COMBINED TECHNIQUES OF IMMUNOCYTOCHEMISTRY AND $[^3\text{H}]$ THYMIDINE AUTORADIOGRAPHY FOR ANALYZING THE TIME COURSE OF PEPTIDE NEURON PRODUCTION

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A combined method of immunocytochemistry and $[^3\text{H}]$ thymidine autoradiography was devised to analyze the time of production of chemically characterized neurons. The method consisted of the following steps: 1) $[^3\text{H}]$ thymidine injection in pregnant animals, 2) immunocytochemistry using peroxidase and anti-peroxidase (PAP) techniques, and 3) autoradiography. The technical problems of these methods are discussed.

Over twenty years ago, it was proved that matrix cells synthesize DNA and go through mitosis and also become neuroblasts (4, 5). Because thymidine is a specific component of DNA, an injection of radioactive thymidine exclusively labels cells in the DNA synthesis phase (S-phase) of their cell cycle at the time of injection (9). When the matrix cells in their final cell cycle are labelled with $[^3\text{H}]$ thymidine, their progeny (neuroblasts or neurons) would be expected to be heavily labelled with the isotope. The administration of $[^3\text{H}]$ thymidine on an indicated day of embryonic life enables us to determine the time of final mitotic division of matrix cells. Previously, there had been several autoradiographic studies on the developing nervous systems, and the time of neuron production in various brain regions had almost been determined (1, 2, 3). However, routine $[^3\text{H}]$ thymidine autoradiography cannot demonstrate the time of production of neurons with specific phenotypes, such as peptide-containing neurons (7).

Recently, immunocytochemical techniques were established (14). Over twenty kinds of neurotransmitter-like substances were visualized in brain tissue using these techniques (6). Thus, we applied $[^3\text{H}]$ thymidine autoradiography and immunocytochemistry to the same tissue sections to determine the time of neurogenesis of chemically identified neurons, particularly peptide-containing neurons. We describe in detail the combined techniques of $[^3\text{H}]$ thymidine autoradiography and immunocytochemistry.

MATERIALS AND METHODS

The animals used in this experiment were ICR strain pregnant mice. The methods described here are presented schematically in Fig. 1.

FIG. 1. A schematic representation of the combined methods of immunocytochemistry and [^3H]thymidine autoradiography applied in the mouse.

[^3H]thymidine injection

The day when a vaginal plug was found in the dams was defined as gestational day 1 (E1). The pregnant dams were injected with [^3H]thymidine (spec. ant. >15 Ci/mmol, New England Nuclear) twice a day subcutaneously with a total dose of $7.5 \mu\text{Ci/g}$ body weight on the indicated day of gestation. The time interval between the first and second injection of the isotope was decided based on matrix cell kinetics; e.g., 4.5 h on day E10, 5 h on day E11 and E12, 7 h on day E13, day E14 and E16, 9 h on day E17 and E18, 20 h on day E20.

Preparation of brain sections

Following injections, the dams continued their pregnancies to delivery at E20. The progeny of at least two dams in the injection group were sacrificed at 8–10 weeks postnatally by cardiac perfusion with a fixative containing 4% paraformaldehyde, 0.2% picric acid and 0.35% glutaraldehyde in 0.1 M phosphate buffer (PB). Brains were removed from the skulls and were postfixed with a fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M PB for 2 days. They were then immersed in the 0.1 M PB containing 20% sucrose for 2 days or more. Frozen sections (6 μm in thickness) were made using cryostat.

Immunocytochemistry

Sections were mounted on chrome-coated glass slides and were air-dried. They were processed for immunocytochemistry by a modified PAP method, the "double bridge" method devised by Vacca *et al.* (16). We adopted this method to increase the immunoreactive density in the perikarya in order to clearly identify immunoreactive neuronal perikarya.

Anti-peptide serums were diluted to various dilutions with 0.1 M phosphate

buffer saline (PBS), for example, anti-vasoactive intestinal peptide (VIP) serums were diluted 1 : 4000. Sections were incubated in diluted anti-peptide serums for 12 hr at room temperature. Sections were then washed in 0.1 M PBS and transferred to a solution of goat anti-rabbit IgG (Miles, Lab.) 1 : 200 for 1 hr. After rinsing them with 0.1 M PBS, they were soaked in a solution of peroxidase anti-peroxidase (PAP) complex (Dako, Co.) 1 : 200 for 1 hr. Then we repeated the process for anti-rabbit IgG and PAP. Finally, the sections were exposed to 0.02% 3,3'-diaminobenzidine-4HCl substrate (DAB) (Wako Pure, Chem.) solution, containing 0.03% H₂O₂, for about 10 min. At the end of the immunocytochemical process, immunoreactive peptides containing perikarya were colored brown with DAB.

Autoradiographic procedure

In the autoradiographic procedure to visualize incorporated [³H]thymidine, the dipping method was used. After DAB reaction, sections were coated with Ilford L4 emulsion and exposed for 4 weeks in a black box. Development was done with Fuji FD111.

RESULTS AND DISCUSSIONS

The present combined technique of immunocytochemistry and [³H]-thymidine autoradiography enables us to detect neurons with brown-colored immunoreactivity in their cytoplasm and silver grains on each nucleus (Fig. 2). To estimate the time course of neuron production, silver grains on each nucleus of both peptide-immunoreactive and peptide non-immunoreactive neurons were counted; the neurons with more than half of the maximum number of silver grains were regarded as heavily labelled neurons. Because heavily isotope-labelled neurons might be expected as the direct progeny of the matrix cells that were in the final DNA synthesis phase at the time of [³H]thymidine injection, we can decide the time course of neuron production.

Usually approximately 500 peptide-immunoreactive neurons for each nucleus were counted for statistical analysis. The ratio of heavily labelled immunoreactive neurons in each nucleus was calculated.

To clarify the time course of neuron production, the following several points should be considered. The first is the injection dosage and schedule of [³H]-thymidine. Radiation damage to the cells after incorporation of [³H]thymidine has been reported (9). As isotope injection dose increases, toxicity to the cell increases (13), and autoradiographic labelling decreases. Consequently, we decided on a dosage of less than 5 μ Ci/g body weight per injection. After a single injection, the thymidine was incorporated into the matrix cells which were in the process of DNA synthesis. However, thymidine is metabolized and excreted within 2 hr in mammals (9). Considering the generation time and duration of the S-phase, we injected twice a day to increase the labelling indexes of the indicated day of gestation. For example, on the embryonic day 10, the generation time of matrix cells was suspected to be about 7 to 9 hr and the duration of the synthetic phase 4 to 5 hr (8, 10). So the following injection schedule on E10 was decided upon as follows: 5 μ Ci/g body weight injection was first given at 10:00 a.m. and 4.5 hr later, a second

FIG. 2. A section of mouse suprachiasmatic nucleus on day E12 stained for VIP and developed for autoradiography of $[^3\text{H}]$ thymidine. Nuclei were colored blue with hematoxylin. The arrow shows a heavily isotope-labelled immunoreactive neuron. Arrowheads indicate isotope-labelled non-immunoreactive neurons. $\times 850$

injection of $2.5 \mu\text{Ci/g}$ body weight was given. By this dual injection in one day, matrix cells which might not be in the S-phase at the time of the first injection could possibly be in at the time of the second injection.

A defined section thickness is necessary to carry out quantitative evaluations after $[^3\text{H}]$ thymidine autoradiography (11). Because $[^3\text{H}]\beta$ -particles have some effect within a field of a few micrometers, sections of less than $6 \mu\text{m}$ thickness were used for analyzing $[^3\text{H}]$ -labelled neurons. If the sections are thicker than $10 \mu\text{m}$, there is a greater possibility of misreading "labelled immunoreactive cells". For example, non-immunoreactive neurons with an isotope-labelled nucleus near the emulsion layer occasionally encounter immunoreactive neurons which have an isotope non-labelled nucleus in the deeper layer. In this case, the possibility of falsely identifying immunoreactive neurons may increase in proportion to the section thickness, particularly in the regions where immunoreactive neurons are densely packed. Another reason that thick sections are not suitable is that in a thick section, "true" isotope-labelled immunoreactive neurons distant from the emulsion layer may be identified as "non-labelled immunoreactive neurons", because β -particles in their nucleus cannot reach the emulsion layer. Contrary to the previous example, misreading "non-labelled immunoreactive cells" may be greater in this case.

In the DAB reaction step to color immunoreactive peptides, we had to work carefully so as not to strongly stain immunoreactive products. As Sternberger indicated, DAB forms polymetric products (14, 15) which become heap-like figures,

and these strong products may interfere with silver grain counting under light microscopy.

Paying careful attention to these technical points, our present method combining [^3H]thymidine autoradiography and immunocytochemistry will be a useful method for studying the production sequence of the functionally identified neurons. By using this technique, we showed the production sequence of vasopressin-containing neurons differs according to the nucleus in which they exist (12), and further, that there is a time lag between the stages of neuron production and peptide synthesis in VIP-containing or vasopressin-containing neurons in the suprachiasmatic nucleus (Okamura, in preparation).

ACKNOWLEDGEMENTS

This work was supported in part by grants (No. 59770053 and No. 57370002) from the Ministry of Education, Science and Culture, Japan.

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