

Production of Monoclonal Antibody to Oxytocin for Immunohistochemical Use[†]

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= Abstract = This study was performed to develop a hybrid cell line secreting antibody to oxytocin for immunohistochemical localization of oxytocin neuron in the brain.

Synthetic oxytocin was conjugated to bovine thyroglobulin and used in immunization of Balb/c mice. The spleen cells from immunized mice were fused with NS₁ murine myeloma cells using polyethylene glycol, and the hybrid cells were selected by HAT medium.

A radiobinding test was used to detect the presence of antibody in the culture fluid of hybrids, and specificity of the antibodies was characterized by both modified enzyme linked immunosorbent assay and immunocytochemical method.

Two hybrid cell lines were found to secrete specific anti-oxytocin antibodies.

These monospecific antibodies did not cross-react with arginine vasopressin or vasotocin.

By light immunocytochemistry these antibodies were able to localize differentially the oxytocin neurons from the vasopressin neurons in the paraventricular nucleus, supraoptic nucleus, median eminence and posterior pituitary gland.

Key Words: *Oxytocin, Monoclonal antibody, Immunocytochemistry, Hypothalamus, Neurohypophysis*

INTRODUCTION

The posterior pituitary hormones, oxytocin and vasopressin, are synthesized by neurons in the hypothalamus. These substances are transported within axonal fibers which form the hypothalamo-hypophyseal tract through the median eminence to the posterior lobe of the pituitary gland (Bargmann 1968; Heller 1974; Lederis 1974). This hypothalamo-hypophyseal system has been utilized as a model of neuroendocrine system for the study of biosynthesis, transport and secretion of peptides for a long time. Oxytocin and vasopressin could be visualized by the conventional staining (Gomori method), while other peptides were not able to be demonstrated histologically (Scharrer and Scharrer 1954; Bullock and Petrusz 1984). Since the introduction of immunocytochemical techniques into

the field of peptide localization in early 1970s, these hormones within neuronal components have been investigated with more specific and sensitive methods using antisera to these peptides. In addition to a finding of the distribution of oxytocin and vasopressin neurons within the hypothalamus, their axonal projection to extrahypothalamic regions in the brain, spinal cord as well as neurohypophysis are also found (Swanson and McKellar 1979; Sawchenko 1982). Immunocytochemical staining requires large amounts of highly specific antisera which are usually obtained by injecting a purified antigen into an animal and then testing the specificity of antisera.

Even when a highly purified antigen is used as immunizing agents, the resulting antiserum is a mixture of many different immunoglobulins with different antigenic specificities and distinct biological functions. In order to prove the specificity of antibody for the homologous antigen, it is necessary to absorb out non-specific antibodies with cross-reacting macromolecules. In case of oxytocin

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which has amino acid structure similar to vasopressin or even vasotocin, the majority of antigenic determinants of these peptides are likely to be common. Therefore, absorbing an antiserum made against oxytocin with vasopressin and vasotocin generally removes a major portion of antibody from the antiserum (Swaab *et al.* 1977). Unless the animal happens to respond in a particular powerful manner to the single antigenic determinant unique to oxytocin, there would be very small amount of antibody left after absorption. There is also a lack of reproducibility, not only from animal to animal, but from bleeding to bleeding.

In 1975 Köhler and Milstein developed a fundamentally new technique for production of antibodies of predetermined specificity in tissue culture. This procedure involved obtaining spleen cells from an immunized mouse, and fusing them to establish tumor cells. They were then grown in tissue culture so that the resulting hybrids could retain a desired monoclonal antibody production of normal cells as well as the growth characteristics of tumor cells.

Monoclonal antibodies have distinctive advantages for immunocytochemistry, since they eliminate heterogeneity of antibodies. They can selectively detect the homologous antigen among the multiple tissue antigens, be present as a continuous supply and also be applied to radioimmunocytochemistry (Cuello *et al.* 1980).

In the field of peptide immunocytochemistry monoclonal antibodies to substance P (Chan-Palay 1979; Cuello *et al.* 1979), tyrosine hydroxylase (Ross *et al.* 1981), serotonin (Consolazione *et al.* 1981), vasopressin (Hou-Yu *et al.* 1982; Cho *et al.* 1984), progesterone receptor (Dicker *et al.* 1984) have been reported at this time.

In this study we have attempted to develop a monoclonal antibody against oxytocin by the hybrid technique and applied it for the cellular localization of oxytocin in the tissue preparation of the brain and pituitary gland.

MATERIALS AND METHODS

1. Antigen preparation

Synthetic oxytocin (Peninsular Laboratories, M.W. 1007) was conjugated to bovine thyroglobulin (Sigma Chemical, M.W. 690,000), the immunogenic carrier protein, by carbodiimide technique (Odell *et al.* 1972).

To a solution of bovine thyroglobulin (12.4 mg)

in 1 ml of distilled water were added 1 ml of oxytocin (2 mg) and 1 ml of carbodiimide (1.84 mg). This mixture was stirred for 15 min and left at room temperature for 18 hrs. The mixture was then reacted with an equal volume of 1M hydroxylamine for an additional 5 hrs in order to free any esters formed with unreacted urea. The mixture was dialyzed through dialyzer tubing (Thomas 3787-D12, M.W. Cutoff 12,000) against 0.15M PO_4 buffer, pH 7.4, overnight. The volume inside the bag was measured, aliquoted, and kept frozen.

2. Immunization

Ten Balb/c mice were immunized with conjugated oxytocin emulsified with an equal volume of complete Freund's adjuvant. Generally, 0.2 ml of emulsion containing 12 μg of oxytocin was given to each mouse by intraperitoneal injection. The injections were repeated at 2-week interval over a period of 10 weeks and the presence of antibody in the tail vein was tested by a binding assay with ^{125}I -oxytocin and immunocytochemical staining on sections of the rat hypothalamus. Each sensitized animal was boosted intravenously with a dose of antigen containing 12 μg of oxytocin in saline 3 days prior to fusion.

3. Hybridization

The murine plasmacytoma line, NS₁, was used for cell fusion. This line was maintained in culture in Dulbecco's modified Eagle's medium (DMEM, M.A. Bioproducts) supplemented with fetal calf serum, hypoxanthine, thymidine, and antibiotics (HT medium). For obtaining enough numbers of healthy rapidly dividing cells by the day of fusion, 60 ml of DMEM was seeded with these cells at a density of $1 \times 10^5/\text{ml}$ 3 days before fusion. The immunized mouse was sacrificed by cervical dislocation and then soaked in ethanol so as to minimize the risk of contamination from the skin. The spleen was removed aseptically and placed in 5 ml of DMEM in a petri dish. The cell suspension was prepared by holding the spleen with blunt forceps and gently teasing cells from the capsule into the medium using curved forceps.

The calculated volume of NS₁ cell suspension was added to the spleen cell suspension in a 50 ml conical tube (Falcon) at NS₁ spleen cell ratio of 1:10, and then mixed by gently swirling the tube. The mixture was spun down and the supernatant discarded. The cell pellet was then disrupted by gentle tapping and 1 ml of 30% polyethylene glycol (PEG 1000, Baker) was added slowly over the

course of 1 min. The cells were pelleted by spinning at 500 g for 3 min and then suspended with 6 ml DMEM and transferred to two petri dishes containing 12 ml of HT medium. The following day after fusion, the HT medium was replaced with 50 ml of HAT medium (hypoxanthin 11.3 μ g/ml, aminopterin 0.15 μ g/ml, thymidine 3 μ g/ml) and the fused cell suspension was distributed into microwells of our microtiter plates using 0.2 ml suspension per microwell and placed in a humidified 37°C incubator containing an atmosphere supplemented with 6% CO₂. The plates were then left undisturbed in the incubator for at least 3 days. Thereafter the plates were inspected to identify hybrids with an inverted microscope. The supernatant for screening was removed from the cultures in which hybrid colonies were of sufficient size to be readily visible by the naked eye.

The primary screening of culture was done by a binding test with ¹²⁵I-oxytocin. The positive cultures were transferred to 24-well plates and then to 100 mm petri dishes. When cell counts reached 1×10^7 , the media were collected, and the cells were stored in a liquid nitrogen freezer. The specificity of positive cultures was tested by an enzyme-linked immunosorbent assay (Vandervelde 1978), and finally by immunocytochemical staining (Nakane 1968).

4. Radiobinding test

To 0.1 ml of culture supernatant or mouse anti-serum the same volume of ¹²⁵I-oxytocin tracer (40,000 cpm) was added in 5 ml test tube, incubated at 37°C for 1 hr, and kept overnight in the refrigerator. The following day, 0.1 ml of goat anti-mouse Ig G (Acurate Chemical) was added to the reaction mixture, agitated and then incubated. The mixture was spun and the supernatant discarded. The radioactivity of the precipitate was counted in a gamma counter.

5. Enzyme-linked Immunosorbent assay (ELISA)

Antigen (oxytocin) and its analogs (arginine vasopressin and vasotocin) were adsorbed to the wells of a microtiter plate (20 μ g/well). Appropriate amount of oxytocin and its analogs were diluted in sodium bicarbonate buffer pH 9.6 containing 1 % bovine serum albumin to a final volume of 0.2 ml per well and kept in a refrigerator at least 24 hrs before use. The microwells were washed five times in a washing buffer (phosphate-buffered saline containing 0.05 % Tween 20). Supernatant (0.1 ml each) from hybridoma cultures were incu-

bated in the coated wells at 4°C for 24 hrs.

After washing, peroxidase-labelled anti-mouse Ig G was incubated in the wells for 30 min at 37°C to allow complex formation between the conjugate and mouse immunoglobulin linked to the wells. Finally, 0.1 ml of *o*-phenylene diamine solution (400 μ g/ml of phosphate citrated buffer, pH 5, containing 0.003 % H₂O₂) was added as a substrate for horseradish peroxidase to obtain a dark brown product. The absorbance of the reaction product was measured by the automatic ELISA reader.

6. Immunocytochemical staining

Adult Sprague-Dawley rats were flushed, under ether anesthesia, with phosphate-buffered saline via the left ventricle followed by perfusion with a mixture of 2 % glutaraldehyde and 2 % paraformaldehyde. The brains were removed from the skull and sliced into 0.5 cm thick frontal blocks. The blocks containing the diencephalon were further fixed in the same fixative at 4°C for 24-48 hrs. The blocks were embedded in paraffin by routine procedures, and 7 μ m thick sections were cut in the coronal plane.

The paraffin sections were dewaxed and hydrated by conventional procedures. Sections were incubated with culture supernatants (1:200) at 4°C for 24 hrs in a humidified chamber. As a control, sections were incubated with the same culture supernatants previously adsorbed with either oxytocin, vasopressin or vasotocin (10 μ g each in 1 ml diluted antibody). Slides were washed three times in phosphate-buffered saline, and excess moisture was then removed from the area around each section before adding 0.1 ml of biotinylated anti-mouse Ig G (Vector Laboratory) at a dilution of 1:200 and followed by 40-50 min incubation. After washing, tissue sections were incubated with 0.1 ml of avidin-biotin-peroxidase and finally with diaminobenzidine substrate solution (75 mg of 3,3-diaminobenzidine in 100 ml Tris buffer containing 0.003 % H₂O₂). Slides were dehydrated and mounted.

To test the cross-reactivity of antibodies simultaneously by identification and localization of two tissue antigen, oxytocin and vasopressin, in a single section, double antibody staining was carried out as follows (Vandesande and Dierickx 1975). The sections were allowed to go through the diaminobenzidine step for detection of oxytocin as described previously. Thereafter they were eluted for 1 hr in several changes of glycine-HCl buffer at pH

7.2 to remove all bound immunoglobulins. Monoclonal anti-vasopressin (Cho *et al.* 1984), biotinylated antimouse Ig G, avidin-biotin-peroxidase complex were sequentially applied on the tissue sections, which were then stained to detect vasopressin using 4-chloro-1-naphthol as chromogen. The 4-chloro-1-naphthol solution was prepared by dissolving 100 mg 4-chloro-1-naphthol in 100 ml absolute alcohol. Then 190 ml Tris HCl, 0.05 M, pH 7.6, buffer was added.

The resulting white precipitate was removed by filtration. H₂O₂ was added to the filtrate until it contained 0.003% H₂O₂. Finally, the stained section was mounted with glycerol gelatin.

RESULTS

Antibodies were detected in the sera of eight of ten immunized mice by a radiobinding test 4-10 weeks after the start of immunization.

Sera from two (No. 4 and 8) mice showed the

best binding to ¹²⁵I-oxytocin, at a dilution of 1:1000, hence those two mice were taken for cell fusion (Table 1).

The hybrids were first visible approximately a week after the fusion and grew at different rates. The well growing hybrids were found in 53 wells out of a total of 246 wells in which the fusion mixture was planted (Table 2). Six of those hybridized cell lines produced antibodies to oxytocin by radiobinding test. The binding percentages (bound/total radioactivity) were 21-60 % (Table 3). The specificity of these 6 hybridomas was tested by an enzyme-linked immunosorbent assay using oxytocin and its analogs, arginine vasopressin and vasotocin. Two hybrid cell lines (81F4 and 82D6) were found to produce specific anti-oxytocin, while antibodies from other four cell lines showed cross-reactivities with either vasopressin or vasotocin (Fig. 1).

These 81F4 and 82D6 cell lines were recloned

Table 1. Bindings of mice sera to ¹²⁵I-oxytocin after sixth boost of conjugated oxytocin

	Mice immunized						
	1	3	4	6	7	8	9
Binding % of sera (Bound/Total)	^a 20.6	68.2	59.4	13.2	61.0	70.1	48.6
	^b 6.3	36.6	42.3	4.8	23.5	63.1	17.5
	^c 4.8	6.7	8.6	3.9	5.2	29.4	4.0

Dilution of antisera: a=1:100; b=1:1,000; c=1:10,000. Background bindings were less than 3 %.

Table 2. Frequency of hybrids selected with HAT medium

Total well dispended with fusion mixture	Wells with growing hybrids	Wells with anti- body secreting hybrids
246	53 (21.5%)	*6 (2.4%)

* Detected by radiobinding test

Table 3. Bindings of hybrid supernatants to ¹²⁵I-oxytocin

Hybrid supernatant	81A3	81E3	81F4	82D5	82D6	83C5
Binding % (Bound/Total)	50.3	21.5	34.4	22.6	60.1	29.8

Background bindings were less than 3 %.

and used in immunocytochemistry for detection of oxytocin in tissue. Sections of rat brain stained by indirect immunocytochemical reaction with these antibodies showed a strong brown colored reaction in the hypothalamus and completely free of non-specific background at a dilution of 1:200 (Plate 1 & 2). The perikarya and their fine nerve fibers were clearly revealed in the paraventricular and supraoptic nuclei, median eminence as well as in the neurohypophysis (Plate 3 & 4). This reaction product in the neuronal elements was not observed in controls in which the antibody was preincubated with excess of oxytocin, while preincubation with either vasopressin or vasotocin did not affect the staining intensity of the antibody.

In the double stained tissue sections two separate types of neuronal perikarya and of nerve fibers were simultaneously demonstrated brown stained oxytocin and grayish blue stained neurons (Plate 5

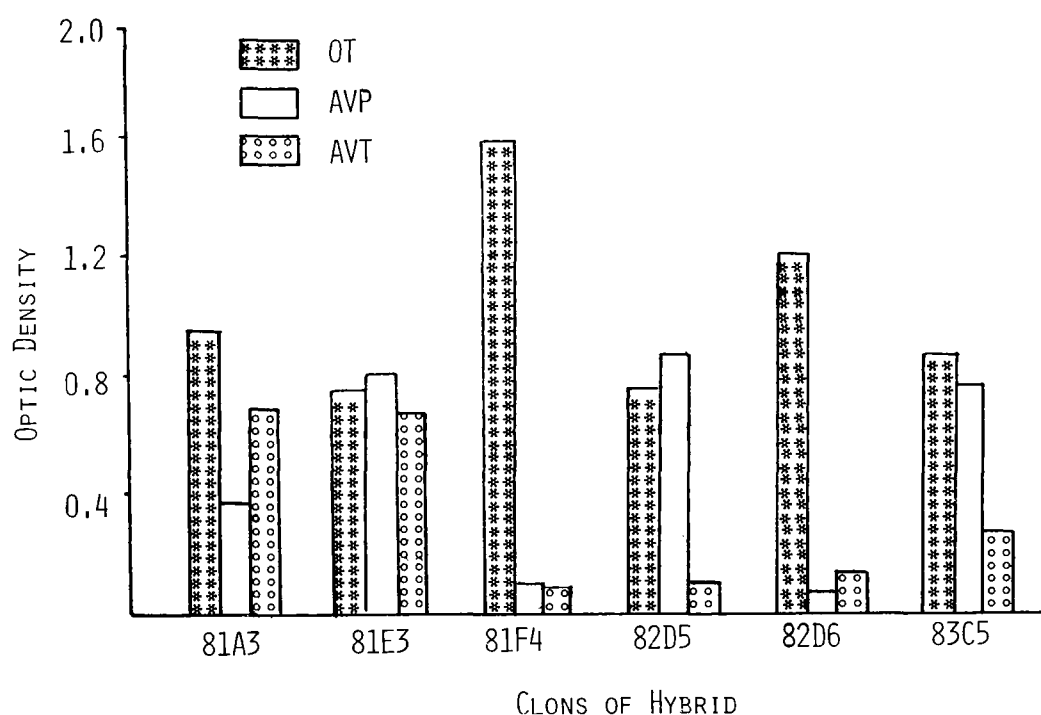


Fig. 1. Immunoreactivities of hybrid supernatant with arginine vasopressin (AVP), oxytocin (OT) and arginine vasotocin (AVT) tested by modified ELISA method.

& 6). No mixed types of staining were observed in a neuron.

DISCUSSION

The present work summarizes our attempts to produce a monoclonal antibody against oxytocin and its initial application to immunocytochemistry. There have been reports on antibodies raised conventionally to oxytocin (Zimmerman *et al.* 1973; Swanson 1977; Chan-Palay 1979). These antisera had, however, a limitation in the antibody specificity since they showed a considerable cross reaction with arginine vasopressin or vasotocin which share one or two amino acid differences among their octapeptide amino acid sequences (Gorbman *et al.* 1983).

In our experiment, it was also shown that antibodies from four of six cell lines which secreted anti-oxytocin had strong cross-reactivities with either arginine vasopressin or vasotocin. The specificity of new antibody should be established in order to evaluate the usefulness of the antibody. In case of immunocytochemistry the specificity has been usually proven with blocking test in which the antiserum is absorbed to the homologous antigen. The limitation of the absorption test was illustrated very well with a homozygous diabetes insipidus (Brattleboro) rats that lack arginine vasopressin by genetical defect (Boer *et al.* 1980). Although anti-vasopressin serum could be absorbed totally with arginine vasopressin, this antibody stained the

hypothalamo-neurohypophyseal system of the diabetes insipidus rat. This staining appeared to be caused by cross reaction with the structurally closely-related oxytocin (Van Leeuwen 1980). Therefore, it appears that the absorption test alone is insufficient, and that other complementary approaches are necessary to prove antibody specificity for the homologous antigen.

A wide variety of techniques have been developed for detection of antibodies in hybridoma culture supernatants. Immunocytochemical technique is inherently much more informative than most of the alternative screening methods such as radiobinding test or ELISA method. Radiobinding or ELISA method provided only single numerical value for each positive supernatant without any indication of the constituents to which the antibody is binding. In contrast, the immunocytochemical reaction pattern of a monoclonal antibody on a tissue section represents a highly characteristic features which often allows the specificity of a new antibody to be identified at the primary screening stage.

A rapid radiobinding test was employed in this experiment. As a large number of samples were required to be tested as early as possible at the right time. Out of 53 clones, the supernatant of six showed specific bindings to ^{125}I -oxytocin. The specificity of these six hybridomas was studied by both ELISA and immunocytochemical methods.

Two cell lines produced specific antibodies to oxytocin but antibodies of the other four cell lines

showed cross-reactions with either arginine vasopressin or vasotocin. Checking the cross reactivities of the antibody with either arginine vasopressin or vasotocin is greatly significant to evaluate the usefulness of the antibody for immunocytochemical application, because those three peptides, oxytocin, arginine vasopressin and vasotocin, are synthesized together in the hypothalamo-neurohypophyseal system, and closely related in their amino acid sequence (Gorbman *et al.* 1983). However, antibodies from our two cell lines (81F4 and 82D6) were able to demonstrate the perikarya, fine nerve fibers in the hypothalamus, median eminence and posterior pituitary gland, which were abolished by preincubation with an excess of oxytocin. The specificity of these antibodies was further tested by double antibody staining using a monoclonal anti-vasopressin and two different chromogens.

It was revealed that oxytocin and vasopressin were localized in separate neurons and there was no part of either perikarya or nerve fibers which responded to both two antibodies. These results demonstrated that our antibodies were monospecific for oxytocin, and suitable for immunocytochemical detection and characterization of oxytocin in the brain tissue. It is also suggested that monoclonal antibody technique can provide an attractive method for producing exquisitely specific antibodies against low molecular weight neuroactive compounds, and be a much valuable tool for the investigation of these substances in tissue preparation.

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LEGENDS FOR PLATES

- Plate 1. Micrography of the rat hypothalamus coronal section treated with monoclonal anti-oxytocin (84F4). Brown-colored oxytocin neurons are selectively localized in the supraoptic nucleus. OC: Optic Chiasm. x40
- Plate 2. Oxytocin neurons in the paraventricular neurons were also selectively stained brown in this dark-field micrograph. V3: Third Ventricle. x40
- Plate 3. High magnification of oxytocin neurons colored brown by treated anti-oxytocin and diaminobenzidine substrate. x1,000.
- Plate 4. High magnification of vasopressin neurons colored grayish blue by treatment of anti-vasopressin and 4-Cl-1-naphthol substrate. x1,000
- Plate 5. Simultaneous demonstration of two types of neurons. Oxytocin neurons (brown) and vasopressin neurons (grayish blue) are separately localized. Note that no mixed stained neurons are visible in this double antibody staining. Rat paraventricular nucleus. X450
- Plate 6. Simultaneous demonstration of two types of nerve fibers. Oxytocin fibers (brown) and vasopressin fibers (grayish blue) are separately localized in this double antibody staining. Note that no staining reactions are visible in the surrounding pars intermedia. Rat pituitary gland. X450.

= 국문초록 =

Oxytocin에 대한 단세포군 항체 생산 및 면역조직화학적 이용에 관한 연구

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Hybridoma technique을 이용하여 특이성이 높은 단세포군 항체를 개발함으로써 뇌조직 절편에서 oxytocin 분비세포를 vasopressin 분비세포와 감별 염색할 목적으로 oxytocin을 bovine thyroglobulin과 결합시켜 Balb/c 생쥐에 면역시키고 항체가 생성됨을 확인한 다음 polyethylene glycol을 매체로 하여 비장세포와 형질세포종 세포(NS₁)와의 hybrid를 만들었으며 이들로부터 항체분비 여부와 그 특이성을 radiobinding test, ELISA, 면역조직화학반응 등에 의하여 검정한 결과 다음과 같은 결론을 얻었다.

1. HAT 배지에 의하여 선택 배양된 hybrid 중 oxytocin에 반응하는 항체를 분비하는 것은 6종이었다.

2. 2종의 세포주 (hybrid 81F4, 82D6)에서 분비되는 항체는 oxytocin과 특이적 결합을 보였으나 나머지 4종은 oxytocin 외에 arginine, vasopressin, vasotocin 등과도 결합하는 교차반응을 보였다.

3. 흰쥐의 뇌조직 절편상에서 시행한 면역조직화학반응에서 이 항체들은 시상하부의 oxytocin 분비세포를 vasopressin 분비세포와 명확히 감별염색할 수 있었다.

이상의 연구 결과로 미루어볼 때 본 실험에서 생산된 항체는 조직절편상에서 oxytocin 분비세포를 특이적으로 염색할 수 있는 성능을 가진 것으로 판단된다.

