CELLULAR INTERACTION BETWEEN FIXED AND LIVING CELLS

Transfer of Radioactive Materials from Living Cells to Fixed Cells

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ABSTRACT

Transfer of radioactive materials to fixed cells from an overlying layer of living cells has been examined to determine whether fixed cells can act as acceptors of glycosyltransferases of living cells. After the incubation of living cells lying upon fixed cells along with radioactive precursor, the living cells were removed by EDTA treatment, and the radioactivity associated with the fixed cells was determined.

Lipids, proteins, and carbohydrates were found to be transferred from the living cells to the fixed cells. The amount of radioactivity transferred to the fixed cells was dependent on the number of both fixed and living cells and increased with the time of incubation. When fixed cells were treated with chloroform-methanol before the addition of living cells, the transfer of both lipids and proteins to the fixed cells decreased drastically, but only a slight decrease in carbohydrate transfer was observed.

Most of the radioactive materials transferred from the living cells labeled with glucosamine or fucose to chloroform-methanol-treated fixed cells were solubilized by trypsin but not by the detergents tested. Approximately 55% of the materials transferred from the cells labeled with glucosamine could be solubilized by hyaluronidase and chondroitinase, and the rest was solubilized by neuraminidase and a glycosidase mixture. The treatment of chloroform-methanol-extracted fixed cells with trypsin caused a significant decrease in the transfer from cells labeled with glucosamine.

When nucleotide sugars were used as the radioactive precursor, no significant amount of radioactivity was transferred to the fixed cells.

KEY WORDS fixed cells living cells glycosylation membrane fusion glycosyltransferases CMP-sialic acid lipids

The cell surface has been widely found to have an important role in many biological processes including cell-cell recognition and intercellular adhesion (10, 19). Malignant transformation of cultured cells is characterized by alterations in several growth-related properties, often accompanied by changes in membrane components such as glycoproteins (5, 21, 27) and glycolipids (3, 22). A
number of investigators have demonstrated that glycosyltransferases which catalyze the transfer of mohosaccharide residues from nucleotide sugars to acceptor glycoproteins or glycolipids are present on the cell surface (8, 14, 28). Others have questioned, however, whether the glycosyltransferases are located on the cell surface (7).

Roth and White have suggested that highly contact-inhibited 3T3 cells catalyze the transfer of galactose from uridine diphosphate galactose to acceptors on adjacent cells (trans-glycosylation), whereas non-contact-inhibited 3T12 cells catalyze this transfer to acceptors located on the same cells (cis-glycosylation) (20). Utilization of exogenously added nucleotide sugar in this experiment has, however, been questioned (2, 4). Furthermore, membrane fusion is thought to be a frequent and important event in the life of most eukaryotic cells (9, 12, 13, 16), and exchange of materials can be expected between adjacent cells. Therefore, if living cells are employed for the acceptors of glycosyltransferases, the exchange of membrane components between cells should make the experimental results obscure. Thus it must be difficult to distinguish trans or cis-glycosylation.

In the present work, we examined fixed cells to determine whether they can act as acceptors of glycosyltransferases. Our results indicate that various components including carbohydrates were transferred to fixed cells from living cells.

MATERIALS AND METHODS

Cell Culture

Nil 2Cl cells, a clone of Nil hamster embryo fibroblasts (22, 23), and A31-714 cells, a recloned line of A31 (6), were grown in Eagle's minimal essential medium (MEM, Nissui Seiyaku Co., Tokyo, Japan) supplemented with 10% calf serum in plates (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) under the conditions of humidified 5% CO₂-95% air at 37°C. Except when stated, experiments were carried out with Nil 2Cl cells.

Preparation of Fixed Cells and Radiolabeling Procedures

Square (9 × 35 mm) or round (25 mm) cover slips (Lux Scientific Corp., Newbury Park, Calif.) were placed in 60-mm or 30-mm plates, respectively, and cells were placed on them. The number of cells on the cover slips was determined with a Coulter counter (Coulter Electronics Inc., Hialeah, Fla.) after dispersing the cells with 0.05% trypsin/EDTA solution (2% EDTA wt/vol in Dulbecco's phosphate-buffered saline minus Ca²⁺ and Mg²⁺). At an appropriate density, the cells were fixed with 3% glutaraldehyde EM (TAAB Laboratories, Reading, England) for 30 min as already described (24). To extract lipids from the fixed cells, the cells were treated twice with chloroform-methanol (2:1) and washed twice with methanol and twice with phosphate-buffered saline. The cover slips with fixed cells were placed in plates, and living cells were placed upon them. A radioactive precursor was added to the culture medium at the time of this inoculation. Leucine-free F-10 medium with 10% calf serum or glucose-free MEM with 10% calf serum was used for leucine or glucose labeling, respectively.

Determination of Radioactivity Transferred to Fixed Cells

After incubation of fixed cells with living cells in the presence of the radioactive precursor, the cover slips were rinsed once with EDTA and incubated in EDTA solution (2 ml) for 30 min to remove the living cells. The suspension of cells was gently pipetted over the surface of the fixed cells to detach the living cells, and the detached cells were kept in ice until counted. The procedure was repeated, and then the surface of the fixed cells was rinsed three times with 1 ml of phosphate-buffered saline. Living cells removed by this method were pooled, and the TCA-insoluble radioactivity was determined. The fixed cells were washed thoroughly with running tap water to insure removal of all living cells. No living cells could be found by microscopy after this washing. No significant amount of fixed cells was removed from the cover slips by these vigorous treatments. The radioactivity remaining bound to the fixed cells was determined by using Bray's scintillator. Protosol (New England Nuclear, Boston, Mass.) was used to solubilize cells labeled with ³H-labeled precursors.

Characterization of Radioactive Materials Transferred to Lipid-Extracted Fixed Cells

After the removal of the living cells, the radioactive components remaining attached to the fixed cells were characterized by treating them with the following enzymes: trypsin (type IV, Sigma Chemical Co., St. Louis, Mo.; 5 mg/ml in phosphate-buffered saline); chondroitinase ABC (Seikagaku Kogyo Co., Tokyo; 0.25 U/ml in 0.1 M acetate, pH 6.5, 0.5 mM MgCl₂, and 0.5 mM CaCl₂ buffer); gastropod α-L-fucosidase (0.5 U/ml in 0.1 M acetate, pH 4.0, 0.1 M NaCl buffer); testicular hyaluronidase (Sigma type VI, 300 U/ml in 0.1 M acetate, pH 4.8, 0.1 M NaCl buffer); neuraminidase from Vibrio cholerae (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.; 100 U/ml in 9 mM potassium phosphate, pH 6.5, 0.5 mM MgCl₂, and 0.5 mM CaCl₂ buffer); gas tropod α-L-fucosidase (0.5 U/ml in 0.1 M acetate, pH 4.0, 0.1 M NaCl buffer); gastropod glycosidase mixture (0.1 M acetate, pH 4.0, 0.1 M NaCl buffer).
Autoradiography

For autoradiographic analysis of fixed cells, chloroform-methanol-treated fixed cells were overlaid with living cells and incubated in the presence of \(^{3}H\)glucosamine. After the removal of the living cells, the fixed cells were treated with hyaluronidase and chondroitinase for 1 h, respectively, as described in Materials and Methods. Then the cover slips were dipped in Sakura NR-M2 Emulsion (Konishiroku Photo Ind. Co., Tokyo, Japan), dried, and exposed for 6 d at 4°C. The cover slips were developed and stained with hematoxylin and eosin.

Analysis of Lipids

Lipids of the fixed cells and of the living cells were extracted and analyzed by thin-layer chromatography according to the method described previously (17).

Materials

\(^{14}C\)PALMITATE (59 mCi/mmol), \(^{14}C\)GLUCOSAMINE (59 mCi/mmol), \(^{3}H\)JUCOSAMINE (20.7 Ci/mmol), L-\(^{3}H\)FUROSE (12.1 Ci/mmol), \(^{3}H\)GLUCOSE (4.9 mCi/ mmol), L-\(^{3}H\)LUECINE (280 μCi/mmol), \(^{3}H\)CMP-SIALIC ACID (2,330.0 μCi/mmol), \(^{14}C\)GDP-FUCOSE (174 mCi/ mmol), and \(^{3}H\)UDP-GUAROSAMINE (6.6 Ci/mmol) were purchased from New England Nuclear. α-L-FUCOSIDASE and the glycosidase mixture were gifts from Dr. T. Muramatsu (Kobe University, Hyōgo, Japan) (11). Tunisicamycin was a gift from Dr. G. Tamura (University of Tokyo, Tokyo, Japan) (25).

RESULTS

Fixation of Cells and Cultivation of Living Cells on Fixed Cells

Cells grown on cover slips were fixed with glutaraldehyde. These cells did not come off the cover slips when treated with 0.05% trypsin/2% EDTA solution at 37°C overnight and washed with running water. Therefore, it was possible to separate fixed cells from living cells after incubation of living cells on the fixed cells. Our preliminary experiments showed that cells fixed with methanol, acetic acid, or TCA came off the cover slips by the following day if the fixed cells were incubated with living cells. When cells were fixed with osmium tetroxide, they remained attached to the cover slips more than a week, even in the presence of living cells. However, they tended to detach from the cover slip by the pipetting over the surface of the fixed cells. Thus glutaraldehyde-fixed cells were used for the following experiments. The shape of glutaraldehyde-fixed cells remained unchanged after the extraction of lipids with chloroform-methanol or mild acid hydrolysis (0.05 N H₂SO₄, at 80°C for 1 h).

No significant difference was found in the growth rate of cells on fixed cells and on the bare plate (24).

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Transfer of Radioactive Materials from Living Cells to Underlying Fixed Cells

When fixed cells were incubated with radioactive palmitate (Fig. 3A), leucine (Fig. 3B), glucosamine (Fig. 3C), or glucose (data not shown) in the absence of living cells, no significant radioactivity was incorporated into the fixed cells. Fixed cells also did not take up proteins labeled with glucosamine, fucose, or leucine after 24-h incubation.
Transfer of radioactive materials from living cells to underlying fixed cells was observed when cells were incubated with radioactive glucose (Fig. 1), palmitate (Fig. 3A), leucine (Fig. 3B), glucosamine (Fig. 3C), or fucose (Fig. 3D). The incorporation of each radioactive precursor into living cells increased linearly, at least up to the period indicated after the inoculation, and concomitant increase was observed in the amount of radioactivity transferred from living cells to fixed cells (Figs. 1 and 3). The amount of radioactivity transferred to fixed cells was dependent on the number of both fixed (Fig. 2A) and living cells (Fig. 2B).

**Effect of Chloroform-Methanol Treatment of Fixed Cells on the Transfer of Radioactive Materials from Living Cells**

The effect of the extraction of lipids from fixed cells was examined with respect to the amount of radioactivity transferred from living cells to fixed cells. The attachment of living cells to the surface of chloroform-methanol-treated fixed cells was not significantly changed from that of those on the nontreated fixed cells (data not shown). The amount of radioactive materials transferred to chloroform-methanol-treated fixed cells was drastically reduced when living cells were labeled with palmitate, leucine (Fig. 3A and B), or glucose (data not shown), but only a slight decrease was observed when living cells were labeled with either glucosamine or fucose (Fig. 3C and D).

When a varying number of chloroform-methanol-treated fixed cells were overlaid by the same number of living cells and the cells were labeled with [3H]glucosamine, the amount of radioactivity transferred to the fixed cells was dependent both on the number of fixed cells and on the time of incubation (Fig. 4).

**Characterization of Radioactive Materials Transferred to Fixed Cells; An Analysis of Chloroform-Methanol-Extractable Radioactivity**

The proportion of chloroform-methanol-extractable radioactivity of both living and fixed cells was measured and is shown in Table I. When the cells were labeled with either palmitate or glucose, a significant portion (77 or 21%, respectively) of the radioactivity transferred to fixed cells was extractable with the solvent. In contrast, little or no significant radioactivity was extracted by the treatment when the cells were labeled with glucosamine or fucose. If fixed cells were treated with chloroform-methanol before inoculation with living cells, the solvent-extractable radioactivity in the fixed cells was reduced significantly (about one-fifth of nontreated fixed cells) when glucose or glucosamine was a radioactive precursor. A reduction in the solvent-extractable radioactivity was also observed when palmitate was used as a precursor, although the ratio of the solvent-extractable to the nonextractable radioactivity was not significantly altered.

The composition of the lipids transferred to the fixed cells was compared with that of the overlying living cells (Table II). In these experiments, cells were labeled with either palmitate or glucose for 2
Table I

Cell-Associated Radioactivity Extractable by Chloroform-Methanol (C-M)

| Pretreatment of cells with C-M | Fixed cells | Living cells |
|-------------------------------|-------------|--------------|
|                               | C-M extractable cpm (%) | C-M nonextractable cpm (%) | C-M extractable cpm (%) | C-M nonextractable cpm (%) |
|                               | (%)         | (%)          | (%)                       | (%)                         |
| +                             | 880 (62.2)  | 535 (37.8)   | 97,620 (71.7)             | 38,617 (28.3)               |
| +                             | 906 (62.9)  | 534 (37.1)   | 113,400 (71.4)            | 45,471 (28.6)               |
| −                             | 6,100 (77.8)| 1,743 (22.2) |                        |                            |
| −                             | 3,816 (74.9)| 1,279 (25.1) |                        |                            |
| +                             | 28 (4.1)‡  | 623 (95.9)   |                        |                            |
| +                             | 33 (4.9)‡  | 634 (95.1)   |                        |                            |
| −                             | 414 (21.3)§| 1,534 (78.8) | 7,821 (11.5)            | 60,031 (88.5)              |
| −                             | 465 (21.9) | 1,678 (78.1) | 8,286 (11.2)            | 65,800 (88.8)              |
| +                             | 16 (0.9)¶  | 1,793 (99.1) |                        |                            |
| +                             | 22 (1.1)¶  | 1,991 (98.9) | 4,100 (12.1)            | 27,500 (87.9)              |
| −                             | 143 (4.9)¶ | 2,786 (95.1) | 3,268 (12.2)            | 22,804 (87.8)              |
| −                             | 108 (3.8)¶ | 2,709 (96.2) |                        |                            |
| −                             | 32 (1.4)¶  | 2,196 (98.6) | 88 (0.6)                | 15,785 (99.4)              |
| +                             | 29 (1.1)¶  | 2,602 (98.9) | 98 (0.7)                | 13,453 (99.3)              |

Cells were fixed at confluence on square cover slips and treated with chloroform-methanol. They were inoculated with living cells and, after the appropriate time of incubation the living cells were removed and trapped on glass-fiber filters. Both the living and fixed cells were dried down, and lipids were extracted with chloroform-methanol (2:1). Aliquots of extracts were counted in Toluene-2,5-diphenyloxazole-(PPO) 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP).

* 3.1 x 10⁵ cells per cover slip were laid over and labeled with [¹⁴C]palmitate (1 μCi/ml) for 2 d.
† 1.5 x 10⁸ cells per cover slips were laid over and labeled with [¹⁴C]glucose (0.5 μCi/ml) for 4 d.
§ The cell density of fixed and overlying living cells was 3.9 x 10⁶ and 3.1 x 10⁵ cells per cover slip, respectively. Cells were labeled with [¹⁴C]glucose (0.5 μCi/ml) for 2 d.
¶ 2 x 10⁵ cells per cover slip were laid over and labeled with [¹⁴C]glucosamine (0.8 μCi/ml) for 3 d.
|| Cells were fixed at a density of 1.2 x 10⁶, 6 x 10⁶ living cells per round cover slip were laid over them and labeled with [³²P]fucose (2.5 μCi/ml) for 3 d. After the extraction of lipids, both the living and fixed cells were dissolved in Protosol and counted in Toluene-PPO-POPOP.

or 3 d, respectively. The proportion of radioactivity that moved to the solvent front was 78.7 and 41% in lipids extracted from fixed cells and 23.9 and 6% in lipids extracted from living cells when cells were labeled with glucose or palmitate, respectively. When palmitate was the radioactive precursor, the proportion of radioactivity in the Forssman glycolipid (GL-5) and globoside (GL-4) was significantly lower in the fixed cells than in the living cells. In contrast, no significant difference, except for dihexosylceramide (GL-2), was found in the proportion of radioactivity between fixed and living cells when cells were labeled with glucose. The proportion of radioactivity on fixed cells that remained in living cells whether the cells were labeled with glucose or palmitate.

Characterization of Radioactive Materials Transferred to Chloroform-Methanol-Treated Fixed Cells

To examine the transfer of sugars from living cells to fixed cells, chloroform-methanol-treated fixed cells were used. In Table III are shown the effects of various detergents on solubilization of the radioactivity transferred to fixed cells from cells labeled with glucosamine. Approximately 72% of the radioactivity was solubilized by trypsin treatment within 2 h. In contrast, at least 70% of the radioactivity remained on fixed cells when they were treated with detergents. When cells were grown on bare cover slips, ~97% of radioactivity incorporated into living cells was removed.
TABLE II
Lipid Composition of Fixed and Overlying Living Cells Labeled with Either [14C]Glucose or [14C]Palmitate

| Lipids    | [14C]Glucose                  | [14C]Palmitate          |
|-----------|------------------------------|-------------------------|
|           | Fixed cells                  | Living cells            | Fixed cells | Living cells |
| SM        | 14.7                         | 12.6                    | 43.3        | 27.9        |
| PC        | 34.0                         | 29.7                    | 44.4        | 54.3        |
| PE        | 14.5                         | 17.9                    | 5.2         | 8.9         |
| PI        | 3.4                          | 3.5                     | 1.7         | 2.4         |
| GL-5      | 22.0                         | 25.3                    | 0.5         | 4.0         |
| GL-4      | 1.1                          | 0.8                     | 0.1         | 0.5         |
| GL-3      | 2.5                          | 0.6                     | 0.8         | 0.1         |
| GL-2      | 1.5                          | 1.3                     | 1.9         | 1.5         |
| GL-1      | 6.6                          | 7.9                     | 1.9         | 2.6         |

Cells grown on 100-mm plates were fixed at confluence. 3 x 10^6 cells were laid over them and labeled with either [14C]glucose (1 μCi/ml) or [14C]palmitate (1 μCi/ml) for 2 or 3 d, respectively. Lipids were extracted from fixed and living cells separately and analyzed on the thin-layer plate. The quantities represent cpm x 100 divided by total amount of radioactivity incorporated into identified phospholipids and glycolipids. SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; GL-5, Forssman glycolipid; GL-4, globoside; GL-3, trihexosyiceramide; GL-2, dihexosylceramide; GL-1, monohexosylceramide; GMα, hematosiside.

by EDTA treatment (Fig. 3 C, day 3) and 68% of the remaining radioactivity was released by 1% SDS.

The distribution of the radioactivity transferred to fixed cells was compared with that of living cells by autoradiography (Fig. 5). Grains were sparsely distributed on the fixed cell surface and no significant amount of grains was found on bare cover slips (Fig. 5A). The density of grains was much higher in living cells than in fixed cells (compare Fig. 5A and B).

Characterization of the radioactive materials transferred to fixed cells was made by investigating their sensitivities towards various enzymes.

TABLE III
Release of Radioactive Materials Transferred to Fixed Cells by Various Detergents

| Treated with:          | % Released |
|------------------------|------------|
| Trypsin                | 72         |
| 1% SDS                 | 28         |
| 1% Deoxycholate        | 32         |
| 1% Triton X-100        | 12         |
| 9 M Urea               | 6          |
| Phosphate-buffered saline | 6         |

Fixed cells were prepared as described in the legend to Fig. 6. The overlying living cells were labeled with [3H]glucosamine (2.5 μCi/ml) for 3 d. After removal of the living cells, the fixed cells were treated with hyaluronidase and chondroitinase and then were incubated in various detergents at 37°C. Within 1 h of incubation, the release of radioactivity from fixed cells by detergents leveled off. The radioactivities released and remaining on the cover slips were determined at 2 h after incubation (the release by trypsin was still increasing).

When glucosamine was the radioactive precursor, radioactive materials transferred to fixed cells were released by trypsin, hyaluronidase, or chondroitinase (Fig. 6A). The amount of radioactivity released by trypsin was still increasing after 3 h of incubation. The radioactivity released by either hyaluronidase or chondroitinase increased up to 15 min of incubation and then leveled off. No further radioactivity was released by the addition of fresh chondroitinase or hyaluronidase. Almost 45% of the total radioactivity transferred still remained in fixed cells after hyaluronidase and chondroitinase treatment. Part of the remaining radioactive substances was solubilized by neuraminidase as shown in Fig. 7. The release continued up to 2 h of incubation and then leveled off. The amount of radioactivity released by this treatment corresponded to 4% of the total radioactivity initially found in the fixed cells. In a separate experiment, fixed cells were incubated with the glycosidase mixture after treatment with hyaluronidase, chondroitinase, and neuraminidase. More than 80% of the remaining radioactivity was re-
FiguRe 6 Characterization of radioactive components associated with fixed cells. Cells were fixed at confluence (1.2 × 10^6 cells/round cover slip) and treated with chloroform-methanol. Living cells (5.8 × 10^6 cells/cover slip) were laid over them and labeled either with [14C]glucosamine (1 μCi/ml) or [3H]fucose (5 μCi/ml) (for fucosidase examination, 10 μCi/ml) for 3 d. The living cells were removed and the fixed cells were incubated with trypsin (0.1%, 1:250; Difco Laboratories, Detroit, Mich.) (x); α-L-fucosidase (0.5 U/ml) (△); hyaluronidase (300 U/ml) (○); chondroitinase (0.25 U/ml) (□); acetate buffer (■); and Tris buffer (△) as described in experimental procedures. At indicated times, aliquots of the incubation solutions were counted in Bray's scintillation solution.

leased by treatment with the glycosidase mixture. When the overlying living cells were labeled with fucose, the radioactive materials associated with the fixed cells were released by trypsin or fucosidase but not by hyaluronidase or chondroitinase (Fig. 6B).

Effects of Pretreatment of Fixed Cells with Trypsin or Mild Acid on the Amount of Radioactive Materials Transferred

The treatment of fixed cells by mild acid before inoculation with living cells did not result in a significant increase in the amount of radioactive materials transferred, although the amount of transferred materials was always slightly higher in acid-treated fixed cells when glucosamine was the radioactive precursor (Table IV).

In contrast, when fixed cells were treated with trypsin the transferred materials decreased significantly (Table IV). When [3H]glucosamine-labeled fixed cells (2 μCi/ml, labeled for 3 d; fixed, lipid extracted) were incubated with trypsin, 29% of the radioactivity was solubilized from the fixed cells under the same conditions described in the legend to Table IV.

How Are the Radioactive Materials Transferred to Fixed Cells?

We next attempted to determine whether sugars were transferred from exogenously added nucleotide sugars to fixed cells by glycosyltransferases on the living cell surface. We used a large excess (1,000 times molar concentration) of nonradiolabeled sugars to prevent the uptake of free sugars. As shown in Table V A, no significant amount of radioactivity was transferred to fixed cells within 4 h. The incorporation of radioactivity into the living cells was also very low. If the concentration of nonradioactive free sugars was reduced (100, 50, and 0 times molar concentrations of nucleotide sugars), the incorporation of radioactivity into living cells increased significantly in the case of CMP-sialic acid. However, no significant radioactivity was transferred to the fixed cells (Table V B).
TABLE IV
Effect of Pretreatment of Fixed Cells with Trypsin or Mild Acid on the Amount of Radioactive Materials Transferred from Living Cells

| Pretreatment          | Radioactive precursor | cpm Transferred to fixed cells |
|-----------------------|-----------------------|------------------------------|
| Acid hydrolysis       | [3H]GlcNAc           | 1,410                        |
|                       | [3H]Fuc              | 1,065                        |
|                       | -                    | 730                          |
| Trypsin†              | [3H]GlcNAc           | 2,000                        |
|                       | -                    | 7,920                        |

Cells were fixed on round cover slips at confluence, except as indicated, and were treated with chloroform-methanol. After treating the fixed cells with mild acid hydrolysis or trypsin, living cells (6 x 10⁶ cells/cover slip) were laid over them and labeled with [3H]glucosamine (2 μCi/ml) or [3H]fucose (2 μCi/ml) for 3 d. The radioactivity associated with the fixed cells was determined after the fixed cells were treated with hyaluronidase and chondroitinase when glucosamine was the radioactive precursor. The values are an average of two cover slips.

† Cells were fixed at sparse density (1.0 x 10⁵ cells/cover slip) and were heated for 1 h at 80°C either in 0.05 N H₂SO₄ or in H₂O.

In contrast, when prelabeled living cells (labeled with either [3H]glucosamine or [3H]fucose) were layered upon fixed cells, a certain amount of radioactive materials was transferred to fixed cells within 3 h (Table VI). This suggests that 3 h of incubation is sufficient for the transfer of materials to the fixed cells.

In the present study, we have shown that lipids, proteins, and carbohydrates could be transferred to glutaraldehyde-fixed cells from overlying living cells. It is unlikely that the living cells, or a part of the living cells, continue to be adherent to the surface of fixed cells after the removal of living cells because only a portion of the radioactivity associated with fixed cells is released by incubation with various detergents. Furthermore, autoradiographic studies have revealed that the density of label in cells was significantly different between fixed and overlying living cells. If the radioactivity associated with fixed cells was due to residual living cell fragments, we should see cell fragments with a high density of grains. In addition, the transfer of radioactive proteins or lipids to fixed cells was greatly reduced when fixed cells were pretreated with chloroform-methanol. The ratio of the solvent-extractable to nonextractable counts of radioactivity transferred was significantly different from that of living cells, except when palmitate was used as a radioactive precursor.

The mechanism of transfer of radioactive materials may be classified into two major categories: the nonspecific transfer and the specific transfer mediated by enzymes. The nonspecific transfer includes the exchange of lipids, membrane fusion, and the deposition of secreted materials from the living cells onto the surface of fixed cells.

Lipids may be exchanged directly between fixed and living cells or indirectly through the culture medium (15). It was possible that membrane fragments from living cells fused to the membranes of fixed cells, since high molecular weight components labeled with leucine were transferred to fixed cells (data not shown). Pagano and Huang have demonstrated that artificial lipid vesicles fuse with the membranes of glutaraldehyde-fixed cells (12). This nonspecific transfer could be greatly decreased when fixed cells were treated with the solvent.

When the overlying living cells were labeled with glucose or glucosamine, radioactive materials which are sensitive to hyaluronidase or chondroitinase were accumulated on the fixed cells. A small amount of radioactive materials was also deposited on the surface of bare cover slips. Roblin et al. (18) and Culp (1) have demonstrated that glycosaminoglycans and proteins are left bound to the tissue culture substrate after the removal of cells by ethylene-bis oxyethylene-nitrolo tetraace-
Incubation of Fixed and Living Cells in the Presence of Nucleotide Sugars

### TABLE V

| Substrates                  | Nil 2C1 cells |             |             | A31-714 cells |             |
|-----------------------------|---------------|-------------|-------------|---------------|-------------|
|                             | µCi/ml        | cpm In fixed cells | cpm In living cells | cpm In fixed cells | cpm In living cells |
| [H]CMP-sialic acid          | 1             | 21          | 59          | 24            | 49          |
|                             | 2             | 19          | 62          | 30            | 79          |
|                             | 4             | 28          | 110         | 32            | 93          |
| [C]GDP-fucose               | 0.5           | 82          | 154         | 53            | 47          |
| [H]UDP-N-acetylglosamine    | 1             | 51          | 211         | —             | —           |
|                             | 2.5           | —           | —           | 48            | 82          |
|                             | 5             | 111         | 314         |               |             |

### TABLE VI

Transfer of Radioactive Materials from Prelabeled Living Cells to Fixed Cells in 3h

| Radioactive precursor | cpm In fixed cells | cpm In overlaying living cells |
|-----------------------|--------------------|-------------------------------|
| [H]GlcNAc             | 1,343              | 28,725                        |
| [H]Fuc                | 225                | 2,257                         |

Nil 2C1 cells were labeled either with [H]glucosamine (2 µCi/ml) or [H]fucose (2 µCi/ml) for 3 d. Cells were harvested with EDTA and resuspended in MEM and laid upon fixed cells prepared as described in the legend to Table V. They were incubated for 3 h. Radioactivity associated with fixed and living cells was determined as described in Materials and Methods. Values are an average of two determinations.

Most of the transferred materials labeled with either glucosamine or fucose are linked to proteins of fixed cells since they are solubilized by 1% SDS (data not shown). Most of the transferred materials labeled with either glucosamine or fucose are linked to proteins of fixed cells since they are solubilized by trypsin treatment. We think that it is unlikely, although not conclusive, that most of the sugars are transferred to acceptors of fixed cells by surface glycosyltransferases on living cells, since no significant amount of radioactivity was transferred to fixed cells from exogenously added nucleotide sugars. Moreover, the removal of sialic acid or fucose from fixed cells did not significantly affect the amount of radioactivity transferred to fixed cells from exogenously added nucleotide sugars. Although those authors could not completely rule out the existence of surface sialyl-transferases, they showed that most of the sialic acid incorporation by cells was due to free sialic acid uptake.
We, also, have found that when nonradiolabeled sugars were not present in incubation mixtures, the incorporation of radioactivity into living cells increased significantly. However, the uptake of free sugars may not account solely for the incorporation since the incubation did not decrease proportionally with increasing concentration of free sugars.

Dolichol carrier lipids may not play an important role in the transfer since tunicamycin did not reduce the transfer specifically.

The transfer was drastically decreased by trypsin treatment of the fixed cells. This presumably is caused by a decrease in the acceptors on fixed cells.

How radioactive materials are transferred to fixed cells is not known at the present. It was found, however, that when fixed cells were incubated at 37°C (but not at 2°C in the conditioned medium prepared from cells labeled with glucosamine, fucose, mannose, or leucine, the radioactive materials were transferred to the fixed cells. At most, ~19% of the transferred radioactivity was released by 1% SDS. Characterization of the activity in the conditioned medium is being carried out (H. Sakiyama, manuscript in preparation). In short, the activity is not dialyzable; it remains in the supernate of 105,000 g and is sensitive to heat (56°C) and trypsin. No significant radioactivity was transferred to fixed cells when nucleotide sugars were added to the conditioned medium of nonlabeled cells. It is possible that at least a part of the materials is transferred from living cells to fixed cells through the activity found in the conditioned medium.

The authors are grateful to Dr. O. Ohno, Dr. H. Takazawa, Dr. P. W. Robbins, and Dr. S. Sakiyama for their helpful suggestions and discussions, and Dr. T. Terasima and Dr. M. Seki for their interest and support. We also thank Miss Yasukawa for her technical help.

This work was supported by the Grant for Cancer Research Program (No. 101051 and 001017) from the Japanese Ministry of Education, Science and Culture.

Received for publication 25 July 1978, and in revised form 15 January 1979.

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