Transfection of KB Cells by Polyethylene Glycol-induced Fusion with Erythrocyte Ghosts Containing Adenovirus Type 2 DNA

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SUMMARY

Cell ghosts were formed by hypotonic haemolysis and subsequent isotonic re-sealing of human erythrocytes in the presence of adenovirus type 2 DNA. The ghosts entrapped virus DNA with an efficiency which depended on the salt concentration employed during haemolysis and on the concentration of the DNA itself. The entrapped DNA was largely protected from digestion by deoxyribonuclease I and could be recovered intact by phenol extraction. Erythrocyte ghosts containing $^{32}$P-adenovirus type 2 DNA were fused with KB cells during brief treatment with polyethylene glycol (PEG). Following fusion, counts equivalent to an average of 25 molecules of labelled DNA were micro-injected and transported to each KB cell nucleus. Less than 1/4 as many DNA counts were recovered from nuclei when PEG treatment was omitted. A direct immunofluorescent assay demonstrated virus replication in some cells following their fusion with DNA-containing ghosts. The efficiency of transfection was considerably lower than that expected from the large number of successfully micro-injected DNA molecules. This suggests that most of the micro-injected DNA molecules were degraded before a successful infection could be completed.

A procedure for rapidly and efficiently micro-injecting macromolecules into large numbers of cultured cells has been described (Loyter et al. 1975; Rechsteiner, 1975; Schlegel & Rechsteiner, 1975; Wasserman et al. 1976; Kriegler & Livingston, 1977; Kriegler et al. 1978). The method entails the incubation of erythrocytes in hypotonic buffer causing them to swell, lose their haemoglobin content and become cell ghosts. Other macromolecules which are added to the hypotonic solution diffuse into the ghosts. Restoration of isotonic conditions results in cell shrinkage and stable entrapment of the new cell contents. The packaged macromolecules may then be 'micro-injected' into other cells by promoting the fusion of those cells with the loaded ghosts. In this report, we present our observations regarding the loading of erythrocyte ghosts with adenovirus type 2 DNA, micro-injection of the DNA into KB cells and the successful transfection of the recipient cells by the infected DNA.

$^{3}$H-thymidine or $^{32}$P-labelled adenovirus type 2 DNA, mol. wt. $23 \times 10^6$ (Craig & Raskas, 1974; Straus et al. 1979) was successfully entrapped in ghosts by employing the technique described by Wasserman et al. (1976) and Kriegler & Livingston (1977). Briefly, fresh human erythrocytes were washed by repeated centrifugation in solution A (160 mM-NaCl, 20 mM-Tricine-NaOH, pH 7.4) and resuspended at a final concentration of 10% (v/v) in solution A containing 0.5 mg/ml cytochrome c and the desired amount of DNA. The suspension was dialysed at 4 °C for 2 h against a dilution of buffer B (160 mM-NaCl, 40 mM-Tricine-NaOH, pH 7.4). For example, $0.25 \times B$ was made by addition of 3 vol. of sterile distilled water to 1 vol. of B. The ghosts were re-sealed by the addition of 1/20 vol. of 2.2 M-NaCl, 0.2 M-MgSO$_4$ and incubation in a shaker bath at 37 °C for 30 min. They were
then pelleted by centrifugation at 12,000 rev/min for 15 min at 4 °C and washed three times in solution A.

The uptake of labelled DNA into ghosts was appreciable when hypotonic lysis was performed in solutions whose tonicity was less than that of 0.5 × B. Maximal uptake of DNA molecules per cell was observed in cells dialysed against distilled water; however, those cells were exceedingly fragile and unsuitable for further experiments. All subsequent preparations of erythrocyte ghosts were dialysed against a solution whose osmolarity was approx. 25% of isotonic buffer (0.25 × B).

The incorporation of DNA into ghosts depended upon the concentration of the DNA in the preparation. Using labelled DNA it was demonstrated that ghosts prepared in a 0.22 μg/ml DNA solution entrapped an average of 0.6 molecules per cell. In the presence of 10 μg/ml DNA an average of about 10 molecules were incorporated into each cell. At high concentrations the uptake levelled off with only 180 molecules packaged per cell when a solution of 492 μg/ml was employed. This suggests that the sites of entry into the cells or the effective internal capacities of the cells were being saturated. The entrapped DNA was stably associated with the ghosts. The DNA counts were not removed by repeated washing of the ghosts in isotonic solutions or tissue culture medium. Deoxyribonuclease I treatment of the washed ghosts rendered an average of only 6 ± 2.4% (mean ± s.d.) of the DNA counts TCA soluble. An average of 88 ± 6% of DNA counts added to empty, pre-formed ghosts were solubilized under similar conditions. The packaged DNA could be quantitatively recovered from erythrocyte ghosts by extraction in phenol, chloroform and isoamyl alcohol (Tibbetts et al. 1973). The recovered DNA was found to be intact.

Sedimentation of the DNA in gradients of neutral or alkaline sucrose revealed that over 80% of the molecules were intact (data not shown).

Fusion of erythrocyte ghosts and KB cells was induced with polyethylene glycol 1000 (PEG) using the protocol of Kriegler & Livingston (1977). The method was tested using washed ghosts prepared in the presence of FITC-conjugated rabbit anti-human IgG. KB cells which were successfully fused to fluorescent ghosts displayed definite cytoplasmic fluorescence, indicating that micro-injection of antibody into the recipient cells had occurred (data not shown). Without PEG treatment an occasional erythrocyte was observed adhering to the KB cell surface but no cytoplasmic fluorescence could be discerned. Addition of virus DNA (150 μg/ml) to the antibody solution did not interfere with the successful packaging of fluorescent immunoglobulin into ghosts, the induction of fusion by PEG or the development of KB cell cytoplasmic fluorescence.

Micro-injection of DNA into KB cells was more difficult to assay directly. As outlined in Table 1, an attempt was made to recover labelled DNA from cells fused with ghosts. In initial experiments, virus DNA counts in whole cells were determined. Approx. \(5 \times 10^8\) ghosts containing \(5 \times 10^4\) ct/min of \(^{32}\)P-virus DNA were fused with \(2 \times 10^6\) KB cells. After repeated washings to remove unattached ghosts, 1.5 to 2.0% of input counts remained associated with the KB cells. This procedure could not distinguish between injected DNA and uninjected DNA remaining in ghosts attached to the cell surface. As an alternative, cell nuclei were isolated and their content of labelled DNA was determined (Straus et al. 1979). The nuclear pool of virus DNA counts is not grossly contaminated with the uninjected molecules and therefore provides a minimal estimate of cell uptake.

Erythrocyte ghosts containing \(^{32}\)P-adenovirus type 2 DNA were added to KB cell suspensions. One set of samples was fused by PEG treatment. Twenty h after plating, the nuclei of each sample were isolated and \(^{32}\)P-DNA counts determined. Table 1 shows the uptake of counts into KB cell nuclei in each sample. Nuclei from the PEG-treated sample contained over four times the TCA precipitable counts as the nuclei from the untreated
Table 1. Micro-injection of 32P-adenovirus type 2 DNA into KB cells and its subsequent transport to the nucleus*

<table>
<thead>
<tr>
<th>DNA trapped in ghosts</th>
<th>DNA recovered from nuclei</th>
<th>% DNA in ghosts recovered from nuclei</th>
<th>Virus DNA molecules/nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of KB cells</td>
<td>Ct/min</td>
<td>Molecules</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>2 x 10⁶</td>
<td>4.8 x 10⁴</td>
<td>7.0 x 10⁹</td>
</tr>
<tr>
<td>+</td>
<td>2 x 10⁶</td>
<td>4.8 x 10⁴</td>
<td>7.0 x 10⁹</td>
</tr>
</tbody>
</table>

* Aliquots (0.1 ml) of 5 x 10⁶ ghosts, each containing 4.8 x 10⁴ ct/min of 32P-adenovirus type 2 DNA, were added to suspensions of KB cells. Each KB cell suspension contained 2 x 10⁶ cells in 0.2 ml of serum-free medium; 0.3 ml of a 50% PEG solution in medium was added to each of two suspensions while 0.3 ml of medium alone was added to the remaining two suspensions. After 1 min incubation at room temperature, 3 ml of medium was added and each sample was plated on 60 mm plastic dishes and incubated at 37 °C. Eight h later the medium was replaced with medium supplemented with 10% foetal calf serum. At 20 h after fusion the cells were trypsinized, nuclei extracted and acid precipitate counts determined. The data represent averages for each pair of samples.

The ability of the DNA-containing erythrocyte ghosts to infect KB cells was determined by a fluorescent focus assay (FFA) (Thiel & Smith, 1967) employing FITC-conjugated rabbit anti-adenovirus type 2 IgG (prepared in this laboratory). In a series of control experiments, a stock of adenovirus 2 was titrated by the FFA (Table 2). The resulting titre correlated well with that calculated for the same stock by the standard plaque assay (Green et al. 1967). High input multiplicities of the same virus stock were completely neutralized by treatment with rabbit anti-adenovirus type 2 IgG (incubation at 37 °C for 30 min) or by heating to 56 °C for 10 min. Heat treatment of adenovirus rapidly destroys virus coat protein associations which are critical to infectivity (Russell et al. 1967). Using the known mol. wt. of adenovirus 2 DNA (2.3 x 10⁷) and an estimated 10 to 100:1 particle to p.f.u. ratio for this virus (Green et al. 1967), an approximate infectivity of 2.7 x 10⁷ fluorescence-forming units (f.f.u.)/μg was calculated for the virion-associated DNA (Table 2).

Erythrocyte ghosts were formed in the presence of 150 μg/ml of adenovirus type 2 DNA and fused in suspension with KB cells. Based upon parallel experiments in which 32P-virus DNA was added as a marker to the same total amount of DNA, it was determined that 1.5 μg of DNA were packaged. Triplicate dilutions of ghosts containing 0.3, 0.03 or 0.003 μg of encapsulated DNA were fused with KB cells. The results of the FFA indicate that a small fraction of cells was successfully transfected with adenovirus DNA (Table 2). In the presence or absence of empty ghosts, purified virus DNA failed to result in a detectable number of infected cells.

Our data demonstrate that transfection was achieved by the erythrocyte-associated DNA (Table 2). No infectivity was detected for naked DNA or empty ghosts, with or without PEG treatment (some data not shown). The infectivity of DNA-ghosts was reduced about 80%, by heat. The observed reduction in infectivity following heat treatment may be the result of thermal damage to some ghosts. Had the infectivity been the result of contaminating adenoviruses, it would have been totally abolished by the heat treatment. The infectivity of the DNA-ghosts was also reduced by treatment with deoxyribonuclease I. Contaminating naked DNA would have been rendered completely non-infectious by similar treatment. The nearly 60% reduction in infectivity of the DNA-ghosts may indicate that the nuclease
Table 2. Infectious yield of adenovirus type 2 DNA*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment</th>
<th>ng DNA/dish</th>
<th>f.f.u./dish†</th>
<th>f.f.u./μg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virions</td>
<td>–</td>
<td>~ 0.12</td>
<td>3.2 x 10⁴</td>
<td>~ 2.7 x 10⁶</td>
</tr>
<tr>
<td>Heat</td>
<td>~ 31.2</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>–</td>
<td>2000</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Empty ghosts</td>
<td>–</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Empty ghosts + DNA</td>
<td>–</td>
<td>2000</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DNA-ghosts</td>
<td>–</td>
<td>3</td>
<td>1</td>
<td>330</td>
</tr>
<tr>
<td>–</td>
<td>30</td>
<td>8</td>
<td>260</td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>300</td>
<td>26</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Heat</td>
<td>300</td>
<td>5</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>DNase</td>
<td>300</td>
<td>11</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

* Confluent KB cell monolayers growing in 35 mm dishes were infected with dilutions of purified virions which had or had not been heated (virions). KB cell suspensions (10⁶ cells/0.2 ml) were treated for 1 min with 0.2 ml of 50 % PEG in medium (mock), in medium containing 2 μg of purified DNA, medium with about 5 x 10⁶ empty ghosts, medium with about 5 x 10⁶ empty ghosts and 2 μg of naked virus DNA, or ghosts containing virus DNA (DNA-ghosts). Three different 10-fold dilutions of DNA ghosts were employed, ranging from 5 x 10⁵ ghosts containing about 3 ng DNA to 5 x 10⁶ ghosts containing 300 ng DNA. The dilution with most DNA-ghosts was also tested after heating at 56 °C for 10 min, or treatment with deoxyribonuclease I (25 μg/ml, 37 °C for 60 min). After treatment with PEG all suspensions were diluted with 3 ml medium and plated. Six to 8 h later medium was replaced with medium containing serum. Twenty-seven h after injection or fusion, monolayers were fixed and assayed by direct immunofluorescence microscopy.

† Fluorescence forming units per dish (mean results for three experiments).

‡ Estimated, see text.

Damaged a small portion of many DNA molecules because only 6 % of ghost-associated counts are solubilized by that treatment. Alternatively, the nuclease may have interfered with transfection directly. We have found that serum proteins severely inhibit fusion of ghosts with cells, an observation which thwarted our ability to use anti-viral IgG treatment in control experiments. It is possible that other proteins, including deoxyribonuclease I, might also inhibit fusion and thereby lessen the infectivity of a preparation of DNA-containing ghosts.

Adenovirus DNA coated naturally by its capsid protein shell is infectious at a level of more than 10⁶ p.f.u./μg (Table 2). Naked DNA, however, is far less infectious. A standard method of transfecting KB cells with adenovirus type 2 DNA yields an average of 14 p.f.u./μg of DNA (Chinnadurai et al. 1978). Reasons for the extremely low infectivity are not known but may include degradation of DNA in the extracellular milieu. The 100- to 1000-fold augmented infectivity observed with the adenovirus DNA–protein complex is compatible with this as well as with other explanations (Chinnadurai et al. 1978). The terminal proteins may protect the genome from extracellular exonuclease activity. Microinjection of erythrocyte-bound DNA into KB cells was viewed as a means of avoiding this potential hazard. Up to 330 f.f.u./μg of DNA was achieved by ghost-mediated microinjection of DNA into KB cells. In spite of this result there remains a vast differential between transfection efficiency and virus infectivity.

Our data suggest that a substantial hazard to the injected DNA resides in the cytoplasm of the recipient cell. A large number of DNA molecules were successfully injected into KB cells with the present system (Table 1). The low level of infectivity observed, however, indicates that the great majority of injected molecules were unable to initiate a successful infection. Cytoplasmic degradation of injected DNA probably abolished the bulk of its potential infectivity. Kaltoft et al. (1976) reported that about 50 % of tRNA which was micro-injected by erythrocyte ghosts into mouse cells was degraded within 6 h. Transfer RNA, however, functions in the cytoplasm, unlike adenovirus DNA whose expression requires its transport through the cytoplasm and into the nucleus. Our data reveal that only
1/3 to 1/2 of counts in ghosts which fuse to KB cells are recoverable from the nucleus. Presumably transport of naked DNA is inefficient, thereby presenting an additional barrier to successful transfection. Finally, experiments employing micro-electrode injection of individual cells have indicated the necessity for direct insertion of DNA into the nucleus to assure its efficient biological expression (Brown & Gurdon, 1977).

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