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In Vivo Gene Gun-Mediated DNA Delivery into Rodent Brain Tissue

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Various types of gene transfer into live tissues have been tried. However, *in vivo* gene transfer into brain tissue or neuronal cells without virus vector has required a great effort. Particle-mediated gene transfer into live brain tissue was thought to be impossible because of its fragility and the mechanical problem of a previous type of gene gun. In addition, particle-mediated DNA transfer into monolayer-cultured cells without mechanical damage has been difficult. We successfully transferred DNA into rodent live brain tissue and also into monolayer-cultured cells without mechanical damage by using a new type of gene gun and also confirmed gene expression in the brain. This new method represents another variation of gene transfer into the brain.

Gene transfer methods are classified into two types, viral and nonviral. A viral transfer method has a risk in itself, because transfected viruses may revert to the wild type even if they are replication-defective viruses. In addition, neutralizing antibodies against viruses such as herpes and adenovirus exist naturally in adults, and it is possible that these antibodies might interfere with the transfection of the viruses. To avoid these disadvantages of viral vectors, it is desirable that highly efficient nonviral gene transfer methods be available. In terms of nonviral gene transfer, electroporation (1) is very hard to use for brain or cultured neuronal cells because of its potent cytotoxicity toward neuronal cells and possible danger of causing convulsions. Liposomes (2) also are cytotoxic. To express foreign genes in nondividing neuronal cells, DNA transfection methods such as phosphate calcium precipitation can be used, but are not very effective. Therefore, DNA microinjection into the nucleus or generation of transgenic mouse are available for gene transfer into neuronal cells. However, the microinjection technique is technically difficult, and it takes long time to generate a transgenic mouse.

A particle-mediated gene transfer method, the so-called "gene gun," was first applied to the transformation of plants tissues (3), which have strong cellular walls. At high velocity, DNA-coated gold particles can be injected efficiency by such guns into target organs, tissues, or single cells.

The Helios gene gun system (Bio-Rad) has been used for transfection of DNA into organitypic cultures of brain (4–6). However, the particle bombardment with a strong stream of helium gas causes severe damage to monolayer-cultured cells (7) or to a fragile tissue such as brain. The minimum gas pressure of the Helios gene gun is 100 psi, which can destroy the center of the target. The presently described PIGG-3 system was designed as a new apparatus for gene delivery into soft tissues. The principle of this system is as follows: A bullet accelerated by helium gas strikes the upper surface of a metal plate which is dented by the impact, and particles on the lower surface of the metal plate are delivered to the target by the shock from the bullet. So no harmful impact of helium gas can injure the target cells or tissues.

Here we present a first report of successful gene transfer into live rodent brain tissue by use of this improved of type gene gun, PIGG-3, without severe brain damage.

MATERIALS AND METHODS

PIGG-3 gene gun. The PIGG-3 system (Nippon Ikakikai Seisakusyo, Japan), which can release particles of adequate speed without gas impact on the target, was used. The plates, 5 mm in thickness, were autoclaved. The bullet and plate were also made by Nippon Ikakikai Seisakusyo. The gold particles (Tokurikihonten, Japan) were 1 μm in diameter.

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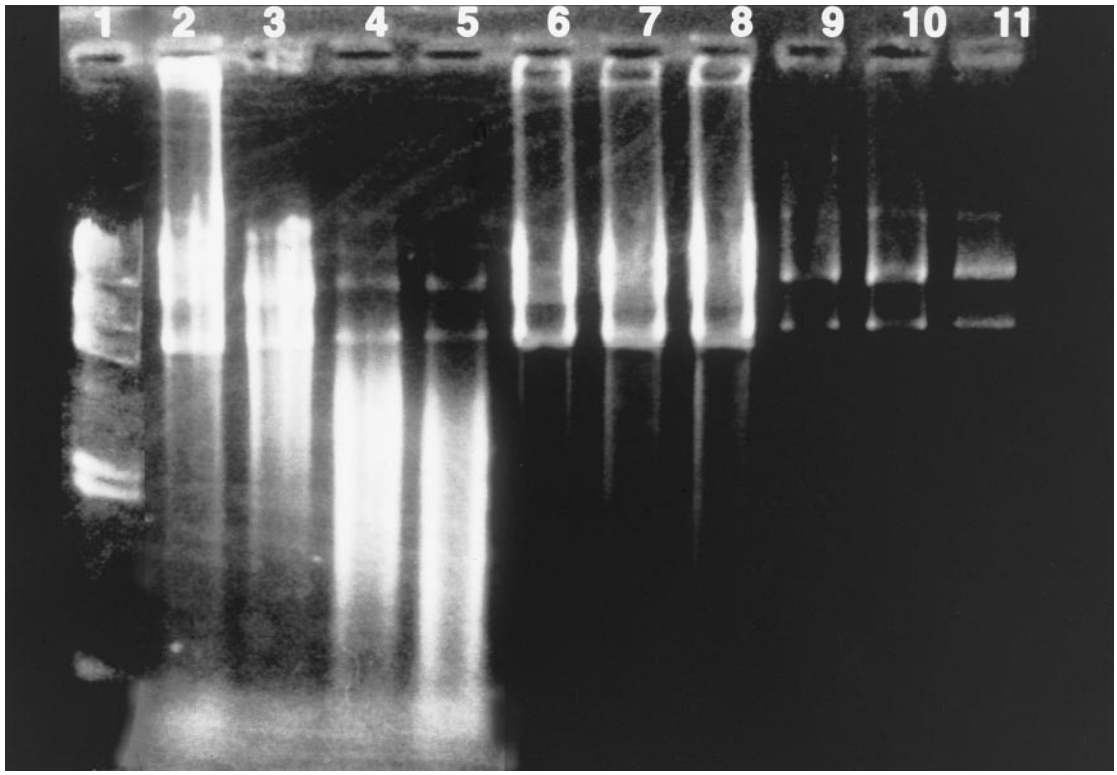


FIG. 1. Stability of DNA on gold particles treated with sonication. Lane 1, λ HindIII digestion. Lane 2, untreated DNA. Lanes 3–5, soluble DNA. Lanes 6–8, dissolved DNA in TE after sonication of DNA-coated gold particles. Lanes 9–11, free DNA in TE after sonication of precipitated DNA. Duration of treatment with Sonifer 250: 2 s (lanes 3, 6, 9), 5 s (lanes 4, 7, 10), 10 s (lanes 5, 8, 11). Free DNA in TE was destroyed in longer duration of the treatment, but DNA on gold particles and precipitated DNA were stable even with long treatment.

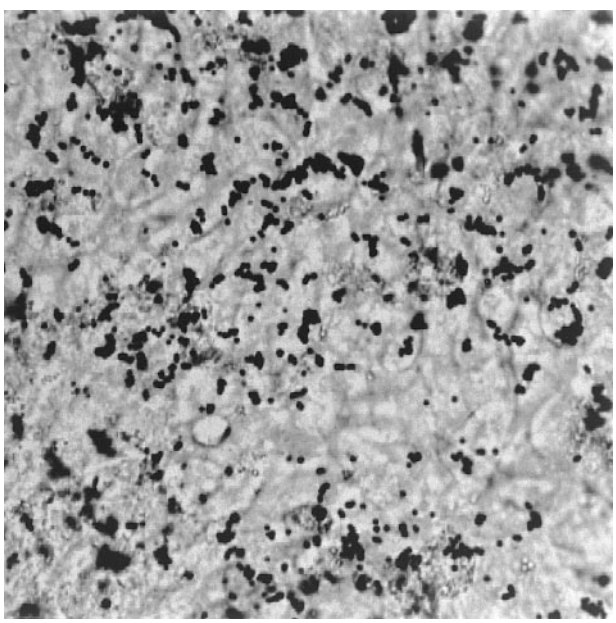


FIG. 2. The center of a 203G cell culture subjected to gene transfer by use of the gene gun of the PIGG-3 system operated at the maximum pressure of 160 psi. Black dots are gold particles. No mechanical damage was shown (×100).

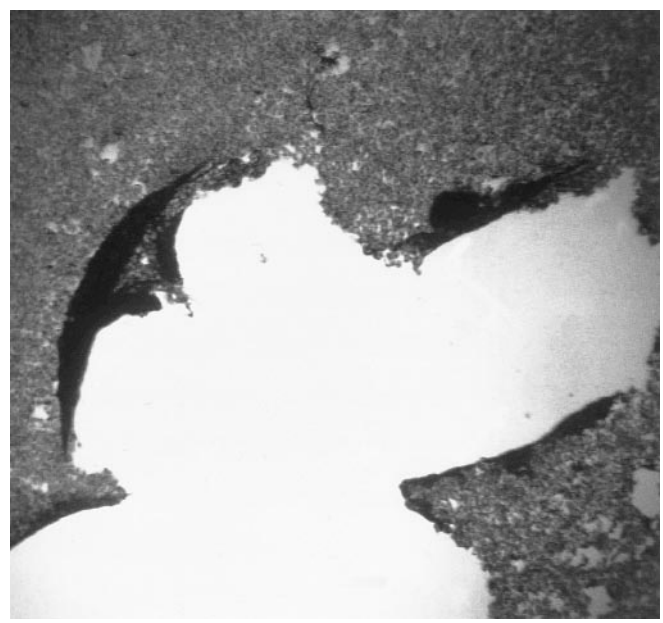


FIG. 3. The center of a 203G cell culture subjected to gene transfer by the Helios-type gene gun. Remarkable gas mediated damage to the center of the culture is evident. The pressure was 100 psi (×40).

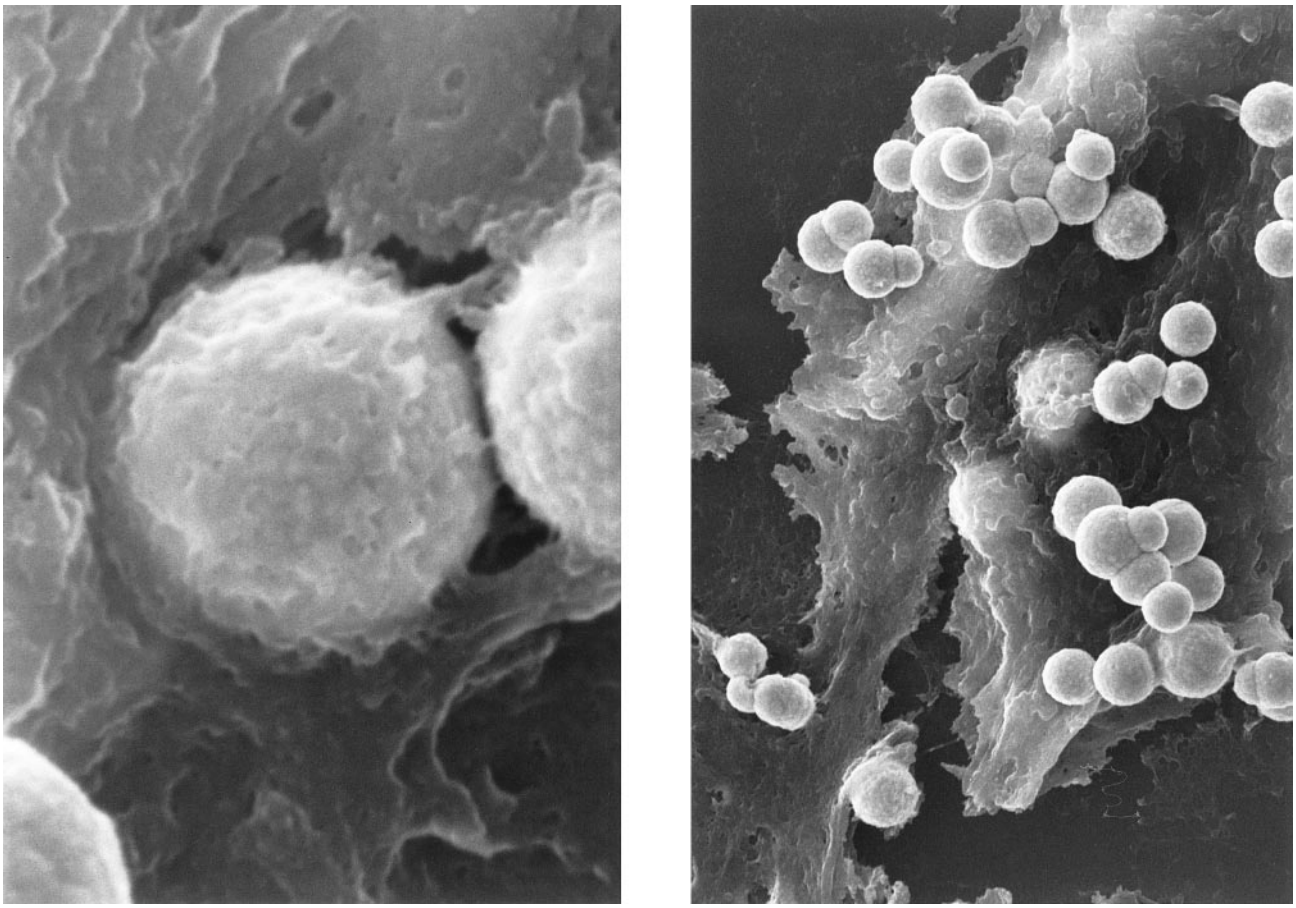


FIG. 4. Scanning electron microscope pictures of a 203G cell into which DNA on gold particles was injected by the PIGG system. Particles in the cell are observed. However, not all particles penetrated the cell, particularly agglutinated particles. One or more gold particles are detected in one cell at this pressure of 160 psi. Left, ×10000; right, ×5000.

Preparation of injected DNA with the gene gun. The expression vectors injected with the gene gun were pCMV β (Clontech, Japan) and pEFEGFP, reconstituted from pEF-BOS (8), encoding green fluorescence protein, GFP, derived from pEGFP-N1 (Clontech, Japan). Before the shot, the DNA was attached to the gold particles. The procedure for their preparation is described as follows: Five milligrams of gold particles were added to 400 μg of 100% ethanol and then sonicated for 5 s with a Sonifier 250 (Branson, Japan). After centrifugation and drying, the dry weight was measured. Then, 20 μl of TE, Tris-EDTA, was added and sonication conducted for 5 s. DNA 20 μg was added to the particles 20 μl of the particles in TE and precipitated with 100 μl ethanol and 4 μl of 3 M CH_3COONa. After centrifugation, the supernatant was removed. DNA on particles was added to 100 μl of 100% ethanol and sonicated for 2 s. Finally, 10 μl of the solution containing DNA on gold particles was painted onto the metal. Before shooting at the target, we sonicated the solution containing the DNA on gold particles in order to disperse the particles for equal gene delivery to the target cells. The stability of DNA on the gold particles or DNA pellet was examined by inspection of DNA bands after electrophoresis on 0.6% agar in TBS. The stability was compared with that of free dissolved DNA. Five or ten seconds of sonication was employed to examine the stability of DNA on the gold particles.

Gene transfer to cultured cells via the PIGG-3 system. A mouse glioma cell line, 203G (9), was used for transfection with pCMV β and pEFEGFP. 203G cells were cultured in monolayer fashion with DMEM containing 10% FCS. At the semi confluent stage, the injection was performed with the gene gun of the PIGG3-system. The injection pressure was set at 160 psi. The green fluorescence was observed by fluorescence microscopy 24 h and 7 days after the transfection.

In addition, primary cultures of neuronal cells obtained from Wistar rat brain were used for transfection with pCMV β. Cortex neural cells were prepared as described previously (10). Wistar rats (of 17–18 days' gestation) were anesthetized with diethyl ether and killed by transection of the aorta. The cerebral hemispheres were removed from 8–12 fetuses and placed in cold Leibovitz L-15 medium. After removal of the meninges, the hippocampi were dissected out, and the cortices were minced and incubated in PBS with 0.25% trypsin for 12 min at 37°C. The cortical tissue was rinsed once and dissociated in 1:1 mixture of Dulbecco modified Eagle's and Ham's F-12 media supplemented with 5% FCS. Twenty-four hours after plating, the medium was changed to a serum-free medium (Dulbecco modified Eagle's and Ham's F-12 media supplemented with transferrin, insulin, progesterone, and selenite). For the present study, neural cells cultured for 7 days were used.

X gal staining was performed 24, 48, and 96 h after the pCMV β transfection. The medium was removed just before the injection and replaced as soon as possible after the transfection. The distance from the plate to the target was 5 mm.

The relationship between the pressure of released gold particles conjugated with DNA and the mechanical damage to the cultured cells was examined. The pressure was elevated up to a maximum of 160 psi. The mechanical damage was assessed by observation of the

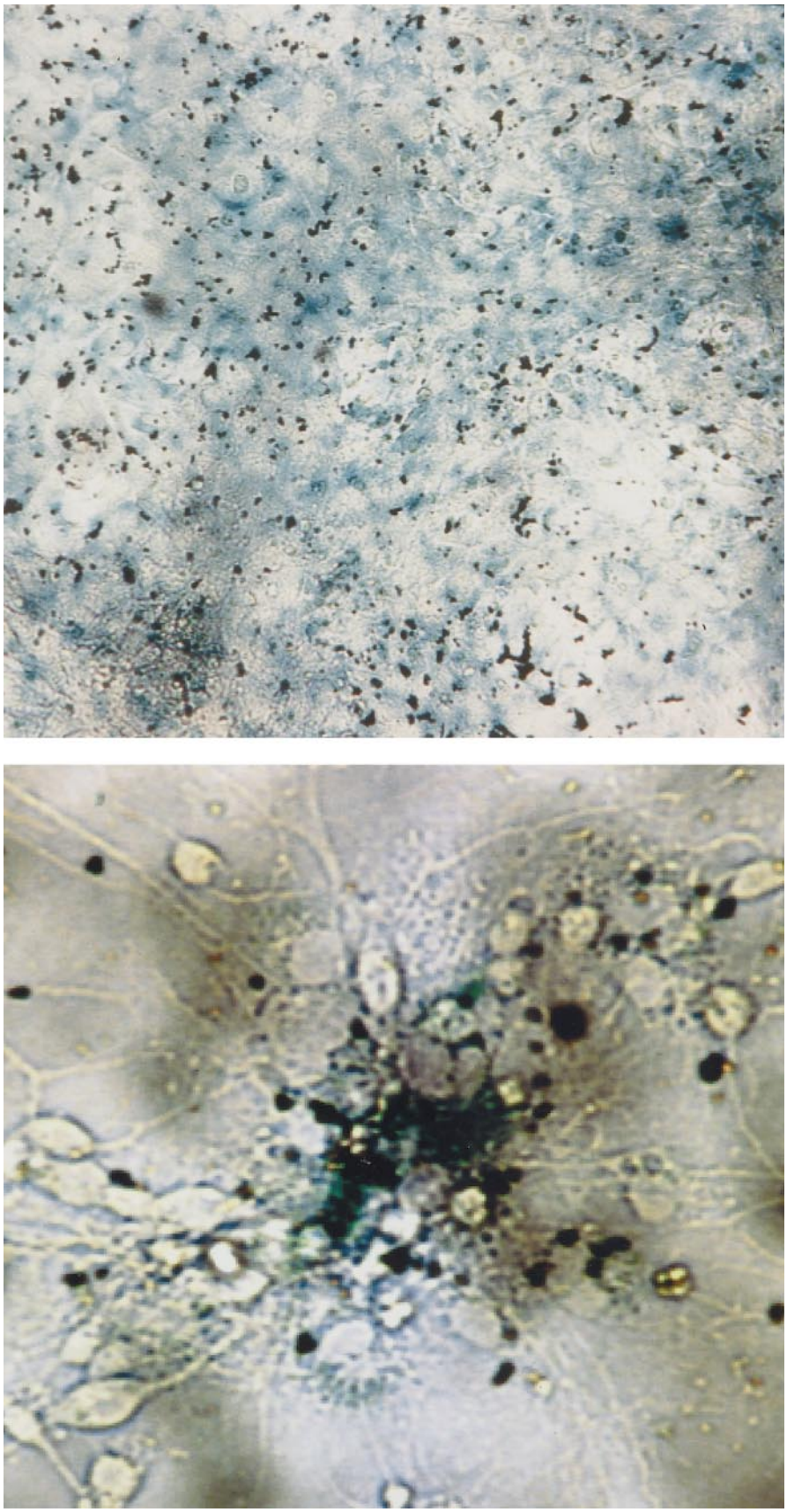
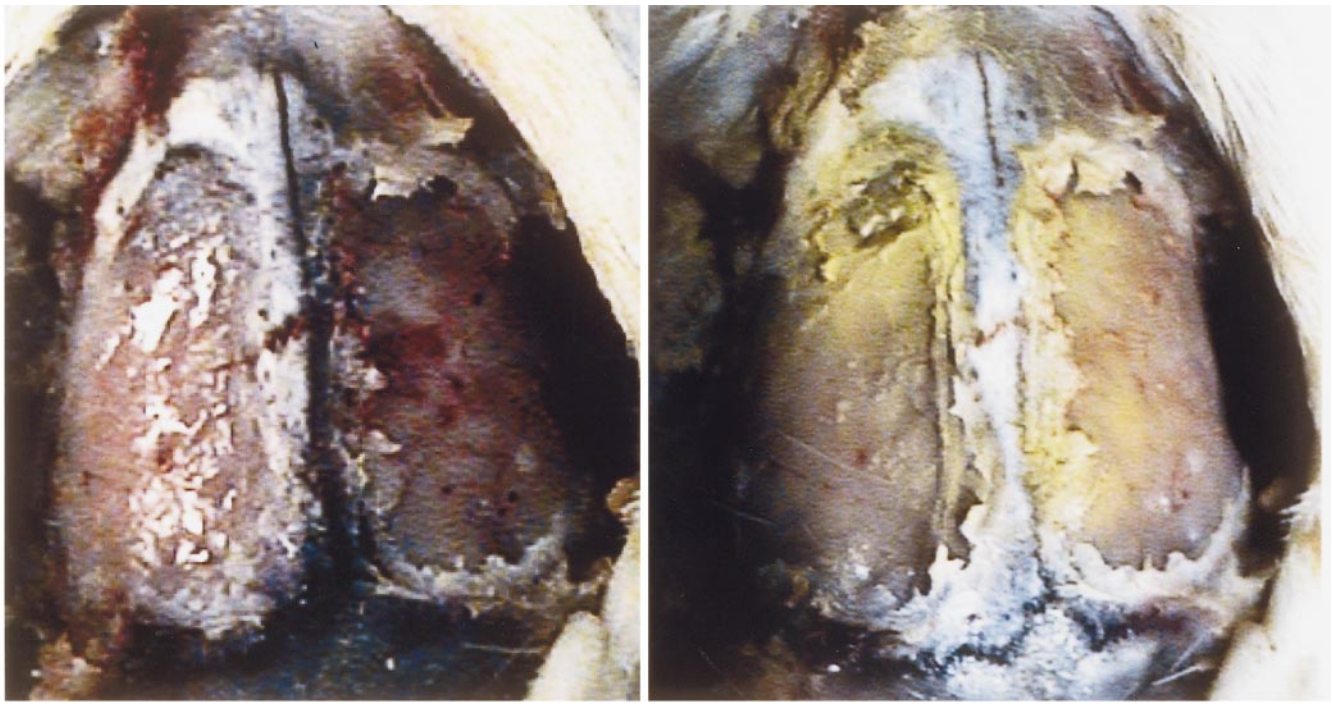


FIG. 5. Expression of LacZ on cultured primary neural cells and 203G cells. pCMV β was transferred to the cells by the PIGG system. Approximately 30% of the neurons and almost 100% of the 203G cells expressed lacZ, as found by Xgal staining, 24 h after the transfection. Black dots are gold particles. Upper: 203G cells (×40). Lower: neurons (×400).



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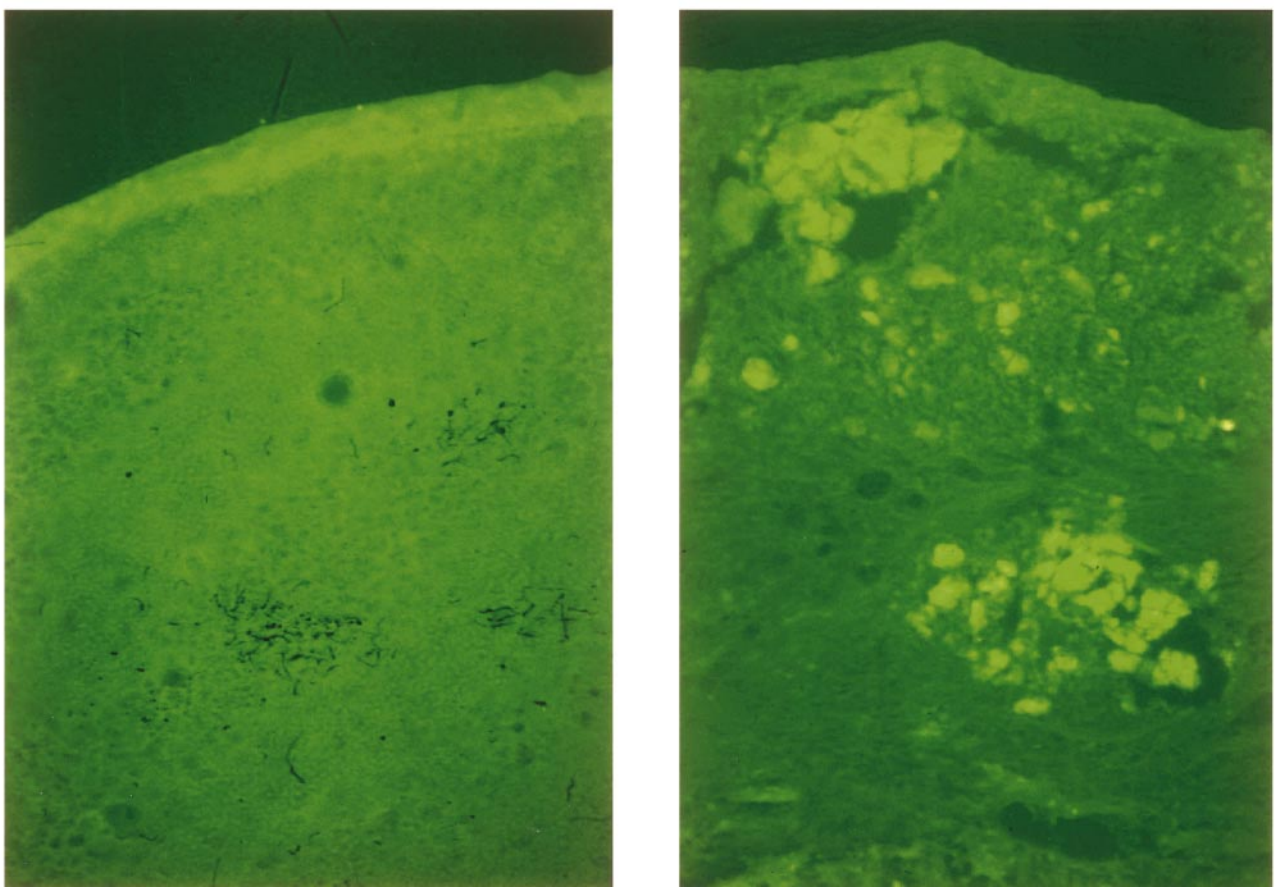

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FIG. 6. Pictures of craniectomy of rodent skull with preservation of the superior sagittal sinus before (left) and after (right) administration of DNA on gold particles. The surface of the brain appears to be gold in color after the administration (right).

FIG. 7. Fluorescent pictures of brain in horizontal section. Left: GFP plasmid on particles was administered by the PIGG system, and green fluorescence in the live brain tissues of all rats was detected. The expression was found 24 h and 7 days after the administration. Right: Only particles were administered. No fluorescence was detected in the brain tissues. Original magnification, ×40.

cultured neuronal cells under light and scanning electron microscopes (S-3500N Hitachi, Tokyo).

The Helios-type gene gun. The Helios Gene Gun System (Nippon Bio-Rad Laboratories, Japan) was used. DNA-coated gold particles in a cartridge were accelerated by pressurized He gas and were transferred to the monolayer cultured 203G cells. The pressure was set at 100, 150, 200, or 250 psi. Details of the method used are described in the manual for the Helios Gene Gun or were given previously (7).

Gene transfer to rodent brain surface. Ten rats were given 3 mg/g mannitol before surgery to reduce the intracranial pressure. Under general anesthesia with ether and hyperventilation, oval-shaped (12 mm × 7 mm) bilateral craniectomies were performed on 5 Wistar rats with preservation of the superior sagittal sinus. The dura matter was carefully removed under a microsurgical scope, and the cortical surface was exposed. The GFP expression vector was delivered by this PIGG-3 system. The injection pressure was set at 160 psi. After the injection, an artificial dura was placed on the brain, the skin was sutured and then the rats were returned to their cages. Twenty-four hours or 7 days after the delivery, the rats were decapitated and the removed brains were rapidly frozen and sectioned at 20 μm with a cryostat. Expression of GFP was examined by fluorescence microscope.

Immunohistochemical analysis. After blocking with PBS plus 5% nonfat dry milk, the sliced sections of the GFP-transfected brains were labeled with mouse anti-glial fibrillary acidic protein (GFAP) mAb (Dako, Code No. M761) as a marker for astrocytes or rabbit anti-neurofilament 200 mAb (Sigma, Product No. N4142) as a marker for neurons. The sliced sections were washed twice with PBS and added blocking buffer containing secondary antibody which was phycoerythrin conjugated anti-mouse Igs or Texas-Red conjugated anti-rabbit Igs. Specificity of antibody binding was examined by staining with isotype-matched antibodies as negative controls for first mAb and followed by staining with fluorescence conjugated second Ab.

Approval of the animal study. This study was approved by our institutional animal care and use committee.

RESULTS

Stability of DNA on Gold Particles

There was no difference in particle dispersion between 5 and 10 s of sonication as judged by observation with a light microscope. Therefore, 5-s sonication was sufficient time to disperse the particles. Electrophoresis of sonicated DNA on gold particles showed a definite band of DNA. On the other hand, electrophoresis of sonicated free DNA showed a dispersive pattern of DNA. These results showed that 5-s sonication broke free dissolved DNA but that DNA on gold particles was stable against 5- or 10-s sonication (Fig. 1).

Little Mechanical Damage to Monolayer-Cultured Cells Caused by the PIGG-3 System

When the center of monolayer cultures was targeted by the PIGG-3 system at the maximum pressure of 160 psi, 203G cells or neural cells subjected to the gene gun mediated DNA delivery showed little mechanical damage when observed by light or electron microscopy. Gold particles were detected in the cells at the pressure of 160 psi (Figs. 2 and 4); however, they rarely penetrated the cell membrane at a pressure of 130 psi. In addition, we found that aggregated particles forming bigger complexes could injure the targets. To reduce this injury, we sonicated the gold particles just before painting onto the metal plate. 203G cells shot with particles from the Helios Gene Gun were lost in the central area of the culture even at the lowest pressure, 100 psi (Fig. 3).

Gene Transfer to Monolayer-Cultured Cells

In the gene transfer to monolayer cells with the PIGG3-system, the cultured cells, even if shot by the maximum pressure of 160 psi, showed no mechanical damage. Primary cultured neurons of rat brain and 203G cells were transfected with pCMV β. 203G cells were also delivered the GFP gene in pEFEGFP. Protein expression of lacZ was found in approximately 30% of the primary neurons and in almost 100% in 203G cells (Fig. 5). GFP was expressed strongly in 203G (data not shown).

In Vivo Gene Transfer to Rat Brain

Craniectomy preserving superior sagittal sinus to expose the rat cortical surface was performed. Green fluorescence were detected in all horizontal sections of brains on the side where pEFEGFP was delivered by the PIGG-3 system in horizontal section. The expression period was at least 7 days. On the other hand, no fluorescence was detected when only particles were administered to the opposite side of the same rat's brain (Figs. 6 and 7). Protein expression was found to occur to a depth of 100 μm from the surface in coronal sections and was detected in astrocytes (Fig. 8), but few GFP were shown in neurons (data not shown).

DISCUSSION

We compared the transfection of neurons in primary culture by the gene gun to that by herpes virus. Most of the neurons expressed lacZ protein after herpes virus transfection, but the cells were shrunk or lost their dendrites because herpes virus itself was cytotoxic (data not shown). Only 30% of the neurons expressed the lacZ gene delivered by the gene gun were detected, but the neurons were not remarkably changed morphologically after the transfection (Fig. 5).

Gene gun-mediated gene transfer can directly administer DNA into the nucleus of a cell and can lead to expression of foreign genes even in nondividing neuronal cells. This type of transfer method has several advantages: simple and rapid procedure of transfection, availability for all species of cells, small volume of DNA, many target cells hit by one shot, different from the microinjection method, and higher efficiency of gene transfection rate than attenuated by electroporation or lipofection. Thompson *et al.* reported that the

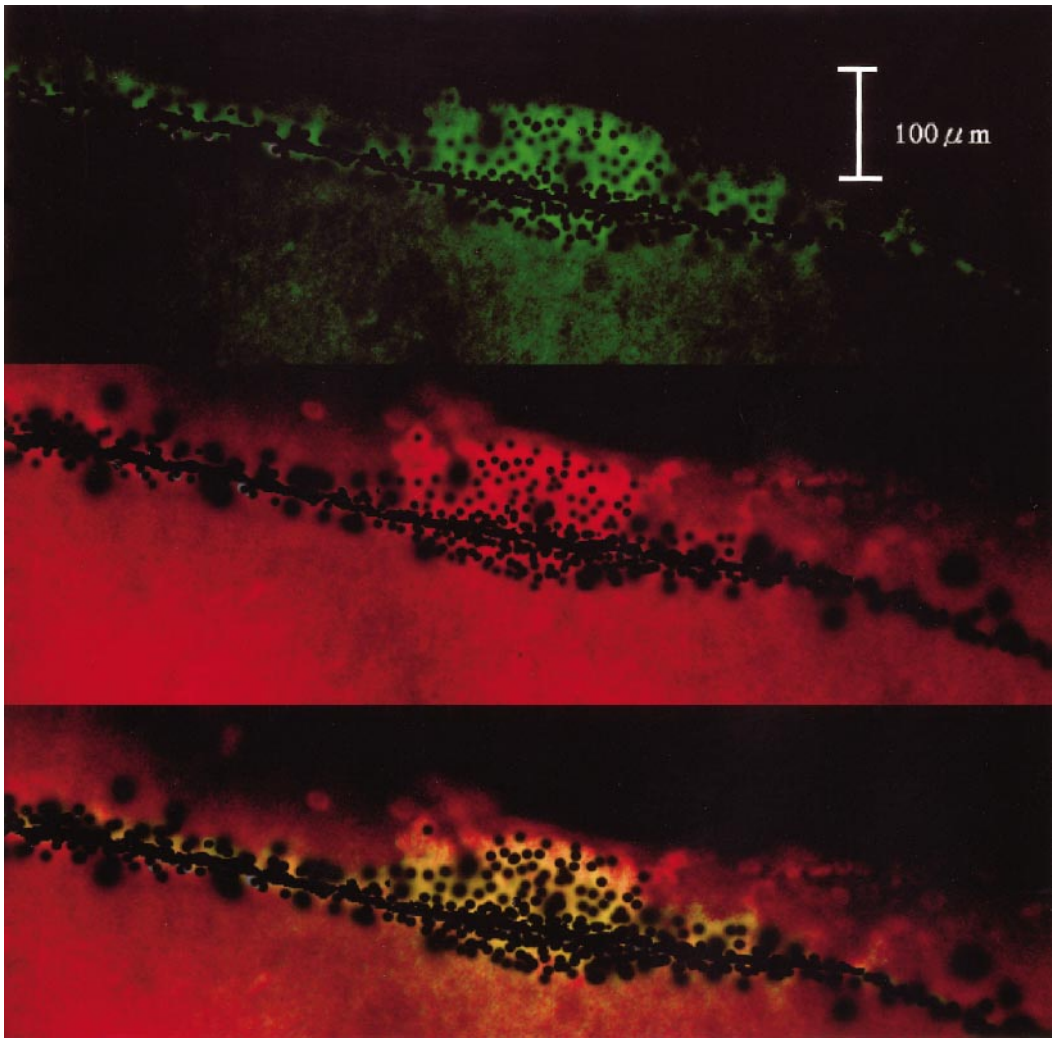

FIG. 8. GFP plasmid was administered by the PIGG system. Fluorescent pictures of brain in coronal section. Upper: GFP expression were seen in 100 μm depth from the surface. Middle: Immunofluorescence showing staining with anti-GFAP. Secondary antibodies were phycoerythrin-conjugated anti-mouse Igs. Lower: The cells expressing GFP colocalized with GFAP-expressing astrocytes. Original magnification, $\times$400.

luciferase activity by gene-gun mediated gene transfer was 2000 to 10000 times greater than that by other gene transfer systems (11).

It has been very hard to transfer DNA to the brain of living mammals, for this brain surface is so fragile to receive a particle-mediated gene from a conventional gene gun such as the Helios type (Bio-Rad) (4, 5). Bombardment with particles in a strong stream of helium gas, which occurs with these guns, causes severe damage to cultured cells or tissues (7). Approximately 130 psi is the minimum pressure of the Helios gene gun and even it cause severe damage to the center of the target.

The PIGG-3 system has a great advantage when used on the fragile target tissues as well as on monolayer cultured cells compared with the Helios-type one. That is, it can release particles of sufficient speed without the gas impacting the target, as described earlier. Thus, no harmful gas injury to the target cells or tissues occurs.

Even using this system, we found that the target was damaged when aggregated particles were present. The cells were pressed by them and given a critical injury in monolayer cultured cell. Furthermore, aggregated particles were so heavy that they could penetrate deeper in brain tissue or agarose gel as previously described (7). The routes which they passed through resulted in laceration of the tissue. To reduce these injuries, we dispersed the particles by sonication just prior to paint to the metal. Although dissolved DNA in buffer was broken by a few seconds of sonication, the DNA on particles or precipitated DNA was stable against even ten seconds sonication. Thus we established a method for very low damage gene transfer with this gene gun.

Most of the cells that expressed GFP in brain tissue were astrocytes (Fig. 8), but not neurons. The neuronal transfection using the particle-mediated gene transfer

system has been reported (4–6). However, in our *in vivo* system, we could not transfer gene into neurons. The reason may be thought as follows. The particles were able to reach 100 μm in depth from the surface of the brain, which was the molecular layer. The molecular layer, which are the most upper layer of the cerebrum in histological analysis, consist of the glia cells and process of the neurons, but few neuronal cell bodies. So it was eligible that the expression was found in only glia cells. We do not know how long the protein can be expressed, although expression was still detected at least 7 days after the transfection.

Previously *in vivo* gene gun-mediated gene transfer to many organs was reported, the target organs being skin (12), oral mucosa (13), liver, pancreas (14), muscle (15), kidney, mammary gland (16), and testis (17).

This PIGG-3 method should significantly expand the usefulness of particle-mediated gene transfer into fragile tissues, especially brain, for gene therapy. For example, tumor suppressor gene (18), tumor angiogenesis factor inhibitor gene (19), and cytokine genes such as interferon gene (2, 20) and interleukin gene (21) may be useful for tumor therapy. After removal of a malignant brain tumor, the use of the PIGG-3 system for injection of such genes into the wall of the cavity formed by the removal of the tumor by a gene gun may be effective for killing invasive tumor cells. Also, stereotaxic injection of tyrosine hydroxylase gene (22) into the striatum with a gene gun may be effective against Parkinson's disease. Other applications of gene transfer to brain tissue with such a gene gun can also be envisioned.

Gene transfer using the PIGG-3 gene gun may well prove to be very useful as an alternative method for gene transfer into brain tissue.

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