Poly-L-Ornithine–Mediated Transfection of Human Keratinocytes

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Human keratinocytes are notoriously difficult to transfect. We have optimized a method for introducing plasmid DNA into keratinocytes that pairs the polycation poly-L-ornithine with a dimethylsulfoxide (DMSO) shock. The optimum poly-L-ornithine conditions for keratinocyte transfection entailed incubating the cells with 12 μg/ml poly-L-ornithine and 10 μg DNA for 6 h, followed by a 4-min 25% DMSO shock. Based on kinetic studies, 1 h is enough time to produce 10% positive cells in transient transfections, which increases up to an average of 20% after 6 h. Transfected cells survive passaging, and marker plasmids and selection can be used to yield stable transfectants at a rate twofold higher than in cells transfected with polybrene and DMSO. Transient transfection rates were significantly higher using poly-L-ornithine/DMSO than with the polybrene/DMSO or polybrene/glycerol methods previously reported. Overall, transfection mediated by poly-L-ornithine provides an efficient and inexpensive means of transiently or stably introducing DNA into keratinocytes. ***Key words: polycation/polybrene. J Invest Dermatol 105:668–671, 1995***

The ability to transfect cells with a desired gene construct is a cornerstone technique of molecular research. We were interested in developing an efficient and economical transfection method for primary keratinocytes that gave reproducible results while being nontoxic. Previously published methods, such as electroporation, lipofection [1], or calcium phosphate precipitation [2], either fail to produce high transfection rates or have undesirable associated factors such as high cost or the induction of differentiation in the transfected keratinocytes.

Polycation-mediated introduction of DNA into cells seems to avoid most of these drawbacks. The polycations were initially used to enhance retroviral adsorption to cells [3], and Kawai and Nishizawa [4] first paired polybrene with an osmotic shock to introduce DNA into mammalian cells. Polybrene has been reported to yield 10% transfection of human keratinocytes when paired with either a glycerol [1,5] or dimethylsulfoxide (DMSO) shock [1]. Another polycation, poly-L-ornithine, has been used with a DMSO shock to transfect 3T3 cells [6] and both proliferating and quiescent Chinese hamster ovary cells [7]. We report here a method of transfecting primary human keratinocytes using poly-L-ornithine and a 25% to 27.5% DMSO shock to yield an average efficiency of 20%. The method is useful for developing stable transfectants, and in as little as 1 h produces 10% transient transfectants.

MATERIALS AND METHODS

Plasmids The plasmid pCMV-β-gal was provided by Dr. Kingsman (University of Oxford) and contains the bacterial *lacZ* gene under the control of the cytomegalovirus promoter. The plasmid pSV2neo provides resistance to the neomycin analogue G418 sulfate (Life Technologies, Gaithersburg, MD). The plasmids were grown in chemically competent HB101 bacteria and purified by polyethylene glycol precipitation.

Cell Culture Human epidermal keratinocytes were collected from neonatal foreskins and maintained in Keratinocyte Growth Medium (KGM; Clonetics) in the absence of a layer of feeder cells. Primary keratinocytes were trypsinized and passaged 2 d before transfection to yield a confluence of 30% to 50% on the day of transfection.

Polycation Transfection Poly-L-ornithine (P3655) and polybrene (hexadimethrine bromide; H9268) were obtained from Sigma Chemical Co. Sterile stock solutions were prepared by dissolving the polycations in sterile water at a concentration of 10 mg/mL and storing for up to 4 months at 4°C. To transfect keratinocytes, the cells were first washed with 37°C phosphate-buffered saline, and then the transfection mixture was added. Except as noted in the text, the transfection mixture consisted of 1.8 ml KGM, including 12 μg/ml polycation, and 10 μg DNA per 60-mm plate. The cells were incubated at 37°C and rocked gently every 1.5 h for the duration of the transfection to ensure an even distribution of the transfection mixture. After the allotted time, the transfection mixture was removed and the DMSO or glycerol solution (expressed as v/v in KGM) was added for 4 min at room temperature (see text for conditions). The cells were then immediately washed twice with 37°C Ham's F12 medium (Gibco BRL), and 37°C KGM was added to each plate. After 40 h, the cells were stained for β-galactosidase activity. Mass ratios were determined by dividing the amount of DNA added to each plate by the total amount of polycation used on that plate. Using the transfection mixture described above as an example, the mass ratio would equal (10 μg DNA)/(1.8 ml medium × 12 μg/ml polycation) = 0.46.

Stable Transfection For cells transfected on 60-mm tissue culture plates, we used a transfection mixture of 1.8 ml KGM with 12 μg/ml polycation and 10 μg pSV2Neo to provide resistance to the neomycin analogue G418. Cells were transfected as described above using a 27.5% DMSO shock. After 24 h, the cells were split to 100-mm plates in KGM. Selection began 24 h after the cells were passaged. After 2 weeks of selection, the number of colonies 2 mm in diameter or greater was counted. Transfections from the β-gal passaging experiment were performed as described under *Polycation Transfection*.

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Table I. Optimum DNA–Poly-L-Ornithine Mass Ratio[a]

Amount of pCMV–β-gal (μg)	Mass Ratio[b]	Percent Transfected Cells[c]
0.0	0.0	0.0
5.0	0.23	5.4 (1.9)
10.0	0.46	15.7 (3.7)
15.0	0.69	10.2 (4.9)
20.0	0.93	7.6 (1.0)

[a] Pooled keratinocytes from multiple foreskins on 60-mm plates were transfected for 6 h with 12 μg/ml poly-L-ornithine and varying amounts of pCMV–β-gal in 1.8 ml KGM. Cells were shocked for 4 min in 25% DMSO and washed twice before adding fresh KGM. The results represent two experiments, with three plates per DNA concentration.

[b] The mass ratio of pCMV–β-gal to poly-L-ornithine.

[c] Values are given as mean (± SD) for 12 representative fields from each plate.

Staining Transfected cells were fixed and stained for β-gal activity as described [8]. Ten to 12 defined fields per plate were counted to determine the percentage of keratinocytes transfected, with at least two fields from each quadrant. Statistical tests were performed using SigmaStat 1.0 (Jandel Corp., San Rafael, CA).

RESULTS AND DISCUSSION

Poly-L-Ornithine–Mediated Transfection Experiments were initially carried out on 60-mm plates, but the method has been adapted to 22-mm and 100-mm plates without loss of efficiency. The conditions for each experiment are noted in the text.

Transfection Optimization The amount of plasmid DNA was varied with a constant concentration of poly-L-ornithine to determine the optimum DNA-polycation ratio for transfecting keratinocytes. Between 5 and 20 μg of pCMV–β-gal was added to the standard transfection mixture, giving DNA–poly-L-ornithine mass ratios of 0.23, 0.46, 0.69, and 0.93 **(Table I)**. The mass ratio of 0.46 (10 μg DNA) produced the highest number of transfectants. As the amount of DNA used in the transfection was increased, the percentage of β-gal–positive clones decreased gradually. The mean values for the percent transfected cells were statistically significantly different by a one-way analysis of variance test ($p = 0.000796$). These results are in agreement with work by Bond and Wold [6] using poly-L-ornithine on different cell types, though their protocol did not include a DMSO shock and yielded significantly lower numbers of transfectants.

Our initial experiments and published reports [1,4] suggested that the optimum tolerable concentration of DMSO for mammalian cells is between 20% and 30%. We tested DMSO concentrations of 0%, 20%, 22%, 24%, 26%, and 30% to determine which level would prove most effective with poly-L-ornithine in transfecting primary human keratinocytes. Although 30% DMSO caused cell death, a shock of 26% DMSO produced the highest transfection rate, with an average of 21.5% β-gal–positive keratinocytes **(Table II)**. However, even this concentration of DMSO produced vacuolization and membrane disintegration in the keratinocytes, which was absent at the 22% DMSO level **(Fig 1)** and was present at a low level at 24%. A one-way analysis of variance test showed the differences of the means to be statistically significantly different ($p = 0.000088$), and the optimum DMSO concentration and the related cytotoxicity were similar to those found for the optimum DMSO concentration when transfecting keratinocytes with polybrene [1]. Notably, in the course of our experiments we used two different batches of DMSO and found that there was a small but significant change in the optimal DMSO concentration for maximal transfection efficiency. Experiments using 30% and 25% DMSO from one batch gave results similar to those with 26% and 22%, respectively, from another batch of DMSO. Because of this, we recommend that DMSO be aliquoted and stored at −70°C once an optimum concentration is determined for a specific batch of DMSO.

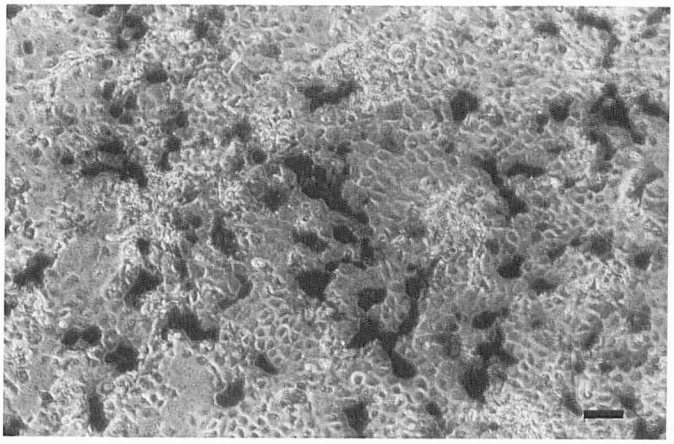

Figure 1. Poly-L-ornithine/22%-DMSO–mediated transfection of pCMV–β-gal into primary human keratinocytes. Cells were stained for β-gal activity 40 h after transfection. A 4-min 22% DMSO shock produced little cytotoxicity. *Bar*, 100 μm.

Kinetics of Transfection We added a DNA/ornithine mixture to primary human keratinocytes for varying lengths of time before the DMSO shock to determine the rate of transfection **(Table III)**. The maximum number of transfectants occurred at 4 h, while the 4-, 5-, and 6-h time points were closely grouped. Although the number of transfected cells does appear to vary with time, a one-way analysis of variance test failed to show a statistically significant difference among the mean values ($p = 0.282$). Notably, however, up to 10.9% of the keratinocytes expressed β-gal activity at the 1-h time point, showing the convenience and versatility of the method. Cells that were incubated for more than 6 h with the transfection mixture before a DMSO shock began to show physical effects of ornithine toxicity, including increased vacuolization and membrane disintegration.

Table II. Optimum DMSO Concentration[a]

DMSO Concentration (%)	Percent Transfected Cells[b]
0.0	0.0
20.0	5.6 (3.3)
22.0	11.6 (4.1)
24.0	17.1 (3.0)
26.0	21.5 (4.3)
30.0	Cell lysis

[a] Pooled keratinocytes from two foreskins on 60-mm plates were transfected in triplicate for 6 h with 12 μg/ml poly-L-ornithine and 10 μg pCMV–β-gal in 1.8 ml KGM. Cells were shocked for 4 min in varying concentrations of DMSO and washed twice before adding fresh KGM.

[b] Values are given as mean (± SD) for 12 representative fields from each plate.

Table III. Kinetics of Transfection by Poly-L-Ornithine[a]

Time (h)	Percent Transfected Cells[b]
1	8.4 (2.5)
2	9.7 (2.2)
3	13.5 (6.6)
4	15.2 (1.9)
5	13.6 (1.0)
6	14.0 (3.8)
7	9.9 (5.7)
8	9.8 (3.0)

[a] Pooled keratinocytes from three foreskins on 60-mm plates were transfected in triplicate for varied lengths of time with 21 μg poly-L-ornithine and 10 μg pCMV–β-gal in 1.8 ml KGM. Cells were shocked in 25% DMSO for 4 min and washed twice before adding fresh KGM.

[b] Values are given as mean (± SD) for 12 representative fields from each plate.

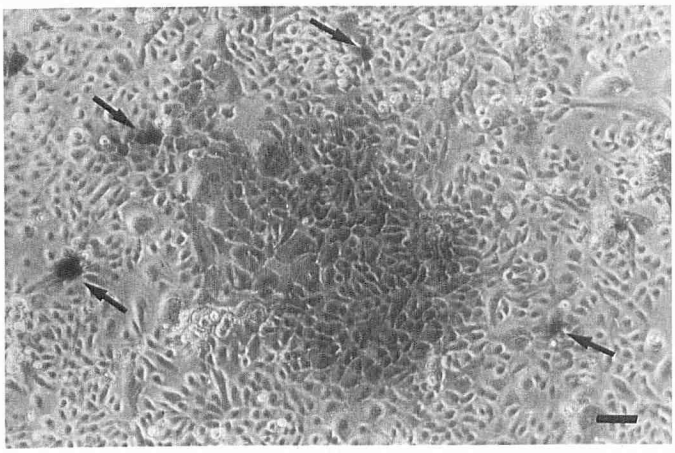

Figure 2. Passaging of poly-L-ornithine/25%-DMSO–transfected primary human keratinocytes. Transfectants were passaged and permitted to reach confluence before staining for β-gal activity. Note the large stained region of cells, in contrast to the staining of single cells (*arrows*). *Bar*, 100 μm.

Table IV. Comparison of Transfection Methods: Polybrene/Glycerol Versus Poly-L-Ornithine/DMSO[a]

Conditions[b]	Mass Ratio[c]	Percent Transfected Cells[d]
Polybrene (10 μg/ml)[e]		
2.5 μg DNA, 0.5 ml	0.50	0.6 (0.2)
2.5 μg DNA, 1.0 ml	0.25	0.7 (0.4)
Poly-L-ornithine (12 μg/ml)[f]		
2.5 μg DNA, 0.5 ml	0.42	19.6 (6.0)
1.25 μg DNA, 0.5 ml	0.21	3.6 (0.8)[g]
Poly-L-ornithine (6 μg/ml)		
1.25 μg DNA, 0.5 ml	0.42	16.9 (2.2)

[a] Primary human keratinocytes from one foreskin were seeded 50,000 cells/well on 2–12-well plates and transfected at 30% to 40% confluence.

[b] Conditions given are per well. CMV–β-gal was used as the reporter plasmid, and transfections were carried out in F12 medium.

[c] The mass ratio of pCMV–β-gal to the total amount of polycation used.

[d] Values are given as mean (± SD) for 10 representative fields from each of four wells per condition.

[e] The concentration of polybrene was kept constant at 30 μg/ml and was paired with a 4-min 15% glycerol shock.

[f] The concentration of poly-L-ornithine was either 6 or 12 μg/ml and was paired with a 4-min 25% DMSO shock.

[g] These conditions were markedly cytotoxic.

Keratinocyte Survival The ability of transfected keratinocytes to survive passaging was examined because of the observed cell toxicity at some ornithine and DMSO concentrations. Keratinocytes were transfected as described (see *Materials and Methods*), trypsinized and passaged 1:1 after 40 h, and then permitted to reach confluence before being stained for β-gal activity (3 d after passaging) **(Fig 2)**. The two 60-mm plates from the split were estimated to be 3% to 4% positive for β-gal activity, while two duplicate plates that were stained before passaging were 15.5% and 16.5% β-gal positive. Therefore, the poly-L-ornithine method is capable of introducing a target construct into keratinocytes, and the resulting transfectants are viable for passaging and further cell division.

Comparison With Polybrene-Mediated Transfection We were interested in comparing ornithine-mediated transfection of human keratinocytes with the polybrene-mediated transfection reported by others [1,5,9]. In an effort to mimic the conditions of other studies [5,9], we used 22-mm plates for transfections rather than the 60-mm plates used in other experiments. Keratinocytes from the same foreskin were transfected on 12-well plates (22-mm–diameter wells) with either polybrene paired with a 15% glycerol shock or poly-L-ornithine paired with a 25% DMSO shock **(Table IV)**. Poly-L-ornithine at 12 μg/ml (a DNA–poly-L-ornithine mass ratio of 0.42) yielded the best results, with more than 25% of the cells in one well staining positively for β-gal activity and with a 19.6% average for that experimental condition. Lowering the DNA concentration without adjusting the poly-L-ornithine concentration (mass ratio of 0.21) resulted in marked cytotoxicity and produced low transfection rates. This low transfection frequency at a mass ratio of approximately 0.21–0.23 was reproducible, as seen in **Table I**. These results confirm reports that cells may have a certain tolerable DNA/ornithine ratio [6]. Scaling back on the amount of both polycation and DNA that was added to the transfection mixture to maintain the mass ratio at 0.42 yielded respectable expression rates for poly-L-ornithine (16.9% average) with no evident cytotoxicity. A scaled-back transfection reaction would be useful if only small quantities of DNA are available. The keratinocytes exposed to the polybrene and glycerol shock appeared healthy, yet failed to express published levels of β-gal activity. Our protocol did differ from that published on several accounts [9]. First, we used transfection-mixture volumes of 0.5 ml and 1.0 ml, which bracketed the 0.75 ml reported, but it is important that the quantity of DNA and the polybrene concentrations were the same in each case. Second, our keratinocytes were grown in serum-free KGM rather than serum-free medium (Gibco BRL) and were never exposed to feeder cells. Finally, we did not add sodium butyrate to increase plasmid expression.

Another polybrene transfection method uses a DMSO shock rather than a glycerol shock [1]. The authors paired a 27% DMSO shock with 10 μg/ml polybrene to transfect keratinocytes [1]. However, the results of the study were expressed as enzyme activity rather than percentage of cells transfected, so direct comparisons are not possible. To compare polybrene and poly-L-ornithine when both polycations were paired with a DMSO shock, we performed transient and stable transfection experiments. Pooled keratinocytes from the same foreskins were transfected in an identical manner, the only difference being which polycation was used. Poly-L-ornithine–mediated transfection produced a five- to sixfold higher transient transfection rate and a twofold higher stable transfection rate than polybrene-mediated transfection **(Table V)**. The stable transfection rate was lower than the transient transfection rate. This is most likely due to the integration of the plasmid required for maintenance of G418 resistance, which could be the limiting factor in forming stable transfectants, as a number of integration events will result in nonexpression of the G418 gene. This experiment demonstrates that poly-L-ornithine paired with a DMSO shock may be used to form stable transfectants and that under conditions identical to those tested here, poly-L-ornithine transfects keratinocytes more effectively than polybrene.

Table V. Comparison of Transfection Methods: Transient and Stable Transfections by Poly-L-Ornithine/DMSO and Polybrene/DMSO[a]

	Experiment 1	Experiment 2
Poly-L-ornithine/DMSO		
Transient[b]	17.0% (2.8)	19.2% (3.5)
Stable[c]	47.7 (10.2)	38 (3.5)
Polybrene/DMSO		
Transient[b]	3.2% (1.4)	3.3% (1.1)
Stable[c]	20.0 (2)	18.7 (3.5)

[a] Transfections were performed as described under *Polycation Transfection*, with the same conditions being used for poly-L-ornithine as for polybrene.

[b] Values are given as mean (± SD) for 10 representative fields from each of three replicate plates per condition.

[c] Transfected keratinocytes were split from 60-mm to 100-mm tissue culture plates (split ratio of 1:3, based on approximate surface area of the plates) and selected as described. Values are given as mean (± SD) for the number of colonies at least 2 mm in diameter from three replicate plates per condition.

Conclusion We present a transfection method for human epidermal keratinocytes that is economical, time and labor efficient, and consistently yields a 15% to 20% transfection efficiency. Our preferred method has a 4–6-h incubation using 60-mm plates and pairs a transfection mixture of 12 μg/ml poly-L-ornithine, 10 μg DNA, and 1.8 ml KGM with a 4-min, 25% to 27.5% DMSO shock. The optimum percentage of DMSO may vary between batches, so testing of each batch is advised. It is important that the poly-L-ornithine–mediated procedure may be adapted to a variety of tissue culture plate sizes, from 22 mm to 100 mm. However, one must maintain a mass ratio (DNA to total poly-L-ornithine added) of 0.42–0.46 when adjusting the transfection to different-sized plates to obtain an optimum transfection. With the correct DMSO concentration, this method produces little cytotoxicity and can be used to produce stable transfectants. Poly-L-ornithine–mediated transfection should permit easier manipulation and genetic characterization of primary human keratinocytes.

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FIRST ANNOUNCEMENT

We are pleased to announce that Skin Therapy Forum III will be held at the University of Wales College of Medicine, Cardiff, on 7–9 October 1996. This is the third meeting in the series and is a British Association of Dermatologists sponsored event. It is intended to be a very practically orientated two and a half day meeting, and will discuss many day to day problems which confront the dermatologist. The meeting is being organised by Professor R. Marks of Cardiff and Professor W.J. Cunliffe of Leeds.

The programme will consist of invited lectures, free communications, poster papers, and lunchtime debates. Sessions will include the following: The Treatment of Autoimmune Disorders, Wound Care in Dermatology, Surgical Techniques, Laser Treatments, The Management of Disease of Hair and Nails, Psoriasis, The Management of Skin Disease in Childhood, Eczematous Disorders, and Acne and Rosacea.

For further details and abstract forms please contact Professor R. Marks, Professor of Dermatology, University of Wales College of Medicine, Heath Park, Cardiff, CF4 4XN, United Kingdom.