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MICROINJECTION OF ARGINASE INTO ENZYME-DEFICIENT CELLS WITH THE ISOLATED GLYCOPROTEINS OF SENDAI VIRUS AS FUSOGEN

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A method of introducing enzymes into the cytoplasm of fibroblasts in culture is described. Erythrocytes obtained from normal and arginase-deficient individuals were loaded with arginase in vitro and fused to arginase-deficient mouse and human fibroblasts. Erythrocyte ghost-fibroblast fusion was quantified by a ^{14}C-radioactive assay for arginase in solubilized fibroblasts. Fusion was successfully induced by Sendai virus and also by the isolated glycoproteins of Sendai virus. After fusion the arginase activity associated with the fibroblasts was 700–1500 U of arginase/mg of cell protein; this enzyme activity was 5- to 10-times higher than that normally found in the fibroblasts. The enrichment in arginase activity indicated that between four and ten ghosts had fused per fibroblast. The use of isolated viral proteins to mediate the transfer of enzymes into cells in vivo might alleviate clinical complications inherent in the use of whole virions. The enzyme replacement technique described in this report for a hyperargininemic model cell system should be applicable to the group of inborn errors of metabolism characterized by deficiency of an enzyme normally localized in the cytoplasmic compartment of cells.

Introduction

Particles or macromolecules trapped within erythrocyte ghosts can be introduced into other cells by virus-induced fusion, a process termed ultramicroinjection [1]. This process introduces material directly into the cytoplasm of the recipient cell and therefore potentially could be used to effect enzyme replacement for inborn errors of metabolism caused by cytoplasmic enzyme deficiencies. Among these disorders are phenylketonuria, galactosemia, adenosine deaminase deficiency, and hyperargininemia (see Ref. 2). The latter disorder is characterized by deficiency of arginase activity primarily in liver which causes progressive mental and physical retardation in affected individuals [3]. Ultramicroinjection of thymidine kinase into thymidine kinase deficient cells resulted in the in vitro correction of the metabolic deficiency [4]. Since Ihler et al. [5] first proposed that erythrocytes could serve as in vivo carriers of exogenous proteins, several enzyme replacement attempts have been made in an individual with Gaucher's disease [6] by this technique. Injected erythrocyte ghosts loaded with human placental β-glucosidase had a circulating half-life of 5–10 days. Although the few treatments produced no demonstrable effects on the patient, the therapeutic value of this enzyme replacement technique over the long-term is unknown. There have been only a limited number of enzyme replacement trials by ultramicroinjection and as yet a successful method of thera-

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Abbreviations: SDS, sodium dodecyl sulfate; U, units of enzyme activity. Sendai virus is also known as Hemagglutinating Virus of Japan (HVJ).

peutic treatment for inborn errors of metabolism has not been demonstrated.

The ultramicroinjection method of mediating an intercellular transfer of active enzyme from ghosts to nucleated cells offers an attractive alternative to direct injection of enzyme or enzyme-loaded ghosts into patients. The paramyxoviruses are a family of membrane-enveloped viruses that fuse with host plasma membranes and, when added to cells in large numbers, promote fusion between cells. Currently microinjection methods are based upon whole Sendai virus or poly(ethylene glycol) to promote cell-cell fusion. Both of these fusogens could produce undesirable side effects in vivo. Viral particles, even when inactivated, could have detrimental effects on cells and will ultimately stimulate an immune reaction, while the concentration of poly(ethylene glycol) used to promote cell-cell fusion in vitro could never be achieved in vivo.

Sendai virus has a simple membrane protein composition; only three proteins (HN, F, and M) are present and two form the glycoprotein spikes seen by electron microscopy. The spikes HN and F are embedded in a bilayer of lipid, which is derived from the host cell [7,8]. The HN protein has hemagglutinating and neuraminidase activities and is involved in the cell recognition process [9–11]. The F protein is involved in hemolysis, cell fusion and virus penetration [12–15]. In a separate study, we found that after the HN and F proteins are extracted from Sendai virus by a standard procedure [13] and treated with Amberlite XAD-2 resin to remove Triton X-100 and lipids, the protein preparation retains hemagglutinating and cell-cell fusion activities [16]. In this study we exploit the fusion ability of the HN and F protein solution to develop a model system for enzyme replacement therapy in hyperargininemia due to liver and erythrocyte arginase deficiency.

Materials and Methods

Biochemicals. [*Guanido*-^{14}C]Arginine (spec. act. 54.3 Ci/mol) was obtained from Amersham-Searle Corp. Bovine serum albumin (Fraction V, essentially fatty acid free), bovine liver arginase (EC 3.5.3.1), and horse heart cytochrome C (Type IIA) were purchased from Sigma. Prosil®-28 (PCR Research Chemicals, Gainsville, FL) was used to siliconize glassware for erythrocyte handling. Hemotrol®, a hemoglobin standard, was obtained from Clinton Laboratories, Santa Monica, CA.

Cells and Cell Counts. Human skin or mouse (3T6 or 3T3) fibroblasts were grown in 75-cm^2 tissue culture flasks (Corning Glass Works, New York, NY). The monolayer cultures were grown at 37°C in Dulbecco's minimal essential medium (MEM) plus 10% fetal calf serum. Cells were plated 12–18 h before use.

Erythrocyte ghosts or tissue culture cells in suspension were counted with a Spencer Hemocytometer (No. 1492 Brightline Counting Chamber, American Optical Co., Buffalo, NY) or a Royco Tissue Cell Counter (Model 920A, San Francisco, CA). Tissue culture cells in monolayer culture were counted with a Falcon Cell Counting Grid (No. 3020; Becton, Dickinson and Co., Oxnard, CA).

Virus and Viral Glycoproteins. Sendai virus was propagated in 10–11-day-old embryonated eggs. Allantoic fluid was harvested 48–72 h after infection and virus purified under sterile conditions by the procedure of Markwell and Fox [17]. Purity of the virus was checked by SDS-polyacrylamide gel electrophoresis and electron microscopy. The titer of the suspensions was determined by hemagglutination titration [18] with formalin-fixed chicken erythrocytes [19].

The method of Scheid and Choppin [13] for isolation of Sendai viral glycoproteins was followed with minor modifications. Purified virus (0.5–1.5 mg of protein/ml) in 1 M KCl and 0.01 M sodium phosphate buffer (pH 7.2) was treated with Triton X-100 at a final concentration of 2%. A sample of the partially solubilized virus was iodinated with ^{125}I as described by Markwell and Fox [20] then returned to the pool of virus. The viral sample was incubated for 1 h at 4°C. The suspension was centrifuged at 10 000 × *g* for 20 min. The supernatant was removed and centrifuged at 200 000 × *g* for 1 h to obtain a second pellet. The supernatant was then dialyzed against 0.01 M sodium phosphate buffer (pH 7.2) to remove KCl and to precipitate the matrix (M) protein. The dialysate was centrifuged at 10 000 × *g* for 20 min and the supernatant recovered. All fractions were analyzed by SDS-polyacrylamide gel electrophoresis and radioautography. The final supernatant containing the HN and F glycoproteins was incu-

bated with Amberlite XAD-2 resin (Rohm and Haas, Philadelphia, PA) at 4°C or 25°C to remove the Triton X-100 [21]. The incubations were continued until absorbance readings at 282 nm with time remained constant. Typically, two or three changes of resin were required. Before use, the resin was washed exhaustively with methanol followed by water and 10 mM sodium phosphate buffer (pH 7.2). The binding capacity of the washed resin was approx. 0.3 g of Triton X-100 per g of wet beads. The quantity of resin used was based on the amount of Triton initially used to solubilize the virus.

Arginase assay. Arginase activity was measured by the conversion of [*guanido*-^{14}C]arginine to urea and ornithine, with subsequent release of $^{14}CO_2$ from the urea by urease [22]. The $^{14}CO_2$ was trapped on filter paper soaked with 8% NaOH and counted in scintillation fluid containing 12.0 g of 2,5-diphenyloxazole and 0.3 g of *p*-bis(2[5-phenyloxazolyl])benzene in 1 liter of absolute methanol and 2 liters of toluene. One unit of arginase activity was defined as 1 nmol of arginine cleaved per 120 min.

Loading of erythrocyte ghosts. Human erythrocytes (type O) were isolated from freshly drawn, heparinized blood. Whole blood was centrifuged at 1 000 × *g* for 10 min at 4°C. The supernatant fraction and buffy coat were removed by aspiration. The pellet was washed four times in 10 vol. of phosphate-buffered saline (5 mM sodium phosphate buffer (pH 7.4), 0.15 M NaCl). Erythrocytes were loaded by the dialysis method described by Dale et al. [23]; cytochrome C was used in the loading buffer as suggested by Wasserman et al. [24]. A 5-ml packed volume of the washed erythrocytes was gently mixed with 4 ml of a filtered bovine arginase solution (10 mg/ml) or bovine serum albumin solution (10 mg/ml) in phosphate-buffered saline containing cytochrome C (0.5 mg/ml). The bovine arginase specimens were assayed and found to have a specific activity of (0.5–2.0) · 10^5 U/mg protein. Erythrocyte-protein mixtures were placed in dialysis tubing and dialyzed for 90 min at 4°C against 5 mM sodium phosphate buffer, pH 7.4, while rotated with a Rugged® rotator (Kraft Apparatus, Inc., Mineola, NY) at 4–5 rev./min. The dialysis tubing, which contained the lysed cells, was transferred to a tube containing phosphate-buffered saline and rotated for 30 min at 25°C to reseal the erythrocytes. The erythrocyte ghosts were placed in centrifuge tubes and annealed at 37°C for 15 min before pelleting at 12 000 × *g* for 15 min. The supernatant was removed and the pellet washed three times with phosphate-buffered saline. Suspensions of enzyme-loaded ghosts (average 1.4 · 10^8 cells/ml, $n = 7$) were made with phosphate-buffered saline as diluent. These were stored at 4°C and used within 2 h for fusion. The number of cells, arginase activity, protein content [25] and hemoglobin concentration [26] were determined for each ghost suspension.

Erythrocyte-mammalian cell fusion experiment. The experiment followed the procedure of Furusawa et al. [27] with minor modifications detailed here. The culture medium (minimal essential medium) was removed from the fibroblasts in monolayer culture and the cells washed twice with cold phosphate-buffered saline. Either fusogen or phosphate-buffered saline was added to the cells (3 ml/flask) and they were incubated for 10 min at 4°C. The incubation medium was removed and the cells rinsed once with cold phosphate-buffered saline. The enzyme-loaded erythrocyte ghost suspension was added to the cells (5 ml/flask) and after 10 min at 4°C was removed by aspiration. Warm phosphate-buffered saline (37°C) with 1 mM $CaCl_2$ and 0.1 mM $MnCl_2$ was added (5 ml/flask) and the cultures incubated at 37°C for 20 min, the fusion period for the cells. The incubation medium was aspirated from the culture flasks and the cells were thoroughly washed three times with phosphate-buffered saline, once with 10 mM sodium phosphate buffer (pH 7.4) for 10 s, and finally with phosphate-buffered saline to remove the lysed erythrocytes. The fibroblasts were detached with trypsin (0.025% in phosphate-buffered saline, 10 min, 37°C), diluted in serum-free culture medium and collected by centrifuging (1 000 × *g* for 10 min). The culture medium was aspirated and the cell pellet was suspended in 0.5 ml of phosphate-buffered saline. A portion was removed for a cell count. The remaining cell suspension was brought to 0.4% Triton X-100 concentration. Samples of the solubilized cells were assayed for arginase and for protein content.

Microscopic examination of the fibroblasts after the washing procedures, whether the cells were treated with fusogens or not, revealed no erythrocytes (or ghosts) adhered to the fibroblasts.

Results

Arginase levels in erythrocytes and arginase-loaded erythrocyte ghosts

The erythrocytes of five normal individuals contained 166 ± 39 U of arginase/10^6 erythrocytes (range 149–214 U/10^6 erythrocytes) before loading. These were loaded with either solutions of bovine arginase (spec. act. $2 \cdot 10^5$ U/mg) or bovine serum albumin. Those loaded with arginase contained 320 ± 77 U of arginase/10^6 ghosts (range 225–417 U/10^6 ghosts). This represented a 93% increase in arginase activity (i.e., 154 U). Those loaded with bovine serum albumin contained 119 ± 22 U of arginase/10^6 ghosts (range 93–148 U/10^6 ghosts) representing a 28% decrease in arginase activity. The erythrocytes lost an average of 31.2 ± 16% ($n = 8$) of their hemoglobin content during loading. When erythrocytes were taken through the entire loading procedure but maintained in isotonic buffer throughout, only 1–3% leakage of hemoglobin was observed. Thus, the handling of the erythrocytes during the loading procedure is gentle.

Erythrocytes from an arginase-deficient individual had an arginase activity of 0.26–0.8 U/10^6 erythrocytes, negligible when compared to the physiological levels found in normal individuals. When these arginase-deficient erythrocytes were loaded with bovine arginase, a specific activity of 201 ± 33 U of arginase/10^6 ghosts was obtained, a level within physiological range. To determine the amount of arginase nonspecifically adsorbing to the erythrocyte surface, erythrocytes from an arginase-deficient individual were taken through the loading protocol as described except maintained in isotonic buffer throughout. The initial arginase activity of the erythrocytes was 0.8 U/10^6 erythrocytes. This rose to 1.2 U/10^6 erythrocytes after exposure to the loading buffer containing arginase and after washing. This indicates that little of the arginase (i.e., 0.4 U/10^6 erythrocytes) is associated with the erythrocytes by adsorption or some other process. As additional evidence, the arginase activity of loaded-ghosts subjected to proteolytic treatment (0.025% pronase, 10 min, 37°C) was the same within experimental error as that of untreated, washed loaded-ghosts. Arginase, when treated with pronase, loses activity.

TABLE I

FUSION OF FIBROBLASTS WITH ERYTHROCYTE GHOSTS INDUCED BY SENDAI VIRUS

Erythrocytes were from an arginase-deficient individual and were used either as intact cells or after loading with bovine liver arginase (spec. act. $0.9 \cdot 10^5$ U/mg). The intact erythrocytes contained 0.41 U of arginase per 10^6 cells; the arginase-loaded erythrocytes contained 207 U/10^6 ghosts. The fibroblasts were treated with approx. 400 HAU/ml of Sendai virus and carried through the fusion protocol. Values are the mean of triplicate samples.

Additions to fibroblasts treated with Sendai virus	Arginase activity per mg of cell protein (units/mg)	
	3T3	3T6
None	142	184
Intact erythrocytes	128	180
Bovine serum albumin-loaded erythrocyte ghosts	167	158
Bovine serum albumin-loaded erythrocyte ghosts plus free arginase [a]	156	190
Arginase-loaded erythrocyte ghosts	979	1469
Fusion index [b]	5.5	7.8

[a] Buffer containing 0.5 mg of arginase (48 411 U) was added to the erythrocyte ghost suspension just before its addition to the fibroblasts.

[b] Values are the average number of arginase-loaded ghosts fused per fibroblast. Fibroblast numbers were derived from protein determinations.

Arginase content in fibroblasts after fusion with erythrocyte ghosts

The extent of cell fusion was measured by the transfer of arginase from arginase-loaded erythrocyte ghosts to mouse 3T6 or 3T3 cells or human skin fibroblasts from the hyperargininemic individual. These cell lines have little endogenous arginase activity. The net increase in arginase activity of the fibroblasts after fusion (total cell numbers obtained by count or by protein determinations), allowed us to calculate reproducibly the average number of erythrocyte ghosts fused per fibroblast, a parameter we call the fusion index.

Results from two separate experiments in which arginase-loaded ghosts were fused to mouse 3T6 and 3T3 cells with Sendai virus as a fusogen are shown in

Table I. Control samples demonstrate that background arginase activity was low and also that arginase did not enter the cells by any process other than fusion with ghosts. In addition to the controls depicted in Table I, 3T6 fibroblasts also were treated with Sendai virus then with arginase in solution. After treatment, the arginase activity of the fibroblasts was similar to normal cell background levels (data not shown). Thus, changes in cell permeability occurring after virus-cell fusion [28] do not facilitate arginase entry. Any arginase, either adhered to cell surfaces or associated with the cells in such a manner as to be in the final assay mixture, was destroyed by trypsin at the cell detachment step in the experimental protocol (data not shown). From 700 to 1 500 U of arginase activity per mg of cell protein was found in fibroblasts after exposure to Sendai virus and arginase-loaded ghosts. This level of activity was seven to ten times higher than that of the highest control value. Fusion indices of 5.5 and 7.8 were determined. Typically, fusion indices of 4 to 10 were obtained; however, occasionally fusion indices as high as 14 were observed. Similar fusion data were obtained when skin fibroblasts from the hyperargininemic individual were used as recipient cells with fusion indices of 5.3 and 6.2.

TABLE II

FUSION OF 3T6 FIBROBLASTS WITH ERYTHROCYTE GHOSTS INDUCED BY THE HN AND F GLYCOPROTEINS OF SENDAI VIRUS

Erythrocytes were from an arginase-deficient individual and were used either as intact cells or after loading with bovine liver arginase (spec. act. $0.05 \cdot 10^5$ U/mg). The intact erythrocytes contained 0.26 U of arginase per 10^6 cells; the arginase-loaded erythrocytes contained 155 U/10^6 ghosts. The fibroblasts were treated with approx. 170 HAU/ml of viral HN and F fusogen (≃8 μg total protein) and carried through the fusion protocol. Values are the mean of triplicate samples.

Additions to fibroblasts treated with HN and F protein	Arginase activity per mg of cell protein (units/mg)
None	70
Intact erythrocytes	35
Bovine serum albumin-loaded erythrocyte ghosts	39
Bovine serum albumin-loaded erythrocyte ghosts plus free arginase [a]	48
Arginase [a]	40
Arginase-loaded erythrocyte ghosts	732
Fusion index [b]	5.0
Fusion index per μg of HN and F protein	0.63
Arginase-loaded erythrocyte ghosts plus Sendai virus [c]	870
Fusion index [b]	7.0
Fusion index per μg of HN and F protein	0.20

[a] Buffer containing 0.5 mg of arginase (2585 U) was added to the erythrocyte ghost suspension just before its addition to the fibroblasts, or to the fibroblasts directly.

[b] Values are the average number of arginase-loaded ghosts fused per fibroblast. Fibroblast numbers were derived from protein determinations.

[c] Whole Sendai virus was used in place of the viral HN and F fusogen at a concentration of approximately 400 HAU/ml (≃87 μg total protein or 34 μg HN and F protein).

Results from a second series of fusion experiments in which the viral HN and F protein preparation was used as the fusogen are shown in Table II. A fusion index of 5.0 was obtained with the 3T6 cells and the new fusogen. As an additional control, Sendai virus was substituted for the HN and F proteins as a fusogen and the cells were exposed to the arginase-loaded ghosts; a fusion index of 7.0 was obtained. In this particular experiment fusion levels induced by the two different fusogens were compared by dividing the respective fusion index of each by the HN and F protein content of the preparation. HN and F constitute approx. 39% of the total virus protein by weight [20,29]. These calculations revealed that the HN and F protein solution was three times more efficient than the whole virus in stimulating fusion per μg of HN and F proteins added.

Fibroblasts were plated at a low density 12–18 h before use to keep homologous cell-cell fusion to a minimum. Although Sendai virus does induce fusion between intact erythrocytes, it fails to induce fusion between erythrocyte ghosts [30–32]; thus, heterologous cell-cell fusion between erythrocyte ghosts and fibroblasts was favored in our system. As previously established, cell viability is maintained after microinjection [33].

Discussion

The evidence presented here and earlier [16] shows that not only Sendai virus, but also the two coat proteins, HN and F, are able to induce fusion between erythrocytes (or ghosts) and fibroblasts. The evidence for the cell-cell fusion rests not only on the transfer of arginase from erythrocytes (or ghosts) to the fibroblasts, but also on the transfer of hemoglobin into the cytoplasm of the fibroblasts [16] and on the absence of any evidence for the adherence of erythrocytes (or ghosts) to surfaces of the fibroblasts.

No delivery system tried to date in enzyme replacement therapy for inborn errors of metabolism has proven to be practical or effective for long term use. Several methods have been tried including direct injection of enzyme and injection of enzyme-loaded erythrocytes or liposomes. In some studies efforts have been made to direct the enzyme to specific tissues or to expose blood and body fluids to enzyme immobilized on solid supports [34–38]. The major problem encountered with all these attempts has been the relatively short half-life of the enzyme. Free enzyme is usually removed by reticuloendothelial cells or is digested in the lysosomes of liver cells and continued injection can elicit an immunologic response in the patient. In addition, free or immobilized enzyme may react with other proteins in the circulation and be inactivated.

Most studies of enzyme replacement have focused on deficiencies of lysosomal enzymes since the cellular fate of injected enzyme typically is to be taken up by lysosomes [34]. Enzyme replacement in disorders of cytoplasmic enzymes presents a problem of a different kind in that the enzyme has to be delivered to the cytoplasm.

Until now, cytoplasmic delivery methods have relied on chemicals or whole Sendai virus to promote fusion of cells with appropriately loaded erythrocyte ghosts. Ferritin, thymidine kinase, latex spheres, bacteriophage, antibody, adenovirus type 2 DNA, bovine serum albumin, and tRNA have all been transferred to recipient cells by the ultramicroinjection technique [4,39–43]. It is preferable, however, to exclude use of whole virus when designing methods for enzyme replacement in man. The isolated viral glycoprotein fusogen described in this report contains no viral genetic material. In addition, it is not part of a reconstituted viral system with phospholipid vesicles. This feature is important because reconstituted membrane systems or protein-free liposomes can stimulate an immunogenic response in vivo [44,45]. Almeida et al. [45] have reported that influenza virus is normally highly immunogenic but that the purified protein subunits are not. When the protein subunits were reconstituted with lipid, however, the particles formed were immunogenic. If this phenomenon extrapolates to the paramyxovirus system, then the protein fusogen preparation described in this study has better prospects for in vivo use than whole virus or liposomes, an advantage augmented by the fact that the isolated protein particles are more effective as a fusogen than the whole virus based on equal amounts of HN and F by weight.

We have chosen arginase-deficiency as a model system for our studies because arginase has been shown to be free in the cytoplasm [46] and because arginase is a relatively stable enzyme with no complex requirements for cofactors. Like Adriaenssens and coworkers [47], we demonstrated that physiological levels of enzyme can be loaded into ghosts and that enzyme activity is stable in the ghosts.

Although we have demonstrated the effectiveness of our fusogen preparation for mediating in vitro correction of cellular arginase-deficiency, further studies are required before this approach can be considered safe or practical to try in man. The type of replacement protocol we envision for in vivo trials would entail: (1) enzyme loading of erythrocytes from an affected animal, (2) incubation of the loaded ghosts with the HN and F fusogen preparation, and (3) injection of the ghosts back into body circulation. Fusion of arginase-loaded ghosts with target liver cells would effect enzyme transfer in the case of hyperargininemic animals [48]. Such a protocol should allow for repeated injections with a low probability of stimulating an immunologic response to the added enzyme or fusogen-coated ghost carrier. Based on the success of our model trials, an in vivo delivery method based on our protein fusogen preparation may ultimately offer a significant option in the treatment of human arginase deficiency and other cytoplasmic enzyme disorders.

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