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Electrofusion of individual animal cells directly to intact corneal epithelial tissue

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An electromechanical process was developed to electrofuse human and nonhuman cultured cells directly to rabbit corneal epithelial tissue in vitro and in situ. This new process was utilized successfully to incorporate functional gonococcal membrane attachment receptors from human lymphoma cells into superficial rabbit corneal epithelium. Thus, cell–tissue electrofusion biotechnology may be employed to establish unique and novel animal models for investigating receptor-mediated processes in vivo.

Introduction

Current concepts in many biomedical and biological sciences will be broadened significantly when custom made bioengineered laboratory animals become routinely available. Phenotypically unique animals could display distinctive features that are normally absent in the unmodified animal species. Such novel in vivo models would be especially useful in studies of receptor-mediated processes. For example, bioengineered animals that express host- or tissue-specific microbial attachment receptors should be susceptible to infection by those etiological agents to which the unaltered animals are naturally resistant. In this regard, suitable animal models for gonorrhea are nonexistent because pathogenesis is initiated by *Neisseria gonorrhoeae* binding to host-specific attachment receptors found exclusively on human cell membranes; nonhuman animal cell membranes lack these receptors.

In order to create an in vivo infectivity model for the obligate human pathogen, the basic idea was conceived of electrofusing human cells that contain gonococcal attachment receptors directly to intact epithelial tissue of anesthetized animals. This new notion of 'cell–tissue' electrofusion extends considerably the recently developed biotechnology of electrofusing individual cells to one another in vitro [1–11]. Therefore, the present study was initiated to transfer functional human membrane gonococcal receptors from cultured cells directly to rabbit superficial corneal epithelium. New cell–tissue electrofusion methods were devised to achieve this first step toward developing a clinically relevant animal model for gonorrhea and other host-specific infectious diseases.

Results and Discussion

Initial studies were performed in vitro on the stage of an inverted microscope with human U937 lymphoma cells and excised rabbit corneas as depicted in Fig. 1. In a typical experiment, an excised cornea was positioned anterior side up over the grid electrode in the well slide. A low ionic strength solution was added to the well to cover the corneal surface. A U937 cell suspension prepared in this solution was also deposited into the well and, by capillary action, into the small volume located at the tip of the disc electrode holder. With the micromanipulator, the cell suspension was positioned over the central corneal area and lowered until the insulating ring at the holder tip made contact with the tissue or with the cell suspension in the well. When the Zimmermann a.c./d.c. power supply was used, an a.c. dielectrophoretic field was produced that forced the juxtaposition of dipole induced human cells closely to the rabbit corneal epithelium. After cell–tissue alignment was observed microscopically, d.c. pulses were delivered to electrofuse the human cells directly to the rabbit cells in the intact corneal epithelium. In experiments where the BTX Transfector 800 was used, the human cells were

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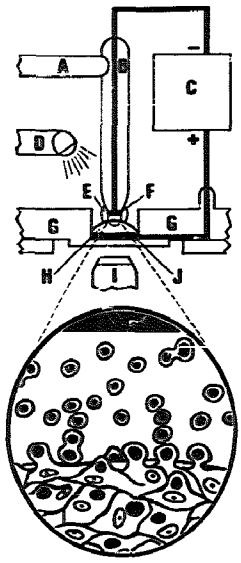

Fig. 1. Diagram of instrumentation, custom built equipment, and biological materials for observing and performing the process of cell–tissue electrofusion in vitro. In the upper portion of the figure, a micromanipulator (A) secured and positioned the disc electrode holder (B) whose disc electrode (F) was connected electrically to either a d.c. pulse generator or to an a.c./d.c. power supply (C). A fiber optic light source (D) provided oblique illumination onto the microscope stage. A translucent molded epoxy insulating ring (E) surrounded the bottom tip of the holder where the recessed platinum disc electrode (F) was housed. This created a small volume below the electrode which effectively retained a suspension of individual cells by capillary action. A transparent well slide (G) was outfitted with a removable platinum grid electrode (J) that was electrically bonded to a post cemented to the slide. A conductive lead was connected from the post to the power supply. An excised rabbit cornea (H) was placed on the grid electrode located on the bottom of the well. Due to the oblique illumination, the superficial epithelium on the transparent rabbit cornea resembled a "cobblestone street" in appearance when viewed through a long focal microscope objective (I). The lower portion of the figure diagrammatically illustrates the space between the electrode and the cornea where electrofusion of individual human lymphoma cells (with dark nuclei) occurs directly to rabbit epithelial cells (with light nuclei) to form human–rabbit somatic cell hybrids within the intact superficial corneal tissue.

allowed to settle onto the tissue surface by gravity in the absence of an a.c. dielectrophoretic field. The BTX pulse generator was then activated to deliver d.c. pulses which fused the human cells to the rabbit corneal epithelium. Fig. 2 illustrates representative scanning electron microscopic (SEM) evidence that cell–tissue electrofusion was achieved in vitro with both instruments. These photomicrographs also illustrate some of the various stages in the formation of somatic human–rabbit cell hybrids within the corneal epithelium.

Cell–tissue electrofusion experiments were performed next in situ with killed rabbits. With the Zimmermann instrument, we observed initially that Joule heating caused some corneal tissue damage when AC dielectrophoretic fields were generated in situ for more than 20 seconds. In order to avoid this problem, we explored whether mechanical pressure could substitute for a.c. dielectrophoresis to juxtapose human cells close enough to the rabbit epithelium for d.c.-induced fusion to occur. We found that the simultaneous administration of mechanical pressure and d.c. pulses was sufficient to electrofuse human and nonhuman cells directly to intact rabbit corneal epithelium under in situ or in vitro conditions where very minimal or no a.c. fields were applied. A.c. fields alone did not produce cell–tissue fusion in the absence of d.c. pulses. Furthermore, fusions between adjacent superficial corneal epithelial cells were not observed to occur, even if these rabbit cells were fused to human cells.

We then examined whether human gonococcal receptors remain functional in the histologically modified corneal epithelium after being incorporated by cell–tissue electrofusion. After electrofusing human HL60 or U937 lymphoma cells directly to the rabbit tissue in situ, the corneas were excised and employed in qualitative gonococcal adherence assays. Fig. 3 illustrates the attachment of *N. gonorrhoeae* Pgh 3-2 to corneal surfaces indicating that the incorporated human receptors were not destroyed by the electrofusion process. In contrast to the early fusion stages (Fig. 2), Fig. 3 also illustrates that human and rabbit plasma membranes appeared to be completely coalesced due to the extended incubation times required to perform the adherence assays.

Specificity studies were conducted to determine whether Pgh 3-2 organisms attach exclusively to human gonococcal receptors transferred by electrofusion in situ. It is conceivable that these microorganisms could adhere nonspecifically to corneas modified by fused cells derived from any animal species or by the electrofusion process itself. Control experiments that utilized gonococcal adherence assays with excised normal unmanipulated corneas (i.e., not exposed to either animal cells or d.c. fusion pulses) showed that Pgh 3-2 bacteria attached only to human donor corneas and not to corneas obtained from rabbits, sheep and baboons. When d.c. fusion pulses were administered to rabbit corneal epithelium in situ in the absence of individual animal cells, Pgh 3-2 organisms failed to attach to the tissue during the assays. These bacteria also failed to adhere to rabbit corneal epithelium histologically modified by electrofused rabbit, monkey or murine cells. In contrast, rabbit corneal epithelium to which gonococcal receptor-containing human cells had been electrofused did bind the pathogen specifically.

In order to determine whether our newly developed electromechanical process is either lethal or causes oc-

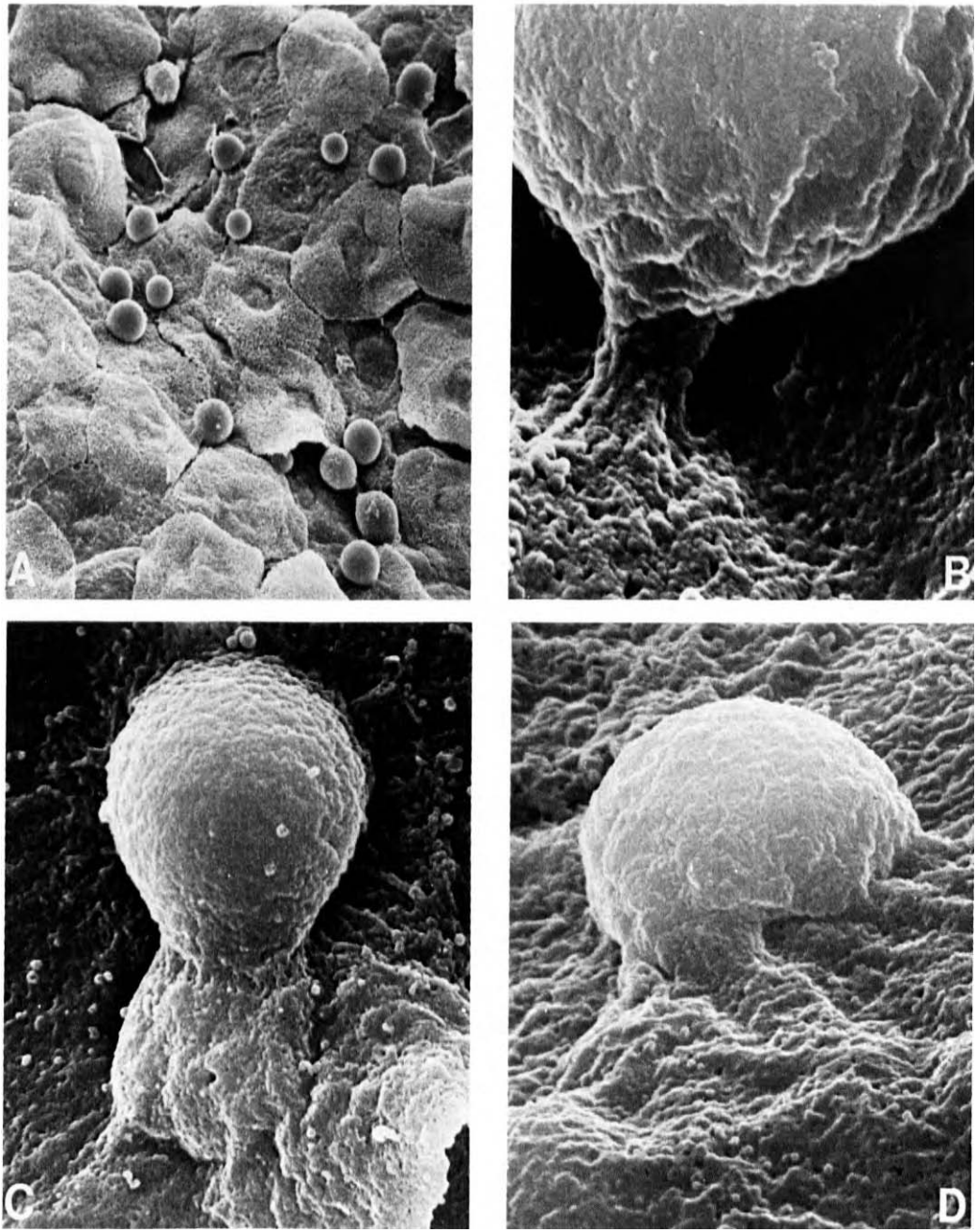

Fig. 2. Electrofusion of human U937 lymphoma cells directly to superficial rabbit corneal epithelium. During the initial 10 min after applying the d.c. fusion pulses, the histologically modified corneas were removed from the well slide, washed free of unfused cells in PBS, and prepared for scanning electron microscopy. Panel A: Corneal surface (1300×) showing a representative distribution of 14 smooth spherically shaped human cells electrofused to the superficial layer of rabbit epithelium. Panel B: Plasma membrane 'stalk' that forms very shortly after electrofusion occurred between the upper smoother human cell and the lower microvilli covered rabbit cell surface (15000×). Panels C (6000×) and D (8600×) illustrate coalescing human–rabbit plasma membranes of somatic cell hybrids beyond the initial stages of their formation in the intact corneal epithelium.

Fig. 3. Membrane receptor mediated attachment of *N. gonorrhoeae* Pgh 3-2 to somatic human–rabbit cell hybrids formed within rabbit corneal epithelium by electrofusion in situ. After electrofusing human HL60 cells to corneal epithelial tissue, the corneas were excised, employed in gonococcal adherence assays and examined by scanning electron microscopy (2000×).

ular inflammation, eyes of rabbits anesthetized with nitrous oxide were subjected to the same fusion conditions employed in situ, i.e., mechanical pressure plus delivering three d.c. pulses in the absence and presence of individual animal cells. After observing the minimal muscle reflex responses that accompanied each electrical stimulus, all rabbits recovered from the anesthesia thus surviving the electrofusion process. Furthermore, no ocular inflammatory responses developed, either on the day the experiments were performed or for several days thereafter.

Scanning electron microscopic evidence presented in this report lends credence to our theoretical approach for developing a bioengineered animal model for gonorrhea. Functional human gonococcal receptors, capable of binding *N. gonorrhoeae* Pgh 3-2, survive a nonlethal, noninflammatory electromechanical process that produces the formation of somatic human–rabbit cell hybrids within superficial epithelial tissue of the laboratory animals. Thus, the model becomes a distinct possibility should the incorporated human receptors remain functional over the one week period before immunological rejection occurs.

One significant finding revealed that tissue damage due to heat produced by a.c. dielectrophoresis can now be eliminated completely by employing mechanical pressure to achieve close cell–tissue juxtaposition prior to electrofusion. With this information, cell–tissue electrofusion biotechniques can now be developed further for many potential in vivo applications. Future research goals could include: (a) establishing bioengineered animal models for other microbial receptor-mediated processes, such as HIV infectivity; (b) developing novel site-specific drug or cloned gene delivery systems by liposome–tissue electrofusion techniques; and (c) utilizing new tissue–tissue electrofusion methods for certain surgical procedures. Thus, future applications of cell–tissue electrofusion biotechnology should contribute significantly to extending our present concepts in a variety of diverse bioscience disciplines.

Materials and Methods

Instrumentation. Instruments employed in this study included an Olympus Model CK inverted tissue culture microscope with its overhead light source removed, a Fiber-Lite illuminator (Dolan-Jenner Ind., Woburn, MA), a micromanipulator (Brinkmann Inst., Westbury, NY), and either a BTX Transfector 800 d.c. pulse generator (Biotechnologies and Experimental Research, Inc., San Diego, CA) or a Zimmermann Cell Fusion System a.c./d.c. power supply (Amsco, Erie, PA). Both electronic instruments were used without the electrodes, chamber devices, and other accessories that are normally utilized for performing electroporation or cell–cell electrofusion in vitro.

Custom built equipment. These materials for the in vitro experiments included the plexiglas well slide with its attached coverslip and metal post, a 3 mm diameter platinum disc electrode in its insulated holder, the epoxy insulating ring secured to the holder tip, and the removable grid electrode designed to fit into the hole of the well slide over the coverslip. The grid electrode consisted of six 24 gauge platinum wires flatly spaced 2 to 3 mm apart in a regular parallel and perpendicular planar array over a circular area 10 mm in diameter. Custom designed leads were also constructed to connect the disc and grid electrodes to the power sources. A custom built eye shaped electrode was employed for the in situ experiments as described below under electrofusion procedures.

Animal cell lines. For the initial in vitro experiments and subsequent in situ studies, the following cell lines were used: human U937 histiocytic lymphoma cells (ATCC CRL 1593; American Type Culture Collection, Rockville, MD); human HL60 promyelocytic leukemia cells (ATCC CCL 240); murine WEHI-3 myelocytic leukemia cells (ATCC TIB 68); Vero monkey kidney

cells (ATCC CCL 81); and, rabbit skin cells [12]. The nonadherent U937, HL60 and WEHI-3 cells were grown in suspension cultures at 37°C in Costar plastic tissue culture flasks containing DMEM (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (Flow Laboratories, McLean, VA) in an atmosphere of 95% air and 5% CO_2. The adherent monkey and rabbit cells were grown as monolayer cultures at 37°C in Eagle's MEM (GIBCO) supplemented with 5% newborn bovine calf serum (Flow). The adherent cells were detached from the plastic flasks by the action of 0.25% trypsin (GIBCO). Prior to electrofusion, cell suspensions were washed twice by centrifugation (200 × *g*, 4° C, 10 min) in either a low ionic strength solution (0.3 M D-mannitol or 0.3 D-glucose each containing 6 mM L-histidine, 0.5 mM $MgCl_2$ and 0.5 mM $CaCl_2$) for the in vitro experiments or a high ionic strength phosphate-buffered saline solution (PBS; pH 7.4; 150 mM NaCl; 3.4 mM KCl; 10.1 mM Na_2HPO_4; and 1.8 mM KH_2PO_4) for the in situ experiments. For in vitro experiments, the final washed sedimented pellets were resuspended in the low ionic strength solutions at cell densities that ranged from 10^7 to 10^8/ml.

Animal corneal tissue. In most experiments, corneal tissue was obtained from adult New Zealand white rabbits (3 to 4 kg) that where housed, fed, watered and handled in compliance with NIH regulations [13]. Rabbits were killed by i.v. administration of pentobarbital overdoses. Animal corneas were excised by standard procedures employed routinely for collecting corneas from human donors. Corneas excised from rabbits, sheep and baboons were used immediately after collection or placed at 8°C in modified M-K corneal storage medium [14] lacking serum, gentamycin and phenol red (Aurora Biologicals, Buffalo, NY) for up to 3 days prior to use. Human donor corneas obtained from tissue banks were likewise maintained in this modified M-K medium after collection. Prior to use, 10 mm diameter corneal buttons were punched out of the tissue with a sharpened cork borer on a Teflon block.

Cell–tissue electrofusion procedures. Prior to applying d.c. fusion pulses with the Zimmermann instrument, the a.c. dielectrophoresis cell–tissue alignment parameters employed in vitro and in situ to juxtapose human cells to rabbit corneal epithelial tissue varied from experiment to experiment. Parameters ranged from a.c. amplitude voltages of < 0.1 V to 20 V at frequencies of 1 to 2 MHz with the instrument duty cycle set between 10% and 20% for < 1 s or for up to 30 s. Low ionic strength solutions were used in vitro to retard the rate of Joule heating that accompanied a.c. dielectrophoresis. The d.c. electrofusion parameters employed with both the BTX and the Zimmermann instruments were kept constant throughout this study. Parameters consisted of delivering three separate d.c. pulses, each 20 V in amplitude and 20 μs in duration at 1 Hz.

For the in situ experiments, human and nonhuman cultured cells were washed in PBS and collected by centrifugation onto Millipore filters (Type HAWP, diameter = 8 mm; New Bedford, MA) positioned at the bottom of the tubes. After discarding the supernatants, the filters containing about 10^7 cells were removed with forceps and placed cell side down on the PBS-washed corneal surfaces of freshly killed rabbits. A custom built titanium electrode, housed in an insulated handle, was held manually against the upper cell free side of the filters. This concave eye shaped electrode was machined to reflect the radius of curvature of the rabbit corneal surface. The other electrode was attached to the buccal mucosa of the dead animals. Leads from the a.c./d.c. power supply were connected to both electrodes; impedences measured about 3 kiloohms between the electrodes. After applying 600 to 700 g/cm^2 of mechanical pressure to the filters with the hand held electrode, a < 0.1 V a.c. dielectrophoretic field was induced for < 1 s before delivering the d.c. pulses. Due to the electronics of the Zimmermann instrument, this very weak and very brief a.c. electrical field was required in order to activate the circuitry for delivering the d.c. fusion pulses. After the Millipore filters were removed, the corneas were excised, and the tissues were washed free of unfused cells with PBS and prepared for scanning electron microscopy (SEM).

Scanning electron microscopy methods. Tissues were fixed overnight at 4°C in 2.5% phosphate buffered glutaraldehyde (pH 7.2). After soaking the corneas in buffer without the fixative for 30 min, the tissues were dehydrated at 4°C through a graded series of ethanol. The dehydrated corneas were rinsed twice for 5 min each in 100% hexamethyldisilazane (Electron Microscopy Sciences, Inc., Ft. Washington, PA). The samples were dried at ambient temperature in a desiccator under vacuum. The dried tissues were mounted on sample holders and coated with 150–200 Å gold-palladium with a Denton Desk-1 cold ion sputter coater. The prepared corneas were examined and photographed with a JEOL JSM-35 scanning electron microscope.

Qualitative gonococcal adherence assays. These assays were performed with excised human and nonhuman corneas together with piliated *N. gonorrhoeae* Pgh 3-2. The excised corneas were placed anterior side up in Nunc plastic tissue culture wells (diameter = 16 mm) and covered with modified M-K medium. These tissues were incubated in the absence of gonococci at 35°C for 30 min before initiating the assays. For these experiments, the bacteria were grown at 35°C on GC medium base agar plates supplemented with 1% Isovitalex (DIFCO, Detroit, MI) in an atmosphere of 95% air plus 5% CO_2. Gonococcal cell suspensions (about 10^8 colony forming units (cfu)/ml) were prepared by flooding plates containing fresh overnight stable type 2 colonies with modified M-K medium. In order to initiate the assays,

bacterial suspensions were added to the wells containing excised corneas. Control corneas received sterile medium that had been flooded onto sterile plates. After allowing the microorganisms to incubate with the tissue for 30 min at 35°C, the corneas were removed from the wells, washed free of unattached and nonreceptor bound tissue associated bacteria, and prepared for scanning electron microscopy. Direct observations revealed that control corneal surfaces exposed to the sterile medium were completely devoid of all microorganisms, including normal flora. Scanning electron microscopy illustrated clumps of piliated Pgh 3-2 organisms bound to the corneal epithelium only if human gonococcal receptors were present. Thus, our qualitative assays clearly distinguished whether or not gonococci attached to corneal epithelial tissues in an 'all-or-none' fashion.

These assay conditions were established empirically by performing the following experiments: Time-course studies utilizing cfu measurements indicating that Pgh 3-2 organisms remained 100% viable for 3 h in modified M-K medium or in brain-heart-infusion broth which served as the control. With the aid of scanning electron microscopy, a wash procedure (i.e., vortexing corneas vigorously in PBS for 30 s) was developed to completely remove all nonreceptor associated Pgh 3-2 bacteria that had settled over a 1 h incubation period onto epithelial surfaces of normal corneas excised from rabbits, sheep and baboons. In contrast, scanning electron microscopy revealed many randomly distributed clumps that contained Pgh- 3-2 organisms that remained firmly attached to the epithelial surfaces of human donor corneas that were incubated and washed in the same manner. It was therefore assumed that these microorganisms adhered specifically to functional gonococcal attachment receptors located exclusively on human cell membranes. Separate time-course studies utilizing quantitative scanning electron microscopy techniques determined that maximum numbers of Pgh 3-2 organisms had adhered to human corneal epithelial surfaces between 15 and 30 min after exposing the tissue to the bacteria.

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