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# Reduction of gap junctional conductance by microinjection of antibodies against the 27-kDa liver gap junction polypeptide

**(electrotonic coupling/cell–cell communication/hepatocytes/myocardial cells/neurons)**

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ABSTRACT **Antibody raised against isolated rat liver gap junctions was microinjected into coupled cells in culture to assess its influence on gap junctional conductance. A rapid inhibition of fluorescent dye transfer and electrical coupling was produced in pairs of freshly dissociated adult rat hepatocytes and myocardial cells as well as in pairs of superior cervical ganglion neurons from neonatal rats cultured under conditions in which electrotonic synapses form. The antibodies have been shown by indirect immunofluorescence to bind to punctate regions of the plasma membrane in liver. By immunoreplica analysis of rat liver homogenates, plasma membranes, and isolated gap junctions resolved on NaDodSO$_4$/polyacrylamide gels, binding was shown to be specific for the 27-kDa major polypeptide of gap junctions. This and similar antibodies should provide a tool for further investigation of the role of cell–cell communication mediated by gap junctions and indicate that immunologically similar polypeptides comprise gap junctions in adult mammalian cells derived from all three germ layers.**

Cells in most tissues can communicate directly with each other through channels isolated from extracellular space; morphologically, these channels are intramembrane particles, identified in clusters as gap junctions (1, 2). It is clear that gap junctional communication in excitable tissues provides for electrical signaling. The role that this pathway plays in inexcitable cells is less clear but, minimally, it would provide for buffering of intracellular metabolite concentrations and permit less precise control on levels of enzymes involved in metabolism (3).

Demonstration that an antibody against liver gap junction protein blocked gap junctional channels would be a useful confirmation that the protein forms the coupling pathway. The finding of immunological (and functional) crossreactivity in a variety of differentiated tissues would indicate a degree of homology among these integral membrane proteins. An inhibiting antibody could also provide a valuable tool in the investigation of the physiological significance of coupling, which remains ambiguous in cells that do not have action potentials to be electrotonically transmitted (however, see ref. 4).

The isolated gap junctions that have been characterized most thoroughly are those from mouse (5) and rat (6) liver; both are comprised of 27-kDa polypeptides. A recently reported procedure enabled the isolation of milligram quantities of the junctions from rat liver, which, when injected into a sheep, led to the production of an antibody specific for the 27-kDa polypeptide (7). In this study, we demonstrate that microinjection of this antibody inhibits both dye transfer and electrical coupling between coupled pairs of mammalian hepatocytes, myocardial cells, and superior cervical ganglion neurons. A preliminary account of these data has appeared (8).

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## METHODS

**Preparation of Antibody.** Gap junctions were isolated from rat liver plasma membranes by a procedure (7) that extracts plasma membranes with alkali instead of detergents (5, 9, 10) to enrich for gap junctions. Briefly, plasma membranes were extracted with cold 20 mM NaOH. Centrifugation of this material at 48,000 × $g$ ($r_{max}$) for 10 min yielded a stratified pellet, the lower part of which was used for purification of gap junctions on discontinuous sucrose gradients.

Antibodies to the gap junction polypeptide were raised by injecting a sheep with purified gap junctions in complete Freund's adjuvent and were affinity purified before use (7).

Conditions for polyacrylamide gel electrophoresis, immunoreplica analysis, and indirect immunofluorescence localization of antibody binding to frozen sections of liver are detailed elsewhere (7).

**Tissue Preparation.** Isolated pairs of hepatocytes were obtained from adult rat liver by using the dissociation procedure of Seglen (11). Briefly, the liver was perfused at 37°C with Ca-free buffer followed by an oxygenated solution containing collagenase. Cells were then dispersed mechanically and passed through a nylon mesh, and parenchymal cells were separated by centrifugation. Cells were generally plated at a density of about 0.5 million per tissue culture dish (35-mm diameter). Normal medium was modified minimal Eagle's medium (MEM) with the addition of antibiotics, hydrocortisone, and insulin (12). Cells obtained in this way are roughly spherical soon after isolation but quickly attach to the plastic dish and flatten over the first 6–8 hr in culture. We chose pairs of cells less than 3 hr after isolation for this study; such cells are invariably coupled with respect to ions and dye molecules and possess gap junctions on their intercellular aspects (13).

Pairs of adult rat ventricular myocytes were obtained by using the dissociation procedure of Wittenberg and Robinson (14) in which heart was perfused with collagenase in low Ca solution and then incubated in low Ca for 1 hr. Normal medium was MEM/Joklik (DM-317, K & K) in which $KH_2PO_4$ was replaced with Hepes and to which essential amino acids were added. Electrotonically coupled cell pairs are commonly encountered among the dissociated cells (cf. ref. 15).

Superior cervical ganglion neurons were dissociated from neonatal rats by using a trypsin triturition dissociation procedure. Nonneuronal cells were eliminated by treatment with cytosine arabinonucleoside and tissue was grown for 2–3 wk

Abbreviations: Bt$_2$cAMP, $N^6$,$O^{2'}$-dibutyryl-cAMP; LY, Lucifer yellow CH; 6-CF, 6-carboxyfluorescein.

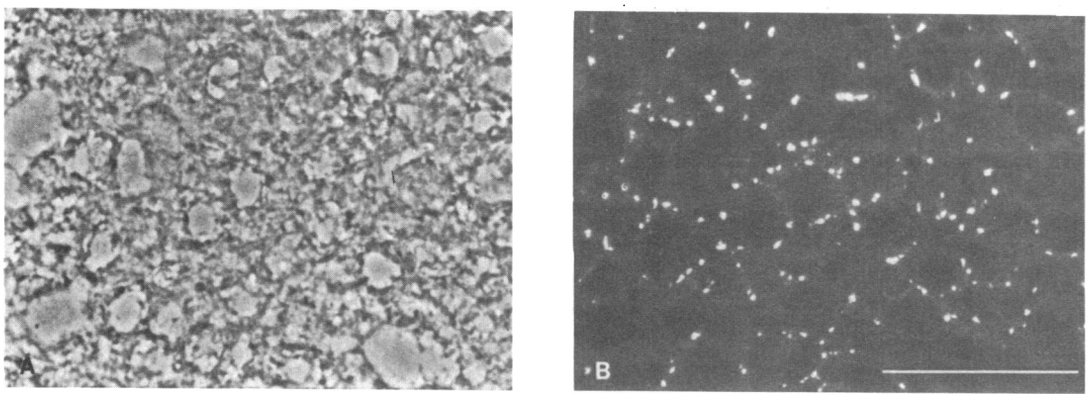


FIG. 1. Indirect immunofluorescence analysis of antibody binding to frozen sections of rat liver. Cryostat sections of rat liver were prepared, blocked with 3% rabbit serum in phosphate-buffered saline, and incubated with affinity-purified antibody (5 $\mu$g/ml). Binding of primary antibody was detected by incubation with fluorescein-conjugated rabbit anti-sheep IgG. (*A*) Phase-contrast image. (*B*) Fluorescence image. (Bar = 50 $\mu$m.)

in a serum containing medium as described (16). Twelve to 24 hr before these experiments, $N^6$,$O^{2'}$-dibutyryl-cAMP ($Bt_2$cAMP, final concentration = 1 mM) and caffeine (1 mM) were added to each dish, resulting in development of electrotonic coupling (17).

**Electrophysiology.** For most studies, one cell of a pair was impaled with a microelectrode (20–70 MΩ) filled with 3 M KCl or, in the case of myocytes, with 150 mM potassium aspartate and 10 mM EGTA buffered with Hepes to pH 7.4. The other cell was impaled with an electrode containing freshly thawed antibody. Antibody was diluted 1:10 to 1:20 in either a filtered (0.22 $\mu$m) solution of 2% Lucifer yellow CH (LY) in 150 mM LiCl or a filtered solution of 2% 6-carboxyfluorescein (6-CF) in 150 mM KCl buffered to pH 7.6 with 10 mM Hepes. Both electrodes were connected to high-impedance electrometers with active bridge circuits (W-P Instruments, New Haven, CT). In addition, the antibody electrode was connected to a Picospritzer (General Valve, Fairfield, NJ) with which brief pressure pulses could be applied. After cell impalement the bridge balance control was adjusted to cancel the initial very fast shift in the recorded voltage, leaving a slower approximately exponential decay (time constant, generally about 10 msec) in response to small hyperpolarizing current pulses (generally <20 nA, 100-msec duration). Current was recorded by a virtual ground current monitor in series with the bath electrode.

Conductances of junctional ($g_j$) and nonjunctional ($g_{nj}$) membranes were calculated from input and transfer resistances obtained in response to current pulses passed alternately in the two cells (18). Pressure injection of the fluorescent solution generally employed 5–20 psi (1 psi = 6.89 kPa), 10- to 100-msec pulses, which produced no detectable volume change in the cells. Fluorescence change in the injected cells was qualitatively similar to that accompanying a 100-msec, 20- to 100-nA hyperpolarizing pulse to that electrode.

## RESULTS

**Antibody Characteristics.** Specificity of the affinity-purified antibody was examined by indirect immunofluorescence staining and by electrophoretic transfer blot analysis. In frozen sections of rat liver, antibody binding was localized to punctate regions of the plasma membrane (Fig. 1*B*). No binding was observed when preimmune IgG was used (7). This pattern of binding is consistent with that expected based upon the observed distribution of gap junctions (19–21). The polypeptide responsible for antibody binding was identified as the 27-kDa gap junction polypeptide in electrophoretic transfer blots of rat liver homogenates, plasma membranes, and purified gap junctions (Fig. 2, lanes a, b, and c, respectively). The homogenate sample was added to $NaDodSO_4$-containing solubilizing buffer within 1 min of death of the animal, and electrophoresis was begun within 4 min. In the case of the homogenate, only the 27-kDa gap junction polypeptide was detected in the electrophoretic transfer blot (lane a), whereas for the isolated plasma membranes and purified gap junctions (lanes b and c), the 47-kDa dimer (5, 22) was also detected, as were higher molecular mass aggregates in the gap junction sample (lane c).

**Electrotonic Coupling.** Pairs of freshly dissociated rat hepatocytes are generally well coupled (Fig. 3*A*, start of record). The larger downgoing deflections in $V_1$ and $V_2$, which alternate between cells, represent voltages when current was passed in the cell from which the recording was done. The height of the solid black region between deflections shows transfer voltages. A single injection of antibody was made

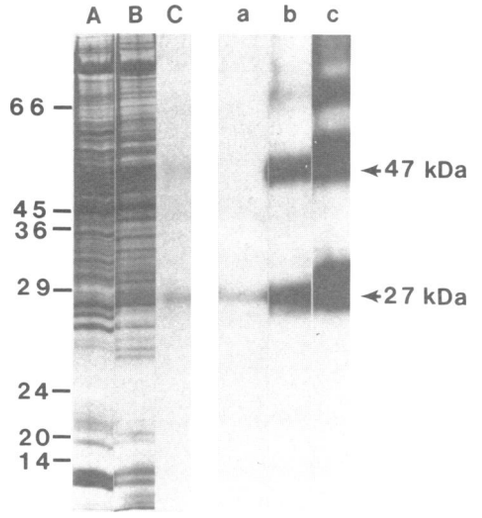


FIG. 2. $NaDodSO_4$/polyacrylamide gel and electrophoretic transfer blot analysis of rat liver homogenate, plasma membranes, and gap junctions. Samples of rat liver homogenate (lane A), plasma membranes (lane B), and isolated gap junctions (lane C) were resolved by electrophoresis in 12.5% $NaDodSO_4$/polyacrylamide gels and stained with Coomassie brilliant blue. An unstained duplicate of each of these lanes, except for lane C in which a sample diluted 1:20 was applied, was electrophoretically transferred to nitrocellulose (lanes a–c). The nitrocellulose replica was incubated first with affinity-purified antibody and then with rabbit anti-sheep IgG; binding of primary antibody was localized by autoradiography subsequent to incubation with $^{125}$I-labeled protein A. The locations of the molecular mass markers (Sigma) (shown in kDa) and the 27-kDa gap junction polypeptide and its 47-kDa dimer are indicated.

with a brief (20 msec) pressure pulse (arrow, injection confirmed visually by observation of fluorescence change caused by coinjected 6-CF). This injection caused a progressive reduction in electrical coupling, indicated by reduction in transfer voltages and an increase in input voltage in the second cell. These decreases in coupling were due primarily to decrease in $g_j$ rather than to increase in $g_{nj}$ values, as indicated by the values for $g_j$ plotted in Fig. 3*E*. In another hepatocyte pair in the same dish, injection of preimmune serum produced no change in $g_j$ (Fig. 3 *D* and *E*). In this case, the protein concentration was five times that of the antibody solution, and 20 pulses were given (at the large open arrow), each of which produced a fluorescence increase.

In a pair of ventricular myocytes, a single pressure injection of gap junction antibody decreased electrical coupling (Fig. 3*B*), whereas in other pairs larger injections of antibody prepared against nonjunctional membranes had no effect (not shown).

In electrotonically coupled superior cervical ganglion neurons, injection of gap junction antibody also decreased coupling (Fig. 3*C*). At the first arrow a single pressure pulse caused little or no antibody to be injected (no fluorescence change was seen); at the second arrow, dye and antibody were injected, producing a reduction in $g_j$. Larger injections of preimmune serum at a protein concentration five times higher than in the antibody experiments had little effect (not shown). With both treatments injected neurons remained excitable.

Bathing hepatocyte pairs in a highly concentrated antibody solution (pressure perfusing the antibody solution over the cells through a pipette with a broken tip) had no effect on $g_j$.

**Dye Coupling.** Gap junctions in many tissues are known to be permeable to the fluorescent dye LY (cf. ref. 23). In Fig. 4, following a single pressure injection of LY along with preimmune serum, fluorescence was quickly detected in both cells of the hepatocyte pair (Fig. 4, pair on lower right, photographs taken within 1 min after injection). In contrast, little or no dye movement was seen after coinjection of LY and the gap junction antibody even after 5 min (Fig. 4, pair on upper left). These two photographs were obtained from a series of dye–antibody experiments on a single dish of cells in which 15 cell pairs were injected with dye plus nonjunctional antibody, then 15 pairs were injected with dye plus gap junction antibody, and then 5 pairs were injected with dye plus nonjunctional antibody. Dye passage was absent or

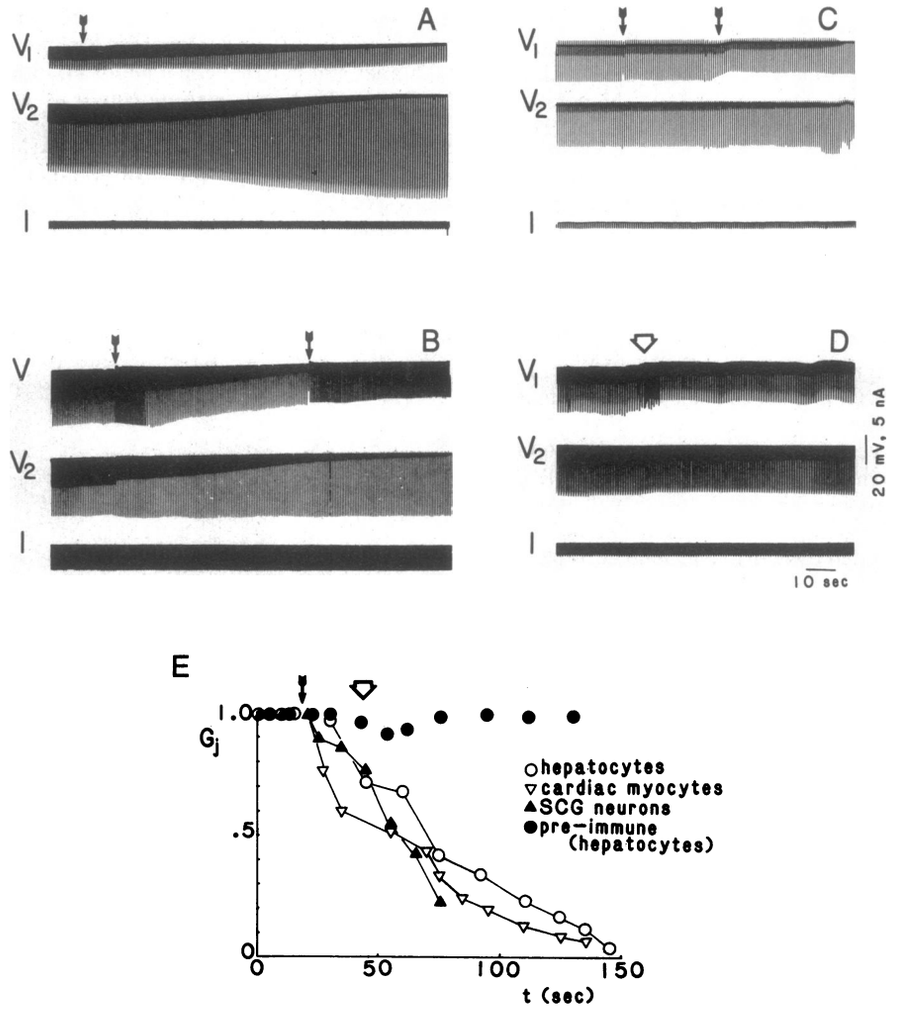


FIG. 3. Electrical coupling between pairs of hepatocytes, cardiac ventricular myocytes, and superior cervical ganglion (SCG) neurons is reduced by injection of gap junction antibody; injection of preimmune serum is ineffective. Two upper traces in *A–D* are voltage recordings from first and second cells; lower traces are current pulses measured by a virtual ground amplifier. (*A*) Pressure injection of antibody into a coupled pair of hepatocytes (at the arrow) reduced electrical coupling. (*B* and *C*) Similar injections of this antibody into coupled pairs of cardiac myocytes and SCG neurons also substantially reduced coupling. (*D*) Repeated injections of coupled hepatocytes with preimmune serum (at the open arrow) at five times higher protein concentration (0.4 mg/ml compared with 0.08 mg/ml) had no effect. (*E*) Injections of antibody but not preimmune serum reduced calculated junctional conductance ($g_j$). Values are normalized to initial maximal values. The brief transient reduction by preimmune serum is ascribable to injury by the large injection.

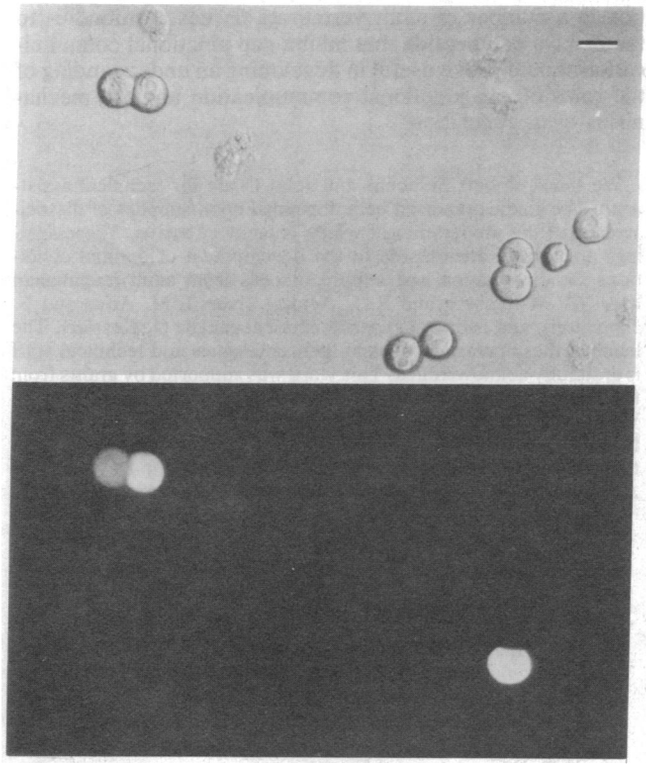

FIG. 4. Injection of one of a pair of hepatocytes with antibody, but not preimmune serum, blocks intercellular transfer of coinjected LY. After a single pressure pulse of LY together with preimmune serum (0.4 mg/ml) fluorescence was quickly detected in both cells of the pair on the upper left (photographs taken within 1 min of the injection). In contrast, no dye movement was seen between a cell pair on the lower right, which had been injected with dye together with the antibody (0.08 mg/ml) 5 min earlier. Fluorescent micrographs were taken with a fluorescein isothiocyanate filter combination; bright field micrographs were taken with Hoffman optics. (Bar = 20 $\mu$m.)

very slight in all cases with gap junction antibody and rapid in all other cases.

A number of experiments were done blind, without the experimenter's knowing the content of the dye injection pipette. In >50 cases and without exception, LY coinjected with preimmune serum passed quickly from cell to cell. In no case (out of >100 cell pairs) was more than a slight passage of dye observed when LY was injected together with junctional antibody in high concentration (40–100 $\mu$g/ml). At lower antibody concentrations (<1 $\mu$g/ml) dye spread was often detected, but we have not yet determined the relations between rate of intracellular transfer and antibody concentration. Dye spread between superior cervical ganglion neurons and between cardiac myocytes (Figs. 5 and 6) is also blocked by junctional antibody but not by preimmune serum.

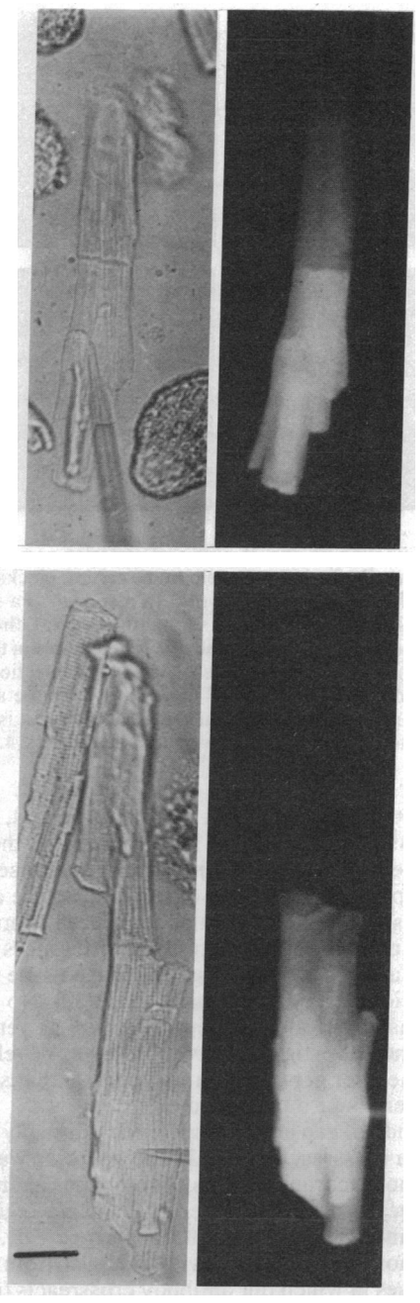

FIG. 5. Injection of one of a pair of cardiac ventricular myocytes with antibody, but not preimmune serum, blocks intercellular transfer of coinjected LY. (*Upper*) After a single pressure pulse injection of one cell with LY together with preimmune serum (0.4 mg/ml), fluorescence quickly spreads from the lower, injected cell to the upper cell (upper right, photograph taken 1 min after injection). (*Lower*) Five minutes after injection of one cell with LY plus antibody (0.08 mg/ml), there is no indication of dye transfer to the neighboring cell. Bright field and fluorescence micrographs as in Fig. 4 (Bar = 20 $\mu$m.)

## DISCUSSION

We show here that microinjected antibody against the liver gap junction polypeptide blocks junctional conductance and dye transfer in liver, heart, and nervous tissue. This antibody binds to punctate regions of the plasma membrane in cryostat sections of liver (Fig. 1*B*) and is specific for the gap junction polypeptide over all other components of liver homogenates and isolated plasma membranes (Fig. 2).

In co-culture, gap junctions and coupling can be established between numerous cell types (24, 25), indicating that compatibility, presumably due to homology, exists in the region of the polypeptide exposed on the cell surface. The inhibition of gap junctional conductance observed upon microinjection of our antibody into myocardial cells (Fig. 5) and superior cervical ganglion neurons (Fig. 6) suggests further similarity in the cytoplasmic domain of the gap junctional polypeptides.

The physiological data support the conclusion, based on immunocytological and immunochemical analyses with this antibody (26), that similar or identical 27-kDa polypeptides

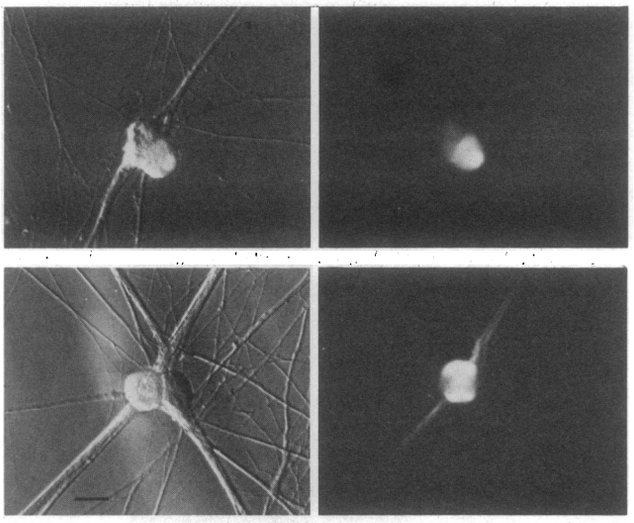

FIG. 6. Injection of one of a pair of superior cervical ganglion neurons with antibody, but not preimmune serum, blocks intercellular transfer of coinjected LY. (*Upper*) In response to a single pressure pulse of LY plus preimmune serum (0.4 mg/ml), the somata of one cell of a pair of neurons fills and dye is detectable in the adjacent somata within 1 min. (*Lower*) Five minutes after injection of a large amount of LY plus antibody (0.08 mg/ml), dye can be seen in processes radiating from the soma but the adjacent soma is unstained. Bright field and fluorescence micrographs as in Fig. 4. (Bar = 20 μm.)

comprise gap junctions in most vertebrate tissues, except for lens fiber tissue. In the case of lens fiber tissue, there is general agreement that there are junctions, comprised of a 26-kDa polypeptide (MP26), that differ in structure and amino acid composition (27–29). Partial sequences from liver and heart gap junctions exhibit significant differences as well as homologies and it has been suggested that a tissue specificity of gap junction polypeptides might exist (30). No antibodies raised against the heart polypeptides have, as yet, been described. Other investigators have, however, developed antibodies to the liver gap junction polypeptide that share some of the properties of our antibody.

In one study of gap junction specificity, broadly consistent with ours, a crossreacting 27-kDa polypeptide was detected in electrophoretic transfer blots of homogenates from a number of tissues and was localized to punctate regions of the plasma membrane by immunofluorescence (31). While no cross-reaction was detected with samples from heart and ovary, tissues in which our antibody crossreacts (ref. 26; unpublished observations), tissue preparation differences might account for this discrepancy.

The present study is generally consistent with the only other reported experiments on microinjection of antibody to rat liver gap junction polypeptide (4). In that report, decrease of gap junctional conductance between amphibian embryonic cells was linked with profound effects on subsequent development. However, electrophoretic transfer blots indicated antibody binding to a 54-kDa polypeptide as well as the 27-kDa polypeptide. Whether the larger polypeptide is a contaminant or a related protein, as the authors suggest, is not certain. There is no binding of the antibody used in the present study to a 54-kDa polypeptide in electrophoretic transfer blots of rat liver samples.

It is clear from this study that the 27-kDa polypeptide found in isolated rat liver gap junctions is intimately involved in gap junctional communication and that antigenically similar polypeptides mediate this form of cell–cell communication in a number of adult vertebrate tissues. Antibodies to the 27-kDa polypeptide that inhibit gap junctional communication should prove useful in developing an understanding of the roles of gap junctional communication and the mechanisms of its regulation.

We thank Robert Skibbens and John Coale for technical assistance. The studies reported here depended upon supplies of dissociated cells, the preparation of which is labor intensive. These findings are largely attributable to the development of optimal conditions for dissociation and culture of cells from adult mammalian heart (B. Wittenberg and R. L. White), liver (I. M. Arias and F. Gatmaitin), and neonatal superior cervical ganglia (J. Kessler). The labor of these investigators and their colleagues and technical staff is gratefully acknowledged. This work was supported by grants from the National Institutes of Health (GM 30667) and the Robert A. Welch Foundation (Q-961) to E.L.H.; Grants NS 16524 and 19830, a New Initiative Award from Grant AM 17702, a Grant-in-Aid from the American Heart Association, and a McKnight Development Award to D.C.S.; and Grants NS 07512 and HD 04248 to M.V.L.B.

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