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Development of a mouse antiperoxidase secreting hybridoma for use in the production of a mouse PAP complex for immunocytochemistry and as a parent cell line in the development of hybrid hybridomas

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Summary. Mouse antibodies are increasingly used as primary antibodies for immunocytochemistry as more mouse monoclonal antibodies are being produced. The localisation of these antibodies by the PAP technique requires mouse antiperoxidase antibody. A monoclonal antiperoxidase would obviate the limitations of production of a polyclonal mouse antiperoxidase. This paper describes the development of a mouse hybridoma producing such an antibody (MAP A6-2) and the use of this antibody to localise a number of mouse primary antibodies by the PAP technique for both light and electron microscopy. The antibodies localised include monoclonal antienkephalin and antityrosine hydroxylase. MAP A6-2 had a higher affinity in immunodiffusion experiments and gives slightly better staining with an horse radish peroxidase of a different type from that used for immunisation. Staining was optimum with horse radish peroxidase type X whereas horse radish peroxidase type VI was used for immunisation. Also described is the production of a HAT sensitive variant cell line allowing the possibility of using this hybridoma as a parent cell line for the production of hybrid hybridomas secreting bi-specific antibodies.

Introduction

Immunohistochemical techniques are widely used for the localisation of tissue antigens. A much favoured method of immunohistochemical staining is the peroxidase antiperoxidase (PAP) technique in which the label, horse radish peroxidase (HRP), is bound by an immunobased system (Sternberger 1970). In methods where the label is directly conjugated to the developing antibody there is loss of activity and therefore staining through denaturation of the immunoglobulins or blocking of the binding site. Both rat (Cuello et al. 1984b) and mouse (Mason et al. 1982) antiperoxidase producing hybridomas have been produced and used successfully in the rat and mouse based immunoperoxidase systems. In this study the aim was to produce a mouse antiperoxidase producing hybridoma cell line for use in peroxidase antiperoxidase immunostaining with mouse primary antibodies. This cell line could also be used for the development of a HAT sensitive antiperoxidase producing cell line. Such a cell line could be used in the production of mouse hybrid hybridomas, secreting bi-specific monoclonal antibodies, similar to those already developed which produce rat bi-specific immunoglobulins (Milstein and Cuello 1983).

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Materials and methods

Immunisations

Immunisations were made in Balb/c mice by multiple site intracutaneous (Vaitukaitis et al. 1971) and intra-peritoneal (I.P.) injections. Mice received 5, 50 or 500 µg horse radish peroxidase (HRP, Sigma type VI) dissolved in distilled H_2O and emulsified 1:1 in complete Freund's adjuvant (Difco). Immunisations were made at weekly intervals for three weeks followed by a fourth immunisation after three weeks. Seven days after the fourth immunisation blood was obtained from the mice and the serum tested for the presence of antibodies by PAP immunocytochemistry (ICC). Three days before fusion an intra-venous boost of HRP dissolved in phosphate buffered saline pH 7.2 (PBS) with no adjuvant was given; the dose being the same as in previous immunisations.

Cell fusion and derivation of variant cell lines

Fusions were carried out essentially by the method of Kohler and Milstein (1975, 1976), using the myeloma cell line NSl (Kohler et al. 1976). NSl was maintained in RPMI 1640 with 10% foetal calf serum (FCS). Myeloma cells and lymphocytes were mixed in a ratio of 10:1 and centrifuged. 1 ml 50% polyethylene glycol (BDH) was added to the pellet over 20–30 seconds and the tube rocked to mix the cells until the total exposure time to PEG was 2 min. Five ml of glucose potassium buffer which contains 8.0 g NaCl, 0.4 g KCl, 3.56 g Na_2HPO_4, 0.78 g NaH_2PO_4, 5.0 g glucose and 0.01 g phenol red/l H_2O (GKN, MRC Cellular Immunology Unit, Oxford) was added over 2–3 min after which the volume was topped up with buffer to 45 ml. The resulting mixture was centrifuged at 100 *g* for 3–5 min, the pellet resuspended in RPMI/10% FCS and distributed 2 × 24 well culture plates (Linbro) at 0.5 ml per well into wells which already contained a feeder layer of 1.5×10^6 cells per well in 0.5 ml RPMI/10% FCS. The following day 1 ml RPMI/10% FCS plus double strength HAT was added to each well. The plates were checked regularly for hybrid cell growth and fed as required by replacing 1 ml medium in each

well with fresh RPMI/10% FCS plus HAT. After 10–14 days supernatant was removed from the wells with hybrid cell growth and tested for antibody content by PAP ICC. Positive hybrids were cloned by limiting dilution and positive clones expanded and frozen in liquid nitrogen.

Cells resistant to 15 μg/ml 8-azaguanine (Sigma) were derived from the antiperoxidase secreting clone, MAP A6-2, in a step wise manner by growing the cells in successively higher doses of 8-azaguanine in RPMI/10% FCS. The concentration of 8-azaguanine was started at 1 μg/ml and was increased slowly to 15 μg/ml. These cells were then cloned by limiting dilution and supernatants from the clones tested for antiperoxidase activity, by PAP ICC for enkephalin, and for sensitivity to hypoxanthine, aminopterin and thymidine (HAT).

Immunocytochemistry

Sera and supernatants were tested for the presence of antiperoxidase activity by PAP immunocytochemistry for enkephalin immunoreactivity using the mouse monoclonal primary antibody NOCl (Cuello et al. 1984a). 10 μm cryostat sections of 4% paraformaldehyde fixed rat brain were incubated overnight at 4° C with NOCl diluted 1:50 in PBS pH 7.2 plus 0.2% Triton X-100 (PBS Triton). Sections were washed for 30 min in PBS Triton and incubated for 1 h at room temperature with rabbit antimouse IgG (Miles) diluted 1:10 (v/v) in PBS Triton. Sections were then incubated with commercial PAP (Sternberger 1970 diluted 1:100 in PBS Triton, or tissue culture supernatants to which 5 μg/ml HRP (Sigma type VI or type X, had been added. Control sections were incubated in a commercial mouse PAP (Sternberger) diluted 1:50 or 1:100. After washing in PBS Triton the sections were incubated for 15 min at room temperature with Tris-buffer pH 7.2 containing 0.06% 3′3′-diaminobenzidine (Sigma) and then for a further 15 min in the same solution containing 0.01% hydrogen peroxidase. Sections were washed in PBS Triton, dehydrated, cleared and mounted. 40 μm vibratome sections were processed free floating using a antityrosine hydroxylase monoclonal antibody, TOH A1.1 (MacMillan, Cuello, Goldstein and Sidebottom, in preparation). These sections were processed as described for light microscopy, or for electron microscopy, essentially as described by Priestley and Cuello (1983). Vibratome sections were immunostained with TOH A1.1 as for light microscopy, but replacing PBS Triton with PBS. After the diaminobenzidine, sections were incubated in 1% OsO_4 (Merck) for 2 h, dehydrated and flat embedded in Durcupan (Fluka). Selected areas of the globus pallidus were re-embedded and cut for EM examination.

Binding in gel media

To visualise the binding characteristics of the antibody MAP A6-2, mouse IgG monoclonal antibodies were immobilised by precipitation in 2% agarose plates (Sigma agarose type II) in barbitone buffer pH 8.6. Adjoining wells were filled with 1:10 (v/v) rabbit anti mouse IgG (Miles) diluted in PBS and hybridoma supernatant from the clone coded MAP A6-2. The plates were incubated overnight at 4° C in humid chambers. Wells were filled with various types of HRP at different concentrations; 50, 5, 0.5, 0.05, 0.005 μg/ml. The plates were further incubated in humid chambers for 6 h at room temperature, washed overnight in PBS, covered with filter paper and dried for 24 h. The plates were subsequently treated as for histochemical detection of peroxidase as described for immunocytochemistry. On other plates adjoining wells were filled with rabbit anti-mouse IgG 1:10 (v/v) and antiperoxidase supernatant, undiluted, 1:10, 1:20, 1:30 and 1:40, left overnight and 5 μg/ml peroxidase added the following day. The plates were then treated as described above.

Results

The mice immunised with the two higher doses of HRP (50 and 500 μg/immunisation) produced antibodies active in immunoenzyme tests to detect enkephalin immunoreactive sites in the globus pallidus of the rat; using the mouse monoclonal antibody, NOCl, as a primary reagent (see methods).

One of the mice which had been given 500 μg HRP per immunisation was used for fusion. Hybrid cell growth occurred in most of the wells of the culture plates and of the supernatants tested by immunocytochemistry for enkephalin sites 6 wells were positive, 4 strongly and 2 weakly out of a total of 15.

The hybrids from 3 strongly positive wells were expanded and frozen and 1 was lost. One of the strongly positive hybrids was cloned by limiting dilution and a clone isolated coded MAP A6-2.

The supernatant from MAP A6-2 has subsequently been used in the PAP procedure for the localisation of several mouse primary antibodies including antienkephalin (NOCl) (Cuello et al. 1984; Figs. 1a, b), antityrosine hydroxylase, TOH A1.1 (MacMillan, Cuello, Goldstein and Sidebottom in preparation, see Figs. 1c, d) and a monoclonal antibody, GTE52, which is expressed differentially in neural cells (MacMillan, Sidebottom and Cuello 1984).

Immunodiffusion experiments showed that MAP A6-2 hybridoma supernatant recognised HRP preparations other than the HRP type VI (Sigma) which was used for immunisation. MAP A6-2 also recognised HRP types I, II, IX and X (Sigma) but did not recognise HRP types VII, VIII nor did it recognise microperoxidase or crude HRP (Sigma) (Table 1). Under the conditions used for immunodiffusion MAP A6-2 showed binding with HRP type IX at a concentration of 50 μg/ml, with HRP types I, II and VI at concentrations down to 5 μg/ml, and with HRP type X there was some degree of binding at a concentration of 0.005μg/ml (Fig. 1). Immunocytochemical staining with MAP A6-2 gave slightly more intense staining with HRP type X than

Table 1. Shows the cross reactivity of MAP A6-2 with various HRP types in immunodiffusion experiments. + + + indicates a very strong reaction, + +, +, +/− and +/− − indicates, reactions decreasing in strength and − indicates no reaction

HRP Conc.	Peroxidase type								
	Micro	I	II	VI	VII	VIII	IX	X	Crude
50 μg/ml	−	+ +	+ +	+ +	−	−	+	+ + +	−
5 μg/ml	−	+	+	+	−	−	−	+ +	−
0.5 μg/ml	−	−	−	−	−	−	−	+	−
0.05 μg/ml	−	−	−	−	−	−	−	+/−	−
0.005 μg/ml	−	−	−	−	−	−	−	+/− −	−

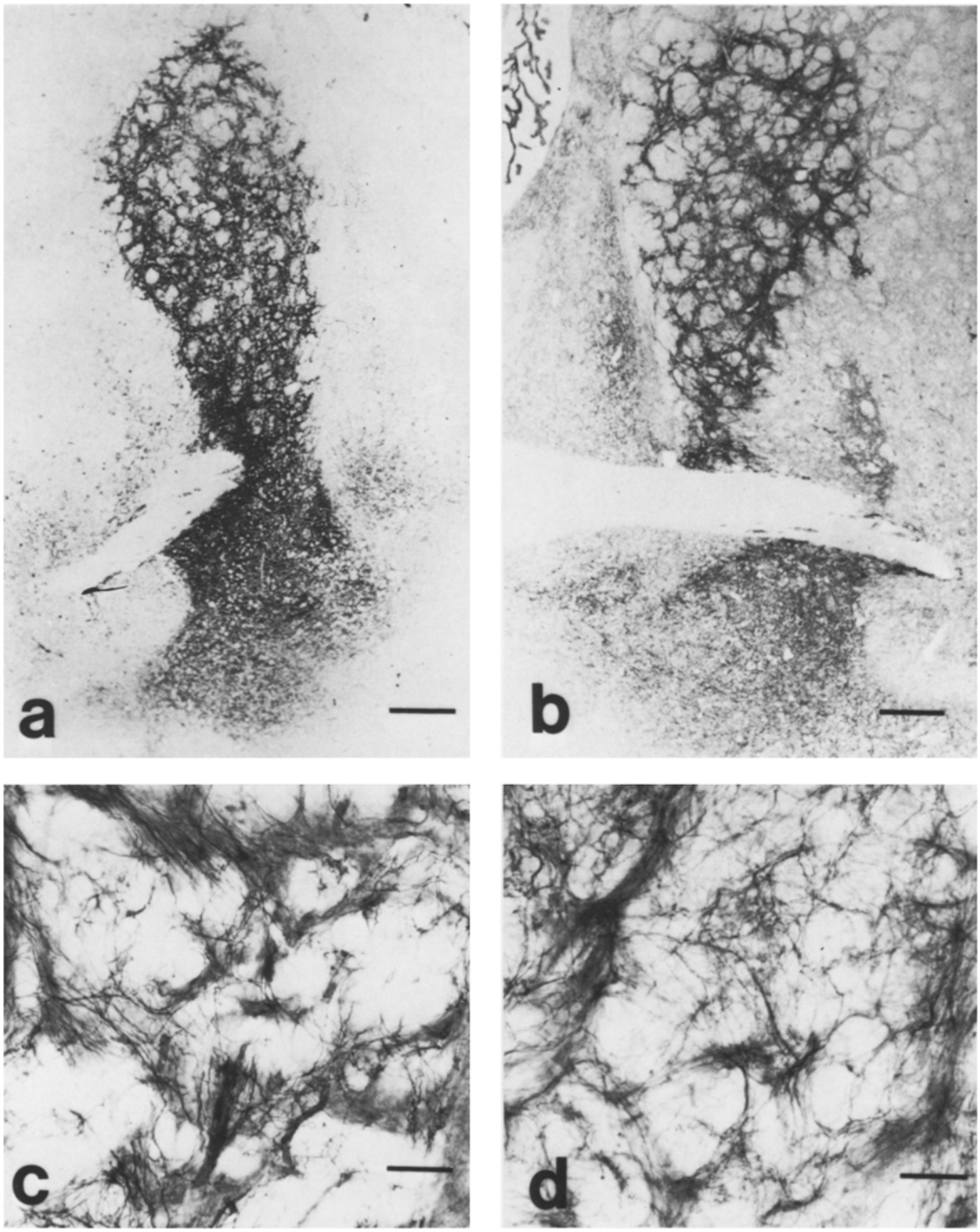

Fig. 1. (**a**) and (**b**) show two 10 μm sections through the rat globus pallidus stained by the PAP procedure to demonstrate enkephalin-like immunoreactivity. (**c**) and (**d**) show two 40 μm sections through rat globus pallidus processed free floating by the PAP technique to show tyrosine hydroxylase-like immunoreactivity. In (**a**) and (**c**) the PAP used was from the cell line MAP A6-2, this was used undiluted and with 5 μg/ml HRP type VI (**a**) and at 1:10 with HRP type X (**c**). In (**b**) and (**d**) the PAP used was from the azaguanine resistant cell line MAP.AG and in both cases it was used undiluted with 5 μg/ml HRP type VI. bars; (**a**) (**b**) = 200 μm, (**c**) = 45 μm, (**d**) = 70 μm

with HRP type VI both at a final concentration of 5 μg/ml. For routine ICC HRP type VI or X was used at a concentration of 5 μg/ml in supernatant diluted 1:10 as this was shown by ICC and Ouchterlony to give an optimum signal to noise ratio.

From MAP A6-2 an azaguanine resistant cell line was derived which was coded MAP.AG. It is sensitive to HAT medium and produces antiperoxidase antibodies which can be complexed with HRP for the detection of T-OH A1.1 and NOCl immunoreactive sites (Figs. 1b, d).

Discussion

The monoclonal mouse antiperoxidase antibody MAP A6-2 has proved to be a very useful and reliable reagent in the localisation of a number of mouse primary antibodies by PAP immunocytochemistry. The staining achieved using MAP A6-2 compared favourably with both commercially available conventional PAP and the monoclonal mouse PAP produced by Mason et al. (1982). The immunoperoxidase staining obtained had a very good signal to noise ratio with little background staining. Monoclonal mouse PAP will become increasingly useful as more mouse primary antibodies are being used in the application of hybridoma technology to immunocytochemistry.

MAP A6-2 has also been used successfully in immunostaining by the PAP procedure for electron microscopy. It has been used for localisation of tyrosine hydroxylase (T-OH) in rat striatum using a monoclonal primary antibody against T-OH (TOH A1.1, MacMillan, Cuello, Goldstein and Sidebottom, in preparation). In these experiments positive immunostaining was localised in axons and terminals of the rat striatum.

The high quality of staining achieved with MAP A6-2 may be due to the smaller size of the PAP complex, a characteristic of the monoclonal PAP produced by Mason et al. (1982) and predicted to be similar for other monoclonal PAP complexes (Cuello et al. 1984b). As a monoclonal antibody recognises only one epitope on the HRP molecule it is likely that the PAP complex consists of two molecules of peroxidase per antibody instead of the conventional complex of three molecules of HRP per two antibody molecules. The smaller PAP complex formed by monoclonal antiperoxidase antibodies endows it with two advantages over polyclonal PAP complexes, firstly the smaller complex is likely to remain stable in solution for longer. This is supported by the fact that once the monoclonal PAP complex is formed with MAP A6-2 it remains stable at 4° C for several months. Secondly penetration of the smaller complex into tissue is likely to be greater, thus enhancing staining. This may be of particular importance in immunostaining for electron microscopy.

A monoclonal mouse PAP has several other advantages over a conventional PAP in that there is a permanent and constant supply of a well characterised antibody which helps to improve the reliability and quality of ICC with conventional techniques.

A hybridoma cell line producing antiperoxidase antibodies also opens possibilities for developing new methods for ICC, particularly in the development of hybrid hybridomas producing bi-specific monoclonal antibodies which simultaneously recognise peroxidase and another defined antigen. This technique has been successfully applied to rat hybrid hybridomas recognising somatostatin and peroxidase (Milstein and Cuello 1983) and more recently substance P and peroxidase (Suresh, Milstein and Cuello, in preparation). These have allowed the introduction of one or two step immunocytochemical staining where the primary antibody incorporates its own peroxidase marker (Milstein and Cuello 1984). Preliminary experiments with MAP A6-2 to derive a cell line resistant to 8-azaguanine are proving successful and it is expected that the HAT sensitive cell line which has been isolated, coded MAP AG, will soon be used in the derivation of mouse hybrid hybridomas producing mouse bispecific antibodies.

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