Transplantation of Immortalized, Nontumorigenic Parotid Acinar Cells into the Allogeneic Rat Parotid Gland and Oral Submucosa (44004)

GREGORY E. KRAUSE,*,†,‡ FRANCISCO G. LA ROSA,*,†,§,|| ARLEN D. MEYERS,†‡ RITA KUMAR,*,† AND KEDAR N. PRASAD*,†,[1]

Center for Vitamins and Cancer Research, Departments of Radiology,† Otolaryngology/Head & Neck Surgery,‡ and Pathology,§ and Webb-Waring Institute for Biomedical Research,|| University of Colorado Health Sciences Center, School of Medicine, Denver, Colorado 80262*

Abstract. **The recent establishment of an immortalized clonal cell line of rat parotid acinar cells (2RSG) by transfecting isoproterenol-stimulated parotid cells with a plasmid vector, pSV3^{neo}, which carries the large T-antigen gene from SV40 virus, afforded the opportunity to develop a model for parotid acinar cell transplantation. Single cell suspensions of 2RSG cells labeled with a fluorescent tracer, DiI, were injected into the parotid gland or oral submucosa of allogeneic adult rats. The grafted cells survived and were functionally viable for at least 30 days. Histological sections revealed no evidence of infiltration of leukocytes or lymphocytes. Grafted cells did not form tumors. Results suggest that allogeneic parotid acinar cell transplantation is a feasible technique in the animal model.** [P.S.E.B.M. 1996, Vol 212]

The recent establishment of an immortalized and nontumorigenic clonal cell line of rat parotid acinar cells (2RSG) in our laboratory afforded the opportunity to develop a model for parotid acinar cell transplantation (1). This cell line was established by transfecting isoproterenol-stimulated rat parotid cells with a plasmid vector, pSV3^{neo}, which carries the large T-antigene gene of SV40 virus (2). Previous characterization of 2RSG has demonstrated that these cells are nontumorigenic in nude mice and produce α-amylase mRNA and α-amylase, parotid secretory protein (PSP) mRNA and PSP, and proline-rich protein mRNA (1, 3). There have been no studies on the feasibility of acinar cell transplants into parotid glands. Such studies may be useful in developing new approaches to the treatment of xerostomia. We now report that when parotid acinar cells were transplanted into allogeneic rat parotid glands, they survived and were functionally viable for at least 30 days after transplantation.

Materials and Methods

2RSG cells were grown in RPMI media containing 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml), and were maintained at 37°C in a humidified atmosphere of 5% CO_2. Mycoplasma free cultures were used in this study.

Prior to transplantation, 2RSG cells were labeled with the fluorescent tracer, 1,1′-dioctadecyl-3,3,3,3′-tetra-methylindo-carbocyanine perchlorate (DiI; Molecular Probes, Eugene, WA) at a concentration of 3.75 μg/ml for 24 hr. Cells were removed from culture dishes by a standard method. The cell pellet was resuspended in Hanks' Balanced Salt Solution 1X (without calcium, magnesium, or phenol red; Mediatech) to yield a density of 50,000 cells/μl. India ink (Davidson's Marking System) was then added to the cell suspension at a 1:30 dilution before transplantation in order to locate the graft site on gross examination and during

[1] To whom requests for reprints should be addressed at Center for Vitamins and Cancer Research, Department of Radiology, University of Colorado Health Sciences Center, 4200 East 9th Avenue, Denver, CO 80262-0278.

Received December 4, 1995. [P.S.E.B.M. 1996, Vol 212]
Accepted February 6, 1996.

0037-9727/96/2122-0160$10.50/0
Copyright © 1996 by the Society for Experimental Biology and Medicine

preparation of frozen sections. The cell suspension was kept on ice for a maximum of 4 hr prior to transplantation.

Twelve male SD rats weighing 300–400 g were anesthetized by intraperitoneal injection of equethesin (3 ml/kg; supplemented as necessary). Injections of 25 μl of the cell suspension containing 2×10^6 DiI-labeled 2RSG cells and India ink, using a 25-gauge needle, were placed bilaterally in the submucosa of the hard palate and floor of mouth. When the injections were properly placed, India ink was visible below the overlying mucosa. An additional group of twelve rats received grafts of 2RSG cells into the parotid gland. A 2-cm midline neck incision and subcutaneous flap exposed the left parotid gland. Then, 20 μl of cell suspension containing 2×10^6 DiI-labeled 2RSG cells and India ink was injected (25-gauge needle) directly into the left parotid gland. The skin was then closed in one layer using 4-0 silk interrupted suture.

Four rats from both the parotid gland and oral submucosa transplantation groups were sacrificed at 1, 7, and 30 days after transplantation. The grafted areas were dissected, embedded immediately in OCT Compound (Miles, Elkhart, IN) and frozen at −20°C. The samples were then stored at −70°C until utilized for frozen sections. Two additional rats were grafted with 2RSG cells and 6 months after transplantation were sacrificed for tumor detection.

Duplicate frozen sections (5-mm thick) from each grafted site were immunostained for α-amylase. Frozen sections were allowed to dry at room temperature for 2 hr, and were then fixed in acetone for 5 min. The fixed preparations were covered with rabbit anti-human α-amylase (A8273; Sigma Chemical Co., St. Louis, MO) diluted 1:80 in 25% Super Block (Sey Tek Laboratories, UT) in phosphate-buffered saline (PBS), and incubated for 60 min. The slides were then washed in PBS for 10 min, followed by a 30-min incubation with fluorescein isothiocyanate (FITC)–conjugated goat anti-rabbit immunoglobulins (Cappel, Durham, NC) diluted 1:40 in 25% Super Block plus 10% normal rat serum. The slides were then washed for 10 min in PBS containing ethidium bromide (1 μg/ml) and mounted with Vectashield (Vector Lab, Burlington, CA) to reduce fading of fluorescence. The slides were then examined under an immunofluorescent microscope with incident light (Zeiss, Jena, Germany). Photographs were taken using Fujichrome 400 (Fuji, Tokyo, Japan) film.

Duplicate frozen sections (5-mm thick) from each grafted site were also immunostained for PSP and SV40 large T antigen. The primary antibody specific for PSP was rabbit anti-human PSP (provided by Dr. Birte Larsen, University of Aarhus, Aarhus, Denmark) diluted 1:100 in 25% Super Block in PUFA and the secondary antibody was FITC-conjugated goat anti-rabbit immunoglobulins diluted 1:40 in 25% Super Block plus 10% normal rat serum. The primary antibody used for large T-antigen staining was a 1:40 dilution of a mouse monoclonal antibody (IgG) anti-SV40 T-antigen (Oncogene Science, CA). The secondary antibody used was a 1:60 dilution of FITC-conjugated goat anti-mouse IgG (Cappel Lab, Durham, NC) containing 10% normal rat serum.

Results

2RSG cells maintained epithelial morphology *in vitro* and grew with a doubling time of approximately 24 hr (Fig. 1D). These cells produced SV40 large T antigen, PSP, and α-amylase (1). In addition, they expressed high levels of α-amylase mRNA, proline-rich protein mRNA, and PSP mRNA, and exhibited β-adrenoceptor– and prostaglandin E_1 receptor–linked adenylate cyclase activity (1, 3).

2RSG cells were grafted into the parotid glands, and the survival of grafted cells was determined at 1, 7, and 30 days after transplantation. Frozen sections of the grafted site of the parotid gland revealed the presence of DiI-labeled grafted cells (Fig. 1C) 1 day after transplantation. These grafted cells were positively stained with the primary antibody to α-amylase (Fig. 1B). The grafted cells without the primary antibody to α-amylase did not stain (Fig. 1A).

After 7 days of transplantation, the grafted cells were easily identified by their red color due to labeling with DiI (Fig. 2B). The surrounding host tissue, by contrast, did not exhibit any specific fluorescent staining (Fig. 2B). The grafted cells were stained with the primary antibody to α-amylase (Fig. 2A, bottom); however, the intensity of staining was much less than that found in the surrounding host tissue (Fig. 2A, top). The addition of India ink into the cell suspension prior to transplantation was useful in identifying grafted site. In addition, the frozen section of the parotid gland revealed the presence of carbon particles demarcating the boundary between grafted cells and the host tissue. The H&E–stained section of parotid gland showed areas of high cellularity with cluster formation at the close proximity to India ink at 7 days after transplantation (Fig. 2D). The grafted cells also survived and remained viable after 30 days of transplantation using the criteria mentioned above.

Similar results were obtained with immunofluorescent staining of grafted cells for PSP (data not shown). When cells were grafted into oral submucosa, and examined at 1, 7, and 30 days after transplantation, the grafted cells remained functional using the criteria described above (data not shown).

During histological examination of each specimen, the carbon particles of India ink provided identification of the graft site. There was no evidence of rejection, lymphocytic infiltration, or any mitotic activity

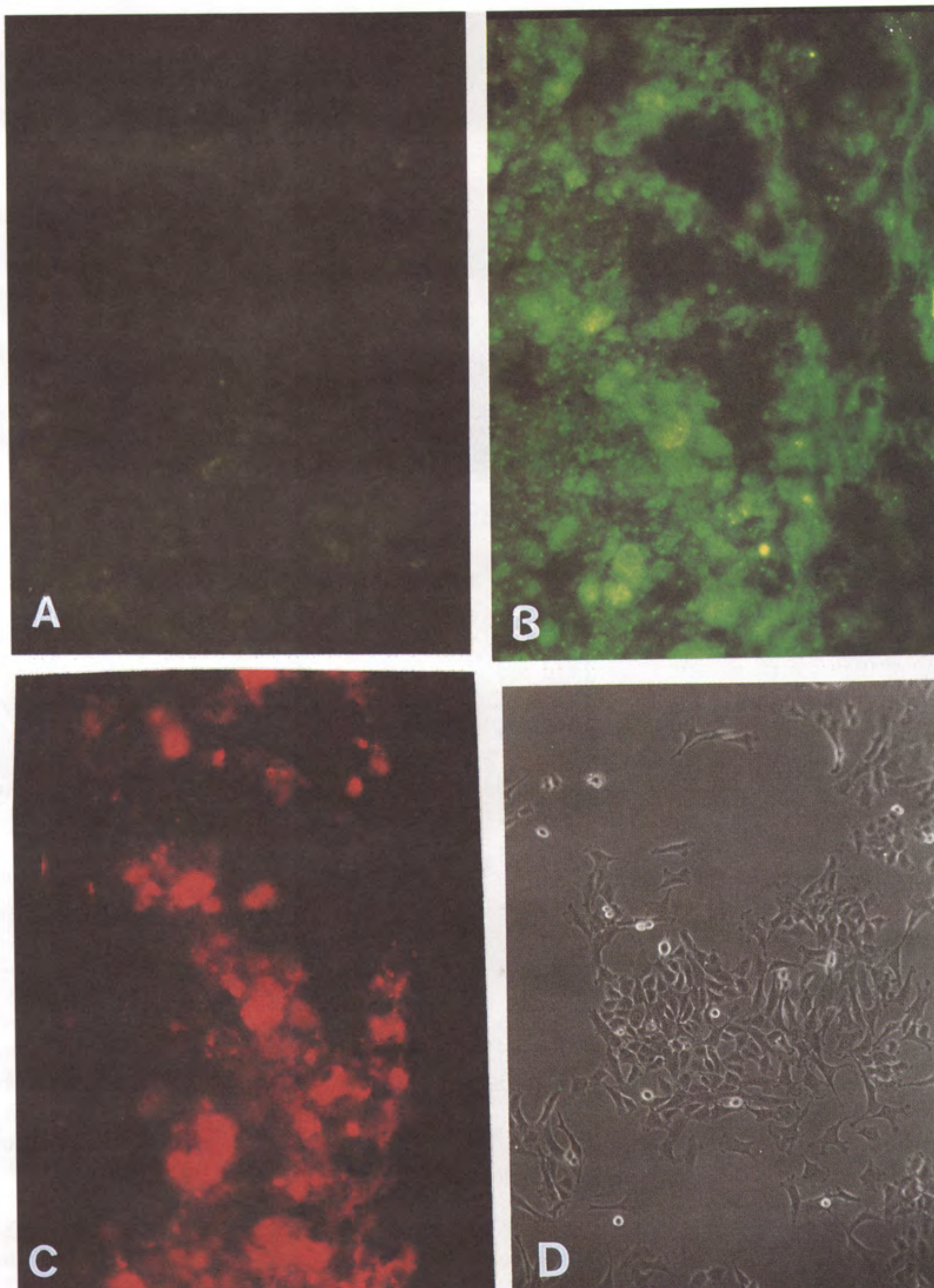

Figure 1. Frozen sections of rat parotid gland obtained after 1 day of transplantation. The DiI-labeled grafted cells appear red (C) when examined under a microscope with a 530–585 filter. The same area stained with the primary antibody to α-amylase shows positive staining (B) and the section stained without the primary antibody to α-amylase shows no staining (A). (D) A photomicrograph of rat 2RSG cells grown in culture. Magnification: ×400 (A–C) and ×200 (D).

within the grafted area of any specimen (Fig. 2D). None of the animals grafted with 2RSG cells had gross or histological evidence of tumor. Two rats, which were transplanted with 2RSG cells, had no gross evidence of tumor when sacrificed 6 months after transplantation.

Discussion

This study shows, for the first time, that the transplantation of allogeneic parotid acinar cells into the rat parotid gland and oral submucosa is feasible. The technique requires minimal surgical intervention, which

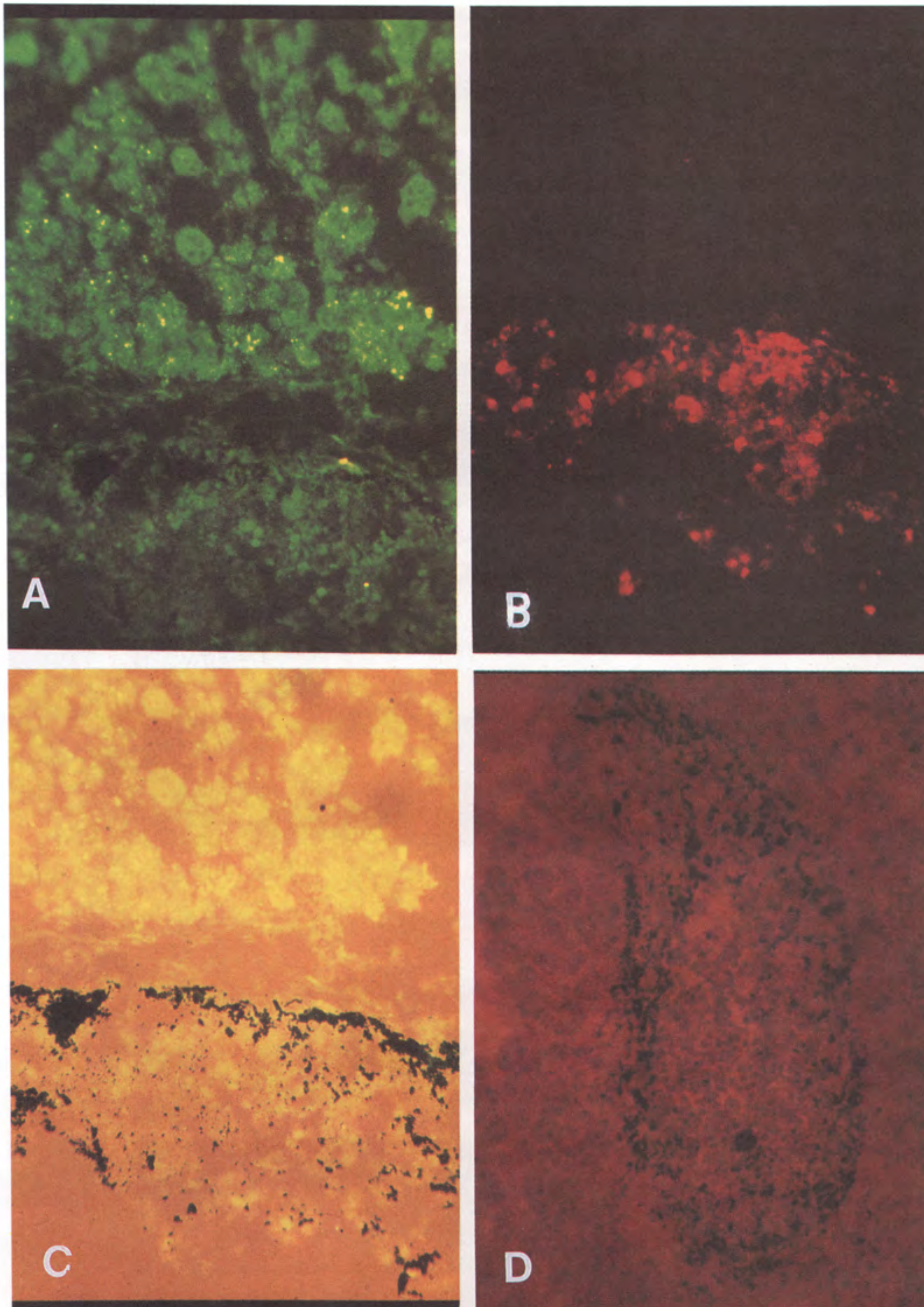

Figure 2. Immunostained frozen sections of rat parotid glands obtained after 7 days of transplantation when examined using combination of a 450–490 filter and conventional transmitted light. The section shows the demarcation of host tissue (C, upper side) and grafted cells (C, lower side) by the carbon particles. The same area when examined using a 530–585 filter combination shows the Dil-stained grafted cells as red (B, lower side) and the host tissue as dark (B, upper side). The same area shows α-amylase–positive host tissue (A, upper side) and α-amylase–positive grafted cells (A, lower side). Histology section of rat parotid gland after 7 days of transplantation shows demarcation of host tissue (D, outside) and grafted cells (D, inside) by carbon particles. Magnification: ×160.

does not produce any visible ill effect on the transplanted animals. Furthermore, this study demonstrates that DiI is effective in the identification of grafted cells in the parotid gland and oral submucosa for at least 30 days. DiI fluorescent labeling of cells grafted to other sites has previously been reported (4, 5). In addition, India ink provided clear identification of the grafted site on gross examination and in H&E sections of both the parotid gland and oral submucosa transplantation sites. India ink was observed in mac-

rophagic cells that were DiI negative. This helped to confirm that the DiI-positive cells were the grafted cells and were not macrophages loaded with graft debris.

The grafted cells survived and were functionally viable, as assessed by immunostaining for α-amylase and PSP, for at least 30 days. In addition, there was no evidence of rejection of the allogeneic grafted cells. The absence of rejection of the grafted cells is most likely due to the lack of donor antigen-presenting cells in the clonal 2RSG cell line (6–8). The grafted cells did not form tumors, suggesting that the immortalized parotid acinar cells are not tumorigenic *in vivo*.

There have been no previous studies to explore the potential of transplantation of parotid acinar cells for the treatment of xerostomia. Xerostomia can result in significant complications including pain, infection, and malnutrition. Acceptable symptomatic improvement is currently achieved in only a limited number of patients. Our preliminary results show that the transplantation of immortalized parotid acinar cells into allogeneic parotid gland or submucosa is feasible, and that the efficacy of parotid acinar cell transplantation for the possible treatment of xerostomia can be explored.

This work was supported by National Institutes of Health Grant DE 09589.

1. Prasad KN, Carvalho E, Edwards-Prasad J, La Rosa FG, Kumar R, Kumar S. Establishment and characterization of immortalized cell lines from rat parotid glands. In Vitro Cell Dev Biol **30A**:321–328, 1994.
2. Southern PJ, Berg P. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J Mol Appl Genet **1**:327–341, 1982.
3. Prasad KN, Kumar S, Carvalho E, Edwards-Prasad J, Kumar R, La Rosa FG, Larsen BB, Ann D. Characterization of human and rat immortalized clones of parotid acinar cells with respect to specific proteins and their mRNAs, and receptor-linked adenylate cyclase. In Vitro Cell Dev Biol **31**:767–772, 1995.
4. Ledley FD, Soriano HE, O'Malley BW Jr., Lewis D, Darlington GJ, Finegold M. DiI as a marker for cellular transplantation into solid organs. Biotechniques **13**:581–586, 1992.
5. Honig MG, Hume RI. DiI and DiO: Versatile fluorescent dyes for neuronal labeling and pathway tracing. Trends Neurosci **13**:333–335, 1989.
6. Snell GD. The homograft reaction. Annu Rev Microbiol **11**:439–458, 1957.
7. Steinmuller D, Hart EA. Passenger leukocytes and induction of allograft immunity. Transplant Proc **3**:673–675, 1971.
8. La Rosa FG, Talmage DW. Major histocompatibility complex antigen expression on parenchymal cells of thyroid allografts is not by itself sufficient to induce rejection. Transplantation **49**:605–609, 1990.