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Two stages of enteropathogenic Escherichia coli intestinal pathogenicity are up and down-regulated by the epithelial cell differentiation

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Accepted in revised form: 19 April 1995

Abstract. Pathogens and eucaryotic cells are active partners during the process of pathogenicity. To gain access to enterocytes and to cross the epithelial membrane, many enterovirulent microorganisms interact with the brush border membrane-associated components as receptors. Recent reports provide evidence that intestinal cell differentiation plays a role in microbial pathogenesis. Human enteropathogenic Escherichia coli (EPEC) develop their pathogenicity upon infecting enterocytes. To determine if intestinal epithelial cell differentiation influences EPEC pathogenicity, we examined the infection of human intestinal epithelial cells by JPN 15 (pMAR7) [EAF+ eae+] EPEC strain as a function of the cell differentiation. The human embryonic intestinal INT407 cells, the human colonic T84 cells, the human undifferentiated HT-29 cells (HT-29 Std) and two enterocytic cell lines, HT-29 glc-/+ and Caco-2 cells, were used as cellular models. Cells were infected apically with the EPEC strain and the cell-association and cell-entry were examined by quantitative determination using metabolically radiolabeled bacteria, as well as by light, scanning and transmission electron microscopy. [EAF+ eae+] EPEC bacteria efficiently colonized the cultured human intestinal cells. Diffuse bacterial adhesion occurred to undifferentiated HT-29 Std and INT407 cells, whereas characteristic EPEC cell clusters were observed on fully differentiated enterocytic HT-29 glc-/+ cells and on colonic crypt T84 cells. As shown using the Caco-2 cell line, which spontaneously differentiates in culture, the formation of EPEC clusters increased as a function of the epithelial cell differentiation. In contrast, efficient cell-entry of [EAF+ eae+] EPEC bacteria occurred in recently differentiated Caco-2 cells and decreased when the cells were fully differentiated. Our results provide evidence that the intestinal cell differentiation could play a dual role in EPEC pathogenesis: it up-regulates intestinal cell colonization and down-regulates intestinal cell invasion.

Introduction

A bacterial pathogen is a microorganism that has the capacity to cause disease (for review see [1, 15]). Entero-virulent microorganisms colonize the intestinal mucosa via bacterial surface-associated products encoded by the virulence genes. After attachment to specific brush border receptors, enterotoxigenic pathogens produce cytotoxic toxins, which bind to their brush border receptors and increase intracellular cAMP and cGMP through stimulation of adenyl cyclase systems. For other enterovirulent bacteria, cell-attachment produces brush border lesions, rearrangements of cytoskeletal proteins, induction of protein phosphorylation, and increases in intracellular calcium concentration and permeability of the tight junctions. Enteroinvasive microorganisms enter the cell, multiply, and cause cell death and exfoliation. As a consequence, rapid migration of crypt cells along the crypt-villus axis occurs and the mucosal surface is reduced and is characterized by a high number of immature intestinal cells, which have a minimal capacity for NaCl-coupled transport, a state of base-line secretion of anions and poor digestive-absorptive functions for nutrients.

To approach in vitro the situation that would mimic in vivo intestinal infection, studies of development of intestinal bacterial pathogenic processes require appropriate cellular models. Indeed, the intestine presents a particular situation due to the rapid epithelial cell renewal, with proliferating crypt cells being undifferentiated and with the differentiation occurring during the crypt-to-villus migration of nondividing cells. The establishment of colon carcinoma cell lines [20] exhibiting characteristics of mature enterocytes or muco-secreting cells has provided an invaluable tool for study of intestinal cell functions, since there are similarities between adenocarcinoma cell lines in culture and small intestinal cells (for reviews see [37, 61]). Recent reports suggest that the enterocytic cell differentiation plays an important role in the development of bacterial pathogenicity. Indeed, for enterotoxigenic bacteria intestinal cell differentiation up-regulates...
bacteria, which develop their pathogenicity by two separate and successive processes such as cell-association and the cell-entry of enteroinvasive bacteria [3]. It regulates the cell-entry of enteroinvasive bacteria [3]. It first stage involves adherence of bacteria to enterocytes followed by cell-entry.

Enterovirulent Escherichia coli (EPEC) causes severe and sometimes persistent infantile and adult diarrhea, particularly in developing countries [52]. Occasionally, EPEC are involved in diarrhea in nurseries and day-care centers in developed countries. Studies of pathogenicity of EPEC have been conducted using non-intestinal and intestinal epithelial cells in vitro (for review see [10]). EPEC promotes a three-stage process of infection. The first stage involves adherence of bacteria to enterocytes in a pattern termed "localized adherence" (LA) involving EPEC adherence factor (EAF). The second stage features signal transduction leading to protein phosphorylation, elevation of intracellular calcium concentrations, and effacement of microvilli. The third stage is characterized by close attachment to the epithelial membrane by intimin, a 94-kDa outer membrane protein encoded by the E. coli attaching and effacing (eae) locus. After these three stages, a subset of bacteria can enter the epithelial cell.

The aim of the present study was to analyse the cell-association and the cell-entry of EPEC with and within human enterocyte-like cells in culture as a function of intestinal cell differentiation. For this purpose, several cultured human colon carcinoma cell lines were used. By quantitative determination using metabolically radiolabeled bacteria, as well as by light, scanning and transmission electron microscopy, we examined here the bacterial colonization of the brush border, the development of the brush border lesion and bacterial entry into the cells.

Methods

Bacterial strains, growth conditions and radiolabeling. The enteropathogenic E. coli strains used were JPN 15 (pMAR7) [EAF+ eae+] and [JPN 15.96] [EAF- eae-] [22]. Before adherence assays, EPEC bacteria were cultured at 37°C for 18 h in Luria broth with appropriate antibiotics. For radiolabeling by 14C-acetic acid (Amersham, 94 mCi/mmol; 100 μCi per 10-ml tube), EPEC bacteria were subcultured twice at 37°C for 24 h in Luria broth without agitation.

Cell culture. Three populations of cultured human colonic adenocarcinoma cell lines were used. Enterocyte-like Caco-2 cells and parental, mainly undifferentiated HT-29 cells, referred to as HT-29 Std, were obtained from Dr. Jorgen Fogh (Sloan Kettering Memorial Cancer Center, Rye, N. USA) [20]. The pluripotent HT-29 Std cell line grown under various metabolic stress conditions is able, after an initial phase of mortality, to undergo growth adaptation to these conditions [62]. This growth adaptation is followed by the emergence of differentiated populations of either absorptive or mucus-secreting cells. Differentiated HT-29 cells, obtained by selection through glucose deprivation [51], maintain their differentiation characteristics when switched back to standard glucose-containing medium, and are referred to as HT-29 glc- cells. Caco-2 cells spontaneously differentiate in culture under standard conditions, i.e. in a standard glucose-containing medium [50].

Cells were routinely grown in Dulbecco modified Eagle's minimal essential medium (DMEM; 25 mM glucose; Eurobio, Paris, France), supplemented with 10% (HT-29 Std, HT-29 glc- cells) or 20% (Caco-2) inactivated (30 min, 56°C) fetal calf serum (Boehringer, Mannheim, Federal Republic of Germany) and 1% nonessential amino acids (Caco-2). For adherence assay, monolayers of Caco-2 cells and HT-29 subpopulations were prepared on glass coverslips which were placed in six-well Corning tissue culture plates (Corning Glass Works, Corning, NY, USA). Cells were seeded at a concentration of 10⁵ (HT-29) and 7.4x10⁴ (Caco-2) cells per cm². For maintenance purposes, cells were passaged weekly using 0.25% trypsin in Ca²⁺/Mg²⁺-free phosphate-buffered saline (PBS) containing 0.53 mM EDTA. Maintenance of the cells and all experiments were carried out at 37°C in a 10% CO₂/90% air atmosphere. The culture medium was changed daily. Cells were used between 20–40 (HT29) and 60–90 (Caco-2) cell passages. Differentiated cells were used for adherence assays at late post-confluence, i.e., after 15 days (Caco-2) and 20 days (HT-29) in culture. Caco-2 cells were used at confluence (5 days, undifferentiated cells), at post-confluence (7 days, when differentiation commences, and 10 days, when differentiation develops), and at late post-confluence (15 days, when differentiated is complete).

INT-407 cells (human embryonic intestine; ATCC CCL 6) were from stock culture of the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in DMEM supplemented with 1% non-essential amino acids and 10% inactivated (30 min, 56°C) fetal calf serum (Boehringer, Mannheim, FRG) at 37°C in a 5% CO₂/90% air atmosphere. Cells were used for adherence assays at confluence, i.e., after 4 days. Human intestinal T₄₅ cells (7) were routinely grown in DMEM (50%) and Ham's F12 medium (50%) supplemented with 2 mM glutamine, 50 mM HEPES, 1% non-essential amino acids and 10% inactivated (30 min, 56°C) fetal calf serum (Boehringer, Mannheim, FRG) at 37°C in a 5% CO₂/90% air atmosphere. Cells were seeded at 3x10⁵ cells per cm². Cells were used for adherence assays at late post-confluence, i.e., after 10 days.

Determination of cell-associated and cell-invaded bacteria. Before the adhesion assay, the cultured intestinal cell monolayers were washed twice with phosphate-buffered saline (PBS). The adherence of EPEC strain to cultured cells was examined as described previously for enterotoxigenic E. coli [5]. To quantify cell-associated bacteria (adhering+invading bacteria), 14C metabolically radiolabelled bacteria were used [30]. Bacteria were suspended in the culture medium and a total of 1 ml (10⁸ CFU cells/ml) of this suspension was added to each well of the tissue culture plates. Incubations were conducted with 1% D-mannose, which inhibits type 1 pili-mediated adhesion. The plates were incubated at 37°C in 10% CO₂/90% air for 180 min. The monolayers were then washed three times with sterile PBS. Cell-associated bacteria and cultured cells were dissolved in a 0.1 N NaOH solution. The level of bacterial adhesion was evaluated by liquid scintillation counting.

Bacterial internalization was determined by quantitative determination of bacteria located within the cells using unlabelled bacteria and an aminoglycoside antibiotic [3]. Bacteria were suspended in the culture medium and 2 ml (10⁶ CFU/ml) of this suspensions was added to each well of the tissue culture plate. The plates were incubated at 37°C in 10% CO₂/90% air, for 180 min. After incubation, the plates were washed three times with sterile PBS and afterwards incubated for 60 min in a medium containing 100 μg/ml gentamicin. Extracellular bacteria are rapidly killed by this method whereas those located within the cells are not. The monolayer was washed three times with PBS and lysed with sterile H₂O. Appropriate dilutions were plated to determine the number of viable intracellular bacteria. Each assay was conducted in triplicate with three successive passages of each cell line. Maintenance of the circumscribed EPEC clusters over the cell surface of cultured intestinal cells was determined. After incubation,
the monolayers were washed five times with sterile PBS, fixed with methanol, stained with the Gram stain, and examined microscopically under oil immersion. Each adhesion assay was conducted in duplicate with cells from three successive passages. For each glass coverslip monolayer, the number of EPEC clusters was counted in 20 random microscopic areas.

**Indirect immunofluorescence.** The state of Caco-2 cell differentiation was evaluated by immunofluorescent staining of the cells with antibody directed against the brush-border-associated marker sucrase-isomaltase (SI) [55]. Cell layers were prepared after cells were grown on glass coverslips, and monolayers were washed three times with PBS and fixed for 10 min at room temperature in 3.5% paraformaldehyde in PBS. Indirect immunofluorescence was performed on unpermeabilized Caco-2 cell layers. Immunoreactivity of SI was detected using polyclonal rabbit antibodies against sucrase-isomaltase purified from Caco-2 cells, obtained from Dr. Alain Zweibaum (INSERM U 178, Villejuif, France). Anti-rabbit fluorescein-coupled goat antiglobulins were from Institut Pasteur Productions (Paris, France). Cell monolayers were incubated with rabbit polyclonal anti-sucrase-isomaltase (diluted 1:200 in PBS) for 45 min at room temperature, washed, and then incubated with FITC-conjugated goat antibodies to rabbit immunoglobulin G. Immunolabeling was examined using a Leitz Aristoplan microscope with epifluorescence. All photographs were taken on Kodak T-MAX 400 black and white film (Eastman Kodak Co., Rochester, NY, USA).

**Scanning and transmission electron microscopy.** For scanning electron microscopy, the cells were grown on glass coverslips. After the bacterial adhesion assay, cells were fixed and treated as previously reported [5]. The specimens were then examined with a Jeol JSM 25S scanning electron microscope. Transmission electron microscopy was performed on cells grown in six-well Corning tissue culture plates as previously reported [50, 51]. Samples embedded in Epon were reembedded in order to make sections perpendicular to the bottom of the flask. The specimens were then examined with a Jeol electron microscope.

**Results**

**Cell-association of EPEC with intestinal cells**

Adherence profiles of [EAF+ eae+] EPEC bacteria to human undifferentiated HT-29 Std cells and differentiated HT-29 glc-/+ cells were examined after apical infection. [EAF+ eae+] EPEC bacteria infected both undifferentiated and differentiated HT-29 cells, i.e., 11.5%±2.5% and 8.8%±1.7% of incubated bacteria remained associated after washing, respectively. As shown in Fig. 1A, [EAF+ eae+] EPEC bacteria adhered diffusely to the multilayer surface of undifferentiated HT-29 Std cells (Fig. 1A). In contrast, localized circumscribed clusters randomly distributed over the monolayer surface were observed on differentiated HT-29 glc-/+ cells (Fig. 1B). As observed by scanning electron microscopy, the EPEC cluster appeared dense and well-organized (Fig. 1C). This result strongly suggests that the intestinal cell differentiation plays a role in the formation of EPEC clusters.

Adhesion of EPEC bacteria was examined as a function of cellular differentiation using the Caco-2 cell line, which provides the most useful tool for the study of cell differentiation, because of its unique ability to spontaneously differentiate upon reaching confluence under normal culture conditions [50]. The state of the intestinal cell differentiation of Caco-2 cells was examined by immunofluorescent labeling of the differentiation-associated brush border hydrolase sucrase-isomaltase (SI). The number of SI-positive cells increased as a function of the days in culture. At confluency (5 days, Fig. 2B), 95%±5% of the cells were undifferentiated without expression of SI-immunoreactivity; positive SI-immunoreactivity increased between 7 and 12 days in culture (Fig. 2C), and at subconfluence (15 days in culture, Fig. 2D).

![Fig. 1A-D. Light and scanning electron micrographs of human intestinal HT-29 Std cells and enterocyte-like HT-29 glc-/+ infected apically with enteropathogenic *Escherichia coli* [EAF+ eae+] JPN 15 (pMAR7). Ultrathin sections of cultured undifferentiated HT-29 Std cells (A) and differentiated HT-29 glc-/+ cells (B), fixed in 3% glutaraldehyde and embedded in Epon for light microscopic examination of sections perpendicular to the bottom of the flask. [EAF+ eae+] EPEC bacteria display high level of diffuse adherence to the multilayer of undifferentiated HT-29 Std cells (A). Circumscribed EPEC clusters randomly distributed over the polarized and well-differentiated HT-29 glc-/+ cell monolayer (B). Scanning electron micrographs of differentiated HT-29 glc-/+ cells infected apically by [EAF+ eae+] EPEC showing clusters of EPEC bacteria (C and D), A, B×100 C×5000; D×10,000.](image-url)
different days of culture (not shown). In undifferentiated cells a diffuse pattern of adhesion was observed. When differentiation commenced, the EPEC adhesion evolved in an aggregative pattern and a few organized EPEC clusters were observed. Moreover, it is important to notice that the EPEC clusters appeared more dense at late postconfluency than at postconfluency, indicating that the numbers of EPEC bacteria associated with the clusters increased after the initiation of cluster formation.

Cell-invasion of EPEC within intestinal cells

The invasive profile of the [EAF+ eae+] EPEC strain was examined as a function of intestinal epithelial cell differentiation using the Caco-2 cell line (Table 2). The kinetics of cell-entry were very different from those of cell-association. Cell-entry occurred within confluent undifferentiated Caco-2 cells (day 5 in culture), increased and reached a maximum at postconfluency (day 10 in culture). Afterwards, cell-entry declined and low numbers of bacteria entered fully differentiated cells (day 15 in culture). In contrast no cell-entry occurred with the [EAF+ eae+] EPEC strain. It was noticed that the Caco-2 cells are more permissive than HT-29 cells for the cell-entry of [EAF+ eae+] strain. Indeed, in undifferentiated HT-29 Std cells 0.22%±0.05% of the total incubated bacteria entered the cells, and 0.45%±0.02% entered the fully differentiated HT-29 gluc+/-cells. No cell-entry into HT-29 Std and HT-29 gluc+/- cells occurred with the [EAF- eae+] EPEC strain.

The entry of [EAF+ eae+] EPEC bacteria into Caco-2 cells was examined by transmission electron microscopy (Fig. 4). In the recently differentiated cells, the [EAF+ eae+] EPEC bacteria interacted with the short-length microvilli and an intimate contact with the cell membrane occurred (Fig. 4A, arrow). EPEC bacteria engaged in direct cell-entry without attaching-effacing lesions, and bacteria were observed in the cytoplasm (Fig. 4A, arrowhead). In fully differentiated cells, the [EAF+ eae+] EPEC bacteria interacted with the microvilli of postconfluent Caco-2 cells (Fig. 4B). The [EAF+ eae+] EPEC bacteria produced attaching-effacing lesions (Fig. 4C) and entered the cells (Fig. 4D and E).

Discussion

Work presented in this communication analyses the cell-association and the cell-entry of [EAF+ eae+] EPEC bacteria in human cultured enterocyte-like cell lines as a function of cell differentiation. With the [EAF+ eae+] JPN 15 (pMAR7) EPEC strain we observed that the localized circumscribed clusters characteristic of EPEC adherence [32, 43, 54] occurred only in differentiated enterocyte-like HT-29 gluc-/+ strain. Moreover, we observed that the cluster-localized binding of EPEC bacteria to Caco-2 cells, which spontaneously differentiate in culture [5], developed in parallel with cell differentiation, disclosed by immunofluorescence labeling of sucrase-isomaltase, a differentiation-associated brush border hydrolase [55].

![Fig. 2A-D. Adhesion of enteropathogenic Escherichia coli to human intestinal Caco-2 cells infected apically as a function of the cell differentiation.](image-url)
EPEC clusters develop as a function of the cell differentiation in cultured human intestinal cells. A–C Examination of the pattern of adhesion of EPEC by scanning electron microscopy in INT407 cells, T54 cryptic cells, and fully differentiated Caco-2 cells. Notice the diffuse and aggregative adhesion of EPEC bacteria in the unorganized cell projection of the confluent undifferentiated INT407 cells (A). In contrast, the characteristic circumscribed EPEC clusters were observed in the T54 cells, showing sparsely distributed short microvilli at their apical domain, and in the brush border of the fully differentiated Caco-2 cells (C). ×5000. D Time-course of EPEC cluster as a function of the day in culture. The number of circumscribed EPEC clusters was microscopically determined in 20 random microscopic areas. Each experiment was conducted in triplicate. The data represent mean values of experiments from three successive passages of Caco-2 cells.

Table 1. Cell-invasion within human cultured intestinal Caco-2 cells of enteropathogenic Escherichia coli [EAF+ eae+] JPN 15 (pMAR7) and [EAF– eae–] JPN 15.96 strains as a function of the day in culture

| Days in culture | JPN 15 (pMAR7) [EAF+ eae+] | JPN 15.96 [EAF– eae–] |
|----------------|---------------------------|-----------------------|
| 5              | 0.9±0.14                  | 0                     |
| 8              | 1.6±0.15                  | 0.05±0.02             |
| 10             | 1.9±0.2                   | 0                     |
| 12             | 1.2±0.12                  | 0                     |
| 15             | 0.5±0.3                   | 0                     |

Each experiment was conducted in triplicate with three successive passages of cultured cells.

It has been widely documented that the adherence of EPEC to eucaryotic nonintestinal epithelial cells is dependent on an adhesive factor encoded by the EAF plasmid (for a review see [10]), and the formation of cluster-localized EPEC microcolonies is due to the bundle forming pili (BFP) encoded by the 60-MDa epec adherence factor (EA) plasmid [9, 24, 25]. Induction of BFP synthesis was recently examined [59]. As observed with HEp-2 cells, EPEC localized adhesion: (i) develops after a latent period and (ii) localized adhesion is induced by the bacterial growth conditions associated with changes in the outer membrane proteins of the bacteria. In contrast, our results suggest that a bacterial response, i.e., the synthesis of BFP, may be triggered by specific contact-dependent signals, which are themselves induced by the expression of an unknown epithelial differentiation-associated receptor. It is well established that during differentiation expression of functional apical or baso-lateral membrane-associated components takes place. For example, expression of microvillar proteins [34, 41], membrane-anchored hydrolases such as sucrase-isomaltase [50, 51] and dipeptidyl peptidase IV [6], apical membrane transporters [37], and basolateral receptors [33, 46, 47] is up-regulated as a function of enterocytic differentiation. Our group and others have recently demonstrated that brush border receptors for the adhesive factors of enterotoxigenic E. coli [5, 28, 29], Clostridium difficile [14] or for E. coli enterotoxin [4, 40] are expressed at the apical side of intestinal cells during cell differentiation. In contrast, we have recently observed that the cell-entry of Yersinia is down-regulated by intestinal cell differentiation [3]. Finally, it has been noticed that viruses interact both with brush border and baso-lateral components to enter the intestinal cells [16–18, 57, 58, 60].

Beneath the localized microcolonies the close association of EPEC bacteria with the apical intestinal cell
A membrane is accompanied by characteristic ultrastructural brush border lesions referred to as "attaching-effacing" (A/E) lesions [31, 32, 43]. The eaeA locus, originally designated aea [8, 26, 27], and the eaeB gene, located downstream of eaeA, are necessary for intimate attachment [11, 12]. It was previously reported that a subset of EPEC bacteria enters within the epithelial cells after developing the close association with the cell membrane, followed by the attaching-effacing lesions (for review see [10]). The cell-entry of [EAF+ eae+] JPN 15 (pMAR7) EPEC within the enterocyte-like Caco-2 cells appears to occur at a very low level that as compared with Yersinia pseudotuberculosis [3]. We observed that the EPEC cell-entry during the differentiation of Caco-2 cells was very different from the EPEC cell-association. Efficient EPEC cell-entry occurred at the beginning of differentiation and then decreased. In contrast, for the JPN 15.96 strain the loss of the EPEC large plasmid, which encodes a positive regulatory factor that increases eaeA expression [26], results in the abolishment of cell-entry. It is clear that the brush border cytoskeletal structure and composition particularly, the assembly of the brush border cytoskeleton from its consistent proteins, evolve during differentiation; [37]. This progressive process occurs in vivo when cells become terminally differentiated along the crypt-villus axis. In vitro throughout differentiation and immediately after confluency, the organization of the apical surface of the cultured intestinal cells undergoes a series of changes [48, 49]. It is possible that at the stage where irregular microvilli are present cell-entry of EPEC bacteria occurs more easily or more rapidly than when the microvilli are fully organized.

The mechanisms whereby EPEC causes diarrhea remain undefined. EPEC causes brush-border lesions [31, 32, 43] and decreases the transepithelial electrical resistance across polarized monolayers of epithelial cells [1]. Moreover, EPEC triggers profound changes in enterocytic cells: rearrangements of the host cell cytoskeleton [31, 53], release of inositol phosphate [13, 21] and
activation of host cell tyrosine kinases [39]. In parallel with this pathogenic effect on mature enterocytes, it is possible that EPEC, by mobilizing the cytoskeletal components, affect the assembly and the spatial organization of the brush-border during intestinal cell differentiation. Indeed, we observed that EPEC invades human cultured intestinal cells when the differentiation commences. Most of the cell cytoskeletal components affected during EPEC attaching-effacing lesions and EPEC cell-entry are involved in the establishment and the maintenance of the epithelial cell polarity [19, 37]. In consequence it may alter the functionality of several brush border components which play a fundamental role in the vectorial movement of ions, water, and macromolecules [35-42].

The human intestinal differentiation-associated receptor for EPEC colonization factor remains to be identified. In addition, it is expected that further studies examining the organization of polarized intestinal cells and function of brush border-associated components during and after EPEC infection will provide greater insight into the detailed mechanism of EPEC diarrhea. Studies addressing these topics are in progress.

Acknowledgements. M.F.B-C was supported by a doctoral fellowship (MFSR). This work was supported by grants from Fondation pour la Recherche Médicale, Association pour la Recherche sur le Cancer, Institut National de la Santé et de la Recherche Médicale and Caisse Nationale d'Assurance Maladies des Travaillers Salariés (to A.L.S.).

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