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GENE DELIVERY

Recombinant *Escherichia coli* as a gene delivery vector into airway epithelial cells

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Abstract

To transfer genes into airway epithelial cells, we have generated auxotrophic *dap Escherichia coli* BM2710 mutant that expresses the invasin of *Yersinia pseudotuberculosis* and the listeriolysin of *Listeria monocytogenes*. *E. coli* BM2710 harboring a plasmid carrying the *gfp* gene was incubated with immortalized normal or cystic fibrosis (CF) airway epithelial cells or with primary bronchial epithelial cells grown as an explant-outgrowth cell culture model. Approximately 2% of immortalized cells expressed GFP. Few primary cells were transfected that were always poorly differentiated and located at the edge of the outgrowth. This was consistent with the expression of β1-integrins only on these cells and with the required interaction for cell entry of *E. coli* expressing the invasin with β1-integrins. The subsequent intracellular trafficking of *E. coli* BM2710 studied by confocal and electronic microscopy showed that the *E. coli*-containing phagosomes rapidly matured into phagolysosomes. This is the first demonstration that recombinant bacteria are able to transfer genes into primary airway epithelial cells, provided that they are able to invade the cells.

Keywords: Bacterial vectors; DNA delivery; Gene therapy; Cystic fibrosis

1. Introduction

Cystic fibrosis (CF) is the most common lethal autosomal recessive disorder in the caucasian population and progressive respiratory failure is the cause of early death in most cases. CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene which encodes a cAMP-regulated chloride channel expressed at the apical membrane of epithelial cells in the airways [1]. Since the cloning of the *CFTR* gene, it seems feasible to treat the respiratory disorder of CF patients by introduction of a normal copy of *CFTR* cDNA into the airway epithelial cells. Viral and nonviral approaches have been used to deliver a

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functional *CFTR* gene into the airway epithelium. Human trials with recombinant adenoviruses and cationic lipids have been initiated. However, problems related to immune response and/or gene transfer efficiency arose and, thus, further refinements of the various vector systems or development of new vectors are needed (for review, see, Ref. [2]).

Direct transfer of functional DNA from bacteria to mammalian cells has been obtained using attenuated intracellular bacteria such as *Shigella flexneri* [3,4], invasive *Escherichia coli* [4], attenuated *Salmonella typhimurium* [5], or *Listeria monocytogenes* [6]. We have constructed a genetically defined invasive strain of *E. coli* K-12 auxotroph for diaminopimelic acid (dap) that undergoes lysis when placed in dap deficient "milieu" such as the one found intracellularly; this strain, BM2710, has been transformed with a plasmid containing the *inv* locus encoding the invasin of *Yersinia pseudotuberculosis* (which allows bacterial entry into non-phagocytic cells expressing β1-integrins) and the *hly* gene coding for listeriolysin O from *L. monocytogenes* (which is responsible for escape of the bacteria or of its content from the entry vesicle). Efficient and stable gene transfer is observed after a 2-h in vitro co-incubation of bacteria with HeLa, CHO, and COS-1 cells. Gene transfer is also described in X-ray-irradiated cells [7]. The advantages of this system are as follows: low cell toxicity, potentially high gene transfer efficiency, broad host range, rare rearrangements in the delivered DNA, and safety since for gene transfer to occur the donor bacteria have to die.

Most in vitro studies on gene transfer with bacterial vectors have been performed on poorly differentiated immortalized cell lines such as HeLa cells or on macrophage cell types. Here, we report the study of the intracellular trafficking of the bacterial vector and its gene delivering ability in explant outgrowth of non-CF bronchial tissue and in immortalized human CF and non-CF airway epithelial cells. Bacterial invasion was observed in β1-integrin expressing cells, i.e. cells at the periphery of the outgrowth and all immortalized epithelial cells. The *E. coli* vector could also transfer, albeit at low efficiency, functional DNA to immortalized and primary airway epithelial cells.

2. Materials and methods

2.1. Strains and plasmids

E. coli BM2710 [4] (*thi-1*, *endA1*, *hsdR17* (r_k^- m_k^+), *supE44*, *Δ(lac)X74, ΔdapAΩcat*, *recA1*) is auxotroph for diaminopimelic acid. Plasmid pGB2 consists of the origin of replication of pSC101 and the *aad3″ 9* gene conferring resistance to streptomycin (Sm^R) and spectinomycin (Sp^R). Construction of plasmids pGB2Ω*inv* (7.6 kb) carrying the *inv* fragment of *Y. pseudotuberculosis* and pGB2Ω*inv–hly* (10.05 kb) containing the *hly* gene of *L. monocytogenes* has been described [7]. Plasmid pEGFP-C1 (pUCΩPcmv-*egfp*, Km^R) (4.7 kb, Clontech, Palo Alto, CA, USA) is composed of the pUC18 and SV40 replication origins, a kanamycin-G418 resistance gene expressed in both bacteria and mammalian cells, and the gene for the green fluorescent protein (GFP) adapted to human codon-usage preferences under the control of the P_{CMV} eukaryotic promotor. Plasmid pAT505 (pUC18Ω*gfp-mut1*, Ap^R) (3.45 kb) consists of the 750-bp *Xba*I–*Pst*I fragment carrying the *gfp*mut1 gene encoding GFP, and cloned into the *Xba*I–*Pst*I sites of pUC18 under the control of the P*lac* promotor. It directs the production of GFP in bacteria.

2.2. Cells and culture conditions

The ΣCFTE29o−, CFBE41o− and 16HBE14o− immortalized human epithelial cells were provided by D.C. Gruenert (University of Vermont, Colchester, VT). The ΣCFTE29o− tracheal epithelial cells and the CFBE41o− bronchial epithelial cells are from CF patients homozygous for the ΔF508 mutation and show no cAMP-dependent Cl transport [8,9]. The 16HBE14o− bronchial epithelial cells, from a non-CF patient [10], retain differentiated epithelial morphology and functions and express levels of CFTR mRNA and protein. These various cell lines were cultured as previously described [8]. HeLa cells were from the American Type Culture Collection (Rockville, MD, USA).

For explant-outgrowth cell culture, non-CF bronchial tissue was collected from 25 patients at the time of open thoracotomy for localized lung tumors. Bronchial tissue had a normal architecture at the light microscopic level. Explant-outgrowth cell culture of human bron-

chial tissue was performed as previously described [11], with modifications described in [12]. Briefly, bronchial tissue was transferred to the laboratory in DMEM/F12 medium supplemented with 20 mM HEPES. Explants (1–2 mm^2) of bronchial tissue were seeded on glass slides coated with type I collagen associated with carbodiimide (Sigma, St Louis, MO, USA) and cultured in DMEM/F12 medium supplemented with 1% Ultroser G (Biosepra, Cergy, France), glucose (55 mM), sodium pyruvate (3 mM), epinephrine (3 μM). After 4–5 days in culture, explants were surrounded by a cell outgrowth that displayed two types of cell differentiation. Cells that were proximal to the explant were differentiated with clusters being ciliated. In contrast, cells at the periphery of the outgrowth were poorly differentiated, as assessed by their nonciliated state, large and flattened morphology, and expression of cytokeratin 14 and/or vimentin [11,12].

2.3. Bacterial cell invasion

One day prior to bacterial invasion, cells were seeded at 5×10^4 cells/well in a six-well plate in DMEM complete culture medium supplemented with 2 mM L-glutamine and 10% fetal calf serum (FCS, Myoclone, Gibco-BRL, Gaithersburg, MD). *E. coli* BM2710 harboring plasmids pGB2, pGB2Ω*inv* or pGB2Ω*inv–hly* and pEGFP-C1 were grown with shaking overnight at 30 °C in brain heart infusion broth (BHI, Difco Laboratories, Detroit, MI) supplemented with 0.5 mM diaminopimelic acid (dap, Sigma) and 25 μg/ml of each spectinomycin and kanamycin, harvested by centrifugation in the late logarithmic phase (LL) of growth (OD_{600} of approximately 4), and resuspended in DMEM with 0.5 mM dap at 10^8–10^9 bacteria/ml. Two milliliters of bacteria at 2.5×10^6 or 5×10^6/ml were added in DMEM containing dap and cells were incubated for 2 h at 37 °C. The cells were then washed three times with DMEM and incubated in complete medium containing 50 μg/ml of gentamicin. A similar procedure was used for invasion of outgrowth cells surrounding bronchial explants cultured for 4–5 days.

2.4. Counts of internalised bacteria

After bacterial invasion, cells were incubated 30 min at 37 °C in culture medium containing 50 μg/ml of gentamicin to kill extracellular bacteria and then washed three times with DMEM. The bacteria were released from the cells with 0.25% deoxycholate and viable counts were determined on BHI agar plates containing 0.5 mM dap and 25 μg/ml of spectinomycin.

2.5. Gene transfer analysis

Two days after invasion, ΣCFTE29o−, CFBE41o−, 16HBE14o− and HeLa cells were trypsinized, washed once in phosphate-buffered saline (PBS) with 2% fetal bovine serum, resuspended in the same medium at 10^6 cells/ml and 3×10^4 cells were analysed by flow cytometry using a FACScan® flow cytometer with CellQuest software (Becton-Dickinson, Mountain View, CA). Cells of explant-outgrowths were fixed in situ in 3% paraformaldehyde at room temperature, mounted in Vectashield-diamidino-phenylindole (DAPI) solution (Vector, Burlingame, CA) and observed with a Leitz epifluorescence microscope (Leica, Wetzlar, Germany).

2.6. Study of intracellular localization of bacteria by confocal microscopy

ΣCFTE29o− cells were seeded on coverslips (3×10^4 cells) in a 24-well plate 1 day prior to bacterial invasion. *E. coli* BM2710 harboring plasmids pGB2Ω*inv–hly* and pAT505 were grown in BHI supplemented with 0.5 mM dap, 25μg/ml spectinomycin and 50 μg/ml ampicillin.ΣCFTE29o− cells or explant-outgrowth cells were incubated with 5×10^5 bacteria/ml in culture medium with dap for 1 h at 4 °C in order to allow for binding of bacteria to the cell membrane without uptake. The cells were washed, incubated at 37 °C in complete culture medium for 30 min, and 50 μg/ml of gentamicin was added to remove extracellular bacteria. At the indicated times, from 15 min to 24 h, the cells were washed twice with PBS, fixed in 3% paraformaldehyde for 15 min at room temperature, incubated for 10 min with 0.1 M glycine in PBS and for 15 min with 0.2% bovine serum albumin (BSA) and 0.05% saponin in PBS. The cells were then incubated for 1 h in the presence of primary antibody (Ab) diluted in 0.2% BSA and 0.05% saponin in PBS. The following primary Ab were used: rabbit anti α5β1-integrin polyclonal Ab (BioValley, Marne la Vallée, France;

dilution: 1:400); mouse monoclonal Ab directed against the human transferrin receptor, clone H68.4 (Zymed Laboratories, San Francisco, CA; dilution: 1:200) or mouse monoclonal Ab directed against the early endosome antigen 1 (EEA1), clone 14 (Transduction Laboratories, Lexington, KY; dilution: 1:200) as markers of early endosomes; mouse monoclonal Ab directed against the lysosomal-associated membrane protein 1 (LAMP-1), clone H4A3 (PharMingen, San Diego, CA; dilution: 1:500) or rabbit polyclonal Ab against cathepsin D (Upstate Biotechnology, Lake Placid, NY; dilution: 1:200) as markers of lysosomes. After incubation with primary antibodies, cells were washed four times with PBS and incubated for 45 min with rhodamine-labeled secondary Ab (Molecular Probes; dilution: 1:200) diluted in PBS. Coverslips were washed with PBS and distilled water and mounted in Vectashield-DAPI solution (Vector laboratories). Cells were examined with an MRC-1024 Bio-Rad confocal system (Hercules, CA, USA) mounted on a Diaphot 300 inverted microscope with laser excitation wavelength calibration at 363, 488 and 568 nm. Serial sections collected at increments of 0.5 μm thick were used to define the intracellular localization of bacteria. Images were obtained with a Kalman acquisition device and processed with Adobe Photoshop, 6.0 software.

In certain experiments, to label more precisely acidic vesicles, 2 h after bacterial invasion cells were incubated for 1 h in the presence of the weakly basic amine LysoTracker Red (Moleculer Probes) that selectively accumulates in cellular compartments with acidic internal pH. The cells were then washed in PBS and distilled water, mounted in Vectashield-DAPI solution and immediately examined by confocal micoscopy.

2.7. Electron microscopy

ΣCFTE29o− cells (5×10^5 cells/well in a six-well plate) were incubated for 1 h at 37 °C in the presence of *E. coli* BM2710 harboring plasmids pGB2Ω*inv–hly* and pEGFP-C1 (15×10^6 bacteria/ml). The cells were washed and incubated at 37 °C in complete culture medium containing 50 μg/ml of gentamicin. At various times, from 30 min after the beginning of bacterial invasion to 24 h, the cells were trypsinized and fixed in suspension with 2.5% glutaraldehyde (TAAB, Aldermaston, UK) in 0.1 M phosphate buffer (pH 7.4) for 10 min at 4 °C and as a pellet for 2 h. After two washings in 0.1 M cacodylate buffer supplemented with 0.15 M sucrose (pH 7.3), the pellets were post-fixed with 2% osmic acid in 0.1 M cacodylate buffer for 30 min at room temperature, stained with 2.5% aqueous uranyl acetate for 30 min at room temperature, then washed and dehydrated in serially graded ethanol before embedding using the Araldite/Epon resin kit (TAAB). Ultrathin sections (70 nm) were cut with a diamond knife Diatome Ultramicrotome and placed on 300×75 mesh copper/rhodium grids to be examined by using a Philips BioTwin CM 120 TEM electron microscope (FEI, Cambridge, UK) operated at 80 kV.

3. Results

3.1. Gene transfer from the E. coli vector to human airway epithelial cells

ΣCFTE29o−, CFBE41o−, 16HBE14o− and HeLa cells were incubated for 2 h in the presence of *E. coli* BM2710 harboring plasmids pEGFP-C1 that directs synthesis of the green fluorescent protein (GFP) in mammalian cells and either the plasmids pGB2Ω*inv*, pGB2Ω*inv–hly*, or the control plasmid pGB2. MOI of 50–100 led to efficient bacterial-cell internalisation (2–5 bacteria/cell 30 min post-invasion, data not shown). After 2 days of culture, cell fluorescence intensity was screened for by flow cytometry analysis. After incubation with bacteria harboring both pGB2 and pEGFP-C1 plasmids (non-invasive bacteria), less than 0.01% airway epithelial cells and HeLa cells expressed GFP (Table 1). After incubation with bacteria harboring both pEGFP-C1 and pGB2Ω*inv–hly* plasmids, the percentage of cells expressing GFP varied from 0.8% to 2.2% (Table 1) demonstrating that invasive bacterial vectors deliver functional genes into these cells. Airway epithelial cell types and HeLa cells displayed similar gene transfer efficiencies. A higher bacteria-to-cell ratio enhanced gene transfer efficiency but resulted in higher cell toxicity (data not shown). As already observed for HeLa cells [7], bacteria expressing only the *inv* gene were able to mediate transfer of genetic material (0.1–0.7% GFP-positive cells). However, for all cell lines studied, higher numbers of GFP-positive cells were consistently obtained when cell invasion

Table 1
Plasmid transfer from *E. coli* to mammalian cells

E. coli BM2710 pEGFP-C1	Bacteria/ml[a]	GFP-positive cells (%)[b]			
		HeLa	16HBE14o−	CFBE41o−	CFTE29o−
pGB2	2.5×10^6	≤0.01	≤0.01	≤0.1	≤0. 01
pGB2	5×10^6	≤0.01	≤0.01	≤0.1	≤0.01
pGB2Ω*inv*	2.5×10^6	0.7±0.1	0.1±0.1	0.1±0.1	0.1±0.1
pGB2Ω*inv*	5×10^6	N.D.	N.D.	0.2±0.1	0.2±0.1
pGB2Ω*inv–hly*	2.5×10^6	1.8±0.3	0.1±0.1	1.0±0.3	0.8±0.1
pGB2Ω*inv–hly*	5×10^6	2.2±0.3	0.3±0.1	1.8±0.3	1.5±0.2

N.D.: not determined.

[a] Cell invasion was performed in 6-well plates plated with 5×10^4 cells/well yielding a MOI of 50–100. Cells were harvested 48 h post-invasion and GFP expression was analyzed by flow cytometry.

[b] Results are expressed as the mean of at least three independent experiments±standard deviation.

was performed with bacteria expressing both invasin and listeriolysin (Table 1).

Gene transfer efficiency was similarly studied in the explant-outgrowth cell culture model. In all cases, very few cells expressed GFP 2 days after bacterial cell invasion (3–5 cells in the whole outgrowth, data not shown). Transfection always occurred at the periphery of the outgrowth in the undifferentiated cells.

3.2. Bacterial cell uptake

A tight interaction between the invasin expressed on the outer membrane of the bacteria and a subset of β1-integrins at the cell surface mediates the uptake of bacteria by non-phagocytic cells. Bacterial uptake was studied by immunocytochemistry in ΣCFTE29o− cells and in the explant-outgrowth cell model with *E. coli* BM2710 harboring plasmids pGB2Ω*inv–hly* and pAT505, a plasmid that directs synthesis of GFP in *E. coli*. All ΣCFTE29o− cells expressed β1-integrin at their cell surface and internalised bacteria as assessed by flow cytometry analysis (data not shown). By contrast, in explant-outgrowth cell cultures, cells expressing β1-integrin were located only at the periphery of the outgrowth in poorly differentiated cells (Fig. 1A and B). When the uptake of GFP-expressing bacteria was studied, fluorescence was mostly observed in cells expressing β1-integrins (Fig. 1C–F).

3.3. Intracellular trafficking: confocal analysis

To study precisely the time course of intracellular trafficking, cells were first incubated in the presence of *E. coli* BM2710 harboring both pGB2Ω*inv–hly* and pAT505 plasmids during 1 h at 4 °C. As expected, bacteria were located at the cell membrane (data not shown). Unbound bacteria were then removed by washings and the cells were incubated at 37 °C for 15 min, 30 min, 1 h, 3 h or 24 h.

To determine the subcellular localization of the bacteria, the early endosomes were labeled using antibodies directed against the human transferrin receptor or the early endosome antigen 1. In explant-outgrowth cells, at early time-points, between 10 and 30 min, fluorescent intracellular bacteria resided in vacuoles that very rarely expressed the human transferrin receptor (Fig. 2). Between 1 and 3 h, bacteria did not reside in vacuoles expressing the human tranferrin receptor. Similar results were obtained with the early endosome antigen (data not shown). These results indicate that shortly after cell internalisation, bacteria reside in vacuoles devoid of early endosomal markers. Similar results were obtained with ΣCFTE29o− cells.

In HeLa cells, within 1–3 h, all intracellular bacteria were localized in vacuolar compartments that expressed the lysosomal membrane glycoprotein LAMP-1 [7]. The lysosomal localization of bacteria was also studied in explant-outgrowth cells with antibodies directed either against LAMP-1 or cathepsin D. Similar results were obtained with the two markers. As shown in Fig. 3, after 15 min, nearly no localization of bacteria was observed in vacuoles expressing LAMP-1 (or cathepsin D, data not shown). The number of fluorescent intracellular bacteria localized in vacuoles expressing LAMP-1 or cathepsin D increased between 30 min and 3 h, and after 3 h, almost all bacteria were localized in these vesicles which tended to cluster around the nucleus (Fig. 3). At later times, the number

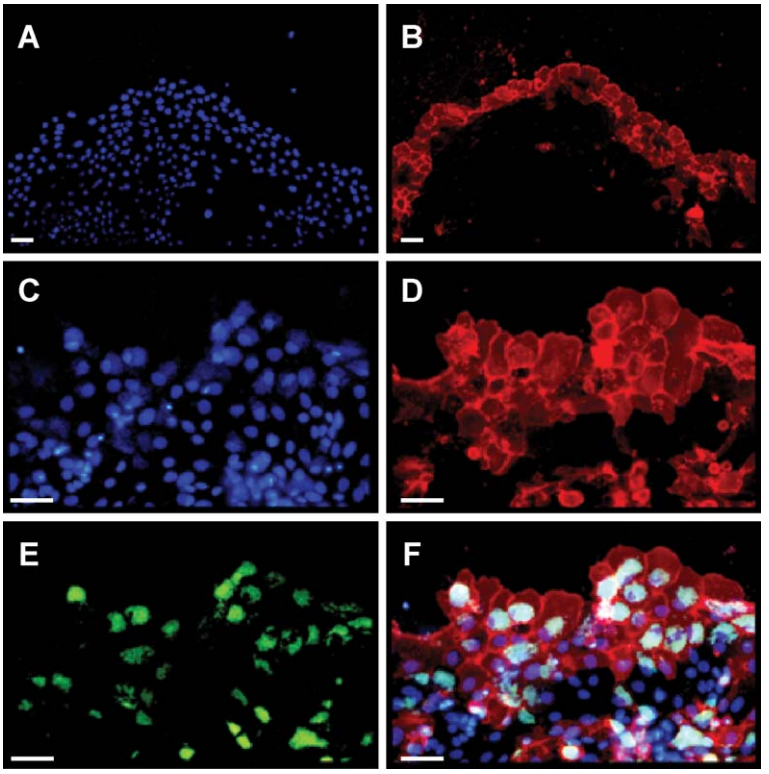

Fig. 1. Expression of β1-integrin by explant-outgrowth cells and bacterial invasion. After invasion for 2 h by *E. coli* BM2710 harboring pGB2Ω*inv*–*hly* and pAT505, explant-outgrowth cells were washed and fixed. β1-Integrin was immunolabeled by anti-α5β1-integrin antibody, followed by rhodamine-conjugated anti-rabbit antibody and appears in red. DAPI-stained nuclei appear in blue. (A, B) Expression of β1-integrin in explant-outgrowth cells at low magnification; (C–F) at higher magnification, localization of bacteria expressing GFP (green, E) in cells expressing β1-integrin (D) and merged images (F) (bar=10 μm).

of fluorescent intracellular bacteria decreased and very few were still observed 24 h after cell invasion; all visible bacteria resided within vesicles expressing LAMP-1 or cathepsin D (data not shown). Results were similar in immortalized ΣCFTE29o− cells. To further identify these vesicles expressing LAMP-1 as

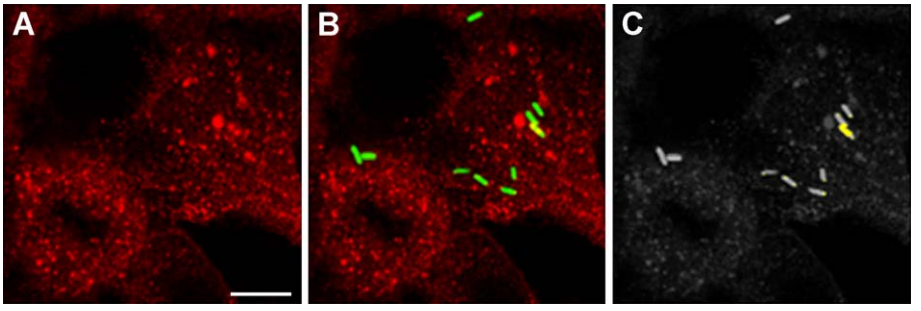

Fig. 2. Endosomal localization of bacteria in explant-outgrowth cells. After invasion for 1 h at 4 °C by *E. coli* BM2710 harboring pGB2Ω*inv*–*hly* and pAT505, the cells were incubated at 37 °C for 30 min and fixed. Endosomes were immunolabeled with anti-transferrin receptor antibody, followed by rhodamine-conjugated anti-mouse antibody. The cells were examined by confocal microscopy: endosomes appear in red (A, B) and bacteria in green (B). The bacteria localized in endosomes appear in yellow (B) and pixel analysis of colocalization of bacteria and endosomes appears in yellow (C) (bar=5 μm).

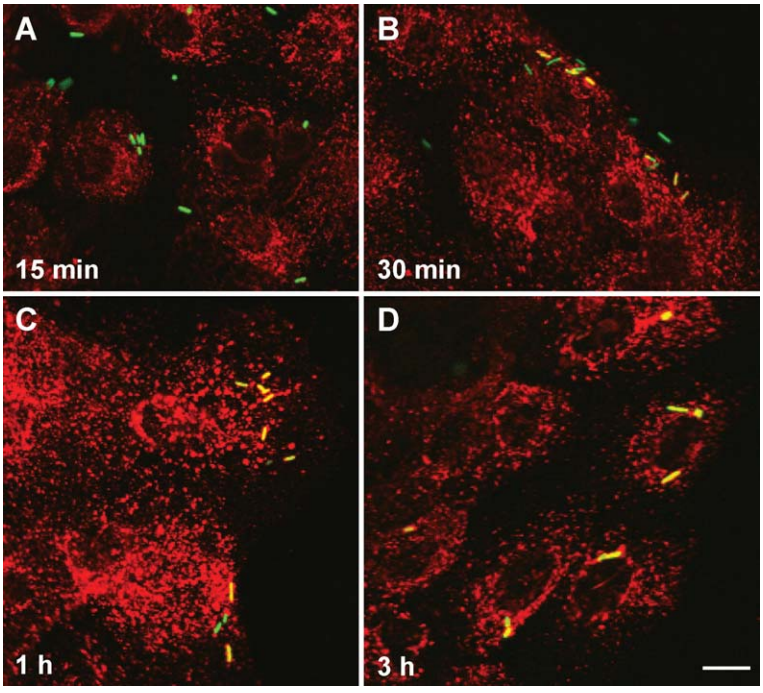

Fig. 3. Lysosomal localization of bacteria in explant-outgrowth cells. After invasion for 1 h at 4 °C by *E. coli* BM2710 harboring pGB2Ω*inv–hly* and pAT505, the cells were incubated at 37 °C for the indicated period of time and fixed. Lysosomes were immunolabeled with anti-LAMP-1 receptor antibody, followed by rhodamine-conjugated anti-mouse antibody. Cells were examined by confocal microscopy: lysosomes appear in red and bacteria in green. Colocalization of bacteria and endosomes appears in yellow (bar=5 μm).

acidic compartments, a weakly basic amine, LysoTracker Red (Molecular Probes, Eugene, OR) which selectively accumulates in cellular compartments with acidic internal pH was used. Three hours after bacterial invasion, all fluorescent intracellular bacteria were localized in vacuoles where the LysoTracker probe had accumulated in ΣCFTE29o− cells (Fig. 4) and in explant-outgrowth cells (Fig. 5). Taken together, these results clearly indicate that quickly after internalisation, bacteria reside in acidic lysosomal compartments.

3.4. Study of intracellular localization by electron microscopy

The intracellular trafficking of *E. coli* BM2710 harboring plasmids pGB2Ω*inv–hly* and pEGFP-C1 was further studied in ΣCFTE29o− cells by electron microscopy (Fig. 6). Thirty minutes after the beginning of cell invasion, bacteria could be observed entering the cells. The plasma membrane progressively enwrapped the bacteria and resulted in a phagosome-like vesicle that was quasi-filled with the bacteria. Similar phagosome-like vesicles were observed after 1 and 3 h. However, after 3 h, bacteria were also present in vesicles with a loose fitting membrane that contained myelin-like concentric systems of membrane characteristic of lysosomes. Moreover, vacuoles could be seen in some bacteria as early as 1 h post-invasion. After 24 h, all bacteria were vacuolar and present in the vesicles observed after 3 h, irregular in shape and containing a heterogeneous material. Although the vesicle membrane was sometimes interrupted at the late stages of the trafficking, a clear lysis of the membrane could not be identified and no bacteria was observed free in the cytosol in the cells which were examined.

4. Discussion

Transfer of eukaryotic expression plasmids to mammalian cells by bacterial vectors has been reported (for

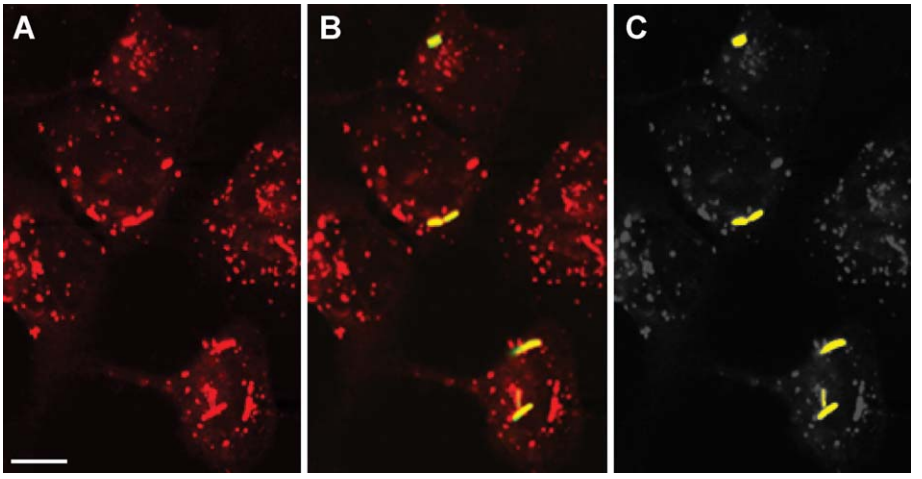

Fig. 4. Localization of bacteria in acidic compartments of ΣCFTE29o− cells. After invasion for 1 h at 4 °C by *E. coli* BM2710 harboring pGB2Ω*inv*−*hly* and pAT505, cells were incubated at 37 °C for 3 h and during the last hour, LysoTracker Red, a weakly basic amine known to accumulate in lysosomes, was added. Living cells were examined by confocal microscopy: the vesicles where LysoTracker Red has accumulated appear in red (A), bacteria appear in green and bacteria localized in the labeled acidic vesicles, in yellow (B). Pixel analysis of colocalization of bacteria and acidic vesicles appears in yellow (C) (bar=5 μm).

review, see Ref. [13]). The main applications of this type of gene transfer are genetic vaccination and gene therapy. We have previously shown that an attenuated invasive *E. coli* was able to deliver genes into HeLa, CHO and COS-1 cells [7]. Here we demonstrate that this strain can deliver DNA into immortalized and primary airway epithelial cells. This represents the first step for the use of bacteria to transfer the *CFTR* cDNA and, ultimately, for CF gene therapy. In immortalized normal or CF airway epithelial cells, gene transfer efficiency was similar to that observed in HeLa cells (Table 1). However, in primary airway epithelial cells, gene transfer efficiency was low and, in order to identify the barriers to efficient gene transfer into these cells, we have studied the uptake and intracellular trafficking of the bacteria in immortalized and primary airway epithelial cells.

E. coli are normally extracellular bacteria. The bacterial vector that we have constructed contains a plasmid with the *inv* gene coding for the invasin of *Y. pseudotuberculosis* and can invade various cell lines [7]. Uptake by non-phagocytic cells of *Y. pseudotuberculosis* results from tight binding of the invasin to a subset of β1-integrins present on the surface of numerous cell types (for review, see Ref. [14]). Expression of α5β1-integrin was observed only in poorly differentiated primary airway epithelial cells at the edge of the explant outgrowth. This is consistent with previous reports indicating that there is no detectable expression of β1-integrins in normal airway epithelium [15] and that they are expressed at the leading edge of poorly differentiated cells during the early phase of epithelial repair [16]. As a consequence, invasive *E. coli* invaded only these cells and gene transfer was observed with a relatively low efficiency consistent with the low number of cells that internalised bacteria. This restricted uptake of invasive *E. coli* in poorly differentiated cells could potentially limit the interest of such strains for gene transfer into the airways. However, in CF, due to bacterial exposure and chronic inflammation, a remodeled surface epithelium has been described [17] and repairing processes involving poorly differentiated cells are likely to be seen neighbouring injured areas of airway epithelium. If the expression of β1-integrins is restricted to poorly differentiated cells in CF airway epithelium as in non-CF one, the use of invasive *E. coli* might prove to be of interest for CF gene therapy.

In airway epithelial cells, the mode of entry of *E. coli* BM2710Ω*inv,hly* is similar to the ‘‘zipper’’ mechanism described for *Y. pseudotuberculosis* in professional macrophages and in less differientiated cell lines such as HeLa cells [14]. As shown by

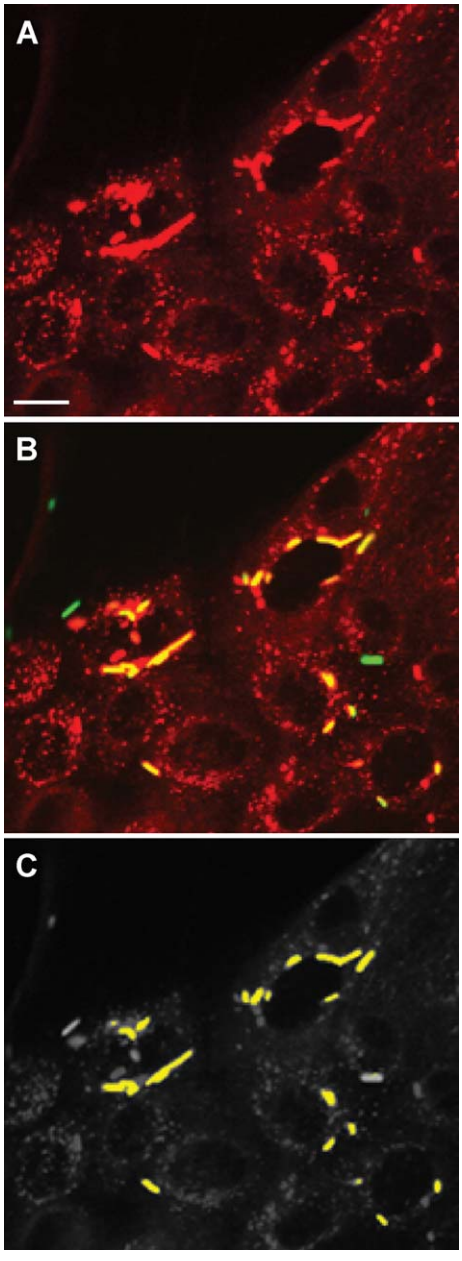

Fig. 5. Localization of bacteria in acidic compartments of explant-outgrowth cells. After invasion for 1 h at 4 °C by *E. coli* BM2710 harboring pGB2Ω*inv*–*hly* and pAT505, cells were incubated at 37 °C for 3 h and during the last hour, LysoTracker Red, a weakly basic amine known to accumulate in lysosomes, was added. Living cells were examined by confocal microscopy: the vesicles where the LysoTracker Red has accumulated appear in red (A), bacteria appear in green and bacteria localized in labeled acidic vesicles, in yellow (B). Pixel analysis of colocalization of bacteria and acidic vesicles appears in yellow (C) (bar=5 μm).

electron microscopy in CF airway epithelial cells, a tight binding between bacterial surface and the host cell membrane results in the engulfment of the bacteria in a close-fitting vacuole (Fig. 6).

Following cell internalisation, bacteria-containing vacuole undergoes maturation steps that mirror the endocytic pathway (for review, see Ref. [18]). Immediately following endocytosis, the early phagosomes acquire markers of early endocytic proteins such as EEA1 and transferrin. During maturation, they lose these markers, fuse with lysosomes, and acquire increasing amounts of lysosomal markers such as lysosome-associated membrane glycoproteins, e.g. LAMP-1, and acid hydrolases, e.g. cathepsin D, and become highly acidified. This maturation pathway which leads to the killing of the ingested bacteria was described for *Y. pseudotuberculosis* in macrophage [19]. We have shown that in HeLa cells, vacuoles containing BM2710Ω*inv,hly* acquire the Lamp-1 marker within 1 h following cell internalisation [7]. In differentiated airway epithelial cells, a similar pathway of maturation of the *E. coli* BM2710Ω*inv,hly* containing vacuoles was observed (Figs. 2 and 3). Certain markers of early endosomes, such as transferrin receptor and EEA1 antigen, were observed at early time points (10 and 30 min) colocalized with the bacteria but at a very rare frequency (Fig. 2). Phagosomes then rapidly acquired lysosomal proteins (cathepsin D and LAMP-1) and were colocalized with LysoTracker Red, a marker of acidic compartments (Figs. 3–5). *E. coli* BM2710 is an auxotrophic *dap* mutant that lyses rapidly in the intracellular vacuole, as shown by electronic microscopy where degraded bacteria were observed as soon as 1 h (Fig. 6). Lysis of the bacterial vector inside the phagolysosome results in the release of the plasmid of interest and of lysteriolysin, a toxin from *L. monocytogenes* that triggers pore formation in the vacuolar membrane and allows subsequent release of plasmid DNA into the cytosol. As in HeLa, CHO and COS-1 cell lines [7], *E. coli* BM2710Ω*inv,hly* resulted in a higher number of transfected airway epithelial cells as compared with *E. coli* expressing the invasin only (Table 1). However, the increase in efficiency was moderate, suggesting that plasmid DNA may also gain access to the mammalian cell cytoplasm via leakage from host cell phagosomes, as it has been proposed for transfer of certain protein antigens from phagosomes to cytosol [20].

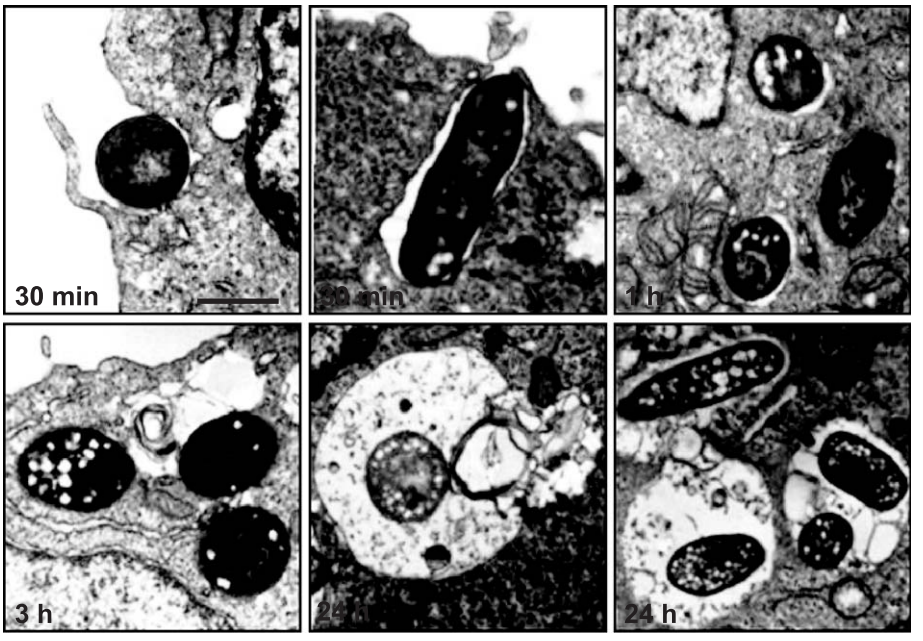

Fig. 6. Electron microscopy analysis of invasion of ΣCFTE29o− cells by *E. coli* BM2710 harboring pGB2Ω*inv*–*hly* and pEGFP-C1. From 30 min to 1 h post-invasion, bacteria enter the cells and are localized in phagosome-like vesicles. Between 3 and 24 h after the beginning of invasion, vacuoles are seen in bacteria and the vesicles containing the bacteria have a loose fitting membrane and contain myelin-like concentric systems of membrane characteristic of lysosomes (bar=700 nm).

Once the plasmid DNA has gained access to the cytosol, it has to reach the nucleus to eventually be expressed. It is well known from studies with synthetic vectors that this step is a limiting one: naked plasmid DNA is rapidly degraded in the cytosol and its entry into the nucleus occurs at low efficiency (for a review, see Ref. [21]). These cytosol and nuclear steps of gene transfer are likely to be important intracellular barriers to efficient gene transfer with bacterial carriers and ways to target the nucleus will have to be engineered. The advantage of our bacterial gene therapy system compared to other usual viral and nonviral gene therapy systems is the possible ''hijacking'' of the bacterial protein synthesis machinery to produce proteins favoring transgene DNA transfer to host cell nucleus.

5. Conclusion

To the best of our knowledge this is the first demonstration that an engineered invasive *E. coli* is able to mediate gene transfer into airway epithelial cells. However, gene transfer efficiency was low, especially into primary airway epithelial cells mostly because of bacterial invasion being restricted to the poorly differentiated cells. Three hours after invasion, all bacteria reached lysosomal compartments where an efficient release of the plasmid of interest is likely to occur. One potential limiting step for an efficient gene transfer with recombinant *E. coli* might be the trafficking of the plasmid DNA to the nucleus once it has gained access to the cytosol. Although more efficient bacterial strains will have to be engineered, advantage might be taken of the massive bacterial colonization observed in CF patients to deliver the normal *CFTR* cDNA via bacterial strains into the airway epithelial cells.

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