II. Materials and Methods

Male Wistar rats (180–200 g body weight) obtained from Japan SLC (Shizuoka, Japan) were maintained on a 12 hr light–12 hr dark lighting schedule for a week with food and water available *ad libitum*. Paraquat (methyl viologen) dichloride hydrate (Aldrich Chemical Co., Milwaukee, WI) was dissolved in physiological saline, and injected intraperitoneally (4 animals/group). At 24 hr after the drug administration, rats were perfused through the ascending aorta with phosphate buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) under anesthesia (sodium pentobarbital, 50 mg/kg, i.p.), and the brain was disected out and immersed in the same fixative overnight at 4°C. The tissue was embedded in paraffin, and 5 μm-thick sections were made using a sliding microtome.

Sections were dewaxed with xylene, rinsed with PBS, and endogenous peroxidase activity was quenched by incubating with 3% hydrogen peroxide in PBS for 5 min. They were incubated with either a mouse monoclonal anti-microtubule associate protein-2 (MAP-2) antibody (1 : 200-dilution; Amersham, Buckinghamshire, England) or a mouse monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (1 : 2000-dilution; Sigma Chemical Co., St. Louis, MO) for 1 hr at 37°C, and the immunostaining was carried out using LSAB2 kit/HRP (Dako Corp., Carpinteria, CA) following the manufacturer's instruction (incubating with the mixture of biotinylated goat anti-mouse IgG and biotinylated goat anti-rabbit IgG antibodies for 30 min, followed by incubation with horseradish peroxidase-labeled streptoavidin for 15 min). The immunostaining was visualized by incubating the sections in the mixture containing 0.02% 3,3′-diaminobenzidine (DAB) and 0.03% hydrogen peroxide in PBS. The coverslip was mounted on a glass slide, then examined and photographed.

III. Results and Discusion

To address the question of whether paraquat could be toxic to glial cells *in vivo*, the effect of this drug on astrocytes in rat hippocampus was examined using immunohistochemical technique. The widespread distribution of the cells expressing the immunoreactivity of GFAP, a marker of astrocytes, was observed in rat hippocampus (Fig. 1a), and the number of these cells was reduced by the administration of paraquat in a dose-dependent manner. The GFAP-immunoreactive cells were evidently reduced by the administration of 25 mg/kg of paraquat (Fig. 1b), and the dramatic reduction of these cells was observed by 50 and 100 mg/kg of the drug (Fig. 1c, d). These observations clearly indicate that the number of astrocytes in the rat brain is reduced by paraquat poisoning, thus suggesting that paraquat can be toxic to not only glial tumor cells *in vitro* but also normal glial cells *in vivo*.

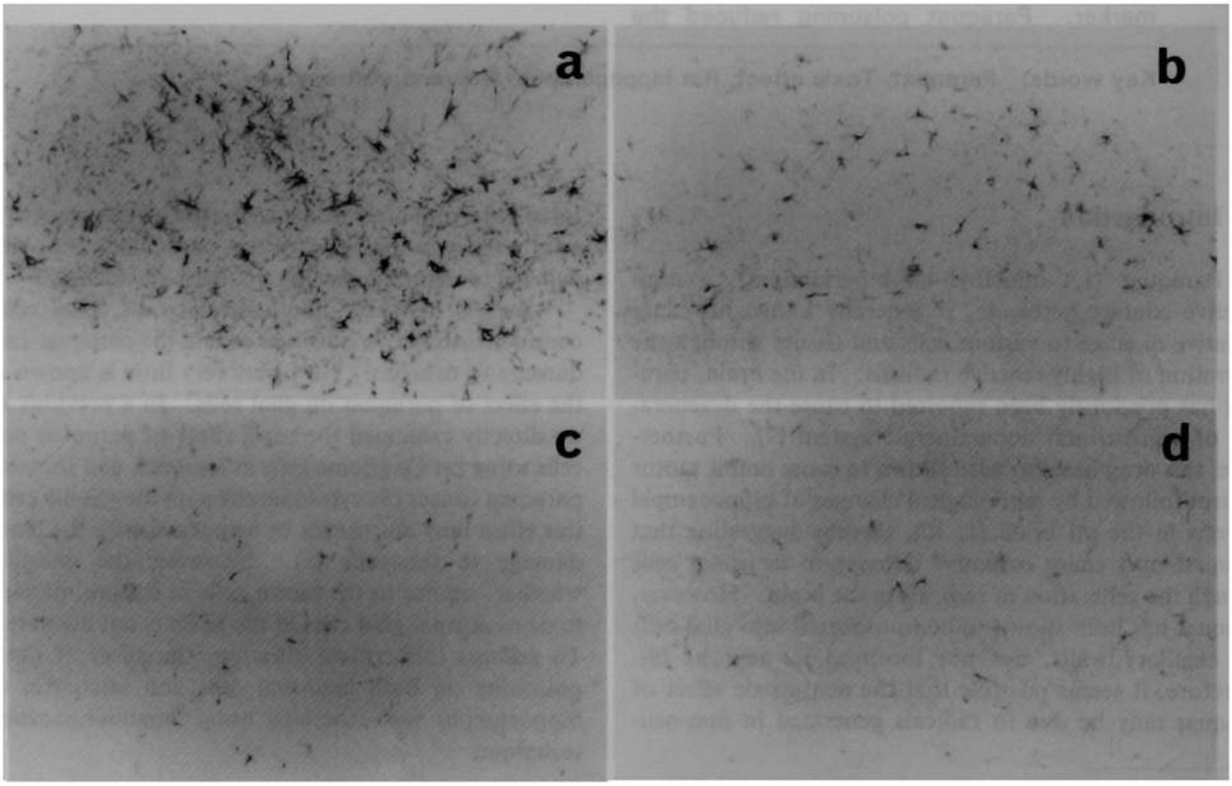

Fig. 1. GFAP-immunoreactive cells in paraquat-poisoned rat hippocampus. Animals were treated with 0 (**a**), 25 (**b**), 50 (**c**) and 100 (**d**) mg/kg of paraquat for 24 hr, and the sections were prepared and GFAP was then stained as described in the text. The CA3 region was shown. ×25.

In addition to glial cells, paraquat was also shown to cause the damage to neuronal cells in rat hippocampus (Fig. 2). The immunoreactivity of MAP-2, a histological marker of intact neuronal cells, was evenly expressed in a whole region of rat hippocampus (CA1/CA2 and CA3 regions were shown in Fig. 2a, b), and loss of the MAP-2 immunoreactivity was observed in CA1/CA2 but not in CA3 regions of hippocampus in the animals treated with 25 mg/kg of paraquat (Fig. 2c, d), indicating that neuronal cells in rat hippocampus are susceptible to paraquat poisoning. Furthermore, the more extensive damage to hippocampal neurons was evidently observed by the administration of 50 mg/kg of the drug (Fig. 2e, f). Contrary to our expectation, the neuronal damage caused by a higher dose of paraquat (100 mg/kg) appeared to be less extensive in comparison with that observed at lower doses (Fig. 2g, h). It therefore seems possible that the paraquat-induced damage to the hippocampal neurons is not simply due to its direct action on neuronal cells, but the mechanism of this unexpected effect is still entirely in question.

Paraquat has been shown to be metabolized by microsomal and mitochondrial enzymes [2–6], thus resulting in the oxidative damage to various cells and tissues through the generation of highly reactive radicals. Furthermore, this drug has been reported to be transported

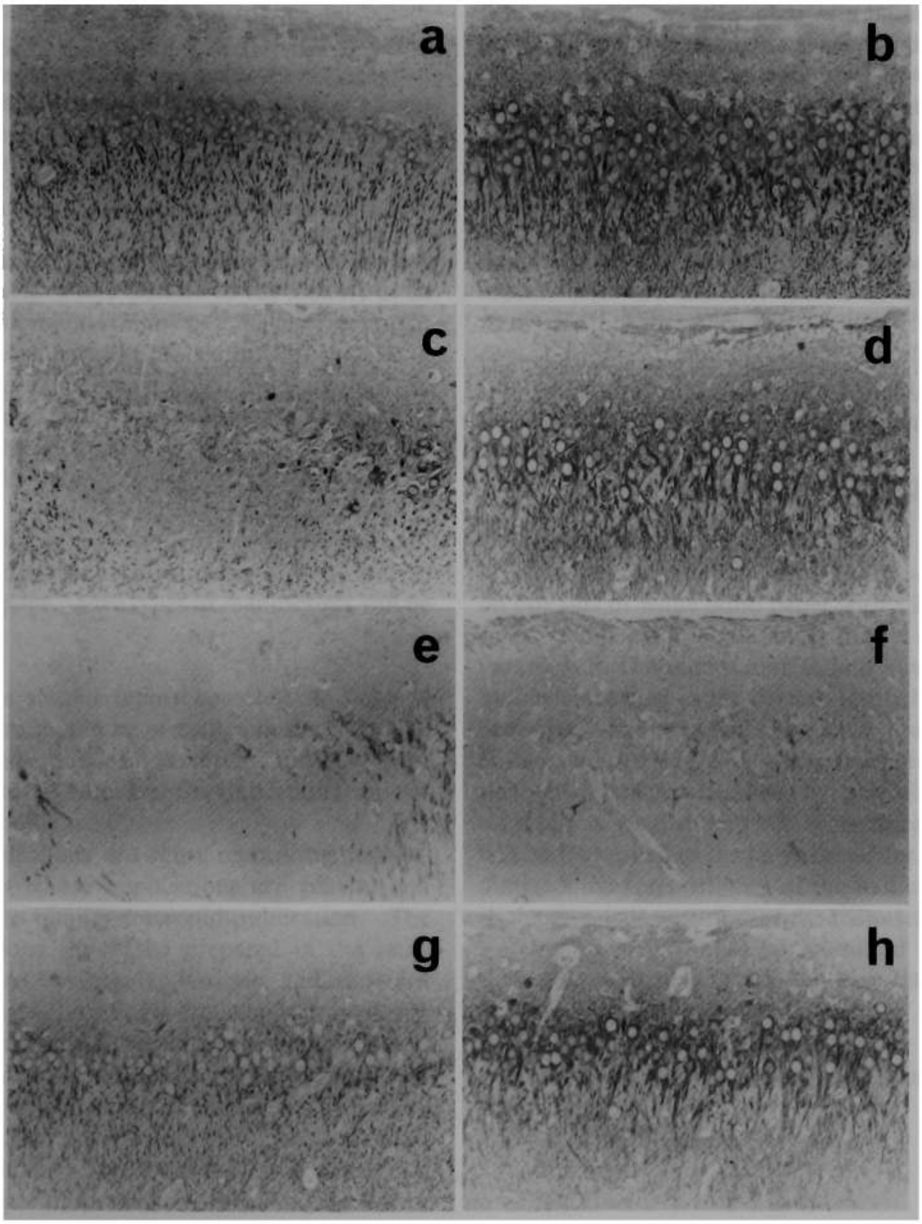

Fig. 2. MAP-2 immunoreactivity in paraquat-poisoned rat hippocampus. Animals were treated with 0 (**a, b**), 25 (**c, d**), 50 (**e, f**) and 100 (**g, h**) mg/kg of paraquat for 24 hr, and the sections were prepared and MAP-2 was then stained as described in the text. The CA1/CA2 (**a, c, e, g**) and CA3 (**b, d, f, h**) regions were shown. × 50.