LIMITATIONS OF THE SCRAPE-LOADING/DYE TRANSFER TECHNIQUE TO QUANTIFY INHIBITION OF GAP JUNCTIONAL INTERCELLULAR COMMUNICATION

SUSAN C. MCKARNS AND DAVID J. DOOLITTLE
Cellular and Molecular Biology Division
R.J. Reynolds Tobacco Company
Winston-Salem, North Carolina

Gap junctional intercellular communication (GJIC) is recognized as playing an important role in normal cell proliferation and development. Chemically induced alteration of GJIC has been proposed to be associated with abnormal cellular growth and/or tumor promotion. Several in vitro assays are currently used to determine the effects of chemicals on GJIC between cultured mammalian cells. One of these assays, the scrape-loading dye transfer (SL/DT) technique, is based on monitoring the transfer of the fluorescent dye Lucifer yellow from one cell into adjacent cells via functional gap junctions. The objective of our study was to evaluate and compare various approaches for quantifying results obtained with the SL/DT technique. Confluent cultures of either WB rat liver epithelial cells or LC-540 rat leydig cells were exposed to the animal tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA), solvent (0.1% ethanol), or culture medium for one hour at 37° C prior to analysis of GJIC. Inhibition of dye transfer was clearly evident following TPA exposure. Quantification of this dye transfer was assessed via four approaches: manually counting the number of labeled cells; measuring the distance of dye travel from the scrape line; quantifying the amount of cellular dye uptake; and determining the distribution of dye away from the scrape line. Our results suggest that while the SL/DT technique can be effectively used as a tool to determine the qualitative presence or absence of GJIC, its use in quantifying changes in GJIC following chemical exposure is limited. Since concentration-dependent responses are critical in chemical testing, application of the SL/DT method should be restricted to a screening assay for qualitatively assessing the presence or absence of GJIC.

1. Address all correspondence to: Susan C. McKarns, Cellular and Molecular Biology, Building 630-2, R.J. Reynolds Tobacco Company, Winston-Salem, NC 27102. Tel: (919) 741-2122.

2. Abbreviations: FBS, fetal bovine serum; GJIC, gap junctional intercellular communication; HBSS, Hank's balanced saline solution; SL/DT, scrape-loading/dye transfer; TPA, 12-O-tetradecanoylphorbol-13-acetate.

3. Key words: scrape-loading/dye transfer assay, gap junctional intercellular communication, TPA.

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Another assay (e.g., electrical coupling, microinjection, metabolic cooperation, radioactive metabolite transfer, or fluorescence redistribution after photobleaching) should be considered to quantify changes in GJIC and construct chemical concentration-response curves.

INTRODUCTION

Gap junctional intercellular communication (GJIC) is important in the regulation of normal cellular growth and development (Loewenstein, 1979, 1981; Lo, 1985). Alteration of normal intercellular communication has been associated with abnormal cellular growth and tumor promotion (Trosko et al., 1982; Fletcher et al., 1987; Trosko et al., 1987; Ye et al., 1990). Thus, assessment of GJIC is often used as a tool to detect potentially adverse biological effects of chemicals and/or chemical mixtures.

Measurement of GJIC has been studied using a variety of techniques including dye transfer through microinjection (Fitzgerald and Murray, 1980), radioactive metabolite transfer (Newbold and Amos, 1981), metabolic cooperation (Yotti et al., 1979), electrocoupling (Yamasaki et al., 1983; Socolar and Loewenstein, 1978), and fluorescence redistribution after photobleaching (Wade et al., 1986; McKarns et al., 1991; McKarns and Doolittle, 1991). These techniques generally require complicated procedures, sophisticated equipment, large sample volumes, or long intervals of experimental time. The scrape-loading/dye transfer technique (SL/DT) has been proposed as a rapid, inexpensive means of measuring gap junctional intercellular communication (El-Fouly et al., 1987).

SL/DT is based on the intracellular introduction of the nonpermeable, fluorescent dye, Lucifer yellow (M.W. 457.2) into a monolayer of cells through a transient cut in the cell membrane (McNeil et al., 1984). The transfer of this dye into adjacent cells via gap junctions is monitored. Lucifer yellow yields a stable high quantum fluorescence which can be readily detected with epifluorescence microscopy (Stewart, 1978, 1981). Lucifer yellow is not cytotoxic at the concentration used in the SL/DT assay and does not diffuse through intact plasma membranes (Stewart, 1978). Thus the transfer of Lucifer yellow between cells is thought to be restricted to passage through functional gap junctions between neighboring, contacting cells.

Several investigators have used the SL/DT assay to determine inhibition of intercellular communication in mammalian cells (El-Fouly et al., 1987; Kavanagh et al., 1987; Suter et al., 1987; Oh et al., 1988; Evans et al., 1988a, 1988b; Flodstrom et al., 1988; Madhukar et al., 1989). The objective of this study was to evaluate and compare various approaches in order to quantify the results generated by the SL/DT technique.

METHODS

Cells

The rat liver epithelial cell line, WB-344 (Tsao et al., 1984) was kindly provided by Dr. J. Trosko (Michigan State University, East Lansing, MI). The rat leydig cell line, LC-540, was

obtained from the American Type Culture Collection (Rockville, MD). Both of these cell lines communicate *in vitro* via gap junctions (Jone et al., 1987; El-Fouly et al., 1987; Bombick, 1990).

Cell Culture

Cells were grown and maintained in Medium D (Earle's balanced salt solution with 50% increase of vitamins and essential amino acids except glutamine, 100% increase of non-essential amino acids, 1mM sodium pyruvate, 5.5 mM glucose, 15.3 mM NaCl, and 11.9 mM $NaHCO_3$). The medium was supplemented with dialyzed, heat-inactivated fetal bovine serum (FBS) and 50 ug/ml gentamycin prior to use. WB cell cultures were supplemented with 5% FBS, while LC-540 cell cultures were supplemented with 10% FBS. The cell cultures were grown and maintained at 37°C in a humidified incubator containing 5% CO_2. Stock cultures were maintained in Corning 75 cm^2 plastic tissue culture flasks. Medium D, FBS, and gentamycin were purchased from GIBCO Laboratories (Grand Island, NY).

Chemicals

TPA and all solvents were purchased from Sigma Chemical Company (St. Louis, MO). Lucifer yellow was purchased from Molecular Probes (Eugene, OR).

Scrape-Loading/Dye Transfer

A modified procedure of the SL/DT assay as described by El-Fouly et al. (1987) was used to assess GJIC. Cells (2×10^5) suspended in 2 ml culture medium supplemented with FBS, were pipetted into Corning 35mm diameter plastic tissue culture plates (GIBCO). Following a 12-hour incubation at 37°C, cells were arrested in the G_0 stage of the cell cycle by removing FBS from the culture medium for 12 hours (Ashihara and Baserga, 1979). Fresh medium containing 5% FBS was added to each plate and the cells were incubated at 37°C for five hours. 12-O-tetradecanoylphorbol-13-acetate (TPA) (dissolved in ethanol) or vehicle control (0.1% ethanol) was added to the plates and incubated at 37°C for one hour. Ethanol (0.1%) and TPA (10 and 100 ng/ml) are not cytotoxic as determined by plasma membrane integrity (McKarns et al., 1991). Following chemical exposure, each plate was gently washed five times with Hank's balanced salt solution supplemented with Mg^{++} and Ca^{++} (modified HBSS) and 2 ml of Hank's balanced salt solution (without Mg^{++} and Ca^{++}) was added to each plate. A size 22 carbon steel surgical blade (Becton Dickinson, Lincoln Park, NJ) fitted to a size 4 handle (Becton Dickinson) was gently pressed into the monolayer in order to induce a "scrape". Seven uniform, evenly spaced vertical cuts were made across each 35mm plate. Great care was taken to keep the depth and width of each cut consistent. The balanced salt solution was immediately removed from the plates and 2 ml of 0.05% Lucifer yellow in modified HBSS was added to each plate. Lucifer yellow was exposed to a minimal amount of room light to avoid fluorescence photobleaching of the dye. Lucifer yellow was removed from the plates after either one or five minutes, depending on the experiment. The cells were washed three times with modified HBSS and immediately fixed at room temperature with 4% phosphate-buffered formalin for 10 minutes. Plates were covered, allowed to dry at room temperature, and

stored at 4°C. Plates were allowed to reach room temperature before cells were analyzed. Fixation and/or storage of the plates at 4°C did not alter the intracellular fluorescence of Lucifer yellow (data not shown).

Quantitative Analyses of Fluorescence Intensity

Fluorescence intensity was determined using an ACAS 470 interactive laser cytometer (Meridian Instruments, Okemos, MI). Instrumentation parameters of 35% photomultiplier tube voltage and 25% scan strength were used to produce 200X digitized psuedoimages of areas containing the scrape and approximately 15 to 20 rows of adjacent, nonscraped cells. The position of the scrape line (vertical) and the size of the area (360 micron^2) were kept consistent among scans within and between experimental groups. Background fluorescence was determined by scanning a 360 micron^2 area distal to the scrape line. Scans of the scrape-loaded cell images were generated and stored on computer disks for data analyses as described below.

Quantitative Analyses of GJIC by Scoring the Number of Labeled Cells

The number of fluorescent labeled cells was scored by two methods. First, the total number of labeled cells adjacent to the scrape line within the fixed area (360 micron^2) was counted manually. Second, the number of rows of labeled cells adjacent to the scrape line was counted manually.

Quantitative Analyses of GJIC by Determining the Total Amount of Fluorescence Uptake

A relative fluorescence intensity value was obtained for each area using the ACAS 470 computer software. Fifteen areas (five plates, three random areas per plate) were analyzed for each of the untreated and solvent control groups. Thirty areas (ten plates, three random areas per plate) were analyzed for each TPA-treated group. Fluorescence intensity data are reported as the mean fluorescence uptake ± the standard deviation and are expressed as relative fluorescence units.

Quantitative Analyses of GJIC by Determining the Distance of Dye Travel Away from the Scrape Line

The average distance of dye travel away from the scrape-loaded cells was determined using the ACAS 470 computer software. This computation was achieved by drawing a series of 50 evenly spaced lines at right angles to the scrape line (i.e., 25 lines on the right-hand side of the scrape and 25 lines on the left-hand side of the scrape). Each line extended from the scrape to the outer edge of the fluorescent cells located furthest from the scrape. The length of each line (in microns) was determined using the ACAS 470 computer software; the line length reflects the distance of fluorescence dye travel into the secondary recipient cells from the initial scrape-loaded cells. Fifteen areas (five plates, three random areas per plate) were analyzed for each of the untreated and solvent control groups. Thirty areas (ten plates, three random areas per plate) were analyzed for each TPA-treated group. The data are reported as the mean distance (microns) of fluorescence travel ± the standard deviation.

Quantitative Analyses of GJIC by Determining the Dye Distribution Away from the Scrape Line

A series of 50 lines was drawn from the scrape line to the outer edge of the cells located furthest from the scrape as described above. Using this series of lines, a profile was generated that described the fluorescence intensity (relative fluorescence units) on the ordinate and the distance of dye travel from the scrape (microns) on the abscissa. This profile graphically illustrates the distribution of Lucifer yellow away from the scrape line. An integrated fluorescence value that assessed the total amount of fluorescence under the profile (fluorescence intensity with respect to distance of dye travel away from the scrape) was generated using the ACAS 470 computer software. An average fluorescence value that represented the total amount of fluorescence with respect to the total area of fluorescence was calculated. Fifteen areas (five plates, three random areas per plate) were analyzed for each of the untreated and solvent control groups. Thirty areas (ten plates, three random areas per plate) were analyzed for each TPA-treated group. The data are expressed as the mean integrated fluorescence value ± the standard deviation.

RESULTS

Timing of Lucifer Yellow Uptake by Cells

Lucifer yellow continues to flow into WB cells (up to 60 seconds) and LC-540 cells (at least 120 seconds) after the scrape is induced into the cell monolayer (Figure 1) indicating that the plasma membrane does not immediately close down following cell scraping. Thus Lucifer yellow may be added to the extracellular medium either before or immediately after the scrape is made. Scraping the cells in the presence of Lucifer yellow is the more traditional method (El-Fouly et al., 1987) and our data confirm that this methodology results in substantial Lucifer yellow uptake. However, scraping the cells in the presence of PBS before adding Lucifer yellow allows for a more uniform and controlled dye exposure among the cells. In the studies reported here, PBS was first added to the plate, the cells were scraped in the presence of PBS, the PBS was removed from the plate and discarded, and then Lucifer yellow was added immediately to each plate. This procedure was carried out one plate at a time in order to minimize, as well as standardize, the time between scraping the cells and adding Lucifer yellow. It is important to note that even in the absence of a scrape line, some Lucifer yellow is incorporated into the cells as evidenced by non-specific background fluorescence (Figure 1).

Effect of TPA on GJIC

An obvious inhibition in the number of fluorescent-labeled cells was observed following exposure when the entire plate of cells was viewed under magnification. This qualitative inhibitory effect of TPA on GJIC in WB and LC-540 cells is in agreement with published data (Bombick, 1990; Flodstrom et al., 1988; Jone et al., 1987). In order to quantify the TPA-induced inhibition of dye transfer, the following parameters were assessed; counting individual labeled cells, counting labeled rows parallel to the scrape line, quantifying the total amount of dye uptake, determining the distance of dye travel away from the scrape line, and calculating the dye distribution from the scrape line.

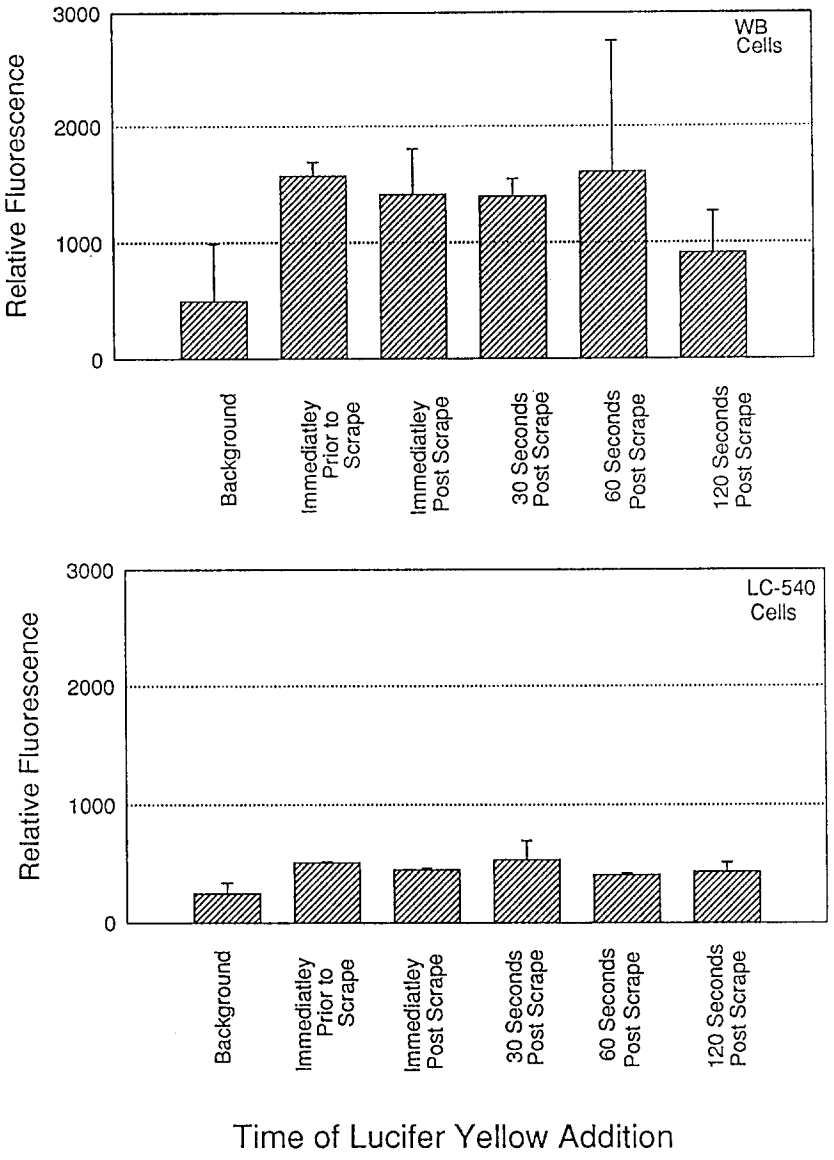

FIGURE 1. Influence of dye exposure timing on Lucifer yellow uptake. Cell monolayers were exposed to fresh culture medium (FBS-free) for one hour at 37°C. Two ml of a 0.05% solution of Lucifer yellow was added to the cells immediately prior to or following (0, 30, 60, and 120 seconds) cell scraping. Dye uptake was assessed by determining the amount of fluorescence within a standardized 360 micron^2 area surrounding the scrape line after a five min Lucifer yellow exposure. The background fluorescence was obtained by determining the fluorescence intensity in areas of non-scraped cells (located distal to the scrape). Fifteen random areas (five plates, three random areas per plate) were analyzed for each untreated and solvent control group. The data are expressed as relative fluorescence units and are reported as mean fluorescence ± the standard deviation.

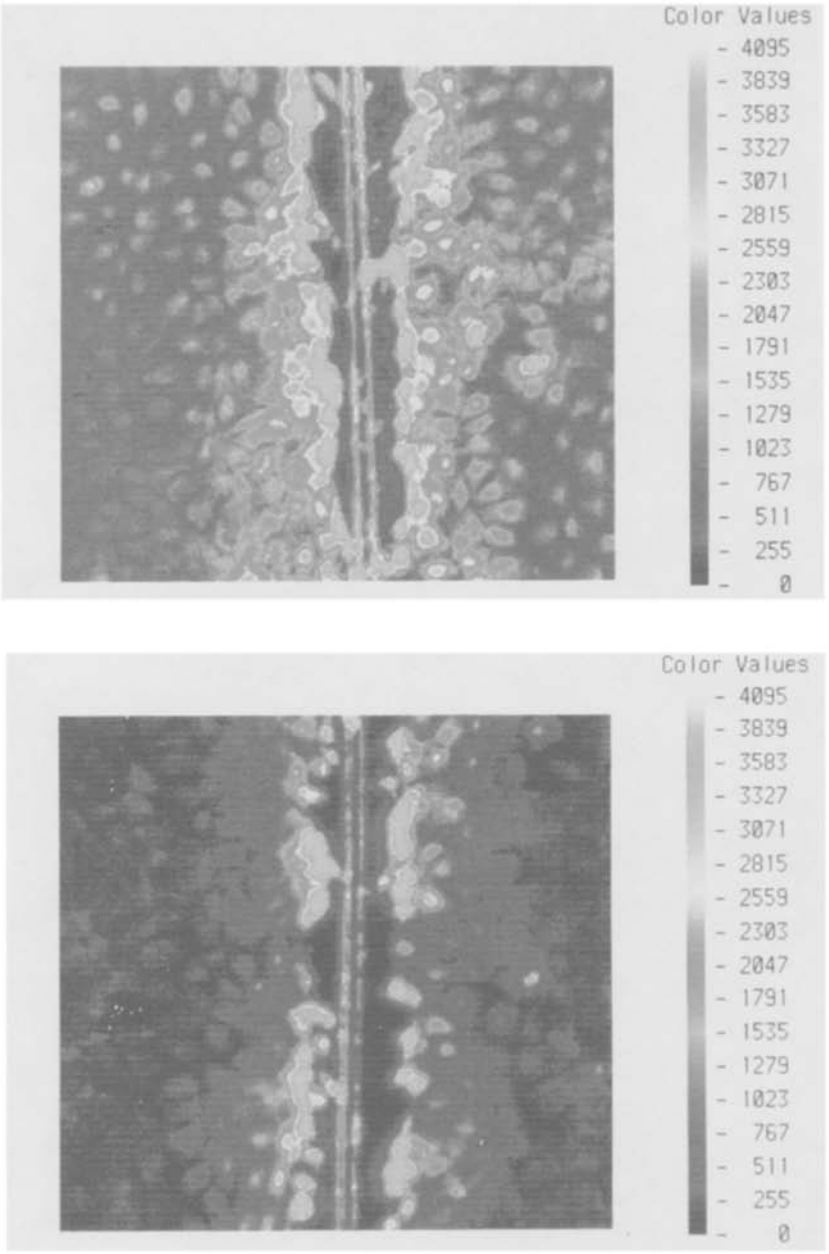

FIGURE 2. Digitized pseudoimages of scrape-loading and dye transfer of Lucifer yellow in a monolayer of untreated WB cells. A) Cells following a five min exposure to 0.05% Lucifer yellow at room temperature. Intercellular communication was determined by measuring the gradient dye transfer among cells; the highest intensity of dye was present along the scrape line. Variation in the number of labeled individual cells and rows of cells can be observed. B) A scan taken from a different area along the same scrape line is illustrated in panel A. Visual observation indicates that a significant variation in GJIC exists among untreated cells.

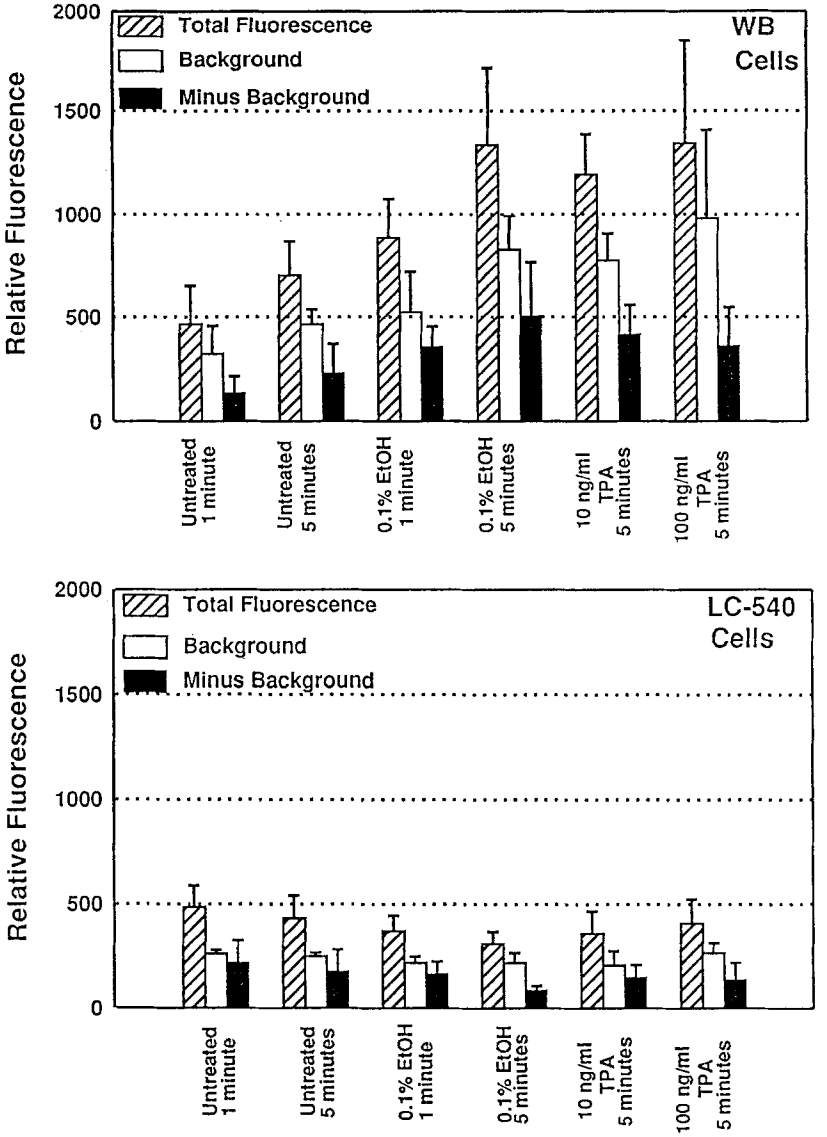

FIGURE 3. Gap junctional intercellular communication in WB and LC-540 cells as determined by the amount of dye uptake. Monolayer cultures were exposed to fresh medium (FBS-free), 0.1% ethanol (solvent control), or TPA for one hour at 37°C prior to scrape loading with Lucifer yellow. Dye transfer was quantified using the ACAS 470 interactive laser cytometer. Untreated and solvent controls were exposed to Lucifer yellow for either one or five minutes, as indicated. TPA-treated cells were exposed to Lucifer yellow for five minutes. Fifteen areas (five plates, three random areas per plate) were analyzed for each untreated and solvent control group. Thirty random areas (ten plates, three random areas per plate) were analyzed for each TPA-treated group. Three nonscraped areas from each plate were scored to obtain the background fluorescence. The mean plate background fluorescence was subtracted from the mean plate total fluorescence to obtain the "minus background" fluorescence value. The data are expressed as relative fluorescence units and are reported as mean fluorescence ± the standard deviation.

Quantitative Analyses of GJIC by Determining the Number of Labeled Cells

Digitized pseudoimages of SL/DT in a monolayer of untreated WB cells following a five minute exposure to Lucifer yellow are shown in Figure 2. This figure illustrates two potential problems with the SL/DT assay—non-uniform dye loading and variable dye transfer into secondary, recipient cells occur among cells in untreated cultures. The highest intensity of Lucifer yellow was always present in the cells located at the scrape line. Variability in the number of fluorescent labeled cells was present along the length of an individual scrape line (Figures 2a and 2b). Additional variability in the extent of label was observed among scrape lines and among areas within the same plate. Figure 2a illustrates an area where cells appear to communicate among several rows adjacent to the scrape line. Figure 2b represents a different area of cells located on the same scrape line and illustrates the fluctuation in the number of labeled cells compared to the area in Figure 2a. Similar variations were observed in TPA and solvent-exposed cells (data not shown). Due to the marked variation in the number of labeled cells, counting the number of individual and/or rows of fluorescent labeled cells is extremely subjective and cannot be justified as a valid method of quantifying GJIC.

Quantitative Analyses of GJIC by Determining the Amount of Dye Uptake

Although a visual inhibition of dye transfer was observed in the TPA-treated cells (as discussed earlier), this inhibition could not be quantified by using the ACAS to determine the total amount of dye transfer into the recipient cells. The total amount of fluorescence uptake was assessed in both cell types (Figure 3). In untreated and solvent control cultures, an increase in the Lucifer yellow exposure from one to five minutes slightly increased Lucifer yellow uptake in the WB cells, but had little effect on Lucifer yellow uptake in LC-540 cells. Based upon these data, all TPA-treated cells were exposed to Lucifer yellow for a five-minute period to allow for maximal dye uptake and subsequent increased assay sensitivity. Relative to untreated controls, 0.1% ethanol slightly enhanced detection of background fluorescence in the WB cells but not in the LC-540 cells. No change in the amount of total fluorescence in the TPA-treated cells was observed when compared to the concurrent solvent control (0.1% ethanol, 5 min exposure).

Quantitative Analyses of GJIC by Determining the Distance of Dye Travel from the Scrape Line

GJIC in LC-540 cells following a one-hour exposure to TPA is shown in Figure 4. These data indicate that determining the distance that the fluorescence travels away from the scrape line is not an adequate method for quantifying chemically induced inhibition of GJIC. TPA did not influence the distance of fluorescence travel relative to the concurrent solvent control (0.1% ethanol, 5 min). Increasing the Lucifer yellow exposure time from one minute to five minutes did not influence the distance the dye travelled away from the scrape line.

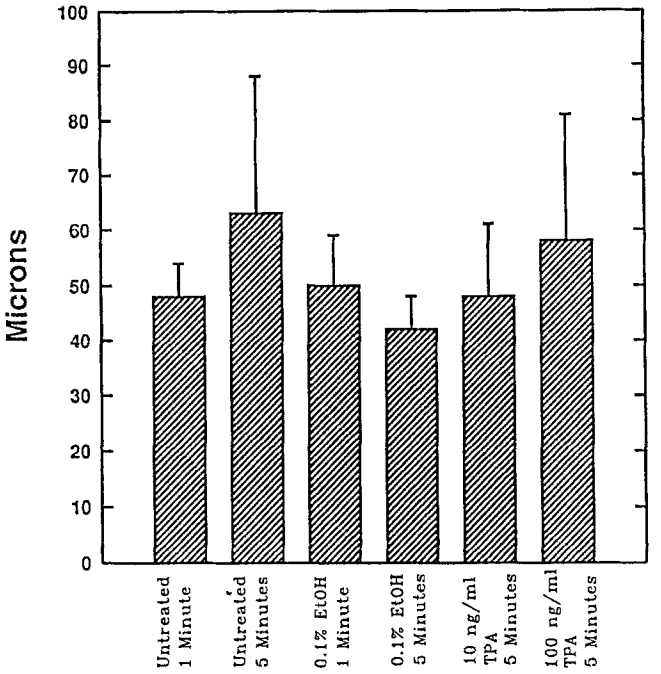

FIGURE 4. Gap junctional intercellular communication in LC-540 cells as determined by quantifying the distance of fluorescence travel away from the scrape line as described in the "Material and Methods" section. Monolayer cultures were exposed to fresh culture medium (FBS-free), 0.1% ethanol (solvent control), or TPA for one hour prior to scrape-loading with Lucifer yellow. Dye transfer was quantified using the ACAS 470 interactive laser cytometer. Untreated and solvent controls were exposed to Lucifer yellow for either one or five minutes, as indicated. TPA-treated cells were exposed to Lucifer yellow for five minutes. Fifteen random areas (five plates, three random areas per plate) were analyzed for each untreated and solvent control group. Thirty areas (ten plates, three random areas per plate) were analyzed for each TPA-treated group. The data are reported as the mean distance (microns) of fluorescence travel ± the standard deviation.

Quantitative Analyses of GJIC by Determining the Dye Distribution Away from the Scrape Line

Results of GJIC in LC-540 cells as determined by quantifying dye distribution away from the scrape are shown in Figure 5. We were unable to quantify a significant inhibition of GJIC following TPA exposure as determined by distribution of Lucifer yellow away from the scrape line. These results suggest that this approach is inappropriate for quantifying the inhibition of GJIC following chemical treatment. TPA treatment did not alter the non-specific background fluorescence relative to the concurrent solvent control (0.1% ethanol, 5 min).

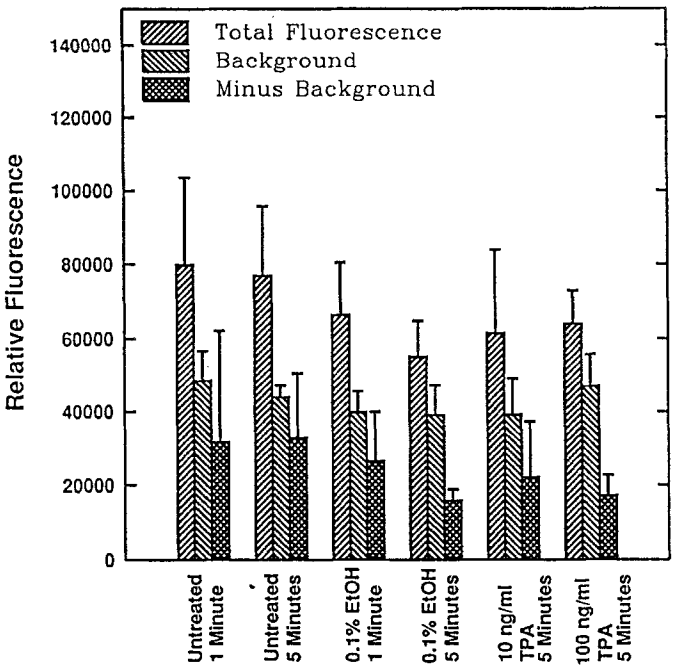

FIGURE 5. Gap junctional intercellular communication in LC-540 cells as determined by quantifying the distribution of dye away from the scrape line as described in the "Material and Methods" section. Monolayer cultures were exposed to fresh medium (FBS-free), 0.1% ethanol (solvent control), or TPA for one hour prior to scrape-loading with Lucifer yellow. Dye transfer was quantified using the ACAS 470 interactive laser cytometer. Untreated and solvent controls were exposed to Lucifer yellow for either one or five minutes, as indicated. TPA-treated cells were exposed to Lucifer yellow for five minutes. Fifteen areas (five plates, thee random areas per plate) were analyzed for each untreated and solvent control group. Thirty areas (ten plates, three random areas per plate) were analyzed for each TPA-treated group. The data are expressed as relative fluorescence units and are reported as mean fluorescence ± the standard deviation.

DISCUSSION

An association between altered GJIC and biological dysfunction has been extensively reported (for review see Klaunig and Ruch, 1990). Because of its relative simplicity and speed, the SL/DT assay (El-Fouly et al., 1987) is selected frequently as the method of choice to determine inhibition of GJIC. The SL/DT assay has been used traditionally as a screening tool to evaluate the qualitative effects of chemicals on GJIC. However, more recently, the SL/DT assay has been used to obtain quantitative concentration-dependent responses of chemically induced inhibition (Suter et al., 1987; Evans et al., 1988a, 1988b; Oh et al., 1988; Loch-Caruso, 1990).

In agreement with other investigators, we have utilized the SL/DT technique to demonstrate a qualitative inhibition of GJIC in WB rat liver cells and MSU-2 human skin fibroblasts following TPA exposure. Specifically, an inhibition of fluorescence distribution around the scrape line can be visually observed when the entire plate of cells is in view, but this is a subjective observation. Our data indicate that the *quantitation* of GJIC as determined by the SL/DT technique is limited. We utilized several parameters in an attempt to quantify the TPA-induced inhibition of GJIC, including: manually counting individual fluorescent labeled cells, manually counting labeled rows of cells lying parallel to the scrape line, quantifying the total amount of dye uptake, determining the distance of dye travel away from the scrape line, and calculating the distribution of the dye as it travels away from the scrape line. These data demonstrate that none of the above described approaches can be used to objectively quantify inhibition of GJIC, thus indicating the limitations of the SL/DT assay. The substantial variability in the fluorescence labeling pattern suggests that quantitative results from the SL/DT assay have the potential to be highly subjective. Therefore, the SL/DT assay should be limited to use as a qualitative indicator of the chemical effects on GJIC.

It should be noted that the SL/DT technique coupled with two-color analyses using flow cytometry has been used to successfully quantify GJIC inhibition (Kavanagh et al., 1987). However, this method is applicable only when a large sample size is available. In addition, analyses of GJIC on individual cells is not possible using this method. Furthermore, the necessity of using a flow cytometry in this assay eliminates many of the advantages (speed and cost) of the SL/DT technique.

We have identified two general concerns associated with quantifying GJIC using the SL/DT assay. The first concern is that the scraped cells do not uniformly label with Lucifer yellow, regardless of the presence or absence of chemical treatment. Other studies have published photographs illustrating similarly inconsistent labeling patterns (El-Fouly et al., 1987; Kavanagh et al., 1987; Suter et al., 1987; Oh et al., 1988; Nicholson et al., 1988; Evans et al., 1988a, 1988b; Madhukar et al., 1989; Loch-Caruso et al., 1990), and a representative example from our laboratory is shown in Figure 2. Several factors may contribute to this lack of uniform dye labeling. For instance, pressing a sharp scalpel into a monolayer of cells imposes a physiological trauma to the cells. McNeil et al. (1984) have shown that in some cases only 50-60% of cells remain viable after scrape-loading. Furthermore, changes in the concentrations of intracellular Ca^{++}, alteration of intracellular pH, and the generation of free radicals frequently occur following cell membrane damage. These factors (i.e., Ca^{++}, pH, free radicals) are modulators of gap junctional intercellular communication (Loewenstein, 1981; Trosko and Chang, 1984; Spray et al., 1986; Saez et al., 1987; Ruch and Klaunig, 1988). The second factor that obviates the use of the SL/DT assay as a quantitative indicator of GJIC is the extensive variability associated with dye transfer into secondary, recipient cells. Since the initial scraped cells do not load up with equivalent amounts of dye initially, variability of dye transfer into the recipient cells is not surprising.

In conclusion, our results indicate that the SL/DT technique may be useful as a qualitative indicator of the presence or absence of GJIC. However, this assay is inappropriate for objectively quantifying chemical effects on GJIC and should not be used to construct chemical concentration-response curves.

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