TRANSLOCATION OF A FLUORESCENT LIPID PROBE BETWEEN CONTACTING CELLS

Evidence for membrane lipid interactions

J. G. COLLARD and A. DE WILDT
The Netherlands Cancer Institute, Antoni van Leeuwenhoek Laboratory, Division of Cell Biology, 108 Sarphatistraat, Amsterdam, The Netherlands

and

M. INBAR
The Weizmann Institute of Science, Department of Membrane Research, Rehovot, Israel

Received 2 March 1978

1. Introduction

Interaction and communication between animal cells are important in the coordinated growth and function of cells and tissues. Direct communication and metabolic cooperation has been detected between cultured cells [1–3], and experimental evidence suggests that exchange of ions or small molecules between touching cells occurs through gap junctions [4,5]. Recent studies on interactions between artificial lipid vesicles and whole cells indicate that lipid components of the vesicle membranes [6–8] and substances trapped inside the vesicles [9,10] can be transferred into the cells. The actual mechanism of the vesicle–cell interaction is not yet quite clear, but fusion and endocytotic processes seem to be involved, depending on the composition of the vesicles [11,12].

In our investigations on membrane lipid interactions between contacting cells we used the lipophilic fluorescent probe DPH (1,6-diphenyl-1,3,5 hexatriene), which can be incorporated into membranes of intact cells. This probe has been used in many investigations to determine the degree of fluorescence polarization (P) and microviscosity ($\tilde{\eta}$) of lipid vesicles, isolated plasma membranes and membranes of intact cells [13–17]. We now use DPH to demonstrate an easy transfer of the fluorescent probe, localized in the membrane lipids, from one cell to another, even when these cells have only transient contact. The possibility that DPH is transferred through the incubation medium was carefully checked and excluded.

We present evidence here for direct membrane lipid interactions between contacting cells, consisting of translocation between intact cells of DPH embedded in the membrane lipid bilayer. The results suggest another way of intercellular communication, namely by direct membrane lipid interactions, in addition to, e.g., the communication through intercellular junctions.

2. Materials and methods

2.1. *Tissue culture*

Swiss albino untransformed- and SV40-virus-transformed 3T3 cells were cultured as in [18]. 3T3 cells were grown to confluency with or without [^{3}H]thymidine (0.05 μCi/ml) in 10% serum in 8 cm Petri dishes (approx. 3×10^6 cells/dish) and used as attached monolayer cells. Suspended cells were obtained by trypsinization of confluent cell cultures (0.02% trypsin in phosphate buffered saline, PBS). After that, the cells were washed twice with trypsin inhibitor in PBS (0.01%). YAC lymphoma cells were grown in vivo as an ascites form of a Moloney virus-induced lymphoma in A strain mice [19].

2.2. *Labelling procedure*

Dishes containing confluent attached 3T3 monolayer cells, previously grown for 48 h in [^{3}H]TdR, were washed 3 times with PBS and subsequently labelled during 30 min at 25°C with a stable aqueous dispersion (5×10^{-6} M) of the fluorescent hydrocarbon 1,6-diphenyl-1,3,5 hexatriene (DPH, Koch Light Labs Ltd) which was practically void of fluorescence [17]. After the labelling procedure the dishes were washed 6 times with excess PBS and used as attached DPH-labelled monolayers in the experiments. Suspended 3T3 cells and/or YAC lymphoma cells were labelled with the same DPH dispersion (2×10^6 cells/ml) during 30 min at 25°C. Next, the labelled cells were washed 4 times with PBS by centrifugation and used as suspended labelled cells. Unlabelled cells, used in the experiments, were treated in the same way except that the labelling procedure was performed with PBS instead of the DPH dispersion.

2.3. *Translocation experiments*

Suspended unlabelled 3T3 cells, 3×10^6, were layered in 3 ml PBS on DPH and [^{3}H]TdR-labelled attached 3T3 monolayers. After incubation at room temperature the suspended cells were collected from the monolayer and next, the monolayer cells were harvested by trypsinization. The fluorescent signal (corrected for scatter) from both cell suspensions was measured in a Zeiss fluorimeter or in an Elscint microviscosimeter. After that, the radioactivity of the samples was measured in a Liquid Scintillation Counter. To that purpose, the cell suspensions were centrifuged. The cell pellets were incubated in 1 ml Soluene-100 (Packard) tissue solubilizer during 2 h at 50°C, diluted with 10 ml Dimilume (Packard) scintillation solution and counted.

Translocation experiments without attached monolayer cells were performed as follows. Carefully washed labelled cells were mixed in known ratio's with unlabelled cells. At different times after incubation the degree of fluorescence polarization (P) at 25°C from the mixed cultures was determined in the Elscint microviscosimeter (MV-1) [17].

3. Results and discussion

Different kinds of cells, either in suspension or as attached monolayers, were labelled in phosphate buffered saline (PBS) with DPH. The excess of DPH was removed after the labelling procedure by extensively washing the plates or cell suspensions. The last wash medium was checked for its capacity to label other cells. No fluorescent signal could be detected.

In one series of experiments the transfer of DPH between cells was measured by the appearance of the fluorescence signal in the unlabelled cells and the concomitant decrease of the fluorescent signal in the labelled cells. Table 1 shows the time-dependent transfer of DPH from DPH-labelled monolayer cells into unlabelled suspended cells layered onto these monolayers. Under these experimental conditions no cells stuck to the monolayer and all suspended cells could be removed completely by washing. On the other hand, practically no radioactivity from the [^{3}H]TdR prelabelled attached monolayers was found in the incubation medium indicating that no cells from the monolayer became detached from the dishes (table 1). The appearance of the fluorescent signal in the unlabelled suspended cells went parallel with a decrease in signal in the labelled cells, whereas the total fluorescent signal from both cells remained constant. Similar results were obtained in the reverse experiments which demonstrated the transfer of DPH from labelled suspended cells to unlabelled cells in monolayers. PBS exposed during 40 min to labelled cells was unable to label other cells. This excluded the possibility of DPH transfer through the incubation medium by leakage or damage of cells (table 1).

The next experiment was also performed to exclude a transfer of DPH through the incubation medium. Unlabelled cells were suspended in PBS or in Ficoll–Isopaque mixture and layered onto labelled monolayers. In addition, a suspension of unlabelled cells was carefully layered on top of a thin film of Ficoll–Isopaque to prevent contact between suspended and attached cells. Under these conditions no exchange of DPH occurred, whereas cells suspended in Ficoll–Isopaque showed approx. 60% less transfer from the monolayers than cells suspended in PBS (table 2). This indicates again that DPH transfer is not due to leakage of DPH from the labelled cells into the medium and that physical cell contact is required for exchange of DPH from one cell to another.

Another approach to show the exchange of DPH between cells it to mix cell suspensions showing a different degree of fluorescence polarization [16,17]

Table 1
Decrease in relative fluorescent signal in unlabelled 3T3 cells layered on DPH labelled attached 3T3 cells

	Relative DPH fluorescence							[^{3}H]TdR radioact. (dpm)
Incubation (min)	0	5	10	15	20	30	40	40
Attached DPH-labelled monolayer[a]	100.0	–	–	–	–	–	–	27 500
Suspended unlabelled cells layered on monolayer	2.8	5.6	8.9	17.2	23.0	27.1	32.0	250
Attached DPH-labelled monolayer[a]	100.0	–	–	–	–	–	98.6	29 000
PBS control without suspended cells	1.2	1.3	1.6	2.3	2.5	2.6	2.9	140
Suspended unlabelled cells incubated during 30 min with PBS from PBS control	–	–	–	–	–	3.1	–	100

[a] Attached DPH-labelled monolayers were prelabelled with [^{3}H]thymidine (0.05 μCi [^{3}H]TdR/ml, 48 h) to exclude the possible release of DPH-labelled cells in the incubation medium

The presented results are average values of 3 identical expt

Table 2
DPH transfer into unlabelled 3T3 cells layered during 30 min on labelled 3T3 monolayer cells

	Relative fluorescent signal after 30 min	
	Unlabelled cells in suspension	Labelled monolayer cells
Control incubation in PBS without unlabelled suspended cells	1.0	100.0
Unlabelled cells layered on labelled monolayer cells in PBS	33.1	69.0
Unlabelled cells layered on labelled monolayer cells in PBS–Ficoll–Isopaque mixture	9.2	88.7
Unlabelled cells layered on labelled monolayer cells on a thin Ficoll–Isopaque film	0.6	98.4

The presented data are average values of 2 identical experiments. The Ficoll–Isopaque film (density 1.077 g/ml) was made by adding 5 ml Ficoll–Isopaque to the labelled monolayers resulting in a layer of 1 mm thickness. Cells were layered on this film in 3 ml PBS. The PBS–Ficoll–Isopaque mixture was made with the same volumes

(*P*) obtained from DPH embedded in the membranes of intact cells. This technique is specially suitable to demonstrate translocation of DPH in cases where no monolayers are available (e.g., different kinds of lymphoid cells), but it requires a difference in the degree of fluorescence polarization between the cells tested. If this difference is too small, the two kinds of cells have to be separated after interacting with each other in order to measure the fluorescent signal in each fraction.

Labelled cells with a known *P* value were mixed with unlabelled cells with a different *P* value. Next, the change in the average degree of fluorescence polarization of the mixed cultures was measured. In this way, the contribution to the *P* value by the unlabelled cells, due to transfer of DPH from labelled cells, could be measured. A representative example of such an exchange between labelled and unlabelled cells is given in fig.1. The change in *P* value in the mixed cell suspensions was already detectable after a few minutes, indicating a rapid transfer of DPH from

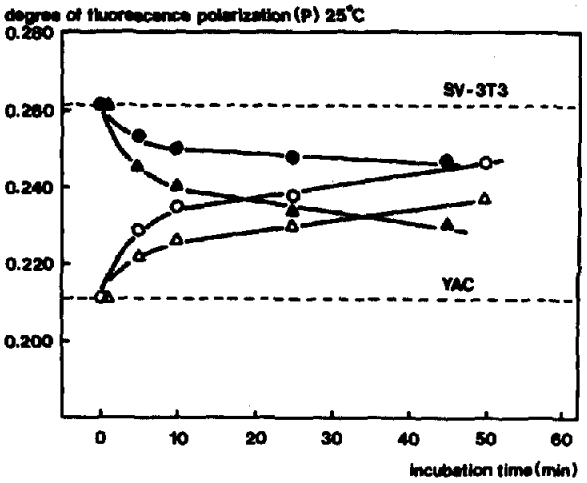

Fig.1. DPH labelled and unlabelled suspended cells were mixed and at different times after incubation, the degree of fluorescence polarization from the mixed cultures was determined at 25°C. (●-●) Labelled SV3T3 with unlabelled YAC cells, ratio 1:2. (▲-▲) Labelled SV3T3 with unlabelled YAC cells, ratio 1:6. (○-○) Unlabelled SV3T3 with labelled YAC cells, ratio 1:2. (△-△) Unlabelled SV3T3 with labelled YAC cells, ratio 1:6. The degree of fluorescence polarization (*P*-value) of pure SV3T3 and YAC cells was 0.261 and 0.211, respectively (dotted lines). The cell volume of SV3T3 was approx. 3.5-times larger than that of YAC cells.

cells with a higher *P* value to cells with a lower *P* value or the reverse, depending on what cells were labelled. Cells artificially suspended with trypsin or EDTA show a similar DPH exchange with labelled cells. Translocation experiments at 0°C in the presence of azide (30 mM), or glutaraldehyde fixation (2%) of cells did not affect the exchange of DPH between cells. This suggests that the process of transfer is not mediated by enzyme catalyzed processes. No cell clumps or cell aggregates were formed during the incubation period indicating that no fusion of cells had occurred. In fact, the DPH exchange between glutaraldehyde fixed cells already indicates that fusion of cells cannot be the cause of the change in the degree of fluorescence polarization of the mixed cultures.

The results in fig.1 indicate that DPH does not transfer preferentially to more fluid (low *P* value) or more rigid (high *P* value) membranes. Similar experiments performed in a viscous medium of 0.5% methyl cellulose in PBS, which hinders cell contact, show that this medium reduced the translocation of DPH in the same way as the Ficoll–Isopaque mixture did in the experiments described in table 2. Translocation of DPH to a greater or smaller extent was found between several kinds of cells. In the experiments pairs of normal and transformed fibroblasts, fibroblasts and tumour ascites cells or normal thymocytes from mice, rats and hamsters were used. In all cases DPH transfer between cells was observed. The most likely explanation for the DPH exchange is that during short cell contact, membrane lipid interactions occur resulting in DPH translocation from one cell to another. The resulting degree of fluorescence polarization of the mixed cell suspensions is dependent on the ratio labelled–unlabelled and on the size of the cells (fig.1). The major parameter that determines the translocation of DPH is possibly the concentration ratio of DPH over the total membrane lipids of the two kinds of cells tested. According to this, the system eventually will reach a steady state situation.

In view of the above data it would be of interest to know how other membrane lipid probes behave during similar cell interactions. It is likely that the degree of exchange depends on the position in and interaction with the lipid bilayer. As yet, it is unclear whether the translocation of DPH occurs by rapid diffusion of DPH only between the contacting mem-

branes or/and by fusion and exchange of small parts of membranes or components of membranes. In both cases direct contact between membrane lipid regions of cells seems to be required. Because glutaraldehyde fixation of cells does not affect the DPH translocation, such 'naked' membrane lipid regions probably are not formed dynamically during cell contact but are present all the time on cells.

A similar exchange of DPH has been found between two populations of artificial membranes mixed with one another [20]. In addition, exchange of DPH and lipid components between liposomes and intact cells have been observed [23]. Recently a transfer of glycolipids [21,22] and phospholipids [24] between membranes of intact cells has been described. Thus it seems that molecules, artificially embedded in the membrane lipid bilayer and possibly also molecules belonging to the cell membrane layer itself (e.g., phospholipid, cholesterol) are able to translocate (c.q. be translocated) from one cell to another. Exchange possibilities of several natural membrane components during transient and functional cell contact, involving, e.g., phospholipids or cholesterol are at the present under investigation.

Acknowledgement

The excellent technical assistance of Mrs N. Harpaz during parts of this work is greatly appreciated.

References

[1] Loewestein, W. R. and Kanno, Y. (1964) J. Cell Biol. 22, 565–586.
[2] Subak-Sharpe, H., Burk, R. R. and Pitts, J. D. (1969) J. Cell Sci. 4, 353–367.
[3] Pitts, J. D. and Simms, J. W. (1977) Expl. Cell Res. 104, 153–163.
[4] Revel, J. P. and Karnovsky, M. J. (1976) J. Cell Biol. 33, C7–C12.
[5] Gilula, N. B., Reeves, O. R. and Steinbach, A. (1972) Nature 235, 262–265.
[6] Huang, L. and Pagano. R. E. (1975) J. Cell Biol. 67, 38–48.
[7] Dunnick, J. K., Rooke, J. D., Aragon, S. and Kriss, J. P. (1976) Cancer Res. 36, 2385–2389.
[8] Dunnick, J. K., Kallman, R. F. and Kriss, J. P. (1976) Biochem. Biophys. Res. Commun. 73, 619–624.
[9] Papahadjopoulos, D., Poste, G. and Mayhew, E. (1974) Biochim. Biophys. Acta 363, 404–418.
[10] Weinstein, J. N., Yoshikami, S., Henkart, P., Blumenthal, R. and Hagins, W. A. (1977) Science 195, 489–491.
[11] Pagano, R. E. and Huang, L. (1975) J. Cell Biol. 67, 49–60.
[12] Poste, G. and Papahadjopoulos, D. (1976) Proc. Natl. Acad. Sci. USA 73, 1603–1607.
[13] Shinitzky, M. and Inbar, M. (1974) J. Mol. Biol. 85, 603–615.
[14] Shinitzky, M. and Inbar, M. (1976) Biochim. Biophys. Acta 433, 133–149.
[15] Barenholz, Y., Moore, N. F. and Wagner, R. R. (1976) Biochemistry 15, 3563–3570.
[16] Van Blitterswijk, W. J., Emmelot, P., Hilkman, H. A. M., Oomen-Meulemans, E. P. M. and Inbar, M. (1977) Biochim. Biophys. Acta 467, 309–320.
[17] Collard, J. G., De Wildt, A., Oomen-Meulemans, E. P. M., Smeekens, J., Emmelot, P. and Inbar, M. (1977) FEBS Lett. 77, 173–178.
[18] Collard, J. G. and Temmink, J. H. M. (1976) J. Cell Biol. 68, 101–112.
[19] Klein, E. and Klein, G. (1964) J. Natl. Cancer Inst. 32, 547–568.
[20] Lentz, B. R., Barenholz, Y. and Thompson, T. E. (1976) Biochemistry 15, 4529–4537.
[21] Huang, R. T. C. (1976) Z. Naturforsch. 31C, 737–740.
[22] Huang, R. T. C. (1977) Z. Naturforsch. 32C, 379–383.
[23] Pagano, R. E. and Huang, L. (1975) J. Cell Biol. 67, 49–60.
[24] Peterson, J. A. and Rubin, H. (1970) Expl. Cell Res. 60, 383–392.