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Intercellular Communication of Transformed and Non-transformed Rat Liver Epithelial Cells

Modulation by TPA

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Gap-junctional intercellular communication of transformed and non-transformed rat liver epithelial cell lines was compared using a dye transfer method in the presence and absence of 12-*O*-tetradecanoylphorbol 13-acetate (TPA). Whereas non-transformed cells (IAR 20, non-tumorigenic in newborn rats and in nude mice) showed very high communication capacity throughout a culture period of 3 weeks, transformed cells (IAR 6-1, tumorigenic in newborn rats and in nude mice) were less able to communicate. Similar correlation between intercellular communication and expression of transformed phenotypes were also found in newly cloned epithelial cell lines, IAR 27 E and IAR 27 F. When TPA was added to culture medium at 100 ng/ml, intercellular communication in all lines tested was reduced within 60 min. However, communication recovered completely from the effect within 10 h after addition of TPA. Further addition of TPA to the cultures every 24 h for 3 weeks had no effect on intercellular communication (measured 30 min after each TPA addition), suggesting that a single application of TPA made these cells refractory to further doses. A known stimulator of gap-junctional communication, db-cAMP, also increased dye transfer in IAR 20 and IAR 6-1 cells. TPA added to db-cAMP-treated cultures of IAR 20 and IAR 6-1 cells inhibited intercellular communication, suggesting that cAMP is not an antagonist of the effect of TPA on intercellular communication in these cell lines. These results are in sharp contrast to those obtained with the fibroblast cell line BALB/c 3T3, in which db-cAMP antagonized TPA effect [1] and inhibition by TPA of intercellular communication was transient only when administered during their growth phase, and was stable and continuous when TPA was applied at confluence [2], and suggest that TPA may not be an effective tumour promoter in rat liver.

Since uncontrolled cell proliferation is an important characteristic of tumour formation and since gap-junctional intercellular communication is believed to control cell proliferation by maintaining homeostasis among cells in a given tissue, there have been numerous studies of the possible role of gap-junctional communication in carcinogenesis (for reviews, see [3, 4]). However, most previous studies have compared the capacity for intercellular communication of tumorigenic and non-tumorigenic cells, and very few attempts had been made to study its role in the *process* of carcinogenesis. In order to do so, we are

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employing cultured cells as a model system [5, 6], and these studies and those from other laboratories provide several lines of evidence that cell–cell interaction plays an important role in the promotion phase of carcinogenesis, including inhibition of intercellular communication by a class of tumour-promoting agents, the phorbol esters [5–8].

Phorbol esters have been shown to inhibit gap-junctional communication in a variety of cells in culture, using different methods to measure intercellular communication: metabolic cooperation assay [9, 10], electrical coupling [11], and a dye transfer assay [1, 12, 13]. However, few studies have been made of the effect of tumour-promoting agents on intercellular communication on epithelial cells [11–14]. The present study was undertaken to examine the effect of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) on rat liver epithelial cells, taking advantage of the availability of a series of established rat liver cell lines with different transformed phenotypes [15, 16]. We have previously reported the effects of TPA on the membranes of some of these cell lines [17]. We report here that TPA inhibits intercellular communication in transformed and non-transformed rat liver epithelial cells; however, the inhibition was transient, and further addition of TPA did not inhibit communication during 3 weeks in culture. These results differ from those obtained with cultured fibroblasts [2].

MATERIALS AND METHODS

Material

Lucifer Yellow CH, dibutyryl adenosine 3′:5′-cyclic monophosphate sodium salt (db-cAMP), caffeine, Triton X-100, L-γ-glutamyl-*p*-nitroanilide and glycylglycine were purchased from Sigma Chemical Co., St Louis, Mo.; TPA was obtained from CCR Co. Ltd, Minneapolis, Minn. Capillary tubes used for microinjection were obtained from A-M Systems Inc., Everett, Wash.

Cell Lines

All cell lines used in this study—IAR 20, IAR 27, IAR6-1—were isolated from liver of BD VI rats [15, 16]. The IAR 27 cells transformed spontaneously and IAR 6-1 transformed in vitro by dimethylnitrosamine as described previously [15, 16]. Characteristics of these cell lines are summarized in table 1.

Cells were cultured in William's E medium supplemented with 10% fetal calf serum (FCS) and L-glutamine, penicillin and streptomycin (100 U/ml), in a 5% CO_2 incubator at 37°C. For experiments, cells were treated with trypsin–EDTA solution and plated onto 60-mm plastic culture dishes at 2×10^5 cells/dish in 4 ml medium. Confluent cultures (3-day-old) were used for studies on intercellular communication; otherwise, cells were maintained by subculturing them once weekly with a medium change every other day.

Measurement of Intercellular Communication by Fluorescent Dye Transfer

Intercellular transfer of fluorescent Lucifer Yellow CH was measured after direct microinjection of the dye into a cell under a phase-contrast microscope and observation of its transfer to neighbouring cells under a fluorescent microscope [1]. A 10% (w/v) solution of Lucifer Yellow CH in 0.33 M lithium chloride solution was transferred to a glass capillary needle which was prepared from a capillary tube using an automatic magnetic puller (Narishige Co., Tokyo, Japan). Individual cells were impaled with capillaries at a site close to the nucleus, and dye was injected continuously for 3–5 sec by hand pressure using an Olympus Injectoscope YF [18]. Transfer of dye to surrounding cells was

monitored under a Leitz phase-contrast fluorescent microscope between 10 and 20 min after injection. Photographs were taken through a phase-contrast fluorescent microscope using Ilford HP5 (ASA 400) films, before or after fixation with glutaraldehyde [3.7% in phosphate-buffered saline (PBS)].

Assay for Tumorigenicity

Suspensions of each cell line (IAR 20, IAR 27, IAR 6-1) were inoculated subcutaneously into 7-week-old female nude mice at concentrations of 10^4–10^7 cells in 0.2 ml of PBS and animals were kept under observation up to 4 months. The tumorigenicity of these cell lines in newborn rats was reported previously [15, 16].

Growth in Soft Agar

This test was performed using a slight modification of the method of MacPherson & Montagnier [19]. 2×10^4 single cells were suspended in 4 ml Eagle's minimum essential medium with added non-essential amino acids and 10% FBS containing 0.3% special Noble agar at 40°C, and the suspension was immediately poured onto a 5-ml basal layer of 0.5% agar and incubated at 37°C. After 3 weeks' incubation, the cultures were examined and scored for the presence of colonies containing four or more cells.

Measurement of γ-Glutamyl-Transpeptidase (GGT)

Transformed and non-transformed cultures seeded at 2×10^6 cells were cultured on 150-mm Petri dishes to obtain approx. 50×10^6 cells for enzymatic assay. Enzyme activity was measured as described by De Young et al. [20], with a slight modification. After removal of the medium, the cultures were rinsed twice with cold PBS (4°C) before detaching the cells with a rubber policeman; the cells were collected by centrifugation (2000 g for 5 min) and pulverized in a glass homogenizer. Homogenates were made up in a solution containing 10% glycerol, 1% Triton X-100 and 5 mM potassium phosphate, pH 7.0 and were spun at 10000 g for 30 min at 4°C.

The enzyme assay, carried out at 25°C, was initiated by adding 0.3-ml samples of homogenate supernatant to 10-ml of assay solution (4.4 mM L-γ-glutamyl-*p*-nitroanilide, 40 mM glycylglycine free base, 11 mM $MgCl_2\times6H_2O$ and 0.1 M *N,N'-bis*hydroxyethylglycine buffer, pH 8.8). After a 30-min incubation, the reaction was terminated by rapid addition with mixing of 2 ml 1.5 M acetic acid. Aliquots of supernatant boiled for 10 min and assayed as above served as blanks for reading in the spectrophotometer (405 nm).

Enzyme activity (nmol *p*-nitroaniline per mg of protein per 30 min) was calculated as described by De Young et al. [20].

RESULTS

Characterization of Transformed Phenotypes of IAR 20, IAR 27 and IAR 6-1 Cells

Prior to studying intercellular communication, we carried out detailed characterization of several lines of rat liver cells in terms of the phenotypes related to transformation. The results are summarized in table 1. In culture, the IAR 20 cell line presents all the characteristics of normal epithelial cells and are attached firmly to the substratum; at confluence, the cells stop growing by contact inhibition and form a monolayer. The common markers of cellular transformation, such as the GGT production, growth in soft agar and tumorigenicity in the nude mice, are absent (table 1). IAR 6-1 and IAR 27, however, have GGT activity, grow in soft agar and produce tumours in nude mice (table 1); parent IAR 27 and IAR 6-1 cells produce undifferentiated sarcomas and carcinomas,

Table 1. *Characteristics of rat liver cell lines used for the study*

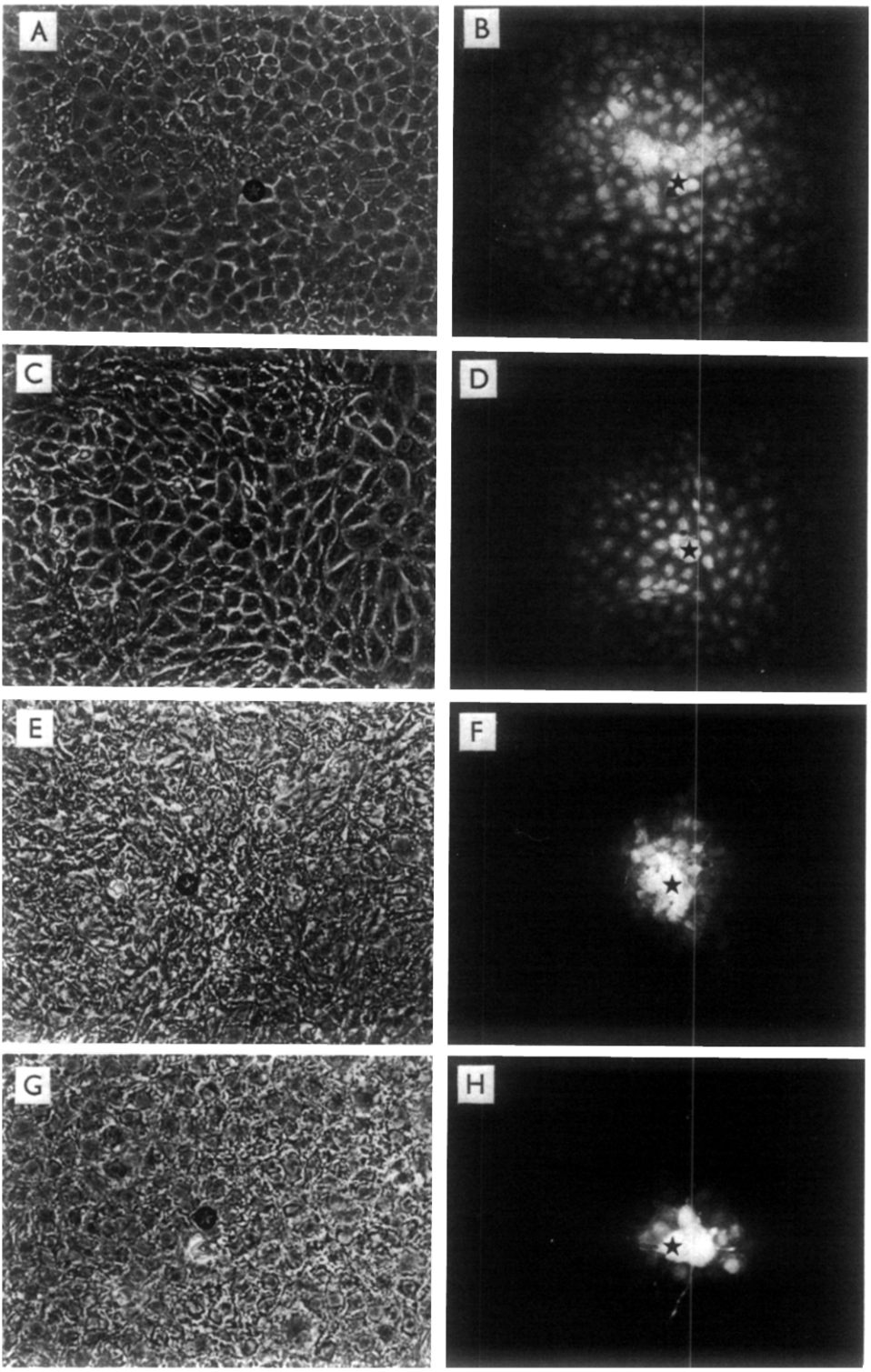
	Origin	Tumorigenicity in		Growth in soft agar	GGT activity (nmoles *p*-nitroaniline mg protein per 30 min	Intercellular communications capacity[b] No. coupled cells per injection
		Newborn rats[a]	Nude mice			
IAR 20	Rat BD VI (10 days)	0/7	0/8	$5/2\times10^4$	125.81	145
IAR 6-1	Rat BD VI (8 weeks) In-vitro transformation by DMN[c]	6/6	6/8	$52/2\times10^4$	617.50	55
IAR 27	Rat BD VI (10 weeks)	4/4	8/10	$251/2\times10^4$	520.00	ND
(IAR 27E)	Subclones	ND	ND	$22/2\times10^4$	222.86	90
(IAR 27F)	from IAR 27	ND	ND	$391/2\times10^4$	1 744.23	30

[a] Data taken from Montesano et al. [15].
[b] Data from fig. 1.
[c] Dimethylnitrosamine.

respectively, in nude mice at injection sites. These two cell lines were shown previously to produce carcinomas in newborn rats [15, 16]. The reason for the production of different types of tumours in nude mice and in newborn rats is not clear at present. Since we found that two different populations of cells existed in IAR 27 cell line, it is important to examine tumorigenicity of these two clonal populations.

The morphology of IAR 6-1 cells varies with the stage of culture. At subconfluence, the cells have irregular shapes, ranging from a nearly normal epithelial type (round and firmly attached to the substratum) to a fibroblastoid type (elongated, bipolar, or stellate, and loosely attached to the substratum). At confluence, the cells become more polygonal and show a mosaic-like pattern. Moreover, in the absence of cell–cell contact inhibition, they form multilayered 'ridges' around monolayered 'islands', giving the characteristic appearance of these confluent cultures. As the cultures age, the upper layers appear to develop trypsin resistance, are weakly attached to the lower layers and may detach themselves spontaneously after 3 weeks in culture. Frequent medium changes (every 48 h) appear to enhance this phenomenon. When the upper layers are

Fig. 1. Communication capacity of rat liver cell lines tested by dye transfer. (*A, C, E, G*) Phase-contrast micrographs of IAR 20, IAR 27 E, IAR 6-1 and IAR 27 F, respectively; (*B, D, F, H*) fluorescence micrographs of (*A, C, E, G*) respectively. ★, Cells injected with Lucifer Yellow CH and photographed 10 min later. Note the morphological aspect of each cell line and the decrease in communication capacity from IAR 20 to IAR 27 F. (A, C, E, G)× 160.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removed and placed in another Petri dish, they grow to form a monolayer and then ridges, which suggests that these cells are still viable.

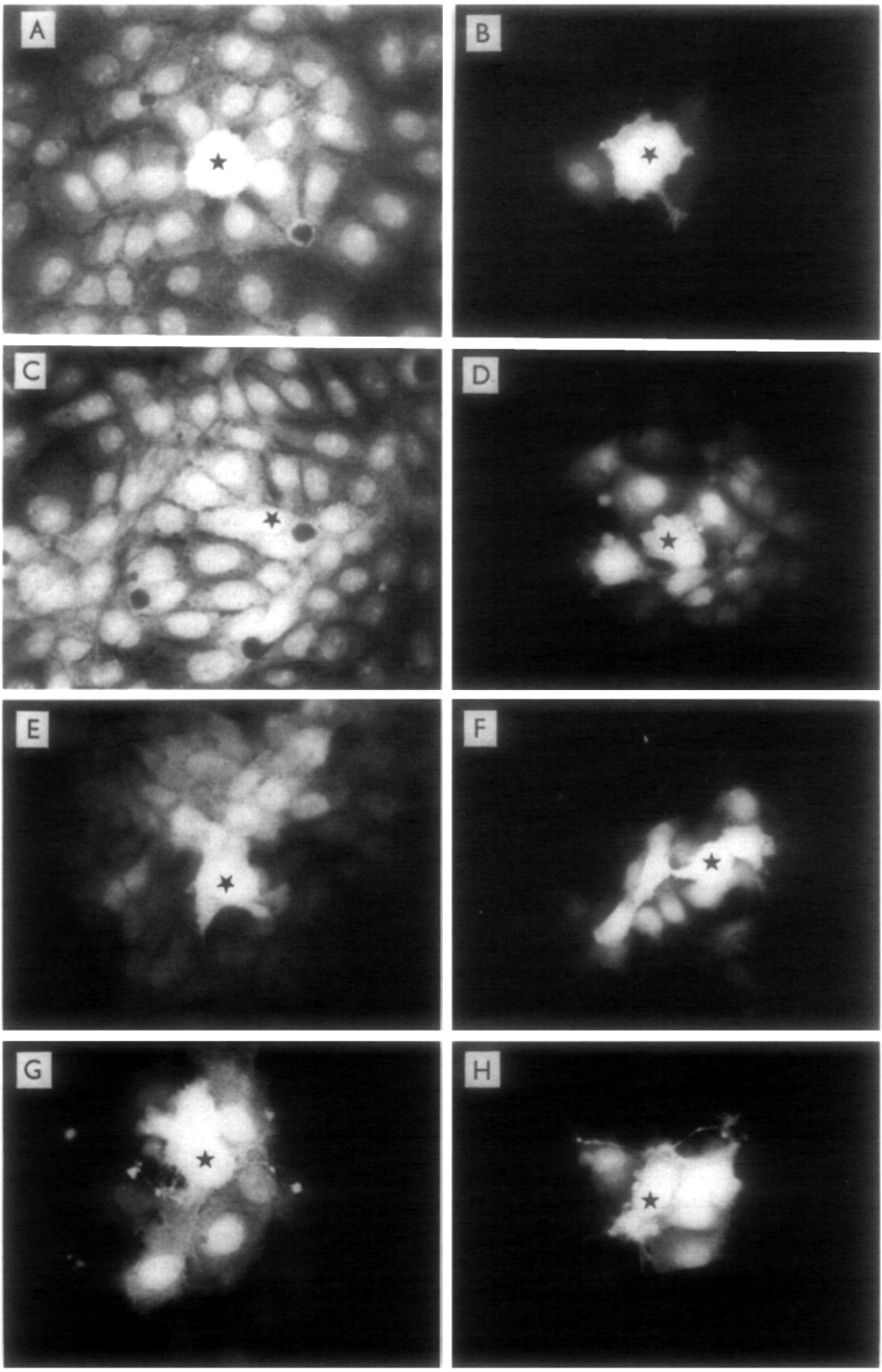
During serial passage of IAR 27 cells, at least two types of cell are observed. We cloned these two cell types and designated them IAR 27 E and IAR 27 F, since the former had epithelial-like morphology, whereas the latter looked like fibroblasts. These two new cell types did not form multi-layered cultures nor ridges. Although the tumorigenicity of these subclones has not been tested, both had GGT activity and grew in soft agar; IAR 27 F showed quite strong responses in these two assays.

Short-Term Effect of TPA on Intercellular Communication of Rat Liver Cell Line, Measured by the Dye-Transfer Method

All the cell lines studied had the capacity to transfer Lucifer Yellow CH into neighbouring cells, suggesting that they are all capable of communication through gap junctions. IAR 20 cells had the highest and IAR 27 F the lowest capacity for such communication (fig. 1). In general, there was an inverse relationship between the communication capacity of cell lines and their expression of transformed phenotypes (table 1).

Figs 2 and 3 depict the effect of a single application of TPA on communication in various lines of rat liver cells. It inhibits communication in all the lines tested, but the extent of inhibition differed: the greatest effect after 1 h incubation with TPA was seen in IAR 20, with 97% inhibition, and the lowest in IAR 27 F, with 59% inhibition. TPA did not alter the morphology of IAR 20 and IAR 27E cells, but it produced 'ridges' or a 'corded' structure of cell aggregates in IAR 6-1 and IAR 27 F cells. A similar effect had been observed previously [17]. Since inhibition of communication is not necessarily accompanied by morphological changes, the effect of TPA on the communication does not appear to be a result of general morphological changes to the cells. Due to cell aggregations, we encountered some difficulty in quantitatively determining the communication capacity in TPA-treated cultures of IAR 6-1 and IAR 27 F cells. Such difficulty was more pronounced when cells were treated at their confluence; however, at earlier stage of cell growth, it was relatively easy to count fluorescent cells, since cell aggregation was less. Since TPA effect shown in fig. 3 was studied with a young culture in which TPA produced little aggregation effect of cells, it appears that TPA inhibition of the communication is not due to morphological change induced by TPA.

Fig. 2. Short-term effect of TPA on intercellular communication of rat liver cell lines. (*A, C, E, G*) Fluorescence micrographs of the IAR 20, IAR 27 E, IAR 6-1 and IAR 27 F communication control respectively. (*B, D, F, H*) Fluorescence micrographs respectively, showing the communication of the IAR 20, IAR 27 E, IAR 6-1 and IAR 27 F cells, 30 min after TPA treatment (100 ng/ml). ★, Cells injected with Lucifer Yellow CH and photographed 10 min later. TPA was applied to confluent cultures, 3 days after seeding at 2×10^5 cells/dish. (*A, C, E, G*) ×400.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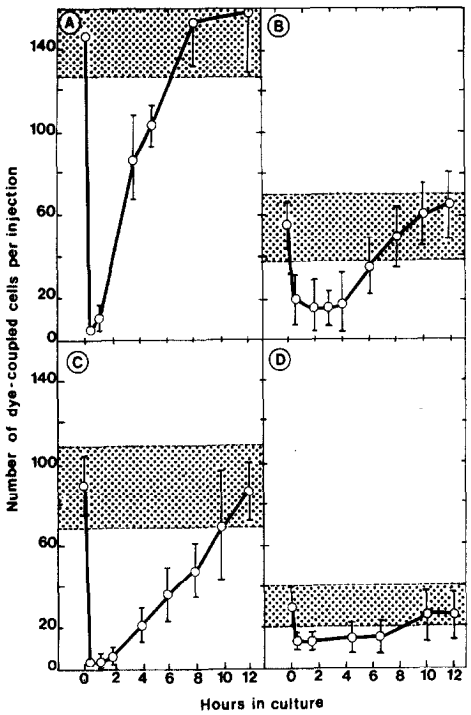

Fig. 3. Short-term effect of TPA on intercellular communication in rat liver cell lines. Cells were treated with 100 ng/ml TPA, and communication was measured by dye transfer at the times indicated. (*A*) IAR 20; (*B*) IAR 6-1; (*C*) IAR 27 E; (*D*) IAR 27 F.

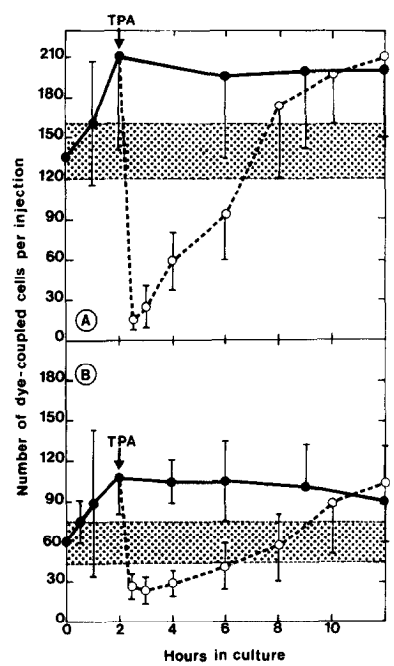

Fig. 4. Effect of db-cAMP on intercellular communication in IAR 20 and IAR 6-1 cells. The cells were treated with 1 mM db-cAMP; 2 h later, some cultures also received 100 ng/ml TPA (indicated by *arrows*). Communication capacity was measured at the times indicated as described in the text. (*A*) IAR 20; (*B*) IAR 6-1. ●, db-cAMP alone; ○, db-cAMP and TPA.

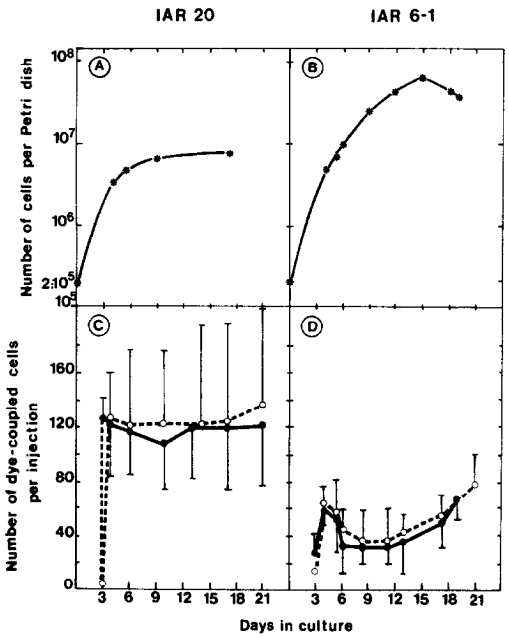

Fig. 5. Growth curves (*A, B*) and long-term effect of repeated additions of TPA on intercellular communication (*C, D*) of IAR 20 and IAR 6-1 cells. Intercellular communication was measured in cells cultured ○, with and ●, without 100 ng/ml TPA. TPA was added every day, and communication was measured 30 min after TPA addition.

After the initial inhibition, all four lines gradually recovered their capacity for intercellular communication (fig. 3). Since repeated additions of TPA did not diminish this capacity, TPA pretreatment probably made the cells refractory to further treatment (see below).

Effect of cAMP on Intercellular Communication in IAR 20 and IAR 6-1 Cells

Since cAMP is known to stimulate gap-junctional communication [21], and since it protects against the effect of TPA on communication in fibroblasts [1], we investigated its effect on rat liver epithelial cells. db-cAMP enhanced the capacity of both non-transformed (IAR 20) and transformed (IAR 6-1) cells to transfer the dye (fig. 4). The effect was maximal about 2 h after addition of db-cAMP, and the increased communication was seen for at least 12 h.

As shown in fig. 4, when TPA was added to db-cAMP-treated cells, there was inhibition of communication; the extent of TPA inhibition was similar to that seen without previous cAMP treatment. These results suggest that cAMP is not a TPA antagonist in rat liver epithelial cells, although it is a potent antagonist in BALB/c 3T3 cells [1].

Long-Term Effect of Repeated Additions of TPA on Communication in Rat Liver Cell Lines

As described above, there was a rapid recovery from the blockage by TPA of intercellular communication (fig. 3). Similar recoveries have been seen in many

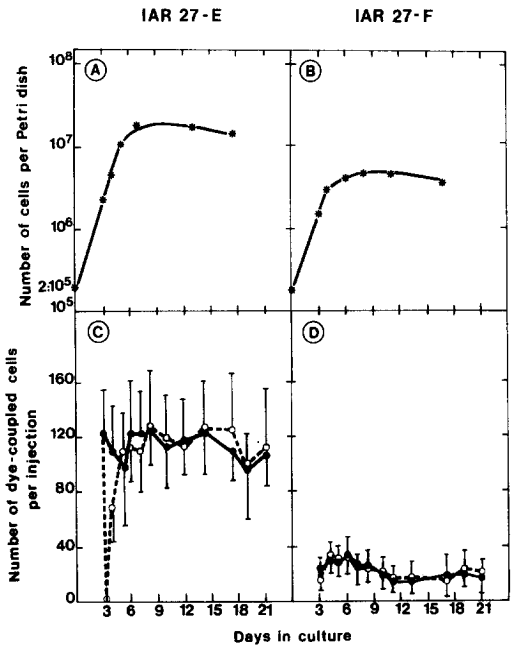

Fig. 6. Comparison of two subclones of IAR 27 cells for (*A, B*) growth; and (*C, D*) communication capacity, with and without repeated treatment with TPA. Cells were treated and communication measured as described in the caption to fig. 3. ●, Control; ○, TPA-treated.

different types of cell [1, 11, 22]. In recent studies with BALB/c 3T3 cells, however, we have shown that, if TPA is added repeatedly to culture media, intercellular communication is inhibited again when the culture reaches confluence, and this inhibition is maintained for a long time [2]. We therefore carried out long-term experiments on rat liver cells (figs 5, 6). No inhibitory effect of repeated applications of TPA was seen in any of the four cell lines, and the level of communication was similar to that in non-treated cultures (figs 5, 6). In these experiments, communication was measured 30 min after each TPA application; it is therefore unlikely that the failure to observe an effect of TPA was due to its metabolism. These results suggest that, if cells are exposed once to TPA, they become resistant to further additions of TPA over long period.

DISCUSSION

We have examined the intercellular communication capacity of various lines of rat liver cells and the effect of TPA. The capacity of these cell lines was relatively greater than that of fibroblasts examined in our laboratory, and the cell lines that exhibited more activity with regard to markers of transformed phenotypes (such as GGT activity, growth in soft agar, tumorigenicity) had a lower communication capacity. These findings are consistent with the idea that transformed cells exhibit more uncontrolled cell proliferation and, therefore, less regulation of cell–cell interaction through gap junctions [2]. This correlation may be restricted

to epithelial cells, since we observed previously that chemically transformed BALB/c 3T3 cells have a capacity to communicate that is similar to or greater than that of non-transformed BALB/c 3T3 cells [23]. Borek et al. [24] reported similar results.

Like many other cells, rat liver cells are also sensitive to inhibition of intercellular communication by TPA. However, the effect of TPA on these cells was transient: after a single application of TPA, there was rapid inhibition of intercellular communication, and then the cells recovered from the effect; further addition of TPA had no effect, even after repeated daily application for 3 weeks. A transient effect of TPA on intercellular communication of cultured rat liver cells was also reported by Walder et al. [25]. In recent studies with BALB/c 3T3 cells, we found that TPA-induced blockage of intercellular communication is only transient when the cells are growing, whereas further addition of TPA beyond confluence of the culture produces a stable and continuous inhibition of communication [2]. Such continuous inhibition of communication by phorbol esters correlates well with their enhancement of cell transformation. It suggests that TPA is not a strong promoter of transformation in rat liver cells. In order to see whether a known promoter in rat liver can produce stable inhibition of communication in IAR 20 cells, we incubated them with phenobarbital (2–200 μg/ml every 2 days) for 3 weeks: there was no effect on the intercellular communication (unpublished results). Further studies are necessary to investigate a possible correlation between blocked intercellular communication and tumour promotion in rat liver.

In order for cells to maintain their transformed phenotype, they must not communicate via gap junctions with surrounding normal cells, since it is reasonable to assume that normal cells neutralize the transformed phenotype by transferring physiological factors necessary for the normal function of cells. Recently, we demonstrated that chemically transformed BALB/c 3T3 cells do not communicate with surrounding non-transformed counterparts [23]. Since study of communication between transformed and non-transformed epithelial cells is hindered by the fact that epithelial cell transformation cannot readily be brought about in culture, we had to carry out a reconstruction experiment, i.e., co-culture of non-transformed (IAR 20) and transformed (IAR 6-1 and IAR 27) cells, to see whether transformed rat liver cells communicate with non-transformed cells. In these studies, we observed no communication between IAR 20 and IAR 6-1 or IAR 20 and IAR 27 F cells, suggesting the existence of selective communication between transformed and non-transformed rat liver cells [26]. It was also noted that there is no communication between chemically transformed and spontaneously transformed cell lines, i.e., IAR 6-1 and IAR 27 E, or IAR 6-1 and IAR 27 F. IAR 27 E cells, which exhibit the least transformed phenotypes, however, communicate with non-transformed IAR 20 cells. These results are consistent with the idea that transformed cells do not communicate with their non-transformed counterparts. It is now important to develop an experimental system in animals in which these

results can be confirmed, in order to see whether our findings on selective communication in vitro are also relevant in vivo.

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