ACQUISITION OF β-GLUCURONIDASE ACTIVITY BY DEFICIENT FIBROBLASTS DURING DIRECT CONTACT WITH LYMPHOID CELLS

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SUMMARY

Fibroblasts deficient in β-glucuronidase acquired high levels of this enzyme when they were co-cultured with concanavalin A-stimulated lymphocytes. Acquired enzyme activity, determined using a single-cell cytochemical assay, was directly proportional to the number of lymphocytes added and persisted for several days in fibroblasts maintained at high density.

Lymphocytes did not secrete significant levels of β-glucuronidase into their culture medium, and did not release other substances able to induce synthesis of the enzyme by the deficient fibroblasts. Nor did β-glucuronidase acquisition result from concanavalin A-mediated uptake of enzyme, since α-methylmannoside did not reduce acquired activity. Moreover, lymphocytes from various sources, whether unstimulated or activated by a different mitogen, bacterial lipopolysaccharide, were equally effective in promoting the appearance of β-glucuronidase.

Deficient fibroblasts did not acquire β-glucuronidase by active endocytosis when co-cultured with lymphocytes, since enzyme extracted from lymphocytes was not itself effective in this respect. Furthermore, mannose 6-phosphate, which did inhibit endocytosis by deficient fibroblasts of exogenous β-glucuronidase prepared from 3T3 cells, had no effect on enzyme acquisition by fibroblasts during their co-culture with lymphocytes. Conversely, inhibitors of protein synthesis and energy metabolism, which did not interfere with endocytosis of exogenous enzyme, abolished the acquisition of β-glucuronidase during co-culture.

Deficient fibroblasts did not acquire β-glucuronidase when they were cultured together with lymphocytes but separated from them by Millipore membranes permeable to exogenous enzyme. Thus, although the mechanism of acquisition is still unclear, the present results suggest that β-glucuronidase is transferred from lymphocytes to deficient fibroblasts by a process in which direct cell-to-cell contact is obligatory.

INTRODUCTION

The metabolic co-operation of cells in vitro leading to correction of inborn errors of metabolism is a well-established phenomenon. For example, Cox, Krauss, Balis & Dancis (1974) showed that fibroblasts from patients with Lesch-Nyhan syndrome, deficient in hypoxanthine-guanine phosphoribosyl transferase (EC 2.4.2.8) and therefore unable to incorporate purines into their nucleic acids, were capable of utilizing hypoxanthine for DNA synthesis when grown in culture with normal fibroblasts. This metabolic co-operation was achieved by the transfer of low molecular weight products of normally active enzyme to the deficient cells rather than of the enzyme itself. Direct cell-to-cell contact was required, however, since correction did not occur when the two cell types were cultured together in the same dish but separated physically, or when conditioned medium from cultures of normal cells...
was added to the deficient fibroblasts. This type of cellular interaction involves rapidly formed gap junctions between cells in contact (Epstein & Gilula, 1977), and the exchange of nucleotides has been shown to occur through these channels of direct communication (Pitts & Simms, 1977).

A different type of metabolic co-operation was observed by Fratantoni, Hall & Neufeld (1968, 1969), who showed that the abnormal $^{35}$S metabolism of fibroblasts from patients deficient in the lysosomal enzyme α-L-iduronidase (EC 3.2.1.76) was corrected when they were cultured together with normal fibroblasts or fibroblasts from patients with a different lysosomal enzyme deficiency. Unlike the Lesch-Nyhan syndrome, however, direct cell contact was not obligatory since the medium in which normal cells or fibroblasts of a different genotype from that of the iduronidase-deficient cells had been cultured was equally effective. Later it was shown that correction resulted from the release of lysosomal enzymes by normal fibroblasts and their subsequent uptake by deficient cells (Fratantoni et al. 1969; Neufeld, Lim & Shapiro, 1975). These observations provided the basis for treatment of a number of inborn errors of metabolism (Dean, Muir, Benson & Button, 1981; Dean et al. 1976, 1979; Desnick, 1980) by enzyme replacement.

Interactions between cell types other than fibroblasts have also been extensively investigated. Direct cell-to-cell contact between lymphocytes and other cell types, described originally by Humble, Jayne & Pulvertaft (1956) as emperipolesis ("inside round-about wandering"), has been shown to be widespread (for a review, see Yoffey & Courtice, 1970), and the cellular and morphological basis of this phenomenon has recently been analysed (Sandilands, Reid, Gray & Anderson, 1978; Reid, Sandilands, Gray & Anderson, 1979). Lymphocytes have also been shown to adhere rapidly to fibroblasts in vitro (Chang, Celis, Eisen & Solomon, 1979) and to transfer radioactively labelled DNA to them (Harris, Olsen & Furner, 1978). A new type of metabolic co-operation between these two types of cells is described here. This involved acquisition of the lysosomal enzyme, β-glucuronidase, by deficient fibroblasts, which required their direct cell-to-cell contact with lymphocytes. Preliminary results have been reported elsewhere (Olsen, Dean, Harris & Muir, 1981).

**MATERIALS AND METHODS**

**Cell culture**

Spleen cells were prepared from 2 to 4-month-old CBA mice as previously described by Olsen & Harris (1975). They were cultured at an initial concentration of $10^5$ cells/ml in RPMI 1640, supplemented with 5% foetal calf serum, 100 μg/ml penicillin, 100 μg/ml streptomycin and $5 \times 10^{-4}$ M-2-mercaptoethanol. Concanavalin A (Con A) or bacterial lipopolysaccharide were also added initially at final concentrations of 2 μg/ml or 50 μg/ml, respectively.

The cultures were incubated for 3 days at 37 °C in an atmosphere of 5% CO₂ in air, after which cell numbers had increased two to three-fold as determined by direct haemocytometer counting. Cell viability was greater than 80% based on the exclusion of Trypan blue, and the proportion of transformed lymphocytes was generally between 60 and 80% of the total cells present. Transformed lymphocytes from the spleens of the AKR and the congenitally athymic nude (nu/nu) mouse strains, and from rabbit spleen, were prepared in the same way. Unstimulated mouse spleen cells in culture did not increase in number and usually contained less than
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20% activated lymphocytes. Human lymphocytes were obtained from fresh defibrinated peripheral blood and separated from polymorphonuclear leukocytes by centrifugation in Histopaque-1077.

Prior to assay or co-culture with fibroblasts, the lymphoid cells were centrifuged at 200 g for 10 min, at 25 °C, resuspended in Eagle's minimal essential medium (MEM) and re-centrifuged. This process was repeated and the final cell concentration was adjusted as required.

Normal human fibroblasts were obtained from umbilical cord following a Caesarian delivery. Fibroblasts from patients with mucopolysaccharidosis type VII (β-glucuronidase-deficient, GM nos. 121 and 151) and from clinically normal people heterozygous for the β-glucuronidase allele (GM nos. 1850 and 2074) were supplied by the Human Genetic Mutant Repository (Camden, N.J.). All fibroblasts were used between passages 5 and 12. The A9 fibroblast cell line, derived from the C3H mouse strain, was supplied by Flow Laboratories (Irvine, Scotland).

Fibroblasts were cultured in 75 cm² flasks containing 20 ml of MEM, supplemented with 10% foetal calf serum, penicillin (100 u/ml) and streptomycin (100 µg/ml). At confluence, they were detached by treatment for 10 min at 37 °C with 0.25% trypsin in phosphate-buffered saline (PBS). Serum was added to a final concentration of approximately 5%. The trypsinized, neutralized cell suspensions were centrifuged, and the cells washed twice with MEM.

For co-culture experiments, 1 ml of the trypsinized suspension containing 1 x 10⁶ to 3 x 10⁴ cells was seeded in 35 mm plastic Petri dishes and incubated until the monolayer reached late logarithmic phase (5 x 10⁴ to 1 x 10⁵ cells per culture dish and 3 x 10⁵ to 5 x 10⁶ cells with A9 fibroblasts). Cells for qualitative histochemical examination were grown directly on sterile glass coverslips (no. 1, 22 mm x 22 mm) placed on the surface of the culture dish.

Direct cell contact with Millipore filter chambers

In co-culture experiments, 10⁷ lymphocytes in 1 ml of medium were added to the washed fibroblast monolayer and incubated for 24 h, unless indicated otherwise. At the end of the culture period, non-adherent lymphocytes, accounting for 90-99% of the number added initially, were removed and the adherent cells were washed gently with warm MEM.

Suspensions of the fibroblasts (plus some contaminating lymphocytes) were obtained for quantitative cytochemical and biochemical assays by treatment of replicate cultures with 0.25% trypsin for 10 min. For histochemical examination, coverslip preparations were either fixed with methanol and stained with Giemsa, or assayed directly for β-glucuronidase activity as described below.

In other experiments, the two cell types were separated by a membrane filter while in the same culture dish. Chambers were constructed from glass rings 20 mm in diameter, fitted at one end with a Millipore membrane (0.22 µm pore size unless otherwise indicated). Three polyethylene legs (2 mm high) were attached to the underside of the filters and the chambers, open at the top, were placed in the culture dishes. The filters were washed extensively with MEM and then distilled water to remove substances toxic to the growth of the fibroblasts. The chambers were sterilized by ultraviolet irradiation.

Cytochemical β-glucuronidase assay

A modification of the procedure of Fishman & Goldman (1965) was used for quantitative cytochemical measurements of β-glucuronidase levels in single cells. The substrate was 11 mg of naphthol ASBI-β-D-glucuronide (ASBI-glucuronide) dissolved in 1 ml of 0.5 M-NaHCO₃. This was made up to 100 ml with 0.1 M-acetate buffer (pH 4.5), and kept frozen until required. Aliquots of the trypsinized cell suspension were washed with PBS, then resuspended in 100 ml of the substrate at 37 °C for 3 h, unless otherwise stated. The cells were then centrifuged, washed with PBS, and placed in 1 ml of a solution of Fast blue RR prepared immediately before use (1 mg per ml in 0.01 M-phosphate buffer, pH 7.4), for 10 min at 4 °C. After coupling, the cells were again centrifuged and washed with PBS, and aliquots were air-dried on slides. These were immersed in methanol for 10 min and then distilled water, dried and mounted in Styrolite. The absorption at 600 nm, of 30-100 individual fibroblasts, were measured on a Vickers Microdensitometer M85 using a B5 mask. All assays of
β-glucuronidase activity acquired by deficient fibroblasts were carried out by this procedure, except in four experiments, as indicated below.

For qualitative assessment of β-glucuronidase activity, washed coverslip preparations of fibroblast monolayers were incubated directly with 1 ml of ASBI-glucuronide for 3 h at 37 °C. After washing, the cells were coupled with Fast blue RR, washed, fixed on slides and mounted as described above.

Biochemical β-glucuronidase assay: homogeneous cell populations

Cell pellets containing not less than either 10⁶ fibroblasts or 10⁵ lymphocytes were subjected to six cycles of freezing and thawing in 1 ml of 0.1 % Triton X-100. After centrifugation, duplicate 50 μl aliquots of the supernatant solution were added to 200 μl of 2 mM-4-methylumbelliferyl-β-D-glucuronide (MeU-glucuronide) in 0.1 M-acetate buffer (pH 4.5). After 1 h at 37 °C, the reaction was stopped by the addition of 10 ml of 0.4 M-glycine buffer (pH 10.4) and the fluorescence at 448 nm was measured on a Locarte Fluorimeter (excitation at 368 nm). One unit of β-glucuronidase was defined as the amount of activity that liberated 1 nm of 4-methylumbelliferone per hour. Specific activity was expressed as units per 10⁶ cells used to prepare the cell-free enzyme extract.

Biochemical β-glucuronidase assay: co-cultured fibroblasts

In four experiments as indicated, the activity acquired by GM151 cells after co-culture with lymphocytes was estimated using the biochemical assay. The adherent cells were washed and incubated with trypsin for 1–2 min at 37 °C, which removed many of the lymphocytes still attached to the fibroblast monolayer. The monolayer was then trypsinized for a further 10 min, as described, and the number of deficient fibroblasts and remaining lymphocytes (less than 1 % of the number added initially) determined by direct counting in a haemocytometer. The total β-glucuronidase activity of these cells, and of a control suspension of lymphocytes cultured alone, was measured using MeU-glucuronide. It was thus possible to calculate and subtract that proportion of the activity due to the few ‘contaminating’ lymphocytes from the total activity of the co-culture. The activity remaining was that acquired by the measured number of deficient fibroblasts, whose specific activity could then be calculated.

Preparation of growth media and cell-free lysates for uptake experiments

Lymphocytes were re-cultured alone in MEM for 24 h at 10⁷ cells/ml, and were removed from their medium by centrifugation at 800 g for 10 min at 25 °C. Non-adherent lymphocytes were similarly removed from co-culture media after 24 h interaction with deficient fibroblasts. The β-glucuronidase activity in the media was assayed by the biochemical procedure and its effect on enzyme acquisition by deficient fibroblasts was examined using the cytochemical assay.

Entire, crude cell lysates of lymphocytes for uptake experiments were prepared by six cycles of freezing and thawing of 10⁶ cells/ml in MEM in the absence of foetal calf serum. No intact cells were observed, and the β-glucuronidase activity was assayed biochemically. Foetal calf serum was added to a final concentration of 10 %, and these lysates were examined for their ability to induce enzyme acquisition by deficient fibroblasts by the cytochemical assay.

Chemicals

The following chemicals were supplied by Sigma Chemical Co. Ltd (Poole, England): concanavalin A; mannose 6-phosphate; α-methylmannoside, puromycin hydrochloride; cycloheximide; carbonylcyanide, m-chlorophenylhydrazone; naphthol ASBI-β-D-glucuronide; 4-methylumbelliferyl-β-D-glucuronide; Fast blue RR; Histopaque-1077; D-saccharo-1,4-lactone; Trypan blue, Giemsa stain. Bacterial lipopolysaccharide prepared from *Serratia marcescens* was obtained from Difco Laboratories (West Molesey, England) and Triton X-100 from Koch-Light Laboratories (Colnbrook, England). Flow Laboratories (Irvine, Scotland) supplied RPMI 1640, Eagles minimal essential medium, heat-inactivated foetal calf serum and trypsin. Styrolite was obtained from Raymond A. Lamb (London, England).
Fig. 1. Adherence of lymphocytes to fibroblast monolayers. Con A-transformed lymphocytes were co-cultured with GM151 fibroblasts for 24 h. After extensive washing of the adherent cells with PBS, the preparation was fixed and stained with Giemsa as described in Materials and Methods. The lymphocytes are smaller, rounded, and more densely stained. ×1000.

RESULTS

Adherence of lymphocytes to fibroblast monolayers

When washed suspensions of Con A-transformed lymphocytes were co-cultured with β-glucuronidase-deficient fibroblasts, large numbers of lymphocytes rapidly became attached to the fibroblasts. Even after extensive washing with PBS, between 1 and 10% of the added lymphocytes remained firmly attached to the monolayer (Fig. 1). The adherent lymphocytes, although closely associated with the fibroblasts, did not appear to be inside them. There was no evidence of any significant degree of phagocytosis of the lymphocytes.

Histochemical localization of β-glucuronidase activity

GM151 cells cultured alone had no detectable β-glucuronidase activity after incubation with ASBI-glucuronide for 6 h. Moreover, no enzyme activity was found under these conditions even when the cultures were kept in stationary phase, in which condition the activity of a number of lysosomal enzymes has been shown to increase in both lymphoid cells (Matsuda et al. 1977) and fibroblasts (Kaplan, 1978; Russell, Russell & Littlefield, 1971; Heukels-Dully & Niermeijer, 1976; Horvat & Acs, 1974). In logarithmically growing GM151 cells co-cultured with lymphocytes for 24 h, β-glucuronidase activity appeared in densely staining lysosome-like bodies within the cytoplasm of the fibroblasts (Fig. 2). Identical patterns of staining were seen when the fibroblasts were cultured in the presence of exogenous β-glucuronidase partially purified from mouse 3T3 fibroblasts (Diment & Dean, unpublished data).
Fig. 2. β-Glucuronidase activity in deficient fibroblasts. Lymphocytes and β-glucuronidase-deficient fibroblasts were co-cultured for 24 h and the adherent cells washed thoroughly with PBS. They were incubated with AS1-glucuronide for 3 h and post-coupled as described in Materials and Methods. β-Glucuronidase has accumulated in the densely stained cytoplasmic vesicles. Note the lymphocyte attached to the uppermost fibroblast. × 250.

The incorporation of exogenous enzyme into lysosomes of β-glucuronidase-deficient cells has been reported previously by Lagunoff, Nichol & Pritzl (1973) and Bach & Liebmann-Eisenberg (1979).

**Single-cell cytochemical analysis of β-glucuronidase**

A variable number of lymphocytes remained attached to the fibroblasts after coculture, as noted above. Trypsinization detached all the adherent cells from the culture dish, and also disrupted the association between the remaining lymphocytes and the fibroblasts. Since it was difficult to remove the contaminating lymphocytes and thereby obtain an homogeneous suspension of fibroblasts, it was not possible to measure the β-glucuronidase activity acquired by the deficient fibroblasts by direct biochemical assay. However, enzyme activity present in individual GM151 cells could be measured accurately by quantitative single-cell cytochemistry, as the fibroblasts in the co-culture were readily distinguishable from the much smaller ‘contaminating’ lymphocytes. Fig. 3A shows the kinetics of the assay of β-glucuronidase acquired by GM151 cells after they had been co-cultured for 24 h with Con A-transformed lymphocytes. All fibroblasts acquired enzyme and showed a Gaussian distribution of activity; there was no indication of an enzyme-negative population of cells. The arithmetic means of the cytochemical absorption measurements demonstrated that the rate of reaction was linear for at least 4 h (Fig. 3B). A standard period of 3 h of incubation with substrate was therefore used in all subsequent experiments. No enzyme activity was detected when 12 mM-D-saccharo-1,4-lactone, a specific inhibitor of β-glucuronidase (Levvy, 1952), was present during the assay.
Comparison of cytochemical and biochemical measurement of β-glucuronidase activity

Cytochemical measurements were compared with those made by direct enzyme assay. Cells used for this purpose were A9 fibroblasts derived from the C3H mouse (Morrow, Greenspan & Carroll, 1949) fibroblasts from two healthy individuals heterozygous for the β-glucuronidase allele (GM nos. 1850 and 2074; Sly, Quinton, McAlister & Rimoin, 1973), and normal human fibroblasts. These were all grown as monolayers to late logarithmic phase and divided into replicates after trypsinization. One set was assayed cytochemically, as described, and the arithmetic means of their β-glucuronidase activities were calculated. The other set was used to prepare enzyme extracts from frozen and thawed pellets. The β-glucuronidase activity of these was assayed directly with MeU-glucuronide and the specific activities (expressed as units per 10⁶ cells used to prepare the enzyme extracts) were calculated. The results in Table 1 show that the means of the single-cell cytochemical assays accurately measured the β-glucuronidase activity in the population of cells as a whole over a wide range. Thus in all subsequent experiments, the cytochemical measurements were converted directly to β-glucuronidase specific activities, using a factor of 0.29 u/10⁶ cells per unit of absorption density.

Relationship between acquired β-glucuronidase activity and number of lymphoid cells

The activity acquired by GM151 cells after 24 h of co-culture was directly proportional to the number of transformed lymphocytes added, up to a maximum of 10⁶ lymphocytes per culture dish (Fig. 4). This optimal number of lymphocytes was therefore used in all subsequent experiments. There was no activity measurable in the deficient fibroblasts when less than 10⁸ lymphocytes were added to the co-culture.

As measured cytochemically in eight experiments, the enzyme activity acquired by 10⁶ GM151 cells after co-culture with 10⁷ lymphocytes was 4.07 units (sp. act. 40.7 u/10⁶ cells ± 2.1 standard error). The acquired activity was also estimated by the biochemical procedure, in which the contribution of the measured number of 'contaminating' lymphocytes was subtracted from the total enzyme activity in the trypsinized co-culture, as described in Materials and Methods. In four experiments, the mean acquired β-glucuronidase activity was thus calculated to be 3.35 units (sp. act. 33.5 u/10⁶ cells ± 9.4 standard error), in good agreement with the cytochemical assay. Biochemical determination of enzyme activity showed that 10⁷ lymphocytes contained 10–40 units β-glucuronidase (sp. act. 1–4 u/10⁶ cells). The level of β-glucuronidase acquired by GM151 cells therefore represented a significant proportion (approximately 10–25%) of the total β-glucuronidase activity in the added lymphocytes: each fibroblast acquired the enzyme activity of an average of at least 10 lymphocytes. This did not arise by phagocytosis of lymphocytes, as reported above. However, it is notable that enzyme levels in the lymphocytes recovered after co-culture, which accounted for an average of 94% of the number of cells added initially, had increased by nearly threefold, whereas the activity in lymphocytes cultured alone remained essentially unchanged. Hence acquisition of β-glucuronidase by GM151 fibroblasts did not deplete the lymphocytes of their enzyme. Moreover, when non-adherent
Fig. 3. Kinetics of the cytochemical assay of β-glucuronidase. Con A-transformed lymphocytes were co-cultured with GM151 cells for 24 h, and the fibroblasts were separated as described in Materials and Methods. The trypsinized suspension was incubated with ASBI-glucuronide at 37 °C. Aliquots were removed at the times indicated and the cytochemical reactions carried out. The absorption densities of 100 individual fibroblasts were measured by microdensitometry. Deficient fibroblasts cultured alone had no β-glucuronidase activity.

A. A suitable range of arbitrary density groups was selected, and the % of the total GM151 cells within each density range was calculated.
lymphocytes, removed by washing after co-culture, were added to a fresh monolayer of GM151 cells and the co-culture repeated, these fibroblasts acquired more than 85% of the activity acquired by GM151 cells during the first co-culture. The lymphocytes were therefore able to induce a further round of enzyme acquisition on subsequent re-culture with fresh, deficient fibroblasts. Thus, the fibroblasts themselves rather than the lymphocytes appeared to limit the transfer of β-glucuronidase activity.
Fig. 4. Effect of increasing numbers of lymphocytes on the acquisition of \( \beta \)-glucuronidase activity by GM151 cells. Con A-transformed lymphocytes were washed and resuspended in complete MEM. Numbers of lymphocytes indicated were added to \( 10^6 \) GM151 cells for 24 h of co-culture, in a final volume of 1.0 ml. The fibroblasts were analysed cytochemically for \( \beta \)-glucuronidase activity, and the means of the single-cell measurements converted to u/10^6 GM151 cells, as shown in Table 1. Deficient fibroblasts cultured alone had no \( \beta \)-glucuronidase activity.

**Persistence of acquired \( \beta \)-glucuronidase activity**

Previous studies showed that there was a direct relationship between the enzyme activity acquired by GM151 cells and the period of interaction with lymphocytes, up to a maximum of 24 h co-culture (Olsen et al. 1981). However, enzyme levels then declined over the next few days even in the continued presence of the lymphocytes (Fig. 5). A similar loss of enzyme activity took place when the lymphocytes were removed after 24 h of co-culture, and replaced with fresh medium alone. Moreover, when the original lymphocytes were replaced with \( 10^7 \) fresh lymphocytes, there was only a small additional increase in enzyme activity after 48 h of co-culture. However, when after 24 h of co-culture with lymphocytes the GM151 cells were cultured alone at sufficiently high cell density to prevent cell division, the acquired \( \beta \)-glucuronidase activity persisted at the original high level.

**Secretion of \( \beta \)-glucuronidase and regulatory factors by the lymphoid cells**

Splenic lymphocytes cultured in the presence of Con A for 72 h did not secrete any detectable \( \beta \)-glucuronidase into their culture medium. However, when collected and re-cultured for a further 24 h at a density of \( 10^6 \) cells/ml, low amounts of activity were detected (3.7-7.3 u/ml in six separate experiments, mean 5.2 u/ml). This level remained unchanged or increased slightly during co-culture for 24 h with
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Fig. 5. Persistence of β-glucuronidase activity acquired by deficient fibroblasts. Lymphocytes were co-cultured with deficient fibroblasts for 24 h, as described in Materials and Methods. Thereafter for 24 h, and in some cases for 48 h: (○) the co-culture was continued; (△) the lymphocytes were replaced with fresh medium; (□) the lymphocytes were replaced with 10^6 fresh lymphocytes; (◇) the lymphocytes were replaced with lymphocyte–fibroblast co-culture medium; (♦) the lymphocytes were removed and the fibroblasts were re-cultured alone at high cell density. Deficient fibroblasts cultured alone had no β-glucuronidase activity.

GM151 cells. No enzyme activity was acquired by the deficient fibroblasts when they were incubated for 24 h with either of these media. Nor was β-glucuronidase activity acquired when fresh medium was supplemented with 3T3 cell enzyme at the level normally found in the lymphocyte and lymphocyte–fibroblast co-culture media (i.e. 5 U/ml). The lymphocyte–fibroblast co-culture medium also had no influence on the β-glucuronidase acquired by the fibroblasts during co-culture for 24 h (Fig. 5). It is therefore unlikely that the appearance of enzyme in the deficient fibroblasts resulted from uptake either of β-glucuronidase or of an inactive precursor secreted by the lymphocytes. Nor could it have been due to the secretion of regulatory factors by the lymphocytes, which might have induced the expression of endogenous fibroblast β-glucuronidase.

Role of Con A-mediated phagocytosis

The influence of high concentrations of Con A on the uptake of lysosomal enzymes (Gonzales-Noriega & Sly, 1978) was examined next, even though the splenic lymphocytes had been thoroughly washed after transformation in vitro with 2 μg/ml of Con A and no additional Con A was included during subsequent co-culture. When added to
the medium at 2 or 100 μg/ml, Con A did not induce any detectable β-glucuronidase activity in GM151 cells cultured either in fresh medium or in medium collected from lymphocyte–fibroblast co-cultures after 24 h. Nor did Con A affect the acquisition of β-glucuronidase by GM151 cells when added directly to lymphocyte–fibroblast co-cultures after 24 h, since in two separate experiments the specific activities were 31.6 and 37.8 u/10^6 cells in the absence, and 27.8 and 25.8 u/10^6 cells in the presence, of 100 μg/ml of Con A. On the other hand, this concentration of Con A did increase uptake from the medium, using 145 u and 49 u of exogenous β-glucuronidase from 3T3 cells, from 17.4 and 17.7 to 28.4 and 40.0 u/10^6 GM151 cells, respectively.

Furthermore, although the glycoside α-methylmannoside dissociates Con A from its cell membrane receptor (Noonan & Burger, 1973) and inhibits Con A-mediated uptake of lysosomal enzymes by fibroblasts (Gonzales-Noriega & Sly, 1978), at a concentration of 25 mM it enhanced enzyme acquisition by GM151 cells when co-cultured with lymphocytes by an average of about 7%. Moreover, when lymphocytes were first washed with medium containing 100 mM-α-methylmannoside and then co-cultured with GM151 cells in the absence or presence of 25 mM-α-methylmannoside, the level of β-glucuronidase acquired by deficient fibroblasts was again slightly greater (6 and 9%, respectively) than that acquired by fibroblasts co-cultured with control untreated lymphocytes. Together these results suggest that the acquisition of β-glucuronidase during co-culture was not potentiated by Con A.

Effects of specific lectin stimulation and the source of lymphoid cells

Lymphocytes from a number of different sources were studied, as shown in Table 2. Con A stimulates interphase T lymphocytes into DNA synthesis and cell division whilst lipopolysaccharide stimulates B cells. The β-glucuronidase levels were increased in both situations (Olsen et al., unpublished data) and the acquisition of enzyme by deficient fibroblasts was promoted by both types of lymphocyte (Table 2). Cultured spleen cells from congenitally athymic nude (nu/nu) mice also responded to lipopolysaccharide, but not to Con A. Nevertheless, spleen cells from such mice incubated with either mitogen subsequently induced high levels of β-glucuronidase activity in GM151 cells. Cultured splenic lymphocytes from the AKR mouse, a strain which has low levels of β-glucuronidase activity (Feder, 1976), are stimulated by both mitogens, and these cells also induced the acquisition of this enzyme by GM151 cells (Table 2). Activation of the lymphocytes by the mitogens was not important, however, since in the absence of any stimulation splenic lymphocytes from all three strains still promoted the appearance of enzyme activity in deficient fibroblasts. Furthermore, lymphoid cells of both human and rabbit origin induced the acquisition of this enzyme by GM151 cells (Table 2).

Specific endocytosis of exogenous enzyme and the effects of inhibitors

The specific endocytosis by fibroblasts of a number of lysosomal enzymes involves recognition of oligosaccharide side-chains on the enzyme possessing mannose 6-phosphate groups (Sando & Neufeld, 1977; Ullrich, Mersmann, Weber & Von Figura, 1978). Mannose 6-phosphate has therefore been widely used as a competitive
**Table 2. β-glucuronidase acquisition by GM151 cells after 24 h of co-culture with lymphocytes: mitogen specificity and source of lymphocytes**

<table>
<thead>
<tr>
<th>Source of lymphocytes</th>
<th>Mitogen stimulation</th>
<th>β-Glucuronidase activity added (units/10⁶ lymphocytes)*</th>
<th>β-Glucuronidase activity acquired (units/10⁶ GM151 cells)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBA mouse spleen</td>
<td>None</td>
<td>17</td>
<td>44:1</td>
</tr>
<tr>
<td></td>
<td>Con A</td>
<td>47</td>
<td>36:3</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>59</td>
<td>61:3</td>
</tr>
<tr>
<td>Nude mouse spleen</td>
<td>None</td>
<td>13</td>
<td>41:2</td>
</tr>
<tr>
<td></td>
<td>Con A</td>
<td>14</td>
<td>51:6</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>33</td>
<td>53:9</td>
</tr>
<tr>
<td>AKR mouse spleen</td>
<td>None</td>
<td>10</td>
<td>48:1</td>
</tr>
<tr>
<td></td>
<td>Con A</td>
<td>18</td>
<td>37:9</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>16</td>
<td>24:7</td>
</tr>
<tr>
<td>Human peripheral blood</td>
<td>None</td>
<td>133</td>
<td>39:4</td>
</tr>
<tr>
<td>Rabbit spleen</td>
<td>Con A</td>
<td>38</td>
<td>47:0</td>
</tr>
</tbody>
</table>

The above data show the results obtained in one representative experiment. LPS, bacterial lipopolysaccharide.

* Assayed biochemically with MeU-glucuronide; 10⁶ lymphocytes added to culture dishes containing 10⁶ GM151 cells.

† Determined cytochemically with ASBI-glucuronide. Deficient fibroblasts cultured alone had no β-glucuronidase activity.

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**Table 3. Effect of various inhibitors on the acquisition of β-glucuronidase by GM151 cells**

<table>
<thead>
<tr>
<th>Additions to fibroblast monolayers</th>
<th>β-Glucuronidase activity (% of control cultures)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Mannose 6-phosphate, 1 mM</td>
<td>12</td>
</tr>
<tr>
<td>Cycloheximide, 0.1 mM</td>
<td>92</td>
</tr>
<tr>
<td>Puromycin, 0.1 mM</td>
<td>92</td>
</tr>
<tr>
<td>Carbonylcyanide, m-chloro-phenyl-hydrazone, 0.01 mM</td>
<td>85</td>
</tr>
</tbody>
</table>

GM151 cells (10⁶ per culture dish) were incubated for 24 h in the presence of the above inhibitors and either: in A, 200 units of exogenous 3T3 enzyme; or in B, 10⁷ lymphocytes containing 15 units of β-glucuronidase. They acquired 29:9 and 49:9 units/10⁶ cells, respectively. Deficient fibroblasts cultured alone had no β-glucuronidase activity.

inhibitor of receptor-mediated uptake by fibroblasts of lysosomal enzymes, including β-glucuronidase (Kaplan, Achord, & Sly 1977; Kaplan, Fischer, Achord & Sly, 1977). The effect of mannose 6-phosphate on the acquisition of β-glucuronidase by GM151 cells is shown in Table 3. At a concentration of 1 mM, it inhibited the uptake of the enzyme prepared from 3T3 cells by 88%, whereas the activity acquired during 24 h of co-culture with 10⁷ lymphocytes was actually increased by about one-third.
Acquisition of β-glucuronidase by direct interaction was more efficient than endocytosis of enzyme prepared from 3T3 cells. Enzyme uptake of 0.05-0.18% per hour was measured in six separate experiments (average 0.10%) using 49-250 units of 3T3 β-glucuronidase, whereas with 10⁷ lymphocytes containing 9-64 units of β-glucuronidase, acquisition of 0.18-0.83% per hour was obtained in 12 separate experiments (average 0.47%). In contrast, it is notable that a frozen and thawed crude cell lysate prepared from 10⁷ lymphocytes, which contained 64 units of β-glucuronidase, produced no detectable activity in GM151 cells when added to culture medium for 24 h. Moreover, 100 u of purified lymphocyte β-glucuronidase (Diment & Dean, unpublished data) was also without effect. Hence, lymphocyte enzyme itself is not taken up by active endocytosis from the medium.

Inhibitors of protein synthesis, puromycin or cycloheximide, did not reduce uptake of exogenous β-glucuronidase by deficient fibroblasts (Table 3), whereas they did inhibit the acquisition of enzyme activity by GM151 cells co-cultured with lymphocytes, suggesting that protein synthesis was involved in the process. There was also a stringent requirement for energy-generating metabolic processes, since uncoupling of oxidative phosphorylation with carbonylcyanide, m-chlorophenylhydrazone (Heytler & Pritchard, 1962) abolished enzyme acquisition during cell-to-cell interaction, but had no effect on the endocytosis of exogenous enzyme. It would therefore appear from these results that enzyme acquisition by fibroblasts during co-culture with lymphocytes occurred by a mechanism other than endocytosis of exogenous β-glucuronidase.

Direct cell-to-cell contact

The requirement for lymphocytes to be in direct contact with the fibroblasts was examined next using Millipore membrane filter chambers to separate the two cell types. In one type of experiment, GM151 cells were grown as monolayers in 35 mm Petri dishes and Con A-transformed lymphocytes (10⁷ cells) were placed in the central chambers for 24 h of incubation. Alternatively, the fibroblasts were first grown directly on the surface of the Millipore filter of the chamber, and the entire chambers transferred to culture dishes already containing 10⁷ lymphocytes. In neither type of experiment was β-glucuronidase activity detected in the GM151 cells after 24 h of co-culture, even when the pore size of the filter was increased from 0.22 μm to 0.8 μm, whereas in the same experiments control cultures of fibroblasts in direct contact with lymphocytes acquired an average activity of 29.9 u/10⁶ cells. Separation of the two types of cell thus prevented the acquisition of β-glucuronidase activity by the deficient cells.

The Millipore membrane was shown to be permeable to 3T3 cell β-glucuronidase. When 200 units were placed inside the chamber, fibroblasts outside it took up enzyme to reach an activity of 24.0 u/10⁶ cells after 24 h, compared with 49.9 u/10⁶ cells when the cells were not separated from the enzyme by the membrane. The presence of lymphocytes in addition to the enzyme inside the chambers had no influence on the uptake of enzyme by fibroblasts outside them. Restricted diffusion of enzyme through the membrane or partial adsorption to it might account for the lower uptake
**Lymphocyte-fibroblast enzyme interchange**

Table 4. Effect of mannose 6-phosphate and Con A on β-glucuronidase activity in partially deficient fibroblasts

<table>
<thead>
<tr>
<th></th>
<th>GM1850 fibroblasts</th>
<th>A9 fibroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt. 1</td>
<td>Expt. 2</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>None</td>
<td>30.7</td>
<td>69.2</td>
</tr>
<tr>
<td>Mannose 6-phosphate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM</td>
<td>27.4</td>
<td>73.7</td>
</tr>
<tr>
<td>Con A, 2 μg/ml</td>
<td>25.3</td>
<td>81.0</td>
</tr>
<tr>
<td>Con A, 100 μg/ml</td>
<td>17.2</td>
<td>8.9</td>
</tr>
</tbody>
</table>

* Assayed cytochemically with ASBI-glucuronide after 24 h of incubation in the absence (A) and in the presence (B) of 10^7 lymphocytes containing 65 and 40 μ of β-glucuronidase in experiments 1 and 2, respectively.

of enzyme by the fibroblasts outside, and gentle rocking of the cultures increased the uptake slightly to 27.3 μ/10^6 cells.

**Acquisition of β-glucuronidase by other fibroblasts**

Exactly analogous results were obtained when fibroblasts from another patient with mucopolysaccharidosis type VII (GM121), also entirely deficient in β-glucuronidase activity, were co-cultured with lymphoid cells.

The effect of direct cell-to-cell contact was examined next using human heterozygous fibroblasts (GM1850) and mouse A9 fibroblasts, which possess measurable but low β-glucuronidase activity. After co-culture with lymphocytes for 24 h, β-glucuronidase in these fibroblasts increased by an average of about 35 and 25 μ/10^6 cells, respectively (Table 4). This was comparable with the mean activity acquired by GM151 fibroblasts after co-culture with lymphocytes, i.e. 40.7 μ/10^6 cells.

As with GM151 cells, this increase in the levels of β-glucuronidase in GM1850 and A9 fibroblasts was unaffected by Con A or mannose 6-phosphate, whereas the uptake of exogenous enzyme by active endocytosis into A9 cells was inhibited by mannose 6-phosphate. Thus, when cultured for 24 h with 100 units of 3T3 cell enzyme, β-glucuronidase activity increased from 29.0 to 50.5 μ/10^6 A9 cells, but when 1 mM-mannose 6-phosphate was present the enzyme level remained at 26.0 μ/10^6 cells. However, the increase in enzyme activity in these cells, after 24 h of co-culture with lymphocytes, did not persist on further incubation when the lymphocytes were removed after 24 h and replaced either with fresh medium or with 10^7 fresh lymphocytes for a further 24 h.
DISCUSSION

The experiments described here show that the genetically deficient human fibroblasts (GM151 and GM121) did not acquire $\beta$-glucuronidase by active endocytosis of enzyme secreted into the medium by lymphocytes. No significant enzyme activity was found in lymphocyte medium or in medium from lymphocyte–fibroblast co-culture. Nor was any $\beta$-glucuronidase acquired by deficient fibroblasts when they were cultured for 24 h in either of these media, although they were capable of active endocytosis of exogenous $\beta$-glucuronidase secreted by 3T3 fibroblasts. Active endocytosis by fibroblasts involves specific recognition of phosphomannosyl groups that are present in many lysosomal enzymes (Kaplan, Fischer, Achord, & Sly, 1977; Sando & Neufeld, 1977), and hence can be inhibited by competition with mannose 6-phosphate. This substance effectively inhibited the uptake of exogenous 3T3 cell $\beta$-glucuronidase by deficient fibroblasts but had no effect on their acquisition of enzyme during co-culture with lymphocytes.

Extracts of disrupted lymphocytes, which contained significant amounts of $\beta$-glucuronidase, also produced no detectable enzyme activity in deficient fibroblasts when added to their culture medium for 24 h. Although a number of other lysosomal enzymes have precursor forms (Hasilik & Neufeld, 1980), which might not be taken up by endocytosis, there does not appear to be any precursor form of $\beta$-glucuronidase, at least in murine macrophages (Skudlarek & Swank, 1979). Although lymphocytes have not yet been studied in this respect, the present experiments showed that $\beta$-glucuronidase from lymphocytes, like that isolated from a number of human tissues (Brot, Glaser, Roozen & Sly, 1974; Glaser, Roozen, Brot & Sly, 1975), has only a low uptake potential. Nevertheless, during co-culture with lymphocytes, deficient fibroblasts acquired high levels of $\beta$-glucuronidase activity.

Receptor-mediated accumulation by macrophages of glycoconjugates containing mannose groups is not dependent on de novo protein synthesis (Stahl et al. 1980) as the specific receptors are thought to be recycled (Tietze, Schlesinger & Stahl, 1980; Goldstein, Anderson & Brown, 1979; Doyle, Hon & Warren, 1979; Tanabe, Pricer & Ashwell, 1979). Endocytosis of exogenous $\beta$-glucuronidase prepared from 3T3 cells was also found to be unaffected by inhibitors of protein synthesis, whereas the acquisition of enzyme during co-culture with lymphocytes was totally dependent on de novo protein synthesis as well as energy supply. However, inhibitors of protein synthesis reduced the level of $\beta$-glucuronidase in lymphocytes cultured alone by 50% in 24 h, and also prevented the threefold increase in lymphocyte enzyme that occurred when they were co-cultured with fibroblasts. Thus, the acquisition of $\beta$-glucuronidase by deficient fibroblasts may be related to concomitant stimulation of new protein synthesis in the lymphoid cells during direct cell-to-cell interaction. It is notable that there was a lack of correlation (Table 2) between the initial levels of $\beta$-glucuronidase in the lymphocytes from different sources and the amount of enzyme acquired by the deficient fibroblasts. This may arise from differences in the degree of stimulation of $\beta$-glucuronidase synthesis by lymphocytes during contact with fibroblasts.
Chang et al. (1979) have noted the adhesion of lymphocytes to (and detachment from) mouse 3T3 fibroblasts in vitro. In the present study, direct contact between lymphocytes and deficient fibroblasts was obligatory, since deficient fibroblasts acquired no enzyme when separated from the lymphocytes by Millipore membranes, which were themselves permeable to the enzyme from 3T3 cells.

Although the mechanism of acquisition of $\beta$-glucuronidase by direct cell contact is unknown, it does not appear to involve Con A-mediated uptake (Juliano, Moore, Callahan & Lowden, 1979). This lectin affects the characteristics of the surface membranes of many cells (Schlessinger et al. 1977; Doetschmann, 1980), including lymphocytes (for a review, see Hume & Weidman, 1980), and induces in them a redistribution of a number of lysosomal enzymes (Hirschorn, Brittinger, Hirschorn & Weissman, 1968). Although Con A stimulated the uptake of exogenous 3T3 cell enzyme from the medium by GM151 cells, it had no effect on enzyme acquisition during direct contact with lymphocytes. This occurred whether or not the lymphocytes had been previously stimulated by the mitogen. Moreover, $\alpha$-methylmannoside, which dissociates Con A from cell membrane receptors (Noonan & Burger, 1973), had no effect on the acquisition of enzyme by fibroblasts in direct contact with lymphocytes that had been treated with Con A.

Lymphocytes secrete a large number of regulatory factors (David & Rocklin, 1978; Waksman, 1978), including some that affect the activity of fibroblasts both directly (Wahl, Wahl & McCarthy, 1978) and indirectly (Hauser & Vaes, 1978). However, it is unlikely that such substances could induce the expression of $\beta$-glucuronidase activity in deficient fibroblasts as these cells are known to produce a cross-reacting but enzymically inactive protein (Bell, Sly & Brot, 1977), apparently as a result of a mutation in the structural gene for $\beta$-glucuronidase (Chern, 1977). Although lymphocyte factors could possibly influence post-translational protein modifications (Tabas & Kornfeld, 1980), other lysosomal enzymes are normal in $\beta$-glucuronidase-deficient fibroblasts (Sly et al. 1973), unlike the situation in I-cell disease (Miller, Freeze & Kress, 1981; Champion & Shows, 1977). However, in addition to the gene determining the amino acid sequence of $\beta$-glucuronidase, there are also several specific regulatory foci involved in the expression of enzyme activity (Paigen, 1981), which might be affected by lymphocyte factors. Nonetheless, in the experiments reported here, substances secreted by lymphocytes into the medium or in extracts of these cells were without effect and did not promote the appearance of $\beta$-glucuronidase activity in the deficient fibroblasts. The cellular origin of the acquired $\beta$-glucuronidase thus remains to be established.

The acquisition of $\beta$-glucuronidase by direct contact with lymphocytes was not restricted to the two deficient human cell lines (GM151 and GM121). Human fibroblasts heterozygous for $\beta$-glucuronidase (GM150) and mouse A9 cells, both of which possess low levels of enzyme, acquired enhanced $\beta$-glucuronidase activity during co-culture with lymphocytes. It is unlikely that gap junctions are involved in the process since only low molecular weight substances can be transferred through them, and moreover, the mouse A9 cell line is not proficient in forming these structures (Gilula, Reeves & Steinbach, 1972; Subak-Sharpe, Bürk & Pitts, 1969; Cox et al.
1974). On the other hand, direct transfer of protein between cells has been estimated to take place at a rate as high as 1% of donor cell protein per hour (Kolodny, 1974). Protein exchange by direct cell-to-cell contact in vivo has also been proposed by Feder (1976) to explain the transfer of \( \beta \)-glucuronidase activity in tetraparental mice, and may explain the presence of \( \alpha \)-mannosidase in some visceral organs and lymphoid tissues of a natural chimeric mannosidosis calf (Jolly et al. 1976).

Lymphoid cells are highly mobile, and establish direct, if temporary, contact with endothelial cells and reticular cells of lymph nodes during circulation (de Bono, 1980). The transient clinical benefit seen after infusion or normal leukocytes into patients with Sanfilippo’s (Moser et al. 1974) and Hunter’s (Knudson, di Ferrante & Curtis, 1971) syndromes, could perhaps have resulted from the transfer of normal enzyme by cell-to-cell contact of donor with patients’ cells.

Acquisition of \( \beta \)-glucuronidase by direct transfer appears to be highly efficient, whilst there was no measurable endocytosis from the medium of enzyme extracted from the lymphocytes. Endocytosis of \( \beta \)-glucuronidase from other sources is very variable; that isolated from human placenta is taken up by fibroblasts at a rate of less than 0.01% per hour (Brot et al. 1974), whereas the rate of uptake of the enzyme extracted from human platelets is 0.2-0.5% per hour (Frankel, Glaser & Sly, 1977; Bach & Liebmann-Eisenberg, 1979). The efficiency of direct enzyme transfer is shown further by the finding that the lymphocytes were able to induce at least a second, full round of \( \beta \)-glucuronidase acquisition in fresh, deficient fibroblasts.

The enzyme acquired by the fibroblasts appeared to be stable, and declined because of cell division rather than intracellular degradation. \( \beta \)-Glucuronidase internalized by endocytosis has also been shown to retain its activity for long periods (Lagunoff et al. 1973; Frankel et al. 1977; Bach & Liebmann-Eisenberg, 1979). Why enzyme activity in the deficient fibroblasts did not increase further, on the addition of fresh lymphocytes or on extended co-culture, cannot be explained at present. It is notable that the level of \( \beta \)-glucuronidase acquired by the deficient human fibroblasts in the present experiments would be more than sufficient to correct the deficiency of mucopolysaccharidosis type VII (Hall, Cantz & Neufeld, 1973).

We are grateful to Roslyn Smith and Christina Clifton for their excellent technical assistance. We also thank Stephanie Diment for her generous gift of \( \beta \)-glucuronidase from 3T3 cells and Dr J. Chayen of the Kennedy Institute for making available the microdensitometer facilities. We acknowledge the support of the Wellcome Trust.

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(Received 22 October 1981)