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Labeling of Neural Cells by Gold-Filled Sendai Virus Envelopes Before Intracerebral Transplantation

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Gold-filled Sendai virus envelopes were fused with cell suspensions from the basal forebrain of fetal rat donors, and the resulting gold-labeled cells were transplanted into the neocortex of adult rat recipients. Not only did large numbers of labeled cells remain intact through 3 months in the neocortex, but sizable numbers migrated subcortically to the recipient's lesioned nucleus basalis region (a distance of 4 to 5 millimeters). Since this technique is capable of labeling most transplanted cells for long periods of time, it may be useful in determining the survival, migration, and connectivity of intracerebrally transplanted tissues.

INTRACEREBRAL TRANSPLANTATION IS well established as an important technique in neuroscience (*1, 2*). For a number of grafting studies, an ability to label neural cell suspensions before transplantation is highly desirable in monitoring the survival, differentiation, and possible migration of transplanted cells. The techniques currently in use involve either fluorescent dye or autoradiographic labeling (*3*). However, these techniques have certain drawbacks, such as the diffusion of dye markers out of neurons after several weeks. We describe a method for labeling neuronal cell suspensions with Sendai virus envelopes that is rapid, capable of labeling most cells, and apparently long-lasting without causing obvious harm to the transplanted cells. Furthermore, we report on the use of this labeling procedure in determining the survival of transplanted fetal rat cells from the nucleus basalis region and their capacity to migrate after intracortical transplantation.

Sendai virus is highly fusogenic to most vertebrate cells and is nonpathogenic to man. The fusion activities of this virus are due mainly to two viral glycoproteins located in the viral envelope. Viral envelopes were obtained by solubilization of intact Sendai virus with the detergent Triton X-100, followed by removal of the detergent through slow dialysis to reconstitute the glycoproteins and lipids into envelopes that are structurally and biologically similar to Sendai virus envelopes (*4, 5*). During the reconstitution process, the viral nucleocapsid was removed and replaced by exogenous colloidal gold particles that became trapped within the reassembled viral envelopes during dialysis. These reassembled viral envelopes were then used as biological syringes to inject colloidal gold into neurons and glial cells during viral envelope–cell fusion.

Colloidal gold–containing Sendai virus envelopes were prepared from Sendai (Cantell) hemagglutinin (Hazleton Research Products, Denver, Pennsylvania) by using modifications of a general procedure by Volsky and Loyter (*5, 6*). Previously frozen or freshly prepared nucleus basalis magnocellularis (nBM) cell suspensions from dissected fetal rat brains (day 18 of gestation) were prepared (*7*) and fused with the colloidal gold–containing viral envelopes (*8*). More than half of the cells within a given nBM suspension were labeled, although we have routinely achieved nearly 100% labeling. Two microliters of this labeled nBM suspension were then transplanted unilaterally into four frontoparietal cortex sites of adult Sprague-Dawley rats (*9*) that had received an ipsilateral excitotoxic lesion of the nBM region 1 to 3 weeks earlier (*10*). Recipients were killed at 10 days, 30 days, or 3 months after transplantation, and their brains were histologically prepared (*11*).

Because of a small, unavoidable contamination of labeled cell suspensions by residual free colloidal gold or unfused gold-containing envelopes, control infusions of either colloidal gold or gold-containing capsules were performed to determine if these contaminants would be taken in by surrounding cells. Results indicate that only a small number of cells near the cortical infusion area (most probably glia and pial cells) will take up free colloidal gold or fuse with reconstituted viral envelopes. Furthermore, the limited number of cells that do take up one of these control infusates remain in the infusion area. It is unlikely that labeled cells are host cells that have phagocytized degenerating transplanted cells since no cellular labeling was observed at infusion sites in two animals with necrotic, nonviable transplants (that is, the colloidal gold released from degenerative transplanted cells was apparently cleared from the brain).

Through 3 months after transplantation, large numbers of grafted, gold-labeled cells were seen at neocortical infusion sites (Fig. 1), although markedly more labeled cells were present at these sites when the nBM

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cell suspensions had been freshly prepared on the day of transplantation than when they had been previously frozen. Nonetheless, nBM cell suspensions that were frozen for 6 months before gold-labeling and transplantation also were present at cortical infusion sites. Labeled cells appear yellow in light-field microscopy (Fig. 2A), and the dark colloidal gold granules they contain are seen at high magnification (Fig. 2, D and E). Moreover, dark-field microscopy provides a striking white image of such labeled cells, which clearly differentiates them from any surrounding unlabeled cells (Fig. 2B). The reassembled Sendai virus envelopes apparently fused with both glial cells and neurons (on the basis of size and presence of a clear nucleolus for neurons), although electron microscopy will be necessary to unequivocally determine that neurons were labeled within these cell suspensions.

Particularly evident in dark-field microscopy was the migration of colloidal gold–labeled cells from cortical infusion sites to the previously lesioned nBM region of the host rat (Fig. 1, A to C). At both 10 and 30 days after grafting, migratory waves of labeled cells could be seen within or around the lesioned nBM area (4 to 5 mm from cortical infusions sites) or in adjacent thalamic regions (Fig. 1, C and D). Labeled cells, apparently en route to these deep subcortical sites, were routinely seen traversing the ipsilateral corpus callosum singularly or in waves, as well as moving through the underlying internal capsule or caudate nucleus, or both (Fig. 1, B and C). Migrating waves of labeled cells were also observed in the contralateral thalamus and contralateral neocortex, these latter waves apparently having traversed the thalamic and cortical midline regions, respectively. We do not know whether the migration of labeled cells to the host's nBM region is due to a natural affinity of transplanted nBM cells for that brain region or to the probable secretion of survival-promoting ("neuronotrophic") factors by cells within the lesioned nBM. Several studies suggest that the source of such factors is reactive astrocytes that proliferate at brain sites shortly after injury (*12*). The migration of labeled cells into several uninjured brain regions suggests a potential for other, nonlesion-related factors to induce transplant cell migration. The migrations indicate a surprising mobility of transplanted immature cells within the adult brain.

The labeling technique described in this report would probably detect sizable migrations of transplanted cells for other neuronal systems, in some of which cell migrations have already been documented by other techniques (*13*). Our labeling method for transplanted cells seems to have a number of

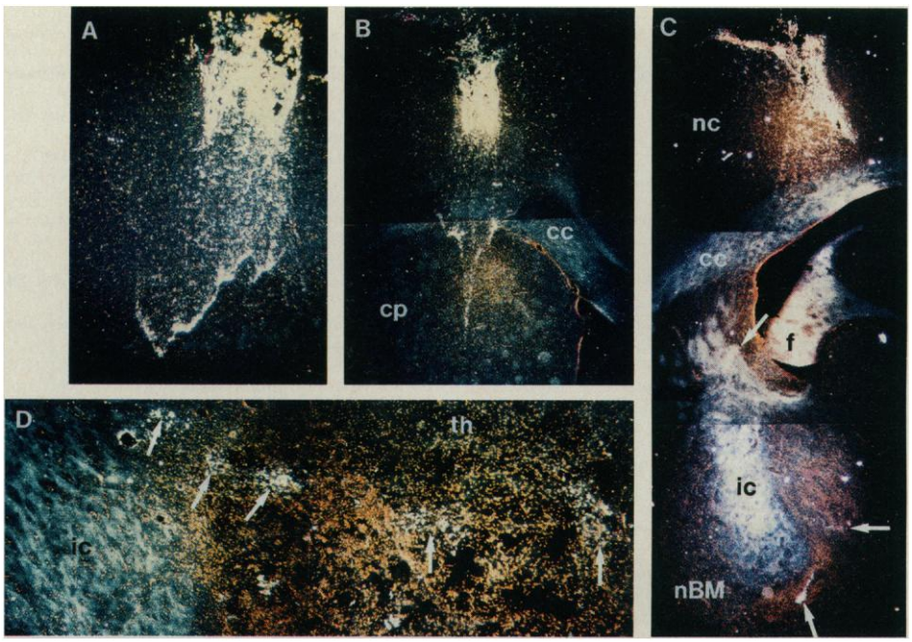

Fig. 1. (**A** and **B**) Two adjacent brain sections seen in dark field that show a gold-labeled nBM transplant in the frontal cortex at 30 days after transplantation. A migratory wave extends into deep cortical areas (A) and the same migratory wave continues through the corpus callosum and caudate nucleus in (B) [(A), ×30; (B), ×12]. (**C**) A montage in dark field showing streams of migrating cells leaving a large nBM transplant in cortex at 10 days after grafting. Several subcortical migratory waves (arrows) can be seen in or adjacent to the internal capsule, and an additional migratory group is present within the lesioned nBM region (×12). (**D**) A dark-field micrograph showing the internal capsule and thalamus on the side of the brain ipsilateral to an nBM transplant that had been placed in the cortex 30 days earlier. A number of migratory waves are shown (arrows) in the thalamus. Cells had been frozen for 6 months before labeling and transplantation (×30). Abbreviations: cc, corpus callosum; cp, caudate-putamen; f, fornix; ic, internal capsule; nBM, nucleus basalis magnocellularis; nc, neocortex; and th, thalamus.

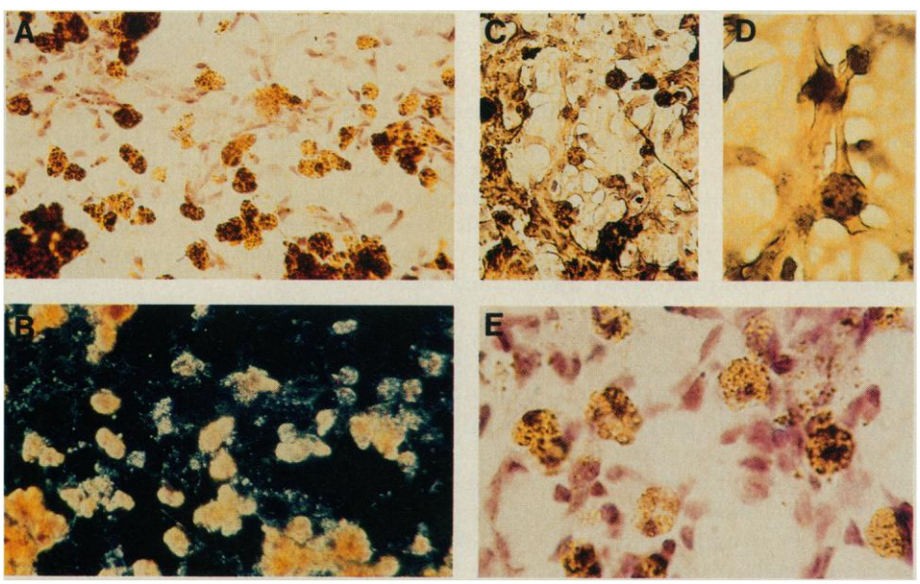

Fig. 2. Light-field (**A**) and dark-field (**B**) micrographs of the same gold-labeled cells within an nBM cell suspension grafted into the neocortex 30 days earlier. The cell suspension had been freshly prepared, and brain sections were counterstained with Mayer's acid hemalum. The dark yellowish appearance of labeled cells in (A) contrasts with their white appearance in (B) [(A) and (B), ×190]. (**C**) A light-field photomicrograph showing nBM cells grafted 10 days earlier. The cells had been frozen for 6 months before gold-labeling and transplantation. This brain section was silver enhanced (Janssen Pharmaceutical kit) for a visualization of neuronal processes (×190). (**D**) A higher magnification of (C) that shows colloidal gold particles in labeled cells and neuronal processes extending from apparent neurons (×480). (**E**) A light-field micrograph of gold-labeled cells within a cortical infusion site at 10 days after grafting. Cells had been frozen for 6 months before labeling and transplantation. The colloidal gold particles within labeled cells give such cells a yellowish cast. Hemalum counterstaining results in a visualization of unlabeled cells (dark pink) and of labeled cell nuclei (×480).

distinct advantages, including an ability to label most cells in a population for long periods of time and the potential to investigate the synaptic connectivity of transplanted neurons through the identification of colloidal gold–containing nerve terminals by means of electron microscopy. In light of recent clinical trials involving the intracerebral transplantation of dopamine-secreting cells to treat Parkinson's disease (*14*) and basic research exploring the possible use of cholinergic transplants to treat Alzheimer's disease (*15*), it would be advantageous to have a labeling technique capable of monitoring the viability and development of transplanted neuronal tissues. The technique described here could be useful clinically in neural transplants and could be helpful in understanding the plasticity and connectivity of transplanted neural tissues.

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6. Approximately 50 to 100 ml of the virus-containing Sendai (Cantell) hemagglutinin are centrifuged at 3000 rpm for 20 minutes and the pellet is discarded. The supernatant is recentrifuged at 37,000*g* for 30 minutes at 4°C and the supernatant discarded. The pellet is then resuspended in 1 ml of solution 1 (100 m*M* NaCl and 50 m*M* tris-base, *p*H 7.4) containing 1 μl of a 10^{-5}*M* stock solution of phenylmethylsulfonylfluoride and 10 μl of a 20% stock solution of Triton X-100. The mixture is left at 20°C for 1 hour, then centrifuged at 100,000*g* for 1 hour at 4°C. The pellet is discarded as it contains viral nucleocapsids. The supernatant is retained and colloidal gold solution is added v/v, followed by dialysis (12,000 molecular weight pore size) against 1 g of Bio-beads SM-2 in 900 ml of distilled water plus 100 ml of solution 2 stock [10 m*M* tris-base, 2 m*M* $CaCl_2$, and 2 m*M* $MgCl_2$ (*p*H, 7.4) to a final volume of 0.5 liter] for 5 hours at 25°C and then for 30 to 35 hours at 4°C. The dialysate is centrifuged at 100,000*g* for 5 minutes at 4°C, and the pellet is resuspended in 1 ml of solution 1. The gold-encapsulated envelopes can be stored at 9°C for several weeks. Colloidal gold preparation consisted of the following: 2.5 ml of 1% AuCl, 15 ml of 1% sodium citrate, and 232.5 ml of water. Boil water, add citrate, and boil vigorously. Then add 2.5 ml of 1% AuCl and reflux for 15 minutes. Store mixture in a sterile dark brown bottle at 9°C. To concentrate colloids, centrifuge at 100,000*g* for 5 minutes at 4°C. Retrieve the soft pellet in a minimal volume. To dilute colloids, add solution 1 to desired volume (usually 1 ml).
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8. We found that a 10% v/v suspension of cells to viral envelopes was appropriate for labeling, with no more than 10 μl of cells in any one incubation tube (1 ml of cell suspension contains approximately 20,000 cells). Thus, for 10 μl of cell suspension, 100 μl of viral envelopes was added. However, each preparation of envelopes may vary. Solution 1 plus 10 m*M* $CaCl_2$ was added to the cell suspension and viral envelopes to make a final volume of 1 ml in each tube. The mixture was then incubated with moderate shaking at 37°C for 1 hour. After incubation, the solution was centrifuged at 750 rpm for 1 minute at 4°C and the supernatant discarded. This washing was repeated several times with solution 1, followed by a final centrifugation at 750 rpm for 1 minute at 4°C and removal of the supernatant. The colloidal gold–labeled cells can then be resuspended in an appropriate buffer. We resuspended them in a 0.9% NaCl, 0.6% glucose solution and transplanted the cells as soon as possible.
9. With the incisor bar set at 2.8 mm below the interaural line, coordinates for the four cell suspension infusions in each animal were as follows: 1.5 mm posterior to bregma (P), 2.0 and 4.0 mm lateral to midline (L), and 1.6 and 1.8 mm vertical (V), respectively; and at 3.5 mm P, 2.0 and 4.0 mm L, and 1.6 and 1.8 mm V, respectively.
10. Recipient rats, weighing 280 to 295 g, were anesthetized with sodium pentobarbital (50 mg/kg) and infused with 1 μl of ibotenic acid (5 μg/ml) at two sites within the nBM by means of the stereotaxic atlas of J. Konig and A. Klippel [*The Rat Brain: A Stereotaxic Atlas of the Forebrain and Lower Parts of the Brain Stem* (Williams & Wilkins, Baltimore, 1963)]. With the incisor bar set at 2.8 mm below the interaural line, infusion coordinates were: 7.0 mm A (anterior to the interaural line), 2.6 mm L, and 6.5 and 5.7 mm V.
11. Animals were perfused with 10% neutral buffered Formalin. Their brains were removed and placed in 10% Formalin for several days before frozen sectioning at 30 to 40 μm. All brain sections were counterstained with Mayer's acid hemalum (40 seconds) for visualization of both labeled and unlabeled cell bodies.
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Cells Process Exogenous Proteins for Recognition by Cytotoxic T Lymphocytes

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Cells exposed to intact, noninfectious influenza virus were shown to be recognized by class I–restricted anti-influenza cytotoxic T lymphocytes (CTLs). Both internal and external proteins derived from virions were processed by cells for CTL recognition. Sensitization required the inactivation of viral neuraminidase activity and could be inhibited by preventing fusion of viral and cellular membranes. These findings are important in designing vaccines to elicit CTL responses, since they demonstrate that cells can process intact, exogenous proteins for recognition by CTLs and suggest that such processing depends on introduction of exogenous proteins into the cytoplasm.

INFLUENZA VIRUS HAS BEEN USED EXtensively to study the recognition of foreign antigens by cytotoxic T lymphocytes (CTLs) restricted by class I molecules of the major histocompatibility complex (MHC) [reviewed in (*1*)]. Seven viral proteins are present in virions (*2*). Two integral membrane proteins, hemagglutinin (HA) and neuraminidase (NA), are embedded in the host-derived lipid envelope. Matrix protein (M1) is believed to form a subenvelope shell enclosing the ribonucleoprotein (RNP) core, which consists of viral RNA complexed with nucleoprotein (NP), and small amounts of three viral polymerases (PA, PB1, and PB2). Viral replication is initiated by delivery of the RNP to the host cell cytoplasm. The HA mediates two functions essential to this process. First, it attaches virus to the cell by binding sialic acid residues present on cellular glycoproteins and glycolipids. Second, after internalization of virus into cellular endosomes, the subsequent acidification triggers conformational alterations in the HA that lead to the fusion of viral and cellular membranes (*3, 4*). Although the NA does not function directly in penetration, its ability to cleave sialic residues prevents irreversible HA-mediated attachment of virions to macromolecules or nonendocytosing cells (*5*).

Early studies showed that de novo synthesis of viral proteins was required for CTL-mediated lysis of influenza virus–infected

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