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INJECTION OF PROTEINS INTO PRIMARY RAT HEPATOCYTES BY ERYTHROCYTE-MEDIATED TECHNIQUES

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SUMMARY: Several erythrocyte-mediated techniques for injecting selected proteins into primary rat hepatocytes were compared. Each method was evaluated as to maintenance of hepatocyte viability, percentage of liver cells receiving protein, and capability of removing non-fused loaded erythrocytes. On the basis of these criteria Sendai virus-induced fusion of pre-loaded erythrocyte ghosts and primary rat hepatocytes in culture was determined to be the best method and most useful for studying degradation of specific proteins.

A major response of parenchymal liver cells to nutritional deprivation is enhanced intracellular protein degradation. This increased catabolism of intracellular protein in hepatocytes, which reaches a maximum of 3.5%/hr as compared to 2.5%/hr in most other cells [1], provides amino acids for gluconeogenesis and for new protein synthesis in other tissues as well as liver. Studies on the breakdown of prelabeled endogenous protein in the perfused liver [2] and primary hepatocyte suspensions [3] or monolayers [4] indicate that the overall rate of proteolysis is regulated by insulin, glucagon and plasma amino acid concentrations. It is also important to determine the effects of hormones and nutrients on selective protein degradation in these cells. Microinjection of specific, radiolabeled proteins into hepatocytes is one possible procedure for doing such experiments. In recent years a number of investigators have used this approach to measure catabolism of individual proteins in hepatoma cells [5,6]. However, established lines of transformed

ABBREVIATIONS: BSA, bovine serum albumin; HRP, horseradish peroxidase; BSS^{+}, balanced salt solution with Ca^{2+}; BSS, balanced salt solution without calcium; HAU, hemagglutinating units; DAB, 3,3'-diaminobenzidine.

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liver cells do not exhibit as many characteristics of liver as primary hepatocytes. Therefore, the purpose of the present study was to determine if current erythrocyte-mediated techniques could be used to microinject proteins into the highly differentiated, non-replicating, primary hepatocyte.

MATERIALS AND METHODS

Hepatocytes were isolated from the livers of 200-250g adult male Wistar rats by collagenase (Worthington, Type II) perfusion [7]. Isolated cells were suspended in L-15 (Leibovitz) medium supplemented with 8.3 mM glucose, 18 mM Hepes, 0.2% bovine serum albumin (Fraction V, Pentex), bovine insulin (5 µg/ml), and 5% newborn calf serum (Flow). Three ml aliquots of this suspension were used to seed 10^6 cells onto glass coverslips in 60 mm tissue culture dishes. The cultures buffered with $NaHCO_3$ were incubated at 37°C in a 5% CO_2 atmosphere. Medium was replaced with fresh L-15 medium without serum after a 4 h attachment period. Experiments were begun 24 h after cell isolation.

Horseradish peroxidase (Type II, Sigma) or [^{125}I]BSA, prepared as previously described [8], was loaded into human erythrocyte ghosts by the rapid lysis technique [9]. In brief 0.1 ml of packed swollen erythrocytes (10^9 cells) was mixed with 0.3 ml of a 10 mM Tris solution (pH 7.4) containing either 1.8 mg of peroxidase or 300 µg [^{125}I]BSA with a specific activity of 2.5-3.6 x 10^5 DPM/µg. After 2 min on ice, 45 µl of a 10 x concentrated Hanks' solution were added and the suspension incubated at 37°C for 60 min. Following 4 washes with 1 x Hanks' solution, the loaded erythrocytes were suspended in Furasawa's BSS$^+$ [10] buffered with 10 mM Tricine (pH 7.4) to give a final concentration of 2.5% v/v. During this procedure 15-20% of the [^{125}I]BSA was trapped in the erythrocyte ghosts. This efficiency of loading indicates that approximately 360 µg of HRP was trapped in 10^9 erythrocytes.

Sendai virus-induced fusions between erythrocytes and monolayer cultures of hepatocytes were performed essentially as described by Furasawa *et al.* [10]. Each monolayer was incubated with 1000 HAU of UV-inactivated Sendai virus in 2 ml BSS for 10 min at 4°C. Following removal of this suspension, cultures were incubated with 2 ml of a 2.5% v/v suspension of the loaded erythrocytes (5 x 10^8 cells) in BSS$^+$ at 4°C for 20 min to allow red cell attachment and then at 37°C for 20 min to allow fusion. Unattached erythrocytes were removed by washing 3 x with Tris-saline (pH 7.4).

Polethylene glycol-induced fusion of erythrocytes and primary hepatocytes was performed as described by Mercer *et al.* [11]. Monolayer cultures were incubated at 37°C with a 3 ml suspension of 10^8 erythrocytes and 150 µg of Phytohemagglutinin-P (Difco) in L-15 medium. After 30 min, the suspension was removed and cultures washed with Ca^{2+} and Mg^{2+} free Hanks' solution. To promote fusion, 2 ml of a 44% polyethyleneglycol (Koch-Light Laboratories, M.W. 6000) in L-15 medium was added to the dish for 60 sec. Following 3 washes with medium, cultures were incubated in fresh L-15 medium for 30 min.

Cultures were then fixed for 30 sec at 22°C in citrate-buffered acetone [12], air dried, and incubated in a staining solution of 0.03% DAB (Sigma) and 0.002% H_2O_2 in Tris-saline for 2 h at 22°C. Following processing for microscopy, cultures were examined under phase contrast and bright field optics and evaluated for percentage of HRP-positive cells.

RESULTS AND DISCUSSION

Several methods for microinjecting proteins into hepatocytes and quantifying the efficiency of microinjection were tested before optimal conditions were determined. Initially Sendai-induced fusion between erythrocytes and freshly isolated hepatocytes in suspension [9] was attempted since this is the method used for most other cells. Liver cells maintained 80-90% viability (as determined by trypan blue exclusion) when suspended in a number of balanced salt solutions including either Mn^{2+} or La^{3+}, cations reported to promote fusion and prevent cell lysis [9,13]. However, when varying concentrations of virus (300-1000 HAU) were added to the suspensions, hepatocyte viability was reduced to 10% or less. Consequently, all subsequent fusions were performed with hepatocytes after 24 h in culture. During this recovery period membrane damage resulting from isolation apparently was repaired. When these cultured cells were incubated with Sendai virus or polyethyleneglycol as described in Materials and Methods, they maintained the same viability as control cultures incubated with balanced salt solutions alone (data not shown).

Many investigators have assayed the extent of microinjection by fluorescence/phase microscopy of cultures injected with fluoresceinated proteins [11]. However, the autofluorescence exhibited by hepatocytes makes it difficult to distinguish between "injected" and "non-injected" cells. Consequently, a method involving microinjection of horseradish peroxidase and microscopic observation of HRP-DAB precipitation product was developed. Maximum cytoplasmic staining intensity and minimum peroxisome staining were achieved by using high concentrations of HRP in the erythrocyte loading medium, rapid fixation of microinjected cells in citrate-buffered acetone, and low concentrations of H_2O_2 in the staining solutions.

Figure 1A shows a typical bright field photograph of a hepatocyte culture following Sendai-induced fusion of hepatocytes with HRP-loaded erythrocytes. There is dark uniform staining of the hepatocyte indicating diffusion of HRP throughout the cytoplasm. This distribution of HRP throughout the recipient cell is even more striking when contrasted with the "non-injected"

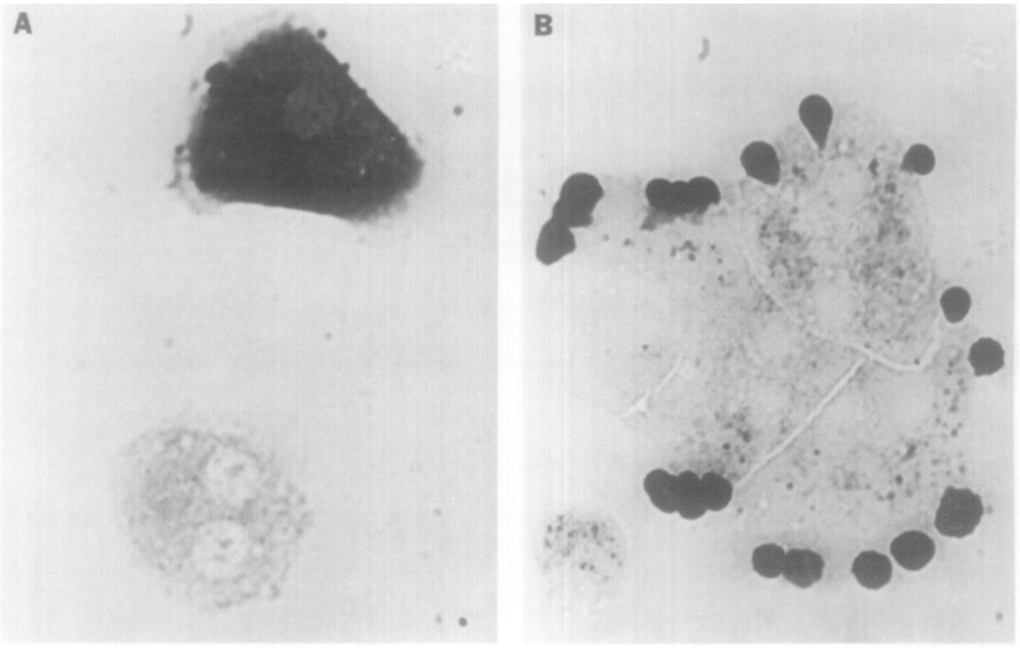

Figure 1. Light micrographs of two hepatocyte cultures following incubation with HRP-loaded erythrocytes and staining with DAB (x 500).

A. Sendai virus present as fusogen during incubation. Note the diffuse distribution of HRP-DAB reaction product throughout the recipient cell (top). The unstained hepatocyte (bottom) indicates no detectable fusion occurred between this cell and loaded erythrocytes.

B. No Sendai virus included during incubation. There is no detectable transfer of HRP from the attached darkly stained erythrocytes to the hepatocytes.

neighboring cell. Approximately 25-35% of the cells in these cultures were positive for HRP-DAB precipitation product. This efficiency of microinjection is comparable to that reported by Zavortink et al. [5] for transformed cells in suspension and Hendil [14] for fibroblasts in monolayer. Figure 1B is a bright field photomicrograph of a control culture, in which HRP loaded erythrocytes were incubated with hepatocyte cultures with no virus present. Since these cultures were not vigorously washed, many darkly stained erythrocytes remained attached to the hepatocytes. However, there was no transfer of HRP into hepatocytes when fusogen was absent.

Since Sendai virus can cause some leakage of loaded protein from erythrocytes, another control was included in these studies to determine if HRP

could be entering the cytoplasm of hepatocytes in some manner other than fusion with pre-loaded erythrocyte. This involved adding 0.90 mg of HRP, or 5 x that loaded into 5 x 10^8 erythrocyte during the rapid lysis procedure, to the BSS^+ during Sendai-induced fusion of hepatocytes with BSA-loaded erythrocytes. Although soluble HRP is taken up by hepatocytes through fluid phase pinocytosis, the endocytosed enzyme remains in pinocytic vesicles or lysosomes [15,16]. It would not be dispersed throughout the cytoplasm unless the conditions of microinjection ruptured these vesicles or rendered the plasma membrane of hepatocytes permeable to protein. In these control cultures, there was some DAB staining of vesicles but no homogenous staining of cytoplasm. These results indicate that during microinjection HRP enters the cytoplasm of viable hepatocytes only by fusion with pre-loaded erythrocytes.

One improvement reported by Mercer et al. [11] for increasing the efficiency of microinjection of monolayer is the use of Phytohemagglutinin-P to attach loaded erythrocytes to cultured cells prior to fusing with polyethyleneglycol. These investigators detected fluoresceinated-BSA in 50% of the fibroblasts in culture. However, in the present study when this method was applied to hepatocyte cultures, the fraction of cells receiving HRP was 25%, which was less than or equal to the efficiency when Sendai virus was used as a fusogen. In addition microscopic observation indicated that a large number of unfused HRP-loaded erythrocytes stayed attached to hepatocytes throughout exhaustive washings (8 x) and a 10 min treatment with 0.17 M NH_4Cl. When [^{125}I]BSA was loaded into the erythrocytes, the amount of radioactivity detected in the cell pellet of mock-fused cultures in which polyethyleneglycol was absent was at least 80% of that detected in cultures where fusogen was present. This demonstrates the difficulty in removing uninjected protein from these cultures, a phenomenon which could lead to subsequent errors in determining the rate of degradation of the selected protein.

On the basis of these results it appears that Sendai-induced fusion of loaded erythrocytes and hepatocytes in culture should be useful for studying

proteolysis or other metabolism of individual proteins in these highly differentiated cells.

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