structural configuration may hamper its interaction with McC10. However, the five monomeric units of the IgM molecule, which are arranged radially, have their Fc portions facing inwards and Fabs extending outwards (Eisen 1980). This arrangement should facilitate, rather than restrict, light chain cross-reactivity. Furthermore, when McC10 was used for the immunocytochemical detection of a mouse IgM primary antibody in fixed tissue preparations, these sites were revealed more readily (Table 1). Further studies might be required to explain this apparent discrepancy in the recognition of kappa light chains of the IgM molecule in different assay systems.

With respect to immunocytochemical detection of mouse primaries in particularly difficult tissue, a light chain specific antibody like McC10 would allow one to use Fab_2 or Fab fragments as primary antibodies to further minimize background staining. Results from this study show that McC10 could be used for the detection of a variety of mouse primary antibodies. Even in difficult tissues like skin and human autopsy samples, specificity and low background staining were obtained despite the use of the whole Ig primary antibody. The enhanced specificity of a direct epitope-specific bi-specific developing reagent like McC10 has been previously documented. Bi-specific anti-human IgG anti-HRP monoclonal antibodies were successfully used in clinical diagnoses of systemic lupus erythematosus and found to be more reliable for the accurate detection of circulating anti-nuclear antibodies in patient sera (Semenenko et al. 1988). In addition, anti anti-rabbit kappa light chain anti-HRP bi-specific monoclonal antibodies proved to be superior to conventional PAP when employed in tissue prone to display high background staining and were used successfully in a sensitive quantitative ELISA for the detection of a biologically active peptide (Kenigsberg et al. 1990). However, this bi-specific antibody, unlike McC10, does exhibit some light chain allotype specificity (Kenigsberg et al. 1990).

In conclusion, bi-specific developing reagents like McC10 overcome the need for generating individual bi-specifics for every antigen of interest and may ultimately serve to improve the quality, reproducibility and reliability of a number of immunobased procedures.

Acknowledgements. We wish to express our gratitude to Drs. A. Ribeiro-da Silva and R. Mena for providing us with unpublished immunocytochemical material, Ms. V. Wei and Mr. S. Cote for valued technical assistance and Mr. A. Forster for the photographic work. This research was funded by grants from MRC (Canada) and Medicorp (Canada).

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