A STUDY OF COMMUNICATION SPECIFICITY BETWEEN CELLS IN CULTURE

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ABSTRACT

We have examined the specificity of communication between cells in culture by co-culturing cells derived from mammalian, avian, and arthropod organisms. Both mammalian and avian culture cells have similar gap junctional phenotypes, while the insect (arthropod) cell lines have a significantly different gap junctional structure. Electrophysiological and ultrastructural methods were used to examine ionic coupling and junctional interactions between homologous and heterologous cell types. In homologous cell systems, gap junctions and ionic coupling are present at a high incidence. Also, heterologous vertebrate cells in co-culture can communicate readily. By contrast, practically no coupling (0-8%) is detectable between heterologous insect cell lines (Homopteran or Lepidopteran) and vertebrate cells (mammalian myocardial or 3T3 cells). No gap junctions have been observed between arthropod and vertebrate cell types, even though the heterologous cells may be separated by less than 10 nm. In additional studies, a low incidence of coupling was found between heterologous insect cell lines derived from different arthropod orders. However, extensive coupling was detected between insect cell lines that are derived from the same order (Homoptera). These observations suggest that there is little or no apparent specificity for communication between vertebrate cells in culture that express the same gap junctional phenotype, while there is a definite communication specificity that exists between arthropod cells in culture.

KEY WORDS: cell communication, communication specificity, gap junctions, arthropod cell cultures, vertebrate cell cultures, ionic coupling

Gap junctional communication is present between cells in most solid tissues (1, 9, 10, 22). However, the communication in vivo is obviously restricted by natural cell contact barriers. For example, intestinal epithelial cells are predictably not communicating with myocardial cells. From limited studies of vertebrate cells in culture, it has not been possible to observe any histiotypic or species specificities for cell communication (9, 27, 32). In fact, under appropriate culture conditions, most communication-competent cells will communicate with other communication-competent cells, regardless of tissue or animal origin.

In previous reports of communication between heterologous cell types in culture (9, 18, 27), the cells were derived from vertebrate organisms that have basically similar gap junctional structures. Thus, a similar gap junctional communication pathway for both cell types may be an essential requirement for communication competence in these studies. Practically all animal organisms,
with the notable exception of arthropods (4, 8, 10, 20, 29, 35) have similar gap junctional structures by ultrastructural analysis (10, 26). Therefore, it seems reasonable to predict that if certain communication specificities exist between cells in culture they may simply result from the presence of gap junctional incompatibilities.

In the present study, we have co-cultured a number of heterologous cell culture populations, both primaries and established cell lines, in order to examine the relationship between gap junctional phenotypes and communication specificity. A preliminary report from this study was presented previously (6).

MATERIALS AND METHODS

Cell Cultures
The cell types used in these experiments are listed in Table I, together with their origin, typical morphology, and normal culture media requirements.

INSECT CELL LINES
The insect cells were grown on 25 cm² plastic Falcon T flasks (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) or 60-mm culture dishes at either 30°C or room temperature in TC 199 MK except where noted. TC 199 MK consists of equal parts of TC 199 (Grand Island Biological Co., Grand Island, N. Y. [Gibco]) and Melnick's Monkey medium A, which was made in the laboratory without HCO₃ and adjusted to pH 7.0 with NaOH. The media was supplemented with 10% fetal calf serum (Gibco), penicillin (100 IU/ml) and streptomycin (50 μg/ml). Confluent cells were removed from the culture vessels for transfer and co-culture experiments by pipetting freshly added medium against the bottom of the dish or flask. The dissociated cells were then placed in 35-mm dishes at appropriate densities for the various studies. The insect cell lines used in this study were generously provided by Dr. Arthur McIntosh (Waksman Institute of Microbiology, Rutgers University, New Brunswick, N. J.).

HOMOPTERA: The AC, MF, and DM cell lines were dissociated either by pipetting or treatment with 0.25% trypsin (Difco Laboratories, Detroit, Michigan) as described previously (25).

LEPIDOPTERA: The TN cells were transferred twice weekly when propagated at 30°C or once a week when maintained at room temperature.

DIPTERA: The ATC-10 cell line was grown in Mitsuhashi and Maramorosch media supplied by Dr. McIntosh. This medium is referred to as M and M in Table I. In addition, these cells could be maintained without growth in TC 199 MK medium.

MAMMALIAN AND AVIAN CELL CULTURES
Mouse 3T3 cells (NIH) and chick embryo fibroblasts (CEF) in first or second passage (34), were kindly provided by Dr. Daniel Rifkin (The Rockefeller University, New York). The 3T3 and CEF cells were grown on 60-mm Falcon dishes at 37°C in a 5% CO₂ atmosphere. The 3T3 cells were cultured in Dulbecco's MEM and the CEF cells in Eagle's MEM (with pyruvate). In both cases, 10% fetal calf serum and penicillin (100 IU/ml)-streptomycin (50 μg/ml) were added to the medium. Confluent cultures were dissociated in 0.25% trypsin (Difco).

| Phylum    | Cell type               | Origin                  | Media         | Reference |
|-----------|-------------------------|-------------------------|---------------|-----------|
| Arthropoda|                         |                         |               |           |
| Insecta   |                         |                         |               |           |
| Homoptera | AC 20 (Agallia constricta) | Minced leafhopper embryo | 199 MK        | 3         |
|           | DMIIB (Dalbulus maidus)  | Minced corn leafhopper embryo | 199 MK        | 24        |
| Homoptera | MF (Macrosteles fascifrons) | Minced leafhopper embryo | 199 MK        | 25        |
| Lepidoptera| TN 368 (Trichoplusia ni) | Minced adult ovaries of cabbage looper | 199 MK | 17        |
| Diptera   | ATC-10 (Aedes aegypti)  | Homogenized mosquito embryos | M + M     | 28, 36    |
| Chordata  |                         |                         |               |           |
| Aves      | CEF (chick embryo fibroblasts) | Minced chick embryos | Eagles's MEM and pyruvate | 34        |
| Mammalia  | 3T3 (mouse fibroblasts)  | Minced mouse embryo (NIH) | Dulbecco's MEM | 19        |
| Mammalia  | MC (myocardial cells)   | Minced neonatal mouse ventricles | Eagle's MEM | This paper |
Primary cultures of myocardial cells from neonatal mouse ventricles were prepared by a modification of the techniques of Blondel et al., (2) and Goshima (14). After removal of the hearts from approx. 30 newborn mice, the ventricles were dissected free and minced into small tissue fragments. The tissue fragments were placed in 10 ml of Ca, Mg-free Dulbecco's phosphate-buffered saline (PBS) (CMF PBS) and incubated in a 37°C shaker bath at 100 strokes/min. After 10 min incubation with shaking, the supernate was discarded. Then the tissue was incubated for 15 min in 10 ml of 0.06% trypsin (Difco) in CMF PBS. After the incubation, the tissue was triturated 12 times with a Pasteur pipette, allowed to settle, and the supernate discarded. The first cell harvest was obtained by adding 10 ml of 0.06% trypsin, shaking for 20 min, triturating 12 times and removing the supernate. The supernate was subsequently filtered through 50-μm Nitex nylon mesh (Tetko, Inc., Elmsford, N. Y.). The filtrate was collected and centrifuged, and the resulting pellet was washed twice in Eagle's MEM containing 10% calf serum (Gibco), 100 IU/ml penicillin and 100 μg/ml streptomycin. The cells were resuspended in 1-2 ml of culture medium and placed at 37°C until they were pooled with successive harvests. The harvesting procedure was repeated twice with the remaining fragments in the flask, and the cells were combined, counted, and plated on 35-mm Falcon dishes at 1.5 × 10^6 cells/dish. After 1 h incubation at 37°C the supernatant was removed from each dish and added to a new 35-mm dish. This differential attachment procedure effectively enriched the myocardial cells by reducing the contaminating endothelial and fibroblastic cells (33). The myocardial cells were used for coculture experiments after a 24-h incubation.

Carmine Labeling Procedure for Co-Cultures

Carmine ingestion was used to label cells in the cocultures for identifying cell types with both light microscopy and electron microscopy (13, 37).

Insect Cells

The AC, MF, DM, and TN cells were labeled with carmine for coculture studies. A stock solution of 20% carmine (Fisher Scientific Co., Chicago, Ill., or that provided courtesy of Michael Stoker [ICRF] Imperial Cancer Research Fund, London, England) was made in distilled water and autoclaved. The carmine was then diluted with TC 199 MK to make a 0.01% to 0.05% solution and centrifuged at 50 g for 3 min. The supernate was removed and centrifuged again. The final supernate containing only the smallest carmine particles was used for labeling. Cells were incubated in the medium containing carmine for 12-24 h at 30°C, dissociated by pipetting, washed twice with TC 199 MK, and were then examined for the extent of dissociation, incidence of labeling, and for the amount of background particles in the medium.

Vertebrate Cells

Solutions of 0.1-0.5% carmine in Dulbecco's MEM were added to dishes of nonconfluent 3T3 cells and incubated at 37°C. 7-24 h later, cells were dissociated, counted, and used for cocultures.

Co-Culture Procedure

Interphyla Co-Cultures

AC-MC: Primary cultures of myocardial cells were incubated for 24 h at 37°C after plating in Eagle's MEM. Carmine-labeled AC cells were then added to the myocardial cell cultures in TC 199 MK medium and incubated for 6-43 h at 30°C; electrical recording was carried out over the next 0-12 h at 30°C or 37°C (see Table III). In another experiment the co-cultures were maintained for 12 h at 37°C for electrical recording during the next 0-12 h at 30°C (Table III).

AC-3T3: AC cells were plated on 35-mm dishes for 2-3 days at 30°C. 3T3 cells were labeled with carmine and added to dishes with AC cells in TC 199 MK. The co-cultures were initially incubated at 37°C in the absence of CO₂ for 14 h, then placed at 30°C, and examined for ionic coupling over the next 12 h period at room temperature with ambient CO₂ levels. In other experiments, after addition of the labeled 3T3 cells, the co-cultures were maintained at 30°C for 12 h, and electrical recording was done over the next 12-h period as described above.

TN-3T3: 3T3 cells were labeled with carmine, dissociated, and mixed with dissociated TN cells. The co-cultures were incubated for 12 h at 30°C in TC 199 MK medium, and electrical coupling was examined over the next 12 h at room temperature.

3T3-CEF: Carmine-labeled 3T3 cells were added to dishes of CEF cells in Eagle's MEM with pyruvate. After 12-h incubation at 37°C, coupling was examined during the next 12-h period at room temperature under ambient CO₂ conditions.

Control Cultures: The AC, MF, DM, and TN cells were plated individually in TC 199 MK, maintained at 30°C, and examined for electrical coupling. The mouse 3T3 cells were dissociated, plated in TC 199 MK, incubated at 37°C for 12 h in the absence of CO₂, and then placed at 30°C for a 12-h period during which electrical recordings were carried out at room temperature. The mouse myocardial cell cultures were placed in TC 199 MK and subjected to the same procedure as the 3T3 cells above, except that electrical properties were examined at 30°C.

Intraphylum-Interorder Co-Cultures

AC-TN: The TN cells were labeled with carmine, dissociated, and added to cultures of previously plated...
AC cells in TC 199 MK. The co-cultures were incubated for 6-24 h at 30°C, and electrical coupling was examined during that time at room temperature.

AC-ATC: ATC and TN cells were dissociated, mixed together, and incubated in 33% TC 199 MK and 66% M and M media for 12 h at 30°C; recording was carried out for the next 12 h at room temperature.

AC-DM: AC cells were labeled with carmine, dissociated, and then added to previously plated DM cells in TC 199 MK. These co-cultures were incubated for 12 h at 30°C and electrical recording was carried out over the next 12 h at room temperature.

INTRAPHYLUM-INTRAORDER

Co-Cultures

AC-MF: Two procedures were used for these co-cultures. Either MF cells were labeled with carmine and added to dishes of AC cells, or carmine-labeled AC cells were added to cultures of MF cells. In both cases the co-cultures in TC 199 MK were incubated at 30°C for 12 h followed by electrical recording for the next 24 h at room temperature.

AC-DM: AC cells were labeled with carmine, dissociated, and then added to previously plated DM cells in TC 199 MK. These co-cultures were incubated for 12 h at 30°C and electrical recording was carried out over the next 12-h period.

Electrophysiology

Culture dishes (35 mm) containing cells were mounted in an aluminum heating block mounted on an inverted microscope (Leitz Diavert). Microelectrodes, made from fiber-filled glass tubing or omega dot tubing (Hilgenberg Glass, Germany), were filled with 2.8 M KCl. The resistances of the microelectrodes varied from 40-100 MΩ. These microelectrodes were bent on a nichrome wire to approx. 45° angles and were connected through Ag/AgCl wires to the input of capacity-compensated DC amplifiers (The Rockefeller University). One microelectrode was used to record the membrane potential, and the other was used for injecting pulses of current and for recording through a bridge circuit. The bridge circuit was driven by Tektronix pulse generators (Philbrick-Bath & Spencer, Inc., Hartford, Conn.) used to monitor current. Membrane potentials and current intensity were displayed on a Tektronix 5111 storage oscilloscope and photographed with a kymograph camera. The image of the cells to be penetrated was projected through the microscope and photographed on 35-mm film. Coupling measurements were analyzed only when a minimum resting potential of −10 mV was recorded from the interacting cells.

Electron Microscopy

Thin-Secti ons of Cell Cultures

All cell cultures were fixed and processed for thin-section electron microscopy on 35-mm culture dishes. The cells were fixed initially by removing the culture medium and immediately adding 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) for 12-15 min at room temperature. All subsequent processing took place at room temperature. After buffer rinses with 0.1 M sodium cacodylate and subsequently veronal-acetate, the cells were fixed for 1 h in veronal-acetate buffered 1% osmium tetroxide. After a veronal-acetate buffer rinse, the cells were treated for 1 h with 3.5% uranyl acetate in veronal-acetate buffer. The cells were rapidly dehydrated in a series of ethanol (70%, 95%, and 100%), impregnated in 100% ethanol:Epon 812 (1:1) overnight, treated with three changes of Epon 812 (30 min each), and placed into a 60°C oven overnight. The embedded cell monolayers were removed from the plastic Petri dishes, and appropriate regions were selected with phase microscopy for re-embedding on polymerized Epon capsules. Thin sections were cut with diamond knives on a Porter-Blum MT-2B ultramicrotome (Sorvall Operations, DuPont Instruments, Newtown, Conn.). The sections were placed on formvar coated 200-400 mesh grids and post-stained with uranyl acetate and lead citrate. All observations were made with a Philips 300 electron microscope at 80 kV.

Freeze-Fracture of Cell Cultures

Cell populations were fixed on 35-mm culture dishes with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 10-15 min at room temperature. After a buffer rinse, the cells were removed from the dishes by scraping with a cover slip or a rubber policeman. The cells were then treated for 2-4 h at room temperature with 25% glycerol in 0.1 M sodium cacodylate buffer before rapid freezing as cell pellets in Freon 22 and storage in liquid nitrogen. The samples were freeze-fractured and replicas made in a Balzers BM 360 apparatus (Balzers AG, Balzers, Liechtenstein) at −115°C. The carbon-platinum replicas were cleaned in bleach and distilled water before mounting on 300-400 mesh grids. All electron microscope observations were made with a Philips 300. The freeze-fracture images were mounted so that the shadow direction is from the bottom to the top of the micrographs.
Solid Tissues

Samples from the guinea pig pancreas, rat liver, and crayfish hepatopancreas were processed for both thin-section and freeze-fracture electron microscopy as small blocks of tissue that were initially fixed in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer. All of the subsequent procedures, with the omission of the monolayer re-embedding, were similar to the previous procedures for cell cultures.

RESULTS

Gap Junctional Phenotypes between Vertebrate Cells and between Arthropod Cells

The gap junctional phenotype that is expressed by vertebrate cells is strikingly different from the structural phenotype between arthropod cells. In thin-section electron microscopy, the two phenotypes look very similar (Fig. 1A and B). The gap junctions are composed of two adjacent plasma membranes that are separated by a 2-4 nm extracellular space or "gap." In thin sections, the vertebrate gap junction is about 16-19 nm wide, while the arthropod junction is slightly increased in width. In freeze-fracture electron microscopy, the differences in the two phenotypes are much more apparent (Figs. 1C and D). The vertebrate gap junction is characterized by a polygonal arrangement of homogeneous intramembrane particles, 8-9 nm in size, that are preferentially associated with the inner membrane half (P-fracture face) (Fig. 1C); a complementary arrangement of pits or depressions is located on the outer membrane half (E-fracture face). In contrast, the arthropod gap junction contains a plaquelike arrangement of heterogeneous intramembrane particles, heterogeneous in size (9-12 nm), variable in packing, i.e., preferentially associated with the outer membrane half (E-fracture face) (Fig. 1D); a complementary arrangement of pits or depressions is found on the inner membrane half (P-fracture face). In the arthropod gap junction, some junctional particles are frequently observed on the P-fracture face (Fig. 1D), while the junctional particles are rarely observed on the E-fracture face in the vertebrate structure.

Homologous Cell Cultures

Insect Cells

Electrophysiological measurements were made on adjacent cells within clusters and indicate that electrical coupling is present between a large percentage of the cells in all of the insect cell lines examined. The percentage of cells coupled varied from 84% for the TN cells to 100% for the MF and DM cells (Table II). Values for the transfer resistance, input resistance, and coupling co-efficient for the respective cell types are included in Table II. These values show great variability, probably resulting from damage during electrode penetration. In many cases, the bridge failed to balance after removal of the microelectrode from the cell, thereby invalidating the electrotonic potential (Vt) measurements made in the cell into which current was injected. Since the coupling co-efficient values cannot be accurately determined from such measurements, only transfer resistance values are provided in Table III, IV, and V.

Each of the insect cell lines used in these studies had unique morphological and behavioral characteristics in culture. The AC cell line was extensively used in these studies because these large epithelial cells are easily penetrated by microelectrodes. However, these cells could not be completely dissociated, and they required 12-24 h to flatten out completely after dissociation. Consequently, large clumps of cells were often surrounded by a peripheral region of flattened cells. The MF and DM cell lines are similar to the AC cell line with respect to epithelial morphology and dissociation difficulty, but the MF and DM cells are smaller and not as vacuolated as the AC cells. In contrast, the TN cells (derived from cabbage looper ovaries) showed dramatic temporal changes in morphology on the culture dish. After complete dissociation, the cells quickly attached to the dish as flattened cells with many cytoplasmic processes and a round prominent nucleus. After 24-36 h on the culture dish, the cells round up and extend 1-2 large cytoplasmic processes before detaching. Coupling measurements were usually made within 24 h of plating the TN cells. The Dipteran ATC-10 cells were distinguished by their small size and round morphology.

Mammalian Cells

Ionic coupling was examined in cultures of primary myocardial cells (mouse) and 3T3 cells (mouse). Microelectrode impalements were made on both small clusters and large clumps of cells, with the latter giving the best recording stability. In these cultures, both the myocardial and 3T3 cells were extensively coupled (Table II).
Figure 1  A composite of the gap junctional phenotypes that are expressed by mammalian and arthropod cells. Fig. 1A and C are thin-section and freeze-fracture images of mammalian gap junctions, while Fig. 1B and D are comparable images of arthropod gap junctions. Note that the thin-section features (A) and (B) are very similar, but the freeze-fracture characteristics (C) and (D) are significantly different. The mammalian gap junctional particles are located on the P-fracture face, while the insect gap junctional particles are on the E-fracture face. (A) Junction between rat hepatocytes. × 192,500. (B) Junction between TN cells, an arthropod cell line. × 194,940. (C) Junction between guinea pig exocrine pancreatic cells. × 100,000. (D) Junctions between cells of the crayfish hepatopancreas. × 100,800.
### TABLE II

**Ionic Coupling in Homologous Cultures**

| Cell cultures | No. of trials | Percentage coupled | Transfer resistance | Input resistance | Coupling coefficient |
|---------------|---------------|--------------------|---------------------|------------------|---------------------|
|               |               | %                  | V<sub>g</sub>/V<sub>g</sub> | V<sub>g</sub> |                      |
| AC            | 53            | 98                 | 13.1 ± 3.1 (52)      | 15.3 ± 3.1 (24)  | 0.43 ± 0.04 (25)    |
| MF            | 12            | 100                | 8.0 ± 1.6 (12)       | 16.4 ± 1.2 (5)   | 0.70 ± 0.10 (5)     |
| DM            | 18            | 100                | 2.0 ± 0.5 (17)       | 3.8 ± 0.6 (4)    | 0.46 ± 0.12 (4)     |
| TN            | 37            | 84                 | 9.4 ± 1.6 (31)       | 21.8 ± 4.3 (17)  | 0.44 ± 0.06 (16)    |
| 3T3           | 18            | 94                 | 6.1 ± 1.3 (17)       | 12.9 ± 2.3 (4)   | 0.34 ± 0.11 (4)     |
| MC            | 23            | 96                 | 4.1 ± 0.8 (19)       | 9.7 ± 2.0 (8)    | 0.32 ± 0.04 (7)     |

**Interphyla Co-Cultures**

**AC-MC**

The myocardial cells were used in this study because their contractile behavior (synchronous vs. asynchronous) could be an indicator of coupling between heterologous cell types in the co-cultures (5, 14). However, synchrony was never observed between beating myocardial cells that were connected by insect cells. Instead, clumps of beating myocardial cells were synchronized by myocardial cells that were interspersed with clumps of AC cells. The AC cell processes frequently extended under and over myocardial cell processes. The myocardial and AC cells were distinguished readily by differences in their morphology and contractile behavior; however, the AC cells were labeled with carmine in order to distinguish the AC cells unequivocally from the mammalian fibroblasts. Approx. 50% of the AC cells were dissociated into single cells and 50–75% were labeled with carmine. Dissociated AC cells attached as clumps of cells, some of which were labeled with carmine. After 12 h incubation, only some of the AC cells flattened out on the dish.

In the co-cultures, labeled AC cells and adjacent beating myocardial cells were chosen for electrode penetration. In initial experiments, the contiguous cells were often isolated single cells. In later experiments, small groups of beating myocardial cells adjacent to clumps of carmine-labeled insect cells were selected for penetrations. Fig. 2A is a photomicrograph illustrating an interaction between contiguous myocardial and insect cells. Initially, the adjacent MC and AC cells were penetrated with microelectrodes, and no coupling was observed between the heterologous cells as indicated in the electrophysiological records (Fig. 2B). The microelectrode was then withdrawn from the MC cell and inserted into an adjacent AC cell. Coupling was observed between the interacting AC cells (Fig. 2C) as indicated by the electrotonic potential in response to current injected in the initial AC cell. Microelectrodes were finally inserted in the MC cell originally impaled and into a nearby MC cell, and these homologous cells were coupled (Fig. 2D). This impalement procedure illustrates the approach that was used to sample most of the ionic coupling in the co-cultures that were used in this study.

In order to optimize the conditions (temperature) for the formation of junctions between insect cells (which grow at 30°C) and mammalian cells (which grow at 37°C), the co-cultures were incubated at either 30°C or at both temperatures for varying periods of time. The results from these experiments (Table II) indicate a low frequency of coupling between the heterologous cells under the conditions that were utilized. The possible interpretations for the coupling found between the heterologous cells will be presented in the Discussion section.

**3T3-AC**

The 3T3 cells were prelabeled with carmine before co-culture with AC cells, since the 3T3 cells were easily dissociated into a single cell population. This procedure produced a high percentage of single 3T3 cells that were labeled with carmine. In all experiments, at least 84% of the 3T3 cells were added as single cells and 90% of the cells contained detectable amounts of carmine. The 3T3 cells interacted with the AC cells and the heterologous cell processes frequently overlapped. A typical interaction between contiguous AC and 3T3 cells is shown in Fig. 3A a phase contrast photomicrograph. The carmine-labeled 3T3 cells in the field are readily appreciated with
Figure 2. Co-culture of insect (AC) and mouse myocardial cells (MC). (A) Photomicrograph of co-culture region of insect cells (AC) and myocardial cells (MC) that was used to examine ionic coupling. The electrophysiological records obtained from this region are in (B), (C), and (D). The bar represents 50 μm whenever it is present in this manuscript. (B) AC* - MC*. A microelectrode was inserted into AC* for simultaneous current injection and voltage recording through a bridge circuit, and a second microelectrode was used to impale the MC* for recording. A pulse of current, whose intensity is indicated in the lower trace, was then injected into the AC* cell producing a large (offscale) deflection of the bridge (middle trace). No detectable voltage deflection was observed in the microelectrode recording from the MC cell (upper trace), thus indicating a lack of ionic coupling between the impaled AC and MC cells. The calibration pulse for all the electrophysiological records in this manuscript represents 5 mV and 20 ms, while the vertical line represents \( 5 \times 10^{-9} \) A. (C) AC* - AC. The microelectrode was removed from the myocardial cell (MC*) and inserted into the adjacent AC cell (AC). A pulse of current injected into the AC cell (AC) produced a bridge deflection in that cell (middle trace) and an electrotonic potential (deflection-upper trace) recorded from AC*. The electrotonic potential indicated the presence of ionic coupling between the two homologous cells. (D) MC* - MC. The microelectrodes were removed from the AC cells and inserted into two myocardial cells (MC* and MC). A current pulse (lower trace) was injected into one myocardial cell (MC*), producing a voltage deflection in that cell (middle trace) and an electrotonic potential in the other myocardial cell (MC) (upper trace), indicative of ionic coupling.
bright-field optics (Fig. 3B). Records of microelectrode impalements indicate that no ionic coupling was detected between contiguous AC and 3T3 cells (Fig. 3C), but ionic coupling was present between the homologous adjacent AC and 3T3 cells (Fig. 3D and E). Varying co-culture conditions did not significantly affect the low incidence of heterologous coupling (Table III).

**Figure 3** Co-culture of insect cell line (AC) and mouse cell line (3T3). (A) Phase-contrast photomicrograph of a region with several AC cells (AC) adjacent to two 3T3 cells (3T3). (B) Photomicrograph using bright-field optics of the same region that is present in Fig. 3A. Note the distinctive appearance of the carmine-labeled 3T3 cells. (C) AC* - 3T3*. The microelectrode inserted into the AC cell (AC*) does not record an electrotonic potential (no deflection-upper trace) in response to a large current pulse (intensity shown in lower trace) injected into the 3T3 cell (3T3*) through a second microelectrode. (D) AC* - AC. The microelectrode has been withdrawn from a 3T3 cell (3T3*) and placed into the cell labeled AC. An electrotonic potential indicative of ionic coupling (deflection on upper trace) is observed in AC* in response to a current pulse (lower trace) injected into AC through a bridge circuit that produced a deflection indicated in the middle trace. (E) 3T3* - 3T3. The microelectrodes were placed in the cells indicated 3T3 and 3T3*. An electrotonic potential (upper trace) is observed in 3T3* in response to current injected (lower trace) into 3T3 through a bridge circuit to produce a deflection indicated in the middle trace.
Table III
Ionic Coupling in Interphyla Co-Cultures

| Cell cultures | Culture conditions | No. of trials | Percentage coupled | Transfer resistance |
|---------------|--------------------|---------------|-------------------|---------------------|
| AC-MC         | (a) 6-12 h at 30°C | 15            | 0                 | 0                   |
|               | (b) 43 h at 30°C   | 9             | 11                | 1.3 (1)             |
|               | (c) 6-12 h at 30°C | 8             | 12.5              | 25 (1)              |
|               | (d) 12 h at 37°C   | 5             | 0                 | 0                   |
| MC-MC         | a, b, c, d         | 15            | 80                | 3.0 ± 0.8 (12)      |
| AC-AC         |                    | 29            | 97                | 5.3 ± 1.3 (27)      |
| AC-3T3        | 12 h at 37°C       | 39            | 8                 | 0.1, 0.5 (3)        |
| 3T3-3T3       |                    | 34            | 97                | 8.1 ± 1.5 (33)      |
| AC-AC         |                    | 31            | 100               | 9.9 ± 3.0 (31)      |
| AC-3T3        | 6-12 h at 30°C     | 30            | 3                 | 0.4 (1)             |
| 3T3-3T3       |                    | 24            | 96                | 6.7 ± 1.4 (23)      |
| AC-AC         |                    | 26            | 100               | 8.3 ± 1.5 (26)      |
| TN-3T3        | 12 h at 30°C       | 11            | 0                 | 0                   |
| 3T3-TN        |                    | 12            | 100               | 5.5 ± 1.9 (12)      |
| TN-TN         |                    | 9             | 100               | 1.6 ± 0.5 (9)       |
| CEF-3T3       | 12 h at 37°C       | 11            | 82                | 5.7 ± 1.9 (9)       |

TN-3T3
Dissociated TN and 3T3 cells (labeled with carmine) were cultured together, and no ionic coupling was detected in any of the TN-3T3 interfaces that were examined (Table III).

CEF-3T3
Carmine-labeled 3T3 cells were added to previously plated CEF cells (Fig. 4). The cells from these two different subphylla readily formed low-resistance junctions with each other in culture (Table III).

Intraphylum-Intraorder Co-Cultures

AC-DM
The DM cells contained vacuoles which were larger and more numerous than the vacuoles characteristic of the AC cells. The two cell types were clearly distinguished by adding carmine-labeled AC cells (50% labeled) to previously-plated DM cells. In these co-cultures, areas containing clumps of labeled AC cells adjacent to enriched regions of unlabeled DM cells were chosen for study. In every AC-DM interface that was examined physiologically, ionic coupling was detected (Table V).

AC-MF
The MF cells could be distinguished by their smaller size and absence of vacuoles. To assist in

TN-3T3
Dissociated TN and 3T3 cells (labeled with carmine) were cultured together, and no ionic coupling was detected in any of the TN-3T3 interfaces that were examined (Table III).

CEF-3T3
Carmine-labeled 3T3 cells were added to previously plated CEF cells (Fig. 4). The cells from these two different subphylla readily formed low-resistance junctions with each other in culture (Table III).

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AC-DM
The DM cells contained vacuoles which were larger and more numerous than the vacuoles characteristic of the AC cells. The two cell types were clearly distinguished by adding carmine-labeled AC cells (50% labeled) to previously-plated DM cells. In these co-cultures, areas containing clumps of labeled AC cells adjacent to enriched regions of unlabeled DM cells were chosen for study. In every AC-DM interface that was examined physiologically, ionic coupling was detected (Table V).

AC-MF
The MF cells could be distinguished by their smaller size and absence of vacuoles. To assist in
Co-culture of chick embryo fibroblasts (CEF) and the mouse 3T3 cell line (3T3). Photomicrograph shows a CEF cell interacting with carmine-labeled 3T3 cells. Inset contains electrophysiological records. A pulse of current (shown in lower trace) injected into the CEF cell produced an electrotonic potential that was recorded from 3T3 (upper trace).

Co-culture of cells from different insect orders. (A) AC(Homopteran)-ATC(Dipteran). Photomicrograph of large AC cells surrounded by smaller ATC cells. (B) ATC(Dipteran)-TN(Lepidopteran). Photomicrograph shows a number of small ATC cells adjacent to larger TN cells. (C) TN(Lepidopteran)-MF(Homopteran). Photomicrograph of TN cells interacting with an MF cell. Only a limited incidence of ionic coupling was detected between the heterologous cell types in these co-cultures.
TABLE IV

Ionic Coupling in Interorder Co-Cultures

| Cell cultures | Culture conditions | No. of trials | Percentage coupled | Transfer resistance |
|---------------|--------------------|---------------|--------------------|---------------------|
| AC-TN         | 6-24 h at 30°C     | 17            | 12                 | 0.05, 0.1 (2)       |
| AC-AC         | “                  | 6             | 100                | 1.2 ± 0.6 (6)       |
| TN-TN         | “                  | 5             | 80                 | 2.1 ± 1.0 (4)       |
| MF-TN         | 12 h at 30°C       | 4             | 25                 | 0.13 (1)            |
| 0-12 h at 30°C|                    |               |                    |                     |
| ATC-TN        | 12 h at 30°C       | 5             | 0                  | 0                   |
| 0-12 h at 30°C|                    |               |                    |                     |
| 199 MK, M and M media |             |               |                    |                     |
| AC-ATC        | 12 h at 30°C       | 21            | 5                  |                     |
| 0-12 h at 30°C|                    |               |                    |                     |
| 199 MK, M and M media |             |               |                    |                     |
| ATC-ATC       | “                  | 8             | 88                 | 2.3 ± 0.9 (7)       |

TABLE V

Ionic Coupling in Intraorder Co-Cultures (Homoptera)

| Cell cultures | Culture conditions | No. of trials | Percentage coupled | Transfer resistance |
|---------------|--------------------|---------------|--------------------|---------------------|
| AC-MF         | 12 h at 30°C       | 20            | 100                | 2.0 ± 0.8 (20)      |
| 0-24 h at 30°C|                    |               |                    |                     |
| AC-DM         | 12 h at 30°C       | 17            | 100                | 7.5 ± 1.8 (17)      |
| 0-12 h at 30°C|                    |               |                    |                     |

distinguishing the cell types, the MF cells were labeled with carmine and added to previously-plated AC cells.

Although only 64% of the MF cells were labeled, a number of cells within a clump of unflattened AC cells would be labeled. This allowed one to distinguish the MF cells which settled out upon the more flattened AC cells. With longer incubations, some of the labeled MF cells on the edges of the clumps would flatten out adjacent to large areas of unlabeled AC cells. With these markers, the two cell types could be recognized. As indicated from the electrophysiological recordings, coupling was always observed between the AC and MF cells (Table V).

The results of these two intraorder co-culture experiments indicate that cells from different species within the Homopteran order can form low-resistance junctions readily with each other. Furthermore, these junctions can be formed not only between flattened cells, but also between clumps of round unflattened cells.

Electron Microscopy

Homologous Cell Cultures

Insect cell lines: Two insect cell lines, the AC (Homopteran) and the TN (Lepidopteran) were chosen for electron microscope examination because they were the most extensively utilized in these studies. Thin-section examination revealed that the AC cells were large, epithelial-like, and they expressed two different junctional structures, the gap junction and the septate junction (Fig. 6). Both junctional structures are very numerous in AC cell cultures, and they are frequently present in close proximity to each other. One striking feature of the AC cells is an unusually high content of cytoplasmic microtubules,
and no detectable region of microfilaments near the cell surface. In contrast, the TN cells are more fibroblastic, and contain only the gap junction in culture. The gap junctions can extend for several micrometers in length between TN cells as illustrated in Fig. 9.

In freeze-fracture replicas, the characteristic intramembrane features of the septate and gap junctions are present between the AC cells (Figs. 7 and 8). The septate junction is characterized by a series of parallel rows of intramembrane particles that correspond to the intercellular septa and are located on the inner membrane half (P-fracture face). These particle rows are complemented by a similar arrangement of pits on the outer membrane half (E-fracture face). In culture, the septate junction is not present as a belt or zonular element around the major somatic portion of the cells, but it normally exists as a beltlike element around interacting processes of adjacent cells. The gap junctions between AC cells contain an aggregate of intramembrane particles, heterogeneous in size, that are preferentially associated with the outer membrane half (E-fracture face) (Fig. 8). The intramembrane particle aggregates are complemented by a similar arrangement of pits on the inner membrane half (P-fracture face) (Fig. 7). Identical gap junctional features are also present in replicas of interacting TN cells.

MAMMALIAN AND AVIAN CELL CULTURES: Since the information has previously been published, it does not seem reasonable to
try to illustrate in this study the thin-section and freeze-fracture characteristics of the gap junctions that are present between mouse myocardial cells, mouse 3T3 cells, and chick embryo fibroblasts. The junctional features have been previously described for these cell types (11, 30, 31). In general, all of these cells express gap junctional structures that are similar to the one illustrated in Fig. 1C: the particles are polygonally packed, homogeneous in size, and preferentially associated with the inner membrane half (P-fracture face).

Co-Cultures

Co-cultures of mouse myocardial cells and carmine-labeled insect AC cells were examined with thin-section electron microscopy after ionic coupling measurements were completed. The cell identities were easily determined on the basis of electron-dense carmine in the AC cells and the presence of myofilaments in the myocardial cells (Fig. 10). The heterologous cell types interact extensively in the co-cultures, and the cells are frequently separated by less than 10 nm (Fig. 11). However, no gap junctions were detected between the heterologous cell types even though gap junctions were frequently observed between homologous cell types in the same culture dish. In co-cultures of 3T3-AC, extensive interactions were also observed between the heterologous cells in thin sections, even though no gap junctions were detected. Furthermore, the frequency of gap junctions between homologous cells did not appear to be altered in the co-culture environment.

Discussion

In this study, we have utilized communication-competent cells in culture to examine the specificity of communication between cells from different organisms. In general, the non-arthropod cells expressed a similar gap junctional phenotype, while the arthropod-derived cells expressed a different gap junctional structure. The results from this co-culture study indicate that there is no apparent specificity for communication between cells derived from different vertebrate organisms (mammalian and avian), while there is a strong indication that a specificity for communication exists between non-arthropod and arthropod cells, as well as between arthropod cells from different Orders. The ability of non-arthropod cells to communicate may be explained on the basis of compatible gap junctional structures expressed by these cells, and the inability of non-arthropod and arthropod cells to communicate may be due to the incompatibility of the two different gap junctional phenotypes. However, the apparent lack of communication between arthropod cells from different Orders cannot be readily explained on the basis of a detectable structural incompatibility.

There have been several reports of communication between vertebrate cells in culture that are derived from different tissues and different organisms (9, 15, 27, 32). In fact, one may predict from those reports, as well as from the observations in this study, that communication-competent vertebrate cells will communicate with other vertebrate cells in culture providing they are viable under the same growth conditions and interact (adhere) closely with each other. It is reasonable to suspect that this ability to communicate is related to the gap junctional structure that these cells share.

Communication was rarely observed in heterologous cultures between communication-competent arthropod and vertebrate cells. We believe that this failure to find extensive ionic coupling reflects an inability of the two different junctional structures to form an effective gap junction. The
low incidence of coupling observed is probably related to a number of factors discussed below. In considering this inability to find extensive communication, a number of pertinent explanations should be entertained. (a) The culture conditions (temperature, media, etc.) are not favorable for the coupling observed between heterologous cells. This is not a likely explanation since the incidence of coupling between homologous cells was exceedingly high within the same culture dish where heterologous cells did not communicate readily. In addition, it should be noted that the detectable heterologous coupling appears to be slightly enhanced by incubations at 37°C. (b) The epithelial (insect) cells are unable to communicate with mesodermally derived (vertebrate) cells in co-culture. This possibility cannot be substantiated on the basis of previously published studies (27) which actually have demonstrated that communication does exist between these two different cell types in co-cultures. However, a possible exception has recently been reported (7). (c) The heterologous cells are unable to sufficiently interact or adhere to each other. Cell adhesion which presumably must precede or accompany junction formation was not directly measured here. However, on the basis of both light and electron microscope observations, the heterologous cells appear to interact as closely as the communicating homologous cell types in co-culture. Moreover, when some of the heterologous cells were dislodged with microelectrodes, adhesion between the cells was evident. Therefore, this is not a likely explanation for the general lack of communication observed.

The communication that was detected between heterologous arthropod and vertebrate cells ranged from a high of 8% between AC-3T3 and AC-MC (incubated at 37°C and then at 30°C) to a low of 0% between TN-3T3 (incubated only at 30°C). This background of coupling is probably not related to the incompatible junctional structures but can be related to other possible explanations. (a) This coupling was the result of spontaneous cell fusion that occurred between heterologous cell types. Spontaneous fusion does occur.

Figure 9 Thin-section of a gap junction between insect cells from the TN cell line. The gap junctions between these cells can be very extensive; in this instance, the junction extends without interruption from the top to the bottom of the micrograph. × 100,000.
Co-culture of the insect AC cell line and mouse myocardial cells.

Figure 10 Thin-section of a myocardial cell (MC) interacting with an insect cell (AC). The insect cell can be positively identified by the electron-dense carmine (C) that is present in a large vacuole. Note the close association between the heterologous cell types. × 19,600.

Figure 11 Thin-section image to illustrate the close interaction between the heterologous cell types in the co-culture. In some regions (arrowheads) the cells are separated by less than 10 nm of intercellular space. No typical cell junctions have been identified between these heterologous cell types. The myocardial cell can be easily identified by the cytoplasmic myofilaments (MF) that are present. × 62,500.
at a low incidence in cell cultures (16, 21), and this could explain some cases of coupling where large transfer resistance values were observed. (b) The heterologous cells are able to form a low-resistance pathway at low incidence, but the low-resistance pathway that is generated is not very efficient, since the two junctional structures are practically incompatible. The low transfer resistance values that have been recorded in some of these heterologous interactions could be consistent with this possibility. (c) The coupling observed between heterologous cells is a result of improper cell identification. This possibility must certainly be entertained since the carmine labeling procedure was never 100% successful, and carmine can be released by one cell and ingested by another cell (37). Also in the case of a misidentification, the transfer resistances would be reasonably similar to those present between the homologous cell types. Misidentification is the most likely explanation for the heterologous coupling that was observed between arthropod and vertebrate cells.

In co-cultures of arthropod cells, the incidence of heterologous coupling was very low when the cells were derived from different insect orders (Homopteran, Lepidopteran, and Dipteran). Since the homologous cell types are all communication-competent and form the arthropod gap junctions, it is clear that the apparent failure to communicate cannot be explained simply on the basis of a structural incompatibility. Other elements, such as membrane composition, adhesion properties, and junctionally related antigens may be involved in generating the coupling pathways. In many arthropod cell cultures, such as the AC cell line, the cells may express multiple junctional contacts, i.e., gap junctions, septate junctions, and desmosomes, and perhaps the coexistence of these various contacts may influence the expression of heterologous communication in the interorder co-cultures.

It is interesting to note, on the basis of limited information, that only a few examples of communication specificity have been documented (7, 23, 32); one is the insect (interorder) specificity found in this study, and another has been reported for sponge cells from the Porifera phylum (23). There are a number of obvious possibilities for explaining communication specificity: (a) Specificity is determined by cell surface-related properties, and the differences in these properties may be larger in the lower phyla, such as Porifera and Arthropoda, and smaller in Chordata. (b) The junctional phenotype has a major role in determining specificity. In this regard, the vertebrate gap junctional structure has been found in the Molluscan phylum (12). Therefore, it would be interesting to examine communication between cells from this phylum with cells from vertebrate organisms. One must consider the possibility that small differences in gap junctional structures, not detected with present techniques, may have a role in conferring specificity such as that observed between arthropod orders. (c) Finally, specificity may result from a combination of factors that may include both the gap junctional structure as well as other cell surface-related properties.

We are indebted to Dr. A. H. McIntosh for providing us with the insect cell lines, for advice, and for reading the manuscript. We wish to thank Dr. D. B. Rifkin for providing the cultures of CEF and 3T3 cells, Dr. M. V. L. Bennett and Ted Lawrence for reading the manuscript and Elena Sphicas and Asneth Klosman for technical assistance.

This research was supported by United States Public Health Service grant HL 16507, the Andrew W. Mellon Foundation, and an award from the Irma T. Hirschl Trust. M. L. Epstein was the recipient of a United States Public Health Service Postdoctoral Fellowship (CA 01621). N. B. Gilula is a Research Career Development Awardee of the National Heart, Lung, and Blood Institute (HL 00110) and an Andrew W. Mellon Foundation Fellow.

Received for publication 1 June 1977, and in revised form 5 August 1977.

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