Highly Efficient DNA Delivery Mediated by pH-Sensitive Immunoliposomes†

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Received April 20, 1989; Revised Manuscript Received July 19, 1989

ABSTRACT: We have previously shown that pH-sensitive immunoliposomes can mediate a target-specific delivery of plasmid DNA to tumor cells grown in a mouse model [Wang, C.-Y., & Huang, L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7851-7855]. The efficiency of delivery in terms of the target cell transformation frequency has now been characterized for both short- and long-term gene expression in a tissue culture system. Herpes simplex virus thymidine kinase (TK) gene was used as a reporter gene. It was placed under the control of the promoter for the rat phosphoenolpyruvate carboxykinase gene, which contains a cAMP regulatory element. Therefore, the expression of the exogenous gene in the target cell, mouse Ltk$^-$ cells, can be regulated by cAMP drugs. The plasmid DNA was encapsulated in liposomes using a detergent dialysis method. The efficiency of gene delivery was optimized with respect to the time course and dose of liposome-associated DNA. The existence of antibody of the liposomes was essential for the maximal level of DNA delivery. Delivery was also dependent on the lipid composition of the liposome. The pH-sensitive lipid composition gave 8-fold higher efficiency than the corresponding pH-insensitive composition. The transformation efficiency of the target cell also depended on the regulation of gene expression; cells incubated with dibutyryl-cAMP and theophylline showed a much higher level of transformation frequency than cells incubated without the drugs. When all liposome and incubation parameters are optimized, the Ltk$^-$ cells showed a 47% efficiency for the short-term transformation, and 2% for the long-term transformation. These levels of efficiency are considerably higher than those obtained with the conventional calcium phosphate precipitation method which was performed in parallel as a comparison.

Delivery of exogenous genes into cells has become an important task for potential therapy in diseases such as inborn errors of metabolism [for a review, see Anderson (1984)]. Recent progress in antisense RNA (Holt et al., 1986, 1988; Hunts et al., 1986; Nishikura & Murphy, 1987) and ribozymes [for a review, see Walbot and Bruening (1988)] as specific molecular reagents to inhibit virus or neoplastic growth has exemplified the need for a proper delivery vehicle. Retrovirus-mediated delivery (Hamer & Leder, 1979; Mulligan et al., 1979), although generally of high efficiency, suffers from the potential pathological side effect and the lack of delivery specificity (Bishop, 1987). Other high-efficiency methods such as electroporation (Neumann et al., 1982), laser microbeam (Tsukakoshi et al., 1984; Tao et al., 1987), and lipofection (Felgner et al., 1987; Felgner & Ringold, 1989) are not useful in vivo. Recently, Wu and Wu (1987, 1988a,b) have developed a promising target-specific delivery vehicle by complexing DNA with an asialoorosomucoid–polylysine conjugate and showed an impressive level of exogenous gene expression in the liver. Delivery to other cell types such as the virus-infected cells and neoplastic cells by this method will have to await further studies.

We have previously shown that the pH-sensitive immunoliposome can mediate a target-specific delivery of an exogenous gene to lymphoma cells grown in a nude mouse model (Wang & Huang, 1987b). The reporter gene used in that study was the *Escherichia coli* chloramphenicol acetyltransferase (CAT)[1] gene placed under the control of a cAMP-responsive eukaryotic promoter, such that the expression of CAT in the lymphoma cells was dependent on the injection of mice with cAMP drugs (Wang & Huang, 1987b). The success of delivery is based on the design of the liposome membrane which becomes destabilized and fusion-active at a mildly acidic pH which takes place when the liposome is endocytosed by the target cells and routed to the acidic endosomes (Connor et al., 1984; Connor & Huang, 1985, 1986, 1987; Collins & Huang, 1987; Wang et al., 1986). Cytoplasmic delivery of the entrapped DNA is presumably the result of liposome–endosome fusion and possibly endosome rupture (Wang & Huang, 1987a).

Although the DNA delivery efficiency of the pH-sensitive immunoliposomes is higher than that of the conventional liposomes, the previous study did not rigorously quantitate the transformation frequency of the target cell (Wang & Huang, 1987b). Furthermore, it was not clear if the pH-sensitive immunoliposome could provide any long-term transformation of the target cells. To address these questions, we have decided to use a selectable reporter gene, herpes simplex virus thymidine kinase (TK) gene, and the mouse Ltk$^-$ cells for a rigorous and quantitative examination of the frequencies of both short- and long-term transformations. We have also compared our results with those obtained with the calcium phosphate method which was done as parallel experiments.

Materials and Methods

Materials. Dioleoylphosphatidylethanolamine (DOPE)[1] and dioleoylphosphatidylcholine (DOPC)[1] were purchased from Avanti (Birmingham, AL). Oleic acid, cholesterol, *n*-octyl glucoside, dibutyryl-cAMP, and theophylline were obtained

† Supported in part by NIH Grant CA 24553 and by a contract from the Martin Marietta Corp.
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[1] Abbreviations: BT, N^6-2′-*O*-dibutyryladenosine cyclic 3′,5′-phosphate (0.5 mM) plus theophylline (1 mM); chol, cholesterol; DOPE, dioleoylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; HAT, hypoxanthine, aminopterin, and thymidine; [^{3}H]CE, hexadecyl [^{3}H]cholestanyl ether; HSV, herpes simplex virus; OA, oleic acid; PEPCK, phosphoenolpyruvate carboxykinase; TK, thymidine kinase; PBS, phosphate-buffer saline (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 1.1 mM Na_2HPO_4, pH 8.0); CAT, chloramphenicol acetyltransferase.

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from Sigma. SM-2 beads were purchased from Bio-Rad Laboratories. Protosol was purchased from NEN Research Products. HAT medium was obtained from Gibco. Plasmid pPCTK-6A, which was a gift from Drs. A. Wynshaw-Boris and R. Hanson, contains the herpes simplex virus thymidine kinase gene, HSV-TK. The endogenous promoter was replaced by the promoter of the rat phosphoenolpyruvate carboxykinase (PEPCK) gene, which contains a cAMP regulatory element (Wynshaw-Boris et al., 1984). The plasmid DNA was isolated by using standard procedures (Maniatis et al., 1982). The OD_{260}/OD_{280} ratio of the plasmid preparations was generally around 1.9. Anti-H2K^k monoclonal antibody, which specifically bound to H2K^k antigen on the surface of mouse L cells, was isolated from ascites fluid generated by the mouse hybridoma 11-4.1 (Oi et al., 1978). The antibody was purified from ascites fluid using protein A–Sepharose affinity chromatography as previously described (Huang et al., 1982). Radioiodination was carried out using chloramine T as described (Huang et al., 1982). Palmitoyl antibody was prepared as previously described (Huang et al., 1982) by using the *N*-hydroxysuccinimide ester of palmitic acid at a molar ratio of 10 to 1. Palmitoyl antibody was dialyzed in PBS containing 0.15% deoxycholate and stored at 0–4 °C.

Immunoliposome Preparation. The detergent dialysis method was used to prepare immunoliposomes as previously described (Wang & Huang, 1987a,b). Briefly, a mixture [10 μmol of lipids containing a trace of hexadecyl [^{3}H]cholestanyl ether (specific activity 1×10^5 cpm/μmol of lipid)] was sonicated in 10 mM Hepes, 1 mM EGTA, and 150 mM NaCl, pH 8. Palmitoyl anti-H2K^k (1/25 of total lipid by weight), octyl glucoside (100 μmol), and 150 μg of DNA were added, and the mixture (0.34 mL) was dialyzed at 4 °C against 10 mM Tris, 1 mM EDTA, and 150 mM NaCl, pH 8, containing washed SM-2 beads (Bio-Rad Laboratories) overnight without stirring. Dialysis was continued with stirring for a total of 48 h. The immunoliposomes were extruded through a polycarbonate filter (0.2 μm) for uniform size distribution. Free, unentrapped DNA was not removed from liposomes. Two different lipid compositions were used; DOPE/chol/OA (4:4:2) is a pH-sensitive composition, and DOPC/chol/OA (4:4:2) is a pH-insensitive composition (Wang et al., 1986).

Cell Incubations. Mouse Ltk$^-$ cells were a kind gift from Dr. Leroy Hood. They were grown in DMEM medium supplemented with 10% fetal calf serum. Approximately 1×10^5 cells in a 60-mm dish were preincubated in serum-free DMEM medium for 4 h before the addition of DNA. Liposome-associated or free DNA (10 μg per dish was used in routine experiments) was added to cells in a total volume of 5 mL in serum-free DMEM medium. Cells were incubated for 3 h at 37 °C, followed by washing 3 times with DMEM medium. Cells were further incubated for 24 h in serum-supplemented DMEM medium with and without 0.5 mM dibutyryl-cAMP (Hayakawa et al., 1984) and 1 mM theophylline (Wynshaw-Boris et al., 1984) before the measurement of TK activity. Calcium phosphate precipitation of DNA was carried out according to Wigler et al. (1978). Salmon sperm DNA (Sigma) was used as a carrier.

Thymidine Kinase Activity. Cells were washed 3 times with cold PBS, scraped, and suspended in PBS. After centrifugation at 15000*g* for 10 min, TK activity was assayed by measuring the phosphorylation of [^{3}H]thymidine according to Wigler et al. (1977). After a 2-h incubation at 37 °C, the reaction was stopped by spotting 50 μL of reaction mixture onto DE-81 paper. After being washed 3 times with 4 mM ammonium formate, the DE-81 papers were dissolved in 1 mL of Protosol, and the radioactivity was counted. Protein was determined by the Lowry assay (Lowry et al., 1951).

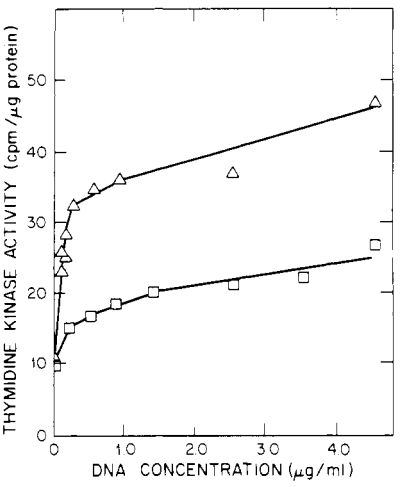

FIGURE 1: Effect of DNA concentration on short-term gene expression. The TK activity was measured in cells transfected with DNA associated with DOPE/chol/OA liposomes containing anti-H2K^k and grown in the presence (△) or absence (□) of BT for 24 h.

Autoradiography. To examine the frequency of short-term transformation, the incorporation of [^{3}H]thymidine into DNA in the transformed Ltk$^-$ cells was carried out by autoradiography according to Schaeffer-Ridder et al. (1982). Cells with silver grains over the nucleus were scored as transformed cells. At least 300 cells were scored in each treatment.

Long-Term Transformation Efficiency. To examine the long-term transformation efficiency, limiting dilutions of treated cells were carried out. Cells were scraped from the culture dishes and seeded in 96-well plates at an average number of one cell per well. Incubation medium supplemented with HAT (1×10^{-2} M hypoxanthine, 4×10^{-3} M aminopterin, and 1.6×10^{-3} M thymidine) was used to select TK$^+$ cells. The cell number in each well was enumerated on days 1, 4, and 12. Only those wells which originally contained one cell per well were examined for cell growth. Long-term transformed cells are defined as those cells which had undergone at least three divisions (i.e., eight cells or more) at day 12.

Results

Association of DNA with Liposome. We have shown in preliminary experiments that free plasmid DNA adsorbed to the preformed "empty", pH-sensitive liposomes can transfect the Ltk$^-$ cells in vitro (Wang & Huang, 1987a), similar to the widely used Lipofectin reagent for in vitro transfection (Felgner et al., 1987; Felgner & Ringold, 1987). Free DNA was without any effect (Wang & Huang, 1987a). Therefore, we have not removed the unentrapped DNA from our liposome preparations for the experiments described here. The liposomes were mostly unilamellar with an average diameter of 0.29 ± 0.08 μm as measured by negative-stain electron microscopy.

Concentration and Time Dependence of Liposome-Mediated Transformation. Transformation of Ltk$^-$ cells with the TK gene was routinely performed by incubating cells with DNA at 37 °C for 3 h. Preliminary experiments showed that 3-h incubation in a serum-free medium was optimal for transformation. The short-term transformation protocol was first optimized with respect to the DNA concentration used in the cell incubation. As can be seen in Figure 1, the expression of the exogenous gene delivered by liposome was DNA concentration dependent. There was a sharp rise in TK activity in the transfected cells at DNA concentrations lower than 0.5

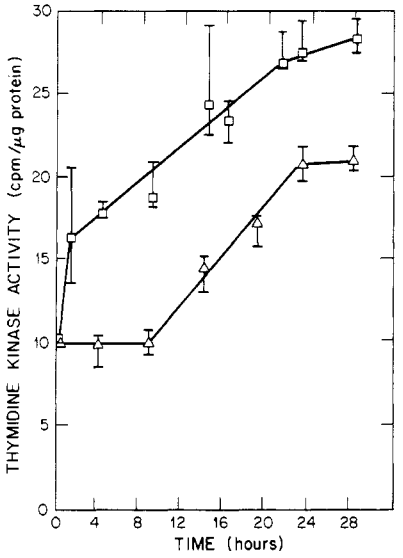

FIGURE 2: Effect of incubation time of transfected cells on the level of TK gene expression. Ltk$^-$ cells transfected with DNA associated with DOPE/chol/OA (4:4:2) liposomes containing anti-H2K^k were grown in DMEM with (□) or without (△) BT.

Table I: Effect of the Lipid Composition of the Immunoliposomes on the Thymidine Kinase Activity of Cells[a]

expt	DOPC[b]	DOPE[b]	DOPE/DOPC
1	12.2	94.2	7.7
2	8.0	78.3	9.8
3	12.6	107.6	8.5
4	10.5	82.5	7.8
5	12.5	81.5	6.5
6	2.6	16.6	6.4
$\bar{x}$ ± SD (no. of obs)	9.7 ± 3.6 (30)	76.8 ± 28.7 (30)	7.8[c]

[a] Cells were incubated in the presence of BT. TK activity is expressed as cpm per milligram of protein. [b] DOPC: liposomes composed of DOPC/chol/OA (4:4:2). DOPE: liposomes composed of DOPE/chol/OA (4:4:2). [c] $p < 0.001$

μg/mL, followed by a more gradual increase of expression at higher concentrations. It is also evident that the expression of the TK was stimulated by BT 1.9–2.5-fold, depending on the DNA concentration used. Thus, the cAMP regulatory mechanism of the plasmid is fully active in the target cells. For subsequent experiments, 10 μg of DNA in 5 mL of DMEM medium per 10^5 cells was used to ensure a sufficient amount of DNA delivery.

The time course of TK expression following the liposome cell incubation was also examined (Figure 2). There was a detectable increase of TK activity in cells incubated with BT as short as 1 h after the liposome incubation. The enzyme activity continued to rise with time and plateaued after 20–24 h. In the absence of BT stimulation, cells showed a lower level of expression with a lag of approximately 9 h. Enzyme activity also reached a plateau after approximately 24 h. Further experiments indicated that TK activity declined when the incubation time was more than 30 h (data not shown). Therefore, an expression time of 24 h was chosen as the optimal time for short-term transformation.

Lipid Composition of Liposome. Effect of liposome characteristics on the short-term expression of plasmid DNA was investigated by using two different lipid compositions. It has been shown that the DOPE/chol/OA (4:4:2) liposomes are pH sensitive while the DOPC/chol/OA (4:4:2) liposomes are not (Wang et al., 1986). As shown in Table I, the measurement of TK activity from cells treated with DNA associated with different liposome preparations varied considerably from experiment to experiment. However, within each experiment, cells treated with pH-sensitive immunoliposomes (DOPE liposomes) always gave higher enzyme activity than cells treated with the pH-insensitive counterpart (DOPC liposomes). The ratio of DOPE/DOPC varied between 6.4- and 9.8-fold in six different experiments with an average ratio of 7.8. It is clear that the pH-sensitive liposome composition is significantly better in the transfection activity than the pH-insensitive composition.

Table II: Effect of the Liposomal Antibody on the Thymidine Kinase Activity of Cells Transformed via DOPE/chol/OA Liposomes[a]

expt	without antibody	with antibody	with antibody/without antibody
1	9.8	81.5	8.3
2	5.8	80.5	13.9
3	26.4	64.5	2.4
4	8.9	76.8	8.6
5	8.5	14.8	1.7
6	10.8	27.4	2.5
$\bar{x}$ ± SD (no. of obs)	11.7 ± 6.8 (30)	57.6 ± 26.6 (30)	6.2[b]

[a] Conditions same as in Table I. [b] $p < 0.001$.

Liposomal Antibody. We also examined the effect of liposome-incorporated antibody on the delivery of DNA using DOPE/chol/OA (4:4:2) liposomes with and without palmitoyl anti-H2K^k antibody (Table II). Again, there was quite a bit of variation in the results of six different experiments. However, liposomes with palmitoyl antibody always gave a higher level of gene expression than liposomes without antibody under otherwise identical conditions. Ratio of "with antibody" to "without antibody" varied from 1.7 to 13.9 with an average of 6.2. Paired t test of the data indicated that the results of the six experiments are statistically significant with $p < 0.001$ (Table II). We conclude that attachment of antibody on the liposome surface is essential for optimal DNA delivery activity of the pH-sensitive liposome.

Comparison with Calcium Phosphate Precipitation Method. The short-term transformation of Ltk$^-$ cells treated with DNA associated with pH-sensitive immunoliposomes was compared with the transformation of the same cells mediated with a conventional transfection method, i.e., calcium phosphate precipitation method. The protocol used for the latter method was already optimized for the Ltk$^-$ cells (Wigler et al., 1978). Thus, the comparison shown in Table III represents the results of two independently optimized methods obtained in well-controlled experiments with both methods carried in parallel. Once again, TK activity measured from these experiments showed considerable variation among different experiments. However, liposome-mediated transfection always resulted in a higher level of enzyme activity than the calcium phosphate mediated transfection. The ratio of the TK activities ranged from 1.4 to 2.8, with an average ratio of 2.3 in nine independent experiments. The higher level of transfection activity with the liposome protocol is probably related to the fact that the liposome treatment of cells was not toxic. When the cells were examined immediately after liposome incubation, 90 ± 5% cells were viable as measured by the trypan blue dye exclusion test. However, only 48 ± 7% of cells treated with calcium phosphate precipitated DNA were viable under otherwise identical conditions.

The short-term transformation frequency was also evaluated with an autoradiography method. Exogenously added [^{3}H]-thymidine is incorporated into nuclear DNA only when the cell expresses a sufficient amount of TK. Thus, autoradiog-

Table III: Comparison of Short-Term Gene Expression in pH-Sensitive Immunoliposome-Mediated and Calcium Phosphate Mediated Transformation[a]

	thymidine kinase activity[b]			% cell-incorporated [^{3}H]TdR		
expt	calcium phosphate	liposome	liposome/calcium phosphate	calcium phosphate	liposome	liposome/calcium phosphate
1	69.1	94.1	1.4	37.4	58.0	1.5
2	34.8	78.2	2.2	34.8	49.0	1.4
3	38.2	106.8	2.8	33.0	43.0	1.3
4	29.6	81.5	2.8	35.2	46.9	1.3
5	30.0	80.5	2.7	36.2	45.7	1.3
6	6.7	15.7	2.3	27.6	43.1	1.6
7	31.8	76.5	2.4	29.0	49.6	1.7
8	36.0	76.8	2.1	43.0	49.8	1.2
9	36.2	79.1	2.2	32.2	43.8	1.4
$\bar{x} \pm$ SD	34.7 ± 15.1	76.6 ± 23.5	2.3[c]	34.3 ± 4.3	47.7 ± 4.4	1.4[c]

[a] Cells were incubated in DMEM containing BT. [b] Activity expressed as cpm per milligram of protein. [c] $p < 0.001$.

Table IV: Clonal Growth of Ltk$^-$ Cells in HAT Medium As Measured by Limiting Dilution

transfection method	BT	no. of wells which originally contained 1 cell	no. of wells with 8 cells or more		long-term transformation efficiency[b]
			day 4	day 12	
liposome[a]	+	1404	8	26	1.9×10^{-2}
liposome[a]	−	936	2	2	2.1×10^{-3}
calcium phosphate	+	1672	2	2	1.2×10^{-3}
calcium phosphate	−	1464	0	0	$<7 \times 10^{-4}$
none	−	953	0	0	

[a] Immunoliposomes composed of DOPE/chol/OA (4:4:2) were used. [b] (no. of wells with 8 cells or more in day 12)/(no. of wells originally containing 1 cell).

raphy of a transformed cell would show silver grains over its nucleus, whereas the nuclei of untransformed cells are not labeled. Table III also shows the results of nine paired experiments comparing the transformation efficiencies of the two transfection protocols using the autoradiography method. The calcium phosphate method showed an average of approximately 34% transformation frequency as compared to approximately 48% for the liposome method. Apparently, the pH-sensitive immunoliposomes have mediated a higher level of short-term transformation than the conventional calcium phosphate precipitation method.

Long-Term Growth of Transformed Cells. It is important that the transfected cells can continuously express the exogenous gene under the proper control of the promoter. Cells treated with liposome-mediated transfection were incubated for 24 h in DMEM medium with or without BT, and then the medium was changed to DMEM supplemented with HAT, a selection medium for cells expressing TK, and cultured for 38 days (Figure 3). The cell number in the plates originally treated with BT showed a small increase for 4 days and then declined. In contrast, the cell number in BT-free plates gradually decreased from the beginning. When BT was added to both types of culture at day 6 (arrows in Figure 3), there was a noted increase in growth rate. Further addition of BT at day 9 had resulted in further enhancement of growth in both types of culture, until BT was depleted in the medium. Cell growth ceased at this point (day 18) followed by a decline in cell number. Further addition of BT to the medium once again stimulated the growth. Thus, it is clear from the data that the transformed cells can undergo long-term growth in a selection medium. However, the survival of cells depends on the proper expression of the TK gene which is regulated by cAMP. In other words, the growth of the transformed cells in the HAT medium was cAMP dependent. This observation is interesting, because growth of the untransformed Ltk$^-$ cells grown in DMEM medium containing no HAT was suppressed by the addition of BT (inset A in Figure 3). This latter growth behavior is similar to other fibroblastic cells in culture (Pastan & Adbya, 1976). Thus, although the growth of the original Ltk$^-$ cells was inhibited by cAMP, the growth of the transformed cells in HAT medium completely depended on cAMP stimulation.

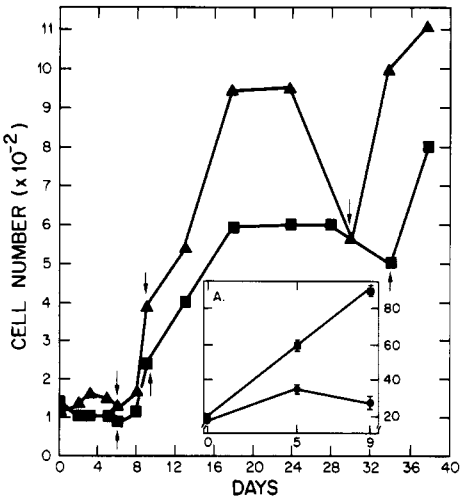

FIGURE 3: Effect of cAMP on growth of cells. Ltk$^-$ cells were transfected with pPCTK-6A via pH-sensitive immunoliposomes and incubated 24 h in DMEM medium in the presence (▲) or absence (■) of BT. After 24 h, the media were changed to HAT medium without BT. Day 0 is the day cells were grown in the HAT medium. Arrows represent the subsequent addition of BT to the medium. Inset A represents growth of untransfected Ltk$^-$ cells in DMEM medium supplemented with 10% FCS in the presence (●) or absence (■) of BT.

Long-Term Transformation Efficiency. The experiment described above indicated that cells transfected with the liposome protocol showed long-term expression of the exogenous gene. In order to quantitate the long-term transformation frequency, clonal cultures with a limiting dilution protocol were carried out in 96-well plates. Growth of cells in HAT medium in those wells containing initially only one cell was followed for 12 days. We have also included the cells which had been

transfected with the calcium phosphate precipitation method, and cells which had not been transfected as parallel controls (Table IV). We have defined the long-term transformation of a cell being that it gives rise to at least three cell divisions in 12 days. Therefore, those wells which contained eight cells or more after 12 days in culture were scored as positive for long-term transformation. As can be seen in Table IV, approximately 2% of the original 1400 wells containing a single cell had been long-term-transformed, if the cells were transfected with the liposome protocol and the growth medium contained BT in addition to HAT. Only two wells could be scored positive for the corresponding clonal culture of cells transfected with the calcium phosphate precipitation protocol; i.e., the frequency was 1.2×10^{-3}. Therefore, the liposome-mediated transfection resulted in a significantly higher level of long-term transformation than the conventional calcium phosphate precipitation method. However, the long-term transformation frequency depended on the gene expression. If BT was not included in the medium, only two clones of eight cells or more were seen after 12 days for the liposome-mediated transfection method. We conclude that it is important to maximize the conditions for gene expression when the transformation efficiency is measured. Control, nontransformed cells gave no long-term transformants.

Also shown in Table IV is the number of clones found at day 4. There were eight clones with eight cells or more for cells transfected with the liposome protocol and incubated with BT in the growth medium. By day 12, more clones had grown to contain eight cells or more under the same conditions. Cells transfected with the liposome protocol but grown in the absence of BT and cells transfected with the calcium phosphate protocol but cultured in the presence of BT both showed small numbers of transformation at day 4, i.e., 0.2 and 0.1%, respectively. However, no other clones had grown to contain eight cells or more by day 12. This result indicates that a sustained high level of expression of the TK gene is required for the establishment of stable, long-term transformants under the selection pressure of HAT.

Discussion

We have previously shown that the gene coding for the *E. coli* chloramphenicol acetyltransferase can be delivered by the pH-sensitive immunoliposome and expressed specifically in the target lymphoma cells grown in nude mice (Wang & Huang, 1987b). Although the effect of liposome composition (lipid and antibody) on the delivery efficiency and target specificity was shown in that study, the transformation efficiency of the target cells was not measured. The purpose of the current study is to carry out these measurements in a well-defined tissue culture system with a selectable reported gene, i.e., viral thymidine kinase gene. Furthermore, we have also studied the role of gene expression in the long-term transformation efficiency of the transfected cells.

Our data have clearly demonstrated high efficiencies of the cells transfected by DNA associated with the pH-sensitive immunoliposome for both short-term and long-term transformations. It has been shown that immunoliposomes are taken up by cells through a receptor-mediated endocytosis pathway and are delivered to the cellular endosomes and lysosomes (Huang et al., 1983). pH-sensitive immunoliposomes are specifically designed to exploit the endocytic pathway and mediate cytoplasmic delivery of fluorescent dye (Connor & Huang, 1985; Wang et al., 1986), anticancer drugs (Connor & Huang, 1986), and diphtheria toxin A fragment (Collins & Huang, 1987). The results of the previous (Wang & Huang, 1987a,b) and present study show that DNA can also be efficiently delivered to target cells by pH-sensitive immunoliposomes. On the basis of previous work in our laboratory (Connor & Huang, 1985, 1986, 1987; Collins & Huang, 1987; Wang et al., 1986), we suggest that a high level of target cell transfection by DNA-containing pH-sensitive immunoliposomes occurs after receptor-mediated endocytosis of the immunoliposome. Cytoplasmic delivery of DNA occurs via an acid-induced fusion of the liposome with the endosome membrane (Connor & Huang, 1987; Wang & Huang, 1987a) similar to the pathway taken by enveloped viruses such as Semliki forest virus (Marsh et al., 1982; White et al., 1981), influenza virus, and vesicular stomatitis virus (White et al., 1981). In addition to liposome–endosome fusion, rupture of endosome due to extensive H_{II}-phase formation may take place which can account for the delivery of the liposome-adsorbed DNA (Wang & Huang, 1987; Collins, 1989). Acid-induced liposome phase transition is the basis for the enhanced delivery efficiency of the pH-sensitive liposomes. Liposomes composed of DOPC/chol/OA do not undergo similar phase transitions at the acidic pH and showed a much lower delivery efficiency (Table I). Furthermore, the attachment of antibody to liposomes is also essential for a high level of transfection efficiency (Table II). We have previously shown that antibody enhances liposome binding to the target cell (Huang et al., 1982) and hence increases the amount of DNA uptake by the cells.

Short-term expression of the delivered gene was examined by monitoring enzymatic activity and [^{3}H]thymidine uptake while long-term expression was determined by monitoring the growth of transfected cells in HAT-containing medium. Approximately 48% of cells treated with pH-sensitive immunoliposomes and grown in the presence of cAMP stimulators took up [^{3}H]thymidine (Table III). Thus, about half of treated cells immediately synthesized thymidine kinase. In the same experiment, it was shown that approximately 34% of the cells treated with calcium phosphate precipitated DNA were labeled with [^{3}H]thymidine. When the short-term expression was assayed as TK activity, there was 2.3-fold more activity expressed by cells transfected with pH-sensitive immunoliposomes than those transfected with calcium phosphate precipitated DNA (Table III). The difference between the degree of increase in TK activity and the percent of cells that showed incorporation of [^{3}H]thymidine suggests that pH-sensitive immunoliposomes not only increase the uptake of DNA by the target cells but also increase to an even greater extent the enhanced expression of DNA delivered. The long-term transformation as measured by the survival of cells in the HAT medium containing cAMP stimulators was approximately 2% (Table IV). This indicated that approximately $^1/_{25}$th of the cells expressing TK activity immediately after liposome treatment sustained the production of TK to survive in the selection medium for a prolonged period of time. This result suggests that the exogenous DNA delivered by pH-sensitive immunoliposomes has been integrated into the host chromosome. In fact, Southern blot analyses of three long-term transformed clones have confirmed this interpretation (data not shown). In any case, both the short-term and long-term transformation efficiencies of the pH-sensitive immunoliposome method were much higher than those of the conventional calcium phosphate precipitation method (Tables III and IV).

Genetic materials have previously been introduced into mammalian cells by calcium precipitation (Wigler et al., 1977, 1978; Graham & Van Der Eb, 1973), DEAE-dextran (Farber et al., 1975), polyornithine (Farber et al., 1975), microinjection (Capecchi, 1980), retroviral vectors (Hamer & Leder, 1979; Mulligan et al., 1979), and electroporation (Neumann et al.,

Table V: Comparison of Liposome-Mediated in Vitro Transfection Methods for Mammalian Cells

lipid composition	liposome method	targeting molecule	inducing agent	cell type	DNA	DNA dose ($\mu g/10^5$ cells)	transformation efficiency: short term (%)	transformation efficiency: long term	ref (first author)
PS/chol[a]	REV	none	Ca^{2+}	Ltk^-	plasmid	50	10	2×10^{-4}	Schaefer-Ridder
PS/chol	REV	none	glycerol, chloroquine	CV-1P	SV40	0.001	?	4×10^{-3}	Fraley
PS/chol	dialysis, Ca^{2+}–cochelate	Sendai		C127	plasmid	?	?	10^{-4}	Mannino
PS	Ca^{2+}–cochelate	none	Mg^{2+}	$FM3Atk^-$	plasmid	1	?	2×10^{-2}	Itani
PS	Ca^{2+}–cochelate	none	spermine, spermidine	$H56tk^-$	*Drosophila*	4	~100	10^{-5}	Somlyai
PC/chol/PS	REV	none		CV-1	SV40	0.001	?	10^{-3}	Rizzo
DOPE/DOTMA[b]	sonication	none		many	plasmid	0.2	25	$\sim 10^{-4}$	Felgner
PC/PS/chol	REV	protein A	electroporation	BJAB	plasmid	0.1	?	2×10^{-6}	Machy
DOPE/chol/OA	dialysis	antibody		Ltk^-	plasmid	10	48	2×10^{-2}	present study

[a] Abbreviations: PS, phosphatidylserine; chol, cholesterol; PC, phosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DOTMA, *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride; OA, oleic acid; REV, reverse-phase evaporation vesicle. [b] Lipofectin.

1982). However, the efficiency of transformation is usually in the range of 10^{-3}–10^{-5} (Anderson, 1984). The low efficiency of transformation may be caused by inefficient delivery, extracellular DNA degradation, or intracellular DNA degradation. pH-sensitive liposomes increase the efficiency of delivery by (a) maintenance of intact DNA in the extracellular environment by protecting the DNA from nucleases, (b) specific and enhanced uptake of liposomes by the target cells via antibody and antigen binding, and (c) efficient cytoplasmic delivery of DNA via liposome–endosome fusion or endosome rupture. Furthermore, the viability of Ltk^- cells after liposome-mediated transfection was much better than that of cells transfected via the calcium phosphate precipitation method. Therefore, it is possible to study the short-term gene expression immediately after the transfection protocol without the need to wait for cell recovery.

There are several different liposome-mediated transfection protocols. It is useful to compare their performance as summarized in Table V. Most of the protocols use the negatively charged phosphatidylserine as the major phospholipid ingredient. The entrapment of DNA is usually mediated by calcium ion induced formation of cochlear structures (Mannino & Gould-Fogerite, 1988; Itani et al., 1987; Somlyai et al., 1985). This method is quite mild, and the resulting liposomes are quite large in size ($d > 0.1$ μm) (Papahadjopoulos et al., 1975). The reverse-phase evaporation method (Szoka & Papahadjopoulos, 1978) is also commonly used for entrapping DNA (Schaefer et al., 1982; Rizzo et al., 1983; Machy et al., 1988; Fraley et al., 1981). We have used a detergent dialysis method which has been shown to also entrap large amounts of nucleic acid (Philippot et al., 1983). While the short-term transformation frequencies of these methods are not always reported, the long-term frequency varies in a wide range of 10^{-6}–10^{-2}, with most methods showing a long-term frequency of 10^{-5}–10^{-4} (Table V). One exception is the method reported by Itani et al. (1987) which shows an excellent efficiency of 2×10^{-2}. The long-term transformation efficiency of our liposome method is the same, highest among all methods reported to date. However, the liposomes of Itani et al. are not target-specific as is the case of the immunoliposomes reported here. The recently reported Lipofectin liposomes differ from all other methods by using positively charged lipids (Felgner et al., 1987; Felgner & Ringold, 1989). Moreover, the transfection procedure does not require entrapment of DNA in the liposomes; thus, the procedure is convenient and rapid (Felgner et al., 1987). However, as can be seen in Table V, the long-term transformation frequency of the method is not high. Recently, we have also developed a similar positively charged liposome system for transfection (Pinnaduwage et al., 1989). Another interesting method reported recently is the use of a combination of liposome and electroporation (Machy et al., 1988). The liposome can be targeted with protein A which binds specifically to cells pretreated with antibody, thus providing a specific delivery of DNA to the target cells (Machy et al., 1988). Unfortunately, the transformation frequency of the method is quite low.

Another important consideration in selecting a method for DNA delivery is the potential for in vivo delivery. Retroviruses have been generally accepted as an efficient vector for this purpose (Anderson, 1984). However, application of vectors in human gene therapy requires safety assurance. Although the viral oncogene can be eliminated by the removal of the encapsulation signal of viruses, the possibility of recombination between vectors and endogenous viral sequences in host cells is still an open possibility and a potential hazard (Bishop, 1987). Compared with retroviruses, liposomes which are made of natural phospholipid are nontoxic and degradable. Previous studies have already demonstrated the utility of liposome carrier for in vivo gene delivery (Nicolau et al., 1983, 1987; Soriano et al., 1983), except that the efficiency of delivery is often too low to yield stable, long-term expression of the exogenous gene (Nicolau et al., 1983). The method described here, however, has demonstrated a large magnitude of improvement in the transformation frequency. This consideration, together with the fact that the delivery is target-specific as shown by our previous work (Wang & Huang, 1987b), has made the pH-sensitive immunoliposome an attractive candidate for gene therapy in vivo.

Acknowledgments

We thank Drs. A. Wynshaw-Boris and R. Hanson for the plasmid pPCTK-6A.

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